Pathogenic potential of human isolates of *Aliarcobacter butzleri*

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Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2º ciclo de estudos)

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agosto de 2021

Dedicatory

To my grandma, Olinda

Acknowledgments

In the end of this bittersweet adventure, I can finally close one more chapter in my life. Luckily, I always was surrounded by amazing people that never made me feel alone or unsupported, and for that, I can only thank you all.

Firstly, I would like to thank my supervisor, Doctor Susana Ferreira for firstly accepting me to be part of her work but also for all the shared knowledge, patience, preoccupation, and encouragement when things would not go as expected. Thank you for being much more than just a supervisor.

I also wanted to thank Doctor Fernanda Domingues, Doctor Mónica Oleastro and Doctor Angelo Luis for all the recommendations and support given.

I would like to thank all my lab co-workers especially to, Ana, Alexandra, Carolina, Cristiana, Filomena, and Igor for all the support that always turned the lab into a place where there was always something new, always someone to talk to, always a place to laugh and sometimes even cry. Because you know, sometimes "something is very wrong". A very special thank you to my ostrich, Francisca for this amazing journey that we made since little freshmen, I could never thank you enough, and I hope we can always walk on the same path for long years to come.

I also need to thank all the members of Desertuna, especially my two "godsons" Eduardo and Carlos for all the support and all the headaches, I owe you guys a lot.

Last, but not least, to my family, that had given everything for me to be where I am, who make me the man that I am, and it is something that I can never repay you. Thank you from the bottom of my heart.

I never had a way for these things, so I ask apologies for all the things not said.

Resumo Alargado

Desde a sua descoberta em 1991 até aos dias de hoje, o género *Aliarcobacter* compreende cerca de 11 espécies reconhecidas, incluindo *Aliarcobacter butzleri*. Esta espécie é descrita como um enteropatogéneo emergente, sendo o quarto organismo relacionado com *Campylobacter* mais frequentemente encontrado em fezes diarreicas no ser humano. Infeções por esta espécie são ainda associadas a dores abdominais, náuseas e diarreia aquosa. Devido não só à sintomatologia associada a esta bactéria, mas também à sua grande prevalência na cadeia alimentar, esta espécie foi desde 2002 incluída na lista de microrganismos considerados um sério risco para a saúde humana pela Comissão Internacional de Especificações Microbiológicas para Alimentos. Apesar de alguns estudos descreverem o potencial patogénico de *A. butzleri,* ainda pouco se sabe sobre os mecanismos virulência deste patógeno, o que pode dificultar o tratamento de infeções causadas pelo mesmo e o seu controlo. Assim, este trabalho teve como objetivo avaliar os diferentes perfis fenotípicos de virulência de quinze isolados de *A. butzleri* com origem em fezes humanas.

Inicialmente, os isolados foram distinguidos usando tipagem por sequenciação de múltiplos *loci* (MLST, do inglês *Multilocus Sequence Typing*), verificando-se que todos os isolados pertenciam a diferentes sequência-tipo (ST), caracterizando-se então por serem geneticamente distintos. Os resultados também apontaram para a alta variabilidade genética que já tem sido descrita nesta bactéria. Em seguida, vários ensaios foram realizados de modo a caracterizar o perfil de fenotípico de virulência de cada isolado clínico.

O perfil de resistência a antimicrobianos tem sido considerado como um elemento-chave na caracterização de agentes patogénicos, possibilitando o uso de um tratamento correto face a uma infeção bacteriana. Neste trabalho, o perfil de resistência para as estirpes em estudo foi realizado através da determinação das concentrações mínima inibitórias de seis antibióticos pertencentes a diferentes classes (ampicilina, ciprofloxacina, tetraciclina, eritromicina, gentamicina e ertapenemo). Esta determinação foi realizada através de difusão em agar com exceção do ertapnemo que foi realizado usando um ensaio de difusão em disco. Os isolados apresentaram seis perfis distintos de resistência, com frequências de resistência superiores para o ertapenemo (100%), a tetraciclina (93,3%) e a ampicilina (86,6%), sendo que a maioria dos isolados apresentou um perfil de multirresistência.

Espécies reativas de oxigénio bem como espécies reativas de azoto estão presentes no meio ambiente, mas são também usadas pelo sistema imunitário do hospedeiro de

modo a proteger-se contra infeções bacterianas. De modo a avaliar a resposta dos isolados clínicos de *A. butzleri*, o perfil de resistência aos stresses oxidativo e nitrosativo foi avaliado usando ensaios de difusão em disco, contendo peróxido de hidrogénio, metil de viologénio ou nitroprussiato de sódio. Os isolados clínicos analisados, apresentaram perfis de resistência semelhantes com pequenas variações de suscetibilidade na presença de metil de viologénio e peróxido de hidrogénio a 3% (stress oxidativo), enquanto nenhuma dos estipes se demonstrou sensível à presença do nitroprussiato de sódio (stress nitrosativo). De seguida de modo a tentar perceber como é que *A. butzleri* é capaz de chegar ao intestino para causar infeção, avaliou-se um conjunto de fatores que estão potencialmente associados com a sobrevivência no trato gastrointestinal. Assim, a sobrevivência em ambiente acídico foi estudada por incubação de cada isolado durante uma hora a pH 4; avaliou-se a presença da enzima urease através da avaliação da sua atividade usando meio contendo ureia, e a resistência a sais biliares por determinação da sua concentração mínima inibitória. Em relação à sobrevivência em pH 4, pôde-se verificar que cinco dos isolados se demonstraram mais sensíveis ao pH ácido, enquanto as restantes apresentaram diferentes níveis de resistência. Também foi verificada a presença de fenótipo positivo para a presença da urease em seis estirpes de *A. butzleri*, embora não tenha sido correlacionado com a resistência ao ácido. Em relação à resistência aos sais biliares, todas as estirpes demonstraram ser resistentes às concentrações descritas para o intestino humano.

Para avaliar a quimiotaxia relacionada com as estirpes, a caracterização do perfil quimiotático dos isolados foi analisada usando nove compostos diferentes representando compostos tais como: hidratos de carbono, aminoácidos e ácidos orgânicos. Todos os isolados apresentaram distintos perfis quimiotáticos, sugerindo uma possível quimioatração por componentes da mucosa intestinal. Considerando que a motilidade confere uma vantagem no processo de infeção, a motilidade foi também avaliada nos isolados clínicos de *A. butzleri*. Esta característica foi então determinada por inoculação em placas de meio com 0,4 % (m/v) de agar, seguido de incubação durante 48 h e posterior medição do halo. Os isolados apresentaram dois perfis distintos, um em que seis isolados apresentaram baixa motilidade e outro grupo mais heterogéneo com valores moderados e altos de motilidade. Para observar possíveis diferenças no morfótipo e produção celulose de cada isolado, a morfologia das colónias de cada estirpe foi observada em placas suplementadas *Congo red* e azul brilhante de Coomassie, bem como em placas suplementadas com *Calcoflour white,* respetivamente. Após análise, todos os isolados parecem apresentar o morfótipo Bdar

(do inglês: "*brown, dry and rough*"), sugerindo a potencial presença de fimbrias e a ausência de produção de celulose.

A capacidade de formação de biofilmes também tem sido descrita como um importante fator na sobrevivência tanto no ambiente como no hospedeiro, sendo capaz de aumentar a resistência ao sistema imunitário e à presença de antibióticos. Para avaliar a capacidade de formação de biofilmes foi usada a metodologia de coloração com violeta de cristal usando placas de 24 poços. Na sua maioria, os isolados apresentaram moderada a alta capacidade de formação de biofilme.

A presença de hemolisinas tem sido reportada em *A. butzleri*, as quais podem conferir às estirpes capacidade hemolítica, sendo capazes de induzir lise de eritrócitos. Para verificar a atividade hemolítica, os isolados foram incubados com eritrócitos humanos. Todos os isolados demonstraram uma capacidade hemolítica fraca, no entanto verificou-se a existência de dois isolados com perfil distinto apresentando valores de capacidade hemolítica superiores. Em *A. butzleri* foi já sugerido um papel do soro humano na sua natureza limitante durante a infeção, sendo assim relevante perceber o comportamento desta bactéria em soro. A sobrevivência no soro humano foi realizada pela incubação com os isolados durante um período de uma hora, onde maior parte dos isolados se apresentaram como sensíveis ao soro humano após 20 minutos de incubação.

Por fim, de modo a estudar a interação bacteriana com a linha de células de adenocarcinoma colorretal humano, Caco-2 (capacidade de adesão e invasão), a linha celular foi infetada com cada isolado clínico de *A. butzleri* com uma multiplicidade de infeção de 200. Todas as estirpes demonstraram ser capazes de aderir a esta linha celular, mas apenas sete isolados apresentaram capacidade de invasão celular.

A. butzleri tem sido descrito como aerotolerante, no entanto exposição a elevados níveis de oxigénio pode ser capaz de induzir stress. Para avaliar os efeitos da exposição a condições de aerobiose, três isolados com perfis fenotípicos diferentes (INSA Cu 29393, INSA 3202 e INSA 3711) foram selecionados para mais estudos. Para avaliar as possíveis diferenças de perfil fenotípico dos isolados de *A. butzleri* quando sujeitos a diferentes condições atmosféricas (aerobiose e microaerobiose) foram realizados ensaios de avaliação da resistência aos stresses oxidativo e nitrosativo, sobrevivência em meio ácido, sobrevivência em soro humano, motilidade, capacidade de formação de biofilme, capacidade hemolítica e capacidade de adesão e invasão em linhas celulares Caco-2. De forma geral, as condições aeróbicas parecem favorecer a capacidade hemolítica e a motilidade dos isolados, apesar de não modificarem a expressão relativa da flagelina A. Para além disso, quando em aerobiose parece ocorrer um desfavorecimento da resistência ao stress oxidativo, e não se observa impacto na

invasão a células do hospedeiro. Nos restantes ensaios foram obtidos resultados variáveis dependentes do isolado em estudo.
Em suma, tendo em conta os resultados obtidos, podemos afirmar que existe uma grande variabilidade fenotípica entre os isolados clínicos de *A. butzleri* estudados, e que muitos dos mecanismos associados às suas características associadas a virulência continuam por explorar.

Palavras-chave

Aliarcobacter butzleri; Virulência; Patogenicidade; Resistência; Perfil quimiotático; Motilidade; biofilmes; Hemólise; Adesão; Invasão; Condições atmosféricas.

Abstract

Since its discovery in 1991, the *Aliarcobacter* genus comprises 11 recognized species, including *Aliarcobacter butzleri*. This species has been described as a pathogen, being the fourth *Campylobacter*-related microorganism most commonly found in human diarrheic samples. Infections by this bacterium are often associated with abdominal cramps, nausea and watery diarrhoea. Although some studies described its pathogenic potential, still not much is known about its virulence mechanisms, which can complicate the treatment of infections caused by this microorganism and its control. In this manner, this work aimed to evaluate the different virulence phenotypic profiles of fifteen human isolates of *A. butzleri*. Initially, all isolates were typed by Multilocus Sequence Typing, which showed that all isolates belonged to different sequence types, pointing towards the genetic diversity of this bacterium. Next, several assays were performed to characterize the virulence phenotypic profile of each clinical isolate by assessing: (i) the antimicrobial profile through determination of the minimum inhibitory concentration (MIC) of six antibiotics of distinct classes; (ii) the susceptibility to reactive oxygen and nitrogen species; (iii) survival in acidic conditions (pH 4); (iv) the presence of the urease enzyme; (v) the resistance to bile salts; (vi) the chemotactic profile of each strain; (vii) the motility; (viii) differences in morphotype; (ix) biofilm formation ability; (x) haemolytic activity; (xi) survival in human serum; and (xii) the interaction between *A. butzleri* with the human colorectal adenocarcinoma cell line, Caco-2. Although, the clinical *A. butzleri* isolates under study, showed similar oxidative and nitrosative stress responses and morphotype, it was observed a great phenotypic variety among the isolates. Various isolates showed a multidrug resistance profile, with higher resistance frequency being observed for the antibiotics: ertapenem (100%), tetracycline (93.3%), and ampicillin (86.6%). Considering the potential survival of the isolates through the gastrointestinal system, the isolates demonstrated diverse resistance profiles against acidic conditions, with six strains having a positive phenotype for the presence of the urease enzyme, but all demonstrated to be resistant do the bile salts concentrations found in the human gut. Distinct chemotactic profiles indicating a possible interaction with compound of the human mucosa were observed. The isolates under study demonstrated low motility or a diverse profile of higher motility, and different profiles of biofilm formation classified from weak to strong biofilm formers. The *A. butzleri* isolates showed weak haemolytic activity and low survival in the human serum, with exception of one. Only seven strains demonstrated to be capable of invading Caco-2 cells, while all showed to be adherent to the cell line despite at different levels.

The effect of aerobic conditions in the phenotype has also been studied on three isolates presenting distinct phenotypic profiles (INSA Cu 29393, INSA 3202 e INSA 3711). To evaluate the differences in the phenotypic profile when subjected to different atmospheric conditions (aerobiosis and microaerophilia), a similar characterization to the one done for assessment of the phenotypic profile of the clinical isolates was performed. Overall, the aerobic conditions seemed to have a positive effect in the haemolytic activity and motility of the isolates, although having no impact on the flagellin A expression. Further, the aerobic conditions also negatively affected the oxidative stress resistance of the isolates, with the remaining assays showing a strain-specific response.

In conclusion, taking into account the results obtained, we can acknowledge that a great phenotypic variability can be found among the clinical isolates of *A. butzleri* studied, which may be associated with a different host-bacterium interaction.

Keywords

Aliarcobacter butzleri; Virulence; Pathogenicity; Resistance; Chemotactic profile; Motility; Biofilm; Haemolysis; Adhesion; Invasion; Atmospheric conditions

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List of Abbreviations

BA	Blood agar
bdar	Brown, dry and rough
bp	Base pair
cDNA	Complementary DNA
CDT	Cytolethal distending toxin
CFU	Colony forming unit
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FBS	Foetal bovine serum
HIV	Human Immunodeficiency Virus
ICMSF	International Commission on Microbiological Specifications for Foods
IL-8	Interleukin 8
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MLST	Multi-Locus Sequence Typing
MOI	Multiplicity of infection
$OD_{620\ nm}$	Optical density at wavelength of 620 nm
PBS	Phosphate Buffered Saline
pdar	Pink, dry and rough
qPCR	Real-time polymerase chain reaction
rdar	Red, dry and rough
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Rotations per minute
saw	Smooth and white
ST	Sequence type
T3SS	Type III secretion system
T4SS	Type IV secretion system

TLR-4	Toll-like receptor 4
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
ZOT	*Zonula occludens* toxin

Chapter 1: Introduction

1.1 Genus *Aliarcobacter*

1.1.1 History and Taxonomy of the *Aliarcobacter* genus

Aliarcobacter was first isolated in 1977 by Ellis and colleagues describing a spiral-shaped bacterium, recovered from aborted bovine and porcine foetuses. Initially, the *Aliarcobacter* genus, at the time included in the historically developed *Arcobacter* genus, was included in *Campylobacter* genus being described as an aerotolerant *Campylobacter* with the capacity to grow in lower temperatures (Ferreira et al., 2016).
In 1991, Vandamme et al. (1991) conducted a phylogenetic study with the analysis of the partial *16S rRNA* gene sequences, proposing the historically developed genus *Arcobacter* to accommodate two aerotolerant *Campylobacter* species: *Campylobacter cryaerophila* and *Campylobacter nitrofigilis* (now known as *Aliarcobacter cryaerophilus* and *Arcobacter nitrofigilis,* respectively) (Vandamme et al., 1991). Later, Vandamme et al. (1992b) also proposed that *Campylobacter butzleri* would be added to this genus along with a new species, *Arcobacter skirrowii*. This bacterium was isolated from humans and animals with diarrhoea, and from diarrhoeal faeces of lambs, aborted porcine, ovine, and bovine foetuses, and the prepuce of bulls, respectively (Vandamme et al., 1992b).

Initially, the *Arcobacter* genus was allocated into the *Campylobacteraceae* family; however, a reclassification of the class Epsilonproteobacteria was recently proposed by Waite et al. (2017). After a comparative genomic analysis, it was suggested that the genus *Arcobacter* should be allocated into a novel family, *Arcobacteraceae*, nonetheless it was only in 2020 that this taxonomic modification was validly published (Oren & Garrity, 2020; Waite et al., 2017, 2018). Considering the historically developed *Arcobacter* genus, until now 33 species have been recognized, with four waiting for validly publishing. Nonetheless, a latter study developed by Pérez-Cataluña et al. (2018a), suggested, after an analysis of the core genome of 57 strains, the division of the genus *Arcobacter* into six new genera: *Aliarcobacter, Arcobacter, Halarcobacter, Poseidonibacter, Pseudarcobacter* and *Malaciobacter* (Figure 1).

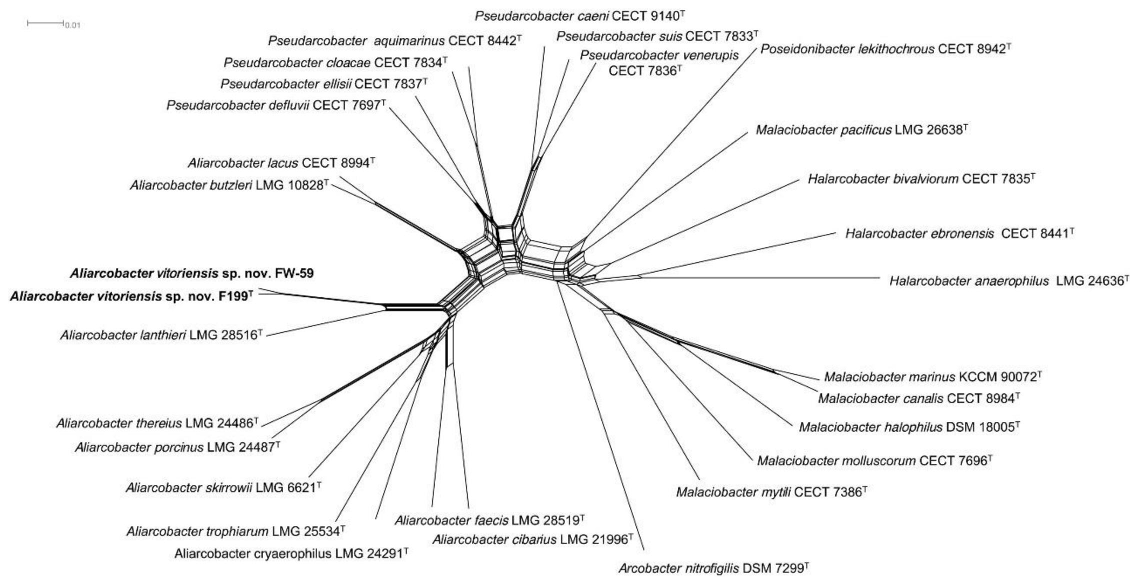

Figure 1 - Split decomposition network constructed with the concatenated sequences of 61 core genes of the *Arcobacteraceae* family (Alonso et al., 2020).

Amongst these genera, *Aliarcobacter* genus has received more attention, and it carries until now nine validly published species: *Aliarcobacter cryaerophilus, Aliarcobacter butzleri, Aliarcobacter skirrowii, Aliarcobacter cibarius, Aliarcobacter thereius, Aliarcobacter trophiarum, Aliarcobacter lanthieri, Aliarcobacter faecis, Aliarcobacter lacus*. With two new published proposed species *Aliarcobacter porcinus* and, *Aliarcobacter vitoriensis* (Table 1) (Alonso et al., 2020; Oren & Garrity, 2020; Pérez-Cataluña et al., 2018a; Figueras et al., 2017).

However, On et al. (2020) have recently proceeded with a revaluation of the *Arcobacter* genus performing a comparative analysis through a genomic, phylogenetic and phenotypic approach of the new six genera in a wider context of the Epsilonproteobacteria class. The authors refuted the previously approved division of genus while supporting the proposal of Waite et al. (2017) recognizing a clear discriminated group from other Epsilonproteobacteria, with the family *Arcobacteraceae* containing a single genus, *Arcobacter* (On et al., 2020).

Table 1 - Species of the genus *Aliarcobacter*, by year and source

Strain	Year	Origin	References
Aliarcobacter cryaerophilus	1985	Aborted bovine foetuses	(Neill et al., 1985)
Aliarcobacter butzleri	1991	Humans and animals with diarrhoea	(Kiehlbauch et al., 1991)
Aliarcobacter skirrowii	1992	Prepuce of bulls	(Vandamme et al. 1992b)
Aliarcobacter cibarius	2005	Chicken carcasses	(Houf et al., 2005)
Aliarcobacter thereius	2009	Aborted pig foetuses and ducks	(Houf et al., 2009)
Aliarcobacter trophiarum	2011	Pig faeces	(De Smet et al., 2011).
Aliarcobacter lanthieri	2015	Pig and dairy cattle manure	(Whiteduck-Leveillee et al., 2015)
Aliarcobacter faecis	2016	Human waste septic tank	(Whiteduck-léveillée et al., 2016)
Aliarcobacter porcinus	2017	Piglet foetuses	(Figueras et al., 2017)
Aliarcobacter lacus	2018	Reclaimed water	(Pérez-Cataluña et al., 2018b)
Aliarcobacter vitoriensis	2020	Carrots and urban wastewater	(Alonso et al., 2020)

Although some debate is still needed to reach a consensus about the taxonomy of the species and genera of the *Arcobacteraceae* family, in this work we will keep the validated nomenclature proposed by Perez-Cataluña et al. (2018), considering the genus *Aliarcobacter* and its species.

1.1.2 General characteristics of *Aliarcobacter* spp.

Cells from *Aliarcobacter* genus are Gram-negative curved rods with 0.2-0.5 µm in diameter and 1 to 3.5 µm long, have a single polar flagellum for motility, do not swarm, and do not produce fluorescent pigments. Species from this genus are chemoorganotrophic without fermentation of carbohydrates, and oxidase and catalase positive. Growth occurs between 15 ºC to 42 ºC and it is inhibited by presence of NaCl 4%, 2,3,5-triphenyltetrazolium chloride (0.04%, w/v) or glycine (1%w/v). Some of its species can grow in presence of safranin (0.05% w/v) or oxgall (1% w/v) and are sensitive to cefoperazone (64 mg/L). The range of DNA G+C content is 26.4–29.4 mol% (Pérez-Cataluña et al., 2018a).

Besides the global characteristics of the genus, *Aliarcobacter butzleri* cells usually have a smaller diameter 0.2 to 0.4 µm wide and 1 to 3 µm long. In blood agar grow mostly as round and whitish colonies with a diameter ranging from 2 to 4 mm after three days of incubation. This bacterium has a weak catalase activity, can reduce nitrate, and grow in Vogel-Bonner medium and MacConkey agar. Some strains can produce DNase and hydrogen sulphide from cysteine. Also, several strains can use organic acids and amino acids as carbon sources (Vandamme, et al. 1992b; Fanelli et al. 2019).

1.2 Prevalence of *Aliarcobacter* spp. in humans

Aliarcobacter spp. can be found in a wide range of environments and hosts, such as in food of animal origin and vegetables, in all sectors of the food industry, water, animals both wild and domesticated, and humans (Šilha et al., 2019b).
Amongst this genus, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii,* and *A. thereius* have been linked to human diseases (Tee et al., 1988; Van den Abeele et al., 2014; Vandamme et al., 1992a; Wybo et al., 2004). In 2002, *A. cryaerophilus* and *A. butzleri* were addended in the list of microbes considered a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

The first report of a microorganism of this genus in human samples was done by Tee et al. (1988), who recovered *Aliarcobacter cryaerophilus* from a stool sample retrieved from a 35-year-old man, with intermittent diarrhoea and abdominal pain (Tee et al., 1988). Although this first report, *A. butzleri* has been demonstrated to be the most prevalent species, being considered the fourth *Campylobacter*-related organism most frequently found in human diarrhoeic samples. Few reports of other species of this genus in human samples have been presented, nonetheless, *A. skirrowii* and *A. thereius* were also found in human faecal samples (Van den Abeele et al., 2014; Wybo et al., 2004).

Aliarcobacter spp. infections are often associated with persistent watery diarrhoea, and abdominal cramps being the two most common symptoms (Table 2). Infections by this genus are also associated with bacteraemia, enterocolitis, peritonitis, and fever (Collado & Figueras, 2011).

Table 2 - Report cases of *Aliarcobacter* spp. in humans, by year, country, age of the patient, clinical symptoms, underlying conditions, and species found.

Year	Country	Age	Clinical symptoms	Underlying conditions	Species	Reference
1988	Australia	35	Chronic diarrhoea	Not specified	*A. cryaerophilus*	(Tee et al., 1988)
1992	Unknown	2	Gastroenteritis	Not specified	*A. butzleri*	(Yan et al., 2000)
1994	Germany	48	Diarrhoea, abdominal cramps	Diabetes mellitus (type I)	*A. butzleri*	(Lerner et al., 1994)
1994	Germany	52	Fever, malaise, arthralgia, diarrhoea, abdominal pain	Rheumatic fever in childhood, asthma bronchial, duodenal ulcer, hyperuricemia, lactose intolerance and alcoholic excesses	*A. butzleri*	(Lerner et al., 1994)
1995	United Kingdom	neonate	Bacteraemia	Mother had prenatal bleeding	*A. butzleri*	(On et al., 1995)
1997	Taiwan	72	Bacteraemia	Chronic renal failure, haemodialysis with arteriovenous fistula	*A. cryaerophilus*	(Hsueh et al., 1997)
2000	Taiwan	60	Bacteraemia	Chronic hepatitis B carrier, liver cirrhosis	*A. butzleri*	(Yan et al., 2000)
2001	Hong Kong	7	Bacteraemia	Suffocation in a mud pool	*A. cryaerophilus*	(Woo et al., 2001)
2002	Hong Kong	69	Fever; abdominal pain	Gangrenous appendicitis	*A. butzleri*	(Lau et al., 2002)
2004	Belgium	73	Chronic diarrhoea	Prosthetic aortic heart valve	*A. skirrowii*	(Wybo et al., 2004)

Table 2 (continuation) - Report cases of *Aliarcobacter* spp. in humans, by year, country, age of the patient, clinical symptoms, underlying conditions, and species found.

Year	Country	Age	Clinical symptoms	Underlying conditions	Species	Reference
2004	Chile	2	Acute gastroenteritis; vomiting; chronic diarrhoea	Not specified	*A. butzleri*	(Fernández et al., 2004)
2004	Chile	1	Acute diarrhoea	Not specified	*A. butzleri*	(Fernández et al., 2004)
2012	Turkey	30	Abdominal pain, acute diarrhoea	Not specified	*A. butzleri*	(Kayman et al., 2012b)
2013	Hong Kong	63	Fever, abdominal pain	Not specified	*Arcobacter spp.*	(Yap et al., 2013)
2014	Spain	26	Chronic diarrhoea; abdominal cramps	Not specified	*A. cryaerophilus*	(Figueras et al., 2014)
2015	USA	85	Fever, hypotension, and chronic, persistent diarrhoea; bacteraemia	Chronic lymphocytic leukaemia	*A. butzleri*	(Arguello et al., 2015)
2017	Costa Rica	27	Bloody watery diarrhoea; acute stomach pain	Chronic diarrhoea	*A. cryaerophilus*	(Barboza et al., 2017)
2020	Spain	86	Intestinal colonization with no signs or symptoms	Arterial hypertension, hypercholesterolemia, osteoporosis, Sjögren syndrome and rosacea	*A. butzleri*	(Jiménez-Guerra et al., 2020)
2021	Ecuador	11 months	Acute, and watery diarrhoea	Malnutrition and anaemia	*A. butzleri*	(Simaluiza et al., 2021)

Also is important to note that the recovery rate of *Aliarcobacter* in human stool samples is somewhat low (Ferreira et al., 2016), with prevalence ranging from 0.01 up to 7.64 %. *A. butzleri* had the highest prevalence value of 7.64 %, followed by *A. cryaerophilus* 0.41%, then *A. skirrowii* 0.04% and lastly *A. thereius* with 0.01 % when summing 14,118 human stools samples analysed for *Aliarcobacter spp.* with an overall prevalence of the genus of 8.11 % (n=1.145) (Table 3).

Until now there is no standard isolation procedure (medium, temperature, atmosphere) able to isolate all *Aliarcobacter* species or a reliable method of identification (Webb et al., 2016). Thus, since no clear detection practices are used in-clinic diagnosis, the recovery rate could be much higher than reported. More research is necessary to understand the distribution and routes of transmission of *Aliarcobacter* spp.

Table 3 - Prevalence studies of *Aliarcobacter* spp. in stool samples by year, country, period of sample collection, prevalence, species, and study description of the positive cases.

Year	Country	Period of sample collection	Prevalence (positives/ total)	Species	Description of the positive cases	Reference
2004	Belgium	1995 - 2002	67/1906	*A. butzleri*	<2 years of age patients; 10 of 61 had underlying disease: four were HIV seropositive, and three were immunocompromised (postrenal graft, celiac disease, and chemotherapy for cerebellar astrocytoma). One had dementia, one had insulin-dependent diabetes mellitus and one had hepatitis C	(Vandenberg et al., 2004)
			10/1906	*A. cryaerophilus*		
2006	France	2002 - 2003	29/2855	*A. butzleri*	Diarrhoea and/or abdominal pain, bloody stools, vomiting and/or fever	(Prouzet-Mauléon et al., 2006)
2007	South Africa	2004 - 2005	20/322	*A. butzleri*	Gastrointestinal complaints/diarrhoea	(Samie et al., 2007)
			9/322	*A. cryaerophilus*		
			6/322	*A. skirrowii*		
2010	Mexico; Guatemala; India	2009	16/201	*A. butzleri*	Travellers' diarrhoea	(Jiang et al., 2010)
2013	Chile	2010 - 2012	2/256	*A. butzleri*	Diarrheic and nondiarrheic people	(Collado et al., 2013)
2014	Portugal	2012	4/298	*A. butzleri*	Patients presenting acute gastrointestinal symptoms	(Ferreira, et al., 2014a)
			1/298	*A. cryaerophilus*		
2014	Belgium	2008 - 2013	49/6774	*A. butzleri*	Outpatients and patients with symptoms of enteritis	(Van den Abeele et al., 2014)
			38/6774	*A. cryaerophilus*		
			2/6774	*A. thereius*		
2016	Canada	2008	892 /1506	*A. butzleri*	Diarrheic and non-diarrheic	(Gorham & Lee, 2016)

1.3 Transmission routes of *Aliarcobacter* spp.

Some of the *Aliarcobacter* species, such as *A. butzleri*, *A. cryaerophilus,* and *A. skirrowii* are considered emerging pathogens with a global distribution. When its presence in the food chain is considered along with its ability to withstand stress and storage conditions, this can become a major public health concern (Ferreira et al., 2019a).

Ingestion of contaminated food or water has been considered as the main route of transmission of *Aliarcobacter* spp., with this being supported by numerous water and food-associated-outbreaks reports. However, the transmission routes have not been completely understood and the association between source and human disease was not always clearly established (Ferreira et al., 2016).

1.3.1 Consumption of contaminated foods

It is thought that the main source of transmission of *Aliarcobacter* spp. is through the consumption of contaminated foods (Zacharow et al., 2015). *Aliarcobacter* spp. can be found all over the food chain, with several surveys on food products showing a high presence of these bacteria in the primary stage, processing, retail, on ready-to-eat products, and supplying waters of food facilities (Ferreira et al., 2019b).

Most of the studies related to the prevalence of *Aliarcobacter* spp. have been focused on meat, with the greatest number of reports being associated with poultry, however, this pathogen can also be found in other types of meat, such as pork, beef, and others. It is thought that some of the cross-contamination related to meat could come from the contact of the carcasses and faeces, or by the contact with scalding waters in the processing stage. We can also find this bacterium in dairy products, such as milk and cheese, that usually are consumed without further processing (Mateus et al., 2021a).

Because some microorganisms of the *Arcobacteraceae* family seems to be native to the marine environments, fish and seafood have also been considered important transmission sources, especially in seashells where we can find a high prevalence of *A. butzleri* and *A. cryaerophilus* (Salas-Massó et al., 2018; Vicente-Martins et al., 2018). This becomes especially important when we consider that much of the seafood is ingested raw with no depuration steps (Ferreira et al., 2019b).

We can also find *Aliarcobacter* spp. in some vegetables, although studies on the presence in this type of food are scarce, some species were found in spinaches, lettuces,

valerian, chards, cabbages, and rockets. Considering that some of these vegetables were ready-to-eat, and no cleaning procedure are needed before consuming, a higher health risk could be associated with its consumption (González et al., 2017; Mottola et al., 2016).

Pointing for the risk associated with the consumption of contaminated food, in Thailand, a study aiming to evaluate the traveller's risk of exposure to certain enteric pathogens, while eating at Bangkok restaurants, had shown a prevalence of 13 % of *A. butzleri*. Each meal had a risk of exposure of 13% and could rise to 75% when 10 meals are consumed (Teague et al., 2010).
There is also a report on a foodborne outbreak associated with *A. butzleri* at a wedding reception in the USA, the results suggested that the origin was a contaminated roasted chicken (Lappi et al., 2013). These outbreaks reinforce the association of food as a possible route of transmission (Ferreira et al., 2017).

1.3.2 Consumption of contaminated water

As mentioned earlier, the consumption of contaminated water is one possible route of transmission of *Aliarcobacter* to humans and animals. These bacteria have been recovered from several environmental water sources, like rivers, lakes, groundwater, or seawater (Collado & Figueras, 2011). Several reports have shown its presence in groundwater and river of many countries, such as Czech Republic, Japan, South Africa, Spain, Turkey, and USA (Collado et al., 2008; Diergaardt et al., 2004; Ertas et al., 2010; Moreno et al., 2004; Morita et al., 2004; Rice et al., 1999; Šilha et al., 2019b; Talay et al., 2016); being one of the most prevalent pathogenic bacterium isolated in groundwater as well as in surface water samples (Shrestha et al. 2019). One potential vehicle for water contamination may be the faeces of livestock or even wild animals, since most of the *Aliarcobacter* species are reported as being commensal in the gastrointestinal tract of many animals (Shrestha et al. 2018).

The first report of an outbreak linked to the consumption of *Aliarcobacter* spp. contaminated water was described at a girl scouts camp in the USA in 1996. After a breakdown in the camp's automated chlorination system, 81 % of the individuals in the camp became ill with nausea, vomits, abdominal cramps, and diarrhoea. Although later tests showed the presence of *A. butzleri* in the well contaminated water, no direct correlation was established in the outbreak analysis (Rice et al. 1999). Another outbreak was registered in 2004, also in the USA, and affected 1,450 residents and visitors of Lake Erie, who reported gastrointestinal illness after consumption of water, from seven wells which tested positive for *Aliarcobacter* spp. (Fong et al., 2007). One

more outbreak in 2008, in Slovenia, affected 408 citizens with diarrhoea, vomiting, fever, abdominal pain, headache, and nausea. This happened due to changes in the water distribution system that in the process had become contaminated, one of the species found in stool samples was *A. cryaerophilus* (Kopilovi et al., 2008).

These outbreaks support the idea that *Aliarcobacter* spp. can be transmitted by the consumption of contaminated waters (Ferreira et al., 2016).

1.3.3 Contact with pets and wild animals

Some species of *Aliarcobacter* spp. can also be found in pets, such as cats and dogs, doves and raccoons suggesting that contact with these animals could be a possible route to human infection (Ferreira et al., 2016). Several studies around the globe have shown distinctive rates of recovery of *Aliarcobacter* species in animals, which could be associated with the diversity of distribution of this bacterium in animals, but also to different sensitivities of the isolation and confirmation procedures used in its recovery (Goni et al., 2017).

In Italy, Fera and colleagues analysed samples of blood and an oral swab from 85 domestic cats and found that 78.8 % tested positive for *Aliarcobacter* species, 76.6 % for *A. butzleri,* and 34.1 % for *A. cryaerophilus* (Fera et al., 2009). Another study, in Belgium with oral swabs and faecal samples from 267 dogs and 61 cats shown that *Aliarcobacter* spp. were present in 2.67 % of dogs and absent in the cat samples (Houf et al., 2008). Also, in Chile, in a study on dogs, a prevalence of 3.3 % was shown by analysis of 60 faeces samples (Fernández et al., 2007). Another study in Turkey analysed 62 rectal swabs from dogs, with no isolation of *Aliarcobacter* spp. (Aydin et al., 2007). A similar study in New Zealand with faecal samples from 50 farm dogs shown one positive isolate for *A. cryaerophilus* and five for *A. butzleri* (Bojanić et al., 2019).

Wild animals may also play an important role in the spreading of these bacteria, since many share urban and suburban environment alongside humans, such as doves, racoons, and rats, but few studies have been carried to evaluate the prevalence of *Aliarcobacter* (Collado & Figueras, 2011). Two studies with more relevance due to the presence of these animals in public spaces, and that their faeces also represent sources of contamination to other animals and also humans, reported a prevalence of 60 % of *Aliarcobacter* spp. in a sample of 10 raccoons (*Rocyon lotor*) and 19 % in a sample of 95 Eurasian collared doves (*Streptopelia decaocto*) (Di Francesco et al., 2014; Hamir et al., 2004). Other examples of the presence of *Aliarcobacter* in exotic or non-

domesticated animal, such as chelonians, lizards, snakes, rabbits, rhinoceros, gazelle, rhea, alpacas, and more recently ostriches have been presented (Ramees et al., 2017; Shange et al., 2020).

1.3.4 Person-to-person contact

Person-to-person contact was suggested as a route of transmission for the first time in 1992 by Vandamme and collaborators, when an outbreak of recurrent abdominal cramps in an Italian school in 1983 affected ten children and one schoolteacher. All the isolates recovered were identified as *A. butzleri*, and presented the same protein profile, serogroup, and phenotypic characteristics, suggesting an epidemiological relationship between the cases. This supports the idea of person-to-person transmission due to the small-time window of successive cases of infection (Vandamme et al., 1992a).

In 1995, another possible association to person-to-person transmission was proposed when a 34-year-old woman with no sign of illness delivered a baby boy with bacteraemia. After a microbiological analysis, it was determined an infection by *A. butzleri*. The source of the infection was unknown, but it was suggested that the infection was probably initiated by prenatal bleeding by the mother implying a possible vertical transmission of *A. butzleri* (On et al., 1995).

1.4 Pathogenesis and virulence factors of *Aliarcobacter butzleri*

A. butzleri infections have been associated with cases of bacteraemia and enteritis in humans, being the most recovered *Aliarcobacter* species from human stools (Table 3). Although some studies have been made to elucidate the potential pathogenicity of *A. butzleri*, there are still some clarifications in need, to understand the true mechanism that could trigger an *A. butzleri* infection. *In vitro* studies have already shown its ability of adhesion and invasion to eukaryotic cells from animal and human origin, and toxin production capable of harming the host (Ferreira et al., 2016). Few *in vivo* studies have been made to determine the pathogenic mechanism associated with *A. butzleri* infection. Most of the studies used murine models and suggested that *A. butzleri* can produce both local and systemic inflammatory immune responses even inducing changes in the regulatory system and matrix-degrading molecules essential to the turnover of the tissue in the intestine and its immune response. Although the pro-inflammatory response produced by *A. butzleri* induce less pronounced sequelae than

C. jejuni, it produced a more defined local and systemic response that commensal *E. coli* (Gölz et al., 2016; Gölz, et al., 2015a; Heimesaat et al., 2016; Heimesaat et al., 2015a). Another study using zebrafish suggested that *A. butzleri* infections could not always correlate with clinical signs or disease *per se*, but can still be responsible for histopathological lesions (Açik et al., 2016). All the studies also suggested that differences on the inflammatory responses can be related to each strain and the diversity of the lipopolysaccharide (LPS) or lipooligosaccharide (LOS) structures that could determine if a strain acts as a commensal or as a pathogen *in vivo* (Gölz et al., 2016; Gölz et al., 2015a; Heimesaat et al., 2016; Heimesaat et al., 2015a). This hypothesis is supported by two studies that studied the interaction of the toll-like receptor (TLR)-4, the main receptor for LPS and LOS of Gram-negative bacteria, with *A. butzleri*, despite its involvement was proven, only recently LPS or LOS was suggested as an important virulence factor in arcobacteriosis (Fanelli et al., 2019; Gölz et al., 2015b; Heimesaat et al., 2015b).

Currently, the complete genome sequencing of several strains had shown great support to *A. butzleri* research, enabling to establish a relationship between its phenotypic characteristics and its genome (Fanelli et al., 2019, 2020; Isidro et al., 2020). Several virulence and metabolic genes found in the *A. butzleri* genome are homologues to other species of bacteria, like *Campylobacter jejuni,* with 30 % protein homologues with best matches to non-epsilonproteobacerial proteins (Miller et al. 2007).

1.4.1 Antimicrobial resistance determinants

Throughout the years the effectiveness of antibiotics has been largely decreasing, with the use of antibiotics (Table 4) in the agriculture, food chain, and poor health practices contributing to the emergence of antimicrobial-resistant organisms (Laxminarayan et al., 2013; Marston et al., 2016).

In the *Aliarcobacter* genus, only a few species have been analysed for their antibiotic susceptibility, namely *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Ferreira et al., 2016). A meta-analysis by Ferreira et al. (2019) analysed the antimicrobial resistance rates of the *Aliarcobacter* genus. This study has shown that *Aliarcobacter* species presented higher frequencies of resistance to ampicillin, amoxicillin, cephalothin, azithromycin, and levofloxacin while presenting a lower resistance to antibiotics such as erythromycin, gentamicin, tetracycline, streptomycin, enrofloxacin or doxycycline (Ferreira et al., 2019).

Table 4 - Major classes of antibiotics, mode of action and examples.

Class	Mode of action	Examples
Β-lactams	Inhibit bacteria cell wall biosynthesis	Penicillins, such as amoxicillin and flucloxacillin; Cephalosporins, such as cefalexin
Aminoglycosides	Inhibit the synthesis of proteins, leading to cell death	Streptomycin, neomycin, kanamycin, paromomycin
Tetracyclines	Inhibit the synthesis of proteins by bacteria, preventing growth	Tetracycline, doxycycline, lymecycline, oxytetracycline
Macrolides	Inhibit the synthesis of protein by bacteria, occasionally leading to cell death	Erythromycin, clarithromycin, azithromycin
Quinolones	Interfere with bacteria DNA replication and transcription	Ciprofloxacin, levofloxacin, trovafloxacin

Although frequently no antibiotic treatment is necessary due to the self-limiting nature of *A. butzleri* infection, the severity and prolongation of the symptoms could justify its use (Collado & Figueras, 2011). In this case, fluoroquinolones, tetracycline, and aminoglycosides, such as gentamicin, kanamycin, and streptomycin, have been recommended for the treatment (Chieffi et al., 2020). However, some studies have reported a high frequency of resistance of some strains to tetracycline, which could be of great concern since this class of antibiotics is one of the most used (Vicente-Martins et al., 2018; Yesilmen et al., 2014).

Until now several genetic determinants of antimicrobial resistance have been identified in the genome of *A. butzleri*. Resistance to β-lactams has been related to the presence of β-lactamases, such as *bla1, bla2, bla3, hcpC* and one putative metallo β-lactamase encoding gene responsible for hydrolysis of β-lactams and modifications in the target site of the antibiotics of this class (Fanelli et al., 2019; Isidro et al., 2020). Resistance to quinolone has been attributed to several point mutations present in the *gyrA* gene that confer different rates of resistance to ciprofloxacin and levofloxacin fluoroquinolones (Abdelbaqi et al., 2007; Fanelli et al., 2019, 2020; Ferreira et al., 2018). Resistance to erythromycin has been also associated with truncated TetR regulator protein related to a single nucleotide polymorphism or presence of insertions sequences (Isidro et al., 2020). Other antimicrobial resistance factors that may be found in the whole genome of *A. butzleri* are chloramphenicol acetyltransferase encoding genes (*cat3* and *wbpD*); macrolide export proteins (*macA* and *macB*); fosmidomycin resistance protein (*fsr*); tetracycline resistance protein (*tetA*); bicyclomycin resistance protein (*bcr*); polymyxin

resistance (*arnA, arnB, eptA*), methicillin resistance (*hlpA*), mupirocin resistance (*ileS*), ciprofloxacin and ampicillin resistance (*relE*), ribosome targeting antibiotic resistance (*rlmN*), streptothricin resistance (*sttH*) and bacitracin resistance (*uppP*) (Fanelli et al., 2019, 2020; Isidro et al., 2020).

In a study by Isidro et al. (2020), the authors found 19 efflux pump systems (EP1–EP19) that may be associated with antibiotic resistance, ten of these systems were found in all strains analysed, with nine of them belonging to the family resistance-nodulation-cell-division (RND) (Isidro et al., 2020). In *A. butzleri* three efflux pumps of the RND family: AreABC, AreDEF, and AreGHI have shown to have a role in the extrusion of diverse antimicrobials agents, such as antibiotics, bile salts, and biocides. The AreABC and AreGHI efflux pumps demonstrated a more active role in antibiotic efflux, with AreGHI with a special role in the presence of bile salts, while AreDEF with a more modest role with less significance in multidrug resistance (Mateus et al., 2021b).

1.4.2 Stress and survival responses of *Aliarcobacter* spp.

Many bacteria can tolerate extreme environmental conditions, or even adapt to different circumstances, such as acidity, pressure, temperature, ultraviolet radiation, dehydration, antibiotic susceptibility, salinity, nutrient depletion and even the presence of oxygen (Kim et al., 2021; Poole, 2012). This is an important feature when considering that pathogens may be exposed to these stresses when colonizing a host, especially gastrointestinal pathogens. In fact, during their passage through the gastrointestinal tract, these bacteria are exposed to pH fluctuations, osmotic stresses, reactive oxygen and nitrosative species (Flint et al., 2016).

After ingestion, bacteria have to survive a range of pH values in the stomach, as pH 2 when in a fasting state, and up to pH 7 a few minutes after the meal intake (Flint et al., 2016). *A. butzleri* can withstand pH values between 4.5 and 8.5, with optimal growth in the range of 6.0 to 7.0 (D'sa & Harrison, 2005; Giacometti et al., 2015). Like *Campylobacter* spp., *A. butzleri* is described as relatively sensible to low pH values (Gaynor et al., 2005). Although the molecular mechanisms behind pH adaptation and survival were not described in *Aliarcobacter* spp. some genes related to the urease cluster, ureD (AB)CEFG, associated with survival to acid stress and host colonization in *Helicobacter pylori* were found (Flint et al., 2016; Isidro et al., 2020; Miller et al., 2007).

Several bacteria can adapt to fluctuations in the osmolarity of the environment, with the hypotonic environments leading to a swelling of the bacterial cell and hypertonic

environments to dehydration and shrinkage of the cells, being this adaptation ability essential to bacterial proliferation and survival (Csonka, 1989; Sleator & Hill, 2002). Hypertonic environments are often related to the food industry, NaCl is one of the most recurring food preservatives used (Desmond, 2006). Until now, a few studies have evaluated the response of *Aliarcobacter* spp. to NaCl in medium, suggesting that only some strains of *A. butzleri* are capable of growth in media containing up to 4 % NaCl or 5% when in non-optimal temperatures (25 ºC) (Cervenka, 2007; D'sa & Harrison, 2005). These values suggest that *A. butzleri* can grow and tolerate higher concentrations of NaCl than *Campylobacter* spp. (1.5 %), having an enhanced survival compared to other *Campylobacter*-like microorganisms (D'sa & Harrison, 2005).

Reactive oxygen species (ROS) are one of the most used strategies by host organism to fight bacterial infections, with several components of the immune system being able to produce ROS and using it to kill or inhibit the growth of pathogens (Imlay, 2008). In response, pathogens encode several detoxification enzymes, such as superoxide dismutase, catalase, alkyl hydroperoxide reductase, to be able to survive the damaging effects of oxidative species found during host colonization (Flint et al., 2016). Despite the lack of studies regarding oxidative stress response in *Aliarcobacter* spp., a catalase peroxidase gene, *katG,* was identified in this genus (Parisi et al., 2019). In *Salmonella* Typhimurium mutations in this gene can lead to a decrease in virulence in a mouse model of infection, pointing to its relevance (Hébrard et al., 2009).

Bacteria also must survive the presence of nitric oxide (NO) and reactive nitrogen species (RNS) during host colonization. These reactive species can be found along the gastrointestinal tract being originated from food ingestion and from the host itself (Flint et al., 2016). To survive, bacteria often resort to globins and NO reductases to defend themselves against the nitrosative species. While *Aliarcobacter* has no data regarding this defence mechanism, in *Campylobacter,* haemoglobin Cgb and nitrate reductase NrfA have been associated with survival against nitrosative species (Weingarten et al., 2008). Haemoglobin Cgb-deficient mutants showed a decrease in resistance to two nitrosating agents and a nitric oxide releaser, and with a *nfrA* deletion leading to a higher sensitivity to different sources of nitrosative reactive species (Elvers et al., 2004; Weingarten et al., 2008). Further studies are needed to reveal the mechanisms behind resistance to nitrosative species.

1.4.3 Chemotactic factors

Chemotaxis is the mechanism that allows motile bacteria to sense and move close or away from a variety of environments, being capable of perceiving chemical gradients.

This mechanism can help bacteria to have higher access to growth substrates, and in some bacteria it also play an important role in pathogenicity (Matilla & Krell, 2018). In 2007, for the first-time chemotactic proteins were described in *A. butzleri*, although its function and pathways still need to be understood, several of these proteins are similar to the proteins found in *Campylobacter* species (Miller et al., 2007). These includes the CheARDB proteins used in a signal transduction pathway, CheY that links the sensory pathway to the flagellar motor and control its rotation, and CheV a docking protein that links methyl-accepting chemotactic proteins (MCPs) to CheA proteins (Table 5) (Bolton, 2015; Matilla & Krell, 2018; Miller et al., 2007).

In *A. butzleri* few studies have been made to establish a relationship between chemotaxis proteins and their effect on the overall response of the bacteria to external stimuli. In 2020, in a study by Fanelli and collaborators, the metabolic fingerprinting of 5 strains of *A. butzleri* was presented using Biolog AN MicroPlates. In this work, the incapacity of the genus to metabolize carbohydrates, as described previously by Vandamme et al. (1992b), and to use organic acids and amino acids as carbon sources was confirmed (Chieffi et al., 2020; Fanelli et al., 2020; Vandamme et al., 1992b). Some of these metabolic substrates include acetic acid, formic acid, fumaric acid, α-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, D, L-lactic acid, L-lactic acid, D-lactic acid methyl ester, L-malic acid, propionic acid, pyruvic acid, pyruvic acid methyl ester, succinamic acid, succinic acid, succinic acid mono-methyl ester, L-alanyl-L-histidine, L-glutamic acid, L-glutamine and L-threonine (Fanelli et al., 2020).

Other chemotactic response in bacteria is the system known as, quorum sensing, it allows bacteria to determine concentrations of compound named autoinducers. These autoinducers allow bacteria of the same species to establish cell-to-cell communication, and to act in synchrony (Rutherford & Bassler, 2012). In *C. jejuni*, this ability is associated to the LuxS-mediated cell-to-cell signalling system, and showed an important role in motility, biofilm formation, host colonization and overall virulence of this species (Elvers & Park, 2002; Golden & Acheson, 2002; Jeon et al., 2003; Plummer et al., 2012; Quiñones et al., 2009). In *A. butzleri, luxS* and *tqsA* related to the LuxS-mediated system were described in 2020 (Table 5), this could suggest a similar relation between its system and the virulence of this species (Fanelli et al., 2019; Isidro et al., 2020).

Table 5 - Putative chemotactic genes of *Aliarcobacter butzleri* identified from genomic studies.

Virulence factor(s)	Encoding gene(s)	References
Chemotaxis proteins: Che A, B, R, V and W	*cheA, cheB, cheR, cheV, cheW*	(Isidro et al., 2020; Miller et al., 2007; Müller et al., 2020; Rovetto et al., 2017)
CheY, response regulator protein controlling flagellar rotation	*cheY1; cheY2; cheY3*	(Isidro et al., 2020; Miller et al., 2007; Müller et al., 2020)
AI-2 biosynthesis enzyme	*luxS*	(Fanelli et al., 2019, 2020; Isidro et al., 2020; Müller et al., 2020)
YdgG, AI-2 transporter	*tqsA*	(Fanelli et al., 2019, 2020)

1.4.4 Motility and flagella

Motility alongside chemotaxis enables bacterium to detect and move towards or away from a variety of environmental signs. This allows to locate nutrients and also enable them to reach their preferred niches for colonization, and so essential to bacterial survival (Josenhans & Suerbaum, 2002). Motility also displays an important role in the colonization and invasion of host cells, protein secretion and biofilm formation to facilitate bacterial survival (Haiko & Westerlund-Wikström, 2013). Similar to what has been described for *C. jejuni,* it is also suggested that *A. butzleri* could also reside with the thick mucus layer limiting the intestinal epithelium (Schönknecht et al., 2020). In *C. jejuni*, motility also enables its migration within the mucus to reach microenvironments more prone to growth and proliferation (Ferrero & Lee, 1988). In *A. butzleri,* this relation is yet to be proven; however, it seems that the flagellar motility is more affected by the viscosity of the medium than in *C. jejuni* (Chaban et al., 2018). As such non-motile *C. jejuni* have demonstrated attenuated colonization of human or animal hosts (Hendrixson & DiRita, 2004). For *A. butzleri,* it was also suggested that higher motility could be associated with higher rates of invasion; however, more studies are necessary to verify this hypothesis (Bruegge et al., 2014).

In *Aliarcobacter* species, motility is attributed to an existing polar flagellum at one or both ends of the bacterial cell (Ho et al., 2008). *A. butzleri* flagellum is constituted by two major parts: a hook-basal body and an extracellular filament. The hook-basal body complex is composed of different proteins including FliF (inner membrane MS ring); FliG, FliM, FliN (C-ring); FlhA, FlhB, FliI, FliP, FliQ, FliR (T3SS, a type 3 secretion system), FliE, FlgB, FlgC (proximal rod); FlgI (P-ring in the peptidoglycan); FlgH (L-ring in the outer membrane); PflA, PflB (flagellar accessory proteins specific to *Campylobacter*); FlgO (flagellar accessory proteins specific to *Vibrio*); MotA (proton-conducting component of the flagellar motor in *Escherichia coli*), and MotB (linker

protein that fastens the torque-generating machinery to the cell wall in *E. coli*) (Figure 2). The extracellular filament is composed of two flagellin subunits, major flagellin protein FlaA, minor flagellin protein FlaB and more recently a *hag* flagellin responsible for regulating the formation of new filaments was described (Table 6) (Chaban et al., 2018; Isidro et al., 2020; Medina et al., 2019).

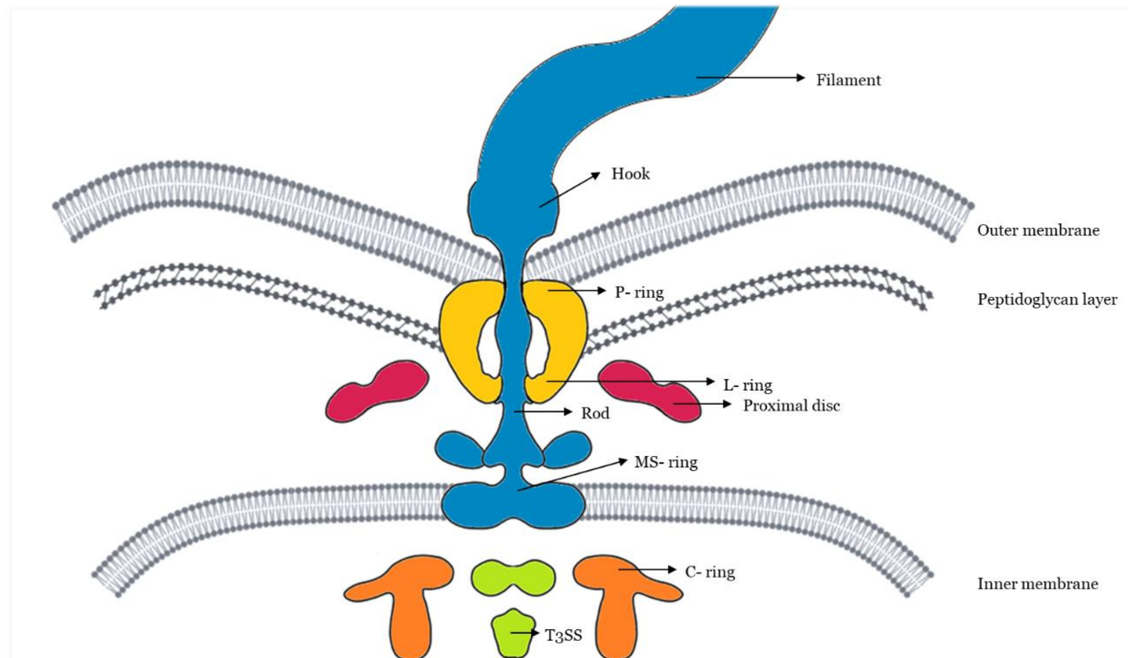

Figure 2 – Suggested flagellar assembly based on the cryo-EM results obtained by Chaban et al., 2018 showing the main components.

A study conducted by Ho et al. (2008) showed that the motility phenotype and transcription of the *fla* genes in the *A. butzleri* wildtype and the *flaA* or *flaB* mutants resemble the same patterns found in *Campylobacter* species, although their expression may not be regulated in the same manner. Also, mutants with no expression on *flaB* gene still have a flagellum with comparable length and were equally motile comparable with the wildtype strain, on the other hand, mutants with no expression of the *flaA* gene resulted in a non-flagellated and non-motile bacterium (Ho et al., 2008). A further study by Medina et al. (2019) aimed to analyse the transcriptional pattern of flagellar genes (*flaA, flaB, flgH, and motA*) during the host interaction with *Acanthamoeba castellanii*. This showed that before infection flagellar genes suffer an up-regulation and then slowly decrease once *A. butzleri* has established as an endosymbiont demonstrating the important role of the flagellum in infection process similarly to previous studies with *C. jejuni* (Medina et al., 2019).

Table 6 - Putative motility and flagella genes of *Aliarcobacter butzleri* identified from genomic studies.

Virulence factor(s)	Encoding gene(s)	References
FlaA, major flagellin protein	*flaA*	(Fanelli et al., 2019, 2020; Miller et al., 2007)
FlaB, minor flagellin protein	*flaB*	(Isidro et al., 2020)
FliF, hook-basal body protein	*fliF*	(Fanelli et al., 2019)
FlgI, P-ring in the peptidoglycan	*flg*I	(Isidro et al., 2020)
FlgH, L ring in the outer membrane	*flgH*	(Isidro et al., 2020)
FlhA, FlhB, FliI, FliP, FliQ, FliR, (T3SS)	*flhA, flhB, fliI, flip, fliQ, fliR*	(Fanelli et al., 2019, 2020)
MotA, catalyses proton transfer protein	*motA*	(Fanelli et al., 2019, 2020; Isidro et al., 2020)
MotB, flagellar motor protein	*motB*	(Isidro et al., 2020)

1.4.5 Biofilm formation factors

Biofilm is described as an organized multicellular community immersed in a self-made matrix capable of adhering to surfaces. These structures are considered ubiquitous and mostly have a heterogeneous nature, often attached to abiotic or biotic surfaces, but can also be found as a non-adherent structure floating in liquids (Berne et al., 2018; Krzyżek et al., 2020). Biofilms are capable of enhancing the survival of bacteria, having a protector effect against host defence mechanisms, antimicrobials, and being responsible for up to 80 % of chronic microbial infections in humans (Magana et al., 2018).

Since 2009, several studies reported the biofilm formation ability of *A. butzleri* being mostly classified as moderate or weak biofilm former (Ferreira et al., 2013; Kjeldgaard et al., 2009). A later study has shown that *A. butzleri* could also produce biofilms in a vast range of temperatures from 5 to 37 ºC (Šilha, et al., 2019a).

Until now 25 proteins related to biofilm production in *Pseudomonas* and *E. coli* were found in the *A. butzleri* genome, but no further studies to evaluate the proteins correlation to biofilm formation were undertaken (Miller et al., 2007). In bacteria from the same order, such as *Helicobacter* spp., biofilm formation has been linked to higher expression of adhesins, lipopolysaccharide, flagella, components of T4SS systems, toxin-antitoxin systems, efflux pumps, enzymes regulating pH and lower expression of the activity of the autoinducer-2 (Krzyżek et al., 2020). Although some homologous genes were found in *A. butzleri*, such as *luxS*, *flaA*, *flgE*, *fliI*, *motA*, and *motB*, no relation has been made regarding their influence in biofilm formation, however its

functions are often related to other main function and mechanisms, such as motility and chemotaxis in other bacteria.

1.4.6 Toxin production

Some bacteria can produce several toxins that can play a role in establishing a successful infection (do Vale et al., 2016). Toxins can be associated with the cell membrane (membrane-bound lipopolysaccharides or lipoproteins), also known as endotoxins or be secreted by the bacteria, exotoxins (Rudkin et al., 2017). Production of toxins can directly induce epithelial secretions, damage the epithelial cells or the intestinal barrier function, recruit secondary cells or mediators, such as macrophages, lymphocytes, and mast cells, which then trigger intestinal secretion, inflammation, and/or damage (Guerrant et al., 1999).

The first report of *A. butzleri* cytotoxic effect was done in 1997, by Musmanno and collaborators who exposed Vero and CHO cells to bacterial culture filtrates (Musmanno et al., 1997). Since then, more authors have found cytotoxic effects on several isolates of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* from diverse origins, detecting toxicity against multiple cell lines (Vero, CHO, HeLa, INT407), with studies suggesting that *Aliarcobacter* is capable of both endotoxin and exotoxin production (Ferreira et al., 2016). A study that detected direct cytotoxicity in HT-29/B6 cells led to the belief that heat-sensitive proteins possibly associated with the cell membrane are responsible for this cytotoxic effect (Bücker et al., 2009). Exotoxin production reports come from the use of filtrates and culture broths that have also displayed cytotoxicity (Bücker et al., 2009; Johnson & Murano, 2002). Also, an *in vivo* study with adult male rats showed the toxicogenic ability of *A. cryaerophilus* by using a cell-free supernatant of two strains, leading to distention of the ileal loops and an increase in electrolytes concentrations (Fernández et al. 1995).

One of the most common cytotoxins associated with *Campylobacter* species is the cytolethal distending toxin (CDT), but until now no presence of the related genes (*cdtABC*) was reported in *A. butzleri* suggesting that another mechanism is underlying its cytotoxicity (Johnson & Murano, 2002; Miller et al., 2007). In 2017, the presence of the zonula occludens toxin (ZOT) in 8 strains of *A. thereius* was reported. This can have an important role in the adhesion and invasion of host cells, since in *Vibrio cholerae* and *Neisseria meningitidis,* this toxin is known to work on intracellular thigh junctions leading to an increase in tissue permeability, but no further reports were found for other *Aliarcobacter* species (Rovetto et al., 2017).

LPS and LOS, also known as endotoxins with a role as virulence factors and also in antibiotic resistance, have been found in *Aliarcobacter*. In *A. butzleri,* clusters related to LOS biosynthesis were found, additionally, *waaC* and *waaF* genes were also reported. The *waaC* encodes a heptosyltransferase involved in the biosynthesis of LOS and *waaF* a lipopolysaccharide heptosyltransferase responsible catalysing the formation of glycosidic bonds between sugar moieties and specific acceptor molecules (Fanelli et al., 2019). In *Campylobacter*, mutations in these genes can lead to an incomplete LOS and increased susceptibility to detergents, bile salts, and hydrophobic antibiotics (Kanipes et al., 2006).

Other important virulence factors expressed by *A. butzleri* are the haemolysins. These compounds can damage cells membrane, even provoking lysis and destruction of nearby cells and tissues, in particular, the red blood cells, often linked to haemolytic activity (Mogrovejo et al., 2020). In *Aliarcobacter* spp., three genes putatively encoding for haemolysins were found: *tlyA* a cytotoxic haemolysin, *hecB* a related haemolysin activation protein and *pldA* outer membrane phospholipase A (Miller et al., 2007). Until now, only one study tried to establish a correlation between the haemolytic activity of six strains of *A. butzleri* and the presence of the virulence genes *tlyA*, *hecB*, and *pldA*. (Ferreira et al., 2014b). Although the six strains were described as weakly haemolytic, no correlation was found between the presence of the genes and haemolytic activity (Ferreira et al., 2014b).

Further characterization of toxins and other cell surface structures is required to understand more about interactions and cytotoxic effects in *A. butzleri*.

1.4.7 Adhesion factors

Adhesion is a critical factor in bacterial pathogenicity, it is often related to the ability to establish the infection of the host cells, tissue colonization, and in some cases can lead to cellular invasion. This ability is frequently mediated by adhesins, glycoproteins present in the bacterium surface, which can recognize different components of the host cell (Pizarro-Cerdá & Cossart, 2006). Two adhesins to fibronectin homologs of proteins related to adhesion processes in *Campylobacter* spp. have been found in *A. butzleri*: CadF, a fibronectin-binding outer membrane protein responsible for internalization in host cells by GTPases, and Cj1349 another fibronectin-protein that promotes bacteria cell contact (Table 6) (Isidro et al., 2020; Parisi et al., 2019). In *Campylobacter* species, CadF revealed to have an essential role for *in vivo* colonization of chickens, since the establishment of a knockout of the *cadF* gene resulted in an impairment in colonizing the chicken cecum; however, this potential relation in *Aliarcobacter* as yet to be

demonstrated (Ziprin et al., 2001). A study conducted in 2013, tried to correlate modifications in *cadF* and *cj1349* genes of *A. butzleri* with the adhesion ability of the strains, but no correlation could be found. The authors justified the results with the small number of isolates analysed, indicating that more investigation is necessary (Karadas et al., 2013). Phospholipase A (PldA) is another outer membrane protein that has been associated with adhesion, although most commonly associated with haemolytic activity in *Campylobacter* and *Helicobacter* species (Table 7). A study has shown that mutants of *C. jejuni* with defects or absence of the gene encoding for PldA protein had a reduced colonization ability and suggested a possible role of protection against immune responses (Dekker, 2000; Ziprin et al., 2001).

Table 7 - Putative adhesion genes of *Aliarcobacter butzleri* identified from genomic studies.

Virulence factor(s)	Encoding gene(s)	References
CadF, outer membrane protein	*cadF*	(Fanelli et al., 2019, 2020; Isidro et al., 2020; Miller et al., 2007; Parisi et al., 2019)
Fibronectin binding protein	*cj1349*	(Fanelli et al., 2019, 2020; Isidro et al., 2020; Miller et al., 2007; Parisi et al., 2019)
Phospholipase A	*pldA*	(Fanelli et al., 2020; Isidro et al., 2020; Miller et al., 2007; Parisi et al., 2019)

Despite the few studies regarding genetic analysis, several studies were conducted to evaluate the adhesion ability in *A. butzleri,* being one of the most frequently studied abilities (Chieffi et al., 2020). Until now, *A. butzleri* has shown the ability to adhere to several cell lines, such as Hep-2, HeLa, INT407, Caco-2, IPI-21, HT-29, HT-29/B6, IPEC-J2 with variable results, possibly correlated to the strains origin and cell lines (Chieffi et al., 2020; Ferreira et al., 2016). Nonetheless, a strain with adhesion properties was more likely to follow the same tendency in other cell lines, while when this ability is non-existent in one cell line a lower interaction was seen in other cell lines (Ferreira et al., 2016).

Two relevant studies conducted by Bücker et al. (2009) with HT-29/B6, and by Karadas et al. (2016) with HT-29/B6 and IPEC-J2 cell lines showed a possible mechanism related to the watery flux type diarrhoea caused by *A. butzleri* (Bücker et al., 2009; Karadas et al., 2016). *A. butzleri* shown to be able to induce epithelial barrier impairment function through changes in tight junction proteins and apoptosis induction of epithelial cells (Bücker et al., 2009). Further, human cells seemed more

susceptible in comparison to porcine cells due to an apparent higher affinity to human cell receptors (Karadas et al., 2016).

1.4.8 Invasion factors

To avoid the extracellular harsh environment and physical stresses imposed by the host, such as pH variations, shear stress from mucosal secretions, blood, and cells of the immune system, several bacteria induce their entry into the target cells (Pizarro-Cerdá & Cossart, 2006). In this way, bacteria can survive, replicate and disseminate to other host tissues through invasion, and establish and maintain a successful infection (Cossart & Sansonetti, 2004).

Flagellum in *Campylobacter*-like bacteria is usually associated with motility, but it is believed that it also plays an important role in the invasion and colonization of host cells (Ho et al., 2008). In *Campylobacter*, the T3SS apparatus present in the flagellum structure had shown an important role in the secretion of proteins relevant for the invasion process. Mutants where the *flhB* and *fliI* genes, related to the T3SS apparatus, were absent, shown a decrease in the invasion ability of the strain and a reduction in protein export, namely the Cia protein (Konkel et al., 1999; Konkel et al., 2004). Meanwhile, there is a lack of studies of the role of this apparatus in *A. butzleri* and its relationship with invasion.

The other two homolog proteins found in *A. butzleri* associated with invasion in *Campylobacter* species are CiaB and IamA (Isidro et al., 2020). Studies with *Campylobacter* mutants revealed a decrease in the invasion ability of the strains when the gene *ciaB* was deleted (Konkel et al., 2004). However, in *Aliarcobacter* the absence of the gene *ciaB* did not indicate a correlation with the invasion ability (Karadas et al., 2013). However, the lack of some virulence genes (*ciaB*, *cadF*, and *cj134*) and the reduced ability to invade Caco-2 cells was reported for *A. thereius* and A. *cibarius* species (Levican et al., 2013). In this way no clear correlation between invasion and the presence of virulence genes *ciaB* and *iamA* was observed for *A. butzleri* (Table 8), with some authors suggesting that other genes may be involved in the invasion process (Ferreira et al., 2016; Ferreira et al., 2014b).

Table 8 - Putative invasion genes of *Aliarcobacter butzleri* identified from genomic studies.

Virulence factor(s)	Encoding gene(s)	References
CiaB, protein involved in adhesion	*ciaB*	(Fanelli et al., 2019, 2020; Isidro et al., 2020; Miller et al., 2007; Parisi et al., 2019)
IamA, invasion associated protein	*iamA*	(Isidro et al., 2020)

Even though several studies report invasion ability in *A. butzleri*, studies involving comparative analyses between genomic characterization and its phenotypic outcome are still scarce, thus a relation to the genes involved in this process is still unknown (Chieffi et al., 2020).

1.4.9 Host immune responses

When pathogens contact the host, a cascade of complex interactions between the host, the pathogen, and the native intestinal microbiota occurs. In the case of mammal hosts, this is mediated by the immune system, which can be categorized into innate and adaptive systems. The innate immune system alongside with the physical barriers (skin and mucous membranes) in the host tissues are the first line of defence, mediating the elimination and protecting against the entry of bacterial pathogens, respectively (Kumar et al., 2013). On the other hand, the adaptative immune response is composed of T cells and B cells responsible to respond to foreign antigens and coordinate a protective immune response more persistent and memorized (Shepherd & McLaren, 2020).

One of the most important innate defences against bacteria is the bacterial activity of the complement in serum (Keo et al., 2011). *Aliarcobacter* species isolated from several sources have shown to be susceptible to serum at different degrees. This behaviour could suggest a similar behaviour to what happens with *C. jejuni*, where strains with systemic origin could be more resistant to complement-mediated killing than enteric strains (Ferreira et al., 2016; Keo et al., 2011; Wilson et al., 2010).

Interleukin 8 (IL-8) is a chemotactic factor, released during the inflammation process, responsible for the attraction of neutrophils, basophils, and T-cells to the inflammation site (Brennan & Zheng, 2007). Two studies showed that *A. butzleri* was able to induce high levels of IL-8 production. Both studies suggested that triggering of local inflammation could result in a more suitable environment for bacterial survival than cellular invasion, since strains associated with persistent diarrhoea could have lower levels of cellular invasion. Still, no correlation with adhesion and invasion abilities was fully established (Ferreira et al., 2014b; Ho et al., 2007). Another study with THP-1 derived macrophages have shown that *A. butzleri* can also stimulate the production of proinflammatory cytokines IL-1, IL-6, IL-8, IL-12 and TNFα (Bruegge et al., 2014), indicating that cells from the innate immune system could recognize *A. butzleri* through toll-like receptors, yet more studies are needed to clarify this mechanism (Bruegge et al., 2014).

Lactoferrin is a glycoprotein capable of indicate neutrophil activity and often used as an indicator of intestinal inflammation, which has been linked to inflammation associated with *A. butzleri* infection (Dai et al., 2007; Samie et al., 2007). A study regarding the prevalence of *Campylobacter* spp., *H. pylori* and *Arcobacter* spp. in stool samples has shown that 55 % of samples positive for *A. butzleri* had higher levels of lactoferrin, a similar trend was observed for 33 % of the *A. cryaerophilus* and *A. skirrowii* positive samples (Samie et al., 2007).

Chapter 2: Objectives

Aliarcobacter butzleri has been described as a pathogenic bacterium, widely spread throughout different environments. In humans, infections with *A. butzleri* have been largely associated with reports of enteritis, and bacteraemia, being included in the list of microorganisms that represent a serious hazard for human health. However, the mechanisms of virulence behind its pathogenic potential and relation to the human host have been relatively underexplored. In this way, the objective of this work was to characterize the different virulence traits of fifteen human isolates of *A. butzleri*.

To achieve this aim, several specific objectives were established:

- To evaluate if the different isolates were genetically distinct;
- To evaluate the resistance profile to antibiotics;
- To study the survival to stress-inducing environments;
- To evaluate the chemotactic profile of each isolate;
- To study the phenotypic profile of the isolates regarding the motility, biofilm formation ability, morphology of the colonies, cellulose production, serum survival, and haemolytic activity;
- To study the adhesion and invasion ability in the human cell line, Caco-2;
- To analyse the differences of aerobic and microaerobic exposure in the pathogenic behaviour.

Chapter 3: Materials and Methods

3.1 Strains used in this study

To evaluate the pathogenic potential of human isolates, in this study fifteen isolates of *A. butzleri* recovered from human faecal samples were used (Table 9).

Table 9 - List of strains used in this study by identification, isolation date and data collected from the patients (gender, age, and underlying conditions).

A. butzleri strain	Gender	Age	Isolation date	Underlying condition
INSA Cu 29393	M	55	03/08/2018	Non-Hodgkin lymphoma immunosuppression
INSA 593	M	84	09/08/2011	Diarrhoea (with mucus and blood), abdominal cramps
INSA 776	F	89	13/05/2011	Diarrhoea, and abdominal cramps
2003/1426	M	44	20/08/2013	Diarrhoea, and fever
INSA 2680	F	67	19/02/2017	Not specified
INSA 2756	M	68	13/04/2017	Not specified
INSA 2808	M	86	30/05/2017	Community-acquired pneumonia and acute decompensated heart failure
INSA 2999	M	1	28/09/2017	Gastroenteritis
INSA 3202	M	83	02/03/2018	Constitutional syndrome
INSA 3711	M	20	06/07/2019	Infectious colitis
INSA 3727	M	79	26/07/2019	Diarrhoea
INSA 3774	M	37	06/06/2019	Not specified
INSA 3800	F	60	30/09/2019	Leukopenia
INSA 3890	F	91	10/12/2019	Fever
INSA 4015	F	91	19/06/2020	Diarrhoea, vomiting, and abdominal cramps

From the group of strains under study, three strains that shown different profiles in preliminary results, *A. butzleri* INSA Cu 29393, INSA 3202, and INSA 3711, were selected to perform a comparative analysis of its virulence profile under microaerophilic and aerobic conditions at 37 ºC.

3.2 Storage and growth conditions

Strains were preserved in cryogenic vials in Brain Heart Infusion medium with 20 % (v/v) glycerol at a temperature of -80 ºC. When needed, strains were inoculated in

plates containing Blood Agar medium (VWR, Belgium) supplemented with 5 % (v/v) defibrinated horse blood (BA) and incubated at 37 ºC in microaerophilic conditions for 24 h. After the incubation, the inoculated plates were used as short store at 4 ºC until necessary, for a maximum period of two weeks. Microaerobic atmosphere (6 % O_2, ±7.1 % CO_2 and 3.6 % H_2) was generated using an Anoxomat system (Anoxomat, The Netherlands).

For the assessment of the virulence profile of each strain, all strains were previously inoculated in Tryptic Soy Agar (TSA) medium for 24 h at 37 ºC in microaerophilic conditions, or aerobic conditions according to the purpose of use. Afterwards, overnight cultures with 16 h were performed in 10 mL of Tryptic Soy Broth (TSB) medium (VWR, Belgium) with an initial optical density at 620 nm ($OD_{620\ nm}$) of 0.02 and incubated in an orbital shaker at 100 rpm at 37 ºC in microaerophilic conditions, or aerobic conditions according to the purpose of use.

To evaluate the gene expression, strains were grown as described before, and the overnight culture was used for starting a culture that was incubated to grow until reaching the mid-exponential phase. To determine the mid-exponential phase for each isolate, growth curves were constructed at least three times independently. For aerobic conditions, the mid-exponential phase was achieved after 7 h and for microaerophilic conditions after 8 h of growth.

3.3 Multi-Locus Sequence Typing (MLST)

To assess and evaluate the allelic profile of the fifteen *A. butzleri* isolates, MLST was used. Firstly, the genomic DNA was extracted from TSA cultures grown for 24 h at 37 ºC, using a GRS Genomic DNA Kit–Bacteria (GRiSP, Portugal). The whole-genome sequencing was performed at the National Institute of Health Dr Ricardo Jorge, using the Illumina MiSeq platform. The MLST was performed *in silico* with the obtained raw reads of each isolate using the MLST 2.0 software (https://cge.cbs.dtu.dk/services/MLST/, accessed on 15 July 2021) based on Achtman's scheme with internal fragments of seven housekeeping genes (*aspA, atpA, glnA, gltA, glyA, pgm, tkt*), and the allelic profiles further confirmed in PubMLST (https://pubmlst.org/).

3.4 Antimicrobial susceptibility profile

To assess the susceptibility profile to different antimicrobial agents, the minimum inhibitory concentration (MIC) was determined to different representatives of the

various antibiotic's classes and to bile salts. The compounds used can be found in Table 10.

Table 10 - List of different antimicrobial agents used to determine the minimum inhibitory concentrations.

	Antibiotic class	Antimicrobial agent	Supplier	Purity (%)	Solvent
Antibiotic	β-lactams	Ampicillin	NzyTech	100	Phosphate buffer, pH=8
	Quinolones	Ciprofloxacin	Sigma-Aldrich	98	NaOH and water
	Tetracyclines	Tetracycline	Sigma-Aldrich	98	Ethanol 95%
	Macrolides	Erythromycin	Sigma-Aldrich	100	Ethanol 95%
	Aminoglycosides	Gentamicin	Sigma-Aldrich	100	Water
Bile Salts		Approximately 50 % sodium cholate, and 50 % sodium deoxycholate salts	Sigma-Aldrich	100	TSB

3.4.1 Agar dilution method

To evaluate the minimum inhibitory concentrations of ampicillin (AMP), ciprofloxacin (CIP), tetracycline (TET), erythromycin (ERY) and gentamicin (GEN), the agar dilution technique was used as described in the CA-SFM/EUCAST (2021) and modified for the determination of the MIC for bile salts.

Thus, in the previous day to the assay, Mueller-Hinton plates supplemented with 5 % of defibrinated horse blood for antibiotics, or TSA plates for bile salts were prepared, with increasing concentrations of each antimicrobial agent and were stored at 4 °C until use. To standardize the density of the inoculum to reach 10^4 colony forming unit (CFU) mL^{-1} per spot on the agar, colonies of each strain were suspended in a saline solution of 0.85 % NaCl (w/v) and adjusted to a density of 0.5 McFarland. A further dilution of the suspension was made (1:100) with 0.85 % NaCl (w/v), and 2 µL inoculated on the surface of each plate and incubated at 37 °C in microaerophilic conditions. Two reference strains were used as control, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29123. The MIC was defined as the lower concentration of each compound that ceased growth of the strain at naked eye. Since no recommendation of breakpoints value is available for *A. butzleri*, the breakpoints of resistance used were those of *C. jejuni* following the European Committee on Antimicrobial Susceptibility Testing

(EUCAST) for ciprofloxacin (>0.5 µg mL^{-1}), erythromycin (>4 µg mL^{-1}), and tetracycline (>2 µg mL^{-1}) and, the ones used for Enterobacterales for the remaining antimicrobial agents, ampicillin (>8 µg mL^{-1}), and gentamicin (>2 µg mL^{-1}). Each assay was performed at least three times independently.

3.4.2 Disc diffusion method

To evaluate the resistance profile to ertapenem (ETP), the disc diffusion technique was used following as described in CA-SFM/EUCAST (2021).

Bacterial suspensions of the fifteen strains were prepared by direct suspension in a saline solution of 0.85 % NaCl of 24 h cultures grown at 37 ºC in microaerobic conditions in BA plates. Turbidity was adjusted to 0.5 McFarland, and the strains were inoculated through the use of a swab in Mueller-Hinton Agar supplemented with 5 % of defibrinated horse blood. After, a disc containing 10 µg of ertapenem was placed on the centre of each inoculated plate. The plates were then incubated at 37 ºC for 48 h in a microaerobic atmosphere. The inhibition zones were analysed according to the values for *Campylobacter* spp. from ISO standard 20776-1:2019, considering as zone diameter breakpoint of resistance <20 mm, since there are no established breakpoints for *Aliarcobacter* species.

3.5 Oxidative and nitrosative stresses

The survival to oxidative stress induced by hydrogen peroxide and methyl viologen and to nitrosative stress induced by sodium nitroprusside was evaluated by disc diffusion as described in Kern et al. (2011) with slight alterations.

The strains of *A. butzleri* were grown as described before (3.2), after which the cells were centrifuged for 5 min at 12,000× g and then resuspended in fresh TSB medium adjusting the number of cells to approximately 10^8 CFU mL^{-1}. Each inoculum was spread onto a TSA plate using a swab, and a sterile cellulose filter disc of 6 mm containing 5 µL of each solution was added to the centre of the plate. The hydrogen peroxide was tested at three different concentrations: 3, 10, and 30 % (v/v), methyl viologen at 125 mM and sodium nitroprusside at 250mM. After, the plates were incubated for 48 h at 37 ºC in microaerophilic conditions, or aerobic conditions according to the condition studied. The halo of inhibition corresponding to the diameter of growth inhibition zones created around the discs was measured. This assay was performed at least three times independently for each strain.

3.6 Urease test

An urease test was performed for the human isolates as described by Isidro et al. (2020) with slight modifications.

The urease test was accomplished using a urease test composed of urea broth (Oxoid, United Kingdom). Strains were grown in TSA plaques for 24 h at 37 ºC in microaerophilic conditions, and 2 mL of the broth in glass tube was heavily inoculated with the culture and incubated at 37 ºC for 48 h. Two negative controls were used, one with no inoculum and other with a negative urease strain, for the positive control a urease positive strain was used. A change of colour from light orange to pink indicated a positive result.

3.7 Acidic stress survival

The survival to acidic stress was performed as described in Isohanni et al. (2013) with slight alterations. The strains of *A. butzleri* were grown as described before (3.2), after which the cells were centrifuged for 5 min at 12,000× g and then resuspended in fresh TSB medium adjusting the number of cells to approximately 10^8 CFU mL^{-1}. Then 300 µL of the inoculum was added to 2700 µL of sterile TSB with a pH of 4, previously adjusted by addition of hydrochloric acid 1 M through a pH meter (Thermo Scientific, United States of America) and incubated at 37 ºC. Viable counts of *A. butzleri* were determined at 0, 20, 40, and 60 minutes of exposure to acid stress (pH 4) by serial dilution in sterile phosphate buffered saline (PBS), pH 7.4 (10 mmol L^{-1}) followed by drop-plate method in TSA and 48 h of incubation at 37 ºC in microaerophilic conditions, or aerobic conditions according to the condition studied. This assay was performed at least three times independently for each strain.

3.8 Chemotactic profile through tube-based assay

Chemotaxis assay was performed by tube-based chemotaxis assay as described in Cain et al. (2019) with slight modifications.

A. butzleri strains were cultured as described above (3.2), after which the cells were collected by centrifugation for 5 min at 12,000× g, washed with sterile PBS (pH 7.4, 10 mmol L^{-1}), and resuspended in 125 µL of sterile PBS and added to 125 µL of 0.4 % PBS-agar to an $OD_{620\ nm}$ of 2 and then transferred into a 2 mL tube and allowed to solidify. Plugs were overlaid with an additional 750 µL of 0.4% (w/v) PBS-agar, which was also allowed to solidify. A sterile cellulose paper disc of 6 mm containing 25 µL of each

"chemoattractant" (Table 11) with a concentration of 2 M in PBS was laid on the surface. Also, a disc containing only PBS was used as a negative control. Tubes were incubated for 72 h at 37 °C under microaerophilic or aerobic conditions according to the condition studied. After the incubation, the discs were removed and 200 µL of triphenyltetrazolium chloride at 0.01 % in PBS was added. An additional incubation for 24 h at 37 ºC in microaerobic or aerobic conditions was performed. Chemotactic behaviour was assessed by the presence of 1,3,5-triphenylformazide (indicative of cellular respiration) and scored based on the intensity and position of the dye (+++, intense at the surface; ++ significant migration; + some diffuse colour; -, no reaction). This assay was performed at least three times independently for each strain.

Table 11 - List of different chemoattractant agent used to assess the chemotactic profile.

Chemoattractant agent	**Supplier**	**Purity (%)**
Pyruvic acid, sodium salt	Honeywell Fluka	99
Sodium L-lactate	Sigma-Aldrich	98
L-Proline	Tokyo Chemical Industry	99
L-Fucose	Tokyo Chemical Industry	97
L-Serine	PanReac	100
Potassium L-Aspartate Hydrate	Tokyo Chemical Industry	97
Sodium hydrogen glutamate monohydrate	VWR	99
Sodium succinate hexahydrate	Fisher Scientific	99

3.9 Motility evaluation

3.9.1 Soft agar motility assay

To evaluate the different motility profiles of the fifteen strains, a motility assay was performed as described in Ferreira et al. (2018) with slight modifications.

The *A. butzleri* strains were grown in TSB as described above (3.2), after which the cells were collected and resuspended in fresh TSB medium to ~10^8 CFU mL^{-1}, as described above. Following, 5 µL of each strain was inoculated by stabbing the centre of semisolid TSA (0.4 % agar) plate. The motility halo was measured after incubation at 37 ºC for 48 h in microaerobic or aerobic conditions according to the condition studied. This assay was performed at least three times independently for each strain.

3.9.2 Expression of the *flaA* gene

The expression of the *flaA* gene under microaerobic and aerobic conditions was evaluated on three *A. butzleri* strains, INSA Cu 29393, INSA 3202, and INSA 3711.

3.9.2.1 Total RNA extraction

The three strains were grown in TSA plates and incubated at 37 ºC in microaerophilic or aerobic conditions for 24 h. Then an overnight culture was made in 10 mL of TSB medium with an initial $OD_{620\ nm}$ of 0.02 and incubated in a shaker for approximately 16 h at 37 ºC and 100 rpm in microaerophilic or aerobic conditions. After, this culture was used to initiate another culture with 15 mL of TSB medium with an initial OD_{620nm} of 0.02, which was incubated in the same conditions. After reaching the mid-exponential phase (8 h in microaerophilic conditions, and 7 h in aerobic conditions), 1 mL of the culture was collected and centrifuged at 12,000× g for 5 minutes at 4 ºC, and the supernatant was discarded. Cells were washed once with 0.85 % NaCl (v/v) and stored at -80 ºC in 500 µL of TripleXtractor (GRiSP, Portugal).

The RNA extraction was performed as recommended by the manufacturer. To assess the quantity and purity of RNA recovered, a nano spectrophotometer was used, and its integrity and purity was evaluated by gel electrophoresis with 1% (w/v) agarose in Tris Acetate-EDTA buffer (1×) and stained with Midori Green (NIPPON Genetics, Germany). The electrophoresis run was accompanied using the molecular weight marker GRS Universal Ladder (GRiSP, Portugal), for 30 minutes at 100 V. The gel was analysed in a transilluminator (UVITEC, United Kingdom).

3.9.2.2 Total RNA treatment with DNase I

To remove possible DNA contaminants from the previous samples, the commercial kit DNase I, RNase-free (Thermo Scientific, United States of America) was used according to the supplier guidelines. In this manner, a reactional mixture was prepared, containing 1 µL of reaction buffer (10×), 1 µL of DNase I and 1 µg of RNA, ultrapure water was used to complete a final volume of 10 µL, then the mixture was incubated at 37 ºC for 30 minutes. Next, to stop the reaction 1 µL of ethylenediaminetetraacetic acid (EDTA) at 50 mM was added and the mixture was incubated at 65 ºC for 10 minutes. Aliquots were stored at -80ºC until use.

3.9.2.3 Complementary DNA (cDNA) synthesis

For the cDNA synthesis, a commercial kit Xpert CDNA Synthesis Mastermix (GRiSP, Portugal) was used. A reaction mixture was made with 9 µL of purified RNA and 10 µL of Master Mix, followed by an incubation period of 5 minutes at 65 ºC. After, 1 µL of RTases was added and another incubation step was made 10 minutes at 25 ºC, 15 min at 50 ºC and lastly 5 min at 85 ºC. After the cDNA synthesis, the cDNA was stored at -20ºC.

3.9.2.4 Determination of *flaA* gene expression

Real-time PCR (qPCR) technique was used to assess the expression of the *flaA* gene in the strains under study. For the qPCR reaction, the commercial kit NZYSpeedy qPCR Green Master Mix (2×) (NZY Tech) was used.

For the amplification reaction, a mixture for each gene was made with 5 µL master mix (2×), 0.4 µM of each set of primers corresponding to the gene *flaA* or *16S rRNA* (Table 12), and 1 µL of cDNA, ultrapure water was added until 10 µL volume was reached.

Table 12 - List of primers used in the real-time PCR reaction, sequence, and size

Primer designation	Primer sequence	Size (pb)	References
flaA_F	5- AGT TGC ACC AGC TGA CAT TT -3	20	(Medina et al., 2019)
flA_R	5- AGT TGG TGA AGG AAG TTC CGA -3	21	
P338_F	5- ACT CCT ACG GGA GGC AGC AG -3	20	(Muyzer et al., 1993)
P518_R	5- ATT ACC GCG GCT GCT GG – 3	17	

After the reaction mixture each sample was incubated in a CFX Real-Time PCR System (Bio-Rad, United States of America) following the program: 2 min at 95 ºC, 40 cycles for 5 s at 95 ºC followed by 30 s at 60 ºC, and at last 5 s in crescent gradient to 95ºC. Levels of purity and dimmer formation were evaluated by the resulting melting curves. The relative expression of *flaA* was analysed by the ΔΔCt method, using the constitutively expressed 16S rRNA gene was used (Ferreira et al., 2021). All qPCR reactions were performed in duplicates.

3.10 Morphotype analyses and evaluation of cellulose production

To observe the morphotype of each strain, the protocol was proceeded as described in Levert et al. (2010).

The *A. butzleri* strains were grown in TSB as described above (3.2), after which the cells were centrifuged for 5 min at 12,000× g and then resuspended in fresh TSB medium adjusting the number of cells to approximately 10^8 CFU mL^{-1}. Ten µL were inoculated in TSA plaques supplemented with Congo red (40 µg mL^{-1}) and Coomassie brilliant blue R-250 (20 µg mL^{-1}) followed by incubation at 37 ºC for 48 h in microaerophilic conditions, or aerobic conditions according to the condition studied.

Similarly, to evaluate the cellulose production of each strain, Calcofluor white plates (TSA plaques supplemented with 0.002% Calcofluor white (v/v)) were used. In this situation, the results were analysed in a transilluminator (UVITEC, United Kingdom) at 365 nm.

3.11 Biofilm formation ability

Biofilm formation ability of each strain was assessed as described in Reeser et al. (2007), with slight modifications.

All the strains were grown as described above (3.2), then 24-well polystyrene plates (VWR) were inoculated with 500 µL of a suspension with 10^8 CFU per well with the overnight cultures after the cells were centrifuged for 5 min at 12,000× g and resuspended in fresh TSB medium. The plates were incubated at 37 ºC for 48 h in microaerophilic conditions, or aerobic conditions according to the condition studied. Following the incubation, the medium was removed, the wells were dried for 1 h at 55ºC, and 1 mL of 0.1 % (w/v) crystal violet was added for 15 min at room temperature. The unbound crystal violet was removed, and the wells were washed three times with distilled water. The wells were dried at 55 ºC for 15 min, and bound crystal violet was solubilized with a 30 % methanol/10 % acetic acid solution. Finally, the absorbance at 570 nm was determined using a microplate reader (Biorad, xMark) to determine the biofilm formation. Each assay was performed with four replicates in at least three independent assays.

3.12 Haemolytic activity

The haemolytic activity of the strains was evaluated as described in Ferreira et al. (2014) with slight modifications.

The strains of *A. butzleri* were grown as described before (3.2), after which the cells were collected, by centrifugation for 5 min at 12,000× g, washed in sterile PBS (pH 7.4, 10 mmol L^{-1}) and the bacterial suspension adjusted to approximately 10^9 CFU mL^{-1} in PBS. Erythrocytes were collected from one healthy donor to a blood collection tube with EDTA, then washed three times in PBS and a stock suspension was prepared in the same buffer. In a 96-well plate with a U bottom, 100 µL of the bacterial suspension and the same volume of 2 % (v/v) erythrocytes were mixed. The plaque was incubated at 37 ºC for 18 h in microaerophilic conditions, or aerobic conditions according to the condition studied. The plate was then centrifuged at 1000× g for 5 min, 100 µL of the supernatant was removed from each well to a 96-well plate and the absorbance at 492 nm was measured in a microplate reader (Biorad, xMark). A negative control (without bacteria) and positive control of total haemolysis (1 % (v/v) Triton X-100) were also included. Each assay was performed with four replicates in at least three independent assays.

3.13 Serum survival assay

Serum bactericidal activity against *A. butzleri* was evaluated as described in O'Shaughnessy et al. (2012), with slight modifications.

For serum recovery, blood was collected from four healthy donors and left to clot at room temperature for 30 min. Then, serum was separated by centrifugation at 2,000 rpm for 10 min at 4 ºC, pooled and frozen in aliquots at – 80 ºC in sterile cryogenic vials. When required, frozen aliquots of serum were thawed on the bench at room temperature. *A. butzleri* strains were cultured and washed as described above (3.2) and the bacterial suspension was adjusted to approximately 10^8 CFU mL^{-1} in PBS. In microtubes with 180 µL of pooled human serum, 20 µL of the bacterial suspension was added, and incubated at 37 ºC. Viable counts of *A. butzleri* were determined at 15, 30, 45, and 60 min of exposure to the serum by serial dilution in 0.85 % (w/v) NaCl followed by drop-count plating in TSA, and incubation for 48 h at 37 ºC in microaerophilic conditions, or aerobic conditions according to the condition studied. This assay was performed at least three times independently for each strain.

3. 14 Adhesion and invasion in a Caco-2 cell line

The adhesion and invasion assays were performed as described in Ferreira et al. (2014) with slight modifications.

The Caco-2 human intestinal epithelial cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) nonessential amino acids, 100 μg mL^{-1} of streptomycin, and 100 U mL^{-1} of penicillin. The cells were grown in tissue culture flasks maintained in incubators at 37 ºC in 5 % CO2 and 95 % air. Medium was replaced every two days until cells reached at a semi confluent state of about 80 %. Before preparation for experiments, cells were trypsinized and 10 μL of cells were collected and mixed with 10 μL of trypan blue, and the viable cells were counted in a hemocytometer. Following, the cells were seeded in 24-well polystyrene plates (VWR) with 1×10^5 cells per well and left to multiply for 48 h at 37 ºC in 5 % CO2 and 95 % air.

Bacterial strains were grown as described before (3.2), after which the cells were collected by centrifugation (5 min at 12,000× g), washed and resuspended in the medium used for Caco-2 cell culture, but without antibiotic at 37 ºC. The culture density was estimated by serial dilution and plating in TSA plates. 500 μL of each strain (approximately 10^7 CFU per well) were added to Caco-2 cells and incubated for 3 h, to allow adhesion and invasion to occur, with multiplicity of infection (MOI) of 200. Following this period, the cells were washed three times with 500 μL of sterile PBS to remove unbound bacteria and the number of interacting (adherent and internalized) bacteria was determined by lysing the Caco-2 cells with the addition of 500 μL Triton X-100 at 0.1 % (v/v) for 5 min, followed by plate count of bacteria. For bacterial invasion measuring, after the previous described 3 h of incubation and washing steps, 500 μL of medium containing 125 μg mL^{-1} of gentamicin was added to each well for 1 h to kill extracellular bacteria. Then, cells were washed three times with 500 μL of sterile PBS and lysed with the addition of 500 μL Triton X-100 at 0.1 % (v/v) in each well for 5 min. The released intracellular bacteria were enumerated by plating serial dilutions of the lysates in TSA plates. In this assay, three replicates per assay were used in at least three independent measurements. The results are shown by percentage of adhesion and invasion.

Chapter 4: Results and discussion

4.1 Pathogenic and virulence traits of the clinical isolates of *Aliarcobacter butzleri*

Since 2002, *A. butzleri* has been seen as an emergent pathogen considered a serious hazard to human health, capable of causing enteritis, severe diarrhoea, septicaemia, and bacteraemia to humans. The lack of studies relating its virulence mechanisms and genome, jeopardize the understanding of this bacterium as a pathogen and could lead to an increase in difficulty on the treatment of infections related to *A. butzleri,* but also on its control. In this study, we aimed to find differences among the phenotypic profiles of each isolate and suggest possible mechanisms associated with its virulence traits.

4.1.1 Multi-locus Sequence Typing

MLST is a widely used technique for bacterial typing. Its principle relies on a comparison of internal fragments of protein-encoding housekeeping genes. For each fragment, a random integer is attributed to unique sequence (alleles), this unique combination of alleles at each locus can be described as an allelic profile. This unique allelic profile then specifies one the sequence type (ST) (Bartual et al., 2005; Larsen et al., 2012).

To assess the allelic profile of each isolate, *in silico* analysis was performed using the MLST 2.0 (Multi-Locus Sequence Typing) software, an online bioinformatic toll created by the Center of Genomic Epidemiology. The results obtained for each strain were compiled in the Table 13.

Table 13 - Allelic profile of the fifteen clinical isolates of *Aliarcobacter butzleri*.

Strain	***Allelic profile***							**ST**
	aspA	*atpA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	
INSA Cu 29393	~366	43	35	~54	~540	79	~17	New ST
INSA 593	~40	~147	26	22	~705	~239	31	New ST
INSA 776[a]	-	-	-	-	-	-	-	-
2003/1426	8	8	8	8	9, 191	9	8	47
INSA 2680	20	2	11	27	~165	~87	14	New ST
INSA 2756	30	5	9	9	120	10	9	-[b]
INSA 2808	26	24	17	20	67	22	28	113

Table 13 (continuation)- Allelic profile of the fifteen clinical isolates of *Aliarcobacter butzleri*.

Strain	***Allelic profile***							**ST**
	aspA	*atpA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	
INSA 2999	203	64	227	40	~776	~273	~333	New ST
INSA 3202	160	125	26	60	~455	~367	73	New ST
INSA 3711	37	5	11	30	624	16	72	-[b]
INSA 3727	74	8	1	60	191	200	~206	New ST
INSA 3774	28	5	7	7	167	7	6	116
INSA 3800	30	5	5	30	~778	35	4	New ST
INSA 3890	73	12	1	9	220, 623[c]	10	179	676
INSA 4015	55	~61	~26	48	~745	~223	32	New ST

[a] not sequenced
[b] alleles identified, but no identification of the ST in the database
[c] multiple perfect hits found.

Analysing the results obtained, twenty one possible new alleles can be found, with only five alleles with less the 100% identity and/or coverage. Unfortunately, phylogenetic analyse, the identification of the ST or submission to the database was not possible due to lack of a curator of the database. Further, the *A butzleri* INSA 776 isolate was not possible to undergo whole-genome sequencing and subsequent analysis. However, earlier studies with enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) pointed to a different genomic profile than the other strains. From these results, we may conclude that all the strains presented genetically diverse profiles.

Also, when searching through the PubMLST data base the identified ST, we could observe that the same ST found in the strains 2003/1426, INSA 2808, INSA 3774, and INSA 3800 were also found in strains isolated from France in human stools; from Thailand in chicken offal or meat, Thailand from pork offal or meat, and from Spain in human stools respectively.

4.1.2 Assessment of the minimum inhibitory concentration and resistance profile in clinical isolates of *Aliarcobacter butzleri*

Since antibiotic discovery, medicine was revolutionized forever. Its initial accomplishment led many to believe that all bacterial infections could be successfully controlled and eliminated. However, throughout the years, an excessive and inappropriate use of antibiotics resulted in the emergence of several bacterial strains resistant to the most used antibiotics in clinical practice (Maugeri et al., 2019). Thus,

the antimicrobial resistance profile is considered one of the key elements in pathogen characterization, being essential to define the most appropriate course of treatment to patients in clinic (Hornischer & Häußler, 2016).

To assess the resistance profile of each isolate under study, one representative antibiotic of six different classes was used (Table 14).

Table 14 - Minimum inhibitory concentration, breakpoints and resistance profile to antibiotics of the fifteen clinical isolates of *Aliarcobacter butzleri*.

A. butzleri strain	**MIC (μg mL^{-1})**					**Inhibition halo (mm)**	**Resistance profile**
	AMP	TET	ERY	GEN	CIP	ERT	
INSA Cu 29393	64	4	4	2	≤0.06	9.4	AMP, TET, ERT
INSA 593	128	4	>32	0.5	>32	6.0	AMP, TET, ERY, CIP, ERT
INSA 776	64	4	16	1	0.125	9.7	AMP, TET, ERY, ERT
2003/1426	64	4	2	1	≤0.06	12.1	AMP, TET, ERT
INSA 2680	32	4	4	0.5	4	9.6	AMP, TET, CIP, ERT
INSA 2756	16	4	4	0.5	>8	11.8	AMP, TET, CIP, ERT
INSA 2808	64	4	>32	≤0.125	≤0.06	8.5	AMP, TET, ERY, ERT
INSA 2999	64	4	4	0.5	≤0.06	7.4	AMP, TET, ERT
INSA 3202	64	4	2	2	≤0.06	11.2	AMP, TET, ERT
INSA 3711	32	8	4	0.5	≤0.06	9.5	AMP, TET, ERT
INSA 3727	64	4	2	0.5	≤0.06	8.8	AMP, TET, ERT
INSA 3774	16	4	8	0.5	≤0.06	10.2	AMP, TET, ERY, ERT
INSA 3800	2	4	4	0.5	≤0.06	6.0	TET, ERT
INSA 3890	8	2	2	0.5	≤0.06	11.4	ERT
INSA 4015	64	4	4	0.5	≤0.06	7.3	AMP, TET, ERT
Breakpoint[a] (R)(mg/ml)	> 8	> 2	> 4	> 2	> 0.5	<20	-

[a] Since no recommendation of breakpoints is available for *A. butzleri*, the breakpoints used were those of *C. jejuni* following EUCAST or when lacking in this interpretative criterion, the ones used for Enterobacterales. For the classification of ertapenem resistance the ISO standard 20776-1 values for *Campylobacter* were used, corresponding to a halo inhibition <20.
AMP: ampicillin, TET: tetracycline, ERY: erythromycin, GEN: gentamicin, CIP: ciprofloxacin, ERT: ertapenem.

Analysing the results obtained (Table 14), the highest frequency of resistance was observed to ertapenem for 100 % of the strains tested, followed by tetracycline and ampicillin, with values of 93.3 and 86.6 %, respectively.

When comparing to results from other studies concerning resistance to the class of β-lactam antibiotics in human isolates, we can also observe high percentages of resistance

to ampicillin, with resistance rates ranging from 21.3 to 100 % (Kayman et al., 2012a; Mandisodza et al., 2012; Pérez-Cataluña et al., 2017; Van den Abeele et al., 2016; Vandenberg et al., 2006). These results seem to corroborate the values shown by this work. The already stated high resistance to ampicillin in *A. butzleri* is thought to be associated with the presence of β-lactamases genes encoded in its genome, yet its true implication in the resistance mechanism still needs further study (Miller et al., 2007; Pérez-Cataluña et al., 2017).

In the case of the carbapenem class, no other studies were made using ertapenem in *A. butzleri* with human isolates. Only one study with imipenem and meropenem was performed, having a resistance rate of 0 %, which represent opposite values compared to what we found in this work (Fera et al., 2003). The presence of metallo β-lactamases and plasmid-associated Oxa-48 has been associated with resistance to carbapenems. Genes coding for these enzymes have been described in *A. butzleri* genome, what can justify the results obtained. It is also to note that a rise in carbapenem resistance has been seen for species of the *Enterobacteriaceae* family in the last years. This could indicate a potential explanation to the results obtained in this work, although more studies are needed to reach that conclusion (Fanelli et al., 2019; Gupta et al., 2011; Marston et al., 2016).

In the case of the tetracycline class, much lower frequencies of resistance have been reported from human isolates in other studies, with values from 0 to 42.9 %, (Kayman et al., 2012a; Mandisodza et al., 2012; Pérez-Cataluña et al., 2017; Van den Abeele et al., 2016; Vandenberg et al., 2006), in contrast with the 93.9% of resistance found in this work. Tetracyclines are one of the antibiotic classes that is not often used in *Campylobacter* enteritis, but that have been suggested for the treatment of infections associated with *Aliarcobacter* species (Ferreira et al., 2016). The meta-regression analysis done by Ferreira et al. (2019a) demonstrated an increase on the resistance trend regarding this antibiotic (Ferreira et al., 2019a), which may be associated with the values obtained here. This high resistance rate could represent a great concern to public health and possibly discarding the use of this antibiotic as a treatment option for this type of infections.

In the case of erythromycin and ciprofloxacin, lower resistance rates of 26.6 and 20.0 % were found, respectively. In other studies, for human isolates, erythromycin resistance frequency has been described as ranging from 0 to 23.6%, which is consistent with the rates found in this work. In fact, macrolides such as erythromycin have been largely recommended in the clinic treatment of infections linked with this genus due to the association to low rates of resistance (Ferreira et al., 2016; Ferreira et al., 2019a;

Kayman et al., 2012a; Van den Abeele et al., 2016). As for ciprofloxacin, resistance rates reported in other studies regarding human isolates can range from 0 to 12.6%, representing lower values compared to the values obtained in this work. Similarly to macrolides, fluoroquinolones have been also suggested as a possible antibiotic for the treatment of *A. butzleri* infections. However, throughout the years an increase trend of resistance to some antibiotics of this class have been reported (Ferreira et al., 2016; Ferreira et al., 2019a; Kayman et al., 2012b; Van den Abeele et al., 2016)., that could support the results obtained by this work.

Finally, for gentamicin we can observe a null resistance to this antibiotic, with two strains presenting values at the breakpoint. This is in line with what has been published, where resistance rates range from 0 to 1.6 %. For this reason, aminoglycosides have been suggested a choice treatment for severe *Aliarcobacter* spp. infections (Brückner et al., 2020; Ferreira et al., 2019a; Kayman et al., 2012a; Mandisodza et al., 2012; Pérez-Cataluña et al., 2017; Šilha et al., 2017; Van den Abeele et al., 2016; Vandenberg et al., 2006).

Regarding the multidrug resistance, we can observe that thirteen strains seem to present a multiple resistance profile. A multidrug resistant strain is characterized by having resistance to three or more distinct classes of antibiotics (Le et al., 2012). This multiple resistance profile was already reported in isolates from diverse origins, such as food samples, water environments (Fanelli et al., 2020; Vicente-Martins et al., 2018). One study analysing multidrug resistance in *A. butzleri* isolates reported that 85.7 % of the human isolates presented this profile, similarly to the values found in the present study (86.6%) (Šilha et al., 2017). This multidrug resistance profile can be concerning, since it can jeopardize the treatment of a more severe infection (Ferreira et al., 2016). Also, the absence of international criteria relating the breakpoints of susceptibility to antimicrobials for the *Aliarcobacter* genus and the lack of standardized methods for its evaluation can difficult a comparison between works (Ferreira et al., 2016; Ferreira et al., 2019). In this manner, it is also important the creation of a standardized method with specific breakpoints to evaluate the antimicrobial resistance of *A. butzleri*, resulting in more cohesive and significant data.

4.1.3 Susceptibility to oxidative and nitrosative stresses of clinical isolates of *Aliarcobacter butzleri*

When grown in the presence of oxygen, bacteria are inevitable in contact with ROS. As previously referred, these reactive species are capable of damaging intracellular macromolecules, such as DNA, proteins, lipids, and even leading to cell death (Oh et al.,

2015). Considering that ROS can be used by the host immune system against bacterial infections, the bacteria had to find a way to survive in these conditions. As a result, pathogens encode numerous detoxification enzymes, enabling their survival in the environment and during host colonization (Flint et al., 2016). Two agents are commonly used to evaluate the effects of the oxidative stress in bacteria, hydrogen peroxide capable of producing hydroxyl radical, and methyl viologen a superoxide generator (Comtois et al., 2003). Since both agents could trigger different detoxifications pathways, we verified the susceptibility of the fifteen isolates when exposed to these substances (Figure 3).

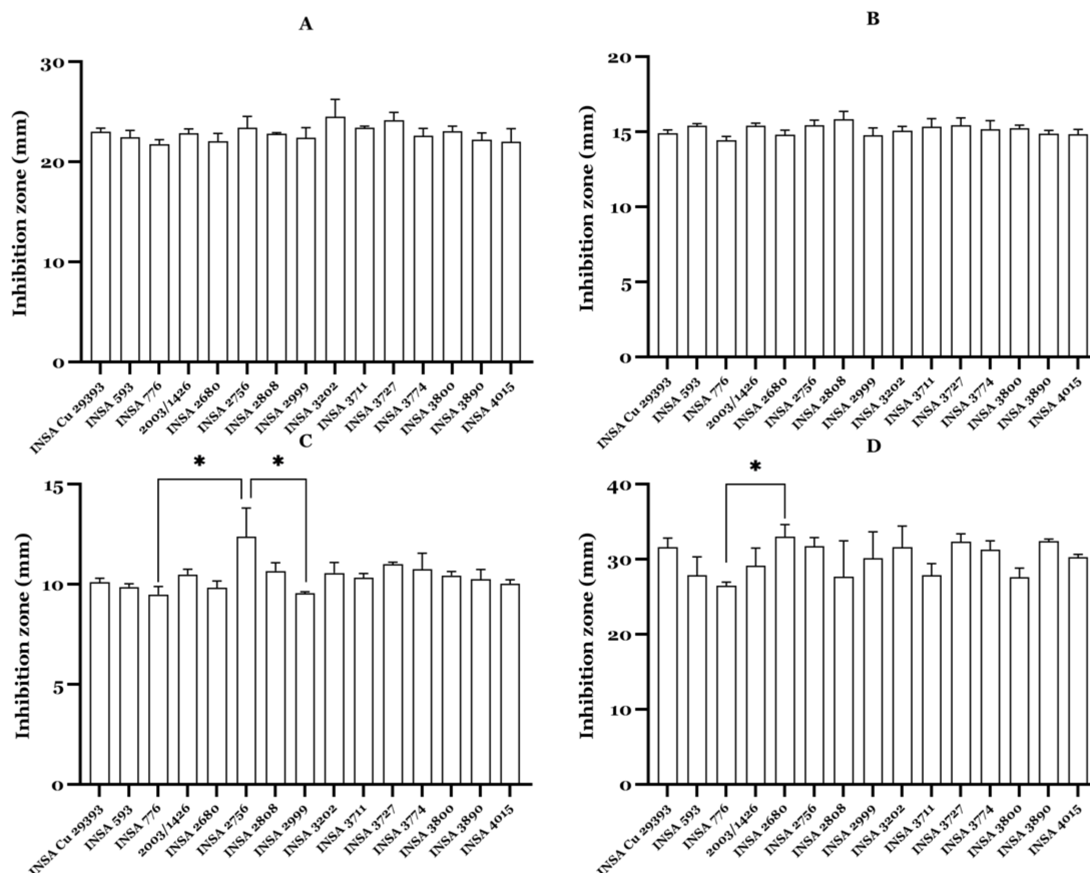

Figure 3 - Susceptibility of the fifteen clinical isolates of *Aliarcobacter butzleri* to oxidative stress when exposed to (A) 30 %, (B) 10 %; or (C) 3 % of hydrogen peroxide; or (D) 125 mM of methyl viologen. Results were analysed using a one-way ANOVA test. The data shown represent the mean ± standard deviation of at least three independent assays. $*p < 0.05$

When we analyse the data obtained, no significant differences were found in presence of 30 % and 10 % of hydrogen peroxide, among the fifteen strains. In the presence of 3 % of hydrogen peroxide only the *A. butzleri* INSA 2756 showed to be more susceptible comparing with the strains INSA 776 and INSA 2999. While in the case of the methyl

viologen only the strain INSA 776 has shown to be significantly more resistant than the strain INSA 2680.

Only two other studies approached the susceptibility of *A. butzleri* to hydrogen peroxide. When comparing to the results obtained, lower inhibition values can be found for the three concentrations tested in this work (Ferreira et al., 2018; Mateus et al., 2021b). In other studies, with species such as *C. jejuni,* slightly higher values of inhibition can be found, with inhibition halos from 20 mm at concentrations of 3 % H_2O_2 to 39 mm in concentrations around 30 % of H_2O_2 (Atack & Kelly, 2008; Baillon et al., 1999; Wainwright et al., 2005). However, differences in both studies can be associated to the volume applied on the disc, and the more microaerophilic nature of the *C. jejuni*, respectively, offering little comparison to the results obtained. In *Campylobacter* species this resistance is often linked to the presence of catalase (KatA) and peroxidase (AhpC) proteins. Although the same peroxidase can be found in *A. butzleri* genome, a different catalase gene has been described, encoding for catalase-peroxidase KatG (Flint et al., 2016; Miller et al., 2007). In other species, such as *E. coli,* the KatG and AhpC have been described as the major detoxifiers of exogenous and endogenous hydrogen peroxide, capable of offering a full peroxide resistance in similar disc diffusion assays (Uhlich et al., 2009). This possibly explain the different resistance associated with hydrogen peroxide found in *A. butzleri* compared to *C. jejuni,* possibly suggesting a response more similar to *E. coli*; however, further studies need to be made to explore this hypothesis (Miller et al., 2007).

When accounting to the methyl viologen, similar studies in *C. jejuni* showed inhibition zones ranging from 10 mm at a concentration of 50 mM and 37 mm with concentrations of 116 mM, describing a similar resistance pattern found in this work (Atack & Kelly, 2008; Wainwright et al., 2005). This similarity with *C. jejuni* can be attributed by both species only encoding a single superoxide dismutase, SodB (Miller et al., 2007). This could lead to believe that *A. butzleri* can share a similar detoxification of superoxide species to *C. jejuni*.

Nevertheless, more studies are required to understand the mechanisms associated with resistance of oxidative stress in *A. butzleri*.

Although reactive oxygen species are more common, reactive nitrogen species can also be found when colonizing the host. These reactive species can be found not only in the innate immune system, but also in the gastrointestinal tract from the host. Once again to establish a successful infection, pathogens needed to evolve to protect themselves

against nitrosative stress. The most important protein family associated with this feature is the bacterial globins (Flint et al., 2016).

When analysing the results obtained by disc diffusion, using sodium nitroprusside (NO^+ donor), none of the strains showed an inhibition zone around the disc.

In similar works done on other species, such as from the genera *Campylobacter* and *Wolinella*, we can find small inhibitions zones around 13 and 7 mm respectively when sodium nitroprusside is used (Atack & Kelly, 2008; Kern et al., 2011). In both species' survival and resistance to nitrosative reactive species is usually linked to the existence of globins, and cytochrome c nitrite reductases, but in *A. butzleri* no proteins or genes related to this feature were yet described (Flint et al., 2016; Miller et al., 2007).

The absence of an inhibition zone in this assay could also be attributed to the low sensitivity of the assay (Kern et al., 2011) or the nature of the compound used. Sodium nitroprusside cannot generate NO^+ spontaneously, requiring light or a reductive metabolism, being considered an unpredictable effect of the extent and rates of NO release (Bowman et al., 2011). It is possible that *A. butzleri* did not possess the reductive metabolism needed to the release of NO from the sodium nitroprusside, what could also explain the results obtained. Despite the results obtained, more studies are needed to elucidate the mechanisms behind nitrosative reactions and *A. butzleri*.

4.1.4 Gastrointestinal virulence related factors in clinical isolates of *Aliarcobacter butzleri* (survival to acidic conditions, urease phenotype and bile salts resistance)

Pathogens can be exposed to several stressful conditions while establishing a successful infection into a new host. Gastrointestinal pathogens, such as *A. butzleri*, need to pass through the stomach and survive to the low range of pH values found, but also to the barrier that are bile salts (Chiang, 2013; Flint et al., 2016).

This low pH is capable of injury the outer cell membrane, deregulating cell homeostasis, and resulting cell death. Some bacteria to survive exposure to acidic conditions can upregulate the expression of defensive enzymes, such as urease, thus helping the pH homeostasis within the cell and ensuring its survival (Flint et al., 2016).

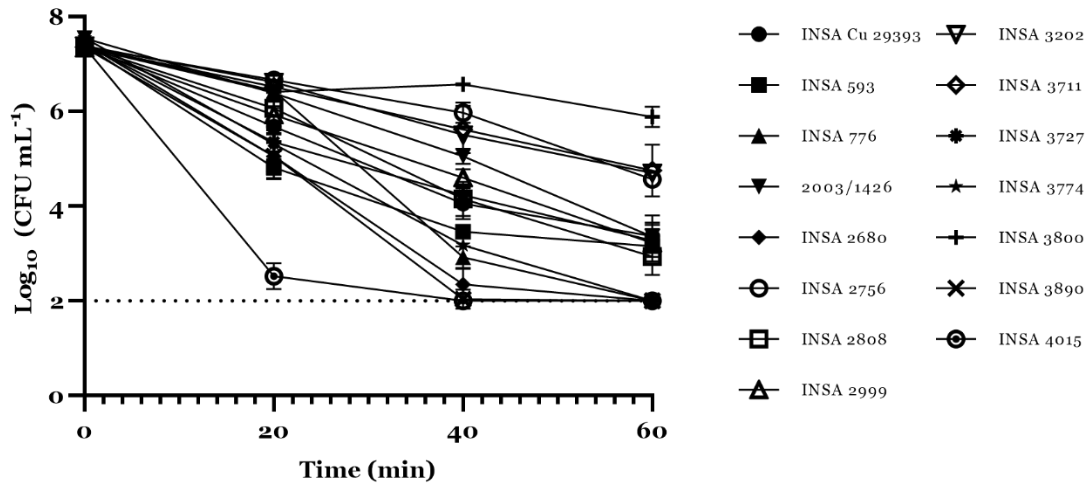

Figure 4 - Effect of acidic stress in the survival of the fifteen clinical isolates of *Aliarcobacter butzleri* when exposed to TSB medium at pH 4. Dashed line represents the detection limit of the assay. Results were analysed using a two-way ANOVA test. The data shown represent the mean ± standard deviation of at least three independent results.

By the analysis of the results obtained, and presented in Figure 4, we can observe that after 20 minutes of incubation at pH 4 only the *A. butzleri* INSA 4015 isolate showed a significant reduction compared to all the others. While at 40 minutes, the same strain showed a reduction below the detection limit. After 60 minutes, four different profiles of resistance can be observed. The first profile is constituted by the *A. butzleri* isolates INSA 776, INSA 2680, INSA 3774, and INSA 3890 having a reduction of cultivable cells below to the detection limit. A second group of more susceptible isolates is constituted by the strains INSA Cu 29393, INSA 593, 2003/1426, INSA 2808, INSA 2999 and INSA 3727 with a reduction by half on the initial Log 10 (CFU mL^{-1}). While the remaining isolates presented a reduction of about 37 % of the initial population (INSA 2756, INSA 3202, and INSA 3711) or of 19.9 % for the isolate INSA 3800.

Few other studies have been made to characterize the bacterial resistance to acidic conditions of *A. butzleri*. One study conducted in 2004, showed that no growth could be observed after 48 hours in pH below 5. While another study, later in 2013, has shown that the strain tested could not survive after one hour at a pH value of 4 (D’sa & Harrison, 2005; Isohanni et al., 2013). From the results obtained from this work, we can observe that some strains were able to survive after one hour of incubation at a pH of 4, showing more resistance to acidic conditions.

In species related to *A. butzleri,* such as *H. pylori,* the urease has a well-characterized role as direct defence mechanism against acidic conditions. This mechanism not only include the cytoplasmic urease, but also an H^+ gated urea channel and a membrane-bound carbonic anhydrase. Through this system, *H. pylori* can uptake urea present in

the stomach, and hydrolyse it to urea to ammonia and carbonic acid, acting as a buffering agent maintaining the periplasm and cytoplasm at a pH of 6.1. This enables its survival and even colonization of the human stomach (Sachs et al., 2006). Although the urease cluster ureD(AB)CEFG is often found in *A. butzleri,* until now, the other components such as the urea channel (UreI) or the membrane-bound carbonic anhydrase have not been described (Isidro et al., 2020).

For the functional urease assay in Table 15, we can observe a urease-positive phenotype in six strains while the remain nine presented a negative urease phenotype.

Table 15 - Results obtained from the urease test of the fifteen strains of *Aliarcobacter butzleri.*

Urease test result	Nº of strains	***A. butzleri* strains**
Positive	6	INSA Cu 29393, INSA 593, INSA 776, 2003/1426, INSA 3202, INSA 4015
Negative	9	INSA 2680, INSA 2756, INSA 2808, INSA 2999, INSA 3711, INSA 3727, INSA 3774, INSA 3800, INSA3890

Comparing to the resistance to the acid medium, no correlation seems to exist between the presence of urease and the resistance to acidic conditions.

In *Campylobacter,* most of the species have no encoding genes for any annotated urease, but can still survive up to 30 minutes in exposure to a pH of 3.5 without loss of cell viability (Le et al., 2012). Studies suggest that, unlike the *H. pylori*, *C. jejuni* has no direct pathway against acid stress survival, relying on the upregulation of heat shock proteins, components of the capsule, and flagellar biosynthesis (Reid et al., 2008). Thus, the results obtained, may suggest, that the acid resistance mechanism of *A. butzleri* may not be dependent on urease, but other proteins, as seen in the case of *C. jejuni,* or it could be associated with an incomplete system similar to *H. pylori*. However, more studies are needed to elucidate the mechanisms behind acid stress survival in *A. butzleri.*

Bile has been often associated with host antimicrobial defence in the human gut, bile is produced in the liver and in normal cases secreted to the duodenum in percentages between 0.2 to 2%. Since it is considered a detergent, during the process of colonizing the human gut, bacteria must overcome and survive the toxicity caused by the presence of bile (Hofmann, 1976; Joffre et al., 2019). Bile is composed mostly of un-conjugated and glyco- or tauro-conjugated bile salts, but also cholesterol, phospholipids, and biliverdin. Bile salts are constituted primarily by bile salt cholate and secondly by bile salt deoxycholate, followed by salts taurocholate and glycocholate (Chiang, 2013).

Table 16 - Minimum inhibitory concentration of the bile salts for the fifteen clinical strains of *Aliarcobacter butzleri*.

MIC (%)	***A. butzleri* strain**
5	INSA 776, 2003/1426, INSA 2680, INSA 3727
10	INSA Cu 29393, INSA 593
>10	INSA 2756, INSA 2808, INSA 2999, INSA 3202, INSA 3711, INSA 3774, INSA 3800, INSA 3890, INSA 4015

Considering the evaluation of the resistance to bile salts, we can observe that most of the isolates were resistant for concentrations of bile salts higher than 2 % (mixture of sodium cholate and sodium deoxycholate), with four strains having a MIC of 5 %, two of 10%, and the remaining nine superior to 10 % (Table 16). Suggesting that the concentrations of bile salts present in the human gut cannot offer sufficient barrier against *A. butzleri* infections.

Since there are no other studies evaluating the effect of bile salts on *A. butzleri*, and the composition of bile salts mixtures used in studies for other enteropathogens is not consistent throughout the studies, few comparisons can be made accurately. Nonetheless, in general it may be stated that several bacteria can tolerate bile, and it may even induce virulence traits (Hay & Zhu, 2016). In *Campylobacter spp.,* exposure to sodium deoxycholate resulted in an up-regulation of virulence and resistance genes, such as *ciaB*, *cmeABC*, and *tlyA*. Homologues of these genes have been described in the *A. butzleri* genome, however the effect of bile salts in their expression has yet to be described (Malik-kale et al., 2008; Miller et al., 2007). Nonetheless, resistance to bile salts in *A. butzleri* should not be discarded, since all isolates presented a MIC above what can be found in the human gut, once more suggesting the pathogenic potential of this bacteria.

4.1.5 Chemotactic behaviour in clinic isolates of *Aliarcobacter butzleri*

Chemotactic and metabolic analysis in bacteria is essential to understand its overall functioning system. Thus, enabling to describe its transcriptome, proteome, and metabolome, by understanding the functioning of the bacterium itself. Also allowing to understand the actions of chemical agents such as, antibiotics, and further comprehend its interactions with the host, limitations, and ecological niches (Guccione et al., 2008).

The results from the chemotactic profile obtained by tube-based chemotaxis assay can be seen in Table 17.

Table 17 - Chemotaxis assay of the fifteen clinical isolates of *Aliarcobacter butzleri* in the presence of pyruvic acid (Pyr), lactate (Lac), fucose (Fuc), proline (Pro), serine (Ser), aspartate (Asp), glutamate (Glu) or succinate (Suc).

***A. butzleri* strain**	**Pyr**	**Lac**	**Fuc**	**Pro**	**Ser**	**Asp**	**Glu**	**Suc**
INSA Cu 29393	+++	+++	+	+	++	+++	+++	+++
INSA 593	+++	+++	-	-	+++	++	+++	+++
INSA 776[a]	++	++	-	-	+++	++	+++	+++
2003/1426	++	+++	-	+	+++	+++	+++	+++
INSA 2680	++	++	+	+	++	++	++	++
INSA 2756	++	++	+	+	+++	++	++	+++
INSA 2808	+++	+++	+	+	+++	+++	++	+++
INSA 2999	++	++	+	-	+++	+	+	+++
INSA 3202	+++	+++	+	+	+++	++	+++	+++
INSA 3711	+++	+++	+	-	+++	+	+	+++
INSA 3727	++	++	-	-	+++	++	+++	+++
INSA 3774	+++	+++	+	++	++	++	+	++
INSA 3800	+++	++	-	-	++	++	+	++
INSA 3890	+++	++	+	+	++	+++	++	++
INSA 4015	+++	++	-	-	++	++	+	++

[+++] intense at the surface; [++] significant migration; [+] some diffuse colour; - no reaction.

When analysing the obtained results (Table 17), we can observe that the compounds with higher chemoattractant behaviour by the fifteen isolates were pyruvic acid, lactate, serine, and succinate, with 100% of the isolates having a strong to a moderate response. These were followed by the response to aspartate and glutamate having mostly moderate responses, and at last fucose and proline with mostly weak or null responses.

Only one other study described responses to pyruvic acid, L-lactate and derivates from the sodium glutamate and sodium succinate in *A. butzleri*, however the objective was related to the metabolic ability of the strains and not its effect on chemotactic behaviour (Fanelli et al., 2020). When comparing to results obtained from other chemotactic assays in *C. jejuni,* similar results can be found (Elgamoudi et al., 2018; Hugdahl et al., 1988). Chemotactic behaviour towards carbon sources, such as serine, aspartate, glutamate could be related to the portion of free proteins present in the mucus of the gut. This attraction to the intestinal mucus layer can also be found not only in *C. jejuni*, but also, *Vibrio cholerae* and *E. coli* (Allweiss et al., 1977; Hugdahl et al., 1988). These results seem consistent with the hypothesis that *A. butzleri* is mainly found in the mucous layer (Schönknecht et al., 2020; Ho et al., 2007). Interestingly serine, aspartate, glutamate and proline were described as the most abundant amino

acids in the chicken faecal matter, where *C. jejuni* is often found. In humans has been described a main presence of aspartate, asparagine and lactate (Backert, 2021).

These results appear to be in concordance with the observed for *A. butzleri,* except in the case of proline. In *C. jejuni* it is thought that proline enters through a transmembrane protein channel PutP (Cj1502), then is oxidized as glutamate by bifunctional proline dehydrogenase PutA (Cj1503) with D1-pyrroline-5-carboxylate (P5C) as an intermediate and enter in the TCA cycle via fumarate (Guccione et al., 2008). However, homologues to these proteins or genes have not been described from *A. butzleri* genome, this could justify its low response rates to proline.

In the same manner, the results obtained for fucose also do not correspond to what we can find in C. *jejuni*. Until recent years, *C. jejuni* was thought to be asaccharolytic, not capable of uptaking and metabolizing glucose or fucose. However, recently has been proven that *C. jejuni* can move and bind to fucosylated structures present in the human gut and possibly metabolize fucose (Dwivedi et al., 2016). Nonetheless, sugar fermentation has yet to be described in *A. butzleri*, and proteins related to fucose interaction still have not been described in its genome (Miller et al., 2007). This can possibly explain the lower results obtained in *A. butzleri*. On the other hand, responses associated with fucose in *A. butzleri* can be related to binding mechanism and not related to metabolism, but no further connection can be made by the present study. Nonetheless, more studies are needed to understand *A. butzleri* metabolic profile and its relation with its chemotactic behaviour and virulence.

4.1.6 Motility of clinical isolates of *Aliarcobacter butzleri*

Bacterial motility is considered a huge advantage in the process of establishing a successful infection. In bacteria, such as *Helicobacter spp*. and *Campylobacter spp*., this flagellar motility is essential for complete pathogenesis. The flagellum also plays an important role, not only in the infection cycle (reaching the host, colonization or invasion, growth and maintenance, and dispersal to new hosts) but also in adhesion, biofilm formation, as an effector molecule secretor, and immune system modulator (Chaban et al., 2015).

The results obtained for the motility of the fifteen *A. butzleri* isolates can be seen in Figure 5.

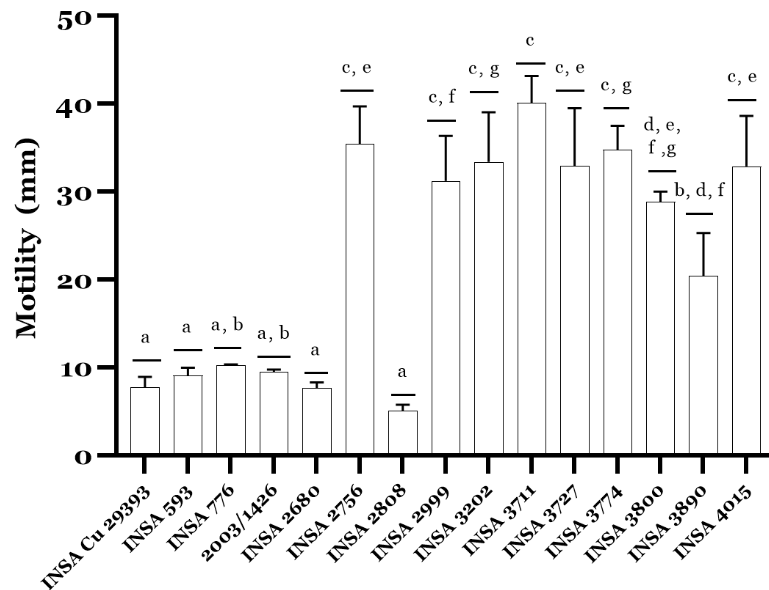

Figure 5 - Motility halo of the fifteen clinical isolates of *Aliarcobacter butzleri* in millimetres. Results were analysed using a one-way ANOVA test. Columns marked with different letters were significantly different ($p < 0.05$). The data shown represent the mean ± standard deviation of at least three independent assays.

Analysing the results obtained (Figure 5) we can observe two very different profiles in motility of the strains. The first group of isolates have low motility (INSA Cu 29393, INSA 593, INSA 776, 2003/1426, INSA 2680, and INSA 2808) with motility diameters between 5 and 10 mm. The next group displayed higher variability in the motility halos between 20 mm and 40 mm, comprising the strains, INSA 2756, INSA 2999, INSA 3202, INSA 3711, INSA 3727, INSA 3774, INSA, 3800, INSA 3890 and INSA 4015.

When comparing to other studies evaluating the motility on *A. butzleri,* we can observe values in the range of 23.8 to 29.3 mm (Ferreira et al., 2018). These values seem to correspond to the range of values of the group that displayed a moderate motility. However, another study found values between 32 and 34 mm, corresponding to the values obtained for the group with high motility in the present work (Mateus, 2019). Despite the results shown, no relation seems to be found with the origin of the strain and the motility displayed.

Since *A. butzleri* flagella is believed to be closely related to the flagella found in *C. jejuni,* when comparing with the range of values found in this species in other studies, we can find similar values to those obtained for the high motility group (Chaban et al., 2018; Zeitouni et al., 2013). Moreover, when analysing the motility profile for clinical *C. concisus* isolates, it has been suggested that variability in motility among strains could be the result of a different flagella composition or different levels of glycosylation

(Ovesen et al., 2017). However, motility is not stationary and can change under different factors, such as the presence of mucin, temperature, pH and viscosity, resulting in lower levels of motility in the laboratory assays than in the host (Ovesen et al., 2017). A similar reason could be applied to the results obtained for the *A. butzleri* isolates, however more studies are needed to understand the different motility patterns and their importance in the mechanism of virulence of *A. butzleri*.

4.1.7 Morphology and cellulose expression of the clinical isolates of *Aliarcobacter butzleri*

Some pathogens depend on their matrix components, such as fimbriae and cellulose for their survival. Cellulose can be produced not only from bacteria but also plants, animals and algae; being one of the most abundant biopolymers in nature. In bacteria, cellulose is produced as a structural component, capable of mechanical and chemical protection (Vestby et al., 2009). On other hand, fimbriae are bacterial surface components capable of establishing contact between the bacteria and surfaces. This contact can be to inanimate surfaces, eukaryotic, or prokaryotic cells, being often related to adhesion and biofilm formation (Römling et al., 1998). This may be associated to colony morphology that can be classified as previously described in *S.* Typhimurium as: rdar (violet colony, expresses curli fimbriae and cellulose); pdar (pink colony, expresses cellulose); bdar (brown colony, expresses curli fimbriae); and saw (no expression of curli fimbriae nor cellulose) (Römling et al., 2000).

The results obtained from the colony morphotype in agar plates supplemented with Congo red and brilliant Coomassie blue are shown in Figure 6.

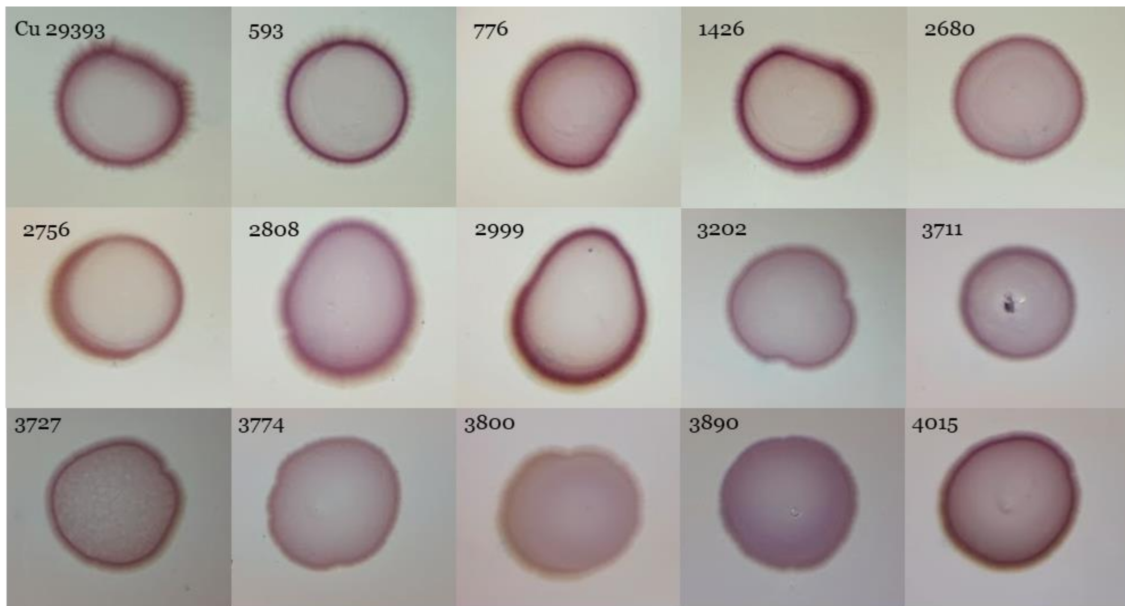

Figure 6 - Morphotypes expressed by the fifteen clinical isolates of *Aliarcobacter butzleri*.

The results obtain from the agar plate supplemented with calcofluor white are shown in Figure 7.

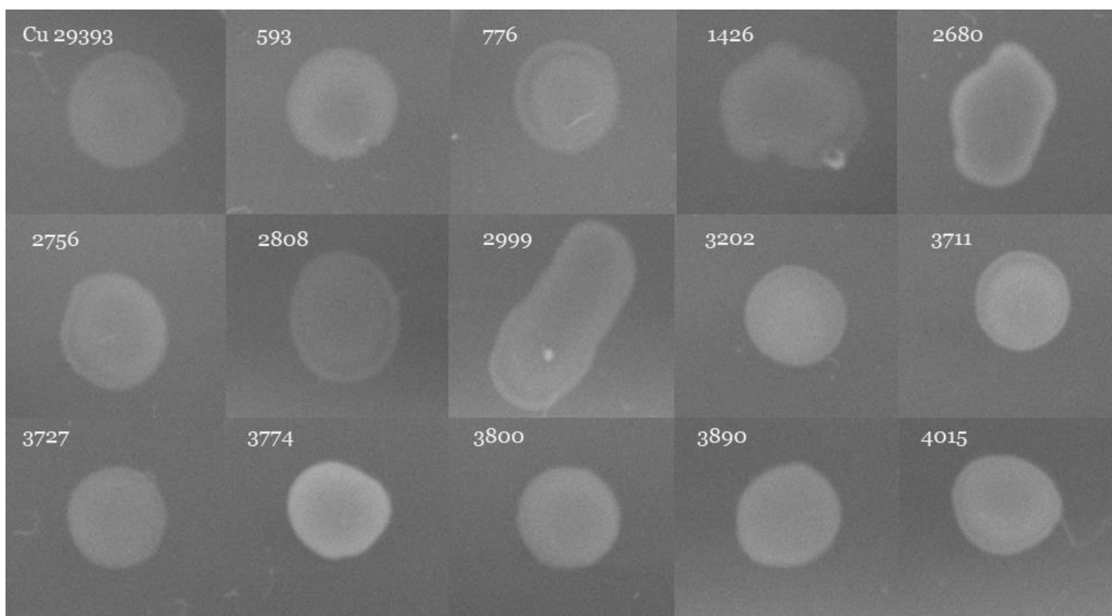

Figure 7 – Analysis of the cellulose expression by fluorescence exposure of the calcofluor white supplemented plate for the fifteen clinical isolates of *Aliarcobacter butzleri*.

When analysing the results obtained, by comparison with results obtained in *E. coli* strains, all the isolates seem to express the Bdar morphotype, capable of only expressing fimbriae and no cellulose, although not all strains seem to be morphological equal (Bokranz et al., 2005; Lajhar et al., 2018). This can be supported by the results obtained by the calcofluor white plates, where none of the isolates presented fluorescence, thus indicating the absence of cellulose production.

Until now, no reports of cellulose production or presence of fimbriae in *A. butzleri* species have been reported, nor in other species of the class of *Epsilonproteobacteria*. However, other enteric pathogens such as *E. coli* possesses fimbriae structures such as type 1 fimbriae and curli fimbriae (Bokranz et al., 2005). In *E. coli*, these fimbriae structures have been reported as mediators in the invasion process of host cells. However, its expression is not a requisite for successful colonization of the human gastrointestinal tract (Bokranz et al., 2005; Gophna et al., 2001).

In *A. butzleri* similar structures could be present on its surface, explaining the obtained results, but further studies are required to evaluate its presence and its function in the virulence of this bacterium.

4.1.8 Biofilm formation by the clinical isolates of *Aliarcobacter butzleri*

Biofilm formation has been described as an important survival factor within the host. Although immune responses are stimulated by infecting bacteria, none of the immune cells involved can enter or eliminate bacteria in a well-established biofilm (Jensen et al. 2010). This bacterial ability has been also linked to food industry, where it can affect the quality and safety of food products and being related with the persistence of a pathogen and be a frequent source of infection (Adetunji et al., 2014; Costerton et al., 1999).

Thus, the biofilm formation ability of the fifteen clinical isolates of *A. butzleri*, under study, was evaluated and the results are represented in Figure 8.

Analysing the results from Figure 8, and applying the categorization used in Stepanović et al. 2000, we can observe that six of the isolates were classified as strongly adherent; five isolates as moderately adherent; three as non-adherent and only one as weakly adherent. The biofilm-forming ability of weak adherent isolate had an average of absorbance at 570 nm of 0.226, Moderately adherent isolates showed a range of biofilm ability varying from 0.287 to 0.508 and strongly adherent isolates a ranged from 0.609 to 1.312.

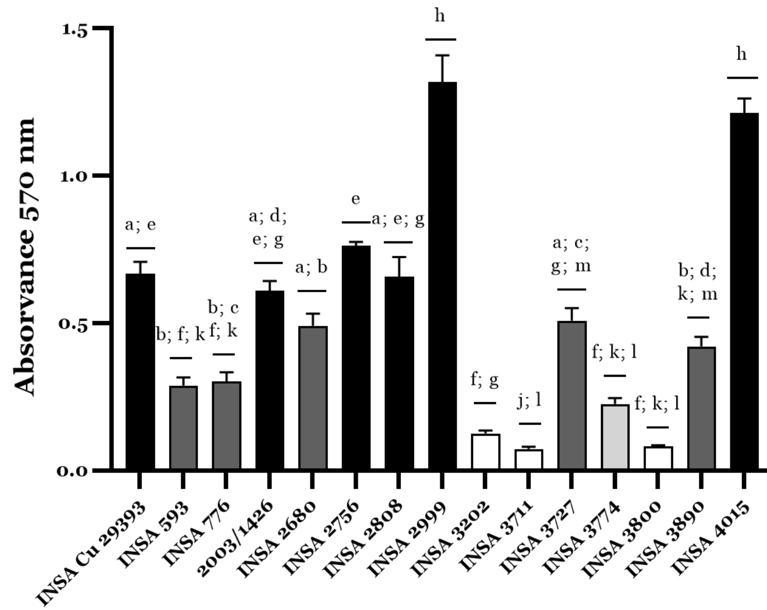

Figure 8 - Biofilm formation ability by the fifteen clinical isolates of *Aliarcobacter butzleri*, evaluated by crystal violet staining. Biofilms were classified according to Stepanović et al., 2000 as strongly adherent (black bars), moderate adherent (dark grey bars), weak adherent (light grey bars) or non-adherent (white bars). Results were analysed using a one-way ANOVA test. Columns marked with different letters were significantly different ($p < 0.05$). The data shown represent the mean ± standard error of the means of at least three independent assays.

When comparing to other studies that evaluated biofilm formation ability in *A. butzleri*, most of the isolates found were classified as weakly adherent. (Ferreira et al., 2013; Ferreira et al., 2014a). Yet, these differences can be attributed to intrinsic factors of each strain, methodology, and material the used (Girbau et al., 2017). This can be further seen in two other studies, where the strain AB 28/11 in 2013 was classified as moderate, and later in 2014 was classified as strong, while using a modified protocol. The same happens when we compare the strains INSA 593, INSA 776 and 2003/1426 present in this study, that presents lower values of biofilm formation compared to a previous study where the strains were used (Ferreira et al., 2013; Ferreira et al., 2014a). Despite the differences in the values found, it seems that clinical isolates have higher biofilm formation ability than other isolates from other sources, being only similar to those of poultry (Ferreira et al., 2013; Girbau et al., 2017).

As mentioned before, biofilm formation in several species seem to be associated with different extrinsic factors including, surface, nutrients availability, atmosphere, and also intrinsic factors of the strain itself. In *H. pylori,* biofilm formation is dependent on adhesins, lipopolysaccharide, flagella T4SS components, efflux pumps, metabolism, cell wall (Krzyżek et al., 2020). Stress mediating proteins have been also related to biofilm

formation in *Acinetobacter baumannii*, with biofilm formation being related with the strain's motility, hydrophobicity, antibiotic resistance, and serum sensitivity (King et al., 2009; Krzyżek et al., 2020). More studies relating the biofilm ability to other virulence factors are needed to understand the nature of *A. butzleri*.

4.1.9 Haemolytic activity of the clinical isolates of *Aliarcobacter butzleri*

Several bacterial pathogens can produce a variety of toxins, such as haemolysins. These are proteolytic enzymes responsible for attacking eukaryotic cells like erythrocytes. Some of these haemolysins are considered virulence factors responsible for increasing the availability of iron by releasing the heme group from erythrocytes (Istivan et al., 2008). Haemolysin's activity is closely linked to haemolytic activity, capable of stimulate the release of inflammatory factors and also result in the sequester of iron from the host, providing iron for bacterial growth (Marchetti et al., 2020; K. Poole & Braun, 1988).

The result obtained for the haemolytic activity of the fifteen strains can be shown in Figure 9.

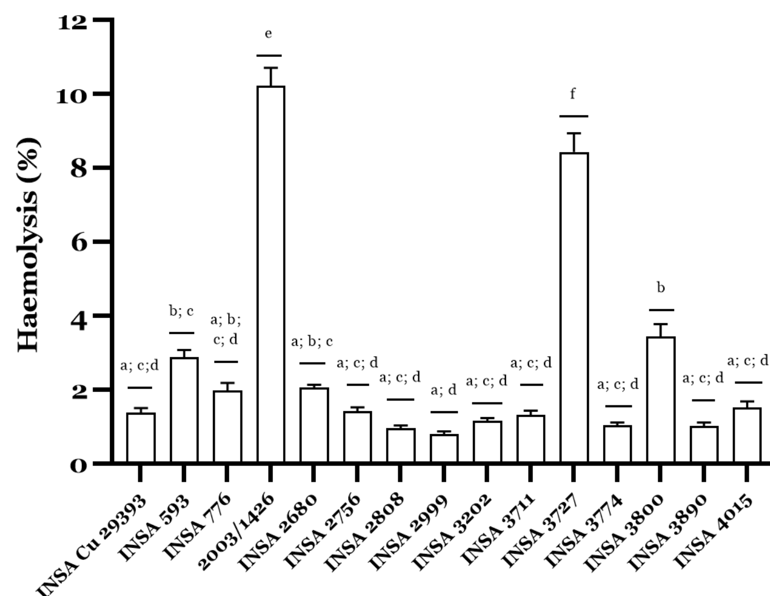

Figure 9 - Haemolytic activity of the fifteen clinical isolates of *Aliarcobacter butzleri* against human erythrocytes. Results were analysed using one-way ANOVA test. Columns marked with different letters were significantly different ($p < 0.05$). The data shown represent the mean ± standard error of the means of at least three independent assays.

When analysing the results obtained, all the isolates presented low values of haemolytic activity, not surpassing the 10 %. Nonetheless, *A. butzleri* INSA 1426 isolate has shown the highest value of the haemolytic activity of 10.2 %, followed by INSA 3727 with 8.4 %, succeeded by the isolates INSA 593 and INSA 3800 with 2.8 and 3.4 %, respectively. The remaining isolates did not present statistical differences on their haemolytic activities, ranging from 0.8 to 2.0%.

Few studies have been made to assess the haemolytic activity of *A. butzleri*, however one study by Ferreira et al. 2014, showed slightly higher values but without reaching 20 % for any strain (Ferreira et al., 2014b). The results obtained seem to agree with Vandamme (1992b) that described most of *A. butzleri* as non-haemolytic, with only a few strains being capable of showing haemolysis in blood agar (Vandamme et al., 1992b).

When comparing to other pathogens, regarding *Campylobacter* spp*.,* haemolytic activity was reported in 71 % isolates from sporadic cases of human diarrhoea, but mostly was classified as weakly haemolytic (Grant et al., 1997; Istivan et al., 2008). This ability has been associated with the presence of the phospholipase A (PldA) that also have been reported in *A. butzleri*. However, no relation with the presence of PldA and a higher haemolytic activity has been described. On other hand, it is believed that enzymatic activity of PldA could depend on the presence of other factors, such as calcium or iron, justifying the low values of haemolysis (Grant et al., 1997; Istivan et al., 2008). Another study with *C. jejuni* isolates from diverse sources, suggested that strains recovered from faeces could express less haemolytic activity than recovered from other sources (Coote et al., 2007). The effect of haemolysins and haemolytic activity in *A. butzleri* is yet to be revealed.

4.1.10 Serum susceptibility of the clinical isolates of *Aliarcobacter butzleri*

A. butzleri is commonly associated with self-limiting watery diarrheal illness in humans, which can suggest that the host innate immune system could actively contribute to the resolution of the infection, similar to what has been observed in *C. jejuni* (Keo et al., 2011). Studies often focus on *A. butzleri* diarrheal pathogenesis, with few studies being made to understand the behaviour of the strains when gaining access to the vascular space and causing systemic infection. The bactericidal effect of the human serum has been recognized throughout the years, leading to loss of viability of the bacterial cells. Resistant strains are usually causative agents of infections involving damaged tissues; accordingly serum resistance have been suggested as an important determinant of virulence (Taylor, 1983).

The resistance of *A. butzleri* clinical isolates to human serum was analysed and the results can be seen in Figure 10.

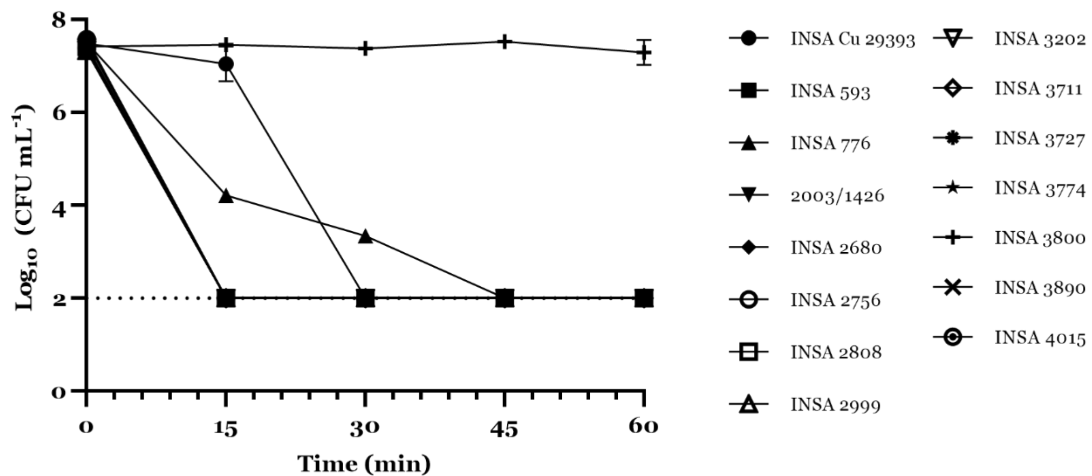

Figure 10 - Survival of the fifteen clinical isolates of *Aliarcobacter butzleri* to human serum. Dashed line represents the detection limit of the assay. Results were analysed using a two-way ANOVA test. The data shown represent the mean ± standard deviation of at least three independent assays.

When analysing the results obtained, we can observe that the majority of the isolates presented a reduction of the number of cultivable cells to values inferior to the detection limit after only fifteen minutes of incubation with human serum. However, the *A. butzleri* isolate INSA Cu 29393 showed to be able to survive until the fifteen-minute mark, and INSA 776 until the thirty-minute mark. Only the *A. butzleri* INSA 3800 showed survival at the one-hour mark, with statistical significance.

Until now, only one other study evaluated the resistance to human serum in *A. butzleri,* in this study only ten strains were evaluated for serum survival with a survival rate of 0.3 % of the CFU mL^{-1} after 60 minutes in contact to human serum. When compared to the results obtained the percentage of survival of all the strains, excluding INSA 3800, the value obtained is much lower than what is suggested by this work (0.0003 %); and when comparing to INSA 3800 resistance rate is much higher (98.9%). However, both suggest a high susceptibility of *A. butzleri* to human serum (Wilson et al., 2010).

In *C. jejuni*, *C. coli* and *C. concisus* similar low resistance values to human serum have been reported; however, the contrary is observed in *C. fetus* strains (Blaser et al., 1985; Kirk et al., 2015). This difference in resistance to human serum is believed to be modulated by the components of the bacterial capsule and lipooligosaccharide (Keo et al., 2011). Further, the resistance of *C. fetus* to human serum is associated with the

existence of acidic high molecular weight proteins on the bacterial surface, capable of disabling the binding of complement component C3b (Thompson, 2002).

A study by Isidro et al., 2020, described the presence of simple sequence repeats known to mediated phase-variable phenotypic changes in adaptation and pathogenicity related to lipopolysaccharide regulation mechanism in *A. butzleri* genome. This can suggest a possible existence of ON and OFF switch proteins capable of modulating this type of response when in contact to human serum. Curiously, the most resistant isolate INSA 3800 was the only reported related to a case of leukopenia (low number of leukocytes present in the bloodstream).

4.1.11 Adhesion and invasion of the clinical isolates of *Aliarcobacter butzleri*

Several studies have been made to characterize the adhesion and invasion abilities in *A. butzleri,* with adhesion being reported as one of the most detectable pathogenic effects in this bacterium (Chieffi et al., 2020). The results obtained for the adhesion and invasion assays in Caco-2 cell line are shown in Figure 11 and Figure 12, respectively.

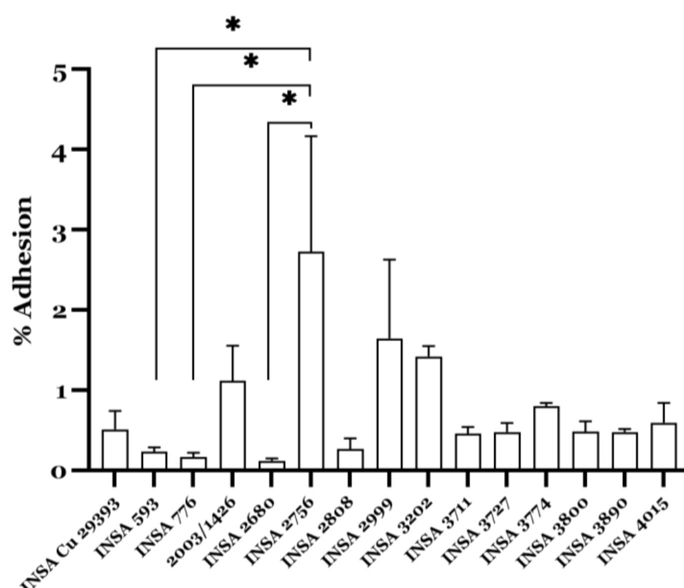

Figure 11 – Percentage of adhesion of the fifteen clinical isolates of *Aliarcobacter butzleri* in Caco-2 cell line. Results were analysed using a one-way ANOVA test. The data shown represent the mean ± standard error of the means of at least three independent assays. $*p < 0.05$

Observing the results obtained for the adhesion ability assays, we can see that all the fifteen strains have adhesion ability with only the strain INSA 2756 showing a

statistically higher adhesion ability than the isolates INSA 593, INSA 776 and INSA 2680.

In comparison to other studies regarding the adhesion ability in *A. butzleri,* all studies reported an adherent phenotype in Caco-2 cell line, corroborating the data obtained from this study. Moreover, similar values of adhesion can be found (Ferreira et al., 2014b; Ho et al., 2007; Karadas et al., 2013; Levican et al., 2013). Until now it seems that *A. butzleri* has a special affinity for this cell line, since adhesion is not always reported in other cell lines, such as HeLa, INT407, and HT-29. This could suggest a link between the structures found in this cell line and the bacterial structures responsible for adhesion.

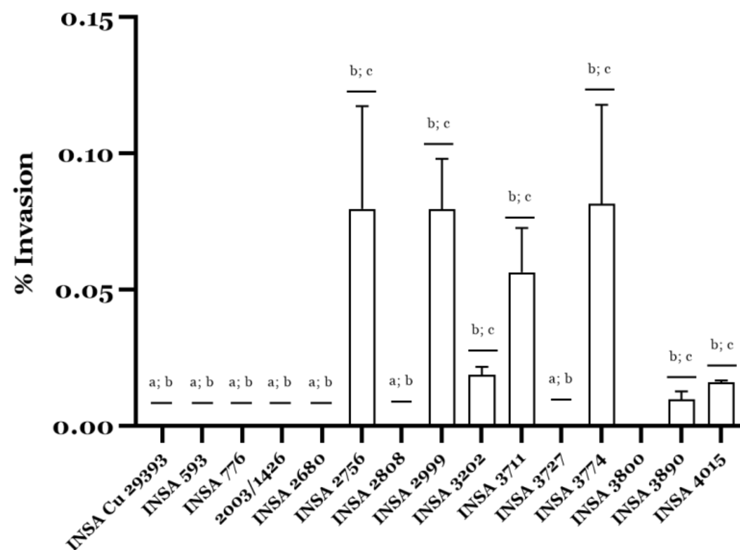

Figure 12 - Percentage of invasion ability of the fifteen clinical isolates of *Aliarcobacter butzleri* of Caco-2 cell line. Results were analysed using a one-way ANOVA test. Dashed line represents the detection limit of the assay. Columns marked with different letters were significantly different ($p < 0.05$). The data shown represent the mean ± standard error of the means of at least three independent assays.

When analysing the results obtained for the invasion ability, we can observe that only seven isolates presented invasion ability, namely *A. butzleri* INSA 2756, INSA 2999, INSA 3202, INSA 3711, INSA 3774, INSA 3890 and INSA 4015. While the remaining isolates seem to have no apparent invasion ability, when considering the detection limit of the method used.

In concordance with other studies with *A. butzleri,* the percentage of isolates able to invade in this study was around 46.6 %, what is in the range reported in other studies, between 16.6% to 100 % for Caco-2 cell line (Ferreira et al., 2014b; Ho et al., 2007; Karadas et al., 2013; Levican et al., 2013). However, lower values of invasion ability

seem to have been reported in human isolates when comparing to other isolates (Ferreira et al., 2014b; Karadas et al., 2013).

Similarly to what we can found in *Campylobacter* species, it seems that adhesion is required for the invasive ability in *A. butzleri*, however, adhesion does not always correlate with the strain capacity to invade host cells (Zheng et al., 2006). Some genes found in *A. butzleri* have been reported to be associated with adhesion and invasion abilities in *Campylobacter* species, such as *ciaB*, *cadF*, and *cj1349*. But until now no relation with their presence or absence and the phenotype observed have been established (Ferreira et al., 2014b). This could suggest the existence of other mechanisms different from what we can observe in *Campylobacter* species, or a mechanism dependent on stimulus present in the host or underlying conditions as seen with the induction of inflammation in Caco-2 cells, which increased the invasion and adhesion abilities of the *A. butzleri* strains (Ferreira, et al., 2014b). Other study suggested that mucous layer present in the intestine could provide a preferable environment for *A. butzleri* than undergoing the process of invasion (Ho et al., 2007).

The overall profile of each strain can be found in Figure 13, where all results from the phenotypic assays are resumed.

Figure 13 - Heatmap resuming all phenotypic results obtained from the previous mentioned assays of the fifteen clinical isolates of *Aliarcobacter butzleri*.

Analysing the data gattered from all the phenotypic assays, we can observe that all the strains possessed great variability and distinct phenotypic profiles. These results support the already described heterogenicity of the genus and its species.

4.2 Effect of the atmospheric conditions in virulence and survival of *Aliarcobacter butzleri*

A. butzleri is considered an aerotolerant organism, having optimal growth conditions in a microaerophilic atmosphere with levels of oxygen from 3 to 10 % and at temperatures of 37ºC. But unlike *Campylobacter* spp., *A. butzleri* can also grow in aerobic conditions at 30 ºC (Vandamme et al., 1992b). When exposed to high levels of atmospheric oxygen, oxidative stress may be induced. Resistance to atmospheric stress is crucial, especially when we consider the oxygen-rich conditions in food processing and food preservation, playing as a crucial factor in the transmission and propagation of the bacterium (Gundogdu et al., 2016; Kim et al., 2015b). This exposure to atmospheric oxygen can also modulate changes in the phenotype and resistance and stress-related mechanisms such as happens to *C. jejuni* (Oh et al., 2015).

To evaluate the effects of the aerobic exposure in *A. butzleri*, three strains were selected by presenting different phenotypes. *A. butzleri* INSA Cu 29393 was selected by having low acid resistance, low motility but being capable of forming strong biofilm and capable of some resistance to serum exposure and by not displaying invasion ability. *A. butzleri* INSA 3202 since it displayed acid resistance, high motility, and capability of cell invasion but incapable of forming biofilm and with no resistance to human serum. *A. butzleri* INSA 3711 displayed low resistance to acid, high motility, but once again incapable of forming biofilm, also displayed cell invasion and with no resistance to human serum.

4.2.1 Effect of aerobic exposure on susceptibility of *Aliarcobacter butzleri* to oxidative and nitrosative stresses

As seen before, ROS accumulation can lead to great damage to the essential's mechanism in the bacteria cell. When considering aerobic conditions more of these reactive oxygen species can be accumulated by the bacteria (Oh et al., 2015). So, we decided to evaluate if the oxidative and nitrosative stress responses were modulated by growth and assay under aerobic conditions. The results obtained for the oxidative stress in both conditions are shown in Figure 14[SMPF1].

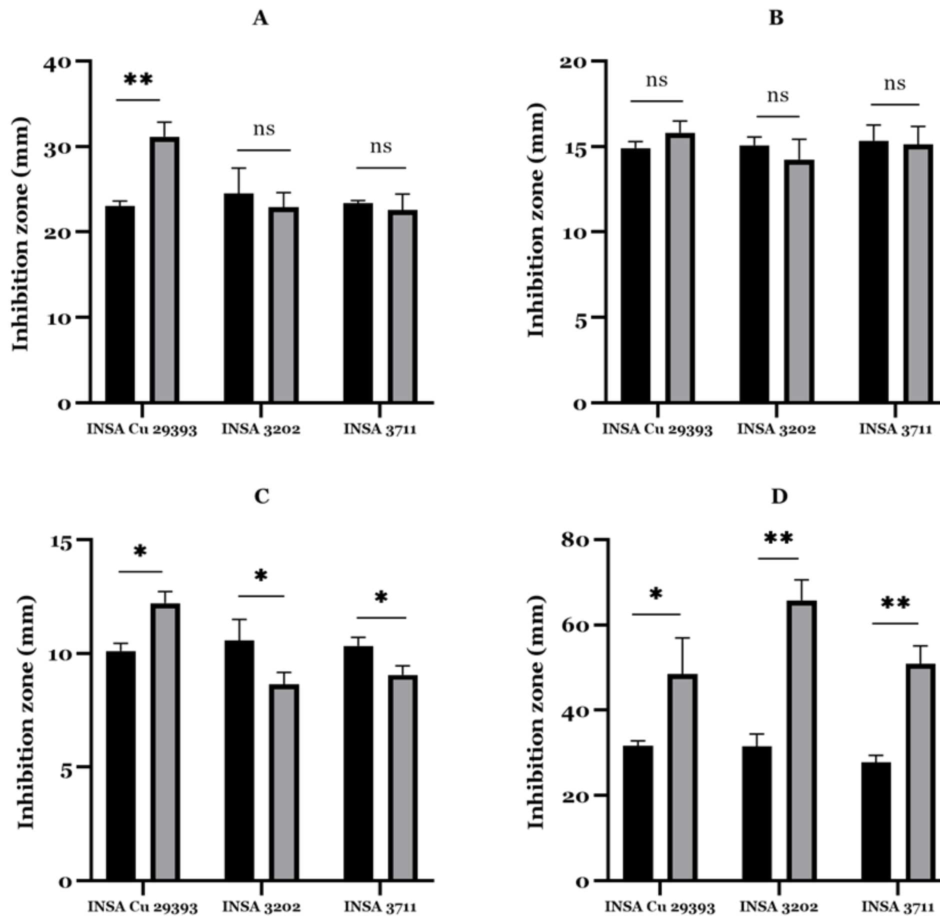

Figure 14 – Susceptibility of three clinical isolates of *Aliarcobacter butzleri* to oxidative stress when exposed to (A) 30%, (B) 10% or (C) 3% of hydrogen peroxide; or (D) 125 mM of methyl viologen, when growth in microaerophilic (black) and in aerobic conditions (grey). Results were analysed using Student's t-test. The data shown represent the mean ± standard deviation of at least three independent assays. $*p < 0.05$, $**p < 0.01$.

We can observe that when exposed to higher concentrations of hydrogen peroxide, only the *A. butzleri* INSA Cu 29393 isolate tend to present higher resistance when grown in microaerobic conditions. On the other hand, both *A. butzleri* INSA 3202 and INSA 3711 isolates seem to be more resistant when growing under aerobic conditions when exposed to 3% hydrogen peroxide. Once in contact with methyl viologen, all strains presented higher resistance when grown in microaerophilic conditions.

When *C. jejuni* was exposed to aerobic conditions an up-regulation of several genes related to oxidative protection, such as *katA*, *sodB*, and *ahpC* was observed. However, ultimately the accumulation of ROS in the aerobic conditions led to lipid oxidization, diminished cell viability and induced the formation of viable-but-non-culturable forms (Kim et al., 2015a; Oh et al., 2015).

We can theorize that same can happen in *A. butzleri* cells. As described before, *ahpC* is present in *A. butzleri* genome and could have a similar role. This would explain why when exposed to hydrogen peroxide, differences are lower than when exposed to methyl viologen. Since AhpC have been pointed as a major detoxifier of exogenous and endogenous hydrogen peroxide in *E. coli* (Uhlich, 2009).

Concerning nitrosative stress, once again no inhibition halo was formed in both conditions, when exposed to sodium nitroprusside by disc diffusion assay. For *C. jejuni,* it was shown that higher concentrations of oxygen can lead to an increase in resistance to nitrosative reactive species. However, this mechanism seems to be related to truncated haemoglobin (*Ctb*) present in *C. jejuni* (Wainwright et al., 2005). This further suggests the existence of other mechanisms that could be present in *A. butzleri*.

4.2.2 Effect of aerobic exposure to acidic stress in *Aliarcobacter butzleri*

During food processing, foodborne pathogens are often exposed to mild acid and aerobic conditions (Murphy et al., 2003). Oxygen exposure has been reported to confer cross-protection to acidic conditions in bacteria, such as *C. jejuni*, *Listeria monocytogenes*, and *H. pylori* (Hill et al., 2002; Murphy et al., 2003; Toledo et al., 2002). Before consumption pathogens can be exposed to aerobic conditions that could lead to changes in the survival response to acid. Considering this, we decided to evaluate if a similar cross-protection effect could be detected in *A. butzleri*.

Results for the acid stress exposure when growth in microaerophilic and aerobic conditions are represented in Figure 15.

When observing the data obtained, no statistical difference was determined for the *A. butzleri* isolate INSA Cu 29393 in both conditions. However, the INSA 3711 isolate only showed significant differences at the twenty-minute mark, while for INSA 3202 presented significant differences after twenty minutes. Overall strains were more resistant to acid conditions when exposed to microaerophilic conditions with exception of the strains INSA 3711. Although for *A. butzleri* INSA Cu 29393 isolate no statistical difference was observed between aerobic and microaerobic conditions, when grown in aerobic conditions after one hour no growth was detected, in contrast to the observed for microaerophilic conditions.

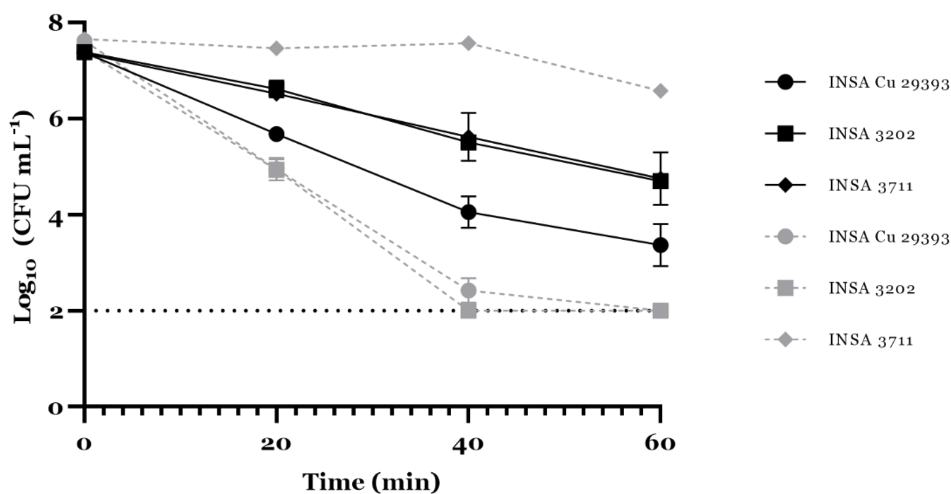

Figure 15 - Effect of acidic stress in the survival of three clinical isolates of *Aliarcobacter butzleri* when exposed to TSB medium at pH 4, while grown in microaerobic (black solid lines) and in aerobic conditions (grey dash lines). Dashed line represents the detection limit of the assay. Results were analysed using a two-way ANOVA test. The data shown represent the mean ± standard deviation of at least three independent assays.

For *C. jejuni*, it was observed a cross-protection effect of the exposure to aerobic conditions in the survival of the species under acid stress. In fact, that bacterial cells were more capable of withstanding further stress-induced by acidic conditions when exposed to aerobic conditions than non-stressed cells. However, different rates of resistance after exposure to aerobic conditions were found. These differences are thought to be strain related, influenced by time of exposure, or even by the phase where the initial stress was applied, although not always with significance (Ma et al., 2009; Caroline Murphy et al., 2003).

Comparing with the results obtained in this study, the *A. butzleri* INSA 3711 isolate seemed to benefit from aerobic exposure, while both strains INSA Cu 29393 and INSA 3202 present an opposite behaviour. As described by Murphy *et al.* (2006), for *C. jejuni,* the strains that are already naturally aerobic do not seem to suffer the same protective effect. This could suggest a further aerobic adaptation from the strains INSA Cu 29393 and INSA 3202 compared to the INSA 3711 however, more studies are needed to reach further conclusions.

4.2.3 Effect of aerobic exposure on the motility of *Aliarcobacter butzleri*

Oxygen levels seem not only to be capable of regulating stress responses and metabolic profiles, but also can have a role in the virulence of Gram-negative bacteria, such as in

the case of motility (Sengupta et al., 2014). Since changes in oxygen levels could result in modification in cellular motility, we decided to evaluate if the same could happen to *A. butzleri*. The results obtained for the motility in both conditions can be seen in Figure 16.

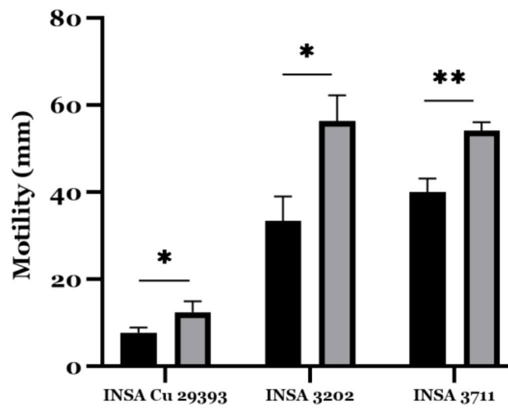

Figure 16 - Motility halo of three clinical isolates of *Aliarcobacter butzleri* in millimetres, when growth under microaerobic (black) and aerobic conditions (grey). Results were analysed using Student's t-test. The data shown represent the mean ± standard deviation of at least three independent results. $*p < 0.05$, $**p < 0.01$

Analysing the results obtained, we can observe that all three strains presented higher motility halos when exposed to aerobic conditions than under microaerophilic conditions. Although some studies evaluate motility of *A. butzleri* at aerobic conditions, none of them used the temperature of 37 ºC (Ferreira et al., 2018; Mateus, 2019). In a study with the aerotolerant *C. jejuni* Bf, the authors did not found visible differences when the strain was exposed to both conditions. In the case of *H. pylori,* when cells are exposed to high levels of oxygen it converts to non-motile coccoid forms (Bronnec et al., 2016; Sengupta et al., 2014). These previous studies point to a opposite result of what is seen in this work potentially associated with the bacterium microaerophilic nature. To further explore the observed trend, and since the absence of *flaA* could lead to a non-motile phenotype (Ho et al., 2008), we further evaluated the levels of expression of *flaA* (major flagellin).

The transcription of the *flaA* was then evaluated by real-time PCR for the three isolates under study. However, the *A. butzleri* INSA 3711 isolate did not seem to amplify the *flaA* gene. After a conventional PCR, using the same primers pair used for real-time PCR, followed by electrophoresis, it can be shown that no amplification was detected (Figure 17).

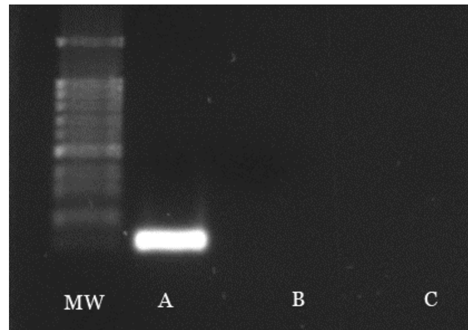

Figure 17 - Electrophoresis agarose gel of the amplification products of the partial *flaA* gene in *Aliarcobacter butzleri*. MW: molecular weight marker; A: positive control (INSA Cu 29393), B: INSA 37111; C: negative control.

This lack of amplification for the *A. butzleri* INSA3711 isolate could be explained by insertions or deletions on the central domain of the flagellins, that do not affect motility. Facing these events, the primers used in this work could be inadequate for the analysis in some strains (Ho et al. 2008).

The transcriptional analysis was then only evaluated for the *A. butzleri* INSA Cu 29393 and INSA 3202 isolates (Figure 18).

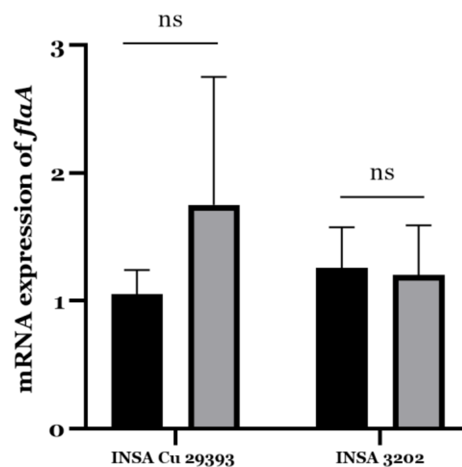

Figure 18 - Relative gene expression levels of the *flaA* in *Aliarcobacter butzleri* INSA Cu 29393 and INSA 3202 isolates, when grown under microaerobic (black) or aerobic conditions (grey). Results were analysed using Student's t-test. The data shown represent the mean ± standard error of the means of at least three independent assays.

When analysing the results obtained, no significant differences for assays at both conditions. These results are in accordance with what was described by Ho et al. (2008), showing that oxygen percentage does not influence the levels of expression of the *flaA* gene. This suggests that other factors could be contributing to the higher levels of motility in aerobic conditions. For other bacteria, as *C. concisus* an increased

motility was observed in the presence of microaerophilic conditions in comparison to anaerobic conditions (Ovesen et al., 2019). The authors suggested that higher levels of oxygen, related to pre-inflammation, could act as a regulatory switch to survival and pathogenesis on the bacteria promoting dissemination (Ovesen et al., 2019). Considering this, the trend observed in our study of augmentation of motility for aerobic atmospheric conditions, may be associated with a potential promotion of dissemination since pre-existing inflammation is thought to promote dissemination to other tissues in *A. butzleri* (Chieffi et al., 2020; Ferreira et al., 2014b).

4.2.4 Effect of aerobic exposure in the morphotype and cellulose production of *Aliarcobacter butzleri*

In S. Typhimurium strains, oxygen tensions play an important role in the expression of the rdar phenotype; and as seen before can have effect on the virulence of this bacterium (U. Gerstel & Römling, 2001; Ulrich Gerstel & Römling, 2003). A similar study was performed for *A. butzleri,* the results for the morphotype and cellulose production in the two studied conditions can be seen in Figure 19.

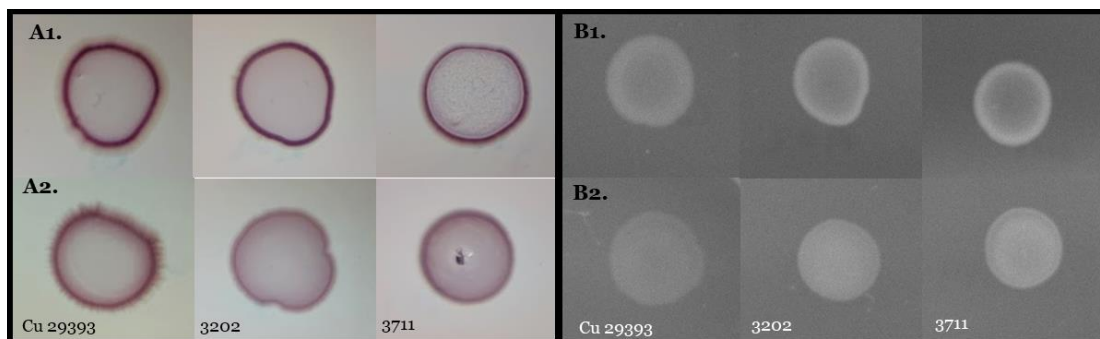

Figure 19 - Different morphotypes (A) and cellulose expression (B) in the *Aliarcobacter butzleri* INSA Cu 29393, INSA 3202, and INSA 3711 isolates under aerobic (1) and microaerophilic conditions (2).

Comparing both results, a more pronounced violet colour is present in aerobic grown bacteria than in microaerophilic conditions. While in the calcofluor white plates, a faint glow can be seen for the strains INSA 3202 and INSA 3711 under aerobic conditions.

As described above, no data regarding the presence of fimbriae or cellulose was reported in *A. butzleri*, thus it is not possible to correlate this information to any known characteristic. For *S.* Typhimurium*,* it is described that microaerophilic conditions can up-regulate the expression of certain genes related to curli and cellulose production, such as *CsgD* and the *agfD* promoter responsible for the rdar phenotype of the species.

However, this only appear to be true when in rich nutrient medium, when in low nutrient medium the inverse seems to occur. This regulatory mechanism also seems to switch between the planktonic state and in a structured community as biofilm (U. Gerstel & Römling, 2001; Ulrich Gerstel & Römling, 2003).

4.2.5 Effect of aerobic exposure in the biofilm formation of *Aliarcobacter butzleri*

Aerobic environments could represent a challenge for microaerophilic bacteria, such as *A. butzleri* and *C. jejuni*. Similarly to *C. jejuni*, *A. butzleri* is widely spread in the environment, and biofilm formation in these conditions could play a significant role in survival of this bacterium, as well as in its persistence in the environment and resistance (Ferreira et al., 2013; Teh et al., 2017).

When studying the effect of the atmospheric conditions on the biofilm formation ability of *A. butzleri*, we can observe that the INSA Cu 29393 and INSA 3202 isolates had higher biofilm formation ability when grown and allowed to form biofilm under microaerophilic conditions (Figure 20). While the isolate INSA 3711 presents higher values of biofilm formation under aerobic conditions.

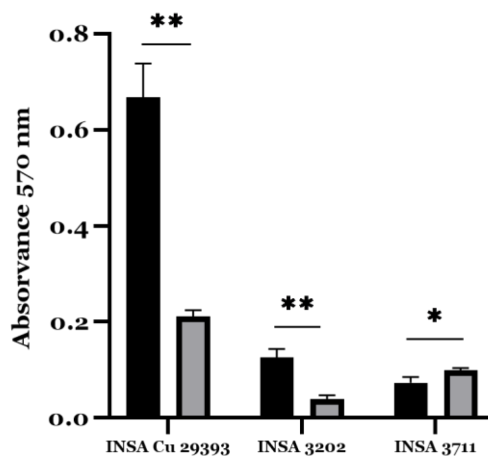

Figure 20 - Biofilm formation ability by three clinical isolates of *Aliarcobacter butzleri*, evaluated by crystal violet staining, when grown under microaerobic (black) and aerobic conditions (grey). Results were analysed using Student's t-test. The data shown represent the mean ± standard error of the means of at least three independent results. $*p < 0.05$, $**p < 0.01$

Comparing to a similar work done by Ferreira et al. (2013), aerobic atmosphere seems to negatively impact biofilm formation. In turn, another study by Girbau et al. (2017) observed that aerobic conditions could favour the formation of biofilm for the majority

of the strains analysed, showing a strain-dependent response could be responsible for the varying results (Ferreira et al., 2013; Girbau et al., 2017). Also, for *C. jejuni* a relation of biofilm formation and temperature and the oxygen availability has been described. The formation of biofilm in microaerophilic conditions was stimulated at higher temperatures, while biofilm formation in aerobic conditions was stimulated by low temperatures (Reeser et al., 2007). Other studies also suggest that increasing levels of oxygen could induce the change from planktonic to a biofilm lifestyle, but the differences in results also could be associated with strain depend-mechanism (Reuter et al., 2010; Zhong et al., 2020).

4.2.6 Effect of aerobic exposure in the haemolytic activity of *Aliarcobacter butzleri*

As previously described, *A. butzleri* is considered weakly haemolytic (Vandamme et al., 1992b), however, the role of differences in oxygen level on this virulence trait has never been studied. To further explore this, we evaluated the influence of aerobic exposure in the haemolytic activity of the strains (Figure 21).

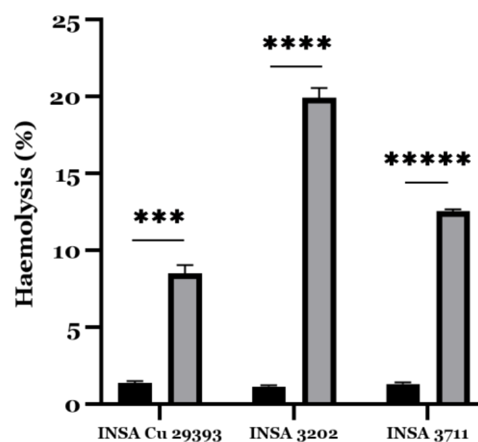

Figure 21 - Haemolytic activity of three clinical isolates of *Aliarcobacter butzleri* against human erythrocytes, when grown under microaerobic (black) and aerobic conditions (grey). Results were analysed using Student's t-test. The data shown represent the mean ± standard deviation of at least three independent results. ***$p < 0.001$, ****$p < 0.0001$, *****$p < 0.00001$

When analysing the data obtained, we can see that all three strains had their haemolytic activity greatly increased when exposed to aerobic conditions. No studies have reported the effects of aerobic atmosphere and the haemolytic activity in any species from the *Campylobacter*eales order and few studies were made to other species.

For other pathogens, such as nontuberculous mycobacteria, a much weaker haemolytic phenotype was observed when exposed to aerobic conditions compared to microaerophilic conditions, which has been associated with phospholipase C (Gomez et al., 2001). Another study suggests that high levels of oxygen can expose eukaryotic cell surfaces, and in this manner make them more susceptible to membrane-damaging and perforating agents, such as haemolysins from bacteria (Ginsburg, 1998), which could explain the obtained results. Also, an overexpression of haemolysins under stress could also be plausible. However, further exploration of these interactions should be studied.

4.2.7 Effect of aerobic exposure in the serum susceptibility of *Aliarcobacter butzleri*

Similarly, to the study of the haemolytic activity of *A. butzleri*, few studies tried to compare the effects of aerobic exposure in the strains' serum resistance. In the same manner we decided to evaluate the effect on serum resistance when the cells were previously stressed by aerobic exposure. The results obtained from the serum resistance of the three strains under microaerobic and aerobic conditions can be seen in the figure 22.

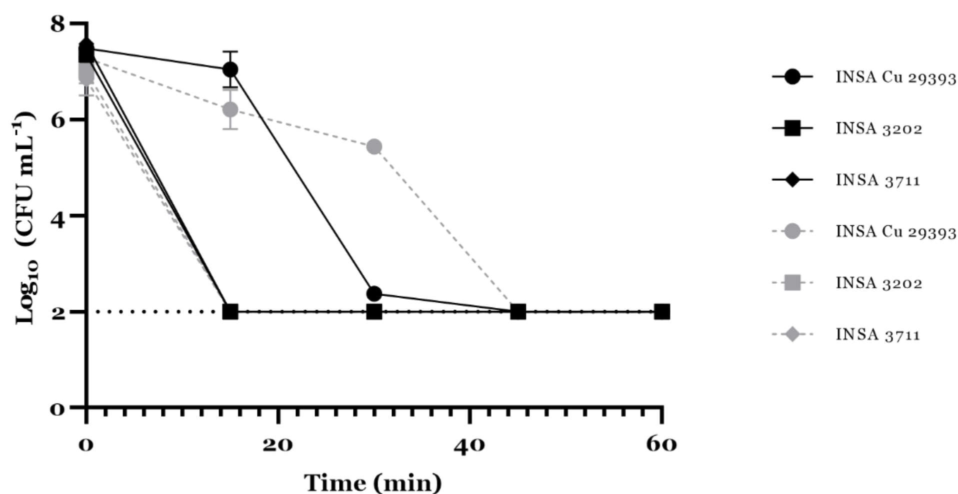

Figure 22 – Survival of three clinical isolates of *Aliarcobacter butzleri* to human serum, when grown under microaerobic (black) and aerobic conditions (dashed grey). Dashed line represents the detection limit of the assay. Results were analysed using Student's t-test. The data shown represent the mean ± standard deviation of at least three independent results.

When analysing the results obtained, only the *A. butzleri* INSA Cu 29393 isolate presented significant increase in survival for aerobic versus microaerobic conditions after incubation for 30 minutes.

Although no studies were made comparing these conditions in species from the *Campylobacter*eales order, one study with *E. coli* suggested that superoxide dismutase *sodA* and *sodB* could be responsible for survival against human serum (Mcmanus & Josephy, 1995). As mentioned before, the presence of the superoxide dismutase SodB was already described in *A. butzleri,* and a possible relation between its presence and serum survival could be suggested in the case of the INSA Cu 29393 isolate (Miller et al., 2007). In the same manner, the existence of a capsule has been also associated with serum resistance in some *E. coli* strains, with an induced low activity in capsule formation by aerobic conditions (Ginsburg, 1998). However, both previous studies suggested that these differences in resistance could be also attributed to an up-regulation of those genes. This implies the presence of other mechanisms associated with serum resistance under aerobic conditions.

4.2.8 Effect of aerobic exposure in the adhesion and invasion abilities of *Aliarcobacter butzleri*

The adhesion and invasion abilities of isolates cultured under different atmospheres are also poorly explored. In this study we decided to explore if the high levels of oxygen could also modulate the adhesion and invasion ability since, changes in the oxygen levels in the gut could influence these abilities in *A. butzleri*. The results for the adhesion and invasion abilities in microaerophilic and aerobic conditions can be seen in the Figure 23.

When analysing the data obtained, we can see that no significant differences are seen regarding a potentiation of adhesion or invasion abilities by either atmospheric condition tested with exception for the strain INSA 3202 where higher percentages of adhesion were observed in microaerophilic conditions. In *A. butzleri* no other study compared the differences between the two conditions and their adhesive and invasive abilities in any cell line.

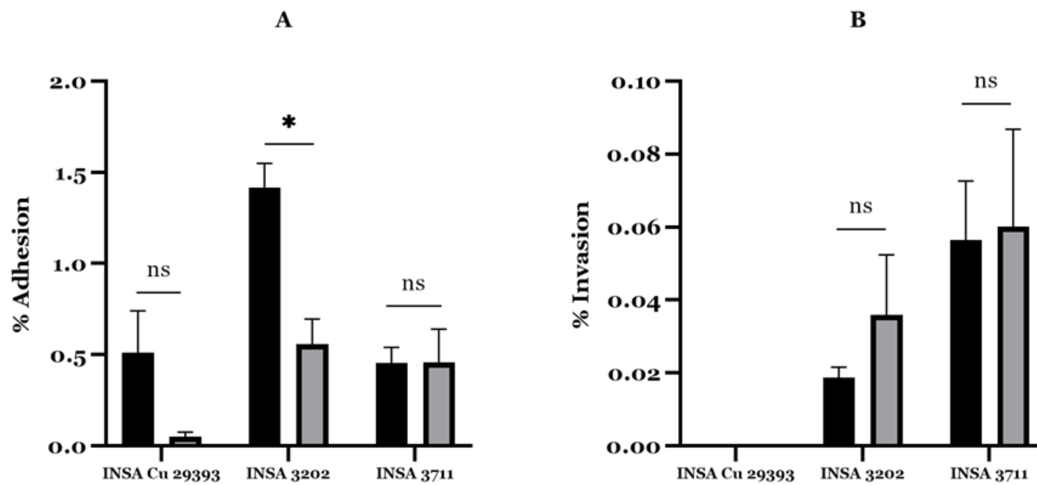

Figure 23 – Percentage of adhesion (A) and invasion (B) of three *Aliarcobacter butzleri* isolates, when grown under microaerobic (black) and aerobic conditions (grey). Dashed line represents the detection limit of the assay. Results were analysed using Student's t-test. The data shown represent the mean ± standard error of the means of at least three independent results.

Results obtained from similar studies in *C. jejuni,* referred that microaerophilic conditions are preferable for invasion and adhesion, and also pointing that microaerophilic conditions are more representative of the *in vivo* conditions (Gundogdu et al., 2011; Mills et al., 2012). These results somewhat agree with the effect of the microaerophilic conditions on the *A. butzleri* isolate INSA 3202 that seem to benefit its adhesive ability, with a similar trend for the isolate INSA Cu 29393, although with no statistical significance. Nonetheless, the observed response seemed to be strain-specific. Further, the higher rates of adhesion and invasion in *C. jejuni* in microaerophilic conditions could be related to the more sensitive nature of this bacterium. Notwithstanding, more strains should be analysed to have a broader view of the phenotypic changes in the adhesion and invasion phenotypes in the presence of aerobic stress.

Chapter 5: Conclusions

Aliarcobacter butzleri is a species belonging to the *Aliarcobacter* genus, recently allocated in the *Arcobactereacea* family. This bacterium has been widely distributed across the environment, and it is suggested that when consumed by humans, it can lead to infections capable of inducing abdominal cramps, or diarrhoea, among other symptomatology. Despite being considered a serious hazard for human health for several years, little is known about the mechanism behind its virulence and pathogenic potential of *A. butzleri*.

Firstly, in this work we started by evaluating the genomic differences of the fifteen clinical isolates of *A. butzleri* by multilocus sequence typing (MLST), confirming that all the fifteen strains presented a distinct genomic profile. This, also point to the high genetic variability of the strains, presenting eight possible novel sequence types.

Through the evaluation of the minimum inhibitory concentration of diverse antibiotics, we observed six different resistance profiles, and with resistance rates of 100% for ertapenem, 93.3 % for tetracycline, 86.6% for ampicillin 86.6%, followed by 26.6% for erythromycin, 20% for ciprofloxacin and with no resistance found for gentamicin. Thus, suggesting the use of gentamicin for treatment of severe infections by *A. butzleri*. However, also 13 strains presented a multidrug resistance profile that could difficult the treatment for these bacteria.

When evaluating to the susceptibility to oxidative and nitrosative stresses, most of the strains presented a similar pattern of resistance suggesting a common mechanism present in all the strains. This could further suggest that the mechanisms of detoxification in *A. butzleri* can be sufficient for its adaptation to oxidative stress, but also its survival against some elements of the immune system.

Whereas in the gastrointestinal virulence factors, most of the strains shown to be capable of survival at pH 4 after one hour of exposure, with six strains having a positive phenotype for the urease test, but no correlation could be made between both the activity of urease and its ability to survive on acidic medium. The minimum inhibitory concentration for bile salts showed that all the strains could survive the normal levels of bile salts present in human gut, since all of them had a MIC superior to 2%. These results suggests that both acidic conditions and bile salt concentrations are incapable of establish a successful barrier against *A. butzleri* infections.

By evaluation of the chemotactic profile of the strains we can observe that all the strains present different chemotactic profiles with special chemoattraction for: pyruvic acid, lactate, serine, and succinate. This is possibly related to the presence of these compounds in the human and chicken gut and also related to the mechanism of attraction of *A. butzleri* towards the mucus layer where it is believed this bacterium is located.

Regarding the motility, seven strains (INSA Cu 29393, INSA 593, INSA 776, 2003/1426, INSA 2680, and INSA 2808) revealed little to no motility, while other strains presented a variable range of motility halos from 20 to 40 mm. This could suggest the variability on flagellar components and different levels of glycosylation between strains, supporting the described heterogenicity in the species. However, motility did not correlate to the biofilm formation ability with majority of the strains being strongly to moderate biofilm producers. Besides all the strains seem to express similar morphotypes, possibly suggesting the existence of curli fimbriae, that until now never had been reported for this species, while none of the strains showed capable of cellulose production.

All of the strains demonstrated to display a weak haemolytic activity as previously described, although the strains 2003/1426 and INSA 3727 demonstrated a higher level of haemolytic activity against human erythrocytes. While in relation to human serum resistance, fourteen strains showed to be susceptible to the human serum. Possible relating to the self-limiting nature already reported for *A. butzleri*, while the *A. butzleri* strain INSA 3800 showed resistance after one-hour exposure. This could suggest the existence of capsule, but also suggest an adaptation to human serum survival, since this strain was found related to a case of leukopenia.

Regarding the interaction of *A. butzleri* strains with the Caco-2 cell line, all the strains showed adhesive capability as described in other studies, while only seven strains demonstrated invasion ability. Suggesting that the mucous once again could be pointed as a preferable environment for *A. butzleri* survival as supported by the results obtained by chemotactic profile.

When comparing microaerobic to aerobic exposure in the pathogenic behaviour, aerobic conditions increased the susceptibility to lower concentrations of hydrogen peroxide and in the presence of methyl viologen. Regarding, the survival to acid stress, the *A. butzleri* strain INSA 3711 seem to benefit from aerobic exposure, while the other two strains showed a decrease in survival. Aerobic conditions largely improve the motility ability in the three selected strains, although did not influence the expression

of *flaA* gene whereas biofilm formation seem to be decreased for the strains INSA Cu 29393 and INSA 3202, the strain INSA 3711 benefited the aerobic condition exposure. The aerobic conditions also greatly increased the haemolytic activity in all of three strains, while its impact on the human serum resistance was minimal, since only benefited survival in the strains INSA Cu 29393 in a time frame of twenty minutes. Aerobic conditions seem to have no direct effect on the invasion ability in any of the strains, however, the adhesion ability of the strain INSA 3202 was diminished, while having no effect for the other two strains studied.

In conclusion, this work has shown the vast variety of phenotypes presented in this species demonstrating the already described heterogeneity of this genus and its species. This work also provided some hints of possible mechanisms associated to the virulence traits in these bacteria; and reported also for the first time the influence of oxygen levels in the modulation of the virulence in these bacteria, demonstrating in this manner the pathogenic potential of *A. butzleri*.

Chapter 6: Future Perspectives

Accounting to the high prevalence of *A. butzleri* and the pathogenic potential of the bacterium shown in this work, by health safety reasons, it becomes crucial to better understand the mechanisms behind its virulence. *A. butzleri* shown to have different virulence phenotypic profiles but still little is known about the mechanisms behind these phenotypes. Thus, an increased knowledge on the pathomechanisms of this bacterium can help us to find strategies to control *A. butzleri*. For this reason, more studies are required as a follow up of this work, such as:

- To analyse the whole genome of each strain and try to establish a relation between the observed phenotype and its genomic background;
- To evaluate the expression of some genes in stress conditions, such as exposure to aerobic stress, while also study the effects in cross-protection.
- To create knockout mutants of the putative virulence genes of interest to validate their role for specific virulence traits;
- To perform more assays with different cell lines and evaluate the different behaviour in adhesion, invasion and survival of the strains, and to further stud the host-bacteria interaction;
- To perform *in vivo* assays to further characterize the responses and mechanisms suggested by the *in vitro* assays.

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Annex I

Poster presentations:

Martins R., Oleastro M., Ferreira S. (2019, December 12-12). Diversidade genética e virulência em estirpes clínicas de *Arcobacter butzleri* [Poster presentation]. IV Jornadas de Educação e Investigação em Saúde, Guarda, Portugal 2019.

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CERTIFICADO

Certifica-se que **Rodrigo Martins, Mónica Oleastro e Susana Ferreira** apresentaram um poster com o título **«Diversidade genética e virulência em estirpes clínicas de *Arcobacter butzleri*»** nas **IV Jornadas de Educação e Investigação em Saúde** realizadas no dia 12 de dezembro de 2019, na Escola Superior de Saúde do Instituto Politécnico da Guarda.

Guarda, 12 de dezembro de 2019

A Diretora da Escola Superior de Saúde
(Prof. Paula Pissarra)

A Presidente do Conselho Pedagógico
(Prof. Doutora Ermelinda Marques)

List of publications

Mateus, C., Martins, R., Domingues, F., Pereira, L., & Ferreira, S. (2021). Prevalence of *Arcobacter*: From farm to retail – A systematic review and meta-analysis. Food Control, 128, 108177