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ACINETOBACTER AND PUBLIC HEALTH: RISKS POSED BY STRAINS ISOLATED FROM FOODS

Thesis submitted to Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology, with specialization in Microbiology

Ana Isabel Teixeira Carvalheira

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Supervisor: Professor Doctor Paula Teixeira

Co-supervisor: Doctor Joana Silva

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To my family, friends and Monk Ledgen

Abstract

Acinetobacter spp. has emerged as a pathogen of a major public health concern due to their increased resistance to antibiotics and their association with a wide range of nosocomial infections. The aim of this work was to gain insight into the food-related ecology and epidemiology of *Acinetobacter* spp. and to compare food and clinical strains regarding biofilm production, resistance to desiccation and disinfectant susceptibility. As there is no standard procedure to recover *Acinetobacter* spp. from food, two selective enrichment media were evaluated for the recovery of low levels of these organisms. Enrichment in Dijkshoorn enrichment medium followed by plating on CHROMagar^{TM} Acinetobacter medium was shown to be a reliable method. Using this procedure, *Acinetobacter* spp. were isolated from 77.9% of fruit (35/50) and vegetables (39/45) samples and from all the meat analysed (50). A high genetic diversity established by pulsed-field gel electrophoresis (PFGE) was observed among the isolates and based on the analysis of the partial sequence of *rpo*B, 181 strains recovered from fruits and vegetables and 156 strains recovered from meat samples were identified as members of eighteen and thirteen distinct species, respectively. *Acinetobacter calcoaceticus* and *Acinetobacter johnsonii* were the most common species (both with the frequency of 26.5%) recovered from fruit and vegetables, while *Acinetobacter guillouiae* (34.9%), *A. johnsonii* (15%) and *A. bereziniae* (12%) were the most common species recovered from meats. *Acinetobacter* spp. belonging to the *A. baumannii* group (11.0% from fuit and vegetables, 18.7% from meats), which is most frequently associated with nosocomial infections worldwide, were also recovered. Most of these strains were resistant to some of the antimicrobials recommended to treat *Acinetobacter* infections such as piperacillin-tazobactam, ceftadidime, ciprofloxacin, as well as to colistin and polymyxin B, the last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter*. Overall, 29.8% of the isolates from fruit and lettuces, and 51.2% of the isolates from meats were classified as multidrug-resistant (MDR), and 4.4% and 9.6% as extensively drug-resistant (XDR), respectively.The taxonomic status of six strains of *Acinetobacter* obtained from meats, was investigated, using a polyphasic analysis, since their partial *rpo*B sequence similarities to other *Acinetobacter* species with validly published names were lower than 95%. The species status of two groups was confirmed by comparative multilocus sequence analysis, including also the *gyr*B, *rec*A and 16S rRNA genes, low (below 95%) whole-genome sequence (WGS) average nucleotide identity (ANI) values and low (below 70%) digital DNA-DNA hybridization (dDDH) similarities between the WGS of the proposed type strains of each novel species and the representatives of the known *Acinetobacter* species. Phylogenomic treeing from core genome analysis supported these results as well as, the coherence of each new species-lineage was supported by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, differentiation of the species at the protein level by cellular fatty acids profiles and by unique and differential combinations of metabolic and physiological properties shared by each novel species. These strains represented two coherent lineages that were distinct from each other and from all known species, and the names *Acinetobacter portensis* sp. nov. (four strains: AC 877^T = CCUG 68672^T = CCM 8789^T as type strain), and *Acinetobacter guerrae* sp. nov. (two strains: AC 1271^T = CCUG 68674^T = CCM 8791^T as type strain) were proposed for these novel species. Control the dissemination of *A. baumannii* is challenging mainly due to its high adaptability to adverse environmental conditions. The biofilm formation ability in silicon and stainless steel, the resistance to desiccation on a stainless steel surface and the susceptibility to eleven commercial antimicrobial products was compared between food (10) and clinical strains (10). The clinical strains were selected based on their presumptive persistent and non-persistent status among 104 isolates recovered from patients in Hospital de São Marcos, Braga. Predominant clones of MDR *A. baumannii* repeatedly isolated in different years (2004 to 2007) or in different months (isolates recovered in 2014) were defined as persistent and strains recovered sporadically (with a PFGE pattern observed only once among all isolates) were defined as non-persistent. There were no significant differences between clinical and food strains since all the strains were able to form biofilm on silicon and stainless steel surfaces, exhibited desiccation resistance capacity ranging from 14 to 77 days and were susceptible to disinfectants at the recommended use concentrations. However, the biofiom-forming capacity of persistent strains was significantly higher than the non-persistant strains on both surfaces. The resistance to desiccation of persistent strains (mean survival time: 65.8 days) was also significantly longer than that of the non-persistent strains (mean survival time: 35.8 days). Therefore, these factors may contribute to their maintenance in the hospital setting. A high intra-species variability in susceptibility to disinfectants was observed for *A. baumannii* and there was no correlation between the efficiency of disinfectants and the origin of the isolates. Moreover, no correlation between antibiotic resistance and biofilm production, resistance to desiccation and disinfectant susceptibility was found.

Therefore, food products may be a potential vehicle of spread in the community and clinical environments of *Acinetobacter* strains resistant to several antibiotics, able to produce biofilm and to survive to desiccation, which may led to nosocomial and community-acquired infections in susceptible individuals.

Keywords: *Acinetobacter* spp., occurrence in foods, antibiotic resistance, taxonomic status.

Resumo

Acinetobacter spp. emergiu como um patógenico de grande relevância para a saúde pública devido ao aumento da resistência aos antibióticos e à sua associação com várias infeções nosocomiais. Este trabalho teve como objetivo obter uma visão sobre a ecologia alimentar e epidemiologia de *Acinetobacter* spp. e comparar estirpes alimentares e clínicas quanto à produção de biofilme, resistência à dessecação e suscetibilidade a desinfetantes. Como não existe um procedimento padrão para recuperar *Acinetobacter* spp. de alimentos, dois meios de enriquecimento seletivo foram avaliados para a recuperação de baixos níveis desses organismos. O enriquecimento em meio Dijkshoorn seguido de plaqueamento em meio CHROMagarTM Acinetobacter mostrou ser um método adequado. Usando este procedimento, *Acinetobacter* spp. foram isolados de 77,9% das amostras de frutas (35/50) e de vegetais (39/45) e de todas as carnes analisadas (50). Foi observada uma elevada diversidade genética entre os isolados, estabelecida por eletroforese em gel de campo pulsado (PFGE) e, com base na análise da sequência parcial de *rpo*B. 181 estirpes recuperadas de frutas e de vegetais e 156 estirpes recuperadas de amostras de carne foram identificadas como membros de 18 e de 13 espécies distintas, respectivamente. *Acinetobacter calcoaceticus* e *Acinetobacter johnsonii* foram as espécies mais comuns (ambas com frequência de 26,5%) recuperadas de frutas e de vegetais, enquanto *Acinetobacter guillouiae* (34,9%), *A. johnsonii* (15%) e *A. bereziniae* (12%) foram as espécies mais comuns recuperadas de carne. *Acinetobacter* spp. pertencentes ao grupo *A. baumannii* (11,0% de frutas e vegetais, 18,7% de carnes), frequentemente associado a infecções nosocomiais em todo o mundo, também foram recuperados. A maioria dessas estirpes era resistente a alguns dos antimicrobianos recomendados para tratar infecções por *Acinetobacter*, como piperacilina-tazobactam, ceftadidima, ciprofloxacina, bem como à colistina e polimixina B, os antibióticos de último recurso para tratar infecções causadas por *Acinetobacter* multirresistentes. No geral, 29,8% dos isolados de frutas e de alfaces e 51,2% dos isolados de carnes foram classificados como multirresistentes (MDR) e 4,4% e 9,6% como extensivamente resistentes aos medicamentos (XDR), respectivamente. A posição taxonómica de seis estirpes de *Acinetobacter* recuperadas de amostras de carne, foi investigada, usando uma análise polifásica, uma vez que as semelhanças entre as suas sequências parciais *rpo*B com outras espécies de *Acinetobacter* com nomes válidos foram inferiores a 95%. Confirmou-se que as estirpes pertenciam a duas novas espécies por análise de sequências multilocus, incluindo também os genes *gyr*B, *rec*A e 16S rRNA, baixos valores (inferiores a 95%) de identidade média de nucleotídeos (ANI) na sequência completa do genoma (WGS) e baixa semelhança (inferior a 70 %) de hibridização digital DNA-DNA (dDDH) entre o WGS das estirpes tipo proposta para cada nova espécie e os representantes das espécies conhecidas de *Acinetobacter*.

RESUMO

A coerência de cada nova linhagem-espécie foi apoiada por espectrometria de massa de ionização e dessorção a laser assistida por matriz, a diferenciação das espécies ao nível de proteínas, pelo perfil de ácidos gordos membranares e pelas combinações únicas e diferenciadoras de propriedades metabólicas e fisiológicas partilhadas por cada nova espécie. Essas estirpes representam duas linhagens que são distintas uma da outra e de todas as espécies conhecidas, e os nomes *Acinetobacter portensis* sp. nov. (quatro estirpes: AC 877^T = CCUG 68672^T = CCM 8789^T como estirpe tipo) e *Acinetobacter guerrae* sp. nov. (duas estirpes: AC 1271^T = CCUG 68674^T = CCM 8791^T como estirpe tipo) foram propostos para essas novas espécies. Controlar a disseminação de *A. baumannii* é um desafio principalmente devido à sua elevada capacidade de adaptação a condições ambientais adversas. A capacidade de formação de biofilme em silicone e em inox, a resistência à dessecação em inox e a suscetibilidade a 11 produtos antimicrobianos comerciais foi comparada entre estirpes alimentares (10) e clínicas (10). As estirpes clínicas foram selecionadas entre 104 isolados recuperados de pacientes no Hospital de São Marcos (Braga) com base na sua presuntiva persistência e não persistência. Os clones predominantes de MDR *A. baumannii* isolados repetidamente em anos diferentes (2004 a 2007) ou em meses diferentes (isolados recuperados em 2014) foram definidos como persistentes e as estirpes recuperadas esporadicamente (com um padrão de PFGE observado apenas uma vez entre todos os isolados) foram definidas como não persistentes. Não foram identificadas diferenças significativas entre as estirpes clínicas e alimentares; todas as estirpes formaram biofilme em superfícies de silicone e inox, resisistiram à dessecação de 14 a 77 dias e revelaram-se suscetíveis aos desinfetantes testados nas concentrações de utilização recomendadas. No entanto, a capacidade de formação de biofilme das estirpes persistentes foi significativamente maior do que a das estirpes não persistentes em ambas as superfícies. A resistência à dessecação de estirpes persistentes (tempo médio de sobrevivência: 65,8 dias) foi também significativamente maior do que a das estirpes não persistentes (tempo médio de sobrevivência: 35,8 dias). Estes fatores poderão contribuir para a sua manutenção no ambiente hospitalar. Foi também observada uma elevada variabilidade intra-espécies na suscetibilidade aos desinfetantes mas não foi revelada correlação entre a eficiência dos desinfetantes e a origem dos isolados. Não foram também encontradas correlações entre a resistência a antibióticos e a produção de biofilme, a resistência à dessecação ou a susceptibilidade a desinfetantes.

Os alimentos podem, então, ser um potencial veículo de disseminação, tanto na comunidade como em ambientes clínicos, de *Acinetobacter* resistente a diversos antibióticos, com capacidade para produzir biofilme e de sobreviver à dessecação, o que pode resultar em infeções nosocomiais e adquiridas na comunidade por indivíduos suscetíveis.

Palavras-chave: *Acinetobacter* spp., ocorrência em alimentos, resistência a antibióticos, posição taxonómica.

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To my family for all the love, motivation, constant support and for always believing in me!

Gratitude!

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Acb	*A. calcoaceticus-A.baumannii*
AFLP	Amplified Fragment Length Polymorphism
ANOVA	One-way analysis of variance
ANI	Average Nucleotide Identity
ANIb	Average Nucleotide Identities based on BLAST
API	Analytical Profile Index
AK	Amikacin
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
BHI	Brain-Heart Infusion
BLAST	Basic Local Alignment Search Tool
CAZ	Ceftazidime
$CaCl_2$	Calcium chloride
CCM	Czech Collection of Microorganisms
CCUG	Culture Collection University of Gothenburg
CFA	Cellular fatty acids
CFU	Colony forming unit
CHCA	α-cyano-4-hydroxycinnamic acid
CH_3COONa	Sodium acetate
CIP	Ciprofloxacin
CL	Colistin
Cl^-	Chlorite
CLB	Cell Lysis Buffer
CLSI	Clinical and Laboratory Standards Institute
$CH_3COONa.3H_2O$	Sodium acetate trihydrate
dDDH	digital DNA-DNA hybridization
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EC	European Commission

EDTA	Ethylenediamine tetraacetic acid
EEA	European Economic Area
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EU	European Union
FAO	Food and Agriculture Organization
FAME	Fatty acid methyl ester
$FeSO_4.7H_2O$	Ferrous sulfate heptahydrate
G+C	Guanine-Cytosine
GC	Gas Chromatography
GGDC	Genome-to-Genome Distance Calculator
*gyr*B	Gyrase subunit-B
H_2O_2	Hydrogen peroxide
ICU	Intensive care unit
IMF	Infant milk formula
IPM	Imipenem
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium Nitrate
LMG	Bacteria Collection Laboratorium voor Microbiologie Universiteit Gent
Log	Logarithm
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MDR	Multidrug-resistant
MEM	Meropenem
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
MHA	Muller-Hinton agar
MI	Minocycline
MIC	Minimum inhibitory concentration
MLSA	Multilocus Sequence Analysis

LIST OF SYMBOLS AND ABBREVIATIONS

NA_2HPO4	Sodium phosphate dibasic
$Na_2HPO_4.H_2O$	di-Sodium hydrogen phosphate dihydrate
ND	not determined
NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium chloride
NIPH	Norwegian Institute of Public Health
OTUs	Operational taxonomic units
OXA	Oxacillin
PB	Polymyxin B
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
PGAP	Prokaryotic Genome Annotation Pipeline
PIP	Piperacillin
R2A	Reasoner's 2A
rDNA	Ribosomal DNA
tDNA	
*rec*A	Recombinase subunit-A
REP-PCR	Repetitive Extragenic Palindromic sequence-based PCR
*rpo*B	RNA polymerase subunit B
16S rRNA	16S ribosomal ribonucleic acid
SAM	Ampicillin-sulbactam
SXT	Trimethoprim-sulphamethoxazole
TE	Tetracycline
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TOB	Tobramycin
TZP	Piperacillin-tazobactam
UK	United Kingdom
UPGMA	Unweighted-Pair Group Method with Average linkages
WHO	World Health Organization
XDR	Extensively drug-resistant

Scope and outline

Acinetobacter spp. have emerged in recent decades as a major cause of nosocomial infections worldwide. Although *Acinetobacter* spp. are mainly associated with hospital-acquired infections, these organisms are also responsible for community-acquired infections associated with relatively high mortality. The role of foods as potential sources of human infections and dissemination of *Acinetobacter* to and within healthcare settings is not yet clear. Therefore, this study aimed to identify/characterize *Acinetobacter Acinetobacter* isolates recovered from food and compare them with clinical isolates.

To achieve this main goal, specific objectives were defined:

- To select a procedure to recover *Acinetobacter* spp. from food in order to gain insight into the food-related ecology and epidemiology of this genus.
- To evaluate the role of ready-to-eat products (lettuces and fruits) as a reservoir of pathogenic species of *Acinetobacter* and their antibiotic resistance.
- To evaluate the prevalence and antibiotic resistance of *Acinetobacter* spp. in meat.
- To determine the taxonomic status of *Acinetobacter* strains using a polyphasic analysis.
- To evaluate the persistence of *A. baumannii* in clinical settings.
- To evaluate and compare the biofilm formation ability, the resistance to desiccation and the susceptibility to disinfectants between food and clinical strains.

This thesis is structured in seven parts which comprises 8 chapters. The different chapters are presented by the order in which the practical work was developed.

Part I contains chapter 1, in which a literature revision on the occurrence of *Acinetobacter* spp. in food products is presented as recognition of the pressing need for a better understanding of food as a significant reservoir of these organisms.

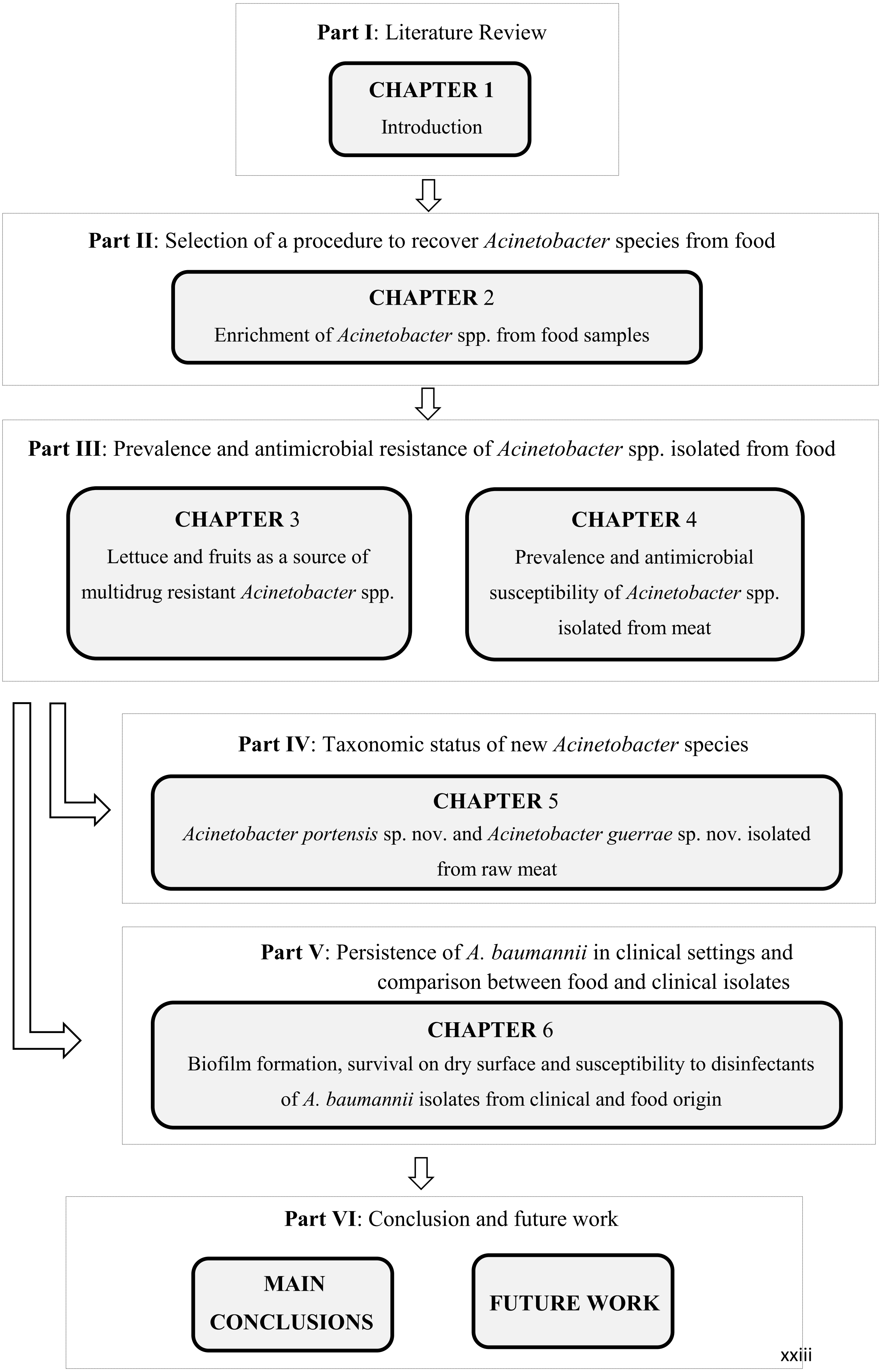
Part II comprises chapter 2 in which the results of the comparison between two acetate mineral media for the recovery of low levels of different *Acinetobacter* species are presented. This approach was then applied using the medium with the highest recovery capacity, followed by direct plating on selective and differential CHROMagar^{TM} Acinetobacter medium, to detect *Acinetobacter* spp. in food samples.

Part III comprises chapters 3 and 4 which are the results of prevalence, diversity and antibiotic resistance of *Acinetobacter* spp. recovered from ready to eat products, including lettuces and fruits, and meat as an indicator of their potential as a risk factor for consumers.

Part IV comprises chapter 5 in which the results of comprehensive characterisations of *Acinetobacter* strains from two proposal novel species (*Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov.) were assed, using polyphasic taxonomic methods relevant for this genus.

Part V comprises chapter 6 in which the results of the evaluation and characterizations of *A. baumannii* strains persistent in hospital are presented and compared with non-persistent clinical strains, as well as with strains isolated from food concerning their biofilm production ability, survival to desiccation and susceptibility to antiseptics and disinfectants commonly used in hospital settings and kitchen canteens.

Part VI comprises the main conclusions of this study and the proposals of future work.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Part VI: Conclusion and future work
MAIN CONCLUSIONS
FUTURE WORK

The work performed and presented in this thesis has resulted in six scientific publications (five published and one to be submitted for publication) and two poster communications:

Chapter 1

Carvalheira, A., Silva, J., Teixeira, P. 2021. *Acinetobacter* spp. in food and drinking water – a review. *Food Microbiology* **95**: 103675.

Chapter 2

Carvalheira, A., Ferreira, V., Silva, J., Teixeira, P. 2016. Enrichment of *Acinetobacter* spp. from food samples. *Food Microbiology* **55**: 123-127.

Chapter 3

Carvalheira, A., Silva, J., Teixeira, P. 2017. Lettuce and fruits as a source of multidrug resistant *Acinetobacter* spp. *Food Microbiology* **64**: 119-125.

Carvalheira A., Ferreira V., Silva J., Teixeira P. 2013. Occurrence of *Acinetobacter* spp. in vegetables. MicroBiotec'13. Portuguese Congress of Microbiology and Biotechnology. Universidade de Aveiro, Portugal, 6 to 8 December 2013.

Chapter 4

Carvalheira, A., Casquete, R., Silva, J., Teixeira, P. 2017. Prevalence and antimicrobial susceptibility of *Acinetobacter* spp. isolated from meat. *International Journal of Food Microbiology* **243**: 58-63.

Chapter 5

Carvalheira, A., Gonzales-Siles, L., Salvà-Serra, F., Lindgren, Å., Svensson-Stadler, L., Thorell, K., Piñeiro-Iglesias, B., Karlsson, R., Silva, J., Teixeira, P., Moore' E. R. B. 2020. *Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov. isolated from raw meat. *International Journal of Systematic and Evolutionary Microbiology* **70**: 4544-4554

Chapter 6

Carvalheira, A., Silva, J., Teixeira, P. 2020. Biofilm formation, survival on dry surface and susceptibility to disinfectants of *A. baumannii* isolates from clinical and food origin (be submitted for publication).

Carvalheira, A., Silva, J., Faustino, A., Teixeira, P. 2015. *Acinetobacter* spp. other than *A. baumannii* associated to nosocomial infections. MicroBiotec'15. Portuguese Congress of Microbiology and Biotechnology. Universidade de Évora, Portugal, 10 to 12 December 2015.

CHAPTER 1

Introduction

CHAPTER 1

Abstract

Acinetobacter spp. has emerged as a pathogen of major public health concern due to their increased resistance to antibiotics and their association with a wide range of nosocomial infections, community-acquired infections and war and natural disaster-related infections. It is recognized as a ubiquitous organism however, information about the prevalence of different pathogenic species of this genus in food sources and drinking water is scarce. Since the implementation of molecular techniques, the role of foods as a source of several species, including the *A. baumannii* group, has been elucidated. Multidrug resistance was also detected among *Acinetobacter* spp. isolated from food products. This highlights the importance of foods as potential sources of dissemination of *Acinetobacter* spp. between the community and clinical environments and reinforces the need for further investigations on the potential health risks of *Acinetobacter* spp. as foodborne pathogens. The aim of this review was to summarize the published data on the occurrence of *Acinetobacter* spp. in different food sources and drinking water. This information should be taken into consideration by those responsible for infection control in hospitals and other healthcare facilities.

1.1. Introduction

Acinetobacter spp. have emerged in recent decades as a major cause of nosocomial infections worldwide (Hanlon, 2005; Knapp et al., 2006; Peleg et al., 2008; Van Looveren and Goossens, 2004; Vanbroekhoven et al., 2004). A wide range of human infections including meningitis, pneumonia, endocarditis, bacteremia, skin and urinary tract infections in immunocompromised patients have been reported (Allen and Hartman, 2005; Falagas and Rafailidis, 2007; Joly-Guillou, 2005; Peleg et al., 2008) and associated with high rates of morbidity and mortality (Dijkshoorn et al., 2007; Joly-Guillou, 2005; Perez et al., 2007; Richet and Fournier, 2006). Although *Acinetobacter* spp. are mainly associated with hospital-acquired infections, these organisms are also responsible for community-acquired infections also associated with relatively high mortality (Chen et al., 2018; Dexter et al., 2015; Peleg et al., 2008; Porter et al., 2014). The recognition of the public health significance of these opportunistic pathogens is particularly related to their propensity for extensive drug resistance and the worldwide emergence of multidrug-resistant strains (Jain and Danziger, 2004; Peleg et al., 2007; Vila et al., 2002). Currently, the genus comprises 65 species, with validly-published names (www.szu.cz/anemec/Classification.pdf) including four pairs of heterotypic synonyms.

Acinetobacter spp. are versatile organisms, non-motile, non-fermentative, non-sporulating, non-fastidious Gram-negative, catalase positive, oxidase negative, strictly aerobic coccobacilli with a DNA G+C content between 39 and 47% (Bergogne-Bérézin and Towner, 1996; Juni, 2005; Peleg et al., 2008; von Graevenitz, 1995). *Acinetobacter* species are ubiquitous and can be found in several ecological niches including environment, animals and human (Doughari et al., 2011). In contrast to many other *Acinetobacter* species, *Acinetobacter baumannii* has been mainly associated with clinical environments and hospital outbreaks (Chu et al., 1999; Fournier and Richet, 2006; Ku et al., 2000; Manikal et al., 2000; Peleg et al., 2008; Towner, 2009). *A. baumannii* and its close relatives, *Acinetobacter calcoaceticus*, *Acinetobacter lactucae* (Rooney et al., 2016), *Acinetobacter nosocomialis*, *Acinetobacter pittii* (Nemec et al., 2011) and *Acinetobacter seifertii* (Nemec et al., 2015) form the *A. baumannii* group which is of greatest importance in clinical settings (Towner, 2009). However, since the implementation of molecular techniques such as RNA polymerase subunit B (*rpo*B) gene sequencing, that allow correct identification of *Acinetobacter* spp. to the species level (Gundi et al., 2009; La Scola et al., 2006), the role of species other than *A. baumannii* in human infections has been elucidated (Fitzpatrick et al., 2015; Krizova et al., 2015; Li et al., 2015; Mostachio et al., 2012; Schleicher et al., 2013; Turton et al., 2010) as well as their presence in non-health care associated niches.

The roles of food and water as potential source of human infections and of food sources of dissemination of *Acinetobacter* to and within healthcare settings is not yet clear (Amorim and Nascimento, 2017; Berlau et al., 1999; Gholam-Mostafaei et al., 2017).

Foods have long been known to be an important reservoir of Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella* spp., and also *Acinetobacter* spp. (Amorim and Nascimento, 2017; Peleg et al., 2008). These organisms have been recovered from a variety of food products such as vegetables, fruit (Carvalheira, et al., 2017b; Ruimy et al., 2010), meat (Carvalheira et al., 2017a; Rafei et al., 2015), fish (Brahmi et al., 2016; Ture et al., 2018), milk (Li et al., 2018; Quigley et al., 2013), cheese (Riquelme et al., 2015; Xue et al., 2018), and drinking water (Narciso-da-Rocha et al., 2013; Vaz-Moreira et al., 2017).

It is recognized that meals served in healthcare facilities can be vehicles of pathogenic bacteria specially for vulnerable individuals and several outbreaks had been reported (reviewed by Lund, 2019). Underlying causes for these outbreaks are diverse and cross-contamination in the kitchens should not be ignored (Konecka-Matyjek et al., 2012; Miller et al., 2018). Moreover, contaminated foods can be a source of dissemination in the health care settings environment. *Acinetobacter* is one of the most commonly isolated bacteria from hospital kitchens with poor systems of sanitation (Gholam-Mostafaei et al., 2017; Lazarević et al., 2013; Stellato et al., 2015). The capacity of *Acinetobacter* spp. to survive for long periods on both dry surfaces and in water (e.g. kitchen sinks), to form biofilms and to resist disinfectants facilitate long-term persistence in the environment (Antunes et al., 2011; Doughari et al., 2011; Gaddy et al., 2009; Jawad et al., 1996; Visca et al., 2011; Weber et al., 2010). Literature regarding the association of *Acinetobacter* spp. with foodborne illnesses is limited and the few reports available are merely related with immunocompromised individuals and other high-risk groups such as young children (Grotiuz et al., 2006; Polanco and Manzi, 2008; Rathinavelu et al., 2003; Regalado et al., 2009; Silva and Lipinski, 2014). Nevertheless, there is evidence to suggest that *Acinetobacter* spp. may colonize the digestive tracts through the consumption of contaminated food. For example, *A. lwoffii* was recognized as capable of inducing gastritis (Rathinavelu et al., 2003) and reported as responsible for community-acquired gastroenteritis (Regalado et al., 2009). *A. baumannii* and *A. calcoaceticus* were isolated from the feces of children under five years of age presenting with acute diarrhea and showed toxic activity to cell cultures (Polanco and Manzi, 2008). The isolation of *Acinetobacter haemolyticus* from feces of infants younger than one year of age were described in two cases of bloody diarrhea: one study reported the production of Shiga toxin 2 (Grotiuz et al., 2006) and the other associated *A. haemolyticus* with bloody diarrhea and haemolytic uremic syndrome (Silva and Lipinski, 2014).

The present review aims to summarize the current literature on the occurrence of *Acinetobacter* spp. in food products as recognition of the pressing need for a better understanding of food as a significant reservoir of these organisms (Amorim and Nascimento, 2017; Tables 1.1-1.5).

1.2. Occurrence of *Acinetobacter* spp. in food and drinking water

1.2.1. Fruits and vegetables

Consumer demands for fresh fruits and vegetables are increasing. The European legislation imposes several microbiological criteria as indexes of food hygiene and safety. These are usually related to the recovery of microorganisms such us *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* (EFSA, 2013). However, other bacteria like *Acinetobacter* spp., which may be present in lower numbers, may influence the quality and safety of these foods. Fruits and vegetables can be consumed without further preparation or treatment and are therefore potential vehicles for the introduction of pathogenic organisms (e.g. *Acinetobacter* spp.) into human beings. Moreover, these ready-to-eat foods have been recognized as a potential route by which these bacteria may be introduced into hospitals with implications for infection control (Berlau et al., 1999). Campos et al. (2019) concluded that disinfection of ready-to-eat products such as vegetables and fruits could be done with vinegar during at least 15 min or AMUKINA® during at least 30 s. Other studies showed that household washing treatments (water rinsing, a mild hypochlorite treatment and dipping in vinegar solution) were inefficient in their removal from salads (Houang et al., 2001; Tatsika et al., 2019).

A summary of the *Acinetobacter* species recovered from fruit and vegetables is presented in table 1.1.

Several studies reported a low prevalence of these organisms on fruits and vegetables. For example, in a study conducted by Hamilton-Miller and Shah (2001) only two isolates of *Acinetobacter* spp. were recovered from 28 samples of several salad vegetables. Oie et al. (2008) analysed the microbial contamination of 36 fruits and 64 vegetable samples, after washing or washing followed by disinfection, and only one isolate of *Acinetobacter* spp. was recovered and identified to the species level as *A. baumannii* using API system. Similarly, Bezanson et al. (2008) and Khiyami et al. (2011) only recovered one and five isolates of *Acinetobacter* spp., respectively, from salad vegetables.

However, other studies reported a higher frequency of isolation of *Acinetobacter* spp., including species belonging to the *A. baumannii* group and multi-drug resistant strains. Berlau et al. (1999) recovered *Acinetobacter* spp. from 17% of 117 fresh fruits and vegetables (apples, melons, cabbages, cauliflowers, lettuce, cucumbers, peppers, mushrooms, radishes, carrots, potatoes and sweet corn); these organisms were at low levels

(50–1000 CFU/g). *A. baumannii* and *A. guillouiae* (each with a frequency of 27%) were the predominant species isolated, followed by *A. calcoaceticus* and *A. pittii* (each at 13%). *A. nosocomialis* was found only once. Most of the isolates belonged to the *A. baumannii* group (56%) and showed higher resistance to ciprofloxacin and gentamicin than the other species.

In Hong Kong, 21 samples of local vegetables were analysed and 51% were culture positive for organisms of this genus. *A. pittii* (33%) was the most common species isolated and *A. baumannii* was identified from one sample (Houang et al., 2001).

In France, 227 *Acinetobacter* isolates were recovered from 399 samples of raw fruits (apple, peach, pear and strawberry) and vegetables (carrot, celery, cucumber, lettuce, radish and tomato) analysed. *A. calcoaceticus* (78.9%) was the species most commonly identified but the species *A. baumannii*, *A. johnsonii*, *A. junii*, *A. lowfii* and *A. haemolyticus* were also recovered. These isolates showed resistance to piperacillin (67%), cefotaxime (63%), ceftazidime (47%), ticarcillin (27%), ticarcillin/clavulanic acid (14%) and piperacillin/tazobactam (10%) (Ruimy et al., 2010).

MDR *A. baumannii* and *A. lwoffii* have been detected in carrots (n=50) originating from crops irrigated with water from the Jakara River in Nigeria, into which domestic, hospital, commercial, and industrial sewage is discharged (Dahiru and Enabulele, 2015). A strain of carbapenem-resistant *A. calcoaceticus* was recovered from vegetables cultivated in Lebanon (Al Atrouni et al., 2016).

In a study conducted by Carvalheira et al. (2017b), *Acinetobacter* spp. were isolated from 86.7% of lettuce and 70.0% of fruit (apples, pears, strawberries, and bananas) samples marketed in Portugal. *A. calcoaceticus* and *A. johnsonii* were the most common species (26.5% each), but other species such as *A. baumannii*, *A. pittii*, *A. seifertii* and *A. nosocomialis* that belong to the *A. baumannii* group were also recovered (11.0%). *A. guillouiae*, *A. beijerinckii*, *A. baylyi, A. lwoffii, A. soli, A. radioresistens, A. ursingii*, *A. parvus*, *A. variabilis*, *A. bereziniae* and *A. junii* were also isolated but less frequently. About 29.8% of the isolates were classified as multidrug-resistant (MDR), 4.4% as extensively drug-resistant (XDR) and the prevalence of MDR strains within the *A. baumannii* group (25%) was similar to other species (30.4%). Another notable result was the resistance of 13.3% of the isolates to colistin, the last resort for treatment of MDR *Acinetobacter.* A third-generation cephalosporin-resistant *Acinetobacter* spp. was found among fruits, vegetables and the agricultural environment in Algeria (Zekar et al., 2017).

In a study conducted by Tatsika et al. (2019) the bacterial communities of commercial rocket and spinach ready-to-eat salads were evaluated by means of 16S rRNA gene high-throughput sequencing. *A. johnsonii* was found in all the samples analysed with a relative abundance of 7.9% among all the recovered isolates from several genus.

Also remarkable is the fact that these microorganisms were found as endophytes (i.e. present within the plant tissue) in papaya (Krishnan et al., 2012) and lettuce samples (Carvalheira, et al., 2017b).

Table 1.1. *Acinetobacter* species recovered from vegetables and fruits.

***Acinetobacter* species**	**Origin of isolation**	**Identification method**	**References**
A. baumannii	Vegetable	Phenotypic	Bezanson et al., 2008; Dahiru and Enabulele, 2015
		16S rRNA, *rpo*B	Ruimy et al., 2010
		*rpo*B	Carvalheira et al., 2017b
		ARDRA	Berlau et al., 1999b; Houang et al., 2001
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
		Phenotypic	Oie et al., 2008
A. baylyi	Fruit	*rpo*B	Carvalheira et al., 2017b
A. beijerinckii	Vegetable	*rpo*B	Carvalheira et al., 2017b
	Fruit	*rpo*B	Carvalheira et al., 2017b
A. bereziniae	Vegetable	ARDRA	Berlau et al., 1999b; Houang et al., 2001
		*rpo*B	Carvalheira et al., 2017b
A. calcoaceticus	Vegetable	MALDI-TOF MS	Zekar et al., 2017
		Phenotypic, *rpoB*	Rafei et al., 2015
		ARDRA	Berlau et al., 1999b
		MALDI-TOF MS, *rpoB*	Al Atrouni et al., 2016
	Fruit	16S rRNA, *rpoB*	Ruimy et al., 2010
		MALDI-TOF MS	Zekar et al., 2017

		rpoB	Carvalheira et al., 2017b
A. guillouiae	Vegetable	ARDRA	Berlau et al., 1999b
		*rpo*B	Carvalheira et al., 2017b
	Fruit	*rpo*B	Carvalheira et al., 2017b
A. johnsonii	Vegetable	16S rRNA, *rpo*B	Ruimy et al., 2010
		16S rRNA	Tatsika et al., 2019
		*rpo*B	Carvalheira et al., 2017b
		Phenotypic	Gennari and Lombardi, 1993
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
		*rpo*B	Carvalheira et al., 2017b
A. haemolyticus	Vegetable	16S rRNA, *rpo*B	Ruimy et al., 2010
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
A. junii	Vegetable	16S rRNA, *rpo*B	Ruimy et al., 2010
		*rpo*B	Carvalheira et al., 2017b
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
A. lwoffii	Vegetable	Phenotypic	Gennari and Lombardi, 1993; Dahiru and Enabulele, 2015
		16S rRNA, *rpo*B	Ruimy et al., 2010
		ARDRA	Berlau et al., 1999b
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
		*rpo*B	Carvalheira et al., 2017b
A. nosocomialis	Vegetable	ARDRA	Berlau et al., 1999b
	Fruit	*rpo*B	Carvalheira et al., 2017b
A. parvus	Fruit	*rpo*B	Carvalheira et al., 2017b
A. pittii	Vegetable	MALDI-TOF MS	Zekar et al., 2017
		Phenotypic, *rpo*B	Rafei et al., 2015
		ARDRA	Berlau et al., 1999b; Houang et al., 2001
		*rpo*B	Carvalheira et al., 2017b
	Fruit	MALDI-TOF MS	Zekar et al., 2017
		*rpo*B	Carvalheira et al., 2017b
A. radioresistens	Fruit	*rpo*B	Carvalheira et al., 2017b
A. schindleri	Fruit	*rpo*B	Carvalheira et al., 2017b

A. seifertii	Fruit	*rpo*B	Carvalheira et al., 2017b
A. soli	Vegetable	Phenotypic, *rpo*B	Rafei et al., 2015
	Fruit	*rpo*B	Carvalheira et al., 2017b
A. ursingii	Fruit	*rpo*B	Carvalheira et al., 2017b
A. variabilis	Fruit	*rpo*B	Carvalheira et al., 2017b
A. venetianus	Vegetable	Phenotypic, DNA-DNA hybridization, AFLP, *rpo*B, ARDRA, tDNA PCR	Vaneechoutte et al., 2009
Acinetobacter spp.	Vegetable	Phenotypic	Jiwa et al., 1981; Gennari and Lombardi, 1993; Hamilton-Miller and Shah, 2001
		ARDRA	Houang et al., 2001
		16S rRNA, *rpo*B	Ruimy et al., 2010
		16S rDNA	Khiyami et al., 2011
		MALDI-TOF MS	Zekar et al., 2017
		*rpo*B	Carvalheira et al., 2017b
	Fruit	16S rRNA, *rpo*B	Ruimy et al., 2010
		*rpo*B	Carvalheira et al., 2017b
		MALDI-TOF MS	Zekar et al., 2017
		16S rRNA	Krishnan et al., 2012

1.2.2. Meat

Food animals have been recognized as a potential reservoir for *Acinetobacter* spp. in different countries including Argentina (Ledesma et al., 2017), China (Wang et al., 2012; Zhang et al., 2013), France (Poirel et al., 2012), Germany (Wilharm et al., 2017), Senegal (Kempf and Rolain, 2012), the UK (Hamouda et al., 2011; Hamouda et al., 2008), Korea (Nam et al., 2009) and Lebanon (Al Bayssari et al., 2015; Rafei et al., 2015); and carbapenemase-producing isolates had also been described (Al Bayssari et al., 2015; Pailhoriès et al., 2016; Poirel et al., 2012; Wang et al., 2012; Webb et al., 2016; Zhang et al., 2013). Nevertheless, studies about the prevalence of *Acinetobacter* spp. in raw meat samples are limited (Carvalheira et al., 2017a; Hamouda et al., 2008; Houang et al., 2001; Lupo et al., 2014; Rafei et al., 2015). While the antimicrobial susceptibility of the isolates was not reported by some authors (Houang et al., 2001), others only refer to the species *A. baumannii* (Hamouda et al., 2008; Lupo et al., 2014).

High recovery rates of *Acinetobacter* spp. from meat samples have been reported in Hong Kong (75% of 36 pork and beef samples; Houang et al., 2001), Lebanon (28% of 50 cow meat samples; Rafei et al., 2015) and Portugal (100% of 50 chicken, turkey, beef and pork samples (Carvalheira et al., 2017a). High genetic diversity in population structures was observed among the genus (Carvalheira et al., 2017a; Rafei et al., 2015) as well as among the species *A. baumannii* (Lupo et al., 2014). A summary of the *Acinetobacter* species recovered from meats is presented in table 1.2.

Diversity of *Acinetobacter* species in meat samples was evaluated by Rafei et al. (2015). Three species, namely *A. baumannii*, *A. pittii*, and *A. bereziniae* were detected by *rpo*B gene sequencing.

In a study conducted by Carvalheira et al. (2017a) thirteen species were identified, by *rpo*B gene sequencing. *A. guillouiae* (34.9%), *A. johnsonii* (15%), and *A. bereziniae* (12%) were the most prevalent but species that belong to the *A. baumannii* group as *A. baumannii* (n=7), *A. pittii* (n=12), *A. seifertii* (n=8), *A. nosocomialis* (n=4) and *A. calcoaceticus* (n=3) were also detected as well as *A. ursingii*, *A. gyllenbergii*, *A. gerneri*, *A. parvus* and *A. radioresistens*.

Marí-Almirall et al. (2019) analysed 138 meat samples from poultry, swine and beef from Lima (Peru) and twelve isolates of the genus *Acinetobacter* were recovered from five different calves meat samples. Strains were identified, by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) and sequencing of the partial *rpo*B gene sequences, as members of the *A. baumannii* group: *A. pittii* (n=9), *A. baumannii* (n=1), *A. dijkshoorniae* (n=1, a later heterotypic synonym of *A. lactucae*; (Dunlap and Rooney, 2018) and one isolate could not be identified to the species level unambiguously.

Other studies only refer to the prevalence of the species *A. baumannii*. While Hamouda et al. (2008) did not detect this species in 27 meat samples (cow, chicken and pork) from retail supermarket chains in Edinburgh, it was recovered by Lupo et al. (2014) in 25% of 248 meat samples (chicken, turkey, veal, beef and pork) in Switzerland, based on MALDI-TOF MS, among which poultry meat was the most frequently contaminated. Besides 22 isolates were identified as *A. baumannii*, by ERIC-PCR method, from 126 animal meat samples (chicken, bovine, camel, turkey and ovine) by Tavakol et al. (2018). *A. baumannii* was also isolated from 20.1% out of 194 different types of raw meat samples (bovine, ovine, caprine, camel, chicken and turkey) in Isfahan (Iran) by Askari et al. (2020) and ovine raw meat was the most commonly contaminated samples (32.1%).

Lupo et al. (2014) suggested the clonal diffusion of *A. baumannii* in the food chain setting and a common source of contamination (i.e., breeding, slaughter, or meat processing and packing) which remained undetermined. Therefore, stricter hygiene and hazard analysis and critical control point principles should be implemented along the entire meat production chain up to the retail setting to identify sources of contamination and prevent potentially pathogenic bacteria from reaching consumers.

Acinetobacter spp. have been described as spoilage organisms of meat products even when stored under refrigeration or following irradiation treatment (Gennari et al., 1992). Saha and Chopade (2001) compared the population of *Acinetobacter* spp. in fresh meat, spoiled meat and meat stored at freezer and refrigerator temperatures, and concluded that *Acinetobacter* spp. were the dominant organisms (38.3%) only in meat stored at refrigeration temperature while in the other cases *Acinetobacter* spp. were the second dominant bacteria (31.6% in fresh meat, 18.5% in spoiled meat and 10.4% in meat stored at freezing temperatures) following *Pseudomonas*. Lee et al. (2017) also investigated the psychrotrophic bacteria isolated from chicken meat to characterize their microbial composition during refrigerated storage and *Acinetobacter* spp. was not recovered using a culture-dependent approach, whereas when bacterial communities were analyzed by an Illumina MiSeq platform, *Acinetobacter* was the third more common genus 3.45% (among 187,671 reads obtained).

The presence of *Acinetobacter* spp. strains resistant to antibiotics in meat may represent an additional concern for public health. Lupo et al. (2014) and Rafei et al. (2015) concluded that *A. baumannii* isolates found in meat were generally susceptible to clinically relevant antibiotics and resistance to colistin, ciprofloxacin, tetracycline, ceftazidime, and piperacillin-tazobactam was sporadically observed. Marí-Almirall et al. (2019) reported a high susceptibility of eleven isolates of the *A. baumannii* group However, *A. baumannii* isolates recovered by Tavakol et al. (2018) were frequently resistant to tetracycline, trimethoprim, cotrimoxazole and gentamicin but just a few isolates displayed low levels of resistance to imipenem, azithromycin, meropenem, rifampin, levofloxacin, ceftazidime and tobramycin. As well, *A. baumannii* strains recovered by Askari et al. (2020) harboured resistance to some of the categories of antimicrobials used to treat *A. baumannii* infections as gentamicin (87.1%), tetracycline (79.5%), erythromycin (74.4%), azithromycin (66.7%), ciprofloxacin (58.9%), trimethoprim/sulphamethoxazole (56.4%) rifampin (51.3%), chloramphenicol (28.2%) and imipenem (17.9%). Conversely, a high number of isolates analysed by Carvalheira et al. (2017a) were resistant to piperacillin-tazobactam (64.9%), ceftadidime (43.5%), ciprofloxacin (42.9%), as well as to colistin (41.7%) and polymyxin B (35.1%). It was also observed that 51.2% of the strains were considered as MDR, 9.6% as XDR and the prevalence of MDR strains within the *A. baumannii* group (38.7%) was lower than the prevalence within the others species identified (54.1%). Moreover, a wide variety of third-generation cephalosporin-resistant species were recovered from fresh pork and chicken meat specimen (n=120) from local supermarkets/butchers across Melbourne (Australia), among which *A. baumannii* complex was the most common (37%; McLellan et al., 2018).

The incidence of antibiotic resistant bacteria in meat samples has been attributed, at least partially, to the extensive use of antimicrobials for treatment, prevention and control of diseases in food-producing animals, since this enhances the antimicrobial selective pressure for strains present (Marshall and Levy, 2011; Müller et al., 2014). According to European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (EMA/ESVAC, 2013), tetracyclines (36.7%) and penicillins (24.5%) were the antimicrobials most often used for food-producing animals across the 26 EU/EEA countries in 2013. Polymixins, aminoglycosides and fluoroquinolones are also used but in a lower percentage (6.1%, 3.7% and 1.9%, respectively). On the other hand, as carbapenems antibiotics are not allowed to treat food-producing animals this may explain the low level of resistance to antibiotics of this class.

The presence of *Acinetobacter* spp. in meat is of special concern since it was demonstrated that it can survive more than 60 min under thermal processing at 60 °C (Campos et al., 2019).

Table 1.2. *Acinetobacter* species recovered from meat.

***Acinetobacter* species**	**Identification method**	**References**
A. baumannii	MALDI-TOF MS	Lupo et al., 2014; McLellan et al., 2018
	16S–23S rRNA	Tavakol et al., 2018
	Phenotypic, *rpoB*	Rafei et al., 2015
	ARDRA	Houang et al., 2001
	rpoB	Carvalheira et al., 2017a
	16S rDNA	Lee et al., 2017
	MALDI-TOF MS, *rpoB*	Marí-Almirall et al., 2019
A. bereziniae	Phenotypic, *rpoB*	Rafei et al., 2015
	rpoB	Carvalheira et al., 2017a
A. calcoaceticus	Phenotypic, *rpoB*	Rafei et al., 2015
	16S rRNA, *rpoB*	Ruimy et al., 2010
	rpoB	Carvalheira et al., 2017a
A. gerneri	*rpoB*	Carvalheira et al., 2017a
A. guillouiae	*rpoB*	Carvalheira et al., 2017a
A. gyllenbergii	*rpoB*	Carvalheira et al., 2017a
A. johnsonii	Phenotypic	Gennari and Lombardi, 1993
	*rpo*B	Carvalheira et al., 2017a
A. lactucae	MALDI-TOF MS, *rpo*B	Marí-Almirall et al., 2019
A. lwoffii	Phenotypic	Gennari et al., 1992; Gennari and Lombardi, 1993
A. nosocomialis	*rpo*B	Carvalheira et al., 2017a
A. parvus	*rpo*B	Carvalheira et al., 2017a
A. pittii	Phenotypic, *rpo*B	Rafei et al., 2015
	*rpo*B	Carvalheira et al., 2017a
	MALDI-TOF MS, *rpo*B	Marí-Almirall et al., 2019
A. radioresistens	*rpo*B	Carvalheira et al., 2017a
A. seifertii	*rpo*B	Carvalheira et al., 2017a
A. ursingii	MALDI-TOF MS	McLellan et al., 2018
	*rpo*B	Carvalheira et al., 2017a

Acinetobacter spp.	Phenotypic	Eribo and Jay, 1985; Gennari et al., 1992;Gennari and Lombardi, 1993
	ARDRA	Houang et al., 2001
	Phenotypic, *rpo*B	Rafei et al., 2015
	*rpo*B	Carvalheira et al., 2017a
	16S rDNA	Lee et al., 2017
	MALDI-TOF MS, *rpo*B	Marí-Almirall et al., 2019

1.2.3. Fish

Acinetobacter spp. were regarded as emerging opportunistic bacteria for aquaculture in different parts of the world. *Acinetobacter* species were isolated from diseased fish in China (Li et al., 2017; Xia et al., 2008) and India (Behera et al., 2017; Rauta et al., 2011), form the altered skin and gills of sharptooth catfish living in the Nile River in Egypt (El-Sayyad et al., 2010) as well as from diseased rainbow trout and common carp cultured in Poland (Kozińska et al., 2014). The association of *Acinetobacter* spp. with bacteremia and gastritis (Kozińska et al., 2014; Turton et al., 2010; Zavros et al., 2002), suggests that fish could also represent the route for potential transmission of pathogens to humans. The species *A. johnsonii, A. lwoffii*, *A. pittii* and *A. baumannii* have been implicated as fish pathogen in the last years (El-Sayyad et al., 2010; Kozińska et al., 2014; Li et al., 2017; Rauta et al., 2011; Xia et al., 2008) however, it is important to highlight that the species identification was assessed by biochemical tests which are recognized as non-reliable methods to identify *Acinetobacter* isolates to the species level (Chang et al., 2005; Dijkshoorn et al., 2007) and by sequencing analysis of 16S rRNA gene fragment; however *rpo*B gene sequencing is recognized as the most accurate method for the identification of *Acinetobacter* species, because of the abundance of *rpo*B polymorphisms in these species (Wang et al., 2014; Gundi et al., 2009; La Scola et al., 2006).

Acinetobacter spp. have been recovered from freshwater fish in China (Houang et al., 2001), Spain (González et al., 2000), Serbia (Ksenija et al., 2017), Turkey (Ture et al., 2018), Norway and Scotland (Holben et al., 2002).

Dekić et al. (2018) suggested that the colonization potential of *Acinetobacter* spp. in freshwater fish is dependent upon its concentration in surrounding water and concluded that closed water bodies where there is constant inflow of water polluted by *A. baumannii* in concentrations above 3 log CFU/mL may represent risk for public health while lower concentration of *A. baumannii* in natural waters represents low colonization potential of freshwater fish.

A summary of the *Acinetobacter* species recovered from fish is presented in table 1.3.

Table 1.3. *Acinetobacter* species recovered from fish.

***Acinetobacter* species**	**Identification method**	**References**
A. baumannii	ARDRA	Houang et al., 2001
	16S rRNA, MALDI-TOF MS	Ksenija et al., 2017
	MALDI-TOF MS	Brahmi et al., 2016
	Phenotypic, 16S rDNA	Xia et al., 2008; Rauta et al., 2011
	Phenotypic	Gu et al., 1996
	16S rRNA	Behera et al. 2017
A. johnsonii	16S rRNA	Ture et al., 2018
	Phenotypic, 16S rDNA	Kozińska et al., 2014
	Phenotypic	Gennari and Lombardi, 1993; González et al., 2000
A. junii	16S rDNA	Holben et al., 2002
A. lwoffii	16S rRNA, MALDI-TOF MS	Ksenija et al., 2017
	Phenotypic, 16S rDNA	Kozińska et al., 2014
	Phenotypic	Gennari and Lombardi, 1993; González et al., 2000; El-Sayyad et al., 2010
A. pittii	Phenotypic, 16S rDNA, *rpo*B	Li and Cao, 2017
Acinetobacter spp.	Phenotypic	Jiwa et al., 1981; Gennari and Lombardi, 1993; González et al., 2000
	ARDRA	Houang et al., 2001
	16S rRNA	Ture et al., 2018

Antibiotic resistance among strains isolated from fish has been reported, including OXA-23-producing strains and multidrug resistance (Brahmi et al., 2016; Kozińska et al., 2014; Xia et al., 2008). Therefore, the use of antibiotics in aquaculture is very undesirable. Moreover, as *Acinetobacter* spp. are recognized as microorganisms commonly transmitting antibiotic resistance genes, they might have a great impact on the resistance transfer in aquaculture.

1.2.4. Milk and Cheese

While the bacterial composition of cows' milk has been extensively studied for quite some time, new developments arising from DNA sequencing technologies have highlighted that the diversity of bacterial populations is greater than that originally thought and *Acinetobacter* was recognized as a member of the core milk microbiota (Kable et al., 2016; Li et al., 2018; Quigley et al., 2013). Using high-throughput sequencing, *Acinetobacter* spp. (21%) were also detected as the second dominant microorganisms in buffalo milk and throughout the manufacture of mozzarella cheese (Ercolini et al., 2012).

Milk storage at refrigeration temperatures reduces the growth of most bacteria except psychrotrophic populations, which can proliferate under cold storage and frequently include *Acinetobacter* spp. (Gennari et al., 1992; Hantsis-Zacharov and Halpern, 2007; Munsch-Alatossava and Alatossava, 2006; Raats et al., 2011; Uraz and Çitak, 1998). *Acinetobacter* spp. were also found in milk samples treated by thermization, microfiltration and addition of carbon dioxide during cold storage (Rasolofo et al., 2010).

Acinetobacter spp. were also identified as part of bacterial biodiversity of a variety of cheeses all over the world including Camembert cheese produced in different states of Australia (Addis et al., 2001), Danish raw milk cheeses (Masoud et al., 2011), Poro cheese (an artisanal Mexican cheese; Aldrete-Tapia et al., 2014), Pico cheese (an artisanal Azorean cheese; Riquelme et al., 2015) and Jianchuan Rushan cheese (Xue et al., 2018). *Acinetobacter* species were also associated with the process of maturation of Camembert cheeses since it was observed that its population increased during the mid–later stages. However, they were detected only after the growth of the yeast *Debaryomyces hansenii*, as well as other bacteria such as *Staphylococcus* and *Micrococcus* species (Addis et al., 2001). In the same study, *Acinetobacter* species were not isolated from two blue-veined cheese varieties, and this was associated with their inability to tolerate the high salt contents of the blue-veined varieties. However, when samples were analyse by culture-independent molecular methods, *Acinetobacter* ssp. were identified among the bacterial community of blue-veined raw milk cheese (Yunita and Dodd, 2018).

Acinetobacter species, including *A. baumannii*, have been isolated from raw cow milk and raw cow milk cheeses (Franciosi et al., 2011; Rafei et al., 2015) suggesting that this species was derived from the raw milk used in production. Most of the *A. baumannii* isolates were not resistant to clinically relevant antibiotics whereas other *Acinetobacter* species exhibited antibiotic resistance to tetracycline, trimethoprim-sulfamethoxazole, ceftazidime and ampicillin-sulbactam (Gurung et al., 2013).

The FAO-WHO microbiological risk assessments of powdered infant formula included *Acinetobacter* spp. as organisms of concern for neonatal health following the consumption of powdered infant formula (FAO/WHO, 2006). Indeed, infant milk formula intrinsically contaminated with *Acinetobacter* spp. has been reported (Araújo et al., 2015; Cawthorn et al., 2008; Chap et al., 2009; Marino et al., 2007; Miled et al., 2010). Multidrug-resistant *Acinetobacter* Abc complex was isolated (17 isolates, 37.8%) from infant milk formula (IMF) in a nursery in Rio de Janeiro, Brazil (Araújo et al., 2015). Most of the isolates presented a multidrug-resistance profile and showed high resistance to ampicillin-sulbactam (88.2%), cefotaxime (82.3%) and trimethoprim-sulfamethoxazole (70.6%); one of the MDR isolates was resistant to imipenem (an antibiotic from the carbapenems class). *Acinetobacter* spp. were also isolated from milk powder produced in Germany and most of the species were identified as *A. baumannii* (n=42) while the remaining isolates (n=5) were identified *A. pittii* (Cho et al., 2018). However, the strains in this study were generally not resistant to the clinically relevant antibiotics, especially tobramycin, ciprofloxacin, cefepime, and meropenem.

Moreover, the ability of these organisms to remain viable during long-term desiccation (two years of storage) in infant formula and to be recovered after reconstitution was demonstrated by Juma et al. (2016) and related to inadequate hygiene practices in the preparation or distribution of infant milk formula. According to the FAO/WHO risk assessment (FAO/WHO, 2006), the number of microorganisms is dramatically reduced when IMF is reconstituted with water heated to at least 70 °C.

A summary of the *Acinetobacter* species recovered from milk products is presented in table 1.4.

Table 1.4. *Acinetobacter* species recovered from milk and cheese.

***Acinetobacter* species**	**Origin of isolation**	**Identification method**	**References**
A. baumannii	Milk	Phenotypic	Munsch-Alatossava and Alatossava, 2006
		16S rRNA	Hantsis-Zacharov and Halpern, 2007
		*rpo*B, *gyr*B	Gurung et al., 2013
		Phenotypic, *rpoB*	Rafei et al., 2015
	Infant Milk Formula	Phenotypic,16S rDNA	Chap et al., 2009
	Cheese	Phenotypic, *rpoB*	Rafei et al., 2015
A. calcoaceticus	Milk	16S rRNA	Hantsis-Zacharov and Halpern, 2007; Rasolofo et al., 2010
	Cheese	Phenotypic	Addis et al., 2001
A. johnsonii	Milk	Phenotypic	Gennari and Lombardi, 1993; Munsch-Alatossava and Alatossava, 2006
		16S rRNA	Hantsis-Zacharov and Halpern, 2007
A. haemolyticus	Milk	16S rRNA	Hantsis-Zacharov and Halpern, 2007
A. lwoffii	Milk	Phenotypic	Gennari et al., 1992; Gennari and Lombardi, 1993
		16S rRNA	Hantsis-Zacharov and Halpern, 2007
		Phenotypic	Gennari and Lombardi, 1993
	Cheese	Phenotypic	Addis et al., 2001

A. pittii	Cheese	Phenotypic, *rpo*B	Rafei et al., 2015
A. rudis	Milk	Phenotypic, G+C, fatty acids, 16S rRNA, rpob, gyrB, DNA-DNA hybridization	Vaz-Moreira et al., 2011
Acinetobacter spp.	Milk	Phenotypic	Gennari et al., 1992; Gennari and Lombardi, 1993; Uraz and Çitak, 1998; Jayarao and Wang, 1999; Ndegwa et al., 2001; Munsch-Alatossava and Alatossava, 2006; Nam et al., 2009
		*rpo*B, *gyr*B	Gurung et al., 2013
		16S rRNA	Franciosi et al., 2011
			Rasolofo et al., 2010
			Quigley et al., 2013
			Kable et al., 2016; Li et al., 2018; Raats et al., 2011
	Infant Milk Formula	Phenotypic	Marino et al., 2007; Araújo et al., 2015
		16S rDNA	Cawthorn et al., 2008; Miled et al., 2010
		16S rDNA, Phenotypic	Chap et al., 2009
	Cheese	Phenotypic	Gennari et al., 1992; Addis et al., 2001
		16S rRNA	Xue et al., 2018
			Aldrete-Tapia et al.,

		2014; Ercolini et al., 2012
	16S rDNA	Masoud et al., 2011; Yunita et al., 2018
	16S rDNA	Riquelme et al., 2015

1.2.5. Drinking water

Acinetobacter spp. are found in unpolluted sites, such as ground, surface or tap water (McKeon et al., 1995; Pavlov et al., 2004). Several studies reported *Acinetobacter* as a persistent genus in tap water, as well as, the presence of different species of this genus in drinking water (Hauer et al., 1999; Leclerc and Moreau, 2002; Narciso-da-Rocha et al., 2013; Van Assche et al., 2019; Vaz-Moreira et al., 2017). A summary of the *Acinetobacter* species recovered from drinking water is presented in table 1.5.

Acinetobacter was reported as abundant in water samples from the drinking water ecosystem in Portugal (Vaz-Moreira et al., 2013) and detected in tap water in levels of 10^4 CFU/mL (Narciso-da-Rocha et al., 2013). Most of the isolates recovered belong to the species *A. johnsonii* and *A. lwoffii* but the species *A. parvus*, *A. tjernbergiae*, *A. pittii* and *A. beijerinckii* were also detected. Although, these isolates had low rates of antibiotic resistance and 80% of the isolates were wild type, the potential of some species to acquire antibiotic resistance and disseminate resistance via drinking water was suggested (Narciso-da-Rocha et al., 2013).

A general occurrence of *Acinetobacter* in drinking water production and distribution systems were also reported in Belgium, reaching real abundances of up to 47.5% in processed water samples and phylogenetic analysis revealed that the *Acinetobacter* sequences were closely related to the species *A. baumannii*, *A. pitti*, *A. nosocomialis*, *A. seifertii*, *A. dijkshoorniae*, *A. johnsonii*, *A. calcoaceticus* and the genomic species "between 1 and 3" (Van Assche et al., 2019).

Moreover, *Acinetobacter* spp. has been associated with waterborne hospital acquired infections (Williams et al., 2013). *A. lwoffii* was detected in the drinking water distribution system of a hospital in Hungary emphasizing that drinking water systems, especially those with stagnant water sections, could be the source of nosocomial infections (Felföldi et al., 2010). Likewise, an outbreak of amikacin and ciprofloxacin resistant *A. baumannii* in Tokai

University hospital's emergency intensive care unit was caused by its colonization in water systems and subsequent spread through oral care using tap water (Umezawa et al., 2015). This outbreak was successfully controlled after replacement of the water system and implementation of daily cleaning of water taps and oral care with a dry method, which highlight the importance of strict management of water systems in critical care areas.

Table 1.5. *Acinetobacter* species recovered from drinking water.

***Acinetobacter* species**	**Identification method**	**References**
A. baumannii	Phenotypic Phenotypic, OXA-51-like genes	Hauer et al., 1999 Umezawa et al., 2015
A. beijerinckii	*rpo*B, *rec*A, *gyr*B	Narciso-da-Rocha et al., 2013
A. johnsonii	*rpo*B, *rec*A, *gyr*B	Narciso-da-Rocha et al., 2013
A. lwoffii	*rpo*B, *rec*A, *gyr*B Phenotypic 16S rDNA	Narciso-da-Rocha et al., 2013 Gennari and Lombardi, 1993 Felföldi et al., 2010
A. parvus	*rpo*B, *rec*A, *gyr*B	Narciso-da-Rocha et al., 2013
A. pittii	*rpo*B, *rec*A, *gyr*B	Narciso-da-Rocha et al., 2013
A. tjernbergiae	*rpo*B, *rec*A, *gyr*B	Narciso-da-Rocha et al., 2013
Acinetobacter spp.	16S rRNA	Vaz-Moreira et al., 2013; Van Assche et al., 2019

The origin of *Acinetobacter* in surface water seems to be influenced by the wastewater treatment process which contributes to the selective increase of antibiotic resistant bacteria and the occurrence of multidrug-resistant bacteria in aquatic environments (Zhang et al., 2009). *A. baumannii* shown to be approximately 1000-times more resistant to chlorine than *E. coli* (Karumathil et al., 2014) and it was demonstrated that the relative abundance of *Acinetobacter* increased in the tap water disinfected with NaClO (Ma et al., 2020). Moreover, *A. calcoaceticus* was shown to be a key microorganism in the resistance to disinfection and resilience of multispecies biofilms formed by drinking water-isolated bacterial species (Simões et al., 2010). This was related to the ability of this bacterium to coaggregate with almost all other bacteria and increasing the opportunity for metabolic cooperation (Simões et al., 2008).

Drinking water deserves attention as a reservoir and vehicle for the dissemination of *Acinetobacter* spp.; the hazardous potential may be aggravated by the reduced antimicrobial susceptibility.

1.3. Occurrence of *Acinetobacter* spp. in food processing environment

Environmental contamination plays an important role in the nosocomial transmission of *Acinetobacter* spp. (Hota, 2004; Otter et al., 2011, 2013) since these bacteria can survive for prolonged periods in the environment (Chemaly et al., 2014; Kramer et al., 2006; Otter et al., 2013; Weber et al., 2010) and many outbreaks have been associated with frequent surface contamination (Aygün, 2002; Cheng et al., 2018; Karageorgopoulos and Falagas, 2008; Yagnik et al., 2019). However, only a few studies investigated the possible role of food in the occurrence of hospital-acquired infections and the transmission of pathogenic bacteria onto the hospital surfaces (Berlau et al., 1999).

Foods can be vehicles of pathogenic microbes and several bacterial species can reside in kitchens, preparation rooms and storage facilities. It was demonstrated that kitchen environments host more microbes than toilets (Donofrio et al., 2012; Marshall et al., 2012; Ojima et al., 2002). The composition of the microbiota in the foodservice environments is influenced by the characteristics of the specific surfaces and by food handling and processing practices. Food contact surfaces contain good substrates for the proliferation of microorganisms since organic residues that accumulate during food processing can create microenvironments for microbial growth representing a relevant source of cross-contamination (Brooks and Flint, 2008; McLandsborough et al., 2006; Simões et al., 2010). Therefore, food services are considered critical to the health and the evaluation of their microbiological status is recognized as fundamental, especially for those where many meals per day are prepared for vulnerable people (Konecka-Matyjek et al., 2012).

Acinetobacter was shown to be a member of the resident microbiota of processing plants. In the study of Lazarević et al. (2013), *Acinetobacter* spp. was one of the most commonly isolated bacteria from working surfaces, equipment, and kitchen utensils in hospital kitchens. Its presence was also detectd in the kitchen sink and kitchen food preparation areas of a typical district hospital in South Africa (Setlhare et al., 2013) and in a teaching hospital in Iran, *Acinetobacter* spp. was isolated from 2.5% of 200 kitchen samples (Gholam-Mostafaei et al., 2017b).

Stellato et al. (2015) evaluated the biogeographical distribution of the microbiota in a hospital cooking center (including food pre-processing rooms dedicated to fish, vegetables, and red and white meat, storage room and kitchen) after the routine cleaning by 16S-based culture-independent high-throughput amplicon sequencing. It was demonstrated that the microbiota of environmental swabs was very complex, including more than 500 operational taxonomic units (OTUs). A core microbiota was common across all areas (more than 70% of the samples analyzed) and included species of *Acinetobacter*. Indeed, *Acinetobacter* was the most abundant genera that occurred in all the samples (13.6%), as well as, the most abundant genera shared between all the zones of the pre-processing area, kitchen, storage area and tools. It was also abundant in kitchen utensils such as colander, steel shovel and cutting board from pre-processing vegetables and occurred in the sink and workbench samples belonging to pre-processing vegetable area and white meat pre-processing area with an abundance above 40%.

Acinetobacter was also reported as the second most frequently isolated genera (17%) from a cafeteria kitchen. Isolates demonstrated strongly or moderately biofilm-forming capacity, exhibited sensitivity to the disinfectants NaClO and H_2O_2 but were significantly resistant to benzalkonium chloride, lactic acid and citric acid (Lim et al., 2017).

Although cleaning and disinfection procedures are essential to assure acceptable hygienic conditions in the food processing environments (Lazarević et al., 2013; Setlhare et al., 2013) they may fall short in the elimination of a well-developed microbiota which tend to settle on sites that are especially difficult to clean due to difficult access, surface irregularities or retention of sticky raw materials. Moreover, cleaning procedures can have a strong impact on the spatial distribution of the microbial communities, as the use of the same cleaning tools can be even a possible vector of bacterial diffusion. For example, kitchen sponges were proven to represent the biggest microbiological hot spots with the capability to collect and spread bacteria over kitchen surfaces (Chen et al., 2001; Donofrio et al., 2012; Greig and Ravel, 2009; Josephson et al., 1997; Kusumaningrum et al., 2003; Marshall et al., 2012; Mattick et al., 2003; Rossi et al., 2013; Scott, 2000). *Acinetobacter* spp., including *A, johnsonii, A. pittii* and *A. ursingii,* were detected as belonging to the bacterial microbiome of used kitchen sponges and sponge sanitation methods appear not to be sufficient to effectively reduce the bacterial load and might even increase the presence of these bacteria (Cardinale et al., 2017).

1.4. Conclusion

Only a few studies worldwide concentrated on the population of *Acinetobacter* spp. in food products. Whereas some studies only refered to the prevalence of the species *A. baumannii*, others did not evaluate the antimicrobial susceptibility of the isolates which is of major importance taking into account the high capacity of transference of antibiotic resistance.

Moreover, among the available data, there could be some misconceptions since the isolation methods used have a huge impact on the recovery of these organisms and some identification techniques such automated systems like Phoenix, Vitek2 and API 20NE are not able to differentiate species among this genus (Abbott and Peleg, 2014; Dijkshoorn et al., 2007; Eveillard et al., 2013; Peleg et al., 2008; Towner, 2009; Visca et al., 2011).

Nevertheless, the occurrence of several species including the *A. baumannii* group, which are the species most frequently associated with nosocomial infections worldwide, has been reported in food products and drinking water. Likewise, resistance of these organisms to several antimicrobials, including carbapenems and even to colistin and polymyxin, the last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter,* represent an additional concern. This highlighted the importance of assessing the role of foods in the occurrence of infections and transmission of these bacteria into the community and hospital environments which is an important step to design and evaluate strategies to control the spread of multidrug-resistant strains, as well as, to control and prevent *Acinetobacter* spp. infection.

The emergence of nosocomial or community-acquired *Acinetobacter* spp. infections is a result of high adaptability to adverse environmental conditions, long-term survival on numerous surfaces and inanimate objects and a high degree of resistance to desiccation, disinfectants and antibiotics. Once “installed”, these organisms are extremely difficult to eradicate. Thus, avoiding the spread of *Acinetobacter* spp. in the environment, in which food and drinking water have an important role, maybe an effective way to prevent or control infections.

Therefore, characterization of *Acinetobacter* isolates e.g. antibiotic resistance profiles and molecular patterns, among isolates recovered from foods, drinking water, hospital environments (including food service areas), and patients’ clinical samples is required to identify and control possible transmission routes.

CHAPTER 2

Enrichment of *Acinetobacter* spp. from food samples

CHAPTER 2

Abstract

Relatively little is known about the role of foods in the chain of transmission of acinetobacters and the occurrence of different *Acinetobacter* spp. in foods. Currently, there is no standard procedure to recover acinetobacters from food in order to gain insight into the food-related ecology and epidemiology of acinetobacters. This study aimed to assess whether enrichment in Dijkshoorn enrichment medium followed by plating in CHROMagar^{TM} Acinetobacter medium is a useful method for the isolation of *Acinetobacter* spp. from foods. Recovery of six *Acinetobacter* species from food spiked with these organisms was compared for two selective enrichment media (Baumann's enrichment and Dijkshoorn's enrichment). Significantly ($p<0.01$) higher cell counts were obtained in Dijkshoorn's enrichment. Next, the Dijkshoorn´s enrichment followed by direct plating on CHROMagar^{TM} Acinetobacter was applied to detect *Acinetobacter* spp. in different foods. Fourteen different presumptive acinetobacters were recovered and assumed to represent nine different strains on the basis of REP-PCR typing. Eight of these strains were identified by *rpoB* gene analysis as belonging to the species *A. johnsonii*, *A. calcoaceticus*, *A. guillouiae* and *A. gandensis*. It was not possible to identify the species level of one strain which may suggests that it represents a distinct species.

2.1. Introduction

The genus *Acinetobacter* currently comprises 38 validly named species (Euzéby, 2015). Of these, the species included in the so called *Acinetobacter calcoaceticus-A. baumannii* (Acb) complex in particular are opportunistic pathogens associated with considerable morbidity and mortality (Peleg and Hooper, 2010). Despite hospital-acquired infections being the most common, reports of community-associated infections caused by strains of the Acb complex have also increased during the past decade (Anstey et al., 2002; Anstey et al., 1992; Bick and Semel, 1993; Nonaka et al., 2014; Visca et al., 2011). The sources and mode of transmission of these pathogens, both in hospital settings and in the community, and their natural reservoir remain largely unknown albeit that *A. baumannii* seems mainly confined to the human and animal host (Dijkshoorn et al., 2007).

Acinetobacter spp. have been recovered from a variety of foods, including vegetables, dairy products, drinks and water (Berlau et al., 1999; Gennari and Lombardi, 1993; Girão et al., 2013; Hamilton-Miller and Shah, 2001; Narciso-da-Rocha et al., 2013) and from food producing animals (Hamouda et al., 2011; Poirel et al., 2012; Wang et al., 2012). However, the importance of food as a source for colonization with acinetobacters is largely unknown. One study among ICU patients indicated that the digestive tract could be a reservoir of multidrug-resistant *A. baumannii* for infections in hospital outbreaks (Corbella et al., 1996). In order to determine the potential of foods as a vehicle of pathogenic *Acinetobacter* spp. transmission to humans, it is important that a standard method is available for their recovery from food. Enrichment cultivation followed by subculturing on differential media is common practice in food microbiology to recover microorganisms that can be present in low numbers.

For the recovery of *Acinetobacter* spp., a vigorously aerated liquid medium at low pH, supplemented with acetate or another suitable carbon source and with nitrate as the nitrogen source and incubation at 30 °C, has proven useful in several studies(Baumann, 1968; Grehn and von Graevenitz, 1978; Guardabassi et al., 1999). Dijkshoorn et al. (1987), and Zanetti et al. (2007) used another mineral medium (Monod and Wollman, 1947), also enriched with sodium acetate. Nutritional studies have also shown that the basic medium of Cruze et al. (1979) enriched with various C-sources can be used to grow acinetobacters (e.g. Nemec et al., 2000).

This study aimed to compare two acetate mineral media for the recovery of low levels of strains of six different *Acinetobacter* species. Enrichment was followed by direct plating on selective and differential CHROMagar^{TM} Acinetobacter medium. This approach was then applied, using the medium with the highest recovery capacity, to detect *Acinetobacter* spp. in food samples.

2.2. Materials and methods

2.2.1. Bacterial strains

Six strains belonging to different species of *Acinetobacter* (Table 2.1) were used in this study. Stock cultures were kept in Tryptone Soya Broth (TSB, Merck, Darmstadt, Germany) supplemented with 30% (w/v) of glycerol at -80 ºC. Working cultures were sub-cultured twice in TSB (1% v/v) and incubated at 30 ºC for 24 h, before use.

Table 2.1. Strains of *Acinetobacter* spp. used in the present study.

Strain code	Specimen	Geographical origin	Year of isolation/ Deposited in a Culture Colection
A. baumannii 002[a]	Perineal sample	Braga, Portugal	2004
A. pittii T1BP1[b]	Tap water	Porto, Portugal	2009
A. lwoffii T7BT5[b]	Tap water	Porto, Portugal	2009
A. johnsonii CCUG 19095^T	Duodenum	-	1986[c]
A. calcoaceticus CCUG 12804^T	Soil	-	1909
A. junii CCUG 889^T	Human urine	-	1970 [c]

[a] Deposited in the private culture collection of Escola Superior de Biotecnologia, Universidade Católica Portuguesa (Porto, Portugal). Identification to species level was based on the analysis of the sequence of the region Z1– Z2 of the gene for RNA polymerase beta-subunit (*rpoB*) according to La Scola et al. (2006).

[b] Isolated by Narciso-da-Rocha et al. (2013)

[c] Deposited in a Culture Colection

CCUG: Culture Collection University of Gothenburg (Göteborg, Sweden)

2.2.2. Comparison of two selective enrichment broths for the recovery of *Acinetobacter* spp.

Baumann's enrichment medium and Dijkshoorn enrichment medium were prepared from basic ingredients, as they are not commercially available (Table 2.2). Baumann's enrichment medium was prepared without the addition of Hutner's mineral base (Cohen-Bazire et al., 1957). According to Towner (2006), this is not necessary as there are sufficient nutrient trace elements in the other medium components.

Acinetobacter species were inoculated independently in 50 mL of both enrichment broths to a final level of ca. 10 CFU/mL and incubated at 30 ºC for 48 h, in an orbital shaker (New Brunswick Scientific Co. Inc., New Jersey, USA) at 150 rpm. After 24 h and 48 h of incubation, aliquots were collected, serially diluted in Ringer solution (Lab M, Lancashire, UK) and plated using the spread plate counting technique onto Tryptone Soya Agar (TSA, Merck). Plates were incubated at 30 ºC, for 24-48 h. Three independent replicates were performed.

Table 2.2. Composition of enrichment broths used for the isolation of *Acinetobacter* species.

Baumann enrichment medium[a]	Dijkshoorn enrichment medium[b]
	1.5 g KH_2PO_4
2 g $CH_3COONa.3H_2O$	16.5 g $Na_2HPO_4.H_2O$
2 g KNO_3,	0.2 g $MgSO_4.7H_2O$
0.2 g $MgSO_4.7H_2O$,	2.0 g NH_4Cl
0.04 M KH_2PO_4/NA_2HPO_4 buffer (pH 6.0)	0.01 g $CaCl_2$ 0.0005 g $FeSO_4.7H_2O$
1 L deionised water	2.0 g CH_3COONa
Autoclaved for 15 min at 121 °C	1 L water deionised
	Adjust pH to 7.5 ± 0.2
	Autoclaved for 15 min at 118 °C

[a] according to Baumann (1968) without the addition of Hutner's mineral base (Cohen-Bazire et al., 1957); according to Towner (2006), this is not necessary as there are sufficient nutrient trace elements in the other medium components.

[b] saline solution according to Monod and Wollman (1947) enriched with 0.2% (w/v) sodium acetate according to Dijkshoorn et al. (1987).

2.2.3. Evaluation of Dijkshoorn enrichment medium for the detection of *Acinetobacter* spp. in a food matrix

2.2.3.1. Sample preparation of an artificially contaminated food matrix

The efficacy of Dijkshoorn's enrichment was evaluated in tomato artificially contaminated (spiked) with *Acinetobacter* spp. (Table 2.1). Six samples containing 50 g of sliced tomato (pre-washed in running tap water) were prepared in sterile stomacher bags (VWR, Leighton Buzzard, UK) and inoculated, separately, with 10 mL suspensions of *ca.*10 CFU/mL of each *Acinetobacter* isolate. Samples were thoroughly mixed, and 25 g were transferred into a new stomacher bag. Uninoculated tomato samples were used as negative control to ensure that species detected did not originate from the samples but from deliberate contamination. At least, three independent replicates were performed.

2.2.3.2. Sample preparation of raw food

Samples of 25 g of unwashed vegetable foods including lettuce, parsley, carrot (with peel) and tomato were weighed into a sterile stomacher bag.

2.2.3.3. *Acinetobacter* spp. detection

For the pre-enrichment, 225 mL of Dijkshoorn enrichment medium was added to each 25 g sample of raw foods. The mixture was macerated in a Stomacher (Interscience, St Nom la Bretêche, France) for 2 minutes and then incubated at 30 ºC in an orbital shaker (150 rpm) for 48 h. After 24 h and 48 h of incubation, one loopful of enrichment broth was streaked onto plates of CHROMagar^{TM} Acinetobacter (CHROMagar, Paris, France), incubated at 30 °C for 24-48 h, and examined for the growth of *Acinetobacter* spp. typical colonies (bright salmon red colonies).

2.2.4. Identification of *Acinetobacter* spp. isolated from raw food samples

2.2.4.1. Confirmation of presumptive colonies

One typical colony representative of each type of morphology and shape was selected and characterized based on phenotypic tests: Gram-stain, catalase and oxidase tests. Gram-negative coccobacilli, oxidase-negative, and catalase-positive isolates were presumptively identified to the genus *Acinetobacter*. Confirmation to genus was performed by screening for the presence of the *Acinetobacter* spp. 16 S rRNA signature, as previously described by Vanbroekhoven et al. (2004). PCRs were carried out with the following program: initial denaturation at 95 °C for 5 min; 30 cycles at 95 °C for 15 s, 58 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 4 min. The DNA template of *A. baumannii* 002 and *Escherichia coli* ATCC 25922 were used in each amplification reaction as positive and negative controls, respectively.

2.2.4.2. Selection of isolates for identification to species level

All isolates belonging to the genus *Acinetobacter* were further genotyped by repetitive extragenic palindromic sequence-based PCR (REP-PCR). Amplification PCRs were performed as described previously (Snelling et al., 1996) with the primer pair REP 1 (5’-IIIGCGCCGICATCAGGC-3’) and REP 2 (5’-ACGTCTTATCAGGCCTAC-3’) in reactions of 25 µL, with 2.5 µL of 10X PCR buffer; 0.05 mM $MgCl_2$; 1 U of Taq DNA polymerase (Thermo Fisher Scientific Inc, *Massachusetts*, United States); 0.05 mM of dNTP’s, 0.25 µM of each primer and 1 µL of bacterial genomic DNA.

Isolates were considered clonally related when fingerprints had all visible bands with the same apparent migration distance. Variations in the intensity or shape of bands were not taken into account.

2.2.4.3. Identification to species level

Species identification of isolates of *Acinetobacter,* representative of each REP-PCR pattern, was based on the analysis of the sequence of the region Z1– Z2 of the gene for RNA polymerase beta-subunit (*rpo*B) according to La Scola et al. (2006). The partial sequences of *rpo*B (902 bp) were amplified with the primers and conditions described before (La Scola et al., 2006), in reactions of 50 µL, with 0.5 U of KAPA HiFi HotStart (Kapa Biosystems, Boston, United States), 1 × KAPA HiFi Fidelity Buffer with 2 mM of $MgCl_2$, 0.2 mM of dNTP’s, 0.2 µM of each primer, Ac696F and Ac1598R, and 0.5 µL of bacterial genomic DNA. After initial denaturation at 95 °C for 2 min, 35 amplification cycles were performed according to the following format: 30 s at 94 °C, 30 s at 48 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C.

PCR products were purified, using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal), according to the supplier's instructions, and the DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). The partial *rpo*B nucleotide sequences were edited manually, using the software Geneious R7 (ver. 7.1.5, Biomatters Limited, New Zealand, USA). Similarity between the *rpo*B nucleotide sequences of the tested isolates and the *rpo*B sequences of the type strains of all *Acinetobacter* species available in the GenBank database (http://www.ncbi.nlm.nih.gov) was calculated by using the MEGA6 software (Tamura et al., 2013). Phylogenetic tree were constructed by the neighbour-joining method (Saitou and Nei, 1987), and bootstrap replicates were performed in order to estimate the reliability of the nodes of the phylogenetic trees. Bootstrap values were obtained from 1000 trees, generated randomly with the MEGA6 (Tamura et al., 2013).

2.2.5. Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine significant differences between media and time of incubation. Tukey's test was applied to compare the mean values. Statistical significance was set at $p < 0.01$. These analyses were performed using SPSS for Windows, 17.0. (SPSS Inc., Chicago, Illinois, USA).

2.3. Results and Discussion

The first objective of the present study was to test two different enrichment media (Baumann’s enrichment and Dijkshoorn’s enrichment) inoculated with the selected *Acinetobacter* species in order to evaluate their ability to improve *Acinetobacter* detection. It was observed that after 24 h and 48 h of incubation in Dijkshoorn enrichment medium, viable cell counts were at least ca. 10^5 CFU/mL and 10^7 CFU/mL, respectively; while in Baumann’s enrichment medium the strains only achieved maximum viable cell counts levels of ca. 10^3 CFU/mL and 10^6 CFU/mL, respectively (Figure 2.1).

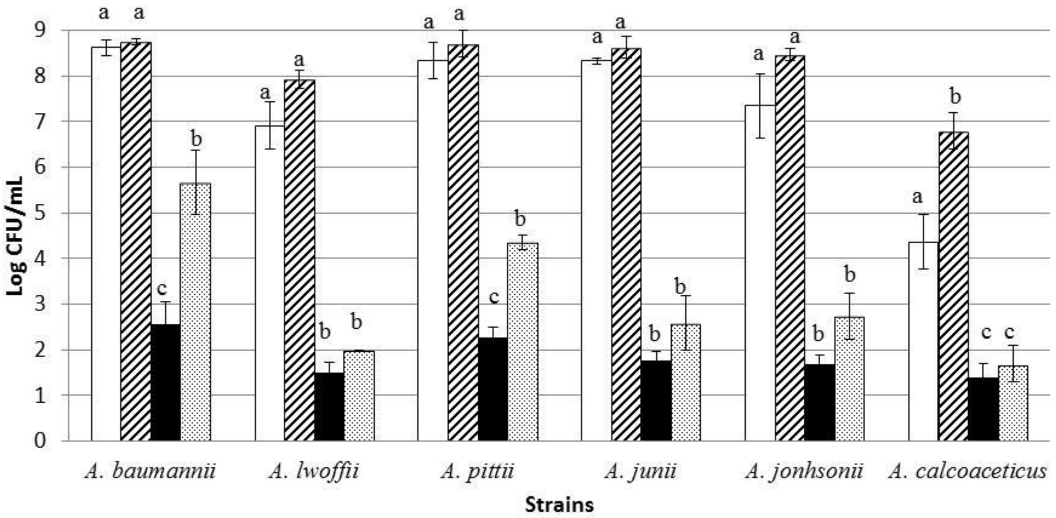


Figure 2.1. Enumeration of *Acinetobacter* species on TSA after enrichment of an initial inoculum level of 10 CFU/mL in: Dijkshoorn enrichment medium after 24 h (□) and 48 h (▨); and Baumann’s enrichment medium after 24 h ( )■nd 48 h ( ).▨

a, b, c - values with different letters are significantly different ($p < 0.01$) between media and time of incubation.

Although most of the authors mention Baumann enrichment medium as that generally used for enrichment Baumann, 1968; Grehn and von Graevenitz, 1978; Guardabassi et al., 1999), a significantly ($p < 0.01$) higher efficacy was observed in the Dijkshoorn's enrichment broth with higher cellular levels. Thereby, Dijkshoorn enrichment medium was selected for isolation of acinetobacters from food samples.

Following 24 and 48 h enrichment of artificially contaminated tomato samples in Dijkshoorn enrichment medium all the inoculated species were recovered on CHROMagar^{TM} Acinetobacter. This is a chromogenic medium designed for the isolation of *Acinetobacter* spp. from samples with heterogeneous native flora (Ajao et al., 2011; Gordon and Wareham, 2009), such as those from food (Gurung et al., 2013). In comparison with other media commonly used for the isolation of *Acinetobacter* spp. CHROMagar^{TM} Acinetobacter has demonstrated a higher sensitivity (Ajao et al., 2011).

Acinetobacter spp. were isolated from all samples of the raw foods analyzed. Among the 17 presumptive colonies of *Acinetobacter* spp. selected, 14 (82%) were confirmed to belong to this genus by screening for presence of the *Acinetobacter* spp. 16 S rRNA signature. Using REP-PCR nine different patterns were identified. The closest related species of each representative isolate of each REP-PCR pattern was further identified by *rpo*B gene analysis as belonging to *A. johnsonii, A. calcoaceticus*, *A. guillouiae*, and *A. gandensis* (Table 2.3, Supplementary Figure 2.1) and more than one species was isolated from a single sample of lettuce (Table 2.3).

It was not possible to identify the strain Ac005 to the species level since its *rpo*B sequence has 90.2% of similarity with *A. calcoaceticus*. According to La Scola et al. (2006) and Krizova et al. (2014), *rpo*B sequence similarity values below 95% indicate distinct species, therefore this isolate may represent a novel species.

Table 2.3. Isolation of *Acinetobacter* spp. from food samples by enrichment in Dijkshoorn´s medium followed by direct plating on CHROMagar^{TM}.

Sample	Nº of presumptive / confirmed *Acinetobacter* spp. isolates	Number of different REP-PCR patterns	Closest related species (isolates code)	% of rpoB sequence similarity (accession number)
Lettuce	7/5	5	*A. guillouiae* (Ac0117, Ac0122) *A. johnsonii* (Ac0013, Ac0015) *A. calcoaceticus* (Ac0110)	99.6 (EU477117) 99.6 (EU477117) 99.0 (EU477113) 99.3 (EU477113) 97.4 (EU477149)
Parsley	2/2	1	*A. calcoaceticus* (Ac0052)	90.2 (EU477149)
Carrot	5/4	2	*A. calcoaceticus* (Ac0611, Ac0624)	97.8 (EU477149) 97.0 (EU477149)
Tomato	3/3	1	*A. gandensis* (Ac0071)	99.0(KJ569689)

2.4. Conclusions

This study demonstrated that Dijkshoorn's enrichment allowed the recovery of a higher number of *Acinetobacter* cells than in Baumann's enrichment medium. Enrichment in Dijkshoorn's medium followed by plating on CHROMagar^{TM} Acinetobacter, appears to be a reliable method for the isolation of *Acinetobacter* spp. from foods, although further testing is required to assess whether all known species of the genus can be recovered by this approach.

CHAPTER 3

Lettuce and fruits as a source of multidrug resistant *Acinetobacter* spp.

CHAPTER 3

Abstract

The role of ready-to-eat products as a reservoir of pathogenic species of *Acinetobacter* remains unclear. The objective of the present study was to evaluate the presence of *Acinetobacter* species in lettuces and fruits marketed in Portugal, and their susceptibility to antimicrobials. *Acinetobacter* spp. were isolated from 77.9% of the samples and these microorganisms were also found as endophytes (i.e. present within the plant tissue) in 12 of 20 samples of lettuces analysed. Among 253 isolates that were identified as belonging to this genus, 181 presented different PFGE profiles, representing different strains. Based on the analysis of the partial sequence of *rpo*B, 175 strains were identified as members of eighteen distinct species and the remaining six strains may represent five new candidate species since their *rpo*B sequence similarities with type strains were less than 95%. *Acinetobacter calcoaceticus* and *Acinetobacter johnsonii* were the most common species, both with the frequency of 26.5%; and 11% of the strains belong to the *Acinetobacter baumannii* group (i.e. *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter seifertii* and *Acinetobacter nosocomialis*), which is most frequently associated with nosocomial infections.

Overall, the strains were least susceptible to piperacillin (80.1%), piperacillin-tazobactam (64.1%), ceftazidime (43.1%), ciprofloxacin (16.6%), trimethoprim-sulfamethoxazole (14.9%), imipenem (14.4%) and colistin (13.3%). The most active antimicrobials were minocycline and tetracycline, with 0.6% and 3.9% of strains resistant, respectively. About 29.8% of the strains were classified as multidrug-resistant (MDR), 4.4% as extensively drug-resistant (XDR) and the prevalence of MDR strains within the *A. baumannii* group (25%) was similar to other species (30.4%). The presence of clinically important species as well as MDR strains in lettuces and fruits may be a threat to public health considering that they may transmit these pathogens to environments such as the community and hospital settings.

3.1. Introduction

Acinetobacter spp. are recognized as major causal agents of nosocomial infections, particularly in severely ill patients in intensive care units or immunocompromised patients (Bergogne-Bérézin and Towner, 1996; Munoz-Price and Weinstein, 2008; Peleg et al., 2008; Villegas and Hartstein, 2003). Although bacteria within the *Acinetobacter baumannii* group (*A. baumannii*, *Acinetobacter nosocomialis*, *Acinetobacter pittii* and *Acinetobacter seifertii*) are the most common organisms that cause healthcare-associated infections worldwide (Dijkshoorn et al., 2007; Joly-Guillou, 2005; Kallen et al., 2010; Maragakis and Perl, 2008; Munoz-Price and Weinstein, 2008; Peleg et al., 2008; Thom et al., 2012), other species, such as *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter ursingii* have been sporadically recovered from clinical specimens and involved in nosocomial infections (Turton et al., 2010).

Acinetobacter species were also associated with community-acquired infections (Falagas et al., 2007; Kang et al., 2012), however the natural reservoir of some species remains unknown. Additionally, the prevalence and antimicrobial resistance of *Acinetobacter* outside the hospital setting has been poorly investigated (Zeana et al., 2003).

Fresh produce consumed raw or minimally processed, such as fruits, salad items and vegetables, has been recognized as an important vehicle for the transmission of human pathogens (Berger et al., 2010; Beuchat, 2002; Brassard et al., 2011; Islam et al., 2004; Newell et al., 2010). The consumption of these foods has been increasing among consumers who prefer healthy diets and provide an appropriate meal for the current lifestyles. As most of these products are eaten without further processing, they are potential vehicles for transmission of contaminants.

Information regarding raw vegetables and fruits as a source of *Acinetobacter* species, particularly those with clinical relevance, as well as their characteristics, are limited (Berlau et al., 1999; Gennari and Lombardi, 1993), and identification techniques used are not sufficiently precise to differentiate species of *Acinetobacter*.

Acinetobacter spp. have the propensity to develop widespread resistance to the major groups of antibiotics (Bergogne-Bérézin and Towner, 1996; Nowak et al., 2010; Park et al., 2009) and have the ability to persist in the environment for long periods (Webster et al., 1998; Wendt et al., 1997) facilitating their survival and spread. Therefore, food contaminated with *Acinetobacter* spp. can be a source of transmission of this organism into domestic and hospital settings and may contribute to horizontal spreading of resistance genes to other pathogens, which constitutes an additional concern for public health.

The aim of this study was to evaluate the prevalence and diversity of *Acinetobacter* spp. in lettuces and fruits, as well as their antibiotic resistance as an indicator of their potential as a risk factor for consumers. This is the first report about the diversity of *Acinetobacter* in foods marketed in Portugal.

3.2. Materials and methods

3.2.1. Samples

Between June 2013 and September 2014, ninety-nine samples of different fresh green leaf lettuces heads (n=45) and fruits (n=55; 15 apples, 13 pears, 13 bananas and 9 strawberries) conventionally produced, were purchased from four supermarkets and two retail greengrocer´s shops located in Porto region (Portugal) and analysed. From each store, no more than one sample of lettuce head or fruit type was collected on the same day.

3.2.2. Isolation method

3.2.2.1. Isolation of *Acinetobacter* spp. from lettuce and fruit samples

Isolation of *Acinetobacter* spp. from lettuce and fruit samples was done according to Carvalheira et al. (2016). Briefly, 25 g of each product, randomly selected without washing or peeling, were added to 225 mL of Dijkshoorn enrichment medium (Carvalheira et al., 2016) and homogenized in a Stomacher (Interscience, St Nom la Bretêche, France) for 2 minutes. After incubation for 24 h at 30 ºC in an orbital shaker (150 rpm; New Brunswick Scientific Co. Inc., New Jersey, USA) one loopful of enrichment broth was plated by streaking on CHROMagar^{TM} Acinetobacter (CHROMagar, Paris, France), incubated at 30 °C for 24-48 h, and examined for the growth of typical red colonies of *Acinetobacter* spp.

3.2.2.2. Isolation of endophytic *Acinetobacter* spp. from lettuce samples

Twenty samples of lettuce were analysed simultaneously in order to evaluate the presence of endophytic *Acinetobacter* spp. The outer leaves were removed by hand and the inner leaves of lettuce were washed in running tap water to remove attached soil. Afterward, surface sterilization was performed according to Pereira and Castro (2014), with some modifications, by stepwise immersion in an 70% ethanol solution for 10 s, fresh sodium hypochlorite solution (2.5% available Cl^-) for 10 min, and 70% ethanol solution for 2 minutes, followed by five rinses in sterile distilled water. To confirm the success of the sterilization process, aliquots of the final rinsing with sterile distilled water were spread onto Tryptic Soy Agar (TSA; Merck, Darmstadt, Germany) and CHROMagar^{TM}

Acinetobacter media. The plates were examined for bacterial growth after incubation at 30 °C for 2 days, and the subsequent analysis for endophytes was done with samples that were not contaminated.

The isolation of *Acinetobacter* endophytes was performed as previously mentioned (section 2.2.1.) with 25 g of the externally sterilized specimens.

6.4.1. **Identification of *Acinetobacter* spp.**

3.2.3.1. Confirmation of presumptive colonies

One typical colony representative of each type of morphology and shape was further sub-cultured on TSA and characterized based on phenotypic tests: Gram-stain, catalase and oxidase tests. Gram-negative coccobacilli, oxidase-negative, and catalase-positive isolates were presumptively identified to the genus *Acinetobacter*. Confirmation to genus was performed by screening for the presence of the *Acinetobacter* spp. 16 S rRNA signature, as previously described by Vanbroekhoven et al. (2004).

3.2.3.2. Selection of isolates for identification to species level

All isolates belonging to the genus *Acinetobacter* were further genotyped by Pulsed-field Gel Electrophoresis (PFGE) according to Chang et al. (2013), using the restriction enzyme *Asc*I (New England Biolabs, Ipswich, MA), with minor modifications: the optical density of bacterial cell suspensions used was adjusted to 1.3 at 600 nm and the minimal amount of *Asc*I was 5U. The PFGE was run in a CHEF Mapper XA (Bio-Rad Laboratories, Hercules, CA), with pulses ranging from 4 to 40 s at 14 °C with 6 V/cm for 21 h. The band patterns were analysed by GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) and the cluster analysis of PFGE profiles was done by the unweighted-pair group method with average linkages (UPGMA), using the Dice coefficient to generate a dendrogram describing the relationship among PFGE profiles. A band position tolerance of 1.0 was selected and the classification of isolates into different *Asc*I patterns was visually validated.

3.2.3.3. Species identification

Species identification was performed for representative isolates of each PFGE pulsotype based on the analysis of the sequence of the region Z1– Z2 of the gene for RNA polymerase beta-subunit (*rpo*B) according to La Scola et al. (2006). The partial sequences of *rpo*B (902 bp) were amplified with the primers (Ac696F and Ac1598R) and conditions described by La Scola et al. (2006). PCR products were purified, using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal), according to the supplier's instructions, and the DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). The partial *rpo*B nucleotide sequences were edited manually, using the software BioEdit 7.1.3.0 (Hall, 1999). Similarity between the *rpo*B nucleotide sequences of the tested isolates and the *rpoB* sequences of the type strains of all *Acinetobacter* species available in the GenBank database (http://www.ncbi.nlm.nih.gov) was calculated by using the MEGA6 software (Tamura et al., 2013). Isolates showing at least 95% *rpo*B gene sequence similarity with a type strain were identified as specific *Acinetobacter* species (Krizova et al., 2014; La Scola et al., 2006).

3.2.4. Antibiotic susceptibility testing

Antimicrobial susceptibility test of all different strains of *Acinetobacter* was performed according to standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2012) using Mueller Hinton agar (MHA, BioMérieux, Marcy-l'Étoile, France). The disk diffusion method was used to test twelve antibiotics: piperacillin (PIP, 100 µg), piperacillin-tazobactam (TZP 100/10 µg), ampicillin-sulbactam (SAM 10/10 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), tetracycline (TE, 30 µg), minocycline (MI, 30 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT 1.25/ 23.75µg) (all from Oxoid, Hants, UK). Colistin (CL) and polymyxin B (PB) susceptibility was evaluated by agar dilution method with antibiotic concentrations ranging from 1 to 8 µg/mL. Cultures were incubated for 24 h at 35 °C and *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* DSM 1117 were used as quality control strains.

The inhibition zones were measured and the strains were categorized as susceptible or resistant based on interpretive criteria according to CLSI guidelines (CLSI, 2012).

3.3. Results and Discussion

Acinetobacter spp. were isolated from 86.7% of lettuce (39/45) and 70.0% of fruit (73.3% of apples (11/15), 77.0% of pears (10/13), 55.6% of strawberries (5/9) and 69.2% of bananas (9/13) samples analysed. These results express the maximum likelihood of colonization since the food samples were analyzed without washing or peeling.

To the best of our knowledge, this is the first report of prevalence of *Acinetobacter* spp. in lettuce and fruit samples marketed in Portugal. Furthermore, there are only a few studies about the presence of *Acinetobacter* in foods, particularly on fruits and lettuces, and the recovery rate of *Acinetobacter* spp. reported, was much lower. Berlau et al. (1999) analysed 117 samples of fruit and vegetables and *Acinetobacter* spp. was isolated in 17% of samples. In a brief study by Hamilton-Miller and Shad (2001), only two isolates of *Acinetobacter* spp. were recovered from fifteen samples of salad vegetables. Oie et al. (2008) analysed the microbial contamination of 36 fruits samples and 64 vegetables samples, after washing or washing followed by disinfection, and only one isolate of *Acinetobacter* spp. was recovered. More recently, Ruimy et al. (2010) analysed the population of Gram-negative bacteria in 399 raw fruits and vegetables and less than 50% were shown to be contaminated with this microorganism. The higher prevalence of *Acinetobacter* spp. in the current study may be explained by different methods of isolation used since unlike other studies a selective enrichment method was used followed by growth on a selective and differential medium (Carvalheira et al., 2016).

Through the screening for the presence of the *Acinetobacter* spp. by the presence of their 16 S rRNA signature, a total of 253 isolates were identified as belonging to the genus *Acinetobacter*. In order to avoid some repetitive isolates from each sample, these isolates were genotyped by PFGE and those having the same PFGE profile were considered clones and excluded from further analysis. Among the isolates, 181 strains (111 and 70 from lettuce and fruit samples, respectively) had a different PFGE profile and constitute the collection of *Acinetobacter* isolates which were further analysed for species diversity and antibiotic resistance. Moreover, a high diversity of PFGE profiles between different samples was observed; indeed only eight profiles were shared between different samples such as six lettuce, one lettuce and fruit (pear), and one between two isolates from fruit (banana).

Based on the analysis of the partial sequence of *rpo*B, 175 out of 181 strains were identified as members of eighteen distinct species (Table 3.1). The remaining six strains could not be identified at the species level as their values of *rpo*B sequence similarity with type strains were less than 95% which indicate distinct species according to La Scola et al. (2006) and Krizova et al. (2014). Therefore, these six strains, which were clustered in five distinct groups, may represent five new candidate species. The closest related species of these six strains comprised the species *A. gyllenbergii, A. venetianus, A. tjernbergiae, A. parvus* and *A. lwoffii*, with *rpo*B sequences similarities ranging from 89.4% to 94.6% (Table 3.1).

Table 3.1. Closest related species of *Acinetobacter* strains based on the *rpoB* sequence analysis, number of strains per sample.

Closest related species (type strain)	**% of *rpoB* sequence similarity (accession number)**	**No. of strains**	**Samples**				
			Lettuce	**Apple**	**Pear**	**Strawberry**	**Banana**
A. calcoaceticus (NIPH 2245^{T})	96.7 - 98.9 (EU477149)	48	39	4	1	1	3
A. johnsonii (NIPH 518^{T})	95.0 - 99.5 (EU477113)	48	32	6	2	4	4
A. guillouiae (NIPH 522^{T})	99.2 - 99.5 (EU477117)	20	14	2	1	3	
A. beijerinckii (NIPH 838^{T})	97.7 - 99.7 (EU477124)	16	13		1	2	
A. pittii (NIPH 519^{T})	96.9 - 100 (EU477114)	12	4	1	4		3
A. baylyi (NIPH 2312^{T})	98.1 - 99.7 (EU477155)	9					9
A. baumannii (NIPH 501^{T})	98.9 - 99.4 (EU477108)	4	4				
A. seifertii (NIPH 973^{T})	99.1 - 99.5 (EU477126)	3					3
A. lwofii (NIPH 512^{T})	97.8 - 99.1 (EU477111)	3		1	2		
A. soli (CCUG 59023^{T})	99.4 - 99.7 (HQ148175)	2					2
A. radioresistens (NIPH 513^{T})	99.2 - 100 (EU477112)	2		2			
A. ursingii (DSM 16037T)	100 (EU477105)	2		2			
A. parvus (NIPH 384^{T})	96.5 (EU477107)	1		1			
A. variabilis (NIPH 546^{T})	98.6 (EU477119)	1					1
A. bereziniae (NIPH 521^{T})	99.1 (EU477116)	1	1				
A. junii (NIPH 511^{T})	99.2 (EU477110)	1	1				
A. schindleri (CIP 107287^{T})	97 (EU477128)	1			1		
A. nosocomialis (LMG 10619^{T})	99.4 (HQ123389)	1					1
Non Identified:							
A. gyllenbergii (NIPH 2150^{T})	93.2* - 93.4* (EU477148)	2	2				
A. venetianus (NIPH 1925^{T})	93.4* (EU477136)	1				1	
A. tjernbergiae (NIPH 2285^{T})	94.1* (EU477153)	1	1				
A. parvus (NIPH 384^{T})	94.6* (EU477107)	1			1		
A. lwofii (NIPH 512^{T})	89.4* (EU477111)	1		1			

**rpo*B sequence similarity values <95 % suggest that these isolates may represent novel species.

Eight and fifteen species of *Acinetobacter* were identified among the strains isolated from lettuce and fruit samples, respectively. Some species were found exclusively in lettuce samples such as *A. baumannii*, *A. bereziniae* and *A. junii* while *A. baylyi, A. seifertii, A. lwoffii*, *A. soli, A. radioresistens*, *A. ursingii*, *A. parvus*, *A. variavilis*, *A. schindleri and A. nosocomialis* were only isolated from fruit samples (Table 3.1).

Beyond the high diversity of strains, a diversity of species in each sample was also observed, ranging from one to three. The commonest species were *A. calcoaceticus* and *A. johnsonii* both at a frequency of 26.5% (48/181) but one of the most important findings may be the identification of nosocomial human pathogens such as *A. baumannii* (n=4), *A. pittii* (n=12), *A. seifertii* (n=3) and *A. nosocomialis* (n=1). These species accounted for 11.0% (20/181) of all strains analysed and belong to the *A. baumannii* group which is most frequently associated with nosocomial infections worldwide (Bergogne-Bérézin and Towner, 1996; Park et al., 2012; Peleg et al., 2008; Wisplinghoff et al., 2012). Although these species are recognized as the most important in terms of pathogenicity, since appropriate techniques for identification to the species level of this genus began to be used, such as *rpo*B gene sequencing, other species that were isolated in this study such as *A. calcoaceticus* (n=48), *A. johnsonii* (n=48), *A. guillouiae* (n=20) , *A. beijerinckii* (n=16) *A. baylyi* (n=9) *A. lwoffii* (n=3), *A. radioresistens* (n=2), *A. ursingii* (n=2), *A. soli* (n=2), *A. parvus* (n=1), *A. variabilis* (n=1); *A. bereziniae* (n=1), *A. junii* (n=1) and *A. schindleri* (n=1) have also been associated with nosocomial infections (Bergogne-Bérézin and Towner, 1996; Bernards et al., 1997; Choi et al., 2006; Dortet et al., 2006; Nemec et al., 2001; Nemec et al., 2003; Seifert et al., 1993, 1994; Turton et al., 2010).

In the study conducted by Ruimy et al. (2010), only seven species of *Acinetobacter* were identified in 399 samples of raw fruits and vegetables; *A. calcoaceticus* (78.9%) was the species most commonly identified. However, the prevalence of *A. johnsonii* (0.9%) as well as the species belonging to the *A. baumannii* group (4.0%) were lower than in the current study. These differences can also be explained by the different isolation methods used between the studies, as already mentioned above.

In contrast to our findings, in the studies of Berlau et al. (1999) and Gennari and Lombardi (1993) the species most recovered from vegetables and fruits were *A. baumannii* and *A. lwoffii*, respectively. However, the methods used in those studies are now recognized as not reliable for identification of *Acinetobacter* to the specie level.

Fruits and lettuces are ready to eat products that are usually rinsed in water or exposed to a mild hypochlorite treatment before consumption; however, once endophytic this microorganism cannot be eliminated. It is known that endophytic bacteria can be found in roots, stems, leaves, seeds, fruits, tubers and also inside legume nodules (Benhizia et al., 2004; Hallmann et al., 1997; Sturz et al., 1997) but this is one of the few studies that report the natural internalization of *Acinetobacter* spp. in lettuce (Jackson et al., 2013).

Acinetobacter spp. endophytes were isolated in 12 of the 20 samples examined (60%) and the species found as endophytes were *A. calcoaceticus* (n=8), *A. guillouiae* (n=1), *A. bereziniae* (n=1), *A. johnsonii* (n=1) and *A. beijerinckii* (n=1). Endophytic *Acinetobacter* spp. in a single lettuce sample was not restricted to a single species but it was shown that they can comprise at least two different species. Indeed, *A. calcoaceticus* and *A. johnsonii* as well as *A. beijerinckii* and *A. johnsonii* were identified in the same samples (data not shown). The genotyping analysis revealed that most of the endophytic isolates presented different PFGE profiles when compared to those isolates from the sample surface; only in three samples was the same PFGE profile identified as being both endophytic and on the surface of lettuce.

The presence of endophytic *Acinetobacter* spp. indicates that lettuces contamination could be due to mere contamination during growth by soil, organic fertilisers and irrigation water (Brandl, 2006; Hamilton et al., 2006; Heaton and Jones, 2008; Tyler and Triplett, 2008). Other sources of contamination can be present during harvest, transportation and further handling, as well as from the ability of pathogens to persist and proliferate in vegetables (Hamilton et al., 2006; Heaton and Jones, 2008) thus indicating that prevention of contamination is paramount to control the presence of *Acinetobacter* spp. in these food products.

Although *Acinetobacter* spp. are recognized as avirulent for healthy humans and cause infections almost exclusively in debilitated patients in hospitals and immunocompromised individuals (Bergogne-Bérézin and Towner, 1996; Villegas and Hartstein, 2003), the remarkable ability of these microorganisms to develop resistance to all clinically relevant antimicrobials is a matter of public health concern. The antimicrobial susceptibility of all the species identified to fourteen antimicrobial agents is presented in Table 3.2 and in supplementary table 3.1. In general, the strains were more resistant to piperacillin (80.1%), piperacillin-tazobactam (64.1%), ceftadidime (43.1%), ciprofloxacin (16.6%), trimethoprim-sulfamethoxazole (14.9%), imipenem (14.4%) and colistin (13.3%).

Minocycline and tetracycline were found to be the most active agents, with only 0.6% and 3.9% of strains being resistant, respectively.

Penicillins such as piperacillin are considered a non-effective antibiotic for the treatment of *Acinetobacter* infections (Jain and Danziger, 2004) which is consistent with the results observed. Overall, strains presented a high resistance to some of the antimicrobials categories used to treat infections caused by antibiotic-susceptible *Acinetobacter* such as cephalosporins, combination of β-lactam with β-lactamase inhibitors (piperacillin-tazobactam) and carbapenems (imipenem) as well as to the fluoroquinolone ciprofloxacin that can be used in combination with the previous antimicrobials. The increased resistance of clinical isolates to that antimicrobial has been widely reported (Lee et al., 2011; Van Looveren and Goossens, 2004). Furthermore, the resistance to colistin, the last resort for treatment of multidrug-resistant *Acinetobacter*, was observed in 13.3 % of the strains. The resistance to this antibiotic in clinical isolates has been reported all over the world (Giamarellos-Bourboulis et al., 2001; Hawley et al., 2008; Ko et al., 2007; Li et al., 2006).

Another notable result is the resistance of the strains to several antimicrobial agents. Indeed, only 24.9% (45/181) of the strains were susceptible to all the antibiotics tested and 29.8% (54/181) of the strains were considered as multidrug-resistant (MDR), i.e. acquired non-susceptibility to at least one agent in three or more antimicrobial categories among the eight different classes of antibiotics used to define MDR (Magiorakos et al., 2012). Moreover, 4.4% (8/181) of the strains are extensively drug-resistant (XDR) i.e. non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (Magiorakos et al., 2012).

Table 3.2. Prevalence of antibiotic resistance of *Acinetobacter* spp. strains

Species	**Number (%) of resistant strains**													
(no. of strains)	**PIP**	**TZP**	**SAM**	**CAZ**	**IMP**	**MEM**	**PB**	**CL**	**AK**	**TOB**	**TE**	**MI**	**CIP**	**SXT**
A. calcoaceticus (48)	46 (95.8)	35 (72.9)	3 (6.3)	12 (25)	4 (8.3)	4 (8.3)	2 (4.2)	3 (6.3)	3 (6.3)	3 (6.3)	3 (6.3)		4 (8.3)	3 (6.3)
A. johnsonii (48)	39 (81.3)	30 (62.5)	3 (6.3)	33 (68.8)	6 (12.5)	4 (8.3)		1 (2.1)	4 (8.3)	2 (4.2)	1 (2.1)		12 (25)	12 (25)
A. guillouiae (20)	17 (85.0)	15 (75.0)		7 (35.0)	10 (50.8)	5 (25.0)		5 (25.0)					3 (15.0)	1 (5.0)
A. beijerinckii (16)	13 (81.3)	12 (75)		12 (75)	1 (6.3)	1 (6.3)	3 (18.8)	5 (31.3)	2 (12.5)	1 (6.3)			1 (6.3)	1 (6.3)
A. pittii (12)	9 (75)	8 (66.7)	1 (8.3)	2 (16.7)	1 (8.3)	1 (8.3)	3 (25)	1 (8.3)	1 (8.3)	2 (16.7)	2 (16.7)	1 (8.3)	2 (16.7)	2 (16.7)
A. baylyi (9)	5 (55.6)	5 (55.6)	3 (33.3)	3 (33.3)		1 (11.1)		1 (11.1)	2 (22.2)	4 (44.4)			4 (44.4)	4 (44.4)
A. baumannii (4)	3 (75)	3 (75)		2 (50)	2 (50)				1 (25)					
A. seifertii (3)	1 (33.3)						3 (100)	1 (33.3)						
A. lwoffii (3)	3 (100)	3 (100)	1 (33.3)	3 (100)									1 (33.3)	1 (33.3)
A. soli (2)														
A. radioresistens (2)	2 (100)	1 (50)												
A. ursingii (2)	1 (50)	1 (50)	1 (50)	1 (50)				1 (50)		1 (50)			1 (50)	
A. parvus (1)								1 (50)						
A. variabilis (1)	1 (100)	1 (100)	1 (100)	1 (100)						1 (100)			1 (100)	1 (100)
A. bereziniae (1)							1 (100)	1 (100)						
A. junii (1)							1 (100)	1 (100)						
A. schindleri (1)														
A. nosocomialis (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)		1 (100)		1 (100)	1 (100)	1 (100)		1 (100)	1 (100)
Non Identified*:														
*A. gyllenbergii** (2)	1 (50)			1 (50)	1 (50)			1 (50)	1 (50)	1 (50)				1 (50)
*A. venetianus** (1)	1 (100)	1 (100)					1 (100)	1 (100)						
*A. tjernbergiae** (1)	1 (100)						1 (100)	1 (100)						
*A. parvus** (1)	1 (100)													
*A. lwoffii** (1)														
Total (181)	**145 (80.1)**	**116 (64.1)**	**14 (7.7)**	**78 (43.1)**	**26 (14.4)**	**16 (8.8)**	**16 (8.8)**	**24 (13.3)**	**15 (8.3)**	**16 (8.8)**	**7 (3.9)**	**1 (0.6)**	**30 (16.6)**	**27 (14.9)**

PIP, piperacillin; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole.

*rpoB sequence similarity values <95 % suggest that these isolates may represent novel species

All species comprise MDR strains as well as strains susceptible to all the antibiotics tested, except for the species in which the number of strains analysed cannot be considered significant since it ranged between one to three such as *A. seifertii*, *A. soli*, *A. radioresistens*, *A. parvus*, *A. variabilis*, *A. bereziniae*, *A. junii*, *A. schindleri* and *A. nosocomialis.* Moreover, most of the species include strains resistant to at least four antimicrobial categories and the species *A. calcoaceticus*, *A. johnsonii, A. pittii, A. baylyi,* and *A. nosocomialis* include strains resistant to six or more antibiotics. *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* (isolated from banana and pear) include strains resistant to all the classes of antibiotics tested (Supplementary table 3.1).

Although it has been reported that strains belonging to the *A. baumannii* group were generally more resistant to antibiotics than other species (Dijkshoorn et al., 2007; Wareham et al., 2008), in the current study the prevalence of MDR strains within the *A. baumannii* group was similar to the prevalence within the other species identified, 25% (5/20) and 30.4% (49/161), respectively. Within the *A. baumannii* group, one strain of *A. pittii* as well as *A. nosocomialis* were resistant to all the classes of antibiotics tested. While the strains of *A. seifertii* were resistant only to one class of antimicrobial and between the strains of *A. baumannii* there was one susceptible to all antibiotics and the others were resistant to one, three and four classes of antibiotics, respectively.

To the best of our knowledge, this is the first study that analysed the antimicrobial susceptibility of a large number of strains isolated from fruits and vegetables to all the categories of antibiotics recommended in CLSI (2012). The incidence of antibiotic resistant bacteria in fruits and vegetables, to imipenem and ceftazidime was previously found to be similar in the study of Ruimy et al. (2010) although the resistance to piperacillin and piperacillin/tazobactam were previously found to be lower (66.8% and 15%, respectively). The large amount of antibiotics used in agriculture, the use of organic fertilisers, such as sewage sludge and manure, and contaminated irrigation water, may lead to the contamination with resistant bacteria from animal and/or human sources (Boehme et al., 2004; de la Cruz and Davies, 2000; Heuer and Smalla, 2007; Lipsitch et al., 2002; McManus et al., 2002; Tenover, 2006; Vidaver, 2002).

Ready to eat fruit and lettuces may therefore be a potential source of resistant *Acinetobacter* strains and provide a route by which these bacteria are introduced into hospital and maybe a source of resistance genes, however, the prevalence and biochemical or genetic mechanisms of resistance have not been analyzed.

3.4. Conclusions

Ready to eat products such as lettuces and fruits are a natural habitat of several *Acinetobacter* species, including the species belonging to the pathogenic *A. baumannii* group such as *A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. seifertii*. Moreover, these food products are a source of strains resistant to some of the categories of antimicrobials used to treat infections caused *Acinetobacter* and some of them are MDR and XDR.

Several studies have shown hospital food to be a potential source of Gram negative rods such as *Klebsiella*, but only in one study performed by Berlau et al. (1999) was suggested the implications of *Acinetobacter* spp. from vegetables in nosocomial infections. However, once introduced into the domestic or hospital environment, these microorganisms, being resistant to desiccation, may persist and multiply to become resident in the environment (Jawad et al., 1996; Wendt et al., 1997).

The presence of clinically important species and multidrug-resistant strains in lettuces and fruits may be a threat to public health considering that they may transmit these pathogens to human beings and to environments that surround them such as the community and hospital settings.

CHAPTER 4

Prevalence and antimicrobial susceptibility of *Acinetobacter* spp. isolated from meat

Abstract

The prevalence and antibiotic resistance of *Acinetobacter* spp. from fifty samples of meat (chicken, turkey, beef and pork) were evaluated. *Acinetobacter* spp. was recovered from all samples and the clonal relatedness of 223 isolates identified to belong to the genus *Acinetobacter* was established by PFGE. A high genetic diversity was observed and 166 isolates from different samples, 141 representing different PFGE profiles, were further identified to the species level by *rpo*B gene sequencing. Thirteen distinct *Acinetobacter* species were identified among 156 isolates. The remaining ten isolates may represent three putatively novel species since *rpo*B sequence homologies with type strains of all available described *Acinetobacter* species, were less than 95%.

The most common species was *Acinetobacter guillouiae* with a prevalence of 34.9%. However 18.7% of the strains belong to the *Acinetobacter baumannii* group (n=31) which include the species *Acinetobacter baumannii* (n=7), *Acinetobacter pittii* (n=12), *Acinetobacter seifertii* (n=8) and *Acinetobacter nosocomialis* (n=4) that are the species most frequently associated with nosocomial infections worldwide.

In general, strains were resistant to some of the antimicrobials most frequently used to treat *Acinetobacter* infections such as piperacillin-tazobactam (64.9% of strains resistant), ceftadidime (43.5%), ciprofloxacin (42.9%), as well as to colistin (41.7%) and polymyxin B (35.1%), the last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter*. The percentage of strains resistant to trimethoprim-sulfamethoxazole, tetracycline, aminoglycosides (amikacin and tobramycin) and ampicillin-sulbactam was greater than 10% (23.2%, 23.2%, 14.3%, 12.5%, 12.5%, respectively). However, resistances to meropenem, imipenem and minocycline were only sporadically observed (8.3%, 1.2% and 1.2%, respectively).

Overall, 51.2% of the strains were considered as multidrug-resistant (MDR) and 9.6% as extensively drug-resistant (XDR). The prevalence of MDR strains within the *A. baumannii* group (38.7%) was lower than the prevalence within the others species identified (54.1%). Therefore, food of animal origin may be a vehicle of spread *Acinetobacter* strains resistant to several antibiotics in the community and in the hospital setting environment. This may led to nosocomial and community-acquired infections in susceptible individuals.

4.1. Introduction

Members of the genus *Acinetobacter* are strictly aerobic non-fermenting Gram-negative cocco-bacilli currently including 39 validly named species (Euzéby, 2016). *Acinetobacter baumannii* group i.e. *Acinetobacter baumannii, A. pittii*, *A. nosocomialis* and *A. seifertii*, includes the species most often associated with nosocomial infections worldwide (Dijkshoorn et al., 2007; Nemec et al., 2015; Peleg et al., 2008). Moreover, these opportunistic pathogens have been implicated in community-acquired infections (Chang et al., 2000; Falagas et al., 2007; Falagas and Rafailidis, 2007; Kang et al., 2012). The ubiquity of *A. baumannii* in nature has been considered a widespread misconception due to difficulties in unequivocal species identification (Dijkshoorn et al., 2007; Eveillard et al., 2013; Peleg et al., 2008; Towner, 2009; Visca et al., 2011). Identification by molecular methods such *rpo*B gene sequencing improved the identification across the genus (Gundi et al., 2009; La Scola et al., 2006). Additionally, with the use of more reliable identification methods, other species outside the *A. baumannii* group, such as *A. lwoffii*, *A.ursingii*, *A. johnsonii* and *A. parvus* have also been implicated in nosocomial infections, and may represent emerging pathogens (Turton et al., 2010).

Owing to its remarkable ability to resist almost all available antimicrobial agents, *Acinetobacter* infections are difficult to treat. Indeed, multidrug-resistant or even pan-drug resistant isolates are increasing alarmingly in the hospital environment (Coyne et al., 2010; Dijkshoorn et al., 2007; Kempf and Rolain, 2012; Peleg et al., 2008; Zarrilli et al., 2013). This characteristic, associated with the ability of these organisms to survive under diverse environmental conditions (Bergogne-Bérézin and Towner, 1996; Fournier and Richet, 2006; Jawad et al., 1996), facilitates their survival and spread. Therefore, it is important to identify and monitor the possible sources and routes of transmission to community and hospital settings. Worldwide, the spread of antibiotic resistant bacteria through food is considered a major public health concern since the food chain has an important role in the dissemination of some important human pathogens (Perreten, 2005; Seiffert, Hilty, et al., 2013; Seiffert, Tinguely, et al., 2013; Verraes et al., 2013). Furthermore, the widespread use of antibiotics in food-producing animals has been linked to the emergence and dissemination of resistant bacteria (Phillips et al., 2004), which can further be spread to community and hospital settings through food.

The prevalence and antimicrobial resistance of *Acinetobacter* species isolated from human clinical isolates has been reported. However, studies about their prevalence in meat samples are limited. *Acinetobacter* spp. has been isolated from veterinary clinical specimens of food animals in the UK (Hamouda et al., 2008), Lebanon (Rafei et al., 2015), France (Poirel et al., 2012) and China (Wang et al., 2012; Zhang et al., 2013); in some cases, carbapenemase-producing isolates were described (Poirel et al., 2012; Wang et al., 2012; Zhang et al., 2013). Nevertheless, only a few studies have reported the presence *Acinetobacter* spp. in raw meat (Hamouda et al., 2008; Houang et al., 2001; Lupo et al., 2014; Rafei et al., 2015; Saha and Chopade, 2001); whereas the antimicrobial susceptibility of the isolates was not determined (Houang et al., 2001; Saha and Chopade, 2001), others refer to the prevalence of the specie *A. baumannii* (Hamouda et al., 2008; Lupo et al., 2014).

The objective of this study was to evaluate the prevalence and diversity of *Acinetobacter* spp. in meat samples, as well as their antibiotic resistance.

4.2. Materials and methods

4.2.1. Samples

Between October 2013 and September 2014, fifty meat samples purchased from five supermarkets located in Porto region (Portugal), belonging to four convenience store groups were analysed. These included chicken breast (n=14), turkey breast (n=12), pork steaks (n=12) and beef steaks (n=12) samples, all without skin and bones. From each store, no more than one sample of each meat type was collected on the same day.

4.2.2. Prevalence of Acinetobacter spp. in meat samples

4.2.2.1. Isolation method

The isolation of *Acinetobacter* spp. from meat samples was done according to Carvalheira et al. (2016). As a low number of cells was expected, 25 g composite samples were analysed as recommended by the EC regulation No. 2073/2005 for *Salmonella* spp. in meat products (EC, 2005). Briefly, 25 g of each product were added to 225 mL of Dijkshoorn enrichment medium and homogenized in a Stomacher (Interscience, St Nom la Bretêche, France) for 2 minutes. After incubation for 24h at 30 ºC in an orbital shaker (150 rpm; New

Brunswick Scientific Co. Inc., New Jersey, USA) one loopful of enrichment broth was plated by streaking on CHROMagar^{TM} Acinetobacter (CHROMagar, Paris, France), incubated at 30 °C for 24-48 h, and examined for the growth of typical red colonies of *Acinetobacter* spp.

4.2.3. Identification of *Acinetobacter* spp.

4.2.3.1. Confirmation of presumptive colonies

One typical colony representative of each type of morphology and shape was further sub-cultured on TSA and characterized based on phenotypic tests: Gram-stain, catalase and oxidase tests. Gram-negative coccobacilli, oxidase-negative, and catalase-positive isolates were presumptively identified to the genus *Acinetobacter*. Confirmation to the genus level was performed by the presence of *Acinetobacter* spp. 16 S rRNA signature, as previously described by Vanbroekhoven et al. (2004).

4.2.3.2. Selection of isolates for identification to the species level

In order to avoid some repetitive isolates from each sample all isolates belonging to the genus *Acinetobacter* were genotyped by Pulsed-field Gel Electrophoresis (PFGE). From each sample, only one isolate of each PFGE profile was selected for further analysis. PFGE was performed according to Chang et al. (2013), using the restriction enzyme *Asc*I (New England Biolabs, Ipswich, MA), with minor modifications: the optical density of bacterial cell suspensions used was adjusted to 1.3 at 600 nm and the minimal amount of *Asc*I was 5U. The PFGE was run in a CHEF Mapper XA (Bio-Rad Laboratories, Hercules, CA), with pulses ranging from 4 to 40 s at 14 °C with 6 V/cm for 21 h. The band patterns were analysed by GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) and the cluster analysis of PFGE profiles was done by the unweighted-pair group method with average linkages (UPGMA), using the Dice coefficient to generate a dendrogram describing the relationship among PFGE profiles. A band position tolerance of 1.0 was selected and the classification of isolates into different *Asc*I patterns was visually validated.

4.2.3.3. Species identification

Species identification was performed for representative isolates of each PFGE profile based on the analysis of the sequence of the region Z1–Z2 of the gene for RNA polymerase beta-subunit (*rpo*B) according to La Scola et al. (2006). The partial sequences of *rpo*B (902 bp) were amplified with the primers (Ac696F and Ac1598R) and in conditions described by La Scola et al. (2006). PCR products were purified, using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal), according to the supplier's instructions, and the DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). The partial *rpo*B nucleotide sequences were edited manually, using the software BioEdit 7.1.3.0 (Hall, 1999). Similarity between the *rpo*B nucleotide sequences of the tested isolates and the *rpo*B sequences of the type strains of all *Acinetobacter* species available in the GenBank database (http://www.ncbi.nlm.nih.gov) was calculated by using the MEGA6 software (Tamura et al., 2013). Isolates showing at least 95% *rpo*B gene sequence similarity with a type strain were identified as specific *Acinetobacter* species (Krizova et al., 2014; La Scola et al., 2006).

4.2.4. Antimicrobial resistance

Antimicrobial resistance of representative isolates of each PFGE profile, including strains with the same PFGE pattern when recovered from different meat samples, was investigated according to the standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2012) using Mueller Hinton agar (MHA, BioMérieux, Marcy-l'Étoile, France). The disk diffusion method was used to test twelve antibiotics: piperacillin (PIP, 100 μg), piperacillin-tazobactam (TZP 100/10 μg), ampicillin-sulbactam (SAM 10/10 μg), ceftazidime (CAZ, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), amikacin (AK, 30 μg), tobramycin (TOB, 10 μg), tetracycline (TE, 30 μg), minocycline (MI, 30 μg), ciprofloxacin (CIP, 5 μg), trimethoprim-sulfamethoxazole (SXT 1.25/ 23.75 μg) (all from Oxoid, Hants, UK). Susceptibility to colistin (CL) and polymyxin B (PB) was evaluated by the agar dilution method with antibiotic concentrations ranging from 1 to 8 μg/mL. Cultures were incubated for 24 h at 35 °C and *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* DSM 1117 were used as quality control strains. The inhibition zones were measured and the strains were categorized as susceptible or resistant according to CLSI interpretive criteria (CLSI, 2012).

4.3. Results and Discussion

4.3.1. Prevalence of *Acinetobacter* spp. in meat samples

In the current study, *Acinetobacter* spp. was isolated from all the meat samples analysed. Previous studies reported recovery rates of 75% (from 36 pork and beef samples, purchased from local markets in Hong Kong; Houang et al. (2001)) and 28% (from 50 cow meat samples from Lebanon; Rafei et al. (2015)). These differences may be explained by the different methodologies used for the isolation. Houang et al. (2001) did not perform a pre-enrichment step; on the other hand, Rafei et al. (2015) used Baumann medium enrichment. In the present study, Dijkshoorn´s medium was used for the enrichment step, which was previously demonstrated to be a medium resulting in high recovery rates (Carvalheira et al., 2016). In addition, the work developed by Rafei et al. (2015) used MacConkey agar medium supplemented with antibiotics (cephadine, amoxicillin, fosfomycin and cycloheximide) after the enrichment step; this fact may be related with the observed limited recovery.

A total of 223 isolates were identified to belong to the genus *Acinetobacter.* To verify the distinctness of the isolates recovered from the same sample at the strain level we performed macrorestriction analysis of genomic DNA by PFGE. Excluding clones recovered from each sample, 166 isolates were selected and constituted the representative group of *Acinetobacter* strains which were further analysed. These isolates were distributed among 141 different PFGE profiles since isolates showing similar PFGE profiles were recovered from different samples and were grouped in 13 clusters (Supplementary Figure 4.1). Indeed, it was observed that one PFGE profile of one strain of *A. pittii* (Cluster F; Supplementary Figure 4.1.) was shared between samples of all kinds of meat. The identification of small clusters constituted by strains that were isolated from different meat samples suggested a common source of contamination for example during breeding, slaughter, meat processing, packing or retail. Moreover, the high genetic diversity of strains isolated from meat samples has also previously been reported by Lupo et al. (2014) but only concerning the species *A. baumannii*.

Based on the analysis of the partial sequence of *rpo*B a high diversity of *Acinetobacter* species from meat samples was observed. Table 4.1 shows the distribution of the *Acinetobacter* species by source.

Table 4.1. Closest related species of *Acinetobacter* strains based on the *rpoB* sequence analysis according to the source of isolation.

Closest related species (type strain)	**% of *rpoB* sequence similarity (accession number)**	**No. of strains**	**Meat Samples**			
			Chicken	**Turkey**	**Beef**	**Pork**
A. guillouiae (NIPH 522^{T})	97.3 - 99.9 (EU477117)	58	15	12	14	17
A. johnsonii (NIPH 518^{T})	98.3 - 99.8 (EU477113)	25	10	10	2	3
A. bereziniae (NIPH 521^{T})	97.4 - 100 (EU477116)	20	8	5	3	4
A. pittii (NIPH 519^{T})	98.1 - 100 (EU477114)	12	5	1	1	5
A. ursingii *(DSM 16037^{T})*	97.1 - 100 (EU477105)	8	1	1	2	4
A. seifertii (NIPH 973^{T})	98.9 - 99.7 (EU477126)	8	4	1	1	2
A. baumannii (NIPH 501^{T})	99.1 - 99.9 (EU477108)	7	3	3		1
A. nosocomialis (LMG 10619^{T})	99.3 - 99.6 (HQ123389)	4	1	1	1	1
A. gyllenbergii (NIPH 2150^{T})	99.5 - 99.7 (EU477148)	4	1		2	1
A. gerneri (NIPH 2282^{T})	99.8 (EU477151)	4	1	3		
A. calcoaceticus (NIPH 2245^{T})	97.5 - 98.7 (EU477149)	3			1	2
A. parvus (NIPH 384^{T})	96.8 (EU477107)	2		1		1
A. radioresistens (NIPH 513^{T})	99.3 (EU477112)	1		1		
Non Identified:						
A. gandensis (ANC 4275^{T})	89.6* - 92.2* (KJ569689)	7	1	1	4	1
A. ursingii (DSM 16037^{T})	93.7* - 94.0* (EU477105)	2	2			
A. tjernbergiae (NIPH 2285^{T})	94.5* (EU477153)	1			1	

**rpo*B sequence similarity values < 95 % suggest that these isolates may represent novel species.

A total of 156 out of 166 strains, were identified as members of thirteen distinct species and *A. guillouiae* (34.9%, 35/166), *A. johnsonii* (15%, 25/166), and *A. bereziniae* (12%, 20/166) were the most prevalent species. One important finding in the present study is the identification of nosocomial human pathogens, i.e. 18.7% (31/166) of the strains were identified as members of the *A. baumannii* group: *A. baumannii* (n=7), *A. pittii* (n=12), *A. seifertii* (n=8) and *A. nosocomialis* (n=4). These are the species most frequently associated with nosocomial infections worldwide (Bergogne-Bérézin and Towner, 1996; Park et al., 2012; Peleg et al., 2008; Wisplinghoff et al., 2012).

The implementation of molecular methods such *rpo*B gene sequencing improved the detection and identification of species other than *A. baumannii* such as *A. johnsonii*, *A. bereziniae*, *A. ursingii*, *A. gyllenbergii*, *A. calcoaceticus*, *A. parvus* and *A. radioresistens* that have also been associated with nosocomial infections (Krizova et al., 2015; Nemec et al., 2009; Turton et al., 2010) and these were recovered in this study (Table 4.1). In contrast, Rafei et al. (2015) detected the presence of just three species namely *A. baumannii* (n=4), *A. pittii* (n=6), and *A. bereziniae* (n=3) from 50 cow meat samples from Lebanon. These differences can also be explained by the different isolation methods used between the studies as mentioned above.

A. baumannii was previously isolated by Lupo et al. (2014) from 62 out of 248 (25%) meat samples (chicken, turkey, veal, beef and pork) based on matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS). Nevertheless, *A. baumannii* was not detected in twenty-seven meat samples (cow, chicken and pork) from retail supermarket chains in Edinburg (Hamouda et al., 2008). It is possible that these results may reflect different levels of compliance with good manufacturing practices in different meat production chains.

The isolation of one species per sample is usually reported (Houang et al., 2001; Rafei at al., 2015). However, in the present study beyond the high diversity of strains it was also observed a diversity of species in each sample ranging from one to four. Indeed, it was possible to recover the species *A. johnsonii*, *A. baumannii*, *A. bereziniae* and *A. nosocomialis* from a sample of chicken meat.

At least 10 strains could not be identified to species level since the similarity of the values of *rpo*B sequence were less than 95% which indicate distinct species according to La Scola et al. (2006) and Krizova et al. (2014). Therefore, these ten strains, which were clustered in three distinct groups, may represent three new species candidates. The closest related species of these ten strains comprised the species *Acinetobacter gandensis* (n=7), *A. ursingii* (n=2), *Acinetobacter tjernbergiae* (n=1), with *rpo*B sequences similarities ranging from 89.6% to 94.5% (Table 1).

4.3.2. Antimicrobial resistance

Antimicrobial resistance of the strains to the tested fourteen antimicrobial agents is shown in Table 4.2 and in Supplementary Table 4.1. In general, strains were resistant to piperacillin (70.8%) which has previously been documented as a non-effective antibiotic for the treatment of *Acinetobacter* infections (Jain and Danziger, 2004); but also to some of the categories of antimicrobials often used to treat *Acinetobacter* infections such as combination of β-lactam with β-lactamase inhibitors (piperacillin-tazobactam, 64.9%), cephalosporins (ceftadidime, 43.5%), as well as to fluoroquinolones (ciprofloxacin, 42.9%) that can be used in combination with the previous antimicrobials. Moreover, it is important to highlight the high numbers of resistant strains to colistin (41.7%) and polymyxin B (35.1%), which are frequently used as last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter*. The percentage of strains resistant to trimethoprim-sulfamethoxazole, tetracycline, amikacin, tobramycin and ampicillin-sulbactam was higher than 10% (23.2%, 23.2%, 14.3%, 12.5%, 12.5%, respectively). Whereas strains resistant to minocycline (1.2%) as well as to carbapenems such meropenem (8.3%) and imipenem (1.2%), were sporadically observed. This observation is in contrast to the reported increasing resistance of clinical isolates to carbapenem (Kempf and Rolain, 2012; Zarrilli et al., 2013).

The incidence of antibiotic resistance bacteria in meat samples has been attributed, at least partially, to the extensive use of antimicrobials for treatment, prevention and control of diseases in food-producing animals, since this enhances the antimicrobial selective pressure for strains present (Marshall and Levy, 2011; Müller et al., 2014). According to European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (EMA/ESVAC, 2013), tetracyclines (36.7%) and penicillins (24.5%) are the antimicrobials most often used for food-producing animals across the 26 EU/EEA countries in 2013. As well, polymixins, aminoglycosides and fluoroquinolones are also used but in a lower percentage (6.1%, 3.7% and 1.9%, respectively). On the other hand, as carbapenems antibiotics are not allowed to treat food-producing animals this may explain the low level of resistance to antibiotics of this class observed in the current study.

Table 4.2. Prevalence of antibiotic resistance among *Acinetobacter* spp. strains isolated from meat samples.

Species	No. (%) of resistant strains													
(no. of strains)	PIP	TPZ	SAM	CAZ	IMP	MEM	PB	CL	AK	TOB	TE	MI	CIP	SXT
***A. guillouiae* (58)**	53 (91.4)	53 (91.4)	9 (15.5)	37 (63.8)		3 (5.2)	16 (27.6)	26 (44.8)	13 (22.4)	8 (13.8)	13 (22.4)		45 (77.6)	22 (37.9)
***A. johnsonii* (25)**	22 (88)	18 (72)	7 (28)	14 (56)	1 (4)	5 (20)			6 (24)	8 (32)	6 (24)		10 (40)	10 (40)
***A. bereziniae* (20)**	12 (60)	12 (60)		6 (30)		1 (5)	19 (95)	18 (90)			9 (45)		6 (30)	3 (15)
***A. pittii* (12)**	6 (50)	6 (50)	3 (25)	4 (33.3)	1 (8.3)	4 (33.3)		2 (16.7)	2 (16.7)	3 (25)	3 (25)		4 (33.3)	
***A. ursingii* (8)**	2 (25)	2 (25)		4 (50)			3 (37.5)	2 (25)						
***A. seifertii* (8)**	7 (87.5)	5 (62.7)		2 (25)			7 (87.5)	8 (100)			3 (37.5)		3 (37.5)	
***A. baumannii* (7)**	3 (42.9)	3 (42.9)		1 (14.3)		1 (14.3)	2 (28.6)	1 (14.3)	1 (14.3)		3 (42.9)			
***A. nosocomialis* (4)**	2 (50)	1 (25)		1 (25)			4 (100)	4 (100)	1 (25)					1 (25)
***A. gyllenbergii* (4)**	4 (100)	2 (50)					2 (50)	3 (75)				1 (25)	1 (25)	
***A. gerneri* (4)**	2 (50)	2 (50)		1 (25)			3 (75)	3 (75)						
***A. calcoaceticus* (3)**	2 (66.7)	1 (33.3)						1 (33.3)						
***A. parvus* (2)**														
***A. radioresistens* (1)**														
Non Identified* (10)	4 (40)	4 (40)	2 (20)	3 (30)			3 (30)	1 (10)	1 (10)	2 (20)	2 (20)	1 (10)	3 (30)	3 (30)
Total (166)	119 (70.8)	109 (64.9)	21(12.5)	73 (43.5)	2 (1.2)	14 (8.3)	59 (35.1)	70 (41.7)	24 (14.3)	21 (12.5)	39 (23.2)	2 (1.2)	72 (42.9)	39 (23.2)

PIP, piperacillin; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole.

*rpoB sequence similarity values < 95 % suggest that these isolates may represent novel species.

Another notable finding is the resistance of the strains to several antimicrobial agents (Supplementary Table 4.1). Indeed, only 13.3% (22/166) of the strains were susceptible to all the antibiotics tested and 51.2% (85/166) were considered as multidrug-resistant (MDR), i.e. acquired resistance to at least one agent in three or more antimicrobial categories among the eight different classes of antibiotics used to define MDR (Magiorakos et al., 2012). Besides, 9.6% (16/166) of the strains were classified as extensively drug-resistant (XDR) i.e. resistant to at least one agent in all but two or fewer antimicrobial categories (Magiorakos et al., 2012). Moreover, at least 50% of the strains of the species *A. guillouiae*, *A. johnsonii, A. bereziniae* and *A. pittii,* and *A. seifertii* were MDR and some of them were resistant to more than 5 classes of antibiotics (Supplementary Table 4.1).

In contrast to that previously reported by Lupo et al. (2014) whereas poultry samples were the most contaminated with *A. baumannii*, in this study no specific prevalence (considering specific kind of meat) of species as well as of MDR strains was observed. Although it has been reported that strains belonging to the *A. baumannii* group were generally more resistant to antibiotics than other species (Dijkshoorn et al., 2007; Wareham et al., 2008) in the current study the prevalence of MDR strains within the *A. baumannii* group (38.7%, 12/31, Supplementary Table 4.1) was lower than the prevalence within all the other species identified (54.1%, 73/135, Supplementary Table 4.1). The acquisition of the MDR phenotype has been suggested as an important factor for the success of these nosocomial pathogens (Imperi et al., 2011).

In general, *A. baumannii* strains were susceptible to almost all the antibiotics tested and only one strain was resistant to four antimicrobial classes. Few studies have reported the antimicrobial resistance of *Acinetobacter* spp. in raw meat and usually related to the species *A. baumannii* (Lupo et al., 2014; Rafei et al., 2015). Lupo et al. (2014) and Rafei et al. (2015) also found that the population of *A. baumannii* outside the hospital was generally susceptible to clinical relevant antibiotics. *Acinetobacter* spp. previously isolated from veterinary clinical specimens of food producing animals were usually susceptible to most of the antibiotics (Hamouda et al., 2011; Hamouda et al., 2008; Rafei et al., 2015) although the detection of carbapenemase-producing *Acinetobacter* spp. had also been reported (Poirel et al., 2012; Wang et al., 2012; Zhang et al., 2013).

4.4. Conclusions

This study reports the occurrence and antibiotic susceptibility of *Acinetobacter* spp. isolates outside the hospital and is one of a limited number of studies exploring this population in meat products worldwide. To the best of our knowledge, this is the first report about the prevalence and antimicrobial susceptibility of *Acinetobacter* spp. in meat retailed in Portugal.

It was demonstrated the presence of several *Acinetobacter* species, including the species belonging to the pathogenic *A. baumannii* group such as *A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. seifertii* on raw meat samples. Moreover, it was concluded that meat is also an important source of strains resistant to some of the categories of antimicrobials used to treat infections caused by *Acinetobacter* (piperacillin-tazobactam, ceftadidime, ciprofloxacin, colistin and polymyxin B) and some of them are MDR and XDR. Additional studies are required to assess the role of resistant strains in the dissemination of resistance genes.

The presence of clinically important species and multidrug-resistant strains in meat may be a threat to public health considering that meat may provide a vector for the spread of these opportunistic pathogen into both community and hospital settings environment.

CHAPTER 5

Acinetobacter portensis sp. nov. and *Acinetobacter guerrae* sp. nov. isolated from raw meat

CHAPTER 5

Abstract

The taxonomic status of six strains of *Acinetobacter* obtained from meat samples, collected from supermarkets in Porto, Portugal, was investigated, using a polyphasic analysis. Partial *rpo*B sequence similarities lower than 95% to other *Acinetobacter* species with validly published names led to the hypothesis that these strains represented novel species. This was confirmed, based on comparative multilocus sequence analysis (MLSA), including also the *gyr*B, *rec*A and 16S rRNA genes, revealing that these strains represented two coherent lineages that were distinct from each other and from all known species. The names *Acinetobacter portensis* sp. nov. (comprising four strains), and *Acinetobacter guerrae* sp. nov. (comprising two strains) are proposed for these novel species. The species status of these two groups was confirmed by low (below 95%) whole-genome sequence average nucleotide identity (ANI) values and low (below 70%) digital DNA-DNA hybridization (dDDH) similarities between the whole-genome sequences of the proposed type strains of each novel species and the representatives of the known *Acinetobacter* species. Phylogenomic treeing from core genome analysis supported these results. The coherence of each new species-lineage was supported by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) differentiation of the species at the protein level, by cellular fatty acids (CFA) profiles, and by unique and differential combinations of metabolic and physiological properties shared by each novel species. The type strain of *A. portensis* sp. nov. is AC 877^T (= CCUG 68672^T = CCM 8789^T) and the type strain of *A. guerrae* sp. nov. is AC 1271^T (= CCUG 68674^T = CCM 8791^T).

5.1. Introduction

The genus Acinetobacter was described initially by Brisou and Prévot (1954) and currently comprises 63 species, with validly-published names (www.szu.cz/anemec/Classification.pdf) including four pairs of heterotypic synonyms. There are also four genomospecies delineated by DNA–DNA similarity (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Dijkshoorn et al., 2007). Most of these species were proposed based on the descriptions of clinical and environmental isolates while descriptions of strains derived from food are less common (www.szu.cz/anemec/Classification.pdf). However, during a study focused on the diversity of *Acinetobacter* spp. in samples of meat (beef, pork, chicken and turkey), we identified novel genomospecies clearly distinct from all known species and other genomospecies of the genus (Carvalheira at al., 2017).

This study carried out comprehensive characterisations and assessed the properties and taxonomic and phylogenetic positions of these strains, using polyphasic taxonomic methods relevant for the genus *Acinetobacter* (Vandamme and Peeters, 2014; Vandamme et al.,1996). Our results indicate that these bacteria represent two novel species genotypically and phylogenetically coherent and distinct from each other and from all known species. The names *Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov. are proposed for these two taxonomic lineages.

5.2. Isolation and ecology

A total of six strains of *A. portensis* sp. nov. (n=4) and *A. guerrae* sp. nov. (n=2) (Table 5.1) were isolated from raw meat samples (purchased from supermarkets in Porto, Portugal), using enrichment cultivation in Dijkshoorn's medium, at 30 ºC, for 24 h, followed by plating on CHROMagar^{TM} *Acinetobacter* at 30 °C, for 24 – 48 h, according to Carvalheira et al. (2017). The typical red colonies of *Acinetobacter* spp. were identified to the genus-level, on the basis of phenotypic (Gram-negative, coccobacilli, oxidase-negative and catalase-positive) and genotypic (16S rRNA gene sequence signatures and similarities) characteristics, according to Vanbroekhoven et al. (2004).

Macrorestriction profile analysis of genomic DNA was performed by pulsed-field gel electrophoresis (PFGE) according to Carvalheira et al. (2017), to verify the diversity of the isolates at the strain level, and showed that these strains yielded unique macrorestriction patterns (Supplementary Figure 5.1). Additionally, the genotypic heterogeneity of the strains studied was supported by differences in DNA-directed RNA polymerase β-subunit (*rpo*B) gene sequences (Carvalheira et al., 2017), which is the best studied single gene taxonomic and phylogenetic marker for the *Acinetobacter* spp. (Krizova et al., 2014; Krizova et al., 2015; La Scola et al., 2006; Nemec et al., 2011; Nemec 2009; Nemec et al., 2010).

Table 5.1. Strains of *Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov.

Strains designation	**Specimen**	**Locality and date of isolation**	**Accession no.**				
			16S rRNA	***rpo*B gene**	***gyr*B gene**	***rec*A gene**	**Genome sequence**
***Acinetobacter portensis* (n=4)**							
AC 877^T (= CCUG 68672^T = CCM 8789^T)	Raw pork meat	Porto, Portugal. September 2014	KX870877	KX885208	KX885192	KX885200	LWRV00000000.1
AC 1335 (= CCUG 68677)	Raw turkey meat	Porto, Portugal. September 2014	ND	KX885213	KX885197	KX885205	ND
AC 1301 (= CCUG 68676)	Raw chicken meat	Porto, Portugal. September 2014	ND	KX885212	KX885196	KX885204	ND
AC 1123 (= CCUG 68673 = CCM 8790)	Raw beef meat	Porto, Portugal. September 2014	ND	KX885209	KX885193	KX885201	ND
***Acinetobacter guerrae* (n=2)**							
AC 1271^T (= CCUG 68674^T = CCM 8791^T)	Raw chicken meat	Porto, Portugal. September 2014	KX870878	KX885210	KX885194	KX885202	LXGN00000000.1
AC 1272 (= CCUG 68675 = CCM 8792)	Raw chicken meat	Porto, Portugal. September 2014	ND	KX885211	KX885195	KX885203	ND

CCUG: Culture Collection University of Gothenburg, Sweden; CCM: Czech Collection of Microorganisms, Brno, Czech Republic. ND, not determined.

5.3. 16S rRNA gene phylogeny

The strains AC 877^T (= CCUG 68672^T = CCM 8789^T) and AC 1271^T (= CCUG 68674^T = CCM 8791^T), were selected, respectively, as the designated type strains of the species *A. portensis* sp. nov. and *A. guerrae* sp. nov.. To determine the 16S rRNA gene sequences the primers, 16F28 (5′-AGA GTT TGA TCK TGG CTC AG-3’) and 16R1494 (5′-NTA CGG YTA CCT TGT TAC GAC-3′) were used for PCR-amplification, and primers, 16F63 (5′-AGG CCT AAC ACA TGC AAG TC-3′), 16SF1103 (5’-TGT TGG GTT AAG TCC CGC AAC-3’) 16SR806 (5′-GGA CTA CCA GGG TAT CTA AT-3′) and 16R1494 were used for Sanger sequencing. PCR-amplification of biomarker genes was performed in volumes of 25 µl, with 1.25 U of Taq PCR Master Mix Kit 250 Units (Qiagen, Hilden, Germany), 1 µM of each amplification primer and 5 µL of bacterial genomic DNA. After initial denaturation, at 95 °C, for 2 min, 35 amplification cycles were performed, according to the format: 30 sec at 95 °C; 1 min at 55 °C; 2 min at 72 °C; and a final extension of 10 min at 72 °C. PCR-products were purified and sequenced (GATC Biotech AG, Konstanz, Germany). The PCR amplification primers target the region corresponding to positions 28 – 1,494 in the *Escherichia coli* 16S rRNA gene sequence numbering (NCBI accession no. J01859).

The full-length 16S rRNA gene sequences were extracted from the available whole-genome sequences of the type strains of *Acinetobacter* species and reference strains of genomospecies (Supplementary Table 5.1) to compare the sequences of the putative new species within the context of all known species of the genus. For *A. modestus,* and *A. piscicola*, only incomplete 16S rRNA gene sequences were available in the genome sequence assemblies. Thus, alternative publicly available, nearly complete sequences were used (Supplementary Table 5.1).

The analysis was carried out, using a region of 1,353 nucleotide positions, corresponding to positions 105 through 1,457 of the 16S rRNA gene sequence of *E. coli*. Evolutionary distances were calculated, using the Maximum Composite Likelihood method (Tamura et al., 2004), with the MEGA6 software (Tamura et al., 2013) and a phylogenetic tree was reconstructed, using the Neighbour-Joining method (Saitou and Nei, 1987) (Supplementary Figure 5.2). The most closely related species to the type strain of *A. portensis* sp. nov., was observed to be *A. celticus* ANC 4603^T (97.7 %) and *A. cumulans* WCHAc060092^T (97.6%). The most closely related species to the type strain of *A. guerrae* sp. nov., was *A. ursingii* CIP 107286^T (99.1%) and *A. beijerinckii* CIP 110307^T (97.3%). Notably, in the case of the comparative analyses of *A. portensis* sp. nov., the similarity values are below 98.5%, which was proposed by Kim et al. (2014) as the threshold for delineating prokaryotic species, confirming that this proposed novel species is distinct from all known species. Concerning *A. guerrae* sp. nov., the 16S rRNA gene sequence similarity value is above the threshold of 98.5%; therefore, the species status was confirmed based on house-keeping gene sequence analyses and whole-genome sequences analysis. The lowest 16S rRNA gene sequence similarities to other *Acinetobacter* spp. between the type strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov. were with the 16S rRNA gene sequence of *A. apis* ANC 5114^T (94.2% and 94.7%, respectively; Supplementary Figure 5.2).

5.4. Genome sequence determination

Representatives of all *Acinetobacter* species, including known genomospecies, except *A. grimontii*, a later synonym of *A. junii* (Touchon et al., 2014), *A. pakistanensis*, a later synonym of *A. bohemicus* (Nemec and Radolfova-Krizova, 2016), *A. guangdongensis*, a later synonym of *A. indicus* (Nemec and Radolfova-Krizova, 2017) and *A. dijkshoorniae*, a later synonym of *A. lactucae* (Dunlap and Rooney, 2018), were included in the genomic analyses (Figures. 5.1, 5.2, Supplementary Figure 5.3). Accession numbers are presented in Supplementary Table 5.1.

The type strains of *A. portensis* sp. nov. AC 877^T and *A. guerrae* sp. nov. AC 1271^T were cultivated overnight, on Columbia agar base with 5% of defibrinated horse blood, at 30 °C. Cells were lysed in CLB buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) supplemented with proteinase K (1 mg/ml), incubating at 56 °C for 1 h. Afterwards, genomic DNA was isolated, using a MagNA Pure Compact Nucleic Acid Isolation Kit version I (Roche Diagnostics, Mannheim, Germany). Libraries for the whole-genome sequencing were prepared, using the TruSeq DNA Nano kit (Illumina, San Diego, CA), with a mean fragment length of 900 bp. Libraries were sequenced on the Illumina MiSeq platform v.3 chemistry, 2*300 bp (SciLifeLab, Stockholm, Sweden). Subsequently, sequence reads were trimmed and assembled *de novo*, using CLC Genomics Workbench v8.5.1 (CLC bio, Aarhus, Denmark). The genome assembly of *A. portensis* sp. nov. AC 877^T yielded 123 contigs of a total size of 2.89 Mb and a GC content of 36.6%. The genome assembly of *A. guerrae* sp. nov. AC 1271^T yielded 34 contigs of a total size of 3.41 Mb and a GC content of 39.2%. The genome sequences of *A. portensis* sp. nov. AC 877^T and *A. guerrae* sp. nov.

AC 1271^T were annotated, using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016), revealing 2,595 and 3,104 coding sequences, respectively.

5.5. Overall genome relatedness indices

The average nucleotide identities based on BLAST (ANIb) (Goris et al., 2007) were determined between the genome sequences of *A. portensis* sp. nov. AC 877^T, *A. guerrae* sp. nov. AC 1271^T and those of the type strains of nearly all *Acinetobacter* species and representatives of genomospecies (Supplementary Table 5.2).

Table 5.2. Highest similarity values between 16S rRNA, concatenated *rpo*B, *rec*A*, gyr*B gene sequences and whole genome sequences of the strains of *Acinetobacter portensis* sp. nov., *Acinetobacter guerrae* sp. nov. and the type or reference strains of known species of the genus *Acinetobacter.*

	Highest similarity values			
Strains designation	**16S rRNA gene**	**Concatenated *rpo*B, *rec*A, *gyr*B gene**	**ANIb**	**dDDH**
***Acinetobacter portensis* (n=4)**				
AC 877^T (= CCUG 68672^T = CCM 8789^T)	97.7 % *A. celticus*	86.6 % *A. cumulans*	78.2 % *A. equi*	23 % *A. cumulans*
AC 1335 (= CCUG 68677)		86.6 % *A. cumulans*		
AC 1301 (= CCUG 68676)		86.7 % *A. cumulans*		
AC 1123 (= CCUG 68673 = CCM 8790)		86.9 % *A. cumulans*		
***Acinetobacter guerrae* (n=2)**				
AC 1271^T (= CCUG 68674^T = CCM 8791^T)	99.1 % *A. ursingii*	87 % *A. ursingii*	86.7 % *A. ursingii*	32.6 % *A. ursingii*
AC 1272 (= CCUG 68675 = CCM 8792)		86.7 % *A. ursingii*		

The ANIb were determined, using the JSpeciesWS (http://jspecies.ribohost.com/jspeciesws) (Richter et al., 2016). The ANIb values (means of reciprocal values; Tables 2 and Supplementary Table 5.2) of the genome sequences of *A.*

portensis sp. nov. AC 877^T and *A. guerrae* sp. nov. AC 1271^T against those of all other species of *Acinetobacter* ranged from 71.26% (*A. qingfengensis* ANC 4671^T) to 78.23% (*Acinetobacter equi* 114^T) and from 71.52% (*Acinetobacter boissieri* ANC 4422^T) to 86.70% (*A. ursingii* CIP 107286^T), respectively. These values are much lower than the suggested threshold of 94–96% sequence similarity, proposed to distinguish bacterial species (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009) and further supports the genomic distinction of *A. portensis* sp. nov. and *A. guerrae* sp. nov. at the species level. These findings were supported by the similarity values from digital DNA-DNA hybridization (dDDH) analyses (Tables 2 and Supplementary Table 5.2), which were determined, using the Genome-to-Genome Distance Calculator (GGDC) web server (Meier-Kolthoff et al., 2013). The dDDH values ranged between 19.2–23% for *A. portensis* sp. nov. AC 877^T and other species of *Acinetobacter* and between 19.1–32.6% for *A. guerrae* sp. nov. AC 1271^T and other species of *Acinetobacter*, all well below the threshold of 70% for belonging to the same species.

5.6. Relatedness with publicly available genome sequences

All genome sequences labelled as *Acinetobacter* sp. that were available in NCBI RefSeq (O'Leary et al., 2016) on 12th January 2020, were downloaded. Subsequently, average nucleotide identity values, based on BLAST (ANIb) between *A. portensis* AC 877^T, *A. guerrae* AC 1271^T and the downloaded genome sequences, were calculated, using JSpeciesWS (Richter et al., 2016). The analyses revealed two genome sequences of strains matching with *A. guerrae*: TUM15565 (GenBank accession number: BKYM00000000; ANIb value: 97.90%) and WCHAc060096 (GenBank accession number: RAXU00000000; ANIb value: 98.26%).

Strain TUM15565 was isolated from human sputum in Kanagawa, Japan, in 2013; the genome sequence has a total length of 3.86 Mb, GC content of 39.5% and 3,465 coding sequences. Strain WCHAc060096 was isolated from hospital sewage in Sichuan, China, in 2018; the genome sequence has a total length of 3.54 Mb, GC content of 39.3% and 3,252 coding sequences. Strains of *A. guerrae* described in the present study were isolated from raw meat in Portugal. However, the finding of these two additional strains isolated from human sputum and hospital sewage in Asia, suggests that the species has a broader geographical and ecological distribution and that the public health risk of *A. guerrae* strains should not be neglected. Therefore, these two additional *A. guerrae* TUM15565 and

WCHAc060096 strains were also included in the genotypic analyses (Figures 5.1, 5.2, Supplementary Figure 5.3).

5.7. Core genome-based phylogenomic analysis

The core genome for the dataset of the novel genome sequences and the reference genomes (listed in Table S1) was determined, using the Roary pan-genome analysis pipeline (Page et al., 2015) with an amino acid identity threshold of 50 %.

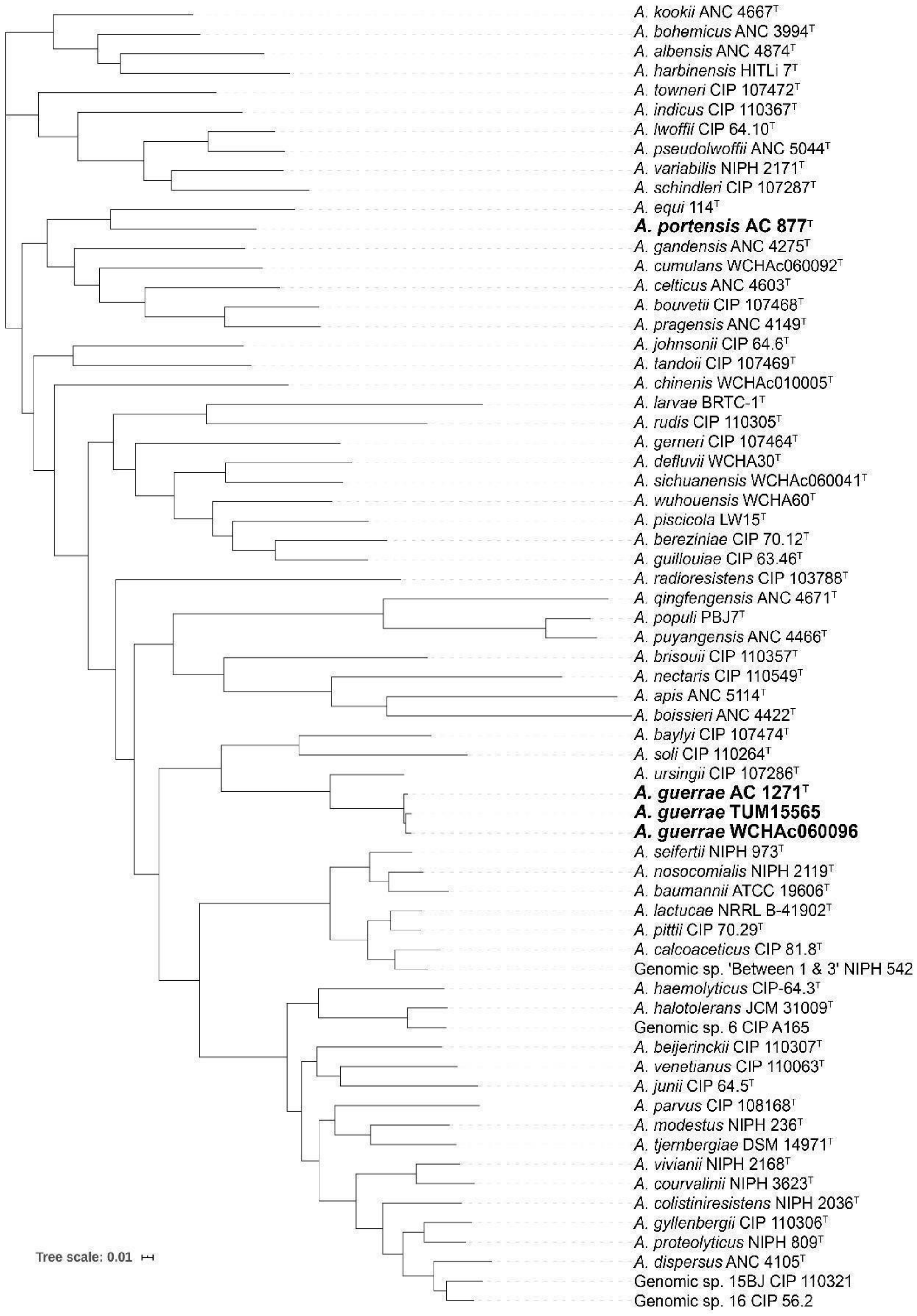


Figure 5.1. Core genome-based phylogenomic tree based on the alignment of 1,007 core genes. Taxa in bold highlight the two proposed novel species.

The resulting core genome alignment, based on 1,007 core genes, was used for construction of a dendrogram of phylogenetic relationships, using FastTree (Price et al., 2009). The core genome tree supported other phylogenetic analyses in that *A. portensis* was observed to be most closely related to *A. equi* while *A. guerrae* is most closely related to *A. ursingii,* sharing an evolutionary clade with *A. baylyi* and *A. soli* (Figure 5.1).

5.8. Multilocus sequence analysis (MLSA)

The intraspecies genotypic coherence of the strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov. was assessed, using multilocus sequence analysis (MLSA), based on the concatenated sequences of genes encoding *rpo*B, recombinase subunit-A (*rec*A) and DNA gyrase subunit-B (*gyr*B).

The partial sequences of *rpo*B of *A. portensis* sp. nov. and *A. guerrae* sp. nov. strains were obtained as previously described (Carvalheira et al., 2017; Krizova et al., 2015). Likewise, the partial sequences of *rec*A were amplified with the primers, RA1 and RA2, as described previously (Nowak and Kur, 1995), and the partial sequences of *gyr*B were obtained, using newly-developed primers, Aci_gyrB-F4: 5′-AHH GAA AAR GCD TAT GAT TCY TC-3′ and Aci_gyrB-R2: 5′-SWH CCR CCH GCN GAR TCR C-3′), under PCR-amplification and Sanger sequencing conditions described above for 16S rRNA gene. Sequence accession numbers are listed in Supplementary Table 5.1.We obtained complete or nearly-complete *rpo*B, *rec*A and *gyr*B gene sequences from GenBank or by extracting them from the available whole-genome sequences of the type strains of *Acinetobacter* species and reference strains of genomospecies to compare these sequence data within the context of the genus (Supplementary Table 5.1). The three gene sequences were also extracted from the two additional database whole-genome sequences of strains of *A. guerrae* and included in the analysis.

The nucleotide sequences of *rpo*B, *rec*A and *gyr*B were individually aligned by MEGA6 software (Tamura et al., 2013) and further concatenated, using the Join Alignments tool of the CLC Genomics Workbench version 12 (QIAGEN Aarhus A/S, Aarhus, Denmark). The phylogenetic tree and evolutionary distances were inferred, by MEGA6 software (Tamura et al., 2013), as described above for 16S rRNA gene sequences. Gene sequence similarity calculations and cluster analyses were carried out for 717 bp, 388 bp and 773/776 bp segments, corresponding to nucleotide positions 3041–3757, 210–597 and 425–1200 of the *rpo*B, *rec*A and *gyr*B coding regions of *A. baumannii* ATCC 19606^{T} (NCBI accession no. ACQB00000000.1), respectively.

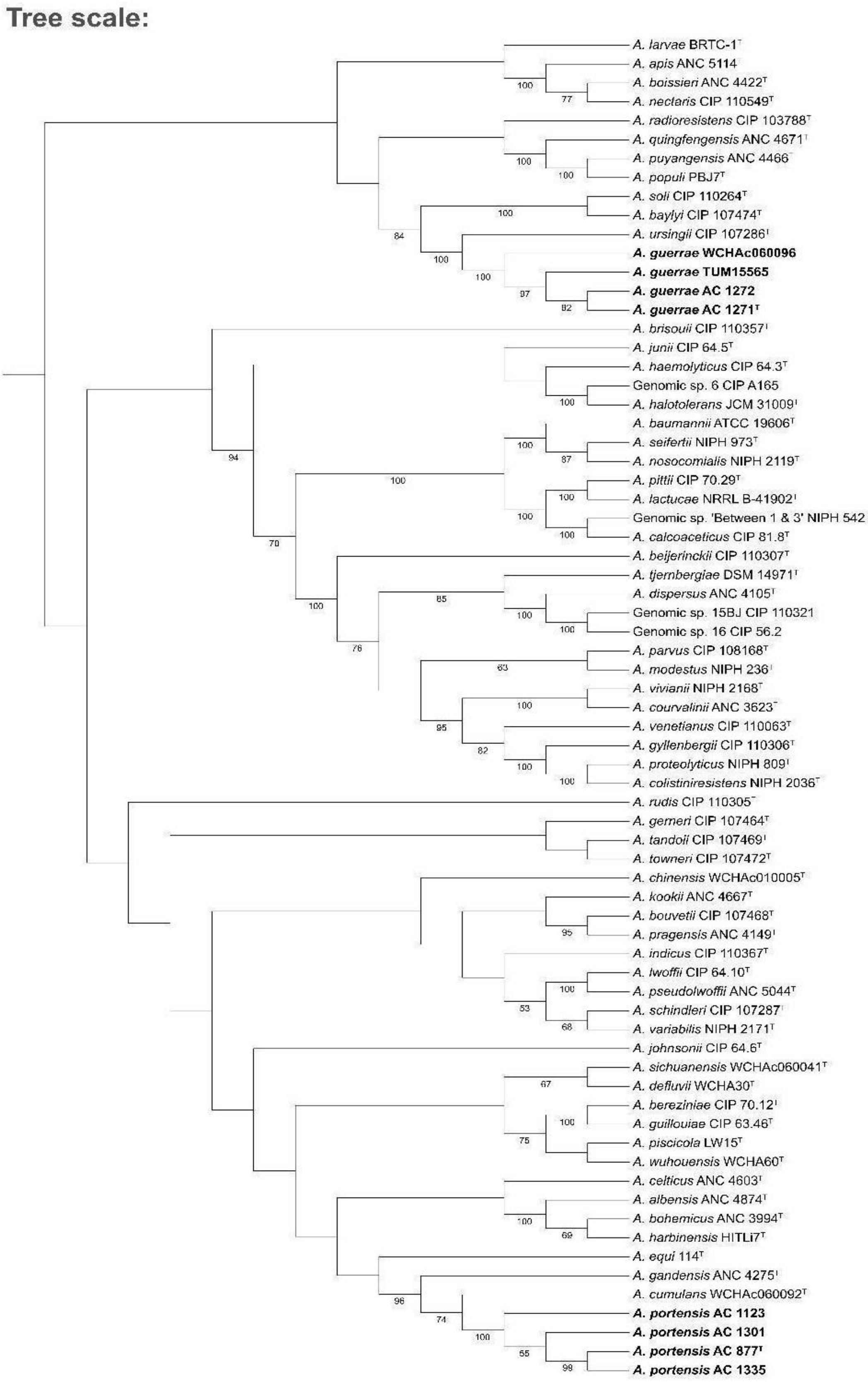


Figure 5.2. Neighbour-joining phylogram based on the concatenated partial *rpo*B, *rec*A and *gyr*B gene sequences of four strains of *Acinetobacter portensis* sp. nov., four strains of *Acinetobacter guerrae* sp. nov., the type strains of the 63 species with validly published name of the genus *Acinetobacter*, and four genomospecies of the genus. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Bootstrap values (> 50%) after 1,000 simulations are shown at branch nodes. All calculations were conducted in MEGA6 (Tamura et al., 2013).

Figure 5.2 shows the results of cluster analysis for the concatenated *rpo*B, *rec*A and *gyr*B sequences of the strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov., and type strains of all species and genomospecies of *Acinetobacter*. As shown in Figure 5.2, the strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov. grouped into two respective clusters, distinct from all known *Acinetobacter* spp., which was supported by high bootstrap values. The intraspecies similarity values (expressed as the percentages of identical nucleotides in corresponding positions in two aligned sequences) of the concatenated sequences of the strains of *A. portensis* sp. nov. ranged from 99.8% to 100% while the similarity values for the strains of *A. guerrae* sp. nov. ranged from 98.4% to 99%. The similarity values between the strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov. with the other species of the genus ranged from 72.4% (*A. radioresistens* CIP 103788^T) to 86.9% (*A. cumulans* WCHAc060092^T), and from 72.8% (*A. apis* ANC 5114^T) to 87% (*A. ursingii* CIP 107286^T), respectively (Table 5.2). High intraspecies similarities were also reflected in *rpo*B individual sequences: 99.4-100% and 99.4-99.6% for the *A. portensis* sp. nov. and *A. guerrae* sp. nov. strains, respectively. The similarity values between *rpo*B individual sequences of *A. portensis* sp. nov. and *A. guerrae* sp. nov. strains with the other species of the genus ranged from 70.7% (*A. puyangensis* ANC 4466^T) to 92.0% (*A. wuhouensis* WCHA60^T), and from 74% (*A. kookii* ANC 4667^T) to 92.2% (*A. ursingii* NIPH 107286^T), respectively (Supplementary Figure 5.3). These results are in agreement with the results of other studies of *rpo*B sequence analyses of *Acinetobacter* species, wherein *rpo*B interspecies similarity values are observed to be below 95% (Krizova et al., 2014; Krizova et al., 2015; Nemec and Radolfova-Krizova, 2016; Nemec and Radolfova-Krizova, 2017; Nowak and Kur, 1995). Therefore, these data further support the distinctness of these two novel group at the species level.

5.9. MALDI-TOF MS

Colonies from fresh cultures (overnight growth on Columbia II agar base, BD BBL, with 5% defibrinated horse blood, at 30 ºC) of each strain, as well as the type strains of the most closely related species (i.e., based on the core genome-based phylogenomic tree analyses) and the type strain of the type species of the genus (i.e., *A. calcoaceticus*) were smeared in duplicate, onto disposable target plates and covered with 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. The MALDI-TOF MS analyses were carried out on a VITEK MS RUO system (Research Use Only configuration; bioMérieux, Marcy l'Étoile, France), using standard settings for routine identifications, within a mass range of 2-20 kDa. Spectra were acquired in automatic mode by accumulating 100 profiles of 5 laser shot cycles each, using the auto quality control of Launchpad 2.9. External calibration of the mass spectra was performed, using *E. coli* (CCUG 10979). The MALDI-TOF MS-based dendrogram was generated, using the correlation distance measure with the mean linkage algorithm of the BioNumerics software platform, version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis demonstrated clear separation of the respective mass spectra of the strains of *A. portensis* sp. nov. and *A. guerrae* sp. nov. from those of the representatives of the closest related members of the genus based on the core genome phylogenetic tree analyses (Figure 5.3).

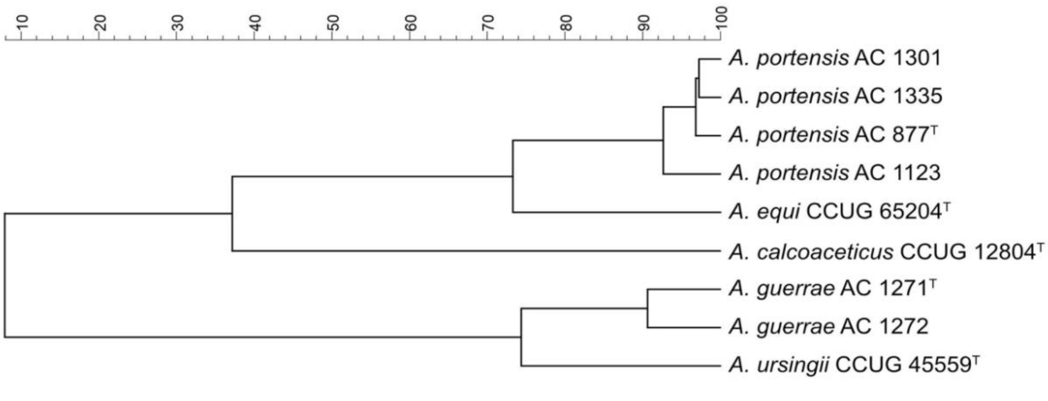


Figure 5.3. Dendrogram based on the MALDI-TOF mass spectra of four strains of *Acinetobacter portensis* sp. nov., two strains of *Acinetobacter guerrae* sp. nov. and the type strains of the most closely related species of the genus *Acinetobacter* (based on core genome-based phylogenomic tree analysis) and the type species of the genus (*A. calcoaceticus*). The dendrogram was constructed using the correlation distance measure with the average linkage algorithm (UPGMA).

5.10. Chemotaxonomy and Physiology

Cellular fatty acid-fatty acid methyl ester (CFA-FAME) analysis of two strains of each novel species (*A. portensis* sp. nov., AC 877^T and AC 1301, *A. guerrae* sp. nov., AC 1271^T and AC 1272) were determined from strain cultures grown for 24 h, at 30 ºC, under aerobic conditions, on Columbia II agar base (BD BBL) with 5% defibrinated horse blood. The bacterial biomass was harvested in stationary phase and treated chemically by alkaline methanolysis afterwards, following a modified version (Zamora et al., 2012) of the method proposed by MIDI (MIDI, Inc., Newark, DE, USA), in order to extract the fatty acid methyl ester (FAME) present in the samples (Sasser, 2001). CFA-FAMEs were identified by Gas Chromatography (GC) by means of a Gas chromatograph HP 5890A Series II (Hewlett-Packard, Palo Alto, CA, USA). The predominant CFA-FAMEs of *A. portensis* strains were observed to be 16:1ω7c and 16:0, while the fatty acids, 18:1ω9c and 16:1ω7c predominated in the CFA composition of *A. guerrae* strains, as well as those of the type strains of related species (Supplementary Table 5.3). Moreover, the novel species could be distinguished from the type strains of the most related species (based on the core genome-based phylogenomic tree analyses) by their CFA profiles. Indeed, *A. portensis* strains could be distinguished from *A. equi* CCUG 65204^T by the higher proportions of the fatty acids 16:1ω7c and 16:0 and lower proportions of the fatty acids 12:0 and 12:0 3-OH and the presence of the fatty acid 17:1ω8c. *A. guerrae* strains could be distinguished from *A. ursingii* CCUG 45559^T by higher proportions of the fatty acid 16:1ω7c, lower proportions of the fatty acid 18:1ω9c and the absence of 14:0 and 16:1ω9c (Supplementary Table 5.3).

Metabolic and physiological features of the strains were assessed, using a set of tests described by Nemec et al. (Nemec et al., 2010), with some modifications and additions. Unless stated otherwise, the cultivation temperature was 30 ºC and results were evaluated after two days incubation. Liquefaction of gelatin was performed, using the Kohn gelatin test. Haemolytic activity was tested on Columbia II agar base (BD BBL) supplemented with 5 % defibrinated horse blood. Growth tests at 20, 30, 37 and 42 ºC were performed in tubes containing 3 ml of Brain-Heart Infusion (BHI) broth (Acumedia) and each tube was inoculated with a drop (30 µl) of cell suspension of standardised turbidity (~10^8 c.f.u. ml^{-1}) prepared in saline from an overnight Blood Agar culture at 30 ºC. NaCl tolerance was assessed in culture medium supplemented with 0.5% to 6% NaCl (w/v), as well as, growth on R2A (Oxoid) and TSA (Acumedia) was also tested at 30 ºC. Utilization of citrate was tested on Simmons' Citrate agar (Scharlab) and was recorded after 2, 4 and 6 days incubation. Tests for the assimilation of the other 27 carbon sources were performed, using the basal mineral medium of Cruze et al. (1979) supplemented with 0.1% (w/v) carbon source. Tubes containing the supplemented medium (3 ml) were inoculated as in the growth-temperature tests and growth on carbon sources was evaluated after 2, 4, 6 and 10 days by visual comparisons between inoculated tubes containing carbon sources and control tubes containing only inoculated basal medium.

Table 5.3. Phenotypic characteristics of the strains of *Acinetobacter portensis* sp. nov. (AC 877^T, AC 1335, AC 1301, AC 1123), *Acinetobacter guerrae* sp. nov. (AC 1271^T, AC 1272), type strains of the most closely related species (based on the core genome phylogenomic tree) and *Acinetobacter calcoaceticus*, the type species of the genus *Acinetobacter*.

Characteristic	***A. portensis***	***A. guerrae***	***A. equi***	***A. ursingii***	***A. calcoaceticus***
Growth at 37 ºC	-	+	+	+	-
Growth at 42 ºC	-	-	ND	-	-
Hemolysis of horse blood	-	-	ND	ND	ND
Hemolysis of sheep blood	ND	ND	-	-	-
Assimilation of:					
trans-Aconitate	-	-	-	-	+
Adipate	-	+	+	+	+
4-Aminobutyrate	-	-	-	-	+
L-Arginine	-	-	-	-	+
Benzoate	-	+	+	-	+
2,3-Butanediol	-	50 (+)	-	-	+
Citrate (Simmons)	+	+	+	+	D
Ethanol	75 (+)	+	-	+	+
Gentisate	25 (-)	+	+	-	-
L-Histidine	+	-	+	-	+
4-Hydroxybenzoate	-	50 (-)	+	+	+
Malonate	-	-	-	-	+
L-Ornithine	-	-	-	-	+
L-Phenylalanine	-	-	-	-	+
Putrescine	-	-	-	-	+

The results of the type strains of *A. equi*, *A. ursingii* and *A. calcoaceticus* were obtained from the study of Hu et al. (2018). All strains grew on acetate, L-aspartate, L-glutamate and DL-lactate whereas no strain liquefied gelatin, acid is not produced from D-glucose or grew on β-alanine, L-arabinose, D-gluconate, D-glucose, histamine, L-leucine, D-ribose, L-tartrate and tryptamine. +, positive; -, negative; numbers, percentages of strains giving a positive reaction; D, mostly doubtful or irreproducible reactions; ND, not determined.

The phenotypic characteristics of *A. portensis* sp. nov., *A. guerrae* sp. nov. and the reference strains of closely related members of the genus according to the core genome-based tree analyses (Table 5.3). The four strains of *A. portensis* were seen to have nearly identical metabolic features, as were the two strains *A. guerrae* seen to exhibit nearly identical metabolic profiles. *A. portensis* sp. nov. differs from *A. equi* 114^T in four characteristics (growth at 37 ºC, assimilation of adipate, benzoate and 4-hydroxybenzoate) and from *A. calcoaceticus* CIP 81.8^T in 11 characteristics (assimilation of *trans*-aconitate, adipate, 4-aminobutyrate, L-arginine, benzoate, 2,3-butanediol, 4-hydroxybenzoate, malonate, L-ornithine, L-phenylalanine and putrescine). While, *A. guerrae* sp. nov. differed from *A. ursingii* CIP 107286^T in two characteristics (assimilation of benzoate and gentisate) and from *A. calcoaceticus* CIP 81.8^T in 10 characteristics (growth at 37 ºC, assimilation of *trans*-aconitate, 4-aminobutyrate, L-arginine, gentisate, L-histidine, malonate, L-ornithine, L-phenylalanine and putrescine) (Supplementary Table 5.3).

The metabolic capabilities of *A. portensis* sp. nov. and *A. guerrae* sp. nov. were compared with those from all *Acinetobacter* species (http://apps.szu.cz/anemec/Phenotype.pdf) and the most useful combinations of characteristics for differentiating *A. portensis* from other members of the genus includes growth on L-aspartate and L-histidine and absence of growth on L-arginine and 2,3-butanediol and at 37 ºC. *A. guerrae* strains could be differentiated from other members of the genus by the combination of growth at 37 ºC, assimilation of L-aspartate and gentisate and absence of assimilation of β-alanine, L-arginine and L-histidine. Although differentiation of *A. guerrae* strains from *A. guillouiae* is not so evident, the type strain of *A. guillouiae* can be differentiated by growth on β-alanine and L-histidine and no growth on gentisate.

Altogether, these data are congruent with the accepted characterisation of bacterial species as a group of strains that shows a high degree of overall similarity and differs considerably from related strain groups, with respect to many independent characteristics (Rosselló-Móra and Amann, 2001).

5.11. Description of *Acinetobacter portensis* sp. nov.

Acinetobacter portensis (por.ten'sis. N.L. masc. n *portensis* pertaining to Porto, a city of Portugal where the first four strains of this species were isolated).

The description is based on the characterization of four strains (Table 5.1). Phenotypic characteristics correspond to those of the genus (Baumann et al., 1968), *i.e.*, cells are Gram-negative, strictly aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli, capable of growth in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen but incapable of denitrification. Colonies on Columbia II Agar Base with 5% defibrinated horse blood after 24 h incubation at 30 ºC are approximately 0.5–1.0 mm in diameter, circular, convex, smooth and bright with entire margins. Growth occurs at temperatures ranging from 20 to 30 ºC, but not at 37 ºC. Growth occurs also on R2A and TSA media, as well as in the presence of NaCl concentrations as high 4.0%. Haemolysis of horse blood was not observed after 24 h at 30 ºC. Acid is not produced from D-glucose and gelatin is not hydrolysed. Acetate, L-aspartate, citrate (Simmons), ethanol (3 strains), gentisate (1 strain), L-glutamate, L-histidine, DL-lactate, are utilised as sole sources of carbon, with growth becoming visible after two days of incubation. No growth occurs on *trans*-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, benzoate, 2,3-butanediol, D-gluconate, histamine, 4-hydroxybenzoate, L-leucine, malonate, L-ornithine, L-phenylalanine, putrescine, D-ribose, L-tartrate or tryptamine within 10 days (Tables 5.3). The predominant cellular fatty acids are 16:1ω7c, 16:0 and 12:0 3-OH.

The strains studied were isolated from beef, pork, chicken and turkey raw meat (Table 5.1). The type strain is AC 877^T (= CCUG 68672^T = CCM 8789^T), isolated in September of 2014 from raw pork meat. The type strain does not grow on gentisate. The GenBank/EMBL/DDBJ accession numbers for the genome, partial 16S rRNA gene, *rpo*B, *gyr*B and *rec*A sequences of AC 877^T are LWRV00000000, KX870877, KX885208, KX885192, and KX885200, respectively.

5.12. Description of *Acinetobacter guerrae* sp. nov.

Acinetobacter guerrae (*guer'rae* N.L. gen. n. *guerrae* of Guerra, named after Francisco Carvalho Guerra, a Portuguese biochemist, for his contributions to the foundation of Escola Superior Biotecnologia of Universidade Católica Portuguesa.

The description is based on the characterization of two strains (Table 5.1). Phenotypic characteristics correspond to those of the genus (Baumann et al., 1968), *i.e.*, cells are Gram-negative, strictly aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli, capable of growth in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen but incapable of denitrification. Colonies on Columbia II agar base with 5% defibrinated horse blood after 24 h incubation at 30 ºC are approximately 0.5–1.0 mm in diameter, circular, convex, smooth and bright with entire margins. Growth occurs at temperatures ranging from 20 to 37 ºC, but not at 42 ºC. Growth occurs also on R2A and TSA media, as well as in the presence of up to 4.5% of NaCl. Haemolysis of horse blood is not observed after 24 h at 30 ºC. Acid is not produced from D-glucose and gelatin is not hydrolysed by strains. Acetate, adipate, L-aspartate, benzoate, 2,3-butanediol (1 strain), citrate (Simmons), ethanol, gentisate, L-glutamate, 4-hydroxybenzoate (1 strain), DL-lactate are utilised as sole sources of carbon, with growth becoming visible after 2 days incubation. No growth occurs on *trans*-aconitate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, D-gluconate, histamine, L- histidine, L-leucine, malonate, L-ornithine, L-phenylalanine, putrescine, D-ribose, L-tartrate or tryptamine within 10 days (Tables 5.3). The predominant cellular fatty acids are 18:1ω9c, 16:1ω7c and 16:0.

The strains studied were isolated from chicken raw meat and the type strain is AC 1271^T (= CCUG 68674^T = CCM 8791^T), isolated in September of 2014 (Table 5.1). The type strain grows on 2,3-butanediol but does not grow on 4-hydroxybenzoate. The GenBank/EMBL/DDBJ accession numbers for the genome, partial 16S rRNA gene, *rpo*B, *gyr*B and *rec*A sequences of AC 1271^T are LXGN00000000, KX870878, KX885210, KX885194 and KX885202, respectively.

CHAPTER 6

Biofilm formation, survival on dry surface and susceptibility to disinfectants of *A. baumannii* isolates from clinical and food origin

CHAPTER 6

Abstract

Acinetobacter baumannii is a major threat to human health and its dissemination is challenging to control mainly due to its high adaptability to adverse environmental conditions. The aim of this study was to investigate the biofilm formation ability in two abiotic surfaces (silicon and stainless steel), the resistance to desiccation on stainless steel surface and the susceptibility to eleven commercial antimicrobial products of ten food and ten clinical strains. Potential associations between the investigated characteristics and antibiotic resistance or persistence in hospital environment were also evaluated.

Clinical and food strains were able to form biofilm on silicon and stainless steel surfaces, exhibited desiccation resistance capacity ranging from 14 to 77 days and were susceptible to disinfectants at the concentrations recommended by the manufacturers. There were no significant differences ($p>0.05$) between clinical and food strains. However, the biofilm-forming capacity of persistent clinical strains was significantly ($p<0.05$) higher than that of the non-persistent strains on both surfaces. The resistance to desiccation of persistent strains (mean survival time: 65.8 days) was also significantly longer ($p<0.05$) than that of the non-persistent strains (mean survival time: 35.8 days). Therefore, these characteristics need to be further investigated to assess their significance as drivers to persistence of particular strains in the hospital setting.

A high intra-strain variability in the susceptibility to disinfectants was observed but no correlation with the origin of the *A. baumannii* isolates was established. This suggests that this variability does not depend on the origin of the isolate but is probably intrinsic to the strain. Moreover, no correlation between antibiotic resistance and biofilm formation, resistance to desiccation and disinfectant susceptibility was found.

These results showed that food products may be an important vehicle of *A. baumannii* transmission to the hospital settings since some of the food strains were able to produce biofilm and survive to desiccation as the persistent clinical strains.

6.1. Introduction

Acinetobacter baumannii has become one of the major causes of sporadic and epidemic nosocomial infections worldwide (Bergogne-Bérézin and Towner, 1996; Maragakis and Perl, 2008; Souli et al., 2008; Villegas and Hartstein, 2003) causing a variety of infections, including bacteremia, pneumonia, meningitis, septicemia, urinary tract infections, wound and skin infections in immunocompromised patients (Bergogne-Bérézin and Towner, 1996; Joly-Guillou, 2005; Maragakis and Perl, 2008; Peleg et al., 2008; Visca et al., 2011). The survival of this pathogen in the healthcare environment has a major role in nosocomial infections. The success of *A. baumannii* is mainly a result of different factors: the resistance to antibiotics (Babaei et al., 2015; Bergogne-Bérézin and Towner, 1996; Chang et al., 2015), the long persistence on dry surfaces (Espinal et al., 2012; Jawad et al., 1996), the ability to form biofilms (Singhai et al., 2012) and the tolerance to commonly used disinfectants (Babaei et al., 2015).

A. baumannii is currently at the top of the priority pathogens list for development of new antibiotics (Tacconelli et al., 2018) as the high multidrug or pandrug resistance of this pathogen is widely recognized as a frequent problem in the clinical settings (Chu et al., 2001; Livermore et al., 2008; Magnet et al., 2001; Rossolini and Mantengoli, 2008).

Besides the resistance to antimicrobial agents, other factor making it challenging to prevent the persistence and spread of *Acinetobacter* in hospital settings is their ability to survive during extended periods of time on abiotic surfaces under desiccated conditions (Espinal et al., 2012; Gayoso et al., 2014; Jawad et al., 1996; Lee et al., 2006; Turton et al., 2004; Wendt et al., 1997) which enables the species to withstand dry environments for months (Dijkshoorn et al., 2007) and facilitating its spread via hospital personnel, infrastructure, and medical devices (Doughari et al., 2011; Roca et al., 2012).

The high capacity of biofilm formation of this pathogen is as well considered an important pathogenic feature in the establishment and spread of nosocomial infections due to the surface colonization of hospital furniture and equipment, indwelling medical devices (such us urinary catheters, central venous catheters and endotracheal tubes) and even gloves of healthcare providers (Cerqueira and Peleg, 2011; Djeribi et al., 2012; Donlan, 2001; Espinal et al., 2012; Gaddy et al., 2009; Harding et al., 2018; Morgan et al., 2010; Rodríguez-Baño et al., 2008; Trautner and Darouiche, 2004). In fact, *A. baumannii* has been reported as one of the most common bacterial causes of biofilm-related contamination of medical devices (Singhai et al., 2012).

The use of antiseptics and disinfectants on environmental cleanliness plays an important role in these infection control practices for the prevention of the spread of *A. baumannii*. The regular use of these products in hospital has raised concerns about its resistances although, contrary to antibiotics, only few reports are available about the susceptibility of

A. baumannii to antiseptics and disinfectants (Babaei et al., 2015; Gnanadhas et al., 2013; Peleg and Paterson, 2006.

All these factors may contribute to the persistence of the species in hospital and community settings and make it challenging to control infection and dissemination of *A. baumannii*.

Some studies reported that hospital meals could be vehicles of pathogenic microbes for vulnerable people (children, elderly, pregnant women and immuno-compromised individuals) and hospital kitchen is considered as the main source of cross-contamination (Lund and O'Brien, 2011; Maguire et al., 2000; Margaret et al., 2010). Although food products have been recognized as a source of *A. baumannii* that could serve as a vector for its dissemination in community and hospital settings (Antunes et al., 2014; Carvalheira, Casquete, et al., 2017; Carvalheira, Silva, et al., 2017; Lupo et al., 2014) to the best of our knowledge, no prior study evaluated the capacity of *A. baumannii* food isolates to form biofilms and their survival to desiccation and susceptibility to antiseptics and disinfectants. Therefore, the aim of this study was to compare clinical and food strains of *A. baumannii* concerning their biofilm production, survival to desiccation and susceptibility to antiseptics and disinfectants commonly used and its correlation with antimicrobial resistance and, in the case of clinical strains, with persistence in hospital settings.

6.2. Material and methods

6.2.1. Bacterial isolates

6.2.1.1. Food isolates

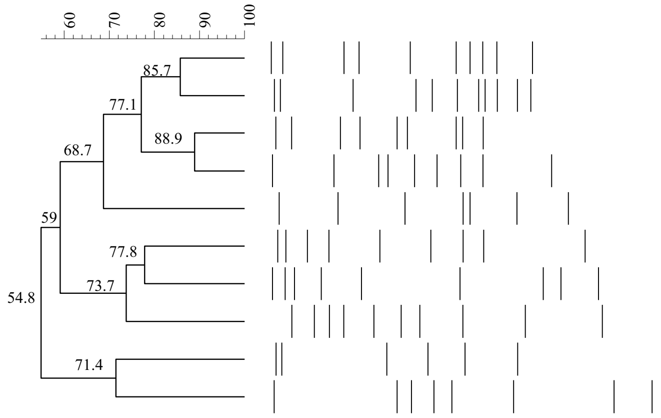
The study was carried out with ten *A. baumannii* strains previously isolated from different food samples (Carvalheira et al. 2017a, b). Figure 6.1. shows the PFGE profiles, source, antibiotic resistance and reference of each strain.

6.2.1.2. Clinical isolates

Clinical isolates of *A. baumannii* (n=104) were recovered between 2004 to 2007 (n=50) and in 2014 (n=54) from patients of Hospital de Braga, a 705-bed tertiary care facility that serves a local population in the north of Portugal. Of the 104 isolates, 29 (27.9%) were derived from sputum and bronchial aspirates, 25 (24.0%) from perineal and rectal swabs, and 22 (21.2%) from urine samples. Twenty two (21.2%) isolates were derived from other sources, including auricular exudate, blood, axillar, skin, mucus, pus, pleural liquid or biopsy and the source of 6 (5.8%) isolates was unreported (Figure 6.2.).

A. baumannii isolates were preserved in Tryptone Soya Broth (TSB, Merck, Darmstadt, Germany) supplemented with 20 % (w/v) glycerol at – 80 ºC for genotypic analysis.

Figure 6.1. Genetic relatedness (with restriction enzymes *Asc*I) of strains isolated from different food sources, its antibiotic resistance and reference of isolation and characterization.



Strain code	Source	Antibiotic Resistance	Reference
F1	Chicken meat	TZP	Carvalheira et al., 2017a
F2	Chicken meat	TZP, MEM	Carvalheira et al., 2017a
F3	Lettuce	TZP,CAZ,IMP,AK	Carvalheira et al., 2017b
F4	Pork meat	PB	Carvalheira et al., 2017a
F5	Lettuce	TZP	Carvalheira et al., 2017b
F6	Lettuce	TZP,CAZ,IMP	Carvalheira et al., 2017b
F7	Turkey meat	TE	Carvalheira et al., 2017a
F8	Lettuce		Carvalheira et al., 2017b
F9	Chicken meat	TE	Carvalheira et al., 2017a
F10	Turkey meat	CAZ, (CL/PB), AK,TE	Carvalheira et al., 2017a

TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole. Antibiotics from the same antimicrobial class were written in brackets.

6.2.1.2.1. Confirmation of species identification and genetic relatedness of clinical isolates

In order to confirm the species identification performed at the hospital setting by VITEK 2, the analysis of the sequence of the region Z1–Z2 of the gene for RNA polymerase beta-subunit (*rpo*B) of each isolate was done according to Carvalheira et al. (2017a).

In order to identify clones and evaluate the persistence of *A. baumannii* strains at the hospital setting, all isolates that were confirmed to belong to this species were further genotyped by Pulsed-field Gel Electrophoresis (PFGE), using the restriction enzyme *Asc*I (New England Biolabs, Ipswich, MA), according to Carvalheira et al. (2017a). The band patterns were analysed by GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) and the cluster analysis of PFGE profiles was done by the unweighted-pair group method with average linkages (UPGMA), using the Dice coefficient to produce a dendrogram describing the genetic relatedness of the isolates. A band position tolerance of 1.0 was selected and the classification of isolates into different *Asc*I patterns was visually validated. Differences in just one band in the PFGE pattern were interpreted as reflecting different PFGE types. Isolates having the same banding patterns were regarded as identical and were assigned to the same type (e.g., type 1).

1.1.2.2 Antimicrobial resistance of clinical isolates

The antimicrobial resistance of isolates representative of the different PFGE types to fourteen antibiotics was evaluated according to the standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2012) using Mueller Hinton agar (MHA, BioMérieux, Marcy-l'Étoile, France). Briefly, disk diffusion method was used to test twelve antibiotics: piperacillin (PIP, 100 µg), piperacillin-tazobactam (TZP 100/10 µg), ampicillin-sulbactam (SAM 10/10 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), tetracycline (TE, 30 µg), minocycline (MI, 30 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT 1.25/ 23.75 µg) (all from Oxoid, Hants, UK). Susceptibility to colistin (CL) and polymyxin B (PB) was evaluated by the agar dilution method with antibiotic concentrations ranging from 1 to 8 µg/mL. Cultures were incubated for 24 h at 35 °C and *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* DSM 1117 were used as quality control strains. The inhibition zones were measured and the strains were categorized as susceptible or resistant according to CLSI interpretive criteria (CLSI, 2012).

6.2.1.2.3 Selection of clinical strains

Ten clinical strains were selected based on their persistence (P, n=5) and non-persistence (Np, n=5) for the following analyses (Figure 6.2) Persistence was defined by the repeated isolation of *A. baumannii* strains with identical molecular subtypes determined by PFGE in different years (among the isolates recovered between 2004 to 2007) or in different months (among the isolates recovered in 2014), while strains recovered sporadically, i.e. when a PFGE pattern was observed only once among all isolates, were considered non-persistent.

6.2.2. Biofilm formation

The capacity of biofilm formation of food strains (n=10) and clinical strains (5 persistent and 5 non-persistence strains) of *A. baumannii* in two abiotic surfaces (silicon and stainless steel) was evaluated according to Orsinger-Jacobsen et al. (2013) with some modification. Before utilization, the coupons were washed with distilled water, immersed in acetone for 3 min, rewashed with distilled water and immersed in ethanol (70%) during 3 min. After rewashing and air-dried, the coupons were sterilized at 121 ºC for 15 min. The sterile coupons were then immersed vertically in 50 mL sterile Falcon tubes (Sarstedt, Nümbrecht, Germany), each containing a single coupon, previously filled with 45 mL of sterile TSB and inoculated with the different strains to achieve a test suspension with approximately 10^7 cells per mL. As all coupons were fitted into Falcon tubes both sides of each coupons were available for bacterial adherence. Static incubation was performed for 48 h at 37 ºC for tubes containing the silicon coupons and at 22 ºC for tubes containing the stainless steel coupons.

After incubation, the coupons were rinsed three times with sterile distilled water and transferred aseptically into a new tube containing 10 mL of sterile phosphate buffer saline (PBS). Biofilms were removed by swabbing on both sides of the coupons followed by vortexing for 1min. Serial decimal dilutions of the suspensions were then performed in PBS and aliquots of 100 µL from each dilution were inoculated onto TSA. After incubation at 30 ºC for 24 h the colony enumeration was performed. Three independent assays were performed for each strain. *Pseudomonas aeruginosa* ATCC 10145 was used as a positive control. A negative control (tubes with coupons and media without bacteria) was also included. Biofilm formation was determined by calculation of Log_{10} of colony-forming units (CFU) per cm^2.

6.2.3. Susceptibility to desiccation

The resistance of *A. baumannii* planktonic cells (ten food strains and ten clinical strains) to desiccation on stainless steel surfaces was evaluated according to Orsinger-Jacobsen et al. (2013) with some modifications. Briefly, each strain was grown in TSB for 24 h at 30 ºC to obtain an inoculum with approximately 10^8 cells per mL. After incubation, 5 mL of each inoculum was centrifuged for 8 min at 5000 rpm, the cells were washed once with 5 mL of sterile distilled water and then resuspended in another 5 mL. Ten drops of 10 µL of each suspension (~ 10^8 cells per mL) were deposited onto sterile stainless steel coupons (prepared as referred above on 6.2.2) placed in sterile Petri dishes at 22 ºC with a relative humidity of 31%.

At regular intervals (once a week for a maximum period of three months) three stainless steel coupons of each strain, separately, were transferred into a 50 mL sterile tube containing 10 mL of sterile PBS and the adhered cells were removed by swabbing followed by vortexing for 30 s. Two fold serial dilutions of the suspensions were performed and aliquots of 100 µL from each dilution (or 500 µL when the viable count was less than 30 CFU) were inoculated onto TSA. After incubation at 30 ºC for 24 h the colony enumeration was performed.

6.2.4. Susceptibility to disinfectants

The sensitivity of *A. baumannii* strains (ten food strains and ten clinical strains) to eleven commercial disinfectants including six commonly used in hospital settings and five used at kitchen canteens were tested (Table 6.1). The minimum inhibitory concentrations (MICs) were determined by the agar dilution method, according to the protocol recommended by the CLSI (2012). Briefly, appropriate dilutions of disinfectant solutions were added to Mueller-Hinton agar. Since there was no standart breakpoints available for antiseptics against *A. baumannii*, we tested a 2 fold dilutions (v/v) from 50% to 0.0195%. The first dilutions testes were 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39% and 0.195%. After an initial MIC was found, subsequent dilutions were prepared in a tighter range to allow further discrimination between strains

Each bacterial culture was adjusted to a turbidity equivalent to that of 0.5 McFarland standard and a 10 µL aliquot of each diluted bacterial suspension with approximately 10^4 cells per mL was spotted onto the agar surface with different disinfectant concentrations and incubated at 35 ºC for 24 h. The MIC was recorded as the lowest concentration of the disinfectant that completely inhibited growth, except for a single colony or a faint haze caused by the inoculum.

Table 6.1. Description, active ingredient and recommended dilutions of each commercial product evaluated.

Disinfectant Code	**Product**	**Description**	**Active ingredient**	**Recommended dilutions**
D1	Anios DDSH	Disinfectant used to clean inert surface	Quaternary ammonium propionate	Pure
D2	Stellisept med	Antiseptic antimicrobial used to disinfection of hands Bactericidal	Castor oil PEG-150 distearate, panthenol, allantoin, Cocoate PEG-7 glyceryl didecildimónio chlorides, sodium citrate didecyldimonium chloride	Pure
D3	Sterillium	Hand desinfectant	propan-2-ol 45 g propan-1-ol 30 g Mecetroniumetilsulfate 0.2g (em 100g)	Pure
D4	Betadine	Iodine solution used for skin	Povidone iodine 50 mg/ml	Pure
D5	Cutasept	Alcohol solution used for skin skin antiseptic bactericidal	propan-2-ol 63.0g (equals 72 vol. %) Benzalkonium Chloride 0.025 g (em 100g)	Pure
D6	Presept and Typol	Disinfectant used to wash the floor	Sodium dichlorisocyanurate 0.5g	0.5 g in 2 L of water (140 ppm)

D7	Sonaril Da5	Biocid Hard Surface Disinfectant Universal disinfectant used on inert surface	Sodium hypochlorite 5%	20 mL/10 L (96 ppm)
D8	Sonaril HB	Bactericide soap cream used to wash hands	Alcohol (5-15%)	pure
D9	Sonaril LM	Manual dishwashing liquid	Alcohol Benzenesulfonic acid (1-methylethyl)-sodium salt (<5%)	6-8 mL/L
D10	Sonaril LAC	Detergent for dishwasher	Sodium hypochlorite Hydroxyde hypochloride	4 g/L
D11	Sonaril DGF	Detergent used for degreasing surface	Sodium hydroxyde	5-10%

5. Statistical analysis

An analysis of variance (one-way ANOVA) was performed to compare the source of isolation (food and clinical strains), persistent and non-persistent clinical strains and antibiotic resistance on biofilm formation, survival on dry surface and susceptibility to disinfectants. All calculations were carried out using the software KaleidaGraph (version 4.04, Synergy Software, Reading, USA).

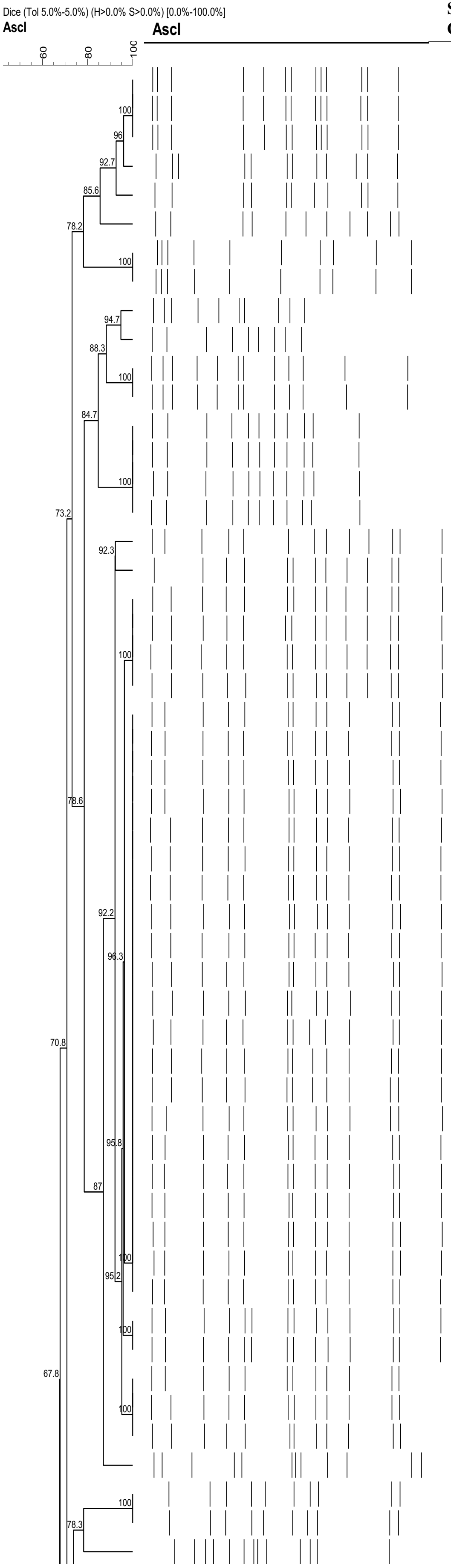
6.3. Results and Discussion

6.3.1. Clinical isolates

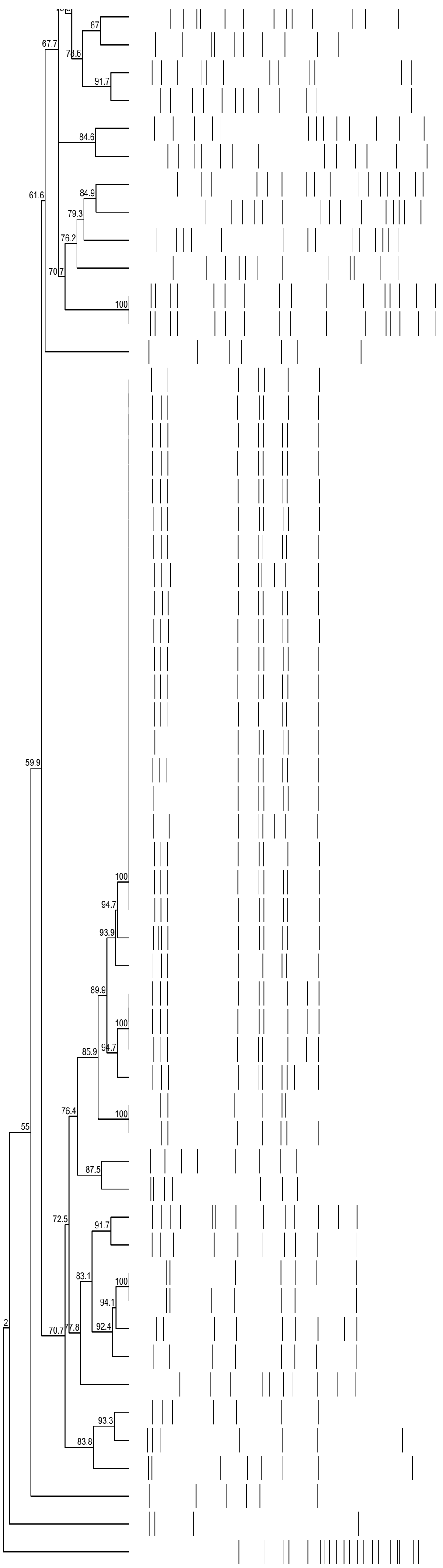
Among the 108 clinical isolates identified as *A. baumannii* by VITEK 2 System (bioMérieux), 16 (14.8 %) were identified as *A. pittii* (n=6), *A. calcoaceticus* (n=4), *A. seifertii* (n=4), *A. nosocomialis* (n=1) and *A. soli* (n=1) by *rpo*B sequencing. VITEK 2 System is a commercial microbial automatic system most frequently used in hospitals for bacterial identification however, as referred by Ling et al. (2001), this system is not a reliable method to *Acinetobacter* species identification, even within the *A. baumannii* group. The use of *rpo*B sequencing (La Scola et al., 2006) has facilitated identification across the genus and it is becoming clear that *A. baumannii* is not the only species that should be a concern to public health (Turton et al., 2010).

The 92 isolates that were confirmed to belong to the species *A. baumannii* were tested for their genetic relatedness using PFGE. The PFGE results (Figure 6.2) showed one predominant PFGE pattern (PFGE type 12) and 16 sporadic PFGE patterns, among the isolates recovered between 2004 to 2007. This prevalent pattern was recovered along this 4 years period. Regarding the isolates recovered during the year 2014, it was also observed one prevalent PFGE pattern (PFGE type 20), recovered over a period of three months, and 17 sporadic PFGE patterns. Between 2004 to 2007, the *A. baumannii* population is represented by prevalent strain of related MDR isolates recovered over 4 years which include 21 isolates, six small groups formed for two or four MDR isolates (one group formed by three isolates recovered in 2004, other group formed by two isolates from 2006, other group formed by isolates recovered in 2004 and 2005, other formed by four isolates recovered in 2007, other formed by four recovered in 2007, and other formed by three recovered in 2004 and 2005) and nine sporadic isolates (only one of them non-MDR). Among the isolates recovered in 2014, there was one dominant clone over 3 months which include 22 MDR isolates, three small groups formed by two or three MDR isolates and 15 sporadic isolates (seven of which were non-MDR).

Similarly to our results, several studies revealed the emergence and persistence of *Acinetobacter* species in hospitals worldwide and the recovery of prevalent clones and sporadic patterns (Baang et al., 2012; Gallego and Towner, 2001; Gonzalez et al., 1998; Jawad et al., 1996; Lambiase et al., 2012; Weisenberg et al., 2011).



Strain Code	Source	Month/ Year of isolation	PFGE type	Antibiotic Resistance
	Nasal	2004	1	(IMP/MEM), AK, (TE/MI), TZP, CAZ, CIP, SXT
	Perineal	2004		
	Bronchial	2004		
Np8	Blood	2006	2	(IMP/MEM), (TE/MI), TZP, CAZ, CIP, SXT, (CL/PL)
	Expectoration	2004	3	(IMP/MEM), (TE/MI), TZP, CAZ, CIP, SXT, (CL/PL)
	Mucus	2007	4	TZP, CAZ, CIP
	Blood	8/2014	-	
	Blood	8/2014	-	
	Pleural liquid	2006	5	(IMP/MEM), TZP, CIP, SXT, PL
Np9	Urine	2007	6	TZP, CAZ, CIP, SXT
	*	2006	7	(IMP/MEM), TOB, TZP, CIP, SXT
	Bronchial	2006		
P1	Rectal	2004	8	TOB, (TE/MI), TZP, CIP, SXT
	Rectal	2004		
	Perineal	2005		
	Inguinal	2005		
	Urine	2006	9	(IMP/MEM), (TE/MI), TZP, CAZ, CIP, SXT
	Urine	2007	10	(IMP/MEM), (TZP/SAM) CAZ, CIP, SXT
P3	Skin	2007	11	(IMP/MEM), (TE/MI), (TZP/SAM) CAZ, CIP, SXT
	Skin	2007		
	Urine	2007		
	Urine	2007		
	Bronchial	2004	12	(IMP/MEM), (TE/MI), (TZP/SAM) CAZ, CIP, SXT, (CL/PL)
	Perineal	2004		
	Bronchial	2004		
	Catheter	2005		
P2	Perineal	2005		
	Perineal	2005		
	Perineal	2005		
	Perineal	2005		
	Perineal	2005		
	Biopsy	2006		
	Urine	2007		
	Skin	2007		
	Expectoration	2007		
	Skin	2007		
	*	2007		
	Nasal	2004		
	Perineal	2004		
	Axilar	2004		
	Expectoration	2004		
	Perineal	2004		
	Perineal	2005		
	Auricular exsudate	2007	13	(IMP/MEM), (TE/MI), (TZP/SAM) CAZ, CIP, SXT
	Blood	2007		
	Perineal	2004	14	(IMP/MEM), AK, (TE/MI), (TZP/SAM) CAZ, CIP, SXT, PL
	Perineal	2005		
	Rectal	2007		
	Urine	5/2014	15	
	Catheter	8/2014	-	
	Expectoration	7/2014	-	
	Nasal	2004	-	



Strain code	Source	Month/ Year of isolation	PFGE type	Antibiotic Resistance
	*	2004	-	
	Urine	7/2014	16	IMP, (TE/MI), TZP, CAZ, CIP
	Urine	5/2014	17	
	Urine	5/2014	18	
	Urine	6/2014	-	
	Skin	8/2014	-	
	Expectoration	6/2014	-	
	Blood	8/2014	-	
	Urine	7/2014	-	
	Blood	7/2014	-	
	Skin	6/2014	-	
	Catheter	7/2014	-	
	Bronchial	8/2014	19	
	Urine	8/2014	20	(IMP/MEM), (AK/TOB), (TE/MI), (TZP/SAM), CAZ, CIP, SXT, CL
	Rectal	8/2014		
P5	Skin	9/2014		
	Rectal	9/2014		
	Rectal	9/2014		
P4	Expectoration	7/2014		
	Expectoration	7/2014		
	Skin	7/2014		
	Urine	7/2014		
	Urine	8/2014		
	Expectoration	8/2014		
	Rectal	8/2014		
	Urine	8/2014		
	Expectoration	8/2014		
	Expectoration	7/2014		
	Rectal	7/2014		
	Urine	7/2014		
	Bronchial	8/2014		
	Bronchial	8/2014		
	Bronchial	8/2014		
	Skin	7/2014		
	Expectoration	7/2014		
	Rectal	8/2014		
	Bronchial	9/2014	21	(IMP/MEM), (AK/TOB), (TE/MI), (TZP/SAM), CAZ, CIP, SXT
	Bronchial	8/2014		
	Skin	8/2014	22	(IMP/MEM), (AK/TOB), (TE/MI), (TZP/SAM), CAZ, CIP, SXT, PL
	Rectal	6/2014	23	
	Expectoration	7/2014		
Np6	Nasal	2004	24	PL
Np10	Skin	8/2014	25	SXT
	Expectoration	9/2014	26	(IMP/MEM), (TE/MI), TZP, CAZ, CIP, PL
	Urine	7/2014	27	(IMP/MEM), (AK/TOB), (TE/MI), TZP, CAZ, CIP, SXT
	Urine	6/2014	28	
	Urine	6/2014		
	Rectal	5/2014	29	(IMP/MEM), TOB, (TE/MI), TZP, CAZ, CIP, SXT
	Urine	6/2014	30	(IMP/MEM), (AK/TOB), (TE/MI), (TZP/SAM), CAZ, CIP, SXT, CL
	Urine	6/2014	31	(IMP/MEM), (TE/MI), TZP, CAZ, CIP
	Rectal	5/2014	32	(IMP/MEM), (AK/TOB), (TE/MI), (TZP/SAM), CAZ, CIP, SXT, CL
	Blood	7/2014	33	
	Blood	7/2014	34	CIP
Np7	Urine	2005	35	AK, TZP,CIP
	Auricular exsudate	7/2014	-	
	Expectoration	8/2014	-	

Figure 6.2. PFGE profiles (with restriction enzymes *Asc*I) of *A. baumannii* clinical isolates, including source, month/year of isolation, antibiotic resistance, pulse field types and selected persistent (P1, P2, P3, P4, P5) and non persistent (Np6, Np7, Np8, Np9, Np10) strains. TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole. Antibiotics from the same antimicrobial class were written in brackets. *unknown, - species other than *A. baumannii.*

6.3.2. Antimicrobial resistance of clinical isolates

Based on the PFGE patterns, 36 representative strains were selected for antimicrobial resistance tests. The antimicrobial resistance patterns of these strains to fourteen antimicrobial agents are presented in Figure 6.2.

In this study, only 13.9% (5/36) of the strains were susceptible to all the antibiotics tested, 52.8 % (19/36) of the strains were classified as extensively drug-resistant (XDR) i.e. non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (Magiorakos et al., 2012), and 25.0% (9/36) of the strains were considered as multidrug-resistant (MDR), i.e. acquired non-susceptibility to at least one agent in three or more antimicrobial categories among the eight different classes of antibiotics used to define MDR (Magiorakos et al., 2012).

Overall, strains presented a high resistance to almost all antimicrobials categories used to treat *A. baumannii* infections. Resistance to ciprofloxacin was the most common (80.6%), followed by piperacillin-tazobactam (77.8%), imipenem (66.7%), ceftazidime (66.7%), trimethoprim-sulfamethoxazole (66.7%), meropenem (63.7%), tetracycline (63.7%), amikacin (33.3%), tobramycin (33.3%), ampicillin-sulbactam (30.6%) and minocycline (27.8%). Colistin and polymyxin B, which are frequently used as last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter,* were found to be the most active agents. However, it is important to highlight that 19.4% and 22.2% of the strains were resistant to colistin and polymyxin B, respectively.

The multidrug and pandrug resistance of *A. baumannii* isolates has been reported all over the world (Bahador et al., 2013; Doi et al., 2015; Giamarellos-Bourboulis et al., 2001; Gupta et al., 2016; Hawley et al., 2008; Inchai et al., 2015; Ko et al., 2007; Lambiase et al., 2012; Li et al., 2006; Long et al., 2009; Potron et al., 2015; Qi et al., 2016; Villalón et al., 2011; Weisenberg et al., 2011). As in the present study, Qi et al. (2016) reported a high prevalence of MDR and XDR isolates (72.4%). Long et al. (2009) also showed that 87.8% of the *A. baumannii* isolates recovered from patients in UCIs from a hospital in Brisbane, Australia, were XDR. Villalón et al. (2011), Weisenberg et al. (2011) and Lambiase et al. (2012) also reported the multidrug resistance of *A. baumannii* isolates collected in Spanish hospitals (mainly from patients hospitalized in intensive care units), New York hospital and ICU of Southern Italy, respectively. However, the studies of Villalón et al. (2011) and Lambiase et al. (2012) reported the susceptibility of all the isolates tested to colistin and 91% of the isolates analysed in the study of Weisenberg et al. (2011) were susceptible to polymyxin B.

A study conducted by Duarte et al. (2016) demonstrated that 12 out of 17 clinical *A. baumannii* isolates collected in a Portuguese hospital were also MDR (Duarte et al., 2016) and the majority of the isolates showed resistance to gentamicin, tobramycin, and trimethoprim/sulfamethoxazole.

Antibiotic resistance itself is associated with an increased potential of some *A. baumannii* strains to persist in hospitals compared with more susceptible strains (Longo et al., 2014; Rodríguez-Baño et al., 2004). Qi et al., (2016) reported that the dominant clones were MDR and the non-MDR population were found to be sporadic strains. However, our results showed that most of the sporadic strains were also MDR.

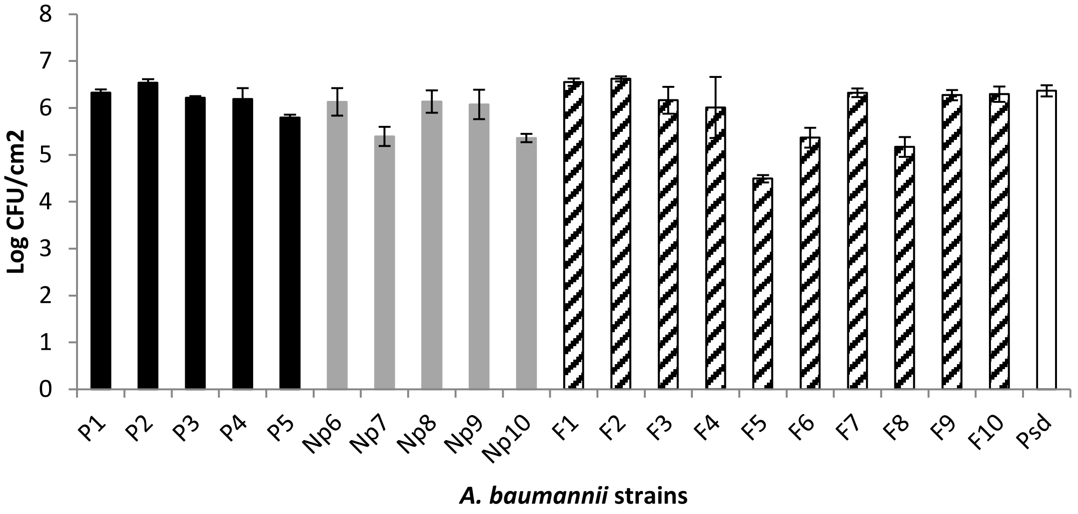
6.3.3. Biofilm formation

The ability of ten food strains and ten clinical strains (five persistent and five non-persistent) to form biofilm on silicon and stainless steel surfaces was evaluated and the results showed that all strains were able to form biofilm on both surfaces (Figure 6.3). *A. baumannii* has been reported as one of the most common bacterial cause of biofilm-related contamination of medical devices (Singhai et al., 2012) and, in agreement to our results, several studies have shown that biofilm-forming ability on different materials is a virulence factor common to a large number (50 to 92%) of *A. baumannii* clinical isolates (Eijkelkamp et al., 2011; Greene et al., 2016a; Gurung et al., 2013; King et al., 2009; Qi et al., 2016; Rao et al., 2008; Rodríguez-Baño et al., 2008). However, to the best of our knowledge, this is the first study that evaluates the biofilm-forming capacity of strains isolated from food samples and our results showed that there were no significant differences on biofilm-forming capacity between food and clinical strains ($p > 0.05$, Figure

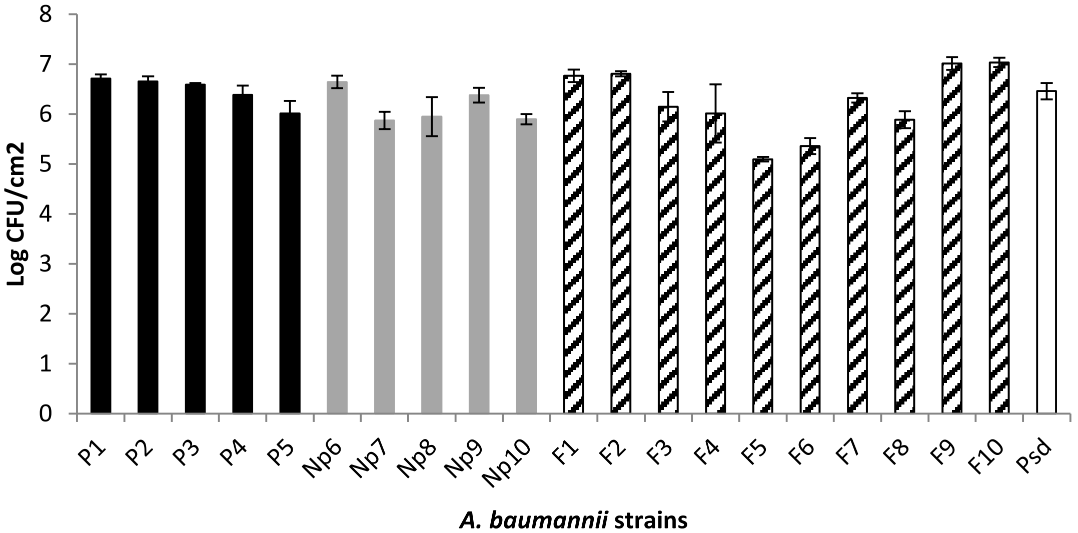
6.3). Other study compared isolates from clinical and environmental and a significant correlation between multidrug resistance and biofilm formation of clinical and environmental isolates was established, i.e., clinical isolates demonstrated a higher ability to form strong biofilm than environmental isolates (Greene et al., 2016b).

Figure 6.3. Capacity of biofilm formation of clinical (persistent P1, P2, P3, P4, P5; non-persistent Np6, Np7, Np8, Np9, Np10) and food (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10) strains of *A. baumannii* in two surfaces: silicon at 37 ºC (a) and stainless steel at 22 ºC (b).

a



b



However, among each group (clinical and food strains) there were significant differences on the two surfaces ($p < 0.0001$). Namely, amongst the clinical strains, the biofilm-forming capacity of persistent strains was significantly higher than the non-persistent strains on the silicon ($p < 0.01$) and on the stainless steel surfaces ($p < 0.05$; Figure 6.3), which could contribute for it persistence. There was also significant differences among the biofilm forming capacity among food strains ($p < 0.01$) on stainless steel surface, ranging between approximately 5 log CFU/cm^2 to 7 log CFU/cm^2, and on the silicon surface, ranging between approximately 4.50 log CFU/cm^2 to 6.6 CFU/cm^2. This also shows the high variability among these isolates which seems to be strain dependent.

Biofilm formation is more associated with MDR *A. baumannii* strains than with the susceptible strains (Badave and Kulkarni, 2015; Bardbari et al., 2017; Bocanegra-Ibarias et al., 2015; Zhang et al., 2016). However, this association remains controversial. In fact, in our study this correlation was observed only for food strains on stainless steel ($p < 0.05$) and silicon ($p=0.067$) surfaces. While, despite the higher antibiotic resistance of clinical strains than food strains there were no significant differences between the biofilm forming capacity concerning the strain sources. In agreement with that, Duarte et al. (2016) also did not observe statistical significance between the ability to form biofilms and antibiotic resistance among the *A. baumannii* isolates recovered from a Portuguese hospital, while 74.7% of the isolates showed biofilm forming ability.

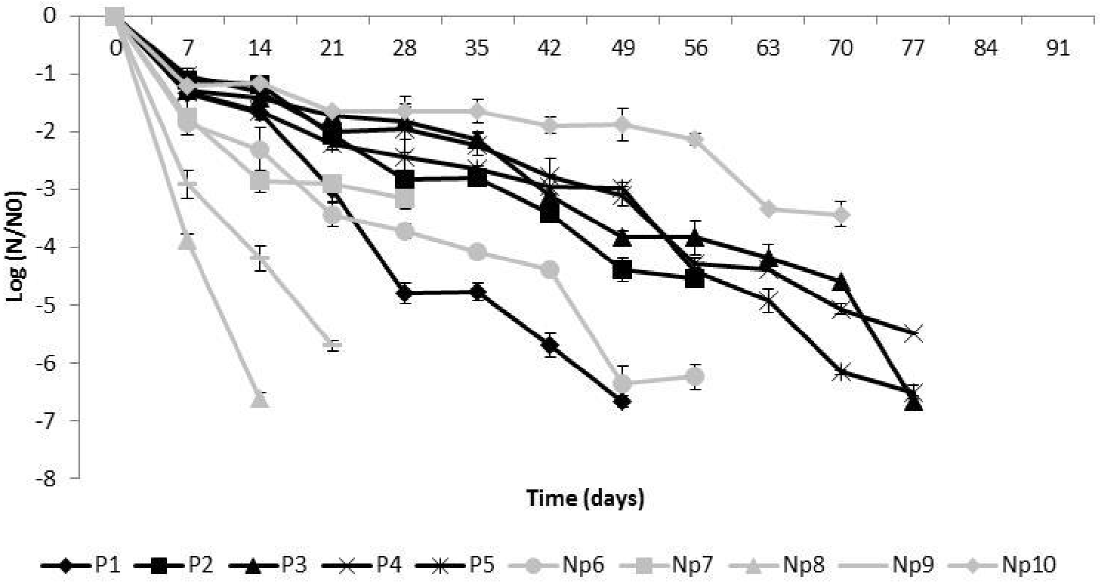
Nevertheless other studies suggest an inverse relationship between biofilm production and antibiotic resistance, indicating that biofilm-forming strains are not as dependent on antimicrobial resistance as non-biofilm-forming strains for survival (Dafopoulou et al., 2015; de Campos et al., 2016; Espinal et al., 2012; Pour et al., 2011; Qi et al., 2016; Rodríguez-Baño et al., 2008). Indeed, a comprehensive study of 272 *A. baumannii* isolates showed that MDR and XDR isolates tended to form weaker biofilms than sensitive strains, suggesting that biofilm acts as a mechanism for bacteria to get a better survival, especially in isolates with resistance level not high enough as its (Qi et al., 2016). However, Salmani et al. (2020) analysed research works published on several international databases from January 2005 up to 31th December 2019 that assessed the correlation between biofilm formations with antibiotic resistance in *A. baumannii* retrieved from wound infections of burn patients and conclude that over 90% of *A. baumannii* strains are capable of producing biofilms which have a significant role in the development of antibiotic resistance in the burn unit since the combined prevalence of MDR isolates was 49.8%.

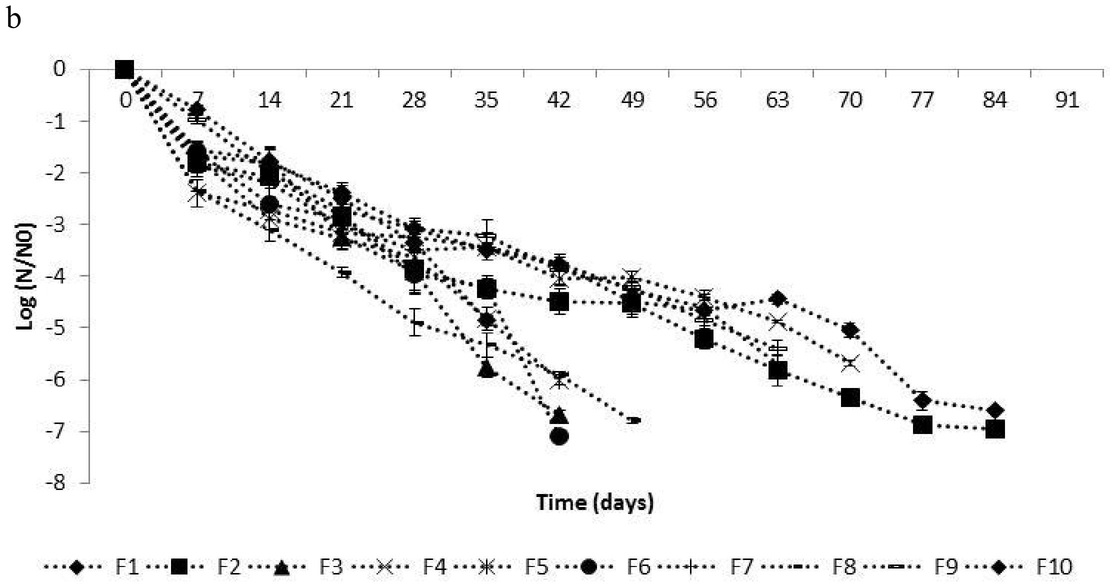
6.3.4. Susceptibility to desiccation

One of the main concerns regarding *A. baumannii* is its ability to persist in the hospital environment on various abiotic materials. In our study, the resistance to desiccation on stainless steel surfaces was investigated and the results (Figure 6.4a and 6.4b) showed that all clinical and food strains exhibited desiccation resistance capacity. The survival times ranged from 14 to 77 days and there was no significant difference between the survival time of clinical and food strains ($p>0.05$). The mean survival time for clinical and food strains was 51.8 days (range: 14 to 77 days) and 54.6 days (range: 35 to 77 days), respectively.

Figure 6.4. Survival time on desiccation conditions of (a) clinical (persistent P1, P2, P3, P4, P5; non-persistent Np6, Np7, Np8, Np9, Np10) and (b) food (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10) *A. baumannii* strains during 3 month.

a





Previous reports showed that *A. baumannii* strains have a considerably different ability to survive on dry surfaces. Wendt et al. (1997) demonstrated that some *Acinetobacter* strains might survive for more than 4 months under dry conditions Catalano et al. (1999) showed that *A. baumannii* strains could be isolated from a hospital bed rail 9 days after an infected patient was discharged from the hospital. Antunes et al. (2011) found that some isolates remaining viable for almost 100 days on glass coverslips surfaces. Orsinger-Jacobsen et al. (2013) have shown that planktonic cells were able to survive between 28 and 42 days on stainless steel.

Among clinical strains, there was a statistically significant difference ($p<0.001$) between the survival time of the persistent and the non-persistent strains. The mean survival time for persistent strains was 65.8 days, which was significantly longer than that of the non-persistent strains (35.8 days). Indeed, tree of the five tested non-persistent strains survived less than 35 days. Therefore, our results suggest that the ability of *A. baumannii* to survive in the hospital environment for prolonged periods may be in part due to its resistance to desiccation.

Although the survival time of clinical persistent strains (mean survival time: 65.8 days; range, 49 to 77 days, Figure 6.4a) was longer than that of food strains (mean: 54.6 days, p=0,051), these last ones showed a significant higher resistance to desiccation ($p<0.005$) than the clinical non-persistent strains (mean survival time: 35.8 days, range of 14 to 70 days, Figure 6.4a). This suggests that once introduced in the hospital settings food strains may also survive for prolonged periods and this increases the risk of cross contamination leading to infection.

Moreover, previous studies suggested that *A. baumannii*'s ability to persist in these conditions is related to its capacity to form biofilms (Gaddy et al., 2009). Espinal et al. (2012) found biofilm-forming strains to be significantly more resistant to desiccation (36 days) than non-biofilm forming strains (15 days). Similarly, Chiang et al. (2018) demonstrated that the desiccation tolerance of *A. baumannii* is significantly correlated with its capacity of biofilm formation, and the mean survival time ranged from 14 to 56 days depending on their capacity to form biofilm.

In our study, this correlation was observed among clinical strains since clinical persistent strains showed to survive longer on desiccation conditions and have higher biofilm forming capacity than clinical non-persistent strains. However, while food strains survived longer on dry surface than clinical non-persistent strains, no significant difference were observed concerning the biofilm forming capacity. Therefore, our results do not demonstrate a clear association between desiccation resistance and biofilm forming capacity as well as no correlation with antibiotic resistance was observed ($p > 0.1$).

6.3.5. Susceptibility to disinfectants

This study aimed to assess the susceptibility of *A. baumannii* strains to commercial disinfectants commonly used in hospital settings and kitchen canteens. All clinical and food strains tested were sensitive to commercial products evaluated at the recommended use concentrations and in their undiluted forms, which revealed the effectiveness of the disinfectants tested. These results are consistent with previous reports, which found no apparent development of resistance to disinfectants among clinically isolated *Acinetobacter* spp. (Ivanković et al., 2017; Kawamura-Sato et al., 2008; Martró et al., 2003; Wisplinghoff et al., 2007). However, other studies have reported reduced susceptibility to commonly used biocides such as alcohols, chlorhexidine, formaldehyde, glutaraldehyde, hydrogen peroxide, hypochlorite, iodine and iodophors, quaternary ammonium compounds (such as benzalkonium), and triclosan (Chen et al., 2009; Fernández-Cuenca et al., 2015; Lin et al., 2017; Wisplinghoff et al., 2007) among clinical isolates of *A. baumannii* (Babaei et al., 2015; Gnanadhas et al., 2013; Peleg and Paterson, 2006).

However, when used diluted, disinfectants were not as effective, which is widely recognized (Lanjri et al., 2017; Liu et al., 2014) and emphasizes the need for using these products in dilutions recommended by the manufacturer. The minimum inhibitory concentrations (MICs) to eleven disinfectants are shown in Table 6.2. The tested strains showed a variety of susceptibility to biocides.

Table 6.2. The minimum inhibitory concentrations (MICs) to eleven commercial disinfectants used at hospital settings and kitchen canteens of clinical (persistent P1, P2, P3, P4, P5; non-persistent Np6, Np7, Np8, Np9, Np10) and food (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10) strains.

	MICs (%)										
A. baumannii strains	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
P1	0.78	0.78	4.20	6.25	6.25	31.50	4.00	18.75	50.00	2.34	1.37
P2	3.12	3.12	4.00	6.00	6.25	32.50	4.69	18.75	37.50	2.34	1.17
P3	2.34	0.78	3.91	6.25	12.50	34.38	4.00	18.75	34.38	2.34	1.17
P4	1.56	0.78	3.91	6.00	6.25	31.50	4.00	18.75	34.38	2.73	1.37
P5	2.34	0.78	3.91	6.25	6.25	32.50	5.00	18.75	37.50	2.73	1.37
Np6	0.78	0.50	4.20	6.00	6.25	31.50	4.00	15.60	43.75	2.34	1.37
Np7	1.56	0.78	4.50	6.25	6.25	32.50	4.00	18.75	50.00	2.73	1.37
Np8	0.78	1.00	4.00	6.00	6.25	31.50	4.69	15.60	43.75	2.73	1.37
Np9	0.50	0.78	4.20	6.00	6.25	31.50	4.00	18.75	43.75	2.34	1.37
Np10	0.39	0.50	4.20	6.25	12.50	32.50	6.25	18.75	43.75	2.73	1.56
F1	1.56	0.50	4.20	6.25	6.25	31.50	5.00	18.75	50.00	2.73	1.56
F2	1.56	0.78	4.20	6.25	6.25	31.50	4.69	15.60	43.75	2.73	1.56
F3	0.50	0.50	4.20	6.00	6.25	31.50	4.00	18.75	43.75	2.73	1.56
F4	0.78	0.50	4.00	6.25	6.25	31.50	4.69	15.60	50.00	2.73	1.56
F5	0.50	0.78	4.50	12.50	6.25	34.38	4.69	25.00	50.00	2.73	1.56
F6	0.39	0.78	4.50	6.25	6.25	36.00	4.00	25.00	43.75	2.73	1.56
F7	0.50	0.78	4.20	12.50	6.25	34.38	5.00	18.75	43.75	2.34	1.37
F8	0.78	0.78	4.20	6.25	12.50	36.00	4.00	18.75	37.50	2.34	1.37
F9	0.50	0.78	4.20	12.50	6.25	35.00	5.50	25.00	43.75	2.73	1.37
F10	0.50	0.25	4.20	12.50	12.50	37.00	5.50	25.00	50.00	2.73	1.56

Clinical strains were significantly more resistant ($p<0.01$) than food strains to Stellisept med ($p=0.02$) and Anios DDSH ($p<0.001$), the two most active disinfectants used in clinical settings. This may be explained by the selection pressure exerted by the use of disinfectants in hospitals and sometimes facilitated by the repeated exposure to sub-inhibitory concentrations (Gilbert and McBain, 2003; Kawamura-Sato et al., 2008). However, food strains were significantly more resistant ($p<0.01$) than clinical strains to six of the eleven disinfectants evaluated (Sterillium, Betadine, Presept + Typol, Sonaril HB, Sonaril LM and Sonaril DGF).

Moreover, clinical persistent strains were more resistant to two of the tested disinfectants (Stellisept and Sonaril HB) than clinical non-persistent strains ($p<0.05$), while these last ones shown to be more resistant to four disinfectants (Anios DDSH, Sterillium, Sonaril LM and Sonaril DGF ($p<0.01$).

Therefore, *A. baumannii* spp. showed high intra-species variability in susceptibility to disinfectants and there was no significant correlation ($p>0.01$) between the efficiency of disinfectants and the origin of *A. baumannii* isolates which suggest that this variability does not depend on the origin of the isolate but is probably intrinsic to the strain. Similar findings for clinical and environmental *A. baumannii* isolates have already been reported (Babaei et al., 2015; Ivanković et al., 2017; Lanjri et al., 2017).

In our study, multidrug resistance phenotype does not clearly link to disinfectants susceptibility. The correlation between reduced susceptibility to disinfectants and multidrug resistance was observed only for two disinfectants (Stellisept med, $p=0.006$; Anios DDSH, $p =0.0001$). In contrast, the opposite correlation was observed to seven disinfectants (Sterillium, $p=0.003$; Betadine, $p=0.01$; Cutasept, $p=0.02$; Presept + Typol, $p=0.0009$; Sonaril Da5, $p =0.02$; Sonaril LM, $p=0.004$; Sonaril DGF, $p < 0.0001$) and the strains most resistant to these disinfectants tended to be less resistant to antibiotics then the sensitive strains.

Likewise, literature about antibiotic/disinfectant cross-resistance among *Acinetobacter* ssp. isolates is inconclusive. Some studies showed no apparent association between resistance to antibiotics and resistance to disinfectants (Ivanković et al., 2017; Martró et al., 2003), while some found a significant correlation (Kawamura-Sato et al., 2010), others found correlations with certain types of antibiotic but not with others (Ali et al., 2014) and some concluded that cross-resistance was possible (Russell et al., 1998). Lin et al. (2017) reported that although there is a high prevalence of multiple antibiotic resistant and biocide resistant isolates in clinical settings it is challenging to establish an overall relationship or cross-resistance between antibiotic resistance and reduced susceptibility to biocides which could be explained by the diversity of genotypes isolated and for the variety of mechanisms of action of the biocides and antibiotics that act on different cellular targets (McDonnell and Russell, 1999).

6.4. Conclusion

Although measures to limit environmental contamination in the hospital and vigorous efforts to prevent patient to patient spread via health care workers are always recommended, an environmental niche that favors MDR *A. baumannii* may exist. The antimicrobial resistance pattern, together with the results of PFGE, strongly suggests a presence of predominant clones of MDR *A. baumannii* and a number of distinct genetically uniform minor clones among the clinical isolates; however most of the sporadic strains were also MDR. Therefore, although these results showed that antibiotic resistance itself could not be associated with increased potential to persist in hospitals, the persistence of dominant clonal group could be due to a combination of factors. Our results show that the biofilm-forming capacity and resistance to desiccation may provide *A. baumannii* with an advantage and ideal niche for its extended prevalence in hospital environment. Indeed, the biofilm-forming capacity of persistent strains was significantly higher than the non-persistent strains on the silicon and on the stainless steel surfaces. As well as, clinical strains exhibited desiccation resistance capacity and the survival time of the persistent strains was significantly longer than that of the non-persistent strains. This may facilitate its colonization and persistence on hospital environmental surfaces, thereby increasing the probability of causing nosocomial infections and outbreaks either by direct contact or horizontal transmission.

However, it was not observed a correlation between persistence and disinfectant resistance since all the tested strains were sensitive to commercial products evaluated at the recommended use concentrations and in its purest forms.

Moreover, there were no significantly difference between clinical and food strains concerning its biofilm forming capacity and resistance to desiccation. This could suggest that if hygienic practices were not effective, this strains may persist in community and hospital settings which highlighted the importance that food products may have as a vehicle of *A. baumannii* whether in contamination of kitchen environments whether in the cross contamination to hospital environments.

The combination of this and other factors, such as the high ability of bacteria to transfer genetic material and the selective pressure present in clinical settings, is a recipe for the proliferation of multiple-resistant bacteria. Further studies including a higher number of isolates are needed to corroborate these findings.

MAIN CONCLUSIONS

Main Conclusions

Currently, there only exist a few studies focused on the diversity of *Acinetobacter* spp. in food products. This work reports the occurrence and antibiotic susceptibility of *Acinetobacter* spp. isolated outside the hospital. It is one of the very few studies exploring this population in ready to eat fruits and vegetables and meat products worldwide. Moreover, to the best of our knowledge, this is the first report about the prevalence and antimicrobial susceptibility of *Acinetobacter* spp. in fruit, vegetables and meat retailed in Portugal.

Whereas some studies only referred to the prevalence of the species *A. baumannii*, others did not evaluate the antimicrobial susceptibility of the isolates which is of major importance taking into account the high capacity of transference of antibiotic resistance.

Moreover, among the available data, there could be some misconceptions since the isolation methods used have a huge impact on the recovery of these organisms and some identification techniques such automated systems like Phoenix, Vitek2 and API 20NE are not able to differentiate species among this genus (Abbott and Peleg, 2014; Dijkshoorn et al., 2007; Eveillard et al., 2013; Peleg et al., 2008; Towner, 2009; Visca et al., 2011).

Therefore, the first aim of this work was to identify a procedure for *Acinetobacter* spp. recovery from food products. Enrichment in Dijkshoorn's medium followed by plating on CHROMagar^{TM} Acinetobacter showed to be a reliable method for this isolation and was used in the following studies. Concerning the identification of *Acinetobacter* spp. to the species level, this was based on the RNA polymerase subunit B (*rpo*B) gene sequencing, which is the best studied single gene taxonomic and phylogenetic marker for this genus (Gundi et al., 2009; La Scola et al., 2006).

It was demonstrated that ready to eat products, such as lettuces and fruits, as well as raw meat samples are a natural habitat of several *Acinetobacter* species, including the species belonging to the pathogenic *A. baumannii* group such as *A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. seifertii*. These food products are also an important source of strains resistant to several antimicrobials, including carbapenems and even to colistin and polymyxin, the last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter*. Moreover, several isolates shown to be MDR and XDR which represent an additional concern.

The presence of clinically important species and multidrug-resistant strains in food products may be a threat to public health considering that meat may provide a vector for the spread of these opportunistic pathogens into both community and hospital settings environment.

The taxonomy of this genus has undergone a wide development over the past years and several new species were described. In this work we assessed the taxonomic status of six strains of *Acinetobacter* obtained from meat samples using a polyphasic analysis and it was confirmed that these strains represented two coherent lineages that were distinct from each other and from all known species. The names *Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov. were proposed for these novel species.

Among this genus, *A. baumannii* is the species most often associated with nosocomial infections. In this work we concluded that an environmental niche that favours MDR *A. baumannii* may exist since the antimicrobial resistance pattern, together with the results of PFGE, strongly suggested the presence of a predominant clone of MDR *A. baumannii* during 4 years, in Hospital de Braga, and a number of distinct genetically uniform minor clones. However, most of the sporadic strains were also MDR, which suggests that increased potential to persist in hospitals may not be associated with antibiotic resistance itself but could be due to a combination of several factors. In this work some potential factors contributing to persistence were evaluated, namely, the biofilm-forming capacity, the resistance to desiccation and the susceptibility to disinfectants and compared among food and clinical isolates. Results showed that all the tested strains were sensitive to commercial products evaluated at the recommended use concentrations and in their purest forms, which revealed the effectiveness of the disinfectants tested and no correlation between persistence and disinfectant resistance was observed. However, the biofilm-forming capacity and resistance to desiccation may provide *A. baumannii* with an advantage and ideal niche for its extended prevalence in hospital environment. Indeed, the biofilm-forming capacity on silicon and on stainless steel surfaces was significantly higher for persistent than for the non-persistent strains. As well as, clinical strains exhibited desiccation resistance capacity ranging from 14 to 77 days and the survival time of the persistent strains (mean survival time: 65.8 days) was significantly longer than that of the non-persistent strains (mean survival time: 35.8 days). This may facilitate their colonization and persistence on hospital environmental surfaces, thereby increasing the probability of causing nosocomial infections and outbreaks either by direct contact or horizontal transmission.

Moreover, there were no significantly difference between clinical and food strains concerning its biofilm forming capacity and resistance to desiccation since food strains were also able to form a high level of biofilm and it survival time ranged from 35 to 77 days (mean survival time: 54.6 days). This highlighted the importance of assessing the role of foods in the occurrence of infections and transmission of these bacteria into the community and hospital environments since if hygienic practices were not effective, these strains may persist in community and hospital settings. Once “installed”, these organisms are extremely difficult to eradicate, thus avoiding the spread of multidrug-resistant *Acinetobacter* spp. in the environment, in which food may have an important role, could be an important way to prevent or control *Acinetobacter* spp. infections.

FUTURE WORK PROPOSALS

Future work Proposals

Although this study was a good starting point for the understanding of food as an important source of MDR *Acinetobacter* spp. which are also resistant to desiccation and forming biofilm, there are still unanswered questions which lead to the proposal of the following suggestions for future research:

- Evaluation of the presence of this genus and antimicrobial resistance in others foods such as fish, dairy products, vegetables, including spinach, broccoli, carrots and olives, in order to increase the insight into the food-related ecology and epidemiology of *Acinetobacter* spp.

- To assess the role of foods as a route of transmission of these bacteria into the hospital environments it is essential to recover and genotype *Acinetobacter* spp. from hospital meals, hospital environments (including food service areas), and patients' clinical samples and compare these isolates with food isolates.

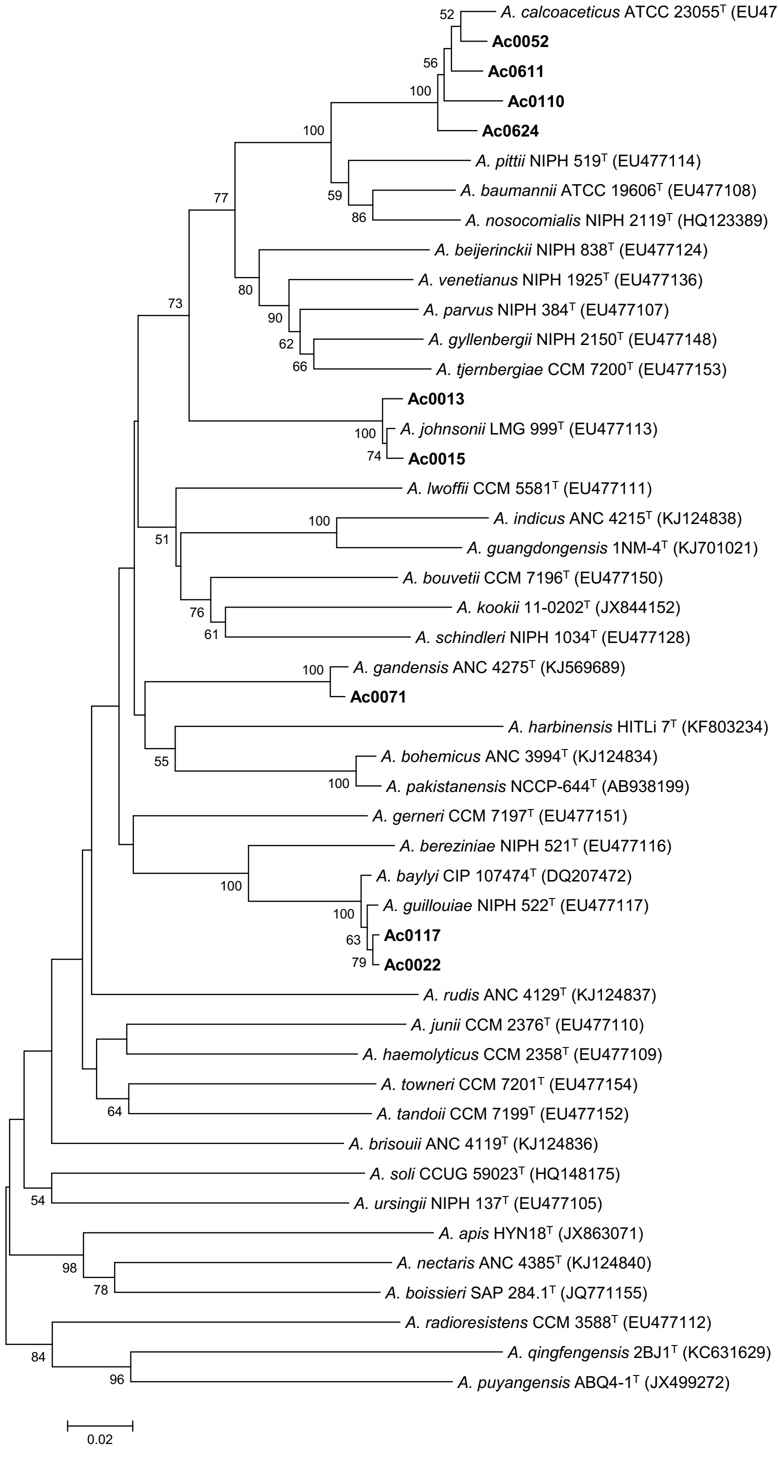
- To evaluate the presence of antimicrobial resistance genes among the food isolates in order to assess the role of resistant strains in the dissemination of resistance genes.

- Further studies including a higher number of isolates are needed to corroborate the findings concerning biofilm forming capacity, resistance to desiccation and susceptibility to disinfectants.

- To assess the resistance to desiccation and susceptibility to disinfectants after biofilm formation.

- To evaluate the capacity of food strains to causa infection in order to assess the role of foods in the occurrence of *Acinetobacter* spp. infections by the evaluation of:
    - the resistance to sub-lethal stress applied in food processes (e.g. milld heat treatments, refrigeration temperatures, disinfection);
    - the survival through the gastrointestinal tract;
    - adhesion and invasion into the epithelial cells of the small intestine;
    - gut microbiome-*Acinetobacter* spp. interaction.

SUPLEMENTARY MATERIAL



Supplementary Figure. 2.1. Neighbor-joining tree based on partial nucleotide sequence of *rpo*B of 9 strains of *Acinetobacter* spp. isolated from food (underlined) and the representatives of the known species of the genus *Acinetobacter* (NCBI accession nos. are given in parentheses). Bootstrap values (>50%) after 1000 replicates are shown at branch nodes. The evolutionary distances were computed and the bar (0.02) is the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura K., 2013).

Suplementary Table 3.1. Antimicrobial resistance profiles of *Acinetobacter* spp.

No. of resistante antimicrobial classes	**Resistance pattern**	**Species (no.) per sample**				
		Lettuce	**Fruit**			
			Apple	**Pear**	**Strawberry**	**Banana**
0		*A. calcoaceticus* (11) *A. johnsonii* (4) *A. guillouiae* (3) *A. baumannii* (1) *A. gyllenbergii* (1)*	*A. calcoaceticus* (2) *A. johnsonii* (1) *A. ursingii* (1) *A. radioresistens* (1) *A. lwoffii* (1)*	*A. pittii* (3) *A. schindleri* (1) *A. parvus* (1)*	*A. beijerinckii* (2) *A. johnsonii* (1)	*A. pittii* (1) *A. johnsonii* (4) *A. baylyi* (4) *A. soli* (2)
1	CAZ			*A. johnsonii* (1)		
	CL		*A. parvus* (1)			
	PB					*A. seifertii* (2)
	(PB/CL)	*A. junii* (1) *A. bereziniae* (1)				*A. seifertii* (1)
	TZP	*A. calcoaceticus* (18) *A. johnsonii* (1) *A. beijerinckii* (1) *A. baumannii* (1) *A. pittii* (1)	*A. calcoaceticus* (1) *A. pittii* (1) *A. radioresistens* (1)		*A. calcoaceticus* (1) *A. guillouiae* (3)	*A. pittii* (1)
	CAZ	*A. johnsonii* (4)				
	IMP	*A. johnsonii* (2)				
	(PB/CL)	*A. beijerinckii* (1)				
		A. tjernbergiae (1)*				
2	TZP/CAZ	*A. calcoaceticus* (5) *A. johnsonii* (6) *A. beijerinckii* (2)	*A. calcoaceticus* (1) *A. lwoffii* (1) *A. johnsonii* (4)	*A. lwoffii* (1) *A. johnsonii* (1) *A. beijerinckii* (1)		
	CAZ/ MEM			*A. guillouiae* (1)		

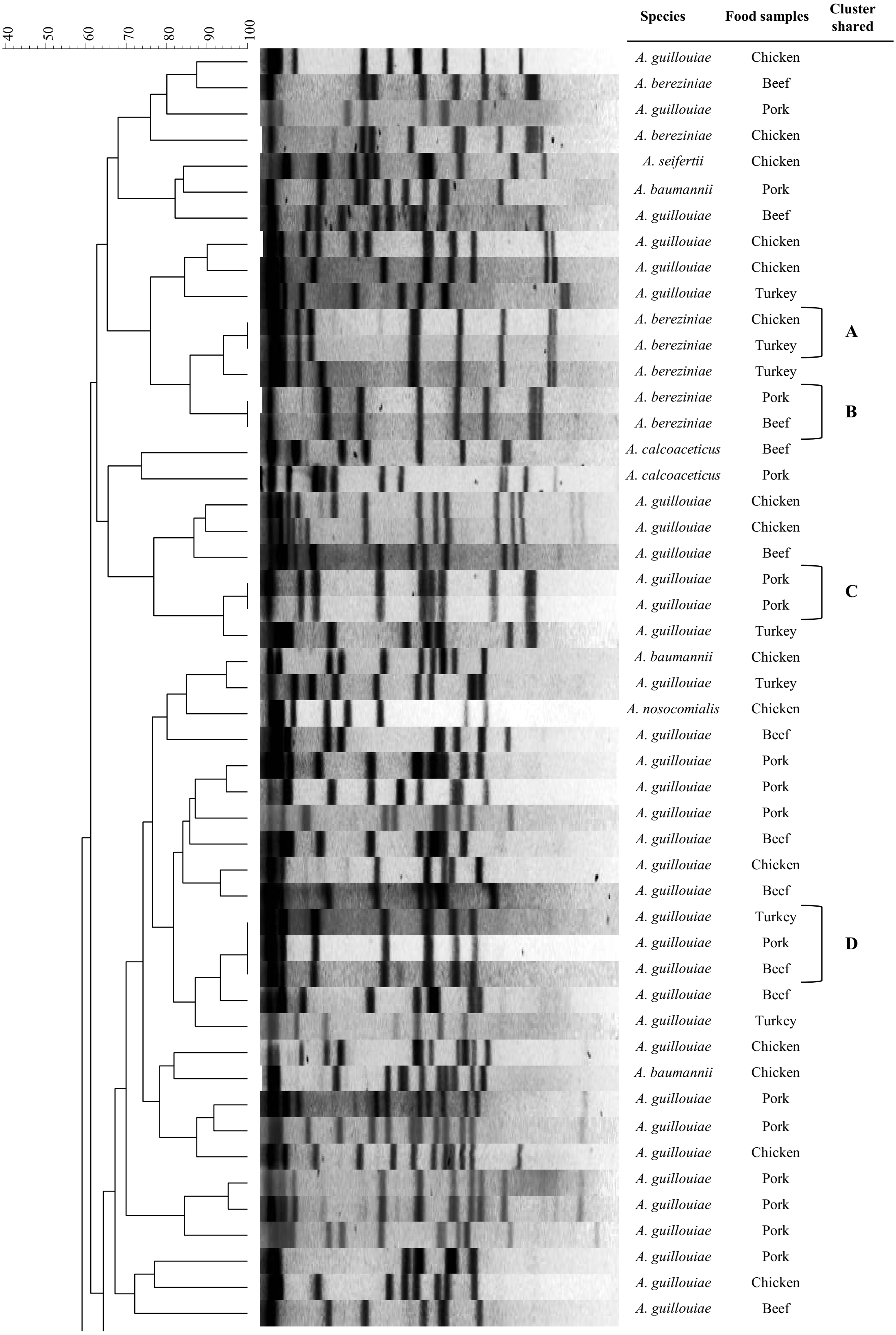
	CAZ/CIP	*A. johnsonii* (1)				
	TZP/IMP	*A. johnsonii* (1) *A. guillouiae* (1)				
	TZP/(PB/CL)	*A. calcoaceticus* (1)			*A. venetianus* (1)*	
	TZP/PB	*A. pittii* (3)				
	TZP/TOB					*A. baylyi* (1)
	TZP/(IMP/MEM)	*A. guillouiae* (2) *A. calcoaceticus* (1)	*A. guillouiae* (1)	*A. calcoaceticus* (1)		
3	IMP/CAZ/PIP	*A. beijerinckii* (1)				
	TZP/CAZ/IMP	*A. calcoaceticus* (1) *A. johnsonii* (1) *A. baumannii* (1)				
	TPZ/CAZ/(IMP/MEM)	*A. johnsonii* (1) *A. guillouiae* (1)	*A. guillouiae* (1)			
	TZP/IMP/CL	*A. guillouiae* (1)				
	TZP/CAZ/CL	*A. calcoaceticus* (1) *A. beijerinckii* (4)				
	TZP/CAZ/PB	*A. beijerinckii* (1)				
	TPZ/CAZ/SXT	*A. johnsonii* (1)				
	TPZ/CAZ/CIP	*A. johnsonii* (1) *A. guillouiae* (1)				
	CAZ/CL/CIP	*A. guillouiae* (1)				
	TZP/CIP/SXT	*A. guillouiae* (1)				
	TZP/TOB/SXT		*A. johnsonii* (1)			
	(TPZ/SAM)/CAZ/SXT	*A. johnsonii* (1)				
4	TPZ/CAZ/IMP/CIP	*A. calcoaceticus* (1)				
	TPZ/CAZ/IMP/AK	*A. baumannii* (1)				
	TPZ/CAZ/CIP/SXT	*A. johnsonii* (2)			*A. johnsonii* (1)	

	TPZ/CAZ/AK/CIP	*A. johnsonii* (1)				
	TPZ/CAZ/AK/SXT	*A. beijerinckii* (1)				
	(TPZ/SAM)/CAZ/CIP/SXT			*A. lwoffii* (1)		*A. baylyi* (1)
	TPZ/CAZ/MEM/(AK/TOB)	*A. beijerinckii* (1)				
	TPZ/CAZ/CL/SXT				*A. johnsonii* (1)	
	TPZ/CAZ/IMP/CL	*A. guillouiae* (2)				
	TPZ/CAZ/CIP/PB	*A. beijerinckii* (1)				
	TPZ/CAZ/IMP/CL	*A. guillouiae* (1)				
	CAZ/IMP/(AK/TOB)/SXT	*A. gyllenbergii* (1)*				
5	(TPZ/SAM)/CAZ/TOB/CIP/PB		*A. ursingii* (1)			
	TPZ/CAZ/TOB/CIP/SXT	*A. johnsonii* (1)				*A. baylyi* (1)
	TPZ/CAZ/TE/CIP/SXT				*A. johnsonii* (1)	
	TPZ/CAZ/(IMP/MEM)/CIP/SXT	*A. johnsonii* (1)				
	(TPZ/SAM)/CAZ/AK/CIP/SXT	*A. johnsonii* (1)				
	(TPZ/SAM)/CAZ/MEM/AK/CIP	*A. johnsonii* (1)				
	(TZP/SAM)/MEM/(AK/TOB)/CIP/SXT					*A. baylyi* (1)
	(TPZ/SAM)/CAZ/TOB/CIP/SXT					*A. variabilis* (1)
6	TZP/CAZ/MEM/AK/CIP/SXT	*A. johnsonii* (1)				
	TPZ/CAZ/TOB/TE/CIP/SXT					*A. pittii* (1)
	(TZP/SAM)/CAZ/CL/(AK/TOB)/CIP/SXT					*A. baylyi* (1)
7	(TZP/SAM)/CAZ/(AK/TOB)/(MI/TE)CIP/SXT/MEM					*A. calcoaceticus* (1)
8	(TZP/SAM)/CAZ/MEM/CL/(AK/TOB)/TE/CIP/SXT					*A. calcoaceticus* (1)
	(TZP/SAM)/CAZ/IMP/PB/(AK/TOB)/TE/CIP/SXT					*A. nosocomialis* (1)
	(TZP/SAM)/CAZ/(IMP/MEM)/CL/(AK/TOB)/TE/CIP/SXT					*A. calcoaceticus* (1)
	(TZP/SAM)/CAZ/(IMP/MEM)/CL/(AK/TOB)/(TE/MI)/CIP/SXT			*A. pittii* (1)		

TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole.

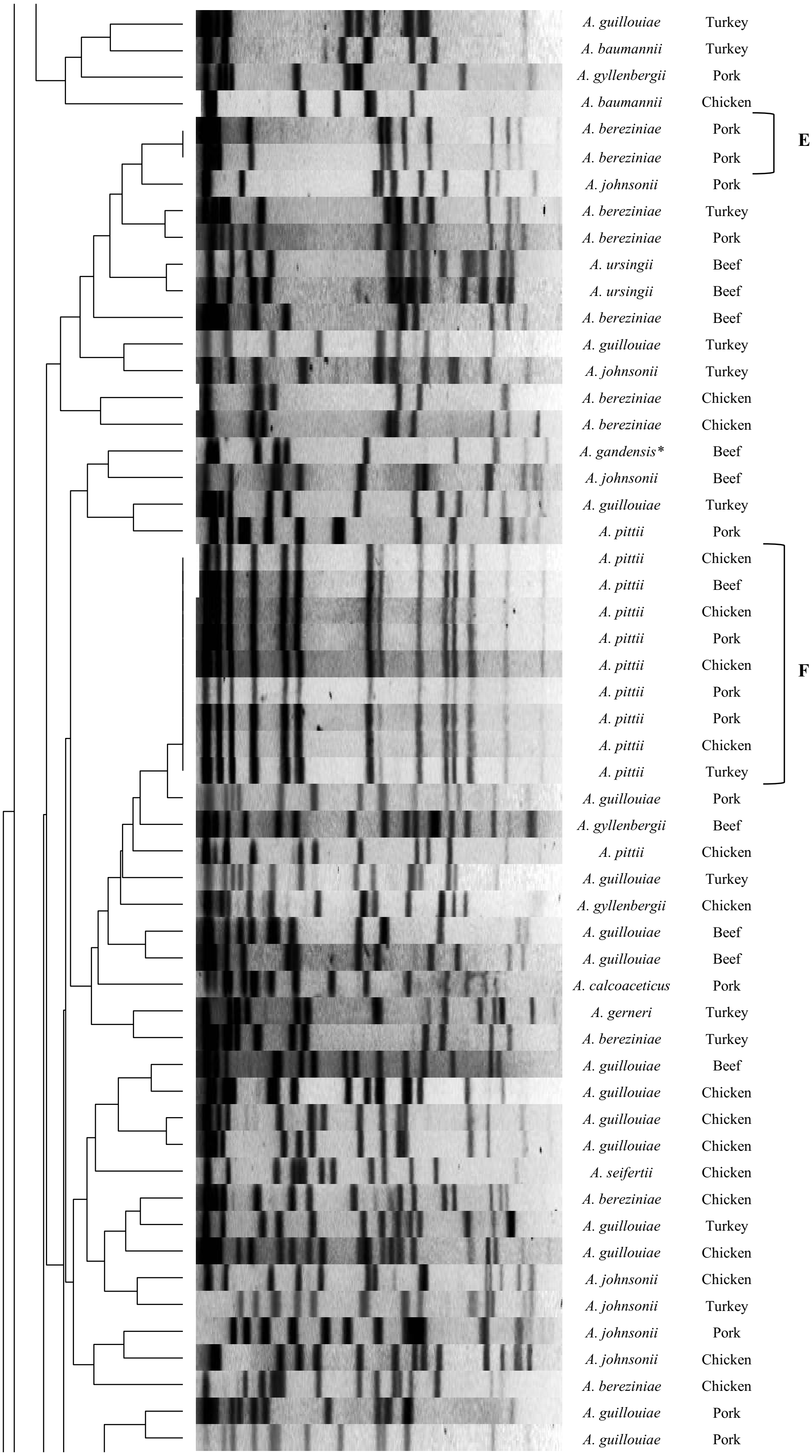
Antibiotics from the same antimicrobial class were written in brackets.

**rpo*B sequence similarity values <95 % suggest that these isolates may represent novel species.

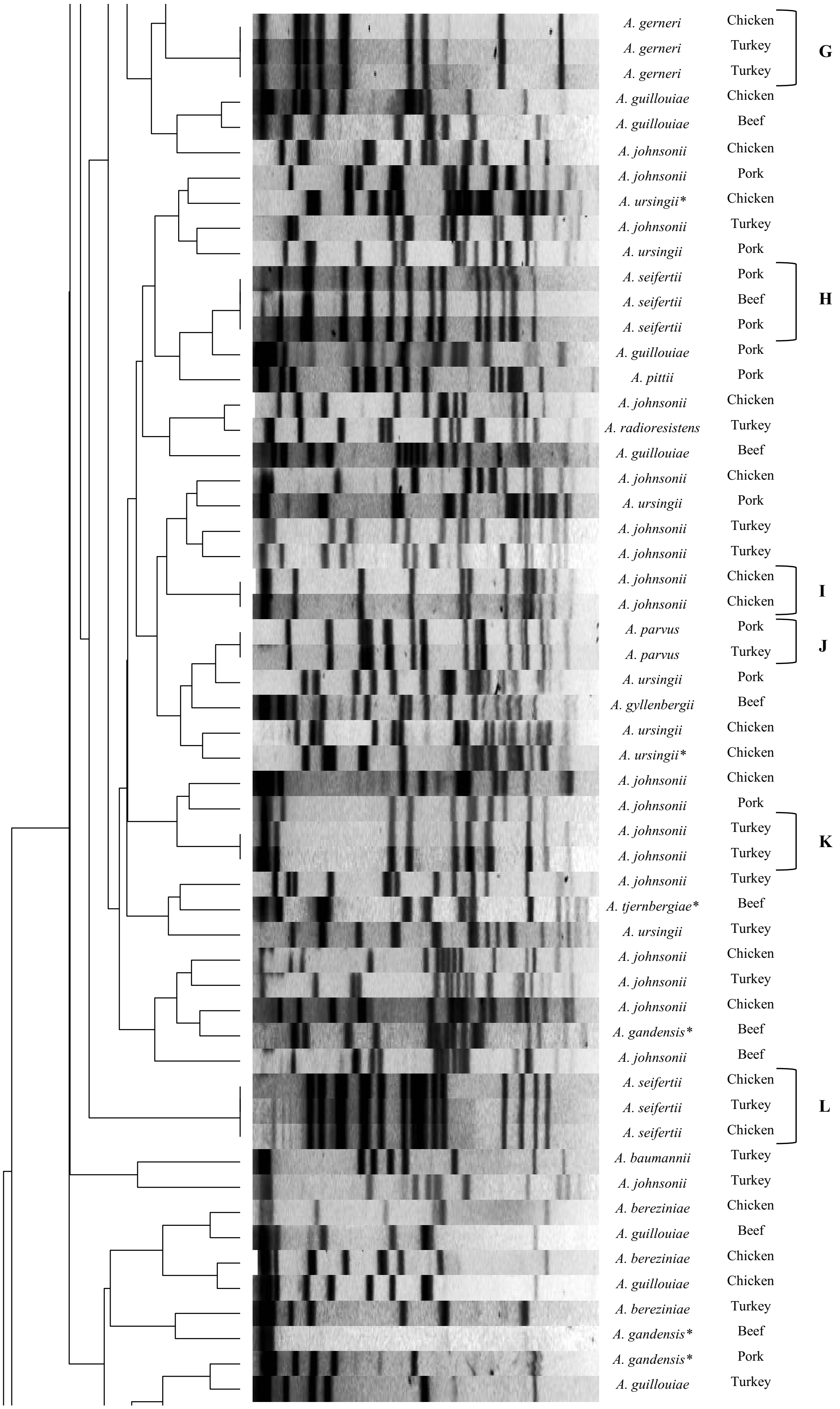


Supplementary Figure 4.1. Dendrogram of 166 *Acinetobacter* spp. isolates from different meat samples obtained with restriction enzymes *Asc*I. Thirteen clusters were shared between different samples and a capital letter was ascribed to each one.

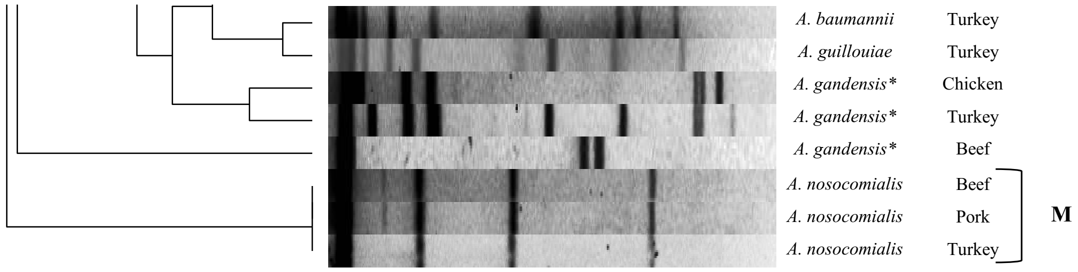
**rpo*B sequence similarity values <95% suggest that these isolates may represent novel species.



Supplementary Figure. 4.1. (continued)



Supplementary Figure. 4.1. (continued)



Supplementary Figure. 4.1. (continued).

Supplementary Table 4.1. Antimicrobial resistance profiles of *Acinetobacter* spp. isolated from different meat samples.

No. of resistante antimicrobial classes	**Resistance pattern**	**Species (no.) per meat sample**			
		Chicken	**Turkey**	**Beef**	**Pork**
0		*A. pittii* (1) *A. guillouiae* (1) *A. gandensis* (1)*	*A. johnsonii* (2) *A. radioresistens* (1) *A. gandensis* (1)* *A. parvus* (1)	*A. johnsonii* (1) *A. gandensis* (2)*	*A. johnsonii* (1) *A. pittii* (4) *A. gyllenbergii* (1) *A. ursingii* (2) *A. calcoaceticus* (2) *A. parvus* (1)
1	TE	*A. johnsonii* (2) *A. baumannii* (1)	*A. guillouiae* (1) *A. baumannii* (1)		
	TZP	*A. johnsonii* (1) *A. baumannii* (1)	*A. johnsonii* (1) *A. guillouiae* (1) *A. baumannii* (1)		*A. johnsonii* (1)
	CAZ	*A. guillouiae* (1)	*A. johnsonii* (1)		*A. ursingii* (1)
	CL	*A. seifertii* (1)	*A. pittii* (1) *A. guillouiae* (1)	*A. ursingii* (1)	
	(CL/PB)	*A. bereziniae* (1) *A. seifertii* (1) *A. nosocomialis* (1)	*A. gerneri* (1) *A. nosocomialis* (1)	*A. bereziniae* (2) *A. gyllenbergii* (1)	*A. bereziniae* (2) *A. nosocomialis* (1)
	PB	*A. gerneri* (1) *A. ursingii* (2)*	*A. guillouiae* (1)	*A. ursingii* (1)	*A. baumannii* (1)
2	CAZ/TZP	*A. johnsonii* (2) *A. guillouiae* (1)			

		A. ursingii (1)			
	CAZ/(TZP/SAM)			*A. johnsonii* (1)	
	TZP/(CL/PB)	*A. bereziniae* (1) *A. guillouiae* (1) *A. seifertii* (1)		*A. tjernbergiae* (1)*	*A. bereziniae* (1)
	CAZ/PL		*A. bereziniae* (1)		*A. ursingii* (1)
	TE/(CL/PB)		*A. bereziniae* (1)		*A. seifertii* (1)
	CAZ/(CL/PB)			*A. bereziniae* (1)	
	TZP/CL	*A. guillouiae* (1) *A. gyllenbergii* (1)	*A. guillouiae* (1) *A. gerneri* (1)	*A. calcoaceticus* (1)	
	CIP/TZP		*A. guillouiae* (2)	*A. guillouiae* (1)	
	MEM/TZP	*A. baumannii* (1)			
3	CAZ/TZP/TE		*A. johnsonii* (1)		*A. bereziniae* (1)
	SXT/TOB/TZP		*A. johnsonii* (1)		
	MEM/TZP/CL	*A. pittii* (1)			
	CIP/TZP/TE	*A. pittii* (1)			
	CIP/TZP/(CL/PB)	*A. bereziniae* (1)	*A. guillouiae* (1)	*A. gyllenbergii* (1)	*A. guillouiae* (1)
	TZP/TE/(CL/PB)		*A. bereziniae* (1)	*A. seifertii* (1)	
	CAZ/MEM/(TZP/SAM)	*A. guillouiae* (1)			
	CAZ/SXT/(TZP/SAM)	*A. guillouiae* (1)			
	CIP/TZP/CL		*A. guillouiae* (1)	*A. guillouiae* (2)	
	CAZ/CIP/TZP	*A. guillouiae* (2)			*A. guillouiae* (3)
	AK/CIP/TZP				*A. guillouiae* (1)
	CAZ/TZP/(CL/PB)		*A. ursingii* (1) *A. gerneri* (1)		
4	CAZ/CIP/TZP/TE	*A. johnsonii* (1)			

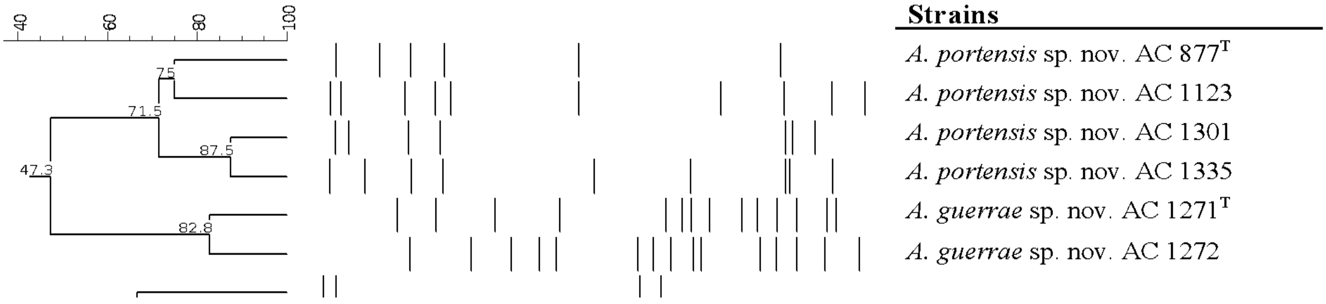
	AK/CIP/SXT/TZP		*A. johnsonii* (1)		
	CAZ/CIP/TOB/(TZP/SAM)	*A. pittii* (1)			
	CIP/TZP/TE/(CL/PB)	*A. bereziniae* (2)			*A. seifertii* (1)
	CAZ/TZP/TE/(CL/PB)	*A. bereziniae* (1)			
	CAZ/CIP/TZP/(CL/PB)	*A. seifertii* (1)	*A. bereziniae* (1) *A. seifertii* (1)		*A. guillouiae* (1)
	(AK/TOB)/CIP/SXT/TZP		*A. guillouiae* (1)		
	MEM/CAZ/(TZP/SAM)/(CL/PB)	*A. guillouiae* (1)			
	CAZ/CIP/SXT/TZP	*A. guillouiae* (1)	*A. guillouiae* (1)	*A. guillouiae* (1) *A. gandensis* (1)*	
	CAZ/CIP/TZP/CL			*A. guillouiae* (1)	
	CIP/TZP/TE/CL			*A. guillouiae* (1)	
	CAZ/CIP/SXT/(TZP/SAM)				*A. guillouiae* (1)
	AK/CAZ/TE/(CL/PB)		*A. baumannii* (1)		
5	CAZ/CIP/SXT/TOB/(TZP/SAM)	*A. johnsonii* (1)	*A. johnsonii* (1)		
	(AK/TOB)/CAZ/CIP/SXT/TZP			*A. guillouiae* (1)	*A. johnsonii* (1)
	CAZ/CIP/SXT/MEM/(TZP/SAM)		*A. johnsonii* (1)		
	CAZ/MEM/TOB/TZP/TE			*A. pittii* (1)	
	AK/CAZ/CIP/MEM/(TZP/SAM)				*A. pittii* (1)
	CAZ/SXT/TZP/TE/(CL/PB)	*A. bereziniae* (1)			
	CIP/SXT/TZP/TE/(CL/PB)		*A. bereziniae* (1)		
	CAZ/CIP/TZP/TE/CL	*A. guillouiae* (1)		*A. guillouiae* (2)	
	CAZ/CIP/SXT/(TZP/SAM)/TE			*A. guillouiae* (1)	*A. guillouiae* (1)
	CAZ/CIP/SXT/TZP/(CL/PB)				*A. guillouiae* (2)
	AK/CIP/SXT/TZP/(CL/PB)				*A. guillouiae* (1)
	CIP/TOB/TZP/TE/(CL/PB)				*A. guillouiae* (1)

	CAZ/CIP/SXT/TOB/TZP		*A. guillouiae* (1)		*A. guillouiae* (1)
	CAZ/CIP/TZP/TE/(CL/PB)			*A. guillouiae* (1)	
	CAZ/CIP/(TZP/SAM)/TE/CL	*A. guillouiae* (1)			
	AK/CAZ/CIP/TZP/CL			*A. guillouiae* (1)	
	AK/CAZ/SXT/TZP/(CL/PB)			*A. nosocomialis* (1)	
6	(IMP/MEM)/(AK/TOB)/CAZ/CIP/(TZP/SAM)/TE	*A. pittii* (1)			
	MEM/(AK/TOB)/CAZ/CIP/SXT/(TZP/SAM)	*A. johnsonii* (1)	*A. johnsonii* (1)		
	MEM/CIP/SXT/TZP/TE/(CL/PB)	*A. bereziniae* (1)			
	AK/CAZ/CIP/SXT/TZP/PB				*A. guillouiae* (2)
	(AK/TOB)/CAZ/CIP/SXT/TZP/CL			*A. guillouiae* (1)	*A. guillouiae* (1)
	AK/CAZ/CIP/SXT/TZP/(CL/PB)				*A. guillouiae* (1)
	(AK/TOB)/CAZ/CIP/SXT/(TZP/SAM)/TE				*A. gandensis* (1)*
	TOB/CAZ/CIP/SXT/(TZP/SAM)/TE			*A. gandensis* (1)*	
7	MEM/(AK/TOB)/CAZ/CIP/SXT/TZP/TE	*A. johnsonii* (1)			
	(IMP/MEM)/(AK/TOB)/CAZ/CIP/SXT/(TZP/SAM)/TE	*A. johnsonii* (1)			
	AK/CAZ/CIP/SXT/(TZP/SAM)/TE/(CL/PB)	*A. guillouiae* (1)			
	AK/CAZ/CIP/SXT/TZP/TE/PB			*A. guillouiae* (1)	
	MEM/(AK/TOB)/CAZ/CIP/SXT/(TZP/SAM)/TE	*A. guillouiae* (1)			

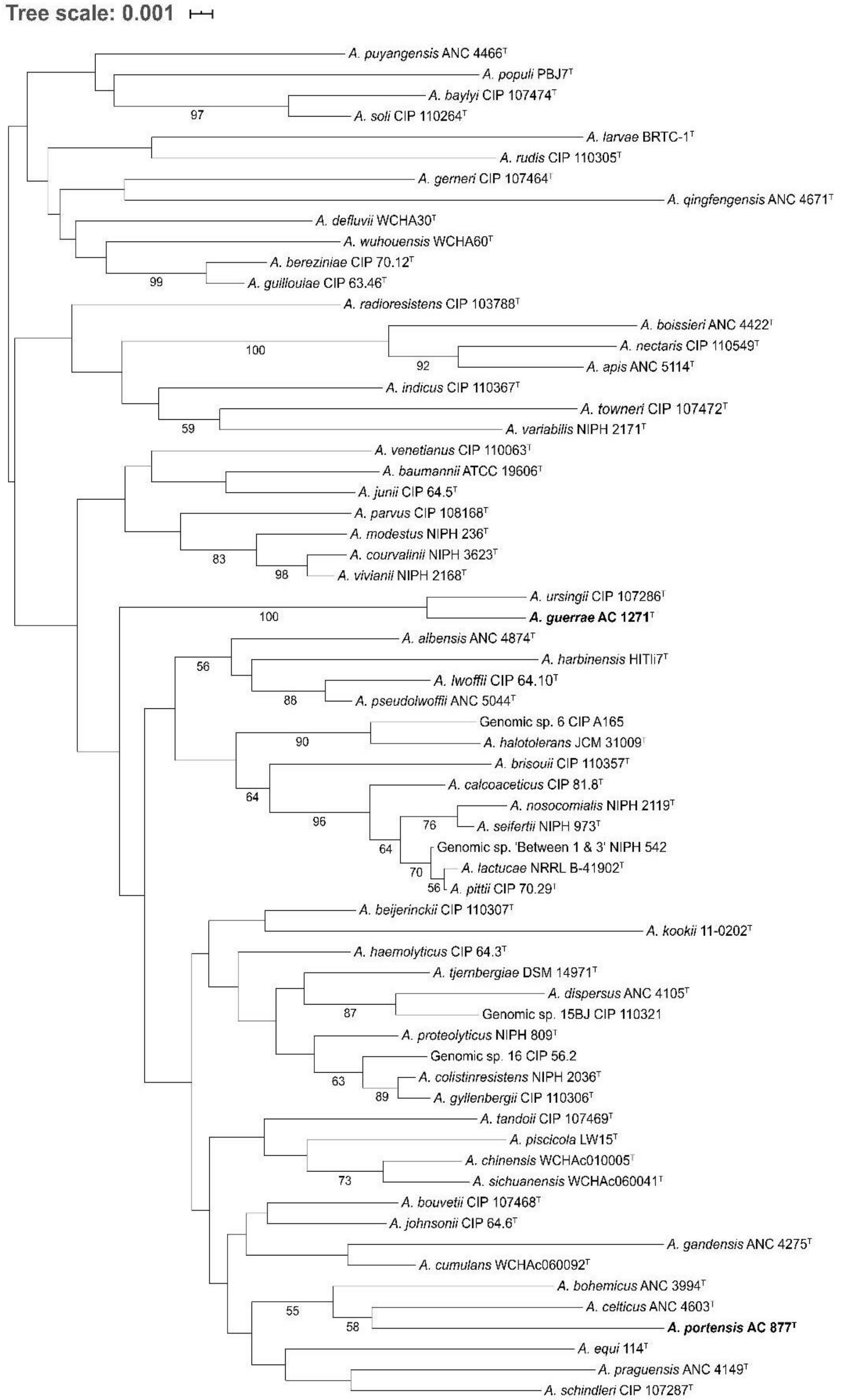
PIP, piperacillin; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PB, polymyxin B; CL, Colistin; AK, amikacin; TOB, tobramycin; TE, tetracycline; MI, minocycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole.

Antibiotics from the same antimicrobial class were written in brackets.

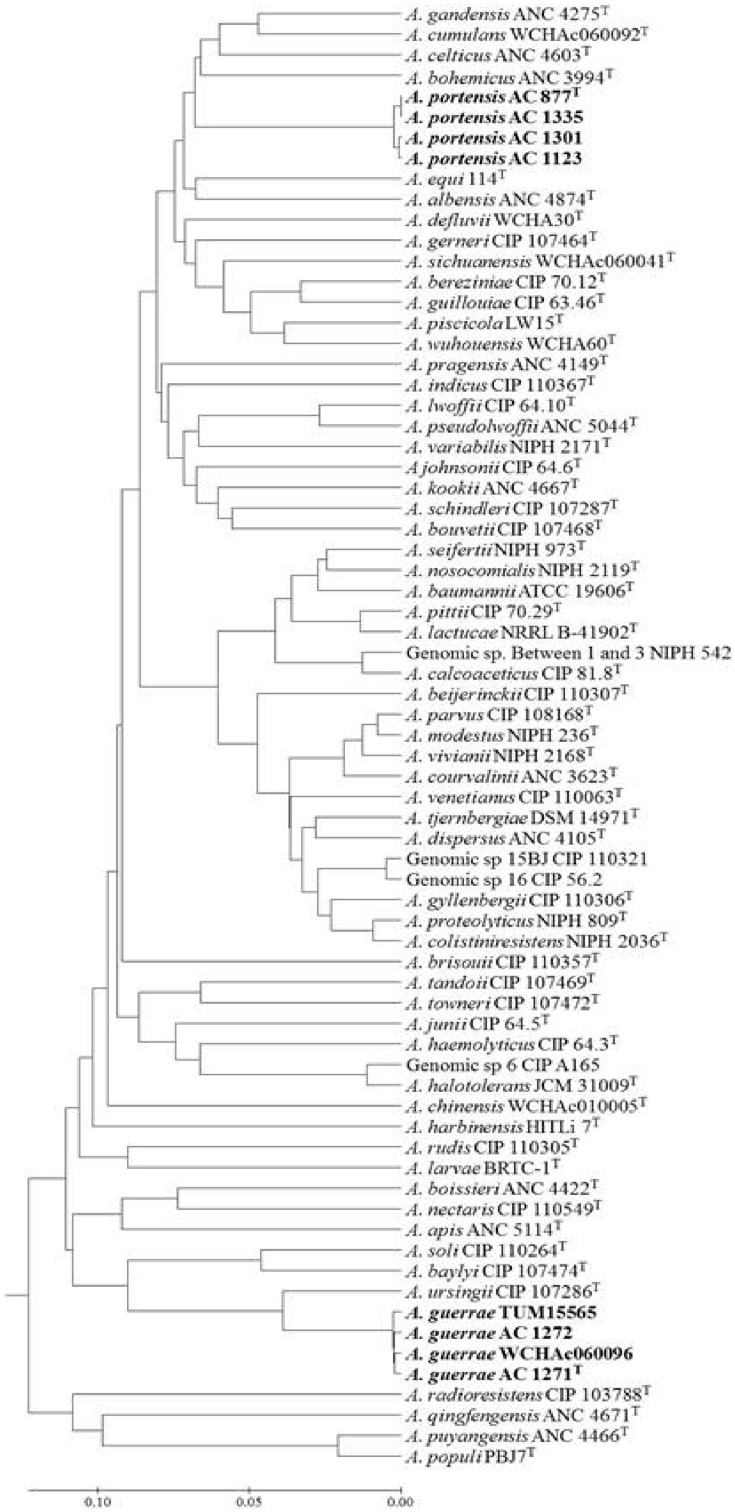
**rpo*B sequence similarity values <95 % suggest that these isolates may represent novel species.



Suplementary Figure 5.1. PFGE patterns of the strains of *A. portensis* sp. nov (AC 877^T, AC 1335, AC 1301, AC 1123) and *A. guerrae* sp. nov. (AC 1271^T, AC 1272) demonstrating their genotypic distinctness at the strain level. PFGE patterns were obtained with restriction enzyme *Asc*I according to Carvalheira et al. (2017a).



Suplementary Figure 5.2. Neighbour-joining tree derived from 16S rRNA gene sequence comparisons showing the relationship of the type strains of *Acinetobacter portensis* sp. nov. and *Acinetobacter guerrae* sp. nov. with type or reference strains of known species of the genus *Acinetobacter.* The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Bootstrap values (>50%) after 1,000 simulations are shown at branch nodes. Bar, 0.001 substitutions per nucleotide site. All calculations were conducted in MEGA6 (Tamura et al., 2013).



Suplementary Figure 5.3. Rooted UPGMA tree based on partial nucleotide sequences of *rpo*B gene of four strains of *Acinetobacter portensis* sp. nov., four strains of *Acinetobacter guerrae* sp. nov. and the type or reference strains of validly published names and genomospecies of the genus *Acinetobacter.* The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). All calculations were conducted in MEGA6 (Tamura et al., 2013).

Suplementary Table 5.1. NCBI accession numbers of genome, 16S rRNA, *gyr*B, *rec*A and *rpo*B sequences of strains of *Acinetobacter portensis* sp. nov., *Acinetobacter guerrae* sp. nov. and the type strains of all species of *Acinetobacter* with validly published names and four genomospecies

NCBI accession numbers					
Strain	**Genome sequence**	**16S rRNA gene**	***gyrB* gene**	***rec*A gene**	***rpo*B gene**
A. albensis ANC 4874^{T}	FMBK00000000.1	-	-	-	KR611814
A. apis ANC 5114^{T}	FZLN00000000.1	-	-	-	JX863071
A. baumannii ATCC 19606^{T}	ACQB00000000.1	-	-	-	EU477108
A. baylyi CIP 107474^{T}	APPT00000000.1	-	-	-	EU477155
A. beijerinckii CIP 110307^{T}	APQL00000000.1	-	-	-	EU477124
A. bereziniae CIP 70.12^{T}	APQG00000000.1	-	-	-	EU477116
A. bohemicus ANC 3994^{T}	APOH00000000.1	-	-	-	KJ124834
A. boissieri ANC 4422^{T}	FMYL00000000.1	-	-	-	JQ771155
A. bouvetii CIP 107468^{T}	APQD00000000.1	-	-	-	EU477150
A. brisouii CIP 110357^{T}	AYEU00000000.1	-	-	-	KJ124836
A. calcoaceticus CIP 81.8^{T}	APQI00000000.1	-	-	-	EU477149
A. celticus ANC 4603^{T}	MBDL00000000.1	-	-	-	KX548350
A. chinensis WCHAc010005^{T}	CP032134.1	-	-	-	-
A. colistiniresistens NIPH 2036^{T}	ATGK00000000.1	-	-	-	-
A. courvalinii ANC 3623^{T}	APSA00000000.1	-	-	-	KT997518
A. cumulans WCHAc060092^{T}	CP035934.2	-	-	-	-
A. defluvii WCHA30^{T}	CP029397.2	-	-	-	KY435935
A. dispersus ANC 4105^{T}	APRL00000000.1	-	-	-	KT997527
A. equi 114^{T}	CP012808.1	-	-	-	KC494699
A. gandensis ANC 4275^{T}	LZDS00000000.1	-	-	-	KJ569689
A. gerneri CIP 107464^{T}	APPN00000000.1	-	-	-	EU477151
A. guillouiae CIP 63.46^{T}	APOS00000000.1	-	-	-	EU477117
A. gyllenbergii CIP 110306^{T}	ATGG00000000.1	-	-	-	EU477148
A. haemolyticus CIP 64.3^{T}	APQQ00000000.1	-	-	-	EU477109
A. halotolerans JCM 31009^{T}	SGIM00000000.1	-	-	-	-
A. harbinensis HITLi 7^{T}	JXBK00000000.1	-	-	-	KF803234
A. indicus CIP 110367^{T}	AYET00000000.1	-	-	-	KJ124838
A. johnsonii CIP 64.6^{T}	APON00000000.1	-	-	-	EU477113
A. junii CIP 64.5^{T}	APPX00000000.1	-	-	-	EU477110
A. kookii ANC 4667^{T}	FMYO00000000.1	-	-	-	KM821031
A. lactucae NRRL B-41902^{T}	LRPE00000000.1	-	-	-	-
A. larvae BRTC-1^{T}	CP016895.1	-	-	-	KU837228
A. lwoffii CIP 64.10^{T}	AYHO00000000.1	-	-	-	-
A. modestus NIPH 236^{T}	APOJ00000000.1	KT997474	-	-	KJ124832

A. nectaris CIP 110549^{T}	AYER00000000.1	-	-	-	KJ124840
A. nosocomialis NIPH 2119^{T}	APOP00000000.1	-	-	-	HQ123389
A. parvus CIP 108168^{T}	APOM00000000.1	-	-	-	EU477107
A. piscicola LW15^{T}	NIFO00000000.1	NR_159919	-	-	-
A. pittii CIP 70.29^{T}	APQP00000000.1	-	-	-	EU477114
A. populi PBJ7^{T}	NEXX00000000.1	-	-	-	KM518646
A. pragensis ANC 4149^{T}	LUAW00000000.1	-	-	-	KX014578
A. proteolyticus NIPH 809^{T}	APOI00000000.1	-	-	-	KJ124841
A. pseudolwoffii ANC 5044^{T}	PHRG00000000.1	-	-	-	-
A. puyangensis ANC 4466^{T}	OANT00000000.1	-	-	-	JX499272
A. qingfengensis ANC 4671^{T}	MKKK00000000.1	-	KC686827	-	KC631629
A. radioresistens CIP 103788^{T}	APQF00000000.1	-	-	-	EU477112
A. rudis CIP 110305^{T}	ATGI00000000.1	-	-	-	KJ124837
A. schindleri CIP 107287^{T}	APPQ00000000.1	-	-	-	EU477128
A. seifertii NIPH 973^{T}	APOO00000000.1	-	-	-	EU477126
A. sichuanensis WCHAc060041^{T}	PYIX00000000.2	-	-	-	-
A. soli CIP 110264^{T}	APPU00000000.1	-	-	-	HQ148175
A. tandoii CIP 107469^{T}	AQFM00000000.1	-	-	-	EU477152
A. tjernbergiae DSM 14971^{T}	ARFU00000000.1	-	-	-	EU477153
A. towneri CIP 107472^{T}	APPY00000000.1	-	-	-	EU477154
A. ursingii CIP 107286^{T}	APQA00000000.1	-	-	-	EU477105
A. variabilis NIPH 2171^{T}	APRS00000000.1	-	-	-	EU477119
A. venetianus CIP 110063^{T}	APPO00000000.1	-	-	-	EU477136
A. vivianii NIPH 2168^{T}	APRW00000000.1	-	-	-	KJ124844
A. wuhouensis WCHA60^{T}	CP031716.1	-	-	-	KY853663
Genomic sp. 6 CIP A165	APOK00000000.1	-	-	-	EU477115
Genomic sp. 15BJ CIP 110321	AQFL00000000.1	-	-	-	EU477133
Genomic sp. 16 CIP 56.2	APPH00000000.1	-	-	-	EU477135
Genomic sp. 'Between 1 & 3' NIPH 542	APSC00000000.1	-	-	-	EU477122
Acinetobacter portensis sp. nov. AC 877^{T}	LWRV00000000.1	KX870877	KX885192	KX885200	KX885208
Acinetobacter portensis sp. nov. AC 1335	*	*	KX885197	KX885205	KX885213
Acinetobacter portensis sp. nov. AC 1301	*	*	KX885196	KX885204	KX885212
Acinetobacter portensis sp. nov. AC 1123	*	*	KX885193	KX885201	KX885209
Acinetobacter guerrae sp. nov. AC 1271^{T}	LXGN00000000.1	KX870878	KX885194	KX885202	KX885210
Acinetobacter guerrae sp. nov. AC 1272	*	*	KX885195	KX885203	KX885211
Acinetobacter guerrae sp. nov. TUM15565	BKYM00000000	-	-	-	-
Acinetobacter guerrae sp. nov. WCHAc060096	RAXU00000000	-	-	-	-

SuplementaryTable 5.2. ANIb and dDDH values between the genome sequence of *Acinetobacter portensis* AC 877^T, *Acinetobacter guerrae* AC 1271^T and other *Acinetobacter* spp.

Strain	**ANIb**		**dDDH**	
	A. ***portensis*** **AC 877^T**	**A.** ***guerrae*** **AC 1271^T**	**A.** ***portensis*** **AC 877^T**	**A.** ***guerrae*** **AC 1271^T**
A. albensis ANC 4874^T	76.74	74.17	21	19.5
A. apis ANC 5114^T	72.00	71.60	19.2	20.6
A. baumannii ATCC 19606^T	74.35	74.94	20.3	20.2
A. baylyi CIP 107474^T	73.49	76.67	20	20.8
A. beijerinckii CIP 110307^T	74.68	74.81	20.7	20.7
A. bereziniae CIP 70.12^T	75.55	73.93	22.4	20.7
A. bohemicus ANC 3994^T	77.44	74.26	21.9	19.6
A. boissieri ANC 4422^T	71.85	71.52	19.7	20.7
A. bouvetii CIP 107468^T	75.16	72.54	20.9	19.5
A. brisouii CIP 110357^T	73.51	73.67	20.7	21.8
A. calcoaceticus CIP 81.8^T	74.51	74.87	20.8	20.2
A. celticus ANC 4603^T	77.16	73.68	21.7	19.4
A. chinensis WCHAc010005^T	75.38	73.10	21.2	20
A. colistiniresistens NIPH 2036 ^{T}	73.89	74.49	21.7	20.3
A. courvalinii ANC 3623^T	73.57	74.56	21	20.7
A. cumulans WCHAc060092^T	77.38	73.38	23	19.9
A. defluvii WCHA30 ^{T}	76.27	74.25	21.9	20.1
A. dispersus ANC 4105 ^{T}	74.31	74.64	20.8	20.8
A. equi 114^T	78.23	73.86	22.2	20.9
A. gandensis ANC 4275^T	77.26	73.49	22.5	19.4
A. gerneri CIP 107464^T	75.75	73.96	22.7	21.2
A. guillouiae CIP 63.46^T	75.65	73.92	22.2	20.7
A. gyllenbergii CIP 110306^T	73.98	74.51	20.6	20.3
A. haemolyticus CIP 64.3^T	74.88	74.72	22	20.4
A. halotolerans JCM 31009^T	74.16	74.53	20.6	19.9
A. harbinensis HITLi 7^T	75.85	73.79	20.8	19.5
A. indicus CIP 110367^T	75.32	73.50	21.1	20.2
A. johnsonii CIP 64.6^T	76.93	73.67	22.5	20.4
A. junii CIP 64.5^T	74.90	74.90	21.9	20.8
A. kookii ANC 4667^T	76.28	73.66	20.8	19.6
A. lactucae NRRL B-41902^T	74.34	74.69	20.4	20.3
A. larvae BRTC-1^T	72.74	72.34	21.2	21.2
A. lwoffii CIP 64.10^T	75.99	73.34	21.8	20

A. modestus NIPH 236^{T}	74.83	74.88	20.7	20.8
A. nectaris CIP 110549^{T}	72.70	72.07	20	20.7
A. nosocomialis NIPH 2119^{T}	74.61	74.93	20.4	20.5
A. parvus CIP 108168^{T}	74.51	74.97	22.1	20.7
A. piscicola LW15 ^{T}	76.23	74.23	21.5	20.3
A. pittii CIP 70.29^{T}	74.48	74.98	20.6	20.6
A. populi PBJ7^{T}	71.42	72.23	21.5	21.6
A. pragensis ANC 4149^{T}	75.18	72.42	20.6	19.1
A. proteolyticus NIPH 809^{T}	74.04	74.66	20.8	20.6
A. pseudolwoffii ANC 5044 ^{T}	75.66	73.47	21.2	19.7
A. puyangensis ANC 4466 ^{T}	71.35	72.07	20.6	21.9
A. qingfengensis ANC 4671^{T}	71.26	72.04	20.3	21.6
A. radioresistens CIP 103788^{T}	73.52	73.29	20.8	19.3
A. rudis CIP 110305^{T}	73.22	72.61	20.8	20.8
A. schindleri CIP 107287^{T}	75.60	73.23	21.2	20.3
A. seifertii NIPH 973^{T}	75.04	74.95	21.1	19.6
A. sichuanensis WCHAc060041^{T}	76.25	74.01	21.7	20.5
A. soli CIP 110264^{T}	72.73	75.22	19.7	19.9
A. tandoii CIP 107469^{T}	76.09	73.85	21.5	20.1
A. tjernbergiae DSM 14971^{T}	74.71	74.99	20.8	20.6
A. towneri CIP 107472^{T}	77.23	74.61	22.1	20.6
A. ursingii CIP 107286^{T}	74.36	86.70	20.7	32.6
A. variabilis NIPH 2171^{T}	76.13	73.29	21.5	19.7
A. venetianus CIP 110063^{T}	74.71	74.79	21.1	20.7
A. vivianii NIPH 2168^{T}	74.07	74.72	21.3	20.7
A. wuhouensis WCHA60 ^{T}	76.72	74.16	22.7	20.9
Genomic sp. 6 CIP A165	74.96	74.72	21.9	20.2
Genomic sp. 15BJ CIP 110321	74.71	74.61	22.2	20.8
Genomic sp. 16 CIP 56.2	74.25	74.59	20.9	20.8
Genomic sp. 'Between 1 & 3' NIPH 542	74.53	74.85	20.8	20.4
Acinetobacter portensis sp. nov. AC 877^{T}	*	73.95	*	19.3
Acinetobacter guerrae sp. nov. AC 1271^{T}	73.95	*	19.3	*

Suplementary Table 5.3. Cellular fatty acid profile of strains of *Acinetobacter portensis* sp. nov. (AC 877^T, AC 1301), *Acinetobacter guerrae* sp. nov. (AC 1271^T, AC 1272), the type strains of respective closely related members of the genus (based on core genome phylogenomic treeing) and the type strain of the type species of the genus.

Fatty Acid	**ECL**	***Acinetobacter portensis***		***Acinetobacter guerrae***		**Reference strains**		
		1	**2**	**3**	**4**	**5**	**6**	**7**
10:0	10.000	4.0	2.9	-	-	tr	-	-
12:0	12.000	8.2	6.1	6.9	6.9	**25.7**	7.1	**19.2**
12:0 2-OH	13.178	3.7	3.5	2.4	2.7	9.0	1.4	12.3
12:0 3-OH	13.455	**10.0**	**8.5**	4.6	4.6	**19.6**	2.9	**20.8**
14:0	14.000	3.5	3.1	-	-	3.0	0.8	-
16:1ω9*c*	15.774	-	-	-	-	-	1	-
16:1ω7*c*	15.819	**49.1**	**47.3**	**20.3**	**21.8**	**24.3**	**17.2**	**19.4**
16:0	16.000	**15.9**	**17.7**	**17.4**	**17.9**	7.5	**19.7**	4.2
17:1ω8*c*	16.792	tr	1.2	-	-	-	-	3.8
17:0	17.000	-	-	-	-	-	-	1.6
18:1ω9*c*	17.769	5.3	7.1	**39.2**	**39.4**	2.6	**42.7**	4.8
18:0	18.000	-	-	1.4	-	-	0.8	-
Summed feature*								
1	14.469	-	-	-	-	-	-	5.2
2	15.485	-	-	4.7	4.5	4.7	2.9	6.1
5	17.724	-	-	3.2	2.2	1.4	2.6	tr
8	17.824	tr	2.5	-	-	-	-	-

*Summed feature 1 contains iso-15:1 H/13:0 3-OH; summed feature 2 contains 14:0 3-OH/16:1 ISO I; summed feature 5 contains 18:2 ω 6,9c/18:0 ANTE; Summed feature 8 contains 18:1 ω 7c/12t/9t. ECL: equivalent chain length. Strains: 1, AC 877^T; 2, AC 1301; 3, AC 1271^T; 4, AC 1272; 5, *A. equi* CCUG 65204^T; 6, *A. ursingii* CCUG 45559 ^{T}; 7, *A. calcoaceticus* CCUG 12804^T. Values are percentage of total fatty acids; tr, trace (<0.7%); -, not detected. The three most predominant components of each strain are highlighted in bold.

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