Comprehensive REVIEWS in Food Science and Food Safety

From hazard analysis to risk control using rapid methods in microbiology: A practical approach for the food industry

Journal:	*Comprehensive Reviews in Food Science and Food Safety*
Manuscript ID	CRF3-2019-0357.R2
Manuscript Type:	Comprehensive Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Ripolles-Avila, Carolina; Ciencia Animal y de los Alimentos Martinez-Garcia, Maria; Universitat Autònoma de Barcelona, Faculty of Veterinary Sciences Capellas, Marta; Universitat Autònoma de Barcelona, ciència animal i desl aliments Yuste, Josep; Universitat Autònoma de Barcelona, ciència animal i desl aliments Fung, Daniel; University of Kansas City Rodriguez-Jerez, José-Juan; Universitat Autònoma de Barcelona, Faculty of Veterinary Sciences
Keywords:	

Prof. Mary Ellen Camire, Ph.D.

Scientific Editor

Comprehensive Reviews in Food Science and Food Safety

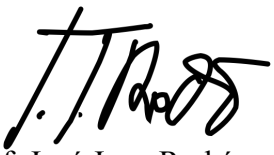
Dear Professor Camire

We send a copy of our paper entitled: "From hazard analysis to risk control using rapid methods in food microbiology: A practical approach to the industry".

Continuing with our research about food mirobiology and food safety, we have ready a new review regarding the detection and quantification of foodborne pathogens by means rapid methods. This is one of the biggest issue for the food industry, and, especially, for the control of pathogens. It is a well-known problem, but under our point of view, currently is not correctly analyzed nor detected.

We would be very appreciated if you may consider this work for its publication in Comprehensive Reviews in Food Science and Food Safety.

Sincerely yours.



Prof. José Juan Rodríguez-Jerez

Barcelona, 17th December 2019

From hazard analysis to risk control using rapid methods in microbiology: A practical approach for the food industry.

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Abstract

The prevention of foodborne diseases is one of the main objectives of health authorities. To this effect, analytical techniques to detect and/or quantify the microbiological contamination of foods prior to their release onto the market are required. Management and control of foodborne pathogens have generally been based on conventional detection methodologies, which are not only time-consuming and labor-intensive but also involve high consumable materials costs. However, this management perspective has changed over time given that the food industry requires efficient analytical methods that obtain rapid results. This review covers the historical context of traditional methods and their passage in time through to the latest developments in rapid methods and their implementation in the food sector. Improvements and limitations in the detection of the most relevant pathogens are discussed from a perspective applicable to the current situation in the food industry. Considering efforts that are being done and recent developments, rapid and accurate methods already used in the food industry will be also affordable and portable and offer connectivity in near future, which improves decision making and safety throughout the food chain.

Keywords: Rapid methods; foodborne pathogens; detection; quantification; control; food industry.

1. Introduction

It is estimated that the world population could potentially reach around 9.8 billion by 2050, which will be paralleled by a significant increase in food demand worldwide (United Nations, 2017). The capacity to satisfy this demand will involve, among other factors, a greater food production. This food should not only be available, easy to access and nutritious, but it should also be safe. Moreover, consumers' growing concern about food safety and quality has compelled the public and private food sectors to develop higher food safety and quality standards (Kotsanopoulos & Arvanitoyannis, 2017; Trienekens & Zuurbier, 2018).

Foodborne disease transmission is one of the most widespread health problems in the contemporary world and an important cause of reduced economic productivity (González-Rivas, Ripolles-Avila, Fontecha-Umaña, Ríos-Castillo, & Rodríguez-Jerez, 2018; Ripolles-Avila, Ríos-Castillo, & Rodríguez-Jerez, 2018). Human diseases caused by the consumption of contaminated products can spread easily and, if there are no appropriate surveillance systems for their control, the number of affected consumers can be significantly high (Hascoët, Ripolles-Avila, Guerrero-Navarro, & Rodríguez-Jerez, 2019; Todd, Greig, Bartleson, & Michaels, 2007). This leads to food scandals, giving rise to a direct reduction in the consumption of some foods and the onset of economic crises, affecting large productive sectors. Consequently, governments and different stakeholders in the food chain have intensified their efforts to adapt and control the large number of existing risks to ensure that food safety and quality are not compromised.

National registration systems, diagnostic methods and official notifications vary

considerably between countries. In the USA, the percentage of cases registered by health authorities is very small compared with the real number of cases among the population, indicating that the actual incidence of foodborne diseases is higher than declared (Arendt et al., 2013; Tack et al., 2019). This fact has also been observed in other countries. The latest report from the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) advises that the inference of the results reported may be greatly influenced by under ascertainment and the underreporting of outbreaks. In 2018, of a total of 359,692 cases of foodborne and waterborne diseases in the EU, 41,203 people were hospitalized and 572 died (EFSA-ECDC, 2019), an increment of 1.01 % of the total cases of foodborne diseases, 1.29 % of the number of hospitalizations and 15.38 % of the number of deaths in comparison with the previous year (EFSA-ECDC, 2018). Therefore, among an EU population of 500,000,000 people, the estimate is approximately one case of foodborne disease per year per 1,390 inhabitants, of which one in 12,135 people are hospitalized and one in a million die. These are low but not negligible numbers. According to the report, the main biological agents involved in European foodborne diseases are *Campylobacter* spp., *Salmonella* spp., shiga-toxin-producing *Escherichia coli* (STEC), and *Yersinia enterocolitica*, representing 68.55 %, 25.54 %, 2.27 % and 1.86 % of the total documented cases, respectively. In the USA, the pathogenic bacteria causing most outbreaks are *Salmonella* spp. (30 %), followed by STEC (6 %), *Campylobacter* spp. (5 %) and *Clostridium perfringens* (4 %) (Dewey-Mattia, Manikonda, Hall, Wise, & Crowe, 2018). While these pathogens are the ones causing most foodborne diseases per year, their trend is mostly downward. The 2019 report compiled by EFSA-ECDC affirms that *Listeria monocytogenes* is the only foodborne pathogen with notably increasing confirmed cases in the last six years, representing the highest number of

deaths in the EU in 2018, with a mortality rate up to 15.6 % (EFSA-ECDC, 2019).

Food products can easily be contaminated along the production chain via non-hygienic surfaces, poor handling or inadequate food processing (Barjaktarović-Labović et al., 2018). If these products are contaminated, not submitted to an exhaustive control and commercialized, outbreaks can occur followed by the associated public and economic burdens. According to the Rapid Alert System for Food and Feed (RASFF), a comparison of the notifications received in 2017 and figures for the previous year showed that the number of original notifications (*i.e.* new hazards including alerts, informative notifications and border rejections) increased by 22 %, follow up notifications by 24 %, and alerts by 11 % (EC, 2018). These data are particularly worrying given that the control system applied in the food industry is precautionary and should work as a way of preventing product contamination. To this effect, the implementation of procedures and guidelines such as Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), Good Hygienic Practices (GHPs) and Hazard Analysis and Critical Control Points (HACCP) is a hugely important legal requirement aimed at preventing foodborne outbreaks (Chaves et al., 2017; Das, Nanda, Das, & Biswas, 2019). Moreover, and in parallel with the above, it is essential to have robust sampling plans and adequate methods to detect pathogens. Microbiological testing is considered as crucial not only for verifying the HACCP system but also for use in baseline studies in food safety policy settings, import and export food safety assessments, trade association studies, assessments of the suitability of incoming raw materials for use in products, and as a contribution to finished product criteria, among many others (Zwietering, Ross, & Gorris, 2014). To prevent potential problems, it is vital to apply analytical methodologies to rapidly evaluate whether the food is within

the legal microbiological safety limits.

This review focuses on evaluating the current status of rapid microbiological methods from the perspective of food microbiology and food safety. Furthermore, whether the methodologies used up to now are suitable and sufficient to detect foodborne pathogens will also be discussed. Accordingly, it aims to guide different food industry sectors in finding the most appropriate and up-to-date methods to analyze their products and consequently assure risk control.

2. Conventional methods, an outdated choice?

Enumeration of viable cells, selective isolation of bacteria in culture media and biochemical assays, considered as conventional microbiological methods, appear to be unattractive methodologies nowadays, especially when compared with new technologies developed for the rapid detection of various pathogenic microorganisms in food products (Hameed, Xie, & Ying, 2018). It is crucial to find rapid ways of detecting foodborne pathogenic and spoilage microorganisms in their initial growth stage to attenuate the number of foodborne outbreaks (González-Rivas et al., 2018; Pinu, 2016) and contribute to reducing the notable food and economic losses due to microbial spoilage (Ripolles-Avila, García-Hernández, Cervantes-Huamán, Mazaheri, & Rodríguez-Jerez, 2019). If fast results are obtained, food can be marketed early without risk and shelf life may be extended by some days. Moreover, microbial detection in the early stages of food processing provides information to control the process efficiently and prevent safety and quality issues (Ripolles-Avila, Cervantes-Huaman, Hascoët, Yuste, & Rodríguez-Jerez, 2019; Ripolles-Avila, Hascoët, Martínez-Suárez, Capita, & Rodríguez-Jerez, 2019). Hence, conventional methods would not fit with this objective. While they are

138 economical, manageable and easy-to-use, they are also time consuming since they rely
139 on microbial growth in different pre-enrichment, selective enrichment and selective
140 plating media (J. W. F. Law, Mutalib, Chan, & Lee, 2015; Rajapaksha et al., 2019). Thus,
141 a minimum of 3-4 days are needed for a preliminary identification of the microorganism
142 present in the food sample, and more than a week to confirm the pathogenic species
143 (Zhao, Lin, Wang, & Oh, 2014), making it impossible to make fast and coherent
144 decisions. Moreover, while these methods involve culture media preparation, serial
145 dilution, plating and colony counting, they tend to be laborious (Jasson, Jacxsens, Luning,
146 Rajkovic, & Uyttendaele, 2010; Mandal, Biswas, Choi, & Pal, 2011). Conventional
147 methods may have low sensitivity, which limits their applicability as they can present
148 false negative results due to the presence of pathogens in a viable but non-culturable
149 (VBNC) state (N. Lee et al., 2014), and the cultivable cells can only be quantified under
150 controlled conditions (*i.e.* selective media, incubation time and temperature, and oxygen
151 availability) (Davis & Mauer, 2010). Despite all these drawbacks, and as indicated almost
152 two decades ago by Fung, (2002) conventional methods are still considered to be the
153 "Gold Standard" for most of the microorganisms involved in food microbiology
154 considering that national and international regulatory agencies must use tests that have
155 legal standing. However, where the pathogenicity capacity must be detected in a widely
156 distributed microorganism, these methods are now starting to be displaced by molecular
157 biology, and especially by quantitative polymerase chain reaction (qPCR). That is the
158 case with Shiga toxin production by different serovars of *E. coli* (ISO/TS 13136-2012),
159 which provides information that is not available with current plating methods since they
160 cannot detect all the clones involved.
161
162 The pathogenic microorganisms most present in confirmed human infections in the EU

in 2018 and the standards followed in their detection are shown in **Table 1**. As previously stated, the actual incidence of foodborne diseases and confirmed cases is notably higher than those declared (Arendt et al., 2013; Tack et al., 2019). Therefore, pathogen detection is clearly failing in food products prior to their exportation from the industry. Moreover, a varying number of days are required for detection and confirmation using official standards performed with conventional methodologies. To overcome these limitations, official control institutions need to validate more sensitive and rapid methodologies. Several authors have reported the enormous importance of rapid techniques as opposed to conventional methodologies (Ankireddy & Kim, 2019; Ferrario et al., 2017; Rohde et al., 2017; Umesha & Manukumar, 2018). A case in point is the FDA's decision to publish the "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds" (2015), including the PCR as a rapid method to be used in food safety and feed control.

3. Overcoming challenges: the era of rapid methods

Research into rapid and alternative methods in food microbiology goes back to 1965 when experts in medical microbiology looking to develop rapid diagnostic methodologies adopted an approach that was quickly imitated by experts in food microbiology (Fung, 1992). Fung (2002) structured the advances on this research field in different decades (**Figure 1**), starting from miniaturization and diagnostic kit development, moving on to immunological test kit development and molecular testing system and PCR applications, and on to more recent advances such as biosensors, proteomics, and spectroscopic techniques such as terahertz radiation, among others. The way of addressing the ecology and diversity of microorganisms in food products to assist risk managers with quality and food safety decisions is also currently undergoing

change, thanks to next generation sequencing (NGS) techniques (Cocolin & Comi, 2005). Nonetheless, despite the research undertaken in this field and the already promising results obtained, these still need to be accepted as official methodologies. Rapid methods may currently be accepted if they are validated by independent institutions such as AOAC, AFNOR, MicroVal or NordVal.

As has long been indicated, rapid methods present a series of important competitive advantages (Fung, 2002; J. W. F. Law et al., 2015; Mandal et al., 2011). For example, they require less time to obtain results and can process a high number of samples per unit of time. Nonetheless, in most cases the use of rapid methods does not exclude the enrichment step of the target microorganisms or the need to obtain pure cultures, and positive results obtained with alternative methods must be confirmed with conventional methods (Fachmann et al., 2016). Furthermore, rapid methods are generally easy to use, accurate (*i.e.* adequate sensitivity and specificity) and economically profitable, despite requiring considerable initial economic investment in some cases (de Boer & Beumer, 1999). In fact, the food industry considers this as one of the main disadvantages when implementing new techniques for pathogen detection (Fung, 2002). Clearly, in the short run the use of new methods leads to an increase in analytical costs mainly due to investment in equipment and also because of the cost of assay kits. In many cases, the wrong approach from the food industry is to focus excessively on the cost and forget the essential objective: food safety and quality, which enables the effective control of risk. Besides the cost of the assay kits and the high investment in terms of equipment including both hardware and software, other factors such as the number and variety of samples, the need for technical and expert staff, and the cost of consumables will also determine the cost-effectiveness ratio for each particular food company.

Rapid methods are usually categorized according to the technique on which they are based. Immunology-based methodologies are based on the specific binding of antigens with antibodies and implies the choice of an appropriate antibody as the determining factor. These include assays such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescent assay (ELFA), latex agglutination assays, immunodiffusion, immunoprecipitation and immunochromatography (Maciorowski, Herrera, Jones, Pillai, & Ricke, 2006). Molecular techniques, discovered in the 1990s, constitute another group of methodologies. They are based on the analysis of genomic markers which are directly associated with nucleic acid sequences. Bacteria have conserved gene sequences, especially those that encode rRNA, which are used not only for bacterial detection but also for taxonomy and phylogeny (Váradi et al., 2017). Another molecular alternative developed in the 1990s is amplification based on the nucleic acid sequence (NASBA), which is performed under isothermal conditions, unlike PCR which requires thermocycling (J. W. F. Law et al., 2015). A new approach of interest is the development of multiplex PCR (m-PCR), which aims to simultaneously detect different foodborne pathogens with the same assay, thus allowing for further cost savings and higher throughput (J. Chen, Tang, Liu, Cai, & Bai, 2012; Villamizar-Rodríguez & Lombó, 2017). Therefore, an m-PCR has recently been developed with common primers for the detection of a total of 11-12 foodborne pathogens (Ying Liu et al., 2019; Tao et al., 2020). Furthermore, some molecular methodologies provide a general overview of microbial genomes, and in metabolic activities (including metabolites and proteins expressed for their production) these are the multi-omic approaches, which combine metagenomics and metabolomics (Franzosa et al., 2016). These methodologies are increasingly being applied and are among the most widely accepted by the food

industry and other researchers (Zhao et al., 2014). Another technology for bacterial pathogen identification is MALDI-TOF mass spectroscopy (MS), which is now used in the most advanced clinical laboratories (Doern & Butler-Wu, 2016). This methodology is based on the absorption of energy by means of the vaporization and ionization of analytes. The ions are separated depending on the time required to reach the detector. In general, most of the bacterial molecules observed using this technique are ribosomal proteins that end up producing an MS spectrum as if it were a peptide mass fingerprint, which can be compared with an online MS database to identify genera and specific species (Váradi et al., 2017). Although these alternative methodologies reduce detection time, in many cases a minimum of 24 h is still needed as an enrichment step to increase the amount of bacteria to detectable numbers (Fachmann et al., 2016). Therefore, "same-day detection" methodologies are currently emerging, optimizing the sample treatment and therefore detection. In this sense, *E. coli* O157:H7 same-day detection has been achieved with a sensitivity of 3.9 and 3.3 CFU/25 g for ground beef and green leafy vegetables, respectively (Garrido-Maestu, Azinheiro, Carvalho, Fuciños, & Prado, 2020).

Alternative methods must be validated for use in food microbiological analysis. Validation consists in demonstrating the capability to accomplish detection and quantification at the same level of sensitivity and specificity as the reference method (Rohde et al., 2017). In the absence of an accepted universal procedure for validating alternative methods, standards such as ISO 16140 and the AOAC guidelines, accepted in the EU and the USA, respectively, are used (Ryan, Roof, Post, & Wiedmann, 2015). However, a negative point to consider is that these allow for a certain flexibility in the experimental design, for example in the choice of target microorganism strains

(Quintela, De Los Reyes, Lin, & Wu, 2019). While the AOAC provides recommendations on the minimum number of strains of target microorganisms (50 strains, except for *Salmonella* spp. detection where 100 strains are recommended), there are no specifications as to which strains should be used. Hence, there may be differences in the sensitivity and effectiveness of detection between methods where the strains used are different. The future trend appears to be multidisciplinary methods: sensitive materials for cell detection, autonomous systems, big data, electronics, supercomputing and machine learning will combine to provide effective diagnostic solutions to detect microorganisms in food products (Váradi et al., 2017).

4. Foodborne pathogen detection and quantification: conventional *vs* rapid methods

4.1. Foodborne bacteria

Different pathogenic bacteria relevant to the food industry will be discussed, and both the different methodologies used for their detection and quantification and the leading alternatives investigated at present will be evaluated. These methodologies are legislated and therefore safe legal limits have been established for the pathogens.

4.1.1. Salmonella spp.

An important zoonotic pathogen transmitted by food is *Salmonella* spp., which causes gastroenteritis and typhoid fever and is able to persist in the environment, soil, water, food and food processing environments through ecological niches (Condell et al., 2012; Eng et al., 2015; Ripolles-Avila, Ríos-Castillo, Fontecha-Umaña, & Rodríguez-Jerez, 2019; Visvalingam, Zhang, Ells, & Yang, 2019). Salmonellosis is caused by two

species of *Salmonella* spp. (*S. enterica* and *S. bongori*). There are six subspecies of *S. enterica*: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. The difference between the strains of *Salmonella* spp. lies in their reaction to antisera, which recognizes different O and H antigens. *S. enterica* ssp. *enterica* inhabits warm-blooded animals, whereas other *S. enterica* subspecies and *S. bongori* are usually commensals of cold-blooded animals (Alakomi & Saarela, 2009). Although the *Salmonella* spp. strains mostly associated with human clinical diseases are those of the *S. enterica* subsp. *enterica*, foodborne diseases have been reported involving all *Salmonella* species and subspecies, except for the subspecies *indica* (Buehler, Wiedmann, Kassaify, & Cheng, 2019). The presence of this pathogen is attributed mainly to foods of animal origin (**Figure 2**). However, *Salmonella* spp. can also be related to other food products, especially when cross-contamination occurs either from contaminated surfaces or poor handling (Jensen, Danyluk, Harris, & Schaffner, 2017; S. Wang et al., 2016). Although reported data are scarce and not representative enough to describe the situation at an EU level, there are food categories with a higher percentage of notifications reported for *Salmonella* spp. than zoonosis involving this pathogen. However, there are food categories where the opposite occurs, with more zoonoses than notifications reported, as may be the case of eggs and egg products. This may indicate difficulties in detecting *Salmonella* spp. in these products, a point which will be discussed in greater depth later.

Microbiology companies have developed and improved technologies to detect this pathogen in food. Since regulation establishes the total absence of *Salmonella* spp. in all foods, these methods must be sufficiently sensitive to detect just one cell in a sample. Furthermore, the time of analysis can vary depending on the enrichment steps used to reach the minimum cell concentration to detect the pathogen. This time is always long

313 in conventional methods (*i.e.,* 18-24 h for pre-enrichment and 18-24 h for selective
314 enrichment), whereas it is shorter in rapid methods since they can detect *Salmonella*
315 spp. from 10^4 cells/ml (K. M. Lee, Runyon, Herrman, Phillips, & Hsieh, 2015).
316
317 Considering that standard culture methods depend on the ability of *Salmonella* spp. to
318 grow in visible colonies, they usually require between 5 to 7 days to be completed since
319 the results must be confirmed by biochemical or serological tests. There are different
320 standard methods published for detecting *Salmonella* spp. in food, feed and
321 environmental samples, including the current ISO horizontal method (ISO 6579:2017)
322 and the Bacteriological Analysis Manual (BAM) of the FDA. They consist in the
323 general enrichment of a food portion in a broth such as buffered peptone water to
324 recover cells injured by heat, cold, acid or osmotic shock (Gracias & McKillip, 2004)
325 and to increase the concentration of target cells, which are generally present in low
326 numbers, heterogeneously distributed and integrated in a mixed microbial population
327 (Odumeru & León-Velarde, 2012). The samples are subsequently incubated in a
328 selective enrichment broth such as Rappaport Vasiliadis Soy broth (RVS) or Muller-
329 Kauffmann Tetrathionate-Novobiocin broth (MKTTn), among others. These media
330 usually contain inhibitory agents capable of stopping or delaying the growth of non-
331 target microorganisms and of stimulating the growth of the target pathogen. The
332 samples are then plated in differential agar media such as xylose-lysine deoxycholate
333 (XLD) agar, bright green (BG) agar or others, which contain particular substrates that
334 can only be degraded by target bacteria such as *Salmonella* spp., or they confer a
335 particular color on the growing colonies (Manafi, 2000). This process involves
336 confirming colonies as presumptively positive, which extends the time to obtain results.
337

Improvements to the standard methodology have been proposed to maximize its efficiency and reduce the time, material and labor costs involved (Nemati, Hamidi, Dizaj, Javaherzadeh, & Lotfipour, 2016). To this end, alternative methods have been developed combining the general and selective enrichment stages in a single step, and later using chromogenic media such as IRIS *Salmonella* (Bioser), RAPID'*Salmonella* (Biokar) or SALMA One Day (BioMerieux), among many others, for detection. These media usually employ a chromogen linked to octanoic acid to detect C8 esterase activity, which is common in all *Salmonella* spp. (Váradi et al., 2017). Due to the great variety of available culture media for *Salmonella* spp. isolation, together with the fact that the choice of method is not common in all laboratories or food companies, the isolation of this pathogen in food products is one of the most variable procedures, involving the addition of new specific, sensitive, rapid ways of detection (Odumeru & León-Velarde, 2012). In general, these chromogenic media produce results a day sooner than conventional methods, although they are not rapid enough to respond to an outbreak caused by *Salmonella* spp. and the subsequent product recall (K. M. Lee et al., 2015). Furthermore, the time needed for pathogen detection when using chromogenic media (between 16 to 48 h) is considered to be a disadvantage since it requires an incubation step to produce enough bacterial cells, and therefore enzymes, for color development. Alternatives based on the use of fluorogenic substrates have been proposed, since it is easier to detect fluorescence than to perceive a color, enabling the detection of microcolonies within 2 h (Váradi et al., 2017).

There are several categories of commercially available rapid methods for *Salmonella* spp. detection including selective media (already discussed), immunology-based assays, nucleic acid-based assays and mass spectrometry, among others (**Table 2**).

Immunoassays are based on specific mono or polyclonal antibodies used for binding somatic or flagellar antigens of *Salmonella* spp. These assays have been widely used and are interesting as routine analyses, partly because of their ability to detect viable but non-culturable *Salmonella* spp. cells (Maciorowski et al., 2006). One example of these assays is Reveal 2.0 for *Salmonella enterica* serovars of somatic groups A-E (Neogen), which enables the rapid recovery of stressed or injured cells with selective enrichment that favors *Salmonella* spp. growth to detectable levels. The principle behind this method is a solution that contains specific anti-*Salmonella* antibodies conjugated to colloidal gold particles. If there are antigens present in the sample, an immune complex with gold conjugate will be further captured and aggregated to a zone of a nitrocellulose membrane, displaying a visible, easily interpretable line. Although the ELISA method was developed decades ago, new techniques based on this principle are still being investigated. For example, Di Febo et al., (2019) developed a *S. enterica* capture ELISA, reporting good results in relation to sensitivity, specificity and accuracy compared to the reference method (ISO 6579). Compared with the conventional techniques discussed above, these immunological techniques are faster and more specific methodologies that not only involve isolating the pathogen if techniques such as immunomagnetic separation (IMS) are included, but can also be easily automated to reduce time and labor (J. Chen & Park, 2018; K. M. Lee et al., 2015). Assays that combine immunology, nanotechnology and changes in temperature have also been developed, including the immune-thermometer assay based on the photothermal effect of graphene oxides for *S.* Typhimurium (S. Du, Wang, Liu, Xu, & Zhang, 2019). Molecular methods, most notably PCR, have also been highly used in the food industry to detect *Salmonella* spp. and other foodborne pathogens in food and environmental samples (Domesle, Yang, Hammack, & Ge, 2018; Garrido-Maestu, Fuciños, et al.,

2019; Garrido-Maestu, Tomás Fornés, & Prado Rodríguez, 2019; Kasturi & Drgon, 2017; Yuejiao Liu, Singh, & Mustapha, 2018; M. Wang et al., 2018). The specificity and sensitivity of the ELISA and PCR assays do not differ from conventional methods, although these parameters are highly dependent on the sample matrix, the background microbiota, and the presence of non-culturable cells and inhibitory substances such as fat, proteins and antibiotics, among others (Alakomi & Saarela, 2009). Their sensitivity can be further increased if sample preparation and purification techniques are included such as the modification of enrichment media or microbial concentration through immunomagnetic separation (K. M. Lee et al., 2015). Although these techniques are more rapid than conventional methodologies, they require sample enrichment, which extends the detection process to at least 24 h. Techniques that combine sample concentration followed by PCR have been developed to resolve this problem and further reduce the length of the detection process. This combination enables the qPCR detection of low levels of *Salmonella* spp., reaching an efficiency of 92 % and lowering the detection limit to approximately 2 cells/ml (Vinayaka et al., 2019). Another recent development that combines sample concentration and detection using a molecular methodology is the one proposed by J. Wang et al., (2018), who reported high sensitivity detection within 10 h when using recombinant *PagC* protein and *PagC* antibody coupled with immunomagnetic beads to capture *Salmonella* spp., and subsequently combined with SYBR Green qualitative PCR detection. Additionally, the development of "same-day detection" assays have been described, with detection times of less than 5 h including the sample treatment steps of enrichment, filtration, centrifugation, and enzymatic digestion, followed by molecular detection techniques (Fachmann et al., 2016).

Other alternatives that can be used for *Salmonella* spp. detection in food matrices have recently gained in popularity. A clear example are optical biosensors, which show high sensitivity and simplicity. Quintela et al., (2019) have developed a colorimetric detection method with a new optical biosensor platform using gold nanoparticles (AuNPs) functionalized with oligonucleotides. This method has high biosensitivity and is able to detect 100 % of the nineteen strains of *Salmonella* spp. tested with high specificity and a minimum detection limit of 10 CFU/ml. Appaturi et al., (2020) have developed a biosensor for accurate, rapid, label-free electrochemical *S. enterica* detection using reduced graphene oxide-carbon nanotubes (rGO-CNT), reporting the same detection limit as in the previous study (10 CFU/ml). Another novel methodology is the Lab-on-Chip techniques, which are able to detect low concentrations of target microorganisms in food samples (Dhar & Lee, 2018). These are based on the capture of pathogens by bio-receptors in microfluidic devices, facilitating the subsequent recognition of the microorganism, minimizing the impact of the complex food matrix and improving the detection limit (Kant et al., 2018). In the case of *Salmonella* spp., a modular lab-on-chip platform that integrates different phases including sample preparation, bacteria capture, thermal lysis, purification of DNA and isothermal DNA amplification has been developed for detecting the pathogen in milk (Tsougeni et al., 2019).

As previously shown in **Figure 2**, there is a higher proportion of zoonosis transmitted by *Salmonella* spp. through eggs and egg products than notifications reported by the RASFF Portal (*i.e.* their detection). This is also evident in bakery products, which could also be related to the use of eggs as an ingredient. This circumstance shows the importance of *Salmonella* spp. detection in this type of matrix, indicating that current

methods used may not be effective enough to detect this pathogen in specific types of products. It has been shown that different *Salmonella* spp. can contaminate both the internal and external egg content, penetrate the eggshell, survive in albumin for more than 24 h, migrate from albumin to yolk, and multiply in the eggs during storage (Crabb, Gilkerson, & Browning, 2019). Failure to detect *Salmonella* spp. in products of this type could be related to the pathogen interaction with the egg components, which would limit its detection. A food matrix is composed of a heterogeneous variety of components such as biochemical compounds, inorganic particles and many others, which can interfere with the detection of food pathogens (Y. Wang & Salazar, 2016). Dwivedi & Jaykus, (2011) suggested that carbohydrates may interfere with nucleic acid amplification methods, and that fat and other particles may interfere with antibody binding. Consequently, more efficient, reliable methods that do not give false negatives must be developed considering the possible interference of food matrices in pathogen detection. In this regard, a novel technology for *Salmonella* spp. detection in egg products that is simple and easy to operate is loop-mediated isothermal amplification (LAMP), which amplifies DNA with high speed, efficiency and specificity under isothermal conditions (Hu, Ma, et al., 2018). LAMP technology has been validated for *Salmonella* spp. detection in different foods including animal feed (D'Agostino et al., 2016; Domesle et al., 2018). The development, named Roka Atlas *Salmonella* Assay, is a primer for detecting the *prot6E* gene that encodes a fimbrial biosynthesis protein and is located in a virulence plasmid of *S. enterica* var. Enteritidis. In fact, this technology was compared with the conventional culture official method, qPCR and different isothermal amplification assays such as MicroSEQ *Salmonella* spp. detection kit (MicroSEQ), 3M Molecular Detection System (MDS) *Salmonella*, Amplified Nucleic Single Temperature Reaction *Salmonella* Assay (ANSR), and Pro-AmpRT *Salmonella*

463 spp. (Pro-AmpRT). It has proven to be the most sensitive technology currently available
464 with relatively better results obtained for *Salmonella* spp. detection in egg products and
465 for detection limit, although no significant differences were found (Hu, Deng, et al.,
466 2018). Das et al., (2019) also compared Roka Atlas assay with the BAX System
467 (DuPont Nutrition & Health) and conventional methodologies for *Salmonella* spp.
468 detection in broiler carcasses, concluding that both molecular methods were as effective
469 as the conventional cultural procedure.
470
471 ***4.1.2. Campylobacter* spp.**
472 *Campylobacter* spp. is the bacterial pathogen that has produced the greatest number of
473 zoonosis in the EU over the last decade (EFSA-ECDC, 2019), and is also one of the
474 most frequent in the USA (Bolton, 2015). The genus *Campylobacter* is a large, diverse
475 group of Gram-negative bacteria capable of producing a series of gastrointestinal
476 problems and autoimmune conditions, including Guillain-Barré syndrome (Humphrey,
477 O'Brien, & Madsen, 2007). Several emerging species of *Campylobacter* are currently
478 associated with human disease, with two species, *C. jejuni* and *C. coli*, responsible for
479 the vast majority of bacterial gastroenteritis in humans worldwide (Whitehouse, Zhao,
480 & Tate, 2018).
481
482 Broiler meat is the most important single source of campylobacteriosis (EFSA-ECDC,
483 2018, 2019), with the rest of food products usually presenting low numbers of this
484 pathogen (Biesta-Peters, Jongenburger, de Boer, & Jacobs-Reitsma, 2019).
485 Contamination usually occurs during slaughter and carcass processing (Humphrey et al.,
486 2007). In fact, the control of *Campylobacter* spp. in chicken farms is still challenging
487 despite more than 30 years of research into ways to manage it (Bolton, 2015). Previous

488 approaches undertaken depend largely on biosecurity, which is often ineffective once
489 broiler flocks become infected in the third or fourth week of rearing (Bolton, 2015;
490 Patriarchi et al., 2009). The control of this pathogen in the food chain is essential, but no
491 effective ways have yet been found, which could be one of the reasons why
492 *Campylobacter* spp. was not partially legislated until 2018. Attempts have been made to
493 develop methods for its control, focusing on the pre-slaughter stages associated with
494 broiler rearing (Hermans et al., 2011; Lin, 2009), including the use of vaccines (A. Liu
495 et al., 2018; Meunier et al., 2017) and probiotics (Saint-Cyr et al., 2017; Smialek,
496 Burchardt, & Koncicki, 2018). Limiting the entry of chicken carcasses highly
497 contaminated by *Campylobacter* spp. into the market would be another effective
498 strategy to reduce the number of cases of foodborne disease produced by these bacteria
499 (Duqué, Haddad, Rossero, Membré, & Guillou, 2019). Therefore, a hygiene criterion
500 for broiler carcasses has been established: a limit of 1,000 CFU/g in carcasses after
501 cooling (EC, 2017). The aim of this new microbiological criterion is to reduce the risk
502 by more than 50 %. For this legal parameter to be met, food producers must improve the
503 hygiene of chicken slaughter to limit cross-contamination, and new control measures
504 that can favor *Campylobacter* spp. inactivation after the slaughter must also be
505 developed. In this regard, it has been suggested that chicken processing steps through
506 rearing, slaughter, cutting and manipulation could be a strategy to address the risk of
507 this pathogen (Duqué et al., 2019).
508
509 Although the greatest presence of *Campylobacter* spp. is in broiler meat, there are also a
510 high number of campylobacteriosis outbreaks related to milk and dairy products which
511 produce very few RASFF notifications probably because the pathogen is difficult to
512 detect (**Figure 3**). This may be related to the presence of low numbers of cells that

might be sub-lethally injured by environmental factors or food preservation techniques, which makes them unable to grow in selective media (Biesta-Peters et al., 2019). This is one of the situations that must be considered when developing methodologies for both *Campylobacter* spp. detection and enumeration. According to RASFF notifications, plant-based foods are beginning to be contaminated by this pathogen. However, no human campylobacteriosis have so far been reported with these food vehicles (EFSA-ECDC, 2018).

The standard method for *Campylobacter* spp. detection in food matrices was published by the ISO in 2006. It consists in an enrichment step in Bolton broth, followed by cultivation in modified charcoal-cefoperazone-deoxycholate agar (mCCDA) and another selective agar media, and subsequent incubation under microaerophilic conditions (ISO 10272-1:2006). However, after an exhaustive review of the effectiveness of this method, the conclusion was reached that it was not optimal for *Campylobacter* spp. detection, leading to underestimation of the real prevalence of this pathogen (Jacobs-Reitsma, Jongenburger, de Boer, & Biesta-Peters, 2019). Hence, the first standard was reviewed, modified and validated, resulting ISO 10272-1:2017, which includes three procedures for *Campylobacter* spp. detection to be employed depending on the expected levels of *Campylobacter* spp. and background microbiota in the food product to be tested. The growing demand for broiler meat and the consequent speed of production make the availability of tools for real-time decision making regarding product safety increasingly important (Khan, Abulreesh, Qais, & Ahmad, 2018; Mottet & Tempio, 2017; Ricke et al., 2019). To this effect, researchers have developed an optimized enrichment medium to facilitate rapid *Campylobacter* spp. cell recovery (Al, Incili, Akcay, & Koluman, 2019), in addition to a large number of different culture

media and rapid techniques for the isolation and characterization of this pathogen in different food matrices (Gharst, Oyarzabal, & Hussain, 2013). For example, Poonlapdecha et al., (2018) developed a system based on *C. jejuni* capture by means of nanoparticles combined with a lateral flow test strip assay for its rapid detection. A compilation of commercial methods used in the industry for *Campylobacter* spp. evaluation in food and the environment is shown in **Table 3**. Although different studies have been carried out for *Campylobacter* spp. isolation in culture, the challenges persist because this pathogen has biological, physiological and metabolic characteristics that can change over time, which markedly influences the sensitivity and specificity of culture-dependent methods (Ricke et al., 2019). For example, under refrigeration and freezing conditions it enters into a state of stress and becomes VBNC, and is consequently not detected by many conventional microbiological techniques (Castro et al., 2018; Schottroff et al., 2018). In this case, molecular methodologies are more advisable. Real-time recombinase polymerase amplification (qRPA) assay for the efficient detection of *C. jejuni* has been developed by targeting the *hipO* gene (Geng et al., 2019; J.Y. Kim & Lee, 2017). However, *Campylobacter* spp. also show metabolically-driven strain-to-strain variations in the expression of virulence factors, which means that if molecular determination of these factors is applied, detection can be problematic because the pathogen cannot express them (Hofreuter, Novik, & Galán, 2008). All this leads to the conclusion that the use of a single methodology for *Campylobacter* spp. detection might not be enough.

Research around the development of rapid, accurate and sensitive diagnosis methods to detect *Campylobacter* spp. in food should continue to assist with the establishment of food safety policies and intervention strategies to eliminate or mitigate the risk to the

563 consumer, thus preventing foodborne outbreaks.

564

565 ***4.1.3. Listeria monocytogenes***

566 *L. monocytogenes* is a ubiquitous microorganism that can be found in a wide range of
567 ecological niches including food production facilities (Buchanan, Gorris, Hayman,
568 Jackson, & Whiting, 2017; Ripolles-Avila, Hascoët, Martínez-Suárez, et al., 2019). It is
569 very adaptable to unfavorable conditions compared with other microorganisms due to
570 its ability to survive temperatures ranging from -0.4 to 50°C, pH between 4.7 and 9.2
571 and high concentrations of sugar (39.4 % sucrose) and salt (10 % NaCl) (Gharst et al.,
572 2013). This is currently the pathogen that presents the highest mortality rates at the
573 European and American levels and there is an increasing trend in the number of cases
574 (Dewey-Mattia et al., 2018; EFSA-ECDC, 2019), especially in some food products
575 (**Figure 4**). Fish and fishery products is the category that presents the highest number of
576 RASFF notifications, and especially smoked salmon, although products such as smoked
577 trout are also starting to stand out (EC, 2018). This coincides with the reported zoonoses
578 in EFSA-ECDC, (2018), with the highest occurrence found in fish and fishery products
579 in the ready-to-eat (RTE) category. The increasing number of cases of *L.*
580 *monocytogenes* associated with RTE food needs to be highlighted. In fact, this
581 relationship has already been observed and addressed with Member States increasing
582 their sampling for most of the RTE food categories in 2017 compared with 2016. In this
583 regard, it must be considered whether the detection systems currently used are efficient
584 enough and how they can be improved to make *L. monocytogenes* control more
585 effective.

586

587 Human listeriosis is an infection with a low incidence but a high mortality rate among

588 populations with compromised immune systems. This fact, along with its connection
589 with stillbirth and miscarriage, makes it a considerable public health problem (Ripolles-
590 Avila, Ríos-Castillo, Guerrero-Navarro, & Rodríguez-Jerez, 2018; Sadekuzzaman,
591 Yang, Mizan, Kim, & Ha, 2017). One of the most important modes of transmission is
592 cross-contamination from food contact surfaces, given that *L. monocytogenes* has the
593 ability to form biofilms. In this case, the control and analysis of food industry surfaces
594 to establish the effectiveness of cleaning and disinfection programs is as important as
595 the analysis of the final product (M. Chen, Wu, Zhang, Wu, & Guo, 2015; J. W.-F.
596 Law, Ab Mutalib, Chan, & Lee, 2015; Ripolles-Avila, Cervantes-Huaman, Hascoët,
597 Yuste, et al., 2019; Ripolles-Avila, Hascoët, Guerrero-Navarro, & Rodríguez-Jerez,
598 2018).
599
600 Detecting *L. monocytogenes* in food and environmental samples traditionally starts with
601 a pre-enrichment step followed by sample enrichment. Pre-enrichment is carried out to
602 recover the microorganisms that may be injured in food samples. Injury would make
603 them vulnerable to the presence of suppressing agents, that are generally included in
604 enrichment broths to inhibit surrounding microbiota, which allows the target
605 microorganism to grow and be subsequently detected (Lammerding & Doyle, 1989).
606 For this reason, and as indicated in the ISO 11290, a pre-enrichment stage in half-Fraser
607 broth is used to promote the recovery of stressed cells in the sample. Fraser selective
608 enrichment broth is then used to promote *L. monocytogenes* growth and suppress the
609 growth of accompanying microbiota through selective agents such as acriflavine, which
610 inhibits Gram-positive bacteria, nalidixic acid, which inhibits Gram-negative bacteria,
611 and lithium chloride, which inhibits enterococci (Jordan, Fox, & Walker, 2014). This
612 step is followed by culture plating. The media initially recommended by the ISO were

Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM), and Oxford agar (OXA). However, detection based on aesculin hydrolysis can give a number of false negative results, especially if other species of *Listeria* spp. are present (Johansson, 1998). In response, in 2004 the ISO amended the isolation protocol for *L. monocytogenes*, adopting the Chromogenic Agar *Listeria* medium according to Ottaviani and Agosti (ALOA) as a selective and differential medium. The detection of the pathogen in this medium is based on a double enzymatic activity. The first, common to all *Listeria* spp., is β-glucosidase activity, which cleaves the chromogenic substrate and produces blue-green colonies; the second, related to pathogenic ability and therefore only in *L. monocytogenes* and *Listeria ivanovii*, is phosphatidylinositol phospholipase C activity, which hydrolyzes the lecithin present in the agar, forming a white precipitation zone around the colony (Reissbrodt, 2004). Suspicious colonies must be confirmed, usually using techniques based on hemolytic properties and sugar fermentation. There are several methods for *L. monocytogenes* detection and/or enumeration, the reference one being the method defined in ISO 11290-1 and ISO 11290-2. A confirmed result is usually obtained in 4-5 days.

Table 4 compiles the methods used in the industry for *L. monocytogenes* evaluation in food and the environment. When deciding about using and implementing a new rapid method, the food industry evaluates the attributes of the method such as accuracy for the intended purpose, speed in productivity and initial, per test, reagents and labor costs (Fung, 2002). Companies that constantly develop new rapid products for microbiological analyses must cover these needs, giving weight to the attributes required by the food industry. A greatly reduced detection limit is generally accompanied by an increase in the cost of the methodology, thus reducing the

possibility of these methods being used by SMEs, which constitute the largest proportion of companies in the European food industry. In this case, there is a risk of reducing the sampling frequency, increasing the probability of non-detection and the release of contaminated batches. However, the worryingly high mortality rate for *L. monocytogenes* (EFSA-ECDC, 2019) and the essential need to detect it correctly without giving false negatives must also be considered. Therefore, reducing analytical time by combing samples for analysis (i.e., pooling analyses) to verify the effectiveness of a food safety management system will be advantageous for laboratories. This type of analysis for *Listeria* spp. and *L. monocytogenes* detection in processed dairy products has already been validated (Jagadeesan, Bastic Schmid, Kupski, McMahon, & Klijn, 2019). Another strategy to shorten the analytical time is the simultaneous use of immunomagnetic separation followed by a molecular detection methodology such as qRPA (IMS-qRPA), achieving results in 24 h with reported detection limits of 6.3 cells/25 g (Garrido-Maestu, Azinheiro, Carvalho, & Prado, 2019).

Intensive research has recently been done on the use of biotechnology to generate new ways of preparing antibodies for *L. monocytogenes*. Some examples are recombinant phage expression antibodies and new recognition elements such as the aptamer, molecular printing polymers and vancomycin, which are starting to be applied in the food industry (A. Liu et al., 2018; Zhang et al., 2016). The analysis of this pathogen will be increasingly effective thanks to these huge advances.

4.1.4. Shiga toxin-producing *Escherichia coli*

One of the most important causes of foodborne diseases worldwide are *E. coli* strains that produce Shiga-type toxins (STEC). Serotype O157:H7 in particular began to be

663 considered as a significant risk to public health in the 1980s when it was associated with
664 outbreaks of gastrointestinal symptoms and hemolytic uremic syndrome (HUS) (Adams
665 et al., 2016). The STEC serogroup level most frequently reported in confirmed cases of
666 infection by this pathogen in Europe is O157 (31.9 %), the proportion of which is
667 decreasing year by year, while other serogroups such as O26, O103 and O91 are
668 increasing (EFSA-ECDC, 2018). STEC infections have significant economic
669 repercussions, leading to annual healthcare costs of over US$ 400 million in the USA
670 (Gossman, Wasey, & Salen, 2019). Furthermore, STEC strains cause around 176,000
671 cases, 2,400 hospitalizations and 20 deaths annually (Incili, Koluman, Aktüre, &
672 Ataşalan, 2019), figures which demonstrate the considerable repercussions for public
673 health. Detection systems must therefore be sufficiently specific, while enabling the
674 wide variety of serogroups involved in the production of these toxins, or other important
675 virulence factors, to be detected. Shiga toxin, the main virulence factor, targets the cells
676 that express glycolipid globotriaosylceramide, interrupting the synthesis of host proteins
677 and causing further apoptotic cell death (Ethelberg et al., 2004).
678
679 With regional variability worldwide, beef and dairy products are the foods with the
680 strongest relationship with the transmission of this pathogen (Pires, Majowicz, Gill, &
681 Devleesschauwer, 2019). These data coincide with the trends observed in annual
682 evaluations of STEC zoonosis at the European level (EFSA-ECDC, 2019). The contrast
683 between the few alerts reported by RASFF relating to the presence of STEC in these
684 products and the high percentage of STEC presence reported by EFSA-ECDC (**Figure**
685 **5**) may indicate that the STEC detection methodologies currently used are not effective
686 for milk and dairy products. On this regard, Incili et al., (2019) have highlighted that the
687 specificity and sensitivity of the ISO methodology for STEC detection in cheese is low

compared to other matrices such as beef, minced lamb, apple puree, soybean sprouts and milk. This could indicate that within the category milk and dairy products, cheese is the product that could pose problems in detecting this pathogen, and if false negative results are obtained a lack of security would be produced.

STEC and specifically *E. coli* O157:H7 detection has traditionally been done on sorbitol MacConkey (SMAC) agar, which contains bile salts, a carbohydrate source, sorbitol and a pH-indicator, neutral red. *E. coli* O157:H7 does not ferment sorbitol, so if this pathogen is present it will grow as colorless colonies, while other *Enterobacteriaceae* appear as pink colonies (March & Ratnam, 1986). However, the use of enrichment broths containing peptone and different antimicrobials such as cefsulodine, cefixime, vancomycin and potassium tellurite improves *E. coli* O157:H7 detection by supplying nutrients and limiting the growth of competing microbiota, thus allowing the pathogen to produce more colonies when subsequently plated on SMAC (Priyanka, Patil, & Dwarakanath, 2016). Among the limitations of this method are the slow response time and the high number of false negatives due to the emerging serotypes capable of fermenting sorbitol (Hirvonen, Siitonen, & Kaukoranta, 2012). This can be partially overcome by using chromogenic media for STEC isolation and their easier discrimination based on color, which has increased specificity and sensitivity (Priyanka et al., 2016), although in some cases has not been proven to be sensitive enough to detect all strains (Hirvonen et al., 2012). All these factors lead us to think that detection systems based on the culture of this pathogen are not entirely reliable, and that other more precise methodologies need to be implemented as reference methods in food matrices.

A compilation of different methods to evaluate the presence of STEC in food and environmental samples is shown in **Table 5**. A recent study focused on *E. coli* O157:H7 concentration by filtration followed by qPCR detection to eliminate the need for microbial culture enrichment concluded that this method is effective and sensitive with a detection limit of 5 CFU/g within 2 h (J.-H. Kim & Oh, 2019). There are also other technologies that combine the different fundamental concepts described above. Optical biosensors stand out among these methodologies, providing rapid, accurate results for detecting multiple pathogens in food matrices (Yoo & Lee, 2016). In general, these biosensors are categorized according to their sensing approach, which may be based on color, fluorescence, impedance or other technologies such as surface plasmon resonance. The latter monitors the change in the refractive index due to biomolecular interactions in the sensor (Rand, Ye, Brown, & Letcher, 2002). This technology includes immobilized antibodies. When antigens interact with antibodies the refractive index of the medium surrounding the sensor is modified, which in turn changes the resonance angle proportional to the change in concentration of the surface-bound antigens (Waswa, Irudayaraj, & DebRoy, 2007). Many molecular techniques have also been developed for *E. coli* O157:H7 detection, which are currently the most commonly used in the food industry (Khezri, Rezaei, Mohabbati Mobarez, & Zolfaghari, 2019; Mahmoud Ouf, Yuan, Singh, & Mustapha, 2017). However, research has intensified into developing multiplex molecular methods in which common pathogens in food matrices whose presence should be controlled are sought as target. For example, enriched multiplex PCR (Tem-PCR) for the simultaneous detection of *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus*, reporting a detection limit of less than $2x10^2$ CFU/ml (Xu et al., 2016); and m-PCR to detect *E. coli* O157:H7, *Cronobacter* spp. and *Salmonella* spp. in milk and milk products, with a

738 reported detection limit of 10^2 CFU/ml (Liang et al., 2019).

739

740 Nanotechnology has also been combined with molecular techniques resulting in
741 developments such as gold nanoparticles-assisted m-PCR assays for the simultaneous
742 detection of *S.* Typhimurium, *L. monocytogenes* and *E. coli* O157:H7, with a reported
743 detection limit of 10-50 pg/µl (J. Du et al., 2020). Furthermore, thanks to the current
744 progress in nanotechnology, the resonance of localized surface plasmon combined with
745 gold nanoparticles (AuNP) and immunological tests has led to the development of many
746 types of biosensors (J. Chen & Park, 2018; Güner, Çevik, Şenel, & Alpsoy, 2017; J.
747 Kim et al., 2018). To this effect, Zhou et al., (2018) demonstrated that these systems
748 coupled with optical fibers and optimized with antimicrobial peptides as recognition
749 elements, and silver nanocomposites with reduced graphene oxide nanoparticles
750 (AgNP-rGO) as assisted signal amplification, are effective in detecting *E. coli* O157:H7
751 in water and juice, reporting a low detection limit ($5x10^2$ CFU/ml). Much effort has
752 recently been put into developing biosensors that can give continuous results for early
753 decision making, and even during food processing. Yao et al., (2018) developed a
754 biosensor for the continuous flow detection of *E. coli* O157:H7 based on impedance,
755 which integrates immune-magnetic nanoparticles for bacteria isolation, urease for
756 biological signal amplification and a microfluidic chip for impedance measurement.
757 This biosensor was able to detect the pathogen in 2 h with a detection limit of $1.2x10^1$
758 CFU/ml. Yu, Chen, Wang, & Li (2018) proposed a quartz crystal microbalance sensor
759 that uses DNA aptamers, which are short molecules of single stranded DNA (ssDNA)
760 or RNA that bind to target molecules with affinity and specificity similar to those of
761 antibodies, but with many competitive advantages over them (Kumar, Malinee,
762 Dhiman, Kumar, & Sharma, 2019; Nimjee, Rusconi, & Sullenger, 2005). This was

763 investigated for *E. coli* O157:H7 and a detection time of 50 min with a detection limit of
764 1.46x10^3 CFU/ml was reported (Yu et al., 2018). Furthermore, Zheng et al., (2019)
765 reported detection limits under optimal conditions as low as 50 CFU/ml in 1 h using a
766 microfluidic biosensor based on the aggregation of gold nanoparticles and smartphone
767 images for the rapid detection of the pathogen.
768
769 ***4.1.5.*** **Other bacteria**
770 There are many other bacterial pathogens that are legislated and should be monitored
771 even though they are not included in the EFSA-ECDC, (2019) report. Among them,
772 *Staphylococcus aureus* toxins stand out, as they are heat resistant, cause food poisoning,
773 and their presence in processed foods is related to poor sanitation (Ripolles-Avila,
774 Hascoët, Ríos-Castillo, & Rodríguez-Jerez, 2019; Valderrama, Dudley, Doores, &
775 Cutter, 2016). Two aspects must be considered, *S. aureus* detection and enumeration in
776 foods and the detection of its toxins. Regarding microorganism detection, molecular
777 methodologies have usually been employed in the food industry, with developments
778 such as LAMP with an analytical sensitivity of 7.6x10^2 CFU/ml (Tian, Feng, & Wang,
779 2018), qPCR combined with propidium monoazide to optimize the analysis and
780 obtaining a detection limit of 5.0x10^1 CFU/ml (Zi et al., 2018), and qRPA which has
781 proved to be as sensitive as real-time PCR while requiring significantly less time (Geng
782 et al., 2018). However, what is legislated is *S. aureus* numbers in food since the amount
783 of staphylococcal toxin required to trigger food poisoning varies from 20 ng to 1 µg,
784 which corresponds to approximately 10^5 CFU/g of *S. aureus* in food (Akkaya, Gök,
785 Kara, & Yaman, 2014). This is the microbiological parameter usually used by the food
786 industry as routine control. Researchers have investigated and developed detection
787 systems for staphylococcal toxins. Wu et al., (2016) suggested that aptamer-based

788 bioassays may be an interesting alternative to implement as a new future trend. In fact,
789 aptamers are an ideal recognition element to be implemented in biosensors. Another
790 method that might be of interest is the detection of staphylococcal toxins through
791 molecularly imprinted polymers-based bioassays (Wu et al., 2016). These have
792 molecular recognition properties given that cavities in the polymer matrix are produced
793 which are directly complementary in shape and size to the target printing molecule
794 (Piletsky et al., 2001). All these methodologies based on the development of biosensors
795 could also be implemented for other toxin-producing bacteria such as *Bacillus cereus*, a
796 Gram-positive bacterium that is very relevant in the food industry. One of the most
797 important characteristics of this pathogen is its ability to produce refractile endospores,
798 which are more resistant than vegetative cells to drying, preservatives, heat and other
799 adverse environmental conditions (Griffiths & Schraft, 2017). Current methods for *B.*
800 *cereus* detection are generally based on culture media plating, which does not detect the
801 toxin (*i.e.* cereulide). Ducrest et al., (2019) indicated that MALDI-TOF MS is a method
802 that may be useful for cereulide detection and would encourage food producers to
803 initiate corrective actions for the immediate recovery of contaminated food.
804 Additionally, molecular methodologies based on m-PCR have been developed for the
805 simultaneous detection of *Salmonella* spp., *S. aureus* and *B. cereus* and promising
806 results have been obtained in pork products (Arunrut, Kiatpathomchai, &
807 Ananchaipattana, 2018).
808
809 There are other spore-forming microorganisms of great interest, especially due to the
810 type of toxin they produce. This is the case of *Clostridium botulinum*, whose neurotoxin
811 has long been of great concern. Among different methods for detecting botulinum toxin
812 in food, only a few of them are validated by official organizations (Thirunavukkarasu et

al., 2018). Tripathi, Upadhyay, & Nara, (2017) indicated that among the diagnostic systems previously used to detect toxins, one of the technologies of interest is lateral flow immunoassay. Systems based on this technology have been developed to detect botulinum toxin up to a level of 1 ng (patented, US 11/223,353). Considering that the risk for *B. cereus* and *C. botulinum* is based on the amount of toxin present, if there is no easy, rapid technique to routinely detect the toxin rather than the bacteria, the risk for these poisonings might be underestimated. Hence, the detection of microbial toxins should be evaluated routinely. *C. perfringens* is also relevant within the genus *Clostridium* spp. because it is a ubiquitous spore-forming bacterium present in the environment and therefore in food processing plants (Brynestad & Granum, 2002). This pathogen produced a total of 108 outbreaks worldwide with confirmed etiology, which produced 5,132 cases and 16 hospitalizations in the USA and Puerto Rico between 2009 and 2015 (Dewey-Mattia et al., 2018). Culture media plating has traditionally been the procedure used for *C. perfringens* detection and identification. Triptose-sulfite-cycloserine agar (TSC) is used for this purpose with 24-48 h of incubation at 37°C under anaerobic conditions and subsequent confirmation of the suspected colonies with biochemical tests such as gelatinase production, nitrate reduction or motility (Hong, 2017). Some methods based on molecular systems and biosensors have been proposed to reduce labor-intensive work and time to result. In this regard, Priya et al., (2018) reported a LAMP assay as a promising tool for the early detection of *C. perfringens* strains in food products. Qian et al., (2018) developed an electrochemical impedance biosensor containing DNA probes that detects DNA extracted from *C. perfringens* in dairy products, providing a possible application for food quality control in the future.

837 *Cronobacter* spp. is a Gram-negative bacteria belonging to the *Enterobacteriaceae*
838 family, which is a relevant pathogen in infant foods and has been linked to numerous
839 cases of meningitis and necrotizing enteritis in babies (Feeneyy, Kropp, O'Connor, &
840 Sleator, 2015). This genus is currently comprised of seven species: *C. sakazakii*, *C.*
841 *malonaticus*, *C. universalis*, *C. turicensis*, *C. dublinensis*, *C. muytjensii* and *C.*
842 *condimenti*. Current data indicates that none of the species can be ruled out as risk-free
843 for infants (WHO-FAO, 2004). Song, Teng, Chen, & Kim, (2018) reviewed the
844 methods employed for *Cronobacter* spp. detection specifically in powdered infant
845 formulas, concluding that although the USFDA and ISO methods are the most widely
846 used standards, they are time consuming since it takes 7 days to obtain results. For this
847 reason, they recommended a combination of conventional methods with new techniques
848 such as selective media combined with PCR, biosensors or fluorescence-based methods.
849 In a recent study, Song et al., (2018) recommended implementing an immunoliposome-
850 based immunochromatographic strip assay in the food industry as an easy alternative to
851 detect *Cronobacter* spp., with a detection limit of 10^5–10^7 CFU/ml. There has also been
852 much interest in the development of multiplex assays for the specific detection of
853 *Cronobacter* spp. and *Salmonella* spp. in powdered milk formula (Hyeon, Park, Choi,
854 Holt, & Seo, 2010; Morlay, Piat, Mercey, & Roupioz, 2016). Another species belonging
855 to the *Enterobacteriaceae* family that is highly relevant nowadays is *Yersinia*
856 *enterocolitica*. Yersiniosis usually causes febrile gastrointestinal disease, although it can
857 also trigger other symptoms and complications such as erythema nodosum and reactive
858 arthritis (Koskinen, Keto-Timonen, Virtanen, Vilar, & Korkeala, 2019). Pigs are the
859 main reservoir of *Y. enterocolitica* and the only animal species from which pathogenic
860 strains have so far been isolated (Bari, Hossain, Isshiki, & Ukuku, 2011). Among the
861 factors that contribute to the presence of this pathogen in foods are its ability to grow at

low temperatures (Gill & Reichel, 1989) and at pH from 4.2 (Stern, Pierson, & Kotula, 1980), and its relative resistance to some preservatives/disinfectants (Bari et al., 2011), making it of considerable concern to food producers. This pathogen is not currently legislated in food, an issue which should be addressed considering that only *Campylobacter* spp., *Salmonella* spp. and *E. coli* STEC are above *Y. enterocolitica* in the number of reported cases per year in Europe (EFSA-ECDC, 2019). The conventional methods for *Y. enterocolitica* detection are specific culture media, although like for the rest of pathogens they have proven to be slow, unreliable and tedious and require further confirmation (Cocolin & Comi, 2005; Luciani et al., 2018). Hence, rapid methods based on immunological and molecular techniques have been optimized to find specific assays. Although these methods are highly accurate, their application in the food industry is limited because the immunological or molecular markers used are unable to precisely discriminate between virulent and nonvirulent strains. In fact, the supposedly small occurrence of *Y. enterocolitica* in food products could be due to the insensitivity of current detection methods (Gupta, Gulati, Bhagat, Dhar, & Virdi, 2015). In this regard, the reference method, ISO 10273, has recently been reviewed and validated in a comparative study comprising 14 different laboratories, presenting high sensitivity for the tested food matrices (raw milk, minced meat and lettuce) at inoculation levels of pathogenic *Y. enterocolitica* 5–10 times above the detection level (Hallanvuo et al., 2019). However, more samples should be investigated, such as different types of products containing pork as an ingredient.

4.2. Foodborne viruses

Various pathologies such as gastroenteritis, acute hepatitis, myocarditis and even meningitis can be caused by foodborne viruses, one of the main causes of foodborne

887 diseases with widespread worldwide distribution (Cauteren et al., 2017; Shukla, Cho,
888 Kwon, Chung, & Kim, 2018). Food safety experts state that a greater number of control
889 measures and detection systems for viruses are required throughout the food chain
890 (Bosch et al., 2018). In recent years, the number of foodborne outbreaks caused by these
891 pathogens has increased, with norovirus as the main cause of foodborne outbreaks in the
892 USA (Dewey-Mattia et al., 2018) and the second in Europe (EFSA-ECDC, 2019).
893 Nowadays, foodborne viruses of most concern are human noroviruses (NoV), due to
894 their relationship with ready-to-eat products contaminated during preparation by food
895 handlers (Hall, Wikswo, Pringle, Gould, & Parashar, 2014; Maunula & von Bonsdorff,
896 2016), the hepatitis A virus (HAV), due both to its abundance and its relationship with
897 the lack of personal hygiene when processing many types of food (Mohd Hanafiah,
898 Jacobsen, & Wiersma, 2011) and, more recently, the hepatitis E virus (HEV), associated
899 with the consumption of meat and meat products (Kupferschmidt, 2016). Viruses can
900 contaminate a great variety of foods before or after harvest. These include bivalve
901 molluscs, fruits, vegetables, salads and ready-to-eat products (Bosch, Pintó, & Guix,
902 2016).
903
904 Different international organizations have highlighted the importance of enteric viruses
905 in the area of food safety, an example of which is the publication of a document on
906 foodborne viruses by the *Codex Alimentarius* Commission (WHO-FAO, 2008). This
907 document includes a series of general principles on food hygiene for virus control,
908 raising the point of the need to develop rapid diagnostic methods and to carry out
909 studies not only to establish the correlation between infectivity and molecular detection,
910 but also to investigate the effectiveness of food processing for the inactivation of enteric
911 viruses. More recently, Price-Hayward & Hartnell, (2016) stated in the Summary

912 Report of the Joint Scientific Workshop on Foodborne Viruses held by the UK Food
913 Standards Agency and EFSA that the control measures currently implemented in the
914 food industry have not been evaluated or are not effective for enteric virus inactivation.
915 Moreover, viruses require special attention because their behavior differs from that of
916 bacteria (Gerba & Pepper, 2019). It is therefore essential to know the real risk of
917 presence of enteric viruses in food, as well as the conditions that guarantee the
918 microbiological safety of food in terms of these viruses.
919
920 In general, foodborne viruses do not readily replicate in cell cultures (Baert,
921 Uyttendaele, & Debevere, 2007), they are found in very low concentrations (Thebault,
922 Teunis, Le Pendu, Le Guyader, & Denis, 2013) and they are not uniformly distributed
923 in food (Rzezutka & Carducci, 2013; Urbanucci, Myrmel, Berg, von Bonsdorff, &
924 Maunula, 2009), making their detection especially complex. Much research has been
925 done on developing effective methods, especially with molecular techniques, and
926 mainly reverse transcription-qPCR (RT-qPCR). The protocol used to analyze the
927 presence of viruses in food consists in an initial stage of isolation of the viruses from the
928 food matrix, followed by concentration and purification, and then nucleic acid
929 extraction, detection and quantification by RT-qPCR (Jean, Blais, Darveau, & Fliss,
930 2001). This procedure is the only one that has been standardized (ISO 15216-1:2017
931 and ISO 15216-2:2013) and validated for various food matrices for NoV and HAV
932 (Lowther et al., 2019). Due to the physico-chemical properties of food products, virus
933 detection in food matrices is a current challenge. For this reason, the ISO method
934 includes certain criteria to prevent false negatives or underestimation (Bosch et al.,
935 2018). Although RT-qPCR has also been used to detect HEV in food, it is not
936 sufficiently developed and needs to be validated and standardized for meat and meat

products, particularly pork, which are the foods that show the greatest risk for HEV transmission (Ricci et al., 2017). Nowadays, there is no ISO method for HEV detection in meat and meat products, so the development of a standard is needed to increase the accuracy and consistency of HEV detection tests to better control the real risk in these products. New metagenomic approaches based on Next Generation Sequencing (NGS) for virus detection have also been proposed (Bachofen, 2018), which should be investigated in depth for subsequent validation and application at the industrial level.

Another obstacle when analyzing food matrices is the difficulty in detecting low virus levels due to limited sample sizes (Bosch et al., 2018). Therefore, new methodologies under development should consider this issue and include possible solutions to avoid false negatives, leading to more efficient surveillance and control programs for the food industry (Nieuwenhuijse & Koopmans, 2017). In this situation, the best control is the application of correct hygiene measures although this is very difficult to implement in practice as proven, for example, by a HAV outbreak first identified in 2016 followed by public reports from a further 30 states, with a total of 27,634 cases and 16,679 hospitalizations registered up to November 1, 2019 (CDC, 2019). Since control through personal hygiene measures appears not to have the expected efficacy, vaccination is suggested as the best way to prevent this infection.

5. Conclusions

Foodborne diseases are one of the most widespread health problems in the world and are considered as an important cause of reduction in economic productivity. Therefore, pathogen control in the food industry before releasing any food product into the market is essential to prevent their occurrence. Many food industries use conventional methods

for food microbiological analysis associated with standards required by public authorities in official controls. However, there are many other techniques that are validated by independent organisms, indicating their effectiveness in food pathogen detection. The food industry is currently under great pressure due to the various food crises that have occurred over the years, so it is very important that pathogen detection methods are as rapid, efficient, sensitive and specific as possible. Furthermore, pathogen detection in some food products is not accurate, inducing false negatives and the consequent release of contaminated batches into the market, leading to food outbreaks. Since lack of sensitivity might be related to the interference of food matrices, method validation must be performed for each specific food product. Recent research aims at developing detection methodologies based on advanced technology such as sensors, which integrate different concepts ranging from molecular and immunological techniques to the use of nanotechnology. Rapid and accurate methods already used in the food industry will be also affordable and portable and offer connectivity in near future, which improves decision making and safety throughout the food chain.

Author Contributions

Dr. Carolina Ripolles-Avila, Faculty of Veterinary Sciences, UAB, Spain: literature search, discussed findings, analyzed data, writing, and conception. Dr. Maria Martinez-Garcia, Faculty of Veterinary Sciences, UAB, Spain: literature search, discussed findings, writing, and conception. Dr. Marta Capellas, Faculty of Veterinary Sciences, UAB, Spain: discussed findings, reviewing, provided critical inputs, and planning. Dr. Josep Yuste, Faculty of Veterinary Sciences, UAB, Spain: discussed findings, reviewing, provided critical inputs, and planning. Professor Dr. Daniel Y.C. Fung,

Kansas State University, USA: conception and planning. Professor Dr. José Juan Rodríguez-Jerez, Faculty of Veterinary Sciences, UAB, Spain: analyzed data, reviewing, provided critical inputs, planning, and conception.

Funding sources

This study was supported by Research Project grants RTI2018-098267-R-C32 from the Ministerio de Ciencia, Innovación y Universidades. The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge Ms. Sarah Davies for the English grammar review. This manuscript also represents a tribute to Professor Dr. Daniel Y. C. Fung, who passed away in December 1, 2019, before the manuscript was completed. Rest in peace.

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1733 **Tables captions**

1734

1735 **Table 1.** Overview of confirmed human infections by the most prevalent pathogens and
1736 the standards followed for their detection in food samples.

1737

1738 **Table 2.** Methodologies used for *Salmonella* spp. detection in food and environmental
1739 samples validated by AOAC.

1740

1741 **Table 3.** Methodologies used for *Campylobacter* spp. detection in food and
1742 environmental samples validated by AOAC.

1743

1744 **Table 4.** Methodologies used for *L. monocytogenes* detection in food and
1745 environmental samples validated by AOAC.

1746

1747 **Table 5.** Methodologies used for STEC detection in food and environmental samples
1748 validated by AOAC.

Figure captions

Figure 1. Timeline of the trends in rapid methods and automation in food microbiology over the last five decades and future predictions.

Figure 2. Main food categories relating to the presence (%) of *Salmonella* spp. in food products. The data were obtained from the Rapid Alert System for Food and Feed (RASFF) Portal and the European Food Safety Authority (EFSA) zoonosis report (EFSA-ECDC, 2018).

Figure 3. Main food categories related to the presence (%) of *Campylobacter* spp. in food products. The data were obtained from the Rapid Alert System for Food and Feed (RASFF) Portal and the European Food Safety Authority (EFSA) zoonosis report (EFSA-ECDC, 2018).

Figure 4. Summary of *Listeria monocytogenes* occurrence in the major Ready-to-Eat (RTE) food categories in the EU from 2013 to 2017. Data obtained from EFSA-ECDC (2018). The data can give an overall idea of what is detected, but an analysis of trend cannot be made due to the variation in the number of samples analyzed.

Figure 5. Main food categories relating to the presence (%) of Shiga toxin producing *Escherichia coli* in food products. The data were obtained from the Rapid Alert System for Food and Feed (RASFF) Portal and the European Food Safety Authority (EFSA) zoonosis report (EFSA-ECDC, 2018).

Figure 1

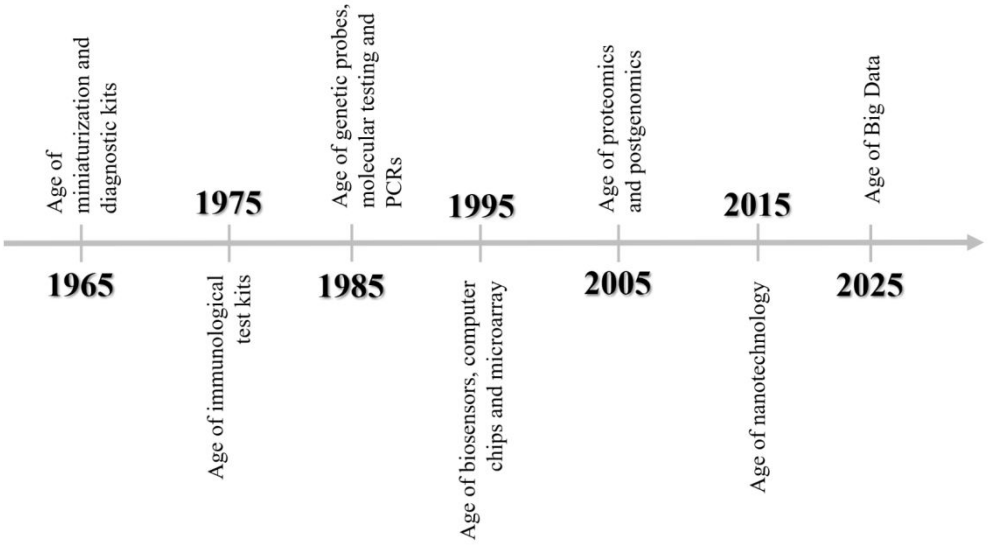

Figure 2

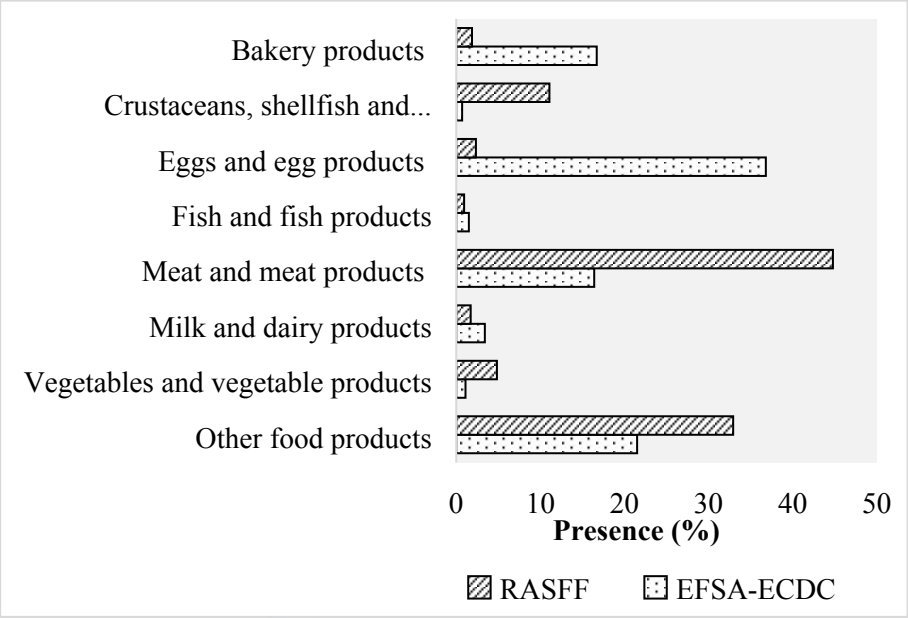

Figure 3

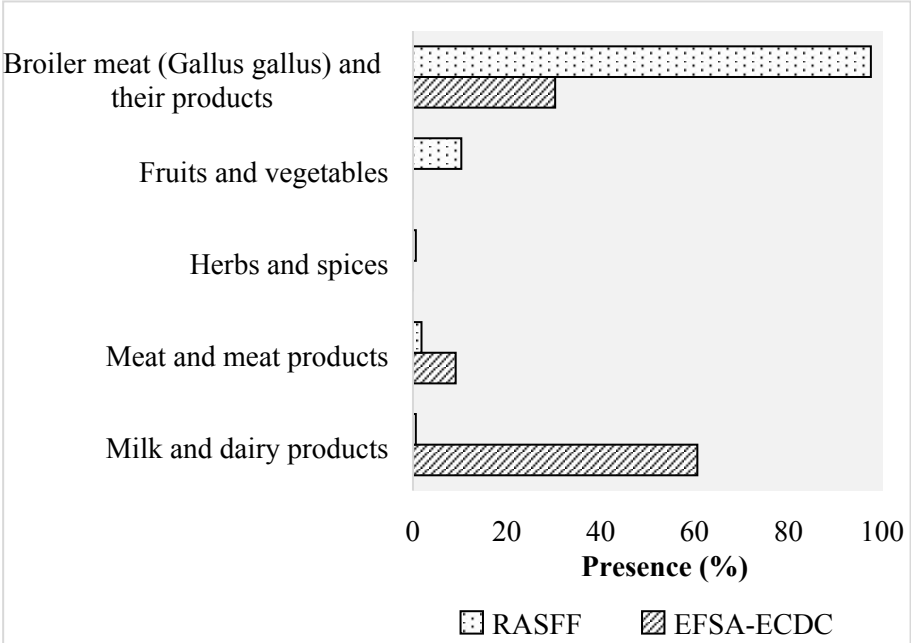

Figure 4

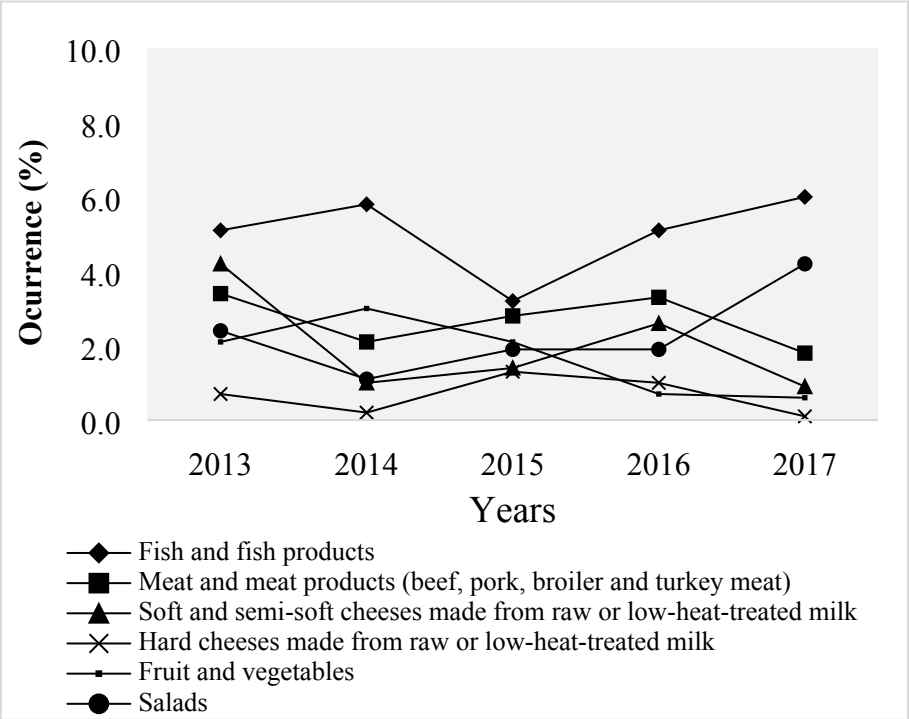

Figure 5

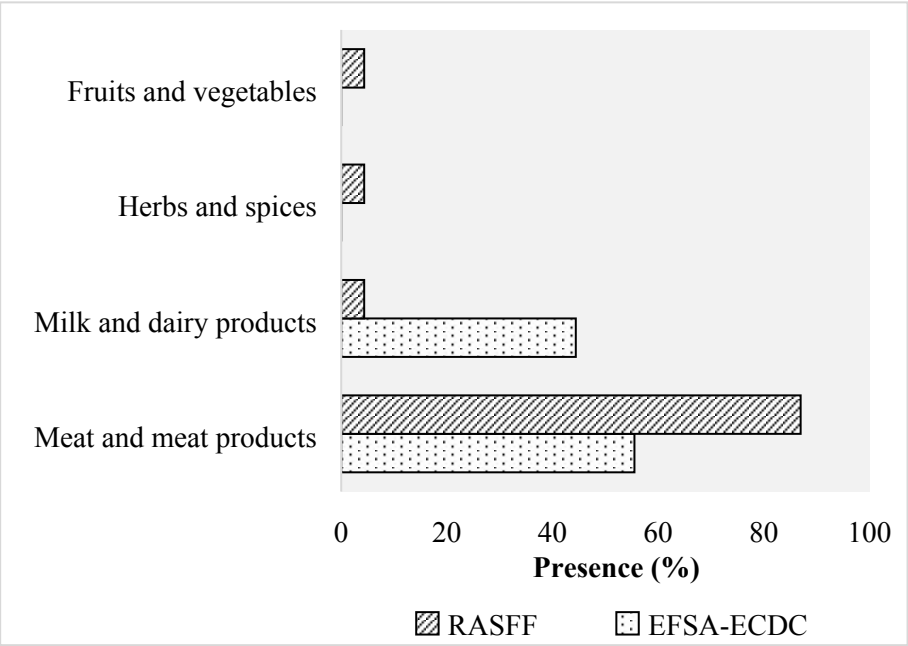

Table 1

Pathogenic microorganism	Number of confirmed human infections in the EU in 2018 (EFSA-ECDC, 2019)	ISO standard	Days for pathogen detection
Campylobacter spp.	246,571	ISO 10272-1:2017	6
Salmonella spp.	91,857	ISO 6579-1:2017	4
STEC*	8,161	ISO 13136:2012	3
Yersinia enterocolitica	6,699	ISO 10273:2017	4
Listeria monocytogenes	2,549	ISO 11290-1:2017	5

*STEC: Shiga toxin-producing *Escherichia coli*.

Table 2

Commercial methods	Manufacturer	Typology	Analysis time (hours)	Basis
1,2 Test	BioControl Systems	Immunological	40-54×	Based on the observation of *Salmonella* spp. motility through the recognition of polyvalent H (flagellar) antibodies. LOD*: 1-4 CFU/test.
DuPont Lateral Flow System	Qualicon Diagnostics	Immunological	22-44α	Combines anti-*Salmonella* antibodies conjugated with colloidal gold particles coated on a membrane surface. LOD: 1-4 CFU/25g.
FoodChek	FoodChek Systems	Immunological	8-21α	Magnetic nanoparticle immunoassay with an automated magnetic reader. Specific enrichment media must be used. LOD: 10^7 CFU/ml, except for *Salmonella* Duisberg which is 10^8 CFU/ml.
Locate	Rhone-Poulenc	Immunological	27	Enzyme immunosorbent assay (EIA). It uses a monoclonal antibody to screen for *Salmonella* heat-stable antigens. LOD: 10^4-10^5 CFU/g.
Microstick-*Salmonella*	Microkit	Immunological	36-48×	Immunolateral test containing latex particles coated with anti-*Salmonella* antibodies. A specific recovery media is required. LOD: 10-16 CFU/25g.
RapidChek Select	Romer Labs	Immunological	22-44α	Lateral flow detection system that contains anti-*Salmonella* antibodies. Includes selective bacteriophage agents. Sensitivity¤: 93.5%.
Reveal 2.0	Neogen	Immunological	24	Lateral flow immunoassay for the detection of *S. enterica* serogroups A-E. Selective enrichment is needed. LOD: 10^6 CFU/ml post-enrichment.
Salmonella Optima	Eurofins GeneScan	Immunological	48-50	ELISA based method. LOD: 10^5 CFU/25g.
Salmonella-Tek ELISA	Organon Teknika	Immunological	46-48	Colorimetric enzyme-linked immunosorbent assay with the use of monoclonal antibodies. LOD: 10^5 cells/ml.
SAS	SA Scientific	Immunological	16-24	Colored particle-conjugated antibody coated to a membrane.

Singlepath	Merck Millipore	Immunological	24-25	Lateral flow test that uses antibody-linked colloidal gold particles to react specifically with its complementary antigenic determinant. LOD: 1-6 CFU/25g.
Solus	Solus Scientific	Immunological	39-49[α]	ELISA test. *S. enterica* subsp. *arizonae* not detected. LOD: 10^5-10^6 CFU/ml post-enrichment.
Tecra	3M	Immunological	36-42[×]	Visual immunoassay based on ELISA test. LOD: 1-5 CFU/25g.
Transia Plate *Salmonella* Gold	BioControl Systems	Immunological	24-48[×]	Sandwich enzyme immunoassay with highly specific antibodies which, if present, create a color change reaction. LOD: 10^5-10^6 cells/ml in the selective enrichment broth.
VIDAS SPT	bioMérieux	Immunological	19-25	Automated qualitative enzyme-linked fluorescent assay based on a novel recombinant phage protein-based technology. LOD: 0.3-1.3 CFU/25 g.
VIP Gold	BioControl Systems	Immunological	24	Single-step visual immunoassay for the detection of motile and non-motile *Salmonella* spp. Sensitivity: > 77 %.
BBL CHROMagar *Salmonella*	BD	Plating	46-48	Mix of chromogenic substrates targeting the esterase activities specific to *Salmonella* spp. Sensitivity: 94 %.
Brilliance	Thermo Fisher Scientific	Plating	16-20	Expression of two enzymatic activities, caprylate esterase and ß-glucosidase. LOD: 1 CFU/25g with a sensitivity of 84 %.
Chromatic *Salmonella*	Liofilchem	Plating	46-48	Chromogenic mix enables differentiation based on the color and morphology of the colonies. It includes Tween 20 to increase microbial growth.
InSite	BC Aplicaciones Analíticas	Plating	24-48	Test employed for surfaces using liquid medium formulated with growth stimulants and selective chromogenic compounds for the development of *Salmonella* spp. LOD: <10 CFU/cm^2.
PDX-SIB	Paradigm Diagnostics	Plating	48	Contains nutrients that are specific energy source metabolized primarily by *Salmonella* spp. LOD: <10 CFU/sample with a sensitivity of 98.5%.
Petrifilm SALX	3M	Plating	68-72	Selective and differential culture media that contains a cold-water- soluble gelling agent. LOD: 1-5 CFU/sample.
RAPID'*Salmonella*	Bio-Rad Laboratories	Plating	46-48	The principle relies on the expression of two enzymatic activities, one of which (C8 esterase) is specific for

				Salmonella spp. Sensitivity: 89-89.9% depending on the incubation period.
SALMA	bioMérieux	Plating	46-48	Mix of chromogenic substrates targeting the esterase activities specific to *Salmonella* spp. Sensitivity: 87.9-97.1% depending on the food matrix.
ANSR	Neogen	Molecular	20-24	Isothermal amplified nucleic acid assay. Detection with fluorescent molecular beacon probes. LOD: 10^4 CFU/ml post-enrichment with a sensitivity of 95%.
Assurance GDS Tq	BioControl Systems	Molecular	12-28[×]	PCR that provides two additional levels of specificity through the use of highly specific primers and probes. Sensitivity: 93.2%.
Atlas	Roka Biosciences	Molecular	25-27	Transcription mediated amplification for the detection of *Salmonella* spp. LOD: < 1 CFU/25 g.
BACGene	Eurofins GeneScan	Molecular	13-24[α]	Real-time PCR presenting a LOD: 1-3 CFU in 25/375 g.
BAX System	DuPont Nutrition & Health	Molecular	24-48[×]	Automated method that uses real-time PCR for the detection of specific DNA fragments unique to *Salmonella* spp. LOD: 1-2 CFU/25g sample.
BAX System Sal Quant	DuPont Nutrition & Health	Molecular	24	Real-time PCR using data to monitor levels of the pathogen and identify changes over time, enabling plant managers to track areas where *Salmonella* spp. is present. LOD: 1-2 CFU/25g sample.
DNAble	EnviroLogix	Molecular	24	Rapid nucleic acid amplification method similar to PCR using a nicking enzyme and a strand displacing polymerase. Sensitivity: 96.1%.
Foodproof	Biotecon Diagnostics	Molecular	24	Assay based on 5'Nuclease and hybridization probes (for sequence specific detection). LOD: 10^3-10^4 CFU/ml in enriched cultures.
GDS *Salmonella* Tq	BioControl Systems	Molecular	10-24[×]	Automated real-time PCR method. Sensitivity: 90.2-91.5%, depending the incubation period.
Gene-Up	bioMérieux	Molecular	24	Real-time PCR method. Sensitivity: 96.2%.
GeneDisc Plate	Pall GeneDisc Technologies	Molecular	10	Real-time PCR method. LOD: 1 cell/25g.

GeneQuence	Neogen	Molecular	24-48	DNA-based assays prepared in a microwell format and a streamlined enrichment protocol. LOD: 1–5 CFU/25g.
InstantLabs	InstantLabs Medical Diagnostics	Molecular	8-16[α]	Hunter accelerated real-time PCR. LOD: 1 CFU/sample.
iQ-Check *Salmonella* II	Bio-Rad Laboratories	Molecular	24	PCR gene sequence amplification and real-time detection of fluorescent signals. It includes an internal inhibition control that is amplified in parallel with the target DNA for a reliable result. Sensitivity: 97.5%.
MDS	3M	Molecular	24	Based on the amplification of nucleic acids using isothermal conditions. Detection through fluorescence. LOD: 1-5 CFU/sample with a sensitivity of 95%.
Mericon	Qiagen	Molecular	24-26	Real-time PCR method. LOD: 0.2-2.2 cells/25 g.
MicroSEQ	ThermoFisher Scientific	Molecular	19	The assay is a single-well, real-time PCR in which pathogen DNA targets are amplified and detected in real time using fluorescent TaqMan probes. LOD: 10^3 CFU/ml.
PolySkope 1.0	PolySkope Labs	Molecular	23-25	Multiplex real-time PCR method. It detects *Salmonella* spp., *L. monocytogenes* and *E. coli* O157. LOD: 0.2-5 CFU/25g.
Probe4*Salmonella*	Biomode2	Molecular	27	FISH method using peptide nucleic acid probes hybridizing to specific ribosomal RNA sequences of the pathogen.
QFast	iMiCROQ	Molecular	24-27	PCR with electrochemical detection of absence/presence in a quantitative way. LOD: 6 CFU/25g.
R.A.P.I.D.	BioFire Diagnostics	Molecular	17	Real-time PCR presenting a LOD: 1 CFU/25 g.
SureFood	CONGEN Biotechnologie	Molecular	16-24	Real-time PCR. LOD: < 5 DNA copies.
SureTect	Thermo Fisher Scientific	Molecular	9-25[α]	Dye-labeled probes target unique DNA sequences which, if present, are detected by real-time PCR. LOD: 1 CFU/sample, although enrichment is needed.
TaqMan	Applied Biosystems	Molecular	27	Real-time PCR. Uses TaqMan chemistries for the detection of the pathogen. LOD: 1-5 CFU/sample.
VereBeef	Veredus Laboratories	Molecular	10	Multiplex PCR microarray-based test. LOD: 10^4-10^5 post-enrichment.

Veriflow	Invisible Sentinel	Molecular	26	PCR detection method coupled with a rapid, visual, flow-based assay. No need for fluorophore-based detection of target amplification. LOD: 10^4 CFU/ml.

[×]Depending on the enrichment step applied.
[α]Depending on the type of food matrix analyzed.
*LOD (limit of detection). The lowest LOD found, in the literature, for all food matrices that were validated, is included.
[¤]The highest sensitivity found, in the literature, for all food matrices that were validated, is included.

Table 3

Commercial methods	Manufacturer	Typology	Analysis time (hours)	Basis
Singlepath	Merck Millipore	Immunological	24-25	See Table 2. LOD: 10^4-10^7 CFU/ml, depending on the serogroup.
Transia Plate	BioControl Systems	Immunological	46	See Table 2. LOD*: 10^5-10^6 CFU/ml.
VIDAS CAM	bioMérieux	Immunological	45-53×	Automated qualitative enzyme-linked fluorescent assay. It has a solid phase receptacle coated with anti-pathogen antibodies adsorbed onto its surface. LOD: 1 CFU/sample.
CampyFood Agar (CFA)	bioMérieux	Plating	48-72×	Media combining a colored indicator with selective agents to turn colonies an orange-red color. Sensitivity¤: 93.2%.
RAPID' agar	Bio-Rad Laboratories	Plating	40-48	Selective chromogenic media for *C. jejuni*, *C. coli* and *C. lari* containing selective agents for yeast and mold inhibition.
ANSR	Neogen	Molecular	20-24	See Table 2. LOD: 10^2 CFU/ml post-enrichment.
BAX System	DuPont Nutrition & Health	Molecular	24-48×	Real-time PCR assay for qualitative results of *C. jejuni*, *C. coli* and *C. lari*. LOD: 10^4 CFU/ml with a sensitivity of >90%.
iQ-Check	Bio-Rad Laboratories	Molecular	24	See Table 2. Sensitivity: 100%.
MDS	3M	Molecular	23-29	See Table 2.
Veriflow	Invisible Sentinel	Molecular	26	See Table 2. LOD: $5x10^3$ CFU/ml of sample.

×Depending on the enrichment step applied.

*LOD (limit of detection). The lowest LOD found, in the literature, for all food matrices that were validated, is included.

¤The highest sensitivity found, in the literature, for all food matrices that were validated, is included.

Table 4

Commercial methods	Manufacturer	Typology	Analysis time (hours)	Basis
DuPont Lateral Flow System	Qualicon Diagnostics	Immunological	46-48	See Table 2. LOD*: 1-4 CFU/25g.
Listeria-Tek ELISA	Organon Teknika	Immunological	46-48	See Table 2.
RapidChek	Romer Labs	Immunological	20-48[α]	See Table 2. Sensitivity[¤]: 100%.
Singlepath L'MONO	Merck Millipore	Immunological	24-25	See Table 2. LOD: $5x10^6$ cells/ml or 1 CFU/sample, depending on the serotype.
Solus	Solus Scientific	Immunological	46-54[α]	See Table 2. LOD: 0.2-1.2 CFU/25g.
Transia Plate	BioControl Systems	Immunological	47	See Table 2. LOD: 1-9 CFU/25g.
VIDAS LMX	bioMérieux	Immunological	26-30	See Table 2. LOD: 0.2-1.8 CFU/25g.
ALOA	bioMérieux	Plating	46-48	Detection of two enzymatic activities: β-glucosidase and phospholipase C. LOD: 0.3-0.7 CFU/25g with a sensitivity of 99.49%.
BBL CHROMagar *Listeria*	BD	Plating	46-48	Detection of two enzymatic activities: β-glucosidase and phospholipase C. LOD: 1-18 CFU/25 g with a sensitivity of 99%.
InSite *L. mono* Glo	Hygiena LLC	Plating	48	Based on β-glucosidase and phospholipase C to differentiate pathogenic species. UV light needed to read fluorescence for *L. monocytogenes*. LOD: < 10 CFU/cm^2.
RAPID'L.mono	Bio-Rad Laboratories	Plating	46-48	Detection of phospholipase C activity and inability to metabolize xylose. Sensitivity: 99.4%.
ANSR	Neogen	Molecular	24-26	See Table 2. LOD: 10^4 CFU/ml post-enrichment.
Assurance GDS Tq	BioControl Systems	Molecular	24	See Table 2. LOD: 0.3-1.3 CFU/25g.
Atlas	Roka Biosciences	Molecular	44	See Table 2. Sensitivity: 93.3%.
BACGene	Eurofins GeneScan	Molecular	21	See Table 2. Sensitivity: 97.5%.
BAX System	DuPont Nutrition & Health	Molecular	40-48	See Table 2. LOD: 10^4 CFU/ml post-enrichment
GDS	BioControl Systems	Molecular	24	See Table 2. LOD: 0.3-1.3 CFU/25g.

Gene-Up	bioMérieux	Molecular	24	See Table 2. LOD: 10^4-10^5 CFU/ml post-enrichment.
GeneDisc Plate *Listeria* DUO	Pall GeneDisc Technologies	Molecular	20	Real-time PCR that detects specific DNA sequences for the simultaneous detection of six species of *Listeria* (*L. monocytogenes, L. grayi, L. innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri*). LOD: 1 cell/sample.
GeneQuence	Neogen	Molecular	30-48	See Table 2. LOD: 1-5 CFU/25g.
IEH System LmG2	IEH Laboratories & Consulting Group	Molecular	27-28	Real-time PCR method.
InstantLabs	InstantLabs Medical Diagnostics	Molecular	22	See Table 2. LOD: 1 CFU/sample.
iQ-Check	Bio-Rad Laboratories	Molecular	24-28	See Table 2. LOD: 10^2-10^3 CFU/ml post-enrichment.
LT *Listeria*	BioFire	Molecular	20	See Table 2.
MDS	3M	Molecular	24-26	See Table 2. LOD: 1-5 CFU/sample.
Mericon	Qiagen	Molecular	18-27[×]	See Table 2. LOD: 1.6-6.6 CFU/25g.
MicroSEQ	ThermoFisher Scientific	Molecular	24-28	See Table 2. LOD: 1-3 CFU/25g.
PolySkope 1.0	PolySkope Labs	Molecular	23-25	See Table 2. LOD: 0.2-5 CFU/25g.
Probe4Monocytogenes	Biomode2	Molecular	42	See Table 2.
QFast	iMiCROQ	Molecular	33-34	See Table 2.
SureTect	Thermo Fisher Scientific	Molecular	23-27	See Table 2. Highly specific detection of the *prfA* gene. LOD: 0.2-1 CFU/25g.
Veriflow	Invisible Sentinel	Molecular	24	See Table 2. Sensitivity: Zero tolerance detection.

[×]Depending on the enrichment step applied.
[α]Depending on the type of food matrix analysed.
*LOD (limit of detection). The lowest LOD found, in the literature, for all food matrices that were validated, is included.
[¤]The highest sensitivity found, in the literature, for all food matrices that were validated, is included.

Table 5

Commercial methods	Manufacturer	Typology	Analysis time (hours)	Basis
DuPont Lateral Flow System	Qualicon Diagnostics	Immunological	8-15[α]	See Table 2. LOD*: 1-4 CFU/25g.
FoodChek	FoodChek Systems	Immunological	8	See Table 2. LOD: 1 CFU/375g.
MultiPath System E	Crystal Diagnostics	Immunological	24-28	Combines liquid crystal technology with antibody-coated paramagnetic microspheres to selectively capture and detect *E. coli* O157. LOD: 1 CFU/375g.
NH Immunochromato	NH Foods	Immunological	18-24	Two-step enrichment assay using immunochromatographic identification. LOD: 1 CFU/25g.
RAPID-B *E. coli* O157	Vivione Biosciences	Immunological	7-10	Bacterial physical properties (*i.e.* size, shape, and transparency) are evaluated with responses to probes and DNA dyes.
Reveal 2.0	Neogen	Immunological	12	See Table 2. LOD: 10^4 CFU/ml post-enrichment.
Singlepath	Merck Millipore	Immunological	24-25	See Table 2. LOD: 1 CFU/sample.
Solus *E. coli* O157 ELISA	Solus Scientific Solutions Ltd	Immunological	18-22	Based on ELISA technology and with just a single enrichment step. LOD: 10^4-10^5 CFU/ml post-enrichment.
Tecra	3M	Immunological	20-26	See Table 2. LOD: 1-5 CFU/25g.
VIDAS ECPT	bioMérieux	Immunological	7-24[α]	See Table 2. LOD: 0.8-1.6 CFU/25g with a sensitivity[¤] between 84.5-89.2%.
VIP Gold EHEC	BioControl Systems	Immunological	8-28[α]	See Table 2. Sensitivity: > 98%.
BBL CHROMagar O157	BD	Plating	24	Chromogenic substrates specific for *E. coli*-associated enzymes. Sensitivity: > 99%.
Chromatic *E. coli* O157	Liofilchem	Plating	18-24	Chromogenic mix enabling the identification of the microorganisms based on the color and morphology of colonies.
PhageDX	Laboratory Corporation of America	Plating	7	Luciferase-expressing recombinant *E. coli* O157:H7 phage. A luminometer is needed to read the results.

RAPID'*E.coli* O157:H7	Bio-Rad Laboratories	Plating	46-48	Selective chromogenic agar based on the simultaneous detection of β-D-glucuronidase, β-D-galactosidase activities, and the ability to ferment sorbitol. Sensitivity: > 99%.
ANSR	Neogen	Molecular	12-26[α]	See Table 2. LOD: 10^4 CFU/ml post-enrichment.
Assurance GDS Tq	BioControl Systems	Molecular	8-12	See Table 2. Sensitivity: > 99%.
Atlas EG2	Roka Biosciences	Molecular	21-27	See Table 2.
BAX System	DuPont Nutrition & Health	Molecular	9-24[α]	See Table 2. LOD: 10^4 CFU/ml post-enrichment.
E. coli O157:H7 Test Kit	BioFire Diagnostics	Molecular	18-22	See Table 2. Sensitivity: 92-100%.
Foodproof	Biotecon Diagnostics GmbH	Molecular	20-28[×]	See Table 2. LOD: 1-10 cells/25g-100g.
Foodproof *E. coli* O157	Biotecon Diagnostics GmbH	Molecular	24	See Table 2. LOD: 1-10 cells/25g.
GDS *E. coli* O157:H7 Tq	BioControl Systems	Molecular	8-10[ß]	See Table 2. Sensitivity: 99%.
Gene-Up	bioMérieux	Molecular	8-24[α]	See Table 2. LOD: 3 CFU/25g.
GeneDisc Plate STEC	Pall GeneDisc Technologies	Molecular	10	Real-time PCR technique for the detection of *stx* and *eae* genes and others specific to the O157 serogroup. LOD: 1 cell/25g of food sample or 1 cell/375g beef.
IEH *E. coli* O157 Test System	IEH Laboratories & Consulting Group	Molecular	10-48[α]	Microorganisms are grown in IEH enrichment medium followed by PCR assay for the presence of *eae*, *stx* and *wzx* genes common to pathogenic STECs. Samples can be run through an immuno-magnetic separation procedure
InstantLabs	InstantLabs Medical Diagnostics	Molecular	12	See Table 2. LOD: 1 CFU/sample.
iQ-Check STEC	Bio-Rad Laboratories	Molecular	12-24[×]	See Table 2. LOD: 1 CFU/25g.
MDS	3M	Molecular	24	See Table 2. LOD: 1-5 CFU/sample.

Mericon STEC-O type	Qiagen	Molecular	23-26	See Table 2. LOD: 10 copies of the respective *E. coli* target gene.
MicroSEQ	ThermoFisher Scientific	Molecular	8-18[×]	See Table 2. LOD: 1-3 CFU/25g.
NeoSeek	Neogen	Molecular	24	PCR coupled with mass spectrometry-based multiplexing to develop a genetic profile for differentiating target strains.
PolySkope 1.0	PolySkope Labs	Molecular	23-25	See Table 2. LOD: 0.2-5 CFU/25g.
RapidFinder STEC Workflow	ThermoFisher Scientific	Molecular	12-18[α]	Two-stage real-time PCR method. LOD: 1-5 CFU/sample.
SAS Molecular Tests	SA Scientific	Molecular	8-20[×]	Loop-mediated isothermal amplification method requiring electrophoresis, and all the steps from amplification to result are done inside one reaction tube.
SureTect	Thermo Fisher Scientific	Molecular	9-25[α]	See Table 2. Sensitivity: 90%.
VereBeef	Veredus Laboratories	Molecular	8	See Table 2.
Veriflow	Invisible Sentinel	Molecular	20	See Table 2. LOD: 1-10^3 CFU/ml.

[×]Depending on the enrichment step applied.
[α]Depending on the type of food matrix analysed.
[ß]Depending on the weight of the sample.
*LOD (limit of detection). The lowest LOD found, in the literature, for all food matrices that were validated, is included.
[¤]The highest sensitivity found, in the literature, for all food matrices that were validated, is included.

RESPONSE TO REVIEWERS

We thank the reviewers and editor for their interest in our work and for all the helpful comments provided in the previous version that highly improved the quality of the manuscript, as well as the opportunity to make the last modifications for your consideration.

As in the previous version, all the changes proposed in the manuscript are highlighted in red so they can be easy to locate.

Editor's Comments to the Author:

Thank you very much for making the requested revisions. I agree with the second reviewer that Figure 2 does not add any important information to the paper and that footnotes should be added to the other figures to explain any abbreviations. On a personal note, because I knew Dr. Fung too, perhaps you could mention in the Acknowledgments that he passed away before the manuscript was completed. This is not required, but I think that people would appreciate seeing him recognized.

Response:

Thank you very much for considering this study. Now, you can find the following modifications on the manuscript:

1) We have removed Figure 2.
2) We have re-codified all figures.
3) We have maintained the rest of figures, but we have included the explanation of the abbreviations used in the figures.
4) We have added some more details on the Tables.
5) We have included your suggestion on the Acknowledgments section. Now, you can find: "*This manuscript also represents a tribute to Professor Dr. Daniel Y. C. Fung, who passed away in December 1, 2019, before the manuscript was completed. Rest in peace.*"

We hope that with all the work performed in both revisions, we could reach a higher quality and it is now worthy of publication.

Reviewer(s)' Comments to Author:

Reviewer #1:

No further comments.

Response:

Thank you, especially for your help in the previous version of the manuscript.

Reviewer #2:

Figure 2 adds little value to the manuscript. Just a picture of a pathogen with arrows pointing to different detection technologies does not inform.

Response:

We have removed Figure 2 from the manuscript, and we have re-codified the rest of the Figures to maintain an order.

Figure 3, 4, 5 and 6 add little value to the manuscript as it's not about rapid methods. If retained in the manuscript the abbreviations must be explained.
Response:
In this case, we prefer to keep these Figures because the manuscript focuses not only on the application of rapid pathogen detection methods but also on risk control of food products. A cross-over of foodborne disease data reported by EFSA and RASFF could show if there are some susceptible matrices in which the detection of a foodborne pathogen is not carried out properly and, thus, batches of products with the presence of certain pathogens are being released to the market. This has been done for each of the 4 pathogens discussed separately in section 4.1. In our opinion, this part enriches the manuscript, since it is intended to go beyond the developments in new analytical methods.

We have included the acronym descriptions in the figure captions. Now, you can find the abbreviations explained. As an example: "Figure 2. Main food categories relating to the presence (%) of *Salmonella* spp. in food products. The data were obtained from the Rapid Alert System for Food and Feed (RASFF) Portal and the European Food Safety Authority (EFSA) zoonosis report (EFSA-ECDC, 2018)".

Reviewer #3:

Authors have did a good job in revising according to all my comments and I am satisfy with the revision. Good luck in publishing the work!
Response:
Thank you, especially for your help in the previous version of the manuscript.

All the best,

Dr. José Juan Rodríguez Jerez
Barcelona, May 08th 2020