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Survival of clinical and food *Acinetobacter* spp. isolates exposed to different stress conditions

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ABSTRACT

Acinetobacter baumanni is recognized as one of the most important agents of nosocomial infections. Other species such as *Acinetobacter lwoffii* have also been associated with such infections. These species can be found in food products, such as vegetables, fruits and meats which can be a source of transmission of these organisms to community and hospital settings. Evidence that hospitals' kitchens are a route of entry of pathogenic and antimicrobial-resistant bacteria was recently demonstrated. This study aimed to determine whether different *Acinetobacter* spp. isolated from human and food samples (lettuce, turkey meat, apple and pear) were resistant to stress conditions often applied in food processes, such as exposure to 60 °C, AMUKINA® and vinegar. Also the influence of food matrices on the behavior of isolates to these stress conditions was evaluated. Treatment with AMUKINA® and vinegar were effective against all clinical and food isolates. Exposure to 60 °C resulted in the reduction of the majority of isolates to values below the detection limit of the enumeration technique, however, it is important to note that most of the reductions only occurred after 30 min of exposure. One food isolate identified as *A. baumanni* was resistant to this thermal treatment and one clinical isolate only decreased 4 log cycles after 1 h. In general, food isolates were demonstrated to be more resistant than clinical isolates and no significant differences ($p > 0.05$) were found between *A. baumanni* and *A. lwoffii* species. With the exception of one food isolate that was more resistant to thermal stress in the presence of turkey meat, the food matrices investigated did not confer protection to the applied stresses. Due to the limited knowledge on this topic, we believe that this study is an important contribution to understanding the behavior of *Acinetobacter* spp. when exposed to treatments commonly applied to foods.

1. Introduction

Acinetobacter spp. are commonly found in water and soil, but can also be found in contaminated areas, sewage, dumpsites, food and animals (Atrouni et al., 2016). The majority of the *Acinetobacter* species are isolated from meat and vegetables, but they can also be found in several types of foods such as fruits, cheese, milk, fish, shrimps, water and rice (Berlau et al., 1999; Carvalheira et al., 2017a, 2017b).

Members of the *Acinetobacter baumannii* group (*A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. seifertii*) are major agents of nosocomial infections (Madigan et al., 2009). Other species non-*A. baumannii*, such as *A. lwoffii*, have been also sporadically been causative agents in such infections (Turton et al., 2010). Cases of bacteremia caused by *Acinetobacter* spp. have been increasingly reported in hospital settings (Chen et al., 2002, 2015).

When introduced into the hospital environment, either via the food from the hospital's kitchens or by the food, mainly fruits, that the visitors offer to the patients (a common practice in Portugal), contaminated foods could be a vehicle for the dissemination of these organisms into hospital settings. Being resistant to desiccation, *A. baumannii* may persist, becoming resident in the hospital environment (Jawad et al., 1998). According to Lazarević et al. (2013), *Acinetobacter* spp. was one of the most commonly isolated bacteria from hospital kitchens with poor systems of sanitation.

As previously mentioned, although the presence of *Acinetobacter* spp. in different foods (Berlau et al., 1999; Carvalheira et al., 2017a, 2017b) has been demonstrated, there is still no evidence that these microorganisms are foodborne pathogens (Amorim and Nascimento, 2017). Moreover, the few studies reporting the association of *Acinetobacter* spp. with foodborne illnesses are merely related with individuals in the risk groups (Grotiuz et al., 2006; Polanco and Manzi, 2008).

Good hygiene and handling practices associated with food

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processing as well as disinfection of ready-to-eat products, such as vegetables and fruits, are very important in order to avoid and/or reduce contamination (Feás et al., 2014). A number of methods to disinfect food are well known. Vinegars, or acetic acid solutions, are commonly used to disinfect fruits and vegetables. Several authors mentioned that treatments with vinegar protect these foods from foodborne pathogens such as *Escherichia coli, Salmonella* Typhimurium and *Listeria monocytogenes* (Rhee et al., 2003; Wu et al., 2000). Commercial chlorine-based products (mainly sodium hypochlorite) are available and used for the same purpose (Feás et al., 2014). AMUKINA® (Angelini) can be used to disinfect fruits and vegetables, but several reports have questioned its efficacy (Bachmann and Earles, 2000; Hinenoya et al., 2015). In other types of food matrices, such as raw meats, the methods applied are quite different. Since pre-history high temperatures (e.g. cooking), are used to prepare and preserve foods. The recommended temperatures to completely destroy bacteria range from 60 °C to 74 °C (Taché and Carpentier, 2013).

The aims of this study were to evaluate: i) if *A. baumannii and A. lwoffii* isolates, recovered from human and food samples, were susceptible to thermal processing methods, such as exposure to 60 °C, and to disinfection methods of vegetables and fruits, such as exposure to AMUKINA® and vinegar, and ii) the influence of food matrices on the behavior of isolates to these stress conditions. Since these microorganisms could be found in foods it is extremely important to know more about the efficacy of these methods on their elimination in order to ensure that these foods cannot be a vehicle of new contamination.

2. Materials and methods

2.1. Origin and growth conditions of isolates

Eleven isolates of *Acinetobacter* spp. deposited in the culture collection of CBQF - *Escola Superior de Biotecnologia* (Table 1) were selected: five recovered from human samples, kindly supplied by *Hospital de S. Marcos* (Braga, Portugal); and six recovered from food products (Carvalheira et al., 2017a, 2017b). Isolates were stored at −80 °C in Tryptic Soy Broth (TSB, Pronadisa, Madrid, Spain) with 30% (v/v) of glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before use in TSB at 30 °C for 24 h.

2.2. Survival of different stress conditions

2.2.1. Inoculum

One colony from TSA incubated at 30 °C for 24 h, was transferred to TSB and incubated in the same conditions. To prepare the final inoculum, the last culture was transferred to fresh TSB (1:10) and incubated at 30 °C for 24 h. Cells were collected by centrifugation (7000 rpm, 10 min; Rotina 35R, Hettich, Germany) and re-suspended in the same volume of sterile quarter strength Ringer's solution (Lab M, Lancashire, United Kingdom) in order to obtain a final level of 10^5–10^6 colony forming units (CFU)/ml. Some strains had a low growth capacity, whereby the inoculum obtained had a final level of 10^3–10^4 CFU/ml.

Table 1
Description of the isolates used in this study.

Isolate	Origin	*Acinetobacter* species	References
A8	Human	*A. baumannii*	kindly supplied by *Hospital de S. Marcos*
A21	Human	*A. baumannii*	
A27	Human	*A. baumannii*	
A46	Human	*A. baumannii*	
A105	Human	*A. lwoffii*	
17.3	Lettuce	*A. baumannii*	Carvalheira et al., 2017 b
18.6	Lettuce	*A. baumannii*	
103.2	Apple	*A. baumannii*	
123.2	Pear	*A. lwoffii*	
133.1	Raw turkey meat	*A. baumannii*	Carvalheira et al., 2017 a
133.2	Raw turkey meat	*A. lwoffii*	

2.2.2. Simulated conditions of stress

Three stress conditions were tested by exposing the isolates to 60 °C and to the presence of vinegar and AMUKINA® (Angelini, Rome, Italy). Aliquots of 0.5 ml of the inoculum (prepared as described above) were placed into glass flasks with i) 49.5 ml of sterile distilled water and kept at 60 °C for thermal stress, ii) 42 ml of sterile distilled water and 7.5 ml of vinegar (15% (v/v)) for acidic stress and iii) 48.5 ml of sterile distilled water and 1 ml of AMUKINA® (1% (v/v) sodium hypochlorite, according to the product instructions) for chemical stress. Glass flasks containing cells exposed to the chemical stress conditions were placed at 25 ± 5 °C. All the samples were taken at time 0 (time of inoculation) and during 15 min (for acidic stress with AMUKINA®) or during one hour (for other stresses). For each experiment an aliquot of 0.5 ml of inoculum was placed into glass flasks with 49.5 ml of sterile distilled water at 30 °C and used as control, to ensure that the effect on survival was due to the different conditions applied. Each experiment was performed in duplicate.

2.2.3. Simulated conditions of stress in the presence of food matrices

Survival of the isolates to the stress conditions was also evaluated in the presence of food matrices: acidic and chemical stresses were in the presence of lettuce, pear and apple and thermal stress only for turkey meat. Moreover, the behavior of clinical isolates was tested in the presence of all food matrices, whereas each food isolate was only studied in the presence of their food source matrix (Table 1).

The food matrices lettuce, pear and apple were superficially disinfected according to Carvalheira et al. (2017b), while turkey meat was triturated and autoclaved.

For the food matrices lettuce, pear and apple, 0.5 ml of each inoculum (prepared as described above) was mixed with 5 g of each disinfected food matrix and, after 24 h of contact in refrigerated conditions (lettuce) or room temperature (fruits), the different stresses - acidic and chemical - were applied as previously described. For turkey meat, 0.5 ml of each inoculum was mixed with 5 g of this matrix and, after 10 min of contact at room temperature (Barbosa et al., 2014), the thermal stress was applied as described above. As controls, 0.5 ml of each inoculum was mixed with each particular matrix in glass flasks and, after the appropriate time of contact, 49.5 ml of sterile water was added and, while were kept at 25 ± 5 °C, all the samples were taken at the same times for each stress condition. To guarantee the efficient disinfection of fruit and lettuce, the same was done, but without the addition of inoculum.

Each experiment was done in duplicate.

2.3. Enumeration

Each sample was diluted in sterile quarter strength Ringer's solution and plated on TSA, in duplicate, by the drop count technique (Miles and Misra, 1938). After incubation at 30 °C for 24 h, the colonies were counted and the CFU/ml calculated.

2.4. Statistical analysis

An analysis of variance was carried out to test the effect of each stress condition on the survival of different *Acinetobacter* isolates as well as any significant effect of the different food matrices used.

Microbial counts were transformed to logarithmic reduction using the equation: log (N/N0), where N is the microbial cell count at a particular sampling time and N0 is the initial cell density. Two independent assays were carried out.

Multiple comparisons were evaluated by Tukey's post-hoc test and all analyses were performed using IBM SPSS Statistics, 24 (IBM Corporation, USA). The mean difference was considered significant at the 0.05 level.

3. Results and discussion

The choice of isolates was made according to their origin - would the clinical *Acinetobacter* spp. isolates be more resistant than food isolates? – and also to their species – would be *A. baumannii* more resistant than *A. lwofii*? Consequently, stress conditions were chosen taking into account the origin of food isolates: i) temperature of 60 °C due to the existence of turkey meat isolates, simulating an inappropriate temperature of cooking (what happens inside a hamburger, for example) and by the existence of food isolates from lettuce, apple and pear ii) the treatment with AMUKINA®, which is a product useful for the disinfection of fruits and vegetables and, according to the manufacturer, is able to eliminate bacteria and germs and iii) the treatment with vinegar, which, besides being commonly used in salad dressings, it is also used for the disinfection of fruit and vegetables.

To our knowledge, this is the first study about the behavior of *Acinetobacter* spp. to various stress conditions to which foods can be exposed in order to reduce/eliminate microbial contamination. *Acinetobacter* spp. are found in foods (Berlau et al., 1999; Carvalheira et al., 2017a, 2017b). If they are resistant to treatments commonly used to eliminate bacterial contaminants from foods, e.g. high temperatures, washing/disinfection, contaminated products may be a vehicle of dissemination of these organisms.

The survival of clinical and food isolates (Arabic numbers), respectively, to each stress condition (capital letters) in the absence of the food matrix is shown in Figs. 1 and 2.

Thermal stress was not effective for all the isolates tested. Clinical isolates (Fig. 1), were reduced to values below the detection limit of the enumeration technique between 30 and 45 min of exposure (Fig. 1; graphs B1 to B4), with the exception of isolate A105 (Fig. 1; graph B5), which was only reduced by 4 log cycles after 60 min. Among food isolates (Fig. 2), despite three of the six isolates (Fig. 2; graphs B3, B4 and B5) being more sensitive to 60 °C than clinical isolates, isolate 133.2 (Fig. 2; graph 6) took 60 min to be reduced by 5 logs and food isolate 17.3 (Fig. 2; graph B1) was able to resist the full 60 min with logarithmic reductions of less than 0.5 log units. Monu et al. (2015) described the efficacy of thermal treatment at 60 °C against *Listeria monocytogenes* and *Salmonella* Typhimurium with D-values between 0.16 and 0.66 min. Nonetheless, exposure to 60 °C was not sufficient to eliminate all of the food *Acinetobacter* isolates tested.

Concerning the exposure to vinegar, all isolates were sensitive and similar reductions were observed among clinical and food isolates ($p > 0.05$). In fact, sensitive clinical isolates were totally reduced to undetectable values after 30 s to 10 min (Fig. 1; graphs C1 to C5), while sensitive food isolates were reduced after 5–10 min (Fig. 2; C1 to C6). Besides the lack of information about the effect of vinegar on *Acinetobacter* spp., Ramos et al. (2014) found that 15% (v/v) white wine vinegar (the same proportion used in this study) had a bactericidal effect against *L. monocytogenes* isolated from lettuce.

Treatment with AMUKINA® was also effective for all isolates, allowing reductions to values below the detection limit of the enumeration technique after 30 s for both clinical (Fig. 1; graphs D1 to D5) and food isolates (Fig. 2; graphs D1 to D6). In the study of Karumathil et al. (2014), the authors tested the effect of five free chlorine concentrations (0.2, 1, 2, 3 and 4 ppm) separately on the survival of 8 clinical multidrug resistant *A. baumannii* during 30, 60, 90 and 120 s. The authors found that all isolates survived the tested conditions and, in addition, a possible chlorine-associated induction of antibiotic resistance was observed (Karumathil et al., 2014).

Comparing the survival after exposure to vinegar and AMUKINA®, despite both being effective, AMUKINA® eliminated *Acinetobacter* isolates more rapidly.

No relation was found between species and origin of isolation ($p > 0.05$). Survival of the isolates to each stress condition was also evaluated in the presence of food matrices.

The effect of turkey meat matrix on the survival of all clinical isolates and two food isolates at 60 °C is shown in Fig. 3. With the exception of one food isolate identified as *A. lwoffii* (133.2), which was reduced by 2 log cycles after 60 min (Fig. 3, graph 6) against 5 log reduction obtained in the same period of time in the absence of the food matrix (Fig. 2, graph 6), in general, this matrix did not confer any protection in the survival of the isolates to this thermal treatment.

Among clinical isolates (Fig. 3, graphs B1 to B5), no significant differences were obtained in their survival ($p > 0.05$). The reductions were below the detection limit of the enumeration technique and occurred earlier than with the absence of turkey meat matrix (Fig. 1). Despite sensitive, reductions below detectable values of clinical isolate A105 (Fig. 3, graph 5) were verified only after 60 min. For the two food isolates, both originating from this meat matrix, only 133.2 (Fig. 3, graph B7) was able to resist this thermal stress after incorporation in

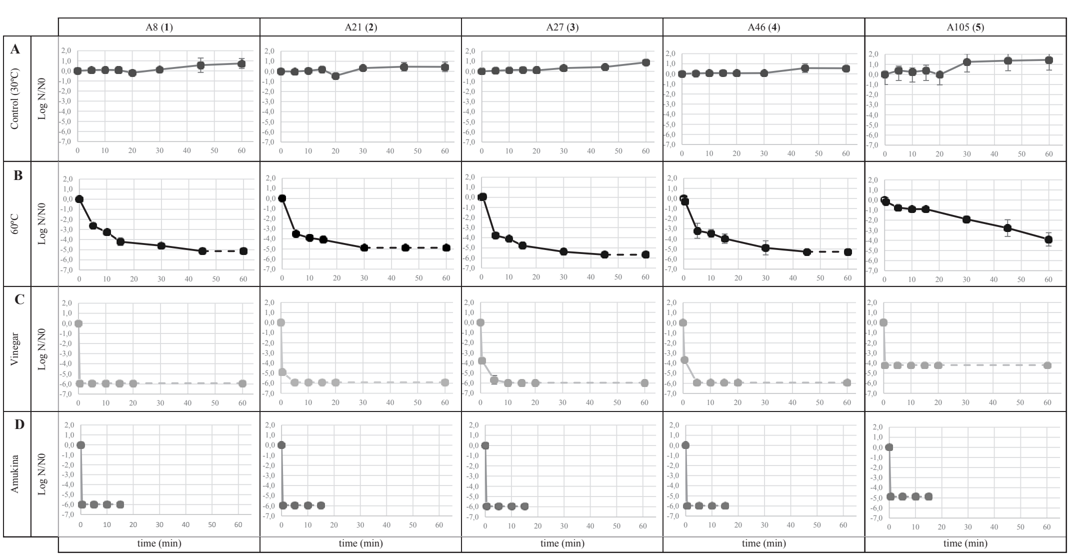

Fig. 1. Logarithmic reduction of clinical *Acinetobacter* spp. (1–5) in the absence of food matrix and for different conditions applied (A, B, C or D): (—) control (30 °C); (—) thermal stress (60 °C); (—) acidic stress (vinegar) and (—) acidic stress (AMUKINA®). The dotted lines mean that the isolate was reduced to values below the detection limit of the enumeration technique.

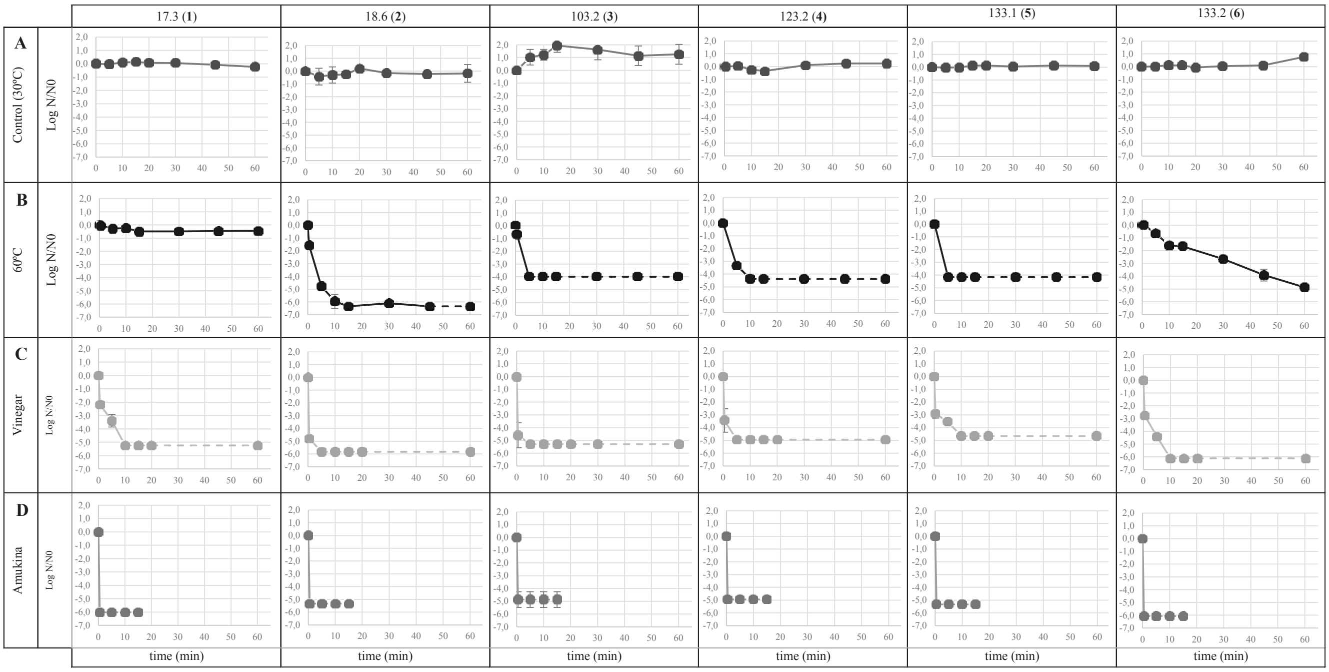

Fig. 2. Logarithmic reduction of food *Acinetobacter* spp. (1–6) in the absence of food matrix and for different conditions applied (A, B, C or D): (—) control (30 °C); (—) thermal stress (60 °C); (—) acidic stress (vinegar) and (—) acidic stress (AMUKINA®). The dotted lines mean that the isolate was reduced to values below the detection limit of the enumeration technique.

that food matrix. Although the negative effects conferred by the treatment with high temperatures leading to nutrient losses (Durek et al., 2014), it was expected that meat could protect isolates because it is a matrix rich in fat, proteins and minerals (DeGeer et al., 2016). However, other authors had mentioned that microorganisms inoculated into different meats did not survive high temperatures (Gurman et al., 2016; Juneja et al., 2001). Juneja et al. (2001) found that different *Salmonella* spp. in chicken broth rapidly exhibited logarithmic reductions at 60 °C, with D-values from 0.83 to 1.02 min.

Once again, it is important to highlight that exposure to 60 °C was not sufficient to eliminate all of the food *Acinetobacter* isolates tested and, more importantly, consumers do not cook the meat for 1 h, much less if they grill a turkey steak. This means that viable *Acinetobacter* spp. present in meat products may be ingested by consumers.

The survival of isolates to vinegar and AMUKINA® exposure was also tested in the presence of pear, apple and lettuce matrices.

Survival of all clinical isolates and one food isolate after vinegar and AMUKINA® exposure in the presence of pear matrix is presented in Fig. 4.

For the treatment with vinegar (Fig. 4; graphs B1 to B6) none of the isolates survived, as had occurred in the absence of any matrix. Two clinical isolates not exposed to a food matrix - A8 and A46, which were reduced to undetectable values before and after 5 min, respectively (Fig. 1, graphs B1 and B4), in the presence of pear matrix their reductions to undetectable values were obtained only after 10 min. However, those differences were not significant ($p > 0.05$).

All isolates were also sensitive to the treatment with AMUKINA® (Fig. 4; graphs C1 to C6), presenting the same behavior without matrix, with reductions to values below the detection limit of the enumeration technique after 30 s. So, pear matrix did not confer any protection, probably due to its composition, which could act as an additional stress.

Concerning the effect of apple matrix, it was observed that the food isolate 103.2, isolated from apple, was not able to survive after exposure of vinegar and AMUKINA® in the presence of this matrix (data not shown). Apparently, the acidic stress conferred by the matrix followed by acidic stresses applied resulted in a lethal condition.

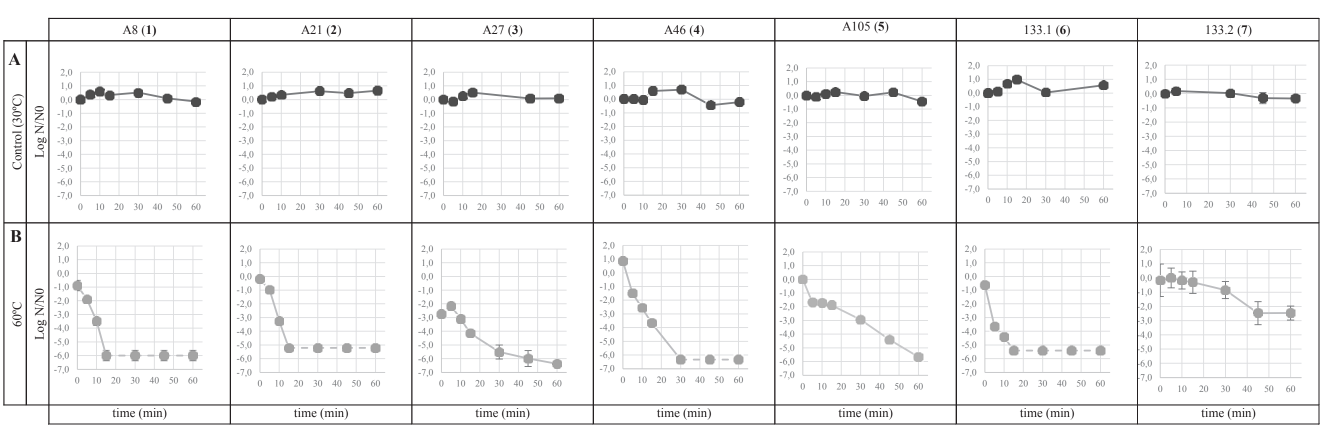

Fig. 3. Survival of all clinical *Acinetobacter* spp. (1–5) and two food *Acinetobacter* spp. (6 and 7) in the presence of turkey meat matrix and for two conditions applied (A and B): (—) control (30 °C) and (—) thermal stress (60 °C). The dotted lines mean that the isolate was reduced to values below the detection limit of the enumeration technique.

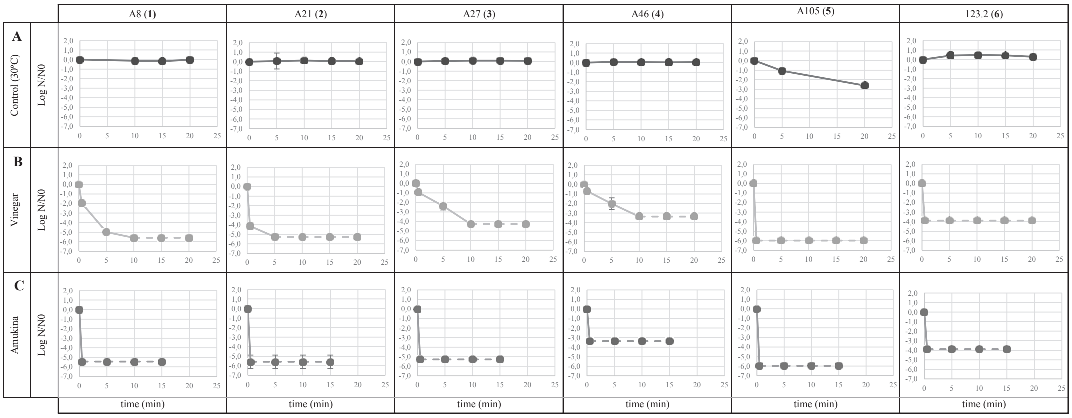

Fig. 4. Survival of all clinical *Acinetobacter* spp. (1–5) and one food *Acinetobacter* spp. (6) in the presence of pear matrix and for different conditions applied (A, B and C): (—) control (30 °C); (—) acidic stress (vinegar) and (—) acidic stress (AMUKINA®). The dotted lines mean that the isolate was reduced to values below the detection limit of the enumeration technique.

Raybaudi-Massilia et al. (2009) had already demonstrated the antimicrobial proprieties of apple juice with the inhibition of growth of *L. monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7 when stored at 20 °C and 35 °C in apple juice.

The survival of all clinical isolates and two food isolates in the presence of lettuce matrix after vinegar and AMUKINA® exposure is shown in Fig. 5. The behavior of isolates treated with vinegar (Fig. 5, graphs B1 to B7) in the presence of lettuce matrix was the same as in the absence of the matrix, since all isolates were reduced to undetectable values ($p > 0.05$). Survival of all isolates was also affected by the treatment with AMUKINA® (Fig. 5; graphs C1 to C7), as previously observed without food matrix (Fig. 1; graphs D1 to D5 and Fig. 2; graphs D1 to D6). This means that also lettuce did not confer any protection.

The treatment with vinegar and AMUKINA® was demonstrated to be a good way to disinfect fruit and lettuce contaminated with *Acinetobacter* spp.. In the study of Gutiérrez-Alcántara et al. (2015), the authors used acetic acid to disinfect two types of tomatoes contaminated with *S.* Typhimurium and *Salmonella* Typhi, and also observed logarithmic reduction, but near 1 log cycle. Besides the different composition of the vegetable matrix used, the authors just used 0.5% (v/v) of acetic acid (Gutiérrez-Alcántara et al., 2015). In the same study, logarithmic reductions of the same order of magnitude were obtained after the use of 10% (v/v) sodium hypochlorite solution as disinfectant (Gutiérrez-Alcántara et al., 2015). However, 1% (v/v) sodium hypochlorite from AMUKINA® used in our study was effective against *Acinetobacter* spp.. Silveira et al. (2017) showed that washing lettuce with 200 mg/l sodium hypochlorite was effective against *Salmonella* Enteritidis. It would be interesting to test lower concentrations of AMUKINA® and confirm whether its ability to inhibit *Acinetobacter* isolates is maintained.

The lack of information about the behavior of *Acinetobacter* isolates through exposure to different stresses means that our results are relevant and could be a starting point to extend this type of study to other *Acinetobacter* isolates.

4. Conclusion

Despite the small number of isolates tested in this study, exposure to vinegar and AMUKINA® was effective for the elimination of the *Acinetobacter* spp. isolates. Therefore, in order to avoid food products being a vehicle of transmission of these organisms, 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. Some *Acinetobacter* spp. can also be eliminated from food products such as meat by thermal processing, at 60 °C, however, 60 min may not be

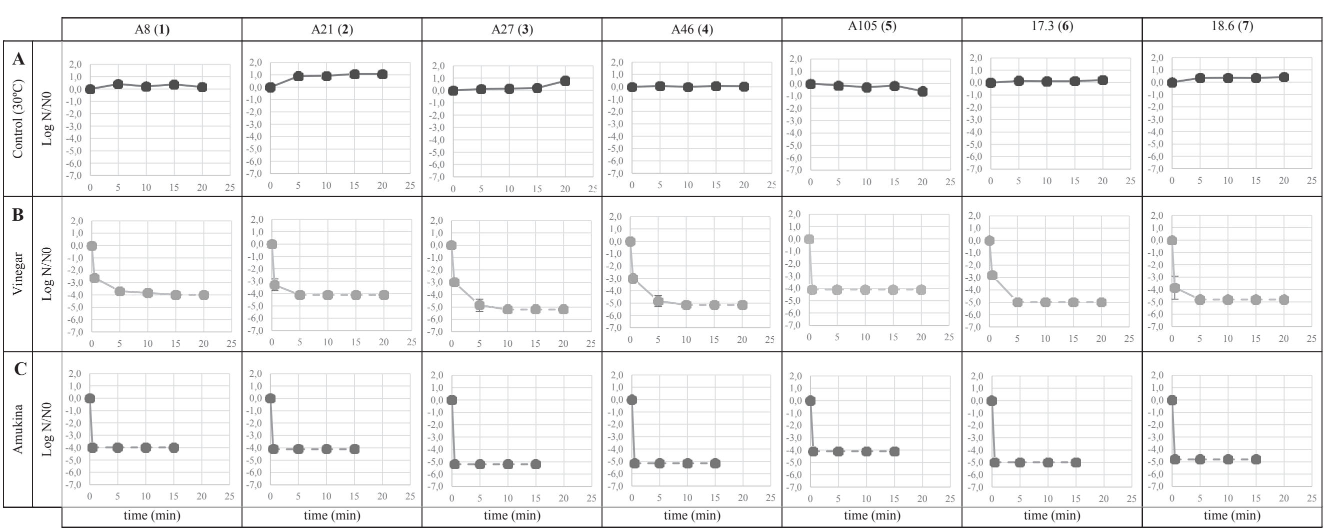

Fig. 5. Survival of all clinical *Acinetobacter* spp. (1–5) and two food *Acinetobacter* spp. (6 and 7) in the presence of lettuce matrix and for different conditions applied (A, B and C): (—) control (30 °C); (—) acidic stress (vinegar) and (—) acidic stress (AMUKINA®). The dotted lines mean that the isolate was reduced to values below the detection limit of the enumeration technique.

enough. One of the *Acinetobacter* food isolates was able to survive more than 60 min at this temperature.

Moreover, any food matrix conferred protection to the survival of the isolates when the different stress conditions were applied, with exception of only one food isolate that was shown to be more resistant to the thermal stress in the presence of turkey meat matrix.

Based on these results, it is important to validate the effect of these same stresses on other *Acinetobacter* strains. In terms of thermal stress, other temperatures and times should also be applied, as well as other concentrations of vinegar and AMUKINA® with other exposure times. Also studying the ability of *Acinetobacter* strains, resistant to these stresses, in surviving through the passage of the gastrointestinal tract would be a good starting point to understand if these microorganisms can reach the intestine and, perhaps, cause infection.

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