Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the prospection of biological hazards of interest in food safety in Spain (2)

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Working group

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Abstract

This report addresses the prospection of biological hazards for some types of food that may pose a risk to the population and that are not currently included in the official control programs in Spain. It completes and updates the 2018 report by the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN, 2018). A number of bacteria that are significant contributors to nosocomial infections due to the increase in the number of multi-resistant strains of *Acinetobacter* spp., *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are listed first. It is also addressed the study of the prevalence and possible control of *Bacillus cereus* and *Cronobacter* spp. presence in cereal flours and others, the revision of *Campylobacter jejuni* and/or *Campylobacter coli* in meats

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other than poultry, as well as the study of Shiga toxin-producing *Escherichia coli*. These latter two biological agents are much better known from the food control perspective, although there are control measures for *Campylobacter* spp. in poultry meat and not in other types of meat such as beef or pork and in the case of *E. coli*, producers of Shiga toxins, the control of this particular type of pathogenic strains in food has not been specifically addressed either. Finally, tick-borne viral encephalitis, which can be transmitted to humans through the consumption of raw milk or raw dairy products, has been indicated as a viral hazard.

The prospective study shows the need to determine the prevalence of multi-resistant bacteria of *Acinetobacter baumannii*, *K. pneumoniae* and *P. aeruginosa* in foods in Spain, especially in ready-to-eat foods such as salads and fresh plant-based foods. This is especially important due to the lack of data on the prevalence of these bacteria in foods in Spain. However, food research is carried out in neighbouring countries. It is also necessary to include *C. jejuni* and/or *C. coli* in the investigations of beef and pork, since the incidence of these foodborne pathogens in humans is not explained solely by the presence of these agents in poultry meat, being their presence in other animals for slaughter also evident. Similarly, outbreaks of Shiga toxin-producing *E. coli* have been reported in Spain over the last 25 years, which makes it advisable to control them in beef, raw milk and leafy vegetables. With regard to *Cronobacter* spp. and *B. cereus*, the importance of these agents can be demonstrated given their survival in powdery materials such as flours of different origins, including cereals, although the reported outbreaks do not seem to indicate a high prevalence. As regards the only viral hazard mentioned, it should be noted that the wide dispersion of the ticks that can transmit this virus, together with the potential consumption of raw milk, makes it advisable to investigate it in raw milk products. However, the study of the actual infective capacity of this virus is not easy to establish with simple analytical methods. With this last exception, research for controlling all these biological hazards in food is possible, with classical or advanced methodologies that are robust enough, available for each case.

Key words

Acinetobacter, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Cronobacter* spp., *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli*, STEC, Shiga toxin, viral encephalitis, ticks, *Ixodes*, multiresistance.

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1. Introduction

Different biological hazards that may pose a risk to the consumer may be present, incorporated or occur along the food chain.

Official control programmes aim to ensure risk-based controls of food safety hazards, but concern only those parameters with maximum established limits on certain foods.

However, there are other hazards of interest to food safety for which there is no specific regulation or such regulation exists, but only for certain foods. This can be the object of prospecting programmes in order to obtain data that, in addition to protecting the consumer from timely exposure to a hazard, enable the performance of a risk assessment.

In addition, identification of new hazards for which significant exposure may occur, or the assessment of the risk arising from new or significantly increased exposure or susceptibility to a known hazard, is important, not only for an eventual control of these emerging hazards, but also to promote research and improve consumers and scientific community awareness.

For this reason, in 2018, the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) published a first report on the prospection of biological hazards of interest to food safety in Spain (AESAN, 2018) in which the following were noted:

- Food-borne virus: norovirus, Hepatitis A virus and Hepatitis E virus in bivalve molluscs and fresh vegetables, and Hepatitis E virus in pork meat by-products.
- Bacteria: *Yersinia enterocolitica* in pork meat, *Vibrio parahaemolyticus* and *V. vulnificus* in fishery products and bivalve molluscs, *Escherichia coli* (non-STEC pathotypes) in fresh vegetables and *Clostridium difficile* in fresh meat.
- Parasites: protozoa (*Toxoplasma* and *Cryptosporidium*) in fresh meat and fresh vegetables.

In order to incorporate more hazards of interest into possible prospective studies, the Scientific Committee is requested to carry out a new review of the hazards of greatest interest in food safety in Spain that do not have a specific regulation, identifying them and pointing out those foods or conditions that, *a priori*, could imply a greater risk for the consumer.

2. Biological hazards and suggested foods

This document contains the prospective study of recent scientific documentation on the presence of certain bacteria that usually carry multiple determinants of resistance to antibiotics habitually used in the treatment of processes caused by such bacteria, such as *A. baumanii*, other multiresistant *Acinetobacter* and *K. pneumoniae* multiresistant in meat, milk, and vegetables and/or ready-to-eat salads, as well as *Pseudomonas aeruginosa* multiresistant in food of plant origin. The presence of *Bacillus cereus* and *Cronobacter* spp. in flours. Also the study of *Campylobacter jejuni* and *Campylobacter coli* in pork beef and other meats. Also the presence of Shiga toxin-producing *E. coli* in beef, raw milk and leafy greens. Finally, as the only viral hazard, tick encephalitis in raw milk and unpasteurised dairy products of goats, sheep, and cattle.

3. *Acinetobacter* spp. multidrug-resistant

3.1 General

The use of antimicrobials both in humans and animals is often the reason behind the increasing number of multidrug-resistant bacteria. Despite the growing number of antimicrobials available for the treatment of diseases in recent years, perhaps as early as in the last decade of the last century, certain microorganisms that initially do not appear to be associated with specific diseases in humans have become "popular". One of these bacteria is *Acinetobacter*. The genus *Acinetobacter* was initially described in 1911 by Beijerinck (1911), being *Acinetobacter baumannii* probably the most known and important species of this genus. It was described in 1986 by Bouvet and Grimont (1986), although the genus *Acinetobacter* currently comprises about 80 species, its allocation at the taxonomic level is complex. A most relevant aspect of this agent is that, while in the last decades of the last century the isolated strains of *A. baumannii* were generally sensitive to most antimicrobial agents, today it is increasingly common to isolate multidrug-resistant strains, a fact that is of global concern. In this regard, the World Health Organization (WHO) has identified antimicrobial resistance as one of the three most important problems for human health (Bassetti et al., 2011).

3.2 Impact of the disease on people

The genus *Acinetobacter* is composed of Gram negative coccobacilli, strictly aerobic, non-fermenting, non-motile and oxidase negative. The species included in the group of *A. baumanii* are the species most frequently related to nosocomial infections worldwide. In fact, *A. baumannii* is included in the acronym ESKAPE which encompasses bacteria capable of "escaping" the action of antimicrobials (Pendleton et al., 2013). In relation to multiresistant bacteria, *A. baumannii* can be considered a paradigmatic microorganism (Souli et al., 2008) and its frequent resistance has been related not only to strains of clinical origin from human patients but also of animal origin (Zhang et al., 2015) and practices sometimes used in the production of slaughter animals (Adeyemi et al., 2022). In this regard, the natural transformation of these bacteria to acquire resistance is also mentioned (Domingues et al., 2019) as well as the participation of certain membrane proteins (Han et al., 2022). In recent decades, the spread of multiresistant strains of *A. baumannii* has resulted in a multiplication of severe cases at the hospital level requiring intensive care (Mc Connell et al., 2013). It can cause pneumonia in humans, with the highest rates of pneumonia mortality occurring in patients on assisted ventilation. This has become of particular interest in the pandemic years because of COVID 19 and its treatment with assisted ventilation in many cases of hospitalised patients with pneumonia. Indeed, there is multiple literature addressing hospital outbreaks of *A. baumannii* in COVID patients (Wu et al., 2021) (Russo et al., 2022). These bacteria also cause bacteremia and endocarditis, among other pathologies, along with the problematic of the survival of the agent in hospital equipment and personnel that have been in contact with it (Mc Connell et al., 2013), which means that many cases can develop at the hospital level and the bacteria can even be transferred from one hospital to another. Within hospitals, the process can be so severe that mortality can be as high as 60 % in patients with pneumonia on assisted ventilation who are infected with multidrug-resistant *A. baumannii* (Ciginskiene et al., 2019).

Treatment of *A. baumannii* infections was simple in the 1970s, but the rapid increase in multiresistances has caused this treatment to evolve. The β-carbapenemic lactams, such as imipenem and meropenem are antimicrobials of choice for these infections. However, colistin is also indicated as an antimicrobial for the treatment of this agent (Asif et al., 2018). In any case, the existence of a high percentage of strains resistant to β-carbapenemic lactams (CRAB, *Carbapenem Resistant Acinetobacter baumannii*) is indicated, so new strategies for treatment are proposed, namely the use of tigecycline (Isler et al., 2019) and minocycline (Nair, 2018) (Isler et al., 2019).

3.3 Prevalence in food

Although a specific transmission from food is not demonstrated, the presence of these bacteria in food poses an inherent risk to humans. This is determined, on the one hand, by the increased number of people susceptible to infections with opportunistic agents (immunosuppressed, elderly, etc.) as well as by the easier distribution of these agents into hospitals and increased spread of resistance to other microorganisms. However, the transmission of *A. baumannii* through drinking water has been documented (Umezawa et al., 2015).

The presence of *A. baumannii* in various types of food is clearly established. So, Guo et al. (2021) indicate that up to 4.52 % of the bacteria present in milk belong to the genus *A.*, with *A. lwoffii* being one of the three most prevalent species in a study that analysed 355 milk samples from various regions of China. Likewise, bacteria of the group *A. baumannii/calcoaceticus* have been found in a total of 39.4 % of milk, yoghurt and cheese samples in Brazil (Rabêlo et al., 2021). *A. baumannii* have also been isolated in traditional fermented African foods obtained from bean oil (Okorie et al., 2017). The development of fermentation as a characteristic element of many foods is key in relation to the microbiota of these types of products (Abouelnaga et al., 2016). The fact that these agents may appear in fermented foods is important, given the increased consumption of functional foods and probiotics supplements. In these cases, the aim is to add living microorganisms that improve or restore the proper functioning of the human digestive system and provide a nutritional and health improvement (FAO/WHO, 2006). Thus, it is worrying that some studies (Mazzantini et al., 2021) have shown of this type of supplement with *Acinetobacter* spp., reporting counts of *A. baumannii* populations above 10^{11} per dose of probiotic (Celandroni et al., 2019). Likewise, *Acinetobacter* has demonstrated an ability to persist in powdery infant formulas (Juma et al., 2016), surviving desiccation.

In relation to the prevalence in meat; studies in raw meat can be found in Switzerland. Lupo et al. (2014) determined 25 % of meat samples positive to *A. baumannii* between November 2012 and May 2013; being chicken meat the most frequently contaminated with this agent. In Portugal, it was identified in multiple types of meat (Carvalheira et al., 2017a), and it is reported that *Acinetobacter* spp. was isolated from all meat samples included in the study (chicken, turkey, beef, and pork). 13 different species were identified, with a great genetic diversity and a prevalence of 18.7 % of the strains belonging to the group of *A. baumannii,* the second most prevalent group out of a total of 223 isolates. In Peru, several *Acinetobacter* species were also isolated in meat (Marí-Almirall et al., 2019). Chinese researchers also show the high prevalence of agents of the genus *Acinetobacter* in pork in the first moments after refrigeration. Although this prevalence is decreasing as the refrige-

ration period increases in favour of *Pseudomonas* (Wang et al., 2022a). In China, the presence of this agent was also investigated in *bacon* with, in this case, lower values, accounting for 8.65 % (Wang et al., 2022b). *Acinetobacter* has also been isolated in fresh products ready for consumption or that do not require a culinary treatment, like the components of salads such as lettuce, or fruits (Carvalheira et al., 2017b). In this case, *Acinetobacter* was isolated in 77.9 % of the samples, with 11 % of the strains isolated belonging to the *A. baumannii* group. Finally, it can be noted that *Acinetobacter* is a ubiquitous genus in water (Vaz-Moreira et al., 2017), having also been detected in drinking water (Van Assche et al., 2019).

Acinetobacter has also been identified in fish, both in Spain (González et al., 2000) and in other countries, in similar proportions to those obtained for other products, and even strains related to diseases in humans have been molecularly determined (Houang et al., 2001).

Evidence of hospital-level contamination of surfaces, water, etc. by this bacterium has been found. However, contamination by these agents also appears on food processing surfaces (Xu et al., 2022), thus justifying the high prevalence that seem to be present in all foodstuffs in general. Prevalence studies in recent years seem to show more consistent results, probably due to the generalisation of studies based on cutting edge techniques of massive sequencing. However, with the exception of the study by González et al. (2000), no food prevalence results were found in Spain for these bacteria.

It is noteworthy that, although the number of publications investigating *Acinetobacter* and *A. baumannii* in humans, animals or food is abundant, in most cases there are no quantitative values of the populations determined for these bacteria, which seem to range between 50 and 1000 CFU/g, from data obtained from plant foods (Berlau et al., 1999).

Most of the studies cited indicate antibiotic resistance studies of *Acinetobacter* or *A. baumanni* strains against various antimicrobials. However, the published results are not clarifying enough. Low prevalence of antimicrobial resistance and absence of multidrug resistance (Marí-Almirall et al., 2019) was observed in meat in Peru. In Switzerland (Lupo et al., 2014) meat-derived *A. baumannii* isolates were generally susceptible to clinically relevant antibiotics without resistance to carbapenemic antimicrobials. The existence of resistance against sulfamethoxazole-trimetroprim, tetracycline and aminoglycosides is also reported, but resistance against meropenem or imipenem (carbapenemic antimicrobials) was only detected sporadically (Carvalheira et al., 2017a). However, these Portuguese authors point out that 51.2 % of the strains were considered resistant to several antimicrobials (MDR, Multi Drug Resistance) and 9.6 % extensively resistant to antimicrobials (XDR, extensively drug resistance), being 38.7 % MDR for the *A. baumannii* group.

3.4 Determination methods

The most recent prevalence studies have been conducted using mass sequencing-based systems (Guo et al., 2021), basing their identification on 16S rRNA sequences. This does not allow determining aspects such as antimicrobial resistance. However, *A. baumannii* was also isolated from food by various culture media within the context of classical microbiology, such as modified Leeds medium for *Acinetobacter* (MLAM, Modified Leeds Acinetobacter Medium) containing vancomycin

(Houang et al., 2001), or selective/chromogenic media for β-lactamase-producing enterobacteria of extended spectrum (Lupo et al., 2014). Perhaps the most widely used culture medium for its isolation is CHROMagar™ *Acinetobacter.* However, microorganisms of this genus have also been isolated from media for counting and isolation of enterobacteria such as MacConkey agar (Marí-Almirall et al., 2019) or MacConkey added with various antimicrobials such as amoxicillin, cephradine and phosphomycin. These media have been compared by Moran-Gilad et al. (2014). Enrichment media for food research within the context of classical microbiology have also been proposed (Carvalheira et al., 2016) such as Baumann's and Dijkshoorn's enrichment media. Preliminary phenotypic identification is simple, and confirmation at the species level by molecular methods including genotyping by pulsed-field electrophoresis and/or study of the sequence of the *rpoB* gene (902 bp) (Rafel et al., 2015) (Carvalheira et al., 2017a). Other identification methods such as MALDI-TOF have also been employed (Toh et al., 2015). Verification of resistance in identified strains can be done by phenotypic methods, following guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2012). There are multiple articles in which the genes responsible for the resistance of *Acinetobacter* are referenced (Tavakol et al., 2018) (van der Kolk et al., 2019).

4. Multidrug-resistant *Klebsiella pneumoniae*

4.1 General

K. pneumoniae is another agent included in the acronym ESKAPE. It is an enterobacteria with a marked ability to become resistant to multiple antimicrobials, undoubtedly one of the main aspects to take into account. Like other bacteria included in the acronym ESKAPE, in the last decade the prevalence of processes caused by this agent and the prevalence associated with strains of *K. pneumoniae* multiresistant to antimicrobials has multiplied (Wyres et al., 2020).

K. pneumoniae is originally an enterobacteria of fermentative metabolism and, in this sense, different from *Acinetobacter* spp.

4.2 Impact of the disease on people

Although classically *K. pneumoniae* was considered a bacterium causing community-acquired lung processes (Ko et al., 2002). Nowadays it is one of the most important bacteria that cause pathological processes in people, apart from pneumonia, being normally at the extraintestinal level and, like the other agents included within the acronym ESKAPE, one of the main nosocomial agents, that is, causing infections at the hospital level. However, although pneumonia and/or urinary processes caused by these bacteria in the hospital are important, there is also a significant number of community-acquired bacteremia caused by this agent (Meatherall et al., 2009).

Likewise, the fact that people carrying *K. pneumoniae* at the intestinal level are a predisposing factor for hepatic abscesses in several countries of Asia has been highlighted (Hartantyo et al., 2020) and this agent has been identified as an aetiological agent in 6 % of cases of hepatic abscesses in some studies in Spain (Barreiro, 2018). The incidence in Spain of people carrying β-broad-spectrum lactamase-producing microorganisms should also be highlighted with *K. pneumoniae* being one of the most frequent microorganisms, with 20 % of the bacteria identified in the study (Díaz-Agero et al., 2019).

Apart from the community-acquired level of this agent from extraintestinal processes and, unlike other agents of mainly nosocomial importance, we should point out that the acquisition of an enteroinvasive variant of *K. pneumoniae* from the consumption of a meat product (hamburger) has been reported (Sabota et al., 1998), with the isolates of the agent at the level of the meat product and from the sick person being concordant. However, in spite of the years that have elapsed, no other similar cases have been confirmed since then. In any case, although indirectly in most cases, the scientific literature frequently relates food (Haryani et al., 2007) (Gundogan and Avci, 2013) (Peng et al., 2021) as well as meat-producing animals (Davis and Price, 2016) (Projahn et al., 2019) (Aguilar-Bultet et al., 2020) as transmitters of this agent.

From all of the above it is clear the existence of classic community-acquired pneumonia and nosocomial cases that include pneumonia and other processes, as well as serious community cases, and the possibility of food transmission. The different cases reported depend, as can be assumed, on the type of strain and more specifically on the virulence of the strain. In this way the strains of this agent can be classified into several groups, some would be the classic *K. pneumoniae*, and others the hypervirulent strains, which are invasive variants responsible for liver abscesses and other severe processes. The latter case involves bacteria with a large number of virulence factors that can be aggravated with the acquisition of multidrug resistance. Processes caused by the classic strains are usually at hospital level, however, those caused by hypervirulent strains are usually originated at community level and those caused by multiresistant hypervirulent strains, have both nosocomial and community origin (Zhu et al., 2021). Studies on the origin of multiple antimicrobial resistance of these agents confirm that the majority of resistance genes against antimicrobials of clinical relevance are acquired horizontally (Wyres et al., 2020).

K. pneumoniae is naturally resistant to ampicillin, but there are also a large number of genes that contribute to the multiple antimicrobial resistance of this bacteria. Also, in *A. baumannii*, acquired resistance to carbapenemic antimicrobials is of major importance and has been related to possible transmission to humans from ready to eat fresh plant foods (Soliman et al., 2021).

4.3 Prevalence in food

As we have pointed out, despite these bacteria being considered as causative agent of nosocomial infections, there are numerous publications in which their prevalence in food is investigated or associated with food safety. Thus, in raw and ready-to-eat products, *K. pneumoniae* was recovered in 21 % of the samples investigated in Singapore, with high percentages of positivity, up to 80 %, in some groups of raw (vegetable) food samples and slightly lower percentages, but reaching 27 %, in ready-to-eat dishes (Hartantyo et al., 2020). More food research has been done in China. Namely, the work of Guo et al. (2016), who determined a prevalence of 9.9 % in food samples contaminated with *K. pneumoniae*, ranging from 7.5 % for frozen raw seafood samples to 13.8 % for fresh raw chicken samples (Guo et al., 2016). Zhang et al. (2018) analysed 1200 samples of ready to eat food sold retail, including vegetables and meats in 24 cities in China. 5 % of the samples were positive to *Klebsiella* spp. and also performed a phenotypic and genotypic characterisation of the strains. In Malaysia, 32 % of food samples obtained from street markets were positive for *K. pneumoniae*

(Haryani et al., 2007). In Oklahoma, USA, the presence of this agent and its resistance to antimicrobials was also investigated in beef, turkey and chicken farms, as well as in meat from shops or supermarkets. More than 70 % of turkey meat samples were positive to multidrug-resistant *K. pneumoniae*, with significantly lower values for beef (15 %) and chicken (30 %) (Kim et al., 2005). In Côte d'Ivoire, the presence of *K. pneumoniae* in restaurants on the university campus in Abidjan has been studied, as well as its profile of resistance to various antimicrobials, being up to 20.8 % in fish soup samples and 37 % in fried fish samples (Kone et al., 2022). Concern for this microorganism has also reached Europe, with a multicentre study being conducted to develop *Klebsiella* detection methodologies and also to study the prevalence in ready-to-eat chicken meat and salads (Rodrigues et al., 2022). The results showed prevalence values between 50 and 60 % in chicken meat and between 20 and 30 % in salads, depending on whether conventional cultivation methods or PCR (Polymerase Chain Reaction) are used. The countries involved were Denmark, Ireland, Italy, France and Austria. These values, compared to those cited in China and other countries, seem relatively high, perhaps also due to the improvement of the methodology used. Although there is a lot of information on the characterisation of strains of clinical origin in our country, we have not found specific studies on the prevalence of this microorganism in food in Spain.

4.4 Methods to detect *Klebsiella pneumoniae*

Some methods for the selective detection of *Klebsiella* spp. date back to the last century (Bagley and Seidler, 1978). But in our century, *K. pneumoniae* was isolated from classic, well-known and relatively non-selective culture media, such as MacConkey agar (Kim et al., 2005), with MacConkey agar still in use to this day. Thus, in the study carried out by Zhang et al. (2018), which is one of those analysing a larger number of food samples, they used nutrient broth to enrich the samples and then MacConkey agar to isolate this agent, isolating colonies with a pink mucoid aspect, subsequently employing classical phenotypic tests such as the use of API 20E kits from bioMérieux®, also in the work of Hartantyo et al. (2020). Blue methylene eosin agar, other classic culture media for enterobacteria has also been employed for the isolation of these food agents (Gundogan and Avci, 2013). However, at present, given the concern about the prevalence of this bacteria in food in Europe, and the lack of genuine standardisation in the culture media and methodologies used, studies have been carried out to define optimal culture methods for the recovery of these bacteria in food, testing foods such as chicken meat and ready-to-eat salads (Rodrigues et al., 2022). In this sense, buffered peptone water and LB broth (lysogeny broth), supplemented with ampicillin, have been used as a diluent for enrichment. This article has also studied selective solid culture media for isolation, including chromogenic commercial media such as that marketed by Sigma® or that marketed by Liofilchem® and also already classic non-commercial media. The final optimised protocol includes the use of buffered peptone water, with incubation at two temperatures and the use of SCAI (non-commercial) agar (van Kregten et al., 1984) (Passet and Brisse, 2015) for isolation. *K. pneumoniae* appears yellow on SCAI agar. However, commercial chromogenic media also perform well, in general, in distinguishing *Klebsiella* spp. from other genera. Apart from systems based on classical microbiology for the isolation and subsequent characterisation of these bacteria, molecu-

lar methods are available for the detection and characterisation, such as the detection of certain genes (Zhang et al., 2018) or the use of MALDI-TOF for identification (Rodrigues et al., 2018).

The determination of antibioresistance classically employs phenotypic methods using the agar diffusion method, as well as the search for specific genetic determinants depending on the resistance of interest (Hartantyo et al., 2020) (Rodrigues et al., 2022). Resistance to carbapenem antimicrobials is of utmost interest.

5. *Pseudomonas aeruginosa* multidrug-resistant

5.1 General information

Pseudomonas spp. is a bacillus between 1.5 and 5 µm in length, and diameter between 0.5 and 1 µm, Gram negative, catalase and oxidase positive, non-fermentative, capable of colonising different environments due to its great capacity of adaptation and development of metabolic routes (Capatina et al., 2022). These bacteria are not spore-forming and despite being strict aerobic, they can metabolise nitrates as an alternative source of electrons, allowing them to grow into anaerobiosis (Park and Sauer, 2021).

The genus *Pseudomonas* includes numerous species, among which stands out *P. aeruginosa*, opportunistic pathogen associated with infections in animals and humans. The proliferation of this microorganism in humid environments and its growth at low temperatures, represents a problem both at hospital and food level. Indeed, the transmission of the pathogen may be associated with contamination of surgical material and aqueous solutions including disinfectants, soaps, or irrigation and dialysis fluids, given their biofilm-forming capacity (Lanini et al., 2011). The bacteria may have different morphological variants as a result of responses to environmental stress, or the acquisition of antimicrobial resistance. In recent years, new strains have emerged that present a large number of virulence factors, associated with resistance to several families of antibiotics. Its infectivity is not so much dependent on the genotype as on the conditions of the host at the time of colonisation (Valentini et al., 2018), the sessile forms being associated with chronic infections and the planktonic with acute ones.

The presence of multidrug-resistant *P. aeruginosa* in food is associated with raw milk, meat or water, but the increase in the prevalence in food of plant origin has increased its relevance in the context of public health. It is also included within the bacteria collected under the acronym ESKAPE because of multiresistances.

5.2 Environmental growth factors

The growth of *Pseudomonas* spp. requires a water activity value above 0.95, while pH values below 5.4 result in its inhibition. As regards temperature, some species can grow at refrigeration temperatures, being of a psychophilic nature, while others can adapt and grow at higher temperatures, up to 42 ºC (Wu and Li, 2015). *P. aeruginosa* is resistant to high concentrations of salt and colourants as well as to a moderate concentration of a wide spectrum of antibiotics. It should be noted that *P. aeruginosa* is able to develop in low concentrations of nutrients, and even in media such as distilled water (Mena and Gerba, 2009). These properties give this pathogen a greater capacity for survival

and adaptation in different environments, mainly humid, promoting its ubiquitous nature and the appearance of nosocomial infections.

5.3 Pathogenicity factors

P. aeruginosa presents several bacterial communication systems or *quorum sensing*, such as *las* and *rhl*, which control the production of different virulence factors, including elastases (*lasB* and *lasA*), alkaline protease *(AprA)*, rhamnolipids involved in biosurfactant synthesis *(rhlAB)*, exotoxin A *(exoA)* with ADP-ribosyltransferase activity, hydrogen cyanide, superoxide dismutase, etc. (Schaber et al., 2004).

Regarding toxin production, the most important is exotoxin A, which inhibits protein synthesis in eukaryotic cells once bound to the receptor. In addition, this toxin has immunosuppressive activity (Michalska and Wolf, 2015). Tissue destruction is promoted by certain enzymes such as elastases that hydrolyse numerous host proteins such as elastin, laminin, fibrinogen, collagen, among others (van der Plas et al., 2016).

The pathogenicity of *P. aeruginosa* is given by the Type III Secretion System (T3SS) through which several virulence factors are produced in the cytoplasm of the host cells (Hausser, 2009). Such a system is modulated by environmental responses, such as decreased Ca^{2+} or contact with eukaryotic cell surfaces.

The ability to form biofilm is itself a virulence factor, associated with antimicrobial resistance, causing infections that are difficult to treat. However, *P. aeruginosa* usually affects damaged tissues in patients with a weakened immune system, so infections rarely occur in healthy individuals.

5.4 Associated symptoms and treatment

The emergence of multidrug-resistant strains of *P. aeruginosa* has been associated with a greater impact on the transmission of hospital acquired infections, with a frequency of around 8 % (EPINE, 2019). Bacterial resistance to known antipsedomonics, including carbapenems with activity against *Pseudomonas* spp. (imipenem, meropenem and doripenem) has made it more difficult to adapt targeted treatment to infection, causing mortality to rise to rates between 35 and 70 %, depending on the location of the infection, prognosis of the underlying disease, initial clinical severity and antibiotic treatment (van Loon et al., 2018). High morbidity and mortality occur in immunocompromised patients, and is also the most frequent cause of chronic respiratory infection in patients with cystic fibrosis. Nosocomial infections generally include pneumonia, bacteremia, surgical wound infection, and urinary tract infections (Kerr and Snelling, 2009) (Sordé et al., 2011). The main risk factors associated with the development of multidrug-resistant *P. aeruginosa* are associated with old age, mechanical ventilation, tracheotomy, and prior use of carbapenems (Cezario et al., 2009). Treatment of resistant *P. aeruginosa* infections should include antimicrobials, selected according to the antibiogram. The administration of colistin, which generates little cross-resistance against other antipseudomonic agents, together with combined antibiotic treatments (beta-lactam and aminoglycosides) seem to be the best alternatives for the treatment of infections, although there are still no sound studies that support a reduction in mortality due to these treatments (Peña et al., 2013).

5.5 Epidemiological and prevalence data

Due to its large reservoir and environmental origin, *Pseudomonas* spp. can be present in foods of plant origin, since the main sources of contamination come from water and soil (Kominos et al., 1972). Indeed, the genus can comprise up to 40 % of the natural microbiota present on the surface of fruits and vegetables, causing approximately 50 % losses of products stored in refrigeration during the post-harvest period.

Although it is not considered one of the main food pathogens, there are studies that report information on antimicrobial resistance of up to 401 isolates of *Pseudomonas* spp., mainly *P. aeruginosa*. Through microdilution and minimum inhibitory concentration methods, one study identified a significant rate of resistance to aminoglycosides in *Pseudomonas* spp. isolate from vegetables and salads (Schwaiger et al., 2011). This study showed resistance to the main antibiotics of clinical origin such as gentamicin, tobramycin, amikacin, ciprofloxacin, colistin, piperacillin, ceftazidime, or cilastatin. Through the limits established by the CLSI scale (CLSI, 2012) and disc diffusion methods, resistance to carbapenems was also observed in isolates from lettuce, cauliflower, carrot, pepper, cucumber and tomato (Allydice-Francis and Brown, 2012). Another study conducted in Portugal isolated a total of 35 positive samples for multidrug-resistant *Pseudomonas* spp. in lettuce, tomato and carrot (Jones-Días et al., 2016). Apparently, *P. aeruginosa* contamination in processed plant-based foods comes more from the raw material than during processing, as demonstrated by Wright et al. (1976) where they isolated 44 % of positive samples for *P. aeruginosa* in salads served in the kitchen of a hospital. Similarly, Correa et al. (1991) obtained a total of 38 and 98 isolates from salad samples and clinical samples, respectively, in a hospital in Brazil, and more than 50 % of the samples had counts above 100 CFU/g. In a more recent study (Ruiz-Roldán et al., 2021), a prevalence of 53.1 % of positive samples for *Pseudomonas* spp. was obtained in raw vegetables, classified into 139 pulsotypes. From the isolates, a total of 37 *P. aeruginosa* strains were recovered, distinguishing a total of 28 sequences and 9 serotypes. The authors demonstrated that the isolates presented, to a large extent, traits associated with virulence factors, so their pathogenicity is considered highly relevant.

5.6 Methodologies for detection

The rapid and correct identification of *P. aeruginosa* is critical in samples of clinical origin, so that the most appropriate treatments can be developed for the prevention and eradication of the infection. Their identification in the laboratory and the determination of their sensitivity to antimicrobials do not usually pose difficulties, with the exception of mucous phenotypes that are usually identified in patients with cystic fibrosis. According to Community legislation and *Codex Alimentarius* guidelines, *P. aeruginosa* should be absent in water and food samples (Tang et al., 2017). For detection, it is necessary to develop methods that determine the presence of cells in planktonic media, as well as in the form of biofilms. In addition to traditional methods of enumeration and detection, methods of molecular, immunological, optical and electrochemical detection and confirmation have been developed, through the direct or indirect presence of *P. aeruginosa* via metabolites or signalling molecules.

Dependent culture techniques, including the ISO method for the detection and enumeration of *P. aeruginosa* in water (ISO, 2008), include the use of selective agars for isolation of *Pseudomonas*

spp. with triclosan (PIA) or cetrimide (PCN). Pyocyanin production is usually determined on various chromogenic agars, but they have the disadvantage of low selectivity, as only 90-95 % of *P. aeruginosa* species produce it (Weiser et al., 2014). Incubation temperatures vary depending on the sensitivity and selectivity of the medium used, being higher than 42 ºC, while for other chromogenic media the results are more reliable at 37 ºC. Incubation periods usually range from 2-5 days under aerobic conditions. However, deep-layer biofilm-forming cells require anaerobic conditions, often not detected by these culture media (Thi et al., 2020).

Molecular techniques, despite their high cost, present greater reliability for the detection of *P. aeruginosa*. These include PCR methods (RT-qPCR and M-CPR), which are capable of detecting DNA sequences as well as 16S rRNA, and associated genes (*oprI*, *oprL*, *algD*, *gyrB*, *ecfX*, *fliC*, *toxA*, *rrl*, *rrs*, and *ETA*) (Thi et al., 2020). In recent years, isothermal amplification methods have been developed such as the Spiral Polymerase Reaction (PSR) or the LAMP method (Loop Mediated Isothermal Amplification), which presents a lower cost of reagents and equipment (Zhang et al., 2013) despite its complex design and interpretation of the results.

Immunoassay techniques include screening for antigen-antibody binding, and usually exhibit high sensitivity and low cost. Various molecules that act as antigens have been used for the detection of *P. aeruginosa* such as exotoxin A, elastase, and alkaline protease, as well as protein F (OprF) and the commercial antigen St-Ag:1-17. Key immunoassay techniques include enzyme-linked immunosorbent assays (ELISA), immunochromatographic assays (ICA), immunofluorescence methods, immunoelectrophoresis, or immunoblot, among others (Tang et al., 2017).

The detection of *P. aeruginosa* cells, as well as other pathogens, is being optimised through the development of optical, electrochemical or piezoelectric biosensors. These biosensors are analytical devices that incorporate a biological material and/or biomolecules (tissues, microorganisms, cellular organelles, enzymes, antibodies, antigens, nucleic acids, proteins, aptamers, biomimetic compounds, synthetic catalysts, conjugated biomolecules and printed polymers, among others) associated with or integrated into a physicochemical transducer or transduction system (Lazcka et al., 2007). The development of electrochemical biosensors is considered the most viable option for the design of Point of Care (POC) devices that can monitor the presence of *P. aeruginosa* in food and environmental samples (Ciui et al., 2017).

6. *Bacillus cereus*

6.1 General information

B. cereus is a Gram-positive sporulated microorganism belonging to the genus *Bacillus* and has been classified as an independent group. This group (*B. cereus sensu lato*) consists of eight formally recognised species: *B. cereus sensu stricto* (or simply *B. cereus*), *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus*, and *B. toyonensis* (Oren and Garrity, 2014). Some species in this group can cause food or clinical diseases, while others do not appear to be pathogens. *B. cereus sensu stricto* is related to food toxinfections and is an important food contaminant due to its wide and frequent distribution in nature and in the food chain.

6.2 Prevalence

B. cereus is widely distributed in nature, so it can be identified in all kinds of food. The increase in recent years in the production and use of flours of different cereals and insects for their incorporation in food makes it necessary to assess the biological risks that may be present.

Different studies have shown its presence in different types of flours. In relation to insect, for example, Grabowski and Klein (2017) identified *B. cereus* in dried or powdered insects, with *Bacillus* spp. counts around 3.0 $\log_{10}$ CFU/g. In a study in soldier flies, which is of great interest for its nutritional value, *B. cereus* was identified, along with other food pathogens (Raimondi et al., 2020). *Bacillus* spp. counts of 2.3-3.2 $\log_{10}$ CFU/g were achieved. In other research on the same insect, foodborne pathogens *Salmonella* and *B. cereus* were identified in larval and/or residue samples (Wynants et al., 2019)

Also Walia et al. (2018) carried out a qualitative risk assessment of cricket dust, in which *B. cereus* was considered as one of the possible biological risks.

Treatments such as lactic fermentation or the use of compounds such as sodium nitrite and sodium lactate in flour worm samples processed therewith resulted in *Bacillus cereus*, *Salmonella* and *Listeria monocytogenes* being below the limit of detection (Borremans et al., 2020).

In general, the studies agree in recommending that, since there is a risk of contamination by *B. cereus* in these products, an appropriate decontamination treatment (by heat or other technology) be applied to ensure the safety of insect meal before it is incorporated into food for human consumption.

6.3 Associated outbreaks

In 2019, a total of 155 outbreaks of toxi-infection caused by this agent were detected in Europe, of which 7 affected people died (EFSA/ECDC, 2021a). A total of 10 European countries reported outbreaks, including Spain. It is worth noting the significant increase in the mortality rate compared to the previous year (from 1 to 7 people, respectively).

In the RASFF (Rapid Alert System Feed and Food) numerous alerts for *B. cereus* have been reported in different products in recent years. Among them were bars made with insect protein, cooked scorpions, an oat drink (29 people with symptoms), sesame seeds, barley grass powder and different spices.

6.4 Associated symptoms

Outbreaks associated with different types of flour have been reported, which is of particular concern when used for infant feeding. Given the small proportion of foods incorporating insect meal, no outbreak of *B. cereus* toxi-infection associated with consumption has yet been described. The expected symptomatology would be the same as that of outbreaks associated with other foods.

6.5 Methodologies for detection

Classical methods include growth in selective media (mannitol, egg yolk and polymyxin, MYP; and agar supplemented with NaCl and glycine, NGKG), Gram stains and sporulation tests, and biochemical methods (API-type galleries).

Molecular methods include PCR, qPCR and RT-qPCR for the molecular typing of the group members, based on rRNA 16S and 23S variable regions (Oliwa-Stasiak et al., 2010). The results show that there is a high degree of homology in the sequences between the strains, and therefore the methods have not allowed an exact differentiation between the different *B. cereus* species included in the group (EFSA, 2016). There are also different techniques for the identification of enterotoxins produced by *B. cereus*.

The differentiation between B. *cereus sensu stricto* and *B. thuringiensis* is based on microscopic phenotypic aspects that are not always conclusive, so the only way to recognise if a strain belongs to the species *B. thuringiensis* is the identification by WGS (EFSA, 2016), which may pose a limitation for the unequivocal detection of *B. cereus sensu stricto* in food.

7. *Cronobacter* spp.

7.1 General information

The genus *Cronobacter* spp. is an emerging pathogen and its study is of increasing interest. In 2002, the International Commission on Microbiological Specifications for Food (ICMSF, 2002) defined *Cronobacter* spp. as "a severe risk to a restricted population, posing a threat of death or long-term chronic sequelae".

Considerable attention has been paid to *Cronobacter* spp. infections in newborns. From 1961, *Cronobacter* spp. was first reported in infants hospitalised in the United Kingdom; in 1979, cases of *Cronobacter* spp. bacteremia were reported among newborns in Macon (United States) and in subsequent years more *Cronobacter* spp. infections were reported in the Netherlands (1983, 1987), Greece (1987), Iceland (1989) and the United States (1989 and 2001). In 2001, a group of researchers, in connection with the study of *Cronobacter* spp. infections, allowed the United States Center for Disease Control and Prevention (CDC) to conduct a screening investigation that identified the contaminated Infant Powder Formula (PIF) as a major source of childhood infections (Strysko et al., 2020).

Despite the attention paid to children's cases, *Cronobacter* spp. can also infect people of all ages. Infections with *Cronobacter* spp. in adults cause mild gastrointestinal symptoms, diarrhoea and urinary tract infections. Elderly (Alsonosi et al., 2015) and immunocompromised adults are the most susceptible to these agents.

WHO recommends breast milk as the best food for infants and its promising health benefits are recognised (Boué et al., 2015) (WHO, 2022). However, infants and newborns who cannot be breastfed for unavoidable reasons are fed reconstituted PIF which is considered an equivalent alternative to breast milk (Barron and Forsythe, 2007) (Kent et al., 2015). In this regard, the quality and safety of PIFs present a major challenge for food manufacturers and must meet the international microbiological criteria of the "Code of Hygiene Practice for Powder Formulas for Infants and Young Children (CAC/RCP 66-2008)". Although PIF is a dehydrated product with reduced water activity, being an unfavourable medium for the growth of most microorganisms, there are still several instances in which contaminated reconstituted PIF has been reported to be involved in various *Cronobacter sakazakii* infections in infants and newborns (Lepuschitz et al., 2019) (Elkhawaga et al., 2020).

As discussed above, the International Commission on Microbiological Specifications for Food (ICMSF, 2002) has described *C. sakazakii* (now the genus *Cronobacter*) as a "serious danger to restricted, life-threatening, or long-term chronic substantial sequelae populations". The agent is especially associated with PIF and infants and newborns. Due to the severity of infections caused by *C. sakazakii*, it is essential to develop rigorous control measures to reduce the risks of contamination at each step during the production of reconstituted PIF following the guidelines and recommendations established by the competent food safety authorities (Lehner et al., 2018).

Cronobacter spp. is included in the European Union Microbiological Criteria Regulation (Regulation 2073/2005). *Enterobacter sakazakii* as a subject to the Food Safety Criterion in "Dehydrated infant formulae and dehydrated dietary foods intended for special medical uses for infants under 6 months" with a Sampling Plan of n= 30, c= 0, m and M limits of absence in 10 g and with the analytical reference method (ISO, 2017a).

7.2 General characteristics and impact

Cronobacter spp. is a genus of Gram-negative bacteria that is part of the enterobacteria, found naturally in nature (soil, water, plants and animals), although in many cases the reservoir is unknown.

Its ability to form biofilms and its resistance to desiccation, compared to other enterobacteria, makes it survive for long periods of time (up to 2 years) in dry environments, such as milk powder and its production environment. Once the preparations are reconstituted, *Cronobacter* spp. multiplies depending on the preparation and storage conditions.

Cronobacter spp. (formerly *E. sakazakii*) is an important group of emerging foodborne opportunistic pathogens. Severe *Cronobacter* spp. infections in infants can lead to necrotising enterocolitis, bacteremia (septicaemia), and meningitis, with long-term complications for survivors, such as neurodevelopmental retardation, hydrocephalus, and permanent neurological damage (Holý and Forsythe, 2014). Low birth weight or immunocompromised infants have a particular risk of contracting infections caused by this pathogen (Hunter and Bean, 2013). In addition, the mortality rate of *Cronobacter* spp.-related meningitis can be up to 40 to 80 % (Friedemann, 2009). However, the epidemiology and rates of infection with *Cronobacter* spp. are not always fully clarified, and only 4 to 6 cases of such infections in infants are typically reported annually to the United States Centers for Disease Control and Prevention (CDC, 2022). Most *Cronobacter* spp. infections occur in the adult population, especially in immunocompromised people, the elderly, and those with medical implants, prolonged hospital visits, or acute, chronic, or severe diseases (Patrick et al., 2014) (Alsonosi et al., 2015).

The bacterial genus *Cronobacter* was previously known as *E. sakazakii* and was first defined as a new genus in 2007 (Iversen et al., 2007). It is a member of the family *Enterobacteriaceae* and is closely related to the genera *Enterobacter* and *Citrobacter*. In recent years, the genus *Cronobacter* has been subject to a number of reviews and there are currently authors who consider that it contains 10 species (Joseph et al., 2012) (Brady et al., 2013). The following species were formally recognised: *C. sakazakii*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, *C. turicensis*, *C. condimenti*, *C. malonaticus*, *C. helveticus*, *C. pulveris* and *C. zurichensis*, including the ancient species described *E. sakazakii*, *E. helveticus*, *E. pulveris* and *E. turicensis* (Holý and Forsythe, 2014), making it difficult to determine

the specific species of *Cronobacter* spp. that were reported prior to the 2007 scientific publications. Holý and Forsythe (2014) classified *Cronobacter* spp. into two main groups: Group 1, which comprises *C. sakazakii* and *C. malonaticus*, which mainly come from clinical samples, and Group 2, which comprises *C. turicensis* and *C. universalis* with a lower reported frequency. *Cronobacter* spp. are ubiquitous bacteria as described by Cawthorn et al. (2008) and El-Sharoud et al. (2009) because the pathogen has been isolated from clinical samples, foods (PIFs, ready-to-eat foods), beverages, water, meat, vegetables and even cheese (Beuchat et al., 2009) (El-Sharoud et al., 2009). The incidence of *Cronobacter* spp. in these food matrices and possible household contamination increase the potential risks of infections in immunocompromised adults, as stated by Baumgartner et al. (2009). The PIF formula is, so far, the only food source that has been clearly epidemiologically linked to outbreaks caused by *Cronobacter* spp. In developed countries, much of the research on *Cronobacter* spp. has focused on the possible presence of these pathogens in PIF feedstocks (El-Sharoud et al., 2009), processing facilities (Mullane et al., 2007) (Proudy et al., 2008) and final products (Mullane et al., 2008).

However, many taxonomic studies point to only 7 different species under the genus *Cronobacter* (formerly *E. sakazakii*), namely *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis* and *C. condimenti* (Iversen et al., 2007, 2008) (Joseph et al., 2012) (Stephan et al., 2014). *C. sakazakii* being the most frequent clinical isolation of the genus *Cronobacter* spp. among all age groups, belonging to the considered Group 1 (Forsythe, 2018). Along with *C. malonaticus* and *C. turicensis*, it is also known as the most pathogenic species causing meningitis, sepsis, and necrotising enterocolitis (NEC) in newborns and infants (Patrick et al., 2014) (Alsonosi et al., 2015) (Finkelstein et al., 2019).

A very interesting work is the one done by Odeyemi and Sani (2019) in Malaysia. They consider that there are very limited studies of this pathogen in that part of the world. Therefore, studies on the incidence, virulence, and antibiotic resistance of *Cronobacter* spp. are imperative. This study aimed to investigate antibiotic resistance, presumed virulence factors and the morphology of multicellular colonies among *C. sakazakii* previously isolated in Malaysia and compared to reference strains.

This study aimed to investigate the alleged phenotypic virulence factors and antibiotic resistance in *C. sakazakii* isolated from PIF and other sources. 9 cultures (referred to as CR1-9) from the researchers' culture collection (*C. sakazakii*) were used, and 3 species of *Cronobacter* were collected: *C. sakazakii* ATCC® 29544™, *C. muytjensii* ATCC® 51329™, *C. turicensis* E866. The isolates were subjected to susceptibility to antibiotics and the following virulence factors: protease, DNAase, hemolysin, gelatinase, motility and biofilm formation, using phenotypic methods. All bacteria were able to form biofilm on agar at 37 °C and were resistant to ampicillin, erythromycin, phosphomycin and sulfamethoxazole. In this study, it was observed that the tested strains formed a weak and, at times, strong biofilm, with morphotypes of dry and rough violet colony (rdar), dry and rough brown (bdar), red and smooth mucoid (rmas) in Congo red agar. rdar expresses curli (amyloid fibrils) and fimbriae, while bdar expresses curli. Both morphotypes of biofilm-forming colonies are commonly found in *Enterobacteriaceae*, including various strains of *Salmonella* spp. This study also reveals new colony morphotypes in *Cronobacter* spp. species. In conclusion, there was a correlation between the pre-

sumed virulence factors and antibiotic resistance among the bacteria analysed. Therefore, further studies on virulence and antibiotic resistance genes are recommended.

In 2004, Iversen et al. (2004) reported on the ability of *Cronobacter* spp. to form biofilms on various surfaces such as glass, stainless steel, silicone, latex and polycarbonate. Binding of cells was observed to occur rapidly with respect to plastic (a hydrophobic material) and hydrophilic materials (Lehner et al., 2006). Nutrient availability and growth medium temperature are important influences on biofilm formation (Kim et al., 2006). It was reported that more than 75 % of *Cronobacter* spp. produced biofilm with infant formula milk, while less than 20 % produced biofilm using dilute soybean tryptone broth (TSB), when both were used as culture medium (Oh et al., 2007). Moreover, temperature influences the formation of biofilms. Kim et al. (2006) reported that *Cronobacter* spp. did not form biofilms at 12 °C in any of the growth media studied. According to a study by Strydom et al. (2012), compounds such as D-galactose, heteropolysaccharide, D-glucose, glucuronic acid, D-fucose and D-mannose increase the biofilm formation of *Cronobacter* spp. (Strydom et al., 2012). In addition, these compounds help to increase the resistance of these bacteria to antibiotics, detergents and other environmental dysgenesis situations. Kim et al. (2007) reported that most disinfectants used in food service kitchens, as well as hospitals and nurseries, are not always effective enough to remove bacterial cells trapped within these organic matrices, thus the formation of biofilms on equipment and in hospital settings increases the risk of infections in infants and children, as well as in immunocompromised adults. Biofilm formation in infant formula milk after 24 hours in enteral feeding tubes has been observed in some studies, increasing the risk of neonatal infection, as these types of tubes can remain *in situ* for several days at normal body temperature while nutrients are administered to infants at 2 or 3 hour intervals (Hurrell et al., 2009).

7.3 Epidemiological data and food prevalence

Infant *Cronobacter* spp. infections have been epidemiologically linked to contaminated PIF; by analysing a large number of PIF samples to verify the presence of *Cronobacter* spp., the occasional existence of this contaminant has been confirmed (Norberg et al., 2012) (Pei et al., 2016). Therefore, PIF surveillance for the presence of *Cronobacter* spp. has become a routine for manufacturers and food control services. Since infant formulas are products that undergo pasteurisation during the manufacturing process and *Cronobacter* spp. cannot survive this heat treatment (Iversen et al., 2004), the contamination observed in the final PIF products suggests that the bacteria probably come from the factory environment or from heat-sensitive micronutrients added after pasteurisation. Furthermore, the occurrence of infections in individuals who did not consume or manipulate PIF suggests that the pathogen may originate from the environment or food other than PIF (Patrick et al., 2014) (Alsonosi et al., 2015). Indeed, research has been conducted on the presence of *Cronobacter* spp. species in powdery dehydrated foods that are not infant formulae, such as plant material and other dehydrated sources (Sani and Odeyemi, 2015). In addition, PIF can also become contaminated in the home environment or elsewhere after opening the container. In various investigations of outbreaks or cases of *Cronobacter* spp. infection in premature infants and newborns,

the microorganism was isolated from blenders, bottle cleaning brushes, and open containers of PIF (Kandhai et al., 2004) (Friedemann, 2009).

Cronobacter spp. can be isolated from a wide range of foods and environments. Investigations of *Cronobacter* spp. isolated from food have included, in addition to studies on PIF or dehydrated (Norberg et al., 2012) (Gicova et al., 2014), also others in flour (Cetinkaya et al., 2013), dried powdered foods, cereals, cereal products (Brandao et al., 2017), spices (Li et al., 2017), herbs (Jaradat et al., 2009) (Garbowska et al., 2015), vegetables (Chen et al., 2016) (Berthold-Pluta et al., 2017), ready-to-eat foods (Vasconcellos et al., 2018), and various foods of animal origin (Sani and Odeyemi, 2015). Environmental sampling has also been performed as previously indicated in the PIF production environment (Sani and Odeyemi, 2015), the domestic environment (Jaradat et al., 2009) and farms (Vojkovska et al., 2016); confirming the ubiquitous distribution of the microorganism and that most *Cronobacter* spp. isolates worldwide originate from plant sources (Chen et al., 2016). However, the ecological characteristics of this microbial agent are little known and extensive and in-depth studies of the habitats of *Cronobacter* spp. are needed to identify the occurrence and characteristics of these pathogens present in the various food categories and the various environments in which they can be identified.

Several studies have shown that the majority of *Cronobacter* spp. isolates worldwide originate from plant sources (Sani and Odeyemi, 2015) (Chen et al., 2016). It has also been reported that a relatively high number of cereals test positive for *Cronobacter* spp. (Friedemann, 2007) (Brandao et al., 2017). Wheat and rice are staple foods of great global importance; rice being considered a primary food in the diet of a large part of the world's population. Lou et al. (2019) studied, in mainland China, the presence of *Cronobacter* spp. in samples of rice, wheat and their derived products, as well as in their environments related to cultivation, processing and consumption. The ultimate objective of the study was to identify and determine possible routes of contamination and understand how to minimise or eradicate such contamination. This study investigated the incidence and distribution of *Cronobacter* in 1245 cereal samples and related environments. 39.1 % (101/258) of the rice-related samples and 46.9 % (98/209) of the wheat-related samples tested positive for that microorganism, and the positive rate differed markedly according to the processing method. *Cronobacter* was found in rice and wheat plants at the tillering, filling and mature stages. Soil, water and swab samples from nearby milling plants were analysed and the results revealed that 6.3 % (7/122) of the rice field water, 49.1 % (28/57) and 62.1 % (41/67) of the swab samples from rice and wheat flour milling plants were positive for *Cronobacter* spp. The subtyping of Pulsed Field Gel Electrophoresis (PFGE) indicated that some strains had a common profile, suggesting their persistence in the environment, possible transmission routes, and cross-contamination in processing. Finally, 18 families were sampled to assess potential risks and it was observed that none of the families that mainly ate rice cooked in water, tested positive for *Cronobacter* spp., however, 66.7 % of the families (6/9) whose staple foods were produced from wheat flour did test positive.

Today, biological measures for the control of foodborne pathogens are becoming more attractive due to the emergence of antimicrobial resistance and consumer awareness of health issues related to food additives and chemical preservatives (Balciunas et al., 2013) (Oliveira et al., 2018). The use

of bioprotective agents such as plant-derived compounds, probiotics, bacteriophages, and/or their metabolites exhibiting antagonistic effects are some of the approaches researched against *C. sakazakii* so far. Just as in recent years numerous reviews have appeared aimed at physicochemical approaches for the inactivation of *C. sakazakii* (Pina-Pérez et al., 2016) (Hu et al., 2018) (Henry and Fouladkhah, 2019), biological methods have not been exhaustively discussed so far.

7.4 Methodology

Various methods for the isolation and identification of *Cronobacter* spp. in food have been standardised. The best known methodologies for the isolation of *Cronobacter* spp. are referenced in the United Sates Food and Drug Administration's Manual of Bacteriological Analysis (BAM-FDA) (Chen et al., 2012) and in ISO 22964:2017 (International Organisation for Standardisation) (ISO, 2017a). Both use a preenrichment step and chromogenic culture media.

In addition, several analytical methods have been found for identifying the genus *Cronobacter* spp. with good accuracy, including commercial kits, PCR, Multilocus Sequence Typing (MLST), which is quite efficient for both microbial identification and typing, and, more recently, the MALDI Source Mass Spectrometer and Flight Time analysis (TOF), known as MALDI-TOF/MS (Joseph et al., 2012) (Forsythe et al., 2014). However, research with MALDI-TOF/MS and *Cronobacter* spp. is still somewhat scarce in the literature. The PFGE allows typing.

7.5 Future steps

Extensive and in-depth ecological studies of the habitats of *Cronobacter* spp. are necessary to identify the occurrence and characteristics of these pathogens present in the various food categories and food environments in which they can be found and, in addition, sufficiently representative sampling is required.

The present emerging pathogen is well studied (given the particular importance of this at-risk population) in PIF and in the environment of its processing factories, but other products such as cereals should also be given importance.

There are usable, verifiable and reference techniques of recognised prestigious organisms, such as in the Bacteriological Analytical Manual (BAM-FDA) of the United States (Chen et al., 2012) or the ISO 22964:2017 standard (ISO, 2017a). However, promising techniques such as MALDI-TOF/MS.

There are a number of physicochemical approaches and food processing techniques based on such approaches for the inactivation and destruction of *Cronobacter* spp. in food. However, it is necessary to deepen the study of bioprotective agents such as plant derivatives, probiotics, bacteriophages and/or their metabolites that cause an antagonistic effect in the microorganism.

8. *Campylobacter* spp.

8.1 General

Campylobacteriosis is a globally distributed zoonosis caused by bacteria of the genus *Campylobacter*. Such bacteria are Gram-negative, "spiral" shaped bacilli, which grow with little oxygen and exhibit their optimal growth at 42-43 °C (AESAN, 2022a). The genus *Campylobacter* comprises 17

species and 6 subspecies, of which the most frequently detected in human diseases are *C. jejuni* (subspecies *jejuni*) (80 %) and *C. coli* (10 %). In humans, campylobacteriosis develops with acute enterocolitis that manifests as malaise, fever, severe abdominal pain, headache, nausea and/or vomiting, and watery or bloody diarrhoea. The incubation period ranges from 1 to 11 days (usually from 1 to 3 days). In most cases, diarrhoea tends to subside on its own. Bacteremia occurs in <1 % of patients with enteritis, and sequelae can also occur such as rheumatoid disorders or peripheral neuropathies such as neuromuscular paralysis of Guillain-Barré syndrome (AESAN, 2012a). In low-income areas, *Campylobacter* infections are common in young children (causing watery diarrhoea rather than bloody diarrhoea that occurs in high-income countries) and are associated with many deaths, as well as stunting and lifelong physical and cognitive impairments (Corcionivoschi and Gundogdu, 2021). In addition, as highlighted by WHO, *C. jejuni* is a pathogen resistant to multiple antibiotics and new therapies are urgently needed (WHO, 2020).

Campylobacteriosis is the most commonly reported foodborne gastrointestinal infection in humans in the European Union since 2005. In 2020, reporting of campylobacteriosis recorded the lowest number of cases since the beginning of surveillance in 2007, due to the impacts of the United Kingdom's withdrawal from the European Union and the COVID-19 pandemic. In 2020, the number of confirmed cases in the European Union amounted to 120 946 (reporting rate of 40.3 per 100 000 inhabitants). In total, 317 outbreaks caused by food contaminated with *Campylobacter* spp. were reported to the European Food Safety Authority (EFSA), including 1319 cases of disease, 112 hospitalisations and no deaths (EFSA/ECDC, 2021b).

Reporting of campylobacteriosis is mandatory in 22 European Union Member States. In 5 Member States, the notification is based on a voluntary system (Belgium, France, Greece, Italy and the Netherlands). In Spain, campylobacteriosis is a Compulsorily Notifiable Disease (CND), as established by Royal Decree 2210/1995, establishing the National Epidemiological Surveillance Network (BOE, 1995). The autonomous communities must individually report confirmed cases.

8.2 Transmission

The main reservoir of *Campylobacter* spp. are birds, as well as cattle, sheep, pigs, rodents, dogs and cats, as well as other mammals and wild birds. The spectrum of reservoirs varies with species: *C. jejuni* is very common, while *C. coli* is more frequently isolated from pigs. The primary acquisition of *Campylobacter* spp. by animals occurs after birth and although it may cause morbidity and mortality, in most cases, colonisation leads to a permanent carrier state.

In 2019, AESAN collected a total of 1951 food analysis results, corresponding to 2018, in which the presence of *Campylobacter* spp. was detected in 512 samples, representing 26.24 % of positive samples with respect to the total. In 2020, the percentage of positives reached 44.04 %, which is a strong increase compared to previous data (AESAN, 2022a).

Food contamination by *Campylobacter* spp. in the European Union is monitored in accordance with Chapter II ("Surveillance of zoonoses and zoonotic agents") of the Zoonoses Directive 2003/99/EC (EU, 2003). The most frequent form of transmission of this zoonosis to humans is through undercooked chicken meat, or ready-to-eat (RTE) foods that have been in contact with raw chicken. In

its assessments, EFSA has found that chickens and chicken meat can directly account for between 20 % and 30 % of cases in humans (EFSA/ECDC, 2021b). Within the RTEs, the most frequently contaminated food with *Campylobacter* spp. was raw milk, confirming the trend of 1 in 100 reported during the period 2016-2019. In addition, the data showed positive results for meat and meat products, followed by fruits, vegetables and juices.

8.3 Epidemiology and antibiotic resistance

In recent years, research aimed at better understanding the pathogenicity and physiology of *Campylobacter* spp. has grown significantly. In addition, several research studies focus on models of infection and/or immunological aspects of *C. jejuni* infection (Corcionivoschi and Gundogdu, 2021).

In relation to studies in farms and slaughterhouses environments, in a recent study carried out in a slaughterhouse in the Valencian Community, to evaluate the epidemiology and resistance of *C. jejuni* to antimicrobials in pork processing, the results showed that all the batches arrived at the slaughterhouse with *Campylobacter* spp. in the faeces, and remained positive during processing and slaughter (42.8 %), even just before delivery to the consumer. Furthermore, 96.3 % of *C. jejuni* isolates (the major species involved in food-borne human infection) were multidrug-resistant strains (Marin et al., 2021).

In another cross-sectional study carried out between 2014-2016 in 301 herds of ruminants from the Basque Country to estimate the prevalence of *C. jejuni* and *C. coli*, and to investigate their susceptibility to antimicrobials, it was found that the risk of excretion of *C. jejuni* was higher in cattle than in sheep (81.2 % versus 45.2 %), while the risk of excretion of *C. coli* was higher in sheep than in cattle (19.1 % versus 11.3 %) (Ocejo et al., 2019). *C. coli* showed higher resistance (94.1 %, 32/34) than *C. jejuni* (65.1 %, 71/109), and resistance was more widespread in dairy cattle isolates than in meat or sheep cattle isolates. Compared to the results obtained 10 years earlier (2003-2005) in a similar study (Oporto et al., 2007), an increase in resistance to fluoroquinolones in *C. jejuni* from beef cattle (32.0 to 61.9 %), and a decrease in resistance to tetracyclines in *C. jejuni* from dairy cattle (75.0 % vs. 43.2 %) were observed. Macrolide resistance was stable at low and restricted rates to *C. coli* from dairy cattle, and all macrolide-resistant *C. coli* showed a pan-resistance pattern.

8.4 Methodologies for detection

The reference method for the detection of *Campylobacter* spp. in products intended for human consumption is defined in ISO 10272-1:2017 (ISO, 2017b). This method contemplates both detection by enrichment, as well as direct sowing on a plate. This standard has three sub-methods:

a) Enrichment detection for samples with low levels of campylobacteria and basal microbiota.
b) Enrichment detection for samples with low levels of campylobacteria and high level of basal microbiota.
c) Direct seeding detection for samples with high campylobacteria content.

Bolton broth is used for procedure (A) and incubated for 4-6 hours at 37 °C and then at 41.5 °C for 44 hours. Preston broth is used for procedure (B) and incubated at 41.5 °C for 24 hours. All these incubations are performed in microaerobiosis. The enriched broths are then seeded in solid media.

For procedure (A) it is seeded on mCCD agar (Modified Charcoal Cefoperazone Deoxycholate) and another selective medium based on selection principles other than mCCD such as, for example, Biorad's RAPID'*Campylobacter* Medium or bioMérieux® CASA and CampyFood agar. For procedure (B) and (C) it is sown exclusively in mCCD. Plates are incubated at 41.5 °C for 44 hours in microaerobiosis. For the confirmation of the suspected colonies, the morphology and mobility are examined, the oxidase test and an aerobic growth analysis is performed at 25 ºC. In addition, biochemical tests such as API Campy galleries or molecular tests can be employed. There is therefore a sufficiently proven methodology which corresponds to a standardised method. The ISO method has recently been successfully used to isolate *Campylobacter* spp. from pork samples (Linn et al., 2021). For the qualitative detection of *Campylobacter* spp. of milk samples, a 1/10 dilution of the milk sample in Bolton enrichment broth was performed and incubated at 37 °C for 48 hours with 5 % CO_2 and then seeded in mCCDA and incubated at 42 °C for 48 hours under 5 % CO_2 atmosphere. To perform counting, 100 µl of milk was directly sown in mCCDA. In addition, other methods have also been developed that combine classical microbiology with other techniques. For example, the VIDAS antibody-based system (bioMérieux®) has been used in matrices such as minced beef or vegetables (Chon et al., 2011). Another method of great interest is qPCR. Stingl et al. (2021) developed a method for the quantification of total viable thermophilic *Campylobacter* spp. with a limit of quantification of 20 genomic equivalents per PCR reaction. Molecular methods have also been developed based on isothermal techniques, which are often easier to apply in the food industry and are commercially available. An example is the ATLAS® *Campylobacter* Campy Detection Assay which is based on the isothermal technique called Transcription Mediated Amplification. This technique only requires a 12-hour enrichment and molecular analysis is performed without the need for a nucleic acid extraction step (Rishi et al., 2021) and is validated, among other matrices, on pork and beef. Regarding the characterisation of the strains, conventional PCR has been used for the detection, in *Campylobacter* spp., of resistance genes such as *tet(O)* or *blaOXA-61* and virulence genes such as *flaA*, *cadF* or *cdtA* among others (Rangaraju et al., 2022).

9. Shiga toxin-producing *Escherichia coli*

9.1 General

According to the ICMSF (1998), intestinal *E. coli* is defined as those strains of *E. coli* that are capable of causing a diarrhoeal disease in humans and animals. In the microorganism, for practical purposes and with clinical interest, a subdivision of the pathogenic forms is performed, considering the mechanism underlying the disease. Four main types of pathogenic *E. coli* have been considered up to about two decades ago: *E. coli* enteropathogenic (EPEC), *E. coli* enterotoxigenic (ETEC), *E. coli* enteroinvasor (EIEC) and *E. coli* enterohemorrhagic (EHEC) (in this type is framed, for example, *E. coli* O157:H7) (Nataro and Kaper, 1998) (Kaper et al., 2004). Subsequently, the pathotypes *E. coli enteroaggregative* (EAEC) and *E. coli* with diffuse adhesion (DAEC) have been added. The EHEC type is also known as verotoxigenic or Shiga toxin-producing (EHEC/VTEC/STEC).

According to a report of the Scientific Committee of AESAN in 2012 (AESAN, 2012b) on prevention measures and recommendations applicable to avoid possible food infections by strains of vero-

toxigenic *E. coli* (VTEC)/producers of Shiga toxins (STEC)/enterohaemorrhagic (EHEC), this group of microorganisms and especially the highly virulent strains of serotype O157:H7, are remarkable pathogens causing pathologies and very serious processes in the human species: Haemorrhagic Colitis (HC) and Haemolytic Uraemic Syndrome (HUS) may occur. Ruminants, in particular cattle, are considered to be the main reservoir of this type of micro-organism (this is a zoonosis), with minced meat, hamburgers and vegetables consumed raw or slightly cooked being considered as the main vehicles of transmission (AESAN, 2012b).

Given the special importance of this agent, the *Instituto de Salud Carlos III* (ISCIII) has developed a "protocol for surveillance of infection by strains of *E. coli* that produce Shiga toxin or verotoxins" (ISCIII, 2016) and, according to this protocol, *E. coli* is classified, on many occasions, into more than 170 serogroups (O), according to the antigenic characteristics of its lipopolysaccharide (LPS) and in serotypes, by the combination of somatic (O) and flagellar (H) antigens. The main serotype considered to be Shiga toxin-producing *E. coli* is O157:H7, but serotypes O26: H11, O76: H19, O91: H14, O103: H2, O111: H8, O113: 114, O118: H16, O128: H2, O145: H28, O146: H21 or O169:H41 (ISCIII, 2016) may also be included (at least at a clinical level). The fundamental and main virulence factor of these strains is the group of cytotoxins called Shiga toxins: Stx1 or VT1 toxin, which can be said to be identical to the toxin produced by *Shigella dysenteriae* type 1, and Shiga toxin Stx2 or VT2, which is very similar and shares identical functional characteristics.

Shiga toxins produced by VETC are detected by the specific cytotoxicity test on Vero cells, whence this group name (VTEC) comes from. Serotypes O157:H7, O104:H4, O26, O103, O11 and O145 are noteworthy in the group (ACSA, 2019).

Annex I of the protocol (ISCIII, 2016) presents a very detailed epidemiological survey, including different epidemiological variables and possible causal foods. Sampling and shipment of samples are included in annex II.

Based on the data collected by the EFSA and the European Centre for Infectious Diseases (ECDC) (EFSA/ECDC, 2021b) food-borne processes (food-toxi-infection) by Shiga toxin-producing *E. coli* in humans are the fourth most frequent zoonoses in the European Union. In 2020, 4446 cases of STEC disease were reported in people (EFSA/ECDC, 2021b) (AESAN, 2022b).

In Spain, VTEC-STEC toxi-infection has been a Compulsory Notifiable Disease (CND) for some years. In 2020, 74 cases of STEC-VTEC were reported in people, with the notification of 269 cases in 2019 (AESAN, 2022b). Table 1 details the outbreaks verified by VTEC/STEC in Spain (Mora et al., 2011) (Sánchez et al., 2014).

Table 1. VETC/STEC outbreaks in Spain

Location	Year	Affected/situation	Seropathotypes	Number of affected
Ibiza	1986	British tourists in a hotel	O157:H7 Stx2*	3 (+ 3 asymptomatic)
Balearic Islands	1994	British tourists	O157:H7 Stx2 Phagotype 2 (PT2)*	-
Álava	1995	Boys in a cottage	O111:H- Stx1	13
Fuerteventura	1997	European tourists in 4 hotels	O157:H7 Stx2 Phagotype 2 (PT2)	14 (3 with HUS*)
Guipúzcoa	1999	Children in a day-care centre	O157:H7	8 (1 with HUS + 6 asymptomatic)
Guipúzcoa	1999	-	O157:H7	2 (1 with HUS + 2 asymptomatic)
Barcelona	2000	Children from 5 schools	O157:H7 Stx2 Phagotype 2 (PT2)	175 (6 with HUS)
Lugo	2003	Family outbreak	O157:H7 Stx1 Stx2 Phagotype 8 (PT 8)	3
Lugo	2003	Family outbreak	O26:H11 *vt*	4
Cáceres	2007	-	O157:H7 Stx2 Phagotype 14 (PT 14)	3
Navarre (Pamplona)	2012	Family outbreak	2 O76 strains:H19 STEC, 6 aEPEC strains	4 (several asymptomatic)

*HUS: Haemolytic Uraemic Syndrome; Stx: Shiga toxin; PT: phagotype. **Source:** (Mora et al, 2011) (Sánchez et al., 2014).

The largest outbreak occurred in 2000 in different schools in Barcelona and was caused by the serotype O157:H7.

Occasionally, food health alerts occur in relation to VTEC, including one initially from France in the recent spring of 2022, concerning contaminated frozen pizzas (STEC-VTEC O26). Another recent food alert has occurred due to the presence of *E. coli* producing Shiga toxins in *Brie* cheese from France (ES 2020/91) (AESAN, 2020).

9.2 Beef and veal

Cattle can be carriers of VTEC strains. This is a proven zoonosis. Mora et al. (2011), in relation to the environment of the city of Lugo, indicated that, in the years prior to 2011, they detected a significant decrease in the prevalence of VTEC-STEC in beef sold in that city. Especially important is the absence of positivity for STEC-VTEC O157:H7 in the period 2005-2009. STEC O157:H7 was detected in 8 cases (0.6 %), and STEC-VTEC no O157 in 146 (10 %) out of 1445 samples analysed between 1995 and 2009. None of the STEC-VTEC strains isolated from cattle in the Lugo area belonged to serotype O104:H4, which was the one involved in the "so-called crisis of cucumbers sold in Germany -actually due to sprouts of fenugreek or alhova-". However, the level of samples contaminated with VTEC

no O157 remained too high, indicating the need for extreme hygiene, control and surveillance along the food chain (AESAN, 2012b).

STEC-VTEC strains may be found in sheep and goats. With regard to cattle, it is necessary to consider the environment they are in and the wild animals in the vicinity. In the study by Mora et al. (2012) it is concluded and confirmed that, based on the results obtained, the populations of deer, wild boar and the fox of North western Spain are carriers of STEC-VTEC strains that are potentially pathogenic to humans and contribute to the maintenance and transmission of VTEC-STEC. Sánchez et al. (2010) performed VTEC-STEC isolates on wild ruminants such as deer, fallow deer, roe deer and mouflon, confirming and confirming that wild ruminants can also be a reservoir of STEC-VETC strains.

9.3 Raw milk

Raw milk can become a problem given that, in the absence of pasteurising heat treatment, there is no, what is called, "terminal sanitising treatment". It is a very dangerous food product if the hygienic conditions of the farm and processing plant are not extreme. The agent may, in certain circumstances, be isolated in cheeses.

Rey et al. (2006) examined 502 samples of dairy products from 64 different ovine and caprine herds and 6 dairy plants in Extremadura. A monthly sampling was carried out between March 2003 and June 2004 and 360 samples of unpasteurised milk obtained from the bulk tank (milk storage tank), 103 samples of fresh cheese curd and 39 samples of cheese were analysed. VTEC-STEC strains were detected in 39 (11 %) of the bulk tank samples, 4 (4 %) of the fresh cheese curd samples, and 2 (5 %) of the cheese samples. O157:H7 serotype was isolated from a bulk tank sample (0.3 %). A total of 9 VTEC-STEC strains (O27:H18, O45:H38, O76:H19, O91:H28, O157:H7, ONT:H7, ONT:H9 and ONT:H21) were identified in that investigation.

There is a study carried out in Extremadura (Sánchez et al., 2010), where 46 isolates of *E. coli* O157:H7 obtained from the faeces of different healthy ruminants (cattle, sheep and common deer) and from unpasteurised goat milk were analysed, characterised and typified over a period of 11 years (1997-2008). These isolations came from previous studies carried out in the autonomous community itself. An atypical strain of *E. coli* O157:H7 (positive sorbitol-glucuronidase fermenter) from deer faeces was detected. The genes encoding the Shiga toxins were present in 69.6 % of the isolates and all of them carried only the *Stx2* gene. The isolates came from nine different phage types, although 67.4 % were restricted to only three: PT14, PT34 and PT54. PT54 was the most prevalent type of phage and was detected in isolates of cattle, sheep and deer. Most isolates came from phage types previously found in strains associated with human infection.

In another study carried out in the community of *Castilla y León*, Caro et al. (2007) characterised 13 VETC-STEC strains isolated from sheep milk products in *Castilla y León*. 8 strains isolated from the milk belonged to serotype O157:H7. 3 STEC strains (2 of serogroup O14 and 1 ONT) were detected in 2 samples (2.4 %) of *Castellano* type cheese, one with about 2.5 months of maturation and the other with a maturation period of 12 months.

Other studies were also carried out in the same community of *Castilla y León* on raw milk and cheeses made from it, taking into account the environment and farm equipment. It has been investigated in

raw goat milk (Álvarez, 2014), raw sheep milk (Otero, 2014) and raw cow milk (Rios, 2018). STEC-VETC strains were isolated in the three types of raw milk, therefore the importance of extreme care in the hygienic conditions of the farms, milking halls and milk processing facilities must be emphasised.

9.4 Green leafy vegetables

Ceuppens et al. (2015) conducted an extensive collaborative study regarding primary production and food safety with respect to green leafy vegetables. The microbiological quality and food safety of green leafy vegetables and strawberries in primary production in different countries, in particular Belgium, Brazil, Egypt, Norway and Spain, were assessed and evaluated by means of counts of *E. coli* (a microorganism marker of hygienic conditions and index of faecal contamination in water) and detection of *Salmonella* spp., Shiga toxin-producing *E. coli* (STEC) and *Campylobacter* spp.

Water samples were more likely to contain pathogens (54 positives out of 950 analyses) than soil (16/1186) and field products (18/977 for green leafy vegetables and 5/402 for strawberries). The prevalence of pathogens also varied markedly according to the geographic region where it was sampled. Irrigation of the fields increased the risk considerably, with Odds Ratio (OR) of 10.9 for *Salmonella* spp. and 7.0 for STEC. There was a significant association between elevated counts of generic reporter *E. coli* and pathogen detection (OR of 2.3 for STEC-VTEC and 2.7 for *Salmonella*). In the investigation it was found that, as expected, *E. coli* generic indicator is an index or indicator microorganism suitable for *Salmonella* spp. and STEC-VTEC, but to a lesser extent for *Campylobacter* spp. The article states that precise guidelines or recommended guidelines on sampling frequency and threshold value for *E. coli* in irrigation water should be taken into account and may differ from region to region. In this research, samples positive for STEC-VTEC appear in water and soil, but not in vegetables and strawberries.

Sampling data related to Spain come from the studies of Castro-Ibañez et al. (2015a, b). A recent and very interesting work is that of Truchado et al. (2021) in relation to the new Euroepan Union-wide standards on water reuse for agricultural irrigation and its relationship with Spanish wastewater treatment plants. Table 2 of said work presents results related to different sampling points. At the entrance of the treatment plants, a remarkable number of samples with detection of STEC-VTEC no O157:H7 and *E. coli* O157:H7 (in both cases 100 % prevalence, in both cases) are observed, and some sample is observed at the culture level that is positive (3/100) to STEC-VETC no O157:H7.

9.5 Methodologies for detection

In terms of detection and identification, cultural methods (especially developed for VTEC/STEC O157:H7, non-O157s are sometimes complicated in their detection and identification), serological methods, conventional PCR and quantitative PCR are used. An extremely useful technique for typing is PFGE.

There is an ISO standard applicable to STEC-VTEC which is ISO/TS 13136:2012: "Microbiology of food and animal feed Real-time Polymerase Chain Reaction (PCR)-based method for the detection of food-borne pathogens. Horizontal method for the detection of Shiga toxin-producing *E. coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups" (ISO, 2012).

Detailed and verified information in relation to methods for detection and characterisation of STEC-VETC can be found in the EFSA report on "STEC and the public health risk posed by contamination of food with STEC" (EFSA, 2020). This report reviews the methods for detecting STEC-VTEC which are: (1) culture-base methods with selective and differential media - among them we have the known MacConkey sorbitol agar -, and use of cell cultures; (2) immunological based methods (such as ELISA kits, immunomagnetic beads, that allow a concentration or enrichment); and (3) molecular methods (detection of Shiga toxin coding genes; this report indicates that a metagenomic approach has recently been made in relation to the direct detection and characterisation of STEC-VETC in stool samples).

The report includes PCR-based methods (conventional and real-time); standardised methods for the detection of STEC-VTEC in food and feed, such as the aforementioned ISO 13136:2021 or ISO 16654:2001 (in said standard there is an immunomagnetic concentration using magnetic beads, in 2018 there is an amendment or modification thereof); methods usable to typify STEC-VETC strains by serotyping and molecular serotyping, and phagotyping (ISO, 2001).

For subtyping and molecular footprint characterisation in epidemiological and population studies, molecular typing methods such as PFGE and MLVA analysis are useful. WGS can be a very useful tool, also typing by SNPs.

Moreover, in the Bacteriological Analytical Manual of the United States (BAM Council, 2022) we find a good and proven description of methods compiled by Feng et al. (2020).

10. Tick-borne viral encephalitis

10.1 General

Tick-Borne Encephalitis (TBE) is a severely notifiable disease in the European Union since 2012 and is now considered endemic in many European countries (Amicizia et al., 2013). In 2019, a total of 3411 cases (0.7 per 100 000) were reported in the European Union (ECDC, 2021), of which 15 cases were associated with 3 food outbreaks, with 80 % of cases hospitalized (EFSA/ECDC, 2021a). These data represent an increase compared to the 0.6 rate that had been described in previous years. TBE cases generally have a seasonal peak in the months of July and August. The reported cases are higher in adult men aged 45-64 years, associated with an increased likelihood of being exposed to ticks during outdoor activities and reduced use of preventive measures (Lindquist et al., 2008) (Jepsen et al., 2019). Spain remains free of the disease, although the main vector, although the tick of the genus *Ixodes* (ECDC, 2020) is present and an imported case associated with dairy products in Estonia has been described (Camprubí et al., 2020).

TBEV (Tick-Borne Encephalitis Virus) is an enveloped RNA virus, belonging to the genus *Flavivirus* within the family *Flaviviridae*. The disease occurs in two distinct phases. A first phase of asymptomatic viraemia or with flu-like symptoms lasting 2 to 8 days. And a second phase that is characterised by the central nervous system involvement, of 2 to 4 weeks duration after infection. The clinical picture may run as meningitis, encephalitis, or meningoencephalomyelitis. A high percentage of these patients (35-58 %) will suffer sequelae and the fatality rate in adult patients is between 1-3 %.

10.2 Transmission

The most common form of transmission in humans is by the bite of a tick, which are the main and reservoir vectors of TBEV. Raw goat, cow and sheep milk can contain the virus and can also be a source of infection for humans. The infectious virus has been isolated from dairy derivatives including yoghurt, butter and cheese (AESAN, 2015).

In Eastern European countries, food transmission by ingestion of raw sheep or goat milk is common, with frequent family outbreaks in this way. In the last ECDC report of 2020, 5 outbreaks of TBEV were detected in Austria and Slovakia, with 12 cases, all of which required hospitalisation due to unpasteurised milk consumption (EFSA/ECDC, 2021b). In this last report, it is highlighted that Slovenia monitored 19 samples of raw goat and sheep milk, not detecting the presence of the virus in any of them.

10.3 Stability and survival of TBEV in milk and dairy products

In general, flaviviruses are relatively sensitive to temperature and detergents, although TBEV is not completely inactivated in goat milk after 30 minutes of treatment at 65 °C and treatments at 100 °C for 3 minutes are necessary to completely eliminate infectivity (Balogh et al., 2012).

In another recent study, Rónai and Egyed (2020) compared the survival of TBEV in different pasteurisation conditions in goat milk and in cheeses made with or without salt. Both pasteurisation methods, at 63 ºC 30 minutes or at 72 ºC, 15 seconds, completely inactivated TBEV infectivity. In parallel, infectious viruses were detected after 10-25 days in raw milk and unsalted processed cheese, depending on the initial virus concentration. The virus survived in raw milk for 3 weeks storage at 4 °C and in unsalted processed cheese for 2 weeks. Both pasteurisation and the presence of salt did not detect the presence of TBEV in goat's milk or in cheeses made with this milk. These findings underscore that the safest and easiest way to avoid milk-borne TBE is to boil/pasteurise the milk before drinking it, and if the consumer insists on raw milk, it is important to immunise animals against TBEV in endemic areas.

10.4 Methodologies for detection

Currently, there are no standardised and validated methods for the qualitative or quantitative detection of TBEV in the most at-risk food matrices, milk and its derivatives. Although different published methods are available describing the methodology for isolating TBEV from milk, in some of them the detection of TBEV antibodies in milk has been used, using ELISA techniques and subsequent confirmation by Western blot (Wallenhammar et al., 2020). Molecular methods have been used for the detection of TBEV in milk and cheese samples, based on a step of isolation of the virus from the food matrix, purification of the viral RNA and subsequently its detection by RT-qPCR (Balogh et al., 2012) (Hennechart-Collette et al., 2022). One of the most important limitations of these molecular techniques is that the positive result of PCR does not confirm the infectivity of the detected virus.

Conclusions of the Scientific Committee

This prospective report addresses the interest of controlling a group of bacteria as well as a viral hazard in foods in Spain. Initially, the first section includes the prospecting on *Acinetobacter* spp., *K. pneumoniae* and *P. aeruginosa* multiresistant. These microorganisms are included within the acronym ESKAPE, which includes bacteria that, due to their multiresistance, cause highly complex clinical conditions in humans. These agents are of common interest in many countries of the world and also in our environment (Europe) with a low number of data from our country regarding their prevalence and populations in food, which makes it very necessary to have them available, also taking into account that the population at risk tends to increase. Based on the initial proposal, where neither *A. baumannii* nor *K. pneumoniae* had initially been considered for salad research specifically, the data reported advise the introduction of this food in prospective searches.

The information reported highlights the importance of *P. aeruginosa* transmission in clinical settings and in food samples of plant origin intended for raw consumption. The emergence of multidrug-resistant strains, together with high morbidity and mortality in the immunocompromised population, make it necessary to develop sufficiently sensitive methods for their detection in food. Likewise, as regards *Campylobacter* spp. in meat, aside from poultry, given the prevalence found in cattle, sheep and pig farms, as well as the increase in the number of positive samples in the studies reported, research in these sources is considered important. Also, although poultry meat has always been identified as responsible for the transmission of this toxic infection, there is still a high number of cases in people which do not come from poultry meat. Likewise, there is evidence of the increase in multiresistance in these bacteria, an additional fact to keep in mind.

The same applies to the data on Shiga toxin-producing *E. coli* in our country, which show that the outbreaks that have occurred make it advisable to control the proposed matrices (beef, raw milk and leafy vegetables).

The cases of diseases caused by *B. cereus* and *Cronobacter* spp. are, in principle, minor, although in both cases the flours and powdery products especially allow the survival of these agents.

Finally, the only viral danger reported, producer of tick-borne encephalitis, is important due to the fact that tick transmitters are widely spread in Spain. On the other hand, its presence, sometimes, in raw dairy products, together with the possibility of the consumption of raw milk, may make it advisable to have more data. The methodology is a drawback in this case, since the investigation of viruses with infective capacity is complex, and the PCR search methods do not provide information on said infectious capacity, although having such information would be, in our opinion, relevant. For the other hazards caused by bacteria covered in this study, robust classical methodology is available as well as advanced methods of control and identification.

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