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Study of molecular and cellular pathogenicity mechanisms of *Arcobacter* species

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Dedicatory

To my family

“If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward.”

Martin Luther King Jr.

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List of Publications

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I. **Ferreira, S.,** Fraqueza, M.J., Queiroz, J.A., Domingues, F.C., Oleastro, M. (2013) Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse. *International Journal of Food Microbiology* **162**, 82–88.

II. **Ferreira, S.,** Silva, F., Queiroz, J.A., Oleastro, M., Domingues, F.C. (2014) Resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus*: Activity and effect on cellular functions. *International Journal of Food Microbiology* **180**, 62-68.

III. **Ferreira, S.,** Júlio, C., Queiroz, J.A., Domingues, F.C., Oleastro, M. (2014) Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients. *Diagnostic Microbiology and Infectious Disease* **78**, 220–225.

IV. **Ferreira, S.**, Queiroz, J.A., Oleastro, M., Domingues, F.C. Comparative pathogenic and genotypic properties of human and non-human *Arcobacter butzleri* strains. *Submitted for publication*

V. **Ferreira, S.**, Queiroz, J.A., Oleastro, M., Domingues, F.C. Insights in the pathogenesis and resistance of *Arcobacter*: a review. *Submitted for publication*

Publications not related to the thesis

I. Luís, A., Breitenfeld, L., **Ferreira, S.,** Duarte, A.P., Domingues, F. (2014) Antimicrobial, antibiofilm and cytotoxic activities of *Hakea sericea* Schrader extracts. *Pharmacognosy Magazine* **10**, 6-13.

II. Duarte, A.F., **Ferreira**, **S.,** Oliveira, R., Domingues, F.C. (2013) Effect of Coriander Oil (*Coriandrum sativum*) on Planktonic and Biofilm Cells of *Acinetobacter baumannii*. *Natural Product Communications* **8**, 673-678.

III. Silva, R., **Ferreira**, **S.,** Bonifacio, M.J., Dias, J.M., Queiroz, J.A., Passarinha, L.A. (2012) Optimization of fermentation conditions for the production of human soluble catechol-O-methyltransferase by Escherichia coli using artificial neural network. *Journal of Biotechnology* **160**, 161-168.

IV. Duarte, A., **Ferreira**, **S.,** Silva, F., Domingues, F.C. (2012) Synergistic activity of coriander oil and conventional antibiotics against *Acinetobacter baumannii*. *Phytomedicine* **19**, 236-238.

V. **Ferreira**, **S.,** Santos, J., Duarte, A., Duarte, A.P., Queiroz, J.A., Domingues, F.C. (2012) Screening of antimicrobial activity of *Cistus ladanifer* and *Arbutus unedo* extracts. *Natural Product Research* **26**, 1558-1560.

VI. Silva, F., **Ferreira, S.**, Duarte, A., Mendonça, D.I., Domingues, F.C. (2011) Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B. *Phytomedicine* **19**, 42-47.

VII. Silva, F., **Ferreira, S.**, Queiroz, J.A., Domingues, F.C. (2011) Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. *Journal of Medical Microbiology* **60**, 1479–1486.

VIII. **Ferreira**, **S.,** Gil, N., Queiroz, J. A., Duarte, A. P., Domingues, F. C. (2011) An evaluation of the potential of *Acacia dealbata* as raw material for bioethanol production. *Bioresource Technology* **102**, 4766–4773.

IX. **Ferreira, S.**, Gil, N., Queiroz, J. A., Duarte, A. P., Domingues, F. C. (2010) Bioethanol from the Portuguese forest residue *Pterospartum tridentatum* – An evaluation of pretreatment strategy for enzymatic saccharification and sugars fermentation. *Bioresource Technology* **101**, 7797–7803.

X. Paulo L., **Ferreira S.**, Gallardo E., Queiroz J.A., Domingues F. (2010) Antimicrobial

activity and effects of resveratrol on human pathogenic bacteria. *World Journal of Microbiology and Biotechnology* **26**, 1533-1538.

XI. Gil, N., **Ferreira, S.**, Amaral, M.E., Domingues, F.C., Duarte, A.P. (2010) The influence of dilute acid pretreatment conditions on the enzymatic saccharification of *Erica* spp. for bioethanol production. *Industrial Crops and Products* **32**, 29–35.

List of scientific communications

Oral scientific communications of this Doctoral work

I. **Ferreira, S.,** Fraqueza, M.J., Queiroz, J.A., Domingues, F.C., Oleastro, M. Characterization of *Arcobacter butzleri* isolates from poultry and slaughterhouse environment - II International Conference on Antimicrobial Research, Lisbon, Portugal, 21-23 November 2012.

Poster presentations of this Doctoral work

I. **Ferreira**, **S.,** Silva, F., Queiroz, J.A., Oleastro, M., Domingues, F.C. Activity and mechanism of action of resveratrol on *Arcobacter cryaerophilus* – V International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld 2013), Madrid, Spain, 2-4 October 2013.

II. **Ferreira**, **S.,** Silva, F., Queiroz, J.A., Oleastro, M., Domingues, F.C. Inhibitory effect of resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus* – *Campylobacter*, *Helicobacter* and Related Organisms (CHRO 2013), Aberdeen, Scotland, UK, 15-19 September 2013.

III. **Ferreira, S.,** Santos, A., Júlio, C., Queiroz, J.A., Domingues, F.C., Oleastro, M. Prevalence of *Campylobacter* and *Arcobacter* species in diarrhoeal faeces from humans in Portugal - *Campylobacter*, *Helicobacter* and Related Organisms (CHRO 2013), Aberdeen, Scotland, UK, 15-19 September 2013.

IV. **Ferreira, S.,** Fraqueza, M.J., Queiroz, J.A., Domingues, F.C., Oleastro, M. Genetic diversity and antibiotic resistance of *Arcobacter butzleri* isolated from poultry and slaughterhouse environment in Portugal - Central European Symposium on Antimicrobials and Antimicrobial Resistance (CESAR 2012), Primosten, Croatia, 23-26 September 2012.

Other oral scientific communications

I. Silva F., **Ferreira S.**, Duarte A., Queiroz J.A., Domingues F.C. Coriander oil antimicrobial activity: a flow cytometric study - 43rd International Symposium on Essential Oils (ISEO), Lisbon, Portugal, 5-8 September 2012.

II. Luís, A., Breitenfeld, L., **Ferreira, S.,** Duarte, A.P., Domingues, F. Antimicrobial and cytotoxic Properties of *Hakea sericea* Schrader methanolic extracts – 1st International Symposium of Pharmacy, Guarda, Portugal, 6 July 2012.

III. Silva, R.R., **Ferreira**, **S.,** Bonifácio, M.J., Dias, J., Queiroz, J.A., Passarinha, L. A. Batch experimental design for optimal temperature, pH and agitation in hSCOMT recombinant production systems - 8th European Symposium on Biochemical Engineering Science (ESBES) ESBES & ISPPP & ISB Symposia 2010, Bologna, Italy, 6-8 September 2010.

Other poster presentations

I. Oleastro, M., Santos, A., **Ferreira, S.,** Rodrigues, A., Júlio, C. *Campylobacter jejuni* infection in Portugal: high rates in hospital settings - 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 10–13 May 2014

II. Oleastro, M., Santos, A., Costa, I., **Ferreira, S.,** Rodrigues, A., Júlio, C Protozoan-associated diarrheal infections in Portuguese patients - 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 10–13 May 2014

III. Afonso, M.S., **Ferreira, S.**, Domingues, F.C., Silva, F. Resveratrol production in bioreactors: assessment of cell physiology states by flow cytometry and plasmid segregational instability by real time q-PCR. Portuguese Congress of Microbiology and Biotechnology' 2013 (MicroBiotec'13), Aveiro, Portugal, 6-8 December 2013.

IV. Afonso, M.S., **Ferreira, S.**, Domingues, F.C., Silva, Resveratrol production in bioreactors using DoE optimization – assessment of physiological states by flow cytometry and plasmid segregational instability by real-time qPCR – V International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld 2013), Madrid, Spain, 2-4 October 2013.

V. Duarte, A., **Ferreira, S.**, Santos, A., Benoliel, J., Martins, A., Fraqueza, M.J., Domingues, F.C., Oleastro, M. Antibiotic resistance and genetic diversity of Human, Food and animal origin *Campylobacter* spp. isolates from Portugal - *Campylobacter*, *Helicobacter* and Related Organisms (CHRO 2013), Aberdeen, Scotland, UK, 15-19 September 2013.

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

VII. Soares, R., **Ferreira, S.**, Pedro, A., Maia, C., Bonifácio, M.J., Queiroz, J., Passarinha, L. Biosynthesis of human membrane-bound catechol-*O*-methyltransferase: optimization using Plackett-Burman and Central Composite Rotatable Design - 9th European Symposium on Biochemical Engineering Science (ESBES), ESBES & ISPPP Symposia 2012, Istanbul, Turkey, 24 -26 September 2012.

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

IX. Silva F., **Ferreira S.,** Queiroz J.A., Domingues F.C. Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action investigated by flow cytometry. - 1st European Congress of Applied Biotechnology, Berlin, Germany, 25-29 September 2011.

X. Silva F., **Ferreira S.,** Duarte A., Mendonça D.I. and Domingues F.C. Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B - IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld 2011), Málaga, Spain, 14-16 September 2011.

XI. Duarte A., **Ferreira S.**, Silva F., Domingues F.C. Synergistic activity between coriander essential oil and conventional antibiotics against *Acinetobacter baumannii* - IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld 2011), Málaga, Spain, 14-16 September 2011.

XII. **Ferreira, S.,** Costa, B., Domingues, F.C. Antimicrobial activity of Coriander Oil - I International Conference on Antimicrobial Research (ICAR2010), Valladolid, Spain, 3-5 November 2010.

XIII. **Ferreira, S.,** Gil, N., Queiroz, J.A., Duarte, A.P., Domingues, F.C. Evaluation of *Acacia dealbata* as a potential biomass resource for bioethanol production - 14th International Biotechnology Symposium and Exhibition, Rimini, Italy, 14-18 September 2010.

XIV. **Ferreira, S.,** Gil, N., Queiroz, J.A., Duarte, A.P., Domingues, F.C. Bioethanol from the Portuguese forest residue *Pterospartum tridentatum* - 8th European Symposium on Biochemical Engineering Science (ESBES), ESBES & ISPPP & ISB Symposia 2010, Bologna, Italy, 6-8 September 2010.

Resumo alargado

O género *Arcobacter* foi proposto em 1991 e faz parte da família *Campylobacteraceae* juntamente com os géneros *Campylobacter* e *Sulfurospirillum*, compreendendo atualmente 18 espécies. Entre as espécies reconhecidas, *Arcobacter butzleri*, *Arcobacter cryaerophilus* e *Arcobacter skirrowii* têm sido associados a doença em humanos e animais. O género *Arcobacter* está amplamente distribuído, tendo sido isoladas espécies em alimentos, água, instalações de processamento e manipulação de alimentos, diversas amostras ambientais, animais e amostras humanas. Em produtos de origem animal, este microrganismo, tem sido isolado com maior prevalência em frangos, seguido de porco e vaca. O consumo de alimentos ou água contaminados com *Arcobacter* é considerado como a via de transmissão mais provável. Assim, o conhecimento sobre a distribuição de *Arcobacter* em alimentos ou ambiente de processamento alimentar, juntamente com a avaliação da variabilidade genética e outras características fenotípicas e genotípicas associadas ao seu potencial de virulência, pode ajudar na compreensão do mecanismo de persistência de *Arcobacter* na cadeia alimentar e contribuir para o seu controlo.

Devido à ausência de estudos prévios sobre a prevalência de *Arcobacter* em Portugal para humanos ou relativos ao sector alimentar, procedeu-se neste trabalho ao seu isolamento a partir de amostras recolhidas de três bandos de frangos e de superfícies da linha de processamento do matadouro. *A. butzleri* foi isolado e identificado em todas as amostras recolhidas, com exceção das amostras correspondentes ao conteúdo cecal. Quarenta e três isolados de *A. butzleri* foram caracterizados por eletroforese em gel de campo pulsado, utilizando as enzimas de restrição *Sma*I e *Sac*II, revelando uma elevada diversidade genética, com os 43 isolados pertencendo a 32 padrões distintos. No entanto, foram identificados genótipos comuns entre diferentes amostras, indicando a possibilidade de ocorrência de contaminação cruzada durante o processo de abate. Verificou-se ainda neste estudo que os isolados de *A. butzleri* apresentaram elevados níveis de resistência aos antibióticos em estudo, com todos os isolados a serem resistentes a pelo menos três antibióticos dos nove testados. Enquanto os 43 isolados demonstraram ser suscetíveis à gentamicina e 2,3 % resistentes ao cloranfenicol, 55,8 % das estirpes foram resistentes à ciprofloxacina. Genotipicamente a resistência à ciprofloxacina foi associada a uma transição de citosina para timina na região determinante de resistência às quinolonas do gene *gyrA*, como previamente descrito. Entre 36 isolados selecionados, 72,2 % apresentaram capacidade de formação de biofilme, o que poderá em parte estar associado à sobrevivência de *A. butzleri* em ambiente de matadouro, provavelmente por favorecer a dispersão e contaminação cruzada ao longo da linha de processamento. A deteção de genes putativos de virulência por PCR demonstrou a presença dos genes *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* e *tlyA* em todos os isolados, com uma deteção variável dos genes *hecA* (75 %), *hecB* (89 %) e *irgA* (42 %). Como neste estudo se encontrou uma elevada prevalência de *A. butzleri* nas amostras estudadas, pressupõe-se que

o frango possa ser considerado uma via de transmissão importante para humanos. Este facto associado a diversos fatores de virulência, como elevada resistência a antimicrobianos, capacidade de formação de biofilmes e presença de possíveis genes de virulência, tornam este microrganismo como um patogéneo potencialmente relevante a nível alimentar.

A potencial transmissão de *Arcobacter* através de alimentos contaminados, a elevada prevalência encontrada para *A. butzleri* e o facto de *A. cryaerophilus* ser a segunda espécie mais comum associada com alimentos, para além da resistência a antimicrobianos comuns e da sua capacidade de sobreviver a tratamentos físicos e químicos, torna relevante encontrar estratégias alternativas para o seu controlo. Assim sendo, o objetivo seguinte foi centrado na avaliação das propriedades antimicrobianas do resveratrol contra *A. butzleri* e *A. cryaerophilus*. O resveratrol exibiu atividade bactericida, levando a uma redução igual ou superior a 3 $\log_{10}$ UFC/mL do inóculo inicial, para células em fase exponencial de crescimento de *A. butzleri* após 6 horas de incubação com uma concentração de resveratrol de 100 µg/mL, ou de 200 µg/mL após 24 horas de incubação com células em fase estacionária. Para as células de *A. cryaerophilus* em fase exponencial de crescimento, o efeito bactericida foi observado após 24 horas com 100 µg/mL, enquanto para as células em fase estacionária, a atividade bactericida foi apenas detetada com 200 µg/mL. Com o objetivo de elucidar o potencial mecanismo de ação deste composto sobre *Arcobacter*, começou por se avaliar o conteúdo intracelular de ADN e atividade metabólica por citometria de fluxo. Após incubação com resveratrol verificou-se uma diminuição em ambos os parâmetros avaliados. Este composto levou também a um aumento na acumulação de brometo de etídio nas células, demonstrando que o resveratrol pode agir como inibidor de bombas de efluxo, e a uma redução da concentração mínima inibitória de resveratrol pelo inibidor de bombas de efluxo fenilalanina-arginina ß-naftilamida. Para avaliar a ação do resveratrol sobre a morfologia e integridade celular de *Arcobacter* usou-se a técnica de microscopia eletrónica de varrimento. Com esta técnica verificou-se que o resveratrol provocou desintegração das células de *A. butzleri* tratadas com concentração mínima inibitória de resveratrol durante 6 horas, enquanto nenhuma alteração morfológica foi observada para as células de *A. cryaerophilus*. Deste estudo concluiu-se que o resveratrol mostrou elevada atividade anti-*Arcobacter* e os resultados obtidos sugerem que esta atividade antibacteriana pode resultar da ação do mesmo sobre diferentes alvos celulares, o que pode por sua vez levar à indução de morte celular, em consequência de danos nas funções celulares. Assim, neste estudo foram realçadas as potencialidades antimicrobianas do resveratrol sobre um patogéneo de origem alimentar, *Arcobacter*. Este aspeto associado ao facto de o resveratrol ser naturalmente sintetizado por plantas em respostas a diversas agressões, e de adicionalmente exibir diversas propriedades descritas como benéficas para a saúde humana e ser um reconhecido antioxidante, encoraja a utilização do resveratrol como um potencial conservante alimentar.

O género *Arcobacter* compreende algumas espécies que têm sido relacionadas maioritariamente com doença gastrointestinal em humanos, no entanto, apesar de algumas serem consideradas patogéneos emergentes, não têm sido vistas como microrganismo de

elevada preocupação para a saúde pública. Diversos estudos de prevalência têm apresentado *A. butzleri* como a quarta espécie mais comum em fezes humanas de indivíduos com gastroenterite, de entre os organismos denominados *Campylobacter-like organisms*. Assim, foi realizado um estudo de prevalência em 298 amostras de fezes de pacientes com diarreia, recolhidas de 22 hospitais portugueses, entre setembro e novembro de 2012. As amostras foram analisadas quanto à prevalência e diversidade de espécies de *Arcobacter* e *Campylobacter*, usando técnicas de deteção molecular. Relativamente às espécies de *Arcobacter*, 1,3 % das amostras foram positivas para *A. butzleri* e 0,3% para *A. cryaerophilus*. Foram encontradas espécies de *Campylobacter* em 31,9 % das amostras de fezes diarreicas, com *C. jejuni* e *C. concisus* sendo as espécies predominantes deste género (13,7 % e 8,0 % , respetivamente). Neste grupo de amostras, *A. butzleri* foi a quarta espécie mais frequente. Estes resultados evidenciam a importância das espécies de *Arcobacter* e *Campylobacter* como agentes etiológicos de gastroenterite aguda entre os pacientes portugueses, afetando particularmente a faixa etária pediátrica. Dado que não existe nenhum trabalho prévio relativo à prevalência e distribuição de *Arcobacter* em Portugal, este trabalho contribui para um conhecimento da epidemiologia local de *Arcobacter*, tal como de espécies de *Campylobacter*.

Embora *A. butzleri* tenha sido implicado em algumas doenças em humanos como diarreia e bacteriémia, ainda são necessários mais estudos para clarificar a patogénese associada a este microrganismo. Assim, o potencial de virulência de *A. butzleri* foi avaliado através da caracterização genotípica e de propriedades patogénicas de três isolados humanos e três não-humanos. Os isolados mostraram suscetibilidade às tetraciclinas e aminoglicosídeos testados, porém exibindo alta resistência às quinolonas. *A. butzleri* demonstrou uma atividade hemolítica fraca e capacidade de formar biofilmes em superfícies de poliestireno. Foram demonstrados níveis de adesão semelhantes aos de *Salmonella* Typhimurium para *A. butzleri* em células Caco-2, sendo que a inflamação pré-existente das células mostrou não ter efeito significativo sobre a capacidade de adesão dos isolados, no entanto, a capacidade de invasão aumentou significativamente para dois dos isolados humanos. *A. butzleri* demonstrou capacidade de sobrevivência intracelular em células Caco-2 e de indução significativa da secreção de interleucina-8, assim como demonstrou capacidade de induzir perturbação da estrutura celular. Não foi encontrada correlação entre a presença de genes putativos de virulência e os diferentes fenótipos de patogenicidade. Estes resultados forneceram novas perspetivas acerca da virulência e potencial patogénico de *A. butzleri*.

Em conclusão, este trabalho avaliou a prevalência de espécies *Arcobacter* tanto em amostras humanas como alimentares o que vem contribuir para um melhor conhecimento acerca da epidemiologia deste microrganismo em Portugal. A sobrevivência e a persistência deste organismo no ambiente foi evidenciada devido à sua capacidade de formação de biofilmes, tal como a sua importância como patogéneo humano foi salientada pela resistência aos antimicrobianos, presença de vários genes de virulência, capacidade de adesão, invasão, sobrevivência intracelular e indução da secreção de citoquinas pró-inflamatórias em células

de epitélio intestinal. Para controlar este microrganismo foi ainda testada uma abordagem baseada no resveratrol, um composto de origem natural com o potencial para atuar como conservante na indústria alimentar. Este trabalho permitiu assim ampliar o conhecimento acerca da epidemiologia e patogenicidade de *Arcobacter,* bem como contribuir para o desenvolvimento de novas estratégias de controlo.

Palavras-chave

Arcobacter; diversidade genética; resistência antimicrobiana; biofilmes; genes de virulência; resveratrol; epidemiologia da infeção humana; patogénese

Abstract

Arcobacter is a genus of Gram-negative, spiral-shaped bacteria in the *Epsilonproteobacteria* class. It was first proposed in 1991 and was included in the *Campylobacteraceae* family together with the genera *Campylobacter* and *Sulfurospirillum*. There are currently 18 species described, among which *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are known to be human and animal pathogens. *Arcobacter* genus shows an unusually wide range of habitats, having been isolated from food, water, food processing and handling facilities, diverse environmental, animal and human samples. In fact, consumption of *Arcobacter*-contaminated food or water is regarded as the most probable cause of infection by this bacterium. Therefore it is important to evaluate its distribution in food or food processing environment, together with the assessment of its genetic variability and other phenotypic and genotypic features associated with virulence potential, to be able to understand the persistence mechanism of *Arcobacter* in the food chain.

Thus, this study intend to evaluate the presence, genetic diversity, and virulence characteristics (namely antibiotic resistance, biofilm-forming capacity and detection of putative virulence genes) of *A. butzleri* strains isolated from poultry and from the environment of a Portuguese slaughterhouse. *A. butzleri* isolates revealed a high genetic diversity, with all isolates showing to be susceptible to gentamicin, in contrast to 55.8% that were resistant to ciprofloxacin. The latter resistance was associated with the presence of a cytosine to thymine transition in the quinolone resistance determining region of the *gyrA* gene. Among selected isolates, 72.2 % presented biofilm-forming ability and in all strains putative virulence genes were detected. These results highlight the relevance of *A. butzleri* relevance as food-borne pathogen.

Taking into account that *Arcobacter* is potentially transmitted through contaminated food and is resistant to common antimicrobials, it is important to develop alternative control strategies that could be both effective and safe for human consumption. Therefore, the antimicrobial properties of resveratrol against *A. butzleri* and *A. cryaerophilus* were studied. Resveratrol exhibited a bacteriostatic or bactericidal activity dependent on cellular growth phase and resveratrol concentration, leading to both DNA content and metabolic activity reduction on *Arcobacter* cells. Resveratrol also showed the ability to act as an efflux pump inhibitor, and to induce cellular damage. Thus, resveratrol showed anti-*Arcobacter* activity, with the results obtained suggesting that this compound inhibits this microorganism through different pathways, which together with resveratrol beneficial properties described for human health may encourage its use as a food preservative.

Some of the species of *Arcobacter* genus have been associated with gastrointestinal disease in humans, however there were a lack of studies evaluating its prevalence in Portugal, with the same happening for non-*Campylobacter jejuni/coli* species. Therefore, the frequency of *Arcobacter* and *Campylobacter* species in faeces from patients with diarrhoea in Portugal was

assessed using a molecular approach. Concerning, *Arcobacter* and *Campylobacter* prevalence and distribution, 298 diarrhoeal samples from Portuguese patients were analysed, 1.3% of the samples were positive for *A. butzleri* and 0.3 % for *A. cryaerophilus*. *Campylobacter* species were found in 31.9 % of diarrhoeic faeces samples, with *C. jejuni* and *C. concisus* being the most prevalent species of this genus (13.7 % and 8.0 %, respectively). In this cohort of samples, *A. butzleri* was the fourth most frequent species. These results evidence the importance of *Arcobacter* and *Campylobacter* species as aetiological agents of acute gastroenteritis among Portuguese patients, affecting particularly the paediatric age group.

Although *A. butzleri* has been implicated in human diseases, much of its pathogenesis and virulence factors remain unclear. Thus, *A. butzleri* virulence potential was also investigated, through the characterization of genotypic and pathogenic properties of human and non-human isolates. The isolates showed to be susceptible to tetracyclines and aminoglycosides, however displaying high resistance to quinolones. *A. butzleri* demonstrated a weak haemolytic activity and the ability to form biofilms in polystyrene surfaces. Adhesion levels similar to *Salmonella* Typhimurium were found for *A. butzleri* on Caco-2 cells, with pre-existing inflammation showing no significant effect on its adherence ability, yet invasion ability showed to vary among the isolates. *A. butzleri* was able of intracellular survival in Caco-2 cells and to induce a significant up-regulation of interleukin-8 secretion, as well as to promote structural cell disturbance. These data brings new insights to *A. butzleri* virulence and highlights its pathogenic potential.

Overall in this work, the prevalence of *Arcobacter* species both in human and food-related samples was evaluated, contributing to understanding the epidemiology of *Arcobacter* in Portugal. The survival and persistence of this organism in the environment was highlighted due to its ability to form biofilms. Its relevance as a human pathogen was underlined by the resistance to antimicrobials, the presence of several putative virulence genes, along with its adherence, invasion, intracellular survival abilities and induction of proinflammatory cytokine secretion in intestinal epithelial cells. Finally, resveratrol was tested as an alternative to control the growth of *Arcobacter*.

This work provided new insights on the epidemiology and pathogenicity of *Arcobacter*, and also identified a natural compound with anti-*Arcobacter* activity, which may contribute for future developments of new control approaches.

Keywords

Arcobacter; genetic diversity; antimicrobial resistance; biofilm; virulence genes; resveratrol; epidemiology of human infection; patogenesis

Thesis Overview

This thesis comprises the research work performed at the Health Sciences Research Centre from the Faculty of Health Sciences of the University of Beira Interior. This work was done in collaboration with the National Reference Laboratory for Gastrointestinal Infections from the Department of Infectious Diseases of the National Institute of Health Dr. Ricardo Jorge in Lisbon.

This thesis is structured in three main parts. The first part includes a general introduction consisting of a review of the literature and state of the art regarding the importance of *Arcobacter* species for public health, detection methods, pathogenesis general aspects, resistance to antimicrobials and potential control strategies as well as the global aims of this work. Part II comprises the experimental results obtained in this work, organized in four chapters corresponding to three original publications and one submitted manuscript. Finally, the part III summarizes the main conclusions from this work and addresses future perspectives for further research in this field.

Index

List of Figures xxxv
List of Tables xxxvii
List of Abbreviations and Acronyms xxxix

Chapter 1

Introduction 1
1. Genus *Arcobacter* 3
2. *Arcobacter* species in food and environment 8
3. Human and animal infections associated with the genus *Arcobacter* 12
4. Isolation and detection of *Arcobacter* 17
    4.1. Conventional bacteriological culture 17
    4.2. Molecular-based detection and identification methods 20
    4.3. Genetic typing and diversity 29
5. Pathogenesis and virulence factors of *Arcobacter* species 33
    5.1. Adhesion and invasion to host cells 34
    5.2. Production of toxins by *Arcobacter* 41
    5.3. Host immune response 42
    5.4. Plasmids 44
    5.5. *In vivo* infection model systems 45
6. Antimicrobial resistance 47
7. *Arcobacter* control strategies 54
8. References 58

Chapter 2

Aims of the thesis 83

Chapter 3

Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse 87
Supplementar material from Chapter 3 97
    1. Characterization of ciprofloxacin-resistant isolates 97
    2. Occurrence of nine virulence-associated genes in Arcobacter butzleri isolated from poultry and slaughterhouse environment 99
    3. Analysis of a Plasmid from Arcobacter butzleri 101
    4. References 103

Chapter 4

Resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus*: Activity and effect on cellular functions 107

Chapter 5

Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients 117

Chapter 6

Comparative pathogenic and genotypic properties of human and non-human *Arcobacter butzleri* strains 127

Chapter 7

Concluding remarks and future perspectives 157

List of Figures

Figure 1. Historical achievements in the *Arcobacter* microbiology (1977–2014).....4

Figure 2. Scanning electron micrographs showing the adhesion (black arrows) and invasion (white arrows) of *Arcobacter* strains to Caco-2 cells. Bar 1 μm (Levican *et al*. 2013b).....35

Figure 3. Schematic representation of virulence mechanisms of *Arcobacter* in intestinal epithelial cells (collected from information acquired on different cell lines) (Collado and Figueras 2011).....40

List of Tables

Table 1. Recognized *Arcobacter* species.....5

Table 2. Differential phenotypic characteristics between the 18 *Arcobacter* species.....7

Table 3. Case-reports of human intestinal and extra-intestinal infections associated with *Arcobacter* spp. (adapted from (Figueras *et al.* 2014)).....14

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application...23

Table 5. Pathogenicity of *Arcobacter* species on different cell lines (*in vitro* assays) (Adapted from (Collado and Figueras 2011) and updated).....36

Table 6. Antimicrobial resistance of *Arcobacter* isolates from different sources.....50

List of Abbreviations and Acronyms

AFLP	Amplified fragment length polymorphism
AMP	Ampicillin
AZT	Azithromycin
BHI	Brain heart infusion
cadF	*Campylobacter* adhesion to fibronectin
CAT	Cefoperazone, amphotericin B and teicoplanin supplement
CET	Cephalothin
cfu	Colony forming units
CHL	Choramphenicol
ciaB	*Campylobacter* invasion antigen
CIP	Ciprofloxacin
CLI	Clindamycin
CLSI	Clinical Laboratory Standards Institute
CMJ	Chicken meat juice medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMJH	Ellinghausen McCullough Johnson Harris
ERIC-PCR	Enterobacterial repetitive intergenic consensus-PCR
ERY	Erythromycin
FISH	Fluorescent *in situ* hybridization
flaA	Flagellin subunit A
FRET-PCR	Fluorescence resonance energy transfer real-time PCR
GEN	Gentamicin
glyA	Serine hydroxymethyltransferase
gyrA	Gyrase A
hecA	Haemagglutinin gene A
hecB	Haemolysin activation gene
HIV	Human immunodeficiency virus
hsp60	Heat shock protein 60
IL-8	Interleukin 8
irgA	Iron-regulated gene A
KAN	Kanamycin
LAMP	Loop-mediated isothermal amplification assay
M.I.C.E.	M.I.C. Evaluator
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MET	Methicillin
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
mviN	Mouse virulence
NAL	Nalidixic acid
NARMS	U.S. National Resistance Monitoring System
ND	Not determined

NR	Not referred
PCR	Polymerase chain reaction
PCR-DGGE	PCR-denaturing gradient gel electrophoresis
PCR-ELISA	PCR-Enzyme-linked immunosorbent assay
PFGE	Pulsed-field gel electrophoresis
pldA	Phospholipase A
QRDR	Quinolone resistance determining region
RAPD-PCR	Randomly amplified polymorphic DNA-PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SMZ/TMP	Sulfamethoxazole-trimethoprim
ST	Sequence type
STR	Streptomycin
TET	Tetracycline
tlyA	Haemolysin A
TSP	Trisodium phosphate
U.S.A.	United States of America
UHT	Ultrahigh-temperature
Upp	Uracil phosphoribosyltransferase
VBNC	Viable but nonculturable

Chapter 1

Introduction

Introduction

1. Genus *Arcobacter*

Arcobacter genus belongs to the family *Campylobacteraceae*, which comprises two other genera *Campylobacter* and *Sulfurospirillum*. *Arcobacter* species have been isolated from several environments and hosts, with this genus becoming a large and diverse group of bacteria currently comprising 18 recognized species (Table 1). Since 2011, a high number of species have been described, representing half of the *Arcobacter* species proposed although the genus has been recognized for more than 20 years.

The first known isolation of a microorganism that was later to be recognized as *Arcobacter* species was done in 1977 (Figure 1), by Ellis who described a *Spirillum*-like organisms from bovine and pig foetuses (Ellis *et al*. 1977, 1978). Initially bacteria belonging to *Arcobacter* genus were included in *Campylobacter* genus, being known as aerotolerant campylobacteria that presented the ability to grow at lower temperatures.

In 1983, it was described a nitrogen-fixing *Campylobacter* strain isolated from the roots of *Spartina alterniflora* Loisel and root-associated sediments from salt marsh, denominated as *Campylobacter nitrofigilis* (Mcclung *et al*. 1983). Neill *et al*. (1985) described a group of aerotolerant strains isolated mainly from animal abortions and proposed a new species of *Campylobacter* named *Campylobacter cryaerophila*. Three years later, *C. cryaerophila* is for the first time reported in human samples (Tee *et al*. 1988), followed by *Kiehlbauch et al*. (1991a) work, which proposed the name *Campylobacter butzleri* to a group of strains mainly isolated from human stool, but also from animals. Nonetheless, *Arcobacter* genus was proposed by Vandamme *et al*. in 1991 (Vandamme *et al*. 1991), including at that time two species: *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus*, that were the first ones to be described as belonging to *Campylobacter* genus.

In 1992, the genus *Arcobacter* was augmented to four species to include *Arcobacter butzleri*, and a new species, *Arcobacter skirrowii,* isolated predominantly from preputial fluids of bulls or from bovine, porcine, and ovine aborted foetuses and diarrheic faeces (Vandamme *et al*. 1992a). Since then, a number of novel species have been described, mainly from environments and animals. Wirsen *et al*. (2002) characterized a coastal marine sulphide-oxidizing autotrophic bacterium, for which phylogenetic analysis placed it in the genus *Arcobacter*; the authors proposed the provisional name of "*Candidatus* Arcobacter sulfidicus", however this species was never formally recognized.

Two new species were recognized in 2005, *Arcobacter cibarius* isolated from broiler carcasses (Houf *et al*. 2005) and *Arcobacter halophilus* recovered from a hypersaline lagoon (Donachie *et al*. 2005), with the last one introducing a new characteristic to *Arcobacter* genus since it is an obligate halophile species. A further twelve new species were recognized, *Arcobacter*

mytili isolated from mussels and brackish water (Collado *et al*. 2009a), *Arcobacter thereius* from the kidneys and liver of pigs abortions and cloacal content of ducks (Houf *et al*. 2009), *Arcobacter marinus* from seawater (Kim *et al*. 2009), *Arcobacter trophiarum* from faecal samples of fattening pigs (De Smet *et al*. 2011a), *Arcobacter defluvii* from sewage (Collado *et al*. 2011), *Arcobacter molluscorum* (Figueras *et al*. 2011a), *Arcobacter ellisii* (Figueras *et al*. 2011b), *Arcobacter bivalviorum* (Levican *et al*. 2012) and *Arcobacter cloacae* (Levican *et al*. 2013a) from mussels, *Arcobacter venerupis* from clams (Levican *et al*. 2012), *Arcobacter suis* from pork meat (Levican *et al*. 2013a) and *Arcobacter anaerophilus* from estuarine sediment (Sasi Jyothsna *et al*. 2013). With the introduction of *Arcobacter anaerophilus*, Sasi Jyothsna *et al*. (2013) suggested an emend in the *Arcobacter* genus description (Figure 1), introducing modifications that allow the inclusion of new species, such as some species that are obligate anaerobes and lack motility, or present different phenotypic features (Table 2).

The analysis of sequences deposited in public databases may lead to the inference of existence of potentially new species of *Arcobacter*, even though some of the sequences being from uncultured clones (Miller *et al*. 2007). This together with the description of several new *Arcobacter* species from extremely different habitats or hosts may indicate that new species will be proposed and recognized in the future (Collado and Figueras 2011).

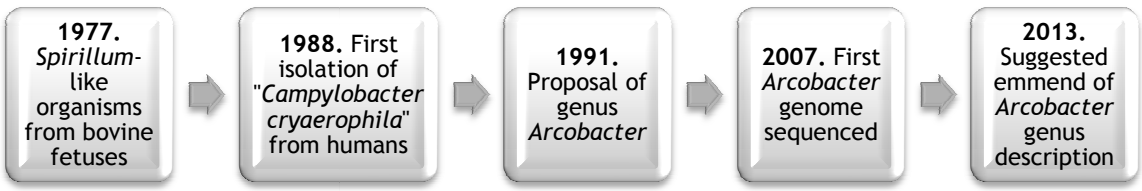

Figure 1. Historical achievements in the *Arcobacter* microbiology (1977–2014).

Table 1. Recognized *Arcobacter* species.

Specie	Source	Country	Year of report	Reference
Arcobacter nitrofigilis[a]	Roots of *Spartina alterniflora* Loisel and in root-associated sediments from salt marsh	Sapelo Island, Georgia	1983	(Mcclung *et al*. 1983)
Arcobacter cryaerophilus[a,c]	Bovine abortions	Several continents	1985	(Neill *et al*. 1985)
Arcobacter butzleri[b]	Humans and animals with diarrhoeal disease	Several continents	1991	(Kiehlbauch *et al*. 1991a)
Arcobacter skirrowii	Preputial fluids of bulls Bovine, porcine, and ovine isolates obtained from aborted fetuses and diarrhoeic faeces.	Several continents	1992	(Vandamme *et al*. 1992a)
Arcobacter cibarius	Broiler carcasses	Belgium	2005	(Houf *et al*. 2005)
Arcobacter halophilus	Hypersaline lagoon	Hawaii	2005	(Donachie *et al*. 2005)
Arcobacter mytili	Mussels (*Mytilus* sp) Brackish water	Spain	2009	(Collado *et al*. 2009a)
Arcobacter thereius	Kidney and liver of Danish pigs abortions Cloacal content of ducks	Denmark	2009	(Houf *et al*. 2009)
Arcobacter marinus	Seawater with seaweeds and starfish	Korea	2010	(Kim *et al*. 2009)
Arcobacter trophiarum	Faecal samples taken rectally from fattening pigs	Belgium	2011	(De Smet *et al*. 2011a)
Arcobacter defluvii	Sewage samples	Spain	2011	(Collado *et al*. 2011)
Arcobacter molluscorum	Mussels (Mytilus sp) and oysters (2009)	Spain	2011	(Figueras *et al*. 2011a)
Arcobacter ellisii	Mussels (Mytilus sp)	Spain	2011	(Figueras *et al*. 2011b)
Arcobacter bivalviorum	Mussels (Mytilus sp)(collected in 2007)	Spain	2012	(Levican *et al*. 2012)
Arcobacter venerupis	Clam (*Venerupis pullastra)*(collected in 2009)	Spain	2012	(Levican *et al*. 2012)
Arcobacter cloacae	Mussels (*Mytilus* sp) and Sewage from the Waste Water Treatment Plant	Spain	2012	(Levican *et al*. 2013a)
Arcobacter suis	Pork meat (collected in 2008)	Spain	2012	(Levican *et al*. 2013a)
Arcobacter anaerophilus	Estuarine sediment	India	2013	(Sasi Jyothsna *et al*. 2013)

[a] First identified as belonging to the genus *Campylobacter* and included in *Arcobacter* genus proposed by Vandamme *et al*. in 1991 (Vandamme *et al*. 1991)

[b] First identified as belonging to the genus *Campylobacter* and included in *Arcobacter* genus by Vandamme *et al*. in 1992 (Vandamme *et al*. 1992a).

[c] An emended description of *C. cryaerophila* was introduced by Kiehlbauch *et al*. in 1991 (Kiehlbauch *et al*. 1991a).

Members of genus *Arcobacter* are small curved Gram negative rods, including S-shaped or helical cells (0.2-0.9 μm wide; 0.5 to 3 μm long). All species present motility by means of a single polar flagellum, and are able to growth under microaerobic conditions, with the exception of the recently described *A. anaerophilus* which has no flagella and is an obligate anaerobe (Vandamme *et al*. 1991, 1992a; 2005; Sasi Jyothsna *et al*. 2013). *A. molluscorum*, *A. ellisii*, *A. suis* and *A. anaerophilus* present some cells with filamentous form up to 7 μm long (Figueras *et al*. 2011a, 2011b; Levican *et al*. 2013a; Sasi Jyothsna *et al*. 2013). All species are oxidase positive, but catalase is absent in some species.

Arcobacter species were generally described as possessing differentiated abilities from *Campylobacter*, namely the ability to grow in aerobic conditions and at temperatures between 15 and 30 ºC (Vandamme *et al*. 1992a); however the increased number of new species may change this principle.

To date, only the genomes of six strains belonging to *Arcobacter* genus were fully sequenced, including three *A. butzleri* strains: (1) human clinical isolate RM4018 (Miller *et al*. 2007), (2) ED-1 isolated from a microbial fuel cell (Toh *et al*. 2011) and (3) 7h1h strain originated from a clinically healthy dairy cow (Merga *et al*. 2013a); as well as, (4) one of *A. nitrofigilis* type strain CI^T isolated from roots of *Spartina alterniflora* Loisel (Pati *et al*. 2010), (5) one *Arcobacter* sp. strain L from a microbial fuel cell, whose species was not identified (Toh *et al*. 2011) and (6) *A. cibarius* isolated from broiler carcasses (LMG21996^T strain) (Adam *et al*. 2014).

Table 2. Differential phenotypic characteristics between the 18 *Arcobacter* species.

Characteristics	*A. nitrofigilis*	*A. cryaerophilus*	*A. butzleri*	*A. skirrowii*	*A. cibarius*	*A. halophilus*	*A. mytili*	*A. thereius*	*A. marinus*	*A. trophiarum*	*A. defluvii*	*A. molluscorum*	*A. ellisii*	*A. bivalviorum*	*A. venerupis*	*A. cloacae*	*A. suis*	*A. anaerophilus*[1]
Motility	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	-
Growth in/on																		
Air at 37 ºC	V(-)	V(+)	+	+	-	+	+	-	+	-	+	+	+	+	-	+	-	-
Microaerobic conditions at 37 ºC	-	V(+)	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	-
MacConkey Agar	-	V(-)	+	-	V	-[a]	+	V(+)	-	V(+)	+	+	V(+)	-	+	+	+	ND
Minimal medium	-	V(-)	+	-	+	-	-	+	-	-	+	-	+	-	+	V	+	ND
NaCl (4 %)	+	-	-	+	-	+	+	-	+	V(-)	-	+	-	+	-	-	-	+
Glycine (1 %)	-	V(-)	V(+)	V(+)	-	+[a,b]	+	+	+	V(-)	-	-	-	-	-	-	-	+
Resistance to cefoperazone (64 mg/L)	-	+	+	+	+	-[a]	-	+	-	+	V(-)	+	-	-	-	-	-	-
Enzyme activities																		
Catalase	+	+	(+)	+	V(+)	-	+	+	-	+	(+)	+	+	+	+	+	+	-
Urease	V(+)	-	-	-	-	-	-	-	-	-	+	-	V(-)	-	+	-	-	-
Nitrate reduction	+	V(-)	+	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	+

Data collected: *A. nitrofigilis* (Mcclung *et al*. 1983; Vandamme *et al*. 1992a; Levican *et al*. 2013a); *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* (Vandamme *et al*. 1992a; Levican *et al*. 2013a); *A. cibarius* (Houf *et al*. 2005); *A. halophilus* (Donachie *et al*. 2005); *A. mytili* (Collado *et al*. 2009a); *A. thereius* (Houf *et al*. 2009); *A. marinus* (Kim *et al*. 2009; Levican *et al*. 2013a; Sasi Jyothsna *et al*. 2013); *A. trophiarum* (De Smet *et al*. 2011a; Figueras *et al*. 2011a); *A. defluvii* (Collado *et al*. 2011; Levican *et al*. 2012); *A. molluscorum* (Figueras *et al*. 2011a; Levican *et al*. 2012); *A. bivalviorum* and *A.venerupis* (Levican *et al*. 2012); *A. cloacae* (Levican *et al*. 2013a) and *A. suis* (Levican *et al*. 2013a); *A. anaerophilus* (Sasi Jyothsna *et al*. 2013).

+, ≥95 % of strains positive; (+), weakly positive; -, ≤10 % of strains positive; V, Variable; V (+), Variable where a majority of strains were positive; V(-), Variable where a majority of strains were negative; ND, Not Determined. All strains were Gram-stain-negative and oxidase positive.[1] Obligate anaerobic growth under 100% Argon, do not grow in blood agar.

[a] Data from reference Figueras *et al*. (2011a), with all tested in medium supplemented with 2 % NaCl. [b]Growth on glycine was previously not observed by Donachie *et al*. (2005).

2. *Arcobacter* species in food and environment

Currently, a total of 18 species are recognized in the genus *Arcobacter*, with *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* being considered the most important ones concerning public health due to their association with foods, water, animal and human diseases. Other *Arcobacter* species have been isolated from diverse animal sources; however their prevalence may be underestimated since detection procedures may not support the isolation or identification of such species. Additional *Arcobacter* species have been isolated mostly from food- and water-related sources, implying its zoonotic potential. *A. cibarius* was isolated from chicken carcasses and pig effluents (Houf *et al*. 2005; Chinivasagam *et al*. 2007) with no report of isolation from humans. *A. thereius* was isolated from ducks and from porcine abortions (Houf *et al*. 2009), as well as from mussels (Levican *et al*. 2014), and was recently reported in human stools (Van den Abeele *et al*. 2013), *A. trophiarum* was reported from faeces of fattening pigs (De Smet *et al*. 2011a) and *A. suis* from pork meat (Levican *et al*. 2013a). Further *Arcobacter* spp. found in environmental samples include *A. nitrofigilis* isolated from the roots of the aquatic plant *Spartina alterniflora* and mussels (Mcclung *et al*. 1983; Collado *et al*. 2009b), *A. halophilus* an obligate halophile from a hypersaline lagoon (Donachie *et al*. 2005), *A. mytili, A. ellisii, A. molluscorum, A. bivalviorum* and *A. venerupis* isolated from shellfish (Collado *et al*. 2009a, 2009b; Figueras *et al*. 2011a, 2011b; Levican *et al*. 2012, 2014), *A. marinus* recovered from marine environments (Kim *et al*. 2009), *A. defluvii* and *A. cloacae* isolated from sewage (Collado *et al*. 2011; Levican *et al*. 2013a) and *A. anaerophilus* from estuarine sediment (Sasi Jyothsna *et al*. 2013). Additionally, operational taxonomic units highly similar to *A. nitrofigilis*, *A. ellisii*, *A. suis* and *A. venerupis* were detected from a spinach-processing plant (Hausdorf *et al*. 2013a).

Concerning *Arcobacter* detection on vegetables, it was found in spinach (Hausdorf *et al*. 2013a, 2013b) and fresh lettuces (Gonzalez and Ferrus 2011). Hausdorf *et al*. (2013a) results indicate an *Arcobacter* transfer to spinach during wash (Hausdorf *et al*. 2013a) with considerable risk of vegetables contamination during the industrial washing process by potential pathogens suspended in the wash water (Hausdorf *et al*. 2011). The fact that most vegetables are consumed raw, together with the reported *Arcobacter* detection in these foods, suggest them as a potential route of transmission of this pathogen. Lee and Choi (2013) evaluated the survival ability of *A. butzleri* when inoculated in apple and pear purees, showing that *A. butzleri* colony forming units (cfu) number significantly decreased during storage at 4 or 20 ºC. The acidic pH, high sugar content, water activity and polyphenols in the purees may contribute to the reduction of *A. butzleri* population (Lee and Choi 2013).

Arcobacter spp. have been frequently identified in healthy cattle and pigs (Öngör *et al*. 2004; Van Driessche *et al*. 2004, 2005; Aydin *et al*. 2007; De Smet *et al*. 2011a; Shah *et al*. 2013), and so they may represent a contamination risk during slaughter (Shah *et al*. 2011a).

Animal origin food products have been suggested as an important source of *Arcobacter* spp. (Collado and Figueras 2011), with a higher prevalence found in poultry (22-73 %), followed by pork (0-35 %) and beef (0-34 %) meat products, being *A. butzleri* the most frequently detected species (Kabeya *et al*. 2004; Rivas *et al*. 2004; Scullion *et al*. 2006; Lee *et al*. 2010; Nieva-Echevarria *et al*. 2013).

Albeit *Arcobacter* frequent isolation from poultry carcasses and meat, *Arcobacter* is uncommonly detected in live birds, being proposed that birds are not reservoir hosts, suggesting postslaughter carcass contamination (Wesley and Miller 2010). This is supported through the rare recovery of *Arcobacter* from the intestinal contents or faeces of chickens and the distribution of this genus in slaughterhouse environment; however the transmission route was not yet clearly demonstrated (Gude *et al*. 2005; Aydin *et al*. 2007; Van Driessche and Houf 2007a; Ho *et al*. 2008).

The *Arcobacter* ability to survive in food animal sources has been described, with *A. butzleri* exhibiting the ability of survive in raw ground pork in a strain dependent manner, yet without cell number increase (D'Sa and Harrison 2005). *Arcobacter* survival was further underlined by Ho *et al*. (2008) who showed that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* may survive to the scalding process conditions (52 ºC, 3 min) and in this way stressing the possibility of cross-contamination during processing in poultry slaughterhouses. Although *Arcobacter* spp. has been isolated with high prevalence percentile from animal carcasses, it appears that the cooling process could reduce the *Arcobacter* incidence significantly. Despite 37 % *Arcobacter* prevalence on the bovine carcasses postevisceration, it was described that after cooling of the carcasses the incidence was reduced to 7 % (De Smet *et al*. 2010). A similar behaviour was observed with broiler carcasses where in pre-chill carcasses the recovery was of 61.3 % with a reduction to 9.6 % post-chilling (Son *et al*. 2007a).

Some studies have reported high prevalence of *Arcobacter* detection on shellfish (Collado *et al*. 2009b; Nieva-Echevarria *et al*. 2013; Levican *et al*. 2014). Levican *et al*. (2014) reported a high diversity of *Arcobacter* species for shellfish with the isolation of eleven different species with the most prevalent species being *A. butzleri* (60.2 %), followed by *A. molluscorum* (21.2 %), showing an overall prevalence of 29.9 %. *Arcobacter* prevalence of 100 % for clams, 41.1 % for mussels, but with no *Arcobacter* recovered from oysters or frozen shrimp was also reported (Collado *et al*. 2009b) and similar values (100 % for clams, 50 % for mussels) were presented in another study (Nieva-Echevarria *et al*. 2013).

Few surveys have investigated *Arcobacter* spp. in raw milk, but the ones that did have reported different prevalence rates: 46 % in Northern Ireland (Scullion *et al*. 2006), 3.2 % in Brazil (Pianta *et al*. 2007), 5.8 % in Malaysia (Shah *et al*. 2012a), 15 % in Finland (Revez *et al*. 2013) and 42.8 % in Spain (Nieva-Echevarria *et al*. 2013), being *A. butzleri* the most prevalent species. *Arcobacter* survival in milk was also evaluated, showing that *A. butzleri* and *A. cryaerophilus* were capable of survival in ultrahigh-temperature (UHT), pasteurized and raw cow's milk stored at 4 or 10 ºC, or even grow in case of *A. butzleri* on UHT and pasteurized

milk at 20 ºC. However, raw milk stored at 20 ºC lead to a decrease in *A. butzleri* and *A. cryaerophilus* count, associated with the pH decrease due to multiplication of competitive microflora. Despite being unlikely that *Arcobacter* spp. survive milk pasteurization or the UHT process, insufficient post-processing hygienic management of milk and cross-contamination are possible (Giacometti *et al.* 2014). The presence of *Arcobacter* spp. in milk has been reported and the evidence of their survival and/or growth ability in milk could have significant public health implications, especially for raw milk consumption (Giacometti *et al.* 2014).

Furthermore, *Arcobacter* spp. has been detected in dairy plants, such as in artisanal and industrial dairy plants in Italy (Giacometti *et al.* 2013; Serraino *et al.* 2013a; Serraino and Giacometti 2014), being detected in surfaces in contact and not in contact with products, and in raw milk used in cheese production (Giacometti *et al.* 2013; Serraino and Giacometti 2014). *A. butzleri* showed to be able to survive production and storage of artisan water buffalo mozzarella cheese, however with a bacterial count of *A. butzleri* decrease during the production process. Moreover, *A. butzleri* was recovered from 12 of 162 cheese samples collected during storage, by enrichment but not by direct plating (Serraino *et al.* 2013b).

Beyond the association of *Arcobacter* with raw food products, its presence was also detected in ready-to-eat food, with a study regarding enteric pathogen in meals served at Bangkok restaurants recommend for tourists presenting a higher prevalence of *Arcobacter* than *Salmonella* or *Campylobacter*. The authors calculated the traveler's risk of exposure to *Arcobacter* as 13 % per meal eaten, rising to 75 % when 10 meals are consumed (Teague *et al.* 2010).

Arcobacter species were found in water samples or on patients in three outbreak reports (Rice *et al.* 1999; Fong *et al.* 2007; Kopilovic *et al.* 2008). The first outbreak occurred in a Girl Scout camp in Idaho, where a breakdown in the automated chlorination system occurred at the same time as the outbreak. The well water presented faecal contamination, with *A. butzleri* being isolated from the ground water samples (Rice *et al.* 1999). In 2004, a groundwater-associated outbreak was reported in Ohio, where a massive groundwater contamination occurred, probably with contaminants from wastewater treatment facilities and septic tanks. *Arcobacter* was isolated from several ground water samples (Fong *et al.* 2007). *A. cryaerophilus* was found in human stool during an outbreak of acute gastroenteritis in Slovenia, that was associated with the local water distribution system, however *Arcobacter* was not confirmed as the aetiological agent (Kopilovic *et al.* 2008). While no specific conclusions were drawn from these outbreaks, *Arcobacter* was either isolated or detected from water or stool from patients where disease was linked to water.

A. butzleri, *A. cryaerophilus* and *A. skirrowii* were detected in different types of environmental water samples such as seawater (Fera *et al.* 2004; Collado *et al.* 2008), estuarine waters (Fera *et al.* 2010a), wastewater (Diergaardt *et al.* 2004; Gonzalez *et al.* 2007a; 2010), surface, river, canal, lakes and spring water (Musmanno *et al.* 1997; Diergaardt

et al. 2004; Morita *et al*. 2004; Collado *et al*. 2008, 2010; Ertas *et al*. 2010; Lee *et al*. 2012), being *A. butzleri* and *A. cryaerophilus* the most commonly isolated species.

Arcobacter spp. frequency isolation from water samples has been directly associated with levels of faecal contamination (Collado *et al*. 2008, 2010; Lee *et al*. 2012) with a greater number of different genotypes occurring in samples more affected by urban sewage presenting higher levels of faecal indicators (Collado *et al*. 2010). *Arcobacter* spp. were detected at different collection points of two sewage treatment plants, including in the tertiary reclaimed water (Rodriguez-Manzano *et al*. 2012), suggesting that contaminated water could be discharged into the effluent, contaminating the water.

Collado *et al*. (2010) reported a frequent *Arcobacter* isolation from river water and from water used for the production of drinking water, however no detection was observed in finished chlorinated water (Collado *et al*. 2010), in consonance with the work evaluating *Arcobacter* resistance to chlorination (Rice *et al*. 1999; Moreno *et al*. 2004). Survival of *A. butzleri* in well water at 5 °C for 16 days was reported, demonstrating that *A. butzleri* can remain viable for a prolonged time in well water (Rice *et al*. 1999), as well as in non-chlorinated drinking water were cells remain viable for more than 35 days with culturable cells being recovered during 16 days (Moreno *et al*. 2004). However *A. butzleri* is sensitive to chlorine inactivation, with membrane integrity and intact nucleic acids remaining after loss of culturability (Rice *et al*. 1999; Moreno *et al*. 2004). Continuous chlorination is necessary to establish a barrier to the spread of infectious agents through a contaminated water source (Rice *et al*. 1999; Moreno *et al*. 2004). However, some studies reported the presence of this microorganism in drinking water (Jacob *et al*. 1993,1998; Ertas *et al*. 2010; Shah *et al*. 2013), treated water (Shah *et al*. 2012b) or water from food plants, such as carrot wash water (Hausdorf *et al*. 2011) or washing water of a spinach-processing plant (Hausdorf *et al*. 2013a).

A. butzleri has also shown to be able to survive in seawater through the induction of viable but nonculturable (VBNC) state, so despite the loss of culturability, cells remain intact with undamaged membranes for almost 270 days, being possible to recover cells to a culturable state, with VBNC induction appearing to be related with starvation more than with temperature (Fera *et al*. 2008). VBNC state may allow *A. butzleri* to survive in the absence of nutrients and persist in the environment until it finds suitable conditions (Fera *et al*. 2008). The presence of *Arcobacter* in water and the ability of the bacteria to grow at low temperatures and survive in environments scarce of nutrients further support its potential as a waterborne pathogen.

Arcobacter spp. was also detected in other environmental samples such as microbial communities in oil field water (Kumaraswamy *et al*. 2011), oil sands formation waters (Hubert *et al*. 2012), activated sludge (Snaidr *et al*. 1997), petroleum-contaminated groundwater (Watanabe *et al*. 2000) or plankton samples (Fera *et al*. 2004).

The improved ability of *A. butzleri* to survive in so many different environments, when compared with other related organisms, such as *Campylobacter*, could be explained by the

high number of respiratory enzymes, chemotaxis proteins and two component systems, together with a number of genes described providing ability to grow under a vast range of atmospheric conditions and lower temperatures, such as more oxygen-stable enzymes, or the presence of novel cold shock and stress proteins (Miller *et al*. 2007).

Also, Merga *et al*. (2013b) observed phenotypic and genotypic evidences suggesting differences in gene carriage amongst *A. butzleri* isolates from different sources that may be related with possible niche adaptation, namely on survival and sensing systems. However the authors stressed the need of more detailed studies with a larger number of isolates from multiple sources in order to help to clarify any relationships (Merga *et al*. 2013b).

The genome sequence and analysis of *A. butzleri* RM4018 strain shows that this bacterium is most likely an environmental microorganism that may be the source of disease by water-mediated food contamination or ingestion of *A. butzleri*-contaminated water (Miller *et al*. 2007).

3. Human and animal infections associated with the genus *Arcobacter*

Due to the expansion of the number of species proposed for inclusion in *Arcobacter* genus in recent years, the data regarding the species distribution in either in humans or animals is still scarce for some species.

Arcobacter spp. was shown to be present in healthy cattle (Van Driessche *et al*. 2003, 2005; Öngör *et al*. 2004; Aydin *et al*. 2007; Shah *et al*. 2013), pigs (Van Driessche *et al*. 2004; De Smet *et al*. 2011b), goats (De Smet *et al*. 2011c; Shah *et al*. 2013), sheep and horses (Van Driessche *et al*. 2003; De Smet *et al*. 2011c).

Arcobacter spp. has been associated with animal diseases or conditions such as abortion or diarrhoea (Vandamme *et al*. 1992a; de Oliveira *et al*. 1997; Higgins *et al*. 1999; On *et al*. 2002; Bath *et al*. 2013). The association of aerotolerant *Campylobacter*-like organisms with bovine and porcine abortions was first described in the late 1970s (Ellis *et al*. 1977;1978). Strains of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from aborted foetuses and placentas of bovine, porcine and ovine sources (Vandamme *et al*. 1992a). A recent report suggests that *A. skirrowii* was the possible cause of abortion in sheep, as it was recovered from both placenta and foetuses (Bath *et al*. 2013). Recover of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* from animal preputial fluid suggests venereal transmission of these bacteria as well (Ho *et al*. 2006a). Since *Arcobacter* spp. were detected also in healthy live newborn piglets (Ho *et al*. 2006b), On *et al*. (2002) suggested that these bacteria may be opportunistic pathogens playing a role in the disease process as opposed to being the primary cause. Moreover, they also suggested that considering the diversity found, certain strains may

act as primary abortifacient pathogens (On *et al*. 2002). *A. butzleri, A. cryaerophilus*, *A. skirrowii* and *A. thereius* have been associated with reproductive disorders, enteritis or mastitis in domestic livestock (De Smet *et al*. 2011c), but have also been recovered from faeces of healthy animals, as well as *A. trophiarum* (Van Driessche *et al*. 2004, 2005; De Smet *et al*. 2011a).

Apart from reproductive disorders, *A. butzleri* has been also associated with enteritis and diarrhoea in pigs, cattle, and horses, whereas *A. skirrowii* was associated with diarrhoea in sheep and cattle (Ho *et al*. 2006a). *A. butzleri* association with diarrhoea in non-human primates was also reported (Anderson *et al*. 1993; Higgins *et al*. 1999), with the histological evaluation of colonic specimens showing mild to moderately severe chronic, active colitis (Anderson *et al*. 1993). *Arcobacter* species were also recovered from pet cats and dogs with oral disease (Petersen *et al*. 2007) and other health and unhealthy exotic animals, such as alpaca, rhea, rhinoceros, gorillas or raccoons (Wesley and Miller 2010). The observation of *Arcobacter* in both healthy and diseased animals has also been reported in other studies, showing *Arcobacter* presence in swine stomach with or without gastric ulcers, and therefore not allowing to establish a role of *Arcobacter* spp. in swine gastric ulceration (Suarez *et al*. 1997; de Oliveira *et al*. 2010).

Transmission routes were not clearly associated with human disease; however consumption of *Arcobacter*-contaminated food or water has been considered a possible mode of transmission. In addition to the water related outbreaks referred in the previous section, an outbreak associated to broasted chicken consume was reported, suggesting that *A. butzleri* should be considered an agent that can cause outbreaks of foodborne illness (Lappi *et al*. 2013). These outbreak reports strengthen the association of food and water as a possible *Arcobacter* route of transmission. The presence of *A. butzleri* and *A. cryaerophilus* in pet cats and dogs (Petersen *et al*. 2007; Houf *et al*. 2008; Fera *et al*. 2009), suggests that the contact with pets can be one potential route of human infection. Possible person-to-person transmission was also suggested due to the successive timing of cases of abdominal cramps in the *Arcobacter*-associated outbreak in an Italian School (Vandamme *et al*. 1992b), or to the possible contraction of infection *in utero* by a neonate (On *et al*. 1995).

In humans, *A. butzleri* and *A. cryaerophilus* infection has been associated with enteritis and occasionally with bacteraemia, with *A. skirrowii* being isolated from diarrhoeic faeces (Table 3); however, *Arcobacter* species have also been found in healthy individuals (Houf and Stephan 2007). Several case-studies of gastrointestinal, along with extra-gastrointestinal disease, have been reported being summarised in Table 3. In some of the cases, *Arcobacter*-associated gastrointestinal illness is self-limiting, not requiring an antibiotic treatment.

Table 3. Case-reports of human intestinal and extra-intestinal infections associated with *Arcobacter* spp. (adapted from (Figueras *et al*. 2014)).

Patients' sex/age (years)	Country	Presentation	Species	Outcome	Reference
Intestinal infections					
M/ 35	Australia	Chronic diarrhoea (6 months)	*A. cryaerophilus*	Not specified	(Tee *et al*. 1988)
3-7 [a]	Italy	No diarrhoea, abdominal pain, occasional vomiting or fever	*A. butzleri*	Recovered 7–10 days after no specific treatment	(Vandamme *et al*. 1992b)
1. M/48	Germany	1. Acute watery diarrhoea (15 days) and abdominal cramps	*A. butzleri*	1. Recovered 3 days after treatment with ofloxacin	(Lerner *et al*. 1994)
2. F/52		2. Chronic diarrhoea (3 weeks) and abdominal cramps		2. Recovered 2 days after treatment with doxycycline	
1. M/2	Chile	1. Acute mucous diarrhoea and vomiting	*A. butzleri*	1. Recovered in 2 days with parenteral fluid therapy, restricted diet but without antimicrobial treatment	(Fernández *et al*. 2004)
2. F/1		2. Chronic diarrhoea (4 months) with abdominal cramps and pain		2. Recovered 10 days after treatment with erythromycin	
M/ 73	Belgium	Chronic diarrhoea (2 months)	*A. skirrowii*	Recovered 10 days after no specific treatment	(Wybo *et al*. 2004)
M/30	Turkey	Acute watery diarrhoea, abdominal pain, nausea and sweating	*A. butzleri*	Recovered 2 days after treatment with ciprofloxacin	(Kayman *et al*. 2012a)
19-90 [b]	US	Diarrhoea, abdominal cramps, fatigue, nausea, chills, body/muscle aches, and/or headache	*A. butzleri*	Recovered 1-6 days after no specific treatment.	(Lappi *et al*. 2013)
M/26	Spain	Persistent bloody and watery diarrhoea (3 weeks)	*A. cryaerophilus*	Recovered 8 days after treatment with amoxicillin/clavulanic acid	(Figueras *et al*. 2014)

Table 3. Case-reports of human intestinal and extra-intestinal infections associated with *Arcobacter* spp. (adapted from (Figueras *et al*. 2014)) (continuation).

Patients' sex/age (years)	Country	Presentation	Species	Outcome	Reference
Extra-intestinal infections					
Neonate	UK	Bacteraemia with hypotension, hypothermia and hypoglycaemia	*A. butzleri*	Recovered 6 days after penicillin and cefotaxime treatment	(On *et al*. 1995)
M/ 72	Taiwan	Bacteraemia and haematogenous pneumonia	*A. cryaerophilus*	Recovered 2 weeks after ceftizoxime and tobramycin treatment	(Hsueh *et al*. 1997)
M/ 60	Taiwan	Bacteraemia with fever and haematemesis	*A. butzleri*	Recovered 4 days after cefuroxime treatment	(Yan *et al*. 2000)
M/ 7	Hong Kong	Bacteraemia	*A. cryaerophilus*	Died on the following day after suffocation in a mud pool	(Woo *et al*. 2001)
F/ 69	Hong Kong	Bacteraemia with fever and lower quadrant pain	*A. butzleri*	Recovered 3 days after cefuroxime and metronidazole treatment	(Lau *et al*. 2002)
F/ 63	China	Peritonitis after repositioning of catheter with fever and abdominal pain	*Arcobacter* sp.	Recovered 2 weeks after treatment with ticarcillin-clavulanate	(Yap *et al*. 2013)

[a] Ten children (6 females and 4 males).

[b] Fifty one case patients (34 females and 17 males)

The largest *Arcobacter* survey was conducted by Vandenberg *et al*. (2004) that performed culture analysis of 67,599 stool specimens during 8 years. A global prevalence of 0.1 % for *A. butzleri* and *A. cryaerophilus* was observed, establishing a significant association between *A. butzleri* detection and persistent or watery diarrhoea when compared with *C. jejuni* infections (Vandenberg *et al*. 2004). *A. butzleri* has been described as the fourth most prevalent *Campylobacter* related organism species found in human diarrhoeic samples (Vandenberg *et al*. 2004; Prouzet-Mauleon *et al*. 2006; Samie *et al*. 2007; Collado *et al*. 2013). In Venda region in South Africa, 6.2 % of the 322 stool samples (diarrhoeic and non-diarrhoeic) analysed, by molecular methods, were positive for *A. butzleri*, 2.8 % for *A. cryaerophilus*, and 1.9 % for *A. skirrowii;* however, the authors were unable to establish a correlation between diarrhoea and any of those species (Samie *et al*. 2007). Collado *et al*. (2013) observed a 1.4 % prevalence of *A. butzleri* positive diarrhoeic samples, with no detection in healthy samples, through the use of a combination of traditional and molecular methods (Collado *et al*. 2013).

When considering *Arcobacter* recovery in human stool samples by cultural methods, reports' values ranged from 0.1 to 2.4 %, for *A. butzleri* and *A. cryaerophilus* (Collado and Figueras 2011). Recent surveys have reported prevalence rates for *Arcobacter* spp. in stool samples within the same range, for example, in Turkey *A. butzleri* was isolated from 1.25 % of the

samples (Kayman *et al*. 2012b) and in New Zealand *A. butzleri* and *A. cryaerophilus* were isolated from 0.9 % of the samples (Mandisodza *et al*. 2012).

Regarding molecular detection methods higher prevalence values were obtained than with cultural methods, with 1.2 % of stool samples being *A. butzleri* positive by fluorescence resonance energy transfer real-time PCR (FRET-PCR) but none of the stool samples was positive for *Arcobacter* species by culture (Abdelbaqi *et al*. 2007a). Another study reported detection rates of 0.7 and 1.4 % for culture and molecular methods, respectively (Collado *et al*. 2013). Despite the higher prevalence obtained by molecular detection methods, in a study from The Netherlands, *A. butzleri* was detected in only 0.4 % of the samples, using a Real-Time multiplex PCR designed to detect *A. butzleri* and *Campylobacter* in stool samples (De Boer *et al*. 2013). *Arcobacter* was also found during a survey in U.S. and European travellers with acute diarrhoea acquired in Mexico, Guatemala and India, being *A. butzleri* detected in 8 % of the samples by molecular methods. However, its co-detection with enterotoxigenic *Bacteroides fragilis* for 81 % of the positive samples hampered to establish *A. butzleri* as the aetiological agent of diarrhoea (Jiang *et al*. 2010).

Some studies have focused on *Arcobacter* prevalence and underlying disease, with no significant difference being observed for patients HIV-positive or negative (Kownhar *et al*. 2007; Samie *et al*. 2007), but correlating infection with type 2 diabetes, indicating that older subjects and those with type 2 diabetes might be at higher risk for *Arcobacter* carriage (Fera *et al*. 2010b).

Regarding *Arcobacter* species detection, both *A. butzleri* and *A. cryaerophilus* have been the most commonly detected ones, with *A. skirrowii* being first isolated from an elderly patient with chronic diarrhoea in 2004 (Wybo *et al*. 2004), and then detected only twice in human stool samples, but in co-detection with other *Arcobacter* or *Campylobacter* species (Samie *et al*. 2007; Patyal *et al*. 2011). Recently, also *A. thereius* was isolated from the stools samples of two patients with clinically confirmed enterocolitis. The first patient was a 3 year old child with recurrent diarrhoea and failure to thrive, and the second was a 29 years old woman with a flare up of Crohn's disease; none of the patients presented other bacterial pathogens in stool samples (Van den Abeele *et al*. 2013).

The more psychrophilic nature of *A. butzleri* (i.e. its ability to survive and proliferate at lower temperatures) when compared with *Campylobacter* spp. have been used as an argument to justify the lower incidence of *A. butzleri*-related food-borne illnesses, due to the possible difficulty on proliferation in some hosts (Miller *et al*. 2007).

4. Isolation and detection of *Arcobacter*

4.1. Conventional bacteriological culture

A diversity of methods have been exploited for the isolation of *Arcobacter* spp., ranging from modified *Campylobacter* and *Leptospira* techniques to those involving *Arcobacter*-specific media (Merga *et al*. 2011), with several enrichment and plating media being recommended for *Arcobacter* isolation. Cultural detection of *Arcobacter* is usually achieved by an enrichment step followed by the use of an isolation medium. The isolation step can be performed by the use of a selective medium or include a filtration of the sample homogenate onto non-selective blood agar plates.

Several media and procedures for *Arcobacter*-specific isolation have been developed over the years, with diverse selective antibiotic being used in both enrichment and isolation steps. De Boer *et al*. (1996) developed an enrichment and plating media using cefoperazone (32 mg/L), piperacillin (75 mg/L), trimethoprim (20 mg/L) and cycloheximide (100 mg/L) as selective agents for *Arcobacter* isolation from poultry, pork and beef meats (De Boer *et al*. 1996).

Johnson and Murano (1999a) developed a new media formulation supplemented with bile salts, cefoperazone (32 mg/L) and 5-fluorouracil (200 mg/L) as selective agents (Johnson and Murano 1999a). These media were compared with several broths and solid media for *Arcobacter* isolation from poultry showed to be most effective in isolating *Arcobacter* with limited contamination (Johnson and Murano 1999b). Other methods using passive filtration were described for *Arcobacter*-specific isolation, for biopsy samples taken from aborted porcine foetuses (On *et al*. 2002), chickens samples (Atabay and Corry 1997; Atabay *et al*. 1998, 2003; Villalobos *et al*. 2013), domestic geese (Atabay *et al*. 2008a) or others (Aydin *et al*. 2007; Ertas *et al*. 2010) allowing the isolation of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. Moreover, the work that showed the highest diversity of *Arcobacter* species isolation (10 recognized species, from 204 shellfish samples) used passive filtration as a subsequent step to enrichment with cefoperazone (8 mg/L), amphotericin B (10 mg/L), and teicoplanin (4 mg/L) (CAT) supplement (Levican *et al*. 2014).

Shah *et al*. (2011b) compared the methods used by De Boer *et al*. (1996) and Atabay *et al*. (1998), where the latter used an enrichment broth using CAT supplement followed by a passive filtration step. It was found that the method of Atabay showed improved results for beef, fresh milk and rectal swabs of cattle concerning *A. butzleri*-, *A. cryaerophilus*- and *A. skirrowii*-isolation.

Through the evaluation of the susceptibility of *A. butzleri, A. cryaerophilus*, and *A. skirrowii* to antibiotics used in selective media, Houf *et al*. (2001a) showed that many of the *A. skirrowii* and *A. cryaerophilus* strains were susceptible to piperacillin at the levels used in some selective media, as well as the inclusion of cefoperazone at 32 mg/L in media could be

detrimental to stressed or injured cells of *A. cryaerophilus* and *A. skirrowi* (Houf *et al.* 2001a). The same group developed a new protocol for isolation of these three *Arcobacter* species through the incorporation of cefoperazone (16 mg/L), amphotericin B (10 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L) and trimethoprim (64 mg/L) to both enrichment and isolation media (Houf *et al.* 2001b). These media was later modified for *Arcobacter* isolation from livestock faeces, by the increase of novobiocin concentration (64 mg/L) and introduction of cycloheximide (100 mg/L) for specificity improvement and fungal growth delay (Van Driessche *et al.* 2003). The latter medium was then validated for *Arcobacter* isolation from poultry intestinal content as well (Van Driessche and Houf 2007a). Houf *et al.* (2007) validated a method for isolation of *Arcobacter* from human stool samples based on the previous method described by the same group for *Arcobacter* isolation from poultry products (Houf *et al.* 2001b). The selective isolation method ensured the growth of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* and suppressed the accompanying flora without hinder *Arcobacter* isolation (Houf and Stephan 2007).

Enrichment has been reported to be necessary in order to isolate *Arcobacter* spp. from poultry carcasses or pig abortions (Atabay and Corry 1997; On *et al.* 2002), with other works also reporting higher recovery rates when using an enrichment step (Van Driessche *et al.* 2003; Gude *et al.* 2005; Houf and Stephan 2007; Van Driessche and Houf 2007b). Nonetheless, this enrichment may reduce the isolation of *A. cryaerophilus* and *A. skirrowii*. Houf *et al.* (2002a) reported the isolation of both *A. butzleri* and *A. cryaerophilus* species by direct plating, but when using the enrichment step only *A. butzleri* was isolated, however with a higher recovery rate than that obtained by direct plating (Houf *et al.* 2002a). In a similar way, without the enrichment step, through direct isolation, De Smet *et al.* (2011b) was able to describe a larger diversity of *Arcobacter* strains, with the isolation of *A. thereius* and *A. trophiarum* through the use of selective media described by Van Driessche *et al.* (2003). The use of direct plating excludes the potential selection occurring during the enrichment phase, which can influence epidemiological results (Houf *et al.* 2001b), however diminishing the recovery of *A. butzleri*, probably due to low contamination charge (Van Driessche and Houf 2007b). This led to the proposal of inclusion of both direct plating and enrichment in the methodology for *Arcobacter* isolation (Atabay and Corry 1997).

Several studies evaluated and compared the differences between culture methods (Atabay and Corry 1998; Golla *et al.* 2002; Scullion *et al.* 2004; Hamill *et al.* 2008; Merga *et al.* 2011; Shah *et al.* 2011b). Two studies have compared the methods described by Houf *et al.* (2001b) and Johnson and Murano (1999b) in retail raw poultry and beef samples (Scullion *et al.* 2004; Hamill *et al.* 2008). Both comparison works have shown a similar detection of *Arcobacter* with the two methods, with an improvement in recovery through sampling of the enrichment broth at 24 h as well as at 48 h of incubation (Scullion *et al.* 2004; Hamill *et al.* 2008). Also, performing the incubation in a microaerobic atmosphere was associated with either a better survival and growth of *Arcobacter* spp. or better suppression of the contaminating flora

(Hamill *et al*. 2008). Merga *et al*. (2011) performed a comparative study concerning *Arcobacter* isolation, from cattle, sheep, and badgers faecal samples, by using five different methods, which corresponded to the combination of three enrichment and two isolation media. The authors tested the enrichment media reported by Houf *et al*. (2001b); Atabay and Corry (1998) and the *Campylobacter* specific enrichment broth described by Kemp *et al*. (2005) containing 5 % (V/V) defibrinated horse blood and cefoperazone (20 mg/L), vancomycin (20 mg/L), trimethoprim (20 mg/L), and cycloheximide (50 mg/L) (Kemp *et al*. 2005). Regarding solid media, they tested the solid medium reported by Houf *et al*. (2001b) and a modified charcoal cefoperazone deoxycholate agar (mCCDA) with CAT supplementation (Kemp *et al*. 2005). The most sensitive and specific method used the enrichment broth reported by Houf *et al*. (2001b) combined with mCCDA with antibiotic supplements (Merga *et al*. 2011).

Another relevant culture conditions to be taken into account are the temperature and incubation atmosphere requirements that may help to differentiate between *Campylobacter* and *Arcobacter*. Some of the *Arcobacter* species will grow optimally at 37ºC microaerophilicaly, while 30 ºC has been shown to support aerobic growth of this bacterium (Johnson and Murano 1999a), however lower temperatures have been used with De Boer *et al*. (1996) applying an incubation temperature of 24 ºC for *Arcobacter* isolation, to reduce cold-sensitive microflora and favouring the selection for *Arcobacter*. Both aerobic and microaerobic conditions have been adopted for the isolation of *Arcobacter* from both clinical and environmental samples (Collado and Figueras 2011). González *et al*. (2007a) observed a slight improvement in the *Arcobacter* isolation rate when performing the enrichment step under microaerophilic conditions, without being able to draw a definitive conclusion concerning the atmospheric incubation conditions, a similar result was observed by Hamill *et al*. (2008).

Incubation time showed also to influence *Arcobacter* species isolation, namely *A. cryaerophilus* and *A. skirrowii* required a longer enrichment and plating incubation time (Van Driessche *et al*. 2003; Van Driessche and Houf 2007a), with some studies reporting an increase in recovery when using both 24 and 48 h of enrichment broth incubation (Scullion *et al*. 2004; Hamill *et al*. 2008).

The success of bacterial cultivation is also dependent on the source of the sample, for example, when isolating *Arcobacter* from environmental samples this process is highly dependent both on the distribution and on the growth state of bacteria at the time of sampling (Fera *et al*. 2010a).

The differences in recovery rates of *Arcobacter* spp. from various sources could in addition be attributed to a number of factors, such as geographic and seasonal divergence or other aspects related to the hygiene at collection sites, but also to differences in the sensitivity and specificity of isolation methods used (Patyal *et al*. 2011). The lack of a standardized method

for cultural isolation of *Arcobacter* spp. may lead to an unsuccessful detection of this bacterium.

4.2. Molecular-based detection and identification methods

As seen in the previous section, detection and identification of *Arcobacter* species by conventional microbiological and biochemical methods may be hampered due to problems associated with the lack of standardized culture methods and to the similarity to *Campylobacter* spp., namely metabolic inertness, jeopardizing a correct identification through conventional phenotypic tests. Therefore, molecular approaches are the most used tools for identification of *Arcobacter* species (Gonzalez *et al*. 2012). DNA-based methods have been described as more suitable for the rapid detection, confirmation and identification to species level of *Arcobacter* and can be more efficient and cost-effective than the classic phenotypic identification methods (Gonzalez *et al*. 2007a; Figueras *et al*. 2008; Gonzalez and Ferrus 2011; Patyal *et al*. 2011). However, the growing number of new *Arcobacter* species has increased the doubt regarding their specificity (Douidah *et al*. 2010), with new molecular detection assays being proposed.

Genus and species PCR-based methods have been used for confirmation of presumptive *Arcobacter* colonies or for *Arcobacter* detection after enrichment or culture independent in several different sort of samples. Some of the most widely used PCR-based methods for *Arcobacter* detection include simplex and/or multiplex Polymerase Chain Reaction (PCR), PCR-Restriction fragment length polymorphism (RFLP) and real-time PCR (Table 4).

The detection rates by PCR-based procedures were higher than with isolation methods, probably due to a low number of cells or to the presence of non culturable cells in samples (Fera *et al*. 2004; Gonzalez and Ferrus 2011; Bullman *et al*. 2012; De Boer *et al*. 2013). Even, when introducing an enrichment step, before PCR-based methods, to increase the level of viable cells, while diluting dead cells and inhibitors, the overall time for detection and identification of the bacterium is less when compared with conventional methods (Gonzalez and Ferrus 2011).

Recently, Levican and Figueras (2013) compared the performance of five PCR based methods with different target regions (16S rRNA, 23S rRNA or *gyrA* genes) (Levican and Figueras 2013). Four multiplex PCR methods ((Houf *et al*. 2000; Kabeya *et al*. 2003a; Pentimalli *et al*. 2009; Douidah *et al*. 2010)) and one 16S rRNA-RFLP method (Figueras *et al*. 2008) were evaluated according to its performance and ability to correctly identify the *Arcobacter* species. The authors performed the comparison evaluating the Douidah *et al*. (2010) method in combination with the *A. trophiarum* PCR method described by De Smet *et al*. (2011a). The

authors have found that none of the methods evaluated were fully reliable, presenting misidentifications of both targeted and non-targeted species, thereby emphasizing the methods limitations. These limitations could be in part explained by the use of limited amount of sequences to primers design and by the posterior description of new species after PCR primers design (Levican and Figueras 2013). From the methods described, the multiplex PCR developed by Houf *et al*. (2000) has been the most used one. Even though being considered one of the most unreliable, this method was 100 % reliable for the identification of *A. butzleri*, with no misidentification with other species (Levican and Figueras 2013). The authors suggested that the use of an updated 16S rRNA-RFLP method (Figueras *et al*. 2012) for the evaluation of *Arcobacter* spp. diversity in different environments. Figueras *et al*. (2012) updated the method previously described by the same group (Figueras *et al*. 2008) introducing the use of two supplementary endonucleases (*MnlI* and *BfaI*) to the *MseI* endonuclease previously used 16S rRNA gene digestion, now allowing the identification of 17 species (all recognized species, except *A. anaerophilus*).

For the purposes of *Arcobacter* spp. identification and prevalence evaluation, the 16S rDNA sequencing has also been used (Woo *et al*. 2001; Lau *et al*. 2002; Chinivasagam *et al*. 2007; Pejchalova *et al*. 2008). The existence of potentially new species from diverse environments can also be inferred from 16S rRNA gene sequences deposited in public databases (Collado and Figueras 2011).

Fluorescence *in situ* hybridization (FISH) has been presented as an alternative to PCR, namely for samples from aquatic environment, as it is not inactivated by sample inhibitors, does not require DNA extraction and positive results can be directly observed in samples. Despite these advantages, the use of FISH does not improve the detection of *Arcobacter* spp., when compared with PCR, nevertheless it can provide valuable information, for example about cell morphology (Moreno *et al*. 2003; Fera *et al*. 2010a).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique has been used in the discrimination between species, also allowing to infer the existence of new species (Levican *et al*. 2012, 2013a), and the identification of *Arcobacter* spp. (Figueras *et al*. 2014). However the technique may show problems on identification due to the lack of proper database systems with an adequate number of bacteria strains in database. Bessede *et al*. (2011) conducted a comparison study between phenotypic conventional methods (API Campy strips), molecular (Fluorescence Resonance Energy Transfer real-time PCR) and MALDI-TOF MS identification. The authors found a misidentification of all *A. butzleri* strains (n=14) used in the study by the use of conventional methods, while a concordance between molecular and mass spectrometry methods was found in the identification of all isolates (Bessede *et al*. 2011).

Several methods have been suggested and developed over the years for a more reliable *Arcobacter* spp. identification. However the prevalence and diversity of *Arcobacter* spp. in

different matrices such as water, food, and faeces may be underestimated due to the limitations of the identification methods, namely the ability to recognize all species using a stand-alone method (Collado and Figueras 2011).

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application.

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
RFLP, Southern blotting (Kiehlbauch *et al.* 1991b)	16S rRNA, 23S rRNA	*A. butzleri*	Animal and human isolates (Isolate identification)	Equal patterns for *A. cryaerophilus* and *A. skirrowii*	Porcine foetuses (isolates identification) (SchroederTucker *et al.* 1996) Reference strains, poultry and porcine isolates (Harmon and Wesley 1997)
PCR (Bastyns *et al.* 1995)	23S rRNA	*Arcobacter* spp., *A. butzleri, A. cryaerophilus* and *A. skirrowii*	Reference strains and field isolates (Isolate identification)	Primers for *A. cryaerophilus* detect both *A. cryaerophilus* and *A. skirrowii*	Chicken and wastewater (enrichment broth) (Gonzalez *et al.* 2007a) Porcine samples (isolates identification) (Scanlon *et al.* 2013)
PCR-RFLP (Cardarelli-Leite *et al.* 1996)	16S rRNA	*A. butzleri*	Animal, human and environmental strains (Isolate identification)	Equal patterns for *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis*	-
PCR (Harmon and Wesley 1996)	16S rRNA	*Arcobacter* spp.	Reference strains (Isolate identification)	Identify *A. butzleri, A. cryaerophilus* and *A. skirrowii*, but with no amplification for *A. nitrofigilis*	Water (isolates identification) (Rice et al. 1999) Chicken samples (enrichment broth) (Ho et al. 2008) Human, animal and food samples (isolates identification and enrichment broth) (Patyal *et al.* 2011) Human stool (isolates identification) (Kayman et al. 2012b) Chicken viscera (suspected colonies) (Villalobos *et al.* 2013)
Multiplex PCR (Harmon and Wesley 1997)	16S rRNA, 23S rRNA	*Arcobacter* spp., *A. butzleri*	Reference strains, poultry and porcine isolates (Isolate identification)	Genus identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.	Water (isolates identification) (Rice *et al.* 1999) Meat and faecal samples of healthy cattle (genus identification of suspected colonies) (Öngör *et al.* 2004) Human stool samples (direct genus identification) (Collado *et al.* 2013)

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application (continuation).

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
PCR-RFLP (Hurtado and Owen 1997)	23S rRNA	*A. butzleri, A. nitrofigilis*	Field strains (Isolate identification)	Equal patterns for *A. cryaerophilus* and *A. skirrowii*	Evaluation of *A. butzleri* DNA integrity (Moreno *et al*. 2004)
PCR-RFLP (Marshall *et al*. 1999)	16S rRNA	*A. butzleri, A. cryaerophilus* and *A. skirrowii*	Reference strains (Isolate identification)	-	Human stool (culture independent) (Samie *et al*. 2007) Groundwater (isolates identification) (Fong *et al*. 2007)
PCR (Gonzalez *et al*. 2000)	16S rRNA	*Arcobacter* spp.	Chicken meat (Enrichment broth)	Detection takes in account *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.	Cattle samples (isolates identification) (Merga *et al*. 2013b)
Multiplex PCR (Houf *et al*. 2000)	16S rRNA, 23S rRNA	*A. butzleri, A. cryaerophilus* and *A. skirrowii*	Poultry (Enrichment broth)	Confusion between *A. cryaerophilus* and *A. skirrowii* with later reported species (Collado and Figueras 2011), (Levican and Figueras 2013).	Plankton and seawater (culture independent) (Fera *et al*. 2004) Chicken rearing and processing samples (culture independent, enrichment broth and suspected colonies) (Gude *et al*. 2005) Chicken and wastewater (Enrichment broth) (Gonzalez *et al*. 2007a) Animal and water (Isolates identification) (Aydin *et al*. 2007) Cat samples (culture independent) (Fera *et al*. 2009) Human, animal and food samples (Enrichment broth) (Patyal *et al*. 2011) Human stool (isolates identification) (Kayman *et al*. 2012b; Houf and Stephan 2007) Human stool (culture independent) (Samie *et al*. 2007; Jiang *et al*. 2010) Chicken samples (enrichment broth and suspected colonies) (Ho *et al*. 2008) Meat and shellfish (isolates identification) (Collado *et al*. 2009b)

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application (continuation).

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
PCR-hybridization (Al Rashid *et al*. 2000)	*glyA*	*A. butzleri*	Reference strains and isolates (Isolate identification)	-	-
PCR-ELISA (Antolin *et al*. 2001)	16S rRNA	*Arcobacter* spp.	Poultry (Enrichment broth)	Allows quantification from 10-10^4 cfu/g of *Arcobacter* spp. in poultry samples	-
Multiplex PCR (Kabeya *et al*. 2003a)	23S rRNA	*A. butzleri, A. cryaerophilus* 1A, *A. cryaerophilus* 1B and *A. skirrowii*	Reference strains and meat or feces isolates (Isolate identification)	DNA concentration must be strictly adjusted to 20 ng/reaction due to nonspecific amplifications with different concentrations. Produce unreliable results for all three of its targeted species (Levican and Figueras 2013).	Livestock (isolates identification) (Kabeya *et al*. 2003b) Broiler samples (isolates identification) (Son *et al*. 2007a)
FISH (Moreno *et al*. 2003)	16S rRNA	*Arcobacter* spp.	Water and Sewage (Culture independent)	Probe from Snaidr *et al*. (1997) Tested to *A. butzleri* , *A. cryaerophilus, A. nitrofigilis, A. skirrowii*	Estuarine waters (without and with enrichment) (Fera *et al*. 2010a)
PCR-RFLP (Karenlampi *et al*. 2004)	*groEL*	*A. butzleri*	Reference strain (Isolate identification)	*A. butzleri* was the only *Arcobacter* species tested	Cattle and badgers faeces (isolates identification) (Merga *et al*. 2011)
PCR-RFLP (Gonzalez *et al*. 2006)	16S rRNA, 23S rRNA	*A. butzleri*	Reference strains and poultry and water (Isolate identification)	*A. skirrowii* and *A. cryaerophilus* shared the same pattern	-
PCR (Neubauer and Hess 2006)	16S rRNA	*Arcobacter* spp.	Reference strains and field isolates (Isolate identification)	Detection takes in account *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.	-
PCR-DGGE (Petersen *et al*. 2007)	16S rRNA	*A. cryaerophilus 1B, A. nitrofigilis*	Pets saliva (Culture independent) Reference strains and field isolates (Isolate identification)	Equal patterns for *A. butzleri, A. cryaerophilus* 1A and *A. skirrowii*. *A. nitrofigilis* pattern shared with *C. gracilis*	Human faeces (culture independent) (Cornelius *et al*. 2012)

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application (continuation).

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
Taqman real-time PCR and multiplex PCR (Brightwell *et al*. 2007)	*rpoB/C*, 23S rRNA	*A. butzleri*, *A. cryaerophilus*	Field isolates from livestock and/or meat processing (Isolate identification)	The m-PCR uses primers CRY1-CRY2 described by Houf *et al*. (2000), for which unspecific reaction has been reported (Collado and Figueras 2011)	-
DNA microarrays (Quiñones *et al*. 2007)	Virulence and housekeeping genes	*A. butzleri*	Animal and human isolates and isolates from chicken package liquid (Isolate identification)	No hybridization with *A. cryaerophilus* or *A. cibarius*	-
FRET real-time PCR (Abdelbaqi *et al*. 2007a)	*gyrA*	*A. butzleri*, *A. cryaerophilus*, *A. cibarius*, *A. nitrofigilis*	Human stool (Culture independent)	Failed the identification of *A. skirrowii*	Human clinical strains (isolates identification) (Bessede *et al*. 2011)
PCR-RFLP (Figueras *et al*. 2008)	16S rDNA	*A. butzleri*, *A. cryaerophilus 1A*, *A. cryaerophilus1B, A. skirrowii, A. cibarius*, *A. nitrofigilis, A. halophilus*	Human, animal, food and environmental isolates (Isolate identification)	Misidentify three species (*A. trophiarum*, *A. thereius*, and some *A. cryaerophilus* strains) as *A. butzleri*. *A. suis*, and *A. defluvii* producing the same pattern; and *A. venerupis*, and *A. marinus* a very similar one (Levican and Figueras 2013)	Human stool (Isolates identification) (Collado *et al*. 2013) Fresh lettuces (Isolates identification) (Gonzalez and Ferrus 2011) Meat and shellfish (isolates identification) (Collado *et al*. 2009b)
PCR (Pentimalli *et al*. 2009)	*gyrA,* 16S rRNA	*A. butzleri*, *A. cryaerophilus, A. skirrowii,* and *A. cibarius*	Chicken meat (Enrichment broth)	Misidentification with non-targeted species being confused with *A. butzleri, A. cryaerophilus*, or *A. skirrowii* (Levican and Figueras 2013)	-
MALDI-TOF MS (Alispahic *et al*. 2010)	Proteins	*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*	Reference strains and human isolates (Isolate identification)	Only 3 *A. butzleri* strains and a single strain each of *A. cryaerophilus* and *A. skirrowii* were tested	-

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application (continuation).

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
Multiplex PCR (Douidah *et al*. 2010)	23S rRNA	*A. butzleri*, *A. cryaerophilus*, *A.skirrowii*, *A. cibarius*, *A. thereius*	Reference strains (Isolate identification)	*A. defluvii* produced the same band than *A. butzleri* (Collado *et al*. 2011) and occurs misidentification of non-targeted species (Levican and Figueras 2013)	Human stool samples (direct identification) (Collado *et al*. 2013) Chicken viscera (isolates identification) (Villalobos *et al*. 2013)
Real-time PCR (Gonzalez *et al*. 2010)	23S rRNA	*Arcobacter* spp.	Chicken and wastewater (Culture independent and enrichment broth)	Uses Bastyns *et al*. (1995) primers	Beach water (culture independent) (Lee *et al*. 2012) Fresh lettuce (isolates identification) (Gonzalez and Ferrus 2011)
PCR (De Smet *et al*. 2011a)	*hsp60*	*A. trophiarum*	Fattening pigs (Isolate identification)	-	-
PCR-RFLP (Figueras *et al*. 2012)	16S rRNA	All recognized species, with exception of *A. anaerophilus*	Diverse origin (Isolate identification)	Update 16S RNA-RFLP previously described by Figueras *et al*. (2008)	Shellfish (isolates identification) (Levican *et al*. 2014)
Quantitative real-time PCR (SYBR® Green I and TaqMan® assay) (Hausdorf *et al*. 2013a)	16S rRNA 23S rRNA	*Arcobacter spp*.	Spinach-processing plant samples (Culture independent)	Detects *A. bivalviorum*, *A. butzleri*, *A. cibarius*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. nitrofigilis*, *A. skirrowii*, *A. thereius*, *A. trophiarum*, *and A. venerupis*. For 16S rRNA-gene assay, *A. halophilus* and *A. marinus* showed a higher Ct value and a reduced efficiency.	-

Table 4. Molecular methods used for *Arcobacter* spp. identification and their application (continuation).

Method (reference)	Gene (s) targeted	Species discriminated	Sample used	Comment	Applications of the method
TaqMan multiplex Real-time PCR (De Boer *et al*. 2013)	*hsp60*	*A. butzleri*	Human faeces (Culture independent)	-	-
PCR (Gonzalez *et al*. 2014)	16S rRNA	*Arcobacter* spp.	Chicken meat (Enrichment broth)	Detects *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius*, *A. trophiarum*, *A. thereius*, *A. defluvii*, *A. ellisii*, *A. mytili*, *A. molluscorum*	-
LAMP (Wang *et al*. 2014)	23S rRNA	*Arcobacter* spp.	Chicken skin (Culture independent)	Detects *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.	-

RFLP, Restriction fragment length polymorphism; PCR, polymerase chain reaction; PCR-ELISA, PCR-Enzyme-linked immunosorbent assay; FISH, Fluorescent *in situ* hybridization; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; FRET real-time PCR, Fluorescence resonance energy transfer real-time PCR; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; LAMP, Loop-mediated isothermal amplification assay.

4.3. Genetic typing and diversity

The characterization of different types of organisms beyond the species is denominated typing. Several traditional typing systems based in phenotypic differences between isolates have been used for bacteria subtyping, such as serotype, biotype, phage-type or antimicrobial resistance typing. However, and since phenotypic typing methods are not variable enough for discriminating between closely related strains, molecular DNA-based typing methods have become widely used for bacterial strain typing due to its high resolution (Li *et al*. 2009; Sabat *et al*. 2013).

A number of genotyping techniques were developed for the genetic subtyping of *Arcobacter* strains, which have been used for discerning about population relatedness, distribution or routes of transmission of this microorganism (Gonzalez *et al*. 2012). These typing methods may be organized into three main groups: DNA banding pattern-, DNA sequencing-, and DNA hybridization-based methods. The first category includes techniques that allow the discrimination of strains according to the size of fragments generated by amplification or enzymatic digestion of genomic DNA; the second group deals with the methods assessing the nucleotide sequence of target regions and the strain discrimination is centred in the study of polymorphisms of the DNA sequences; the last category mainly refers to DNA macro- and microarrays methods (Li *et al*. 2009).

Up to date, a variety of *Arcobacter* typing methods have been used, including amplified fragment length polymorphism (AFLP), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), randomly amplified polymorphic DNA-PCR (RAPD-PCR), pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).

The AFLP method has been used to identify and explore the genetic diversity of *Arcobacter* species. AFLP profiling was for the first time used by On *et al*. (2003) for subtyping of *Arcobacter* species isolated from several sources and countries. The authors found a high degree of heterogeneity (62 types from 72 isolates); however with a clonality trend within *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, the method also allowed the identification of a potential novel *Arcobacter* taxon (On *et al*. 2003). The same group used this methodology to evaluate the genetic diversity of *A. butzleri* (51 types from 73 isolates) (On *et al*. 2004). Similarly, Gonzalez *et al*. (2007b) reported a high genotypic variation by the use of a single enzyme-AFLP based profiling (11 types from 15 isolates). The discriminatory power of this technique was also used for the reassessment of *A. cryaerophilus* taxonomic status (Debruyne *et al*. 2010). The *A. cryaerophilus* is a heterogeneous species that was described as including two subgroups (Kiehlbauch *et al*. 1991a), its taxonomy was reviewed with the employment of AFLP and heat shock protein 60 sequence analysis, and based on that the subgroup nomenclature was suggested to be abandoned (Debruyne *et al*. 2010). Despite the high

discriminatory power described to this AFLP-based profiling, the most used DNA-based typing method for *Arcobacter* spp. strain discrimination is ERIC-PCR.

ERIC-PCR has been vastly used to evaluate the degree of heterogeneity among animal food sources (Houf *et al*. 2002b; Van Driessche *et al*. 2004, 2005; Aydin *et al*. 2007; Van Driessche and Houf 2007b; Ho *et al*. 2008; De Smet *et al*. 2010; Levican *et al*. 2014), water (Collado *et al*. 2010) and human source isolates (Vandamme *et al*. 1993; Kayman *et al*. 2012b), with all the studies reporting a great level of heterogeneity. ERIC-PCR has also been used to establish the genetic relatedness of novel isolates and to help determining the taxonomic position of novel species (Houf *et al*. 2005; Collado *et al*. 2009a, 2011; Houf *et al*. 2009; Figueras *et al*. 2011a). Beyond the diversity evaluation, this technique was also used to establish the source of contamination or possible cross-contamination during food processing. Van Driessche and Houf (2007b) reported a possible cross-contamination of pork carcasses during slaughter and the characterization of *A. butzleri* and *A. cryaerophilus* isolates indicated a contamination of raw pork during processing at retail (Van Driessche and Houf 2007b). ERIC-PCR was also applied to evaluate the possible routes of *Arcobacter* introduction in poultry slaughterhouses through the isolates genetic diversity and relationship between them (Ho *et al*. 2008). Merga *et al*. (2013b) study questioned the reliability of ERIC-PCR as a specific typing method due to the absence of primers binding sites in the two studied *A. butzleri* genome sequences.

Further studies on *Arcobacter* diversity were performed employing RAPD-PCR typing to isolates from chicken carcasses (Atabay *et al*. 2002), fresh lettuces (Gonzalez and Ferrus 2011) or domestic geese (Atabay *et al*. 2008b). Both methods, ERIC-PCR and RAPD-PCR, were further applied for the molecular characterization of *Arcobacter* spp. isolates collected from poultry products (Houf *et al*. 2002b) and in a poultry slaughterhouse in Belgium (Houf *et al*. 2003). However as fingerprints generated with ERIC-PCR were shown to be more reproducible and complex than those obtained with RAPD-PCR (Houf *et al*. 2002b), the latter technique was suggested to be used in the differentiation of almost identical ERIC fingerprints. RAPD-PCR typing is considered limited by its reproducibility, due to the use of a single nonspecific primer and low annealing temperatures, but is a valuable and simple technique able to discern among closely related *Arcobacter* isolates (Gonzalez and Ferrus 2011).

PFGE has been recognized as the "gold standard" for the assessment of isolates interrelationships. This method is an electrophoretic technique used for the separation of large DNA fragments by applying alternating electric fields at different angles. The purified genomic DNA sample is subjected to digestion with a rare cutting restriction endonuclease, generating large DNA fragments that are separated afterwards by "pulsed-field" electrophoresis. The resulting DNA banding pattern reflects DNA polymorphisms at restriction sites, providing a macrorestriction analysis at genome level, which is used to carry out genetic comparisons among isolates (Foley *et al*. 2009; Goering 2010). PFGE has been successfully used for typing *Arcobacter* from foods, food animal sources, processing plants

and human patients (Rivas *et al.* 2004; Son *et al*. 2006; Revez *et al*. 2013; Douidah *et al*. 2014a) and for delineation of the transmission routes (Hume *et al*. 2001; Ho *et al*. 2006b). This method has been used to evaluate the genotypic distribution of *Arcobacter*, by fingerprinting of isolates from broiler carcasses along processing line (Son *et al*. 2006), raw milk samples (Revez *et al*. 2013), ground chicken, pork, beef and lamb meats from retails (Rivas *et al*. 2004), samples from an artisanal dairy plant (Giacometti *et al*. 2013), or samples from dairy cattle farms and beef samples from retail (Shah *et al*. 2012b). The results, point to a large diversity of *Arcobacter* strains, indicating the presence of multiple strains in one sample, but also the presence of common types of *Arcobacter* in different samples, suggesting possible cross-contamination or even persistence in the processing environment. A PFGE characterization conducted in a farrow-to-finish swine farm, showed great amount of genotypic variation suggesting that the animals were colonized by multiple *Arcobacter* genotypes and/or the identified isolates had undergone genomic rearrangement. In that study the authors also found a lack of association between *Arcobacter*-positive sows and nursing piglets (Hume *et al*. 2001). Still, Ho *et al*. (2006b) also used PFGE for genotyping *Arcobacter* spp. to evaluate the transmission route in sows and their offspring on a breeding farm, suggesting a transplacental transmission but also a horizontal transmission from mothers to their piglets. It was proposed that the differences between both studies may be related to the serum levels of maternal antibodies in piglets (Ho *et al*. 2006b). Genetic alteration by mutations or rearrangements in genes that are necessary under stress conditions has been suggested as a possible explanation to the high diversity of *Arcobacter* (Douidah *et al*. 2014a). PFGE was described as more discriminatory than other genotyping techniques, such as ERIC-PCR, AFLP or RAPD-PCR (Son *et al*. 2006; Douidah *et al*. 2014a). Douidah *et al*. (2014a) applied three typing strategies (ERIC-PCR, AFLP and PFGE) for six *Arcobacter* species (*A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius, A. thereius, A. trophiarum*) showing that PFGE was the most discriminatory technique between strains. The authors did not find a correlation between typing pattern and strain isolates origin host, as previously described when using Multi-locus sequence typing (MLST) (Miller *et al*. 2009).

The MLST method has been developed, in order to overcome the lack or poor interlaboratory comparison of some of the molecular typing approaches (Sabat *et al*. 2013). MLST is a typing method based on sequencing of fragments of housekeeping genes (Maiden *et al*. 1998), usually six to eight loci are sequenced, with each unique sequence assigned as a distinct allele and each allelic combination will define a sequence type (ST) (Jolley *et al*. 2004). The most important advantages of DNA sequencing-based genotyping over DNA banding pattern-based methods is its high reproducibility that together with the storage possibility in online databases allows genotyping comparison among laboratories (Li *et al*. 2009). The first MLST scheme typing for five species of *Arcobacter* (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius*) was introduced by Miller *et al*. (2009), and used the seven loci previously employed for *C. jejuni* (Miller *et al*. 2009). The latter study performed typing of

374 *Arcobacter* isolates from different geographic locations and sources, identifying a large number of alleles at each locus, with the majority of the isolates containing a unique ST. Despite the lack of association with either host or geographical source, which may be due to infrequent observation of the same ST, common STs between samples from healthy or diarrhoeal stool samples from children were observed (Miller *et al*. 2009). Despite its advantages, so far, this scheme was only used in four more studies. In the first one, typing of 39 *A. butzleri* isolates from animal farms was performed, and 11 different STs were detected, demonstrating a considerable level of diversity amongst *A. butzleri* isolates. Indeed, none of the STs identified were found on more than one farm or had been previously reported at the time of the study (Merga *et al*. 2011). In another study, also performed in cattle from UK, MLST typing displayed again a high diversity between *A. butzleri* isolates, with 43 different STs identified from 104 isolates. The observed persistent STs suggested a possible survival overtime or re-contamination by the same STs (Merga *et al*. 2013b). Typing by MLST was used by Rasmussen *et al*. (2013) to evaluate persistence and cross-contamination in a Danish broiler slaughterhouse during meat processing. High strain variability was found, with the recurrence of two STs, indicating that persistence or cross-contamination could take place. The heterogeneity of the *A. butzleri* isolates was demonstrated by the relatively high numbers of new alleles and STs; however also expresses the existence of a limited number of profiles in the *Arcobacter* MLST database (Rasmussen *et al*. 2013). MLST scheme was also used for typing an *A. cryaerophilus* isolate from a bloody human diarrhoea sample; the isolate belonged to a new ST, while sharing three known alleles with strains isolated from faeces of patients with gastroenteritis (Figueras *et al*. 2014). A drawback of MLST typing application to *Arcobacter* spp. was presented by Merga *et al*. (2013b) that obtained 410 poor quality sequences comprising *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* isolates, showing that some strains could not be accurately typed using MLST. Nonetheless, MLST typing demonstrated *Arcobacter* spp. heterogeneity, along with the possible cross-contamination and persistence of microorganisms, as supported by other studies using different typing methods.

The existence of considerable heterogeneity among *Arcobacter* isolates, even within the same sample specimen (Houf *et al*. 2002b), together with the existence of overlapping *A. butzleri* genotypes originating from different sources or places (Aydin *et al*. 2007), hampers the establishment of the exact sources of contamination or infection and the clarification of transmission routes.

5. Pathogenesis and virulence factors of *Arcobacter* species

Arcobacter spp. has been recovered from abortions and enteritis in livestock, whereas human infections are mainly associated with enteritis and bacteraemia (Wesley and Miller 2010).

Currently, the pathogenic potential of *Arcobacter* remains relatively unexplored with few studies devoted to clarify the pathogenic mechanisms related to this genus. *In vitro and in vivo* models were used to start unravelling the mechanism that may trigger infection by *Arcobacter*. *In vitro* human and animal cell culture assays have been used to show that several *Arcobacter* species can adhere to and invade eukaryotic cells and can produce toxins that damage host cells. Scarce *in vivo* studies were performed to clarify the pathophysiology and pathogenic potential of *Arcobacter* spp. In addition, data from genome sequencing highlighted several potential markers that may be helpful candidates for the study and understanding of these mechanisms.

The completion of *A. butzleri* RM4018 genome sequence and its analysis in 2007 was an important step for understanding and advancing in *Arcobacter* research (Miller *et al*. 2007). Miller and collaborators (2007) identified several putative virulence factors, some of them presenting homology with *C. jejuni* virulence determinants, but also lacked some of the virulence-associated genes described for this species. Moreover, this work also identified putative virulence determinants homologue to those described for both plant and animal pathogens (Miller *et al*. 2007).

Nine putative genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB*, *irgA*) have received special attention, due to their homology to genes associated to pathogenicity in other microorganisms. The *cadF* and *cj1349* genes encode outer membrane proteins, which promote bacteria to cell contact by adherence to fibronectin (Monteville *et al*. 2003; Flanagan *et al*. 2009), with *cadF* having a role on adherence to host cells and triggering processes that after will induce internalization (Dasti *et al*. 2010). *CiaB* gene is associated to host cell invasion in *Campylobacter* spp. (Konkel *et al*. 1999). A homologue of *mviN* was described as encoding an essential protein involved on peptidoglycan synthesis in *E. coli* (Inoue *et al*. 2008). *PldA* gene encodes an outer membrane phospholipase A related with cell-associated haemolytic activity (Grant *et al*. 1997). *TlyA* gene encodes a haemolysin, which plays a role in the adherence of *C. jejuni* to Caco-2 cells (Salamaszynska-Guz and Klimuszko 2008). *HecA* gene encodes a protein member of a class of adhesins (filamentous haemagglutinin family) important in several animal and plant pathogens (Rojas *et al*. 2002) and *hecB* codes for a related haemolysin activation protein (Miller *et al*. 2007). The *irgA* gene encodes an iron-regulated outer membrane protein, not required for *Vibrio cholerae* virulence (Mey *et al*. 2002), nonetheless an *irgA* homolog was described as having a role in *E. coli* pathogenesis (Johnson *et al*. 2005).

Douidah *et al*. (2012) developed a PCR-assay to detect those nine putative virulence genes and assessed their presence in 319 human and animal *Arcobacter* strains belonging to *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* species. The nine putative virulence genes were all found in 26 of the 182 *A. butzleri* isolates, in contrast neither *A. cryaerophilus* nor *A. skirrowii* strains carried all the nine genes, suggesting a different pathogenic behaviour or an association to higher genome heterogeneity (Douidah *et al*. 2012). In agreement with Douidah *et al*. (2012), Karadas *et al*. (2013) and Tabatabaei *et al*. (2014) also reported the presence of c*adF*, *ciaB*, *cj1349*, *mviN*, *pldA*, and *tlyA* genes in all *A. butzleri* tested strains (Karadas *et al*. 2013; Tabatabaei *et al*. 2014).

5.1. Adhesion and invasion to host cells

Adhesion is an important factor in bacterial pathogenicity, since it is a required step for the establishment of infection, which may be followed by colonization of tissues, and in some cases by bacterial invasion of host cells, followed by intracellular multiplication, dissemination to other tissues, or persistence (Pizarro-Cerda and Cossart 2006). The host cell may act as an active contributor to the adhesion process, having a role beyond being an inert surface (Lu and Walker 2001).

To promote and maintain a successful infection, microbial pathogens possess a set of strategies to invade the host, evade or resist to innate immune response, to cause damage to cells and multiply (Cossart and Sansonetti 2004). The host in turn has a variety of approaches to resist infections, like the activation of intracellular signals leading to a mucosal inflammatory response in intestinal epithelial cells as a response to bacterial invasion. In the end, it is this pathogen/host interaction that will define the development or lack of disease process (Lu and Walker 2001).

Although *Arcobacter* has emerged as a food- and water-borne pathogen, data regarding the pathogenic mechanisms and virulence potential of *Arcobacter* is still scarce, when compared with other enteric pathogens.

Several studies using *in vitro* cultures have been performed to characterize adhesion, invasion (Figure 2) and cytotoxic potential of *Arcobacter* species to host cells, with adherence and cytotoxicity as the most commonly observed effects (Table 5). Overall, the results have shown a considerable variation, which has been correlated with origin of strains and cell lines used in the studies (Ho *et al*. 2007; Collado and Figueras 2011). For instance, *A. cryaerophilus* strains from healthy humans were tested concerning its adherence on Hep-2 and Caco-2 cells, with four out of seven strains showing adhesion to Hep-2 cells but only two adhering to Caco-2 cell line (Houf and Stephan 2007). In contrast, other studies reported that all animal tested strains adhered to Caco-2 and IPI-2I cells (Ho *et al*. 2007; Levican *et al*. 2013b). The ability of

the same strain to adhere or to invade different cell lines was demonstrated for *A. butzleri*, A. *cryaerophilus*, *A. skirrowii* and *A. cibarius*, and despite the observed differences in interaction, the same strain showed similar trends in terms of adhesion and invasion capacities to different cell lines, while strains without adherent or invasive properties for one cell line showed a lower interaction to a different cell line (Ho *et al*. 2007; Houf and Stephan 2007; Karadas *et al*. 2013).

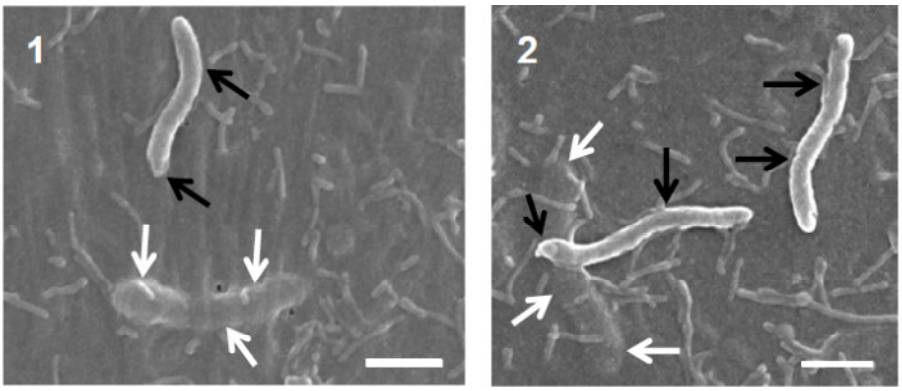

Figure 2. Scanning electron micrographs showing the adhesion (black arrows) and invasion (white arrows) of *Arcobacter* strains to Caco-2 cells. Bar 1 μm (Levican *et al*. 2013b).

The adherence and invasion of 15 of the 18 recognized *Arcobacter* species were evaluated by Levican *et al*. (2013b), with only *A. bivalviorum* species and a single *A. nitrofigilis* isolate from the roots of *S. alterniflora* displaying no adherence on Caco-2 cell line. At least one isolate from each species was considered highly adherent to Caco-2 cells (Levican *et al*. 2013b). Concerning invasion, 10 of the 15 tested *Arcobacter* species demonstrated invasion ability, with the most strongly invasive strains being isolated from faecal sources (animal faeces or sewage), and belonging to A. *trophiarum* (De Smet *et al*. 2011a; Levican *et al*. 2013b), and *A. butzleri* (Levican *et al*. 2013b). This fact do no confirme the results obtained by Ho *et al*. (2007), that found a significantly stronger association of *A. cibarius* with Caco-2 and IPI-2I cells, than for *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* strains, with only one *A. cryaerophilus* strain being able to invade both cell lines (Ho *et al*. 2007). Taking that adhesion and invasion abilities to host cells are required for successful colonization and infection, the reported studies suggest that several species can be considered potential pathogens.

Table 5. Pathogenicity of *Arcobacter* species on different cell lines (*in vitro* assays) (Adapted from (Collado and Figueras 2011) and updated).

Species and cell line[a]	Strain origin	Number of positive /number of tested samples			References
		Adhesion	Invasion	Cytotoxicity	
A. nitrofigilis					
Caco-2	Roots *S. alterniflora*	0/1	0/1	ND	(Levican *et al.* 2013b)
	Animal (mussels)	2/2	2/2	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*2/3 (66.7%)*	*2/3 (66.7%)*		
A. cryaerophilus					
Hep-2	Animal (bovine foetus and swine faeces)	ND	2/2	ND	(Fernández *et al.* 1995)
	Human (faeces)	4/7	ND	ND	(Houf and Stephan 2007)
HeLa	Animal (cattle and poultry)	ND	ND	3/3	(Johnson and Murano 2002)
Vero	Meat (pork, chicken, beef)	ND	ND	2/2	(Villarruel-Lopez *et al.* 2003)
INT407	Animal (cattle and poultry)	ND	ND	3/3	(Johnson and Murano 2002)
Caco-2	Animal (porcine/ovine)	4/4	2/4	ND	(Ho *et al.* 2007)
	Human (faeces)	2/7	ND	ND	(Houf and Stephan 2007)
	Animal (clams, chicken faeces and sheep faeces)	5/5	5/5	ND	(Levican *et al.* 2013b)
IPI-2I	Animal (porcine/ovine)	4/4	1/4	ND	(Ho *et al.* 2007)
	Subtotal (%)	*19/27 (70.4%)*	*10/15 (66.7%)*	*8/8 (100%)*	
A. butzleri					
Hep-2	Sea water	6/17[b]	ND	ND	(Carbone *et al.* 2003)
	Zooplankton	4/4	ND	ND	(Gugliandolo *et al.* 2008)
	Human (faeces)	3/3	ND	ND	(Fernández *et al.* 2010)
	Animal (mussels and faeces from pelican, bovine, duck, dog, sparrow)	33/33	ND	ND	(Fernández *et al.* 2010)
	River water	15/15	ND	ND	(Fernández *et al.* 2010)
	Meat (chicken giblets and carcass)	21/21	ND	ND	(Fernández *et al.* 2010)

Table 5. Pathogenicity of *Arcobacter* species on different cell lines (*in vitro* assays) (Adapted from (Collado and Figueras 2011) and updated) (continuation).

Species and cell line[a]	Strain origin	Number of positive /number of tested samples			References
		Adhesion	Invasion	Cytotoxicity	
A. butzleri					
HeLa	River water	1/18	0/18	ND	(Musmanno *et al*. 1997)
	River water/Human (diarrhoea)/Animal (cattle and poultry	ND	ND	7/7	(Johnson and Murano 2002)
	Sea water	6/17[b]	ND	ND	(Carbone *et al*. 2003)
	Zooplankton	4/4	ND	ND	(Gugliandolo *et al*. 2008)
Vero	River water	ND	ND	17/18	(Musmanno *et al*. 1997)
	Sea water	ND	ND	5/17	(Carbone *et al*. 2003)
	Meat (pork, chicken, beef)	ND	ND	76/80	(Villarruel-Lopez *et al*. 2003)
	Zooplankton	ND	ND	3/4	(Gugliandolo *et al*. 2008)
INT407	River water	1/18	0/18	ND	(Musmanno *et al*. 1997)
	River water/Human (diarrhoea)/Animal (cattle and poultry)	ND	ND	6/6	(Johnson and Murano 2002)
CHO	River water	ND	ND	17/18[c]	(Musmanno *et al*. 1997)
Caco-2	Human (blood)	1/1	0/1	ND	(Ho *et al*. 2007)
	Meat (turkey, duck, pig, chicken and beef)	6/6	6/6	ND	(Levican *et al*. 2013b)
	Animal (Clams, mussels)	4/4	3/4	ND	(Levican *et al*. 2013b)
	Sewage	2/2	2/2	ND	(Levican *et al*. 2013b)
	Human	3/3	3/3	ND	(Karadas *et al*. 2013)
	Meat (chicken)	3/3	3/3	ND	(Karadas *et al*. 2013)
IPI-2I	Human (blood)	1/1	0/1	ND	(Ho *et al*. 2007)
HT-29	Human	2/3	1/3	ND	(Karadas *et al*. 2013)
	Meat (chicken)	2/3	2/3	ND	(Karadas *et al*. 2013)
	Subtotal (%)	*127/176 (72.1%)*	*20/62 (32.3%)*	*131/150 (87.3%)*	

Table 5. Pathogenicity of *Arcobacter* species on different cell lines (*in vitro* assays) (Adapted from (Collado and Figueras 2011) and updated) (continuation).

Species and cell line[a]	Strain origin	Number of positive /number of tested samples			References
		Adhesion	Invasion	Cytotoxicity	
A. skirrowii					
Vero	Meat (pork, chicken, beef)	ND	ND	17/19	(Villarruel-Lopez *et al*. 2003)
Caco-2	Animal (porcine/ovine)	2/2	0/2	ND	(Ho *et al*. 2007)
	Sludge WWTP	1/1	1/1	ND	(Levican *et al*. 2013b)
	Meat (pig)	1/1	0/1	ND	(Levican *et al*. 2013b)
IPI-2I	Animal (porcine/ovine)	2/2	0/2	ND	(Ho *et al*. 2007)
	Subtotal	*6/6 (100%)*	*1/6 (16.7%)*	*17/19 (89.5%)*	
A. cibarius					
Caco-2	Meat (chicken carcass)	1/1	0/1	ND	(Ho *et al*. 2007)
	Piggery effluent	2/2	1/2	ND	(Levican *et al*. 2013b)
	Meat (poultry)	3/3	2/3	ND	(Levican *et al*. 2013b)
IPI-2I	Meat (chicken carcass)	1/1	0/1	ND	(Ho *et al*. 2007)
	Subtotal (%)	*7/7 (100%)*	*3/7 (42.9%)*		
A. mytili					
Caco-2	Brackish water	1/1	0/1	ND	(Levican *et al*. 2013b)
	Animal (mussels)	2/2	0/2	ND	(Levican *et al*. 2013b)
	Subtotal (%)	*3/3(100%)*	*0/3(0%)*		
A. thereius					
Caco-2	Animal (porcine abortion and mussels)	3/3	2/3	ND	(Levican *et al*. 2013b)
	Meat (pig)	1/1	1/1	ND	(Levican *et al*. 2013b)
	Sewage	1/1	1/1	ND	(Levican *et al*. 2013b)
	Subtotal (%)	*5/5(100%)*	*4/5(80%)*		

Table 5. Pathogenicity of *Arcobacter* species on different cell lines (*in vitro* assays) (Adapted from (Collado and Figueras 2011) and updated) (continuation).

Species and cell line[a]	Strain origin	Number of positive /number of tested samples			References
		Adhesion	Invasion	Cytotoxicity	
A. trophiarum					
Caco-2	Animal (pig faeces and chicken faeces)	3/3	3/3	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*3/3(100%)*	*3/3(100%)*		
A. defluvii					
Caco-2	Animals (mussels and pig faeces)	2/2	2/2	ND	(Levican *et al.* 2013b)
	Sewage	6/6	6/6	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*8/8(100%)*	*8/8(100%)*		
A. molluscorum					
Caco-2	Animals (mussels and oysters)	3/3	0/3	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*3/3(100%)*	*0/3(0%)*		
A. ellisii					
Caco-2	Animals (mussels)	3/3	3/3	ND	(Levican *et al.* 2013b)
	Subtotal	*3/3(100%)*	*3/3(100%)*		
A. bivalviorum					
Caco-2	Animals (mussels)	0/3	0/3	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*0/3(0%)*	*0/3(0%)*		
A. venerupis					
Caco-2	Animals (Clams)	1/1	0/1	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*1/1(100%)*	*0/1(0%)*		
A. cloacae					
Caco-2	Animals (mussels)	1/1	1/1	ND	(Levican *et al.* 2013b)
	Sewage	1/1	1/1	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*2/2(100%)*	*2/2(0%)*		
A. suis					
Caco-2	Meat (Pig)	1/1	0/1	ND	(Levican *et al.* 2013b)
	Subtotal (%)	*1/1(100%)*	*0/1(0%)*		

[a] Caco-2, human colorectal adenocarcinoma cell line; CHO, Chinese hamster ovary cell line; HeLa, human cervix carcinoma cell line; Hep-2, human cervix carcinoma cell line (HeLa contamination); INT407, HeLa derivative; HT-29, human colorectal adenocarcinoma cell line; IPI-2I, porcine intestinal epithelioid cell line; Vero, kidney epithelial cell line.

[b] The authors considered strains as non-adherent if the mean adhesion index were <10 microorganism/cell).

[c] The strain that not presented cytotoxic effects showed to induce cell morphological changes related with cytotonic effect.

ND, not determined.

The mechanism by which *A. butzleri* may cause diarrhoea was studied by Bücker *et al.* (2009), who showed that this bacterium was able to induce epithelial barrier impairment in the human colonic carcinoma cells (HT-29/B6), through an increase in macromolecular permeability via paracellular pathway and epithelial resistance decrease. A change in tight-junction protein composition and distribution was identified, with a reduction in expression of sealing-associated proteins such as claudin-1, -5 and -8, which may contribute to diarrhoea after *A. butzleri* infection by a leak flux mechanism (Figure 3).

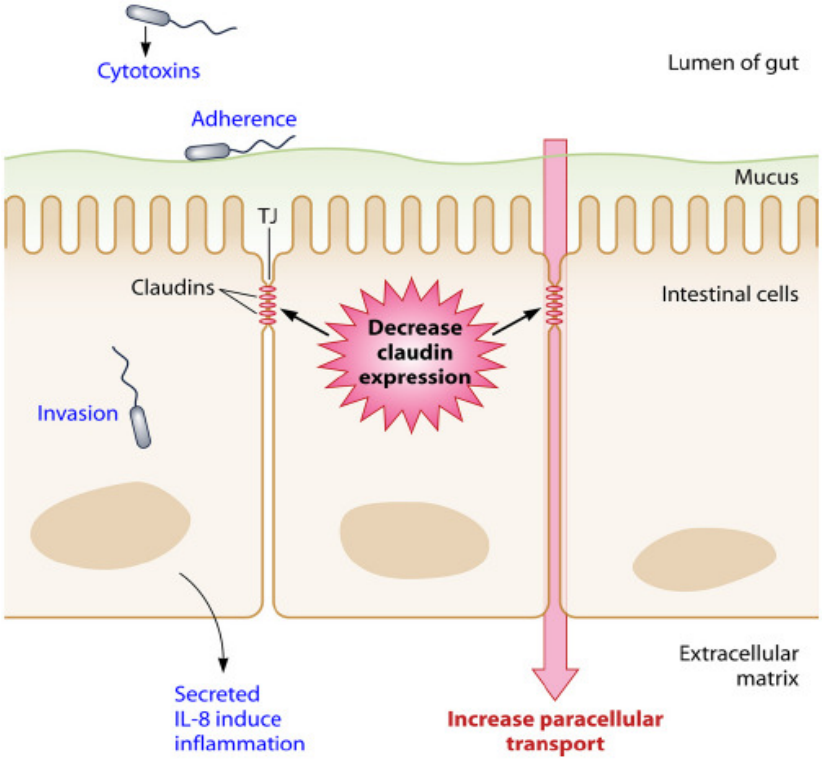

Figure 3. Schematic representation of virulence mechanisms of *Arcobacter* in intestinal epithelial cells (collected from information acquired on different cell lines) (Collado and Figueras 2011).

Since the establishment of a PCR-assay for detection of putative virulence genes, two works attempted to establish whether these putative virulence factors had analogous functions to their homologues in other bacterial species. Karadas *et al.* (2013) determined the presence of putative virulence genes in 52 *A. butzleri* isolates, selecting six of these strains for adherence and invasion assays in HT-29 and Caco-2 cell lines. The *in vitro* assays showed that *A. butzleri* had different adherence and invasion abilities depending on the isolate and on the cell line used. While all isolates adhered and invaded Caco-2 cells only four strains adhered and three invaded HT-29 cells. The strains which showed neither adherence nor invasion on HT-29 cells exhibited lower abilities on Caco-2 cells (Karadas *et al.* 2013). Despite the presence of adhesion and invasion genes in all tested isolates, the adhesion and invasion phenotypes were

different between isolates. Moreover, no correlation was observed between putative virulence gene patterns and adhesion to or invasion of tested cell lines; also modifications in putative functional domains of *ciaB*, *cadF* and *cj1349* showed no correlation with these virulence traits. Those results were potentially explained by the low number of isolates used in the study, being suggested that further *in vitro* and *in vivo* studies are required to clarify the role of these virulence factors in pathogenesis of *A. butzleri* (Karadas *et al.* 2013).

A high prevalence of putative virulence genes for *A. butzleri* was noted by Levican *et al.* (2013b) during the evaluation of the virulence genotype of 15 different *Arcobacter* species. Moreover, for *A. thereius*, *A. mytili* and *A. cibarius*, the authors established a correlation between the lack of invasion of Caco-2 cells and the absence of all virulence genes tested (*ciaB*, *irgA*, *hecA*, *cj1349* and *cadF*) (Levican *et al.* 2013b). However, there was an unclear association between the invasion ability and the presence of virulence-associated genes in this experiment, suggesting that more genes may be involved in the invasion process.

5.2. Production of toxins by *Arcobacter*

Enteric microbial pathogenesis can involve an important array of traits like invasion, signals triggering host inflammation, or secreted factors that damage the host such as toxins and/or by exploiting other environmental host-associated factors to thrive (Kolling *et al.* 2012). The production of toxins is an underlying mechanism by which many bacterial pathogens produce disease. Toxins can directly signal epithelial secretion, damage the epithelial cells or intestinal barrier function, or recruit secondary cells or mediators, which then trigger intestinal secretion, inflammation, or damage (Guerrant *et al.* 1999).

Musmanno *et al.* (1997) described the evidence of cytotoxic activity of *A. butzleri* strains on mammalian cells, through the exposure of two eukaryotic cell lines to broth culture filtrates of 18 strains isolated from river water, among which 17 strains exhibited cytotoxic effects and one showed a cytotonic-like effect (cell elongation) (Musmanno *et al.* 1997). Other authors have shown the cytotoxic effect of five of the 17 tested environmental isolates of *Arcobacter butzleri* against Vero cells (Carbone *et al.* 2003).

In another study by Johnson and Murano (2002), the presence of cytolethal distending toxin (CDT) genes in *Arcobacter* isolates from poultry, cattle, irrigation water and human diarrhoeal samples was investigated, and found no amplification of these genes, according to Miller *et al.* (2007) report for *A. butzleri* RM4018 strain. Nonetheless toxicity to HeLa and INT407 cells was found, with the observation of rapid death and growth inhibition. Thus, the authors suggested that *Arcobacter* has a toxic effect on mammalian cell lines; but probably a toxin different from CDT is involved (Johnson and Murano 2002). Further evidence of toxins production was provided by Villarruel-López *et al.* (2003) who observed a cytotonic and/or

vacuolizing effect of *A. butzleri, A. cryaerophilus* and *A. skirrowii* broth culture filtrates from retail meats in Vero cells; they observed that 94 % of the isolates produced at least one of these two effects, indicating the possible production of an enterotoxin and vacuolizing toxin (Villarruel-Lopez *et al*. 2003). The likely nature of *A. butzleri* toxins was suggested to be a heat-sensitive protein, possible cell associated rather than secreted, that may cause a necrotic effect (Bücker *et al*. 2009).

Various reports of broth culture or similar filtrates extract toxicity (Musmanno *et al*. 1997; Johnson and Murano 2002; Carbone *et al*. 2003; Villarruel-Lopez *et al*. 2003), suggests toxin secretion by *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*; however the use of cell sonicate extracts (Johnson and Murano 2002; Bücker *et al*. 2009) also displayed potential cytotoxic activity. These studies suggest the existence of both cell associated and secreted toxins, which may exhibit different toxicity potential.

The *A. cryaerophilus* toxigenic potential was also demonstrated *in vivo* by Fernández *et al*. (1995) that showed that cell-free supernatant of two *A. cryaerophilus* strains produced distention of the ileal loops with fluid accumulation and an increase in electrolytes concentrations, in inoculated adult Wistar rats (Fernández *et al*. 1995).

Carbone *et al*. (2003) detected a correlation between secretion of toxic factors and adhesion, with 5 of the 6 strongly adhesive *A. butzleri* strains secreting toxin factors able of induce rounding or elongation of Vero cells (Carbone *et al*. 2003). Furthermore, Musmanno *et al*. (1997) demonstrated that the only *A. butzleri* strain showing adhesivity produced an cytotonic-like effect, despite a group of 17 strains was unable to associate with cell surfaces also showed morphological features correlated with cytotoxic effect (cell rounding and nuclear pyknosis) (Musmanno *et al*. 1997).

Despite the several reports on the toxins production by *Arcobacter*, there is still not enough knowledge about it and it remains to be fully understood their nature, regulatory mechanisms and cell targets.

5.3. Host immune response

The entry of a pathogen into the host leads to complex interactions between pathogen-derived molecules and host sensors. In mammalian hosts, the immune system is constituted by innate and adaptive defence mechanisms (Kumar *et al*. 2013). The innate immunity provides the first line of defence against microbial infection, consisting of immediate microbial killing, starting of the local inflammation process and stimulation of the adaptive immune response. The adaptive immunity is characterized by an antigen-specific immunity through B- or T-lymphocytes (Hyun *et al*. 2013). Gastrointestinal infections are a complex mechanism that involves multiple interactions between the host, pathogen and resident

intestinal microbiota. The defence against pathogens is achieved by the physical barriers of the epithelium, which includes tight junctions and mucus, but also through production of immunological functional molecules, like cytokines, chemokines and other signalling molecules (Kunisawa and Kiyono 2013).

In humans, *A. butzleri* and *A. cryaerophilus* have been associated with gastrointestinal disease, with persistent watery diarrhoea as the main symptom associated with *A. butzleri*, although, some cases of bacteraemia have also been attributed to *A. butzleri* and *A. cryaerophilus* (Collado and Figueras 2011). In *C. jejuni*, host innate defences have been suggested to contribute to the resolution of the infection, due to the self-limited nature of the disease, with resolution of disease within several days of onset of symptoms (Zilbauer *et al.* 2008).

The cytokine interleukin-8 (IL-8) is a potent chemoattractant related with immune cells recruitment to the site of local inflammation, with its secretion being associated with diarrhoea development and clearing of infection. The release of intestinal epithelial IL-8 by *C. jejuni* is well established and has been associated with adhesion/invasion ability (Zilbauer *et al.* 2008). One study investigated host cell cytokine responses to four species of *Arcobacter* infection (*A. butzleri*, *A. skirrowii*, *A. cryaerophilus* and *A. cibarius*) in cell culture models using human (Caco-2) and porcine intestinal epithelial cells (IPI-2I) and showed that *Arcobacter* spp. induces the proinflammatory cytokine, IL-8 (Ho *et al.* 2007). The authors showed an upregulation of IL-8 expression 2 h after infection for all the *Arcobacter* strains used in the study, with no correlation established between cell invasion and degree of adhesion (Ho *et al.* 2007). Ho *et al.* (2007) suggested that different virulence mechanisms could exist for distinct *Arcobacter* species and strains. The invasion ability found for an *A. cryaerophilus* strain isolated from an aborted foetus, where vertical transmission was demonstrated, was suggested as a prerequisite for these *Arcobacter* strains. Furthermore, for *A. skirrowii* LMG 6621, a strain isolated from an animal case of persistent diarrhoea, the most appropriate mechanism for strain success should be cell association with trigger of local inflammatory process (Ho *et al.* 2007).

Lactoferrin is one of the first factors released by neutrophils upon encounter with pathogens, limiting microbial proliferation and functioning as a direct microbicidal agent, so being considered an important component of innate host defense system against various pathogens in humans (Actor *et al.* 2009; Vogel 2012). This protein can support proliferation, differentiation, and activation of immune system cells and strengthen the immune response and act to combat excessive inflammation (Adlerova *et al.* 2008), it functions by modulating and affecting responses of both the innate and adaptive immune system (Actor *et al.* 2009). Lactoferrin has been described as an useful faecal marker for prediction and monitoring of intestinal inflammation associated with invasive pathogens (Chen *et al.* 2011). Samie *et al.* (2007) during a study concerning the prevalence of *Campylobacter* spp., *Helicobacter pylori* and *Arcobacter* spp. in stool samples from South Africa, reported that 55 % of *A. butzleri*-

positive stool samples had elevated levels of lactoferrin, with lower percentages (33 %) described for *A. cryaerophilus*- and *A. skirrowii*-positive samples, revealing a possible involvement in inflammatory process (Samie *et al*. 2007).

The intestinal epithelium secretes a variety of antimicrobial peptides, one of which are defensins, small cationic peptides with antimicrobial activity, that protect the intestinal epithelium and regulate the number and composition of the microbiota (Rescigno 2011). ß-defensins have been described as being modulated during *C. jejuni* infection, with possible association with self-limiting disease due to active killing of the bacteria (Zilbauer *et al*. 2005). During a study concerning the differential regulation of ß-defensins 1 and 2 in porcine intestinal epithelial cell line IPI-2I upon *Salmonella* infection, the authors used *A. cryaerophilus* as control, reporting no significant effect on porcine ß-defensins 1 and 2 gene expression (Veldhuizen *et al*. 2006).

Another important innate defence against bacteria achieving access to vascular compartment from mucosal sources is the antibacterial activity of complement present in serum (Keo *et al*. 2011). *A. butzleri* (from chicken liver, bovine faeces, pelican faeces, shellfish, and river water) was shown to be serum sensitive; moreover the bacteria survival in complement-deficient sera highlights the importance of complement-mediated killing (Wilson *et al*. 2010). *A. butzleri* and *A. cryaerophilus* bacteraemia reports (On *et al*. 1995; Hsueh *et al*. 1997; Yan *et al*. 2000; Lau *et al*. 2002) suggest that as described for *C. jejuni* where systemic strains were more resistant to complement-mediated killing than diarrhoeal associated strains (Keo *et al*. 2011). Also *Arcobacter* may have different degrees of serum-susceptibility according to origin, systemic localization or other.

Until the moment, there is no directed study to clarify the host immune response against infection by *Arcobacter*, which remains rather unknown. Further research should be conducted to fully understand *Arcobacter* virulence mechanism and to discern the host response upon *Arcobacter* infection, as well as the possible strategies used by this microorganism to avoid and/or modulate host defence mechanisms.

5.4. Plasmids

The exchange of genetic information between cells allows the transfer of genes and characteristics that potentially can result in the acquisition of one cell phenotype by another. The transferred DNA can be stably maintained as an extrachromossomal element (plasmid), capable of horizontal transmission, being passed to the next cell generation as an autonomously replicating unit independent of the host chromosome, with sizes that may vary from 1 kbp to over 100 kbp. Plasmids can have importance to bacterial pathogenicity, since they may encode for accessory phenotypes, such as antimicrobial or heavy metals resistance genes, or even with small plasmids carrying genes for toxins or large plasmids encoding to

complex virulence phenotypes. A successful transfer of sequences that confer adaptive features may alter the ecology of the recipient bacterium, for example, allowing the colonization of hostile environments, in fact, plasmid-mediated horizontal gene transfer influences bacterial community structure and evolution (Slater *et al*. 2008).

Few studies reported the presence of plasmids in *Arcobacter* (Harrass *et al*. 1998; Toh *et al*. 2011; Douidah *et al*. 2014b). Plasmids of different sizes, ranging from 2.0 to 5.0 kbp, were found in 24 % of the *A. butzleri* isolates obtained from freshly slaughtered broilers, where none of the isolates contained more than one plasmid and all plasmids of the same size showed identical restriction patterns (Harrass *et al*. 1998). Also, a small plasmid containing three protein-coding genes, one encoding for a putative replication protein and the other two hypothetical proteins, was detected in an *Arcobacter* sp. strain isolated from a microbial fuel cell (Toh *et al*. 2011). Additionally, a plasmid from *A. butzleri* NCTC 12481 was sequenced (not published, NCBI Reference Sequence: NC_012733.1).

A recent study evaluated the presence of plasmids in human and animal associated *Arcobacter* species, detecting plasmids in *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* from chicken, pigs and cattle, but not from human, sheep or horse isolates. The sequencing of four small plasmids, three isolated from *A. butzleri* and one from *A. cryaerophilus*, demonstrated the presence of genes coding for putative replication proteins necessary for replication and transfer of the plasmid to a new generation. These small plasmids can be good candidates as vectors to be used in the phenotypic and genotypic *Arcobacter* research, with this possibility being highlighted by the presence of a putative mobilization protein in one of the plasmids (Douidah *et al*. 2014b). In the same work, Douidah *et al*. (2014b) also reported the presence of a large plasmid (27.5 kbp) in an *A. butzleri* isolate from poultry. The aforementioned plasmid sequencing revealed the presence of several putative genes related with the mechanism of the type IV secretion system from *Wolinella succinogenes*, which is referred as possibly having a role in DNA transfer and protein or toxin secretion (Douidah *et al*. 2014b). The presence of plasmids in *Arcobacter* may contribute to its genetic diversity.

5.5. *In vivo* infection model systems

Some *in vivo* models have been used to attempt to replicate clinical diseases and evaluate the virulence attributes of *Arcobacter* spp., mainly *A. butzleri*.

Considering the use of neonatal piglets as animal models in the study of pathogenicity of *C. jejuni* or *H. pylori*, this model was also used to evaluate *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* pathogenicity in 1-day-old caesarean-derived colostrum-deprived piglets, by examination of the duration of faecal shedding and colonization of tissues. The faecal shedding was longer for *A. butzleri*, suggesting intestinal colonization and multiplication of the bacterium, also *A. butzleri* was detected in the majority of the tissues samples (liver,

kidney, ileum, and brain) collected at necropsy. The same was not observed for *A. cryaerophilus* and *A. skirrowii* indicating that these species may fail to penetrate the intestinal barrier, with *A. butzleri* showing to be potentially more virulent (Wesley *et al.* 1996). This same behaviour was not observed using poultry models, where *A. butzleri* was unable to colonize conventional chickens and turkeys poults, in contrast to colonization (65 % overall) and mortality (17 to 54 % according to strains used for infection) observed in Beltsville white turkeys (Wesley and Baetz 1999). The results showed that the invasive capacity and virulence of *A. butzleri* strains were host-dependent with respect to species and breed, and may vary also with the challenge strain used (Ho *et al.* 2006a; Wesley and Miller 2010).

An experimental model was carried out using rainbow trout or albino crosses by infection with *A. cryaerophilus*, isolated from rainbow trout naturally infected. The experimental infection caused several clinical abnormalities with degeneration of opercula and gills, kidney and liver damage, and serous fluid in swollen intestines, ending with animals death (Aydin *et al.* 2000, 2002; Yildiz and Aydin 2006).

In a different study, albino rat were used as laboratory animal model by oral challenge with *A. butzleri*. All infected animals produced diarrhoeal disease, with observation of histopathological lesion such as hepatic necrosis, villous erosion, desquamation, matting and necrosis of the segments of small intestine. The gut marked necrosis of the villi was accompanied by infiltration of leukocytes into the *lamina propria* (Adesiji *et al.* 2009). Infection of rats by oral challenge with a single dose of *A. butzleri* or *A. cryaerophilus* strains resulted in diarrhoea and was accompanied by an electrolyte imbalance and alteration of haematological values, with an increase of leukocytes, neutrophils and lymphocytes. The enhance of inocula in cfu used in oral challenge resulted in a disease symptom amplification from mild ill (10^3 cfu) to diarrhoeal disease (10^6-10^9 cfu), suggesting that disease production may be dose dependent (Adesiji *et al.* 2012). The rat diarrhoea was shown to be self limiting without therapeutic intervention, with continuing faecal shedding of *Arcobacter* up to 5 weeks post infection (Adesiji 2010). These works suggests an aetiologic role of *A. butzleri* and *A. cryaerophilus* in diarrhoeal disease.

Fernández *et al.* (2013) used a mouse intraperitoneal passage model showing an increase of the adhesive ability to Hep-2 cells of low adherent *A. butzleri* strains, after several consecutive passages. The authors also reported an increase in *flaA* gene expression in strains derived from increased number of intraperitoneal passages (Fernández *et al.* 2013). Since adhesion is seen as a required step for the setting of infection, the increase of adhesive ability may be related with an increase of disease expression. The enhancement of disease expression was previously described for *C. jejuni* strains in C57BL/6 IL-10-/- mice following serial passages (Bell *et al.* 2009).

Overall, the virulence mechanism of *Arcobacter* was only superficially studied being important to further follow with *in vitro* and *in vivo* studies in order to establish the actual role of these bacteria as intestinal pathogens.

6. Antimicrobial resistance

Resistance to antimicrobials used in human and veterinary have been reported for *Arcobacter* strains isolated from several sources (Table 6). The narrow amount of studies on *Arcobacter* antibiotic susceptibility, limited to three species, namely A. *butzleri*, *A. cryaerophilus* and *A. skirrowii*, averts any epidemiological consideration but highlights the resistance of the genus. The lack of surveillance studies hampers the clear assessment of the current trends in *Arcobacter* resistance and/or the prevalence of this pathogen worldwide.

Macrolides and fluoroquinolones have been considered for treatment of *Campylobacter* enteritis, with tetracyclines rarely been used, and with aminoglycosides being considered for treatment of serious bacteraemia and other systemic infections (Alfredson and Korolik 2007). The increase in *Campylobacter* resistance has been associated with the exposure of this microorganism to antibiotics used in both animal production and human treatment (Luangtongkum *et al*. 2009).

Several antimicrobials have been suggested for the treatment of *Arcobacter* infections, considering the reported case-studies (Table 3), the choice of the antibiotic class for bacteraemia treatment relied on cephalosporins; however regarding intestinal infections the treatment was less consensual with the use of quinolones, tetracycline, macrolide or even a ß-lactam antibiotic combined with a ß-lactamase inhibitor.

The low resistance rate to fluoroquinolones reported by Vandenberg *et al*. (2006) led the authors to suggest that these could be used for treating severe *Arcobacter* enteritis. In another study, tetracycline had also been proposed as a suitable antibiotic for treatment of *Arcobacter* spp., along with aminoglycosides (Son *et al*. 2007b; Abay *et al*. 2012).

Prior to 2012, ciprofloxacin-resistant *Arcobacter* was rarely observed, with a resistance rate ranging between 0-4.4 % (Table 6); however, some reports from Costa Rica, Pakistan and Malaysia indicated that 12.5-33.4 % of *Arcobacter* isolates from animal, water or environmental sources were resistant to ciprofloxacin (Shah *et al*. 2012c, 2013; Villalobos *et al*. 2013), contrasting with a study from Iran that reported 0 and 1.6% of resistance for *A. cryaerophilus* and *A. butzleri* strains isolated from poultry meat, respectively (Rahimi 2014). Kiehlbauch *et al*. (1992) demonstrated that beyond the susceptibility of *A. butzleri* and *A. cryaerophilus* to ciprofloxacin, all the strains were also susceptible to other fluoroquinolones such as enoxacin, norfloxacin and ofloxacin (Kiehlbauch *et al*. 1992); nonetheless reports of resistance to danofloxacin and enrofloxacin were observed in Malaysia and Turkey (Abay *et*

al. 2012; Shah *et al*. 2013; Unver *et al*. 2013). Fera *et al*. (2003) tested the susceptibility of *Arcobacter* isolated from brackish environment to antimicrobials and reported a reduced susceptibility of *A. butzleri* to fluoroquinolones, when compared with *A. cryaerophilus*, with levofloxacin as one of the best performing fluoroquinolones (Fera *et al*. 2003). Regarding the non-fluorinated quinolone, nalidixic acid, several reports have observed resistant strains to this antibiotic (Table 6).

Concerning cloramphenicol susceptibility, differences between studies have been observed (Table 6), likely reflecting local differences on the use of this antibiotic (Otth *et al*. 2004).

The amplitude of macrolide resistance in *Arcobacter* is very broad, ranging from a prevalence of erythromycin and azithromycin resistance of 0 to 100 % (Table 6).

Despite the low number of tested strains, Scanlon *et al*. (2013) described a high percentage of resistance to aminoglycosides gentamicin and kanamycin (80 %) among *Arcobacter* isolates collected from porcine samples (Scanlon *et al*. 2013). In contrast, other authors have identified lower resistance rates for aminoglycosides (0-26.1 %) (Table 6). Resistance to gentamicin, streptomycin, amikacin and neomycin was not reported for human samples (Kiehlbauch *et al*. 1992; Houf *et al*. 2004; Vandenberg *et al*. 2006; Abay *et al*. 2012; Kayman *et al*. 2012b).

A high resistance rate to ampicillin in *A. butzleri* isolates and less commonly in *A. cryaerophilus* was reported for 78 animal and human isolates (Kiehlbauch *et al*. 1992), being supported by other posterior works (Vandenberg *et al*. 2006; Villalobos *et al*. 2013; Serraino *et al*. 2013a). Table 6 also summarises the more limited activity of ß-lactam antibiotics, as penicillins and cephalosporins, and the good activity of tetracyclines against *Arcobacter*.

Arcobacter spp. resistant to fluoroquinolones, macrolides, chloramphenicol, aminoglycosides, penicillins, tetracyclines and other classes of antibiotics have been reported, with a high rate of multidrug resistance, for *Arcobacter* strains from environmental, animal and human origin (Harrass *et al*. 1998; Fera *et al*. 2003; Kabeya *et al*. 2004; Vandenberg *et al*. 2006; Son *et al*. 2007b; Abay *et al*. 2012; Shah *et al*. 2012c, 2013).

Son *et al*. (2007b) reported the existence of multiple resistances in both *A. butzleri* and *A. cryaerophilus* isolates, with 71.8 % of the *Arcobacter* isolates from broiler carcasses presenting resistance to two or more antimicrobials. Furthermore, the percentage of strains with multiple resistances was shown to be higher in *Arcobacter* than in *Campylobacter* strains (Son *et al*. 2007b). *A. butzleri* was described as being more highly multiresistant (72.9 %) than *A. cryaerophilus* (9.1 %) or *A. skirrowii* (13.3 %) (Kabeya *et al*. 2004). Remarkably high values of multidrug resistance have been described for all of the *A. butzleri* isolates, among human and animal strains (Abay *et al*. 2012), with 80.9 % of the *A. butzleri* isolates from broilers (Harrass *et al*. 1998) being resistant to three or more antibacterial agents.

Differences in the susceptibility patterns could be due to the frequent use of drugs in animals for treatment and/or prophylaxis (Rahimi 2014), but could also be due to the lack of

standardization for *Arcobacter* antimicrobial susceptibility tests and absence of established breakpoints. Several different antimicrobial susceptibility tests have been used to determine the percentage of resistance to antibiotics (Table 6). In addition, the absence of specific breakpoints for defining resistance in *Arcobacter* species, by recommendations, have conducted authors to use different breakpoint criteria, such as the Clinical Laboratory Standards Institute (CLSI) criteria established for *Enterobacteriaceae*, or even for *Staphylococcus* spp. (Vandenberg *et al*. 2006), or the U.S. National Antimicrobial Resistance Monitoring System criteria for *Campylobacter* (Son *et al*. 2007b). Overall, lack of standard sensitivity methods and breakpoints makes the comparison of results of antibiotic resistance patterns or the interpretation of results of antimicrobial resistance of *Arcobacter* spp. more difficult and may lead to some real resistance misclassification.

The only resistance mechanisms described for *Arcobacter* were of chromosomal nature (Abdelbaqi *et al*. 2007b; Miller *et al*. 2007), and till now no genes coding to antimicrobial resistance were identified in plasmids (Douidah *et al*. 2014b).

In *Campylobacter*, the resistance mechanisms described to fluoroquinolones concern the inactivation of the target or efflux of fluoroquinolones (Iovine 2013). Fluoroquinolone resistance was associated with specific point mutations in the quinolone resistance determining region (QRDR) of *gyrA* gene, coding for a subunit of DNA gyrase, in position 254 of the *gyrA* gene, resulting in a cytosine to thymine transition. This mutation was only found in two *A. butzleri* and one *A. cryaerophilus* strains isolated from human stools showing resistance to ciprofloxacin (Abdelbaqi *et al*. 2007b). The lack of the associated mutation in *gyrA* of *A. butzleri* RM4018, during DNA sequencing, suggests that the quinolone resistance mechanism to hydrophobic quinolones may be at uptake level, by increasing of the impermeability or by the activity of efflux pumps (Miller *et al*. 2007). Regarding chloramphenicol resistance, it was proposed that this is probably due to the presence of a *cat* gene encoding a chloramphenicol O-acetyltransferase (Miller *et al*. 2007). Also, for ß-lactam resistance an association was established with the presence of three putative ß-lactamases in *A. butzleri* RM4018 genome, which may be strengthened by the *lrgAB* operon. Furthermore, the absence of *upp* gene (encoding for a uracil phosphoribosyltransferase) in *A. butzleri* RM4018 genome may be liable for 5-fluorouracil resistance (Miller *et al*. 2007).

The increased prevalence of antimicrobial resistance has been attributed to selective pressure created by the exposure of food producing animals to veterinary antimicrobials, together with the over-use of antibiotics by human population. The food contamination with resistant bacteria may also led to a transfer of antibiotic resistance determinants to other pathogenic bacteria conducting to unsuccessful treatments of severe infections (Rahimi 2014).

Table 6. Antimicrobial resistance of *Arcobacter* isolates from different sources.

Country	Source	Year (s)	Species	Isolates studied, n	Resistance (%)														Susceptibility test	Reference
					AMP	ERY	CIP	NAL	GEN	TET	AZT	CLI	MET	CET	SMZ/ TMP	CHL	STR	KAN		
Germany	Broilers	1998 [a]	*Ab*[b]	89	78	0	ND	15	0	2	ND	12	ND	ND	100	11	0	0	Disc diffusion	(Harrass *et al*. 1998)
Turkey	Broiler chickens	2001 [a]	*Ab* [c]	39	64	3	3	0	ND	0	ND	ND	ND	100	100	0	ND	ND	Disc diffusion	(Atabay and Aydin 2001)
Belgium[d]	Human	1995-2001	*Ab*	30	ND	20	0	23	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	Agar dilution	(Houf *et al*. 2004)
			Ac	8	ND	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND		
	Poultry	2001-2002	*Ab*	68	ND	4	4	31	2	ND	ND	ND	ND	ND	ND	ND	ND	ND		
			Ac	20	ND	5	0	5	0	ND	ND	ND	ND	ND	ND	ND	ND	ND		
Japan [e]	Retail meat	1998-1999	*Ab*	96	0	0	ND	64	ND	0	ND	ND	97	95	69	30	0	0	Disc diffusion	(Kabeya *et al*. 2004)
			Ac	11	0	0	ND	9	ND	0	ND	ND	100	36	73	0	0	0		
			As	15	0	0	ND	20	ND	0	ND	ND	100	27	53	7	0	0		
Chile	Cattle, pelicans, duck faeces, mussels, chicken livers and river water	2004 [a]	*Ab*	50	90	2	2	ND	0	0	ND	ND	ND	ND	ND	98	ND	ND	E-test	(Otth *et al*. 2004)
Belgium	Human faeces	1995-2005	*Ab*	61	21	21	3	18	0	0	ND	ND	ND	ND	ND	ND	ND	ND	E-test	(Vandenberg *et al*. 2006)
			Ac	10	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND		

Table 6. Antimicrobial resistance of *Arcobacter* isolates from different sources (continuation).

Country	Source	Year (s)	Species	Isolates studied, n	Resistance (%)														Susceptibility test	Reference
					AMP	ERY	CIP	NAL	GEN	TET	AZT	CLI	MET	CET	SMZ/ TMP	CHL	STR	KAN		
USA	Broiler carcasses	2004	*Ab*	140	ND	4	0	24	0	0	81	90	ND	ND	ND	ND	ND	ND	Broth microdilution [f]	(Son *et al.* 2007b)
			Ac	34	ND	0	3	9	0	0	21	82	ND	ND	ND	ND	ND	ND		
Turkey	Human and animals	2006-2010 (human) NR (animals)	*Ab* [g]	70	79	14	ND	44	0	3	ND	ND	ND	ND	100	ND	3	ND	Disc diffusion	(Abay *et al.* 2012)
Turkey	Human	2010-2011	*Ab*	9	100	0	0	22	0	0	ND	ND	ND	ND	ND	ND	ND	ND	E-test	(Kayman *et al.* 2012b)
Pakistan/ Malaysia	Cattle, beef, milk, water and environmental surfaces	2012 [a]	*Ab* [h]	46	74/ 78	70/ 46	22/ 20	ND	26/ 20	6/9	ND	ND	ND	ND	ND	ND	ND	ND	M.I.C.Evaluator /disc diffsuion	(Shah *et al.* 2012c)
Malaysia	Cattle, floor and water from dairy cattle farms	2012 [a]	*Ab* [i]	20	56	7	33	7	4	7	ND	ND	ND	ND	ND	7	ND	ND	Disc diffusion	(Shah *et al.* 2013)
Italy	In-line milk filters used on milking of cows and buffaloes	2011	*Ab*	22	14	0	0	ND	0	0	0	ND	ND	ND	ND	9	ND	ND	E-test	(Serraino *et al.* 2013a)
			Ac	14	7	0	0	ND	0	0	0	ND	ND	ND	ND	0	ND	ND		

Table 6. Antimicrobial resistance of *Arcobacter* isolates from different sources (continuation)

Country	Source	Year (s)	Species	Isolates studied, n	Resistance (%)														Susceptibility test	Reference
					AMP	ERY	CIP	NAL	GEN	TET	AZT	CLI	MET	CET	SMZ/ TMP	CHL	STR	KAN		
Ireland [j]	Porcine samples	2007-2008	*Ab*	5	ND	80	0	100	80	ND	ND	ND	ND	ND	ND	ND	ND	80	Disc diffusion	(Scanlon *et al.* 2013)
			Ac	5	ND	100	0	100	80	ND	ND	ND	ND	ND	ND	ND	ND	80		
Turkey [k]	Cloacal swab from live domestic geese	2013 [a]	*Ab*	3	67	0	ND	ND	ND	ND	ND	ND	ND	100	ND	67	ND	ND	Disc diffusion	(Unver *et al.* 2013)
			Ac	7	86	0	ND	ND	ND	ND	ND	ND	ND	43	ND	0	ND	ND		
			As	7	71	0	ND	ND	ND	ND	ND	ND	ND	0	ND	29	ND	ND		
Costa Rica	Chicken Viscera	2012 [a]	*Ab*	22	88	0	25	ND	12	0	ND	ND	ND	ND	ND	75	ND	ND	E-test	(Villalobos *et al.* 2013)
			Ac	8	0	25	12	ND	0	0	ND	ND	ND	ND	ND	62	ND	ND		
Iran [l]	Poultry meat	2012-2013	*Ab*	64	58	2	2	22	0	0	95	75	94	100	ND	9	0	0	Disc diffusion	(Rahimi 2014)
			Ac	5	80	0	0	40	0	0	20	40	40	40	ND	20	0	0		
			As	2	100	50	0	0	0	0	0	0	50	50	ND	0	0	0		

Ab-*Arcobacter butzleri*; Ac-*Arcobacter cryaerophilus*; As-*Arcobacter skirrowii*; AMP-ampicillin; ERY- Erythromycin; CIP-Ciprofloxacin; NAL- Nalidixic acid; GEN- Gentamicin; TET- Tetracycline; AZT-Azithromycin; CLI-Clindamycin; MET-methicillin; CET-cephalothin; SMZ/TMP sulfamethoxazole – trimethoprim; CHL-chloramphenicol; STR-streptomycin; KAN-kanamycin. NR – not referred, ND – not determined.

[a] Year of report

[b] All strains were also susceptible to minocycline and neomycin and resistant to cefazolin.

[c] All strains were susceptible to amikacin, danofloxacin, enrofloxacin, nitrofurantoin, oxytetracycline, tobramycin, resistant to aztreonam, cefuroxime,orbenin, oxacillin, penicillin G. *A. butzleri* presented resistance 64.1 % to amoxicillin and amoxicillin-clavulanic acid; of 97.4 % to cefoperazone and mezlocillin.

[d] Resistance to doxycycline for *A. butzleri* from human was 16.7 % and from poultry of 13.2 %, with no *A. cryaerophilus* strain resistant.

[e] All strains were resistant to vancomycin.

[f] Protocol established for the U.S. National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria, using a custom-designed *Campylobacter* panel, providing serial dilutions of each antimicrobial, and a Sensititre® semiautomated system.

[g] All strains were resistant to cefuroxime and rifampin, 20 % to amoxycillin-clavulanic acid, 14.3 % to danofloxacin, 12.9 % to enrofloxacin and 4.3 % to neomycine.

[h] Cefotaxime resistance percentage: M.I.C.E., 69.6 %; disk diffusion, 65.2 %.

[i] Resistance to cefotaxime 33.4 %, enrofloxacin 3.7 %.

[j] *A. butzleri* and *A. cryaerophilus* presented a percentage of resistance to trimethoprim of 80 and 60 %, respectively.

[k] All strains were resistant to vancomycin, cefazolin, cloxacillin, optochin and fusidic acid, and none of the strains were resistant to oxytetracycline, nitrofurantoin, ampicillin sulbactam, ofloxacin,and amikacin. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* presented a resistance of 100, 14.3 and 0 % to amoxycillin, 66.7, 28.6 and 0 % to amoxicillin/clavulanic acid, 66.7, 100 and 14.3 % to mezlocillin, 66.7, 42.8 and 0 % to cefuroxime, 0, 14.3 and 0 % to enrofloxacin, 33.3, 71.4 and 57.1 % to rifampicin, respectively.
[l] Resistance to vancomycin of 98.4 % and 60 % to *A. butzleri* and *A. cryaerophilus*, respectively.

7. *Arcobacter* control strategies

The link of *Arcobacter* spp. with human disease together with its detection and prevalence in food and water, have lead to further studies on the survival ability of this microorganism and to search for new treatments to control and eliminate this bacterium.

Studies on *A. butzleri* growth at cold storage temperature have pointed out a viability decrease with exposure time at 4 ºC, with *A. butzleri* being able to survive over 21 days (Hilton *et al*. 2001) or remaining culturable at 5 ºC for more than 77 days in brain heart infusion (BHI) and chicken meat juice medium (CMJ), however with some loss of culturability. Moreover it was demonstrated that *A. butzleri* was able to form biofilms at 5 ºC, as well as at 10 and 21 ºC (Kjeldgaard *et al*. 2009).

At freezing temperature (-20 ºC) stationary growth phase cells demonstrated a decrease of viability after 24 h, being recovered even after 21 days of exposure to that temperature; however exponential growth phase cells showed to be more susceptible with no recovery after 3 days (Hilton *et al*. 2001). *A. butzleri* showed to be able to survive to prolonged incubation at -20 ºC for 6 months in Ellinghausen McCullough Johnson Harris (EMJH) medium (D'Sa and Harrison 2005).

Hilton *et al*. (2001) reported a temperature of 15 ºC as the minimum detectable growth temperature in BHI, with no detectable growth at 40 ºC (Hilton *et al*. 2001). However other studies showed that *A. butzleri* is able of growth at 10 ºC in EMJH, BHI and CMJ media (D'Sa and Harrison 2005; Kjeldgaard *et al*. 2009).

The inactivation of the *Arcobacter* spp. by heat treatment was also documented in a study showing that decimal reduction times (*D*-values) for *A. butzleri* at 55 ºC was 0.4 and 1.1 min for stationary and exponential growth phase cells, respectively. This shows that cells in the stationary phase were more heat sensitive than those in exponential phase (Hilton *et al*. 2001). However, *Arcobacter* strains were more thermotolerant in ground pork (*D*-value 18.51 min) than in phosphate buffer (*D*-value 5.81 min) at 50 ºC (D'Sa and Harrison 2005). *A. butzleri* and *A. cryaerophilus* showed to be more thermotolerant than *Campylobacter*, with a combination between heat and acid pH diminishing heat resistance. Also, a successive combination of heat (50 ºC) followed by cold shock (4 or 8 ºC) worked synergistically in the reduction of *A. butzleri* cell numbers (D'Sa and Harrison 2005).

Van Driessche and Houf (2008) evaluated the survival of three *Arcobacter* species (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) in water at storage (4 and 7 ºC), room (20 ºC) and scalding temperatures, as applied in commercial pork and poultry slaughter (52, 56 and 60 ºC), in order to evaluate the potential role of water as a vector or source of contamination both in environment and in slaughter and meat-cutting facilities. The three *Arcobacter* species remained viable for a temperature-dependent period of time, with all species surviving for at least 21 days in storage and room temperature tests and when incubated at

scalding temperatures still surviving for several minutes. *A. butzleri* showed a higher survival capacity than the two other species for all temperatures tested. *Arcobacter* survival could be extended by the incorporation of organic material in water at low incubation temperatures (4-20 ºC) (Van Driessche and Houf 2008). The ability to survive to 10 min treatments at 50 ºC had previously been described by Phillips and Duggan (2002).

These bacteria are also sensitive to low pH values and were found to have a pH growth ranging from 5.5 to 8.0, with some strains tolerating a pH 5.0, notably at non-optimal growth temperatures (25 ºC), for up to 2 days (D'Sa and Harrison 2005), being the inhibitory pH dependent on the acid used, growth conditions and growth phase (Hilton *et al*. 2001; Phillips and Bates 2004). Regarding resistance to NaCl levels, it was observed that some strains are able to survive at NaCl levels up to 5.0 %, despite the report of *Arcobacter* spp. growth at levels up to 3.0 or 3.5 % of NaCl, depending on species and strains (D'Sa and Harrison 2005).

The apparent described *Arcobacter* resistance coupled with its ability to survive and grow under aerobic conditions, increases the potential of *Arcobacter* spp. as a food and waterborne pathogen (D'Sa and Harrison 2005). Together with this, the ability to adapt to stressful conditions may later protect them against the same type or different types of stresses; this will allow that bacteria exposed to multiple stresses during their lifecycle in the food chain should yet be capable of survival. It was shown that *A. butzleri* cells when adapted to heat stress, induced by incubation for 2 h at 48 ºC were more resistant to subsequent lethal acid stress (pH 4.0) than non-adapted ones at the 1 h time-point. However, no specific adaptive responses against temperature or acid stresses were found for tested conditions (Isohanni *et al*. 2013).

The resistance and possible specific adaptive or cross protection responses should be taken into consideration when designing food decontamination approaches or processing methods.

The effects of common food preservatives, natural products or physical treatments were also studied in several research works. Lactic and citric acids inhibited growth of *A. butzleri*, with nisin alone or in addition to these two organic acids conferring no meaningful improvement. Also, sodium citrate showed to be effective against this bacterium at concentrations used in food preservation (Phillips 1999). Transient exposure to citric acid or continuous exposure to temperatures of 10 ºC or lower, or both, may be successful in reducing survival of *A. butzleri* with nisin addition providing an enhancement on bacterium reduction. The most effective treatment over 24 h of incubation was suggested to be the use of 100 mM citric acid at 4 ºC followed by incubation in the presence of 500 IU/mL nisin (Phillips and Duggan 2002). *A. butzleri* also showed to be sensitive to ethylenediaminetetraacetic acid (EDTA) and trisodium phosphate (TSP), with EDTA or TSP treatment at 4 ºC, along with a following incubation with nisin displaying no recovery of viable cells (Phillips and Duggan 2001). It was proposed that nisin prevents the recovery of sublethally damaged cells, thus ensuring the maintenance of the initial inhibition (Phillips and Duggan 2001, 2002). When using food models, nisin showed to be ineffective against *A. butzleri* in chicken breast skin, however sodium lactate alone or

in combination with nisin was more effective on chicken than in culture (Long and Phillips 2003).

Červenka *et al*. (2003) reported *A. cryaerophilus* to be more susceptible to weak organic acids, pH decrease and sodium chloride concentration than *A. butzleri*, with both species showing to be sensitive to water activity and pH decrease (Červenka *et al*. 2003)

Additionally, the same group evaluated the action of water activity variation in combination with chemical treatment by weak organic acids in *A. butzleri* growth in culture, identifying a discrepancy between the activities of acetic or citric acid with reduced water activity. The combination of citric acid with reduced water activity led to a reduction in *A. butzleri* cell number; however the use of acetic acid caused an increase on survival, suggesting that the observed difference may be associated to the type of weak organic acid used (Červenka *et al*. 2004).

The potential use of organic acids for the inhibition of *Arcobacter* species in food was confirmed by Skřivanová *et al*. (2011) that described the susceptibility to 17 organic acids, with *A. skirrowii* showing to be the most sensitive followed by *A. cryaerophilus* and *A. butzleri*. Due to its ability to suppress bacterial proliferation and because it does not cause any changes on the appearance and odour of treated raw and cooked meat, benzoic acid revealed to be the most convenient organic acid for chicken skin treatment (Skřivanová *et al*. 2011). Meat decontamination by organic acids rinse is approved in the U.S.A. by the Food Safety and Inspection Service of the U.S., and considering the previous studies, it can be seen as an encouraging method for *Arcobacter* spp. reduction in meat (Skřivanová *et al*. 2011).

The use of irradiation and vacuum-packaging were reported as possible methods for diminishing *A. butzleri* food contamination. Irradiation showed to be an effective treatment against *A. butzleri*, yet this bacterium proved to be more tolerant to irradiation in vacuum-packaged ground pork than C. *jejuni* (Collins *et al*. 1996). Balamurugan *et al*. (2013) reported the ability of *A. butzleri* to survive on vacuum packed chill stored beef, showing that *A. butzleri* culturable cells on sterile beef cores stored under vacuum packaged conditions at −1.5 or 4 °C dropped significantly over the time of the study; however when inoculated in commercial vacuum-packaged beef, natural microflora enhanced *A. butzleri* survival (Balamurugan *et al*. 2013).

Aromatic plants have been used through times for their preservative and medicinal properties, and to grant aroma and flavour to food. Natural compounds have been explored as a source for new drugs and in response to the concern of consumers over the safety of synthetic food additives (Edris 2007; Seow *et al*. 2014). For this reason, the effect of natural compounds with antimicrobial activity has been evaluated against *Arcobacter*.

Spice and medicinal herb extracts were tested with respect to its antimicrobial potential, demonstrating that cinnamon, bearberry, chamomile, sage and rosemary extracts, which are traditionally used for the treatment of gastrointestinal disorders, inhibit the growth of *A.*

butzleri, *A. cryaerophilus* and *A. skirrowii* (Červenka *et al*. 2006). Likewise, lemon, sweet orange and bergamot oils and vapours as well as its components were evaluated against *A. butzleri*. Bergamot essential oil showed the highest inhibitory activity, together with both linalool and citral. The strain susceptibility was variable and the compounds were less effective in food models, with a reduced effectiveness in chicken skin when compared with cabbage leaf (Fisher *et al*. 2007). Irkin *et al*. (2011) investigated the inhibitory activity of commercially marketed essential oils against *A. butzleri* and *A. skirrowii* showing that of rosemary, cinnamon, bay and clove had a strong inhibitory effect on *Arcobacter* strains. The essential oil of rosemary activity led to a complete inhibition of *A. butzleri* on a cooked minced beef system (Irkin *et al*. 2011). Moreover, cinnamaldehyde, thymol, carvacrol, caffeic acid, tannic acid, and eugenol presented antimicrobial activity against *A. butzleri, A. cryaerophilus*, and A. *skirrowii* (Červenka *et al*. 2008a). Regarding essential oils, commercial grade orange-based oils showed to have potential to inhibit the growth of *C. jejuni*, *C. coli*, *A. butzleri* and *A. cryaerophilus*, with *Arcobacter* spp. showing overall less susceptibility than *Campylobacter* spp. (Nannapaneni *et al*. 2009).

Control of *Arcobacter* spp. in food may also be related to cross-contamination due for example to contaminated surfaces. *A. butzleri* was found to be able to survive in polypropylene, glass or stainless steel for several hours, however with no culturable cells detected after 24 h (Červenka *et al*. 2008b), also *A. butzleri* seems to be more desiccation resistant than *Campylobacter* species (Otth *et al*. 2001). The high prevalence of *Arcobacter* in slaughterhouses may also be an indication of a failure of disinfection procedure being used. *A. butzleri* is able to survive in 10 % ethanol in culture, and when attached to stainless steel surfaces 22.7 % of the bacteria remain viable after swabbing with 5 % of ethanol solution. Since ethanol commonly makes part of the cleaning regimes constituents, it was suggested that *A. butzleri* may be able to survive such treatments (Phillips and Bates 2004).

Rasmussen *et al*. (2013) tested the sodium hypochloride tolerance of *A. butzleri* with 31 out of 32 slaughterhouse isolates having a minimum inhibitory concentration (MIC) of 0.5 % which corresponds to 500 ppm of active chloride, which is the maximum recommended working solution for this biocide, with the lowest working concentration of sodium chloride (0.2 %) having no lethal effect on *A. butzleri* even after 20 h of exposure. So considering the disinfection contact time of 10 min, and the working solution recommended concentration, it is likely that *A. butzleri* may survive sanitizing (Rasmussen *et al*. 2013). This is supported by the work of Hausdorf *et al*. (2013a), where genetic diversity of *Arcobacter* spp. on the processing line of a spinach-processing plant suggests that the colonization of the plant by *Arcobacter* strains was not affected by repeated cleaning procedures during production, nor by production stops during the winter time (Hausdorf *et al*. 2013a).

These findings reinforce the requirement for strict hygienic practices, implementation of decontamination technologies and evaluation of the efficacy of control treatments to ensure food safety concerning *Arcobacter* spp.

8. References

Abay, S., Kayman, T., Hizlisoy, H. and Aydin, F. (2012) In Vitro Antibacterial Susceptibility of *Arcobacter butzleri* Isolated from Different Sources. *J Vet Med Sci* **74**, 613-616.

Abdelbaqi, K., Buissonniere, A., Prouzet-Mauleon, V., Gresser, J., Wesley, I., Megraud, F. and Ménard, A. (2007a) Development of a real-time fluorescence resonance energy transfer PCR to detect arcobacter species. *J Clin Microbiol* **45**, 3015-3021.

Abdelbaqi, K., Ménard, A., Prouzet-Mauleon, V., Bringaud, F., Lehours, P. and Mégraud, F. (2007b) Nucleotide sequence of the *gyrA* gene of *Arcobacter* species and characterization of human ciprofloxacin-resistant clinical isolates. *FEMS Immunol Med Microbiol* **49**, 337-345.

Actor, J.K., Hwang, S.A. and Kruzel, M.L. (2009) Lactoferrin as a Natural Immune Modulator. *Curr Pharm Des* **15**, 1956-1973.

Adam, Z., Whiteduck-Leveillee, K., Cloutier, M., Chen, W., Lewis, C.T., Levesque, C.A., Topp, E., Lapen, D.R., Tambong, J.T., Talbot, G. and Khan, I.U. (2014) Draft Genome Sequence of *Arcobacter cibarius* Strain LMG21996T, Isolated from Broiler Carcasses. *Genome Announc* **2**, e00034-14.

Adesiji, Y.O., Emikpe, B.O. and Olaitan, J.O. (2009) Histopathological Changes Associated with Experimental Infection of *Arcobacter butzleri* in Albino Rats. *Sierra Leone J Biomed Res* **1**, 4-9.

Adesiji, Y.O. (2010) Faecal shedding of *Arcobacter* species following experimental infection in rats: Public health implications. *Cent Eur J Med* **5**, 470-474.

Adesiji, Y.O., Seibu, E., Emikpe, B.O., Moriyonu, B.T., Oloke, J.K. and Coker, A.O. (2012) Serum biochemistry and heamatological changes associated with graded doses of experimental arcobacter infection in rats. *West Afr J Med* **31**, 186-191.

Adlerova, L., Bartoskova, A. and Faldyna, M. (2008) Lactoferrin: a review. *Vet Med-Czech* **53**, 457-468.

Al Rashid, S.T., Dakuna, I., Louie, H., Ng, D., Vandamme, P., Johnson, W. and Chan, V.L. (2000) Identification of *Campylobacter jejuni*, *C-coli*, *C-lari*, *C-upsaliensis*, *Arcobacter* butzleri, and A-butzleri-like species based on the *glyA* gene. *J Clin Microbiol* **38**, 1488-1494.

Alfredson, D.A. and Korolik, V. (2007) Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol Lett* **277**, 123-132.

Alispahic, M., Hummel, K., Jandreski-Cvetkovic, D., Nobauer, K., Razzazi-Fazeli, E., Hess, M. and Hess, C. (2010) Species-specific identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser

desorption/ionization time of flight mass spectrometry analysis. *J Med Microbiol* **59**, 295-301.

Anderson, K.F., Kiehlbauch, J.A., Anderson, D.C., Mcclure, H.M. and Wachsmuth, I.K. (1993) *Arcobacter*-(*Campylobacter*)-*Butzleri*-Associated Diarrheal Illness in a Nonhuman Primate Population. *Infect Immun* **61**, 2220-2223.

Antolin, A., Gonzalez, I., Garcia, T., Hernandez, P.E. and Martin, R. (2001) *Arcobacter* spp. enumeration in poultry meat using a combined PCR-ELISA assay. *Meat Sci* **59**, 169-174.

Atabay, H.I. and Corry, J.E.L. (1997) The prevalence of campylobacters and arcobacters in broiler chickens. *J Appl Microbiol* **83**, 619-626.

Atabay, H.I. and Corry, J.E. (1998) Evaluation of a new arcobacter enrichment medium and comparison with two media developed for enrichment of *Campylobacter* spp. *Int J Food Microbiol* **41**, 53-58.

Atabay, H.I., Corry, J.E.L. and On, S.L.W. (1998) Diversity and prevalence of *Arcobacter* spp. in broiler chickens. *J Appl Microbiol* **84**, 1007-1016.

Atabay, H.I. and Aydin, F. (2001) Susceptibility of *Arcobacter butzleri* isolates to 23 antimicrobial agents. *Lett Appl Microbiol* **33**, 430-433.

Atabay, H.I., Bang, D.D., Aydin, F., Erdogan, H.M. and Madsen, M. (2002) Discrimination of *Arcobacter butzleri* isolates by polymerase chain reaction-mediated DNA fingerprinting. *Lett Appl Microbiol* **35**, 141-145.

Atabay, H.I., Aydin, F., Houf, K., Sahin, M. and Vandamme, P. (2003) The prevalence of *Arcobacter* spp. on chicken carcasses sold in retail markets in Turkey, and identification of the isolates using SDS-PAGE. *Int J Food Microbiol* **81**, 21-28.

Atabay, H.I., Unver, A., Sahin, M., Otlu, S., Elmali, M. and Yaman, H. (2008a) Isolation of various *Arcobacter* species from domestic geese (*Anser anser*). *Vet Microbiol* **128**, 400-405.

Atabay, H.I., Unver, A., Otlu, S. and Kalaycioglu, A.T. (2008b) Genotyping of various *Arcobacter* species isolated from domestic geese by randomly amplified polymorphic DNA (RAPD) analysis. *Arch Lebensmittelhyg* **59**, 142-146.

Aydin, S., Gultepe, N. and Yildiz, H. (2000) Natural and experimental infections of *Campylobacter cryaerophila* in rainbow trout: Gross pathology, bacteriology, clinical pathology and chemotherapy. *Fish Pathol* **35**, 117-123.

Aydin, S., Engin, M. and Bircan, R. (2002) A comparative investigation of *Arcobacter cryaerophilus* infection among albino crosses and high- and low-body-weight rainbow trout. *J Aquat Anim Health* **14**, 39-44.

Aydin, F., Gümüssoy, K.S., Atabay, H.I., Içа, T. and Abay, S. (2007) Prevalence and distribution of *Arcobacter* species in various sources in Turkey and molecular analysis of isolated strains by ERIC-PCR. *J Appl Microbiol* **103**, 27-35.

Balamurugan, S., Ahmed, R. and Chambers, J.R. (2013) Survival of *Arcobacter butzleri* on vacuum packaged chill stored beef. *Food Res Int* **52**, 503-507.

Bastyns, K., Cartuyvels, D., Chapelle, S., Vandamme, P., Goossens, H. and DeWachter, R. (1995) A variable 23S rDNA region is a useful discriminating target for genus-specific and species-specific PCR amplification in *Arcobacter* species. *Syst Appl Microbiol* **18**, 353-356.

Bath, G.F., Leask, R., Pettey, K.P. and Coetzee, D.J. (2013) Abortions in sheep associated with *Arcobacter skirrowii i*nfection. *J S Afr Vet Assoc* **84**, Art. #952, 4 pages

Bell, J.A., St Charles, J.L., Murphy, A.J., Rathinam, V.A., Plovanich-Jones, A.E., Stanley, E.L., Wolf, J.E., Gettings, J.R., Whittam, T.S. and Mansfield, L.S. (2009) Multiple factors interact to produce responses resembling spectrum of human disease in *Campylobacter jejuni* infected C57BL/6 IL-10-/- mice. *BMC Microbiol* **9**, 57.

Bessede, E., Solecki, O., Sifre, E., Labadi, L. and Megraud, F. (2011) Identification of *Campylobacter* species and related organisms by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. *Clin Microbiol Infec* **17**, 1735-1739.

Brightwell, G., Mowat, E., Clemens, R., Boerema, J., Pulford, D.J. and On, S.L. (2007) Development of a multiplex and real time PCR assay for the specific detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus*. *J Microbiol Methods* **68**, 318-325.

Bücker, R., Troeger, H., Kleer, J., Fromm, M. and Schulzke, J.D. (2009) Arcobacter butzleri Induces Barrier Dysfunction in Intestinal HT-29/B6 Cells. *J Infect Dis* **200**, 756-764.

Bullman, S., O'Leary, J., Corcoran, D., Sleator, R.D. and Lucey, B. (2012) Molecular-based detection of non-culturable and emerging campylobacteria in patients presenting with gastroenteritis. *Epidemiol Infect* **140**, 684-688.

Carbone, M., Maugeri, T.L., Giannone, M., Gugliandolo, C., Midiri, A. and Fera, M.T. (2003) Adherence of environmental *Arcobacter butzleri* and *Vibrio* spp. isolates to epithelial cells in vitro. *Food Microbiol* **20**, 611-616.

Cardarelli-Leite, P., Blom, K., Patton, C.M., Nicholson, M.A., Steigerwalt, A.G., Hunter, S.B., Brenner, D.J., Barrett, T.J. and Swaminathan, B. (1996) Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. *J Clin Microbiol* **34**, 62-67.

Červenka, L., Zachová, I., Minariková, P. and Vytrasová, J. (2003) Effect of pH and water activity on the growth of *Arcobacter* sp. in culture. *Czech J Food Sci* **21**, 203-209.

Červenka, L., Malíková, Z., Zachová, I. and Vytrasová, J. (2004) The effect of acetic acid, citric acid, and trisodium citrate in combination with different levels of water activity on the growth of *Arcobacter butzleri* in culture. *Folia Microbiol* **49**, 8-12.

Červenka, L., Peskova, I., Foltynova, E., Pejchalova, M., Brozkova, I. and Vytrasova, J. (2006) Inhibitory effects of some spice and herb extracts against *Arcobacter butzleri*, *A-cryaerophilus*, and A-skirrowii. *Curr Microbiol* **53**, 435-439.

Červenka, L., Peskova, I., Pejchalova, M. and Vytrasova, J. (2008a) Inhibition of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* by plant oil aromatics. *J Food Protect* **71**, 165-169.

Červenka, L., Kristlova, J., Peskova, I., Vytrasova, J., Pejchalova, M. and Brozkova, I. (2008b) Persistence of *Arcobacter butzleri* CCUG 30484 on Plastic, Stainless Steel and Glass Surfaces. *Braz J Microbiol* **39**, 517-520.

Chen, C.C., Chang, C.J., Lin, T.Y., Lai, M.W., Chao, H.C. and Kong, M.S. (2011) Usefulness of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea. *World J Gastroentero* **17**, 4218-4224.

Chinivasagam, H.N., Corney, B.G., Wright, L.L., Diallo, I.S. and Blackall, P.J. (2007) Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland. *J Appl Microbiol* **103**, 418-426.

Collado, L., Inza, I., Guarro, J. and Figueras, M.J. (2008) Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ Microbiol* **10**, 1635-1640.

Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P. and Figueras, M.J. (2009a) *Arcobacter mytili* sp nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. *Int J Syst Evol Microbiol* **59**, 1391-1396.

Collado, L., Guarro, J. and Figueras, M.J. (2009b) Prevalence of *Arcobacter* in Meat and Shellfish. *J Food Protect* **72**, 1102-1106.

Collado, L., Kasimir, G., Perez, U., Bosch, A., Pinto, R., Saucedo, G., Huguet, J.M. and Figueras, M.J. (2010) Occurrence and diversity of *Arcobacter* spp. along the Llobregat River catchment, at sewage effluents and in a drinking water treatment plant. *Water Res* **44**, 3696-3702.

Collado, L. and Figueras, M.J. (2011) Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. *Clin Microbiol Rev* **24**, 174-192.

Collado, L., Levican, A., Perez, J. and Figueras, M.J. (2011) *Arcobacter defluvii* sp. nov., isolated from sewage samples. *Int J Syst Evol Microbiol* **61**, 2155-2161.

Collado, L., Gutierrez, M., Gonzalez, M. and Fernández, H. (2013) Assessment of the prevalence and diversity of emergent campylobacteria in human stool samples using a combination of traditional and molecular methods. *Diagn Microbiol Infect Dis* **75**, 434-436.

Collins, C.I., Murano, E.A. and Wesley, I.V. (1996) Survival of *Arcobacter butzleri* and *Campylobacter jejuni* after irradiation treatment in vacuum-packaged ground pork. *J Food Protect* **59**, 1164-1166.

Cornelius, A.J., Chambers, S., Aitken, J., Brandt, S.M., Horn, B. and On, S.L. (2012) *Epsilonproteobacteria* in humans, New Zealand. *Emerg Infect Dis* **18**, 510-512.

Cossart, P. and Sansonetti, P.J. (2004) Bacterial invasion: The paradigms of enteroinvasive pathogens. *Science* **304**, 242-248.

D'Sa, E.M. and Harrison, M.A. (2005) Effect of pH, NaCl content, and temperature on growth and survival of *Arcobacter* spp. *J Food Protect* **68**, 18-25.

Dasti, J.I., Tareen, A.M., Lugert, R., Zautner, A.E. and Gross, U. (2010) *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int J Med Microbiol* **300**, 205-211.

De Boer, R.F., Ott, A., Guren, P., van Zanten, E., van Belkum, A. and Kooistra-Smid, A.M. (2013) Detection of *Campylobacter* species and *Arcobacter butzleri* in stool samples by use of real-time multiplex PCR. *J Clin Microbiol* **51**, 253-259.

De Boer, E., Tilburg, J.J.H.C., Woodward, D.L., Lior, H. and Johnson, W.M. (1996) A selective medium for the isolation of *Arcobacter* from meats. *Lett Appl Microbiol* **23**, 64-66.

de Oliveira, S.J., Baetz, A.L., Wesley, I.V. and Harmon, K.M. (1997) Classification of *Arcobacter* species isolated from aborted pig fetuses and sows with reproductive problems in Brazil. *Vet Microbiol* **57**, 347-354.

de Oliveira, S.J., Bernardi, R.T., Vogt, F.I., Scartezzini, M., Hepp, D. and Lunge, V.R. (2010) Gastric Ulcers in Fattening Pigs: Isolation of *Arcobacter* spp. from Stomachs with Different Severity of Lesions. *Acta Sci Vet* **38**, 351-356.

De Smet, S., De Zutter, L., Van Hende, J. and Houf, K. (2010) *Arcobacter* contamination on pre- and post-chilled bovine carcasses and in minced beef at retail. *J Appl Microbiol* **108**, 299-305.

De Smet, S., Vandamme, P., De Zutter, L., On, S.L., Douidah, L. and Houf, K. (2011a) *Arcobacter trophiarum* sp. nov., isolated from fattening pigs. *Int J Syst Evol Microbiol* **61**, 356-361.

De Smet, S., De Zutter, L., Debruyne, L., Vangroenweghe, F., Vandamme, P. and Houf, K. (2011b) *Arcobacter* population dynamics in pigs on farrow-to-finish farms. *Appl Environ Microbiol* **77**, 1732-1738.

De Smet, S., De Zutter, L. and Houf, K. (2011c) Small ruminants as carriers of the emerging foodborne pathogen *Arcobacter* on small and medium farms. *Small Ruminant Res* **97**, 124-129.

Debruyne, L., Houf, K., Douidah, L., De Smet, S. and Vandamme, P. (2010) Reassessment of the taxonomy of *Arcobacter cryaerophilus*. *Syst Appl Microbiol* **33**, 7-14.

Diergaardt, S.M., Venter, S.N., Spreeth, A., Theron, J. and Brozel, V.S. (2004) The occurrence of campylobacters in water sources in South Africa. *Water Res* **38**, 2589-2595.

Donachie, S.P., Bowman, J.P., On, S.L. and Alam, M. (2005) *Arcobacter halophilus* sp nov., the first obligate halophile in the genus *Arcobacter*. *Int J Syst Evol Microbiol* **55**, 1271-1277.

Douidah, L., De Zutter, L., Vandamme, P. and Houf, K. (2010) Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *J Microbiol Methods* **80**, 281-286.

Douidah, L., de Zutter, L., Bare, J., De Vos, P., Vandamme, P., Vandenberg, O., Van den Abeele, A.M. and Houf, K. (2012) Occurrence of putative virulence genes in *Arcobacter* species isolated from humans and animals. *J Clin Microbiol* **50**, 735-741.

Douidah, L., De Zutter, L., Bare, J. and Houf, K. (2014a) Towards a Typing Strategy for *Arcobacter* Species Isolated from Humans and Animals and Assessment of the In Vitro Genomic Stability. *Foodborne Pathog Dis* **11**, 272-280.

Douidah, L., De Zutter, L., Van Nieuwerburgh, F., Deforce, D., Ingmer, H., Vandenberg, O., Van den Abeele, A.M. and Houf, K. (2014b) Presence and analysis of plasmids in human and animal associated arcobacter species. *PloS One* **9**, e85487.

Edris, A.E. (2007) Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: A review. *Phytother Res* **21**, 308-323.

Ellis, W.A., Neill, S.D., O'Brien, J.J., Ferguson, H.W. and Hanna, J. (1977) Isolation of *Spirillum*/*Vibrio*-like organisms from bovine fetuses. *Vet Rec* **100**, 451-452.

Ellis, W.A., Neill, S.D., O'Brien, J.J. and Hanna, J. (1978) Isolation of *spirillum*-like organisms from pig fetuses. *Vet Rec* **102**, 106.

Ertas, N., Dogruer, Y., Gonulalan, Z., Guner, A. and Ulger, I. (2010) Prevalence of *Arcobacter* Species in Drinking Water, Spring Water, and Raw Milk as Determined by Multiplex PCR. *J Food Protect* **73**, 2099-2102.

Fera, M.T., Maugeri, T.L., Giannone, M., Gugliandolo, C., Camera, E.L., Blandino, G. and Carbone, M. (2003) In vitro susceptibility of *Arcobacter butzleri* and *Arcobacter cryaerophilus* to different antimicrobial agents. *Int J Antimicrob Ag* **21**, 488-491.

Fera, M.T., Maugeri, T.L., Gugliandolo, C., Beninati, C., Giannone, M., La Camera, E. and Carbone, M. (2004) Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean Sea. *Appl Environ Microbiol* **70**, 1271-1276.

Fera, M.T., Maugeri, T.L., Gugliandolo, C., La Camera, E., Lentini, V., Favaloro, A., Bonanno, D. and Carbone, M. (2008) Induction and resuscitation of viable nonculturable *Arcobacter butzleri* cells. *Appl Environ Microbiol* **74**, 3266-3268.

Fera, M.T., La Camera, E., Carbone, M., Malara, D. and Pennisi, M.G. (2009) Pet cats as carriers of *Arcobacter* spp. in Southern Italy. *J Appl Microbiol* **106**, 1661-1666.

Fera, M.T., Gugliandolo, C., Lentini, V., Favaloro, A., Bonanno, D., La Camera, E. and Maugeri, T.L. (2010a) Specific detection of *Arcobacter* spp. in estuarine waters of Southern Italy by PCR and fluorescent *in situ* hybridization. *Lett Appl Microbiol* **50**, 65-70.

Fera, M.T., Russo, G.T., Di Benedetto, A., La Camera, E., Orlando, A., Giandalia, A., Ruffa, V.F., Lanza, G., Lentini, V., Perdichizzi, G. and Cucinotta, D. (2010b) High prevalence of arcobacter carriage in older subjects with type 2 diabetes. *J Biomed Biotechnol* **2010**, 489784.

Fernández, H., Eller, G., Paillacar, J., Gajardo, T. and Riquelme, A. (1995) Toxigenic and invasive capacities: possible pathogenic mechanisms in *Arcobacter cryaerophilus*. *Mem I Oswaldo Cruz* **90**, 633-634.

Fernández, H., Krause, S. and Villanueva, M.P. (2004) *Arcobacter butzleri* an emerging enteropathogen: Communication of two cases with chronic diarrhea. *Braz J Microbiol* **35**, 216-218.

Fernández, H., Flores, S. and Inzunza, F. (2010) *Arcobacter butzleri* Strains Isolated from Different Sources Display Adhesive Capacity to Epithelial Cells In Vitro. *Acta Sci Vet* **38**, 287-291.

Fernández, H., Flores, S., Villanueva, M.P., Medina, G. and Carrizo, M. (2013) Enhancing adherence of *Arcobacter butzleri* after serial intraperitoneal passages in mice. *Rev Arg Microbiol* **45**, 75-79.

Figueras, M.J., Collado, L. and Guarro, J. (2008) A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. *Diagn Microbiol Infect Dis* **62**, 11-15.

Figueras, M.J., Collado, L., Levican, A., Perez, J., Solsona, M.J. and Yustes, C. (2011a) *Arcobacter molluscorum* sp. nov., a new species isolated from shellfish. *Syst Appl Microbiol* **34**, 105-109.

Figueras, M.J., Levican, A., Collado, L., Inza, M.I. and Yustes, C. (2011b) *Arcobacter ellisii* sp nov., isolated from mussels. *Syst Appl Microbiol* **34**, 414-418.

Figueras, M.J., Levican, A. and Collado, L. (2012) Updated 16S rRNA-RFLP method for the identification of all currently characterised *Arcobacter* spp. *BMC Microbiol* **12**, 292.

Figueras, M.J., Levican, A., Pujol, I., Ballester, F., Rabada Quilez, M.J. and Gomez-Bertomeu, F. (2014) A severe case of persistent diarrhoea associated with *Arcobacter cryaerophilus* but attributed to *Campylobacter* sp. and a review of the clinical incidence of *Arcobacter* spp. *New Microbes New Infect* **2**, 31-37

Fisher, K., Rowe, C. and Phillips, C.A. (2007) The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components in vitro and on food. *Lett Appl Microbiol* **44**, 495-499.

Flanagan, R.C., Neal-McKinney, J.M., Dhillon, A.S., Miller, W.G. and Konkel, M.E. (2009) Examination of *Campylobacter jejuni* Putative Adhesins Leads to the Identification of a New Protein, Designated FlpA, Required for Chicken Colonization. *Infect Immun* **77**, 2399-2407.

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

Fong, T.T., Mansfield, L.S., Wilson, D.L., Schwab, D.J., Molloy, S.L. and Rose, J.B. (2007) Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. *Environ Health Persp* **115**, 856-864.

Giacometti, F., Lucchi, A., Manfreda, G., Florio, D., Zanoni, R.G. and Serraino, A. (2013) Occurrence and genetic diversity of *Arcobacter butzleri* in an artisanal dairy plant in Italy. *Appl Environ Microbiol* **79**, 6665-6669.

Giacometti, F., Serraino, A., Pasquali, F., De Cesare, A., Bonerba, E. and Rosmini, R. (2014) Behavior of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in Ultrahigh-Temperature, Pasteurized, and Raw Cow's Milk Under Different Temperature Conditions. *Foodborne Pathog Dis* **11**, 15-20.

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

Golla, S.C., Murano, E.A., Johnson, L.G., Tipton, N.C., Cureington, E.A. and Savell, J.W. (2002) Determination of the occurrence of *Arcobacter butzleri* in beef and dairy cattle from Texas by various isolation methods. *J Food Protect* **65**, 1849-1853.

Gonzalez, I., Garcia, T., Antolin, A., Hernandez, P.E. and Martin, R. (2000) Development of a combined PCR-culture technique for the rapid detection of *Arcobacter* spp. in chicken meat. *Lett Appl Microbiol* **30**, 207-212.

Gonzalez, A., Moreno, Y., Gonzalez, R., Hernandez, J. and Ferrus, M.A. (2006) Development of a simple and rapid method based on polymerase chain reaction-based restriction fragment length polymorphism analysis to differentiate *Helicobacter*, *Campylobacter*, and *Arcobacter* species. *Curr Microbiol* **53**, 416-421.

Gonzalez, A., Botella, S., Montes, R.M., Moreno, Y. and Ferrus, M.A. (2007a) Direct detection and identification of *Arcobacter* species by multiplex PCR in chicken and wastewater samples from Spain. *J Food Protect* **70**, 341-347.

Gonzalez, A., Ferrus, M.A., Gonzalez, R. and Hernandez, J. (2007b) Molecular fingerprinting of *Campylobacter* and *Arcobacter* isolated from chicken and water. *Int Microbiol* **10**, 85-90.

Gonzalez, A., Suski, J. and Ferrus, M.A. (2010) Rapid and accurate detection of *Arcobacter* contamination in commercial chicken products and wastewater samples by real-time polymerase chain reaction. *Foodborne Pathog Dis* **7**, 327-338.

Gonzalez, A. and Ferrus, M.A. (2011) Study of *Arcobacter* spp. contamination in fresh lettuces detected by different cultural and molecular methods. *Int J Food Microbiol* **145**, 311-314.

Gonzalez, I., Garcia, T., Fernández, S. and Martin, R. (2012) Current Status on *Arcobacter* Research: An Update on DNA-Based Identification and Typing Methodologies. *Food Anal Method* **5**, 956-968.

Gonzalez, I., Fernández-Tome, S., Garcia, T. and Martin, R. (2014) Genus-specific PCR assay for screening *Arcobacter* spp. in chicken meat. *J Sci Food Agr* **94**, 1218-1224.

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

Gude, A., Hillman, T.J., Helps, C.R., Allen, V.M. and Corry, J.E.L. (2005) Ecology of *Arcobacter* species in chicken rearing and processing. *Lett Appl Microbiol* **41**, 82-87.

Guerrant, R.L., Steiner, T.S., Lima, A.A.M. and Bobak, D.A. (1999) How intestinal bacteria cause disease. *J Infect Dis* **179**, S331-S337.

Gugliandolo, C., Irrera, G.P., Lentini, V. and Maugeri, T.L. (2008) Pathogenic *Vibrio*, *Aeromonas* and *Arcobacter* spp. associated with copepods in the Straits of Messina (Italy). *Mar Pollut Bull* **56**, 600-606.

Hamill, S., Neill, S.D. and Madden, R.H. (2008) A comparison of media for the isolation of *Arcobacter* spp. from retail packs of beef. *J Food Protect* **71**, 850-854.

Harmon, K.M. and Wesley, I.V. (1996) Identification of *Arcobacter* isolates by PCR. *Lett Appl Microbiol* **23**, 241-244.

Harmon, K.M. and Wesley, I.V. (1997) Multiplex PCR for the identification of *Arcobacter* and differentiation of *Arcobacter butzleri* from other arcobacters. *Vet Microbiol* **58**, 215-227.

Harrass, B., Schwarz, S. and Wenzel, S. (1998) Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. *Zentralb Vet Reihe B* **45**, 87-94.

Hausdorf, L., Frohling, A., Schluter, O. and Klocke, M. (2011) Analysis of the bacterial community within carrot wash water. *Can J Microbiol* **57**, 447-452.

Hausdorf, L., Neumann, M., Bergmann, I., Sobiella, K., Mundt, K., Frohling, A., Schluter, O. and Klocke, M. (2013a) Occurrence and genetic diversity of *Arcobacter* spp. in a spinach-processing plant and evaluation of two *Arcobacter*-specific quantitative PCR assays. *Syst Appl Microbiol* **36**, 235-243.

Hausdorf, L., Mundt, K., Winzer, M., Cordes, C., Frohling, A., Schluter, O. and Klocke, M. (2013b) Characterization of the cultivable microbial community in a spinach-processing plant using MALDI-TOF MS. *Food Microbiol* **34**, 406-411.

Higgins, R., Messier, S., Daignault, D. and Lorange, M. (1999) *Arcobacter butzleri* isolated from a diarrhoeic non-human primate. *Lab Anim* **33**, 87-90.

Hilton, C.L., Mackey, B.M., Hargreaves, A.J. and Forsythe, S.J. (2001) The recovery of *Arcobacter butzleri* NCTC 12481 from various temperature treatments. *J Appl Microbiol* **91**, 929-932.

Ho, H., Lipman, L. and Gaastra, W. (2006a) *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! *Vet Microbiol* **115**, 1-13.

Ho, T.K.H., Lipman, L.J.A., van der Graaf-van Bloois, L., van Bergen, M. and Gaastra, W. (2006b) Potential routes of acquisition of *Arcobacter* species by piglets. *Vet Microbiol* **114**, 123-133.

Ho, H., Lipman, L., Hendriks, H., Tooten, P., Ultee, T. and Gaastra, W. (2007) Interaction of *Arcobacter* spp. with human and porcine intestinal epithelial cells. *FEMS Immunol Med Mic* **50**, 51-58.

Ho, H.T.K., Lipman, L.J.A. and Gaastra, W. (2008) The introduction of *Arcobacter* spp. in poultry slaughterhouses. *Int J Food Microbiol* **125**, 223-229.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2000) Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. *FEMS Microbiol Lett* **193**, 89-94.

Houf, K., Devriese, L.A., De Zutter, L., Van Hoof, J. and Vandamme, P. (2001a) Susceptibility of *Arcobacter butzleri*, *Arcobacter cryaerophilus,* and *Arcobacter skirrowii* to antimicrobial agents used in selective media. *J Clin Microbiol* **39**, 1654-1656.

Houf, K., Devriese, L.A., De Zutter, L., Hoof, J.V. and Vandamme, P. (2001b) Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. *Int J Food Microbiol* **71**, 189-196.

Houf, K., De Zutter, L., Van Hoof, J. and Vandamme, P. (2002a) Occurrence and distribution of *Arcobacter* species in poultry processing. *J Food Protect* **65**, 1233-1239.

Houf, K., De Zutter, L., Van Hoof, J. and Vandamme, P. (2002b) Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. *Appl Environ Microbiol* **68**, 2172-2178.

Houf, K., De Zutter, L., Verbeke, B., Van Hoof, J. and Vandamme, P. (2003) Molecular characterization of *Arcobacter* isolates collected in a poultry slaughterhouse. *J Food Protect* **66**, 364-369.

Houf, K., Devriese, L.A., Haesebrouck, F., Vanderberg, O., Butzler, J.P., Hoof, J.V. and Vandamme, P. (2004) Antimicrobial susceptibility patterns of *Arcobacter butzleri* and *Arcobacter cryaerophilus* strains isolated from humans and broilers. *Microb Drug Resist* **10**, 243-247.

Houf, K., On, S.L.W., Coenye, T., Mast, J., Van Hoof, J. and Vandamme, P. (2005) *Arcobacter cibarius* sp nov., isolated from broiler carcasses. *Int J Syst Evol Microbiol* **55**, 713-717.

Houf, K. and Stephan, R. (2007) Isolation and characterization of the emerging foodborn pathogen *Arcobacter* from human stool. *J Microbiol Method* **68**, 408-413.

Houf, K., De Smet, S., Bare, J. and Daminet, S. (2008) Dogs as carriers of the emerging pathogen *Arcobacter*. *Vet Microbiol* **130**, 208-213.

Houf, K., On, S.L., Coenye, T., Debruyne, L., De Smet, S. and Vandamme, P. (2009) *Arcobacter thereius* sp nov., isolated from pigs and ducks. *Int J Syst Evol Microbiol* **59**, 2599-2604.

Hsueh, P.-R., Teng, L.-J., Yang, P.-C., Wang, S.-K., Chang, S.-C., Ho, S.-W., Hsieh, W.-C. and Luh, K.-T. (1997) Bacteremia caused by A*rcobacter cryaerophilus* 1B. *J Clin Microbiol* **35**, 489-491.

Hubert, C.R., Oldenburg, T.B., Fustic, M., Gray, N.D., Larter, S.R., Penn, K., Rowan, A.K., Seshadri, R., Sherry, A., Swainsbury, R., Voordouw, G., Voordouw, J.K. and Head, I.M. (2012) Massive dominance of *Epsilonproteobacteria* in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. *Environ Microbiol* **14**, 387-404.

Hume, M.E., Harvey, R.B., Stanker, L.H., Droleskey, R.E., Poole, T.L. and Zhang, H.B. (2001) Genotypic variation among *Arcobacter* isolates from a farrow-to-finish swine facility. *J Food Protect* **64**, 645-651.

Hurtado, A. and Owen, R.J. (1997) A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. *J Clin Microbiol* **35**, 2401-2404.

Hyun, J., Kanagavelu, S. and Fukata, M. (2013) A unique host defense pathway: TRIF mediates both antiviral and antibacterial immune responses. *Microbes Infect* **15**, 1-10.

Inoue, A., Murata, Y., Takahashi, H., Tsuji, N., Fujisaki, S. and Kato, J. (2008) Involvement of an Essential Gene, *mviN*, in Murein Synthesis in Escherichia coli. *J Bacteriol* **190**, 7298-7301.

Iovine, N.M. (2013) Resistance mechanisms in *Campylobacter jejuni*. *Virulence* **4**, 230-240.

Irkin, R., Abay, S. and Aydin, F. (2011) Inhibitory effects of some plant essential oils against *Arcobacter butzleri* and potential for rosemary oil as a natural food preservative. *J Med Food* **14**, 291-296.

Isohanni, P., Huehn, S., Aho, T., Alter, T. and Lyhs, U. (2013) Heat stress adaptation induces cross-protection against lethal acid stress conditions in *Arcobacter butzleri* but not in *Campylobacter jejuni*. *Food Microbiol* **34**, 431-435.

Jacob, J., Lior, H. and Feuerpfeil, I. (1993) Isolation of *Arcobacter butzleri* from a drinking water reservoir in eastern Germany. *Zbl Hyg Umweltmed* **193**, 557-562.

Jacob, J., Woodward, D., Feuerpfeil, I. and Johnson, W.M. (1998) Isolation of *Arcobacter butzleri* in raw water and drinking water treatment plants in Germany. *Zbl Hyg Umweltmed* **201**, 189-198.

Jiang, Z.D., Dupont, H.L., Brown, E.L., Nandy, R.K., Ramamurthy, T., Sinha, A., Ghosh, S., Guin, S., Gurleen, K., Rodrigues, S., Chen, J.J., McKenzie, R. and Steffen, R. (2010) Microbial etiology of travelers' diarrhea in Mexico, Guatemala, and India: importance of enterotoxigenic *Bacteroides fragilis* and *Arcobacter* species. *J Clin Microbiol* **48**, 1417-1419.

Johnson, L.G. and Murano, E.A. (1999a) Development of a new medium for the isolation of *Arcobacter* spp. *J Food Protect* **62**, 456-462.

Johnson, L.G. and Murano, E.A. (1999b) Comparison of three protocols for the isolation of *Arcobacter* from poultry. *J Food Protect* **62**, 610-614.

Johnson, L.G. and Murano, E.A. (2002) Lack of a cytolethal distending toxin among *Arcobacter* isolates from various sources. *J Food Protect* **65**, 1789-1795

Johnson, J.R., Jelacic, S., Schoenin, L.M., Clabots, C., Shaikh, N., Mobley, H.L.T. and Tarr, P.I. (2005) The IrgA homologue adhesin Iha is an *Escherichia coli* virulence factor in murine urinary tract infection. *Infect Imm* **73**, 965-971.

Jolley, K.A., Chan, M.S. and Maiden, M.C. (2004) mlstdbNet - distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics* **5**, 86.

Kabeya, H., Kobayashi, Y., Maruyama, S. and Mikami, T. (2003a) One-step polymerase chain reaction-based typing of *Arcobacter* species. *Int J Food Microbiol* **81**, 163-168.

Kabeya, H., Maruyama, S., Morita, Y., Kubo, M., Yamamoto, K., Arai, S., Izumi, T., Kobayashi, Y., Katsube, Y. and Mikami, T. (2003b) Distribution of *Arcobacter* species among livestock in Japan. *Vet Microbiol* **93**, 153-158.

Kabeya, H., Maruyama, S., Morita, Y., Ohsuga, T., Ozawa, S., Kobayashi, I., Abe, M., Katsube, Y. and Mikami, T. (2004) Prevalence of *Arcobacter* species in retail meats and antimicrobial susceptibility of the isolates in Japan. *Int J Food Microbiol* **90**, 303-308.

Karadas, G., Sharbati, S., Hanel, I., Messelhausser, U., Glocker, E., Alter, T. and Golz, G. (2013) Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. *J Appl Microbiol* **115**, 583-590.

Karenlampi, R.I., Tolvanen, T.P. and Hanninen, M.L. (2004) Phylogenetic analysis and PCR-restriction fragment length polymorphism identification of *Campylobacter* species based on partial *groEL* gene sequences. *J Clin Microbiol* **42**, 5731-5738.

Kayman, T., Atabay, H.I., Abay, S., Hizlisoy, H., Molva, C. and Aydin, F. (2012a) Human acute gastroenteritis associated with *Arcobacter butzleri*. *Clin Microbiol Newsletter* **34**, 197-199.

Kayman, T., Abay, S., Hizlisoy, H., Atabay, H.I., Diker, K.S. and Aydin, F. (2012b) Emerging pathogen *Arcobacter* spp. in acute gastroenteritis: molecular identification, antibiotic susceptibilities and genotyping of the isolated arcobacters. *J Med Microbiol* **61**, 1439-1444.

Kemp, R., Leatherbarrow, A.J.H., Williams, L.J., Hart, C.A., Clough, H.E., Turner, J., Wright, E.J. and French, N.P. (2005) Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. *Appl Environ Microbiol* **71**, 1876-1882.

Keo, T., Collins, J., Kunwar, P., Blaser, M.J. and Iovine, N.M. (2011) *Campylobacter* capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. *Virulence* **2**, 30-40.

Kiehlbauch, J.A., Brenner, D.J., Nicholson, M.A., Baker, C.N., Patton, C.M., Steigerwalt, A.G. and Wachsmuth, I.K. (1991a) *Campylobacter-Butzleri* Sp-Nov Isolated from Humans and Animals with Diarrheal Illness. *J Clin Microbiol* **29**, 376-385.

Kiehlbauch, J.A., Plikaytis, B.D., Swaminathan, B., Cameron, D.N. and Wachsmuth, I.K. (1991b) Restriction-Fragment-Length-Polymorphisms in the Ribosomal Genes for Species Identification and Subtyping of Aerotolerant *Campylobacter* Species. *J Clin Microbiol* **29**, 1670-1676.

Kiehlbauch, J.A., Baker, C.N. and Wachsmuth, I.K. (1992) *In vitro* susceptibilities of aerotolerant *Campylobacter* isolates to 22 antimicrobial agents. *Antimicrob Agents Ch* **36**, 717-722.

Kim, H.M., Hwang, C.Y. and Cho, B.C. (2009) *Arcobacter marinus* sp. nov. *Int J Syst Evol Microbiol* **60**, 531-536.

Kjeldgaard, J., Jørgensen, K. and Ingmer, H. (2009) Growth and survival at chiller temperatures of *Arcobacter butzleri*. *Int J Food Microbiol* **131**, 256-259.

Kolling, G., Wu, M. and Guerrant, R.L. (2012) Enteric pathogens through life stages. *Front Cell Infect Microbiol* **2**, 114.

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

Kopilovic, B., Ucakar, V., Koren, N., Krek, M. and Kraigher, A. (2008) Waterborne outbreak of acute gastroenteritis in a costal area in Slovenia in June and July 2008. *Euro Surveill* **13**, pii: 18957.

Kownhar, H., Shankar, E.M., Rajan, R., Vengatesan, A. and Rao, U.A. (2007) Prevalence of *Campylobacter jejuni* and enteric bacterial pathogens among hospitalized HIV infected versus non-HIV infected patients with diarrhoea in southern India. *Scand J Infect Dis* **39**, 862-866.

Kumar, S., Ingle, H., Prasad, D.V. and Kumar, H. (2013) Recognition of bacterial infection by innate immune sensors. *Crit Rev Microbiol* **39**, 229-246.

Kumaraswamy, R., Ebert, S., Gray, M.R., Fedorak, P.M. and Foght, J.M. (2011) Molecular- and cultivation-based analyses of microbial communities in oil field water and in microcosms amended with nitrate to control H_2S production. *Appl Microbiol Biotechnol* **89**, 2027-2038.

Kunisawa, J. and Kiyono, H. (2013) Immune regulation and monitoring at the epithelial surface of the intestine. *Drug Discov Today* **18**, 87-92.

Lappi, V., Archer, J.R., Cebelinski, E., Leano, F., Besser, J.M., Klos, R.F., Medus, C., Smith, K.E., Fitzgerald, C. and Davis, J.P. (2013) An outbreak of foodborne illness among attendees of a wedding reception in Wisconsin likely caused by *Arcobacter butzleri*. *Foodborne Pathog Dis* **10**, 250-255.

Lau, S.K.P., Woo, P.C.Y., Teng, J.L.L., Leung, K.W. and Yuen, K.Y. (2002) Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis. *J Clin Pathol-Mol Pa* **55**, 182-185.

Lee, M.H., Cheon, D.S., Choi, S., Lee, B.H., Jung, J.Y. and Choi, C. (2010) Prevalence of *Arcobacter* Species Isolated from Retail Meats in Korea. *J Food Protect* **73**, 1313-1316.

Lee, C., Agidi, S., Marion, J.W. and Lee, J. (2012) *Arcobacter* in Lake Erie beach waters: an emerging gastrointestinal pathogen linked with human-associated fecal contamination. *Appl Environ Microbiol* **78**, 5511-5519.

Lee, M.H. and Choi, C. (2013) Survival of *Arcobacter butzleri* in Apple and Pear Purees. *J Food Safety* **33**, 333-339.

Lerner, J., Brumberger, V. and Preac-Mursic, V. (1994) Severe diarrhea associated with *Arcobacter butzleri*. *Eur J Clin Microbiol Infect Dis* **13**, 660-662.

Levican, A., Collado, L., Aguilar, C., Yustes, C., Dieguez, A.L., Romalde, J.L. and Figueras, M.J. (2012) *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. *Syst Appl Microbiol* **35**, 133-138.

Levican, A. and Figueras, M.J. (2013) Performance of five molecular methods for monitoring *Arcobacter* spp. *BMC Microbiol* **13**, 220.

Levican, A., Collado, L. and Figueras, M.J. (2013a) *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage. *Syst Appl Microbiol* **36**, 22-27.

Levican, A., Alkeskas, A., Gunter, C., Forsythe, S.J. and Figueras, M.J. (2013b) Adherence to and invasion of human intestinal cells by *Arcobacter* species and their virulence genotypes. *Appl Environ Microbiol* **79**, 4951-4957.

Levican, A., Collado, L., Yustes, C., Aguilar, C. and Figueras, M.J. (2014) Water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of *Arcobacter* spp. from shellfish. *Appl Environ Microbiol* **80**, 385-391.

Li, W.J., Raoult, D. and Fournier, P.E. (2009) Bacterial strain typing in the genomic era. *FEMS Microbiol Rev* **33**, 892-916.

Long, C. and Phillips, C.A. (2003) The effect of sodium citrate, sodium lactate and nisin on the survival of *Arcobacter butzleri* NCTC 12481 on chicken. *Food Microbiol* **20**, 495-502.

Lu, L. and Walker, W.A. (2001) Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *Am J Clin Nutr* **73**, 1124s-1130s.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. and Zhang, Q. (2009) Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol* **4**, 189-200.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J.J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. and Spratt, B.G. (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *P Natl Acad Sci USA* **95**, 3140-3145.

Mandisodza, O., Burrows, E. and Nulsen, M. (2012) *Arcobacter* species in diarrhoeal faeces from humans in New Zealand. *N Z Med J* **125**, 40-46.

Marshall, S.M., Melito, P.L., Woodward, D.L., Johnson, W.M., Rodgers, F.G. and Mulvey, M.R. (1999) Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol* **37**, 4158-4160.

Mcclung, C.R., Patriquin, D.G. and Davis, R.E. (1983) *Campylobacter-Nitrofigilis* Sp-Nov, a Nitrogen-Fixing Bacterium Associated with Roots of *Spartina-Alterniflora* Loisel. *Int J Syst Bacteriol* **33**, 605-612.

Merga, J.Y., Leatherbarrow, A.J., Winstanley, C., Bennett, M., Hart, C.A., Miller, W.G. and Williams, N.J. (2011) Comparison of *Arcobacter* isolation methods, and diversity of *Arcobacter* spp. in Cheshire, United Kingdom. *Appl Environ Microbiol* **77**, 1646-1650.

Merga, J.Y., Winstanley, C., Williams, N.J., Yee, E. and Miller, W.G. (2013a) Complete Genome Sequence of the *Arcobacter butzleri* Cattle Isolate 7h1h. *Genome Announc* **1**, pii: e00655-13.

Merga, J.Y., Williams, N.J., Miller, W.G., Leatherbarrow, A.J., Bennett, M., Hall, N., Ashelford, K.E. and Winstanley, C. (2013b) Exploring the Diversity of *Arcobacter butzleri* from Cattle in the UK Using MLST and Whole Genome Sequencing. *PloS One* **8**, e55240.

Mey, A.R., Wyckoff, E.E., Oglesby, A.G., Rab, E., Taylor, R.K. and Payne, S.M. (2002) Identification of the *Vibrio cholerae* enterobactin receptors VctA and IrgA: IrgA is not required for virulence. *Infect Immun* **70**, 3419-3426.

Miller, W.G., Parker, C.T., Rubenfield, M., Mendz, G.L., Wösten, M.M.S.M., Ussery, D.W., Stolz, J.F., Binnewies, T.T., Hallin, P.F., Wang, G., Malek, J.A., Rogosin, A., Stanker, L.H. and Mandrell, R.E. (2007) The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. *PloS One* **2**, e1358.

Miller, W.G., Wesley, I., On, S.L., Houf, K., Mégraud, F., Wang, G., Yee, E., Srijan, A. and Mason, C.J. (2009) First multi-locus sequence typing scheme for *Arcobacter* spp. *BMC Microbiol* **9**, 196.

Monteville, M.R., Yoon, J.E. and Konkel, M.E. (2003) Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology* **149**, 153-165.

Moreno, Y., Botella, S., Alonso, J.L., Ferrus, M.A., Hernandez, M. and Hernandez, J. (2003) Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. *Appl Environ Microbiol* **69**, 1181-1186.

Moreno, Y., Alonso, J.L., Botella, S., Ferrús, M.A. and Hernandez, J. (2004) Survival and injury of *Arcobacter* after artificial inoculation into drinking water. *Res Microbiol* **155**, 726-730.

Morita, Y., Maruyama, S., Kabeya, H., Boonmar, S., Nimsuphan, B., Nagai, A., Kozawa, K., Nakajima, T., Mikami, T. and Kimura, H. (2004) Isolation and phylogenetic analysis of *Arcobacter* spp. in ground chicken meat and environmental water in Japan and Thailand. *Microbiol Immunol* **48**, 527-533.

Musmanno, R.A., Russi, M., Lior, H. and Figura, N. (1997) In vitro virulence factors of *Arcobacter butzleri* strains isolated from superficial water samples. *Microbiologica* **20**, 63-68.

Nannapaneni, R., Chalova, V.I., Crandall, P.G., Ricke, S.C., Johnson, M.G. and O'Bryan, C.A. (2009) *Campylobacter* and *Arcobacter* species sensitivity to commercial orange oil fractions. *Int J Food Microbiol* **129**, 43-49.

Neill, S.D., Campbell, J.N., Obrien, J.J., Weatherup, S.T.C. and Ellis, W.A. (1985) Taxonomic Position of *Campylobacter-Cryaerophila* Sp-Nov. *Int J Syst Bacteriol* **35**, 342-356.

Neubauer, C. and Hess, M. (2006) Detection and identification of food-borne pathogens of the genera *Campylobacter*, *Arcobacter* and *Helicobacter* by multiplex PCR in poultry and poultry products. *J Vet Med B* **53**, 376-381.

Nieva-Echevarria, B., Martinez-Malaxetxebarria, I., Girbau, C., Alonso, R. and Fernández-Astorga, A. (2013) Prevalence and genetic diversity of arcobacter in food products in the north of Spain. *J Food Protect* **76**, 1447-1450.

On, S.L.W., Stacey, A. and Smyth, J. (1995) Isolation of *Arcobacter-Butzleri* from a Neonate with Bacteremia. *J Infect* **31**, 225-227.

On, S.L., Jensen, T.K., Bille-Hansen, V., Jorsal, S.E. and Vandamme, P. (2002) Prevalence and diversity of *Arcobacter* spp. isolated from the internal organs of spontaneous porcine abortions in Denmark. *Vet Microbiol* **85**, 159-167.

On, S.L.W., Harrington, C.S. and Atabay, H.I. (2003) Differentiation of *Arcobacter* species by numerical analysis of AFLP profiles and description of a novel *Arcobacter* from pig abortions and turkey faeces. *J Appl Microbiol* **95**, 1096-1105.

On, S.L.W., Atabay, H.I., Amisu, K.O., Coker, A.O. and Harrington, C.S. (2004) Genotyping and genetic diversity of *Arcobacter butzleri* by amplified fragment length polymorphism (AFLP) analysis. *Lett Appl Microbiol* **39**, 347-352.

Öngör, H., Çetinkaya, B., Açik, M.N. and Atabay, H.I. (2004) Investigation of arcobacters in meat and faecal samples of clinically healthy cattle in Turkey. *Lett Appl Microbiol* **38**, 339-344.

Otth, L., Wilson, M. and Fernández, H. (2001) Desiccation resistance in *Arcobacter butzleri*. *Braz J Microbiol* **32**, 311-312.

Otth, L., Wilson, M., Cancino, R. and Fernández, H. (2004) In vitro susceptibility of *Arcobacter butzleri* to six antimicrobial drugs. *Arch Med Vet* **36**, 207-210.

Pati, A., Gronow, S., Lapidus, A., Copeland, A., Glavina Del Rio, T., Nolan, M., Lucas, S., Tice, H., Cheng, J.F., Han, C., Chertkov, O., Bruce, D., Tapia, R., Goodwin, L., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Detter, J.C., Rohde, M., Goker, M., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Klenk, H.P. and Kyrpides, N.C. (2010) Complete genome sequence of *Arcobacter nitrofigilis* type strain (CI). *Stand Genomic Sci* **2**, 300-308.

Patyal, A., Rathore, R.S., Mohan, H.V., Dhama, K. and Kumar, A. (2011) Prevalence of *Arcobacter* spp. in humans, animals and foods of animal origin including sea food from India. *Transbound Emerg Dis* **58**, 402-410.

Pejchalova, M., Dostalikova, E., Slamova, M., Brozkova, I. and Vytrasova, J. (2008) Prevalence and diversity of *Arcobacter* spp. in the Czech Republic. *J Food Protect* **71**, 719-727.

Pentimalli, D., Pegels, N., Garcia, T., Martin, R. and Gonzalez, I. (2009) Specific PCR Detection of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, and *Arcobacter cibarius* in Chicken Meat. *J Food Protect* **72**, 1491-1495.

Petersen, R.F., Harrington, C.S., Kortegaard, H.E. and On, S.L.W. (2007) A PCR-DGGE method for detection and identification of *Campylobacter*, *Helicobacter*, *Arcobacter* and related *Epsilobacteria* and its application to saliva samples from humans and domestic pets. *J Appl Microbiol* **103**, 2601-2615.

Phillips, C.A. (1999) The effect of citric acid, lactic acid, sodium citrate and sodium lactate, alone and in combination with nisin, on the growth of *Arcobacter butzleri*. *Lett Appl Microbiol* **29**, 424-428.

Phillips, C.A. and Duggan, J. (2001) The effect of EDTA and trisodium phosphate, alone and in combination with nisin, on the growth of *Arcobacter butzleri* in culture. *Food Microbiol* **18**, 547-554.

Phillips, C.A. and Duggan, J. (2002) The effect of temperature and citric acid, alone, and in combination with nisin, on the growth of *Arcobacter butzleri i*n culture. *Food Control* **13**, 463-468.

Phillips, C.A. and Bates, P. (2004) The survival of *Arcobacter butzleri*, an emerging human pathogen, in the presence of acids or ethanol. *Nutr Food Sci* **34**, 210-215.

Pianta, C., Passos, D.T., Hepp, D. and Oliveira, S.J. (2007) Isolation of *Arcobacter* spp from the milk of dairy cows in Brazil. *Cienc Rural* **37**, 171-174.

Pizarro-Cerda, J. and Cossart, P. (2006) Bacterial adhesion and entry into host cells. *Cell* **124**, 715-727.

Prouzet-Mauleon, V., Labadi, L., Bouges, N., Ménard, A. and Megraud, F. (2006) *Arcobacter butzleri:* Underestimated enteropathogen. *Emerg Infect Dis* **12**, 307-309.

Quiñones, B., Parker, C.T., Janda, J.M., Miller, W.G. and Mandrell, R.E. (2007) Detection and genotyping of *Arcobacter* and *Campylobacter* isolates from retail chicken samples by use of DNA oligonucleotide arrays. *Appl Environ Microbiol* **73**, 3645-3655.

Rahimi, E. (2014) Prevalence and antimicrobial resistance of *Arcobacter* species isolated from poultry meat in Iran. *Br Poult Sci (in press)*.

Rasmussen, L.H., Kjeldgaard, J., Christensen, J.P. and Ingmer, H. (2013) Multilocus sequence typing and biocide tolerance of *Arcobacter butzleri* from Danish broiler carcasses. *BMC Res Notes* **6**, 322.

Rescigno, M. (2011) The intestinal epithelial barrier in the control of homeostasis and immunity. *Trends Immunol* **32**, 256-264.

Revez, J., Huuskonen, M., Ruusunen, M., Lindstrom, M. and Hanninen, M.L. (2013) *Arcobacter* Species and Their Pulsed-Field Gel Electrophoresis Genotypes in Finnish Raw Milk during Summer 2011. *J Food Protect* **76**, 1630-1632.

Rice, E.W., Rodgers, M.R., Wesley, I., Johnson, C.H. and Tanner, S.A. (1999) Isolation of *Arcobacter butzleri* from ground water. *Lett Appl Microbiol* **28**, 31-35.

Rivas, L., Fegan, N. and Vanderlinde, P. (2004) Isolation and characterisation of *Arcobacter butzleri* from meat. *Int J Food Microbiol* **91**, 31-41.

Rodriguez-Manzano, J., Alonso, J.L., Ferrus, M.A., Moreno, Y., Amoros, I., Calgua, B., Hundesa, A., Guerrero-Latorre, L., Carratala, A., Rusinol, M. and Girones, R. (2012) Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. *Water Sci Technol* **66**, 2517-2523.

Rojas, C.M., Ham, J.H., Deng, W.L., Doyle, J.J. and Collmer, A. (2002) HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana clevelandii* seedlings. Proc Natl Acad Sci USA. **99**, 13142-13147.

Sabat, A.J., Budimir, A., Nashev, D., Sá-Leão, R., van Dijl, J.M., Laurent, F., Grundmann, H., Friedrich, A.W., on behalf of the ESCMID Study Group of Epidemiological Markers (ESGEM) (2013) Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveill* **18**, 17-30.

Salamaszynska-Guz, A. and Klimuszko, D. (2008) Functional analysis of the *Campylobacter jejuni cj0183* and *cj0588* genes. *Curr Microbiol* **56**, 592-596.

Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M. and Guerrant, R.L. (2007) Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. *J Infect* **54**, 558-566.

Sasi Jyothsna, T.S., Rahul, K., Ramaprasad, E.V., Sasikala, C. and Ramana, C.V. (2013) *Arcobacter anaerophilus* sp. nov., isolated from an estuarine sediment and emended description of the genus *Arcobacter*. *Int J Syst Evol Microbiol* **63**, 4619-4625.

Scanlon, K.A., Cagney, C., Walsh, D., McNulty, D., Carroll, A., McNamara, E.B., McDowell, D.A. and Duffy, G. (2013) Occurrence and characteristics of fastidious *Campylobacteraceae* species in porcine samples. *Int J Food Microbiol* **163**, 6-13.

SchroederTucker, L., Wesley, I.V., Kiehlbauch, J.A., Laron, D.J., Thomas, L.A. and Erickson, G.A. (1996) Phenotypic and ribosomal RNA characterization of *Arcobacter* species isolated from porcine aborted fetuses. *J Vet Diagn Invest* **8**, 186-195.

Scullion, R., Harrington, C.S. and Madden, R.H. (2004) A comparison of three methods for the isolation of *Arcobacter* spp. from retail raw poultry in Northern Ireland. *J Food Protect* **67**, 799-804.

Scullion, R., Harrington, C.S. and Madden, R.H. (2006) Prevalence of *Arcobacter* spp. in raw milk and retail raw meats in northern Ireland. *J Food Protect* **69**, 1986-1990.

Seow, Y.X., Yeo, C.R., Chung, H.L. and Yuk, H.G. (2014) Plant Essential Oils as Active Antimicrobial Agents. *Crit Rev Food Sci* **54**, 625-644.

Serraino, A., Florio, D., Giacometti, F., Piva, S., Mion, D. and Zanoni, R.G. (2013a) Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy. *J Dairy Sci* **96**, 2801-2807.

Serraino, A., Giacometti, F., Daminelli, P., Losio, M.N., Finazzi, G., Marchetti, G., Zambrini, A.V. and Rosmini, R. (2013b) Survival of *Arcobacter butzleri* during production and storage of artisan water buffalo mozzarella cheese. *Foodborne Pathog Dis* **10**, 820-824.

Serraino, A. and Giacometti, F. (2014) Short communication: Occurrence of *Arcobacter* species in industrial dairy plants. *J Dairy Sci* **97**, 2061-2065.

Shah, A.H., Saleha, A.A., Zunita, Z. and Murugaiyah, M. (2011a) *Arcobacter* – An emerging threat to animals and animal origin food products? *Trends Food Sci Tech* **22**, 225–236.

Shah, A.H., Saleha, A.A., Murugaiyah, M. and Zakaria, Z. (2011b) Assessment of sensitivity, specificity and species discriminatory power of four culture-based isolation methods of *Arcobacter* spp. *Afr J Microbiol Res* **5**, 3753-3759.

Shah, A.H., Saleha, A.A., Murugaiyah, M., Zunita, Z. and Memon, A.A. (2012a) Prevalence and distribution of *Arcobacter* spp. in Raw Milk and Retail Raw Beef. *J Food Protect* **75**, 1474-1478.

Shah, A.H., Saleha, A.A., Zunita, Z., Cheah, Y.K., Murugaiyah, M. and Korejo, N.A. (2012b) Genetic characterization of *Arcobacter* isolates from various sources. *Vet Microbiol* **160**, 355-361.

Shah, A.H., Saleha, A.A., Zunita, Z., Murugaiyah, M. and Aliyu, A.B. (2012c) Antimicrobial susceptibility of an emergent zoonotic pathogen, *Arcobacter butzleri*. *Int J Antimicrob Ag* **40**, 569-570.

Shah, A.H., Saleha, A.A., Zunita, Z., Murugaiyah, M., Aliyu, A.B. and Jafri, N. (2013) Prevalence, Distribution and Antibiotic Resistance of Emergent *Arcobacter* spp. from Clinically Healthy Cattle and Goats. *Transbound Emerg Dis* **60**, 9-16.

Skřivanová , E., Molatova, Z., Matenova, M., Houf, K. and Marounek, M. (2011) Inhibitory effect of organic acids on arcobacters in culture and their use for control of *Arcobacter butzleri* on chicken skin. *Int J Food Microbiol* **144**, 367-371.

Slater, F.R., Bailey, M.J., Tett, A.J. and Turner, S.L. (2008) Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiol Ecol* **66**, 3-13.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. and Schleifer, K.H. (1997) Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Appl Environ Microbiol* **63**, 2884-2896.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J. and Harrison, M.A. (2006) Genetic diversity of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *J Food Protect* **69**, 1028-1033.

Son, I., Englen, M.D., Berrang, M., Fedorka-Cray, P.J. and Harrison, M.A. (2007a) Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *Int J Food Microbiol* **113**, 16-22.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J. and Harrison, M.A. (2007b) Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. *Int J Antimicrob Ag* **29**, 451-455.

Suarez, D.L., Wesley, I.V. and Larson, D.J. (1997) Detection of *Arcobacter* species in gastric samples from swine. *Vet Microbiol* **57**, 325-336.

Tabatabaei, M., Aski, H.S., Shayegh, H. and Khoshbakht, R. (2014) Occurrence of six virulence-associated genes in *Arcobacter* species isolated from various sources in Shiraz, Southern Iran. *Microbi Pathogenesis* **66**, 1-4.

Teague, N.S., Srijan, A., Wongstitwilairoong, B., Poramathikul, K., Champathai, T., Ruksasiri, S., Pavlin, J. and Mason, C.J. (2010) Enteric Pathogen Sampling of Tourist Restaurants in Bangkok, Thailand. *J Travel Med* **17**, 118-123.

Tee, W., Baird, R., Dyall-Smith, M. and Dwyer, B. (1988) *Campylobacter cryaerophila* isolated from a human. *J Clin Microbiol* **26**, 2469-2473.

Toh, H., Sharma, V.K., Oshima, K., Kondo, S., Hattori, M., Ward, F.B., Free, A. and Taylor, T.D. (2011) Complete Genome Sequences of *Arcobacter butzleri* ED-1 and *Arcobacter* sp. Strain L, Both Isolated from a Microbial Fuel Cell. *J Bacteriol* **193**, 6411-6412.

Unver, A., Atabay, H.I., Sahin, M. and Celebi, O. (2013) Antimicrobial susceptibilities of various *Arcobacter* species. *Turk J Med Sci* **43**, 548-552.

Van den Abeele, A.M., Vogelaers, D. and Houf, K. (2013) *Arcobacter thereius* Isolations from Human Stool: Another *Arcobacter* Queuing for a Role as Zoonotic Pathogen? In *17th International Workshop on Campylobacter, Helicobacter and Related Organisms*. Aberdeen, Scotland, UK.

Van Driessche, E., Houf, K., Hoof, J.V., De Zutter, L. and Vandamme, P. (2003) Isolation of *Arcobacter* species from animal feces. *FEMS Microbiol Lett* **229**, 243-248.

Van Driessche, E., Houf, K., Vangroenweghe, F., Nollet, N., De Zutter, L., Vandamme, P. and Van Hoof, J. (2004) Occurrence and strain diversity of *Arcobacter* species isolated from healthy Belgian pigs. *Res Microbiol* **155**, 662-666.

Van Driessche, E., Houf, K., Vangroenweghe, F., De Zutter, L. and Van Hoof, J. (2005) Prevalence, enumeration and strain variation of *Arcobacter* species in the faeces of healthy cattle in Belgium. *Vet Microbiol* **105**, 149-154.

Van Driessche, E. and Houf, K. (2007a) Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. *Poult Sci* **86**, 744-751.

Van Driessche, E. and Houf, K. (2007b) Characterization of the *Arcobacter* contamination on Belgian pork carcasses and raw retail pork. *Int J Food Microbiol* **118**, 20-26.

Van Driessche, E. and Houf, K. (2008) Survival capacity in water of *Arcobacter* species under different temperature conditions. *J Appl Microbiol* **105**, 443-451.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* Taxonomy - Emendation of Generic Descriptions and Proposal of *Arcobacter* Gen-Nov. *Int J Syst Bacteriol* **41**, 88-103.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., van den Borre, C., Higgins, R., Hommez, J., Kersters, K., Butzler, J.P. and Goossens, H. (1992a) Polyphasic Taxonomic Study of the Emended Genus *Arcobacter* with *Arcobacter-Butzleri* Comb-Nov and *Arcobacter-Skirrowii* Sp-Nov, an Aerotolerant Bacterium Isolated from Veterinary Specimens. *Int J Syst Bacteriol* **42**, 344-356.

Vandamme, P., Pugina, P., Benzi, G., Van Etterijck, R., Vlaes, L., Kersters, K., Butzler, J.P., Lior, H. and Lauwers, S. (1992b) Outbreak of Recurrent Abdominal Cramps Associated with *Arcobacter-Butzleri* in an Italian School. *J Clin Microbiol* **30**, 2335-2337.

Vandamme, P., Giesendorf, B.A., van Belkum, A., Pierard, D., Lauwers, S., Kersters, K., Butzler, J.P., Goossens, H. and Quint, W.G. (1993) Discrimination of epidemic and sporadic isolates of *Arcobacter butzleri* by polymerase chain reaction-mediated DNA fingerprinting. *J Clin Microbiol* **31**, 3317-3319.

Vandamme, P., Dewhirst, F.E., Paster, B.J. and On, S.L.W. (2005) Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Hoste, Segers, Tytgat and De Ley 1991a, 99VP emend. Vandamme, Vancanneyt, Pot, Mels, Hoste, Dewettinck, Vlaes, Van Den Borre, Higgins, Hommez, Kersters, Butzler and Goossens 1992b, 355. In *Bergey's Manual of Systematic Bacteriology* eds. Brenner, D.J., Kreig, N.P., Staley, J.T. and Garrity, G.M. pp.1161-1165. New York: Springer.

Vandenberg, O., Dediste, A., Houf, K., Ibekwem, S., Souayah, H., Cadranel, S., Douat, N., Zissis, G., Butzler, J.P. and Vandamme, P. (2004) *Arcobacter* species in humans. *Emerg Infect Dis* **10**, 1863-1867.

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, J.P. and Dediste, A. (2006) Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium. *J Antimicrob Chemoth* **57**, 908-913.

Veldhuizen, E.J., Hendriks, H.G.C.J.M., Hogenkamp, A., van Dijk, A., Gaastra, W., Tooten, P.C.J. and Haagsman, H.P. (2006) Differential regulation of porcine beta-defensins 1 and 2 upon *Salmonella* infection in the intestinal epithelial cell line IPI-2I. *Vet Immunol Immunop* **114**, 94-102.

Villalobos, E.G., Jaramillo, H.F., Ulate, C.C. and Echandi, M.L.A. (2013) Isolation and Identification of Zoonotic Species of Genus *Arcobacter* from Chicken Viscera Obtained from Retail Distributors of the Metropolitan Area of San Jose', Costa Rica. *J Food Protect* **76**, 879-882.

Villarruel-Lopez, A., Marquez-Gonzalez, M., Garay-Martinez, L.E., Zepeda, H., Castillo, A., De La Garza, L.M., Murano, E.A. and Torres-Vitela, R. (2003) Isolation of *Arcobacter* spp. from retail meats and cytotoxic effects of isolates against Vero cells. *J Food Protect* **66**, 1374-1378.

Vogel, H.J. (2012) Lactoferrin, a bird's eye view. *Biochem Cell Biol* **90**, 233-244.

Wang, X., Seo, D.J., Lee, M.H. and Choi, C. (2014) Comparison of conventional PCR, multiplex PCR, and loop-mediated isothermal amplification assays for rapid detection of arcobacter species. *J Clin Microbiol* **52**, 557-563.

Watanabe, K., Watanabe, K., Kodama, Y., Syutsubo, K. and Harayama, S. (2000) Molecular characterization of bacterial populations in petroleum-contaminated groundwater discharged from underground crude oil storage cavities. *Appl Environ Microbiol* **66**, 4803-4809.

Wesley, I.V., Baetz, A.L. and Larson, D.J. (1996) Infection of cesarean-derived colostrum-deprived 1-day-old piglets with *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii*. *Infect Immun* **64**, 2295-2299.

Wesley, I.V. and Baetz, A.L. (1999) Natural and experimental infections of *Arcobacter* in poultry. *Poult Sci* **78**, 536-545.

Wesley, I. and Miller, W.G. (2010) *Arcobacter*: an opportunistic human food-borne pathogen? In *Emerging Infections 9* eds. Scheld, W.M., Grayson, M.L. and Hughes, J.M. pp.185-211. Washington: ASM Press.

Wilson, M., Otth, R., Aron, H. and Fernández, H. (2010) Susceptibility of *Arcobacter butzleri* to human blood serum. *Arq Bras Med Vet Zootec* **62**, 232-235.

Wirsen, C.O., Sievert, S.M., Cavanaugh, C.M., Molyneaux, S.J., Ahmad, A., Taylor, L.T., DeLong, E.F. and Taylor, C.D. (2002) Characterization of an autotrophic sulfide-oxidizing marine Arcobacter sp. that produces filamentous sulfur. Appl Environ Microbiol **68**, 316-325.

Woo, P.C.Y., Chong, K.T.K., Leung, K.W., Que, T.L. and Yuen, K.Y. (2001) Identification of *Arcobacter cryaerophilus* isolated from a traffic accident victim with bacteremia by 16S ribosomal RNA gene sequencing. *Diagn Microbiol Infect Dis* **40**, 125-127.

Wybo, I., Breynaert, J., Lauwers, S., Lindenburg, F. and Houf, K. (2004) Isolation of *Arcobacter skirrowii* from a patient with chronic diarrhea. *J Clin Microb* **42**, 1851-1852.

Yan, J.J., Ko, W.C., Huang, A.H., Chen, H.M., Jin, Y.T. and Wu, J.J. (2000) *Arcobacter butzleri* bacteremia in a patient with liver cirrhosis. *J Formos Med Assoc* **99**, 166-169.

Yap, D.Y., Kwan, L.P., To, K.K. and Chan, T.M. (2013) *Arcobacter* peritonitis after fluoroscopic repositioning of a Tenckhoff catheter. *Perit Dial Int* **33**, 222-223.

Yildiz, H. and Aydin, S. (2006) Pathological effects of *Arcobacter cryaerophilus* infection in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Acta Vet Hung* **54**, 191-199.

Zilbauer, M., Dorrell, N., Boughan, P.K., Harris, A., Wren, B.W., Klein, N.J. and Bajaj-Elliott, M. (2005) Intestinal innate immunity to *Campylobacter jejuni r*esults in induction of bactericidal human beta-defensins 2 and 3. *Infect Immun* **73,** 7281-7289.

Zilbauer, M., Dorrell, N., Wren, B.W. and Bajaj-Elliott, M. (2008) *Campylobacter jejuni*-mediated disease pathogenesis: an update. *T Roy Soc Trop Med H* **102**, 123-129.

Chapter 2

Aims of the thesis

Aims of the thesis

Despite several publications concerning the prevalence of *Arcobacter*, there is a lack of studies concerning the Portuguese case and more research is required on the evaluation of the genetic variability, phenotypic features (like the antimicrobial resistance or biofilm-forming ability) and on the pathophysiology of disease associated with this microorganism. Therefore, the main goal of this work was the evaluation of the prevalence of *Arcobacter* spp. in Portuguese food-related and human samples, together with the evaluation of genetic and phenotypic characteristics, as well the pathogenic potential of *A. butzleri*, and to consider the possible use of resveratrol to control *Arcobacter* spp. This work was developed according to the following specific objectives:

1. To evaluate the distribution, genetic diversity, antibiotic resistance and biofilm-formation ability of *Arcobacter* isolated from poultry and from the environment of a Portuguese slaughterhouse.

2. To study the antimicrobial properties of resveratrol against *A. butzleri* and *A. cryaerophilus* as well as it potential cellular targets.

3. To determine the prevalence and diversity of *Arcobacter* and *Campylobacter* spp. in faeces from patients with diarrhoea in Portugal.

4. To study the virulence potential of *A. butzleri*, through the characterization of genotypic and pathogenic properties of human and non-human isolates.

Chapter 3

Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse

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Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse

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ABSTRACT

The genus *Arcobacter* is an emerging pathogen associated with several clinical symptoms. This genus is widely distributed and has been isolated from environmental, animal, food and human samples, where poultry is considered the major source. In this study, forty three *Arcobacter butzleri* strains isolated from poultry and environment of a Portuguese slaughterhouse, were characterized by pulsed field gel electrophoresis (PFGE) and assessed for antimicrobial susceptibility and ability to form biofilms. PFGE patterns obtained using restriction enzymes *SmaI* and *SacII* revealed high genetic diversity, with 32 distinct PFGE patterns. Most of *A. butzleri* isolates presented multiple antimicrobial resistance, exhibiting four different resistance profiles. All 43 isolates were susceptible to gentamicin and 2.3% were resistant to chloramphenicol, in contrast to twenty four (55.8%) that were resistant to ciprofloxacin. Among 36 selected isolates, 26 strains presented biofilm-forming ability, which was dependent on the atmosphere and initial inoculum density.
Overall, the results showed that *A. butzleri* displays a high genetic diversity, and presents resistance to several antibiotics, which together with its biofilm formation ability may represent a potential hazard for foodborne infections and a considerable risk for human health.

1. Introduction

The genus *Arcobacter* was proposed and included in the family *Campylobacteraceae* in 1991 (Vandamme et al., 1991). The genus currently consists of fifteen recognized species (Vandamme et al., 1992; Donachie et al., 2005; Houf et al., 2005; Collado et al., 2009; Houf et al., 2009; Figueras et al., 2010; Collado et al., 2011; Kim et al., 2010; De Smet et al., 2011; Figueras et al., 2011; Levican et al., 2012), of which *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* have been associated with human and animal illness (Ho et al., 2006).

Arcobacter spp. has been isolated from food, water, food processing and handling facilities, and human samples. *Arcobacter* has commonly been isolated from products of animal origin with the highest prevalence found in chickens, followed by pork and beef (Ho et al., 2006). Indeed, consumption of *Arcobacter*-contaminated food or water is regarded as the most probable transmission route to human and animals (Collado and Figueras, 2011).

Studies have documented the presence of *Arcobacter* spp. in the poultry slaughterhouse environment, along equipments' processing line, including the processing water (Houf et al., 2002a, 2003; Gude et al., 2005), as well as on chicken carcasses at different processing stages of slaughter (Son et al., 2007a), and at retail level (Atabay and Corry, 1997; Atabay et al., 1998). Conflicting results regarding the *Arcobacter* presence in the poultry intestine samples have been reported (Atabay and Corry, 1997; Houf et al., 2002a; Atabay et al., 2006; Van Driessche and Houf, 2007; Ho et al., 2008). Nonetheless, the source of poultry contamination is not so far clear.

The broad distribution of *A. butzleri* has been associated with a high genetic diversity, with multiple genotypes being found in a single location, or even in a single animal (Houf et al., 2003; Son et al., 2006).

Additionally to a vast distribution and variability, resistance to common antimicrobial agents was also observed for *Arcobacter* (Kabeya et al., 2004; Son et al., 2007b; Fera et al., 2003; Vandenberg et al., 2006), constituting a concern among *Campylobacteraceae* family.

In most settings (natural, industrial, or clinical), bacteria are usually found in biofilms rather than in the planktonic state. Biofilms also provide important environmental reservoirs for pathogenic bacteria (Parsek and Singh, 2003), supporting their survival in stressful environments, including food processing facilities and slaughterhouses (Chmielewski and Frank, 2003). Bacteria belonging to the genus *Arcobacter* were isolated from biofilms on the carapace of a live lobster, on the sub-water surfaces of the holding facility (Welsh et al., 2011), and on a multispecies anaerobic biofilm inside a reactor (Fernández et al., 2008). Additionally, *A. butzleri* can attach to several water pipes materials (Assanta et al., 2002) and may reside and proliferate in the

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slaughterhouse environment, due to its ability to form biofilm under chilled conditions (Kjeldgaard et al., 2009).

In this study we have analyzed the distribution, genetic diversity, and virulence associated characteristics, namely antibiotic resistance and biofilm-formation ability, of *Arcobacter* isolated from poultry and environment of a Portuguese slaughterhouse.

2. Materials and methods

2.1. Sample collection

During March 2011, three flocks were sampled at the slaughterhouse: one flock from extensive indoor production system, and two flocks of intensive production systems. The poultry production was characterized as extensive indoor production system when: i) animals stocking density was of 12 birds/m^2 or 25 kg/m^2; ii) the animals had no access to exterior and iii) the birds were not slaughtered before 56 days old. For intensive production systems: i) the animals stocking density was of 14–15 birds/m^2 or up to 42 kg/m^2, ii) with no access to exterior and iii) birds were slaughtered at 5–7 weeks of age. From each flock, ten pre-chilling neck skin and ten caeca were randomly taken. In addition, and also for each flock, 15 mL of water draining off from hanging broiler carcasses (carcass drippings) were collected immediately after the first wash following evisceration. All flocks were slaughtered on the same day at the same plant.

Four samples with approximately 1000 cm^2 were collected with surface collection sponges all along the slaughter line, namely from: i) a ramp before the chilling tunnel at the slaughterhouse; ii) the wall of the evisceration area of extensive indoor production flocks; iii) a conveyor belt after chilling tunnel and iv) a support table.

2.2. Sample processing

Sample processing was performed using the media and *Arcobacter* isolation procedure described by Ho et al. (2008) with slight modifications. Briefly, for the isolation of *Arcobacter*, a representative composite sample by flock was conceived as a pool of 10 samples randomly collected of neck skin or caecum (after removal of feces), totalizing 25 g of sample. Then, 225 mL of *Arcobacter* broth (Oxoid, Hampshire, England) with cefoperazone, amphotericin B, teicoplanin (CAT) selective supplement (Oxoid, Hampshire, England) and novobiocin (Sigma, St Louis, USA) (32 mg/L) (from now on referred to as *Arcobacter* enrichment broth) were added and this mixture was homogenized in a Stomacher® Labblender 400. The surface collection sponges were also homogenized with 225 mL of *Arcobacter* enrichment broth. The carcass drippings were added in a 1:1 proportion with *Arcobacter* enrichment broth. All the homogenates were incubated microaerobically (Anoxomat®, MART Microbiology BV, Drachten, The Netherlands) for 72 h at 30 °C.

From each homogenate, 50 μL was dropped on a cellulose-nitrate membrane filter (0.65 or 0.45 μm) placed on selective blood agar plates (Brain heart infusion agar supplemented with 5% (v/v) defibrinated horse blood (Probiologica, Belas, Portugal) and CAT selective supplement, with or without novobiocin (32 mg/L)). After 1 h incubation at 30 °C, in aerobic atmosphere, the filters were removed and filtrates evenly distribute over the agar surface with a sterile loop of inoculation. These plates were incubated for 48 h at 30 °C under microaerophilic conditions.

The faeces were removed from the caecum and the internal surface was scraped. This caecal content was used in direct plating on selective blood agar plates.

After incubation, at least three colonies suspected to be *Arcobacter* spp. were transferred from selective blood agar plates to blood agar plates (blood agar base Nº 2 (Oxoid, Hampshire, England) supplemented with 5% (v/v) defibrinated horse blood), and incubated for 48 h at 30 °C, under microaerophilic conditions.

2.3. Arcobacter multiplex PCR

For identification of the isolates, an *Arcobacter* species specific multiplex PCR assay was performed using 3 μL of lysed bacteria and the primers SKIR, ARCO, BUTZ, CRY1, and CRY2, previously described (Houf et al., 2000). Amplification products were analyzed by electrophoresis in 1.5% agarose in 0.5x TBE buffer (0.9 M Tris, 0.9 M Boric acid, 0.02 M EDTA pH 8.0). Gels were stained with GelRed and visualized by a UV gel documentation system. *A. butzleri* LMG 6620, *A. cryaerophilus* LMG 10244 and *A. skirrowii* LMG 6621 were used as positive controls.

2.4. Pulsed field gel electrophoresis (PGFE)

Genomic DNA fingerprints of all isolates were determined using PFGE, according to the protocol described in Campynet (http://campynet.vetinst.dk/PFGE.html), but using 50 μg/mL of proteinase K in the preparation of DNA plugs.

Agarose embedded DNA was digested overnight at 25 °C with 20 U of *SmaI*. The isolates with undistinguishable *SmaI* patterns were further digested with 20 U of *SacII*, for 4 h at 37 °C.

The restriction fragments of *Arcobacter* isolates were separated by PFGE on 1.4% agarose gels in 0.5x TBE buffer. Electrophoresis was performed in a CHEF-DR III system (Bio-Rad Laboratories), at initial switch time of 5 s; final switch time of 40 s; angle of 120°; gradient of 6.0 V/cm; at 14 °C; running for 22.5 h for *SmaI* and 26 h for *SacII*. After electrophoresis, gels were stained in 1 μg/mL of ethidium bromide solution for 30 min and then washed with distilled water. Gels were visualized with a UV gel documentation system.

The PFGE patterns of *Arcobacter* were analyzed by InfoQuest FP software (version 5.10) to determine strain relatedness. The optimization setting was 1 and 0.54% and the band position tolerance was 1.8 and 1.0% for restriction with *SmaI* and *SacII*, respectively. Cluster analysis was performed by the Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA).

2.5. Antibiotic susceptibility

The minimum inhibitory concentration (MIC) was determined for the following nine antibiotics: Ampicillin, Ciprofloxacin, Vancomycin, Trimethoprim, Piperacillin, Cefoperazone, Chloramphenicol, Gentamicin and Amoxicillin.

Arcobacter isolates grown on blood agar plates for 24 h were suspended in 0.85% (w/V) NaCl and adjusted to a turbidity of 0.5 McFarland (~10^8 CFU/mL, determined by colony count). These cell suspensions were diluted 1:100 on Mueller-Hinton (MH) broth to obtain a density of about 10^6 CFU/mL. Serial two-fold dilutions of each antibiotic were prepared in a 96-well plate (50 μL per well) and inoculated with 50 μL of the bacterial suspension. The plates were incubated at 37 °C for 48 h under microaerophilic conditions. The MIC was confirmed spectrophotometrically at 620 nm, using a cut-off of 0.05. Each experiment was repeated at least three times at each test concentration and the modal MIC values were selected.

To date, no recommendation of breakpoint values for *Arcobacter* is available. The breakpoints used for ampicillin, ciprofloxacin, chloramphenicol, gentamicin and amoxicillin were those of *Campylobacter* species following the National Antimicrobial Resistance Monitoring System criteria (CDC, 2006, 2010). Interpretative criteria for piperacillin, vancomycin, trimethoprim and cefoperazone followed the Clinical and Laboratory Standards Institute protocol M100 (CLSI, 2005).

2.6. Biofilm formation assay and biofilm quantification

The ability of *Arcobacter* strains to form biofilms was assayed using a previously described method, with slight modifications (McLennan et al., 2008; Gaynor et al., 2007). Briefly, cells were grown overnight in MH broth at 37 °C and diluted to an optical density (OD) at 620 nm

of 0.20 (~10^9 CFU/mL) and 0.02 (~10^8 CFU/mL). Of these dilutions, 100 μL was inoculated into 96-well microtiter polystyrene plates, and incubated for 48 h at 37 °C, in microaerophilic and in aerobic conditions. MH broth was used as negative control. After incubation, 25 μL of a 1% crystal violet (CV) solution in 100% ethanol was added to the wells, and incubated at room temperature for 15 min. The wells were then rinsed thoroughly five times with distilled water. Biofilms were quantified by dissolving the remaining CV with a solution composed of 30% methanol and 10% acetic acid, and absorbance was measured at 570 nm.

Biofilm formation by *Arcobacter* was categorized according to the classification system described by Stepanovic et al. (2000).

3. Results

3.1. Isolation and identification of Arcobacter from collected samples

A total of 43 *Arcobacter* spp. isolates were recovered from all the samples collected from the slaughterhouse surfaces, carcass drippings, caecum and neck skin from the three flocks analysed. However, none was isolated from caecal content samples. All the isolates were identified as *A. butzleri* by multiplex PCR.

3.2. Pulsed field gel electrophoresis (PFGE)

A. butzleri isolates and three *A. butzleri* reference strains were further characterized by PFGE, and the cluster analysis of the DNA banding patterns revealed wide heterogeneity among these isolates.

The *SmaI*-PFGE fingerprints contained three to eight bands, clustering strains with similarities varying between 36.4 and 100%. Two PFGE lineages were observed, each including one of the two typed reference strains, which were subdivided into 11 subclusters at 90% similarity, resulting in 28 distinct *SmaI* profiles (Fig. 1).

Overall, among 43 *A. butzleri* isolates, 15 unique and 11 common *SmaI*-PFGE fingerprints were identified. From the 11 common PFGE types observed among 28 isolates, six were observed in isolates from the same source. Even so, within the same flock, genetically different isolates coming from carcass neck skin, caecum and carcass drippings were detected, sharing a similarity as low as 36.4%.

Although not sharing any epidemiological link, the reference strain *A. butzleri* LMG 9869 presented 85.7% similarity with two isolates (both isolates with the same DNA pattern), while the closest isolates to the *A. butzleri* LMG 6620 shared 53.6% similarity with it.

The 28 isolates not distinguished by *SmaI* restriction were further evaluated using a second restriction enzyme (*SacII*) (Fig. 2). *SacII* digestion generated between three and six DNA fragments and produced 17 PFGE distinct fingerprints from the 28 isolates (Fig. 2). The dendrogram analysis showed some strikingly dissimilar isolates, such as AB 17/11 with a similarity of 11% with all other isolates independently of the source. The overall PFGE analysis considering the *SmaI* and *SacII* digestion of *A. butzleri* isolates yielded 32 distinct pulsetypes, demonstrating a high genetic heterogeneity of this species (Table 2).

3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests for nine antibiotics were performed in the 43 *A. butzleri* isolates obtained in this study and the results are resumed in Table 1. All isolates except one (97.7%) exhibited simultaneous resistance to at least six antimicrobials, namely ampicillin, vancomycin, trimethoprim, piperacillin, cefoperazone and amoxicillin. Additionally, one strain presented resistance to chloramphenicol, whereas resistance to ciprofloxacin was observed in 24 of the 43 isolates (55.8%). Susceptibility to gentamicin was observed for all the 43 *Arcobacter* isolates. Isolates exhibiting undistinguishable patterns with the two restriction enzymes, but displaying different ciprofloxacin resistance phenotypes were identified (Table 2).

3.4. Biofilm formation

Biofilm formation tests were performed in the 36 of the 43 *A. butzleri* isolates either presenting different pulsetypes, similar PFGE patterns but different origin, or even different resistance patterns. The influence of the atmosphere (microaerophilic or aerobic) and initial OD of the culture on the improvement of *A. butzleri* biofilm formation was assessed. Under the experimental conditions that included incubation in microaerophilic atmosphere and initial OD at 620 nm of 0.2, a total of 26 of the 36 (72.2%) isolates analyzed formed biofilms. Twenty one (58.3%) isolates were categorized as weakly adherent and five (13.9%) were included in the category of moderately adherent. Four of the latter were recovered from samples from intensive production systems flocks. The biofilm-forming ability of weak adherent isolates ranged from 0.174 to 0.287 (average of 0.226 ± 0.029) units of OD at 570 nm. Moderately adherent isolates showed a range of biofilm ability varying from 0.422 to 0.634 (average of 0.552 ± 0.083). Decreasing the concentration of the initial inoculum to 0.02 changed the categorization of one isolate (AB 10/11) from moderately adherent (OD at 570 nm of 0.422 ± 0.058) to weakly adherent (OD at 570 nm of 0.253 ± 0.033).

In aerobic conditions, starting with an initial OD at 620 nm of 0.02, none of the 36 isolates showed biofilm formation ability, while when increasing the turbidity to 0.2, seven isolates adhered to the tested surface, although presenting a weak adherence. *A. butzleri* reference strain LMG 10828 presented strong adherence to the polystyrene plate under all microaerophilic tested conditions, and under aerobic incubation starting with an OD at 620 nm of 0.2. In contrast, *A. butzleri* LMG 9869 was categorized as weakly and non-adherent under microaerophilic and aerobic atmosphere, respectively, while *A. butzleri* LMG 6620 changed from moderately to weakly adherent, when changing initial inoculum from 0.2 to 0.02 (Fig. 3). In this study, no association between the ability of biofilm formation, PFGE pattern or antibiotic resistance phenotype was found.

4. Discussion

Arcobacter spp., mostly *A. butzleri*, are frequently isolated from products of animal origin, with the highest prevalence occurring on poultry products, although the true occurrence of this potential pathogen in foods might be underestimated (Cervenka, 2007). In this study, poultry and environment from a Portuguese slaughterhouse were surveyed for the presence of *Arcobacter* spp.. All carcass neck skin, caecum, water draining off from hanging broiler carcasses and slaughterhouse processing line surfaces were positive for *A. butzleri*, but no recovery was achieved from caecum content samples. This issue has been controversial as some studies reported no recovery of *Arcobacter* spp. from intestine content (Houf et al., 2002a; Gude et al., 2005), while others reported its presence in the intestinal content of broiler flocks, albeit with very different frequencies (Atabay and Corry, 1997; Atabay et al., 2006; Van Driessche and Houf, 2007; Ho et al., 2008). These discrepant results may reflect different methodological aspects, such as detection methods, the limited number of caeca content analysed or even the place of collection.

In contrast, the high prevalence of *Arcobacter* spp. on chicken carcasses and slaughterhouse environment has been described (Houf et al., 2002a, 2003; Gude et al., 2005). Gude et al. (2005) suggested that *Arcobacter* spp. seems to colonize the abattoir environment and/or chicken carcasses during processing; nevertheless it appeared not to belong to normal flora of chickens during rearing. Moreover, Ho et al. (2008) suggested that *Arcobacter* spp. are imported into slaughterhouses with the contents of chicken's guts and spread from the intestines to carcasses during processing in the slaughterhouses. This suggestion was based on the detection of same *Arcobacter* genotypes in both intestines and carcasses of the same flock. In the present study, *Arcobacter* PFGE patterns from caecum and carcass neck skin, carcass

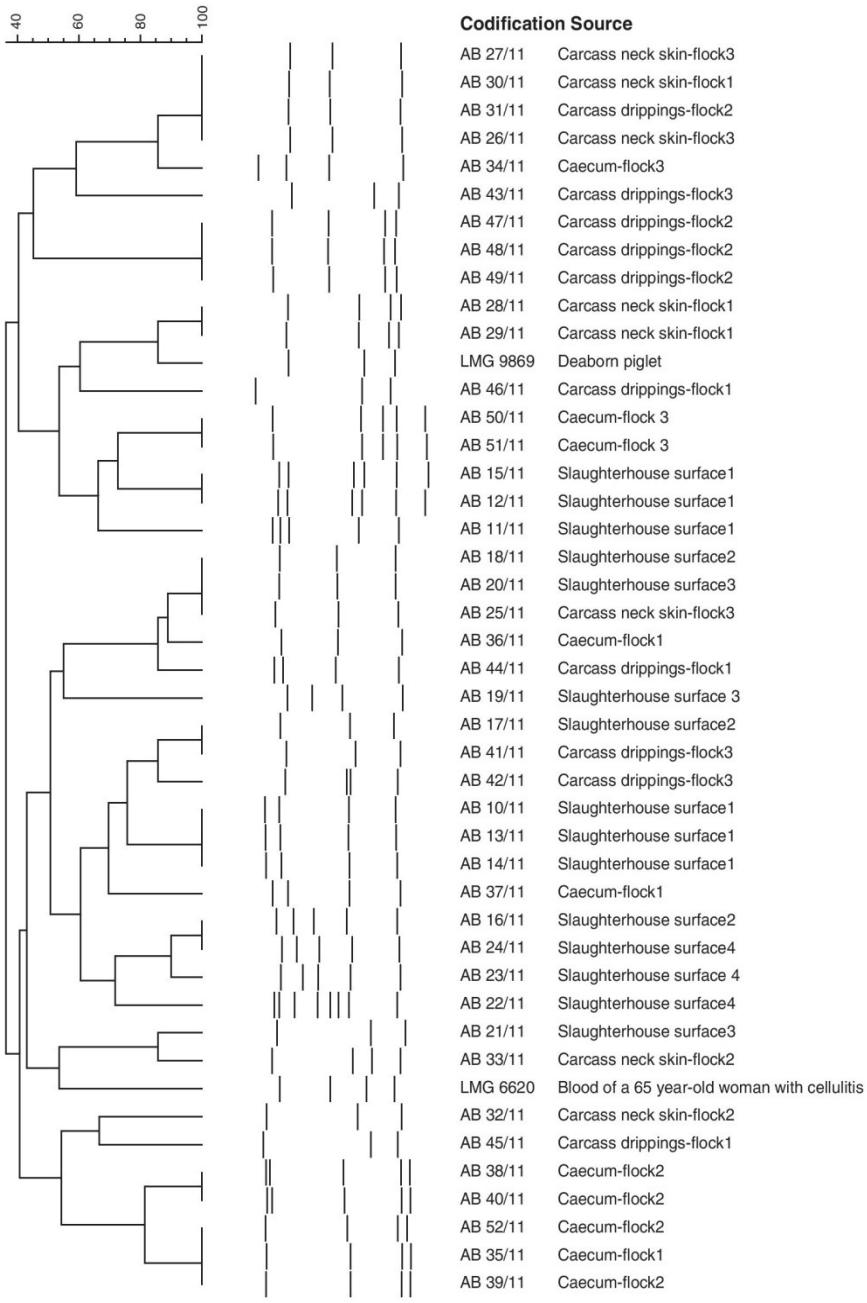

Fig. 1. Dendrogram of *Arcobacter butzleri* isolates based on pulsed-field gel electrophoresis profiles using *SmaI*. The dendrogram was generated with InfoQuest FP software from cluster analysis using UPGMA and the Dice similarity coefficient.

drippings or any of the tested slaughterhouse surfaces appeared not to be correlated. The high genetic heterogeneity among the *Arcobacter* poultry isolates has been explained by multiple sources of contamination, the presence of multiple genotypes in a single animal and a high degree of genomic recombination (Houf et al., 2002b). Another explanation for this high genetic diversity may be the cross-contamination among slaughtered chickens within one flock and from flocks of different farms (Ho et al., 2008). Accordingly, *SmaI* and *SacII* analysis indicates the putative existence of cross-contamination along the slaughterhouse processing line. Despite the high genetic variability of *A. butzleri*, the existence of undistinguishable pulsetypes between different collection samples, such as isolates from surface samples of different collection areas of slaughterhouse, may support the aforementioned. The isolates obtained from the pool of skin neck presented different genotypes from those of caecum, however when considering the caecum's isolates, one same genotype was isolated from flock 1 and 2, both from intensive production system but from different farms. The possible cross-contamination seems to be unavoidable due to slaughterhouse logistics, leading to a high distribution of contamination. However, the contamination exclusively through slaughter equipment may not explain the

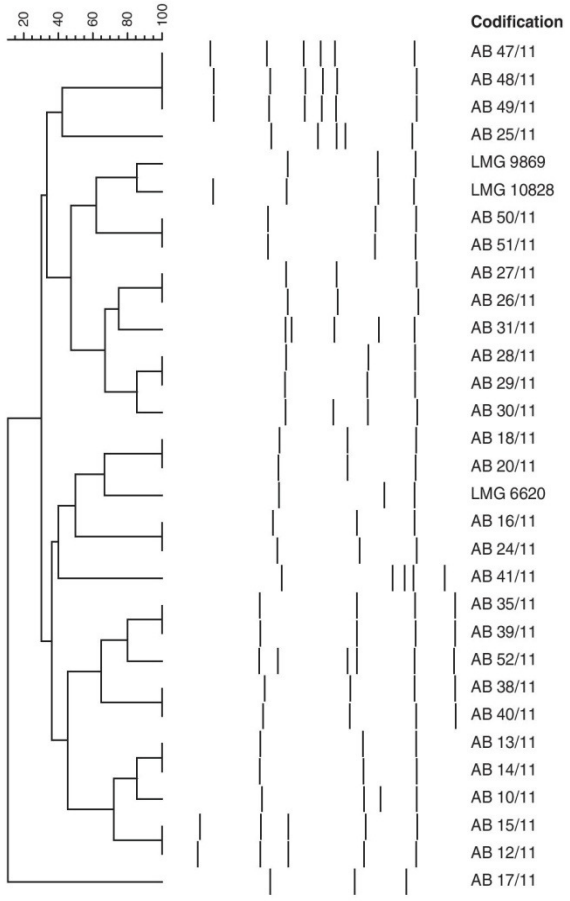

Fig. 2. *SacII*-pulsed field gel electrophoresis genotypic patterns diversity obtained among the 28 *Arcobacter butzleri* isolates not discriminated with *SmaI*. The dendrogram was generated with InfoQuest FP software from cluster analysis using UPGMA and the Dice similarity coefficient.

high levels of contamination found in poultry products (Houf et al., 2002a).

The extended use of antibiotics for disease control in modern food animal production, leads to a spread of antibiotic resistant bacteria capable of causing infections in animals and humans (Bronzwaer et al., 2008). In the present study, *A. butzleri* isolates displayed a high degree of resistance to several antibiotics, which may constitute an intrinsic resistance, as for vancomycin, but it may be acquired by prolonged antibiotic exposure, as is the case of quinolones. In contrast, low levels of resistance were found against chloramphenicol (2.3%), and all strains were susceptible to gentamicin, in agreement with previous studies (Atabay and Aydin, 2001; Houf et al., 2004; Otth et al., 2004; Vandenberg et al., 2006; Son et al., 2007b; Abay et al., 2012).

Table 1
Antibiotic resistance profile of the 43 *Arcobacter butzleri* isolates.

Antibiotic	Resistant breakpoint	MIC_{50} (mg/L)[a]	MIC_{90} (mg/L)[b]	MIC range	N. (%) of resistant isolates
Ampicillin	≥32	128	256	1/>256	42 (97.7)
Ciprofloxacin	≥4	4	>8	0.03/>8	24 (55.8)
Vancomycin	≥32	>512	>512	512/>512	43 (100)
Trimethoprim	≥16	>512	>512	>512	43 (100)
Piperacillin	≥128	512	>512	8/>512	42 (97.7)
Cefoperazone	≥64	>512	>512	16/>512	42 (97.7)
Chloramphenicol	≥32	8	16	4/32	1 (2.3)
Gentamicin	≥8	0.5	1	0.125/1	0 (0)
Amoxicillin	≥32	64	128	1/128	42 (97.7)

[a,b] MIC_{50} and MIC_{90} indicate the concentration (mg/L) at which 50% and 90% of the isolates tested were inhibited by the antibiotic, respectively.

Concerning chloramphenicol, differences in the susceptibility to this antibiotic have been reported, where several authors found high chloramphenicol resistance in human (98%) (Otth et al., 2004) or retail meats isolates (30.2%) (Kabeya et al., 2004), whereas Atabay and Aydin (2001) reported chloramphenicol susceptibility for 100% of isolates from broiler chickens in Turkey.

Houf et al. (2004) found that *A. butzleri* strains isolated from poultry had decreased susceptibility to ciprofloxacin when compared to human strains, relating this fact with the use of fluoroquinolones for treatment of poultry. The high prevalence of ciprofloxacin resistance observed in our work, in contrast to low levels or even absence of resistance reported by others (Houf et al., 2004; Son et al., 2007b), as well as the high level of resistance to amoxicillin, is likely associated with the intensive use of these antibiotic groups in birds' rearing in Portugal (Anonymous, 2010). Studies held in Italy and Turkey also reported high level of resistance to amoxicillin for *Arcobacter* isolates (Fera et al., 2003; Atabay and Aydin, 2001).

Resistance to ampicillin has been reported previously for *A. butzleri* isolated from different sources. A resistance rate of 64.1% was reported for *A. butzleri* isolates recovered from broiler chickens in Turkey (Atabay and Aydin, 2001), which is below the level of resistance observed in the present study (97.7%). Nonetheless, the lack of a standardized method for antibiotic susceptibility determination and breakpoint recommendations for *Arcobacter*, make it difficult to compare results from different studies. Even so, the present study shows high levels of resistance of *A. butzleri* strains isolated in one-day work from the environment of one Portuguese slaughterhouse and from the poultry of three different farms. High level of antibiotic resistance was reported for other bacterial genus isolated from Portuguese poultry or poultry products, such as *Listeria* spp. (Antunes et al., 2002), *Salmonella* spp. (Antunes et al., 2003), *Campylobacter* spp. (Mena et al., 2008) and Enterococci (Novais et al., 2005).

Biofilm formation ability has been associated with bacterial virulence, colonization, environmental survival and antibiotic resistance (Gaynor et al., 2007). The ability of *Arcobacter* to adhere to inert surfaces in the form of biofilms (Kjeldgaard et al., 2009) and to attach to water pipes materials (Assanta et al., 2002), together with the high prevalence of *Arcobacter* spp. in slaughterhouse equipment, makes the evaluation of the biofilm-forming ability of *Arcobacter* isolates a relevant subject.

The majority (58.3%) of the *A. butzleri* isolates studied for its ability to form biofilms were defined as weakly adherent, and 27.8% did not show *in vitro* adherence. Moreover, a negative effect of aerobic atmosphere in biofilm formation was observed, similarly to what has been described for *Campylobacter jejuni* (Reeser et al., 2007). However, there is no consensus, as Reuter et al. (2010) showed that under aerobic conditions, *C. jejuni* biofilm formation increased. Nonetheless, the overall results support the ability of *Arcobacter* strains to form biofilms, and the presence of other bacteria in slaughterhouse environment may suggest that *A. butzleri* may incorporate a pre-established biofilm as a secondary colonizer, as was previously described for *C. jejuni*, (Hanning et al., 2008).

All except one of the five moderately adherent isolates were collected in samples from intensive production systems flocks, suggesting a possible association between biofilm forming ability and broiler production system. The adherence of *A. butzleri* to surfaces, albeit weak, may in part explain the high prevalence and survival of *Arcobacter* in slaughterhouse environment. Biofilm formation may also provide explanation for the discrepancies between the low prevalence of *Arcobacter* spp. in chickens gut, and the high prevalence in chicken carcasses and on slaughterhouse equipment. This suggests that *A. butzleri* may reside and proliferate in the slaughterhouse environment and contaminate

Table 2
Biofilm-forming ability, PFGE pattern, resistance phenotype and specimen source of 36 selected *Arcobacter butzleri* isolates and the three reference strains.

Strain code	Specimen source	PFGE pattern		Resistance phenotype	Biofilm-forming ability[a]
		SmaI[b]	*SacII*[c]		
AB 28/11	Carcass neck skin-flock1	A	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Moderately adherent
AB 11/11	Slaughterhouse surface 1	B	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 50/11	Caecum-flock3	C	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 12/11	Slaughterhouse surface 1	D	1	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 15/11	Slaughterhouse surface 1	D	1	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 47/11	Carcass drippings-flock2	E	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 26/11	Carcass neck skin-flock3	F	2	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 30/11	Carcass neck skin-flock1	F	3	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Moderately adherent
AB 31/11	Carcass drippings-flock2	F	4	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Moderately adherent
AB 34/11	Caecum-flock3	G	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 43/11	Carcass drippings-flock3	H	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 18/11	Slaughterhouse surface 2	I	5	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 20/11	Slaughterhouse surface 3	I	5	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 25/11	Carcass neck skin –flock3	I	6	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 36/11	Caecum –flock1	J	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Moderately adherent
AB 44/11	Carcass drippings-flock1	K	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 19/11	Slaughterhouse surface 3	L	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 17/11	Slaughterhouse surface 2	M	7	Cip, Van, Tmp	Weakly adherent
AB 41/11	Carcass drippings-flock3	M	8	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 42/11	Carcass drippings-flock3	N	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 10/11	Slaughterhouse surface 1	O	9	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Moderately adherent
AB 13/11	Slaughterhouse surface 1	O	10	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 37/11	Caecum –flock1	P	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 16/11	Slaughterhouse surface 2	Q	11	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 24/11	Slaughterhouse surface 4	Q	11	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 23/11	Slaughterhouse surface 4	R	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 22/11	Slaughterhouse surface 4	S	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 32/11	Carcass neck skin-flock2	T	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 45/11	Carcass drippings-flock1	U	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 38/11	Caecum-flock2	V	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 35/11	Caecum-flock1	W	12	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 39/11	Caecum-flock2	W	12	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
AB 52/11	Caecum-flock2	W	13	Amp, Van, Tmp, Pip, Cfp,Amx	Non-adherent
AB 21/11	Slaughterhouse surface 3	X	–[d]	Amp, Cip, Van, Tmp, Pip, Cfp, Amx	Non-adherent
AB 33/11	Carcass neck skin-flock2	Y	–[d]	Amp, Van, Tmp, Pip, Cfp,Chl, Amx	Non-adherent
AB 46/11	Carcass drippings-flock1	Z	–[d]	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
LMG 6620	Human blood sample	Aa	14	Amp, Van, Tmp, Pip, Cfp	Moderately adherent
LMG 9869	Deadborn piglet	Bb	15	Amp, Van, Tmp, Pip, Cfp, Amx	Weakly adherent
LMG 10828	Human Diarrhoeal faeces	N.T.	16	Amp, Van, Tmp, Pip, Cfp, Amx	Strongly adherent

N.T. Non-typable.
Flock 1 and Flock 2 were from intensive production systems; Flock 3 was from extensive indoor production system.
Amp, ampicillin; Cip, ciprofloxacin; Van, vancomycin; Tmp, Trimethoprim; Pip, piperacillin; Cfp, Cefoperazone; Chl, Chloramphenicol; Amx, amoxicillin.
[a] Microaerophilic incubation and initial OD at 620 nm of 0.2.
[b] *SmaI* PFGE pattern types are arbitrarily designated alphabetically.
[c] *SacII* PFGE pattern types are arbitrarily designated numerically.
[d] Not tested with *SacII*.

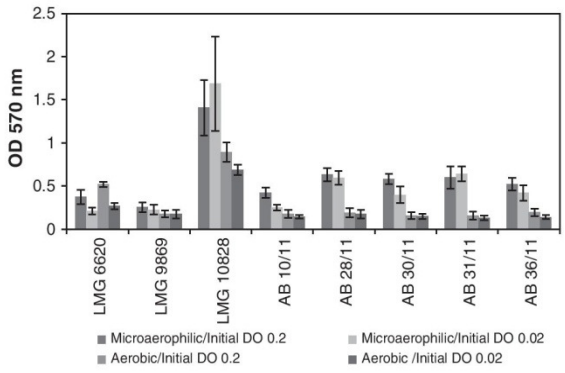

Fig. 3. Biofilm formation by the three *Arcobacter butzleri* reference strains and the 5 isolates presenting moderately adherence, according to type of atmosphere and initial inoculum. Values are expressed as mean + SD. All experiments were carried out at least in triplicate and repeated in at least two independent sets of experiments.

meat products, by biofilm formation under chilled conditions (Kjeldgaard et al., 2009). However, studies on *Arcobacter* spp. biofilm formation are still scarce (Kjeldgaard et al., 2009), and much work is needed to assess its truly importance as a virulence determinant and its impact in the survival and spread of this pathogen.

The findings of the current study confirm the high degree of genetic heterogeneity observed among *A. butzleri* isolates, along with the occurrence of multiple drugs resistance. Adherence ability of *A. butzleri* to surfaces can possibly favour dispersion and cross-contamination along the slaughterhouse processing line. These data contribute for understanding the survival and persistence mechanisms of this organism in the environment and on its relevance as a human pathogen in the food chain.

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References

Abay, S., Kayman, T., Hizlisoy, H., Aydin, F., 2012. *In vitro* antibacterial susceptibility of *Arcobacter butzleri* isolated from different sources. Journal of Veterinary Medical Science 74, 613–616.

Anonymous, 2010. Relatório de Monitorização do Consumo de Antimicrobianos — Portugal (Direcção Geral de Veterinária), Portugal.

Antunes, P., Reu, C., Sousa, J.C., Pestana, N., Peixe, L., 2002. Incidence and susceptibility to antimicrobial agents of *Listeria* spp. and *Listeria monocytogenes* isolated from poultry carcasses in Porto, Portugal. Journal of Food Protection 65, 1888–1893.

Antunes, P., Reu, C., Sousa, J.C., Peixe, L., Pestana, N., 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. International Journal of Food Microbiology 82, 97–103.

Assanta, M.A., Roy, D., Lemay, M.J., Montpetit, D., 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. Journal of Food Protection 65, 1240–1247.

Atabay, H.I., Aydin, F., 2001. Susceptibility of *Arcobacter butzleri* isolates to 23 antimicrobial agents. Letters in Applied Microbiology 33, 430–433.

Atabay, H.I., Corry, J.E.L., 1997. The prevalence of campylobacters and arcobacters in broiler chickens. Journal of Applied Microbiology 83, 619–626.

Atabay, H.I., Corry, J.E.L., On, S.L.W., 1998. Diversity and prevalence of *Arcobacter* spp. in broiler chickens. Journal of Applied Microbiology 84, 1007–1016.

Atabay, H.I., Waino, M., Madsen, M., 2006. Detection and diversity of various *Arcobacter* species in Danish poultry. International Journal of Food Microbiology 109, 139–145.

Bronzwaer, S., Aarestrup, F., Battisti, A., Bengtsson, B., Duran, S.P., Emborg, H.D., Kahlmeter, G., Mevius, D., Regula, G., Sanders, P., Teale, C., Wasyl, D., De Smet, K., Edo, J.T., Tuell, P., Deluyker, H., Makela, P., Authority, E.F.S., 2008. Harmonised monitoring of antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from food animals in the European Union. Clinical Microbiology and Infection 14, 522–533.

CDC, 2006. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): 2003 Human Isolates Final Report. U.S. Department of Health and Human Services, CDC, Atlanta, Georgia.

CDC, 2010. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2009. U.S. Department of Health and Human Services, CDC, Atlanta, Georgia.

Cervenka, L., 2007. Survival and inactivation of *Arcobacter* spp., a current status and future prospect. Critical Reviews in Microbiology 33, 101–108.

Chmielewski, R.A.N., Frank, J.F., 2003. Biofilm formation and control in food processing facilities. Comprehensive Reviews in Food Science and Food Safety 2, 22–32.

Clinical and Laboratory Standards Institute, 2005. Performance standards for antimicrobial susceptibility testing; fifteenth informational supplement. CLSI Document M100-S15 Clinical and Laboratory Standards Institute, Pennsylvania, USA.

Collado, L., Figueras, M.J., 2011. Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. Clinical Microbiology Reviews 24, 174–192.

Collado, L., Cleenwerck, I., Stefanie, V.T., Paul, D.V., Figueras, M.J., 2009. *Arcobacter mytili* sp. Nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. International Journal of Systemic and Evolutionary Microbiology 59, 1391–1396.

Collado, L., Levican, A., Perez, J., Figueras, M.J., 2011. *Arcobacter defluvii* sp. nov., isolated from sewage samples. International Journal of Systematic and Evolutionary Microbiology 61, 2155–2161.

De Smet, S., Vandamme, P., De Zutter, L., On, S.L.W., Douidah, L., Houf, K., 2011. *Arcobacter trophiarum* sp. nov. isolated from fattening pigs. International Journal of Systematic and Evolutionary Microbiology 61, 356–361.

Donachie, S.P., Bowman, J.P., On, S.L.W., Alam, M., 2005. *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. International Journal of Systematic and Evolutionary Microbiology 55, 1271–1277.

Fera, M.T., Maugeri, T.L., Giannone, M., Gugliandolo, C., Camera, E.L., Blandino, G., Carbone, M., 2003. *In vitro* susceptibility of *Arcobacter butzleri* and *Arcobacter cryaerophilus* to different antimicrobial agents. International Journal of Antimicrobial Agents 21, 488–491.

Fernández, N., Díaz, E.E., Amils, R., Sanz, J.L., 2008. Analysis of microbial community during biofilm development in an anaerobic wastewater treatment reactor. Microbial Ecology 56, 121–132.

Figueras, M.J., Collado, L., Levican, A., Perez, J., Solsona, M.J., Yustes, C., 2010. *Arcobacter molluscorum* sp. nov., a new species isolated from shellfish. Systemic and Applied Microbiology 34, 105–109.

Figueras, M.J., Levican, A., Collado, L., Inza, M.I., Yustes, C., 2011. *Arcobacter ellisii* sp nov., isolated from mussels. Systematic and Applied Microbiology 34, 414–418.

Gaynor, E.C., Candon, H.L., Allan, B.J., Fraley, C.D., 2007. Polyphosphate kinase 1 is a pathogenesis determinant in *Campylobacter jejuni*. Journal of Bacteriology 189, 8099–8108.

Gude, A., Hillman, T.J., Helps, C.R., Allen, V.M., Corry, J.E.L., 2005. Ecology of *Arcobacter* species in chicken rearing and processing. Letters in Applied Microbiology 41, 82–87.

Hanning, I., Jarquin, R., Slavik, M., 2008. *Campylobacter jejuni* as a secondary colonizer of poultry biofilms. Journal of Applied Microbiology 105, 1199–1208.

Ho, H., Lipman, L., Gaastra, W., 2006. *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent. Veterinary Microbiology 115, 1–13.

Ho, H.T.K., Lipman, L.J.A., Gaastra, W., 2008. The introduction of *Arcobacter* spp. in poultry slaughterhouses. International Journal of Food Microbiology 125, 223–229.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J., Vandamme, P., 2000. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiology Letters 193, 89–94.

Houf, K., De Zutter, L., Van Hoof, J., Vandamme, P., 2002a. Occurrence and distribution of *Arcobacter* species in poultry processing. Journal of Food Protection 65, 1233–1239.

Houf, K., De Zutter, L., Van Hoof, J., Vandamme, P., 2002b. Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Applied and Environmental Microbiology 68, 2172–2178.

Houf, K., De Zutter, L., Verbeke, B., Van Hoof, J., Vandamme, P., 2003. Molecular characterization of *Arcobacter* isolates collected in a poultry slaughterhouse. Journal of Food Protection 66, 364–369.

Houf, K., Devriese, L.A., Haesebrouck, F., Vanderberg, O., Butzler, J.P., Hoof, J.V., Vandamme, P., 2004. Antimicrobial susceptibility patterns of *Arcobacter butzleri* and *Arcobacter cryaerophilus* strains isolated from humans and broilers. Microbial Drug Resistance 10, 243–247.

Houf, K., On, S.L.W., Coenye, T., Mast, J., Hoof, J.V., Vandamme, P., 2005. *Arcobacter cibarius* sp nov., isolated from broiler carcasses. International Journal of Systematic and Evolutionary Microbiology 55, 713–717.

Houf, K., On, S.L.W., Coenye, T., Debruyne, L., De Smet, S., Vandamme, P., 2009. *Arcobacter thereius* sp. nov., isolated from pigs and ducks. International Journal of Systematic and Evolutionary Microbiology 59, 2599–2604.

Kabeya, H., Maruyama, S., Morita, Y., Ohsuga, T., Ozawa, S., Kobayashi, I., Abe, M., Katsube, Y., Mikami, T., 2004. Prevalence of *Arcobacter* species in retail meats and antimicrobial susceptibility of the isolates in Japan. International Journal of Food Microbiology 90, 303–308.

Kim, H.M., Hwang, C.Y., Cho, B.C., 2010. *Arcobacter marinus* sp. nov. International Journal of Systematic and Evolutionary Microbiology 60, 531–536.

Kjeldgaard, J., Jørgensen, K., Ingmer, H., 2009. Growth and survival at chiller temperatures of *Arcobacter butzleri*. International Journal of Food Microbiology 131, 256–259.

Levican, A., Collado, L., Aguilar, C., Yustes, C., Dieguez, A.L., Romalde, J.L., Figueras, M.J., 2012. *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. Systematic and Applied Microbiology 35, 133–138.

McLennan, M.K., Ringoir, D.D., Frirdich, E., Svensson, S.L., Wells, D.H., Jarrell, H., Szymanski, C.M., Gaynor, E.C., 2008. *Campylobacter jejuni* biofilms up-regulated in the absence of the stringent response utilize a calcofluor white-reactive polysaccharide. Journal of Bacteriology 190, 1097–1107.

Mena, C., Rodrigues, D., Silva, J., Gibbs, P., Teixeira, P., 2008. Occurrence, identification, and characterization of *Campylobacter* species isolated from Portuguese poultry samples collected from retail establishments. Poultry Science 87, 187–190.

Novais, C., Coque, T.M., Costa, M.J., Sousa, J.C., Baquero, F., Peixe, L.V., 2005. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. Journal of Antimicrobial Chemotherapy 56, 1139–1143.

Otth, L., Wilson, M., Cancino, R., Fernández, H., 2004. *In vitro* susceptibility of *Arcobacter butzleri* to six antimicrobial drugs. Archivos De Medicina Veterinaria 36, 207–210.

Parsek, M.R., Singh, P.K., 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annual Review of Microbiology 57, 677–701.

Reeser, R.J., Medler, R.T., Billington, S.J., Jost, B.H., Joens, L.A., 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. Applied and Environmental Microbiology 73, 1908–1913.

Reuter, M., Mallett, A., Pearson, B.M., Van Vliet, A.H., 2010. Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. Applied and Environmental Microbiology 76, 2122–2128.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J., Harrison, M.A., 2006. Genetic diversity of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. Journal of Food Protection 69, 1028–1033.

Son, I., Englen, M.D., Berrang, M., Fedorka-Cray, P.J., Harrison, M.A., 2007a. Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. International Journal of Food Microbiology 113, 16–22.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J., Harrison, M.A., 2007b. Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. International Journal of Antimicrobial Agents 29, 451–455.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., Svabic-Vlahovic, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiological Methods 40, 175–179.

Van Driessche, E., Houf, K., 2007. Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. Poultry Science 86, 744–751.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R., De Ley, J., 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy — emendation of generic descriptions and proposal of *Arcobacter* Gen-Nov. International Journal of Systematic Bacteriology 41, 88–103.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., Van Den Borre, C., Higgins, R., Hommez, J., Kersters, K., Butzler, J.L., Goossens, H., 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. International Journal of Systematic Bacteriology 42, 344–356.

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, J.P., Dediste, A., 2006. Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium. Journal of Antimicrobial Chemotherapy 57, 908–913.

Welsh, J.E., King, P.A., MacCarthy, E., 2011. Characterization of a biofilm bacterium from a recirculation system for European lobster (*Homarus gammarus*). Aquaculture 318, 458–463.

Supplementary material from Chapter 3

This chapter contains additional results that were not explored in the manuscript. Each result is preceded with a short description of the methodology used, ending with the description of the results and a brief conclusion.

1. Characterization of ciprofloxacin-resistant isolates

Multiple mechanisms for antibiotic resistance have been reported for *Campylobacter,* including modification (mutation) of target genes and active efflux pump systems (Iovine 2013). *Arcobacter* resistance to ciprofloxacin was associated with amino acid substitutions in the *gyrA*-encoding subunit of the DNA gyrase within the DNA-binding domain in a region termed the *quinolone resistance determining region* (QRDR). The Thr-85-Ile amino acid substitution in the QRDR showed to be sufficient to confer a resistant phenotype in *A. butzleri* and *A. cryaerophilus* (Abdelbaqi *et al*. 2007).

Genomic DNA was extracted using the Wizard Genomic DNA purification Kit (Promega, Wisconsin, USA) from cultures grown 24 h at 37 ºC in microaerophilic conditions on Blood agar plates. Sequencing of the QRDR of the *gyrA* gene was performed using the primers F-QRDR (5'-TGGATTAAAGCCAGTTCATAGAAG-3') and R2-QRDR (5'-TCATMGWATCATCATAATTTGGWAC-3') reported by Abdelbaqi *et al*. (2007) for the amplification and sequencing on both strands of the 344-bp resulting PCR product. PCR was performed in a 12.5 µL reaction mixture consisting of 0.5 µM of each primer, 1× PCR buffer, 200 µM of each deoxynucleotide triphosphate, 2.8 mM MgCl2, 0.8 U of BIO-X-ACT Short DNA polymerase (Bioline, London, UK), and 5 µL of template DNA. The thermal cycling conditions were: 1 cycle at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 30 s, followed by a final extension step of 10 min at 72 °C. The amplified products were resolved by gel electrophoresis and the presence of PCR products was visualized under ultraviolet transillumination. PCR products were purified using the ExoSAP-IT (GE Healthcare, Uppsala, Sweden). DNA sequencing was performed by the dye termination method using the Big Dye Terminator v1.1 Sequencing standard kit (PE Applied Biosystems Chemistry, Foster City, CA, USA) and the Automated Sequencer Genetic Analyser ABI-Prism 3130 xl (PE Applied Biosystems).

As referred in Paper I, among the 43 *A. butzleri* isolates 24 were resistant to ciprofloxacin (MIC≥4 µg/mL), presenting MIC values ranging from 4 to >8 µg/mL. Sequencing of the QRDR of the gyrA gene were performed in the 36 of the 43 *A. butzleri* isolates that either presented different pulsetypes, similar PFGE patterns but different origin, or even different resistance patterns. All isolates resistant to ciprofloxacin carried the described mutation in position 254 of the *gyrA* gene. This cytosine to thymine transition led to an amino acid substitution in

position 85 of a threonine to an isoleucine of the deduced corresponding protein. This substitution has been related to the quinolone resistance, since the mutation was absent in the susceptible strains (Table S1).

This result led us to conclude that the Thr-85-Ile amino acid substitution in the GyrA-encoding subunit of DNA gyrase is the primary contributor to the ciprofloxacin resistance in *A. butzleri*.

Table S1. Ciprofloxacin minimum inhibitory concentration, *gyrA* mutation and specimen source of 36 selected *Arcobacter butzleri* isolates and the three reference strains.

Strain code	Source	Ciprofloxacin MIC (mg/L)	GyrA mutation Thr-85-Ile
AB 10/11	Slaughterhouse surface 1	8	Present
AB 11/11	Slaughterhouse surface 1	4	Present
AB 12/11	Slaughterhouse surface 1	0.06	Absent
AB 13/11	Slaughterhouse surface 1	4	Present
AB 15/11	Slaughterhouse surface 1	8	Present
AB 16/11	Slaughterhouse surface 2	0.06	Absent
AB 17/11	Slaughterhouse surface 2	8	Present
AB 18/11	Slaughterhouse surface 2	0.125	Absent
AB 19/11	Slaughterhouse surface 3	0.06	Absent
AB 20/11	Slaughterhouse surface 3	0.06	Absent
AB 21/11	Slaughterhouse surface 3	8	Present
AB 22/11	Slaughterhouse surface 4	0.06	Absent
AB 23/11	Slaughterhouse surface 4	0.06	Absent
AB 24/11	Slaughterhouse surface 4	4	Present
AB 25/11	Carcass neck skin –flock3	8	Present
AB 26/11	Carcass neck skin –flock3	8	Present
AB 28/11	Carcass neck skin –flock1	>8	Present
AB 30/11	Carcass neck skin –flock1	>8	Present
AB 31/11	Carcass washing water-flock2	>8	Present
AB 32/11	Carcass neck skin –flock2	>8	Present
AB 33/11	Carcass neck skin –flock2	0.125	Absent
AB 34/11	Caecum -flock3	0.06	Absent
AB 35/11	Caecum –flock1	0.06	Absent
AB 36/11	Caecum –flock1	4	Present
AB 37/11	Caecum –flock1	4	Present
AB 38/11	Caecum –flock2	0.03	Absent
AB 39/11	Caecum –flock2	0.06	Absent
AB 41/11	Carcass washing water-flock3	>8	Present
AB 42/11	Carcass washing water-flock3	>8	Present
AB 43/11	Carcass washing water-flock3	8	Present
AB 44/11	Carcass washing water-flock1	0.25	Absent
AB 45/11	Carcass washing water-flock1	0.5	Absent
AB 46/11	Carcass washing water-flock1	0.125	Absent

Table S1. Ciprofloxacin minimum inhibitory concentration, *gyrA* mutation and specimen source of 36 selected *Arcobacter butzleri* isolates and the three reference strains (continuation).

Strain code	Source	Ciprofloxacin MIC (µg/mL)	GyrA mutation Thr-86-Ile
AB 47/11	Carcass washing water-flock2	>8	Present
AB 50/11	Caecum-flock3	0.125	Absent
AB 52/11	Caecum-flock2	0.06	Absent
LMG 6620	Human blood sample	0.06	Absent
LMG 9869	Deadborn piglet	0.06	Absent
LMG 10828	Human Diarrhoeal faeces	0.06	Absent

2. Occurrence of nine virulence-associated genes in *Arcobacter butzleri* isolated from poultry and slaughterhouse environment

A. butzleri was isolated with a high prevalence from poultry and slaughterhouse environment and due to the consumption of *Arcobacter*-contaminated food being one of the most probable transmission route to human and animals, the occurrence of nine putative virulence genes was determined.

The *A. butzleri* RM4018 genome sequencing revealed the presence of putative virulence determinants (Miller *et al*. 2007). Douidah *et al*. (2012) developed PCR-based assays for nine putative virulence genes, allowing the evaluation of their distribution (Table S2).

A. butzleri culture and genomic DNA extraction was performed according the described above. Detection of putative genes was performed by PCR according to Douidah *et al*. (2012), with some modifications. Briefly, all PCR were carried out in a reaction volume of 25 µL containing 2.5 µL 10×PCR buffer, 200 µM of each deoxyribonucleotide triphosphate, 0.5 µM of each primer, 2.5 mM of $MgCl_2$, 1U Taq DNA polymerase (Invitrogen) and 5 µL of template DNA. An initial denaturation step at 94 ºC for 3 min was followed by 32 cycles at 94 ºC for 45 s, primer annealing at 56 ºC for 45 s for the primer sets *ciaB*, *cj1349*, *hecA*, *irgA*, *mviN* and at 55 ºC for 45 s for the primer sets *cadF*, *hecB*, *pldA*, *tlyA*, and an elongation at 72 ºC for 45 s, followed by a final extension step of 3 min at 72 ºC. All PCR products were resolved by gel electrophoresis and visualized under ultraviolet transillumination. Each target was distinguished based on the respective amplicon size (Table S2).

Table S2. Primers used for detection of *A. butzleri* putative genes, according to Douidah *et al.* (2012)

Target gene	Primer pair	Sequence of primers (5' to 3')	Amplicon size (bp)
cadF	*cadF*-F	ttactcctacaccgtagt	283
	cadF-R	aaactatgctaacgctggtt	
ciaB	*ciaB*-F	tgggcagatgtggatagagcttgga	284
	ciaB-R	tagtgctggtcgtcccacataaag	
cj1349	*cj1349*-F	ccagaaatcactggcttttgag	659
	cj1349-R	gggcataagttagatgaggttcc	
irgA	*irgA*-F	tgcagaggatacttggagcgtaact	437
	irgA-R	gtataaccccattgatgaggagca	
hecA	*hecA*-F	gtggaagtacaacgatagcaggctc	537
	hecA-R	gtctgttttagttgctctgcactc	
hecB	*hecB*-F	ctaaactctacaaatcgtgc	528
	hecB-R	cttttgagtgttgacctc	
mviN	*mviN*-F	tgcacttgttgcaaaacggtg	294
	mviN-R	tgctgatggagcttttacgcaagc	
pldA	*pldA*-F	ttgacgagacaataagtgcagc	293
	pldA-R	cgtctttatctttgctttcaggga	
tlyA	*tlyA*-F	caaagtcgaaacaaagcgactg	230
	tlyA-R	tccaccagtgctacttcctata	

In this work, six (*cadF*, *ciaB*, *cj1349*, *mviN*, *pldA*, *tlyA*) of the nine putative virulence genes were present in all *A. butzleri* isolates, in accordance with the results presented in three previous works (Douidah *et al.* 2012; Karadas *et al.* 2013; Tabatabaei *et al.* 2014) (Table S3). Levican *et al.* (2013) showed that all the *A. butzleri* strains presented the *cadF* gene and that 91.7 % had *ciaB* and *cj1349* genes (Levican *et al.* 2013). In this work, thirteen (36.2 %) of *A. butzleri* isolates carried all nine genes, in contrast to the 13 and 15 % detected by Douidah *et al.* (2012) and Karadas *et al.* (2013), respectively. Also, the global detection of *irgA*, *hecA* and *hecB* genes was higher than the one reported by other authors (Douidah *et al.* 2012; Karadas *et al.* 2013; Levican *et al.* 2013).

Table S3. Presence of putative virulence genes in *A. butzleri* isolates

A. butzleri source	Number of isolates	Number of strains generating a specific gene amplicon								
		cadF	*ciaB*	*cj1349*	*irgA*	*hecA*	*hecB*	*mviN*	*pldA*	*tlyA*
Slaughterhouse surfaces	14	14	14	14	12	13	11	14	14	14
Intensive production system flocks	15	15	15	15	2	10	15	15	15	15
Extensive indoor production system flocks	7	7	7	7	1	4	6	7	7	7

3. Analysis of a Plasmid from *Arcobacter butzleri*

Few studies have reported the presence of plasmids in *Arcobacter* spp. (Harrass *et al*. 1998; Toh *et al*. 2011; Douidah *et al*. 2014).

A plasmid was extracted from one of the isolated *A. butzleri* strains from the slaughterhouse environment, and sequence analysis was performed in order to assess the presence of virulence-associated genes or antibiotic resistance-associated genes. The strain of *A. butzleri* AB10/11 was isolated from a poultry slaughterhouse surface. The isolate was propagated in blood agar when needed and incubated for 48 h at 37 ºC, under microaerophilic conditions.

Plasmid DNA was recovered from the isolate using the JetQuick Plasmid DNA purification kit (Genomed, Lisboa, Portugal). The isolated plasmid was restricted with *HindIII* followed by cloning of resulting bands into the pFLAG-CMV2 vector (Sigma, St Louis, USA) using a T4 DNA ligase (Promega, Wisconsin, USA). Ligation reactions were subsequently transformed into *Escherichia coli* TOP10 (Invitrogen) through heat shock transformation. The inserted fragment was sequenced using the Automated Sequencer Genetic Analyser ABI-Prism 3130 xl (PE Applied Biosystems). In order to complete the sequencing, the primer walking strategy was used. Open reading frames were identified using both Rapid Annotation System Technology (RAST) server (http://rast.nmpdr.org) (Aziz *et al*. 2008) and GeneMark.hmm for Prokaryotes, Version: 3.19 (http://exon.biology.gatech.edu/).

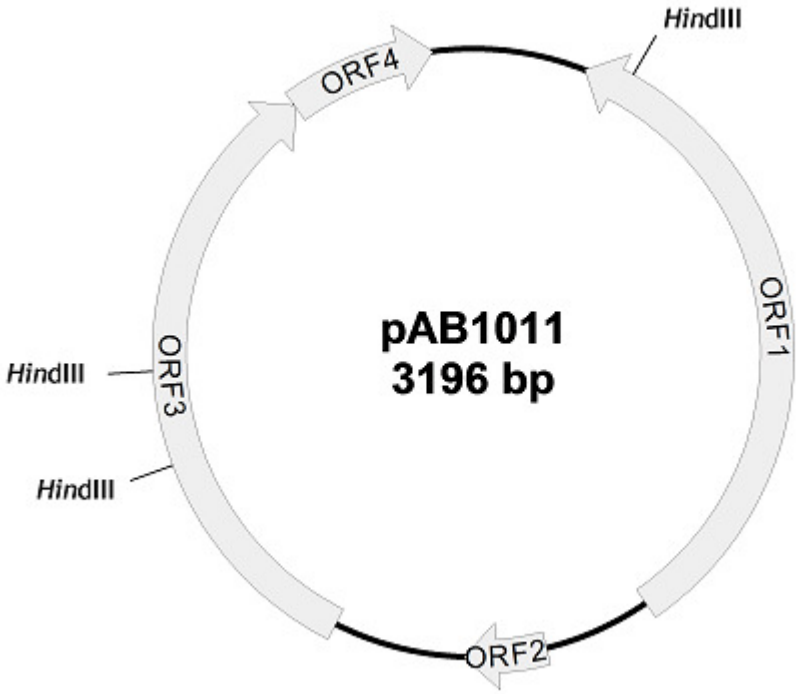

Figure S1. Physical map of plasmid pAB1011. The four putative ORFs are indicated by arrows. The position of the *Hind III* restriction enzyme is also depicted.

Examination of complete nucleotide sequence of the plasmid pAB1011 revealed that it is 3196 bp in length with a mean GC content of 30.2 %. The plasmid contained four putative open reading frames (ORFs), covering 79.4 % of the plasmid genome. The four ORFs were predicted to encode putative proteins. According to the protein sequence homology, ORF1, ORF2 and ORF4 encode hypothetical proteins. The ORF3 encodes a putative protein of 347 amino acids which showed 26 % identity and 45 % similarity with a replication protein A from *Salmonella enterica* subsp. enterica serovar Heidelberg str. SL476 (Accession number YP_002045467.1); if compared within the order Campylobacterales, the protein presented a 29 % identity and 46 % similarity with a plasmid replicase from *Sulfurimonas autotrophica* DSM 16294 (Accession number YP_003891252.1). The putative replication protein encoded by ORF3 contains two conserved domains, one of replicase, which belongs to a family of bacterial plasmid DNA replication initiator proteins common to Gram negative bacteria (Giraldo and Diaz-Orejas 2001) and a Primase-C Terminal-1 (PriCT-1) domain.

Sequences of cryptic plasmids in *A. butzleri* can be found in the NCBI Database with sizes varying from about 2 kbp to 5 kbp (Accession numbers: NC_012733.1; NC_017193.1; KF740633.1; KF740632.1; KF740631.1 and KF955987.1). The sequence of the plasmid pAB1011 was subjected to analysis using the NCBI BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST) and showed no significant similarity with none of the six sequences deposited in NCBI Database for *A. butzleri*. The overall function of these

cryptic plasmids remains unknown and more studies are needed to understand their importance.

4. References

Abdelbaqi, K., Ménard, A., Prouzet-Mauleon, V., Bringaud, F., Lehours, P. and Mégraud, F. (2007) Nucleotide sequence of the *gyrA* gene of *Arcobacter* species and characterization of human ciprofloxacin-resistant clinical isolates. *FEMS Immunol Med Microbiol* **49**, 337-345.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. and Zagnitko, O. (2008) The RAST server: Rapid annotations using subsystems technology. *BMC Genom* **9**.

Douidah, L., de Zutter, L., Bare, J., De Vos, P., Vandamme, P., Vandenberg, O., Van den Abeele, A.M. and Houf, K. (2012) Occurrence of putative virulence genes in *Arcobacter* species isolated from humans and animals. *J Clin Microbiol* **50**, 735-741.

Douidah, L., De Zutter, L., Van Nieuwerburgh, F., Deforce, D., Ingmer, H., Vandenberg, O., Van den Abeele, A.M. and Houf, K. (2014b) Presence and analysis of plasmids in human and animal associated arcobacter species. *PloS One* **9**, e85487.

Giraldo, R. and Diaz-Orejas, R. (2001) Similarities between the DNA replication initiators of Gram-negative bacteria plasmids (RepA) and eukaryotes (Orc4p)/archaea (Cdc6p). *P Nat Acad Sci USA* **98**, 4938-4943.

Harrass, B., Schwarz, S. and Wenzel, S. (1998) Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. *Zentralb Vet Reihe B* **45**, 87-94.

Iovine, N.M. (2013) Resistance mechanisms in *Campylobacter jejuni*. *Virulence* **4**, 230-240.

Karadas, G., Sharbati, S., Hanel, I., Messelhausser, U., Glocker, E., Alter, T. and Golz, G. (2013) Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. *J Appl Microbiol* **115**, 583-590.

Levican, A., Alkeskas, A., Gunter, C., Forsythe, S.J. and Figueras, M.J. (2013) Adherence to and invasion of human intestinal cells by *Arcobacter* species and their virulence genotypes. *Appl Environ Microbiol* **79**, 4951-4957.

Miller, W.G., Parker, C.T., Rubenfield, M., Mendz, G.L., Wösten, M.M.S.M., Ussery, D.W., Stolz, J.F., Binnewies, T.T., Hallin, P.F., Wang, G., Malek, J.A., Rogosin, A., Stanker,

L.H. and Mandrell, R.E. (2007) The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. *PloS One* **2**, e1358.

Tabatabaei, M., Aski, H.S., Shayegh, H. and Khoshbakht, R. (2014) Occurrence of six virulence-associated genes in *Arcobacter* species isolated from various sources in Shiraz, Southern Iran. *Microbi Pathogenesis* **66**, 1-4.

Toh, H., Sharma, V.K., Oshima, K., Kondo, S., Hattori, M., Ward, F.B., Free, A. and Taylor, T.D. (2011) Complete Genome Sequences of *Arcobacter butzleri* ED-1 and *Arcobacter* sp. Strain L, Both Isolated from a Microbial Fuel Cell. *J Bacteriol* **193**, 6411-6412.

Chapter 4

Resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus*: Activity and effect on cellular functions

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Resveratrol against *Arcobacter butzleri* and *Arcobacter cryaerophilus*: Activity and effect on cellular functions

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ABSTRACT

The frequent isolation of *Arcobacter butzleri* and *Arcobacter cryaerophilus* from food samples makes it imperative to search for potential compounds able to inhibit the development of these bacteria. Taking this into consideration, this study focuses on the antimicrobial activity of resveratrol and its mechanism of action against *A. butzleri* and *A. cryaerophilus*. The activity of resveratrol was assessed by a microdilution method and time–kill curves. Resveratrol effect on cellular functions was assessed by flow cytometry evaluating intracellular DNA content and metabolic activity. Ethidium bromide (EtBr) accumulation in the presence of resveratrol was also evaluated, as well as the susceptibility to resveratrol in the presence of phenylalanine-arginine β-naphthylamide (PAβN). Scanning electron microscopy (SEM) was used to further evaluate cell damage caused by resveratrol. Resveratrol presented MIC values of 100 and 50 μg/mL to *A. butzleri* and *A. cryaerophilus*, respectively. Based on the time–kill curves, resveratrol exhibited bactericidal activity, leading to a ≥ 3 $\log_{10}$ CFU/mL reduction of initial inoculums, for *A. butzleri* exponential phase cells incubated for 6 h with 1× MIC or with 2× MIC after 24 h for stationary phase cells. For *A. cryaerophilus* cells in exponential growth phase, 99.9% killing was achieved after 24 h incubation with 2× MIC, whereas, for stationary phase cells, bactericidal activity was only detected after incubation with 4× MIC. Incubation with resveratrol led to a decrease in both intracellular DNA content and metabolic activity. An increase in the accumulation of EtBr was observed in the presence of resveratrol, and the efflux pump inhibitor PAβN reduced the MIC of resveratrol. SEM analysis revealed disintegration of *A. butzleri* cells treated with resveratrol, whereas no morphological alteration was observed for *A. cryaerophilus* cells. Resveratrol has a good anti-*Arcobacter* activity, and the results obtained suggest that this compound could act through several different mechanisms in the inhibition of this microorganism. The results encourage the use of this compound for the development of potential strategies to control *Arcobacter* in food products.

1. Introduction

Arcobacter spp. belong to ε-subdivision of proteobacteria and together with the genera *Campylobacter* and *Sulfurospirillum* compose the Campylobacteraceae family. Comprising motile Gram negative small curved rods, including S-shaped or helical cells, this genus has been isolated from extremely diverse habitats (Vandamme et al., 2005). Currently, *Arcobacter* genus is composed of 18 species (Collado and Figueras, 2011; Figueras et al., 2011; Levican et al., 2012, 2013; Sasi Jyothsna et al., 2013) of which *Arcobacter butzleri* and *Arcobacter cryaerophilus* have been associated with gastrointestinal and extra-gastrointestinal disease, and are considered emerging enteropathogens and potential zoonotic agents (Collado and Figueras, 2011). *Arcobacter* have been found in human, animals, food such as poultry, cattle, milk, retail meat, shellfish, and ready-to-eat meals or water, with a frequent detection in products of animal origin (Collado and Figueras, 2011). Due to the frequent isolation of *Arcobacter* species from food and water, it has been suggested that these are the most probable transmission routes to humans and animals of this microorganism (Collado and Figueras, 2011). Additionally, *Arcobacter* spp. have shown to be resistant to common antimicrobials (Collado and Figueras, 2011), namely those relevant for treatment of Campylobacteraceae human infections, such as erythromycin and ciprofloxacin, and in addition, this pathogen is also able to survive several physical and chemical treatments (Cervenka, 2007). These resistance profiles may compromise this pathogen control and together with its high prevalence in the food chain make it important to find alternative control strategies, for which different physical and chemical treatments (Cervenka, 2007; Skrivanova et al., 2010), or natural products (Cervenka et al., 2006; Fisher et al., 2007; Nannapaneni et al., 2009) have been proposed.

It is known that plants produce a great number of small molecules that help them fight off infections successfully (Klancnik et al., 2012). Therefore it is worthwhile to investigate if natural antimicrobials can be valid alternatives to synthetic molecules. Resveratrol (3,4′,5

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0168-1605/

trihydroxystilbene) is a naturally occurring phytoalexin synthesized by plants in response to phytopathogenic microorganisms or other injurious substances (Sadruddin and Arora, 2009). This naturally occurring molecule is considered one of the most promising ones for application in nutrition and medicine (Kiselev, 2011) as it exhibits several properties beneficial to human health, among which are antioxidant, anti-inflammatory, antiatherosclerotic, antidiabetic or cardioprotective activities (Sadruddin and Arora, 2009). Resveratrol also presents antimicrobial activity against bacteria, yeasts and fungi (Paulo et al., 2011a) and is even capable to inhibit relevant bacterial virulence factors, such as the urease activity of *Helicobacter pylori* (Paulo et al., 2011b; Wang et al., 2006), swarming, cell invasion ability and hemolysin activity (Wang et al., 2006). The capacity of resveratrol to inhibit cellular adhesion and cytokine production in intestinal epithelial cells as a response to *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* Scott A adhesion has also been described (Selma et al., 2012).

Although some studies refer to antimicrobial properties of resveratrol, its action mechanisms are still largely unknown. Thus, the aim of this study focuses on the antimicrobial properties of resveratrol as well as its potential cellular targets, by using *A. butzleri* and *A. cryaerophilus* as exemplary cases.

2. Materials and methods

2.1. Bacterial organisms and handling

Two reference strains, *A. butzleri* LMG 10828 and *A. cryaerophilus* LMG 10829 were obtained from BCCM/LMG Bacteria Collection (Belgian Co-ordinated Collections of Micro-organisms, Ghent University, Belgium). The bacterial strains were stored in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol at −80 °C. Prior to susceptibility testing, each strain was inoculated on blood agar (blood agar base (Oxoid, Hampshire, England) supplemented with 5% (v/v) defibrinated horse blood) to ensure optimal growth and purity.

2.2. Determination of minimum inhibitory concentration (MIC)

A. butzleri LMG 10828 and *A. cryaerophilus* LMG 10829 were grown on blood agar plates for 18 h at 30 °C in microaerobic conditions (6% O_2, ± 7.1% CO_2, 3.6% H_2, 83% N_2). The broth microdilution method was used for measuring the MIC for resveratrol. Serial two-fold dilutions of resveratrol (from 400 to 3.125 μg/mL) were prepared in a 96-well plate (50 μL per well) in Tryptic Soy Broth (TSB, Liofilchem, Italy) with a maximum dimethyl sulfoxide (DMSO) concentration of 1.5% that was used to increase the solubility. Inoculum suspensions were prepared from blood agar cultures, and a diluted bacterial suspension was added to each well, to give a final concentration of about 10^6 colony-forming units (CFU)/mL, confirmed by viable counts. The microtiter plate was incubated for 48 h at 30 °C in microaerobic conditions and growth was visually assessed. At least three independent assays in duplicated wells were performed and the modal MIC values were selected.

2.3. Time–kill curves

Bacterial strains were exposed to final resveratrol concentrations of 1, 2 and 4× MIC over time. An exponentially or stationary-growing culture of each species was obtained by incubation at 30 °C and 150 rpm, in TSB medium. The growth phase was determined by conducting growth curves prior to time–kill assays. *Arcobacter* inoculums were added to test tubes containing resveratrol, DMSO or culture medium to reach a final concentration of 10^6 CFU/mL in 1 mL final volume. Tubes were incubated at 30 °C and viable counts were performed after 0, 2, 4, 6, 8, and 24 h of incubation. At these time intervals, 20 μL was sampled, serially diluted with TSB and plated onto blood agar by drop plate method, with a lower limit of detection of 2 $\log_{10}$ CFU/mL.

Bactericidal activity was defined as a reduction of 99.9% of the total number of CFU/mL in the original inoculum and bacteriostatic activity as the maintenance or reduction of less than 99.9% of the original inoculum concentration.

2.4. Flow cytometry

2.4.1. Exposure of Arcobacter to resveratrol

A. butzleri LMG 10828 and *A. cryaerophilus* LMG 10829 suspensions were obtained from an exponentially-growing culture of each strain on TSB at 30 °C and 150 rpm. This cell suspension was used to inoculate tubes containing resveratrol concentrations ranging from 50 to 200 μg/mL according to the MIC value obtained for each strain to obtain a final cell density of 1×10^6 CFU/mL. The tubes were incubated at 30 °C and 150 rpm for 2 h for 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining or 6 h in the case of deep red-fluorescing bisalkylaminoanthraquinone number five (DRAQ5) staining. The incubation time for DRAQ5 was longer to enable cell growth and DNA replication to a greater extent. At the end of each incubation period, samples were centrifuged at 10,000 *g* for 5 min at room temperature, washed and resuspended in sterile media for CTC staining. For DRAQ5 staining, cells were first fixed with 70% ethanol as previously described (Silva et al., 2010).

Control experiments were also carried out in parallel: cells were incubated in TSB with or without DMSO addition (solvent control) and handled under the same conditions.

2.4.2. Staining procedures for flow cytometry

In order to assess respiratory activity, cellular suspensions (1×10^6 CFU/mL) were incubated with 5 mM CTC (Polysciences, Inc., Warrington, PA) in TSB for 1 h at 30 °C and 150 rpm in the dark. Afterwards, cells were washed once with PBS and stained with 5 μM SYTO® 9 for total cell staining. The suspension was incubated in the dark for 15 min, washed once in PBS and resuspended in the same buffer for subsequent flow cytometric acquisition. Negative and positive CTC staining controls consisted of stationary-growing cells, heat-killed for 30 min at 70 °C and exponentially-growing cells, respectively.

For the analysis of intracellular DNA content, fixed cell suspensions (1×10^7 CFU/mL) were washed with PBS and incubated with 7.5 μM DRAQ5 (Biostatus Limited, Leicestershire, UK) for 30 min in the dark (Silva et al., 2011a). All experiments were conducted at least in duplicate.

2.4.3. Flow cytometry method

Bacterial samples were acquired on a CyAn ADP (Beckman Coulter Inc., Brea, CA) flow cytometer. Acquisition was performed with Summit Software (Beckman Coulter Inc., Brea, CA). The acquisition was based on light scatter and fluorescence signals resulting from 25 mW solid state laser illumination at 488 nm and 60 mW diode illumination at 642 nm. Fluorescence signals were collected by FL1 (530/40 nm, SYTO® 9), FL4 (680/30 nm, CTC) and FL8 (665/20 nm, DRAQ5) bandpass filters. Light scattering, SYTO® 9 and CTC fluorescence measurements were acquired logarithmically; whereas DRAQ5 fluorescence was acquired in a linear scale. Threshold was set on FL1 (SYTO® 9) (to exclude noise, other particles and debris). Cells were gated according to light scatter parameters. Sample acquisition was operated at a flow rate of no more than 300 events per second and a total of 10,000 and 20,000 events were acquired for each CTC or DRAQ5-stained sample, respectively. Data analysis was performed using FCS Express version 4 Plus Research Edition (De Novo Software).

2.5. Effect of resveratrol on the accumulation of ethidium bromide

To measure the level of ethidium bromide (EtBr) accumulation in *A. butzleri* and *A. cryaerophilus*, the bacteria were grown in TSB for 4 h, harvested, washed with PBS and resuspended to a final OD_{620}

of 0.2 in PBS. The cells were then transferred to a 96-well black plate with clear bottom for fluorescence (Greiner Bio-One, Germany) and incubated for 10 min at 37 °C. Resveratrol was added to cell suspensions at final concentrations of 1×, 1/2× and 1/4× MIC. Then, EtBr was added to the suspension at a final concentration of 2 μg/mL. Fluorescence was recorded every minute, after automatic sample homogenization, during 30 min of incubation at 25 °C, with a Spectramax Gemini XS spectrofluorometer (Molecular Devices LLC, USA) at excitation and emission wavelengths of 530 and 600 nm, respectively. Each assay was performed on three independent days, with duplicate determinations each time.

2.6. Efflux pump inhibitor (EPI)

To investigate the contribution of efflux pump activity in resveratrol resistance, both *Arcobacter* species were tested with resveratrol in presence and absence of L-phenylalanine-L-arginine-β-naphthylamide (PAβN) (Sigma, St Louis, USA). MICs were determined by the broth microdilution method in the absence and in the presence of PAβN, at 5 μg/mL. Microdilution tests were performed with PAβN only to confirm that the compound had no inhibitory effect on bacterial growth for the tested strains.

2.7. Scanning electron microscopy (SEM)

Non-treated cells and cells treated with 1× MIC of resveratrol for 6 h were harvested by centrifugation at 10,000 *g* for 5 min. The cells were washed with 0.1 M sodium cacodylate buffer solution, resuspended in the same buffer with 2.5% (v/v) glutaraldehyde and kept for 1 h at room temperature. Fixed bacteria were transferred to poly-D-lysine-coated glass coverslips and allowed to sediment for 1 h at room temperature. The coverslips were washed with sodium cacodylate buffer and cells were post-fixed in 2% (v/v) osmium tetroxide for 1 h. Thereafter, coverslips were washed three times in cacodylate buffer and dehydrated through a series of graded ethanol solutions (30, 50, 70, 80, 90%, for 5 min each) and three times with 100% ethanol, 10 min each. Samples were then critical-point-dried, sputter-coated with gold and examined with a scanning electron microscope (Hitachi S-2700).

3. Results

3.1. Anti-Arcobacter activity of resveratrol

The MIC values listed in Table 1 show the effect of resveratrol against *A. butzleri* and *A. cryaerophilus*. For the time–kill analysis, resveratrol was added to exponential and stationary-growing cells at concentrations corresponding to 1×, 2× and 4× MIC values. The highest DMSO concentration used in 4× MIC assay, used as solvent control, showed no inhibition for stationary growth phase cells or for *A. cryaerophilus* exponential phase cells, with only a maximum reduction of 0.4 $\log_{10}$ CFU/mL for *A. butzleri* exponential phase cells (data not shown). *Arcobacter* cell culturability was progressively reduced for both species; however, the killing kinetics showed a higher resveratrol susceptibility of *A. butzleri* when compared to *A. cryaerophilus* (Fig. 1). Overall, exponential-growing cells exhibited more susceptibility to resveratrol than cells in stationary phase. For instance, for *A. butzleri* exponential phase cells, incubated for 6 h with 1× and 2× MIC or for 4 h with 4× MIC, resveratrol induced a decrease in cultivable counts presenting bactericidal activity (≥3 $\log_{10}$ CFU/mL reduction in initial inoculum) (Fig. 1A); in stationary phase cells, resveratrol was only bactericidal for cells treated with 2× or 4× MIC after 24 and 8 h, respectively.

Table 1
Resveratrol minimum inhibitory concentration in the absence and presence of PAβN (5 μg/mL).

Antimicrobial and addition	MIC (μg/mL)	
	A. butzleri LMG 10828	*A. cryaerophilus* LMG 10829
Resveratrol	100	50
+ PAβN	6.25	12.5

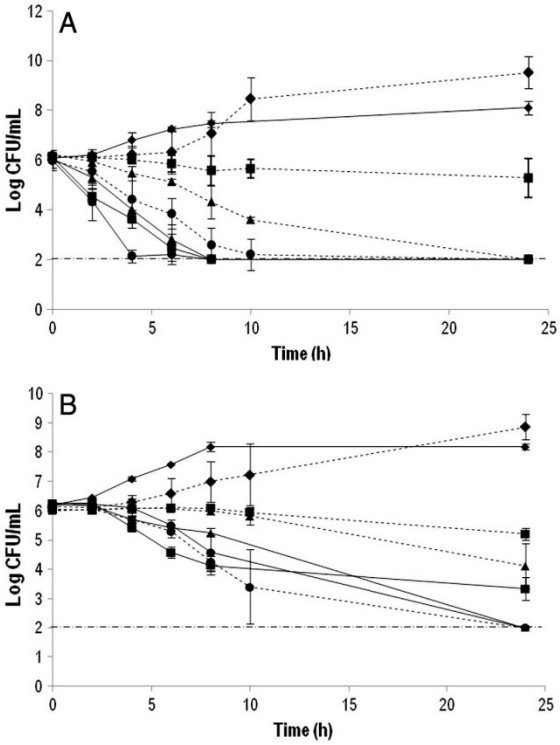

Fig. 1. Time–kill curves of resveratrol activity against *A. butzleri* LMG 10828 (A) and *A. cryaerophilus* LMG 10829 (B) for cells in exponential (full line) or stationary (dashed line) phases of growth. Growth control (♦); 1× (■); 2× (▲) and 4× (●) the MIC, over a 24 h period.

For *A. cryaerophilus* cells in exponential growth phase, 99.9% killing was achieved after 24 h incubation with 2× and 4× MIC, whereas, for stationary phase cells, bactericidal activity was only detected with a concentration of 4 × MICwas only detected after incubation with 4× MIC (Fig. 1B).

3.2. Effect of resveratrol on metabolic activity and DNA content

The antibacterial effect of resveratrol against *A. butzleri* and *A. cryaerophilus* was further evaluated by using flow cytometry after dual staining with SYTO® 9 and CTC, for the determination of metabolic activity, or after single staining with DRAQ5 to ascertain the intracellular DNA content. Fig. 2 shows the dual parameter dot blots of the fluorescence intensities of SYTO® 9 and CTC when *A. butzleri* and *A. cryaerophilus* were exposed to resveratrol. *A. butzleri* CTC-reducing cells significantly dropped from 84.9 ± 3.8 to 40.4 ± 8.3% after 2 h of incubation with 100 μg/mL resveratrol ($P < 0.001$); however no significant difference was obtained when resveratrol concentration increased to 200 μg/mL (44.4 ± 5.6%). Also, for *A. cryaerophilus* the results indicated a significant reduction of CTC-reducing cells from 90.2 ± 1.4% to 22.0 ± 2.4, 27.0 ± 4.1 or 17.7 ± 0.5% ($P < 0.01$), when cells were incubated with 1×, 2× or 4× MIC, respectively. The DMSO percentage used in the assays did not cause any change in cells' metabolic activity, as can be seen by

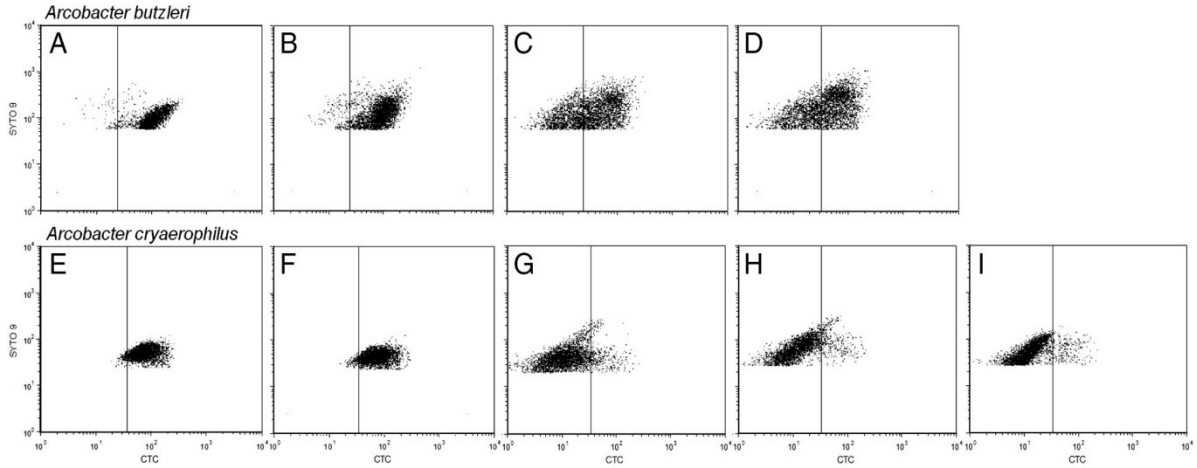

Fig. 2. Dual parameter dot plots of CTC stained *A. butzleri* LMG 10828 and *A. cryaerophilus* LMG 10829 evaluated by flow cytometry after 2 h of incubation with medium (A, E), with maximum DMSO concentration used in the assays (B, F) or with resveratrol at concentrations of 1× MIC (C, G), 2× MIC (D, H) or 4× MIC (I).

the almost identical percentages of CTC-stained cells obtained for growth and DMSO control samples for both species (Fig. 2B and F).

To evaluate modification on intracellular DNA content caused by resveratrol, a fluorescence histogram was acquired following staining with DRAQ5 (Fig. 3). The median fluorescence intensity of the cells showed a pronounced reduction with resveratrol incubation, which is displayed as a shift of the histogram toward lower FL8 channel numbers. This effect was more pronounced for *A. butzleri* cells. Also, the peak's coefficient variation values increased with resveratrol treatment. The incubation of *A. cryaerophilus* with 50 μg/mL of resveratrol originated a histogram where two peaks were identified, suggesting the presence of cells that were still in division.

3.3. Ethidium bromide accumulation and role of efflux pumps in susceptibility to resveratrol

Ethidium bromide accumulation assays were used to demonstrate the effect of resveratrol on intracellular substrate accumulation. Fig. 4 represents the fluorescence intensity obtained from EtBr accumulation within 30 min. For each species, the accumulation activity of resveratrol was evaluated at the corresponding MIC and at sub-inhibitory concentrations of 1/2× and 1/4× MIC.

Using the described fluorometric assay, it was shown that resveratrol increased intracellular EtBr concentration in a dose-dependent manner. Indeed, an increase in uptake of EtBr of 1.5 and 2 folds was observed after the treatment of cells with 50 or 100 μg/mL of resveratrol within 12 or 6 min, for *A. cryaerophilus* and *A. butzleri*, respectively. In addition, the role of efflux in the resistance of *Arcobacter* to resveratrol in the presence of PAβN was also analyzed. Resistance to resveratrol decreased in the presence of PAβN, with a reduction of 16 and 4 folds in MIC values for *A. butzleri* and *A. cryaerophilus*, respectively (Table 1).

3.4. Cell damage study by SEM

The effect of resveratrol on *Arcobacter* morphology was evaluated by comparing untreated and resveratrol-treated cells with 1× MIC value at 30 °C for 6 h. In the absence of resveratrol, bacteria showed a regular morphology (Fig. 5A, C). For *A. butzleri*, morphological alterations were observed, with some cells appearing disintegrated while others were undamaged (Fig. 5B), whereas for *A. cryaerophilus*, cellular morphology was preserved after resveratrol treatment (Fig. 5D).

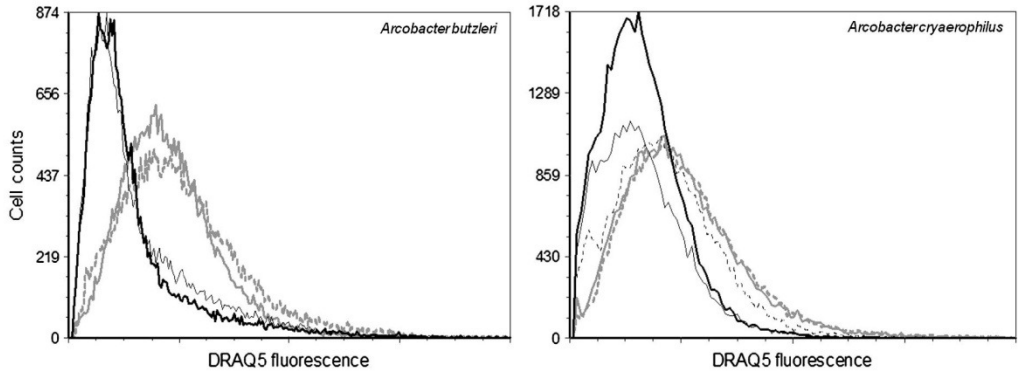

Fig. 3. Histograms showing the distribution of DRAQ5 fluorescence intensity in linear scale using DRAQ5-marked *A. butzleri* LMG 10828 (A) and *A. cryaerophilus* LMG 10829 (B) cell suspensions after 6 h incubation with: only media (gray full line); media with DMSO maximum concentration (dashed gray line); or resveratrol at concentrations of 50 μg/mL (dashed black line), 100 μg/mL (thin black line) or 200 μg/mL (thick black line). A total of 20,000 events were collected for this analysis.

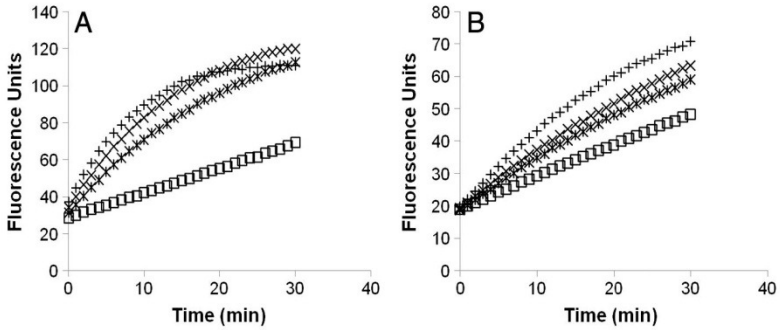

Fig. 4. Time-course accumulation of EtBr in presence of 1× (+), 1/2× (×) or 1/4× (*) resveratrol in *A. butzleri* (A) and *A. cryaerophilus* (B). Bacterial cells in buffer containing DMSO was used as control (□).

4. Discussion

Arcobacter species are recognized as emerging human pathogens, with water and food being associated to transmission to human and animals. Food product analysis has shown that, in general, *A. butzleri* is the most prevalent species, followed by *A. cryaerophilus* (Collado and Figueras, 2011). *Arcobacter* detection in food products, associated with the increased antimicrobial resistance of food-borne pathogens has created a demand for new strategies to control these microorganisms.

Resveratrol antibacterial activity has been described in a number of studies showing either a bacteriostatic (Paulo et al., 2010) or bactericidal effect (Brown and Jiang, 2013; Docherty et al., 2007; Martini et al., 2011; Nawrocki et al., 2013). The present study discusses, for the first time, the antimicrobial activity of resveratrol against *A. butzleri* and *A. cryaerophilus* and elucidates about the resveratrol mechanism of action on these bacteria.

The susceptibility to resveratrol was different between *A. butzleri* and *A. cryaerophilus*; a similar difference in resveratrol susceptibility was previously described for other bacteria even between different classes of the same species, with *Haemophilus ducreyi* class II strains being more susceptible to resveratrol than class I strains (Nawrocki et al., 2013). *A. butzleri* and *A. cryaerophilus* seemed to be more susceptible to resveratrol than other Gram-negative bacteria (Paulo et al., 2010), showing a similar behavior to that described for *H. pylori* (Brown and Jiang, 2013).

Based on the time–kill curves, resveratrol exhibited a bacteriostatic or bactericidal activity that was dependent on cellular growth phase and resveratrol concentration. Moreover, a reduction of the bactericidal effect was observed when resveratrol was added to cells in the stationary phase, in contrast to the addition to exponentially growing cells, revealing a more pronounced effect for *A. butzleri*. This indicates that actively growing cells are more susceptible to resveratrol.

Flow cytometry studies were further used to evaluate the effect of resveratrol against *Arcobacter*. Bacterial cells with electron transport system activity or respiratory activity are able to reduce CTC to an insoluble fluorescent CTC-formazan product that accumulates inside the cells (Silva et al., 2011b). For CTC-formazan fluorescence analysis, two regions of CTC-formazan (FL4) relative fluorescence were established, depending on the intensity of the fluorescence of positive and negative

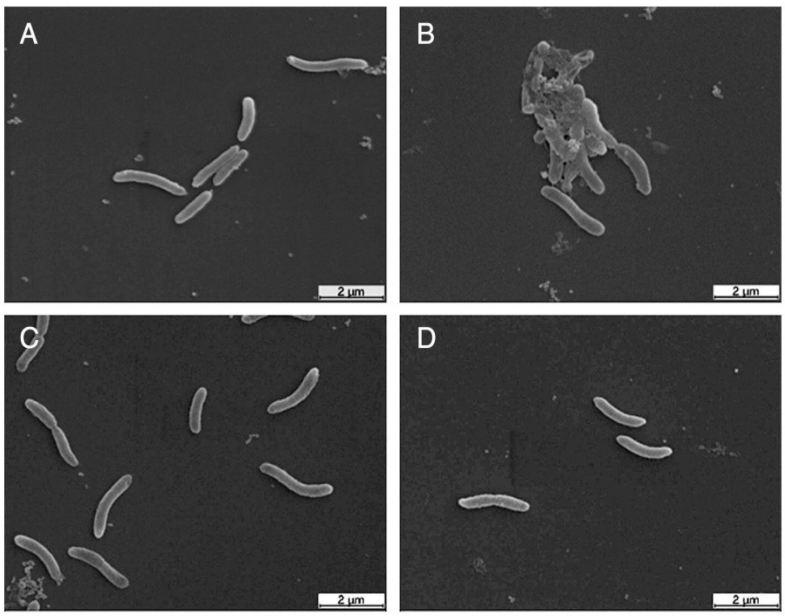

Fig. 5. Scanning electron microscopy images of the antibacterial effect of resveratrol against *A. butzleri* (A: without, B: with 100 µg/mL resveratrol) and *A. cryaerophilus* (C: without, D: with 50 µg/mL resveratrol).

controls. After 2 h of incubation, resveratrol caused a significant decrease on *Arcobacter* metabolic activity with a reduction of about 50% and 80% for *A. butzleri* and *A. cryaerophilus*, respectively (Fig. 2), with no significant decrease in the percentage of CTC-stained cells when the resveratrol concentration was increased. These results are in agreement with the ones obtained for the time–kill assays using exponential growth cells, for which, after 2 h of incubation with resveratrol, no significant differences in the reduction of culturable cells were observed between the several concentrations tested (Fig. 1). Albeit time–kill curves showed a faster decrease of *A. butzleri* culturable cells, this compound seemed to have a more pronounced effect on the metabolic activity of *A. cryaerophilus*, without any significant reduction of culturability. Other authors have also reported that metabolic activity reduction may not be directly related to a decrease in the number of culturable cells. For instance, Bouhdid et al. (2009) obtained very different growth inhibition values (99.99% vs 50.48%) in cells showing similar percentages in CTC-positive cells (11.74% vs 17.09%). This may be explained by the fact that, although CTC staining allows the detection of active metabolic bacteria, cells with a low respiratory activity may not be detected as CTC-positive (Bouhdid et al., 2010; Parthuisot et al., 2000).

Owing to the previous description of resveratrol impact in DNA replication of some microorganisms (Docherty et al., 1999; Paulo et al., 2010), we further investigated resveratrol action in bacterial DNA synthesis using a DNA-specific fluorescent stain, DRAQ5. DRAQ5 fluorescence intensity of resveratrol-treated cells was significantly lower in comparison to untreated cells, with a reduction in the mean fluorescence values. In contrast to the results obtained for CTC staining, DRAQ5 staining results revealed that resveratrol had a more pronounced effect on *A. butzleri* cells. The results obtained herein further support the evidence that resveratrol has, in fact, an important action on DNA synthesis, even in Gram-negative bacteria. *Trans*-resveratrol has been described as inhibiting DNA synthesis possibly at the level of DNA polymerase activity (Stivala et al., 2001), suggesting that, through this mechanism, resveratrol could interfere in DNA synthesis in *Arcobacter* as well. Taking SEM results into account, the reduction of intracellular DNA can also be related with the loss of intracellular material, associated with the presence of some cells presenting disintegration for *A. butzleri*, after 6 h of resveratrol exposure. This could result in an impairment of bacterial growth, thus explaining the low culturability values obtained. Additionally, SEM images seemed to corroborate the two peak distribution seen for DRAQ5 histogram concerning MIC exposure of *A. cryaerophilus* cells, since some dividing cells were still observed.

The results of the SEM assays suggest that the first target for resveratrol action on the bacterial cell was not the membrane, since a prior reduction of metabolic activity and intracellular DNA content was observed. Therefore, it can be proposed that resveratrol acts against different targets that may lead to the induction of cell death and lysis due to the impairment of several cellular functions.

Accordingly, a recent study suggested that part of resveratrol activity against *Salmonella* was linked to chelation of outer membrane stabilizing divalent cations; however this compound was not capable of sufficient destabilization of the outer membrane to cause increase of novobiocin susceptibility (Plumed-Ferrer et al., 2013). Brown and Jiang (2013) reported the lack of effect of resveratrol upon *H. pylori* outer membrane integrity.

Taking into account the global results, it can be suggested that resveratrol can affect *Arcobacter* cells in a dose- and time-dependent manner, requiring the attainment of a minimal concentration before resveratrol could be lethal to *Arcobacter* spp.

Given the fact that resveratrol affected *Arcobacter* metabolic activity and that efflux activity is an energy-dependent process, we further investigated resveratrol ability to cause accumulation of EtBr in cells. The results showed a marked increase in EtBr uptake suggesting that resveratrol can act as an efflux pump inhibitor in *Arcobacter* species. Similarly to our results, resveratrol activity as ethidium bromide efflux inhibitor was previously described in *Mycobacterium smegmatis* (Lechner et al., 2008).

Efflux pumps have been described as responsible for Gram-negative bacteria's resistance to resveratrol, with PAβN showing a more evident effect in the reduction on MIC of resveratrol (Jung et al., 2009; Tegos et al., 2002), or other phenolic compounds (Klancnik et al., 2012), when compared with other efflux pump inhibitors. On the other hand, Jung et al. (2009) showed that an AcrB knockout *E. coli* was eightfold more sensitive to resveratrol than the strain with functional efflux systems, suggesting that AcrAB–TolC efflux systems may be important for the survival of bacteria in the presence of resveratrol (Jung et al., 2009). Considering this, we used PAβN, which is described as an effective inhibitor of Resistance-Nodulation-Division (RND) pumps (Nikaido, 2011), to evaluate the influence of the efflux pump systems, namely RND pumps, in resveratrol resistance in *Arcobacter*. Our results showed that PAβN caused an enhancement of the activity of resveratrol with the reduction of MIC by 16 and 4 folds in *A. butzleri* and *A. cryaerophilus*, respectively, showing the contribution of RND pumps in *Arcobacter* resistance to resveratrol.

Therefore, resveratrol demonstrated a simultaneous function as a putative EPI and also contributing to an increase of bacterial susceptibility in the presence of other EPI. This dual role was also described for carnosic acid (Klancnik et al., 2012; Ojeda-Sana et al., 2013).

Overall, these results suggest that resveratrol may affect DNA synthesis in both the *Arcobacter* species studied, leading to an increase of cells with lower DNA content, which can result in an impairment of cell division. Concomitantly, the higher cellular stress caused by resveratrol resulted in a marked decrease in *Arcobacter* metabolic activity. Taken together, these two resveratrol effects can result in cell division impairment and enzyme inhibition due to a decrease in the cell energetic state. Also, the scanning electron microscopy results, together with flow cytometry and efflux pump inhibition assays, suggest that the antibacterial activity of resveratrol may result from the action of resveratrol upon several targets and not from direct membrane perturbation.

The negative perception of consumers on chemical preservatives has triggered food industries to the search of natural products as safer preservative alternatives (Seow et al., 2014), which taken together with the highlighted antimicrobial activity of resveratrol against an emergent food pathogen such as *Arcobacter* and its well-known antioxidant activity, encourages further investigations to consider its use as a promising food preservative.

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References

Bouhdid, S., Abrini, J., Zhiri, A., Espuny, M.J., Manresa, A., 2009. Investigation of functional and morphological changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Origanum compactum* essential oil. J. Appl. Microbiol. 106, 1558–1568.

Bouhdid, S., Abrini, J., Amensour, M., Zhiri, A., Espuny, M.J., Manresa, A., 2010. Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Cinnamomum verum* essential oil. J. Appl. Microbiol. 109, 1139–1149.

Brown, J.C., Jiang, X., 2013. Activities of muscadine grape skin and polyphenolic constituents against *Helicobacter pylori*. J. Appl. Microbiol. 114, 982–991.
Cervenka, L., 2007. Survival and inactivation of *Arcobacter* spp., a current status and future prospect. Crit. Rev. Microbiol. 33, 101–108.
Cervenka, L., Peskova, I., Foltynova, E., Pejchalova, M., Brozkova, I., Vytrasova, J., 2006. Inhibitory effects of some spice and herb extracts against *Arcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii*. Curr. Microbiol. 53, 435–439.
Collado, L., Figueras, M.J., 2011. Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. Clin. Microbiol. Rev. 24, 174–192.
Docherty, J.J., Fu, M.M.H., Stiffler, B.S., Limperos, R.J., Pokabla, C.M., DeLucia, A.L., 1999. Resveratrol inhibition of herpes simplex virus replication. Antivir. Res. 43, 145–155.
Docherty, J.J., McEwen, H.A., Sweet, T.J., Bailey, E., Booth, T.D., 2007. Resveratrol inhibition of *Propionibacterium acnes*. J. Antimicrob. Chemother. 59, 1182–1184.
Figueras, M.J., Levican, A., Collado, L., Inza, M.I., Yustes, C., 2011. *Arcobacter ellisii* sp. nov., isolated from mussels. Syst. Appl. Microbiol. 34, 414–418.
Fisher, K., Rowe, C., Phillips, C.A., 2007. The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components in vitro and on food. Lett. Appl. Microbiol. 44, 495–499.
Jung, C.M., Heinze, T.M., Schnackenberg, L.K., Mullis, L.B., Elkins, S.A., Elkins, C.A., Steele, R. S., Sutherland, J.B., 2009. Interaction of dietary resveratrol with animal-associated bacteria. FEMS Microbiol. Lett. 297, 266–273.
Kiselev, K.V., 2011. Perspectives for production and application of resveratrol. Appl. Microbiol. Biotechnol. 90, 417–425.
Klancnik, A., Mozina, S.S., Zhang, Q., 2012. Anti-*Campylobacter* activities and resistance mechanisms of natural phenolic compounds in *Campylobacter*. PLoS One 7, e51800.
Lechner, D., Gibbons, S., Bucar, F., 2008. Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*. J. Antimicrob. Chemother. 62, 345–348.
Levican, A., Collado, L., Aguilar, C., Yustes, C., Dieguez, A.L., Romalde, J.L., Figueras, M.J., 2012. *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. Syst. Appl. Microbiol. 35, 133–138.
Levican, A., Collado, L., Figueras, M.J., 2013. *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage. Syst. Appl. Microbiol. 36, 22–27.
Martini, S., Bonechi, C., Rossi, C., Figura, N., 2011. Increased susceptibility to resveratrol of *Helicobacter pylori* strains isolated from patients with gastric carcinoma. J. Nat. Prod. 74, 2257–2260.
Nannapaneni, R., Chalova, V.I., Crandall, P.G., Ricke, S.C., Johnson, M.G., O'Bryan, C.A., 2009. *Campylobacter* and *Arcobacter* species sensitivity to commercial orange oil fractions. Int. J. Food Microbiol. 129, 43–49.
Nawrocki, E.M., Bedell, H.W., Humphreys, T.L., 2013. Resveratrol is cidal to both classes of *Haemophilus ducreyi*. Int. J. Antimicrob. Agents 41, 477–479.
Nikaido, H., 2011. Structure and mechanism of RND-type multidrug efflux pumps. Adv. Enzymol. Relat. Areas Mol. Biol. 77, 1–60.
Ojeda-Sana, A., Repetto, V., Moreno, S., 2013. Carnosic acid is an efflux pumps modulator by dissipation of the membrane potential in *Enterococcus faecalis* and *Staphylococcus aureus*. World J. Microbiol. Biotechnol. 29, 137–144.
Parthuisot, N., Catala, P., Lemarchand, K., Baudart, J., Lebaron, P., 2000. Evaluation of ChemChrome V6 for bacterial viability assessment in waters. J. Appl. Microbiol. 89, 370–380.
Paulo, L., Ferreira, S., Gallardo, E., Queiroz, J.A., Domingues, F., 2010. Antimicrobial activity and effects of resveratrol on human pathogenic bacteria. World J. Microbiol. Biotechnol. 26, 1533–1538.
Paulo, L., Oleastro, M., Gallardo, E., Queiroz, J.A., Domingues, F.C., 2011a. Antimicrobial properties of resveratrol: a review. In: Mendez-Vilas, A. (Ed.), Science Against Microbial Pathogens: Communicating Current Research and Technological Advances. Formatex Research Center, pp. 1225–1235.
Paulo, L., Oleastro, M., Gallardo, E., Queiroz, J.A., Domingues, F., 2011b. Anti-*Helicobacter pylori* and urease inhibitory activities of resveratrol and red wine. Food Res. Int. 44, 964–969.
Plumed-Ferrer, C., Vakevainen, K., Komulainen, H., Rautiainen, M., Smeds, A., Raitanen, J.E., Eklund, P., Willfor, S., Alakomi, H.L., Saarela, M., von Wright, A., 2013. The antimicrobial effects of wood-associated polyphenols on food pathogens and spoilage organisms. Int. J. Food Microbiol. 164, 99–107.
Sadruddin, S., Arora, R., 2009. Resveratrol: biologic and therapeutic implications. J. Cardiometab. Syndr. 4, 102–106.
Sasi Jyothsna, T.S., Rahul, K., Ramaprasad, E.V., Sasikala, C., Ramana, C.V., 2013. *Arcobacter anaerophilus* sp. nov., isolated from an estuarine sediment and emended description of the genus *Arcobacter*. Int. J. Syst. Evol. Microbiol. 63, 4619–4625.
Selma, M.V., Larrosa, M., Beltran, D., Lucas, R., Morales, J.C., Tomas-Barberan, F., Espin, J.C., 2012. Resveratrol and Some glucosyl, glucosylacyl, and glucuronide derivatives reduce *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* Scott A adhesion to colonic epithelial cell lines. J. Agric. Food Chem. 60, 7367–7374.
Seow, Y.X., Yeo, C.R., Chung, H.L., Yuk, H.G., 2014. Plant essential oils as active antimicrobial agents. Crit. Rev. Food Sci. Nutr. 54, 625–644.
Silva, F., Lourenço, O., Pina-Vaz, C., Rodrigues, A.G., Queiroz, J.A., Domingues, F.C., 2010. The use of DRAQ5 to monitor intracellular DNA in *Escherichia coli* by flow cytometry. J. Fluoresc. 20, 907–914.
Silva, F., Lourenço, O., Maia, C., Queiroz, J.A., Domingues, F.C., 2011a. Impact of plasmid induction strategy on overall plasmid DNA yield and *E. coli* physiology using flow cytometry and real-time PCR. Process Biochem. 46, 174–181.
Silva, F., Ferreira, S., Queiroz, J.A., Domingues, F.C., 2011b. Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. J. Med. Microbiol. 60, 1479–1486.
Skrivanova, E., Molatova, Z., Matenova, M., Houf, K., Marounek, M., 2010. Inhibitory effect of organic acids on arcobacters in culture and their use for control of *Arcobacter butzleri* on chicken skin. Int. J. Food Microbiol. 144, 367–371.
Stivala, L.A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., Forti, L., Pagnoni, U.M., Albini, A., Prosperi, E., Vannini, V., 2001. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J. Biol. Chem. 276, 22586–22594.
Tegos, G., Stermitz, F.R., Lomovskaya, O., Lewis, K., 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. 46, 3133–3141.
Vandamme, P., Dewhirst, F.E., Paster, B.J., On, S.L.W., 2005. Genus II. *Arcobacter* Vandamme, Falsen, Rossau, Hoste, Segers, Tytgat and De Ley 1991a, 99VP emend. Vandamme, Vancanneyt, Pot, Mels, Hoste, Dewettinck, Vlaes, Van Den Borre, Higgins, Hommez, Kersters, Butzler and Goossens 1992b, 355. In: Brenner, D.J., Kreig, N.P., Staley, J.T., Garrity, G.M. (Eds.), Bergey's Manual of Systematic Bacteriology. Springer, New York, pp. 1161–1165.
Wang, W.B., Lai, H.C., Hsueh, P.R., Chiou, R.Y., Lin, S.B., Liaw, S.J., 2006. Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. J. Med. Microbiol. 55, 1313–1321.

Chapter 5

Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients

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Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients

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ABSTRACT

The present study was conducted to investigate the prevalence and diversity of *Arcobacter* and *Campylobacter* spp. in 298 stool samples of patients with diarrhoea, collected from 22 Portuguese hospitals, between September and November 2012. Detection of *Arcobacter* and *Campylobacter* spp. was performed using molecular-based detection techniques, such as real-time fluorescence resonance energy transfer PCR, species-specific PCR, and sequencing of amplified PCR products. Overall, 1.3% of the samples were positive for *Arcobacter butzleri* and 0.3% for *Arcobacter cryaerophilus*. *Campylobacter* spp. were found in 31.9% of diarrhoeic faeces. *Campylobacter jejuni* and *Campylobacter concisus* were the most prevalent species (13.7% and 8.0%, respectively). The prevalence of *Arcobacter* and *Campylobacter* spp. was significantly different between children and adults (39.7% versus 22.8%, $P = 0.003$). We underline the high prevalence of these pathogens in diarrhoeal samples among Portuguese patients, with particular relevance in the paediatric age group.

1. Introduction

The family *Campylobacteraceae* comprises the genera *Arcobacter*, *Campylobacter*, and *Sulfurospirillum*. Currently, the *Arcobacter* genus comprises 18 species of which *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* have been associated with gastrointestinal infections in humans (Collado and Figueras, 2011; Figueras et al., 2011; Levican et al., 2012, 2013; Sasi Jyothsna et al., 2013).

Several members of *Campylobacter* genus were described as causative of bacterial gastroenteritis. In fact, *Campylobacter jejuni* and *Campylobacter coli* are considered the most frequent cause of intestinal infections worldwide (Moore et al., 2005). The number of confirmed cases of campylobacteriosis in the European Union (EU) has significantly ($P < 0.001$) increased during the period from 2008 to 2011, rising from 190,579 to 220,209 confirmed cases. In 2011, the EU notification rate was 50.28 per 100,000 habitants. Moreover, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in EU from 2005 to 2011 (EFSA and ECDC, 2013).

A number of different *Campylobacter* spp. other than *C. jejuni* and *C. coli* have also been isolated from patients with diarrhoea, namely, *Campylobacter concisus* and *Campylobacter upsaliensis*, which were described as the principal emerging *Campylobacter* spp. (Man, 2011; Vandenberg et al., 2006). The clinical relevance of non–*C. jejuni*/*coli* *Campylobacter* spp. or *Arcobacter* spp. as potential enteric human pathogens may be underestimated due to the employment of culture-based detection that generally fails to detect these organisms and/or even because cells can enter into a viable but non-culturable state (de Boer et al., 2013; Bullman et al., 2012; Lastovica, 2006). The potential limitations of routine culture methods have lead to the increased use of molecular approaches for detection and identification of these species in faecal samples (Abdelbaqi et al., 2007; de Boer et al., 2013; Bullman et al., 2012; Fera et al., 2010; Man et al., 2010; Samie et al., 2007).

Cabrita et al. (1992) evaluated the prevalence of *C. jejuni* and *C. coli* in samples from Portuguese patients with diarrhoea, collected between 1984 and 1989, using traditional culture methods. To date, this was the only study regarding the prevalence of *Campylobacter* spp. in diarrhoeal faeces from Portuguese patients. Moreover, data about the frequency of *Arcobacter* and non–*C. jejuni*/*coli* species are still lacking. Therefore, this study aimed at determining the prevalence and diversity of *Arcobacter* and *Campylobacter* spp. in faeces from patients with diarrhoea in Portugal, using a molecular approach, as well as searching for a possible association between the prevalence of a particular species with patient's demographic data, patient's symptoms, and diarrhoea presentation.

2. Materials and methods

The study was carried in faecal samples from patients with diarrhoea, collected from different hospital wards/clinical specialties from 22 Portuguese hospitals, distributed from North to South, with a good geographic coverage. Faecal samples were collected from

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patients presenting acute gastrointestinal symptoms, from September to November 2012. At the time of sampling, patient's symptoms (abdominal pain, vomiting, or fever) and physical presentation of diarrhoeic samples (bloody or watery) were recorded for follow-up evaluation. The studied samples were randomly selected by patient's age and gender and by geographic region, from those diarrhoeal samples arriving in that period at the hospital laboratories. At arrival, the samples were immediately frozen at −20 °C, and then, selected samples were transported to the national reference laboratory of gastrointestinal infection at the National Institute of Health Dr Ricardo Jorge (INSARJ) and kept at −80 °C until DNA extraction.

2.1. DNA extraction

DNA was extracted with the Specific B protocol on the NucliSens easyMAG system using the NucliSens magnetic kit (bioMérieux, Marcy l' Étoile, France). Before DNA extraction, faeces samples were pre-treated, by mixing the sample (500 μL) with lysis buffer 1 (1.5 mL), followed by vortexing and centrifugation at 13000 rpm in order to recover at least 200 μL of supernatant fraction to be used for DNA extraction.

2.2. Real-time fluorescence resonance energy transfer (FRET)–PCR for the identification of Arcobacter spp.

All samples were subjected to real-time PCR, based on the FRET technology and melting curve analysis, for the identification of *A. butzleri*, *A. cryaerophilus*, *Arcobacter nitrofigilis*, and *Arcobacter cibarius*, as described by Abdelbaqi et al. (2007), with minor modifications, using 3 mmol/L of $MgCl_2$, and performing 55 amplification cycles.

2.3. Detection of Campylobacter spp. in faecal specimens using genus-specific PCR

Campylobacter genus–specific primers (C412F and C1228R) targeting an 816-bp fragment within the 16S rRNA gene of *Campylobacter* spp. were used to detect *Campylobacter* spp. in faecal specimens (Linton et al., 1996). The PCR reactions were performed in a 12.5 μL reaction mixture consisting of 0.5 μmol/L of each primer, 1×PCR buffer, 200 μmol/L of each deoxy-nucleotide-triphosphate, 1.5 mmol/L $MgCl_2$, 0.8 U of BIO-X-ACT Short DNA polymerase (Bioline, London, UK), and 2.5 μL of template DNA. The thermal cycling conditions were: 1 cycle at 95 °C for 5 min, 40 cycles at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 1 min, followed by a final extension step of 10 min at 72 °C. The amplified products were resolved by gel electrophoresis; the presence of PCR products was visualized under ultraviolet transillumination.

2.4. Real-time FRET-PCR for the identification of C. jejuni, C. coli, and C. fetus

All positive samples for *Campylobacter* genus–specific PCR were subjected to real-time PCR based on FRET, targeting the region of *gyrA* gene outside the quinolone resistance–determining region for the identification of *C. jejuni* and *C. coli* with a biprobe (Ménard et al., 2005). A second assay using the same biprobe and PCR conditions, but different PCR primers, was used to identify the presence of *C. fetus*. Reaction mixture and PCR and hybridisation reactions were performed as previously described (Ménard et al., 2005).

2.5. Detection of C. concisus, C. upsaliensis, and Campylobacter lari in faecal specimens using species-specific PCR

Detection of *C. concisus*, *C. upsaliensis*, and *C. lari* in faecal specimens was performed using *Campylobacter* spp.–specific primers outlined in previous works (Man et al., 2010; Wang et al., 2002). The PCR reactions were executed according to the above described for *Campylobacter* genus–specific PCR, but using the annealing temperatures of 62, 52, and 54 °C for *C. concisus*–, *C. upsaliensis*–, and *C. lari*–specific PCR, respectively.

2.6. Sequence identification

For all samples positive for *Campylobacter* genus–specific PCR, but negative for the 6 species-specific assays, the *Campylobacter* genus–specific PCR fragment was both strands sequenced, using the same PCR primers, by the dye termination method using the Big Dye Terminator v1.1 Sequencing standard kit (PE Applied Biosystems Chemistry, Foster City, CA, USA) and the Automated Sequencer Genetic Analyser ABI-Prism 3130 xl (PE Applied Biosystems). Prior to the sequencing reaction, PCR products were purified using the ExoSAP-IT (GE Healthcare, Uppsala, Sweden). The sequences were compared to gene sequences of known identities using the NCBI BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST).

2.7. Statistical analysis

The results of the study were analyzed with the SPSS software Version 18 (SPSS Inc., Chicago, IL, USA), using the Pearson chi-square test to determine the association between the different qualitative PCR results and patient's parameters, such as gender, age, and symptoms, including diarrhoea presentation. In case of statistical limitations of chi-square test, Fisher exact test was used. The *P* value of <0.05 was deemed statistically significant. In a second phase, we have corrected the level of significance for multiple testing using the Bonferroni correction, considering a total of 3 comparisons. In this case, a *P* value of <0.017 was deemed statistically significant.

3. Results

3.1. Clinical information of the study group

A total of 298 diarrhoeal faecal samples, collected between September and November 2012, were analyzed for the presence of *Arcobacter* and *Campylobacter* using molecular detection methods. The patients' mean age was 26 years (ranging from 3 weeks to 99 years), and 148 (49.7%) patients were male gender. The number of paediatric (age <18 years old) samples analyzed was 184 (61.7%), of which 55.4% of the patients were from the paediatric ward, 33.1% from the paediatric emergency unit, and the remaining samples from other services. Concerning the samples collected from adult patients, 52.6% were from emergency units or observation services, 31.6% from gastroenterology ward or consultation, and the remaining from other services.

Clinical information was obtained regarding patients' symptoms and physical presentation of the samples. In addition to diarrhoea, 38 of the 298 patients suffered from abdominal pain, 57 had vomiting, 14 had dehydration, and 41 had fever as a symptom. Thirty-four (11.4%) of the patients also had bloody diarrhoea, 46 (15.4%) had watery diarrhoea, and four of the 298 samples were classified as both watery and bloody.

3.2. Frequency detection of Arcobacter spp.

Overall, the frequency detection of *Arcobacter* and *Campylobacter* was 33.2% corresponding to 99 positive out of 298 samples (Table 1).

A total of 5 (1.7%) samples were positive for *Arcobacter* spp.; 4 (1.3%), positive for *A. butzleri*, among which 1 was detected from a 69-year-old male and 3 from children with ages of 2 and five years old; 1 (0.3%) sample detected from a 9-year-old boy was positive for *A. cryaerophilus*. In addition to *A. butzleri*, the sample of a 2-year-old boy

Table 1
Prevalence of *Campylobacter* and *Arcobacter* spp. in the studied population, according to age, gender, and geographic region.

	Positive samples for *Arcobacter* spp. and *Campylobacter* spp. (% within group)/(% of total)	No. positive samples by specie (% within group)/(% of total)						
		A. butzleri	*A. cryaerophilus*	*C. jejuni*	*C. coli*	*C. fetus*	*C. concisus*	Other *Campylobacter* spp.
0–4 years (n = 121)	43 (35.5)/(14.4) [a,b,c]	2 (1.7)/(0.7)	0 (0.0)/(0.0)	18 (14.9)/(6.0)	3 (2.5)/(1.0)	0 (0.0)/(0.0)	11 (9.1)/(3.7)	15 (12.4)/(5.0)
5–18 years (n = 63)	30 (47.6)/(10.1)	1 (1.6)/(0.3)	1 (1.6)/(0.3)	16 (25.4)/(5.4)	0 (0.0)/(0.0)	0 (0.0)/(0.0)	2 (3.2)/(0.7)	10 (15.9)/(3.4)
19–64 years (n = 57)	14 (24.6)/(4.7) [d]	0 (0.0)/(0.0)	0 (0.0)/(0.0)	4 (7.0)/(1.3)	0 (0.0)/(0.0)	1 (1.8)/(0.3)	7 (12.3)/(2.3)	3 (5.3)/(1.0)
≥65 years (n = 57)	12 (21.1)/(4.0)	1 (1.8)/(0.3)	0 (0.0)/(0.0)	3 (5.3)/(1.0)	0 (0.0)/(0.0)	0 (0.0)/(0.0)	4 (7.0)/(1.3)	4 (7.0)/(1.3)
Female (n = 150)	47 (31.3)/(15.8) [e]	1 (0.7)/(0.3)	0 (0.0)/(0.0)	16 (10.7)/(5.4)	1 (0.7)/(0.3)	1 (0.7)/(0.3)	17 (11.3)/(5.7)	13 (8.7)/(4.4)
Male (n = 148)	52 (35.1)/(17.4) [f]	3 (2.0)/(1.0)	1 (0.7)/(0.3)	25 (16.9)/(8.4)	2 (1.4)/(0.7)	0 (0.0)/(0.0)	7 (4.7)/(2.3)	19 (12.8)/(6.4)
North (n = 36)	12 (33.3)/(4.0)	0 (0.0)/(0.0)	0 (0.0)/(0.0)	3 (8.3)/(1.0)	1 (2.8)/(0.3)	1 (2.8)/(0.3)	3 (8.3)/(1.0)	4 (11.1)/(1.3)
Centre (n = 89)	23 (25.8)/(7.7) [a, c]	2 (2.2)/(0.7)	0 (0.0)/(0.0)	6 (6.7)/(2.0)	1 (1.1)/(0.3)	0 (0.0)/(0.0)	9 (10.1)/(3.0)	8 (9.0)/(2.7)
Great Lisbon (n = 147)	53 (36.1)/(17.8) [b]	2 (1.4)/(0.7)	1 (0.7)/(0.3)	27 (18.4)/(9.1)	1 (0.7)/(0.3)	0 (0.0)/(0.0)	8 (5.4)/(2.7)	17 (11.6)/(5.7)
South (n = 26)	11 (42.3)/(3.7) [d]	0 (0.0)/(0.0)	0 (0.0)/(0.0)	5 (19.2)/(1.7)	0 (0.0)/(0.0)	0 (0.0)/(0.0)	4 (15.4)/(1.3)	3 (11.5)/(1.0)
Total (n = 298)	99 (33.2)	4 (1.3)	1 (0.3)	41 (13.7)	3 (1.0)	1 (0.3)	24 (8.0)	32 (10.7)

[a] One sample was positive for *C. concisus* and *C. ureolyticus*.
[b] Three samples were positive for *C. jejuni* and *C. concisus*.
[c] One sample was positive for *A. butzleri*, *C. jejuni*, and *C. concisus*.
[d] One sample was positive for *C. jejuni* and *C. concisus*.
[e] Two samples were positive for *C. jejuni* and *C. concisus*.
[f] Two samples were positive for *C. jejuni* and *C. concisus*, one was positive for *A. butzleri*, *C. jejuni*, and *C. concisus* and one for *C. concisus* and *C. ureolyticus*.

was also positive for 2 *Campylobacter* spp., *C. jejuni* and *C. concisus* (Table 1); this patient presented fever, a symptom that was not observed in any other of the patients with positive samples for *Arcobacter* spp.

In total, 4 paediatric samples were PCR positive for *A. butzleri* or *A. cryaerophilus* corresponding to 2.2% of the studied children, compared to a prevalence of 0.9% on the adult population.

3.3. Frequency detection of Campylobacter spp.

The samples were also screened for the presence of *Campylobacter* spp. by the use of a genus-specific PCR. A total of 95 samples (31.9%) gave a positive PCR result.

In addition to *C. jejuni* and *C. coli*, other *Campylobacter* spp., such as *C. concisus*, *C. upsaliensis*, *C. fetus*, and *C. lari*, have also been described as consistently isolated from patients with diarrhoea (Man, 2011), all the positive samples for *Campylobacter* genus–specific PCR were subjected to specific PCR targeting those species.

The detection of *Campylobacter* spp. in the 298 diarrhoeic samples showed the presence of *C. jejuni* (13.7%), *C. concisus* (7.4%), *C. coli* (1.0%), and *C. fetus* (0.3%) (Table 1). Using the corresponding species-specific PCR, neither *C. upsaliensis* nor *C. lari* was detected in this cohort of samples. Among the 95 samples positive for the *Campylobacter* genus–specific PCR, 32 were negative in the species-specific assays. In those cases, PCR fragments were subjected to sequencing revealing high level of similarity (between 98 and 100% similarity) to a range of *Campylobacter* spp. (Table 2), namely, *Campylobacter showae* (0.3%), *Campylobacter hominis*, and *Campylobacter ureolyticus* (1.0% each) and *Campylobacter gracilis* (3.7%). Additionally, in 2 cases (0.7%), sequencing of the genus-specific PCR yielded sequences presenting 98 and 99% similarity with *C. concisus*, although the samples were negative in the species-specific PCR, rising the prevalence of *C. concisus* to 8.0%.

Overall, among the *Arcobacter*- or *Campylobacter*-positive stool samples, *C. jejuni* was by far the most common identified species (41.4%; 41/99), followed by *C. concisus* (24.2%; 24/99) (Table 1).

The distribution of the 99 positive samples for *Arcobacter* and *Campylobacter* organisms among the 298 diarrhoeal stool specimens analyzed is depicted in Fig. 1.

3.4. Arcobacter and Campylobacter prevalence according to age, gender, and geographic region

To evaluate if the prevalence of *Arcobacter* and *Campylobacter* spp. was associated with age (Tables 1 and 2, and Fig. 2), gender, or geographic region of sample provenience (Table 1), we compared the distribution of positive samples according to these 3 criteria.

When considering the distribution of both genus by age groups, there was a significant difference between children (<18 years old) and adult patients (39.7% versus 22.8%, $P = 0.003$), the same trend being observed for each genus, *Campylobacter* spp. (38.0% versus 21.9%, $P = 0.004$) and *Arcobacter* spp. (2.2% versus 0.9%), although in this latter case with no statistical significance probably due the small number of positive cases.

Specifying by species, 34 of the 184 paediatric samples (18.5%) were positive for *C. jejuni* in contrast to only 7 positive among the 114 adults samples (6.1%) ($P = 0.003$), and the 3 *C. coli*–positive samples were all detected in samples from children aged between 0 and 4 years old. Concerning the overall prevalence of other *Campylobacter* spp. detected by sequencing of amplified genus-specific PCR product,

Table 2
Prevalence of *Campylobacter* spp. in 4 different age groups by sequencing of the amplified genus PCR product.

Age group	Prevalence of *Campylobacter* spp. (% within age group)					
	C. showae	*C. hominis*	*C. gracilis*	*C. ureolyticus*	Mixed sequences[a]	Overall
0–4 years (n = 121)	1/121 (0.8)	2/121 (1.7)	2/121 (1.7)	2/121 (1.7)	8/121 (6.7)	15/121(12.5)
5–18 years (n = 63)	0/63 (0.0)	1/63 (1.6)	3/63 (4.8)	1/63 (1.6)	5/63 (7.9)	10/63 (15.9)
19–64 years (n = 57)	0/57 (0.0)	0/57 (0.0)	2/57 (3.5)	0/57 (0.0)	1/57 (1.8)	3/57 (5.3)
≥65 years (n = 57)	0/57 (0.0)	0/57 (0.0)	4/57 (7.0)	0/57 (0.0)	0/57 (0.0)	4/57 (7.0)
Total	1/298 (0.3)	3/298 (1.0)	11/298 (3.7)	3/298 (1.0)	14/298 (4.7)	32/298 (10.7)

A minimum of 98% of similarity was considered to define a species.
[a] Contains sequences from multiple *Campylobacter* spp.

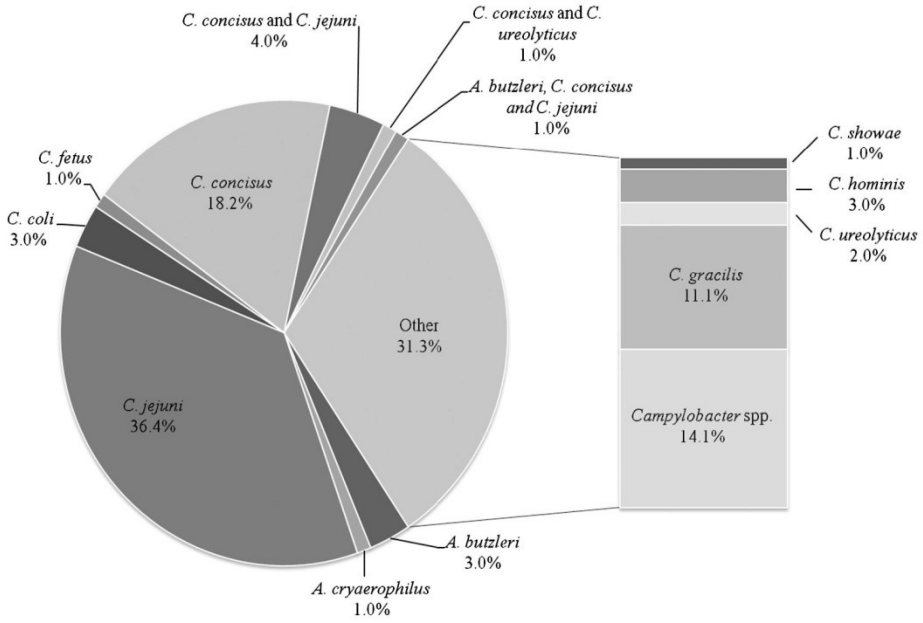

Fig. 1. Distribution of the 99 samples, among the 298 diarrhoeal stool specimens analyzed, which are positive for *Arcobacter* and *Campylobacter* organisms.

a significant difference between children and adults was also observed (13.6% versus 6.1%, $P = 0.04$). On the whole and considering the multiple testing adjustment, a highly significant association was found between genus *Campylobacter* and the species *C. jejuni* present in children's diarrhoeal samples.

Regarding the two most frequently found species, *C. jejuni* and *C. concisus*, the highest prevalence for *C. jejuni* infection was observed on both the age groups from 0 to 4 years (14.9%) and 5 to 18 years (25.4%), while the highest detection values for *C. concisus* was observed on young children (0–4 years old) (9.1%), but also on patients aged between 19 and 64 years old (12.3%) (Table 1). Concerning the other *Campylobacter* spp. detected by genus-specific PCR, there was no overall difference among the children and adult groups, although the prevalence of *C. gracilis* was higher among patients aged above 65 years.

Table 1 further indicates the occurrence of different species according to gender; however, only *C. concisus* was found to be more prevalent in females (11.3% versus 4.7%, $P = 0.036$).

We performed a third analysis grouping the samples according to the hospital geographical origin into 4 main regions: North, Centre, great Lisbon, and South. The highest prevalence of *Campylobacter*- and *Arcobacter*-positive samples was found for samples coming from the south of the country (42.3%) and the lowest for the centre (25.8%), but overall, no significant difference was observed.

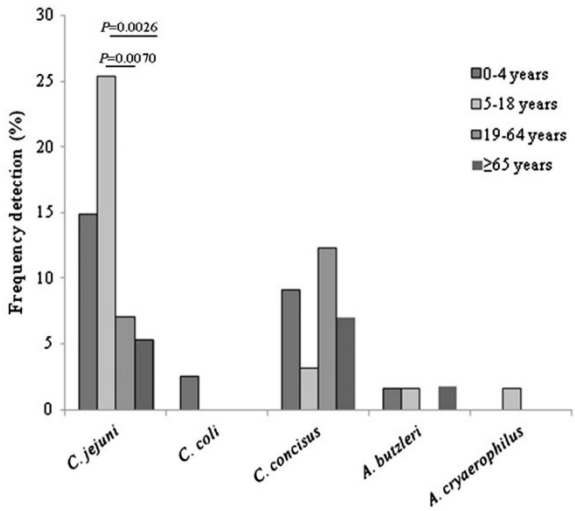

Fig. 2. Distribution *C. jejuni*, *C. coli*, *C. concisus*, *A. butzleri*, and *A. cryaerophilus*, according to age in patients with diarrhoea.

3.5. Association of Arcobacter and Campylobacter infection with clinical presentation

In general, *Arcobacter* and *Campylobacter* infection and *C. jejuni* infection were significantly associated with bloody diarrhoea ($P = 0.027$ and $P < 0.0001$, respectively), fever ($P = 0.003$ and $P < 0.001$, respectively), and abdominal pain ($P = 0.047$ and $P = 0.004$, respectively), while no association was observed with watery diarrhoea. Furthermore, a strong correlation between vomiting and *C. concisus* was found ($P = 0.007$). In addition, 2 of the 4 patients infected with *Arcobacter*, one with *A. butzleri* and other with *A. cryaerophilus*, suffered both from vomiting and abdominal pain. Considering the low prevalence of the species detected by sequencing of amplified PCR product, the association between infection and clinical symptoms was done taking those species as a group, but did not allow us to establish a correlation between the 2 variables.

Considering the multiple testing adjustment, infection with *C. jejuni* was significantly associated with bloody diarrhoea, fever, and abdominal pain, while infection with *C. concisus* was found to be significantly associated with vomiting.

4. Discussion

The prevalence and pathogenic potential of *Arcobacter* spp. have been investigated more thoroughly in the last decade (Abdelbaqi et al., 2007; de Boer et al., 2013; Collado et al., 2013; Pejchalova et al.,

2008; Prouzet-Mauleon et al., 2006; Vandenberg et al., 2004, 2006); also, the number of non–*C. jejuni*/*coli* species recognized as important human pathogens has recently risen (Bullman et al., 2011, 2012; Maher et al., 2003; Man, 2011).

In this study, the prevalence of *Arcobacter* and *Campylobacter* spp. was evaluated in diarrhoeic samples, by the use of real-time FRET-PCR, conventional PCR, and PCR products sequencing. To our knowledge, this is the first study regarding *Arcobacter* and non–*C. jejuni*/*coli* prevalence in diarrhoeic faeces in Portugal.

In this cohort of samples, *A. butzleri* was the fourth most frequent species (Table 1) presenting a detection frequency of 1.3%, which is similar to the recently reported incidence on diarrhoeic samples, using molecular detection methods, in France (Abdelbaqi et al., 2007) and in Chile (Collado et al., 2013). However, prevalence of *Arcobacter* spp. seems to vary greatly. For example, in a study from Venda region in South Africa, also by molecular detection, 6.2% of the 322 stool samples analyzed were positive for *A. butzleri*, 2.8% for *A. cryaerophilus*, and 1.9% for *A. skirrowii*; however, no direct correlation with diarrhoea was found with any of these species (Samie et al., 2007). In contrast, using the same diagnostic approach, an aetiology study of travelers' diarrhoea of US and European patients with acute diarrhoea acquired in Mexico, Guatemala, and India reported a prevalence of *A. butzleri* infection of 8% (Jiang et al., 2010). Typically, the reported values of prevalence of *Arcobacter* spp. determined by molecular methods are superior than those using culture methods (Collado and Figueras, 2011). Nevertheless, a recent study from The Netherlands (de Boer et al., 2013), using a real-time multiplex PCR, examined 493 stool samples from patients with suspected infectious gastroenteritis and only found 0.4% positive for *A. butzleri*. In the present study, *Arcobacter* spp. infection was detected in children, and 1 case was detected in an aged person (>65 years). On the other hand, even affecting the more susceptible individuals, children, and aged persons, the symptoms associated with the infection were milder than the ones observed in patients infected by *Campylobacter* spp., especially *C. jejuni*. Taken together and considering that the studied samples were all isolated in hospital setting, these data may suggest that prevalence of *Arcobacter* spp. may be higher than here reported.

Campylobacteriosis in humans is regularly associated to *C. jejuni* followed by *C. coli*, and *C. lari* (EFSA and ECDC, 2013), but at least 10 non–*C. jejuni*/*coli* species have been detected from patients with gastroenteritis (Man, 2011). As expected, in this study, *C. jejuni* was the most frequently found species in the stool samples analyzed, with an overall frequency of 13.7%, which is similar to that reported in other studies concerning its prevalence in human diarrhoeic samples (Collado et al., 2013; Samie et al., 2007). Nonetheless, the rates of detection are variable, which is evidenced in studies reporting lower prevalence rates, such as 3.4% in Southern Ireland (Bullman et al., 2011), using molecular detection, and 8.2% in Iceland, using culture methods (Hilmarsdottir et al., 2012).

In contrast with the common finding of *C. coli* as the second most prevalent *Campylobacter* spp., some studies (de Boer et al., 2013; Collado et al., 2013) and our results show that *C. concisus* was more frequently detected than *C. coli* (8.0% versus 1.0%). The high prevalence of *C. concisus* (Table 1) comes along with a high rate of co-detection (25%) in agreement with other authors (de Boer et al., 2013; Nielsen et al., 2012a). Besides the co-detection of *C. concisus* with either *C. jejuni* (4 samples) or *C. ureolyticus* (1 sample), there was a multiple detection of *C. concisus*, *C. jejuni*, and *A. butzleri*. The simultaneous presence of several *Arcobacter* and/or *Campylobacter* spp. was also described by other authors (Collado et al., 2013; Jiang et al., 2010; Samie et al., 2007).

Collado et al. (2013) found a significant association between the detection of *C. concisus* and diarrhoea. However, in our study, there was a lack of a control group comprised by healthy individuals, which hampers the establishment of a potential correlation between *C. concisus* and enteric disease. Even so, we observed a significant association of *C. concisus* and vomiting ($P = 0.007$). In accordance, a recent study reported that 40.6% of the patients infected with *C. concisus* presented upper gastrointestinal symptoms, such as vomiting, highlighting that *C. concisus* infection may be understood not as an enteritis, but as a gastroenteritis (Nielsen et al., 2012b). Further studies, including healthy control groups, are needed to address unequivocally the clinical relevance of this organism. In addition, the genetic diversity among *C. concisus* has been proposed as an approach to clarify its ability to cause intestinal disease (Man, 2011).

Regarding other campylobacteria presence, some reports describe a low prevalence (2/11,314 diarrhoeic stool samples) of *C. upsaliensis* (Nielsen et al., 2012a) or even no detection of *C. lari* or *C. upsaliensis* on analyzed samples (Engberg et al., 2000). In a study from Germany, several *Campylobacter* spp. were involved in human infections, although the reported rates were low, *C. coli* (8.1%), *C. lari* (1%), *C. upsaliensis* (0.07%), and *C. fetus* (0.02%) (Stingl et al., 2012). In the current study, neither *C. upsaliensis* nor *C. lari* were detected, and *C. fetus* was only detected in a single sample.

In general, it is observed that *Campylobacter* infection varies according to age and gender, seasonally and even geographically (FAO/WHO, 2009). In our study, we evaluated *Campylobacter* and *Arcobacter* distribution by patient's age, gender and geographical provenience of the sample. As expected, prevalence of *Campylobacter* spp. was higher in children (Fig. 2). Indeed, our rate of detection in this age group was 38.0% for *Campylobacter* spp. and 18.5% for *C. jejuni*, which was considerable higher than those reported in other studies from European countries. Floch et al. (2012) found 25 (24 *C. jejuni*) positive out of 609 stool samples from French patients with community-acquired enteric infection, giving an overall prevalence of 4.1%, being the detection similar among adults and children (<16 years); another study evaluating the epidemiology of severe gastroenteritis in children, aged from 6 months to 5 years living in Québec rural areas, found a prevalence of *Campylobacter* infection of 11.2% (Levallois et al., 2013). Looking at low-income countries, higher rates of infection by *Campylobacter* spp., especially *C. jejuni*, are reported among under 5-year-old ill children, such as 15.4% in Northwest Ethiopia (Lengerh et al., 2013) and 21% in Malawi (Mason et al., 2013). Moreover, in a study conducted in Chile (Collado et al., 2013), 31.6% of the 57 diarrhoeic samples of patients under 5 years old analyzed were positive for *Campylobacter* spp. and/or *Arcobacter* spp., which is similar with our rate of infection in this age group (35.5%).

Thus, based on our results, with a considerable national coverage, Portugal presents *Campylobacter* detection rates that seem more close to developing countries than to European countries; however, this statement must be done carefully, since several factors should be considered, such as the number of samples tested, differences in studied populations, and even the influence of the detection method used, traditional culture versus molecular detection, as addressed by Collado et al. (2013). Generally, it seems that males have a higher incidence rate of *Campylobacter* infections than females (FAO/WHO, 2009), but our study showed otherwise. Indeed, we found an association between female gender and *C. concisus*. Nevertheless, *C. jejuni* was more frequent in males (16.9%) than in females (10.7%), the same trend being observed for *C. coli*, *A. butzleri*, and *A. cryaerophilus* (Table 1).

The high detection of *Arcobacter* and *Campylobacter* spp.–associated infections found in this study gains relevance if we consider that all samples were collected in hospital setting, either from hospitalized patients or from patients that attended care services due to the severity of the symptoms.

In conclusion, this study, conducted in Portugal for the first time, highlights the prevalence and diversity of *Arcobacter* and *Campylobacter* spp. found in diarrhoea samples, with special emphasis on the paediatric age group. These findings also contribute for the understanding of the epidemiology of *Arcobacter* and *Campylobacter* in Portugal and in Europe. However, despite the existing evidence that

Arcobacter spp. and non–*C. jejuni/coli* may be involved in gastrointestinal diseases, further studies, performed on different geographical regions, including developed and developing countries, are needed to demonstrate the importance of these organisms as an aetiological agent of the enteric disease.

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References

Abdelbaqi K, Buissonnière A, Prouzet-Mauleon V, Gresser J, Wesley I, Mégraud F, Ménard A. Development of a real-time fluorescence resonance energy transfer PCR to detect *Arcobacter* species. J Clin Microbiol 2007;45:3015–21.

Bullman S, Corcoran D, O'Leary J, O'Hare D, Lucey B, Sleator RD. Emerging dynamics of human campylobacteriosis in Southern Ireland. FEMS Immunol Med Microbiol 2011;63:248–53.

Bullman S, O'Leary J, Corcoran D, Sleator RD, Lucey B. Molecular-based detection of non-culturable and emerging campylobacteria in patients presenting with gastroenteritis. Epidemiol Infect 2012;140:684–8.

Cabrita J, Pires I, Vlaes L, Coignau H, Levy J, Goossens H, et al. *Campylobacter*-Enteritis in Portugal—Epidemiologic Features and Biological Markers. Eur J Epidemiol 1992;8:22–6.

Collado L, Figueras MJ. Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. Clin Microbiol Rev 2011;24:174–92.

Collado L, Gutiérrez M, González M, Fernández H. Assessment of the prevalence and diversity of emergent campylobacteria in human stool samples using a combination of traditional and molecular methods. Diagn Microbiol Infect Dis 2013;75:434–6.

de Boer RF, Ott A, Güren P, van Zanten E, van Belkum A, Kooistra-Smid AM. Detection of *Campylobacter* species and *Arcobacter butzleri* in stool samples by use of real-time multiplex PCR. J Clin Microbiol 2013;51:253–9.

EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal 2013;11. 250 pp.

Engberg J, On SLW, Harrington CS, Gerner-Smidt P. Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for campylobacters. J Clin Microbiol 2000;38:286–91.

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization). Risk assessment of *Campylobacter* spp. in broiler chickens: technical report. Microbiological Risk Assessment Series 2009;12. 132 pp.

Fera MT, Russo GT, Di Benedetto A, La Camera E, Orlando A, Giandalia A, et al. High prevalence of *Arcobacter* carriage in older subjects with type 2 diabetes. J Biomed Biotechnol 2010:489784.

Figueras MJ, Levican A, Collado L, Inza MI, Yustes C. *Arcobacter ellisii* sp nov., isolated from mussels. Syst Appl Microbiol 2011;34:414–8.

Floch P, Goret J, Bessede E, Lehours P, Mégraud F. Evaluation of the positive predictive value of a rapid immunochromatographic test to detect *Campylobacter* in stools. Gut Pathog 2012;4:17.

Hilmarsdottir I, Baldvinsdottir GE, Harethardottir H, Briem H, Sigurethsson SI. Enteropathogens in acute diarrhea: a general practice-based study in a Nordic country. Eur J Clin Microbiol Infect Dis 2012;31:1501–9.

Jiang ZD, Dupont HL, Brown EL, Nandy RK, Ramamurthy T, Sinha A, et al. Microbial etiology of travelers' diarrhea in Mexico, Guatemala, and India: importance of enterotoxigenic *Bacteroides fragilis* and *Arcobacter* species. J Clin Microbiol 2010;48:1417–9.

Lastovica AJ. Emerging *Campylobacter* spp.: The tip of the iceberg. Clin Microbiol Newsl 2006;28:49–56.

Lengerh A, Moges F, Unakal C, Anagaw B. Prevalence, associated risk factors and antimicrobial susceptibility pattern of *Campylobacter* species among under five diarrheic children at Gondar University Hospital, Northwest Ethiopia. BMC Pediatr 2013;13:82.

Levallois P, Chevalier P, Gingras S, Dery P, Payment P, Michel P, Rodriguez M. Risk of infectious gastroenteritis in young children living in Quebec rural areas with intensive animal farming: results of a case–control study (2004–2007). Zoonoses Public Health 2013. http://dx.doi.org/10.1111/zph.12039.

Levican A, Collado L, Aguilar C, Yustes C, Dieguez AL, Romalde JL, Figueras MJ. *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. Syst Appl Microbiol 2012;35:133–8.

Levican A, Collado L, Figueras MJ. *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage. Syst Appl Microbiol 2013;36:22–7.

Linton D, Owen RJ, Stanley J. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. Res Microbiol 1996;147:707–18.

Maher M, Finnegan C, Collins E, Ward B, Carroll C, Cormican M. Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of feces. J Clin Microbiol 2003;41:2980–6.

Man SM. The clinical importance of emerging *Campylobacter* species. Nat Rev Gastroenterol Hepatol 2011;8:669–85.

Man SM, Zhang L, Day AS, Leach ST, Lemberg DA, Mitchell H. *Campylobacter concisus* and other *Campylobacter* species in children with newly diagnosed Crohn's disease. Inflamm Bowel Dis 2010;16:1008–16.

Mason J, Iturriza-Gomara M, O'Brien SJ, Ngwira BM, Dove W, Maiden MC, Cunliffe NA. *Campylobacter* infection in children in Malawi is common and is frequently associated with enteric virus co-infections. PLoS One 2013;8:e59663.

Ménard A, Dachet F, Prouzet-Mauleon V, Oleastro M, Mégraud F. Development of a real-time fluorescence resonance energy transfer PCR to identify the main pathogenic *Campylobacter* spp. Clin Microbiol Infect 2005;11:281–7.

Moore JE, Corcoran D, Dooley JS, Fanning S, Lucey B, Matsuda M, et al. *Campylobacter*. Vet Res 2005;36:351–82.

Nielsen HL, Ejlertsen T, Engberg J, Nielsen H. High incidence of *Campylobacter concisus* in gastroenteritis in North Jutland, Denmark: a population-based study. Clin Microbiol Infect 2012a;19:445–50.

Nielsen HL, Engberg J, Ejlertsen T, Bucker R, Nielsen H. Short-term and medium-term clinical outcomes of *Campylobacter concisus* infection. Clin Microbiol Infect 2012b;18:E459–65.

Pejchalova M, Dostalikova E, Slamova M, Brozkova I, Vytrasova J. Prevalence and diversity of *Arcobacter* spp. in the Czech Republic. J Food Prot 2008;71:719–27.

Prouzet-Mauleon V, Labadi L, Bouges N, Menard A, Megraud F. *Arcobacter butzleri*: underestimated enteropathogen. Emerg Infect Dis 2006;12:307–9.

Samie A, Obi CL, Barrett LJ, Powell SM, Guerrant RL. Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. J Infect 2007;54:558–66.

Sasi Jyothsna TS, Rahul K, Ramaprasad EV, Sasikala C, Ramana CV. *Arcobacter anaerophilus* sp. nov., isolated from an estuarine sediment and emended description of the genus Arcobacter. Int J Syst Evol Microbiol 2013. (in press).

Stingl K, Knüver M-T, Vogt P, Buhler C, Krüger N-J, Alt K, et al. Quo Vadis?—Monitoring *Campylobacter* in Germany. Europ J Microbiol Immunol 2012;2:88–96.

Vandenberg O, Houf K, Douat N, Vlaes L, Retore P, Butzler JP, Dediste A. Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium. J Antimicrob Chemother 2006;57:908–13.

Vandenberg O, Dediste A, Houf K, Ibekwem S, Souayah H, Cadranel S, et al. *Arcobacter* species in humans. Emerg Infect Dis 2004;10:1863–7.

Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, et al. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J Clin Microbiol 2002;40:4744–7.

Chapter 6

Comparative pathogenic and genotypic properties of human and non-human *Arcobacter butzleri* strains

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Submitted

Comparative pathogenic and genotypic properties of human and non-human *Arcobacter butzleri* strains

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Abbreviated running headline: Pathogenicity of *Arcobacter butzleri*

Abstract

Aims: To evaluate *Arcobacter butzleri* virulence by the study of its genotypic and pathogenic properties.

Methods and Results: A total of six *A. butzleri* isolates were used in this study, comprising three strains from poultry or slaughterhouse environment and three strains of human origin. The minimum inhibitory concentrations of 13 antibiotics were determined, the isolates showed to be susceptible to tetracyclines and aminoglycosides, with non-human isolates being resistant to quinolones. The ability to form biofilms was variable among the strains, and all of them showed a weak haemolytic activity. Nine putative virulence genes were detected by PCR, with *cadF, ciaB, cj1349, mviN, pldA, tlyA* being identified in all strains. High levels of adhesion were observed for *A. butzleri* on Caco-2 cells, with pre-existing inflammation showing variable effect on invasion observed. *A. butzleri* isolates were able to survive intracellularly and to induce an up-regulation of interleukin-8 secretion and structural cell rearrangements.

Conclusions: *A. butzleri* showed *in vitro* proinflammatory properties, together to adherence and invasion abilities, and also of intracellular survival in Caco-2 cells, which may be associated with distinctive pathogenic potentials among strains.

Significance and Impact of Study: These data brings new insights on *A. butzleri* virulence and highlights its pathogenic potential.

Keywords: *Arcobacter butzleri*, virulence, adhesion, invasion, intracellular survival, inflammation

Introduction

The genus *Arcobacter* belongs to the family *Campylobacteraceae* of the class E*psilonproteobacteria*, which also comprises the genera *Campylobacter* and *Sulfurospirillum*. It is constituted by 18 recognized species, isolated from environmental, animal and human sources (Sasi Jyothsna *et al*. 2013).

Among the *Arcobacter* species, three have been implicated on human illness, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. *A. butzleri* is the species most often correlated with human disease, with the majority of the human cases reported being associated with enteritis (Wesley and Miller 2010), being persistent or watery diarrhoea the predominant symptoms (Vandenberg *et al*. 2004). Among *Campylobacter* spp and related organism, it has been described as the fourth most prevalent pathogen found in diarrhoeic samples (Vandenberg *et al*. 2004; Prouzet-Mauleon *et al*. 2006; Samie *et al*. 2007; Collado *et al*. 2013; Ferreira *et al*. 2014).

A. butzleri transmission routes, whose biology is not tightly associated with any particular host or hosts, are not clearly associated with human disease, however it is described as a probable environmental organism, that can cause disease through either water-mediated food contamination, or ingestion of contaminated water (Miller *et al*. 2007). The presence of *A. butzleri* in pet cats and dogs (Petersen *et al*. 2007; Houf *et al*. 2008; Fera *et al*. 2009), suggests that the contact with these animals can be one potential route of human infection. Possible person-to-person transmission was also suggested due to the successive timing of cases of abdominal cramps outbreak in an Italian School (Vandamme *et al*. 1992a), or to the possible contraction of infection *in utero* by a neonate (On *et al*. 1995).

Despite the established relation between *A. butzleri* and human disease, currently the pathogenic potential of *Arcobacter* has remained relatively unexplored with few studies focused on clarifying the pathogenic mechanisms related to this genus. Several *in vitro* studies have been performed to characterize adhesion, invasion and cytotoxic potential of *Arcobacter* species to host cells, with adherence as one of the most commonly observed effects. Nevertheless, a considerable variation in adherence has been observed, correlated with the origin of strains and cell lines used in the different studies (Ho *et al*. 2007; Collado and Figueras 2011).

The completion of *A. butzleri* RM4018 genome sequence identified several putative virulence factors, some of them presenting homology to *C. jejuni* virulence determinants (Miller *et al*. 2007). Since then, two studies tried to establish a relation between the presence of these genes and the adhesion and invasion ability of *Arcobacter* isolates on cell line models (Karadas *et al*. 2013; Levican *et al*. 2013).

Also, the lack of surveillance studies hampers the clear assessment of the current trends in *Arcobacter* resistance profiles and/or the prevalence of this pathogen worldwide. Although

Arcobacter enteritis appears to be self-limiting, the severity or prolongation of the symptoms may support the use of antibiotic treatment (Collado and Figueras 2011). Several works have shown that *A. butzleri* strains can present multidrug resistance (Harrass *et al*. 1998; Kabeya *et al*. 2004; Son *et al*. 2007; Abay *et al*. 2011), namely to some of the antibiotics recommended for treatment of *Campylobacter* human infection, such as erythromycin and ciprofloxacin (Vandenberg *et al*. 2006; Shah *et al*. 2012, 2013; Villalobos *et al*. 2013).

In the present study, we aimed to get further insight into *A. butzleri* virulence. Experiments were designed to perform first a characterization of genotypic and pathogenic properties of *A. butzleri* isolates from human and non-human origin, and secondly to clarify the ability of *A. butzleri* to adhere, to invade and to survive within the intracellular environment of cultured intestinal epithelial cells. Expression of the proinflammatory cytokine interleukin-8 (IL-8) and the pathogenic potential of *A. butzleri* under the effect of pre-existing inflammation were also evaluated.

Materials and Methods

Bacterial strains and growth conditions

A total of six *A. butzleri* isolates were used in this study (Table 1), comprising three strains from poultry or slaughterhouse environment (Ferreira *et al*. 2013) and three strains of human origin (Table 1). *Salmonella enterica* subsp. *enterica* (*ex* Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium ATCC 13311 was used as control strain for adhesion and invasion assays. Isolates were stored at -80 ºC in brain heart infusion (Liofilchem, Roseto degli Abruzzi, Italy) containing 20% glycerol. The isolates were subcultured on blood agar base (Oxoid, Hampshire, England) supplemented with 5 % (V/V) defibrinated horse blood (Oxoid, Hampshire, England) (BA) and incubated at 37 ºC in microaerobic atmosphere (6 % O_2, ±7.1 % CO_2, 3.6 % H_2, 83 % N_2) for 24 h. Control strain was cultured in LB agar at 37 ºC for 24 h. For growth in liquid medium, *A. butzleri* were transferred to 10 ml of Mueller Hinton broth (MHB) (Liofilchem) and incubated at 37 ºC in microaerobic atmosphere for 24 h, while *S.* Typhimurium was cultured in aerobic conditions at 37 ºC.

Antimicrobial activity

The minimum inhibitory concentrations (MIC) of 13 antibiotics were determined in microplates as previously described (Ferreira *et al*. 2013). The antibiotics tested included ampicillin, ciprofloxacin, vancomycin, trimethoprim, cefoperazone, piperacillin, choramphenicol, gentamicin, amoxicillin, erythromycin, doxycycline, tetracycline and nalidixic acid.

Haemolytic activity

The haemolysis ability of *Arcobacter* isolates were tested in human erythrocytes as previously described with slight modifications (Robinson *et al*. 1990; Kalischuk and Inglis 2011). *A. butzleri* strains were grown in MHB as described above, after which the cells were centrifuged (5 min, 10 000 g), washed in a sterile phosphate-buffered saline, pH 7.4 (10 mmol l^{-1}) (PBS) and then resuspended in the same buffer. Erythrocytes were washed three times in PBS and a suspension was prepared in the same buffer. Regarding the haemolytic activity assay the bacterial suspension was adjusted to an OD_{620nm} of 1.0, and then serially 2-fold diluted in PBS. *A. butzleri* suspension dilutions (100 µl) and the same volume of 2% (V/V) erythrocytes suspension were mixed in a 96-well plate with a V bottom. The plate was then incubated at 37 ºC under microaerobic conditions for 18 h. The plates were centrifuged at 1000 g for 5 min, 100 µl of the supernatants was removed from each well to a 96-well plate and the absorvance at 492 nm was measured in a microplate reader. A negative control (without

bacteria) and a positive control of total haemolysis (1% (V/V) Triton X-100) were also included.

Biofilm formation ability

The biofilm formation ability assays of *A. butzleri* isolates were performed as previously described (Ferreira *et al.* 2013), with the following modifications: biofilm formation was initialized using 24 h-grown cultures, and biofilms were washed three times after staining.

Virulence genes

Genomic DNA was extracted using the Wizard Genomic DNA purification Kit (Promega, Wisconsin, USA) from for 24 h-grown cultures at 37 ºC, in microaerophilic conditions, on Blood agar plates. Detection of putative virulence genes was performed by PCR according to Douidah *et al.* (2012), with some modifications. Briefly, all PCRs were carried out in a reaction volume of 25 µl containing 12.5 µl Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 0.5 µmol l^{-1} of each primer, 2 mmol l^{-1} of $MgCl_2$, and 5 µl of template DNA. An initial denaturation step at 95 °C for 5 min was followed by 32 cycles at 95 °C for 45 s, primer annealing at 56 ºC for 45 s, for the primer sets *ciaB*, *cj1349*, *hecA*, *irgA*, *mviN*, and at 55 ºC for 45 s for the primer sets *cadF*, *hecB*, *pldA*, *tlyA*, an elongation of 45 s at 72 °C, and a final extension step of 10 min at 72 ºC. The amplified products were resolved by gel electrophoresis; and visualized with GreenSafe (NZYTech, Lisbon, Portugal) under ultraviolet transillumination.

Adhesion and invasion assays

The Caco-2 human intestinal epithelial was maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma, St Louis, USA) supplemented with 10% (V/V) foetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), 1 % (V/V) nonessential amino acids, 100 µg ml^{-1} streptomycin, and 100 U ml^{-1} penicillin. The cells were maintained at 37 °C in 5 % CO_2 and 95 % air, and were grown in tissue culture flasks at 37 ºC in a 5 % CO_2 humidified atmosphere, with regular changes of medium. For Caco-2 cell co-culture experiments, cells were seeded at 4.5×10^4 cells by well and grown in 24-well plates for 7 days at 37 ºC in a 5 % CO_2 and 95 % air.

The *A. butzleri* and *S.* Typhimurium broth cultures were centrifuged (5 min, 10 000 g) and cells were washed and resuspended in warm (37 ºC) medium used in Caco-2 cell culture, but without antibiotic. The culture density was estimated by serial dilution and plating onto Blood Agar or LB plates. Strains (about 10^8 CFU/well) were added to Caco-2 cells and incubated for 3 h, to allow adhesion and invasion to occur. Following this period, the cells were washed

three times with PBS to remove unbound bacteria and the number of interacting (adherent and internalized) bacteria was determined by lysing the cells with the addition of 1% (V/V) Triton X-100 for 5 min, followed by plate count bacteria. For bacterial invasion measuring, medium containing 125 µg/ml of gentamicin was added for 1 h to kill extracellular bacteria. Then, cells were lysed with 1% (V/V) Triton X100, and the released intracellular bacteria were enumerated by plating serial dilutions of the lysates on blood agar or LB plates. Results were expressed as the mean±SD of the $\log_{10}$ CFU ml^{-1} of adherent (interacting-internalized bacteria) or internalized bacteria, for three replicates in at least three independent measurements.

When applicable, prior to infection, Caco-2 cells were washed with PBS and treated with 40 ng ml^{-1} TNF-α (ImmunoTools) for 1 h prior to adherence and invasion assays (Man *et al*. 2012).

Intracellular survival in Caco-2 cells

For intracellular survival assays, bacterial cells were co-cultured with Caco-2 cells for 3 h, washed three times with PBS, incubated with 125 µg ml^{-1} gentamicin for 1 h, washed again with PBS and incubated with a reduced concentration of gentamicin (10 µg ml^{-1}) for 20 h. The cells were then washed three times in PBS, and the epithelial cells were lysed as described in the previous section.

Quantification of Interleukin-8

After 24 h of infection of Caco-2 cells with the bacterial suspensions, the level of secreted IL-8 cytokine was measured in cell's supernatants by using a ELISA kit (Human IL-8 / CXCL8 ELISA Kit) (Sigma). The assay was conducted according to the manufacturer's protocol.

Actin visualization

This assay was performed for one poultry associated strain (AB28/11) and one human strain (INSA593). The cells were cultured and infected as described for the interaction assays. After 3 h of exposure, the cells were washed three times with PBS, fixed for 10 minutes with paraformaldehyde at room temperature. These were washed again with PBS and permeabilized for 5 min with 0.1% Triton X100. The cells were then washed and incubated with 20% FBS in PBS with 0.01% of Tween 20 (PBS-T) for 1 h. After PBS-T washing, cells were incubated overnight at 4 ºC with primary ß-actin monoclonal antibody in a final dilution 1:2000 (Sigma, St Louis, USA), followed by incubation with an Alexa 488-labelled secondary antibody at a final dilution 1:1000 (Molecular Probes, LifeTecnhologies), for 1 h at room temperature in the dark. After washing, Hoechst 33342 was used. Following three washes, the

coverslips were mounted in microscope slides with Entellan (Merck, Darmstadt, Germany) and examined using a LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical significance was determined by the t-Student test. In all cases differences were considered significant at $P<0.05$.

Results

In the present study, three *A. butzleri* isolates recovered from poultry or poultry slaughterhouse environment and three isolates recovered from human stools were characterized regarding its genotypic and pathogenic properties.

Table 1 shows the minimum inhibitory concentration of 13 antimicrobials against the six isolates used in this work, showing that all isolates were resistant to vancomycin, trimethoprim and nalidixic acid, with none of them presenting resistance to chloramphenicol, gentamicin, erythromycin, doxycycline and tetracycline. All human strains were susceptible to ciprofloxacin while all non-human strains were resistant. The most susceptible isolate was the AB 17/11 strain isolated from the slaughterhouse surface, which presented susceptibility to 10 of the 13 antibiotics tested.

A. butzleri isolates exhibited low haemolytic activity as defined by the percentage of lysis of human red blood cells, when compared to the positive control (Table 2).

Concerning biofilm formation ability, both the human isolate INSA593 and the AB17/11 isolated from poultry slaughterhouse environment, showed weak biofilm formation ability, while the poultry isolate AB28/11 exhibited a strong adhesion to polystyrene surfaces, in the tested conditions.

The presence and distribution of nine putative virulence genes was investigated for the six *A. butzleri* isolates, with all the genes being detected in the strains from poultry or poultry slaughterhouse environment. Regarding the human isolates, both *irgA* and *hecB* genes were not identified in INSA593 and INSA 776 isolates, but were found in 1426.2003 isolate, in which *hecA* was not detected (Table 2).

The six *A. butzleri* isolates were tested for their ability to adhere to or invade Caco-2 cells (Table 3), with all isolates displaying these abilities. Two human isolates, INSA593 and 1426.2003, showed a significantly lower adhesion than the other *A. butzleri* isolates and the control strain. *A. butzleri* isolates from non-human related sources were significantly more invasive than human isolates ($P<0.0001$), showing an invasion capacity similar to *Salmonella*; however the poultry associated AB36/11 strain showed lower invasion ability than the AB28/11 strain, also isolated from poultry ($P=0.02$). When evaluating the effect of pre-existing inflammation on the adhesion and invasion ability of *A. butzleri* strains, a small increase in invasion ability was observed for two human isolates, INSA593 and INSA 776 ($P=0.02$ and $P=0.01$, respectively), when compared with invasion levels recorded for these two strains without the addition of TNFα to Caco-2 cells previous to infection. After Caco-2 pre-treatment with TNFα, both poultry isolates exhibited similar invasion ability, in contrast to what was observed without pre-treatment (Table 3).

Considering that *A. butzleri* invasion of the intestinal epithelium cells was strain specific, we decided to explore other pathogenic mechanism that would be implicated in the ability of *A. butzleri* to cause disease.

The *Salmonella* control strain showed intracellular replication ability. Concerning *A. butzleri*, four isolates revealed to be able to survive within Caco-2 cells; however the three poultry or environmental-associated strains (AB17/11, AB28/11 and AB36/11) showed a significant reduction of cell culturability after 24 h pos-infection, while no reduction was observed for the human-associated INSA593 strain. The other two human isolates, INSA 776 and 1426.2003, could not survive in Caco-2 cells or viable bacterial cells count was below the detection limit (Fig. 1).

The bacterial proinflammatory effect was assessed by measuring IL-8 secretion by intestinal epithelial cells, after 24 h of *in vitro* co-culture. *A. butzleri* induced IL-8 secretion by Caco-2 cell line, with a significant increase when compared to basal secretion of uninfected cells (Fig. 2). Mean values of *A. butzleri*-induced IL-8 secretion were significantly higher for human strains than for poultry and poultry slaughterhouse environment isolates (1363±488 pg ml^{-1} vs 734±282 pg ml^{-1}, P=0.004).

The effect of *A. butzleri* AB28/11 and INSA593 strains on the organization of the ß-cytoplasmatic actin was evaluated and compared with uninfected cells. While the staining of uninfected Caco-2 cells showed a continuous fine meshwork through cells (Fig. 3A), cells exposed to *A. butzleri* strains for 3 h lost their normal organization, showing cellular rearrangement (Fig. 3D, G). Regarding the nuclei staining, a uniform fluorescent nuclear staining through the nuclei was observed both in uninfected Caco-2 cells and in the *A. butzleri*-infected ones (Fig. 3B, E, H).

Discussion

A. butzleri is present in several food and food-processing plants (Collado *et al*. 2009; Ferreira *et al*. 2013; Giacometti *et al*. 2013; Hausdorf *et al*. 2013; Serraino and Giacometti 2014), as well as in water (Rice *et al*. 1999; Fong *et al*. 2007; Kopilovic *et al*. 2008; Collado *et al*. 2008, 2010) and human samples (Vandenberg *et al*. 2004; Prouzet-Mauleon *et al*. 2006; Samie *et al*. 2007; Collado *et al*. 2013; Ferreira *et al*. 2014). In humans, *A. butzleri* has been detected in stools from cases of enteritis or in patients with bacteraemia (Collado and Figueras 2011); however, its pathogenicity has not yet been fully studied.

The low resistance rate to fluoroquinolones reported by Vandenberg *et al*. (2006) led the authors to suggest that these may be used for treating severe *Arcobacter* enteritis. Moreover, tetracycline had also been proposed to be a suitable antibiotic for the treatment of *Arcobacter* spp. infection, along with aminoglycosides (Son *et al*. 2007; Abay *et al*. 2011). However, high values of ciprofloxacin resistance have been reported for *Arcobacter* isolates from animal, water or environmental sources, ranging from 12.5 to 55.8 % (Shah *et al*. 2012, 2013; Ferreira *et al*. 2013; Villalobos *et al*. 2013). In this study, all non-human strains were resistant to ciprofloxacin, in contrast to human strains. Both strains groups were susceptible to gentamicin, tetracycline and doxycyline, presenting however a high percentage of resistance to ampicillin, cefoperazone, piperacillin and amoxicillin as previously described (Ferreira *et al*. 2013).

In agreement with Vandamme *et al*. (1992b) who described that most of *A. butzleri* were non-haemolytic, with few strains showing haemolysis in blood agar, we showed that the whole cells of tested isolates were weakly haemolytic, under the conditions of our assay (Table 2). This is in accordance with what has been described for campylobacters that are considered only weakly haemolytic and not forming readily identifiable zones of haemolysis on blood agar plates (Grant *et al*. 1997). Cell-associated haemolytic activity has been related with an outer membrane phospholipase A encoded by *pldA* gene (Grant *et al*. 1997). This enzyme may be also associated with haemolysis in *A. butzleri*, as it was present in all isolates exhibiting haemolytic activity. In addition, *tlyA* gene was detected in all isolates. This gene encodes a haemolysin shown to be involved in adherence of *C. jejuni* to Caco-2 cells. However the *tlyA* mutant in this species displayed no difference in haemolytic activity from wild type strain, despite the role on haemolysis described for other bacteria (Salamaszynska-Guz and Klimuszko 2008). The *hecB* gene encodes a related haemolysin activation protein (Miller *et al*. 2007) being detected in four of the six tested strains; however no correlation with haemolysis was found in this study.

The ability of *A. butzleri* to adhere to inert surfaces in the form of biofilms was formerly described (Kjeldgaard *et al*. 2009; Ferreira *et al*. 2013). Given that biofilm formation is reported to benefit bacterial survival, colonisation and protection from host immune responses and antibacterial therapies (Donlan 2002), the biofilm forming-ability of *A. butzleri*

isolates was evaluated. All strains were able to form biofilms, nonetheless with no association with adhesion or invasion ability.

Adhesion is a required step for the establishment of infection, which may be followed by colonization of tissues, and in some cases by bacterial invasion of host cells, intracellular multiplication, dissemination to other tissues, or persistence (Pizarro-Cerda and Cossart 2006). Previous studies demonstrated variable adhesion and invasion abilities of *A. butzleri* to eukaryotic cell lines, considering different strain origins (animal-, food-, environmental or human sources) and cell lines used (Hep-2, HeLa, Vero, INT407, Caco-2, IPI-2 and HT-29) (Musmanno *et al.* 1997; Carbone *et al.* 2003; Villarruel-Lopez *et al.* 2003; Ho *et al.* 2007; Gugliandolo *et al.* 2008; Fernández *et al.* 2010; Karadas *et al.* 2013; Levican *et al.* 2013).

In the present study, we analysed the ability of *A. butzleri* strains to adhere to Caco-2 cell line. The human-associated strains, INSA593 and 1426.2003, showed significantly lower adherence ability than the remaining four strains, which displayed an adhesion similar to that of *Salmonella*. All strains presented invasion ability, with human isolates being significantly less invasive than the non-human *A. butzleri* strains, which displayed similar invasion ability as *Salmonella*. Despite the discrepancies with some works reporting a non-adherent phenotype of *A. butzleri* to HeLa, INT407 or HT-29 human cell lines (Musmanno *et al.* 1997; Karadas *et al.* 2013), all studies using Caco-2 cell lines reported adhesion for all tested strains, corroborating our results. Moreover, those studies reported that 89 % of the strains (17/19) were able to invade Caco-2 cells (Ho *et al.* 2007; Karadas *et al.* 2013; Levican *et al.* 2013). The differences regarding adherence and invasive phenotypes displayed by diverse *Arcobacter* species has been correlated with the origin of the strains and the cell lines used in different studies (Ho *et al.* 2007; Collado and Figueras 2011). This variation was supported by Karadas *et al.* (2013) through the evaluation of six *A. butzleri* isolates on Caco-2 and HT-29 cell lines, showing higher rates of invasion in Caco-2 cells. Considering that adhesion and invasion abilities to host cells are required for successful colonization and infection, our results also point out the pathogenic potential of *A. butzleri*.

Despite the presence of adhesion and invasion genes in all tested isolates, no correlation was recognized with the different adhesion and invasion phenotypes between isolates, suggesting that other factors may be involved in these processes. Similarly, Karadas *et al.* (2013) observed no correlation between putative virulence gene patterns, and adhesion or invasion abilities. However, when analysing adhesion and invasion ability of fifteen of the 18 recognized *Arcobacter* species, Levican *et al.* (2013) observed a correlation between the absence of virulence genes and the lack of invasion of Caco-2 cells by *A. thereius*, *A. mytili* and *A. cibarius*.

Despite the lack of reports of *A. butzleri* isolation from inflammatory intestinal disease, the presence of inflammation has been shown to increase the invasion ability *C. concisus*, while the morphological changes induced by inflammation does not influence *C. ureolyticus* abilities to attach or invade Caco-2 cells (Burgos-Portugal *et al.* 2012; Man *et al.* 2012). We tested the

influence of a pre-existing inflammation of epithelial cells upon adhesion and invasion abilities. In cell culture and animal models, tumour necrosis factor-α was shown to induce, barrier defects comparable to some inflammatory bowel diseases (Hering *et al*. 2012). We therefore used this proinflammatory cytokine to induce inflammation in Caco-2 cells. As previously described for *C. ureolyticus* (Burgos-Portugal *et al*. 2012), no increase was observed in adherence ability of *A. butzleri* to Caco-2 cells, however a significant increase in the level of invasion was observed for two human-associated isolates (INSA593 and INSA776), in accordance to what was described for *C. concisus* (Man *et al*. 2012).

Intracellular survival has been suggested to contribute both to virulence and cell damage, as well as to be associated with diverse pathogenesis-related functions, including persistence, immune evasion, increased virulence or even failure of antibiotic treatment (Gaynor *et al*. 2005). To our knowledge no previous works on survival of *A. butzleri* within eukaryotic cells has been reported. It remains to be described what happens to *A. butzleri* after invading the host cell. In this study we investigated the intracellular survival of *A. butzleri* strains in Caco-2 cells. We showed a strain-dependent variation in the ability of *A. butzleri* to survive intracellularly, with animal and environmental strains displaying higher ability than human isolates. However the reduced number of tested strains from each group does not allow drawing conclusions without further experiments. The *S.* Typhimurium control strain was able to replicate inside Caco-2 cells, as previously reported (Horne *et al*. 1997), while no replication was observed for the *A. butzleri* tested strains. This is consistent with what has been described for *C. jejuni*, which typically loses viability within epithelial cells during the first 24 h, showing no evidence of intracellular replication (Buelow *et al*. 2011).

In addition to adhesion and invasion to Caco-2 cells, our results showed that *A. butzleri* strains stimulated significantly high levels of IL-8 secretion, compared to uninfected cells, irrespectively of adherence or invasion levels. This lack of correlation is in accordance with what was observed by Ho *et al*. (2007), who suggested that for strains isolated from cases of persistent diarrhoea, local inflammation triggering could be more suitable for pathogen successful survival than cellular invasion (Ho *et al*. 2007). In accordance with this idea, the mean value of secreted IL-8 for human strains, which showed to be less invasive, was significantly higher than the mean value for the poultry and environmental *A. butzleri* isolates ($P<0.01$).

Specific staining of β-actin, a cytoplasmatic isoform primarily expressed in nonmuscle cells revealed a cellular structure rearrangement of Caco-2 cells due to the infection with *A. butzleri*. This was more evident for Caco-2 cells exposed to the poultry isolate (AB28/11), which presenting higher levels of invasion when compared with the human strain (INSA593). The uniform fluorescent nuclear staining and lack of condensation or fragmentation of nuclear material is consistent with the absence of apoptosis for the initial times of infection, described by Bücker *et al*. (2009) who observed effects in epithelial apoptosis only after 48 h.

Overall, we consider that the results of this work emphasize the role of *A. butzleri* as a human pathogen, as it demonstrates that *A. butzleri* strains possess several genotypic and phenotypic features associated with pathogenicity. The proven *in vitro* proinflammatory properties, together with the different abilities to adhere to, invade Caco-2 cells or survive intracellularly in these cells, may be associated with distinctive pathogenic potentials among strains. However, the low number of isolates may hamper us to clearly establish this association. In future work, more studies must be conducted to understand the function of cytoskeletal filamentous actin rearrangement in the *A. butzleri* invasion course and the process of adhesion and invasion ability by this microorganism.

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Conflict of Interest:

No conflict of interest declared.

References

Abay, S., Kayman, T., Hizlisoy, H. and Aydin, F. (2011) In Vitro Antibacterial Susceptibility of *Arcobacter butzleri* Isolated from Different Sources. *J vet Med Sci* **74**, 613-616.

Bucker, R., Troeger, H., Kleer, J., Fromm, M. and Schulzke, J.D. (2009) *Arcobacter butzleri* Induces Barrier Dysfunction in Intestinal HT-29/B6 Cells. *J Infect Dis* **200**, 756-764.

Buelow, D.R., Christensen, J.E., Neal-McKinney, J.M. and Konkel, M.E. (2011) *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol* **80**, 1296-1312.

Burgos-Portugal, J.A., Kaakoush, N.O., Raftery, M.J. and Mitchell, H.M. (2012) Pathogenic potential of *Campylobacter ureolyticus*. *Infect Immun* **80**, 883-890.

Carbone, M., Maugeri, T.L., Giannone, M., Gugliandolo, C., Midiri, A. and Fera, M.T. (2003) Adherence of environmental *Arcobacter butzleri* and *Vibrio* spp. isolates to epithelial cells in vitro. *Food Microbiol* **20**, 611-616.

CDC (2006) *National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): 2003 Human Isolates Final Report*. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC.

CDC (2010) *National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2009*. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC.

CLSI (2005) *Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI document M100-S15 Clinical and Laboratory Standards Institute*. Pennsylvania, USA.

Collado, L., Inza, I., Guarro, J. and Figueras, M.J. (2008) Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environ Microbiol* **10**, 1635-1640.

Collado, L., Guarro, J. and Figueras, M.J. (2009) Prevalence of *Arcobacter* in Meat and Shellfish. *J Food Protect* **72**, 1102-1106.

Collado, L., Kasimir, G., Perez, U., Bosch, A., Pinto, R., Saucedo, G., Huguet, J.M. and Figueras, M.J. (2010) Occurrence and diversity of *Arcobacter* spp. along the Llobregat River catchment, at sewage effluents and in a drinking water treatment plant. *Water Res* **44**, 3696-3702.

Collado, L. and Figueras, M.J. (2011) Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. *Clin Microbiol Rev* **24**, 174-192.

Collado, L., Gutierrez, M., Gonzalez, M. and Fernández, H. (2013) Assessment of the prevalence and diversity of emergent campylobacteria in human stool samples using a combination of traditional and molecular methods. *Diagn Microbiol Infect Dis*. 75, 434-436.

Donlan, R.M. (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**, 881-890.

Douidah, L., de Zutter, L., Bare, J., De Vos, P., Vandamme, P., Vandenberg, O., Van den Abeele, A.M. and Houf, K. (2012) Occurrence of putative virulence genes in *Arcobacter* species isolated from humans and animals. *J Clin Microbiol* **50**, 735-741

Fera, M.T., La Camera, E., Carbone, M., Malara, D. and Pennisi, M.G. (2009) Pet cats as carriers of *Arcobacter* spp. in Southern Italy. *J Appl Microbiol* **106**, 1661-1666.

Fernández, H., Flores, S. and Inzunza, F. (2010) *Arcobacter butzleri* Strains Isolated from Different Sources Display Adhesive Capacity to Epithelial Cells In Vitro. *Acta Sci Vet* **38**, 287-291.

Ferreira, S., Fraqueza, M.J., Queiroz, J.A., Domingues, F.C. and Oleastro, M. (2013) Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse. *Int J Food Microbiol* **162**, 82-88.

Ferreira, S., Julio, C., Queiroz, J.A., Domingues, F.C. and Oleastro, M. (2014) Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients. *Diagn Microbiol Infect Dis* **78**, 220-225.

Fong, T.T., Mansfield, L.S., Wilson, D.L., Schwab, D.J., Molloy, S.L. and Rose, J.B. (2007) Massive microbiological groundwater contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. *Environ Health Persp* **115**, 856-864.

Gaynor, E.C., Wells, D.H., MacKichan, J.K. and Falkow, S. (2005) The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol Microbiol* **56**, 8-27.

Giacometti, F., Lucchi, A., Manfreda, G., Florio, D., Zanoni, R.G. and Serraino, A. (2013) Occurrence and genetic diversity of *Arcobacter butzleri* in an artisanal dairy plant in Italy. *Appl Environ Microbiol* **79**, 6665-6669.

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

Gugliandolo, C., Irrera, G.P., Lentini, V. and Maugeri, T.L. (2008) Pathogenic *Vibrio, Aeromonas* and *Arcobacter* spp. associated with copepods in the Straits of Messina (Italy). *Mar Pollut Bull* **56**, 600-606.

Harrass, B., Schwarz, S. and Wenzel, S. (1998) Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. *J Vet Med* **45**, 87-94.

Hausdorf, L., Neumann, M., Bergmann, I., Sobiella, K., Mundt, K., Frohling, A., Schluter, O. and Klocke, M. (2013) Occurrence and genetic diversity of *Arcobacter* spp. in a spinach-processing plant and evaluation of two *Arcobacter*-specific quantitative PCR assays. *Syst Appl Microbiol* **36**, 235-243.

Hering, N.A., Fromm, M. and Schulzke, J.D. (2012) Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *J Physiol* **590**, 1035-1044.

Ho, H., Lipman, L., Hendriks, H., Tooten, P., Ultee, T. and Gaastra, W. (2007) Interaction of *Arcobacter* spp. with human and porcine intestinal epithelial cells. *FEMS Immunol MedMicrobiol* **50**, 51-58.

Horne, S.M., Kottom, T.J., Nolan, L.K. and Young, K.D. (1997) Decreased intracellular survival of an *fkpA* mutant of *Salmonella* typhimurium Copenhagen. *Infect Immun* **65**, 806-810.

Houf, K., De Smet, S., Bare, J. and Daminet, S. (2008) Dogs as carriers of the emerging pathogen *Arcobacter*. *Vet Microbiol* **130**, 208-213.

Kabeya, H., Maruyama, S., Morita, Y., Ohsuga, T., Ozawa, S., Kobayashi, I., Abe, M., Katsube, Y. and Mikami, T. (2004) Prevalence of *Arcobacter* species in retail meats and antimicrobial susceptibility of the isolates in Japan. *Int J Food Microbiol* **90**, 303-308.

Kalischuk, L.D. and Inglis, G.D. (2011) Comparative genotypic and pathogenic examination of *Campylobacter concisus* isolates from diarrheic and non-diarrheic humans. *BMC Microbiol* **11**:53.

Karadas, G., Sharbati, S., Hanel, I., Messelhausser, U., Glocker, E., Alter, T. and Golz, G. (2013) Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. *J Appl Microbiol* **115**, 583-590.

Kjeldgaard, J., Jørgensen, K. and Ingmer, H. (2009) Growth and survival at chiller temperatures of *Arcobacter butzleri*. *Int J Food Microbiol* **131**, 256-259.

Kopilovic, B., Ucakar, V., Koren, N., Krek, M. and Kraigher, A. (2008) Waterborne outbreak of acute gastroenteritis in a costal area in Slovenia in June and July 2008. *Euro Surveill* **13** (34):pii=18957.

Levican, A., Alkeskas, A., Gunter, C., Forsythe, S.J. and Figueras, M.J. (2013) Adherence to and invasion of human intestinal cells by *Arcobacter* species and their virulence genotypes. *Appl Environ Microbiol* **79**, 4951-4957.

Man, S.M., Kaakoush, N.O., Leach, S.T., Nahidi, L., Lu, H.K., Norman, J., Day, A.S., Zhang, L. and Mitchell, H.M. (2012) Host attachment, invasion, and stimulation of proinflammatory cytokines by *Campylobacter concisus* and other non-*Campylobacter jejuni Campylobacter* species. *J Infect Dis* **202**, 1855-1865.

Miller, W.G., Parker, C.T., Rubenfield, M., Mendz, G.L., Wösten, M.M.S.M., Ussery, D.W., Stolz, J.F., Binnewies, T.T., Hallin, P.F., Wang, G., Malek, J.A., Rogosin, A., Stanker, L.H. and Mandrell, R.E. (2007) The complete genome sequence and analysis of the epsilonproteobacterium *Arcobacter butzleri*. *PloS one* **2**, e1358.

Musmanno, R.A., Russi, M., Lior, H. and Figura, N. (1997) *In vitro* virulence factors of *Arcobacter butzleri* strains isolated from superficial water samples. *New Microbiol* **20**, 63-68.

On, S.L.W., Stacey, A. and Smyth, J. (1995) Isolation of *Arcobacter-Butzleri f*rom a Neonate with Bacteremia. *J Infect* **31**, 225-227.

Petersen, R.F., Harrington, C.S., Kortegaard, H.E. and On, S.L.W. (2007) A PCR-DGGE method for detection and identification of *Campylobacter*, *Helicobacter*, *Arcobacter* and

related Epsilobacteria and its application to saliva samples from humans and domestic pets. *J Appl Microbiol* **103**, 2601-2615.

Pizarro-Cerda, J. and Cossart, P. (2006) Bacterial adhesion and entry into host cells. *Cell* **124**, 715-727.

Prouzet-Mauleon, V., Labadi, L., Bouges, N., Menard, A. and Megraud, F. (2006) *Arcobacter butzleri*: Underestimated enteropathogen. *Emerg Infect Dis* **12**, 307-309.

Rice, E.W., Rodgers, M.R., Wesley, I., Johnson, C.H. and Tanner, S.A. (1999) Isolation of *Arcobacter butzleri* from ground water. *Lett Appl Microbiol* **28**, 31-35.

Robinson, J., Goodwin, C.S., Cooper, M., Burke, V. and Mee, B.J. (1990) Soluble and cell-associated haemagglutinins of *Helicobacter* (*Campylobacter*) pylori. *J Med Microbiol* **33**, 277-284.

Salamaszynska-Guz, A. and Klimuszko, D. (2008) Functional analysis of the *Campylobacter jejuni cj0183* and *cj0588* genes. *Curr Microbiol* **56**, 592-596.

Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M. and Guerrant, R.L. (2007) Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: studies using molecular diagnostic methods. *J Infect* **54**, 558-566.

Sasi Jyothsna, T.S., Rahul, K., Ramaprasad, E.V., Sasikala, C. and Ramana, C.V. (2013) *Arcobacter anaerophilus* sp. nov., isolated from an estuarine sediment and emended description of the genus *Arcobacter*. *Int J Syst Evol Microbiol* **63**, 4619-4625.

Serraino, A. and Giacometti, F. (2014) Short communication: Occurrence of *Arcobacter* species in industrial dairy plants. *J Dairy Sci* **97**, 2061-2065.

Shah, A.H., Saleha, A.A., Zunita, Z., Murugaiyah, M. and Aliyu, A.B. (2012) Antimicrobial susceptibility of an emergent zoonotic pathogen, *Arcobacter butzleri*. *Int J Antimicrob Agents* **40**, 569-570.

Shah, A.H., Saleha, A.A., Zunita, Z., Murugaiyah, M., Aliyu, A.B. and Jafri, N. (2013) Prevalence, Distribution and Antibiotic Resistance of Emergent Arcobacter spp. from Clinically Healthy Cattle and Goats. *Transbound Emerg Dis* **60**, 9-16.

Son, I., Englen, M.D., Berrang, M.E., Fedorka-Cray, P.J. and Harrison, M.A. (2007) Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. *Int J Antimicrob Agents* **29**, 451-455.

Vandamme, P., Pugina, P., Benzi, G., Van Etterijck, R., Vlaes, L., Kersters, K., Butzler, J.P., Lior, H. and Lauwers, S. (1992a) Outbreak of Recurrent Abdominal Cramps Associated with *Arcobacter-Butzleri* in an Italian School. *J Clin Microbiol* **30**, 2335-2337.

Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., van den Borre, C., Higgins, R., Hommez, J., Kersters, K., Butzler, J.P. and Goossens, H. (1992b) Polyphasic Taxonomic Study of the Emended Genus *Arcobacter* with *Arcobacter-Butzleri* Comb-Nov and *Arcobacter-Skirrowii* Sp-Nov, an Aerotolerant Bacterium Isolated from Veterinary Specimens. *Int J Syst Bacteriol* **42**, 344-356.

Vandenberg, O., Dediste, A., Houf, K., Ibekwem, S., Souayah, H., Cadranel, S., Douat, N., Zissis, G., Butzler, J.P. and Vandamme, P. (2004) *Arcobacter* species in humans. *Emerg Infect Dis* **10**, 1863-1867.

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, J.P. and Dediste, A. (2006) Antimicrobial susceptibility of clinical isolates of non-jejuni/coli campylobacters and arcobacters from Belgium. *J Antimicrob Chemoth* **57**, 908-913.

Villalobos, E.G., Jaramillo, H.F., Ulate, C.C. and Echandi, M.L.A. (2013) Isolation and Identification of Zoonotic Species of Genus *Arcobacter* from Chicken Viscera Obtained from Retail Distributors of the Metropolitan Area of San Jose', Costa Rica. *J Food Protect* **76**, 879-882.

Villarruel-Lopez, A., Marquez-Gonzalez, M., Garay-Martinez, L.E., Zepeda, H., Castillo, A., De La Garza, L.M., Murano, E.A. and Torres-Vitela, R. (2003) Isolation of Arcobacter spp. from retail meats and cytotoxic effects of isolates against Vero cells. *J Food Protect* **66**, 1374-1378.

Wesley, I. and Miller, W.G. (2010) *Arcobacter*: an opportunistic human food-borne pathogen? In *Emerging Infections 9* eds. Scheld, W.M., Grayson, M.L. and Hughes, J.M. pp.185-211. Washington: ASM Press.

Table 1. Source of *Arcobacter butzleri* strains and minimum inhibitory concentration of different agents tested against them.

Strain	Source	MIC (µg/ml)												
		AMP	CIP	VAN	TMP	CFP	PIP	CHL	GEN	AMX	ERY	DOX	TET	NAL
AB 17/11	Slaughterhouse surface	1	8	>512	>512	8	16	4	0.5	1	0.125	0.25	0.5	>32
AB 28/11	Poultry carcass neck skin	128	>8	>512	>512	512	512	8	1	64	4	1	2	>32
AB 36/11	Poultry caecum	128	4	>512	>512	512	>512	4	0.25	64	0.125	0.125	0.25	>32
INSA 593	Male patient, 84 years, presenting diarrhoea with mucous and blood and abdominal pain.	256	0.062	>512	>512	>512	>512	8	0.5	64	0.25	0.125	0.5	>32
INSA 776	Female patient, 89 years, presenting diarrhoea and abdominal pain.	128	0.062	256	>512	512	512	2	0.5	32	0.5	0.25	0.5	>32
1426.2003	Patient with diarrhoea [b]	32	0.031	256	>512	256	512	4	0.5	16	0.125	0.125	0.5	>32
Breakpoints (R) (µg/ml) [a]		≥32	≥4	≥32	≥16	≥64	≥128	≥32	≥8	≥32	≥32	≥16	≥16	≥64

[a] Since no recommendation of breakpoints values is available for *A. butzleri*, the breakpoints used were those of *Campylobacter* following the National Antimicrobial Resistance Monitoring System criteria (CDC 2006, 2010) or when lacking in this interpretative criteria, the ones used for *Enterobacteriaceae* according to the Clinical and Laboratory Standards Institute protocol M100 (CLSI 2005). [b] The strain was provided by the Centre National de Référence des Campylobacters in Bordeaux, France

Table 2. Haemolysis, biofilm formation and putative virulence genes of *Arcobacter butzleri* isolates

Strain	Haemolysis (%)	Biofilm		Putative virulence genes
		Abs 570nm	Class.	
AB 17/11	11.7±0.2	0.372±0.051	Weak	*cadF; ciaB; cj1349; irgA; hecA; hecB; mviN; pldA; tlyA*
AB 28/11	14.3±2.8	1.233±0.256	Strong	*cadF; ciaB; cj1349; irgA; hecA; hecB; mviN; pldA; tlyA*
AB 36/11	9.8±3.8	0.653±0.156	Moderate	*cadF; ciaB; cj1349; hecA; hecB; mviN; pldA; tlyA*
INSA 593	17.6±4.6	0.325±0.062	Weak	*cadF; ciaB; cj1349; hecA; mviN; pldA; tlyA*
INSA 776	8.3±2.3	0.483±0.093	Moderate	*cadF; ciaB; cj1349; hecA; mviN; pldA; tlyA*
1426.2003	15.7±6.1	0.596±0.098	Moderate	*cadF; ciaB; cj1349; irgA; hecB; mviN; pldA; tlyA*

Table 3. Adhesion and invasion of *Arcobacter butzleri* isolates to Caco-2 cells without or with TNFα incubation previous to infection

Strain	Adhesion (Log_{10}CFU/ml)	Invasion (Log_{10}CFU/ml)	Pre-treatment with TNFα	
			Adhesion (Log_{10}CFU/ml)	Invasion (Log_{10}CFU/ml)
AB17/11	6.61±0.05[a]	5.17±0.11[a,b,c]	6.95±0.52[a,b]	5.70±0.51[a]
AB28/11	6.64±0.09[a]	5.36±0.24[b]	6.88±0.42[a,b]	5.48±0.15[a]
AB36/11	6.48±0.17[a]	4.77±0.44[c]	6.67±0.29[b]	5.19±0.33[a]
INSA593	5.99±0.31[b]	2.07±0.45[d]	5.76±0.36[c]	2.99±0.54[b,*]
INSA776	6.51±0.16[a]	2.01±0.27[d]	6.00±0.41[c]	2.93±0.30[b,*]
1426.2003	5.79±0.43[b]	2.21±0.26[d]	5.51±0.34[c]	2.41±0.54[b]
***S*. Typhimurium**	6.6±0.08[a]	5.20±0.44[a,b,c]	7.20±0.13[a,*]	5.48±0.12[a]

Mean values marked with different letters were significantly different, for comparisons within the same column ($p < 0.05$).
* Significantly higher value when compared to cells without incubation with TNFα, previous to infection

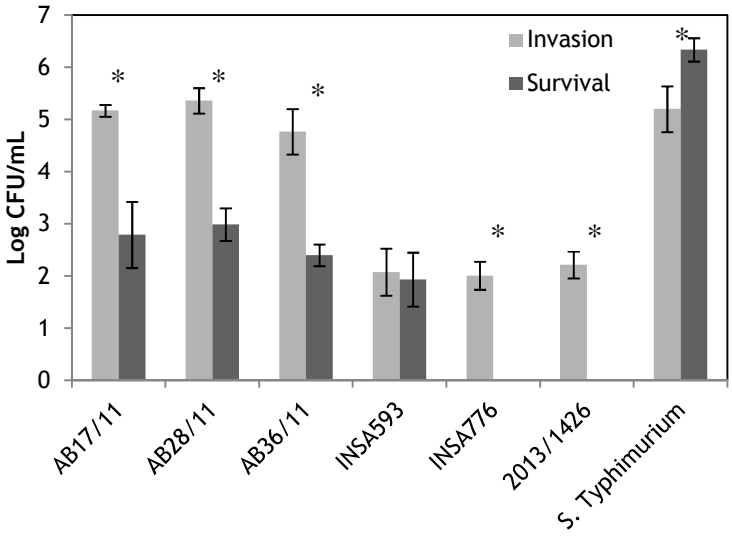

Figure 1. Intracellular survival of *A. butzleri* strains and *Salmonella* Typhimurium in Caco-2 cells.
* Differences between invasion and intracellular survival log counts were statistically significant.

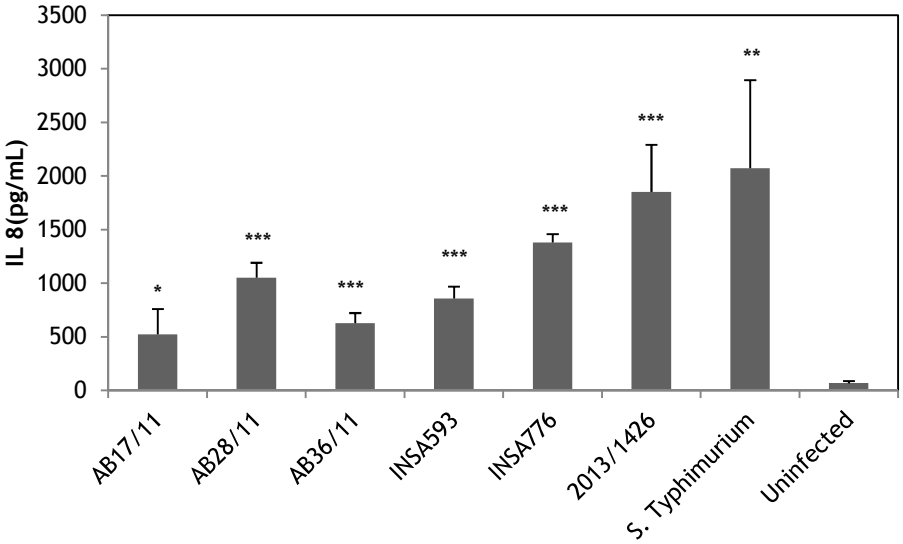

Figure 2. IL-8 production by Caco-2 cells measured in cell's supernatant after 24 h of co-infection. Results were calculated as the mean values (±SEM) of three independent experiments. * P=0.01, ** P=0.004 and ***P<0.0001 versus uninfected Caco-2 cells.

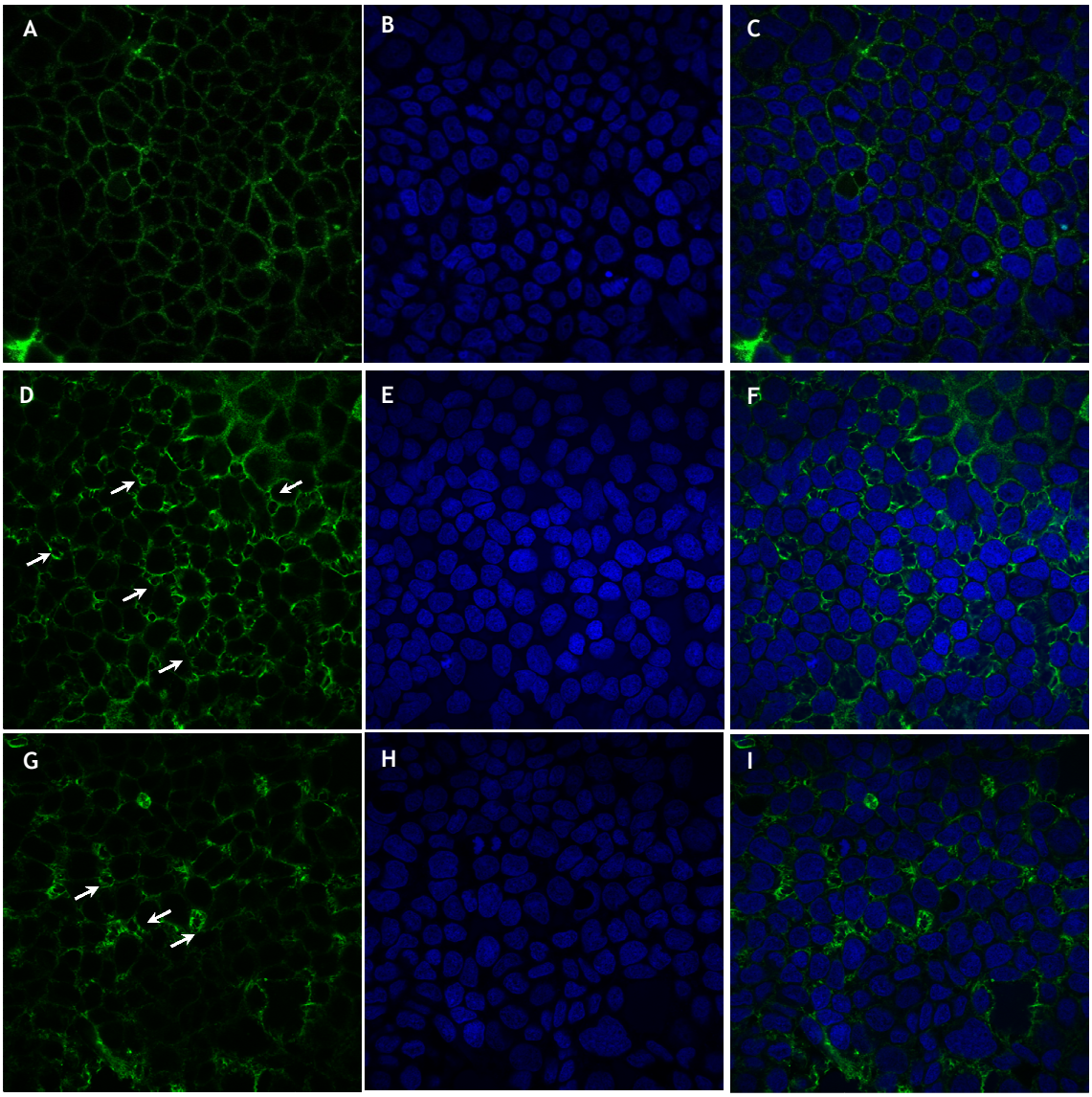

Figure 3. Effects of *A. butzleri* AB28/11 and INSA593 on actin organization of Caco-2 cells. Uninfected Caco-2 cells (A, B, C), exposed for 3 h to AB28/11 (D, E, F) or INSA593 (G, H, I) showing ß-actin staining, Hoechst staining (used to stain nuclei) and merged staining, respectively.

Chapter 7

Concluding remarks and future perspectives

Concluding remarks and future perspectives

As of today, the *Arcobacter* genus comprises 18 recognized species with some of them being considered emerging pathogens. The exact sources of human *Arcobacter* infections in Portugal are still not clear, as there is no previous study concerning *Arcobacter* prevalence in our country neither in humans nor in the food chain. In order to understand the genetic diversity, antimicrobial resistance and biofilm-forming ability of *A. butzleri* isolates from poultry and environment of a Portuguese slaughterhouse we isolated *Arcobacter* from carcass neck skin, caecum, caecal content and water draining off from hanging broiler carcasses from three different flocks and from the slaughterhouse processing line surfaces. We found *A. butzleri* in all analysed samples, except for caecum content samples. Trough the genetic typing by pulsed field gel electrophoresis of the isolates, we found a high genetic heterogeneity with the typing of 43 isolates resulting in 32 distinct pulsetypes. There were still undistinguishable pulsetypes between different collection samples, indicating possible cross-contaminations during the slaughter process. As for antimicrobial resistance, *A. butzleri* isolates showed high levels of resistance with all isolates being resistant to at least three antibiotics and 42 (97.7 %) to six or more antibiotics, of the nine tested. However, only one strain presented resistance to chloramphenicol and all the tested isolates were susceptible to gentamicin. In addition, 55.8 % of the isolates were resistant to ciprofloxacin. Resistant strains presented the previously described cytosine to thymine transition in the quinolone resistance determining region of the *gyrA* gene, a mutation associated with ciprofloxacin resistance. The high prevalence of ciprofloxacin and amoxicillin resistance could be associated with the intensive use of these groups of antibiotics in birds' rearing in Portugal. Moreover, most of the strains showed the ability to form biofilms, which may in part explain the high prevalence and survival of *Arcobacter* in slaughterhouse environment, possibly favouring dispersion and cross-contamination along the processing line. Putative virulence genes detection showed that *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA* were present in all *A. butzleri* isolates, with a variable detection of *hecA* (75 %), *hecB* (89 %) and *irgA* (42 %) genes. By taking into consideration the prevalence of *A. butzleri* in the collected samples, poultry could be considered an important transmission route to humans, which together with the presence of putative virulence associated characteristics as antimicrobial resistance, biofilm formation ability and the presence of presumed virulence genes highlights its relevance as a potential food-borne pathogen.

Considering the high prevalence of *A. butzleri* found in the first part of this study and that *A. cryaerophilus* is the second most commonly reported *Arcobacter* species, we proceeded to research the inhibitory ability of resveratrol as a natural compound focusing on its antimicrobial activity and mechanism of action against *A. butzleri* and *A. cryaerophilus*. Resveratrol showed bactericidal or bacteriostatic activity depending on growth phase and compound concentration. Flow cytometry studies showed resveratrol to cause a significant

decrease on *Arcobacter* metabolic activity and a reduction in intracellular DNA content. Our results showed that resveratrol can act as an efflux pump inhibitor in both *Arcobacter* species. Likewise, scanning electron microscopy observations complement the results suggesting that the antibacterial activity of resveratrol may result from the action of the compound upon assorted targets, which may lead to the induction of cell death due to impairment of cellular functions. This work highlighted the potential use of resveratrol as an alternative food preservative, which has the additional advantage of being a naturally synthesized compound by plants in response to various stresses, displaying several beneficial properties for the human health, including its antioxidant activity. Altogether, these properties may encourage resveratrol use as a food preservative.

Some *Arcobacter* species have been associated mainly with gastrointestinal diseases in humans; however despite some species being considered emergent pathogens, they have not been regarded as microorganisms of major public health concern. Although our main objective was to evaluate the prevalence and distribution of *Arcobacter* species in faeces from patients with diarrhoea in Portugal, we also proceeded with a survey concerning *Campylobacter* species in the same samples. In the studied cohort of 298 samples, *A. butzleri* was the fourth most frequent species with a detection frequency of 1.3 %. Further *A. cryaerophilus* was detected in one sample from a 9-year-old boy. The detection of *Campylobacter* spp. showed the presence of *C. jejuni* (13.7 %), *C. concisus* (8.0 %), *C. coli* (1.0 %), and *C. fetus* (0.3 %) in the analysed stool samples, with a highly significant association found between *C. jejuni* and diarroeal children´s samples. Regarding the association with clinical presentation, it was found that *C. jejuni* infection was associated with bloody diarrhoea, fever, and abdominal pain, while infection with *C. concisus* was found to be associated with vomiting. These results support the importance of *Arcobacter* and *Campylobacter* species as aetiological agents of acute gastroenteritis among Portuguese patients, affecting particularly the paediatric age group. Since no previous work concerning *Arcobacter* prevalence and distribution have been reported, the results obtained from this study can contribute to understand the local epidemiology of *Arcobacter* and *Campylobacter* species.

Based on the results obtained in the Chapters 3 and 5 we proceeded with the investigation to further clarify *A. butzleri* pathogenesis and the mechanism that may assist infection by this microorganism. We assessed the virulence potential of *A. butzleri* isolates through the evaluation of various phenotypic and genotypic characteristics. Isolates showed a high susceptibility (100 %) for chloramphenicol, gentamicin, erythromycin and both tetracyclines tested, however with high resistance for quinolones. Likewise, all displayed a weak haemolytic activity and the ability to form biofilms. In relation to the detection of the nine investigated putative virulence genes, the *A. butzleri* strains studied showed 100 % prevalence for *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA*, with a variable detection of *hecA*, *hecB* and *irgA* genes, as described in the Chapter 3. The ability of human and non-human *A. butzleri* isolates to adhere, invade and intracellularly survive in a human intestinal epithelial

cell line, as well as to induce inflammatory response through stimulation of interleukin 8 secretion was demonstrated. In general, non-human strains displayed a higher invasion and survival ability. Pre-existing inflammation showed to favour the invasive potential of two of the human strains with no significant impact on remaining isolates. No correlation was found between the presence of putative virulence genes and differences on pathogenic potential. These data brought new insights into *A. butzleri* virulence and highlights its pathogenic potential.

Overall this research showed the distribution of *Arcobacter* in both poultry and slaughterhouse environment, as well as in diarrhoeal faecal samples from Portugal. Also this study contributed to the knowledge of strains heterogeneity and of their virulence potential, which is essential to recognize the relevance of *A. butzleri* as a food-borne pathogen. Finally, the study of the inhibitory activity of resveratrol against *Arcobacter* species can contribute to further studies concerning to the development of new control approaches.

For further works, it could be interesting to evaluate the significance of poultry as a source of *Arcobacter* in Portugal by studying its occurrence in meat products and at retail. Moreover, the analysis of *Arcobacter* presence could be extended to other food and environmental related samples. The effective use of resveratrol in food systems will require further investigation, namely concerning the improvement of this compound stability and solubility and to evaluate its potential as a food preservative on food model systems and to identify their effective concentrations in food matrices. Furthermore, the *Arcobacter* prevalence in humans should be expanded throughout the different seasons of the year in order to address to a possible peak of infection. In addition, a control group comprised of healthy individuals should be introduced, which may assist in the establishment of this organism as an aetiological agent of the enteric disease. Additional studies should be undertaken to characterise the virulence traits and to confirm their true role in *Arcobacter* infection, for instance, through the use of mutants of specific genes, such as the putative virulence genes formerly presented and complemented mutants on *in vitro* assays, or even experimentally in animal models. Also, the use of a mucus-secreting intestinal epithelial cell model may help to understand the infection process and should be more suited to the study of cells-pathogen interaction.