BACTERIAL FOODBORNE DISEASES: A LITERATURE REVIEW ON DETECTION BY POLYMERASE CHAIN REACTION-BASED ASSAYS

DOENÇAS DE ORIGEM ALIMENTAR CAUSADAS POR BACTÉRIAS: UMA REVISÃO DA LITERATURA SOBRE A DETECÇÃO POR ENSAIOS DE REAÇÃO EM CADEIA DA POLIMERASE

JOSIELE **RAIMUNDO**[1], JOÃO ALENCAR **PAMPHILE**[2], RAVELY CASAROTTI **ORLANDELLI**[3*]

1. Bióloga, Especialista em Biotecnologia e Bioprocessos pela Universidade Estadual de Maringá; **2.** Biólogo, Doutor em Genética e Melhoramento de Plantas pela ESALQ/Universidade de São Paulo. Docente do Departamento de Biotecnologia, Biologia Celular e Genética da Universidade Estadual de Maringá; **3.** Bióloga, Doutora em Biologia Comparada (Biologia das Interações Orgânicas) pela Universidade Estadual de Maringá; Docente do Colegiado de Ciências Biológicas da Universidade Estadual do Paraná – campus Paranavaí.

*Avenida Gabriel Esperidião, S/N, Jardim Morumbi, Paranavaí, Paraná, Brasil. CEP: 87703-000. ravelycasarotti@gmail.com

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ABSTRACT

Foodborne diseases are mainly caused by bacteria and occur through the ingestion of contaminated food or water. Due to their increasing occurrence, these diseases represent an important public health problem worldwide. This article aimed to review the recent literature on the main foodborne pathogens and their detection by polymerase chain reaction-based assays. A bibliographic survey was performed through the analysis of books and articles available in various databases. National and international literature data highlighted that the conventional techniques used to detect foodborne pathogens usually require at least three days to provide the results, which implies the use of molecular techniques that allow faster and less laborious results, being sensitive and specific methods for detecting foodborne pathogens when compared to traditional culture-based methods.

KEYWORDS: Food contamination, molecular techniques, PCR detection, multiplex PCR, real-time PCR.

1. INTRODUCTION

Foodborne diseases (FBD) represent an increasing and important public health problem worldwide[1-3]. FBD refers to a syndrome caused by the ingestion of food or water contaminated with pathogens or their toxins, usually consisting of anorexia, nausea, vomiting and/or diarrhea with or without fever[4]. Contamination can occur at any point along the food production chain, mainly due to the precarious hygiene habits during the food preparation or consumption[1,5].

Health surveillance authorities are responsible for carrying out epidemiological survey of FBD outbreaks to inform the population about the seriousness of this problem, in view of reducing their incidence. Data of Brazilian Ministry of Health[6] report that FBD resulted in >17,000 hospitalizations between 2007 to 2016, with higher incidence in the Southeast (43.6%), followed by the South (24.6%), Northeast (19.8%), North (7.1%) and Central West (6.2%) of Brazil.

FBD are caused by several pathogens (as bacteria, protozoa, viruses and fungi); among them, bacteria are the most important etiologic agents. These diseases are subdivided into two categories: foodborne intoxications and foodborne infections. The first ones are caused by ingestion of food contaminated with toxins mainly produced by *Clostridium botulinum*, *Clostridium perfringens* and *Staphylococcus aureus*. Foodborne infections are caused by the ingestion viable cells that promote the microbial growth inside the host organism, being mainly caused by *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp., *Shigella* sp., *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Yersinia enterocolitica*[7,8].

Food Safety Management Systems in the food industry comprise quality control and quality assurance activities, and the evaluation of the performance of the implemented actions. The food safety control and regulation occur at federal level and may occur at regional level (states and cities)[3]. Moreover, the industry Self-Control Program needs to include microbiological and physicochemical analyzes to assure the quality and safety of the final products.

Conventional culture-based methods usually require at least three days to provide the results. So, the development of laboratory-friendly molecular techniques has many advantages for the detection of foodborne pathogens (FBP): they are more sensitive and rapidly tools that can detect FBP after few hours instead of days, the organism viability is no longer necessary and the true positivity rate of some organisms in some diseases is higher than previously thought based on culture results[9]. In view of these advantages, herein, a literature review describes the main FBP and their detection by polymerase chain reaction (PCR)-based assays.

2. MATERIAL AND METHODS

A literature review was performed through the analysis of reference books and articles available in several databases (CAPES Periodicals Portal, PubMed,

SciELO and ScienceDirect). The bibliographic survey considered, mainly, recent scientific studies conducted by national and international research groups. The following keywords were used: bacterial pathogens, food contamination, foodborne diseases, foodborne pathogens, molecular techniques, polymerase chain reaction, multiplex PCR and real-time PCR.

3. LITERATURE REVIEW

Main causal agents of foodborne intoxications

Clostridium genus comprises large, gram-positive, endospore-forming and obligately anaerobic rod-shaped bacteria. *C. botulinum* can easily grow in any low-acid (pH >4.7) canned foods causing botulism[10], which is related to neurotoxins that block the acetylcholine release, a chemical necessary for transmitting nerve impulses across synapses. Frequent symptoms are fatigue, nausea, dizziness, generalized weakness and double vision; however, the botulinum toxin can cause a progressive flaccid paralysis and death due to cardiac and respiratory failure. In the early 1800s, botulism was known as the sausage disease; nowadays, sausages rarely carry botulism due to nitrites added for preventing the bacterial growth after endospores germination[11].

C. perfringens strains are classified into five biotypes (A-E) based upon their production of alpha (α), beta (β), epsilon (ε) and iota (¡) toxins: type A only produces α toxin; type B produces α, β and ε toxins; type C produces α and β toxins; type D produces α and ε toxins, and type E produces α and ¡ toxins. These toxins cause a panoply of manifestations ranging from histotoxic infections (as gas gangrene) to intestinal infections[12]. Its transmission is related to the storage or cooking of food at an inadequate temperature and occurs through ingestion of undercooked meats or contaminated sauces, soups and pasta[7].

Although it is part of the normal human microbiota, certain strains of *S. aureus* produce enterotoxins that inflame the intestinal lining and inhibit the water adsorption. Bacterial mechanisms of virulence and adaptations to escape from the human immune system include surface proteins that enable the pathogen to attach to host tissue, secreted enzymes and toxins that damage the cell membrane by cytolytic action[13]. *S. aureus* are relatively resistant to heat and drying[14] and easily transmissible through the contaminated hands of food handlers. These organisms multiply and release toxins in uncooked or inadequately cooked foods (mainly cream pies, dairy products and poultry products), especially if they are unrefrigerated. Contamination is difficult to detect because no changes occur in the appearance, taste or odor of foods[14].

Major causal agents of foodborne infections

Since 1950, The World Health Organization and the Food and Agriculture Organization of the United Nations consider Salmonella as the most common and important zoonosis[15]. These gram-negative, facultatively anaerobic and non-endospore-forming bacilli normally are grouped into only two species: *Salmonella bongori* and *Salmonella enterica*; the last one includes more than 2,000 serotypes. Salmonellosis is an enterocolitis or other gastrointestinal disease caused by any variants of *Salmonella*, which symptoms include nausea, abdominal pain, and diarrhea and begin 12 to 36 hours after eating contaminated food; also, septic shock can occur in infants and in the elderly[11]. The prevalence of *Salmonella* sp. in swine slaughtering environment and pork production has been a worldwide concern, and contamination can occur at any point along the production chain, as recently shown by Neitzke *et al.* (2017)[16].

E. coli is a gram-negative, facultatively anaerobic, rod-shaped and coliform bacterium that normally inhabits the intestinal tract of animals, including humans, and has a beneficial effect on organism: reduces the multiplication of harmful bacteria and synthetize a considerable number of vitamins[17]. However, some strains can cause a variety of gastroenteritis, being grouped into six types: enterohemorrhagic, enteroinvasive, enteropathogenic, enterotoxigenic and diffuse adherent. Pathogenic *E. coli* are serotyped based on their O (somatic), H (flagellar), and K (capsular) surface antigen profiles[18]. Symptoms vary according to the pathogenic strain, generally including diarrhea. Infection occurs primarily through consumption of contaminated foods, such as raw or undercooked ground beef, unpasteurized milk, and contaminated water[8,11].

Shigellosis or bacillary dysentery – caused by gram-negative and facultatively anaerobic bacilli from *Shigella* genus – is acquired by fecal-oral route through contaminated food and water. Symptoms include diarrhea with bloody stools, vomiting and abdominal pain. *S. dysenteriae* produces an endotoxin that irritates the intestinal wall; after invading the epithelial cells, the bacterium excretes a neurotoxin that causes acute gastrointestinal derangement. Other pathogenic species are *S. sonnei*, *S. flexneri* and *S. boydii*[7,8,11].

L. monocytogenes is a gram-positive, rod-shaped, and facultatively anaerobic bacterium that causes listeriosis: a gastrointestinal disease characterized by fever, diarrhea, nausea and vomiting, and may lead to endocarditis, conjunctivitis, bacteremia, meningitis and other clinical manifestations. Ready-to-eat meat, fresh soft cheeses, unpasteurized dairy products, and inadequately pasteurized milk are the major vehicles for this FBP, even when foods are refrigerated at 4 ^{o}C[7,8]. The presence of *Listeria* strains in food handling areas of products of animal origin is a concern to health surveillance agencies because the bacterial control is often hampered by their ability to grow and survive even under adverse conditions[19].

Vibrio genus includes the slightly curved, gram-negative rod species with a single polar flagellum. *V. cholerae* causes cholera: the most common human infectious disease in developing countries, transmitted almost exclusively via water. *V. parahaemolyticus*, can contaminate raw fish, shellfish, oysters and

crustaceans; being an important cause of gastroenteritis in countries where raw fish is widely consumed. Symptoms include abdominal pain, vomiting and watery stools. Treatment by antibiotics and rehydration is usually e ective. *V. vulnificus* can also be found in seafood, but it is frequently causes very dangerous infections of minor skin lesions incurred in coastal sea waters[8,11].

Previously classified as *Vibrio* spp., the *Campylobacter* genus was proposed in 1963 by Sebald and Veron, and comprises gram-negative, microaerophilic, spirally curved and flagellated bacteria[20] that adapt well to the intestinal environment of animal hosts. *Campylobacter* is the major FBP frequently associated with human bacterial gastroenteritis in the world[21], which is caused by *C. jejuni* and *C. coli*, and characterized by copious diarrhea, foul-smelling feces, fever, and abdominal pain; large quantities of fluid can be lost, so dehydration and fluid and electrolyte imbalances are common among the populations most affected[14]. Typical symptoms include fever, diarrhea, nausea and abdominal pain; acute gastroenteritis typically results in a bloody diarrhea[8]. Control measures are based on: good practices of hygiene for food preparation, suitable heat treatment and prevention cross-contamination.

Yersinia gastroenteritis or yersiniosis are mainly caused by *Y. enterocolitica*, a facultatively anaerobic, gram-negative bacillus that can grow at refrigerator temperatures of 4 ºC, being transmitted via contaminated meat and milk. Symptoms include diarrhea, fever, headache, and a severe abdominal pain that can cause a misdiagnosis of appendicitis. Treatment with antibiotics and oral rehydration may be helpful; adults usually recover in one or two weeks, but children may take longer treatment[11].

Detection of FBP by polymerase chain reaction PCR-based assays

PCR is a polymerase-dependent repetitive thermal reaction that can generate amplification and obtainment of copies of a DNA segment. Seven components are required: a sample of target DNA, a pair of synthetic nucleotides (primers), phosphate deoxyribonucleotides (dNTPs), DNA polymerase enzyme, buffer, magnesium (Mg^{2+}) and water. They are placed into a test tube in a thermocycler, which increase and decrease the temperature according to a schedule.

Initially the double stranded DNA molecule is heated, and the paired strands denature, allowing the primers access to the single stranded DNA (ssDNA) templates. Then the reaction mixture is cooled; primers select and hybridize to their complementary positions on the ssDNA templates. Finally, the DNA/primer solution is heated. In the presence of the heat stable polymerase, PCR buffer, dNTP's and Mg^{2+} molecules, the replication procedure begins. The target DNA is doubled with each repetition of this cycle; after about 30 cycles, the reaction will yield more than 1 million copies of DNA fragments. PCR products are analyzed by agarose gel electrophoresis[22].

The infective dose of *Salmonella* in food samples may be as small as 1,000 bacteria, so PCR assays are the best detection method, giving results after few hours[11] whereas conventional methods usually include >16 h of culture enrichment and for-seven days for providing results[1,23]. In this context, Fachmann *et al.* (2017)[23] developed and a PCR-based method for detection of *Salmonella* in pork meat in less than 5 h, with relative accuracy, sensitivity, and specificity of 81.4, 95.1, and 97.9%, respectively. Furthermore, in a recent study[24], 150 samples from fresh and processed chicken meat were subject of a PCR assay for the detection of *Salmonella* invasion gene (*invA*). Overall, 37 samples (24.6%) were *Salmonella*-positive, and this gene was a suitable PCR target with potential diagnostic application, as also reported by other recent studies (Table 1).

Palma *et al.* (2016)[25] conducted a PCR assay to investigate the presence of *L. monocytogenes* strains in beef. Five primer pairs were chosen to detect the expression of the following target genes: *lmo*0737, *Imo*1118, ORF2819, ORF2110 and *prs*. As results, 11 bacterial strains were detected in beef samples (serotypes 4b and 1/2c); two bacterial strains belonging to serotype 1/2a were detected in slaughterhouse environment. In addition to the five genes mentioned, the *hlyA* gene encoding for listeriolysin O (a secreted pore-forming toxin) represents a conventional genetic marker for the identification of *L. monocytogenes* in food matrices, however, by using the conventional PCR protocol available in literature, non-specific PCR amplifications can generate false-positive results[26]. In view of this, Godínez-Oviedo *et al.* (2017)[26] developed a modified PCR protocol using LM1 and LM2 primers, and reported that the optimized conditions (initial denaturation of 5 min at 94 ºC, 25 cycles of 30 s at 94 ºC, 25 s at 60.5 ºC and 25 s at 72 ºC, and a final extension of 5 min at 72 ºC) can be used to confirm *L. monocytogenes* gene strains without producing false-positives.

In comparison with conventional PCR, the multiplex PCR (mPCR) technique decreases the number of reactions needed to detect the desired targets in a sample, and can further reduce the reagent costs by testing for multiple pathogens in each reaction. Primers should be chosen with similar annealing temperatures, allowing a rapid detection of pathogens that cannot currently be distinguished by testing for a single target and it allows the detection of multiple DNA markers associated with the same pathogen, reducing the potential for false-positive and false-negative results[49,50]. As disadvantages, the mPCR has reduced sensitivity, resulting in the need for a longer enrichment time to increase target concentrations. Besides, this method generally relies on a single primer pair for detection of each target pathogen, possibly resulting in failure to detect a target pathogen because of the presence of a polymorphism[50].

Table 1. An overview on studies carried out over the last years (2012-2017) aiming the detection of foodborne pathogens by PCR-based assays.

Technique; Target genes	Pathogens	Food matrix	Reference
PCR*; sent and fimA*	*Salmonella enterica* serovars Enteritidis and Typhimurium	Raw poultry meat	Afzal *et al.* (2015)[27]
PCR; *hly*	*Listeria monocytogenes*	Raw bovine milk	Agostini *et al.* (2012)[28]
PCR; *rfbE* and *fliC*	*Escherichia coli*	Minas frescal cheese	Carvalho *et al.* (2014)[29]
PCR; *stx1*, and *stx 2* and *eae*	*E. coli*	Artisanal mozzarella cheese	Cardoso and Marin (2017)[30]
PCR; *femA*	*Staphylococcus aureus*	Raw bovine milk	Diedrich *et al.* (2013)[31]
PCR; *acbZ, bglA, cat, dapE, dat, ldh* and *lhkA*	*L. monocytogenes*	Vegetables, pork, chicken, beef, mutton, fish, cooked meat, icecream	Wang *et al.* (2012)[32]
PCR; *iam, flaA, cadF, virB11, cdtA, cdtB* and *cdtC*	*Campylobacter jejuni* and *Campylobacter coli*	Beef, pork, chicken and poultry raw meat	Wieczorek *et al.* (2012)[33]
PCR and mPCR; *hblCDA* and *nheABC*	*Bacillus cereus*	Raw mutton, raw chicken and chicken biryani	Fayaz *et al.* (2017)[34]
mPCR; STM4495 and SEN1392	*Salmonella* spp. and *Yersinia* spp.	Raw broiler chicken	Anju *et al.* (2014)[35]
mPCR; *nuc*	*Staphylococcus hyicus, Staphylococcus intermedius* and *S. aureus*	Artificially contaminated milk	Gandra *et al.* (2016)[36]
mPCR*; invA*, flicC and IE	*S.* Enteritidis and *S.* Typhimurium	Broiler chicken swabs	Paião *et al.* (2013)[37]
mPCR*;* 16S rRNA and *hlyA*	*Listeria* spp. and *L. monocytogenes*	Raw vegetables	Tang *et al.* (2017)[38]
mPCR*; mdh, toxR, vvhA* and *colH*	*Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio alginolyticus*	Seafood	Xu *et al.* (2017)[39]
mPCR*;* LMOf2365-2365, *Vick* and *xcd*	*L. monocytogenes, S. aureus* and *Salmonella* spp.	Cold food dishes	Yu *et al.* (2016)[40]
qPCR*; hly, uidA* and *invA*	*L. monocytogenes, E. coli* and *Salmonella* spp.	Fresh-cut vegetables	Elizaquível *et al.* (2012)[41]
qPCR*; fliC*$_{h7}$ and *rfbE*	*E. coli*	Ready-to-eat meat products	Gordillo *et al.* (2014)[42]
qPCR*; plc*	*Clostridium perfringens*	Raw milk	Hernández *et al.* (2017)[43]
qPCR*; ttr*	*Salmonella* spp.	Raw chicken breast	Hyeon and Deng (2017)[44]
qPCR*; rplP*	Enterobacteriaceae and non-Enterobacteriaceae strains, altogether 15 species	Fresh vegetables, ready-to-eat raw seafoods and raw meats	Takahashi *et al.* (2017)[45]
multiplex qPCR; *stx1, stx2, eae* and *iroB*	*E. coli* and *S. enterica* Thompson	Strawberries, basil and lettuce	Delbeke *et al.* (2015)[46]
multiplex qPCR; *ipaH, nuc, hlyA, tlh* and *invA*	*Shigella* spp., *S. aureus, L. monocytogenes, V. parahaemolyticus* and *S. enterica*	Pork, beef, meat, chicken, fish and shellfish	He *et al.* (2016)[47]
multiplex qPCR; *tlh, orgC* and *hlyA*	*Salmonella* spp., *L. monocytogenes and V. parahaemolyticus*	Raw shrimp	Zhang *et al.* (2015)[48]

PCR = Polymerase Chain Reaction; mPCR = Multiplex PCR; qPC = Real Time PCR.

Wang *et al.* (2015)[51] developed a novel mPCR assay for simultaneously detection of *Salmonella* spp., *Shigella* spp., and *S. aureus* in pasteurized milk and ground beef, by amplification of *invA* (invasion gene), *ipaH* (invasion plasmid antigen) and *nuc* (thermostable nuclease gene). The interference of dead cells during the mPCR assay was eliminated by combining sodium deoxycholate treatment with propidium monoazide treatment before DNA extraction. The authors concluded that mPCR represents a convenient tool for identifying FBP by simultaneously amplifying more than one target gene in the same reaction system, in agreement to the data reported by other recent studies (Table 1).

Chukwu *et al.* (2016)[52] employed a mPCR assay for detecting the presence of genes encoding the production of α, β, ε, ¡ toxins and enterotoxins by *Clostridium* species. The food product analyzed includes meat and meat products, milk products, vegetables, canned foods and local honey sampled in Lagos State, Nigeria. C. *perfringens* was the most common species isolated (found in 58% of food samples) and all of them harbored the gene (*cpa*) for α toxin, whereas one strain also harbored the enterotoxin gene (*cpe*). In addition, two *C. botulinum* isolates were identified, both harboring neurotoxin A gene (BoNt/A).

The isolation and identification of *Campylobacter* strains are laborious and difficult due to the growth demands and the phenotypic similarity between species[20]. In view of this, Premarathne *et al.* (2017)[21] employed a culture-based detection of *Campylobacter* strains in cattle and beef meat sampled in Selangor, Malaysia, followed by a mPCR assay for confirming *C. jejuni* and *C. coli* isolates by testing simultaneously three target genes (*cadF*, *ceuE* and oxidoreductase subunit genes). As results, *Campylobacter* prevalence rates were: 33% in cattle fecal samples, 14.2% in raw beef from wet market and 7.5% in raw beef from the hypermarket; the mPCR allows to identify 55% of the strains as *C. jejuni*, 26% as *C. coli*, and 19% as other *Campylobacter* species. Furthermore, Wysok and Wojtacka (2018)[53] recently investigated the virulence genes related to the adherence and invasion ability of *Campylobacter* spp. from cattle and swine in Poland, revealing that three genes (*flaA*, *cadF* and *racR*) were

associated with the adherence of *C. jejuni* and *C. coli*, while the gene *iam* was related to their invasion ability.

The possibility of real-time detection by the qPCR (real-time quantitative polymerase chain reaction) method has revolutionized the process of quantification of nucleic acids fragments. It allows an accurate and specific quantification with greater reproducibility, being a sensitive method for quantification of individual species, which could be very relevant to the diagnosis of microbial pathogens and genetic diseases. The qPCR assay requires a thermocycler with an optical system to capture fluorescence and a computer with a software that captures the data and performs the final analysis of the reaction. Fluorescence emission generates a signal that increases in direct proportion with the amount of PCR products. Fluorescence values are recorded during each cycle and represent the amount of amplified product[54].

Advantages of qPCR include short assay times, low reagent usage, and exceptionally rapid heating/cooling rates, integration of multiple processing modules and a lower risk of contamination, allowing a sensitive and specific identification of microbial pathogens[54-57]. As reported by Peruzy *et al.* (2017)[58], the qPCR represents a sensitive, specific and rapid tool to investigate the distribution of *yadA*, *virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* virulence genes in *Y. enterocolitica* strains. The *ystB* gene, which codes for the enterotoxin *ystB*, was found in all non-pathogenic strains. The most common virulence-associated gene in pathogenic strains was *ystA*, considered the best target gene to be amplified for evaluating the presence of pathogenic biotypes.

Maddocks and Jenkins (2016)[59] emphasized that qPCR machines may have four or more channels that dictates how many labeled primer pairs can be used in a PCR assay, which is very useful for applications of multiplex qPCR protocols. It allows assessing the expression of multiple genes at the same time or genes of multiple origins in the same sample. In this context, Silva et al. (2016)[60] employed the multiplex qPCR assay to identify and quantify *Vibrio parahaemolyticus* in 130 samples of fresh oysters (*Crassostrea gigas*), 215 mussels samples (*Perna perna*) and 222 samples of seawater from different regions of cultivation of bivalve shellfishes in the seacoast of Santa Catarina, Brazil. The target genes assessed were *Rox* (to confirm the species), *tdh* and *trh* genes of pathogenicity. As results, the occurrence of *V. parahaemolyticus* in oysters and mussels was low, 10.76% and 11.62% of the samples, respectively; higher incidences were observed in seawater (18%). All strains of *V. parahaemolyticus* were confirmed genotypically through the detection of *tlh* gene (that encodes thermolabile hemolysin) by multiplex qPCR.

As highlighted by Iwobi *et al.* (2012)[61], although the classical methods employing cultural, biochemical, cytological and immunological assays are still being commonly practiced, molecular techniques play important role for the detection of FBP due to the need for a rapid, specific, sensitive and less laborious detection. PCR-based methods allows rapid and initial screening, together with the classical techniques for reliable end-identification of the pathogens. Furthermore, the PCR and its variations allow a rapid detection of nonculturable bacteria, being advantageous over culture-based methods.

3. CONCLUSION

The foodborne disease outbreaks reach people from different ages and social classes. Molecular techniques are important diagnostic tools and represent a great advance for the microbial detection, identification, serotyping and quantification. The PCR-based assays have higher sensitivity when compared to conventional methods, allowing the obtainment of faster and less laborious results. In this review article, we described the main foodborne pathogens and the main molecular techniques used to detect them. Despite the literature data, there are still much to be explored regarding the inclusion of PCR-based assays in food safety management systems.

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