IMMUNOLOGICAL AND BIOSSENSORY TECHNIQUES FOR DETECTION OF *Salmonella* spp. IN FOOD DERIVED FROM FISH FARMING – REVIEW

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ABSTRACT: Salmonellosis is the world's most common foodborne illness. In Brazil, foods contaminated by salmonella lead the statistics. Therefore, the aim of this study is, through biotechnological knowledge, to compile alternative and innovative techniques for the detection of salmonella in foods, such as fish-farming derivatives, immunological and biosensorial techniques. This is a descriptive exploratory data survey of a qualitative nature, aiming at data analysis. Research and data collection were carried out in bibliographic databases: Academic Google, Scielo, CAPES journals and institutional repositories using specific descriptors - in Portuguese and English, with words and terms separated by the Boolean operators 'AND' and 'OR'. Some innovative and alternative methods are available to identify the presence of salmonella in food. Immunological and biosensory techniques, despite being less frequent in the scientific literature than molecular methods, are techniques that present high specificity and sensitivity. These techniques have been the most developed alternative methods in fish in recent years. And, they can employ both molecular and immunological techniques in biorecognition, which is characterized as an advantage of not having a requirement for pre-enrichment of the sample. According to the literature found, the techniques covered in this study are quick to respond, which speeds up decision-making by researchers and technicians, which makes the techniques very promising for industrial application.

KEYWORDS: Detection methods. Fish. Food poisoning. *Salmonella* spp. Salmonellosis.

TÉCNICAS IMUNOLÓGICAS E BIOSSENSORIAIS PARA DETECÇÃO DE *Salmonella* spp. EM ALIMENTOS DERIVADOS DA PISCICULTURA – REVISÃO DE LITERATURA

RESUMO: A salmonelose é uma enfermidade de maior ocorrência no mundo veiculada por alimentos. No Brasil, alimentos contaminados por salmonelas lideram as estatísticas. Por isso, o objetivo desse estudo é através dos conhecimentos biotecnológicos compilar técnicas alternativas e inovadoras para a detecção de salmonelas em alimentos, como os derivados da piscicultura, as técnicas imunológicas e biossensoriais. Trata-se de um estudo de levantamento de dados descritivo exploratório de de caráter qualitativo, visando à análise dos dados. As pesquisas e coletas de dados foram realizadas nas bases bibliográficas: Google Acadêmico, Scielo, periódicos da CAPES e repositórios institucionais utilizando os descritores específicos - nos idiomas português e inglês, com palavras e termos separados pelos operadores booleanos 'AND' e 'OR'. São disponibilizados alguns métodos inovadores e alternativos para identificação da presença de salmonelas em alimentos. As técnicas imunológicas e biossensoriais, apesar de serem menos frequentes na literatura científica do que os métodos moleculares são técnicas que apresentaram elevada especificidade e sensibilidade. Essas técnicas têm sido os métodos alternativos mais desenvolvidos em peixes nos últimos anos. E, podem empregar tanto técnicas moleculares como imunológicas no biorreconhecimento, o que se caracteriza como vantagem de não haver requerimento de pré-enriquecimento da amostra. Conforme a literatura encontrada, as técnicas abordadas por esse estudo apresentam rapidez de resposta o que agiliza as tomadas de decisões dos pesquisadores e técnicos, o que torna as técnicas bastante promissora para aplicação industrial.

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PALAVRAS-CHAVE: Infecção alimentar. Métodos de detecção. Pescado. *Salmonella* spp. Salmonelose.

TÉCNICAS INMUNOLÓGICAS Y BIOSENSORIALES PARA DETECCIÓN DE *Salmonella* spp. EN ALIMENTOS DERIVADOS DE PISCICULTURA - REVISIÓN DE LITERATURA

RESUMEN: La salmonelosis es la enfermedad transmitida por alimentos más común del mundo. En Brasil, los alimentos contaminados por salmonelas lideran las estadísticas. Por tanto, el objetivo de ese estudio fue a través de conocimientos biotecnológicos recopilar técnicas alternativas e innovadoras para la detección de salmonelas en los alimentos, como los derivados de la piscicultura, las técnicas inmunológicas y biosensoriales. Se trata de una encuesta de datos exploratorio descriptivo de carácter cualitativo, cuyo objetivo es el análisis de datos. Las investigaciones y recopilaciones de datos se realizaron en bases de datos bibliográficas: Google Académico, Scielo, revistas CAPES y repositorios institucionales utilizando descriptores específicos, en portugués e inglés, con palabras y términos separados por los operadores booleanos 'AND' y 'OR'. Se encuentran disponibles algunos métodos innovadores y alternativos para identificar la presencia de salmonela en los alimentos. Las técnicas inmunológicas y biosensoriales, a pesar de ser menos frecuentes en la literatura científica que los métodos moleculares, son técnicas de alta especificidad y sensibilidad. Esas técnicas han sido los métodos alternativos más desarrollados en peces en los últimos años. Y pueden emplear técnicas tanto moleculares como inmunológicas en el biorreconocimiento, que se caracteriza por la ventaja de no tener un requisito de preenriquecimiento de la muestra. Según la literatura encontrada, las técnicas abordadas en este estudio son de rápida respuesta, lo que agiliza la toma de decisiones por parte de investigadores y técnicos, lo que hace que las técnicas sean muy prometedoras para la aplicación industrial.

PALABRAS CLAVE: Infección alimentaria. Métodos de detección. Pescado. *Salmonella* spp. Salmonelosis.

Introduction

In recent years, the world population has significantly increased its average consumption of fish, having doubled its consumption in the last 50 years (DANTAS FILHO *et al*., 2021; FAO, 2018;). However, along with the increase in demand for fish, there was an increase in cases of salmonellosis, which harms the marketing of fish and public health (GAZAL *et al*., 2018). One of the main bacterial agents distributed in the aquatic ecosystem, members of the Enterobacteriaceae and Aeromonadaceae families stand out (EVANGELISTA; LUCIANO, 2021; HUBER *et al*., 2004). The Enterobacteriales order harbors a large group of pathogenic bacteria, such as the genera Salmonella, Shigella, Edwardsiella and some serotypes of *Escherichia coli*. Among the bacteria of the Enterobacteriales *Salmonella* sp. stands out, a ubiquitous bacterium that is able to survive in different environments (McADAM, 2020; OLIVEIRA; VAZ, 2018). Salmonellosis in fish is closely related to its creation, as well as to the environment of its industrialization, as a result of inefficient hygiene practices, equipment and inadequate food handling (FERNANDES *et al*., 2018).

Salmonella are facultative anaerobic, non-spore-forming Gram-negative, being mostly mobile (except *S.* Pullorum and S. Gallinarum) by means of peritrichal flagella. Its growth temperature can vary between 5 to 46° C, with an optimal temperature at 37° C (POPOFF; LEMINOR, 2005). According to some molecular studies, the genus Salmonella is divided into two species, *Salmonella* (S.) *enterica* and *S. bongori*. The species *S. enterica* is subdivided into six subspecies designated by Roman numbers: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI). The genus Salmonella has 2579 different serotypes (serovars) identified by the Kauffmann-White scheme, based on the bacterial composition of its somatic (O), flagellar (H) and capsular (Vi) antigens. The species *S. bongori* (V) is composed of 22 serotypes and S. enterica comprises 2557 serotypes (S. enterica subspecies I = 1531, *S. enterica* subspecies II = 505*, S. enterica* subspecies IIIa = 99, *S. enterica* subspecies = 99, *S. enterica* subspecies IIIb = 336, *S. enterica* subspecies IV = 73 and *S. enterica* subspecies VI = 13) (GRIMONT; WEILL, 2007).

The test to identify the presence of salmonella in food is a requirement of the health authorities that regulate food safety in their respective countries. Conventional techniques, based on classical cultural methods, are considered sensitive and reliable. However, they depend on a complex sequence of steps and require several days for the result (ANDREWS *et al*., 2016; EVANGELISTA; LUCIANO, 2021). Recent advances in technologies for detecting and identifying the presence of microorganisms have made available more agile, sensitive and specific alternatives to conventional methods. And, these are often referred to as alternative methods, terms commonly used to describe a variety of tests that include miniaturized biochemical kits, immunological assays, DNA/RNA based tests, and combinations with cultural methods (GAZAL *et al.,* 2018).

According to epidemiological data presented above, salmonella contamination is the most frequent cause of foodborne disease outbreaks. The determination of this pathogen is mandatory in food products for human consumption and can be performed using conventional methods or alternative methods, also called rapid methods (LIEVENS *et al*., 2011). The development of alternative methods for detecting salmonella is of great importance for food safety and the maintenance of public health. In addition, there is a strong industrial demand for compliance with legislation and the rapid release of food products to the market (DEMERTZIS; ILIADIS, 2015; EVANGELISTA; LUCIANO, 2021).

Given these assumptions, the aim of this study is, through biotechnological knowledge, to compile alternative and innovative techniques for the detection of salmonella in

foods, such as fish-farming derivatives, immunological and biosensorial techniques.

Development

Methodology

This study is a bibliographic research carried out by consulting the database of CAPES journals and institutional repositories. The survey of information carried out is characterized as exploratory descriptive, also of a qualitative nature, aiming at the analysis and crossing of data between several articles and literature related to the studied topic (DANTAS FILHO *et al*., 2020; PONTES, 2019).

The data survey was carried out from April to July 2021, based on questions raised on the subject, and about 50 studies were consulted, with scientific articles, books and references found in electronic databases and bibliographic bases: Google Scholar, Amazon, Scielo, and others. The criteria adopted for the searches were publications in the last ten years in scientific journals with a consolidated technical and editorial staff, and which have a focus and scope related to the theme. In addition, having a link with a higher education institution and qualis concept (2013-2016) at least B2 in the area of Interdisciplinary assessment.

To collect the information, the following descriptors were searched: *Salmonella* spp., food infection, salmonellosis, fish chain, food pathogens, fish health, fish microbiology, epidemiology, legislation, detection methods, immunological and biosensorial techniques; in Portuguese and English, with words and terms separated by Boolean operators 'AND' and 'OR' according to the search objectives in each topic of this review article.

Results e Discussion

Techniques for detection of *Salmonella* ssp.

Molecular tests employ a bacterial nucleic acid sequence as a target for detection and correspond to 47% of validated methods (FSIS, 2016). And, it is the category of alternative methods that has grown the most in recent years. Therefore, as it is a technique often mentioned in the literature, it was not considered in this study.

Immunological Techniques

Experiments based on immunological methods use mono or polyclonal antibodies to identify the presence of salmonella in animal products. Therefore, the antibodies mentioned above can identify antigens in different food matrices (MELO *et al*., 2018). The assays performed through immunological tests involve the ELISA test (Enzyme Linked Immuno in the Sorbent Assay), latex agglutination tests and immunodiffusion assays, as shown in Table 1.

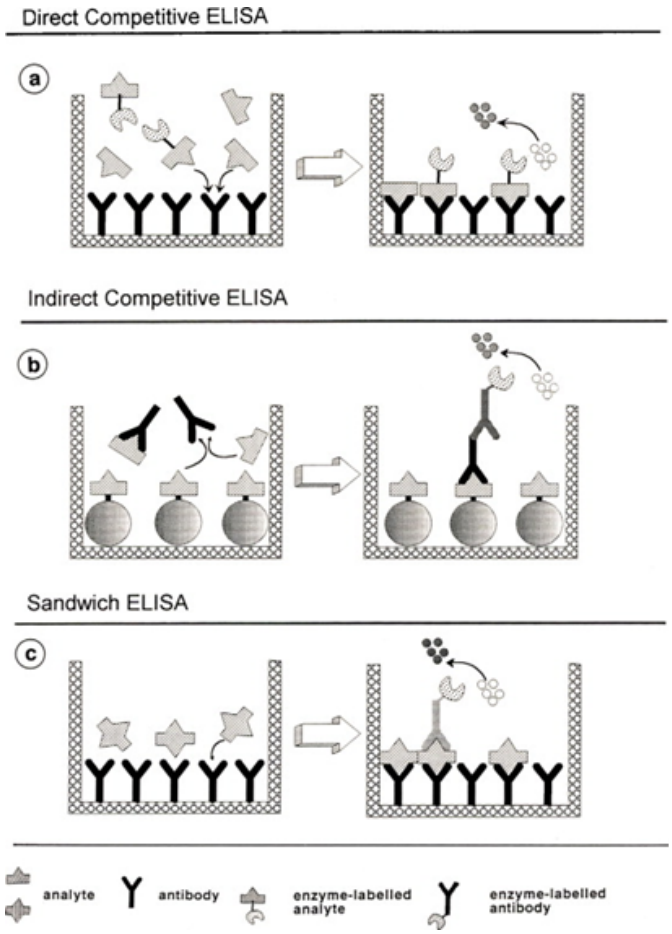
Table 1: Immunological assays available to identify the presence of salmonella in food.

V	Manufacturers Names	Available techniques	Application Modes
3M™ TECRA™ *Salmonella* Visual	3M Microbiology	ELISA	All foods
Assurance Gold EIA *Salmonella*	BioControl Systems, Inc.	ELISA	All foods
BioControl 1-2 TEST	BioControl Systems, Inc.	immunodiffusion	All foods
LOCATE® *Salmonella Assay Kit*	Rhone-Poulenc	ELISA	All foods
Salmonella ELISA Test SELECTA, RayAl Salmonella SELECTA	Diatek AG	ELISA	All food for humans and animals
Salmonella – Tek ELISA Test System	Organon Teknika Corp.	ELISA	All foods
Singlepath® *Salmonella*	Merck KigaA	Immunochromatograph	Skimmed milk powder, raw ground beef, ground turkey, frozen cooked peeled shrimp, dried coconut, black pepper and kibble
TAG 24 *Salmonella*	BioControl Systems, Inc.	ELISA	All food for humans and animals
FoodChek *Salmonella*	FoodChek System Inc.	Lateral flow immunoassay	Liquid and shell eggs, egg whites, powdered eggs, egg yolks, stainless steel, plastic rubber, ceramic tiles and sealed concrete
VIP *Gold for Salmonella Assay*	BioControl Systems, Inc.	Lateral flow immunoassay	All foods
TRANSIA PLATE *Salmonella Gold*	BioControl Systems, Inc.	ELISA	All food for humans and animals and environmental samples
VIDA® *Easy Salmonella*	BioMérieux, S.A.	ELISA	All food for humans and animals and environmental samples (except agricultural environment)

Source: Melo *et al*. (2018).

It should be noted that the ELISA test is the most widely applied category of assays aimed at detecting salmonella in animal products. What's more, this method is available in a significant number of kits (Food Safety and Inspection Service, 2016) on the market, which are based on different ELISA formats (Figure 1). In a homogeneous class immunological method experiment, labeled antibodies and antigens are mixed freely in the detection system. Therefore, the modulation happens as a conversion in the activity of the marker, but for that, the antigens need to bind to the antibodies. In general, they show a change in the color of the sample (MELO *et al*., 2018; SILVA *et al*., 2018).

Figure 1: ELISA formats: (a) Direct competitive, (b) Indirect competitive and (c) Sandwich.



Source: Aspland and Marie-Claire Hennion, 1997.

Generally, immunological assays are heterogeneous, such as a salmonella-specific antigen that binds to its antibody that is located immobilized on a solid food matrix. The antigen-antibody complex is formed and this can be seen by the change in color, which is caused by the enzymatic cleavage of a chromogenic substrate, allowing the identification of the presence of bacteria in the sample to be identified (VALDERRAMA *et al*., 2015). Regarding the immunological assay reagents, they are unbound components, that is, they are washed away. So, in this way, the response is obtained through the markers, and this is proportional to the amount of analyte in the experimental sample evaluated (MELO *et al*., 2018; SILVA *et al*., 2018).

Experiments with immunological methods can still be considered as competitive or non-competitive. The most administered in laboratories are those used by the sandwich method and the competitive method. In the non-competitive sandwich assay, the primary antibodies are immobilized on a surface (MELO *et al*., 2018; VALDERRAMA *et al*., 2015). Following the addition of the sample with the antigen, a labeled conjugated antibody is added to the system. However, in an experiment with the competitive method, competition occurs between the free labeled antibodies, more specifically in a limited amount. As well as the antigen fixed to a base, or between the labeled antigen from the experimental sample and a limited amount of antibodies (MELO *et al*., 2018; SILVA *et al*., 2018).

In this context, it should be noted that agglutination requires latex particles wrapped with antibodies that react with the antigens on the surface of the bacteria's cells, in this case salmonella, forming visible aggregates for the identification of positive samples. The tests are specific and easy to handle, in addition to their significant reliability (VALDERRAMA *et al*., 2015). Typically, these tests have been performed as confirmatory analysis techniques, as opposed to screening tests. Alternatively, there are several kit options on the market that have this technique (Food Safety and Inspection Service, 2016). Which employs immunodiffusion reaction, the kit 1-2 Test© (Biocontrol) (SILVA *et al*., 2018).

As far as the device is concerned, there are two chambers: the first is the inoculation chamber, in which the pre-enriched food sample is added, and the second is the motility chamber, in which bacterial growth and reaction with flagellar antibodies occur (LEE *et al*., 2015). A priori, the sample is pre-enriched for 24 hours. In this way, the sample unit is enriched and inoculated in the inoculation chamber. The inoculated salmonella cells move to the motility chamber, where the antigen-antibody complex is formed (VALDERRAMA *et al*., 2015). Then, when the result is positive, it evidences the formation of a remarkable three-dimensional immunoband after an average time of 24 to 30 hours (AOAC Official Method 989.13, 1998).

The immunological experiments conducted are comparatively more agile as well as more specific than conventional methods. Furthermore, by associating techniques such as immunomagnetic separation (IMS), with the possibility of automation, it becomes faster and more practical, especially for sampling units available in large quantities (LEE *et al*., 2015). Among immunological methods, those based on ELISA express specificity and sensitivity comparable to conventional methods and are the most used. ELISA experiments express detection limits between 10^4 and 10^5 CFU mL^{-1}, levels normally understood after pre-enrichment of the evaluated sample (LEE *et al*., 2015; MELO *et al.,* 2018).

However, it must be admitted that these methods have some limitations for identifying the presence of salmonella, especially in very moist foods such as fish. For in immunological assays it is necessary to conduct a previous enrichment of

the sample, in order to obtain the adequate number of cells, in order to increase the analysis time. Furthermore, cross-reactions with phylogenetically close antigens possibly occur. As well as antigen variations, sensitivity limits for some food sample matrices, and also the high cost of assay automation (SILVA *et al*., 2018). But without a doubt, the sensitivity and specificity of these methods strongly depend on the microbiota of the sample, the complexity of the food matrix and the inhibitory substances. Which can be exemplified, heavy metals, antibiotics, polysaccharides, proteins, lipids and other organic compounds (MELO *et al*., 2018).

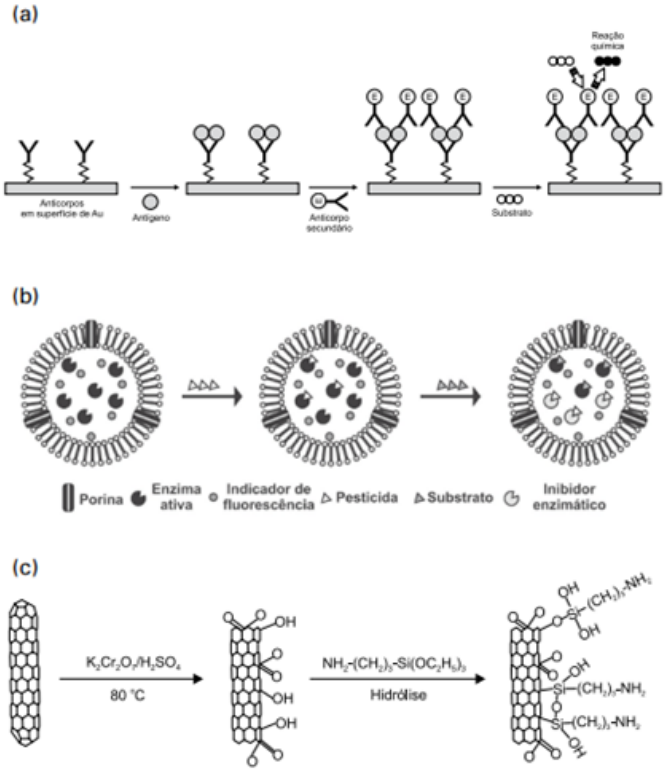
Biosensory techniques

Biosensors are bioelectronic devices capable of detecting analytes, with agility both quantitatively and qualitatively (FURTADO *et al*., 2008; SILVA *et al*., 2018). This technique is developed by two main components, a bioreceptor, a molecule that specifically interacts with the analyte, and a transducer, which transforms the response of the bioreceptor-analyte interaction into an electrical signal (SILVA *et al*., 2018). There are different types of transducers, the most used are: optical, piezoelectric and electrochemical (FURTADO *et al*., 2008). Biosensors with electrochemical transducers have been reported more frequently in the detection of pathogens (ARORA *et al.,* 2013).

These devices are widely used in different areas of knowledge and are an agile response alternative for the detection of pathogenic bacteria (VELUSAMY *et al*., 2010). Furthermore, recent research has shown the development of biosensors capable of detecting the presence of microorganisms or their metabolites in an agile and accurate way with a lower detection limit than conventional methods (MELO *et al*., 2018).

The improvement in the elaboration of biosensors occurs together with the increase in the molecular and biochemical understanding of the analytical response and its supporting technologies (VALDERRAMA *et al*., 2015). That said, one can currently find miniaturized, affordable and easy-to-administer devices on the market. There are nowadays different categories of biosensors that are classified by the type of immobilized biological molecules, by their interaction with the analytic through the analytic response or by the transducer (Figure 2).

Figure 2: Methods of immobilization of biological material: (a) covalent bonding, (b) microencapsulation and (c) physical adsorption.



Source: Furtado *et al*. (2008).

Melo *et al.* (2016) carried out a study on electrochemical immunosensors designed to detect salmonella, whose limits and detection times for the devices are shown in Table 2. In order to compare with molecular and immunological methods, immunosensors stand out more in relation to the period for detection. Table 2 shows the agility in obtaining response from the devices, with detection periods of up to 6 minutes, and low detection limits, with devices capable of detecting up to three mL^{-1} cells (MELO *et al*., 2018). Such information can be obtained without enriching the sample, which is often necessary for the molecular and immunological methods previously presented.

Table 2: List of electrochemical immunological sensors to identify the presence of salmonellae.

Types of technique	Detection Limit Parameter	Period for identification	References
Impedimetric	10 UFC mL^{-1}	3h	Pournaras *et al*. (2008)
	5x10^2 UFC mL^{-1}	6 min	Nandakumar *et al*. (2011)
	10^5 UFC mL^{-1}	2h	Mantzila *et al*. (2008)
	5x10^2 UFC mL^{-1}	1h	Dong *et al*. (2013)
	10^2 UFC mL^{-1}	40 min	Yang *et al*. (2009)
	3 células mL^{-1}	Not reported	Ma *et al*. (2014)
Amperometric	6 UFC mL^{-1}	Not reported	Zhu *et al*. (2014)
	10^6 UFC mL^{-1}	3h	Delbato *et al*. (2006)

Amperometric	143 células mL^{-1}	1.5h	Afonso *et al*. (2013)
	20 células mL^{-1}	Not reported	Salam and Tothil (2009)
	$5x10^3$ UFC mL^{-1}	50 min	Liébana *et al.* (2009)
	1.9510^3 UFC mL^{-1}	Not reported	Hu *et al*. (2014)
	13 células mL^{-1}	1h	Freitas *et al*. (2014)
	$5x10^4$ UFC mL^{-1}	1h	Brandão *et al*. (2013)
	10 UFC mL^{-1}	125 min	Melo *et al*. (2016)
Conductometric	7.9x10 UFC mL^{-1}	10 min	Muhammad-Tahir and Alocilja (2003)
Potentiometric	119 UFC mL^{-1}	Not reported	Dill *et al*. (1999).

Source: Melo *et al*. (2018).

An immunosensor capable of identifying the presence of salmonella in an agile way, and specifically and with a low detection limit was developed (MELO *et al*., 2016). In that same study, the technique of self-assembled monolayers was used to alter the surface of gold electrodes, using the thiol cysteamine. Furthermore, Protein A was applied for the oriented immobilization of the primary antibody through covalent bonds. It should be emphasized here that the biosensor response curve expressed a qualitative behavior with a detection limit of only 10 CFU mL^{-1} and an identification period of 125 min (GONÇALVES *et al*., 2014).

Furthermore, cross-reaction tests against *Escherichia coli* and *Citrobacter freundii* strains expressed high specificity of the device developed (GONÇALVES *et al*., 2014). The good application capacity of the immunosensor in foods, such as fish and milk, was proven (BRITO, 2020; MELO *et al.* 2016), and it can be evaluated in other food matrices (BENETTI, 2009; MATACA, 2014; MONTEIRO, 2018; SILVA JÚNIOR, 2017). It is noteworthy, according to Mataca (2014), Monteiro (2018) and Brito (2020), biosensory techniques have been the most developed alternative methods in fish in recent years.

Before concluding, it is important to point out that these alternative methods were designed to detect a specific target. In ways that make them faster and more suitable for application in quality control and food safety. That is, agile screenings are usually carried out in a considerable number of samples, in order to detect a specific analytical (SILVA *et al*., 2018). However, positive results detected by alternative methods are considered only presumptive. Therefore, they need confirmation by an official standard method (GONÇALVES *et al*., 2014; MELO *et al*., 2018).

Finally, the alternative methods covered in this work can be certified by independent institutions to validate the identification of microorganisms in food (AOAC, AFNOR, MicroVal, NordVal) or regulatory agencies (FSIS, FDA BAM, EFSA, ANVISA, ISO) (GONÇALVES *et al*., 2014). Furthermore, they can only be administered in food matrices for which they have been certified, adopting the validation conditions, which guarantees the reliability of the method used by the researcher and/or applicator (LEE *et al*., 2015).

Conclusions

Some innovative and alternative methods are available to identify the presence of salmonella in food, based on different biotechnological techniques. The techniques highlighted by this work are immunological and biosensorial. Because, despite these methods being less frequent in the scientific literature than molecular methods, they are techniques that present high specificity and sensitivity.

It should be noted that biosensory techniques have been the alternative methods most developed in fish in recent years. And, they can employ both molecular and immunological techniques in biorecognition, which is characterized as an advantage of not having a requirement for pre-enrichment of the sample. According to the literature found, the techniques addressed in this work are quick to respond, which speeds up decision-making by researchers and technicians, which makes the techniques very promising for industrial application in the detection of bacteria in moist foods.

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