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IMPLICATION OF *ACINETOBACTER* SPP. IN FOODBORNE ILLNESSES

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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À minha querida tia Célia,
Por todo o amor e dedicação.
E por me ter ensinado que
A sabedoria nunca é demais.

Resumo

O género *Acinetobacter* inclui uma grande variedade de espécies Gram-negativo que têm sido objeto de vasto e longo estudo. Têm sido isolados de solos, esgotos, água, frutos, vegetais, animais e humanos (especialmente áreas mucosas); no entanto, nem todas as espécies são consideradas patogénicas. Não obstante, *Acinetobacter* spp. são conhecidos como patogénicos nosocomiais responsáveis por um número crescente de infeções em indivíduos cuja imunidade está comprometida ou pacientes internados em unidades de cuidados intensivos (UCI). Recentemente, levantou-se a hipótese de que estes microrganismos poderiam também ser responsáveis por infeções transmitidas pela ingestão de alimentos contaminados, no caso de ingestão de matrizes alimentares contaminadas. Para testar esta hipótese, um total de 264 isolados clínicos de *Acinetobacter* spp. foram genotipados por Eletroforese em Gel de Campo Pulsado (PFGE), tendo obtido um total de 115 perfis distintos. Foi selecionado um isolado de cada um dos perfis (n=115) para os estudos posteriores. Foi determinada a suscetibilidade destes isolados a antibióticos, tendo sido observado que 68 isolados apresentavam resistências múltiplas (MDR). No ensaio para avaliação da resistência ao stress ácido (pH 2,5) observou-se que a maioria dos isolados foram resistentes. Com base nestes resultados, foram selecionados 14 isolados para posterior ensaio em condições gastrointestinais simuladas. Onze isolados demonstraram ser sensíveis a estas condições (redução de 4 ciclos logarítmicos), mas para três isolados, foi observada uma elevada taxa de recuperação ao final de 4 horas de ensaio. Dois desses isolados mais resistentes foram submetidos a um ensaio de cultura celular com células CaCo-2: um isolado exibiu uma taxa de invasão 50% superior à do controlo (*Listeria monocytogenes* invasiva), embora a taxa de adesão não tenha sido muito elevada (15%), enquanto o outro isolado exibiu apenas 0.2% de eficiência de invasão. De acordo com este estudo é possível concluir que *Acinetobacter* spp. pode resistir a condições gastrointestinais e, apesar de mais testes serem necessários para o comprovar, poderá ser um potencial patogénico de origem alimentar.

Palavras-chave: *Acinetobacter* spp.; doença de origem alimentar; resistência a antibióticos; condições gastrointestinais simuladas; colonização do trato gastrointestinal.

Abstract

The genus *Acinetobacter* includes a large variety of Gram-negative species that have been widely studied for a long time. They had been isolated from soil, sewage, water, fruits, vegetables, animals and humans (especially mucous areas); however, not all species are considered as pathogenic. Nevertheless, *Acinetobacter* spp. are known as nosocomial pathogens responsible for causing a high number of infections in immunocompromised individuals or patients in intensive care units. Recently, there has been a hypothesizis whether these microorganisms could also be responsible for foodborne diseases if contaminated food matrices are ingested.

In order to test this hypothesizis, a total of 264 isolates of *Acinetobacter* spp. collected from patients were genotyped by Pulsed-Field Gel Electrophoresis (PFGE), and 115 different profiles were obtained**.** One isolate from each cluster was selected (n=115) for subsequent assays. Antibiotic susceptibility characterization was further performed, and 68 isolates out of 115 were classified as multi-drug resistant (MDR). An acidic stress resistance assay (pH 2.5), simulating the stomach environment, was performed and demonstrated that most of the isolates were resistant. So, 14 isolates were selected for further exposure to simulated gastrointestinal conditions.

Eleven isolates were more sensitive (4 log cycles reduction) to the simulated digestion process. However, for three isolates, a high recovery rate was observed at the end of 4 hours' assay. Two of the most resistant isolates were further studied in a cell culture assay with CaCo-2 cells: one isolate exhibited a 50% higher invasion rate than the control, even though the adherence rate was not that high (15%), whereas the other isolate exhibited a 0.2% invasion efficiency. According to this study, it can be concluded that *Acinetobacter* spp. have the ability to withstand gastrointestinal conditions. While further tests are required to confirm this, they could potentially be considered as foodborne pathogens.

Keywords: *Acinetobacter* spp.; foodborne disease; antibiotic resistance; simulated gastrointestinal conditions; gastrointestinal tract colonization.

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1. Introduction

1.1. The *Acinetobacter* genus

Acinetobacter, initially described by a Dutch microbiologist in 1911 as *Micrococcus calcoaceticus*, was first isolated from the soil using a minimal media enriched with calcium acetate (Beijerinck, 1911). Over the years, *Acinetobacter* spp., underwent several names and classifications, sometimes even being associated with another genus, such as *Achromobacter*, which, in 1923 included non-pigmented, Gram-negative, aerobic saprophytes and both motile and non-motile species (Ingram and Shewan, 1960; Juni, 1978). In 1954, Brisou & Prevot proposed that non-motile achromobacters should be considered species of the newly defined genus *Acinetobacter*, since "–akineto" comes from a Greek word that means "without movement" (Brisou and Prevot, 1954), and since then, it is recognised as such classification (Schreckenberger, 2003). With the introduction of the oxidase test in diagnostic bacteriology, it was noted that organisms previously classified as strains of *Acinetobacter* included both oxidase-positive and oxidase-negative species (Henriksen, 1952; Juni, 1978).

Later on, in 1968, Baumann and his research group concluded that *Micrococcus calcoaceticus*, *Alcaligenes hemolysans*, *Mima polymorpha*, *Moraxella lwoffi*, *Herellea vaginicola* and *Bacterium anitratum* belonged to a single genus and could not be separated into different species based on their phenotypical characteristics (Baumann et al., 1968; Howard et al., 2012). Finally, in 1971, the Subcommittee on the Taxonomy of Moraxella and Allied Bacterial agreed that the genus *Acinetobacter* should only include oxidase-negative strains based on Baumann's results (Lessel, 1971).

Therefore, *Acinetobacter* genus currently belongs to *Proteobacteria* phylum, γ-*Proteobacteria* class, *Pseudomonadales* order and *Moraxellaceae* family (Jung and Park, 2015). It comprises Gram-negative coccobacilli with approximately 1.5 μm length and a varying shape depending on the growth phase (Visca et al., 2011). It also includes characteristics such as strictly aerobic, glucose non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G + C content of 39% to 47% and variable metabolism (Howard et al., 2012; Juni, 1978).

Most species exhibit an easy growth on simple media, forming a domed colony with 2 mm diameter and a pale-yellow colour associated. They are typically mesophilic

bacteria, which means they grow at moderate temperatures between 20 ºC and 45 ºC and their optimal range is between 30 ºC and 49 ºC (Schiraldi and De Rosa, 2016). Clinical isolates exhibit a better growth rate at 37 ºC, while the environmental isolates prefer lower temperatures – water isolates appear to be psychrotrophic (Breuil et al., 1975; Visca et al., 2011).

The taxonomy of this group remained ambiguous for a few years due to the lack of biochemical markers. However, based on the relatedness between bacteria in DNA hybridisation, in 1986, Bouvet and Grimont distinguished 12 genospecies, which included 74 out of the 85 studied. Formal species names were attributed to some of them *Acinetobacter calcoaceticus* (Genospecies 1, which included eight glucose oxidising strains that could not grow at 44 °C and were isolated from soil); *Acinetobacter baumannii* (Genospecies 2, which contained most glucose oxidisers strains that were able to grow at 44 ºC); *Acinetobacter haemolyticus* (Genospecies 4, which included 23 hemolytic and proteolytic strains that were able to utilise DL-4-aminobutyrate but not DL-lactate); *Acinetobacter junii* (Genospecies 5*,* which included strains that were unable to oxidise glucose and able to utilise DL-lactate and L-histidine but not glutarate or azelate), *Acinetobacter johnsonii* (Genospecies 7, which included 23 strains that were unable to grow at 37 °C and to oxidise glucose and utilised only a few carbon sources)*; and Acinetobacter lwoffii* (Genospecies 8, containing mostly glucose-negative strains that utilise azelate but not Simmons citrate, glutarate, L-histidine, L-aspartate, L-leucine, p-alanine, or 2,3-butanediol). Those without a formal name were often indistinguishable from the other groups: Genospecies 3 included strains that were able to oxidise glucose and grow at 41 ºC but not at 44 ºC; Genospecies 6 included three hemolytic and proteolytic strains that were unable to utilise DL-lactate, malonate, or DL-4-aminobutyrate; Genospecies 9 could not be distinguished from Genospecies 8; and Genospecies 10 (four strains), 11 (four strains), and 12 (three strains) were distinguished by their nutritional patterns.

About ten years ago, there were 26 species of *Acinetobacter* recognised by DNA-DNA hybridisation technique and nine genomic groups (Howard et al., 2012). However, every year, new species of *Acinetobacter* are reported and validated (Qin et al., 2021) and recently, some of the genomic groups had been reorganized: genospecies 10 is now called *Acinetobacter bereziniae*, genospecies 11 is now *Acinetobacter guillouiae*, *Acinetobacter* genomic species 13TU is now *Acinetobacter nosocomialis* and *Acinetobacter* genospecies 3 is now *Acinetobacter pittii* (Adewoyin and Okoh, 2018). Nowadays and

until the end of this study, there are more than 80 described and validated species (https://lpsn.dsmz.de/genus/acinetobacter, last accessed at 23-09-2023).

However, identification of *Acinetobacter* to the species level is complicated and phenotypic characteristics alone have proven to be insufficient or inadequate to differentiate genomic strains (Vijayakumar et al., 2019; Visca et al., 2011). Although it is a difficult step to overcome, accurate identification is essential, especially for pathogens like *A. baumannii*, *A. pittii* and *A. nosocomialis* that are very similar but differ in their biological and pathological characteristics (Vijayakumar et al., 2019). So, previous studies only based on chemotaxonomy should be interpreted with caution (Visca et al., 2011).

1.2. *A. calcoaceticus–A. baumanii* complex

Among the total groups, *A. calcoaceticus*, *A. baumanii*, *Acinetobacter* genospecies 3 ("*A. pittii*") and *Acinetobacter* genomic species 13TU (now called "*A. nosocomialis*") are relatively more important and relevant according to their pathogenicity, virulence and distribution, as well as phenotypically similar and closer related according to DNA-DNA hybridisation studies, which leads to a so-called "*A. calcoaceticus–A. baumanii* complex" (ACB complex) (Gerner-Smidt et al., 1991; Howard et al., 2012). However, this combination is not interesting from a clinical viewpoint because it combines three of the most important pathogens in community-acquired and nosocomial infections with an essential soil species – *A. calcoaceticus* (Howard et al., 2012). Many studies and researches have been conducted to differentiate these groups from each other to better understand their epidemiology, ecology and pathogenicity (Visca et al., 2011).

Recently, two other species were added to ACB complex due to their similarity with the other three species belonging to the group: *Acinetobacter seifertii* (previously known as genomic species close to 13TU) and *Acinetobacter dijkshoorniae* (closely related to *A. pittii*). Both are associated with human diseases (Vijayakumar et al., 2019).

1.3. *Acinetobacter* sources

Species included in this genus are widely distributed and often considered ubiquitous, allowing their isolation from soils (Adewoyin and Okoh, 2018; Baumann et al., 1968), water samples (Vaz-Moreira et al., 2017) and sewage (Qin et al., 2020). They

are also frequently found in animals and humans (Henriksen, 1976; Juni, 1978; La Scola and Raoult, 2004; Peleg et al., 2008), where acinetobacters are generally considered part of normal skin microbiota, mucous membranes and respiratory secretions (Munoz-Price and Weinstein, 2008). However, it is also recovered from immunocompromised individuals or the ones who are in intensive care units, being the major infection cause in this area and, therefore, despite not all the species are considered pathogens, some of them are important disease- and nosocomial agents, such as *A. baumannii*, *A. haemolyticus* and *A. calcoaceticus* (Almasaudi, 2018; Howard et al., 2012; Juni, 1978; Munoz-Price and Weinstein, 2008). In turn, *A. lwoffii* resembles *Helicobacter pylori*, which causes persistent stomach inflammation due to its capacity to colonise the mouse stomach but also induces hypergastrinemia, gastritis and increases gastric epithelial cell numbers (Rathinavelu et al., 2003).

In epidemiological studies, acinetobacters were also found as part of human skin microbiota (as mentioned above), more properly on mucous areas, even in non-hospitalised individuals (Munoz-Price and Weinstein, 2008; Peleg et al., 2008). In fact, it was even isolated from the forehead, nose, hand or toe webs of healthy people (Seifert et al., 1997). The most frequent species were *A. lwoffii*, *A. johnsonii*, *A. junii* and *Acinetobacter* genomic species 3. Moreover, if the carrier rate in healthy volunteers was 44%, in ill people, *Acinetobacter* species transmission was even higher – 75% (Peleg et al., 2008; Seifert et al., 1997). In contrast, *A. baumannii*, the most important nosocomial pathogen within acinetobacters (Carvalheira et al., 2017b; de Amorim and dos Santos Nascimento, 2017; Gordon and Wareham, 2010; Peleg et al., 2008), is rarely found on human skin; however, it was recovered from 22% of body lice sampled from homeless people, which can represent another reservoir (La Scola and Raoult, 2004).

Another risk group seems to be military personnel and armed forces members who are quietly exposed to infections in conflict areas (Griffith et al., 2007; Munoz-Price and Weinstein, 2008; Peleg et al., 2008). However, Griffith and his group of researchers did not detect skin carriage of the *A. calcoaceticus–A. baumannii* complex from 303 samples of 102 U.S active-duty soldiers deployed in Iraq – probably due to the use of a non-enrichment recovery culture or an extremely long transport time – and 38 of the total sample even suggesting a previous injury colonisation (Griffith et al., 2007). Other studies have shown that the dry and sandy conditions associated with conflict areas are ideal for *A. baumannii*, making it the main source of infection among injured soldiers (Control and Prevention, 2004). Besides that, season variation also interferes with acinetobacters

infections since warmer and more humid ambient air and/or some potentially preventable environmental contaminants, such as condensate from air-conditioning units, are acceptable and possible explanations for outbreaks during summer (Munoz-Price and Weinstein, 2008; Smith, 1979).

Moreover, related to prevalence in some areas, community-acquired infections with acinetobacters are uncommon but have been reported in tropical or subtropical climates, such as Australia and Asia (China and Taiwan). The reason is still undiscovered, but it may be due to favourable temperature and humidity conditions to bacterial growth. In addition, patient-associated conditions, for instance, chronic obstructive pulmonary disease, renal disease, diabetes mellitus, heavy smoking and excessive alcohol consumption could play a role (Munoz-Price and Weinstein, 2008; Petrosillo et al., 2014).

Therefore, *Acinetobacter* spp. have emerged as important nosocomial agents over the past three decades, with questionable pathogenicity. This is attributed not only to their intrinsic resistance but also to their capacity to adapt to different environments (Falagas and Kopterides, 2006; Munoz-Price and Weinstein, 2008).

1.4. Food as acinetobacters reservoir

On the other hand, *Acinetobacter* spp. have also been recovered from a variety of foods such as vegetables, dairy products, pre-cooked foods, drinks, water and from food-producing animals (Berlau et al., 1999a; Carvalheira et al., 2016; Gennari and Lombardi, 1993; Hamilton-Miller and Shah, 2001; Narciso-da-Rocha et al., 2013; Wang et al., 2012). However, there is no direct correlation between the type of food and the species found.

According to Rathinavelu et al. (2003), *A. lwoffii* and *A. johnsonii* are the predominant species found in foods and, besides that, the authors claim that *Acinetobacter* species are even able to grow on bacon, chicken, egg and fish stored under refrigerated conditions and following irradiation treatment (Rathinavelu et al., 2003). In a more recent review mentioning the different isolated species according to their source, *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii*, *Acinetobacter parvus*, *Acinetobacter radioresistens* or *Acinetobacter soli* were also among the species found in food (Adewoyin and Okoh, 2018).

Since *Acinetobacter* spp. tend to develop resistance to the major groups of antibiotics (Avila-Novoa et al., 2019) and have the ability to persist in the environment

for long periods (Almasaudi, 2018), this facilitates their survival and spread. Therefore, contaminated food with *Acinetobacter* spp. can be a transmission source into hospitals and clinical facilities or even in domestic environments. Also, it may contribute to the horizontal transferring of resistance genes to other pathogens (Carvalheira et al., 2017b).

1.4.1. Fruits and vegetables

Despite *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* being the most common bacteria related to food safety and quality (EFSA, 2013), bacteria like *Acinetobacter* spp. present especially on ready-to-eat products such as fruits and vegetables that do not require any processing, can also have an impact, even in low levels (Carvalheira et al., 2021).

These findings can represent a major problem since consumption of fresh products, raw or minimally processed, is increasingly common, representing a transmission route for human pathogens (Brassard et al., 2011; Carvalheira et al., 2017b). In fact, most people believe that consuming fresh produce is safe and that most food-borne illness outbreaks are caused by foods primarily of animal origin (Brassard et al., 2011).

In 2016, 100 samples each of fresh potatoes, carrots, and lettuce from different farms were analysed in a study carried out in the United States of America and the authors concluded that, although *A. baumannii* was only present in a few samples, all the recovered isolates were resistant to imipenem, ceftriaxone, erythromycin, and streptomycin, which are very commonly used antibiotics (Karumathil et al., 2016). Similar findings were achieved previously by Berlau et al. (1999) in a study conducted in the United Kingdom, where *Acinetobacter* spp. were recovered from 17% of 77 samples of fresh fruits and vegetables, and most of the isolates were identified as *A. baumannii* species, exhibiting higher resistance to ciprofloxacin and gentamicin than the other species found.

Conversely, in a study conducted by Carvalheira et al. (2017b) in Portugal, species of acinetobacters were highly prevalent, being isolated from 86.7% of lettuce samples and 70.0% of fruits (apples, pears, strawberries and bananas). About 29.8% of the isolates were classified as multidrug-resistant (MDR), and 13.3% were not susceptible to colistin, the last resort for the treatment of MDR *Acinetobacter* infections.

1.4.2. Meat

Food animals have been recognized as a potential reservoir for *Acinetobacter* spp. in different countries (Hamouda et al., 2011; Kempf and Rolain, 2012; Rafei et al., 2015; Zhang et al., 2013), although there are not many deep studies about this microorganism's prevalence in raw meat samples (Carvalheira et al., 2017a; Lupo et al., 2014; Rafei et al., 2015). Some studies only refer specifically to *A. baumannii* isolated from raw meat samples: Lupo et al. (2014) found that almost 25.0% of the analysed samples were contaminated with this microorganism, especially poultry meat (Lupo et al., 2014) and Askari et al. (2020) found the same pathogen in 20.1% of the 194 different types of raw meat samples analyzed, with ovine meat samples being the most contaminated (Askari et al., 2020). Other studies show high recovery rates of different *Acinetobacter* species, such as a 75% recovery rate on 36 pork and beef samples in Hong Kong (Houang et al., 2001), 28% recovery rate on 50 cow meat samples in Lebanon (Rafei et al., 2015) and 100% recovery rate on 50 chicken, turkey, pork and beef samples in Portugal (Carvalheira et al., 2017a). In fact, Carvalheira et al. (2017a) isolated 13 different species of *Acinetobacter* from those raw meat samples, including, for example, *A. baumannii*, *Acinetobacter bereziniae*, *A. calcoaceticus*, *Acinetobacter parvus*, *A. johnsonii* or *Acinetobacter radioresistens*, whereas Rafei et al. (2015) isolated three species from cat, goat and cow meat samples: *A. baumannii*, *A. pittii* and *A. bereziniae* (Rafei et al., 2015).

In a similar way to what happens in fruits and vegetables, the isolates from meat samples also exhibit resistance to a wide variety of antibiotics, including tetracyclines, gentamicin, trimethoprim, among others (Askari et al., 2020; Carvalheira et al., 2017a; Lupo et al., 2014). This can be justified by the extensive use of antimicrobials for treatment, prevention and control of diseases in food-producing animals. In fact, according to the European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (EMA/ESVAC), 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. In contrast, carbapenem antibiotics are not allowed to treat food-producing animals, which can explain the susceptibility to antibiotics of this class (European Medicines Agency, 2015).

Besides antibiotic resistance, one of the major concerns is that *Acinetobacter* spp. present in meat samples appear to survive more than 60 min under thermal processing at 60 ºC (Campos et al., 2019). Therefore, stricter hygiene procedures, hazard analysis and critical control point principles should be implemented along the entire meat production

chain to prevent *Acinetobacter* spp. contamination, as well as from other pathogens (Lupo et al., 2014).

1.4.3. Dairy products

The most abundant bacterial taxa in the core milk microbiome are *Streptococcus*, *Staphylococcus* and unidentified members of the order *Clostridiales*. However, *Acinetobacter* spp. were also found, so it is considered as part of that core microbiome (Kable et al., 2016). Also, *Acinetobacter* spp. were identified as part of bacterial biodiversity of a variety of cheeses (Addis et al., 2001; Riquelme et al., 2015; Xue et al., 2018).

While some authors claim that acinetobacters contribute to the milk flavour, odour or texture (Pangallo et al., 2014), others describe them as opportunistic pathogens (Gurung et al., 2013). In fact, MDR strains of *Acinetobacter* were found in infant milk formula served to debilitated or premature infants in a public hospital in Rio de Janeiro, Brazil (Araújo et al., 2015).

1.4.4. Drinking water

Water quality is directly related to microbial and chemical composition, source and the different processes applied (Van Assche et al., 2019). Several studies have shown that *Acinetobacter* is a frequent genus in both tap and drinking water (Van Assche et al., 2019; Vaz-Moreira et al., 2017), especially *A. johnsonii* and *A. lwoffii* (Narciso-da-Rocha et al., 2013).

In a Portuguese study, high levels of acinetobacters in tap water were detected; however, 80% of the isolates were wild-type for the antibiotics tested, which means low rates of acquired resistance mechanisms. Nevertheless, the authors claim that it is possible for some species to acquire antibiotic resistance throughout the water distribution systems (Narciso-da-Rocha et al., 2013). Likewise, in Belgium, *Acinetobacter* spp. were detected in drinking water production and distribution systems and they were one of the most abundant taxa found (Van Assche et al., 2019).

Several studies indicate that microbial growth and detection of acinetobacters in drinking water distribution systems can represent a major problem since most of the species are MDR and can originate nosocomial infections when associated with waterborne hospital-acquired infections (Heéger et al., 2010; Umezawa et al., 2015;

Williams et al., 2013). Besides that, *A. calcoaceticus* not only easily coaggregates with other bacteria but facilitates the association between species that normally would not aggregate without its presence. This represents a metabolic cooperation opportunity and, in a multispecies community, the presence of *A. calcoaceticus* enhances biofilm formation in drinking water (Simões et al., 2008).

1.5. Virulence Factors

The capacity to establish successfully, not only on surfaces, but also in food matrices and become a pathogen by this way is probably due to manifold capacities of acinetobacters such as being good competitors with microbiota, ability to form biofilms, adhering and colonise epithelial cells and to resist through desiccation periods (de Amorim and dos Santos Nascimento, 2017). In fact, many *A. baumannii* strains can persist on both moist and dry surfaces: some acinetobacters can persist longer than *E. coli* on dry surfaces and be able to remain viable for more than four months, as well as survive for more than 20 days on glass surfaces while set at room temperature (Almasaudi, 2018). Such capacity to survive on dry surfaces for a long time attributes additional advantage as an environmental contaminant, as well as biofilms and adhesion to human cells provide a higher disease incidence and survival rate (de Amorim and dos Santos Nascimento, 2017).

Despite all the research, the exact virulence factors of this emergent pathogen are still unknown; however, it is considered a low-virulence organism (Almasaudi, 2018; Peleg et al., 2008) and the full genome sequencing showed that this organism shelters a considerable number of putative virulence-associated genes (Smith et al., 2007). There are a few potential factors already identified, such as biofilm ability, capacity to adhere and invade host cells and resistance to the harshest conditions mentioned above, as well as cell surface hydrophobicity, outer membrane proteins (OMPs), toxic slime polysaccharides, verotoxins, iron uptake and host cells death (Almasaudi, 2018; Jin et al., 2011).

Upon virulence factors, one in particular is considered the most important to Gram-negative bacteria: Outer Membrane Proteins (OMPs), responsible for pathogenesis and adaptation to host cells. Particularly, OmpA in *A. baumannii* is the most abundant surface protein and promotes the apoptosis of eukaryotic cells (Almasaudi, 2018; Choi et al., 2008). It is also involved in biofilm formation, which enhances virulence, drug

resistance as well as survival rate even through unfavourable conditions (Gordon and Wareham, 2010; Howard et al., 2012).

In this research, only biofilm ability and capacity to adhere and invade host cells will be focused.

1.5.1. Biofilm formation capacity

A biofilm is a community of multiple bacterial cells associated with each other and with biotic or abiotic surfaces arranged in a 3D structure. On the outside, this matrix comprises carbohydrates, nucleic acids, proteins and other macromolecules (Costerton et al., 1999). It begins with surface association and adherence, followed by the multiplication of the constituent organisms, then the adherence of other species, and finally, the production of extracellular polymeric substances (Stoodley et al., 2002).

The most common factors that can influence biofilm formation are nutrient availability; bacterial appendages (pili and flagella); bacterial surface components as outer membrane proteins and adhesins; quorum sensing and macromolecular secretions as polysaccharides, nucleic acids, among others (Gaddy and Actis, 2009).

Environmental signals, such as metal cations, not only play a role in controlling the formation of biofilms but can be also a resource for pathogenic acinetobacters which are able to utilize resources from biotic and abiotic environments in order to successfully survive. Plenty of molecules are secreted, including siderophore acinetobactin, responsible for iron uptake. All the molecules involved depend on the strain (Gordon and Wareham, 2010; Howard et al., 2012).

In this case, biofilm production involves quorum sensing signal and expression regulation by some two-component regulatory system. It is a several steps pathway, but it begins with attachment to a surface by pilli and fimbriae intervention, followed by microcolony formation and full structures development. Meanwhile, exopolysaccharide (Biofilm Associated Protein – BAP), an important constituent of mature biofilm that suppresses the activity of neutrophils, is produced, stabilizes the structure and contributes to serum resistance. However, other proteins, such as phospholipase D and C, have been shown to contribute to virulence as well. While phospholipase D is important for resistance to human serum, epithelial cell evasion and pathogenesis, phospholipase C enhances toxicity to epithelial cells (Gaddy and Actis, 2009; Gordon and Wareham, 2010; Howard et al., 2012).

1.5.2. Host cell adherence and infection

Adherence of bacteria to epithelial cells is crucial first to colonise host cells and then infect them (Gyimah and Panigrahy, 1988; Lee et al., 2006). It is defined as the formation of a mechanically stable bond between host cells and bacteria, and it is a two-step process initiated by non-specific and transitory adsorption to the host cell. Later, the specific adhesins bind to complementary receptors on the cell membrane in a highly specific interaction (Gyimah and Panigrahy, 1988). Adhesins represent the bacterial surface components that connect to the receptors, the complementary structure of host cells (Beachey, 1981).

As mentioned above, *Acinetobacter* spp. can adhere to different surfaces and remain viable for long periods (Almasaudi, 2018). In a nosocomial environment, these organisms may be able to colonise medical devices through biofilm and then easily infect patients by the ability to colonise their skin and mucosal surfaces, which is a problem (Berlau et al., 1999b; Dijkshoorn et al., 1987; Lee et al., 2006).

After establishing the adherence bonds with host cells, pathogens such as *A. baumannii* must invade it. There are two ways to achieve that: zipper-like mechanism and trigger mechanism. The first requires direct interaction between bacterial ligands and the host cell surface receptors and involves local cytoskeletal rearrangement at the invasion spot. The second one is initiated by the injected bacterial effector proteins delivered by the type III secretion system that regulate cytoskeleton dynamics and induce rearrangements such as membrane ruffles (Choi et al., 2008; Lee et al., 2006).

Choi et al. (2008) developed a study demonstrating that *A. baumannii* invades cells by the zipper-like mechanism and, once inside host cells, acinetobacters survive within vacuoles that are not submitted to fusion with lysosomes or endosomes. However, future studies are needed to understand these mechanisms more accurately. This pathogen tends to invade more epithelial cells from the respiratory tract than non-respiratory ones, which can be supported by a higher binding capacity of OmpA to respiratory epithelial cells and leads to a possible explanation for the prevalence of this bacteria in the respiratory tract as well as the associated infections (Choi et al., 2008).

1.6. Antibiotic Resistance

Acinetobacter spp. have been highly studied due to their increasing antibiotic resistance, and they can be classified as multidrug-resistant (MDR), extensive drug-

resistant (XDR), and pan-drug resistant (PDR), depending on the respective degree of resistance (Almasaudi, 2018). MDR *Acinetobacter* spp. refers to being resistant to at least one agent in at least three of these antimicrobial categories: Aminoglycosides; Antipseudomonal carbapenems; Antipseudomonal fluoroquinolones; Antipseudomonal penicillins + β-lactamase inhibitors; Extended-spectrum cephalosporins; Folate pathway inhibitors; Penicillins + β-lactamase inhibitors; Polymyxins; Tetracyclines. XDR refers to the isolate that is non-susceptible to at least one agent in all two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories). PDR is defined as resistant to all agents in all antimicrobial categories (i.e., no agents tested as susceptible for that organism) (Magiorakos et al., 2012).

Besides that, this pathogen belongs to a unique class of Gram-negative bacteria labelled as "naturally transformable", meaning it tends to incorporate foreign genetic material, including antibiotic-resistance genes (Almasaudi, 2018). In fact, these genes can occur naturally in the microorganism, considered as intrinsic, or they can be acquired, which is by far the most common way. The intrinsic antibiotic genes are caused by a spontaneous gene mutation that occurs naturally, independent of the selective pressure caused by antibiotics. Subsequently, it can be transferred vertically between bacteria. The most widely known example of intrinsic antibiotic resistance is the multi-drug resistant phenotype that Gram-negative bacteria (which include *Acinetobacter* spp.) exhibit by not being susceptible to different classes of antibiotics, such as carbapenems, third generation of cephalosporins or tetracyclines, that are effective against the Gram-positive ones. On the other hand, antibiotic resistance genes can be acquired in two different ways: gene mutation caused by selective pressure from antibiotics and environment, which leads to an adaption response from a previously susceptible bacterium, or it can occur by accepting antimicrobial gene from other bacteria – horizontal transfer genes (HTG) (Alanis, 2005; Cox and Wright, 2013).

1.7. Objectives

The main objective of this work was to understand whether *Acinetobacter* spp. are capable of causing foodborne illnesses when contaminated food matrices are ingested. Since a large number of isolates were studied, Pulsed-Field Gel Electrophoresis was used to select isolates representative of different clusters. Selected isolates were characterised regarding antibiotic resistance and ability to survive under acidic conditions. Finally, a

small number of carefully selected isolates were tested regarding their ability to survive under the adverse conditions of the digestive tract and adhere and invade epithelial gut cells.

2. Materials and Methods

2.1. Samples source and growth conditions

Two hundred and sixty-four clinical *Acinetobacter* spp. isolates were kindly provided by Hospital de São João (Porto) and Hospital São Marcos (Braga) and were the basis for this study. All of them were isolated in Trypticase Soy Agar medium (TSA; Biokar Diagnostics), a non-selective medium, enriched with Yeast Extract (YE; Biokar Diagnostics) and incubated overnight at 30 ºC.

Isolates were stored at -80 ºC in Trypticase Soy Broth Medium (TSB; Biokar Diagnostics) with 30% (v/v) of glycerol, which is a cryoprotectant and prevents cell damage during freezing (Lorsch, 2013) and sub-cultured twice before using in TSB at the previously mentioned conditions.

2.2. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to (Carvalheira et al., 2017b; Chang et al., 2013) with some modifications described below to genotype all clinical isolates.

2.2.1. Plugs preparation

Isolates were plated on TSA-YE medium and incubated under the previously described conditions. After incubation, the colonies' swabs were resuspended in 3 mL of Cell Suspension Buffer (CSB; 100 mM Tris HCl, 100 mM EDTA, pH 8.0), and the optical densities (O.D) of all solutions were measured at a 600 nm wavelength and adjusted concentrations to 1.3 absorbance. It was also prepared 1% SeaKem® Gold Agarose (Lonza, USA) solution and maintained at 56 ºC and 150 rpm, in a shaker water bath to avoid solidification, as well as CSB containing tubes. Then, it was added a 20% SDS (Bio-Rad Laboratories, Hercules, California) solution to the agarose solution and mixed.

Each bacteria suspension was transferred into a new eppendorf tube as well as the agarose solution, added Proteinase K (Frilabo, Portugal; 20 mg/mL stock) and carefully homogenised. Finally, the total volume of each mixture was immediately dispensed into plug moulds and set at room temperature for 15 min to solidify. After solidification, plugs with isolate were placed into the respective tube containing CSB and Proteinase K and incubated at 56 ºC, 150 rpm, for 2 h to lysis cells and exposed bacteria DNA.

Each plug was washed twice with sterile ultra-pure water and four times with TE buffer (Bio-Rad Laboratories; 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). At the end of all washing steps, each plug was stored in TE buffer at 4 ºC until used.

2.2.2. Restriction digestion

DNA plugs were digested with 10U AscI (New England Biolabs, Ipswich, USA) for 3 h at 37 ºC after a pre-restriction step with Cutsmart® buffer (New England Biolabs) for 10 min at 37 ºC. As control group, plugs of *Salmonella* Braenderup digested with 10U XbaI (ThermoScientific, USA), after a pre-restriction step with 5U Tango buffer (ThermoScientific), for 3 h and 10 min at 37 ºC, respectively, were used. After the initial incubation, the mixture was discarded and followed by the restriction step.

2.2.3. PFGE running conditions

After restriction, it was prepared a 1% SeaKem® Gold Agarose (Lonza, USA) gel in order to embed plugs in the wells and separate the restricted genomic DNA fragments by an electrophoresis assay in a CHEf-DR III System (Bio-Rad Laboratories). It occurred in the following conditions: 0.5 TBE buffer (Bio-Rad Laboratories) previously refrigerated and covering the gel for a total 21 hours running, with pulses ranging from 4 to 40 s, with 6 V/cm, an included angle of 120º and 14 ºC temperature.

2.2.4. Data analysis

Gel was stained with ethidium bromide, and the image was obtained through Gel Doc™ (Bio-Rad Laboratories) and ImageLab (Bio-Rad Laboratories) program. Then, data treatment was carried out using BioNumerics™ software (bioMérieux, Marcy-l'Étoile, France), where all fragments were normalised, compared within databases and a cluster analysis was done by the unweighted-pair group method with average linkages (UPGMA), using the Dice coefficient to obtain a dendrogram according to similarities and differences between PFGE profiles, which describe the correlation between the isolates. A 2.0 band position tolerance and cut-off of 95% were considered.

2.3. Antibiotic resistance characterisation

2.3.1. Inoculum preparation

One hundred fifteen isolates of *Acinetobacter* were previously selected based on PFGE profiles. They were cultured in TSA-YE medium at 30 ºC overnight, after being sub-cultured twice in TSB-YE medium at the same conditions, whereas control strains – *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* DSM 1117 – were grown on TSAYE at 37 ºC for 24 hours, under aerobic conditions. Inocula were prepared by suspending some colonies (from clinical isolates grown on TSA-YE) in sterile Ringer's solution (Biokar Diagnostics) in order to obtain turbidity equivalent to 0.5 in McFarland, which corresponds to 1.5×10^8 CFU/mL. Then, bacterial culture was carefully swabbed into the entire surface of the agar plate of Muller-Hinton medium (MHI, Biokar Diagnostics) to guarantee that inoculum is evenly distributed to perform the antibiogram assay.

2.3.2. Antimicrobial susceptibility

The disk diffusion method was used to test the antimicrobial susceptibility of the isolates. Using an antibiotic disk-dispenser, twelve different antibiotic disks were stamped over TSA-YE plates with suspension already swabbed and incubated at 30 ºC (for isolates) and 37 ºC (for control strains). The next day, plates were examined and, isolates were classified as "sensitive", "intermediate" and "resistant" depending if there was a halo (i.e., a cleared zone) around the disk or not and the diameter of that halo/inhibition zone. That classification is attributed when comparing measures obtained with respective reference values, and usually, the larger the halo, the more susceptible the bacteria is.

Halos were measured (mm) with SCAN 500 version 8.3.1.0 (Interscience, St Nom la Bretèche, France), which automatically compares and classifies the isolates according to reference values from Clinical and Laboratory Standards Institute (CLSI, 2018). Multi-resistance was considered for all the isolates that were resistant to at least two of the twelve antibiotics used, which were Tobramycin (TM, 10 mg), Piperacillin-Tazobactam (TZP, 100/10 mg), Imipenem (IPM, 10 mg), Minocycline (MIN, 30 mg), Ceftazidime (CAZ, 30 mg), Meropenem (MEM, 10 mg), Tetracycline (TE, 30 mg), Trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 mg), Amikacin (Ak, 30 mg), Piperacillin (PIP, 100

mg), Ciprofloxacin (CIP, 5 mg) and Ampicillin-sulbactam (AMPS, 10/10 mg), all from Oxoid (Hants, UK).

2.4. Screening of acid resistance

2.4.1. Inoculum preparation

Initially, isolates were cultivated in TSA-YE medium and set at 30 ºC overnight, as previously mentioned. After that, one colony was resuspended into 5 mL of the respective liquid medium – TSB-YE (previously described) and incubated at the same conditions. Finally, to prepare the final inoculum, 50 µL of the last culture was transferred to a new tube with 5 mL of fresh TSB-YE medium and incubated overnight at 30 ºC.

One milliliter of each isolate suspension was collected from the TSB-YE medium and discarded into a labelled 1.5 mL tube. Cells were washed twice with Ringer's solution (Biokar Diagnostics) and centrifugated (MiniSpin, Eppendorf) at 150 rpm for 8 min.

2.4.2. Acid and Ringer solutions

Buffer Peptone Water (Biokar Diagnostics) solution was prepared, and the pH value was adjusted to 2.5 by adding 6 N HCl, under continuous agitation. Then, the volume was upped to 1 L, and the medium was autoclaved. This solution simulated an acid condition to bacteria in a matrix during a one-hour assay. Other than that, sterile Ringer solution was also prepared in order to perform further serial dilutions.

2.4.3. Matrix preparation

Turkey minced meat was taken as a matrix in this study. The day before the assay, 5 g of autoclaved minced meat was inoculated with 0.5 mL inoculum (prepared within the previously mentioned conditions) to a final concentration of about 10^7 CFU/g and set overnight at room temperature.

2.4.4. One-hour acidic condition assay

For each 5 g of contaminated turkey meat (section 2.4.3), 50 mL of BPW medium, previously heated-up in a water bath at 30 ºC with agitation, was added and the first sample was collected (100 µL) – T0 – which corresponds to time "zero". This was repeated for every isolate and at every 15 minutes, corresponding successively to times "T15", "T30", "T45" and "T60". Between those sampling points, every flask was replaced in the shaker water bath to maintain conditions.

2.4.5. Enumeration

Viable cell counts were determined by preparing serial decimal dilutions in sterile Ringer solutions, plated on TSA-YE medium and incubated overnight at 30 ºC. Colonies were counted to calculate CFU/g.

2.5. Simulated gastrointestinal tract

2.5.1. Inoculum and matrix preparation

Inoculum was prepared as previously described: one colony was transferred from TSA-YE into 10 mL of TSB-YE and incubated overnight at 30 ºC. Then, a 1:100 dilution was made by transferring 0.1 mL of the previous culture into 9.9 mL of fresh TSB-YE medium. Cells were washed twice by centrifugation (150 rpm, 8 min, 4 ºC; MiniSpin, Eppendorf), resuspended in 10 mL sterile Ringer solution (Biokar Diagnostics) and mixed to obtain an inoculum level of about 10^8 CFU/mL.

Turkey minced meat was taken as a matrix again and prepared as previously mentioned. Meat was inoculated with 10 µL of each cell suspension and set overnight at room temperature.

2.5.2. Simulated gastrointestinal conditions

The simulated gastrointestinal tract conditions were performed according to (Minekus et al., 2014) with peristaltic movements simulated through agitation. Oral fluid (previously heated at 37 ºC) was distributed into tubes with the contaminated meat (1:1), homogenised and then samples (100 µL) were immediately collected. The gastric phase was then simulated by adding 2 mL of gastric fluid, with a pH value of 3, adjusted using

6 N HCl, and 25 mg/mL of pepsin (Sigma-Aldrich, USA). Tubes were kept at 37 ºC, with 130 rpm agitation and samples (100 µL) were collected after 1 h and 2 h. After 2 h of exposure to simulated gastric conditions, 4 mL of simulated intestinal fluid were added to every remaining tubes as well as 10 g/L of bile salts (Sigma-Aldrich, USA), and 2 g/L of pancreatin (Sigma-Aldrich), under 45 rpm agitation. At this stage, the pH was adjusted to 7 by the addition of sodium bicarbonate (NaHCO3). Samples (100 µL) were again collected at 1 h and then after 2 h (after 3 and 4 h of experiments).

During the whole process, tubes were kept at 37 ºC with agitation, not only to simulate the peristaltic movements, as previously mentioned, but also to promote homogenisation. Enzymes' solutions were prepared right before use, dissolving in the respective fluid, and kept on ice to maintain their activity.

All assays were conducted in duplicate for each isolate. As a positive control, a tube containing 8 mL of BPW and contaminated meat was not exposed to simulated gastrointestinal conditions but was maintained at 37 ºC for 4 h with agitation. Samples were also collected at the end of the incubation time.

Enumeration was performed as previously described.

2.6. Cell culture

2.6.1. Cells growth conditions

Human colon adenocarcinoma cell lines Caco-2 (American Type Culture Collection ECACC 86010202) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Waltham, USA) supplemented with 10% (v/v) of Fetal Bovine Serum (FBS, BioWest, Nuaillé, France), 1% of Non-Essential Amino Acids (NEAA), 1% of Sodium Pyruvate and 2 mg/mL of Penicillin-Streptomycin solution (Pen-strep, Lonza, Veriers, Belgium), which will constitute the complete medium.

Initially, right after defrosting, cells were incubated as monolayers in 25 cm^2 cell culture flasks (T25, Sarstedt, Nümbrecht, Germany) for 2 days at 37 ºC in a humidified atmosphere containing 5% CO_2, and then scaled-up to 75 cm^2 cell culture flasks (T75, Sarstedt). Whenever 80% confluence was reached, old DMEM medium was removed, cells were washed twice with phosphate-buffered saline (PBS), and then Trypsin solution (Gibco) was added for 15 min to detach the cells. Then, a new fresh DMEM medium was added, and the concentration of cells was calculated.

Cell lines used in the assays had more than 15 passages, and three independent replicates were performed for each studied isolate.

2.6.2. Bacterial adherence assay

Caco-2 cells were incubated for 48 h in a 24-well cell culture plate in a density of 1×10^5 cells/well. After incubation, cells were washed twice with PBS and added new DMEM without antibiotic (900 µL). After that, 100 µL of bacterial culture, previously washed with PBS and containing 10^7 CFU/mL, were added to the monolayer of epithelial cells and incubated at 37 ºC, for 2 h, with 5% of CO_2 atmosphere. Later, contaminated cells were washed three times with PBS and lysed with 1 mL of 0.2% (v/v) Triton (Sigma Aldrich). *Lactobacillus rhamnosus* GG was used as positive control, and one well with DMEM was used as negative control.

2.6.3. Bacterial invasion assay

For the invasion assay, Caco-2 cells were incubated for 48 h in a 24-well cell culture plate to obtain $1{,}0 \times 10^5$ cells/well, then were washed twice with PBS and added new DMEM medium without antibiotic (900 µL). After that, 100 µL of bacterial culture (containing 10^7 CFU/mL) were added to each well and incubated at 37 ºC, for 1 h, with 5% of CO_2 atmosphere. Later, the medium was removed and substituted by fresh DMEM plus 40 µg/mL of gentamicin (Lonza) and incubated again for 90 min at the same conditions. This step guarantees that bacteria adhering to monolayer are killed without affecting those that could penetrate the cells and cause infection. Then, cells were washed three times with PBS and lysed with 0.2% (v/v) of Triton solution (Sigma Aldrich). In this case, *Listeria monocytogenes* ESB2959 was used as a positive control, and one well of the plate with DMEM medium was used as a negative control.

2.6.4. Enumeration

In both adherence and invasion assays, the content of each well was collected, and serial dilutions were performed with Ringer's solution. Isolates of *Acinetobacter* spp. were cultured in TSA-YE medium and incubated overnight at 30 ºC. Positive control samples of *L. monocytogenes* were cultured in the same medium but incubated at 37 ºC

for 24 h, and *L. rhamnosus* GG isolates were plated in De Man, Rogosa and Sharpe agar (MRS; Biokar Diagnostics) and incubated for 24 h also at 37 ºC.

3. Results and Discussion

3.1. PFGE clusters profiles

In this study, 264 isolates of *Acinetobacter* spp. were genotyped by PFGE to compare their relatedness. Conversely to conventional electrophoresis, the PFGE method can separate large fragments of DNA (larger than 50kb and up to 10mb) and generate a fingerprint when an electric field is applied and its direction switches periodically in a gel matrix (Parizad et al., 2016; Tenover et al., 1995). After many years of being a reference method for typing of many bacterial species (Peleg et al., 2008), it is still a powerful tool for molecular typing of pathogens such as *A. baumannii*, establishing epidemiological and/or genetic relationships (Neoh et al., 2019; Parizad et al., 2016). In fact, in case of an outbreak, PFGE can also be used to track transmission routes of this pathogen in a hospital and help eradicate it. Therefore, PFGE will remain an interesting technique for small laboratories and hospitals in years to come (Neoh et al., 2019).

Figures 3.1.1, 3.1.2 and 3.1.3 exhibit the dendrogram obtained as well as the correlations between these pathogens. After analysing the different clusters, 115 isolates were chosen to have the most representativity of all profiles in subsequent assays.

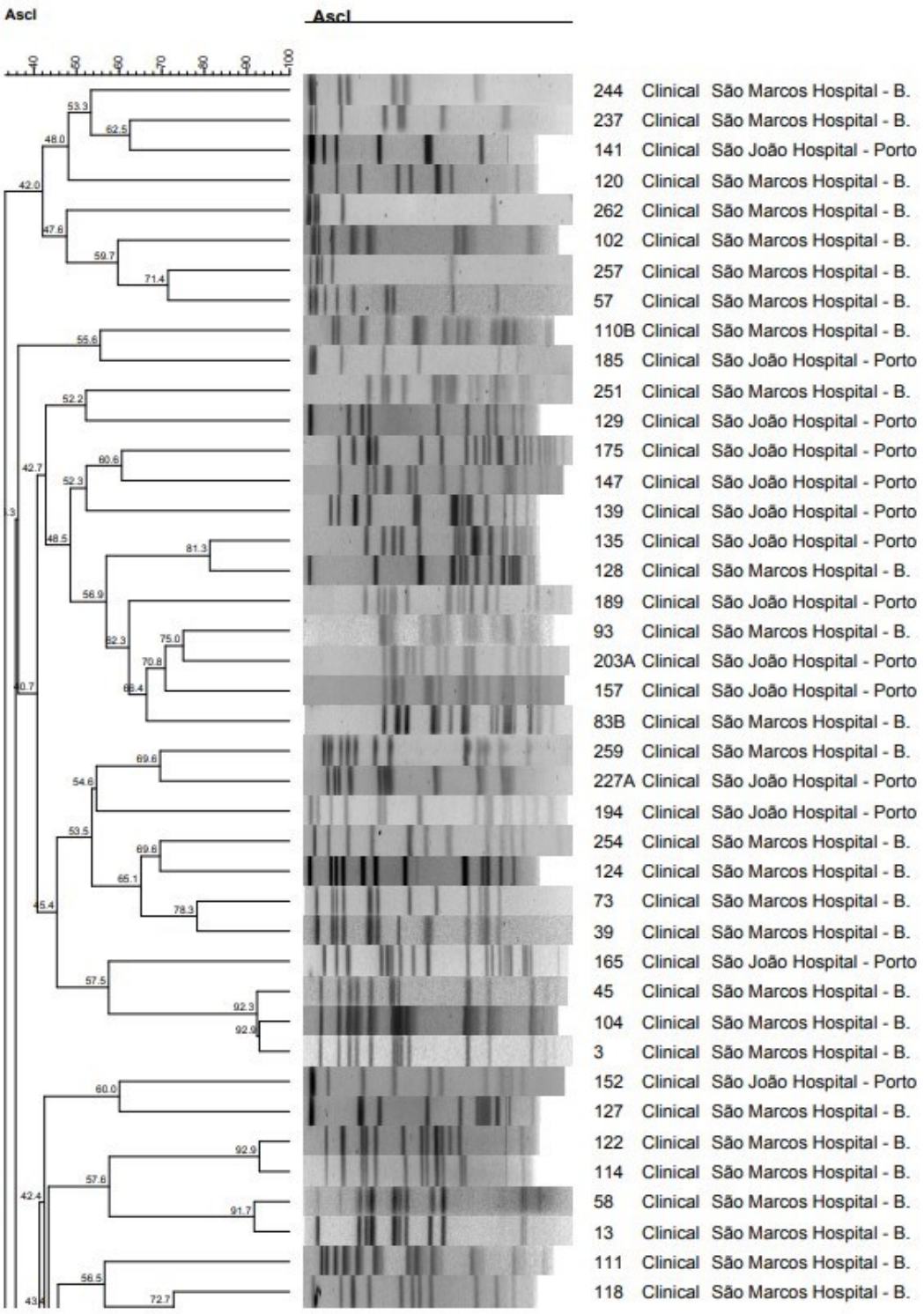


Figure 3.1.1: PFGE profiles of 115 *Acinetobacter* spp. clinical isolates digested with AscI enzyme. 2.0 band position tolerance and cut-off of 95 % were considered.

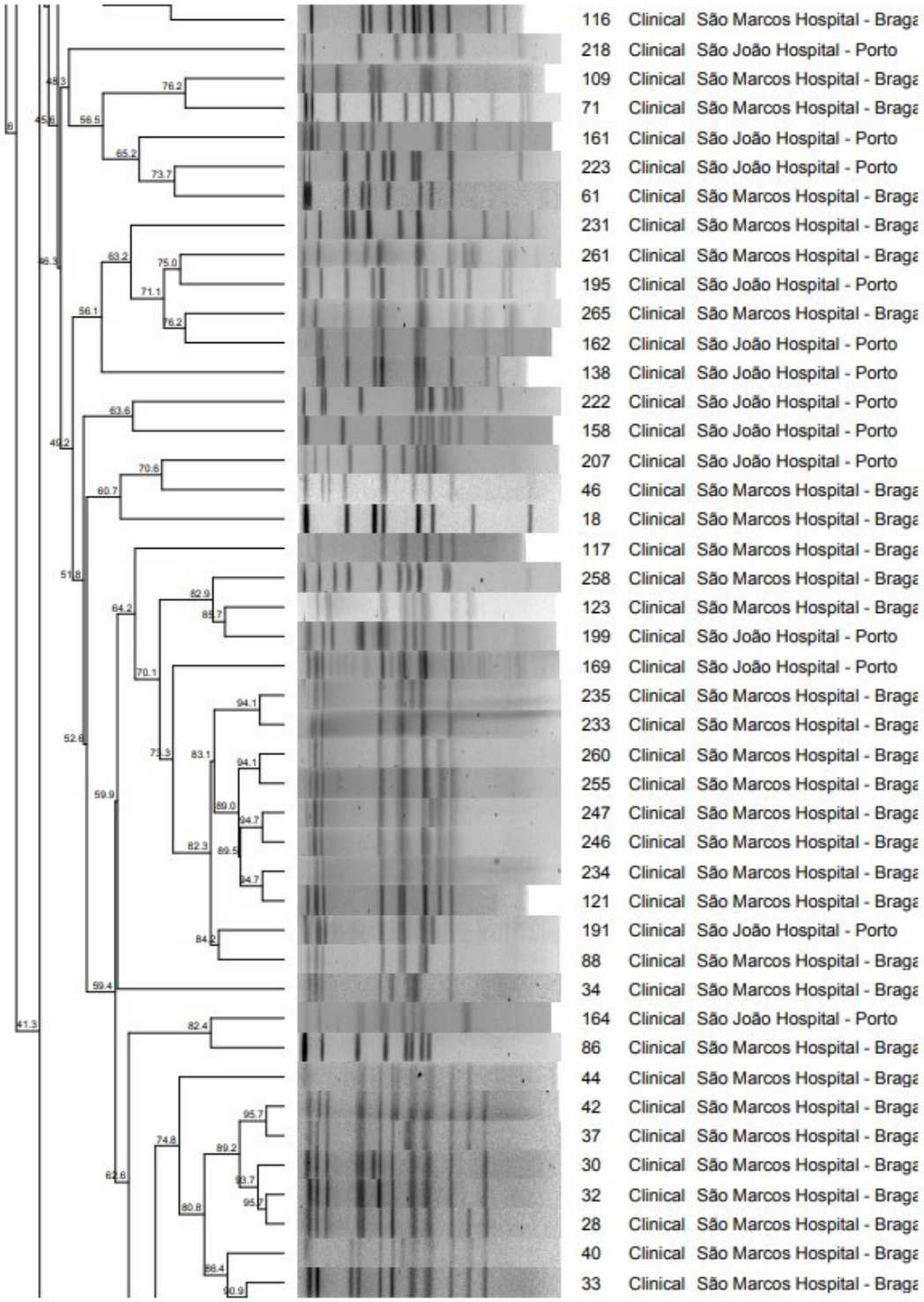


Figure 3.1.2: PFGE profiles of 115 *Acinetobacter* spp. clinical isolates digested with AscI enzyme. 2.0 band position tolerance and cut-off of 95% were considered (continued).

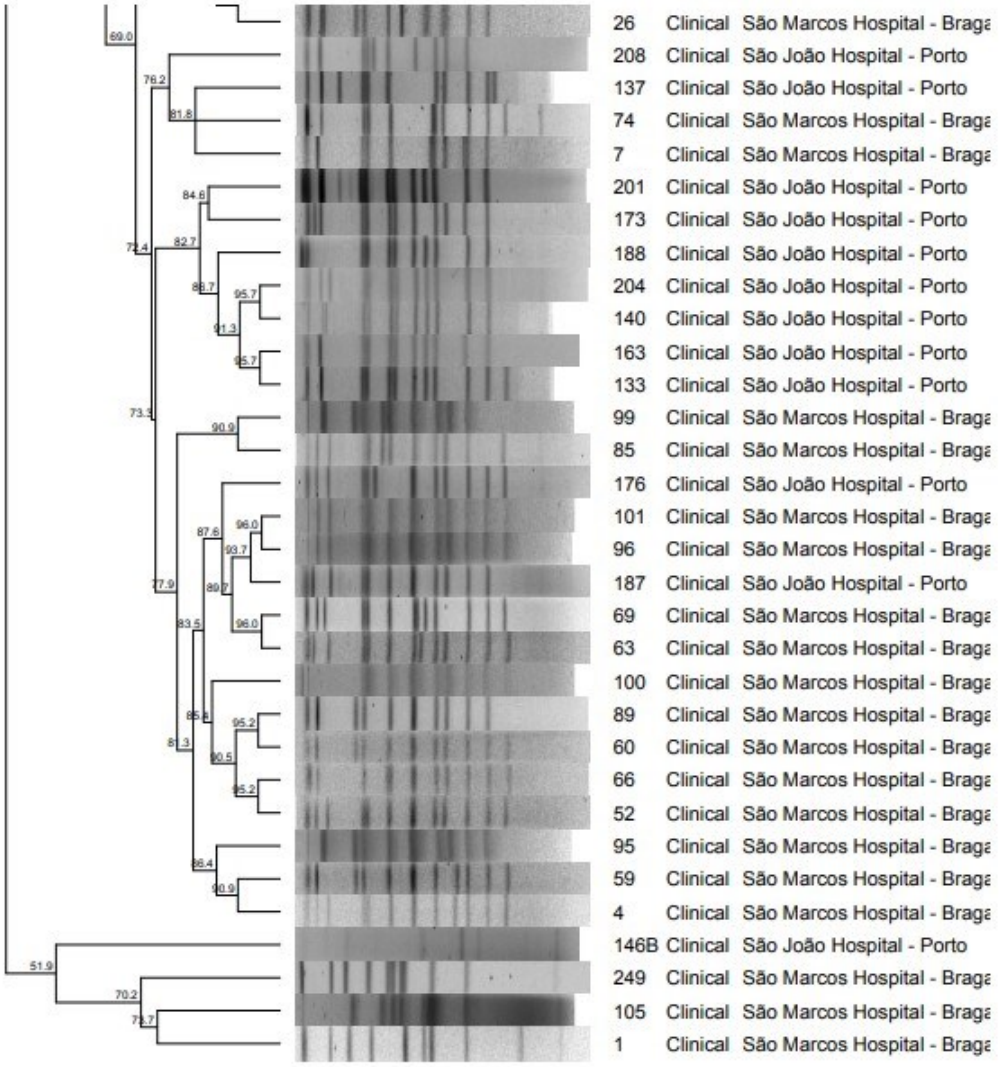


Figure 1.1.3: PFGE profiles of 115 Acinetobacter spp. clinical isolates digested with AscI enzyme. 2.0 band position tolerance and cut-off of 95 % were considered (continued).

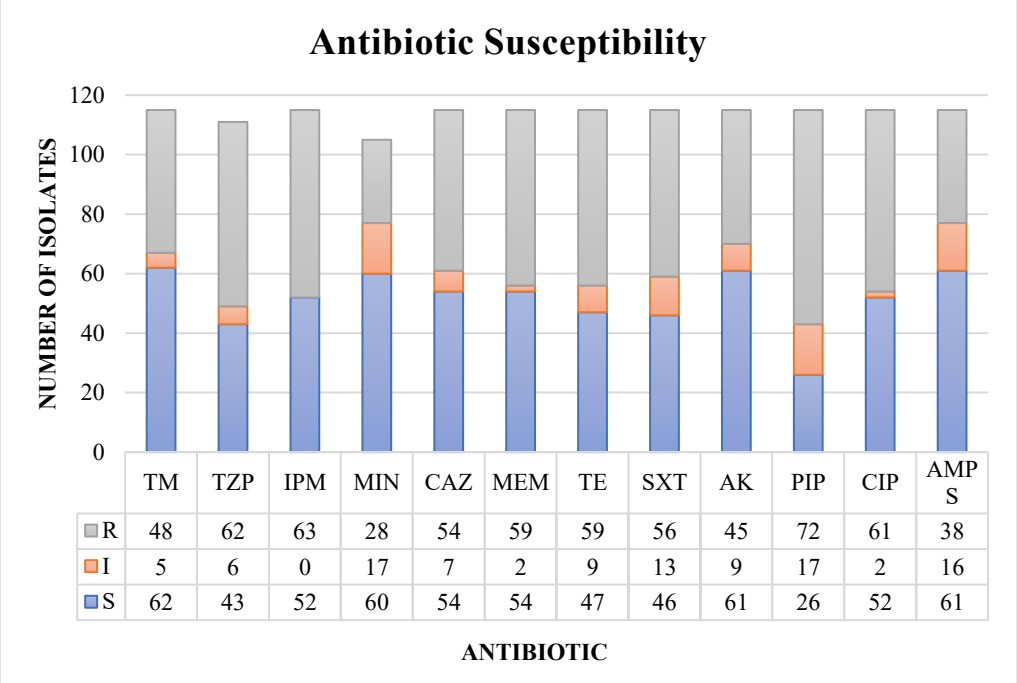
The figures above exhibit the selected isolates representing the 115 clusters originated by PFGE. The high number of different PFGE profiles indicates a high diversity between isolates, although the most closely related belong to the same hospital. This suggests that isolates from different hospitals do not share a common source, i.e., although they came from two different hospitals, they do not have the same transmission route. Similar results were obtained by Carvalheira et al. (2017a) when analysed 166 isolates of *Acinetobacter* spp. from meat samples that generated 141 different genotype clusters (Carvalheira et al., 2017a). In another study, 253 isolates recovered from vegetable and fruit samples were genotyped by PFGE and 181 different profiles were

obtained. Also, a higher discrimination between isolates from different samples was found (Carvalheira et al., 2017b). In 2016, Qi et al. (2016) performed a study with *A. baumannii* strains and also found 167 different patterns and 103 clusters with a similarity of 80% (Qi et al., 2016).

Profiles with more than 95% similarity were considered clones and just one of them was selected to further experiments. Therefore, 115 isolates were selected from each obtained PFGE profile for subsequent assays.

3.2. Antibiotic Characterization

Characterization of each selected isolate regarding antibiotic susceptibility was performed to increase knowledge about their resistance profiles. Figure 3.2.1 shows the number of isolates that were considered resistant (R), intermediate (I) and sensitive (S) to each antibiotic.



	TM	TZP	IPM	MIN	CAZ	MEM	TE	SXT	AK	PIP	CIP	AMPS
R	48	62	63	28	54	59	59	56	45	72	61	38
I	5	6	0	17	7	2	9	13	9	17	2	16
S	62	43	52	60	54	54	47	46	61	26	52	61

Figure 3.2.1: Antibiotic susceptibility of selected isolates to tobramycin (TM), piperacillin-tazobactam (TZP), imipenem (IPM), minocycline (MIN), ceftazidime (CAZ), meropenem (MEM), tetracycline (TE), trimethoprim-sulfamethoxazole (SXT), amikacin (AK), piperacillin (PIP), ciprofloxacin (CIP) and ampicillin-sulbactam (AMPS).

Figure 3.2.1 shows that isolates were less resistant to minocycline (MIN, 24.3%), ampicillin-sulbactam (AMPS, 33.0%), amikacin (AK, 39.1%) and tobramycin (TM, 41.7%). Although minocycline is the most effective antibiotic against *Acinetobacter* spp. among the studied antibiotics, Carvalheira et al. (2017a) found much lower rates: only 1.2% of the strains resisted minocycline (MIN). This antibiotic is a semi-synthetical, second-generation tetracycline. It is effective against Gram-negative and -positive infections and is considered the only tetracycline with neuroprotective activity (Kim and Suh, 2009). Conversely and supporting other findings, Avila-Novoa et al. (2019) found 60.0% resistance rate to amikacin (Avila-Novoa et al., 2019), Qi et al. (2016) found 28.7% (Qi et al., 2016) and resistance to carbapenems has been increasing (Kempf and Rolain, 2012; Zarrilli et al., 2013).

However, isolates exhibit higher resistance rates to imipenem (54.8%), ciprofloxacin (53.0%), meropenem and tetracycline (51.3%), trimethoprim-sulfamethoxazole (48.7%), ceftazidime (46.9%). Moreover, it is important to emphasize the most commonly used antibiotics to treat *Acinetobacter* spp. infections and their respective susceptibility to them, such as ceftazidime (CAZ), a cephalosporin used in therapies against this pathogen and fluoroquinolone ciprofloxacin (CIP). In these cases, resistance rates were similar to what Carvalheira et al. (2017a) found in their study with meat samples – 43.5% and 42.9%, respectively to CAZ and CIP, but meropenem (MEM) resistant rates were totally contradictory (8.3%). Analogous results were obtained for ciprofloxacin (46.6%) in Avila-Novoa et al. (2019) study. In a study by Qi et al. (2016), 59.2% resistance rate was obtained for IPM and MEM, 65.8 for CIP, 60.7% for TE and 59.9% for SXT (Qi et al., 2016). In fact, 100% resistance rate to CAZ and 91% resistant to CIP were reported by other investigators (Di Domenico et al., 2017).

Lastly, the highest resistance rates were exhibited to piperacillin – 62.6% – and 17 isolates (14.8%) already exhibited intermediate resistance. Piperacillin (PIP) belongs to the penicillin group and to a major class called β-lactam antibiotics, and it is a large-spectrum penicillin (Strayer et al., 1994). However, the dissemination of β-lactamase-producing bacteria has decreased its use, and to invert this trend, one of the strategies developed was a combination of piperacillin and tazobactam (an inhibitor of β-lactamase), which results in broad-spectrum activity against many pathogens (Strayer et al., 1994). In fact, in the United Kingdom, between 2008 and 2013, the prescription of this penicillin/β-lactamase inhibitor combination increased almost 95% (Hansen et al., 2019). It is possible to observe in Figure 3.2.1 that the number of isolates that were

resistant to this piperacillin-tazobactam combination (TZP) decreased to 53.9%; however, it is still the third highest resistance rate among the used antibiotics. Similar results were found in a study performed by Carvalheira et al. (2017b) analysing isolates recovered from lettuce and fruits: 80.1% and 64.1% resistance rates to piperacillin and piperacillin-tazobactam, respectively, were found (Carvalheira et al., 2017b). In another study performed by the same authors but with isolates recovered from raw meat, 70.8% and 64.9% resistance rates were found, respectively (Carvalheira et al., 2017a). Qi et al. (2016) found a 63.3% resistance rate to piperacillin; however, the combination of piperacillin-tazobactam was not studied (Qi et al., 2016). Avila-Novoa et al. (2019) evaluated resistance profiles of acinetobacters and claimed that 100% of the *A. baumannii* isolates were resistant to piperacillin-tazobactam (TZP) (Avila-Novoa et al., 2019).

Another important finding is that 19 out of 115 isolates (16.5%) were susceptible to all of the antibiotics investigated, and 68 out of 115 isolates (59.0%) were resistant to at least two of the tested drugs, which were considered as multidrug resistant (MDR) isolates. Carvalheira et al. (2017b) found similar susceptibility rates – 24.9% of strains were susceptible to all the tested drugs, but only 29.8% were considered as MDR. Conversely, in their other study, 13.3% of strains were susceptible, but 51.2% were considered as MDR (Carvalheira et al., 2017a). Avila-Novoa et al. (2019) considered that 100% of the *A. baumannii* were MDR strains (Avila-Novoa et al., 2019). Besides that, 26 isolates (22.6%) were even resistant to all of the antibiotics, which are considered as extensive drug-resistant (XDR), according to the previously considered definition by Magiorakas et al. (2012). It is not possible to consider pan-drug resistant (PDR) because the polymyxin antimicrobial class was not tested in this study.

Resistance of important bacterial pathogens to common antimicrobial therapies and the emergence of multidrug-resistant bacteria are increasing alarmingly and have become an emergent problem, especially on *Acinetobacter* spp. due to not only surviving in different abiotic conditions but also to its capacity to acquire and accumulate genetic information that confers resistance to antibiotics and a MDR phenotype to the major drug groups (Carvalheira et al., 2017b; de Amorim and dos Santos Nascimento, 2017). In fact, alternative therapies with other antibiotics, such as colistin, which seems to be effective against MDR Gram-negative bacteria, including *A. baumannii* infections, have been developed (Kempf and Rolain, 2012; Zarrilli et al., 2013).

3.3. Simulation of acid conditions

An initial screening for resistance to an acidic environment aimed to distinguish between the isolates that could best survive in acidic conditions to be further tested in the simulated gastrointestinal tract assay.

Table 3.3.1 shows the logarithmic reduction of each isolate throughout an hour of exposure to acidic conditions.

Table 3.3.1: Logarithmic reduction (Log N/N0; where "N" represents the final counts and "N0" represents counts at time 0) of each isolate during one hour assay (immediately after adding the acidic solution: 0 minutes and after 15, 30, 45 and 60 minutes of exposure).

	Isolates	**0 min**	**15 min**	**30 min**	**45 min**	**60 min**
Log N/N0	1	-1.7	-4.9	-4.9	-5.2	-6.1
	3	-1.7	-4.5	-5.8	-4.8	-4.3
	4	-1.7	-4.5	-4.6	-4.5	-5.9
	7	-1.7	-3.5	-4.1	-4.7	-4.7
	13	-1.7	-3.0	-3.4	-5.2	-3.7
	18	-1.7	-4.0	-4.8	-5.8	-4.8
	26	-1.7	-3.4	-3.7	-3.8	-4.2
	28	-1.7	-3.4	-4.4	-4.0	-4.1
	30	-1.7	-2.3	-6.1	-3.9	-6.1
	32	-1.7	-3.1	-6.1	-4.7	-5.2
	33	-1.7	-3.1	-4.1	-4.8	-5.8
	34	-1.7	-3.5	-4.7	-4.9	-5.2
	37	-1.7	-2.3	-3.5	-4.3	-4.3
	39	-1.7	-3.1	-6.0	-4.7	-5.0
	40	-1.7	-3.0	-4.3	-4.3	-3.1
	42	-3.0	-3.4	-4.7	-4.7	-4.7
	44	-2.7	-4.1	-5.4	-5.4	-5.4
	45	-2.7	-4.6	-5.8	-5.8	-5.8
	46	-2.8	-4.6	-5.3	-5.7	-5.3
	52	-3.2	-5.4	-6.1	-6.3	-6.4

	Isolates	0 min	15 min	30 min	45 min	60 min
Log N/N0	57	-4.3	-4.8	-6.0	-7.8	-5.8
	58	-2.8	-4.9	-5.8	-4.9	-5.8
	59	-8.2	-7.2	-8.2	-6.9	-6.0
	60	-8.1	-4.3	-5.3	-5.4	-5.4
	61	-3.6	-5.6	-6.4	-6.4	-6.6
	63	-4.6	-5.8	-6.4	-6.2	-6.2
	66	-5.4	-6.2	-6.3	-6.9	-6.6
	69	-5.3	-5.3	-7.3	-5.9	-6.1
	71	-4.6	-5.0	-4.9	-4.9	-5.3
	73	-4.1	-4.9	-6.2	-6.1	-6.2
	74	-2.5	-3.1	-3.7	-5.7	-4.2
	83B	-7.0	-7.0	-7.0	-7.0	-7.0
	85	-3.4	-4.2	-4.3	-4.3	-4.4
	86	-3.5	-4.4	-4.5	-4.5	-4.5
	88	-7.4	-9.4	-6.9	-6.9	-7.1
	89	-7.9	-7.9	-5.5	-5.6	-5.7
	93	-2.7	-8.4	-8.4	-8.4	-8.4
	95	-2.7	-7.4	-5.6	-8.4	-7.1
	96	-2.7	-5.8	-7.4	-6.4	-6.3
	99	-2.7	-5.5	-5.5	-5.5	-6.0
	100	-3.8	-5.1	-4.8	-4.8	-4.7
	102	-3.9	-6.1	-8.4	-5.9	-6.3
	104	-1.8	-7.5	-6.2	-5.7	-5.5
	105	-4.6	-5.9	-8.4	-8.4	-8.4
	109	-2.7	-3.7	-3.7	-3.7	-3.8
	110B	-3.8	-5.6	-5.7	-5.9	-6.1
	111	-2.7	-3.8	-3.7	-3.8	-3.7
	114	-3.1	-4.1	-4.8	-4.7	-5.1
	116	-2.8	-3.9	-4.8	-4.9	-5.4
	117	-3.4	-4.7	-4.8	-4.9	-5.2
	118	-3.6	-5.7	-5.0	-5.5	-5.6
	120	-2.7	-5.0	-3.9	-4.1	-4.2

	Isolates	0 min	15 min	30 min	45 min	60 min
Log N/N0	121	-3.2	-5.0	-5.0	-5.2	-5.3
	122	-3.4	-4.8	-5.1	-8.4	-5.5
	123	-3.6	-5.4	-4.6	-4.8	-4.9
	124	-3.5	-5.1	-4.5	-5.6	-4.4
	127	-3.7	-5.5	-7.7	-7.7	-6.7
	128	-3.1	-4.7	-4.0	-4.0	-4.3
	129	-3.2	-4.6	-4.9	-4.8	-4.9
	133	-3.0	-4.8	-4.4	-4.5	-5.0
	135	-4.4	-7.3	-7.3	-7.3	-7.3
	137	-3.2	-4.5	-4.4	-4.7	-5.1
	138	-3.0	-4.0	-4.0	-4.0	-4.1
	139	-3.4	-4.5	-4.0	-4.1	-4.1
	140	-3.3	-4.4	-4.5	-4.3	-4.5
	141	-2.9	-4.9	-4.7	-4.9	-5.0
	146B	-1.8	-2.8	-2.8	-2.8	-2.8
	147	-4.0	-3.8	-4.2	-4.0	-4.2
	152	-7.6	-5.3	-5.5	-5.4	-5.5
	157	-7.5	-7.5	-6.5	-7.5	-7.5
	158	-7.8	-4.3	-4.9	-4.9	-4.8
	161	-7.3	-5.5	-5.6	-5.3	-5.3
	162	-7.5	-4.1	-4.4	-4.7	-5.0
	164	-7.7	-4.5	-4.8	-5.1	-5.4
	165	-4.8	-7.7	-7.7	-7.7	-7.7
	169	-3.2	-4.3	-4.5	-4.8	-5.0
	173	-3.1	-4.8	-4.2	-4.6	-4.9
	175	-7.5	-4.3	-4.7	-5.7	-5.3
	176	-3.5	-4.2	-4.3	-4.5	-4.5
	185	-3.2	-5.3	-4.3	-4.5	-5.0
	187	-4.3	-5.5	-6.6	-7.5	-7.5
	188	-3.4	-4.2	-4.2	-4.4	-4.7
	189	-7.8	-7.8	-7.8	-6.9	-7.8
	191	-3.7	-3.1	-4.0	-4.3	-4.6

	Isolates	0 min	15 min	30 min	45 min	60 min
Log N/N0	194	-7.6	-6.1	-7.6	-6.4	-7.6
	195	-3.6	-4.5	-4.4	-4.3	-4.6
	199	-7.3	-7.3	-7.3	-7.3	-7.3
	201	-3.2	-7.2	-7.2	-7.2	-7.2
	203A	-6.7	-6.7	-6.7	-6.7	-6.7
	204	-7.5	-7.5	-7.5	-7.5	-7.5
	207	-7.7	-7.7	-7.7	-7.7	-7.7
	208	-7.0	-7.0	-7.0	-7.0	-7.0
	218	-3.9	-5.9	-6.2	-6.2	-7.2
	222	-2.7	-6.5	-8.5	-8.5	-8.5
	223	-2.3	-7.5	-7.5	-7.5	-7.5
	227A	-5.0	-5.0	-5.0	-5.0	-5.0
	231	-2.8	-7.6	-6.1	-6.7	-5.6
	233	-2.9	-7.7	-7.7	-7.7	-7.7
	234	-7.6	-7.6	-7.6	-7.6	-7.6
	235	-4.0	-6.6	-7.5	-7.5	-6.3
	237	-3.3	-5.0	-5.3	-5.5	-5.5
	244	-2.9	-6.0	-7.7	-6.2	-5.2
	246	-4.4	-6.7	-7.7	-7.7	-6.7
	247	-7.7	-7.7	-7.7	-7.7	-7.7
	249	-4.9	-8.1	-8.1	-8.1	-7.2
	251	-3.0	-3.0	-3.0	-3.0	-3.0
	254	-7.8	-7.8	-7.8	-7.8	-7.8
	255	-3.9	-4.9	-5.9	-6.6	-7.1
	257	-3.8	-6.7	-6.7	-6.0	-6.0
	258	-3.9	-7.0	-6.4	-7.0	-6.0
	259	-3.4	-5.3	-6.2	-6.2	-6.0
	260	-3.6	-7.9	-6.2	-6.2	-6.5
	261	-4.4	-7.4	-7.4	-6.1	-7.4
	262	-2.5	-6.2	-6.2	-5.3	-4.9
	265	-2.9	-6.7	-6.7	-6.7	-6.7

Most of the isolates exhibited resistance to acidic conditions (Table 3.3.1). Notably, isolates such as 109, 111 and 146B were particularly resistant by the acid right from the start of the assay, showing reductions of 2.7 (for the first two isolates) and 1.8 (for the latter) log cycles. After 60 minutes, they exhibited an additional reduction of approximately only 1 log cycle, indicating that they were among the most resistant isolates to acidic stress conditions. Other isolates were more susceptible to the acid right at the beginning of the assay, with reductions between 7.3 and 7.7 log cycles, but were able to recover during the 60-minute exposure, such as isolates 60, 89, 158, 162 and 164, exhibiting a recovery between 2.2 and 2.9 log cycle, which means that acidic conditions only cause damage or injury to bacterial cells. On the other hand, some isolates were more susceptible to the acidic solution immediately after adding the solution (time 0, such as 203, 297 and 247) with 7.5, 7.7 and 7.7 log cycle reductions, respectively, and were not able to recover after 60 minutes, exhibiting the same logarithmic values.

Some studies about the capacity of *Acinetobacter* spp. to survive in acidic pH conditions can be found in the literature. Dekic et al. (2018) exposed *A. baumannii* isolates to pH 5.0 for two days, and no multiplication or decline in bacterial cell numbers were observed; however, when subjected to pH of 2.0, they exhibited the most lethal rate, in which isolates were able to survive up to 3 hours (Dekic et al., 2018). Indeed, this study is not consistent with the present study, as most isolates exhibited resistance, even though some isolates showed reductions of more than 6 log cycles. Exposure to an acidic pH of 2.5 only lasted one hour, which could not be enough. However, vinegar solution was used in another study to evaluate its disinfection power against *Acinetobacter* spp. recovered from turkey meat samples, and it proved to be effective in eliminating these pathogens after at least 15 minutes of exposure (Campos et al., 2019), which also corroborates the susceptibility of *Acinetobacter* spp. to acidic conditions.

To further evaluate the behaviour through simulated gastrointestinal conditions, 14 isolates resistant to acidic conditions were selected. However, sensitive isolates from 199 to 265 were also selected to increase knowledge about their behaviour when exposed to basic pH values from intestinal fluids.

3.4. Simulation of gastrointestinal conditions

To investigate a potential foodborne origin, gastrointestinal conditions were simulated for 14 *Acinetobacter* isolates in a four-hour assay, which included simulated saliva, gastric fluid, and intestinal fluid. The results are presented in Figure 3.4.1.

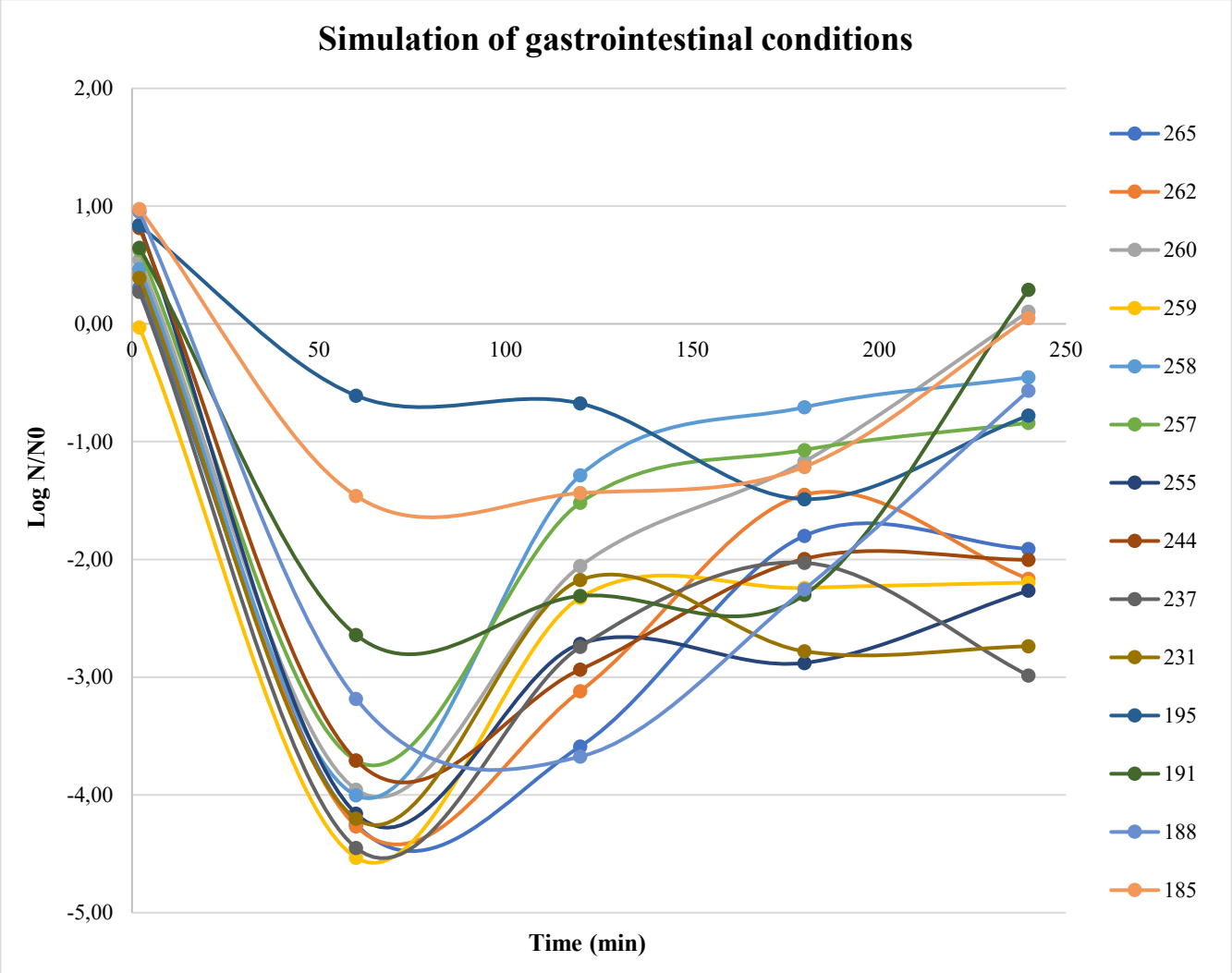


Figure 3.4.1: Logarithmic reductions (Log N/N0) of 14 isolates in each sampling point: immediately after oral fluid was added; after 1 h and 2 h of gastric phase simulation and after 1 h and 2 h of intestinal phase simulation.

This assay was performed for each isolate incorporated in a turkey meat matrix. A solid food matrix has been demonstrated to protect bacteria through the gastrointestinal passage, especially when subjected to acidic stomach conditions. In fact, the low pH of

gastric secretions has been recognised as a primary defense against foodborne enteric pathogens (Cunha et al., 2016; Waterman and Small, 1998). This protection depends on different factors such as its protein and fat level components as well as the acidity of the human stomach, which depends on physiological variables that include previous food intake. Nevertheless, increasing the pH value of gastric secretions has been associated with increased survival rates of some foodborne pathogens (Waterman and Small, 1998).

Two isolates (185 and 195) were the most resistant to gastric conditions. Contrarily, isolate 259 was the most sensitive, with 4.5 log cycles reduction after one hour in contact with gastric fluid. Isolate 258 was also affected by the acidic conditions of the gastric phase (4.0 log cycles reduction). However, it was able to recover and reach successfully, at the end of the experiment, almost the same number of cells, representing a recovery rate of 96.7%. To our knowledge, there is no explanation in literature for this behaviour, but a possible justification is that acid solution causes a stress condition on bacterial cells, which promotes a latency stage and, whenever favourable conditions such as neutral pH values, these microorganisms are able to recover.

Bacteria have different described stages such as “viable”, which means they are active and readily culturable; “dormant” when they are inactive but ultimately culturable; “active but nonculturable”; and “dead” when they are also inactive and nonculturable (Kell et al., 1998). In this assay, a so-called “dormancy stage” might be observed, which is a reversible status where bacteria reduce their metabolic activity and persist for long periods without cellular division. At this point, cells are not “alive” in the sense of being able to form a colony when plated on an agar medium; when conditions are more favourable, they can revert to a state of “aliveness” as so defined (Kaprelyants et al., 1993).

When these pathogens were exposed to alkaline pH from intestinal fluid, most were able to recover. Isolates 237 and 267 exhibited a slow recovery (less than 1.00 log cycle) in the first minutes, and then viable cell counts reduced 4.5 and 4.3 log cycles, respectively, after 60 minutes. However, in the last 120 minutes, they were able to recover and log cycle reduction was 1.9 and 3.0, respectively. Nevertheless, in some cases (isolates 195, 231 and 255), a reduction was already observed after 60-minute exposure to the intestinal fluid (with a pH value of 7.0) and only recovered in the last 60 minutes of the experiment. A possible justification for this can be the exposure to bile salts, which are biological components of bile and have detergent properties that can induce membrane damage and cause oxidative stress to the DNA. This can constitute an

antimicrobial role in the same way bile plays *in vivo,* and resistance to this biological component is crucial to further intestinal colonization (Sánchez et al., 2007).

In addition, to our knowledge, there is no performed study with *Acinetobacter* spp. subjected to simulated digestive process conditions such as the present one. However, a few studies suggest gastrointestinal tract colonization by these pathogens. A study performed in 2008 showed that seven intensive care units' patients were positive for imipenem resistant *A. baumannii* (IRAB) in 26 out of 50 perirectal surveillance cultures from these patients with bacteremia, which suggests that gut colonization generally precedes the blood infection in this population (Thom et al., 2010). The authors also claimed that patients may be colonized and infected with two different isolates of a particular bacterium (Thom et al., 2010), as well as multiple colonization sites with posterior infection had been demonstrated. Another study with a different population showed the recovery of *A. baumannii*, *A. haemolyticus* and *A. junii* from neonates' gut with and without sepsis and, therefore, authors claimed these bacteria are gastrointestinal tract colonizers. Besides that, the isolates of *A. baumannii* were subsequently analysed and exhibited high rates of multidrug- and/or carbapenem-resistance (Roy et al., 2010). More recently, a study not only shows the *A. baumannii* capacity to colonise gut epithelium, but also suggests that Secretory Immunoglobulin A (SIgA), a protein responsible for immune defence on mucosal surfaces and that contributes to GI tract homeostasis, affect that colonization due to a significant reduction in bacterial colonization (almost an 80% decrease in bacterial attachment) in the absence of SIgA (Ketter et al., 2018).

In conclusion, in the present study all isolates exhibit a recovery behaviour throughout time, regardless of whether they are more or less affected by acidic conditions of the stomach. For that reason, isolates 258 and 260 were considered for the following experiments due to being more resistant and more likely to adhere and colonise the intestinal gut cells.

3.5. Cell Culture

3.5.1. Adhesion assay

Back to the 1970s, several epithelial cell lines from gastrointestinal tumours were studied, but one of them was particularly noteworthy– CaCo-2 cells (**Ca**ncer **co**li-**2**), because of its unique ability to spontaneously differentiate (both structurally and

functionally) whenever confluence was reached. Jorgen Fogh established it from a colorectal adenocarcinoma in 1977 (Fogh et al., 1977). Since then, it has been widely used as a model in cell culture assays because, as these cells can grow in monolayers, investigators consider that CaCo-2 cells lack problems with multilayering (Briske-Anderson et al., 1997).

As previously mentioned, adherence of bacteria to epithelial cells is crucial to colonise and infect host cells. *Acinetobacter baumannii* is able to colonize human skin and mucosal membranes, which means that these organisms somehow can successfully adhere to the cells (Lee et al., 2006).

In order to understand if two *Acinetobacter* isolates could adhere to human epithelial cells (CaCo-2), an important feature to cause a foodborne illness, an adherence assay was performed, and the results are presented in Figure 3.5.1.1. *Lactobacillus rhamnosus* GG was used as the positive control (adherence of 100%), and the obtained results were compared to it.

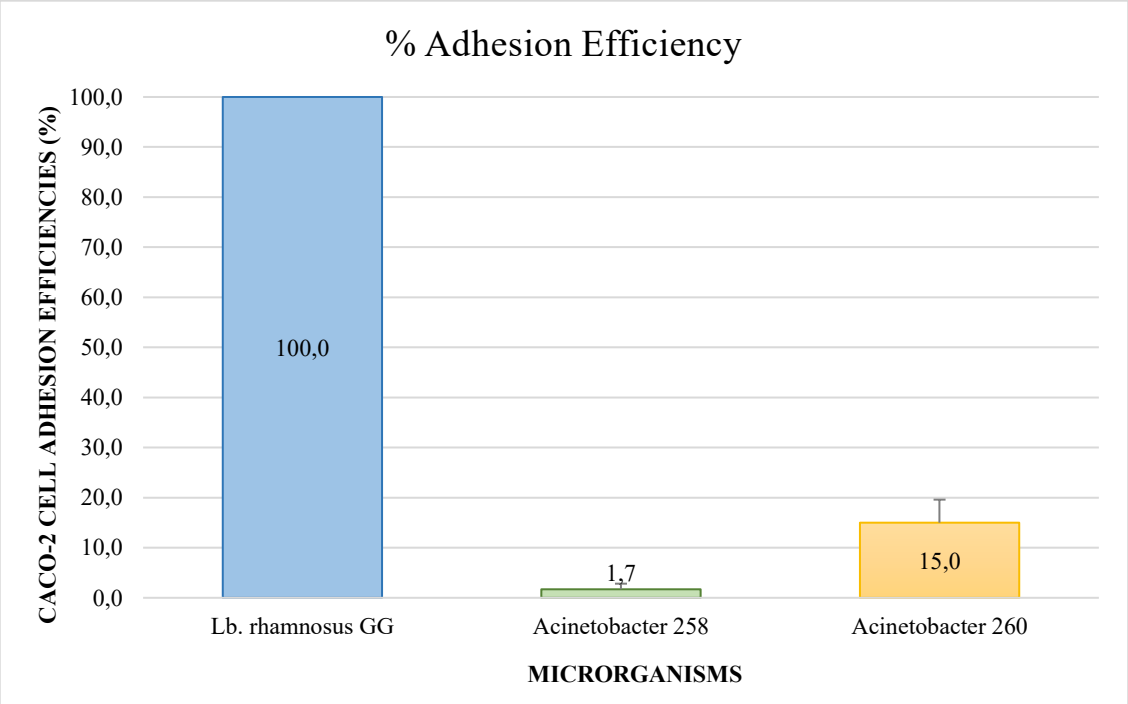


Figure 3.5.1.1: Adherence efficiency of *Acinetobacter* isolates 258 and 260 to CaCo-2 cells in comparison with the control *Lb. rhamnosus* GG. Error bars represent ± standard deviation.

After 2 h of incubation, the adhesion efficiencies of *Acinetobacter* 258 and 260 were 1.7 ± 1.1% and 15.0 ± 4.6% (Figure 3.5.1.1), respectively. *Acinetobacter* 260 was

about 80% less effective in adherence to CaCo-2 cells compared to positive control *Lb. rhamnosus* GG, but ten times more efficient than *Acinetobacter* 258 to adhere. Nevertheless, these values are even lower compared to the study developed by Lee et al. (2006) where the authors found adhesion percentages of 20 – 30% of clinical isolates of *A. baumannii* to bronchial epithelial cells with 1–2 bacteria per infected cell. In the same study, two types of adherence were observed: dispersed adherence to the cell's surface, the most frequent one, and clusters adherence in localized areas of the surface. Besides that, adherence mechanisms may involve structural features. One was the firm attachment of the bacteria to smooth areas of the epithelial cell surface, and the other mechanism was the entrapment of the bacteria by long protrusions extending from the epithelial cell surface; however, this last was detected in uninfected cells, which suggests that the presence of bacteria did not induce their formation. Authors also conclude there was a significant correlation between the clonal lineage and the percentage of infected cells due to one of them being more adherent than the other. Consequently, a wide variation in adherence among the strains was proved (Lee et al., 2006), which is concordant with this case since isolates 258 and 260 belong to two different clusters with 70.1% similarity (Figure 3.1.2) and exhibit different adhesion capacities (isolate 260 is 10% more efficient in adherence to a CaCo-2, comparing to the other isolate).

3.5.2. Invasion assay

In addition to cell adhesion, the invasion process has a crucial role in the pathogenesis of *Acinetobacter* spp. and, like many pathogenic bacteria, it can invade non-phagocytic cells and evolve to survive within the host cells. Although the underlying mechanisms of invasion are not completely explored until now, *A. baumannii* Outer Membrane Protein A (AbOmpA) seems to be the key to infection due to its capacity to interact with epithelial cells, induce apoptosis and facilitate the dissemination of bacteria into the bloodstream (Choi et al., 2008).

Figure 3.5.2.1 shows the results of invasion experiments for *Acinetobacter* isolates 258 and 260. *Listeria monocytogenes* 2959 was used as the positive control during the assays, which means that it was considered a 100% successful invasion, and the results obtained for *Acinetobacter* isolates were compared to it.

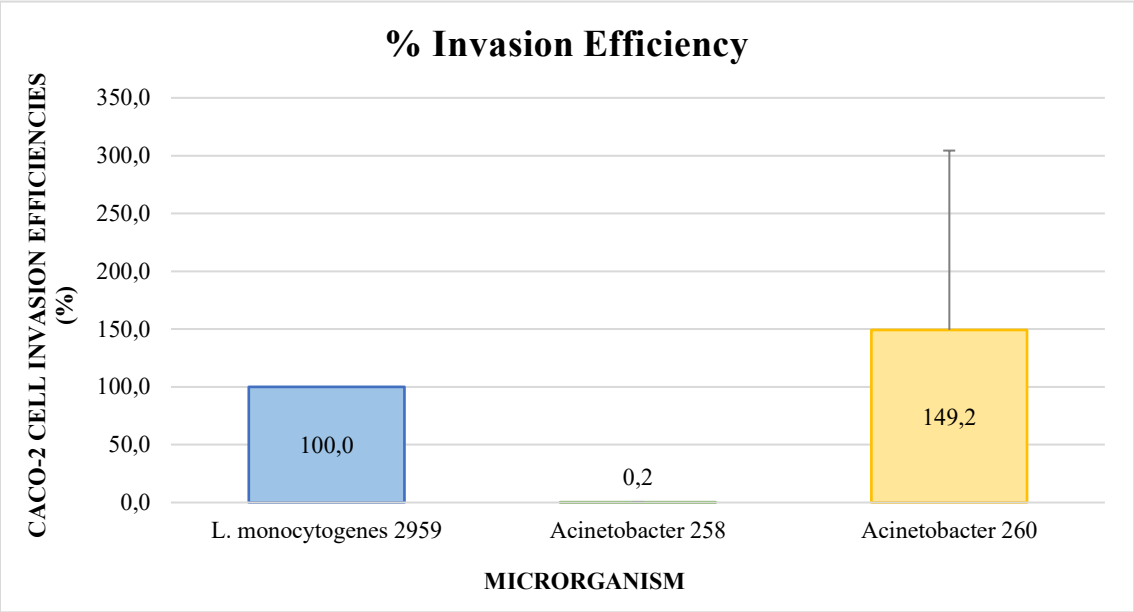


Figure 3.5.2.1: Invasion efficiency of *Acinetobacter* isolates 258 and 260 to CaCo-2 cells in comparison with the control *L. monocytogenes* 2959. Error bars represent ± standard deviation.

Isolate 258 exhibited an invasion efficiency of 0.2 ± 0.3%, while isolate 260 showed a notably higher invasion efficiency of 149.2 ± 155.2% in successfully invading CaCo-2 cells. Since both isolates exhibit low adherence rates (Figure 3.5.1.1), especially isolate 258 which was even less effective, the invasion rate was expected to be similar due to difficulties bonding to the cell membrane and consequently affect the invasion capacity. Relatively to isolate 258, this hypothesis was confirmed, with invasion efficiency nearly null, but a percentage of 149.2 ± 155.2 was achieved by isolate 260, which means that its capacity to invade epithelial cells was almost 50% higher than the positive control. In fact, Choi et al. (2008) reported that *A. baumannii* has a clear potential to invade epithelial cells, especially those derived from respiratory tract, which can explain its prevalence in this site. According to this study and bacteria morphology, *A. baumannii* invades cells by a zipper-like mechanism, as already mentioned (Choi et al., 2008). However, the established connection was loose and unstable – as demonstrated by Lee at. al. (2006) study – which suggests low adherence capacity by these pathogens, so further invasion is also jeopardize (Lee et al., 2006). Besides that, this study showed that after losing the connection, the cell membrane is extended to wrap around the bacteria,

but a microtubule inhibitor appears to also play a role by significantly inhibiting *A. baumannii* invasion to the cells (Choi et al., 2008).

However, the high standard deviations observed are one of the limitations of this technique; its low reproducibility can occur due to many variables used, such as bacteria, medium features or the technique itself (Baker, 2016; Liu et al., 2023).

Low adhesion and invasion to epithelial cells by *A. baumannii* may contribute to a less successful pathogenesis of this opportunistic microorganism that usually infects critically ill patients (Peleg et al., 2008). However, further studies should be developed with the same isolates (more replicates) and other isolates to increase knowledge about *Acinetobacter* ability to adhere and invade CaCo-2 cells.

4. General conclusions

4.1. Conclusion

Despite being a widely distributed species, the major concern is in clinical and hospital environments where *Acinetobacter* spp. are frequently present and often exhibit multi-drug resistance. Nevertheless, some species have been isolated from a variety of foods, leading to another concern: whether they can also be a foodborne disease agent or not. Literature on this topic is quite limited, but in this study, clinical isolates were submitted to several different assays in order to understand if *Acinetobacter* spp. can cause food-borne disease.

In this study, 264 clinical *Acinetobacter* isolates were genotyped by PFGE and 115 different profiles were obtained, which indicates a large variety and diversity among isolates. After antibiotic susceptibility characterization, 68 isolates out of 115 were multi-drug resistant, and 26 isolates exhibited a resistant pattern to all antibiotic classes tested.

Furthermore, when submitted to acidic conditions (pH 2.5) for a one-hour assay, the majority of the isolates were resistant, however some isolates were susceptible to the acid right at the beginning of the assay (7 log cycles reductions), but were able to recover at the end of the assay, such as isolates 60, 89, 158, 162 and 164. Fourteen isolates were chosen (including some that were not resistant to acidic conditions), and subjected to simulated gastrointestinal conditions to evaluate their capacity to survive. Those isolates were submitted consecutively to oral, gastric and intestinal fluids. Eleven isolates were more sensitive (4 log cycles reduction), but for three isolates, a high recovery rate was observed at the end of 4 hours' assay. Since no explanation was found in the literature, we consider the possibility of cells being able to enter a dormancy stage where metabolic rates are reduced when submitted to unfavourable conditions and when the environment changes, they can recover and be "alive". However, it is notorious that almost all of the isolates were able to resist gastrointestinal conditions, which leads to the conclusion that, even protected by fat or protein present in the food matrix, *Acinetobacter* spp. can go through the digestive system and remain viable.

Finally, the most resistant isolates to the previous experiment were studied in a cell culture assay with epithelial cells in order to understand if they are capable of adhering and invading, which is a potential cause of food-borne disease. According to this study, one isolate (*Acinetobacter* 260) exhibited a 50% higher invasion rate than the

control (an invasive strain of *L. monocytogenes*), even though the adherence rate was not that high (15%).

These claims and the lack of more literature about this topic make it possible to conclude that *Acinetobacter* spp. can resist gastrointestinal conditions. However, not every species will probably be able to infect epithelial gut cells and cause food-borne disease. In other words, not every species may be considered a food-borne disease agent; it will depend on their ability to invade cells, similar to what has already been observed with *A. calcoaceticus,* which is not considered a pathogenic microorganism.

4.2. Limitations to the study

Due to methodological and chronological incompatibilities, some aspects that would be interesting to study were not fully explored in this study.

Firstly, only resistant isolates to the simulated gastrointestinal tract were considered in a subsequent cell culture assay to evaluate their adherence and invasion capacity. Secondly, these two assays were developed independently and not consecutively, which means that bacterial cells used in cell culture experiments were grown in TSA-YE medium and not the resultant cells subjected to the simulated gastrointestinal conditions.

Additionally, due to Covid-19, it was difficult to fulfil the schedules since limitations in time and number of people in the laboratory were imposed, which caused a huge difficulty since the gastrointestinal procedure and cell culture are time-consuming methods. Also, due to those limitations and timings, we shorted the number of isolates submitted to the simulated gastrointestinal tract and cell culture assays.

4.3. Future Perspectives

Acinetobacter spp. are widely studied in the clinical context and accepted as nosocomial pathogens. However, developing more work within these bacteria regarding their hypothetical foodborne origin is also necessary. It would be interesting to perform more gastrointestinal assays with the remaining isolates in order to understand if they exhibit the same behaviour and to establish a connection between the resistance to harsh digestion conditions and different clusters originated by PFGE genotyping tools. In addition, this study should also include *Acinetobacter* isolates that have been isolated

from different foods (belonging to the laboratory's culture collection) to ascertain whether they behave similarly.

Another determinant step would be submitting the isolates to a gastrointestinal simulated process and then using the recovered cells in a further cell culture step to understand their capacity to adhere and invade even after injury, which is the most accurate scenario *in vivo* and close to reality.

Additionally, it would be interesting to understand the biggest barriers that bacteria face throughout digestive processes as well as the resistance mechanism present in these organisms, i.e., the reason(s) why most of the isolates present a reduction in viable cell numbers and then are able to recover or, conversely, why some isolates are not able to recover or are not even sensitive.

Furthermore, submitting other isolates to adherence and invasion cell culture assays is necessary, considering that only two isolates were tested and the discrepancy between the results. It is crucial to understand whether they can colonise and invade epithelial cells and whether this ability is related to the different profiles. It would also be interesting to understand why some strains are unable to colonize epithelial cells.

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