Monitoring microorganisms' growth using multisensor electrochemical devices

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Dedication

To my dear Father

Who was there behind me to guide me, to give me all that I ask, to be my father, is the greatest gift that I can have, the one who sacrificed his life to enlighten me the good path of knowledge and fills me of love and tenderness since my young age. My father gave me the most beautiful gift: he believed in me!

To my dear Mother

She who gave me life, her love and her affection, I dedicate this memory for her encouragement and her affection for me, for her infallible support and her sacrifice unceasingly in search of my happiness.

To my dear sister and my dear brother

Who find here the proof of all my gratitude and my eternal appreciation.

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Abstract

Some microorganisms contribute beneficially in processing, safety and quality of certain food products. However, many microorganisms are involved in processes that cause undesirable effects on food, or on the health of consumers, leading to spoilage or to occurrence of foodborne diseases. For that, microbiological surveillance of food corresponds to an area of great interest to ensure the quality and the safety of food to prevent foodborne diseases. Indeed, for reasons related to sampling, methodology and distribution of the microorganisms in the matrix, microbiological analysis for itself does not guarantee the safety of a final product analyzed. For that, a possible promising alternative to the traditional diagnostic methods in the electronic sensors such as the E-tongues that has been used for different applications in food and pharmaceutical industries, they have been useful for the detection of bacterial contamination or diagnosis of infections.

The aim of the present study was the detection and discrimination of microorganism that played an important role in food and environmental areas, namely *E. coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *S. aureus*. In this context, electronic tongues (E-tongues) have been employed for the detection and screening of microorganisms. Thus; the use of a potentiometric E-tongue, comprising lipid polymeric sensor membranes, together with unsupervised and supervised chemometric tools (e.g., principal component analysis, PCA; linear discriminant analysis, LDA; and. multiple linear regression models, MLRM) was evaluated aiming to explore the advantages of these innovative (bio)sensing devices for microorganism's recognition and discrimination, in aqueous solutions.

Our results showed that the potentiometric signals profiles acquired by the 40 E-tongue sensors allowed a satisfactory unsupervised recognition of *P. aeruginosa* and *E. faecalis*, contrary to *E. coli* and *S. aureus*, showed a clear over-plotting. Still to further assess the E-tongue classification capability, a LDA was performed since it represents the most discriminant and non-redundant sensors selected by the SA algorithm. The supervised discriminant model allowed to classify 100% of the original grouped data. Overall, the unsupervised and supervised classification performances clearly showed the potential use of the E-tongue as an accurate and fast recognition device of the four microorganisms studied.

Key words: microorganisms, sensors, E-tongues, PCA, LDA

Resumo

Alguns microrganismos contribuem para a segurança e qualidade de certos produtos alimentares. No entanto, outros grupos de microrganismos causam efeitos indesejáveis nos alimentos provocando a sua deterioração ou inclusive dando origem a doenças de origem alimentar colocando em risco a saúde dos consumidores. Neste contexto, a vigilância microbiológica dos alimentos é uma área de grande relevância de forma a garantir a qualidade e a segurança dos alimentos. Contudo, as técnicas analíticas convencionais utilizadas na deteção de microrganismos em alimentos são caras e demoradas. Alternativamente, podem ser aplicadas outras técnicas, nomeadamente línguas eletrónicas (LE), para cumprir essa tarefa crítica.

Com este estudo pretendeu-se estudar a capacidade de deteção e discriminação de microrganismos que desempenham um papel importante nas áreas alimentares e ambientais, nomeadamente *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* e *Staphylococcus aureus*. Para tal, utilizou-se uma LE potenciométrica e o seu desempenho de deteção foi avaliado recorrendo a ferramentas quimiométricas não supervisionadas e supervisionadas (análise principal de componentes, ACP; análise discriminante linear ADL).

Os resultados mostraram que os sinais potenciométricos adquiridos pelos sensores da LE permitem reconhecer satisfatoriamente e não supervisionado a *P. aeruginosa* e *o E. faecalis*, ao contrário da *E. coli* e *S. aureus* A capacidade de classificação da LE foi ainda avaliada pela ADL, com vista a identificar os sensores não redundantes e com maior potencial discriminante. O modelo discriminatório supervisionado permitiu classificar 100% dos dados originais. Globalmente, os desempenhos de classificação confirmaram a possível utilização da LE como um dispositivo de reconhecimento preciso e rápido dos quatro microrganismos estudados.

Palavras-chave: Microrganismos, Língua electrónica potenciométrica, ACP, ADL

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1. Introduction

1.1- Framework

Microorganisms survive in many environments including food, water, soil, human body, and animals (Erdem et *al*., 2019), being the related number of diseases very high.

Although the scientific and technological development. diseases caused by microorganisms are still a worldwide health problem. and countries until now haven't been able to stop the spread of these foodborne diseases; on the contrary, it is growing each year (Zhang et *al*., 2019).
According to Zhang et *al*. (2019). foodborne diseases can be divided into four categories:

- The food poisoning that occurs after eating contaminated food either with toxic or harmful substances (Inoue et *al*., 2018; Jiang and Huang, 2018).
- The allergic diseases that occur due to certain type of food (Markevych et *al*., 2017).
- The infectious diseases. like dysentery (Berhe et *al*., 2019) and zoonotic diseases (Zhu et *al*., 2013).
- Chronic toxicity diseases that are due to the ingestion of a large number of toxic substances (Yu et *al*., 2012).

In foods, different microorganisms can be found, some of them being used in food's production. On the other hand, other microorganisms can be responsible for food spoilage or foodborne disease. Indeed, many kinds of pathogens produce toxins and other cell metabolites causing foodborne diseases, namely *Escherichia coli*, *Vibrio cholera*, *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium perfringens* (Oliver et *al*., 2010).

For that, the detection of foodborne diseases is of utmost relevance being commonly employed four main methods: culture-based microbiological methods, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and microarray techniques. Rapid methods have also been employed including nucleic acid based methods, antigen-antibody based methods, biosensors, and bacteriophage-based methods. Nowadays, however, biosensor based methods have been progressively more employed since they allow a fast detection of foodborne pathogens (Bavisetty et *al*., 2018).

1.2- Objectives

The main aim of the present study was the detection and discrimination of microorganism that played an important role in food and environmental areas, namely *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Although conventional methods like plating techniques are still the most widely used, is needed to develop fast, ease of use, green and cost effective screening detection methodologies. In this context, the development of electrochemical sensors including electronic tongues (E-tongues) has emerged in the last decades, being recognized as promising bioinspired detection tools for the screening of microorganisms. Thus; the use of a potentiometric E-tongue, comprising lipid polymeric sensor membranes, together with unsupervised and supervised chemometric tools (e.g., principal component analysis, PCA; linear discriminant analysis, LDA) was evaluated aiming to explore the advantages of these innovative (bio)sensing devices for microorganism's recognition and discrimination, in aqueous solutions.

2. Bibliographic review

World's Health Organization (WHO) defines food-borne disease (FBD) as a "disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water" (Le Loir et *al*., 2003).

According to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), in 2018, foodborne outbreaks increased when compared to 2017; being reported 5098 foodborne outbreaks with 48365 illnesses. 4588 hospitalizations. and 40 deaths (The European Union One Health 2018 Zoonoses Report. 2019).

Microbiological analysis is essential to obtain information on hygienic and sanitary conditions during the manufacturing, storage, distribution and to directly assess product quality, shelf-life and guarantee the public health.

2.1- Microorganisms and their role in food quality

Food products, such as meat, milk, vegetables, fish, and many others, contain autochthonous microorganisms, i.e., which are part of the natural microbiota of these products. Thus, their presence is expected, and some of them are essential in the production of some food products such as yogurts, cheese, sausage. During the manufacturing processes, the food products suffer various manipulations being in contact with different surfaces (equipment, hands, packages, etc.,) more or less contaminated with microorganisms, thus allowing their contamination with undesirable microorganisms, which can affect not only the quality of the food but also endanger human health. In a general way, the microbiological quality is conditioned, first, by the quantity and type of microorganisms initially present (raw material and production processes) and then by the multiplication of microorganisms in food products, which depends on the intrinsic factors of the food matrix, such as the nutrients, pH, water activity; environmental factors, such as temperature, relative moisture; and, processing issues, such as heating, cooling or drying. These factors can promote the microorganisms' proliferation on food or decrease the multiplication of spoilage microorganisms and or foodborne pathogens (Hamad, 2012).

Foodborne diseases of microbiological origin can be caused by variety of agents and may be divided into two major classes: food infection and food intoxication (Hamad, 2012).

Food infection occurs when the food contaminated with pathogenic, invasive, food poisoning bacteria are eaten. These bacteria then proliferate in the human body and eventually cause illness. Food intoxication occurs from the ingestion of preformed toxic substances which accumulate during the growth of certain bacterial types in foods (Hamad, 2012).

2.2- Microbiological criteria

The food industry is a solid sector that is regulated, with the objective of ensure the supply of safe products for human health minimizing foodborne diseases. In each food product, the microbiological analysis is related to microbiological criteria that define the acceptability of the food or the processes used to produce it. These criteria are established by the legislation of each country. In the EU, Commission Regulation (EC) No 2073/2005 establishes the microbiological food safety criteria and hygiene criteria throughout the production chain. and at the international level these criteria are set according to the Codex Alimentarius Commission (FAO/WHO). According to the Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, the microbiological criterion is: "a criterion defining the acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number of micro-organisms, and/or on the quantity of their toxins/metabolites, per unit(s) of mass, volume, area or batch;". Microbiological criteria were created with the main objective of protecting consumer health. These should serve as support for make decisions based on evidence generated from microbiological determinations. As stated by Commission Regulation (EC) 2073/2005 "The safety of foodstuffs is mainly ensured by a preventive approach. such as implementation of good hygiene practice and application of procedures based on hazard analysis and critical control point (HACCP) principles. Microbiological criteria can be used in validation and verification of HACCP procedures and other hygiene control measures". Such controls can be guided by microbiological criteria for foods that include safety parameters related to foodborne microbial pathogens and hygiene indicators.

2.2.1- Hygienic indicator microorganisms

Direct testing for detecting pathogens is not feasible to carry out at industrial level, since it is very expensive and pathogens are usually present in foods in low amounts, requiring their identification, the use of numerous physiological/ biochemical tests (Buchanan and Oni, 2012).
Microbiological analysis is being employed for the detection of indicator microorganisms, which when present in a certain concentration, indicate exposure to conditions that can lead to the

proliferation of pathogenic microorganisms (Buchanan and Oni, 2012). Due to the complexity of food matrices and their interaction with microorganisms, it is difficult to find an ideal indicator microorganism, being the most frequently used indicator microorganisms the following ones (Buchanan and Oni, 2012):

- Total coliforms have been employed to indicate fecal contamination and sanitation in some matrices such as raw ground meats. water and spices.
- Thermotolerant fecal coliforms are used to specify fecal contamination. sanitation and also temperature abuse in raw ground meats, water and seafood.
- *Enterobacteriaceae* is used to indicate fecal contamination and verify sanitation programs specifically in dry products, in matrices such as powdered infant formula and ready to eat foods.
- *E. coli* is used in matrices like seafood, water, and ready to eat foods.
- *S. aureus* is employed in matrices for food coming from food service establishments, fermented meat, and fermentation failures of dairy products.
- *Enterococcus* spp. is used in matrices such as produced frozen foods.
- *Listeria* spp. is employed in the evaluation of environmental zones of food facilities and refrigerated ready to eat foods.
- *S. enterica* is used to control enteric bacteria in raw meat and poultry.

2.2.1.1- *Enterococcus faecalis*

E. faecalis is a Gram-positive, catalase-negative coccus (Opera and Zervos, 2007). Cells are predominant and can exist singly, in pairs, or in short chains. They are facultative anaerobes, non-sporulating and very tolerant to extreme temperatures, salinity, and pH. Therefore, they grow in 6.5% NaCl broth at pH 9.6 and at temperatures ranging from 10 to 45°C, with optimum growth at 35°C (Teixeira and Facklam, 2003).

Enterococci are considered as a part of the flora of humans and animals. *Enterococcus faecalis* is considered an indicator of fecal contamination, although they also belong to the natural microbiota of many fermented products (dairy products, meat and vegetables), being *E. faecalis* one of the dominant species. Indeed, *E. faecalis* plays an important role in the development of organoleptic characteristics and safety of fermented products, once it is a producer of bacteriocin, with antimicrobial activity against foodborne pathogens namely against *Listeria* and spoilage bacteria

(Franz, Holzapfel & Stiles, 1999), for these, some strains are used as starter culture. On the other hand, they are currently rank among the most prevalent resistant hospital pathogens worldwide, and can serve as a reservoir for virulence traits and antimicrobial resistance (Giraffa, 2002; Andrighetto et *al*., 2001).
The enterococci strains that colonize foods or purposefully used as starters have been repeatedly found to harbor virulence traits and drug resistance genes (Opera and Zervos, 2007).

The cell envelope of Gram-positive microorganisms involves a large number of proteins that after secretion become linked to the cell wall and are then exposed toward the external environment (Hancock, Murray & Sillanpää, 2014).

2.2.1.2- *Staphylococcus aureus*

S. aureus is a Gram-positive, spherical staphylococci arranged in clusters, resembling to a bunch of grapes, catalase positive and oxidase negative. *S. aureus* is an aerobic and facultative anaerobic organism that can grow in a wide range of temperatures (7° to 48.5°C; optimum 30 to 37°C), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15% NaCl.
S. aureus does not form spores but contributes in the contamination of food products during its preparation and processing (Kadariya et *al*., 2014). It is a commensal and opportunistic pathogen that can cause wide infections, such as superficial skin infections and severe, and eventually deadly, invasive diseases (Lowy, 1998). This ubiquitous bacterium is observed as an important pathogen due to combination of "toxin-mediated virulence, invasiveness, and antibiotic resistance." The pathogen is armed with battery of virulence factors that facilitate to establish infections in the hosts. The organism is well known for its ability to acquire resistance to various antibiotic classes (Kadariya et *al*., 2014).

A typical foodborne disease caused by *S. aureus* has a rapid onset following ingestion of contaminated food (usually 3–5 hours). This is due to the production of one or additional toxins by the bacteria during growth at permissive temperatures (Le Loir, Baron & Gautier, 2003). Symptoms include hyper salivation, nausea, vomiting, and abdominal cramping with or without diarrhea. Foods that have been frequently implicated in SFD are meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, especially cream-filled pastries and

cakes, and sandwich fillings (Le Loir, Baron & Gautier, 2003; Argudin et *al*., 2010; Tamarapu et *al*., 2001).

2.2.1.3- *Escherichia coli*

E. coli is a Gram-negative, rod-shaped, non-sporulating and facultative anaerobic bacterium that is found in the lower intestine of warm-blooded organisms (endotherms) (George and Garrity, 2005). Cells are about 2 µm long and 0.5 µm in diameter, and the cell volume range from 0.6 to 0.7 μm^3 (Kubitschek, 1990). The growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, dimethyl sulfoxide and trimethylamine N-oxide (Ingledew and Poole, 1984).

Most *E. coli* strains are harmless, they colonize the gastrointestinal tract of humans and animals as a normal flora. There are some strains that have evolved into pathogenic *E. coli* by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for product recalls (Hudault et *al*., 2001; Vogt and Dippold, 2005). This pathogenic *E. coli* can be categorized based on sero-groups, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper et *al*., 2004; Nataro and Kaper, 1998).
Three major virulence factors of *E. coli* O157:H7 have been identified including Shiga toxins, the locus of enterocyte effacement considered as products of the pathogenicity island, and pO157 (Lim et *al*., 2010).

Human infection caused by *E. coli* O157:H7 ranging from asymptomatic cases to death can present a broad clinical spectrum. However, it can also be spread directly from person to person, particularly in child day-care facilities, and from animal to person. Infections have been documented from people visiting petting zoos, dairy farms, or camp grounds where cattle have previously grazed (Heuvelink et *al*., 2002; Johnson et *al*., 1999).

2.2.1.4- *Pseudomonas aeruginosa*

Pseudomonas species are Gram-negative, aerobic bacilli with the measure of 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum, and some strains have two or three flagella (Baron, 1996).

The genus *Pseudomonas* holds more than 140 species, in which most of them are saprophytic with more than 25 species are related with humans. Most pseudomonads known to cause disease in humans are associated with opportunistic infections, *P. aeruginosa* and *P. maltophilia* account for approximately 80% of pseudomonads recovered from clinical specimens. Because of the frequency with which it is involved in human disease. *P. aeruginosa* has received the most attention. Even though it hardly causes disease for healthy individuals, it is considered as a major threat to hospitalized patients, particularly those with fundamental serious diseases such as cancer and burns (Baron, 1996).

Pseudomonas species normally inhabit soil, water, and vegetation and can be isolated from the skin, throat, and stool of healthy persons. They often colonize hospital food, sinks, taps, mops, and respiratory equipment. Spread is from patient to patient via contact with fomites or by ingestion of contaminated food and water (Baron, 1996).

P. aeruginosa is a non-fermentative aerobe that derives its energy from oxidation rather than fermentation of carbohydrates. Although able to use more than 75 different organic compounds, it can grow on media supplying only acetate for carbon and ammonium sulfate for nitrogen. Furthermore, although an aerobe, it can grow anaerobically, using nitrate as an electron acceptor. This organism grows well at 25° to 37° C, but can grow slowly or at least survive at higher and lower temperatures. In addition to its nutritional versatility, *P. aeruginosa* resists to high concentrations of salt, dyes, weak antiseptics, and many commonly used antibiotics. These properties help explaining its ubiquitous nature and contribute to its preeminence as a cause of nosocomial infections (Baron, 1996).

2.3- Cell wall structure of Gram-positive and Gram negative microorganisms

The cell envelope of bacteria is composed of multilayered structure when its main purpose is to protect microorganisms from the aggressive environment. Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, that itself is surrounded by an outer membrane that consists of lipopolysaccharide. On the other hand, Gram-positive bacteria are surrounded by layers of peptidoglycan that are thicker than is found in Gram-negative bacteria (Silhavy, Kahne & Walker, 2010).

There are two principal layers in the envelope in Gram-negative bacteria; firstly, the outer membrane (OM) and secondly, the peptidoglycan cell wall. Starting with the OM like other

membranes is an assymetric, with phospholipids in the inner leaflet and lipopolysaccharide in the outer leaflet (Kamio and Nikaido, 1976). The lipopolysaccharide molecules are composed of three moieties namely lipid A, a core oligosaccharide O-chain, being O-chain highly variable even between strains. LPS is a well-known molecule since it is responsible for the endotoxic shock associated with the septicemia caused by Gram-negative organisms (Raetz and Whitfiel, 2002). Regarding the peptidoglycan layer which is made up of repeating units of the disaccharide N-acetyl glucosamine-N-actyl muramic acid, and they are cross-linked by penta-peptide side chains (Vollmer et *al*., 2008).

Since, the outer membrane plays a major role in protecting Gram-negative organisms from the environment by excluding toxic molecules and providing an additional stabilizing layer around the cell, the peptidoglycan mesh surrounding Gram-negative cells is relatively thin (Silhavy, Kahne & Walker, 2010). Along these layers of peptidoglycan are extended anionic polymers, also named as teichoic acids, which are composed mostly of glycerol phosphate, glucosyl-phosphate, or ribitol phosphate repeats. One category of these polymers that consists the wall teichoic acids (TAs), are covalently attached to peptidoglycan; the other category, consists of the lipoteichoic acids, are affixed on the head groups of membrane lipids (Neuhaus and Baddiley, 2003). Combining the two categories, these polymers can count for over 60% of the mass of the Gram-positive cell wall, making them a major contribution to the structure and function of the envelope. There are many differences between bacteria species keeping in mind the peptidoglycan details structure, but the most notable difference is related to the composition of peptide cross links between glycan strands and with the high variability in structure and chemical composition of TAs (Vollmer, 2008; Vollmer et *al*., 2008). Studies carried out on *S. aureus* have shown that the composition of surface-expressed proteins can change immediately depending on environmental cues or the growth conditions of the microorganism, giving the important role of the cell envelope in adapting to the local environment (Pollack and Neuhaus, 1994).

The differences on the structure and chemical composition of the microorganisms' cell walls, e.g., different chemical different compounds, could result into numerous and different cell wall-sensor interactions, leading to specific sensors' signal fingerprints, and thus, to the capability of recognizing and distinguishing different microorganisms (Saylan et *al*., 2019).

2.4- Methods used to detect foodborne pathogens

2.4.1- Conventional methods

Conventional methods are based on culturing microorganisms on agar plates followed by standard biochemical identifications to detect foodborne bacterial pathogens existing in food (Mandal et *al*., 2011). These methods are usually inexpensive and simple but they can be time consuming as they depend on the capability of the microorganisms to grow in different culture media for example; pre-enrichment media, selective enrichment media and selective plating media. Indeed, conventional methods require two to three days for the preliminary identification and more than a week to confirm the species of the pathogens (Zhao et *al*., 2014). Also, they can be laborious as the preparation of culture media, the inoculation of plates and colony counting demands time (Mandal et *al*., 2011).

On the other hand, rapid methods are sensitive enough to detect pathogens present in low numbers in food. Sensitivity is considered important because only one pathogen in food has the risk to cause infection. These methods are more time-efficient, labor saving and prevent human errors (Mandal et *al*., 2011). However, rapid methods have their advantages and limitations. Generally, rapid detection methods are classified into nucleic acid-based, biosensor-based and immunological-based methods (Zhao et *al*., 2014).

2.4.1.1- Nucleic acid-based methods

Nucleic acid-based methods detect a specific DNA or RNA sequences in the target pathogen. The toxin-related genes in these pathogens can be detected by nucleic acid-based methods (Zhao et *al*., 2014). Nucleic-acid based methods detect the specific genes in the target pathogens resulting in preventing uncertain or wrongly interpreted results. The recent nucleic acid-based methods described are simple polymerase chain reaction (PCR), multiplex polymerase chain reaction (mPCR), real-time/quantitative polymerase chain reaction (qPCR), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and microarray technology.

PCR allowed the recognition of a distinct bacterial pathogen present in food by detecting a specific target DNA sequence (Velusamy et *al*., 2010). PCR have been employed to detect numerous foodborne pathogens like *Listeria monocytogenes*. *E. coli* O157: H7, *S. aureus*, *Campylobacter*

jejuni, *Salmonella* spp. and *Shigella* spp. (Cheah et *al*., 2008 ; Lee et *al*., 2008; Alves et *al*., 2012; Chiang et *al*., 2012; Zhou et *al*., 2013).

2.4.1.2- Immunological based methods

Immunological tests are based on the interaction between the antigen and the antibody to detect the bacteria's presence. Indeed, enzyme-linked immunosorbent assay (ELISA) is considered one of the most known immunological techniques. Multiplexed immunoassays have been demonstrated to successfully detect a mixture of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes* (Zhang, 2013).

2.4.2-Sensor based methods

2.4.2.1- Electrochemical techniques: E-tongues

In 1995, the concept of electronic tongue was introduced, and it was used for both qualitative and quantitative determinations (Legin et *al*., 2000; Vlasov et *al*., 2005). Electronic tongues are based on an array of sensors that have low selectivity and cross sensitivity towards the desired compounds in the sample. These analytical devices usually comprise an array of chemical sensors being not specific to any compound but, aiming instead, to provide an electrochemical profile of the matrix under analysis (Zhao et *al*., 2011).

The gathered information can allow establishing a digital fingerprint of the matric, which is extracted using appropriate multivariate pattern recognition tools. Indeed, they became a research topic due to their potential applications in the rapid evaluation of the safety and quality of food. In comparison with other analytical methods, they are easy to build and easy to use, cost-effective providing a fast analysis, being in some cases a non-invasive/non-destructive analytical method (Ghasemi- Varnamkhasti et *al*., 2018).

The electronic tongue (E-tongue) is a multisensory device that allows to obtain non-redundant information of a liquid matrix, being both commercial and lab-made devices used in several fields (Cetó, Voelcker & Prieto-Simón, 2016; Rodriguez-Méndez et *al*., 2014; Song et *al*., 2014). The E-tongue consists of three components: firstly, an automatic/manual sampler which is featured mostly in the commercial systems; secondly, an array of sensors with different selectivity and sensitivity and lastly, a chemometric software to process the signals recorded by the sensors' arrays and to

provide either qualitative or quantitative results (Ciosek and Wróblewski, 2007; Kalit et *al*., 2014; Tahara and Toko, 2013).

The most common E-tongues are based either in potentiometric, voltammetric techniques. Potentiometric methods are based on measuring the potential between two electrodes in the absence of an external flow of current (Ghasemi- Varnamkhasti et *al*., 2018). Potentiometric electrochemical devices sensors can be ion selective membranes or polymeric membranes (eg, lipid membranes…), involving a mixture of sensor additive chemical compounds, plasticizers and adequate polymeric matrix, applied using appropriate techniques to a solid support (Dias et *al*., 2017). The type of sensors and relative composition included in the E-tongue are established according to their capability to distinguish the different basic tastes and their levels (Baldwin et *al*., 2011). Voltammetric sensors are based on a variable that is introduced into the system where the electroactive compounds that are present in the sample are oxidized or reduced, that leads eventually to the generation of a flow of anodic or cathodic current (Ghasemi- Varnamkhasti et *al*., 2018). they are an array of noble metal working electrodes (eg, gold, palladium, platinum, and silver) and electrodes with many coating membranes (eg, polymers and epoxy-graphite) (Dias et *al*., 2017).

E-tongues have been used in the analyses of foods, water quality (Martinez-Bisbal et *al*., 2017), wines (Cleto et *al*., 2012), urine for disease detection and sensing of explosive materials. Electrical impedance spectroscopy (EIS) is considered as a very potent analytical tool. It consists on measuring the impedance of the sample for a certain range of frequencies. EIS has been employed in many different fields such as health (Braun et *al*., 2017; Halter et *al*., 2008), food engineering (Watanabe et *al*., 2018; Masot et *al*., 2010) and materials characterization (Masot et *al*., 2010; Zhao, Wang & Hammond, 2011). Voltammetric E-tongues have been used for recognizing the quality of milk due to microbial growth, classification of different types of fermented milk (Winquist et *al*., 2000), recognition of milk adulteration (Paixao and Bertotti, 2009) and monitoring of off-flavors in the incoming milk (Winquist et *al*., 2005).

Potentiometric E-tongues are usually employed to monitor storage time of foods and beverages, like pork (Gil et *al*., 2011), fish (Gil et *al*., 2008) and red wine (Parra et *al*., 2006).
Potentiometric sensors are most often used in the development of E-tongues with various applications: fermentation processes monitoring, identification of the botanic origin of honey,

evaluation of the impact of micro-oxygenation in the process of wine aging in the presence of oak chips, etc. (Dias et *al*., 2015 ; Gerstl, Joksch & Fafilek, 2013 ; del Valle et *al*., 2014; Mednova et *al*., 2009; Peris and Escuder-Gilabert, 2013; Schmidtke et *al*., 2010).

E-tongues have been largely employed in the field of food analysis: such as quality control, during the process monitoring (Martin et *al*., 2012), in the shelf-life investigation (Rodriguez-Mendez et *al*., 2009); environment monitoring pesticide residue detection (Valdés-Ramirez et *al*., 2009), and water quality monitoring (Mourzina et *al*., 2001); medical and pharmaceutical applications: for example, disease diagnosis (Arias, Perry & Yang, 2010; Chen et *al*., 2009), and development of liquid dosage forms (Woertz et *al*., 2010). Indeed, they mainly can be used also to detect and distinguish microorganisms and to monitor the growth of foodborne pathogens (Zhao et *al*., 2011).

Through miniaturizing and automating. electronic tongues can be used for on-line, in-line or real-time analyses. Another advantage of E-tongues is that they can be used as a non-destructive analytical method (Khan et *al*., 2016; Cetó, González-Calabuig & del Valle, 2015; Medina-Plaza et *al*., 2015).

Throughout the years, researchers succeeded in using E-tongues in many fields. Wei et *al*. (2012) successfully used the voltammetric E-tongue to monitor the storage time of the unsealed pasteurized milk and to investigate whether the total bacterial count and viscosity of milk samples could be predicted by the E-tongue. Martinez- Bisbal et *al*. (2019) monitored the degradation process of microalgae after the concentration stage at the end of the production process using a voltammetric E-tongue and impedance spectroscopy. Han et *al*. (2014) successfully used a commercial E-tongue system coupled with linear and nonlinear multivariate regression algorithms for the accurate and easy determination of total viable counts (TVC) in fish during its storage. They showed also that E-tongue together with back propagation neural network (BP-NN) has a great potential for rapid, convenient and accurate determination of fish microbiological quality during storage. Söderstrom et *al*. (2003) indicated that an E-tongue could be used for the recognition of different microbial species and to reveal food contamination. Zhao et *al*. (2011) successfully used the smart tongue to analyze the growth of molds in liquid medium and differentiate four mold species (*Aspergillus*, *Penicillium*, *Mucor*, and *Rhizopus*), and to detect changes in the medium that occurred during mold growth and to discriminate between different types of molds during the exponential growth phase. Ruiz-Rico et *al*. (2013) used voltammetric E-tongues to evaluate the

shelf-life of cod stored in cold conditions, being able to successfully discriminate between fresh (days 0 and 1) and spoiled fish more than 4 days of storage. Indeed, the statistical models obtained with the E-tongue data could predict certain physico-chemical and microbial parameters namely total volatile basic (TVB) and mesophilic bacteria, two of the best fish spoilage indices. Abu-Khalaf and Abu Rumaila (2020) used E-tongues to classify different *Fusarium* strains. They concluded that the E-tongue could be used as a new tool for microorganism's classification at the strain level. Goméz et *al*. (2019) used an E-tongue to detect *E. coli* bacteria and discriminate it from other bacteria, as well as to assess *E. coli* concentrations. They also showed that the device could be successfully applied to differentiate: *E. coli*, *P. aeruginosa* and *Klebsiella oxytoca* up to 99.4% variance of the data group. Al Ramahi et *al*. (2019) evaluated the feasibility of an E-tongue in the diagnosis of some bacterial infections and to establish the minimum time required for the detection of the microorganism responsible for the infection. They successfully were able to identify bacterial cultures of *E. coli*, *S. aureus* and *P. aeruginosa* at times shorter than those required by classical culture based methods.

2.4.2.2- Biosensor techniques

Biosensor is an analytical device that consists of two fundamental elements: a bioreceptor and a transducer. The bioreceptor is responsible to recognize the analytical target that either can be a: Biological material for example: enzymes, antibodies, nucleic acids and cell receptors, or a material that had been derived biologically like: aptamers and recombinant antibodies, or biomimic: imprinted polymers and synthetic catalysts.

The transducer that transforms the biological interactions into a measurable electrical signal can be optical, electrochemical, mass-based, thermometric, micromechanical or magnetic (Velusamy et *al*., 2010; Zhao et *al*., 2014). The recent biosensors that are usually used to detect foodborne pathogens are optical, electrochemical and mass-based biosensors (Zhang, 2013; Zhao et *al*., 2014).

2.4.2.2.1- Optical biosensors

Waswa et *al*. (2007) used SPREETA biosensor for the detection of *E. coli* O157:H7 in milk, apple juice and ground beef. In addition, Son et *al*. (2007) and Lan et *al*. (2008) *Salmonella Enteritidis* and *Salmonella Typhimurium* were also detected by SPREETA biosensor. Besides, Leonard et *al*. (2004) detected *Listeria monocytogenes* by BIACORE 3000 biosensor. Bokken et

al. (2003); Waswa et *al*. (2006) and Wang et *al*. (2011) successfully detected *Salmonella* Group B, D, and E. *Escherichia coli* O157:H7 and *Salmonella Enteritidis* by BIACORE biosensor.

2.4.2.2.2- Mass-based biosensors

Mass-based or also called as mass-sensitive biosensors are established to detect the small changes in mass. This type of biosensors involves the use of piezoelectric crystal which will get stimulated by a vibration at a certain frequency when induced by an electrical signal of a certain frequency.

2.5-Advantages and limitations of detection methods

Rapid detection methods can be categorized into conventional methods: Nucleic acid based methods that comprise simple PCR, multiplex PCR, and real-time PCR. Biosensors techniques based that can be divided into optical biosensors, electrochemical biosensors and mass based biosensors. And finally, immunological based that involves ELISA. These rapid detection methods have their advantages and their drawbacks. Law et *al*. (2015) summarized the advantages and limitations of these rapid detection methods and they are presented in Table 1.

Table 1: Advantages and limitations of rapid detection methods (Law et *al*., 2015)

Detection method		Advantages	Limitations	References
Nucleic acid-based	Simple PCR	*high sensitivity *high specificity *automated *reliable results	*affected by PCR inhibitors *requires DNA purification	Mandal et *al*., 2011; Zhang et *al*., 2013; Park et *al*.. 2014
	Multiplex PCR	*high sensitivity *high specificity *detection of multiple pathogens *automated	*affected by PCR inhibitors *difficult to distinguish between viable and non-viable cells	Mandal et *al*., 2011; Zhang et *al*., 2013; Park et *al*., 2014

			*primer design is crucial	
	Real-time PCR	*high sensitivity *high specificity *rapid cycling *reproducible *does not require post-amplification producers processing *real-time monitoring PCR amplification products	*high cost *difficult for multiplex real-time PCR assay *affected by PCR inhibitors *difficult to distinguish between viable and non-viable cells	Mandal et *al*., 2011; Zhang et *al*., 2013; Park et *al*., 2014
Biosensor-based	Optical biosensors	*high sensitivity *enables real-time or near real-time detection *label free detection system	*high cost	Ivnitski et *al*., 1999; Mandal et *al*., 2011; Zhang et *al*., 2013
	Electrochemical biosensors	*can handle large numbers of samples *automated *label-free detection	*low specificity *not suitable for analyzing samples with low amount of microorganisms *analysis may interfere by food matrices	Ivnitski et *al*., 1999; Mandal et *al*., 2011; Zhang et *al*., 2013

			Many washing steps	
	Mass-based biosensors	*cost effective *easy to operate *Label free detection *real-time detection	*low specificity *low sensitivity *long incubation time of bacteria *many washing and drying steps *regeneration of crystal surface may be problematic	Ivnitski et *al*., 1999; Mandal et *al*., 2011; Zhang et *al*., 2013
Immunological based	ELISA	*specific *can be automated so that it is more time effcient and labor-saving *allows the detection of bacterial toxins *can handle large numbers of samples	*low sensitivity *false negative results *may result in cross-reactivity with closely related antigens *pre-enrichment is required in order to produce the cell surface antigens *requires labeling of antibodies or antigens	Zhang, 2013; Park et *al*., 2014; Zhao et *al*., 2014

3. Materials and methods

3.1- Microorganisms growth

3.1.1- Bacterial strains

This work aimed to detect and quantify three foodborne pathogens, namely *Staphylococcus aureus* ATCC653 and *Enterococcus faecalis* ATCC29212 both Gram positive spherical-shaped and one Gram-negative rod-shaped bacteria (*Pseudomonas aeruginosa* ATCC15442). Additionally, *Escherichia coli* ATCC29998 was used since it is a broad hygiene/fecal indicator.

3.1.2- Culture medium

Brain Heart infusion medium (BHI) was used for the growth of all microorganisms. To prepare the culture media for the four microorganisms, 11.1 g of Brain Heart Infusion were dissolved in 300 mL of distilled water (Reverse osmosis, Water Storage Tank, PA-E). For each microorganism, eight Erlenmeyer flasks were prepared. The media was autoclaved at 121°C during 15 min.

3.1.3- Inoculum preparation

Each bacterial strain was grown in Erlenmeyer containing 10 mL of Brain Heart Infusion (BHI) (PanReac AppliChem, ITW Reagents) followed by incubation at 37°C on an orbital shaker (Orbital incubator S1500, Stuart) (Figure 1) at 90 rpm for 24h to reach the stationary growth phase of cells. Later, 700 µL of bacterial culture was suspended in 300 µL of glycerol (30%) and the aliquots were frozen at -20°C.

Figure 1: Orbital incubator used in the study

3.1.4- Growth conditions

Bacterial inoculum was prepared as described above, 700 µL of bacterial inoculum was used to inoculate aseptically 300 mL of Brain Heart Infusion in 1L Erlenmeyer flasks. All incubations were carried out at 37 ºC using an orbital shaker (Orbital incubator S1500, Stuart) (Figure 2) adjusted to 90 rpm for 24 h to reach the stationary phase of cells.

Figure 2 : Microorganisms growth

A total of eight independent fermentations were carried out for each microorganism studied. At the end of individually fermentation, a Gram staining was performed, in order to verify the nonexistence of culture contamination. The cell density of suspension was assessed by measuring the optical density (DO) at 560 nm using a spectrophotometer (VWR, UV-3100PC Spectrophotometer) (Figure 3).

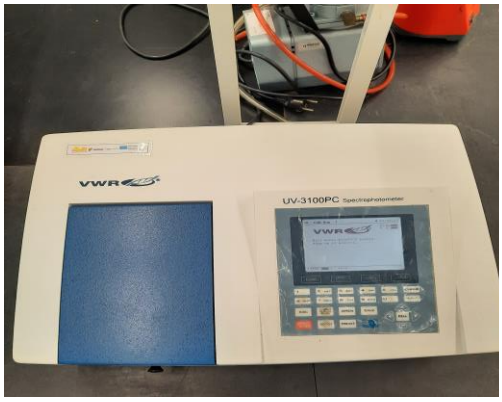

Figure 3: Spectrophotometer used in this study

3.1.5- Determination of dry weight

Biomass yield of each culture growth was assessed by dry weight. For this purpose, each bacterial culture was divided into four 50 mL polypropylene centrifuge tube. Tubes were filled with the same weight and same volume and later, tubes were centrifuged for 10 min at 4000 rpm (Eppendorf, Centrifuge 5810 R). After centrifugation, the liquid phase was removed and the cells were suspended by adding 1 ml of distilled water and transferred to pre-weighed Eppendorf tubes and centrifuged for 5 min at 13000 rpm. The cells harvested were dried in an incubator device (Memmert, Rost frei) at 37°C for 48h and weighed, after cooling in a desiccator, on an analytical balance with accuracy of 0.1 mg (Pioneer TM, OHAUS). The biomass concentration was expressed as a mg of cell dry weight per mL of bacterial culture.

3.1.6- Gram staining

Gram staining was performed as described by Gregersen (1978). Heat-fixed smear on microscope slide was cooled until ambient temperature (~ 20°C) then the crystal violet solution was added on the smear for 20s after that samples were washed with Lugol's iodine (potassium solution with iodine in water) and left for 1min and then a rapid decolorization with ethanol 96%

for 15s followed by washing with distilled water and then smears were counterstaining with safranin solution for 30s. The smears were then washed again and the excess of water was dried with absorbent paper. Observation was carried out by optical microscope at magnification of 1000x.

3.2- Calibration curve

A series of dilutions were carried out aiming to establish a correlation between the OD measured at 560 nm in the spectrophotometer and the biomass content determined by dry weight. The Optical Density of the supernatant discarded after centrifugation of all samples was measured, the aim to evaluate if all cells were harvested after centrifugation.

For each microorganism growth overnight, a set of five serials of two fold dilutions were prepared in 3 mL of final volume (Figure 4) and the cell density of all samples was assessed by measuring the optical density (OD) at 560 nm. Samples for which the absorbance value exceed the linear range determination e.g. OD> 0.7 were re-analyzed at higher dilution, which was the case for the overnight culture and the first two dilutions.

The dilutions made were described in Figure 4.

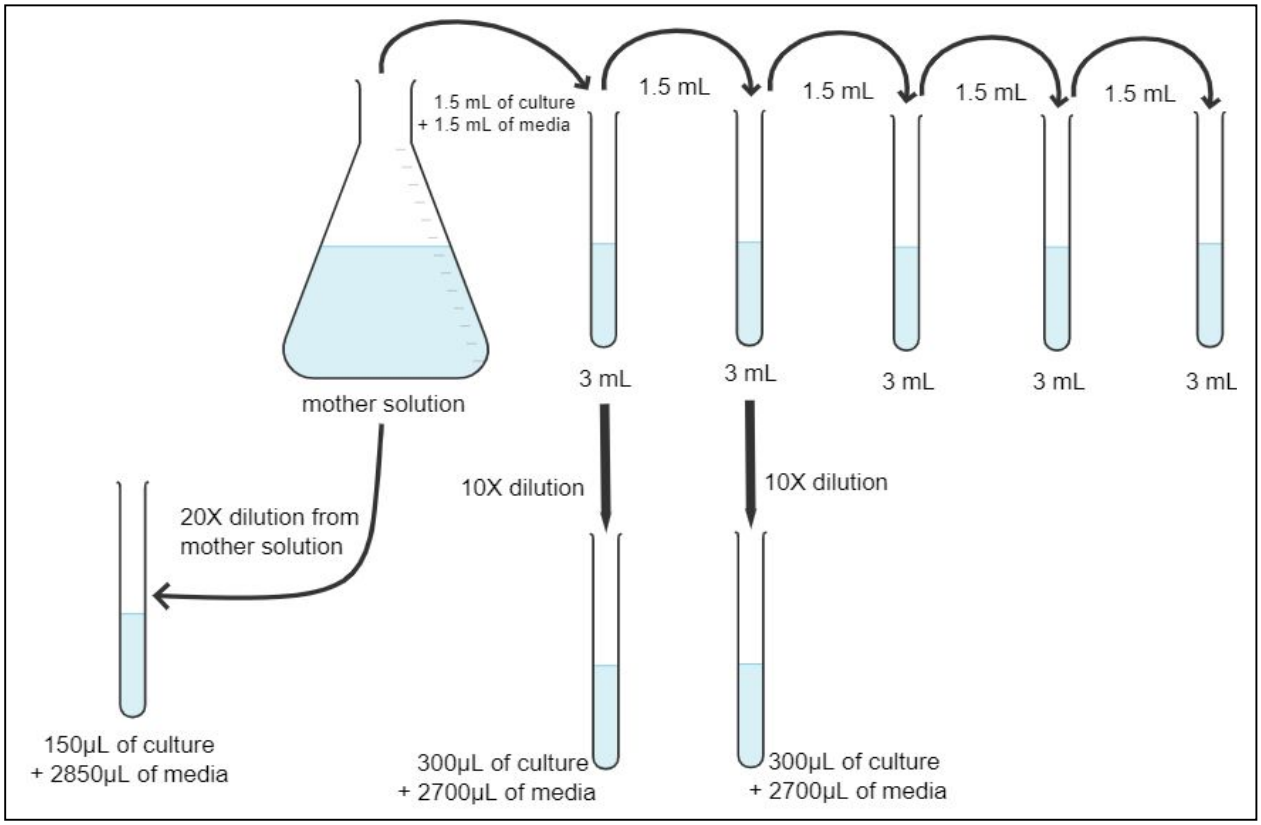

Figure 4: Culture dilutions

3.3- Electronic tongue

3.3.1- Apparatus and sensors composition

A lab-made potentiometric E-tongue was used in this study, which has been previously developed by the research team (Guilherme et *al*., 2020). The device included two potentiometric arrays built in an acrylic cylinder body with a height of 6.5 cm, a diameter of 1.5 cm; wells of 0.5 cm of width and 1mm of depth with support of Araldite epoxy resin and graphite in the proportion of 50% (Figure 5). Each array comprised 20 lipid polymeric cross-sensitive sensor membranes (40 sensors in total), each corresponding to a different mixture of an additive compound (~3%, methyltrioctylammonium chloride, octadecylamine, oleic acid and oleyl alcohol) and a plasticizer (~32%, bis (1-butylpentyl) adipate, dibutyl sebacate, dioctyl phenylphosphonate, 2-nitrophenyl-octyl ether and tris(2-ethylhexyl) phosphate), plus a high molecular weight polyvinyl chloride (PVC, ~65%). Each sensor was coded with a letter S (for sensor) followed by a number related to the sensor array (1: or 2) and a number related to the lipid sensor membrane applied (1 to 20),

corresponding to different combinations of plasticizer and additive compounds, as described in Table 2.

Table 2: E-tongues sensor codes and related composition (%) of the respective lipid membranes (type/pair of additive and plasticizer).

Sensor Code		Plasticizer (~32%)	Additive (~3%)
1st array	**2nd array**		
S1:1	S2:1	Bis(1-butylpentyl) adipate	Octadecylamine
S1:2	S2:2		Oleyl alcohol
S1:3	S2:3		Methyltrioctylammonium chloride
S1:4	S2:4		Oleic acid
S1:5	S2:5	Dibutyl sebacate	Octadecylamine
S1:6	S2:6		Oleyl alcohol
S1:7	S2:7		Methyltrioctylammonium chloride
S1:8	S2:8		Oleic acid
S1:9	S2:9	2-nitrophenyl-octylether	Octadecylamine
S1:10	S2:10		Oleyl alcohol
S1:11	S2:11		Methyltrioctylammonium chloride
S1:12	S2:12		Oleic acid
S1:13	S2:13	Tris(2-ethylhexyl) phosphate	Octadecylamine
S1:14	S2:14		Oleyl alcohol
S115	S2:15		Methyltrioctylammonium chloride
S1:16	S2:16		Oleic acid
S1:17	S2:17	Dioctyl phenylphosphonate	Octadecylamine
S1:18	S2:18		Oleyl alcohol
S1:19	S2:19		Methyltrioctylammonium chloride
S1:20	S2:20		Oleic acid

The sensor membranes were linked to a multiplex Agilent Data Aquisation Switch Unit (model 34970A), and monitored by an Agilent BenchLink Data Logger software. Each assay took 5 min, in which signals recorded the potentiometric signals of 40 sensor membranes. In addition, an Ag/AgCl double junction glass electrode was used as a reference electrode. Later, the E-tongue

was placed in a HCl solution (0.01 M) as it was used also to measure the signals stability during the day or the occurrence of signal drifts.

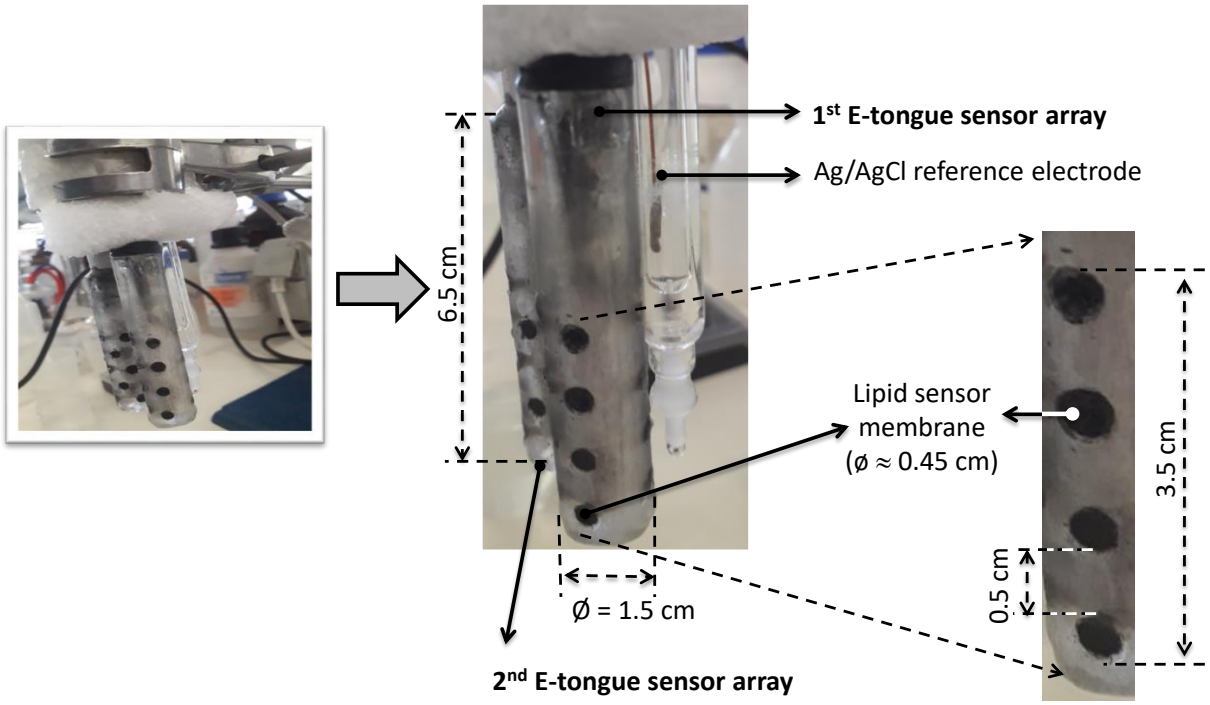

Figure 5 : E-tongue device (adapted from Guilherme et *al*., 2020)

3.3.2- E-tongue microorganism's analysis: sample preparation and potentiometric assays

Samples stored at -20°C were put to room temperature (~20°C) for 20 min then rehydrated with deionized water. At the beginning, 1 ml of deionized water was added to the samples and left for 1h at room temperature. Once the cells were completely re-suspended, the biomass was successively mixed with deionized water in order to ensure that all cells were re-suspended, being obtained a final volume of 100 mL, which allowed immersing the two cylindrical E-tongue arrays. enabling the contact of the sensor membranes with the microorganism's aqueous solutions.

A preliminary E-tongue analysis was carried out on 32 samples (Table 3), in which the solution system was agitated for 5 min and after each E-tongue analysis, the system was smoothly cleaned with deionized water and the remaining water on the sensors' surface was removed with absorbent paper. After five assays, for evaluating the sensors' intra-day signal stability. The E-tongue

potentiometric profiles of solutions of HCl (0.1 M) were recorded ten times in the same day, being all the assays performed within an 8h time period.

Table 3 presents the list of 32 microorganisms' cultures concentrations used for the E-tongue analysis. Since each microorganism undergo two inoculations at different dates, they were labeled as 1st assay and 2nd assay.

Table 3: List of 32 microorganism's cultures and concentrations used for the E-tongue analysis

Microorganisms	**Samples**	**Concentrations (mg/mL)**
E. coli (1st assay)	1.1	0.309
	2.3	0.548
	3.3	0.542
	4.3	0.491
E. coli II (2nd assay)	1.3	0.666
	2.2	0.639
	3.3	0.613
	4.1	0.650
S. aureus (1st assay)	1.1	0.612
	2.4	0.606
	3.4	0.6
	4.2	0.563
S. aureus II (2nd assay)	1.4	0.663
	2.3	0.570
	3.1	0.702
	4.1	0.511
E. faecalis (1st assay)	1.4	0.437
	2.3	0.432
	3.1	0.421
	4.1	0.538
E. faecalis II (2nd assay)	1.1	0.282
	2.4	0.279
	3.3	0.309

	4.2	0.294
P. aeruginosa (1st assay)	1.2	0.326
	2.4	0.330
	3.2	0.333
	4.1	0.324
P. aeruginosa II (2nd assay)	1.3	0.420
	2.2	0.424
	3.1	0.431
	4.1	0.452

A second set of E-tongue assays were conducted for each microorganism in which the solution system was agitated as previously mentioned during 5 min and after five assays the intra-day signal stability was measured with HCl (0.1 M). Samples were selected according to the mass of cells. Two types of assays were made:

- One to evaluate the capacity of the E-tongue to identify and distinguish the four microorganisms under study. For this, in the same day the E-tongue analysis was performed for eight different solutions of each microorganism, that had different concentrations. In total 32 solutions were randomly analyzed. In order, to assess the intra-day signal repeatability, HCl solutions (0.1 M) were also analyzed after the analysis of six microorganisms' solutions. This step also allowed promoting a deeper cleaning of the sensors.
- Secondly, the capability of the E-tongue to quantify the biomass content for each microorganism was evaluated. For this, for each of the four microorganisms 'solutions of re-suspended cells with different concentrations were obtained by combining the cell dried mass of one or more Eppendorf's.

At first, nine solutions were analyzed and on the contrary of the first analysis, samples were mixed together according to the concentrations (Table 4). Secondly, a dilution was made on initial solutions in which 25 ml of initial solution was diluted in 75 ml of deionized water and the rest 75 ml of initial solution was diluted in 25 ml of deionized water to obtain 18 new diluted solutions.

Table 4: List of the nine solutions used for each microorganism according to their concentrations

Microorganism	Samples	Concentrations (mg/mL)
E.coli A (1st assay)	1.2	0.327
	1.4	0.318
	4.1	0.539
E.coli B (1st assay)	2.4	0.566
	4.2	0.562
	4.4	0.562
E.coli C (1st assay)	2.1	0.557
	3.1	0.553
	3.2	0.546
	3.4	0.551
E.coli D (1st assay)	2.2	0.568
E.coli E (1st assay)	1.3	0.333
E.coli II F (2nd assay)	1.4	0.666
	2.1	0.672
	4.2	0.667
	4.3	0.67
E.coli II G (2nd assay)	2.3	0.659
	4.4	0.657
E.coli II H (2nd assay)	3.2	0.641
E.coli II I (2nd assay)	1.1	0.648
	1.2	0.635
	2.4	0.649
	3.1	0.64
	3.4	0.631

Microorganism	Samples	Concentrations (mg/mL)
Ent A (1st assay)	3.2	0.428
	1.3	0.556
	2.1	0.435
	4.3	0.561
Ent B (1st assay)	1.1	0.441
	2.2	0.442
Ent C (1st assay)	2.4	0.499
	3.4	0.467
	3.3	0.435
	4.2	0.493
	4.4	0.591
Ent D (1st assay)	1.2	0.575
Ent II E (2nd assay)	1.4	0.28
Ent II F (2nd assay)	4.1	0.305
	4.3	0.305
	4.4	0.304
Ent II G (2nd assay)	3.1	0.314
	3.2	0.313
Ent II H (2nd assay)	3.4	0.311
Ent II I (2nd assay)	1.2	0.294
	2.1	0.254
	2.2	0.276
	2.3	0.282
	1.3	0.292

Microorganism	Samples	Concentrations (mg/mL)
Staph A (1st assay)	1.2	0.614
	1.3	0.608
	1.4	0.622
Staph B (1st assay)	2.1	0.623
	2.2	0.626
Staph C (1st assay)	2.3	0.604
	3.1	0.612
	3.2	0.597
	3.3	0.607
Staph D (1st assay)	4.1	0.586
	4.3	0.589
Staph E (1st assay)	4.4	0.593
Staph II F (2nd assay)	3.2	0.707
	3.3	0.707
Staph II G (2nd assay)	3.4	0.703
Staph II H (2nd assay)	2.4	0.575
	4.2	0.518
	4.3	0.517
	4.4	0.52
Staph II I (2nd assay)	1.1	0.677
	1.2	0.666
	1.3	0.658
	2.1	0.057
	2.2	0.572

Microorganism	Samples	Concentrations (mg/mL)
Pseud A (1st assay)	1.1	0.333
	1.3	0.354
	3.1	0.364
	3.4	0.377
Pseud B (1st assay)	1.4	0.325
	2.1	0.31
Pseud C (1st assay)	2.2	0.334
	3.3	0.325
	4.2	0.343
	4.3	0.351
	4.4	0.329
Pseud D (1st assay)	2.3	0.317
Pseud II E (2nd assay)	3.4	0.429
Pseud II F (2nd assay)	2.4	0.454
	4.3	0.452
	4.4	0.456
Pseud II G (2nd assay)	4.2	0.436
	1.4	0.492
Pseud II H (2nd assay)	1.1	0.472
	2.1	0.479
	2.3	0.478
	3.2	0.461
	3.3	0.483
	1.2	0.447

3.4- Statistical analysis

The calibration curves between the OD and the biomass contents were established by single linear regression analysis. This technique was also applied to evaluate the response of each E-tongue sensor membrane to re-suspended cell aqueous solutions containing different known microorganisms' concentrations. Also, the E-tongue qualitative and quantitative performance

towards the microorganisms' differentiation and concentration evaluation was further assessed by applying multivariate statistical methods.

The classification performance of the E-tongue regarding the identification and differentiation of the four microorganisms under study was evaluated using principal component analysis (PCA) and linear discriminant analysis (LDA) coupled with the meta-heuristic simulated annealing (SA) variable selection algorithm (Bertsimas et Tsitsiklis, 1993; Kirkpatrick et *al*., 1983). The SA meta-heuristic algorithm was employed to identify the best sub-set containing the lower number of non-redundant E-tongue sensors (Veloso et *al*., 2016). The LDA performance was checked using two internal cross-validation (CV) variants, namely the leave-one-out CV (LOO-CV) and the repeated K-fold-CV (K equal to 4 folds, allowing to keep 25% of the initial data for validation purposes, being the data randomly split for 10x). The former is considered an over-optimistic internal validation technique and the latter aims to overcome this possible limitation and to minimize overfitting risks. Variable scaling and centering procedures were implemented as data normalization procedures. The PCA and LDA models outputs were graphically evaluated using 3D plots of the three most significant principal components (PC) or linear discriminant functions (LD), being calculated the sensitivity values (i.e., the percentage of samples correctly classified into the pre-established groups) for the latter approach.

4. Results and discussion

4.1- Biomass determination by dry weight

A total of eight independent fermentations were carried out for each microorganism studied. At the end of individually fermentation, a Gram staining was performed in order to verify the nonexistence of culture contamination. The Gram staining showed absence of any contamination and confirmed the purity of our cultures (Figure 6).

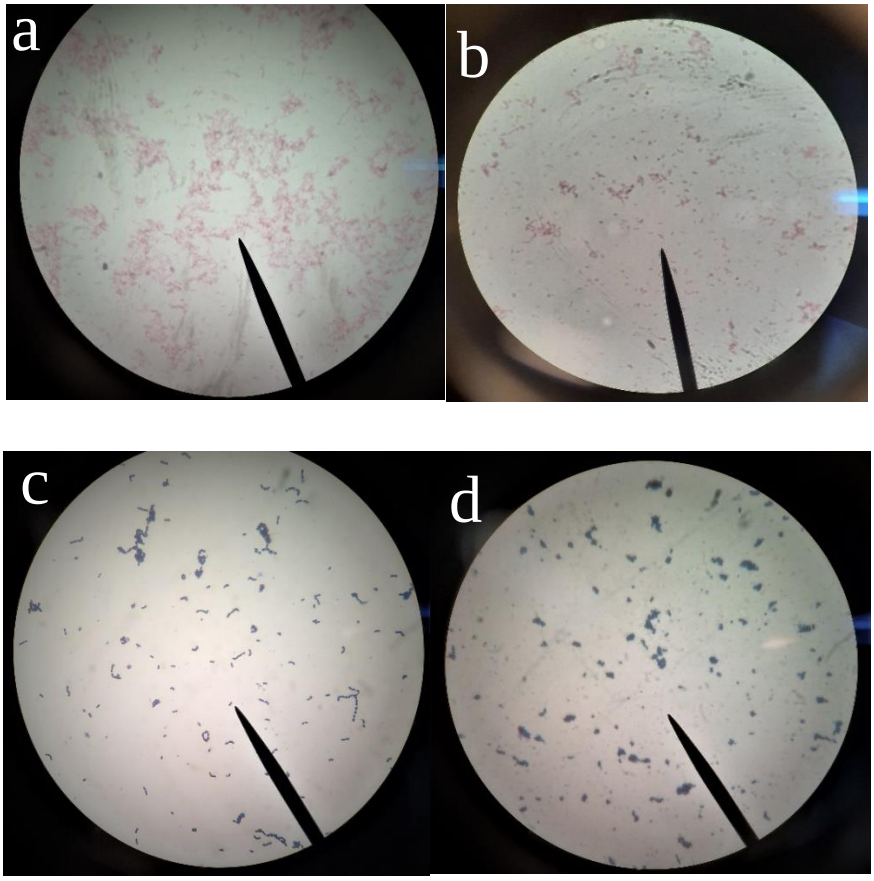

Figure 6: Gram staining of (a) *E. coli;* (b) *P. aeruginosa;* (c) *E. faecalis;* (d) *S. aureus* at magnification of 1000x in optical microscope

In Table 5 are presented the results of mean content± standard deviation of the dry weight of the eight fermentations for the four microorganisms used in the study.

Table 5: Dry weight of the four microorganisms in the eight fermentations

Microorganism	Dry weight (mg/mL)
E. coli	1.21±0.11
E. faecalis	0.98±0.09
P. aeruginosa	0.84±0.02
S. aureus	1.41±0.09

E. coli and *S. aureus* present the higher mean content of dry weight in the eight fermentations performed. On the other hand, *E. faecalis* and *P. aeruginosa* showed the lowest mean content of dry weight in the eight fermentations.

4.2- Calibration curve

Microbial dry weight is a standard biomass parameter that is employed in research and fermentations studies. However, the method for its determination is time consuming and laborious. This work aimed to relate the microbial biomass determined by dry weight and absorbance of culture measured at 560 nm in the spectrophotometer, a faster and easier procedure. For this purpose, for each overnight culture, a set of five serials of two fold dilutions were prepared and the cell density of all samples was assessed by measuring the optical density. The relationships between absorbance (560 nm) and dry weight (mg/mL) for all four microorganisms studied are displayed in Figure 7. For all microorganisms, the absorbance was a very good predictor of dry weight, as demonstrated by R2 that is close to 1.

Unfortunately, we observed that the centrifugation was not enough to harvest all the cells present in the culture, making it impossible to use the values obtained by spectrophotometry in the next work. This error can occur due to the non-precision of the micropipette, always keeping in made that such error can be made and we cannot recover all the cells from the culture.

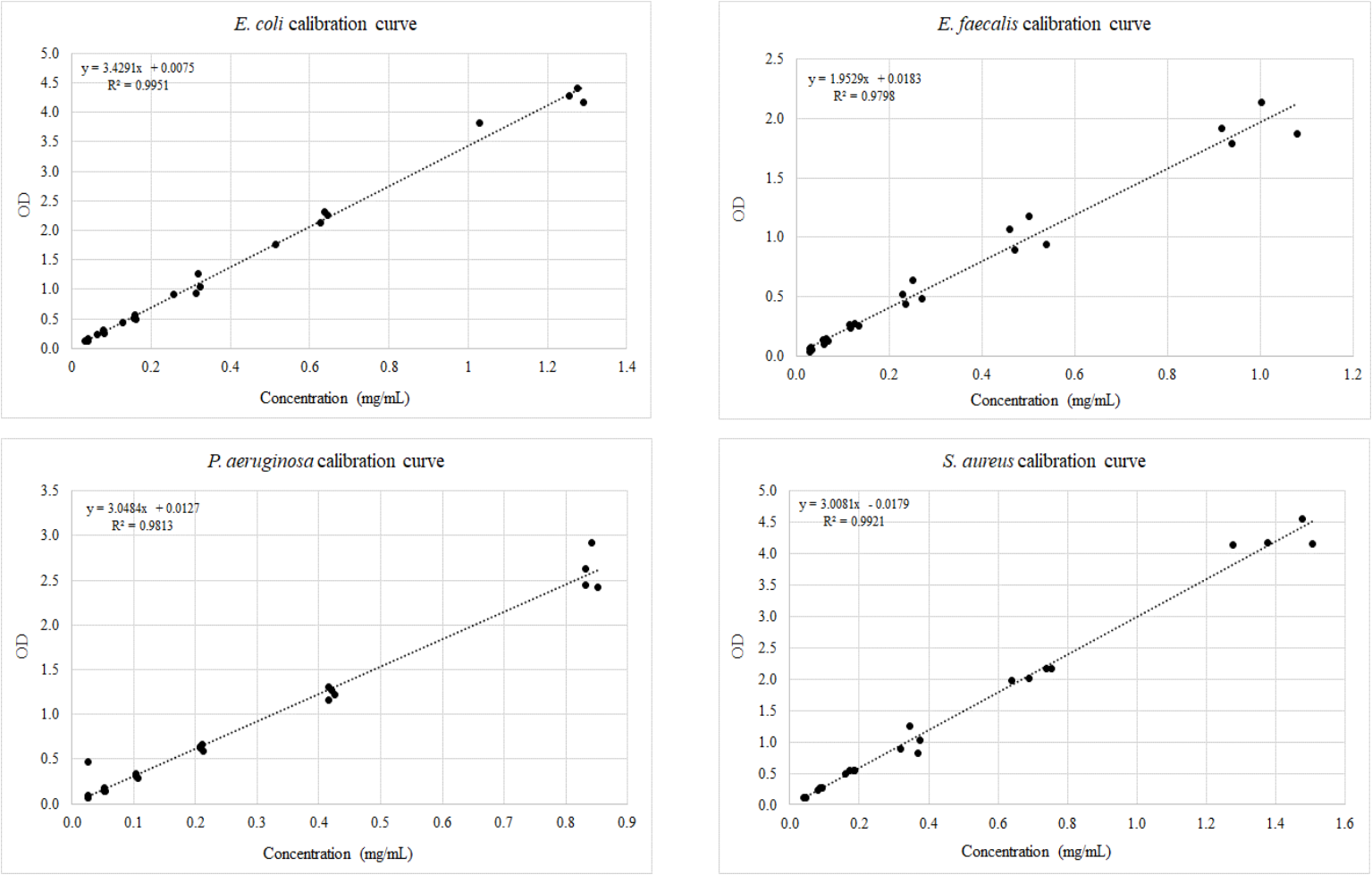

Figure 7: Calibration curve of the eight fermentations recorded between the OD measured at 560 nm and the microbial biomass of the four microorganisms

4.3- E-tongue measurements

4.3.1- E- tongue signals mean value and coefficient of variation

The rehydration of the samples was done carefully to make sure that all the cells that were conserved at -20°C were hydrated. At first, the rehydration was made by adding 1 mL of deionized water to all samples and left for 1h at room temperature. After 1h, the samples were vortexed, once the cells were completely re-suspended, the biomass was successively mixed with deionized water in order to ensure that all cells were re-suspended, to obtain a final volume of 100 mL. The rehydration of the samples was done successfully and allowed us to control the kinetic movement of the microorganisms.

In the Figure 8 below, the signals observed are mean values of eight independent assays for the four microorganism's aqueous solutions. In the present work, it was observed that the signals for all four microorganisms showed a maximum mean value of 350 mV at S2:20. The figures showed that the lowest signal mean value for the four microorganisms was observed at S1:13 with a value around 0.2 mV.

The coefficient of variation was calculated for the four microorganisms according to the equation: Coefficient of variation= (standard deviation/mean value) *100. Our study showed that *E. faecalis* present the highest value of coefficient of variation at the signal S1:13 with a value of 70.6 %. The lowest coefficient value was observed for *S. aureus* with a value of 0.5 %.

Although in general the signals recorded by each of the 40 E-tongue sensors did not varied substantially with all the microorganisms (CV ≤ 3%). On the other hand, some sensors showed a different response depending on the solution concentration (3 ≤ CV ≤30%), and this could be tentatively used to quantify the amount of *E. coli*, *E. faecalis*, *P. aeruginosa* and *S. aureus* in the solution.

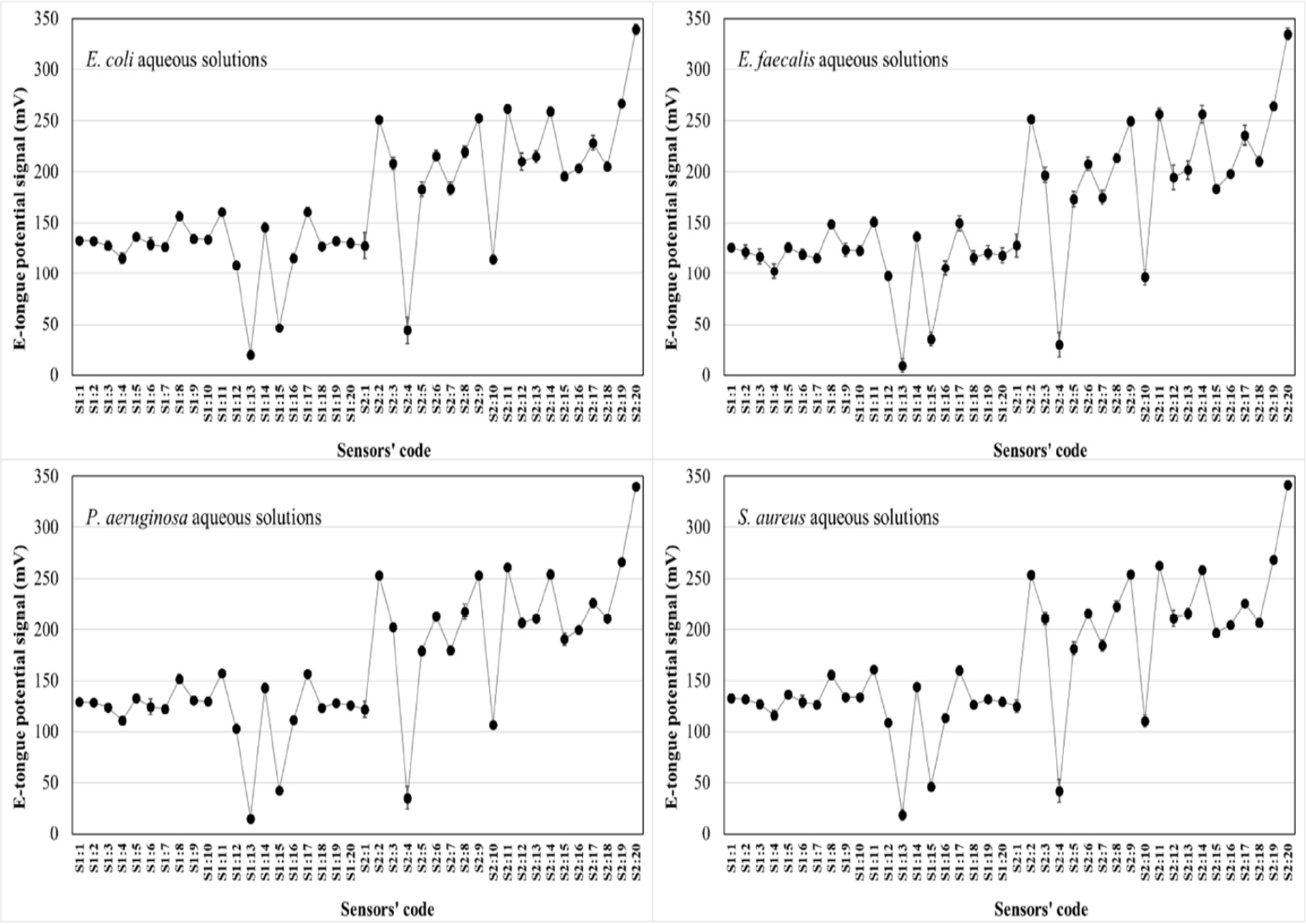

Figure 8: E-tongue potentiometric signal profiles recorded during the analysis of aqueous solutions of the four microorganisms

Before proceeding with the E-tongue analysis, a gram staining was performed for the microorganisms in order to assure the absence of lyse in the cells and to make sure that all the cells were rehydrated. The gram staining showed an absence of a lyse of cells for microorganisms but after running the E-tongue analysis on the four microorganisms (8h), a gram staining was performed again to confirm the state of the cells. We observed that *E. coli*, *S. aureus* and *E. faecalis* cells were intact but on the contrary, *P. aeruginosa* gram staining showed a lyse in the cells that can be due to the rehydration by the deionized water that could've affected the cells (Figure 9).

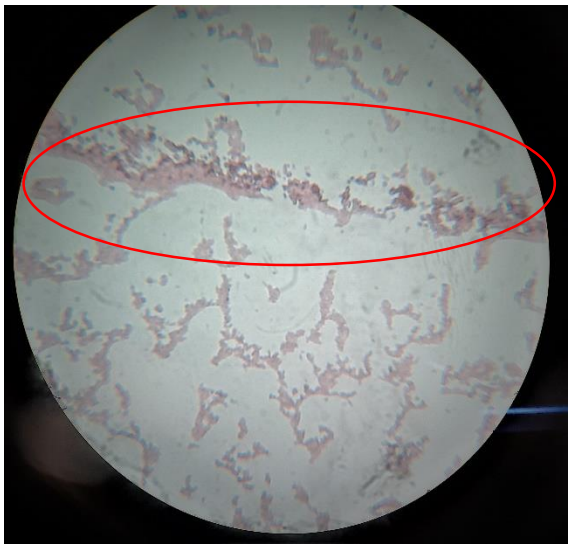

Figure 9: *P. aeruginosa* cell lyse after E-tongue analysis

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4.3.2- Microorganisms recognition and differentiation based on E-tongue potentiometric profiles

The possibility of recognizing and differentiating the four microorganisms under study, based on the potentiometric fingerprints recorded by the E-tongue during the analysis of solutions containing different amounts of each microorganism, was evaluated. For each microorganism, eight independent aqueous solutions were prepared by re-suspending known amounts of dry weight cells in 100 mL of deionized water in which the biomass ranged from E. *coli*: 0.309 to 0.666 mg/mL; *E. faecalis*: 0.279 to 0.538 mg/mL; *S. aureus*: 0.511 to 0.702 mg/mL; and, *P. aeruginosa*: 0.324 to 0.435 mg/mL, having in total 32 aqueous independent solutions analyzed in the same day, allowing a minimum signals' drifts and to avoid the need of complex signals' pretreatments. For the four studied bacteria, the E-tongue signals recorded by the 40 sensor lipid membranes varied within the same potential range (0.2 to 350 mV). Even so, the results from the PCA (Figure 14) showed that the potentiometric signals profiles acquired by the 40 E-tongue sensors allowed a satisfactory unsupervised recognition of *P. aeruginosa* and *E. faecalis*, contrary to *E. coli* and *S. aureus*, showed a clear over-plotting. Additionally, to assess the E-tongue classification capability, a LDA was performed, being the most discriminant and non-redundant sensors selected by the SA algorithm. The best E-tongue-LDA-SA model, which first three discriminant functions (LDs) explained 100% of the data variability, was established based on 12 E-tongue sensors (1st sensors array: 1st E-tongue array: S1:2, S1:5, S1:7, S1:8 and S1:9; 2nd sensors array: S2:4, S2:9, S2:10,

S2:11, S2:13, S2:14 and S2:18). The supervised discriminant model allowed to correctly classify 100% of the original grouped data (Figure 10). Moreover, the model showed sensitivities of 100% for the LOO-CV and of 98±5% for the repeated K-fold-CV internal-validation procedures. It should be noticed that, for the latter CV variant, which ensures that at each run 25% of the dataset are used for validation (i.e., 2 solutions of each microorganism, totalizing 8 among 32 independent samples, were kept aside for validation purposes). A misclassification was only observed between *E. faecalis* and *P. aeruginosa*. The unsupervised and supervised classification performances clearly pointed out the potential use of the E-tongue as an accurate and fast recognition device of the four microorganisms studied, being possible to predict its use as a preliminary quality/safety control tool, taking into account that these microorganisms are typical water-food contamination indicators. Indeed, Al Ramahi et *al*. (2020) used a commercial potentiometric E-tongue (α- Astree device with seven chemically modified solid potential sensors) to cluster different bacteria groups (*E. coli*, *P. aeruginosa* and, *S. aureus*) at different growing time-periods (from 15 to 24 h) to which the discrimination indices ranged from 83 to 96%. Also Au-Khalaf and Rumaila. (2020) used an α- Astree device trying to differenciate with PCA eight Fusarium isolates, which partially succeeded since the isolztes were clustered into main groups.

Also, ET has been tested for monitoring the quality of food such as milk; the taste sensor was capable to discriminate reliably between fresh and spoiled milk when it was stored at room temperature based on PCA (Al Ramahi et *al*., 2020).

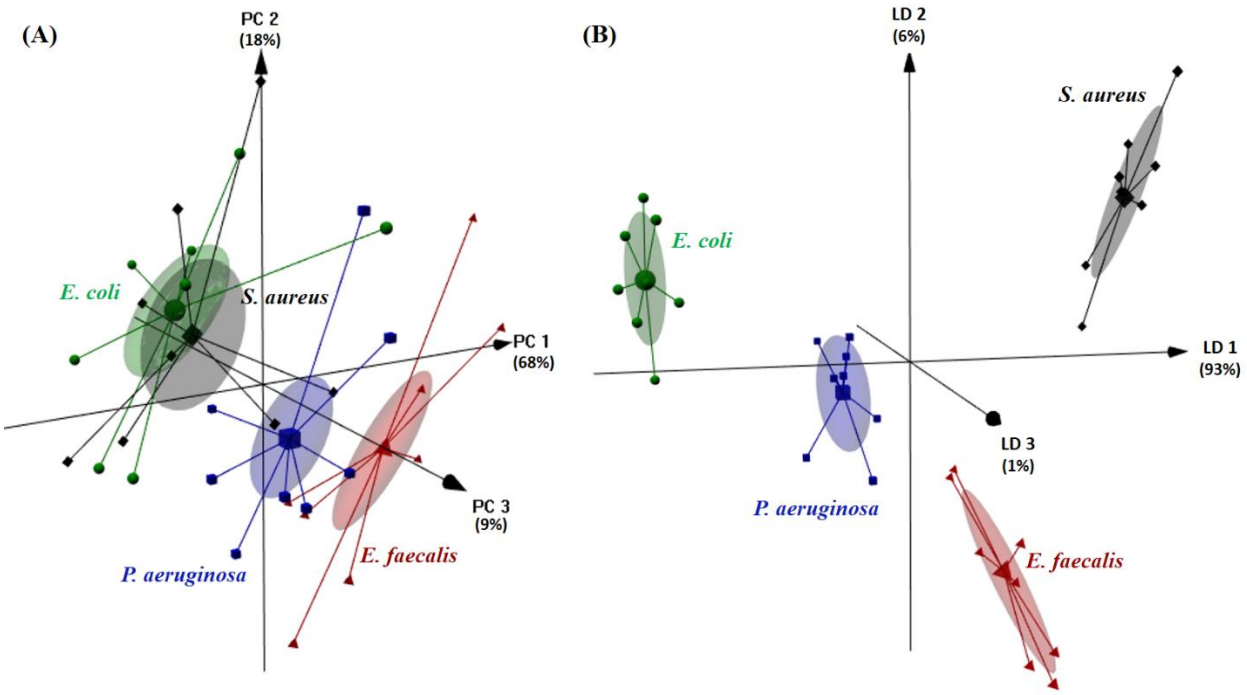

Figure 10: Differentiation (3D plots) of four common water-food contamination microorganisms based on the E-tongue signal profiles acquired during the analysis of aqueous solutions of *E. coli* (0.309 to 0.666 mg/mL, symbol ●), *E. faecalis* (0.279 to 0.538 mg/mL, symbol ▲), *S. aureus* (0.511 to 0.702 mg/mL, symbol ♦) and *P. aeruginosa* (0.324 to 0.435 mg/mL, symbol ■): (A) Unsupervised PCA recognition based on the potentiometric signals of 40 E-tongue sensors; and, (B) Supervised LDA classification based on the potentiometric signals of a set of 12 non-redundant E-tongue sensors, selected by the SA algorithm

5. Conclusion

In this study, the detection and discrimination of the four microorganisms studied (*E. coli*, *P. aeruginosa*, *E. faecalis* and, *S. aureus*) was determined by the use of a potentiometric E-tongue, comprising lipid polymeric sensor membranes, together with unsupervised and supervised chemometric tools (e.g., principal component analysis, PCA; linear discriminant analysis, LDA; and, multiple linear regression models, MLRM) was evaluated aiming to explore the advantages of these innovative (bio)sensing devices for microorganism's recognition and discrimination, in aqueous solutions. Our findings showed that the PCA that the potentiometric signals profiles acquired by the 40 E-tongue sensors allowed a satisfactory unsupervised recognition of *P. aeruginosa* and *E. faecalis*, contrary to *E. coli* and *S. aureus* that showed a clear over-plotting. In which an LDA was further performed and was able to correctly classify 100% of the original selected data.

The differentiation between different types of microorganisms was achieved by the mean use of E-tongues. The findings of this study shows that ET can identify microorganisms at times shorter than conventional methods.

References

Abu-Khalaf, N., Abu Rumaila, B., 2020. Electronic tongue and BOX-PCR for categorization of different Fusarium strains. Plant Cell Biotechnol. Mol. Biol. 21, 121–128.

Al Ramahi, R., Zaid, A.N., Abu-Khalaf, N., 2019. Evaluating the potential use of electronic tongue in early identification and diagnosis of bacterial infections. Infect. Drug Resist. 12, 2445–2451.

Alves, J., Marques, V.V., Pereira, L.F.P., Hirooka, E.Y., Oliveira, T.C.R.M.D., 2012. Multiplex pcr for the detection of *Campylobacter* Spp. and *Salmonella* Spp. in chicken meat. J. Food Saf. 32, 345–350.

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., Swings, J., Dellaglio, F., 2001. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. J. Dairy Res. 68, 303–316.

Argudín, M.Á., Mendoza, M.C., Rodicio, M.R., 2010. Food Poisoning and *Staphylococcus aureus* enterotoxins. Toxins 2, 1751–1773.

Baldwin, E.A., Bai, J., Plotto, A., Dea, S., 2011. Electronic noses and tongues: applications for the food and pharmaceutical industries. Sensors 11, 4744–4766.

Bavisetty, S.C.B., Vu, H.T.K., Benjakul, S., Vongkamjan, K., 2018. Rapid pathogen detection tools in seafood safety. Current Opinion in Food science. 20, 92–99.

Berhe, H.W., Makinde, O.D., Theuri, D.M., 2019. Co-dynamics of measles and dysentery diarrhea diseases with optimal control and cost-effectiveness analysis. Appl. Math. Comput. 347, 903–921.

Bertsimas, D., Tsitsiklis, J., 1993. Simulated annealing. Stat. Sci. 8, 10–15.

Bokken, G.C.A.M., Corbee, R.J., Van Knapen, F., Bergwerff, A.A., 2003. Immunochemical detection of *Salmonella* group B, D and E using an optical surface plasmon resonance biosensor. FEMS Microbiol. Lett. 222, 75–82.

Braun, R., Mangana, J., Goldinger, S., French, L., Dummer, R., Marghoob, A., 2017. Electrical Impedance Spectroscopy in skin cancer diagnosis. Dermatol Clin. 35 (4), 489-493.

Cetó, X., Céspedes, F., Pividori, M.I., Gutiérrez, J.M., Valle, M. del, 2011. Resolution of phenolic antioxidant mixtures employing a voltammetric bio-electronic tongue. Analyst 137, 349–356.

Cetó, X., González-Calabuig, A., Valle, M., 2015. Use of a bioelectronic tongue for the monitoring of the photodegradation of Phenolic Compounds. Electroanalysis. 27 (1), 225–233.

Cetó, X., Voelcker, N.H., Prieto-Simón, B., 2016. Bioelectronic tongues: New trends and applications in water and food analysis. Biosens. Bioelectron. 79, 608–626.

Cheah, Y.-K., Salleh, N.A., Lee, L.-H., Radu, S., Sukardi, S., Sim, J.-H., 2008. Comparison of PCR fingerprinting techniques for the discrimination of *Salmonella enterica* subsp. enterica serovar Weltevreden isolated from indigenous vegetables in Malaysia. World J. Microbiol. Biotechnol. 24, 327–335.

Chiang, Y.-C., Tsen, H.-Y., Chen, H.-Y., Chang, Y.-H., Lin, C.-K., Chen, C.-Y., Pai, W.-Y., 2012. Multiplex PCR and a chromogenic DNA macroarray for the detection of *Listeria monocytogens*, S*taphylococcus aureus*, *Streptococcus agalactiae*, E*nterobacter sakazakii*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Salmonella* spp. and *Pseudomonas fluorescens* in milk and meat samples. J. Microbiol. Methods 88, 110–116.

Ciosek, P., Wróblewski, W., 2007. Sensor arrays for liquid sensing – electronic tongue systems. Analyst 132, 963–978.

Dias, L.G., Veloso, A.C.A., Sousa, M.E.B.C., Estevinho, L., Machado, A.A.S.C., Peres, A.M., 2015. A novel approach for honey pollen profile assessment using an electronic tongue and chemometric tools. Anal. Chim. Acta 900, 36–45.

Dias, L.G., Meirinho, S.G., Veloso, A.C.A., Rodrigues, L.R., Peres, A.M., 2017. Electronic tongues and aptasensors. Bioinspired Materials for Medical Applications, pp. 371-402.

Ercole, C., Del Gallo, M., Mosiello, L., Baccella, S., Lepidi, A., 2003. *Escherichia coli* detection in vegetable food by a potentiometric biosensor. Sens. Actuators B Chem. 91, 163–168.

Erdem, Ö., Saylan, Y., Cihangir, N., Denizli, A., 2019. Molecularly imprinted nanoparticles based plasmonic sensors for real-time *Enterococcus faecalis* detection. Biosens. Bioelectron. 126, 608–614.

Esteban-Fernández de Ávila, B., Pedrero, M., Campuzano, S., Escamilla-Gómez, V., Pingarrón, J.M., 2012. Sensitive and rapid amperometric magnetoimmunosensor for the determination of *Staphylococcus aureus*. Anal. Bioanal. Chem. 403, 917–925.

Franz, C.M., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1–24.

Garrity, G., Brenner, D.J., Krieg, N.R., Staley, J.R. (Eds.), 2005. Bergey's Manual® of Systematic Bacteriology: Volume 2: The Proteobacteria, Part B: The Gammaproteobacteria, 2nd ed, Bergey's Manual of Systematic Bacteriology. Springer US.

Gay Martín, M., de Saja, J.A., Muñoz, R., Rodríguez-Méndez, M.L., 2012. Multisensor system based on bisphthalocyanine nanowires for the detection of antioxidants. Electrochimica acta. 68, 88–94.

Gerstl, M., Joksch, M., Fafilek, G., 2013. Designing a simple electronic tongue for fermentation monitoring. J. Anal. Bioanal. Tech. S12:002.

Ghasemi-Varnamkhasti, M., Apetrei, C., Lozano, J., Anyogu, A., 2018. Potential use of electronic noses, electronic tongues and biosensors as multisensor systems for spoilage examination in foods. Trends Food Sci. Technol. 80, 71–92.

Gil, L., Barat, J., Baigts, D., Martínez-Máñez, R., Soto, J., García-Breijo, E., Aristoy, M., Toldrá, F., Llobet, E., 2011. Monitoring of physical–chemical and microbiological changes in fresh pork meat under cold storage by means of a potentiometric electronic tongue. Food Chemistry. 126 (3), 1261-1268.

Gil, L., Barat, J.M., Garcia-Breijo, E., Ibañez, J., Martínez-Máñez, R., Soto, J., Llobet, E., Brezmes, J., Aristoy, M.-C., Toldrá, F., 2008. Fish freshness analysis using metallic potentiometric electrodes. Sens. Actuators B Chem. 131, 362–370.

Giraffa, G., 2002. Enterococci from foods. FEMS Microbiol. Rev. 26, 163–171.

Goméz, J.K.C., Acevedo, C.M.D., García, R.O., 2019. Application of an E-tongue and E-nose for a rapid *E. coli* detection in a drinking water treatment plant, in: 2019 IEEE International Symposium on Olfaction and Electronic Nose (ISOEN). Presented at the 2019 IEEE International Symposium on Olfaction and Electronic Nose (ISOEN), pp. 1–4.

Gutiérrez-Capitán, M., Vila-Planas, J., Llobera, A., Jiménez-Jorquera, C., Capdevila, F., Domingo, C., Puig-Pujol, A., 2014. Hybrid electronic tongues based on microsensors applied to wine quality control, in: 2014 IEEE SENSORS. Presented at the 2014 IEEE SENSORS, pp. 2130–2133.

Halter, R.J., Schned, A., Heaney, J., Hartov, A., Schutz, S., Paulsen, K.D., 2008. Electrical impedance spectroscopy of benign and malignant prostatic tissues. J. Urol. 179, 1580–1586.

Hamad, S.H., 2012. Factors affecting the growth of microorganisms in food, in: Progress in food preservation. John Wiley & Sons, Ltd, pp. 405–427.

Han, F., Huang, X., Teye, E., Gu, H., 2015. Quantitative Analysis of Fish Microbiological Quality Using Electronic Tongue Coupled with Nonlinear Pattern Recognition Algorithms. J. Food Saf. 35, 336–344.

Hancock, L.E., Murray, B.E., Sillanpää, J., 2014. Enterococcal cell wall components and structures. Enterococci: from commensals to leading causes of drug resistant infection [Internet]. Massachusetts Eye and Ear Infirmary.

Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, J.T.M., van Oosterom, R., Edink, K., van Duynhoven, Y.T.H.P., de Boer, E., 2002. *Escherichia coli* O157 infection associated with a petting zoo. Epidemiol. Infect. 129, 295–302.

Hudault, S., Guignot, J., Servin, A., 2001. *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. Gut 49, 47–55.

Ingledew, W.J., Poole, R.K., 1984. The respiratory chains of Escherichia coli. Microbiol. Rev. 48, 222–271.

Inoue, H., Suzuki, T., Hyodo, M., Miyake, M., 2018. Evaluation of multinomial logistic regression models for predicting causative pathogens of food poisoning cases. J. Vet. Med. Sci. 80, 1223–1227.

Ivnitski, D., Abdel-Hamid, I., Atanasov, P., Wilkins, E., 1999. Biosensors for detection of pathogenic bacteria. Biosens. Bioelectron. 14, 599–624.

Jiang, L., Huang, T., 2018. Food poisoning associated with ingestion of wild wasp broods in the upstream region of the Lancang river valley, Yunnan province, China. Toxicon Off. J. Int. Soc. Toxinology 145, 1–5.

Johnson, RP.; Wilson, JB.; Michel, P.; Rahn, K.; Renwick, SA.; Gyles, CL.; Spika, JS., 1999. Human infection with verotoxigenic *Escherichia coli* associated with exposure to farms and rural environments. In: Stewart, CS.; Flints, HJ., editors. Escherichia coli O157 in Farm Animals. CABI. p. 147-168

Kadariya, J., Smith, T.C., Thapaliya, D., 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. BioMed Res. Int. 2014, 827965.

Kalit, M., Marković, K., Kalit, S., Vahcić, N., Havranek, J., 2014. Application of electronic nose and electronic tongue in the dairy industry. Mljekarstvo 64, 228–244.

Kamio, Y., Nikaido, H., 1976. Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium. Biochemistry, 15 (12), 2561–2570.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123–140.

Khan, Md.R.R., Khalilian, A., Kang, S.-W., 2016. A high sensitivity IDC-electronic tongue using dielectric/sensing membranes with solvatochromic dyes. Sensors 16 (5), 668.

Kirkpatrick, S., Gelatt, C.D., Vecchi, M.P., 1983. Optimization by simulated annealing. Sci., 220 671–680.

Kubitschek, H.E., 1990. Cell volume increase in *Escherichia coli* after shifts to richer media. J. Bacteriol. 172, 94–101.

Lan, Y., Wang, S., Yin, Y., Hoffmann, W.C., Zheng, X., 2008. Using a surface plasmon resonance biosensor for rapid detection of *Salmonella typhimurium* in chicken carcass. J. Bionic Eng. 5, 239–246.

Law, J.W.-F., Ab Mutalib, N.-S., Chan, K.-G., Lee, L.-H., 2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Front. Microbiol. 5, 770.

Le Loir, Y., Baron, F., Gautier, M., 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. GMR 2, 63–76.

Learn-Han, L., Yoke-Kqueen, C., Salleh, N.A., Sukardi, S., Jiun-Horng, S., Chai-Hoon, K., Radu, S., 2008. Analysis of S*almonella Agona* and *Salmonella Weltevreden* in Malaysia by PCR fingerprinting and antibiotic resistance profiling. Antonie Van Leeuwenhoek 94, 377–387.

Legin, A., Rudnitskaya, A., Vlasov, Y., Natale, C., Mazzone, E., D'Amico, A., 2000. A D' pplication of electronic tongue for qualitative and quantitative analysis of complex liquid media. Sens. Actuators B-Chem. - Sens. ACTUATOR B-CHEM 65, 232–234.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T., O'Kennedy, R., 2003. Advances in biosensors for detection of pathogens in food and water. Enzyme Microb. Technol. 32, 3–13.

Lim, J.Y., Li, J., Sheng, H., Besser, T.E., Potter, K., Hovde, C.J., 2007. *Escherichia coli* O157:H7 colonization at the rectoanal junction of long-duration culture-positive cattle. Appl. Environ. Microbiol. 73, 1380–1382.

Lowy, F.D., 1998. *Staphylococcus aureus* Infections. N. Engl. J. Med. 339, 520–532.

Mandal, P.K., Biswas, A.K., Choi, K., and Pal, U.K., 2011. Methods for rapid detection of foodborne pathogens: an overview. American Journal of Food Technology, 6:87-102

Markevych, I., Standl, M., Lehmann, I., Berg, A. von, Heinrich, J., 2017. Food diversity during the first year of life and allergic diseases until 15 years. J. Allergy Clin. Immunol. 140, 1751-1754.e4.

Martínez-Bisbal, M.C., Carbó Mestre, N., Martínez-Máñez, R., Bauzá, J., Alcañiz Fillol, M., 2019. Microalgae degradation follow up by voltammetric electronic tongue, impedance spectroscopy and NMR spectroscopy. Sens. Actuators B Chem. 281, 44–52.

Martínez-Bisbal, M.C., Loeff, E., Olivas, E., Carbó, N., García-Castillo, F.J., López-Carrero, J., Tormos, I., Tejadillos, F.J., Berlanga, J.G., Martínez-Máñez, R., Alcañiz, M., Soto, J., 2017. A voltammetric electronic tongue for the quantitative analysis of quality parameters in wastewater. Electroanalysis 29, 1147–1153.

Masot, R., Alcañiz, M., Fuentes, A., Schmidt, F.C., Barat, J.M., Gil, L., Baigts, D., Martínez-Máñez, R., Soto, J., 2010. Design of a low-cost non-destructive system for punctual measurements of salt levels in food products using impedance spectroscopy. Sens. Actuators Phys. 158, 217–223.

Medina-Plaza, C., García-Hernández, C., de Saja, J.A., Fernández-Escudero, J.A., Barajas, E., Medrano, G., García-Cabezón, C., Martin-Pedrosa, F., Rodriguez-Mendez, M.L., 2015. The advantages of disposable screen-printed biosensors in a bioelectronic tongue for the analysis of grapes. LWT - Food Sci. Technol. 62, 940–947.

Mednova, O., Kirsanov, D., Rudnitskaya, A., Kilmartin, P., Legin, A., 2009. Application of a potentiometric electronic tongue for the determination of free so2 and other analytical parameters in white wines from new zealand.1137, 263–266.

Muñoz-Berbel, X., Vigués, N., Jenkins, A.T.A., Mas, J., Muñoz, F.J., 2008. Impedimetric approach for quantifying low bacteria concentrations based on the changes produced in the electrode-solution interface during the pre-attachment stage. Biosens. Bioelectron. 23, 1540–1546.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11, 142–201.

Neuhaus, F.C., Baddiley, J., 2003. A continuum of anionic charge: structures and functions of d-alanyl-teichoic acids in gram-positive bacteria. Microbiol. Mol. Biol. Rev. 67, 686–723.

Oliver, S.P., 2010. My, how time flies: Foodborne pathogens and disease to begin its seventh year with publication frequency to increase in 2010. Foodborne Pathog. Dis. 7, 1.

Oprea, S.F., Zervos, M.J., 2007. *Enterococcus* and its association with foodborne illness, in: Simjee, S. (Ed.), Foodborne Diseases, Infectious Disease. Humana Press, Totowa, NJ, pp. 157–174.

Paixão, T.R.L.C., Bertotti, M., 2009. Fabrication of disposable voltammetric electronic tongues by using Prussian Blue films electrodeposited onto CD-R gold surfaces and recognition of milk adulteration. Sens. Actuators B Chem. 137, 266–273.

Pal, S., Ying, W., Alocilja, E.C., Downes, F.P., 2008. Sensitivity and specificity performance of a direct-charge transfer biosensor for detecting *Bacillus cereus* in selected food matrices. Biosyst. Eng. 99, 461–468.

Park, S.H., Aydin, M., Khatiwara, A., Dolan, M.C., Gilmore, D.F., Bouldin, J.L., Ahn, S., Ricke, S.C., 2014. Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. Food Microbiol. 38, 250–262.

Parra, V., Arrieta, Á.A., Fernández-Escudero, J.A., Íñiguez, M., Saja, J.A. de, Rodríguez-Méndez, M.L., 2006. Monitoring of the ageing of red wines in oak barrels by means of a hybrid electronic tongue. Anal. Chim. Acta, Papers presented at the 4th SYMPOSIUM IN VINO ANALYTICA SCIENTIA 563, 229–237.

Peris, M., Escuder-Gilabert, L., 2013. On-line monitoring of food fermentation processes using electronic noses and electronic tongues: a review. Anal. Chim. Acta 804, 29–36.

Pollack, J.H., Neuhaus, F.C., 1994. Changes in wall teichoic acid during the rod-sphere transition of Bacillus subtilis 168. J. Bacteriol. 176, 7252–7259.

Raetz, C.R.H., Whitfield, C., 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71, 635–700.

Rodríguez-Méndez, M.L., Gay, M., Apetrei, C., De Saja, J.A., 2009. Biogenic amines and fish freshness assessment using a multisensor system based on voltammetric electrodes. Comparison between CPE and screen-printed electrodes. Electrochimica Acta 54, 7033–7041.

Rodriguez-Méndez, M.L., Medina-Plaza, C., García-Hernández, C., de Saja, J.A., Fernández-Escudero, J.A., Barajas-Tola, E., Medrano, G., 2014. Analysis of grapes and wines using a voltammetric bioelectronic tongue: Correlation with the phenolic and sugar content, in: 2014 IEEE SENSORS. Presented at the 2014 IEEE SENSORS, pp. 2139–2142.

Ruiz-Rico, M., Fuentes, A., Masot, R., Alcañiz, M., Fernández-Segovia, I., Barat, J.M., 2013. Use of the voltammetric tongue in fresh cod (Gadus morhua) quality assessment. Innov. Food Sci. Emerg. Technol. 18, 256–263.

Schmidtke, L.M., Rudnitskaya, A., Saliba, A.J., Blackman, J.W., Scollary, G.R., Clark, A.C., Rutledge, D.N., Delgadillo, I., Legin, A., 2010. Sensory, chemical, and electronic tongue assessment of micro-oxygenated wines and oak chip maceration: assessing the commonality of analytical techniques. J. Agric. Food Chem. 58, 5026–5033.

Silhavy, T.J., Kahne, D., Walker, S., 2010. The Bacterial Cell Envelope. Cold Spring Harb. Perspect. Biol. 2 (5).

Söderström, C., Winquist, F., Krantz-Rülcker, C., 2003. Recognition of six microbial species with an electronic tongue. Sens. Actuators B Chem. 89, 248–255.

Son, J.R., Kim, G., Kothapalli, A., Morgan, M.T., Ess, D., 2007. Detection of *Salmonella* enteritidis Using a Miniature Optical Surface Plasmon Resonance Biosensor. J. Phys. Conf. Ser. 61, 1086–1090.

Song, H.S., Jin, H.J., Ahn, S.R., Kim, D., Lee, S.H., Kim, U.-K., Simons, C.T., Hong, S., Park, T.H., 2014. Bioelectronic tongue using heterodimeric human taste receptor for the discrimination of sweeteners with human-like performance. ACS Nano 8, 9781–9789.

Tahara, Y., Toko, K., 2013. Electronic tongues–A review. Sens. J. IEEE 13, 3001–3011.

Tamarapu, S., McKillip, J.L., Drake, M., 2001. Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products. J. Food Prot. 64, 664–668.

Teixeira, L. M. and Facklam, R. R., 2003. *Enterococcus.* In: *Manual of Clinical Microbiology* (Murray, P. R., Baron, E. J., Jorgensen, J. H., Pfaller, M. A., and Yolker, H. Y., eds.), 8th edn, ASM, Washington, DC, pp. 422–429.

The European Union One Health, 2018 Zoonoses. Report (2019) EFSA Journal 17 (12:5926)

Valdés-Ramírez, G., Gutiérrez, M., Del Valle, M., Ramírez-Silva, M.T., Fournier, D., Marty, J.-L., 2009. Automated resolution of dichlorvos and methylparaoxon pesticide mixtures employing a Flow Injection system with an inhibition electronic tongue. Biosens. Bioelectron. 24, 1103–1108.

Veloso, A.C.A., Dias, L G., Rodrigues, N., Pereira, J.A., Peres, A.M., 2016. Sensory intensity assessment of olive oils using an electronic tongue. Talanta, 146: 585–593.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C., 2010. An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol. Adv. 28, 232–254.

Vlasov, Y.A., O'Boyle, M., Hamann, H.F., McNab, S.J., 2005. Active control of slow light on a chip with photonic crystal waveguides. Nature 438, 65–69.

Vogt, R.L., Dippold, L., 2005. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June-July 2002. Public Health Rep. Wash. DC 1974 120, 174–178.

Vollmer, W., 2008. Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol. Rev. 32, 287–306.

Vollmer, W., Blanot, D., Pedro, M.A.D., 2008. Peptidoglycan structure and architecture. FEMS Microbiol. Rev. 32, 149–167.

Wang, Y., Ye, Z., Si, C., Ying, Y., 2011. Subtractive inhibition assay for the detection of E. coli O157:H7 using surface plasmon resonance. Sensors 11, 2728–2739.

Waswa, J., Irudayaraj, J., DebRoy, C., 2007. Direct detection of E. Coli O157:H7 in selected food systems by a surface plasmon resonance biosensor. LWT - Food Sci. Technol. 40, 187–192.

Waswa, J.W., Debroy, C., Irudayaraj, J., 2006. Rapid detection of *Salmonella enteritidis* and *Escherichia coli* using surface plasmon resonance biosensor. J. Food Process Eng. 29, 373–385.

Watanabe, T., Ando, Y., Orikasa, T., Kasai, S., Shiina, T., 2018. Electrical impedance estimation for apple fruit tissues during storage using Cole–Cole plots. J. Food Eng. 221, 29–34.

Wei, Z., Wang, J., Zhang, X., 2013. Monitoring of quality and storage time of unsealed pasteurized milk by voltammetric electronic tongue. Electrochimica Acta 88, 231–239.

Winquist, F., Holmin, S., Krantz-Rülcker, C., Wide, P., Lundström, I., 2000. A hybrid electronic tongue. Anal. Chim. Acta 406, 147–157.

Woertz, K., Tissen, C., Kleinebudde, P., Breitkreutz, J., 2010. Rational development of taste masked oral liquids guided by an electronic tongue. Int. J. Pharm. 400, 114–123.

Yu, J., Wu, J., Zhang, Y., Guo, L., Cong, X., Du, Y., Li, J., Sun, W., Shi, J., Peng, J., Yin, F., Wang, D., Zhao, P., Wang, J., 2012. Concurrent highly pathogenic porcine reproductive and respiratory syndrome virus infection accelerates *Haemophilus parasuis* infection in conventional pigs. Vet. Microbiol. 158, 316–321.

Zhang, G., 2013. Foodborne pathogenic bacteria detection: An evaluation of current and developing methods. The Meducator 1 (24).

Zhang, Z., Zhou, J., Du, X., 2019. Electrochemical biosensors for detection of foodborne pathogens. Micromachines 10, 222.

Zhao, G., Lin, X., Dou, W., Tian, S., Deng, S., Shi, J., 2011. Use of the smart tongue to monitor mold growth and discriminate between four mold species grown in liquid media. Anal. Chim. Acta 690, 240–247.

Zhao, X., Lin, C.-W., Wang, J., Oh, D.H., 2014. Advances in rapid detection methods for foodborne pathogens. J. Microbiol. Biotechnol. 24, 297–312.

Zhou, B., Xiao, J., Liu, S., Yang, J., Wang, Y., Nie, F., Zhou, Q., Li, Y., Zhao, G., 2013. Simultaneous detection of six food-borne pathogens by multiplex PCR with a GeXP analyzer. Food Control 32, 198–204.

Zhu, D., Zhao, X.-Y., Yao, Y., Dai, F.-F., He, H., Li, R.-Q., Jin, R.-H., Liang, L.-C., Li, N., 2013. A new factor influencing pathogen detection by molecular assay in children with both mild and severe hand, foot, and mouth disease. Diagn. Microbiol. Infect. Dis. 76, 162–167.