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Evaluation of the potential risk associated to the presence of antibiotic resistant microorganisms in food products

Ana Alexandra da Costa Ribeiro

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Evaluation of the potential risk associated to the presence of antibiotic resistant microorganisms in food products

Autor

Ana Alexandra da Costa Ribeiro

Orientador(es)

Doutor Alejandro Garrido-Maestu, International Iberian Nanotechnology Laboratory (INL)

Doutora Marta Prado, International Iberian Nanotechnology Laboratory (INL)

Prof. Doutora Sandra Marlene Mota, Centro de Investigação em Saúde e Ambiente (CISA), Escola Superior de Saúde do Instituto Politécnico do Porto (ESSIP.Porto)

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Resumo

O uso excessivo e indiscriminado de antibióticos é uma das principais causas para o aumento das bactérias resistentes. No entanto, através deste estudo, foi também possível traçar uma plausível ligação entre os parâmetros de higiene microbiológica e a presença de bactérias produtoras de β-lactamases de espetro estendido (ESBL) nas saladas prontas a comer (RTE). As saladas podem ser contaminadas antes, durante e após o processamento, através do contacto direto ou indireto com bactérias patogénicas. Dessa forma, quando estas bactérias resistentes são ingeridas, através de alimentos contaminados, podem contribuir para a propagação dos genes ESBL, fazendo com que antibióticos, como a cefotaxima, percam a eficácia.

Esta tese tem como objetivo avaliar a presença de microrganismos resistentes à cefotaxima nas saladas RTE. Para tal, utilizaram-se duas marcas comercialmente disponíveis, a marca Continente e a marca Vitacress e, ainda, uma salada orgânica. Estas amostras demonstraram uma elevada concentração de microrganismos resistentes à cefotaxima. Foi possível verificar que as bactérias isoladas a partir destas amostras eram produtoras de ESBL, sendo as bactérias do género *Pseudomonas* as mais frequentes. Estudos posteriores, realizados através da análise de qPCR e PCR convencional, demonstraram a presença de genes ESBL em 29.7 % (62/209) dos isolados analisados. Tendo-se verificado que os genes bla_{ACC}, bla_{SHV} e bla_{VEB} foram os mais prevalentes. Assim sendo, constatou-se que as saladas RTE, apesar de serem alimentos nutritivos e integrarem uma dieta saudável, podem constituir um potencial veículo de transmissão de bactérias resistentes.

Palavras-chave: Bactérias Gram-negativo, Antibióticos, ESBL, Cefotaxima, saladas RTE

Abstract

The excessive and indiscriminate use of antibiotics is one of the main causes for the increase in resistant bacteria. However, through this study, it was also possible to trace a plausible link between microbiological hygiene parameters and the presence of extended spectrum beta-lactamase (ESBL) producing bacteria in *ready-to-eat* (RTE) salads. Salads can be contaminated before, during and after processing through direct or indirect contact with pathogenic bacteria. Thus, when these resistant bacteria are ingested, through contaminated food, they can contribute to the spread of ESBL genes, causing antibiotics, such as cefotaxime, to lose efficacy.

This thesis aims to evaluate the presence of cefotaxime-resistant microorganisms in RTE salads. For this purpose, two commercially available brands were used, the brand Continente and the brand Vitacress, and also an organic salad. These samples showed a high concentration of microorganisms resistant to cefotaxime. The bacteria isolated from these samples were found to be ESBL producers, with bacteria from the genus *Pseudomonas* being the most frequent. Further studies, performed using qPCR and conventional PCR analysis, demonstrated the presence of ESBL genes in 29.7 % (62/209) of the isolates analyzed. The bla_{ACC}, bla_{SHV} and bla_{VEB} genes were found to be the most prevalent. Therefore, it was concluded that RTE salads, despite being nutritious foods and integrating a healthy diet, may be a potential vehicle for transmission of resistant bacteria.

Keywords: Gram-negative bacteria, Antibiotics, ESBL, Cefotaxime, RTE salads.

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Abbreviations list

ARMs – Antimicrobial Resistant Microorganisms

BJM – Bush-Jacoby-Medeiros

ESBL – Extended-Spectrum β-Lactamases

KPC – *Klebsiella pneumonie* Carbapenemase

MBLs – Metallo β-Lactamases

MRSA – Methicillin-Resistant *Staphylococcus aureus*

NAG – N-Acetyl Glucosamine

NAM – N-Acetyl Muramic Acid

NB – Nutrient Broth

PBP – Penicillin-Binding Protein

PCA – Plate Count Agar

PCR – Polymerase Chain Reaction

qPCR – Real-Time Polymerase Chain Reaction

RTE – Ready-to-Eat

SME – Serratia Marcescens Enzyme

TSA – Trypto-Casein Soy Agar

Tyr – Tyrosine

WHO – World Health Organization

1. Introduction

Food is essential for human life, for this reason food security is a basic human right. Everyone needs nutritious and safe food, as it not only improves individual health, but also the health of the population in general, and for this reason, people today seek to consume green leafy foods of biological origin (1). In order to facilitate the ingestion of this type of more nutritious food, the need arose to produce food already prepared for consumption, such as ready-to-eat salads (RTE). The growing interest in this type of food has also led to an increase in the number of foodborne illnesses (2) this is because these types of vegetables are eaten fresh without the need for cooking or other methods that help eliminate or reduce the level of microbial contaminants, increasing potential exposure to foodborne pathogens and consequent infection (3). Illness caused by contaminated foods is serious issue and it is responsible for thousands of hospitalizations and deaths every year, which is why it is so important to track and detect these pathogenic bacteria (4).

There is a wide range of microorganisms that can be present in food, however, several studies showed that only a few are capable of causing disease, such as *Salmonella* Typhi, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, among others (2).

Infectious diseases were major threat for human populations however, with the discovery of antibiotics the treatment of these diseases improved (5). Antimicrobials emerged to combat infections caused by pathogenic microorganisms. They are used to inhibit bacterial growth or to kill bacteria, and are therefore classified based on their bacteriostatic and bactericidal effect (6).

However, the increased use of antibiotics in hospital settings, in food production and processing, the lack of development of new antibiotics, or the lack of alternative compounds, have contributed to development of resistance to these drugs (7). The World Health Organization (WHO) has classified antibiotic resistance as one of the top three threats to public health in recent decades (5). It is estimated that 700,000 people have died worldwide of infections caused by drug-resistant bacteria, 25,000 in the European Union, and this number is expected to increase up to 10 million by 2050 (8).

In order to decrease infections caused by antimicrobial resistant microorganisms (ARMs), it is important to understand which microorganisms are present, which ones exhibit resistance, which are the sources of this resistance, and which mechanisms drive the acquisition of the resistance by the bacteria (9).

1.1. Antimicrobial Agents: β-Lactam Antibiotics

Antibiotics can be classified into different groups according to their function, that is, according to their target in the bacterial cell, thus differentiating into:

- Inhibitors of nucleic acid synthesis;
- Inhibitors of protein synthesis;

- Inhibitors of cell wall synthesis;
- Inhibitors of cell membrane biosynthesis (10).

Among the mechanisms of antimicrobial action mentioned, β-lactam antibiotics inhibit cell wall synthesis.

The β-lactams stand out from other antimicrobials because they are currently the most widely used and prescribed class of antibiotics in the world. This happens since these antimicrobials are effective and well tolerated, and only a small percentage of patients have an allergic response (11).

The β-lactam antibiotics are structurally composed of a β-lactam ring, which gives the group its name. This ring can appear alone or conjugated, so there are currently four main classes of these antimicrobials that are used as therapeutics (12). Three groups share a bicyclic structure, such as the penicillins whose β-lactam ring is conjugated to a thiazolidine ring, the cephalosporins which the β-lactam ring is associated with a six-membered dihydrothiazine ring, and the carbapenem in which the β-lactam ring is fused to a five-membered pyrrole ring. The fourth class has a monocyclic structure and are known as the monobactams (Figure 1) (13).

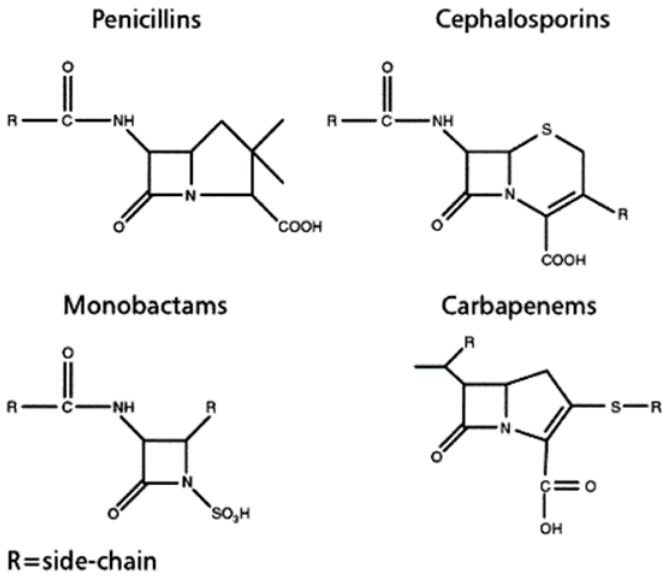

Figure 1 *– Structure of the β-lactam antibiotics* (31).

1.1.1 Cephalosporins

Cephalosporins are the most widely used class of β-lactam antibiotics in hospitals (14), and, this is due to the fact that these drugs have a low toxicity and a broad spectrum, especially in infections caused by Gram-negative bacteria (15). There are five generations of cephalosporins, and they have varying antibacterial activity. First generation cephalosporins have a spectrum of action on Gram-positive bacteria, including penicillinase-producing *Staphylococcus aureus*. The first generation includes the antimicrobials cefadroxil and cefazolin (16). The second generation cephalosporins have a broader antimicrobial activity, and several species are sensitive to this class, such as, *Enterobacteriaceae*, *Proteus*

and *Bacteroides* (17). The third generation cephalosporins is the least active against Gram-positive bacteria, however, it is more active against Gram-negative microorganisms and have greater stability against β-lactamases. The following drugs are part of this class of cephalosporins: cefdinir, cefixime and cefotaxime. The fourth and fifth generations of cephalosporins are active against a wide range of Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). This class includes the drugs cefepime (fourth generation), ceftaroline, and ceftolozane/tazabactam (fifth generation) (16).

As mentioned previously, cefotaxime is a third-generation cephalosporin, and, it is one of the most widely used drugs in human medicine. For this reason, this antimicrobial is the target of study in this thesis (18). Cefotaxime is a semisynthetic cephalosporin. This antibiotic is the only one among the available cephalosporins that has activity against most *Enterobacteriaceae*, including *Escherichia coli*, *Shigella* and *Haemophilus influenzae*. This drug was reported to be more effective than other cephalosporins for the treatment of *Pseudomonas however,* many isolates of this genus are resistant. (19).

1.2. Mode of action of β-lactam antibiotics

The entry of β-lactam antibiotics into the bacterial cell is directly related to the cell wall (20). The cell wall consists essentially of peptidoglycan polymers, which are responsible for maintaining the shape and rigidity of the cell. The function of β-lactam antibiotics is to inhibit the enzymes involved in cell wall synthesis, that is, they prevent the synthesis of peptidic bridges between the chains that constitute peptidoglycan, thus inhibiting the synthesis of this compound (21). Peptidoglycan consists of alternating sugars of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) joined by β-(1,4) bonds. Cell wall synthesis occurs in three phases. The first phase happens in the cytoplasm where the precursors essential for peptidoglycan are formed. In the second phase, these precursors are transported to the cytoplasmic membrane, and the release of the uridine nucleotides from the precursors takes place and the binding of the NAG to the NAM occurs. During the third phase the peptidoglycan polymer formation is terminated. Each NAM is bound to a small peptide, which differs between bacterial species, this peptide ends in D-alanyl-D-alanine. Penicillin-binding protein (PBP) is an enzyme that is responsible for removing the D-terminal D-alanyl-D-alanine from the process so that it can bind to another peptide via an amino acid bridge, and this reaction is catalyzed by the transpeptidase domain of PBPs. The carboxypeptidase and transpeptidase activity of the PBPs are required for peptidoglycan formation (22). The β-lactam antibiotics have a β-lactam ring very similar to the D-alanyl-D-alanine of the NAM peptide and thus the PBPs misuses this ring to continue forming the bacteria cell wall. This binding of PBPs to the β-lactam ring makes this enzyme unable to catalyze other transpeptidation reactions resulting in disruption of the cell wall and subsequent cell lysis (Figure 2) (21).

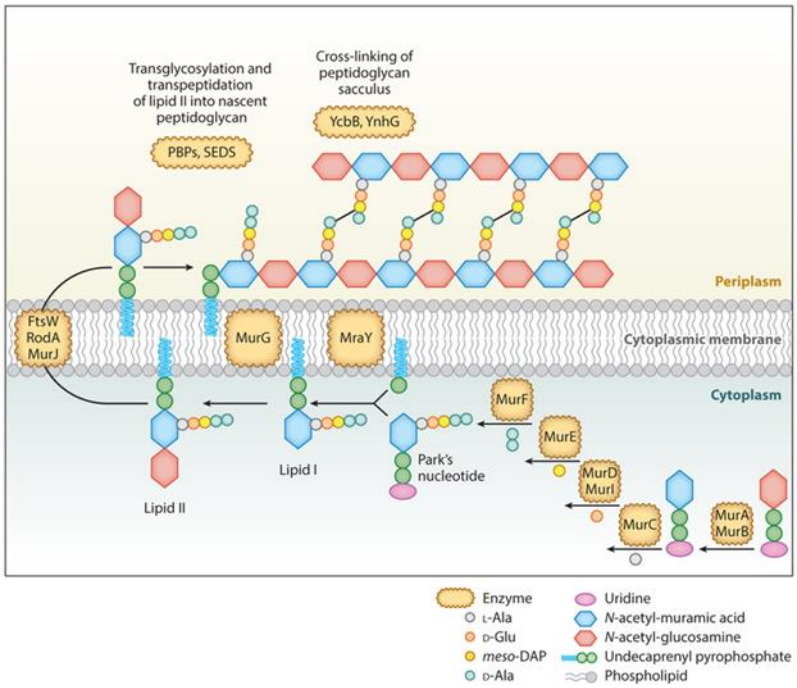

Figure 2 *- Biosynthesis pathway of peptidoglycan* (56).

1.3. Origin of β-lactam resistance

Microorganisms can exhibit several types of resistance, that is, they can demonstrate one of three fundamental phenotypes: intrinsic resistance, acquired resistance, or susceptibility. Most antimicrobial compounds are produced naturally, and for this reason bacteria have developed mechanisms to overcome their action to survive. These microorganisms are considered "intrinsically" resistant to one or more antibiotics, and this natural resistance is exhibited by all the members of a given species (5).

Acquired resistance can result from the mutation of regulatory genes or from acquisition and recombination of exogenous genetic material. In the case of regulatory gene mutations, a subset of susceptible bacterial cells develops mutations in genes, resulting in cell survival in the presence of the drug. Once a resistant mutant emerges the antibiotic will only eliminate the susceptible population with resistant bacteria predominating. In horizontal gene transfer bacteria use three strategies, transformation, transduction and conjugation. Transformation is the simplest form of gene transfer, but only a few species can "naturally" incorporate DNA to develop resistance. Transduction refers to the transfer of genetic material between bacteria mediated by a virus, i.e. bacteriophage (phage). Conjugation, uses mobile genetic elements to share genetic information with other bacterial cells. The most important mobile genetic elements are plasmids, integrons and transposons. The phenotype resulting from acquired resistance will not be present in all individuals of the same species, but will only be present in individuals of a bacterial strain derived from a susceptible organism (5,23).

1.4. Mechanisms of resistance to β-lactam antibiotics

Over the years, the misuse of antibiotics has resulted in the development of resistance mechanisms by several microorganisms against β-lactam antibiotics. These include: target modification, cytoplasmic membrane impermeability, efflux pumps, or enzymatic inactivation of the antibiotic (24).

1.4.1 Target modification

One of the most common mechanisms of resistance arises by changes in its target site. The interaction of the antimicrobial with the target molecule is very specific, small changes can have a large effect on this binding (25). These target site changes can consist of: point mutations in the genes encoding the target site; enzymatic changes to the binding site; substitution or deviation from the original target; or even, and most common with β-lactams, amino acid substitutions in PBPs, where transpeptidases are altered. Regardless of the type of alteration, in the end there will always be a decrease in the affinity of the antibiotic for the target site (26).

1.4.2 Cytoplasmic membrane impermeability

The permeability properties of the outer membrane have a major impact on the susceptibility of Gram-negative bacteria to antibiotics. There are two main mechanisms that relate antibiotic resistance to porins: outer membrane changes, where severe loss/reduction of porins occurs, and also the replacement of one or two major porins by another, or the function of porins is altered due to specific mutations, thus reducing their permeability and conferring resistance to this type of drug (27).

1.4.3 Efflux pumps

Efflux pumps are a resistance mechanism that is not independent, meaning that it is always associated with other resistance mechanisms. Resistance is usually caused by increased production of the proteins that make up the pump, and this happens as mutations can occur in the transcriptional repressors of these proteins. These mutations can also lead to an increase in the efficiency of antibiotic transport to the outside of the cell, making the bacteria resistant to the action of the drug. These types of mechanisms are also associated with multidrug resistance phenomena because they cover a wide range of antibiotics (28).

1.4.4 Enzyme Production: β-lactamases

The most commonly used mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria is the production of enzymes, these are known as β-lactamases, and they degrade the antibiotic (26). These enzymes are produced by both Gram-positive and Gram-negative bacteria (29) however,

Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, that produce the most clinically important β-lactamases (30). The genes encoding the production of β-lactamases can be found on the bacterial chromosome or in plasmids. The β-lactamases of chromosomal origin are universal, that is, they are present in specific bacterial species, while the β-lactamases that are encoded by plasmids are transferable between bacterial species. There is also the possibility that the genetic transfer of these genes is through transposons, since they can transport the genes of β-lactamases from plasmids to the chromosome (31)

These enzymes have the ability to hydrolyze the β-lactam ring (24) and they do this through two mechanisms: through a covalent acyl-enzyme intermediate formed between the β-lactam molecule and the serine of the active site, or through a hydrolysis reaction that is facilitated by one or two zinc ions that are present in the active site of the metallo β-lactamases (MBLs) (13,32).

1.5. Classification of β-lactamases

Although all β-lactamases catalyze the same reaction, certain types of these enzymes have been isolated and characterized based on amino acid sequence homology and biochemical characteristics (33). It can be concluded that these enzymes do not comprise a single homogeneous group, but can be subdivided into different classes. There are two systems that classify these enzymes. One is based on the activities of the β-lactamases, the Bush-Jacoby-Medeiros system (BJM), and the Ambler system that is based on sequence information (30). In the Bush-Jacoby-Medeiros system, enzymes are divided into 4 groups, Groups 1 and 2 include the extended-spectrum β-lactamases (ESBL) that are encoded on the chromosome or in plasmids, respectively. Enzymes belonging to group 3 are specific for carbapenem antibiotics, and finally those belonging to group 4 are enzymes that are weakly inhibited by the β-lactamase inhibitor clavulanate (31). The Ambler System divides β-lactamases into 4 different classes, which are called A, B, C and D, they are distinguished not only based on sequence, but also by differences in the mechanism of hydrolysis. There is yet another division among these 4 classes: classes A, C and D comprise all β-lactamases that have serine at the active site, and are therefore called serine- β-lactamases, whereas enzymes that belong to class B comprise a group in which zinc metalloenzymes are present, called MBL's. The most important of these enzymes are TEM, SHV, CTX-M and KPC from class A; NDM and VIM from class B; and CMY and ACC from class C. And yet, the enzymes in class D are all called oxacillinase (OXA) (30).

1.5.1 Cass A β-lactamases (Group 2 BJM)

Class A β-lactamases are normally encoded by plasmids, however, they can also be found on the bacterial chromosome (34). As a rule, these enzymes are susceptible to certain β-lactamase inhibitors,

such as clavulanate, tozabactam, and sulbactam (21). Class A enzymes comprise a wide range of proteins with very distinct catalytic activities, enzymes in this class can be divided into 3 types: narrow-spectrum β-lactamases, ESBL's, and serine Carbapenemases. Within the narrow-spectrum β-lactamases are the enzymes TEM-1 (in honor of the patient where it was isolated) and SHV-1 which are responsible for hydrolyzing penicillin and some lower-generation cephalosporins.

However, there is also a type of β-lactamases in this class that are clinically relevant and have been extensively studied, the ESBL's, which include the CTX-M (26). ESBL's have emerged, not only because of the increasing number of β-lactamases in more common pathogens, such as *E. coli* and *Klebsiella pneumoniae*, but also because of selective pressure, which has caused other microorganisms, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*, to acquire ESBL's. Thus, SHV- and TEM-type enzymes have undergone point mutations in the bla_{TEM-1} and bla_{SHV-1} genes that lead to amino acid changes, causing these enzymes to be able to hydrolyze many oxymino-cephalosporins, such as cefotaxime and ceftazidime, which are third-generation cephalosporins (21). Another type of ESBL's already mentioned, is CTX-M. This enzyme arose by plasmid transfer of pre-existing chromosomal ESBL genes. It has been found in several *Kluyvera* species, suggesting that insertion sequences and bacteriophages mobilized bla_{Klu} genes, in conjugative plasmids (13). These enzymes can hydrolyze cefotaxime faster than ceftazidime (35) and are responsible for most of the global resistance to advanced generation cephalosporins in *Enterobacteriaceae*. There are also other enzymes that belong to this group, but they are less common and are encoded in intregons, for example, GES-1 from *K. pneumoniae* and VEB-1 which is present in *P. aeruginosa* and *A. baumannii* (34).

Another type of β-lactamases that belong to class A are those that can hydrolyze carbapenems. The main carbapenemases of this class are KPC (*Klebsiella pneumoniae* carbapenemase), SME (*Serratia marcescens* enzyme), GES and Nmc-A/IMI. These can be encoded chromosomally (SME and Nmc-A), by plasmids (KPC and GES) or both (IMI) (13). The carbapenemases hydrolyze not only this class of antimicrobials, but also penicillins, cephalosporins, and aztreonam (34). The enzyme responsible for most of the resistance to these types of drugs is KPC, where the bla_{KPC} gene is carried on plasmids containing Tn 4401 (21). The plasmids that contain this gene vary in size and carry additional genes that can lead to resistance to other types of drugs, such as fluoroquinolones and aminoglycosides, making the treatment of infections caused by KPC-producing pathogens more limited (34).

1.5.2 Cass B β-lactamases (Group 3 BJM)

Class B of the β-lactamases is also known as the class of MBLs since, this class uses a metal ion (zinc) as a cofactor for the nucleophilic attack on the β-lactam ring rather than a serine residue like the other classes (26). These enzymes provide resistance to penicillins, cephalosporins, carbapenem, and clinically available β-lactamase inhibitors but have little or no resistance against monobactams (21,30).

The *bla*$_{MBL}$ genes can be in the chromosome, in plasmids and in integrons. Some microorganisms such as *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* produce these enzymes encoded by mobile elements, while *Bacillus* spp., *Chryseobacterium* spp. and *Stenotrophomonas maltophilia* they are chromosomally encoded (21).

MBLs' can be divided into 3 subclasses: B1, B2 and B3, based on structural characteristics, zinc affinity for the two binding sites and hydrolysis characteristics. Subclasses B1 and B3 include the enzymes IMP, VIM, NDM and SPM and CAU-1, GOB-1 and FEZ-1, respectively. These two subclasses are characterized by the presence of two zinc atoms in the active center of the enzyme, and can hydrolyze a wide spectrum of antimicrobials. Enzymes of subclass B2 differ from the other two mentioned above in that they have only one zinc at the center and hydrolyze a small spectrum, i.e. they preferentially hydrolyze carbapenems (36,37) The most clinically relevant MBLs are NDM, VIM and IMP, belonging to subclass B1. VIM and IMP are mainly included in the integron structure and are subsequently integrated into the chromosomal DNA and plasmid DNA in association with the transposon. Regarding the NDM enzyme, the *bla*$_{NDM-1}$ gene is present in a plasmid and not found in the integron structure. At present, it is the most common and emerging MBLs worldwide, thus in view of limitation in treatment, this class has posed a major threat to public health (34,37).

1.5.3 Class C β-lactamases (Group 1 BJM)

Class C β-lactamases, also known as AmpC are generally encoded on the chromosome of many *Enterobacteriaceae* and a few other microorganisms (38). However, AmpC enzymes that are carried by plasmids have become increasingly prevalent, especially in *Klebsiella* and *Salmonella* species (34). The production of these enzymes is low, conferring only resistance to the early generation aminopenicillins and cephalosporins. However, when bacteria are exposed to certain drugs such as ampicillin, amoxicillin, cefazolin, and cephalothin, they can produce the enzyme and become resistant to some of them (21). Also, spontaneous mutations in AmpC regulatory genes lead to overproduction of these enzymes, causing microorganisms to develop resistance to the extended spectrum cephalosporins such as the oxyimino-cephalosporins, cefotaxime, ceftriaxone, ceftazidime, and even carbapenems (13) due to insertions, deletions, or amino acid substitutions (35).

The main plasmid-mediated extended spectrum AmpC families are CMY (cephamycin hydrolysis β-lactamase), MIR (Miriam Hospital β-lactamase), MOX (moxalactam hydrolysis β-lactamase), LAT (latamoxef hydrolysis β-lactamase), FOX (cefoxitin-hydrolyzing β-lactamase), DHA (Dhahran Hospital in Saudi Arabia β-lactamase), ACT (AmpCtype β-lactamase), ACC (Ambler C class β-lactamase) and FCE (*Citrobacter freundii* β-lactamase) where they have minor differences in amino acids. Different mutations of either chromosomal or plasmid-encoded AmpC enzymes increase the catalytic efficiency for the substrates (13).

1.5.4 Class D β-lactamases (Group 4 BJM)

Class D is a class of serine β-lactamases also known as the OXA class due to the fact that its first enzymes have a higher hydrolysis rate for penicillin oxacillin than for benzylpenicillin, unlike classes A and C. *OXA* genes can be found on both the chromosomes and plasmids of different bacterial species such as *Acinetobacter*, *Shewanella*, *Pseudomonas* and *Burkholderia*, but many of these enzymes can be transferred to plasmids, posing a greater clinical threat (39). The OXA enzymes are resistant to inhibition by clavulanate, sulbactam and tazobactam, except for OXA-2 and OXA-32 which are inhibited by tazobactam, but not by sulbactam and clavulanate. Also, sodium chloride at certain concentrations inhibits some enzymes that hydrolyze carbapenems, this due to the presence of a tyrosine (Tyr) residue at position 144, which facilitates binding to sodium chloride (21). The first OXA enzymes that were identified had activity against some low-spectrum cephalosporins (40) however, this class now includes enzymes that confer resistance to both extended-spectrum cephalosporins (ESBL type OXA) and carbapenems (Carbapenemases type OXA), with a wide range of sensitivities to the inhibitors (30). Enzymes capable of hydrolyzing ESBL's are derived from OXA-10, having between 1 and 9 amino acid substitutions. These types of ESBL's have greater resistance to ceftazidime than to cefotaxime or aztreonam, but there are exceptions, as microorganisms that produce the OXA-1 or OXA-31 enzymes can be susceptible to ceftazidime but resistant to cefepime (35). There are 5 groups that are recognized as OXA carbapenemases, and the main bacterial species involved in the production of these enzymes is *A. baumannii*, since the genes are located on its chromosome. This bacterium can produce four groups of these enzymes, OXA-23, OXA-24/40, OXA-51 and OXA-58. The OXA-48 group is found in plasmids of some enterobacteria, thus posing an additional clinical challenge (30,35).

1.6. Objetives

The main objective of this project is to assess the presence of antibiotic resistance microorganisms in RTE salads. To achieve this goal, the following specific goals will be addressed:

- To enumerate and isolate cefotaxime-resistant Gram-negative bacteria present in RTE salads;
- To screen for specific ESBL resistance genes;
- To discuss the potential public health risk from the presence of these microorganisms in RTE salads.

2. Materials and Methods

2.1. Experimental design and sampling

The samples under study in the current project were RTE salads. Two different commercial brands, were selected purchased from local suppliers, along with organic lettuce acquired from a local farm. Two different batches per brand were selected for this study, and 8 samples per batch were analyzed, making a total of 16 samples per brand, and an overall total of 32. In the organic salad only 8 samples were analyzed (Table 1). The salads of both brands consisted of green leaf lettuce, purple leaf lettuce and arugula. The organic lettuce consisted only of green leaf lettuce.

All salads were handled within the expiration date, and all visibly damaged leaves were excluded. During the process the samples were stored at a temperature of 4°C until analysis (Figure 3).

Table 1 – *Brands and batches used in the study.*

Trial	Brand	Batch
1	Continente	96105279152
2	Continente	96106109152
3	Vitacress	L – 1427
4	Vitacress	L-1529
5	Organic Salad	
The organic salad refers to collected in the local farm		

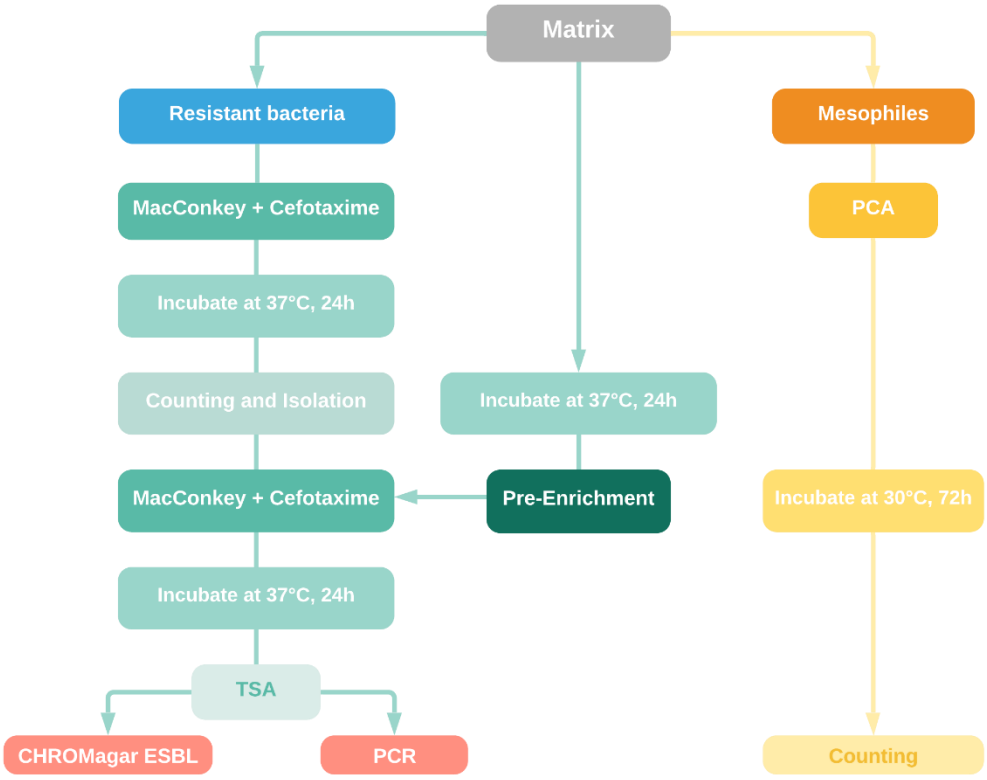

Figure 3 *- Schema of the experimental design*

2.2. Cefotaxime-resistant bacteria and mesophilic microorganisms enumeration

To enumerate bacteria resistant to cefotaxime and mesophilic microorganisms, ten-fold serial dilutions were prepared up to dilution 10^{-5}. For this, 25 g of each RTE salad sample were mixed with 225 mL of Luria Bertani broth (LB, PanReac AppliChem) and homogenized for 30 s in a Stomacher 400 circulator (Seward Limited, West Sussex, UK). The samples were then plated on Plate Count Agar (PCA, Biokar Diagnostics S.A., France) and MacConkey Agar (Difco) supplemented with 4µL/mL of cefotaxime (Fisher BioReagents). The PCA plates were incubated at 31 °C for 72 h and the MacConkey plates were incubated at 37 °C for 24 h. Additionally, the bags containing the original matrixes, were incubated at 37 °C for 24 h. After the pre-enrichment period, they were streaked on MacConkey supplemented with cefotaxime as indicated previously, and the plates were incubated at 37 °C for 24 h. Colonies with different morphologies observed on MacConkey plates, either from the enumeration or the pre-enrichment cultures, were re-isolated on the same medium to obtain pure cultures; these were incubated at 37 °C for 24 h. A total of 5 colonies from each sample were selected and re-isolated onto plates containing Trypto-Casein Soy Agar culture medium (TSA, Biokar Diagnostics S.A., France) and finally incubated at 37 °C for 24 h.

Pure isolated cultures from TSA were streaked on ChromAgar ESBL medium (CHROMagar™), and incubated at 37 °C for 24 h. The same TSA, pure cultures were used for to prepare DNA extracts for molecular analysis.

2.3. DNA extraction and quantification

For genotypic characterization of the isolates under study, DNA extraction was performed and the extracts were quantified spectrophotometry. The pure cultures on TSA were resuspended in 4 mL of Nutrient Broth medium (NB, Biokar Diagnostics S.A., France) and incubated at 37 °C for 24 h. One mL of the fresh culture were centrifuged at 16000× g for 5 min. The supernatant was discarded and the pellet was resuspended in 1 mL of TE 1X buffer (10 mM Tris–HCL, 1 mM EDTA, pH 7.5) and centrifuged under the same conditions previously mentioned. The pellet obtained was then resuspended in 200 µL of the same buffer and placed in a dry bath (Thermomixer comfort, Eppendorf AG, Germany) set at 99 °C for 10 min at 1000 rpm. Next, the suspension was centrifuged again, at 4°C for 5 min at 16000× g. Finally, the supernatant was collected and transferred to a clean tube.

The extracted DNA was quantified and its purity was determined attending to the 260 / 280 and 260 / 230 absorbance ratios with a NanoVue plus spectrophotometer (Biochrom Ltd, Cambridge, UK).

2.4. Polymerase Chain Reaction (PCR) and Electrophoresis

To presence of specific genes encoding for β-lactamases was assessed by PCR. Two different types of PCR were performed: Real-time PCR (qPCR) and conventional PCR. Conventional PCR was performed only on samples that demonstrated the presence of the resistance gene in qPCR.

The PCR reactions were performed in a final volume of 20 µL with the following components: nuclease-free water (Thermo Scientific, Waltham, MA, USA), 10 µL of PowerUp™ SYBR™ Green Master mix (Applied Biosystems, Foster City, CA, USA) and 400 nM Primers (IDT, USA) (Table 2). Regarding conventional PCR, the protocol used was similar to the previous one, however with a change in the Master Mix, as 10 µL of LongAmp® Taq (New England BioLabs® inc, MA, USA) was used. Five µL of the template were added per reaction.

The thermal profile selected for both PCRs consisted in 2 min at 50 °C followed by 2 min at 95 °C for polymerase activation (hot start), and then 50 cycles of dissociation at 95 °C for 30 s, 55 °C for 35 s, and extension at 72 °C for 90 s with a final extension at 72 °C for 7 min. In qPCR runs an additional step, this was the Melt Curve Stage, in which the reaction is heated to 95 °C for 15 s, followed by 60 °C for 1 min and ending at 95 °C for 15 s measuring the fluorescence every 0.3 °C/s. The qPCR reactions were run in a QuantStudio 5 (Applied Biosystems™, Foster City, CA, USA) and a StepOnePlus™ Real-Time PCR (Applied Biosystems™, Foster City, CA, USA). Conventional PCR reactions were run in a Veriti Thermocycler (Applied Biosystems™, Foster City, CA, USA).

Five μL of the amplified products were analyzed by 2 % agarose gel electrophoresis (Agarose Electrophoresis grade, NZYTech) prepared in Sodium Borate buffer (SB) containing 2 μL of GreenSafe (NZYTech); The SB buffer was prepared by adding 8 g NaOH and 47 g boric acid in 1 L of water. The NZYDNA Ladder VI, with fragment sizes ranging from 50 to 1500 bp, (NZYTech) was added to the gel to verify the size of the amplified products. The electrophoresis was run at 300 volts for 30 min. Visualization was performed in Gel Doc EZ Imager (BIO-RAD laboratories, Inc., USA).

Table 2 – *Primers used to amplify ESBL genes.*

Primer name	Primer sequence	Amplicon size (bp)	Reference
*bla*TEM-F	ATGAGTATTCAACAT TTC CG	840	(9)
*bla*TEM-R	CCAATGCTTAATCAG TGA GG		
*bla*OXA-1-F	ATGAAAAACACAATACATATCAACTTCGC	820	(9)
*bla*OXA-1-R	GTGTGTTTAGAATGGTGATCGCATT		
*bla*OXA-2-F	ACGATAGTTGTGGCAGACGAAC	602	(9)
*bla*OXA-2-R	ATYCTGTTTGGCGTATCRATATTC		
*bla*CTXM pan-F	TTTGCGATGTGCAGTACCAGTAA	500	(9)
*bla*CTXM pan-R	CGATATCGTTGGTGGTGCCATA		
*bla*SHV-F	TTCGCCTGTGTATTATCTCCCTG	854	(9)
*bla*SHV-R	TTAGCGTTGCCAGTGYTCG		
*bla*VEB-F	ATTTAACCAGATAGGACTACA	1000	(9)
*bla*VEB-R	CGGTTTGGGCTATGGGCAG		
*bla*DHA con-F	TGATGGCACAGCAGGATATTC	997	(9)
*bla*DHA con-R	GCTTTGACTCTTTCGGTATTCG		
*bla*ACC-like-F	AGCCTCAGCAGCCGGTTAC	818	(9)
*bla*ACC-like-R	GAAGCCGTTAGTTGATCCGG		
*bla*CMY-F	ATGATGAAAAAATCGTTATGC	1200	(9)
*bla*CMY-R	TTGCAGCTTTTCAAGAATGCGC		
Y – T and C (pyrimidine); R – G and A (purine)			

2.5. Statistical analysis

For statistical analyses, to find out whether the $\log_{10}$ concentrations of mesophiles and resistant bacteria followed a normal distribution, a Kolmogorov–Smirnov test was applied. The mesophilic concentrations were found not to follow a normal distribution, and a non–parametric test, Kruskal–Wallis, followed by Dunns *post hoc*, was applied to examine the concentration differences between the batches. Resistant bacteria, on the other hand, followed a normal distribution, so a parametric test, the ANOVA test followed by Tukey *post hoc*, was applied to examine the concentration differences between the batches. All statistical analyses and associated figures were done in GraphPad Prism v.8.0.1 software (CA, USA).

3. Results

3.1. Classic microbiology analysis

A total of 40 samples of RTE salads were analyzed, of which 32 samples belonged to two commercially available brands (Continente and Vitacress) and 8 samples were collected from a local producer (organic). To evaluate the microbiological quality of these salads the concentration of mesophile microorganisms was determined by plating on PCA and incubating at 30 °C for 72 h. The determination of the presence of Gram-negative bacteria resistant to antibiotics was performed plating the samples on MacConkey supplemented with cefotaxime (third generation cephalosporin) and incubating at 37 °C for 24 h. The initial suspensions were further incubated at 37 °C for 24 h and then plated on MacConkey as detailed above, in order to determine the presence of ARM present at very low initial concentration.

The presence of mesophiles was evaluated, not only between different brands (16 samples per brand), but also between different batches (8 samples per batch) of the same brand. Therefore, the mean mesophile concentration (expressed as $\log_{10}$ CFU per gram) in the brands Continente was 6.9 ± 0.4 CFU/ g, the Vitacress brand was 7.3 ± 0.4 CFU/ g and the organic salad had a mean mesophile concentration of 7.3 ± 0.4 CFU/ g (Table 3). The statistical analysis revealed that there was no significant difference between the commercial brands and the organic salads.

The batch A of the brand Continente showed significant differences compared to batch B of the same brand and with the 2 batches of the brand Vitacress and the organic salad, revealing a lower concentration of mesophiles. (Figure 4).

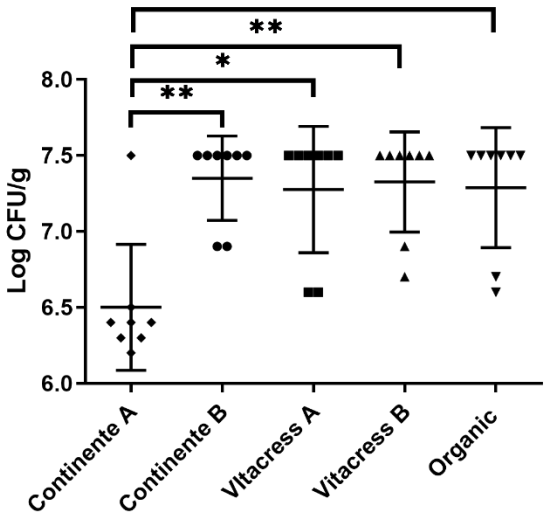

Figure 4 *– Mean concentration (log10 CFU per g) of the mesophiles obtained in the study. The results are present with the corresponding standard deviation (black spike). Comparison between brands and lots. *p<0.05 **p<0.01.*

As previously stated, the presence of cefotaxime-resistant bacteria was also analyzed, revealing a mean concentration of 5.3 ± 0.7 CFU/ g in the Continent brand, 6.6 ± 0.4 CFU/ g in the Vitacress brand and 5.4 ± 0.6 CFU/ g in the organic salad (Table 4). Thus, it should be noted that the prevalence of bacteria resistant to cefotaxime was significantly higher in the brand Vitacress than in Continente ($P<0.01$) and the same was true between Vitacress and the organic salad ($P<0.05$). Statistical differences were observed in the concentration of bacteria resistant to cefotaxime between batches of different brands. Batch A of the brand Continente showed a significantly lower concentration compared to the batch B of the brand Vitacress, while the latter batch showed a significantly higher concentration compared to the organic salad. (Figure 5).

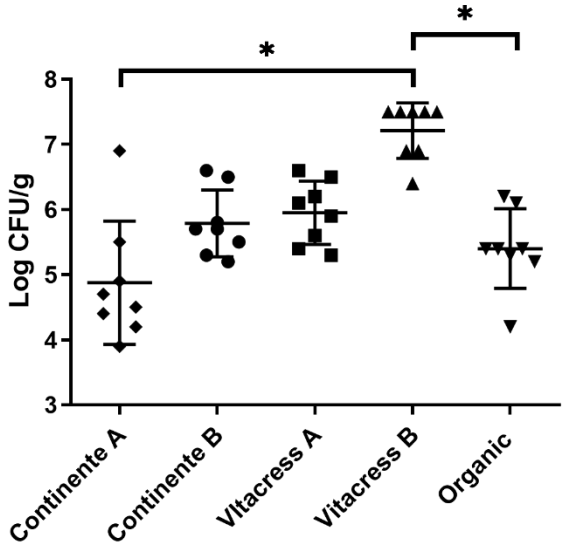

Figure 5 *– Mean concentration (log10 CFU per g) of cefotaxime resistant bacteria obtained in the study. The results are present with the corresponding standard error (black spike). Comparison between brands and lots. **p<0.01; ****p<0.0001.*

After this quantification, the cefotaxime-resistant colonies were re-isolated according to their morphological characteristics. The typical morphologies observed on MacConkey were large and small pink colonies, large and small colorless colonies, and, less frequently, pink colonies with a large and small halo. In this study enrichments were also done, to try to isolate colonies with different morphologies, however, the colonies obtained as well as the genes identified were the same, so this step did not affect the overall results obtained.

The presence of ESBL was confirmed by streaking the colonies obtained in MacConkey with cefotaxime on CHROMagar ESBL. This medium allows the identification of different ESBL bacterial species by development of different colors produced. All the isolates streaked on this medium produced cream-colored translucent colonies, which is typical for the presence of *Pseudomonas* according to the medium manufacturer.

3.2. Detection and characterization of β-lactamases genes

A total of 209 Gram-negative isolates resistant to cefotaxime, were analyzed, of which 18.2 % (n=38) belonged to batch A and 19.6 % (n=41) to batch B of Continente; 19.1 % (n=40) and 22.0 % (n=46) isolates belonged to batches A and B from Vitacress, respectively, and 21.1 % (n=44) belonged to organic salad. All the isolates in this study were subjected not only to a qPCR test, but also to a conventional PCR, the latter being performed on colonies that revealed only amplification in the qPCR and no band in the agarose gel.

3.2.1 Detection and characterization of ESBL genes in different brands

In the present study, a total of 79 bacterial colonies were isolated from the brand Continente, 86 cefotaxime-resistant bacterial colonies were isolated from the brand Vitacress, and 44 colonies were isolated from the organic salads.

In the qPCR analysis, it was observed that 81 % (n=64/79) of the isolates from Continente showed amplification that is, peaks in the melting curve, for the nine genes under study. However, as qPCR is a technique which has a higher amplification efficiency with small fragments of DNA, it was decided to confirm amplicon size of the positive isolates through gel electrophoresis. Thus, in the present brand, the agarose gel performed revealed that only 3 isolates had the presence of the specific band of the bla_{ACC} resistance gene, these being the isolates CA4LC, ECA8SC and ECB6SP. Subsequently, a conventional PCR analysis was also performed, using a different mastermix, designed to detect larger DNA fragments. In this PCR only the isolates that obtained amplification in the qPCR were subjected to analysis. Thus, in only 27.9 % (n=22/64) of the isolates the presence of resistance genes was confirmed, where it was possible to identify only 3 of the 9 genes under analysis, being them, the bla_{ACC} gene, identified in 79.17 % (n=19/22)

of the isolated bacteria. It should be highlighted that in isolates where resistant genes were identified, none corresponded to those with bands in the qPCR electrophoresis. In addition, the *bla*$_{SHV}$ gene was detected in 16.7 % (n=4/22) of the isolates, and the *bla*$_{OXA2}$ present in 4.17 % (n=1/22) (Figure 6 and 7).

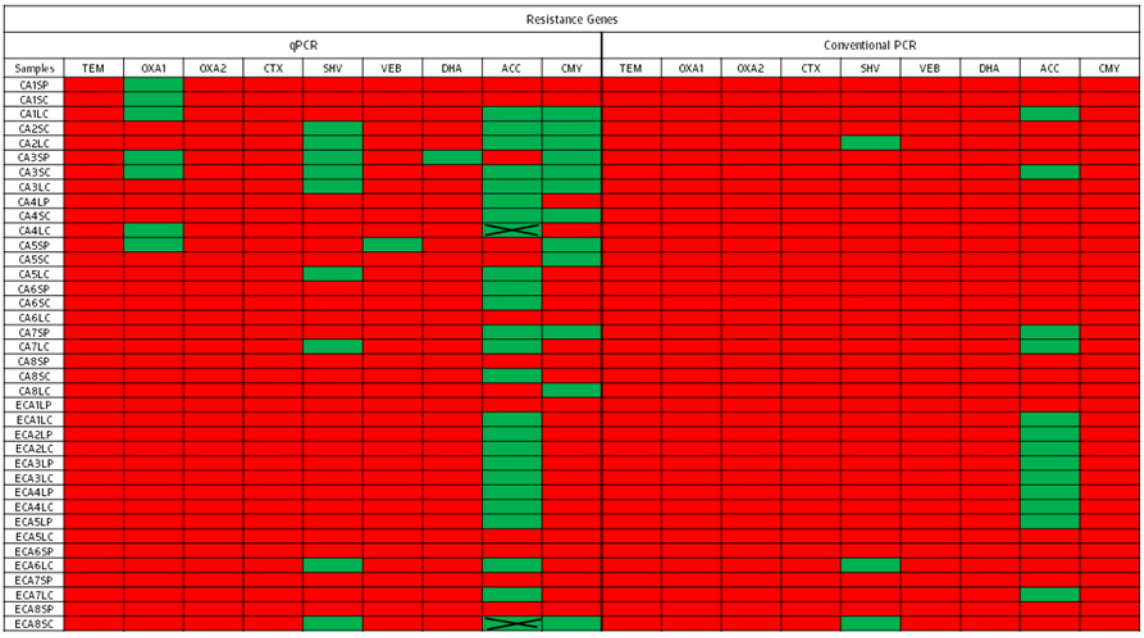

Figure 6 *– Identification of the resistance genes present in the isolates from batch A of the brand Continente by qPCR and conventional PCR. Resistance gene not detected (appear in red), resistance gene detected (are shown in green), and resistance gene detected and confirmed in qPCR are represented with a black "X".*

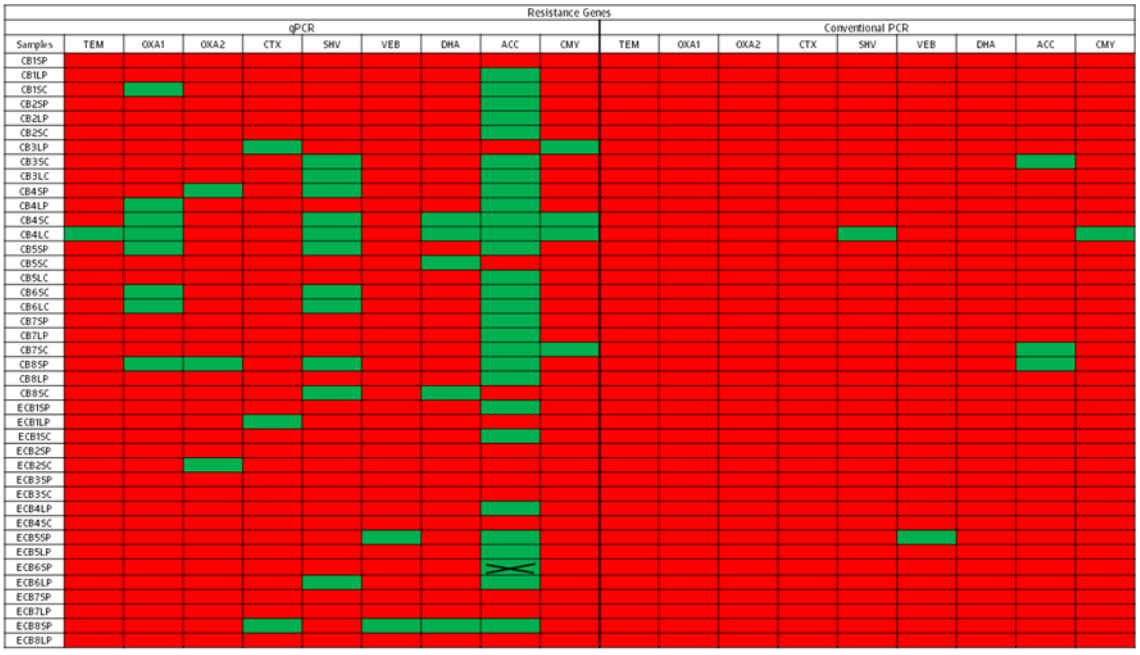

Figure 7 *– Identification of the resistant genes present in the isolates from batch B of the brand Continente by qPCR and conventional PCR. Resistance gene not detected (appear in red), resistance gene detected (are shown in green), and resistance gene detected and confirmed in qPCR are represented with a black "X".*

Regarding the brand Vitacress was subjected to the same type of analysis, and the qPCR revealed that 86.1 % (n=74/86) of the isolates had peaks in the melting curve, which indicated the presence of larger fragments. These same isolates were then subjected to agarose gel electrophoresis to confirm the results. The agarose gel showed that only isolate VA6SC had the presence of a characteristic band of the *bla*$_{ACC}$ gene, however, the positivity of this isolate was not confirmed by conventional PCR. Following, to confirm the results, the same isolates underwent conventional PCR analysis, which revealed that only 33.7 % (n=29/74) of the isolates had resistance genes, and only 7 ESBL genes were identified. The most prevalent gene once more was the *bla*$_{ACC}$ with a 55.2 % (n=16/29), followed by the *bla*$_{VEB}$ with a 17.2 % (n=5/29) and the *bla*$_{OXA2}$ which was identified in 13.8 % (n=4/29) of the isolates. Equally prevalent were the *bla*$_{TEM}$, *bla*$_{OXA1}$, *bla*$_{SHV}$ and *bla*$_{DHA}$ genes present in 3.5 % (n=1/29) of the bacteria isolates (Figure 8 and 9).

Figure 8 *– Identification of the resistant genes present in the isolates from batch B of the Vitacress brand by qPCR and conventional PCR. Resistance gene not detected (appear in red), resistance gene detected (are shown in green), and resistance gene detected and confirmed in qPCR are represented with a black "X".*

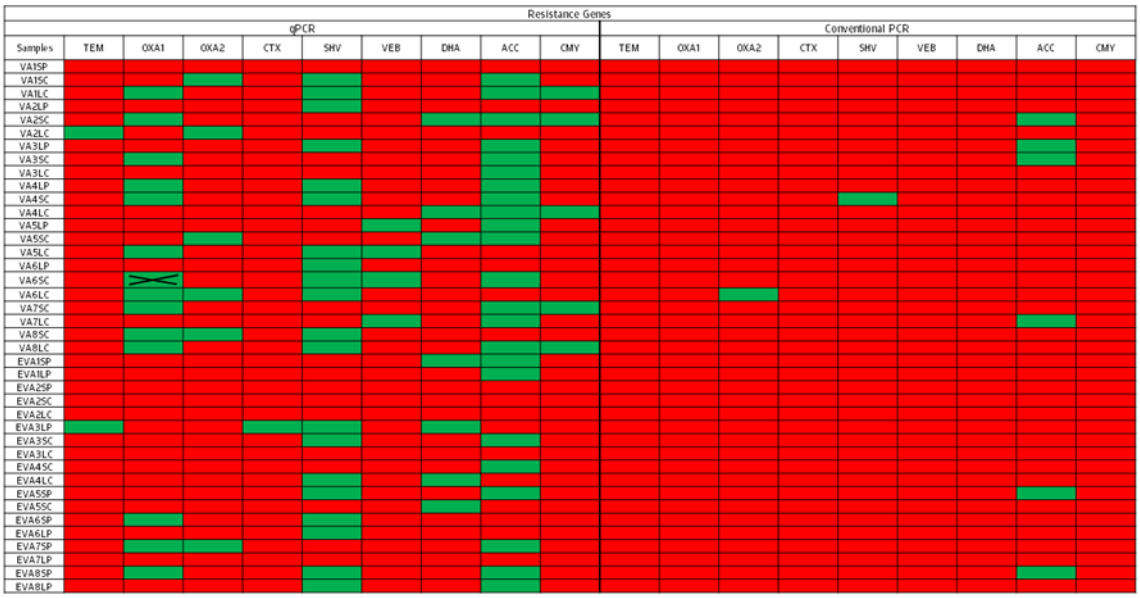

Figure 9 *– Identification of the resistant genes present in the isolates from batch A of the brand Vitacress by qPCR and conventional PCR. Resistance gene not detected (appear in red), resistance gene detected (are shown in green), and resistance gene detected and confirmed in qPCR are represented with a black "X".*

In the organic salad, the qPCR analysis demonstrated that 70. 5 % (n=31/44) of the isolates had peaks in the melting curve. Similar to the brands already presented, to confirm the results an electrophoresis was performed, where only the isolate 04SP showed the presence of a characteristic band of the *bla*$_{ACC}$ gene (818bp) in the agarose gel, which was confirmed by conventional PCR. The conventional PCR analysis confirmed that only 25 % (n=11/31) of the isolates that had demonstrated the presence of genes in the qPCR were positive. In contrast to the commercial brands, the most prevalent gene was *bla*$_{VEB}$ with 54.6 % (n=6/11), subsequently, the *bla*$_{ACC}$ gene in 27.3 % (n=3/11) of the isolates. And less prevalently, the *bla*$_{SHV}$ and *bla*$_{DHA}$ genes present in 9.1 % (n=1/11) of the isolates (Figure 10).

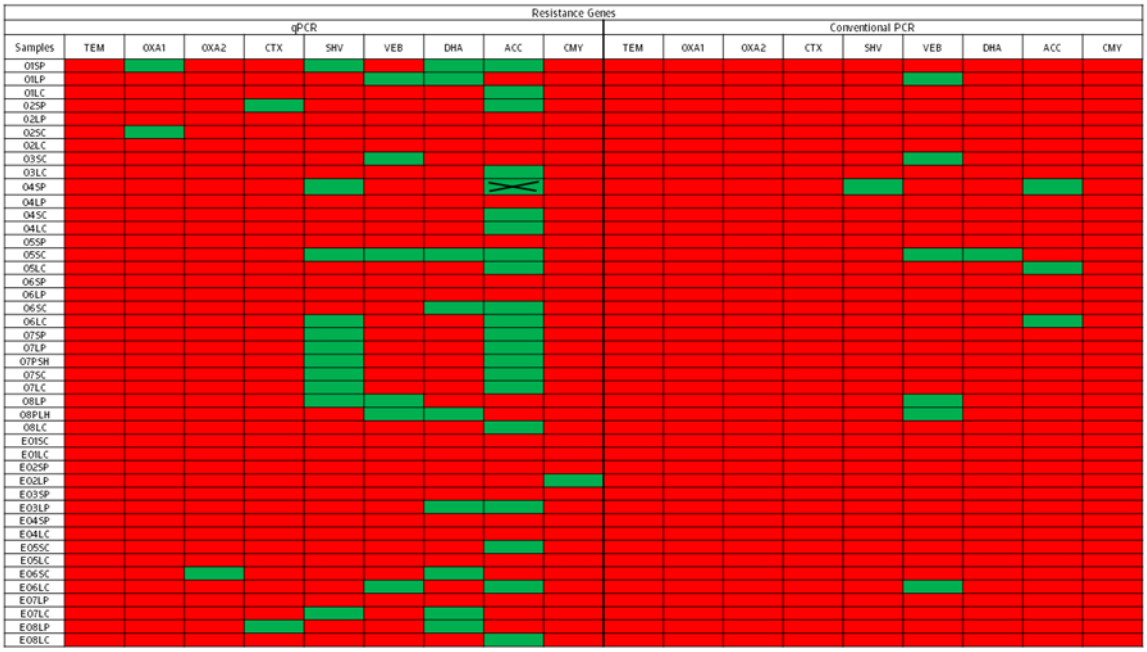

Figure 10 *– Identification of the resistant genes present in the isolates from organic salads by qPCR and conventional PCR. Resistance gene not detected (appear in red), resistance gene detected (are shown in green), and resistance gene detected and confirmed in qPCR are represented with a black "X".*

Comparing the brands attending to the presence of cefotaxime resistance genes, the values obtained by qPCR revealed that Continente and Vitacress did not present any significant difference. The same also applied to the comparison among the brand Continente and the organic salad. However, when compared against Vitacress, the organic salad showed a significantly lower presence of resistance genes than the brand Vitacress (P=0.0328). The analysis by conventional PCR showed no significant difference among the RTE salads tested, regardless they were commercial or organic (P<0.05). Of note, the most prevalent gene in all brands by both qPCR and conventional PCR, was bla_{ACC} (Figure 11, 12, 13 and 14).

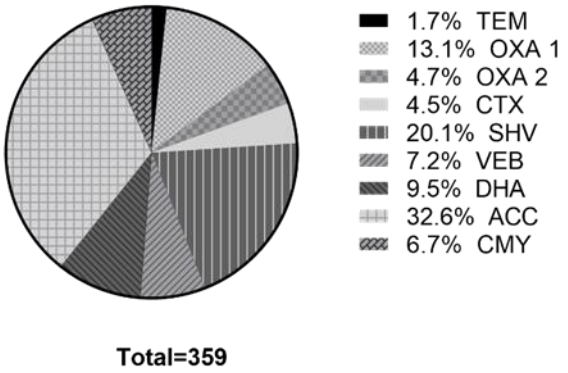

Figure 12 *– Gene prevalence by qPCR*

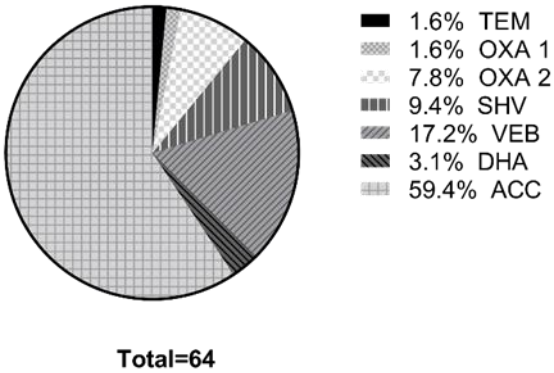

Figure 11 *– Gene prevalence by conventional PCR*

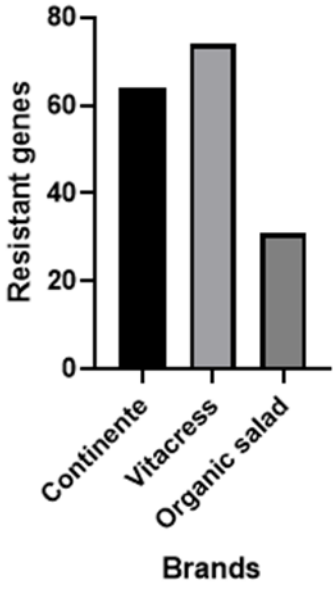

Figure 13 *– Resistance genes identified by qPCR in the 3 salad brands*

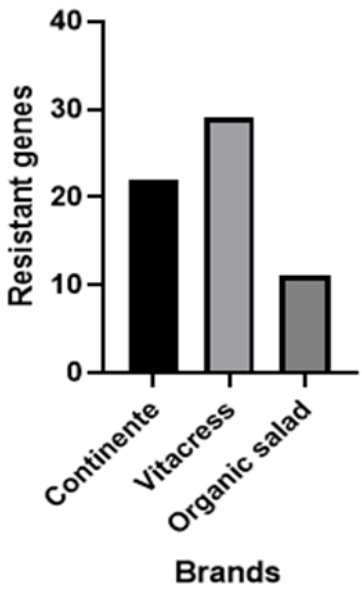

Figure 14 *– Resistance genes identified by conventional PCR in the 3 salad brands*

3.2.2 Detection and characterization of ESBL genes between batches

The prevalence of resistance genes was also compared between each batch. The qPCR analysis of batch A of the Continente revealed that 81.6 % (n=31/38) of the isolates showed the possible presence of resistance genes, and 48.4 % (n=15/31) of the isolates for more than one gene. Electrophoresis was performed in order to confirm the presence of these resistance genes. In this analysis it was detected that the isolates CA4LC and ECA8SC showed in the agarose gel the presence of the characteristic band of the bla_{ACC} gene (818 bp), however, its presence was not confirmed by conventional PCR. Thus, the isolates that showed peaks in the melting curve indicative of the presence of larger fragments were subjected to a conventional PCR, which demonstrated that only 2 genes among the 9 study subjects were present. In this batch it was possible to demonstrate that only 42.1 % (n=16/31) of the isolates actually had the presence of ESBL genes. The most prevalent was the bla_{ACC} gene present in 81.25 % (n=13/16) of the positive isolates and the bla_{SHV} gene present in 18.8 % (n=3/16) (Figure 6).

Regarding batch B, qPCR showed that 80.5% (n=33/41) of the isolates revealed the possible presence of resistance genes, and about 51.5% (n=17/33) of them showed that they could have more than one ESBL gene. After this analysis, the isolates that indicated the presence of larger fragments were subjected to electrophoresis for confirmation of the results. The agarose gel indicated the presence of a characteristic band of the bla_{ACC} gene (818bp) in the isolate ECB6SP, however, its presence was not confirmed by further analysis. In order to confirm the presence of resistance genes among the isolates that had been positive in the qPCR, conventional PCR was performed, as in the previous batch, this revealed that only 14.6 % (n=6/33) of the isolates showed the presence of resistance genes, and of the 9

studied only 4 ESBL genes were identified, these being *bla*$_{ACC}$ present in 50 % (n=3/6) of the isolates in this analysis and *bla*$_{SHV}$, *bla*$_{VEB}$ and *bla*$_{CMY}$ present in 16.7 % (n=1/6) of the isolates that were positive (Figure 7).

In lot A, the brand Vitacress was subjected to the same type of analysis as the previous brand. The results obtained by qPCR revealed that it was possible to observe peaks in the melting curve indicating the probable presence of resistance genes in 85% (n=34/40) of the isolates and, 79.4% (n=27/34) of these isolates could have more than one gene present. Subsequently, in the confirmation analysis by electrophoresis there was only one band in the agarose gel, at 820 bp, characteristic of the *bla*$_{OXA1}$ gene, in the VA6SC isolate. The conventional PCR analysis performed and only 20.0 % (n=8/34) of the isolates that had shown amplification in the previous analysis confirmed the presence of resistance genes, furthermore only 3 resistant genes were detected. The genes identified were *bla*$_{ACC}$ gene in 75.0 % (n=6/8) of the isolates, *bla*$_{OXA2}$ and *bla*$_{SHV}$ present in 12.5 % (n=1/8) of the isolates (Figure 8).

Batch B of the brand Vitacress revealed that 87.0 % (n=40/46) of the isolates showed the presence of peaks on the melting curve indicative of larger DNA fragments, and, 75.0 % (n=30/40) of these isolates were probable to have more than one of these genes present. Unlike previous batches, it was not possible to confirm the presence of any resistant genes by electrophoresis. However, the same isolates when subjected to conventional PCR analysis showed that 45.7 % (n=21/30) had the presence of resistance genes. The *bla*$_{ACC}$ gene was the most prevalent, present in 47.6 % (n=10/21) of the isolates, *bla*$_{VEB}$ was identified in 23.8 % (n=5/21) of the isolates, *bla*$_{OXA2}$ was present in 14.3 % (n=3/21) of the isolates and, finally, the *bla*$_{TEM}$, *bla*$_{OXA1}$ and *bla*$_{DHA}$ genes were present in 4.8 % (n=1/21) of the isolated colonies (Figure 9).

Thus, it was possible to observe that there were no significant differences (P=0.917) in regards to the amount of resistance genes presents by qPCR analysis, among the batches analyzed for the brand Continente. The same observation was made for the batches of the brand Vitacress (P=0.7940).
When the comparison was made based on the results obtained by conventional PCR, there were significant differences between lot A and B of from Continente, as lot A had a higher number of resistance genes compared to lot B (P=0.0065). The same happened for Vitacress, where batch B showed a significantly higher number of resistance genes compared to batch A (P=0.0121) (Figure 15).

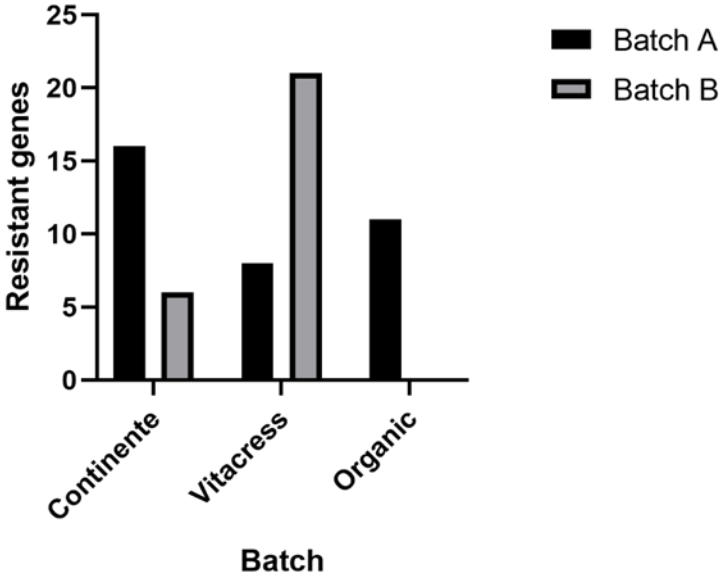

Figure 15 *– Number of resistance genes identified by conventional PCR per batch of each brand considering all the isolates recovered.*

4. Discussion

RTE salads can pose a threat to public health, as they are potential vehicles for the transmission of pathogenic microorganisms, capable of causing foodborne diseases (41). It is important to evaluate the microbiological quality of the foods before they are ready for consumption, thus we performed mesophile counts on the samples under study. It was verified that all analyzed salads had a high concentration of these microorganisms, with no significant difference between commercially available brands, nor between the organic salad. These results corroborate the study described by Campos J. *et al*, who said that these high concentrations of mesophile indicate that hygiene standards may not be being followed, especially during processing, transportation/ or storage (42). According to Abadias M. and collaborators, organic salads would be expected to have significantly lower mesophilic concentrations than RTE salads, because organic salads are not subjected to any processing and are therefore less exposed to new sources of contamination (43). However, Sagoo *et al*, contradicts the previous facts, because the RTE salads, as they are on sale in large commercial surfaces, go through a strict control process, where they are washed and decontaminated, to decrease the microbial load (44). Therefore, the high concentrations of mesophiles obtained in all the samples may not only be related to the fact that these foods grow close to the soil, being in direct contact with the bacteria already present there, but also to improper handling during the processing phase. The transport and storage itself are other factors to be considered. Therefore, it is important to follow the storage and manipulation instructions indicated by the supplier, so that there is no possible increase in the microbiological load (43,44).

Although no significant difference was found among the brands Continente and Vitacress, and between these and the organic salad, the same did not happen with the analyzed batches. Batch A of the brand Continente had a significantly lower concentration of mesophiles compared to the batch B of the same brand and to the batches of the brand Vitacress and the organic salad. The difference between the batches of the same brand may be related to the processing phase, where the conditions are not exactly the same, because despite belonging to the same brand, these processes occurred at different times. The difference found with the other brand's batches and with the organic salad may be related to storage, since bacterial growth is possible during this phase. Torriani S. *et al* found that some mesophile microorganisms can continue to grow at low temperatures. Finally, handling during the experimental phase may also have contributed to this increase (41,45). Regarding the bacteria resistant to cefotaxime, in a first analysis, the salad samples were seeded on MacConkey (selective medium for Gram-negative bacteria) and cultured with the antibiotic under study. The concentrations obtained of these resistant microorganisms were high in all the samples, showing agreement with the studies carried out by Kim H. *et al* (46). Several causes may be associated with these results, such as production. During growth, salads can be contaminated by pathogenic bacteria coming from animals and humans. This contamination can also occur through contact with the soil, that is, the microorganisms already existing in the soil, when indirectly or directly exposed to

antibiotic residues, cause them to be subjected to a high selective pressure leading them to develop resistance, and pass it to the vegetables there. The use of manure and contaminated wastewater is another factor that can lead to the appearance of resistant bacteria in these fresh foods. In addition to these causes, it is important to note that these salads can also be contaminated during processing or even in stores by incorrect handling and storage (47,48).

It was possible to find significant differences in the concentration of resistant bacteria between the brands analyzed. Vitacress had a higher concentration of bacteria resistant to cefotaxime compared to the Continente and the organic salad. This difference may be related to the growth conditions, which were different for all salads, i.e., the soil, manure and water used differed, and in general, the whole production process, packaging and distribution, crucial factors for the development and growth of resistant bacteria. However, as mentioned before, the steps inherent to processing may also be possible sources of contamination, since there is greater handling of the products, increasing the likelihood of contamination. In addition, significant differences were observed among the batches of each brand, with batch A of the brand Continente showing a significantly lower concentration of resistant bacteria compared to batch B of the brand Vitacress, and the latter showing a significantly higher concentration of resistant bacteria compared to the organic salad. The fact that the organic salads have a lower concentration than batch B of the brand Vitacress can be explained by the fact that they do not have the processing phase, which makes them less exposed to contamination. Other reasons that could explain these results have to do with the processing stages that differ from brand to brand and even from batch to batch, since the conditions are not always exactly the same. In addition, factors such as transportation and storage, may have contributed to a higher exposure to resistant microorganisms.

It is worth to mention that the isolates exhibiting phenotypic resistance to cefotaxime on MacConkey agar, regardless the presence of the panel of ESBL genes selected, were confirmed by culturing on CHROMagar ESBL, from which preliminary species/genus indication was obtained. This medium can be used for the detection of Gram-negative bacteria that produce β-lactamases; this recognition is base attending to distinctive color production on the medium. In the present study, all isolates produced a translucent cream-colored, indicative of the presence of *Pseudomonas*. It has been described that these bacteria can grow on raw vegetables at harvest and post-harvest stages (49). Also Pang Z. and co-workers showed that *Pseudomonas* spp. are producers of extended-spectrum β-lactamases, conferring resistance to third-generation cephalosporins such as cefotaxime (50). We performed, preliminary species identification, based on 16S rRNA and MinION sequencing, confirming the presence of this microorganism among the isolates recovered, corroborating the observations from other authors previously mentioned.

Gram-negative bacteria use several processes to inactivate antibiotics. When it comes to β-lactam antibiotics, such as cefotaxime, these bacteria produce certain enzymes, β-lactamases, specifically ESBL,

which give them resistance (51). In this work, nine genes which can confer resistance to cefotaxime, were analyzed. The isolates from RTE salads and organic salad were subjected to qPCR and conventional PCR tests, previously described, which made it possible to identify the ESBL genes present in each of them. It was possible to observe that in almost all samples, 7 of 9 genes used were confirmed, and some of the isolates obtained were positive for more than one gene, which demonstrated a high prevalence of ESBL genes in the analyzed salads. Similarly, to our findings, Kim H. and colleagues, who isolated antibiotic-resistant bacteria in RTE salads, found that the most prevalent genes belonged to ESBL class A, the *bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{SHV}$ genes (46,52). Additional studies realized by Rúben Fernandes et al, also verified that the three genes previously mentioned were the most prevalent in the north of Portugal, more specifically in the Minho region (53). Therefore, the literature contradicts, in part, the results obtained in this study, because the most prevalent genes detected in all brands were *bla*$_{ACC}$ and *bla*$_{OXA1}$ genes in qPCR analysis and *bla*$_{ACC}$ and *bla*$_{VEB}$ genes by conventional PCR analysis. The low prevalence of *bla*$_{TEM}$ or *bla*$_{CTX}$ genes may be explained by the way the study was performed, since the choice of colonies was random, based only on the morphology of the isolates, thus this type of methodology limits the analysis of the microorganisms present in the sample. It should also be noted that this study was done on only two brands and one organic salad, so the samples screened was limited. The *bla*$_{SHV}$ and *bla*$_{VEB}$ genes were also found to be prevalent among the isolates, however, this result would be expected, especially the *bla*$_{SHV}$ gene, since these genes belong to class A of the β-lactamases, a class that besides being widely described and prevalent worldwide in food products, also has genes that are responsible for conferring a high resistance to cefotaxime, as described in previous studies (54,55). Of note, the results obtained by qPCR showed that there is a need for optimization of this protocol, namely redesigning the primers used or even choosing primers where the amplicon size is more suitable, i.e. smaller.

It is worth to note that there were significant differences, both by brand and by batch, regarding the number of isolates identified with resistance genes. All these differences may be related to the number of morphologies, since the more morphologies, the more isolates, therefore, the more resistance genes could potentially be identified.

5. Conclusions

In conclusion, with this project it was possible to confirm the presence of antibiotic resistant microorganisms in foods such as RTE salads and organic salads. We found a high concentration of bacteria resistant to cefotaxime, since it was possible to find, through the ChromAgar ESBL, that they had a phenotype that expressed ESBL genes. A preliminary study indicated that the microorganism present could be *Pseudomonas* spp. Regarding the ESBL genes the most prevalent were bla_{ACC}, bla_{SHV} and bla_{VEB}. The present study demonstrates, that RTE salads have proven to be a potential source of transmission of resistant bacteria and may represent additional risks for individuals at higher susceptibility of developing infections such the elderly and immunosuppressed people.

Since antimicrobial resistance has emerged as a global threat to public health, it is urgent to control the use of antimicrobial agents as well as to develop new antibiotics that are effective. However, we also need to know the possible sources of transmission of resistant bacteria to prevent their spread.

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