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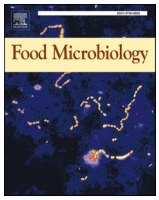

Assessment of the presence of *Acinetobacter* spp. resistant to β-lactams in commercial ready-to-eat salad samples

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ABSTRACT

Acinetobacter baumannii is a well-known nosocomial infection causing agent. However, other *Acinetobacter* spp. have also been implicated in cases of human infection. Additionally, these bacteria are known for the development of antibiotic resistance thus making the treatment of the infections they cause, challenging. Due to their relevance in clinical setups less attention has been paid to their presence in foods, and its relation with infection/dissemination routes. In the current study commercial Ready-To-Eat (RTE) salads were analyzed seeking for antibiotic resistant *Acinetobacter* spp. A preliminary screening allowed us to recover Gram-negative bacteria resistant to β – lactams using cefotaxime, third generation cephalosporins, as the selective agent, and this was followed by identification with CHROMagar™ Acinetobacter and 16S rDNA sequencing. Finally, the isolates identified as *Acinetobacter* spp. were reanalyzed by PCR to determine the presence of nine potential Extended Spectrum β Lactamases (ESBL). Two commercial RTE salad brands were included in the study (2 batches per brand and 8 samples of each batch making a total of 32 independent samples), and compared against an organic lettuce. High concentrations of β – lactam, resistant bacteria were found in all the samples tested (5 log CFU/g). Additionally, 209 isolates were phenotypically characterized on CHROMagar Acinetobacter. Finally, PCR analysis identified the presence of different ESBL genes, being positive for *bla*ACC, *bla*SHV, *bla*DHA and *bla*VEB; out of these, *bla*ACC was the most prevalent. None of the isolates screened were positive for more than one gene. To conclude, it is important to highlight the fact that pathogenic species within the genus *Acinetobacter* spp., other than *A. baumannii*, have been identified bearing resistance genes not typically associated to these microorganisms highlight the importance of continuous surveillance.

1. Introduction

Fresh vegetables are an important source of nutrients and vitamins. The demand for ready-to-eat (RTE) vegetables has increased over the years particularly those which are additive-free (Castro-Ibáñez et al., 2017). However, this seek for "natural" presentations may be behind of the observation of an increased number of foodborne diseases associated to the consumption of fresh produce (Sant'Ana et al., 2014; Thomas et al., 2023). The open nature of the fresh produce chain facilitates contamination of the foods either at the production stage, or harvesting, of post-harvesting, reaching the consumers (Finger et al., 2023; Murray et al., 2017). Irrigation and process water, manure, sewage sludge or wild life can transfer pathogen and antimicrobial resistance bacteria to produce (Koutsoumanis et al., 2021).

Antimicrobial resistance (AMR) is classified among the top 10 public health threat for humanity and the World Health Organization (WHO) has reported that major gaps exist in surveillance related to the emergence of AMR in foodborne bacteria (WHO, 2021). Nowadays, β-lactam antibiotics are the most widely used antibacterial agents to treat infections, they account for 65 % of all the prescriptions for injectable

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antibiotics in the United States (US), among them, cephalosporins represent nearly one half (Bush and Bradford, 2016). Certain cephalosporins have been banned by the Food and Drug Administration (FDA) for their usage in food-producing animals. However, high levels of resistant bacteria, continue to be isolated. In line with this, a specific concern was highlighted by the WHO and the European Centre for Disease Prevention and Control (ECDC) the resistance of Gram-negative bacteria to β-lactams (EFSA & ECDC, 2018; WHO, 2014). Acquired resistance to β-lactams is mainly mediated by the extended-spectrum β-lactamases (ESBL) that confer resistance to all β-lactams except for carbapenems and cephamycins (Coque et al., 2008; Sawa et al., 2020).

The genus *Acinetobacter* is widely distributed in the environment, and of all species that compose the *Acinetobacter calcoaceticus – baumanii* complex, *A. calcoaceticus, A. baumanii and A. pitti* (Villalón et al., 2019), are widely related to nosocomial infections at a global level (Murray et al., 2022). *A. baumannii* isolated in the last decades of the last century were generally sensitive to most antimicrobial agents (Doi et al., 2015). However, the indiscriminate use of antibiotics has exponentially increased the number of multidrug-resistant strains. The global analysis of antimicrobial resistance-related deaths indicates that 132,000 are attributable to this pathogen, being the fourth most common bacterium causing such deaths (Murray et al., 2022). The spread of multidrug-resistant strains of *A. baumannii* in recent decades has resulted in severe hospital cases requiring intensive care (Cavallo et al., 2023; McConnell et al., 2013). The β-lactam carbapenemics, such as imipenem and meropenem, have been antimicrobials of choice in these infections. However, in recent years, resistant strains have increased. In Spain, more than 70 % of the *A. baumannii* strains of the clinical isolates are carbapenem-resistant, indicating the risk posed by infections with this pathogen (Murray et al., 2022). Additionally, it is important to note that *A. baumanii* exhibits natural transformation to acquire resistance (Domingues et al., 2019).

A report on the prospection of biological hazards of interest in food safety elaborated by the Scientific Committee of the Spanish Agency for Food Safety and Nutrition emphasizes the need to determine the prevalence of multi-resistant bacteria as *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *A. baumannii* (Franco Abuín et al., 2023). For instance, a prevalence of 39.4 % *A. calcoaceticus – baumanii complex* (Acb) and 20 % of *A. baumannii* was observed dairy products from Brazil (Rabêlo et al., 2021) and fresh meat from Iran (Askari et al., 2020) respectively. But *Acinetobacter* can be also present in RTE products such as vegetables and fruits. Carvalheira et al. found that the 77.9 % of samples analyzed were positive to *Acinetobacter* with a prevalence of *A. baumannii* of 11 % (Carvalheira et al., 2017). But it is not only important to determine its prevalence but also its resistance. Bezanson et al. observed the presence of antimicrobial resistant *A. baumannii* carrying Class 1 and 2 Type integron, responsible from horizontal transmission of antimicrobial resistance in raw salad vegetables (Bezanson et al., 2008). In a recent study from conducted in organic, fresh produce samples from Spain, *A. baumannii* isolates resistance to β-lactam antibiotics, including carbapenemics imipenem and meropenem, were identified (Jiménez-Belenguer et al., 2023). Also, *A. baumannii* strains from RTE salads showed tolerance to common biocides used in the food industry as sodium hypochlorite or peracetic acid (Fernandes et al., 2022). Up to date, the role of foods a source of human infections, and dissemination, of *Acinetobacter* spp. in healthcare setting is not yet clear (Carvalheira et al., 2021).

The aim of the current study was to evaluate the presence of β – lactam resistant *Acinetobacter* spp. in commercial RTE salads. In addition to this, it was also attempted to elucidate the ESBL determinants involved. In parallel, the results of the commercial presentations were compared with those of organic lettuce collected from a local farmer.

2. Materials and methods

2.1. RTE salads sampling

Two different commercial RTE salad brands, from now on B1 and B2, were purchased from a local supplier in Braga, Northern Portugal. From each brand two different lots were selected and eight independent bags were collected, representing a total of 32 independent samples. In parallel to these, eight organic lettuce, from now on OL, samples were also collected from a local producer (Guimarães, Northern Portugal). All the samples were processed within the expiration date, and all visibly damaged leaves were excluded. During the process the samples were stored at 4 °C until analysis.

2.2. Microbiological analyses

The quality of the RTE salads was assessed based on the concentration of mesophilic bacteria and antibiotic resistant microorganisms (ARM). The colonies recovered from ARM analysis were used for the determination of *Acinetobacter* spp. Fig. 1 summarizes the workflows followed. All samples analyzed consisted on 25 g of RTE salad sample were mixed with 225 mL of Luria Bertani broth (LB, PanReac AppliChem, Barcelona, Spain), homogenized for 30 s in a Stomacher 400 circulator (Seward Limited, West Sussex, UK), and then specific protocols were followed as detailed below.

2.2.1. Mesophilic counts

The enumeration of mesophilic microorganisms was performed as a quality indicator following the ISO 4833 with minor modifications (ISO, 2013). From the initial matrix prepared as detailed above, ten-fold serial dilutions were performed in LB and plated on Plate Count Agar (PCA, Biokar Diagnostics S.A., France). The plates were incubated at 30 °C for 72 h after which all the colonies were enumerated.

2.2.2. Enumeration of antibiotic resistant microorganisms (ARM)

The enumeration of ARM was performed as described by Mir et al. (2016). In brief, taking advantage of the matrix prepared in Materials & Methods (M&M) Sections 2.2 and 2.2.1, the dilutions were plated on MacConkey (Difco, BD & Co., East Rutherford, NJ) supplemented with 4 μg/mL of cefotaxime (Fisher BioReagents), from now on Mac + c. The plates were incubated at 37 °C for 24 h, and after the indicated time all colonies were counted. The rest of the matrix was incubated at 37 °C for 24 h and on the following day a loopful was streaked on Mac + c, the plates were incubated as detailed above.

Five colonies per sample were re-isolated on Mac + c and re-incubated as above. The purified colonies were subcultured on Trypto-Casein Soy Agar culture medium (TSA, Biokar Diagnostics S.A., France) and incubated at 37 °C for 24 h. The TSA-purified colonies were used to prepare glycerol stocks (30 % glycerol 70 % fresh bacterial culture prepared as detailed below).

The assessment of the presence of *Acinetobacter* spp. was performed by recovering the bacteria from the glycerol stocks (scraping the frozen stock with a sterile loop and inoculating fresh Nutrient Broth (NB, Biokar Diagnostics S.A., France) which was incubated at 37 °C overnight). These newly prepared fresh cultures, directly obtained from the glycerol stocks were streaked CHROMAgar™ Acinetobacter (Chrom, CHROMagar Microbiology, Paris, France) to determine the presence of this pathogen. Chrom plates were incubated at 37 °C for 24 h, and screened for typical red colonies.

Fresh cultures from each TSA isolate were prepared by resuspending one single colony in 4 mL of NB. The tubes were incubated at 37 °C for 24 h. The glycerol stocks, for long-term storage of the isolates was performed by mixing the fresh culture with a final concentration of 30 % glycerol and these were stored at −80 °C.

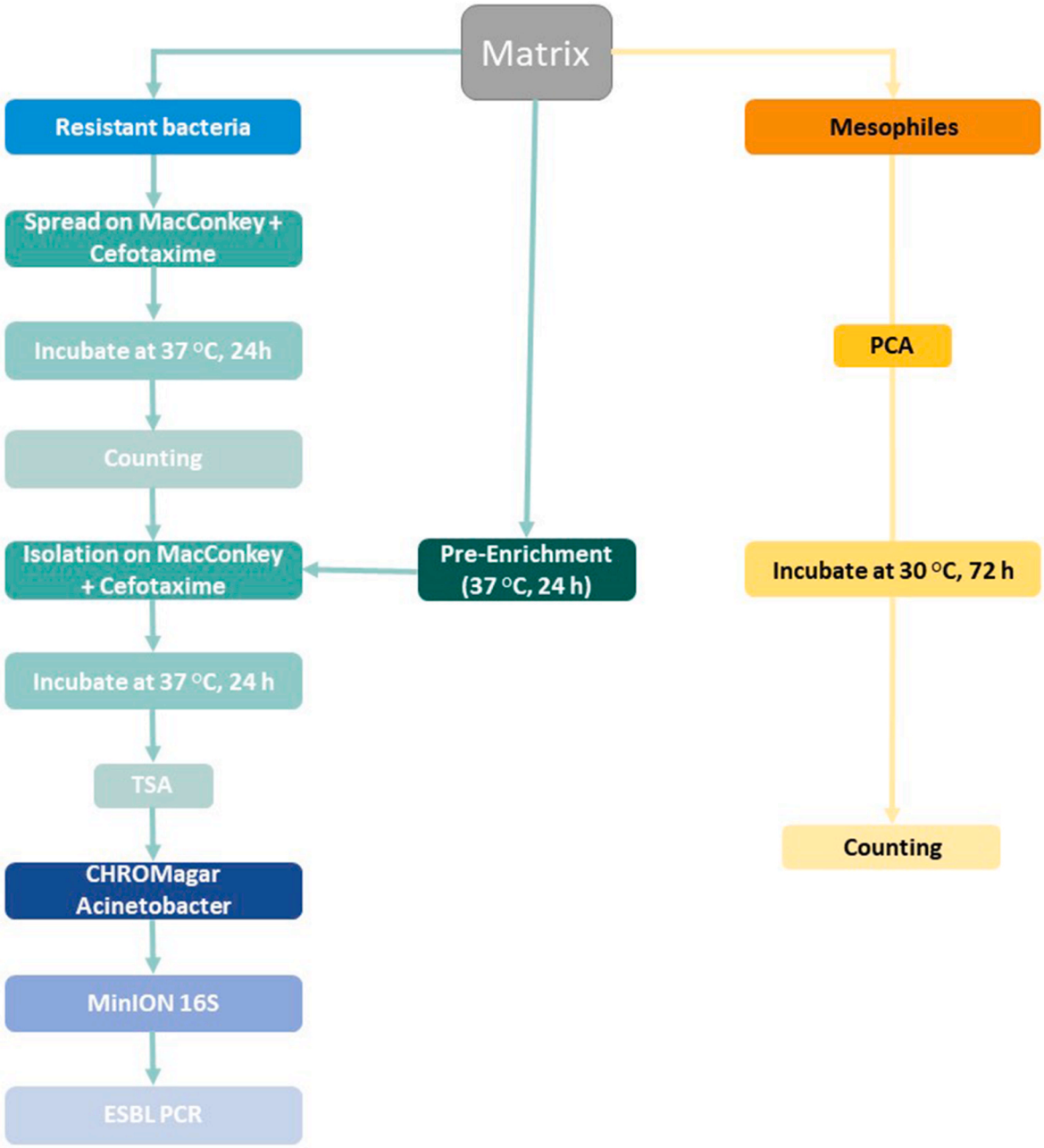


Fig. 1. Workflow of the study.

2.3. DNA extraction

One mL of the fresh culture, prepared as described in M&M 2.2.2, were centrifuged at 16000×*g* for 5 min. The supernatant was discarded and the pellet was resus-pended in 1 mL of TE 1X buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5) and centrifuged under the same conditions. The supernatant was discarded, the pellet was re-suspended in 200 μL of TE 1X and incubated in a dry bath (Thermomixer comfort, Eppendorf AG, Germany) set at 99 °C for 10 min at 1000 rpm. Finally, the suspension was centrifuged for 5 min at 16000×*g* and at 4 °C, the supernatant, containing the DNA, was collected and transferred to a clean tube.

The concentration, and purity of the extracted DNA attending to the 260/280 and 260/230 absorbance ratios, was determined in a NanoVue™ Plus Spectrophotometer (GE Healthcare Europe GmbH).

2.4. Acinetobacter spp. confirmation by 16S MinION sequencing

Colonies presenting typical morphology of *Acinetobacter* spp. on Chrom were confirmed by 16S MinION sequencing. To this end, the DNA obtained from the fresh cultures was used for 16S PCR amplification taking advantage of the primers KCP 812 CAG GCC TAA CAC ATG CAA GTC and KCP 813 GGG CGG WGT GTA CAA GGC (Mir et al., 2016) originally described by Marchesi et al. (1998). The PCR amplification was performed in a final reaction volume of 20 μL composed of 10 μL of LongAmp Taq 2X Master Mix (New England BioLabs, Inc., Ipswich, MA, United States), 400 nM of each primer, 5 μL of template DNA and the remaining volume was filled with nuclease-free water. The reactions were run in a Veriti Thermal Cycler (Applied Biosystems™, Foster City, CA, USA).

After the amplification, the amplicons were used for DNA sequencing with the Rapid Barcoding Kit (SQK – RBK004) following the standard

protocol adapted for Flongle flow cells (FLO – FLG001). The experiments were performed either on a MinION Mk1B or MinION Mk1C from Oxford Nanopore Technologies. The sequencing runs were analyzed the Fastq 16S workflow provided by Oxford Nanopore Technologies in their cloud-based served EPI2ME (https://epi2me.nanoporetech.com/workflows) with the default parameters.

2.5. PCR screening of resistance genes

Those isolates confirmed as *Acinetobacter* spp. in M&M 2.4, were subjected to PCR analyses to assess the presence of a panel of nine different ESBL genes. The primers used to perform the amplification were previously described and are detailed in Table 1 (Kiiru et al., 2012). All primers were purchased from Integrated DNA Technologies (IDT, Integrated DNA Technologies Inc., Leuven, Belgium), the reactions were run in simplex and prepared in a final reaction volume of 20 μL with 400 nM of each primer along with 10 μL of LongAmp® Taq (New England BioLabs® Inc., MA, USA) and 5 μL of template DNA, the remaining volume was filled with sterile milliQ water.

All the reactions were run in a Veriti Thermal Cycler (Applied Biosystems™, Foster City, CA, USA) configured with a thermal profile which consisted of 2 min at 50 °C followed by 2 min at 95 °C and 50 cycles of dissociation at 95 °C for 30 s, annealing at 55 °C for 35 s, and extension at 72 °C for 90 s. After the 50 cycles a final extension step at 72 °C for 7 min was included.

The amplified products were separated by gel electrophoresis. To this end, 5 μL of the corresponding amplicon were mixed with 1 μL of 6 X NZYDNA loading dye (NZYTech, Lisbon, Portugal) and loaded in a 2 % agarose gel (Agarose Electrophoresis grade, NZYTech, Lisbon, Portugal) prepared with Sodium Borate buffer (SB) (Brody and Kern, 2004). The separation was run for 30 min at 300 V and the amplicons were visualized in a GelDoc™ EZ Imager (Bio-Rad Laboratories, Inc., USA). The NZYDNA Ladder VI (NZYTech, Lisbon, Portugal), fragment size from 50 to 1500 bp, was loaded for comparison.

2.6. Statistical analysis

The graphics and statistical analysis were all performed with GraphPad Prism v.8.0.1 software (GraphPad Software, CA, USA). The comparison of the plate count among brands and OL was performed with a one-way Analysis of variance, ANOVA, with a Tukey post hoc. Direct sample comparison was performed with the Kruskal-Wallis test with the Dunns post hoc. For both types of tests, the significance level was set at 0.05 ($p < 0.05$).

Table 1
Primers used to amplify ESBL genes.

Primer	Sequence 5'->3'	Amplicon size (bp)
blaTEM-F	ATGAGTATTCAACAT TTC CG	840
blaTEM-R	CCAATGCTTAATCAG TGA GG	
blaOXA-1-F	ATGAAAAACACAATACATATCAACTTCGC	820
blaOXA-1-R	GTGTGTTTAGAATGGTGATCGCATT	
blaOXA-2-F	ACGATAGTTGTGGCAGACGAAC	602
blaOXA-2-R	ATYCTGTTTGGCGTATCRATATTC	
blaCTXM pan-F	TTTGCGATGTGCAGTACCAGTAA	500
blaCTXM pan-R	CGATATCGTTGGTGGTGCCATA	
blaSHV-F	TTCGCCTGTGTATTATCTCCCTG	854
blaSHV-R	TTAGCGTTGCCAGTGYTCG	
blaVEB-F	ATTTAACCAGATAGGACTACA	1000
blaVEB-R	CGGTTTGGGCTATGGGCAG	
blaDHA con-F	TGATGGCACAGCAGGATATTC	997
blaDHA con-R	GCTTTGACTCTTTCGGTATTCG	
blaACC-like-F	AGCCTCAGCAGCCGGTTAC	818
blaACC-like-R	GAAGCCGTTAGTTGATCCGG	
blaCMY-F	ATGATGAAAAAATCGTTATGC	1200
blaCMY-R	TTGCAGCTTTTCAAGAATGCGC	

Y = T or C; R = G or A. All the primers were reported by Mir et al. (2016) and originally described by Kiiru et al. 1 (Kiiru et al., 2012).

3. Results

3.1. Microbiological analysis

3.1.1. Mesophilic counts

The plate count results are expressed in log Colony Forming Units (CFU) per gram (CFU/g). Considering the average plate counts of all the different samples, sixteen per commercial brand and eight for the organic, B1 had a concentration 6.9 ± 0.4 log CFU/g, B2 of 7.3 ± 0.4 log CFU/g and the OL had 7.3 ± 0.4 log CFU/g. The statistical comparison of means was performed with the Kruskal-Wallis test, and no significant differences were observed neither among both commercial brands nor when compared to the organic presentation. The results of this comparison can be observed in Fig. 2a.

A more in detail analysis focusing on the lots of each brand, eight samples per lot, showed a statistically lower concentration of mesophilic microorganisms in lot 1 of B1 (6.5 ± 0.4 log CFU/g) compared to all the other lots and brands tested (note that all the samples had an average concentration of mesophilic bacteria equal to 7.3 log CFU/g, more precisely, the second batch of B1 had a concentration of 7.3 ± 0.3 log CFU/g, batches 1 and 2 of B2 had plate counts of 7.3 ± 0.4 and 7.3 ± 0.3 log CFU/g respectively, and the OL had an average concentration of mesophiles of 7.3 ± 0.4 log CFU/g). These results are graphically

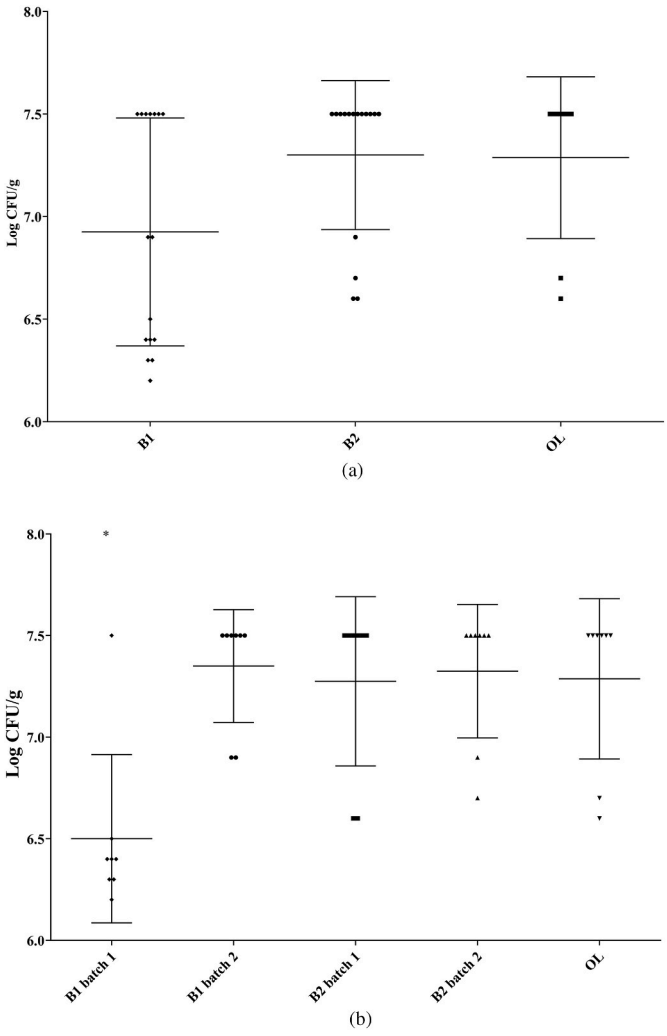


Fig. 2. Mesophilic counts, expressed as log CFU/g, obtained for each brand, B1 and B2, along with the organic lettuce (OL) (a), and the different batches (b). The "*" indicates statistically significant differences by one-way ANOVA analysis ($p < 0.05$).

depicted in Fig. 2b.

3.1.2. ARM enumeration

When we analyze the ARM present in this set of samples, average plate counts on Mac + c of 5.3 ± 0.7, 6.6 ± 0.4 and 5.4 ± 0.6 log CFU/g were obtained for B1, B2 and OL respectively. Out of these, B2 presented statistically lower concentration of ARM as determined by ANOVA, see Fig. 3a.

When the analysis was focused in the different lots, statistical differences were only identified among lot 1 from B1 (4.9 ± 0.9 log CFU/g) compared to lot 2 of B2 (7.2 ± 0.4 log CFU/g), and lot 2 from B2 compared to OL (5.4 ± 0.6 log CFU/g). On the other hand, no differences were observed among both lots of B1 (5.8 ± 0.5 log CFU/g in lot 2 from B1) or between lot 1 from B1 and lot 1 of B2 (6.0 ± 0.4 log CFU/g). as it can be observed in Fig. 3b.

3.1.3. Identification of Acinetobacter spp.

A total of 209 isolates were recovered from Mac + c, composed of 38 and 41 isolates from B1 lots 1 and 2 respectively, 40 and 41 coming from B2 lots 1 and 2, and 42 isolates from OL (it was not possible to recover 7 isolates from the glycerol stocks). Out of these, 180 exhibited typical morphology and color, red, on Chrom corresponding to 19 and 41 from B1 lots 1 and 2, 30 to B2 from each lot, and 39 from the OL. From the potential *Acinetobacter* spp. on Chrom, 8 and 16 isolates from B1, lots 1 and 2, were confirmed as *Acinetobacter* spp. by MinION sequencing (42 and 39 % of the isolates pre-screened were confirmed), while 13 and 17 were confirmed in B2 (43 and 57 % confirmed), and lastly from the OL 15 isolates were confirmed as *Acinetobacter* spp. (38 %). These results are summarized in Fig. 4a and b.

It is worth to note that, except for 2 isolates from the OL, which were identified as *Stenotrophomonas* spp., all the isolates which were non-*Acinetobacter* spp. were identified as *Pseudomonas* spp. Overall, the most abundant species identified was *A. pittii* followed by *A. calcoaceticus*, *A. geminorum*, *A. lactucae* and *A. vivianii*.

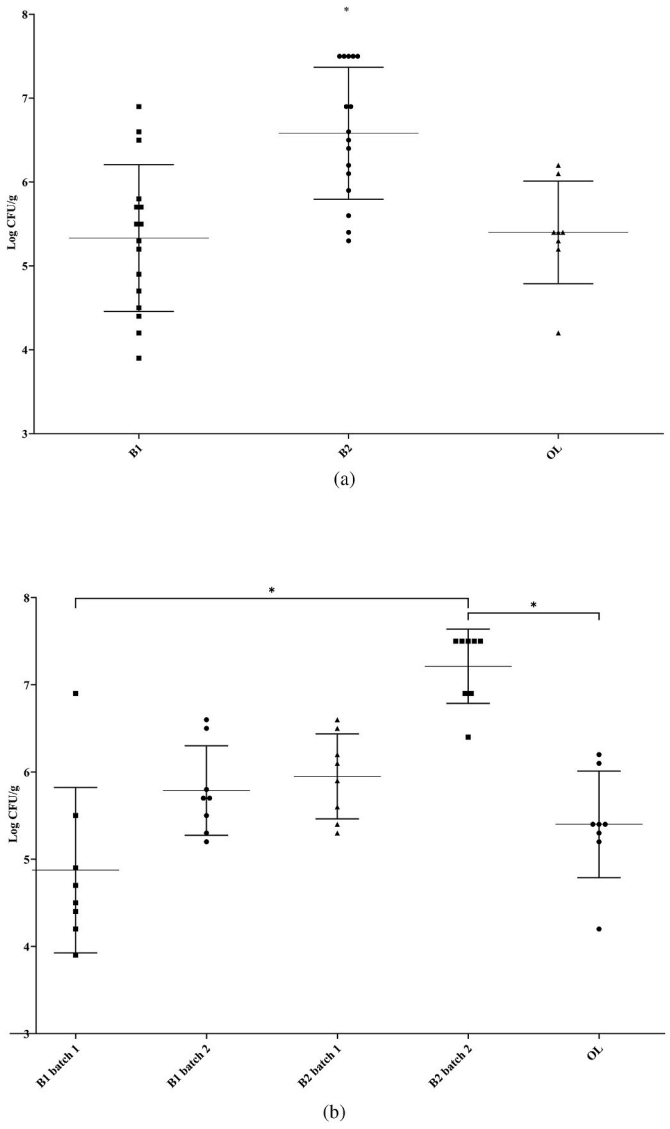


Fig. 3. ARM counts, expressed as log CFU/g, obtained for each brand, B1 and B2, along with the organic lettuce (OL) (a), and the different batches (b). The "*" indicates statistically significant differences by one-way ANOVA analysis ($p < 0.05$).

3.2. PCR screening of resistance genes

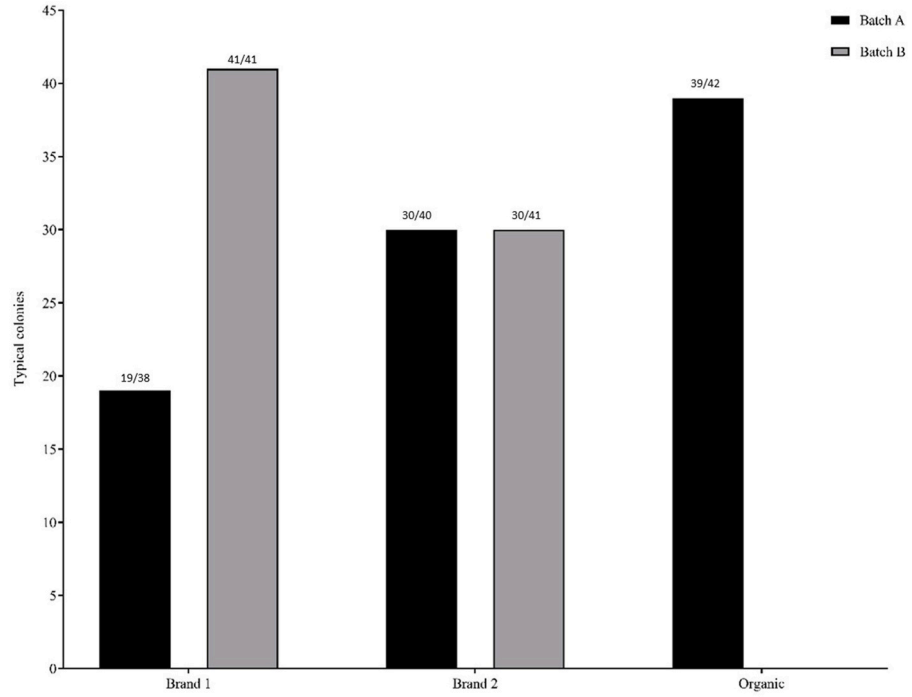
A total of 13 isolates, identified as *Acinetobacter* spp., presented one ARG. It is worth to note that none of the isolates analyzed with the panel selected presented more than one gene. The results indicated that, overall, the most common gene was *bla*ACC with nine isolates positive, six in B1 (five in lot 1 another in lot 2) and three in B2 (two in lot 1 and one in lot 2). In addition to this, in B1 one isolate was positive for *bla*SHV in lot 1 and another one was positive for *bla*VEB in lot 2. In regards to B2, other than *bla*ACC, one isolate was positive for *bla*DHA in lot 2. Finally, when looking into the OL isolates, there was only 1 positive for the ARG panel tested, being this *bla*VEB.

4. Discussion

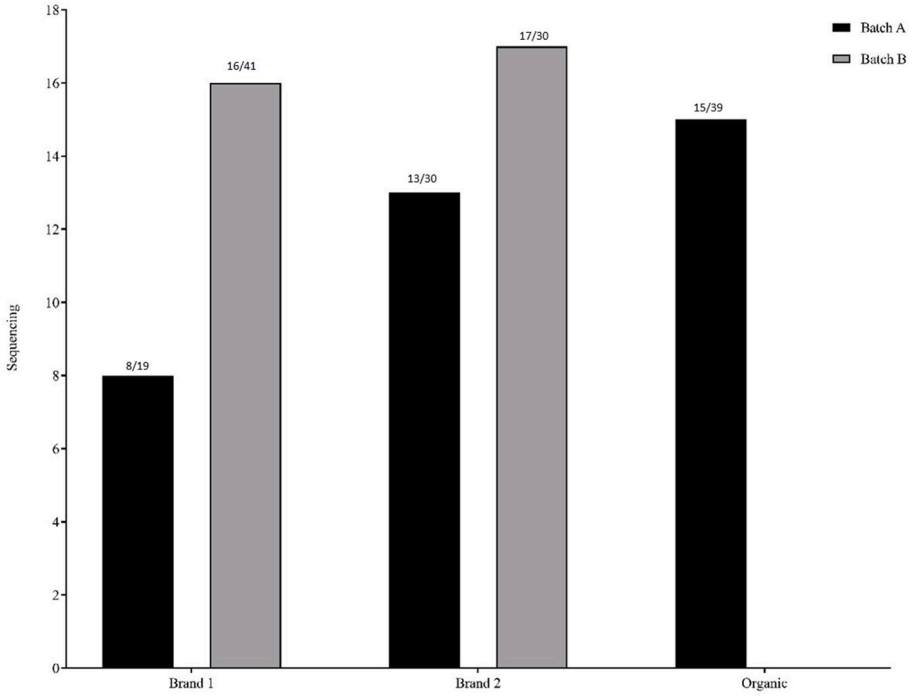
The evaluation of the microbiological quality of RTE salads is an important aspect of food safety as they are consumed directly, many times without even a water rinse by the consumer, thus may serve as carriers of different pathogens causing outbreaks worldwide (Gobin et al., 2018; Kinnula et al., 2018).

Mesophilic microorganisms were enumerated as a quality parameter of the samples. On average, both commercial brands, along with the organic lettuce, showed counts in the range of 7 log CFU/g, without significant differences among them. These counts were in line to those previously reported for this type of product however, differences were observed among commercial and organic presentations. Abadias et al. indicated that lower concentration would be expected in organic salads as they are not as processed as commercial ones (Abadias et al., 2008); on the other hand Sagoo et al., indicated that due to the fact that commercial presentations have to go through more strict quality controls, lower bacterial concentrations would be expected in them in comparison to organic salads (Sagoo et al., 2003). In addition to these, Mantegazza et al. reported on differences among traditional vs organic cultivation however it must be kept in mind that their study was specifically focused on rocket salad analyses (Mantegazza et al., 2023). This contradiction falls within what was reported by Zhang et al., in a surveillance study performed in the US including 14183 samples, a "mixed picture of contamination of organic versus conventional produce" was observed (Zhang et al., 2018). Thus additional studies must be conducted, and the results may be region dependent. It is worth to note that, regardless the mesophile concentration obtained, all these products fulfill the microbiological criteria set for this type of product attending to the European Regulation (European Commision, 2019).

The European Food Safety Authority (EFSA) Scientific Opinion on the role of environment in the emerge and spread of antimicrobial resistance genes through the food chain highlight that there are few studies that evaluates ARM in European plant-based food production environments (Koutsoumanis et al., 2021). This indicates the need for further studies on these foods. All the RTE salad samples analyzed in the current study presented ARM concentrations above 5 log CFU/g. Kim et al., reported a prevalence of 10 % in RTE vegetables however they



(a)



(b)

Fig. 4. Number of typical *Acinetobacter* spp. colonies on Chromagar Acinetobacter recovered from Mac + c and purified on TSA a). Number of isolates presenting typical morphology on Chromagar Acinetobacter and confirmed as *Acinetobacter* spp. by 16S rRNA sequencing b).

focused on ESBL *E. coli* and *K. pneumoniae*, while the one reported here englobed all Gram-negative bacteria (Kim et al., 2015). In line with the previous study, Iseppi et al. reported a low incidence of ESBL in RTE salads as only 20 out of 312 isolates exhibited ESBL phenotype. Differences with regards to the presented methodology may be behind the discrepancies with our findings, which may include the final concentration of cefotaxime (4 μg/mL vs 1 μg/mL), different brand of MacConkey, and differences in productivity/selectivity of the media (Iseppi et al., 2018).

Statistical differences in the plate counts of ARM were only observed among the first lot of B1 with the second lot of B2, and the same second lot of B2 with the OL. Among the commercial presentation, most likely

these differences were associated with batch to batch variations being this observation consistent with the lack of differences when the averages were compared.

Overall, it was observed that roughly 50 % of the *Acinetobacter* spp. presumptive isolates, were confirmed as such. These values correlate to those previously reported by Yusuf et al. who compared different media, namely Chrom against MacConkey, to determine which one was better suited for the isolation of *Acinetobacter* spp. from soil samples (Yusuf et al., 2023). However, the percentages identified in the current study were lower than what was previously reported by Carvalheira et al. in Portugal, 86.7 %. These differences may be related to the fact that they focused their study specifically in lettuce samples while the present one included RTE salads which are composed by a mixture of leafy greens (green and red lettuce, and arugula) (Carvalheira et al., 2017, 2021). It is important to note that, even though *A. baumanii* was not identified in any of the 202 isolates selected from the commercial brands and the organic lettuce, other species belonging to the Acb did show up, namely *A. calcoaceticus* and *A. pittii*, *A. geminorum*, along with *A. lactucae*, all previously reported to be involved in cases of human infection (Villalón et al., 2019; Wolf et al., 2021). Our results support those of Carvalheira et al. who highlighted *A. calcoaceticus* as the most common *Acinetobacter* species isolated in lettuce and fruits from Portugal (Carvalheira et al., 2017). It is important to keep in mind that species identification within the genus *Acinetobacter* spp. remains challenging as recently highlighted by Vasconcellos et al. (2023). This was in line with our observations as the novel species *A. geminorum* was identified in the second lot of B2. In this particular case, reads of *A. pittii* were also identified in the sequencing which representing among both species more than 90 % of all the reads of a given isolate. This is most likely related to the fact that these two species are very close genetically as indicated by Wolf et al. (2021).

The presence of *Pseudomonas* spp. has already been reported on vegetables both, at harvest and post-harvest stages (Tatsika et al., 2019) as they are spoilage bacteria commonly found (Pinto et al., 2015). *Pseudomonas* spp. are known to produce ESBLs which confer resistance to third generation cephalosporins, like cefotaxime. Within this genus *P. aeruginosa* has now for long been known to be particularly problematic to treat in hospital acquired infections due to the characteristic just mentioned (Pang et al., 2019). In the EFSA Opinion on the spread of ARM, *Pseudomonas* spp. *and Acinetobacter* were included as "Antimicrobial-resistant bacteria of highest priority for public health in food-producing environments" because they can carry mobile resistance genes to last resort antibiotics that can be transferred to other gram negative bacteria. This highlights the need to monitor the presence of these bacterial genera in ready-to-eat foods (Koutsoumanis et al., 2021).

In a study conducted in northern Portugal in 2014, *bla*TEM and *bla*CTX-M were the most abundant genes detected in clinical isolates belonging to the family *Enterobacteriaceae.* These isolates belonged to the species *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. aerogenes*, and *C. freundii* (Fernandes et al., 2014). In line with this study, Carvalho et al. reported the presence of *E. coli* bearing *bla*CTX-M isolated from domestic cats (Carvalho et al., 2021). In regards to *Acinetobacter* spp. most studies focus on *A. baumannii* for which the most commonly reported resistance gene in Portugal, and other countries, is *bla*OXA (Da Silva et al., 2004; Grosso et al., 2010). In the present study, a panel nine resistance genes were screened in the isolates identified as *Acinetobacter* spp. including two different *bla*OXA, namely *bla*OXA-1 and *bla*OXA-2. Among the isolates recovered, the most common gene identified was *bla*ACC, nine isolates, followed by *bla*VEB, two isolates, and single isolates were positive for *bla*SHV, *bla*DHA. It is important to note that no single isolate presented more than one gene of the panel tested what would have been of high relevance as previous studies have reported higher phenotypic resistance with certain gene combinations, namely *bla*OXA-1 and *bla*CTX-M, and even though this specific combination was not observed, others bearing *bla*OXA-1 were (Sugumar et al., 2014). The results obtained, in terms of resistance genes, do not fully match those previously reported, however it must be kept in mind that in most cases the studies only focus on *A. baumannii*, which was not identified in the current study, for which *bla*OXA-23 was reported to be one of the most common variants of the gene (Garciglia-Mercado et al., 2020; Hu et al., 2019; Li et al., 2019). These results provide additional insights in regards to the presence of *Acinetobacter* spp., and ARG, coming from foods in northern Portugal, in line with the needs highlighted by Carvalheira et al. (2021) for additional characterization of these bacteria, their antibiotic resistance profiles and molecular patterns coming, not only from clinical samples, but also from foods, water and environment, for better identification and control of transmission routes.

5. Conclusions

In the present study *Acinetobacter* spp. were isolated from commercial RTE salad products from norther Portugal. The workflow followed indicated that the isolates recovered were resistant to cefotaxime thus the isolates recovered, confirmed as *Acinetobacter* spp. by 16S sequencing, were screened for a panel of nine ESBL genes. Of relevance it was noticed that even though *A. baumannii* was not identified, other *Acinetobacter* species also involved in human infections were present in the samples under analysis. In addition to this, even though no single isolate presented more than one resistance gene, there were several positive for *bla*ACC, *bla*SHV, *bla*DHA and *bla*VEB, being *bla*ACC the most common one. The fact that the most prevalent genes observed did not match with those previously reported in northern Portugal highlight the fact that other genes are circulating in the region and may pose an additional risk associated with ARM.

Credit author statement

Ana Costa-Ribeiro: investigation and revision; Sarah Azinheiro: investigation and revision; Sandra Mota: revision and edition; Marta Prado: funding acquisition, revision and editing; Alexandre Lamas: revision and editing; Alejandro Garrido-Maestu: conceptualization, methodology, supervision, validation, writing of the original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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