Microorganisms' discrimination using an electronic nose-chemometric approach

Tarek ZORGANI

Dissertation presented to
Escola Superior de Tecnologia e Gestão
Instituto Politécnico de Bragança
In order to obtain Master's degree in
Chemical Engineering

Supervised by

António Manuel Coelho Lino PERES

Joaquina Teresa Gaudêncio DIAS

Khalil ZAGHDOUDI

BRAGANCA 2023

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 lifeto 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 memoryfor 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 brothers

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

Acknowledgements

Before presenting the results of this work, I had a lot of pleasure in expressing my deep gratitude to all the people who encouraged me and helped in its realization.

This work was done at the “Microbiology” laboratory and at the ‘’GC’’ laboratory at the Escola Superior Agrária de Bragança(ESA-IPB).

I would like to express my sincere thanks to my supervisor, Pr. Antonio Manuel Coelho Peres, auxiliary professor at the ESA-IPB, for agreeing to supervise my work and making it profitable for my training. Special thanks for his proverbial patience and the confidence he has shown in me and despite his many occupations has always been very available, discuss my results and correct my thesis. Special thanks also for the necessary resources that he provided me and without which this work could not have been pursued.

I would also like to thank Pr. Joaquina Teresa Dias, auxiliary professor at the ESA-IPB for her invaluable technical and organizational assistance and to review the results presented here.

I offer my sincere appreciation to Vitor Souza, Nuno Ferreiro, Ana Rodrigues, for their help and friendly behavior towards me during my internship and for making my journey very meaningful.

I extend my sincere thanks to my flat mates, who have stood by my side throughout everything, difficult times and good times.

Immeasurable appreciation and deepest gratitude to everyone in which in one way or another has contributed to make this study possible.

Abstract

The detection/identification of microorganisms is of major relevance for food quality and safety. Traditional analytical procedures (e.g., culture methods, immunological techniques, and polymerase chain reaction), while accurate and widely used, are time-consuming, costly, and generate a large amount of waste. Sensor-based instruments have evolved as quicker and sensitive complementary identification tools for yeasts, bacteria and fungi. Electronic noses (E-noses), in combination with chemometrics, have been effectively employed for the detection/discrimination of different microorganisms, providing a green, quick, cost-effective, and non-destructive/non-invasive assessment. The successful use of the E-noses may be related to the generation of distinctive olfactory fingerprints of certain volatile organic compounds (VOCs) during the microorganism's growth. These devices have already been used to detect/discriminate fungi and bacteria (e.g.,*Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Listeria monocytogenes, Pseudomonas aeruginosa*), namely in milk, juice, soups,goat and pork meat,fruits and vegetables.

Thus, a lab-made E-nose, with nine metal oxide semiconductor sensors, was applied to detect, differentiate, andquantify four common food contamination/quality indicator bacteria, including two Gram positive (*E. faecalis*and *S. aureus*) and two Gram negative *(E. coli* and *P. aeruginosa*). Besides, to support the E-nose performance the volatile profiles generated by these bacteria were also assessed by headspace solid-phase micro extraction gas-chromatography-mass spectrometry. The volatile profiles comprised 15 identified VOCs, being 10 of them emitted by at least one of the four bacteria evaluated, namely two alcohols (1-butanol, and 1-nonanol), three pyrazines (2-ethyl-6-methyl-pyrazine, 3-ethyl-2,5-dimethylpyrazine,and trimethylpyrazine), three terpenes (camphene, D-limonene, and β-pinene), and two other compounds (2,4-thujadiene and indole). The four bacteria could be distinguished using the electrical resistance signals produced by the E-nose in combination with linear discriminate analysis (90% of correct classifications for leave-one-out cross-validation). Additionally, multiple linear regression models, with root mean square errors lower than 4 colony forming units, were successfully established ($0.9428 \leq R^2 \leq 0.9946$). Overall, the E-nose proved to be an effective qualitative-quantitative tool for analyzing bacteria in solid matrices, being foreseen it possible application to solid food matrices.

Keyswords:electronic nose; metal oxide semiconductor sensors; bacteria identification; Gram-positive bacteria; Gram-negative bacteria.

Resumo

A detecção/identificação de microorganismos é de grande relevância para a qualidade e segurança dos alimentos. Os procedimentos analíticos tradicionais (por exemplo, métodos de cultura, técnicas imunológicas e reação em cadeia de polimerase), embora precisos e amplamente utilizados, são demorados, dispendiosos e geram uma grande quantidade de resíduos. Os instrumentos baseados em sensores evoluíram como ferramentas de identificação complementares mais rápidas e sensíveis para leveduras, bactérias e fungos. Os narizes eletrônicos, em combinação com a quimetria, foram efetivamente empregados para a detecção/discriminação de diferentes microorganismos, proporcionando uma avaliação verde, rápida, econômica e não destrutiva/não invasiva. O uso bem-sucedido dos E-noses pode estar relacionado à geração de impressões olfativas distintivas de certos compostos orgânicos voláteis (VOCs) durante o crescimento do microorganismo. Estes dispositivos já foram usados para detectar/discriminar fungos e bactérias (por exemplo, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Listeria monocytogenes, Pseudomonas aeruginosa), nomeadamente no leite, suco, sopas, carne de cabra e de porco, frutas e legumes.

Assim, um E-nose feito em laboratório, com nove sensores semicondutores de óxido de metal, foi aplicado para detectar, diferenciar e quantificar quatro bactérias comuns de contaminação alimentar / indicador de qualidade, incluindo duas Gram positivas (E. faecalis e S. aureus) e duas Gram negativas. (E. coli and P. aeruginosa). Além disso, para apoiar o desempenho do nariz E, os perfis voláteis gerados por essas bactérias também foram avaliados por micro-espectrometria de extracção de gás-cromatografia-massa de fase sólida.Os perfis Voláteis consistiam em 15 VOCs identificados, sendo 10 deles emitidos por pelo menos uma das quatro bactérias avaliadas, ou seja, dois álcoois (1-butanol e 1-nonanol), três pirazinas (2-etil-6-metil-pirazina, 3-etil-2,5-dimetilpyrazina, e trimethylpyrazine), três terpenos (hcampene, D-limonene e-pinene), e outros dois compostos. (2,4-thujadiene and indole). As quatro bactérias poderiam ser distinguidas usando os sinais de resistência elétrica produzidos pelo E-nose em combinação com a análise discriminante linear (90% das classificações corretas para a validação cruzada de abandono-um-out). Além disso, foram estabelecidos com sucesso múltiplos modelos de regressão linear, com erros médios do quadrado raiz inferiores a 4 unidades de formação de colônia ($0{,}9428 \leq R2 \leq 0{,}9946$). No geral, o E-nose provou ser uma ferramenta qualitativa-quantitativa eficaz para analisar bactérias em matrizes sólidas, prevendo-se a possível aplicação a matrizs de alimentos sólidos.

Palavras-chave: nariz eletrônico; sensores de semicondutores de óxido metálico; identificação de bactérias; Bactérias Gram-positivas; Bactéria Gram-negativa.

Table of contents

List of Figures

List of tables

Abbreviations

Brain–heart infusion (**BHI**)

Cluster analysis (**CA**)

Colony-forming unit (**CFU**)

Discriminate function analysis (**DFA**)

Hierarchical cluster analysis (**HCA**)

Linear discriminate analysis (**LDA**)

Luria-Bertani (**LB**)

Metal oxide (**MOX**)

Metal oxide semiconductors (**MOS**)

Multiple linear regression models (**MLRM**)

Phosphate-buffered saline (**PBS**)

Qualitative data analysis (**QDA**)

Support vector machines (**SVM**)

Total viable count (**TVC**)

Uncorrelated LDA (**ULDA**)

Volatile organic compounds (**VOCs**)

The contents of this thesis are based on the following publication:

Dias T., Santos V.S., Zorgani T., Ferreiro N., Rodrigues A.I., Zaghoudi K., Veloso A.C.A., Peres A.M. (2023). A lab-made E-nose-MOS device for assessing the bacterial growth in a solid culture medium. Biosensors, 13(1), article nº 19, 13pp. https://doi.org/10.3390/bios13010019

1. Introduction

The entire food chain can benefit from the early and quick identification of foods contaminated with harmful bacteria as it serves as a diagnostic tool for quality and/or safety. Despite being labor-intensive, expensive, and time-consuming for technicians, conventional culture-based methods, immunological assays, and polymerase chain reaction techniques are frequently utilized as reliable, specific, and sensitive standard techniques (Green et al.,2014; Capuano et al.,2020).

Alternative methods for screening microorganisms (fungi, bacteria, etc.) take into account the identification of particular volatile organic compounds (VOCs) produced during the primary and secondary metabolisms of the microorganisms. Alcohols, aldehydes, hydrocarbons, acids, ethers, esters, ketones, terpenes, and furans are a few examples of these VOCs (Capuano et al., 2020). As a result, the standard analytical tool for the investigation of VOCs is gas chromatography coupled with mass spectrometry, which enables the identification and measurement of the precise volatiles associated with each microorganism (Nieto-Arribas et al., 2011; Tait et al., 2014). Microorganisms release specific volatile chemicals during growth, creating discrete olfactory fingerprints for a given microorganism that can be utilized for identification and discrimination (Bonah et al., 2020). Even while some VOCs might be connected to a specific species, the volatile pattern and quantities emitted by each microorganism can vary depending on the strain, incubation period, and growth circumstances (such as substrate, nutrients, pH, humidity, and temperature) [Capuano et al.,2020; Kladsomboon et al.,2018). Additionally, different bacteria produce variable levels of the same VOCs (such as ethanol and formaldehyde) as they grow (Bonah et al., 2020). For instance, ammonia has been reported as the main VOC produced by *Pseudomonas aeruginosa* or *Staphylococcus aureus*, while acetoin and diacetyl are associated with *Enterococcus faecalis* and *Escherichia coli*, respectively, and methanol, 1-propanol, 1-butanol, and indole are associated with *Escherichia coli* (Nieto-Arribas et al,.2011; Kladsomboon et al.,2018). By assessing the relative quantity of various substances, bacteria may be identified by using pattern recognition analytical tools rather than conventional ones.

As recently reviewed by (Bonah et al., 2020). Electronic noses (E-noses) have become a practical and viable non-invasive alternative for detecting bacterial foodborne pathogens. E-noses are arrays of cross-selective sensors with partial selectivity, capable of encoding high-dimension patterns of VOCs into a smaller-dimension pattern of sensors signals.

In general, many gas sensors, including metal oxide (MOX) or metal oxide semiconductors (MOS), and polymer sensors, have been incorporated into these devices (e.g., polypyrroles, thiophenes, indoles polyaniline, and furan material polymers).

Different classification chemometric tools, such as principal component analysis (PCA), hierarchical cluster analysis (HCA), linear discriminant analysis (LDA) and uncorrelated LDA (ULDA), qualitative data analysis (QDA), support vector machines (SVM), and/or artificial neural networks, have been used to extract the information from the signals profiles generated (ANN). E-noses in combination with multivariate classification techniques have demonstrated a clear potential as screening tools for bacterial foodborne pathogen detection (e.g., *Bacillus cereus, E. coli, E. faecalis, Listeria innocua, P. aeruginosa, S. aureus,* or *Salmonella spp*.) in various media (e.g., Lysogeny broth, Brain-Heart infusion media) or food matrices.

For instance, an E-nose-MOS combined with ULDA permitted the separation of two *E. coli* strains, *Listeria innocua* and *E. faecalis*, cultivated in agar medium (brain-heart infusion, BHI) (with a sensitivity of 97.5% for leave-one-out cross-validation, LOO-CV) (Green et al.,2014). *Salmonella enteritidis*, *Listeria monocytogenes*, and *Salmonella Typhimurium* were suspended in phosphate-buffered saline (PBS) solution, and an E-nose-MOS along with an SVM model and metaheuristic optimization algorithms were able to distinguish between them (Bonah et al., 2019). With 100% prediction accuracy, an E-nose-MOS device with LDA was also used to distinguish between viable and non-viable *Yersinia enterocolitica*, viable and non-viable *E. coli*, or viable *E. coli* from viable *Y. enterocolitica*, cultivated in Luria-Bertani (LB) broth or in skimmed milk (Roda et al., 2016). With sensitivities of 100% (training) and 87% (LOO-CV), respectively, the signals produced by an E-nose-MOS combined with a discriminate function analysis (DFA) or probabilistic neural network (PNN) allowed the correct discrimination of three bacteria in water (*E. coli, P. aeruginosa*, and *Klebsiella oxytoca*) (Carrillo et al.,2019). Detecting and discriminating *L. monocytogenes* and *B. cereus* incubated in Tryptic Soy Broth (TSB) media was also made possible with an E-nose-MOS coupled with SVM, with a prediction accuracy of 84% (Astantri et al., 2020).

The same laboratory-produced E-nose also enabled quick identification of *Salmonella Typhimurium* and *E. coli* cultivated in TSB medium based on an SVM model, providing a classification accuracy of 84% (Prakoso et al., 2021). When a radial basis function (RBF) network model was used, a portable commercial E-nose (Cyrano Sciences' Cyranse 320), with 32 polymer sensors, was able to distinguish between six bacteria (*S. aureus, Haemophilus influenza, Streptococcus pneumonia, E. coli, P. aeruginosa,* and *Moraxella catarrhalis*) with correct classification rates of 98% (Dutta et al.,2006). Two E-noses devices, the e-nose 4000 from Marconi Applied Technology and the model BH-114 from Bloodhound, both used polymer sensor arrays paired with DFA to distinguish between control samples and samples containing *E. coli* or *P. aeruginosa* (Canhoto et al.,2005). To distinguish between bacteria cultivated in LB medium (*Enterobacter cloacae, S. aureus, E. coli, P. aeruginosa,* and *Salmonella enterica*), a hybrid E-nose with organic-inorganic nanocomposite gas sensors and commercial MOS sensors was developed. The five bacteria under study could be distinguished using PCA, and cluster analysis (CA) made it possible to tell the four Gram-negative bacteria apart from the Gram-positive bacteria (Seesaard et al., 2020). Because the gas sensor device can identify the specific volatile chemical fingerprints for each bacterium, it has recently been possible to distinguish between *E. coli, P. aeruginosa, S. aureus,* and *B. subtilis* when cultured in LB medium (Shauloff et al.,2021). In pasteurized milk samples, an E-nose with MOX sensors was also successfully used to distinguish between *Salmonella enterica, Klebsiella pneumonia*, and *E. coli*. The effectiveness of the E-nose accurate categorization was evaluated using three distinct chemometric methods: PCA, LDA, and SVM. With a cross-validation approach, the constructed SVM model had the highest success rate for predictive classification (95%) (Carrillo-Gómez et al., 2021).

Optical E-noses have also been used to identify and classify microorganisms with success. Metallic nanoparticles were used in the development of an optoelectronic nose that enables the rapid detection of 10 Gram-positive and Gram-negative bacteria (*S. aureus, methicillin-resistant S. aureus, Listeria monocytogenes, Streptococcus agalactiae, E. faecalis, E. coli, Klebsiella pneumonia, Proteus mirabilis, E. aerogenes*, and *P. aeruginosa*). All bacteria cultivated on TSB medium, as well as *S. aureus, Listeria monocytogenes, E. coli*, and *Proteus mirabilis* in drinking water samples, could be correctly differentiated with PCA and/or HCA (Bordbar et al., 2020). A near-infrared optical nose with single-wall carbon nanotubes encapsulated in peptides was also suggested for effectively detecting and discriminating *E. coli* and *Klebsiella pneumonia* cultured in LB medium based on PCA (Shumeiko et al.,2022).

However, very few studies have documented the use of E-noses for measuring microorganisms. The viability of employing an E-nose-MOX device to semi-quantitatively measure the levels of *E. coli* contamination in milk samples or drinking water was confirmed by (Carrillo-Gómez et al., 2021).

For each matrix under examination, the signals profiles produced by the gas sensing in conjunction with PCA and/or DFA enabled for the accurate division of the samples into clusters of pre-determined *E. coli* concentrations (i.e., colony forming units per milliliters: from 110-2 to 1107 CFU/mL). Additionally, according to (Tonezzer et al.,2021) an E-nose consisting of a single nanowire gas sensor was able to properly estimate the decimal logarithmic of the total viable count (TVC) during trout fish deterioration with an error rate of less than 5%. The ability of an E-nose-MOX device to assess the emission of VOCs generated by the bacterial breakdown of sardines was also used to estimate the TVC of aerobic bacteria in sardines using partial least squares models (correlation coefficient of 0.91).

In this regard, the current study's objective is to assess the classification and quantification capabilities of a lab-made E-nose using commercial MOS gas sensor for the evaluation of bacteria cultured in solid media. Four target microorganisms—two Gram-positive (*E. faecalis* and *S. aureus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria—were chosen in consideration of their potential use as quality/contamination indicators for food/water samples. Although it required resuspending each dried mass of bacteria in water, which prevents a direct bacteria examination, the study team has previously shown that a potentiometric electronic tongue made of lipid polymeric sensor membranes may be employed for these reasons (Ghrissi et al., 2021). As an alternative, the E-nose-MOS device could be employed as a green, direct (i.e., without requiring any sample's pre-treatment) and non-invasive recognition/counting sensing platform for sniffing bacterial growth in solid media when combined with LDA or multiple linear regression models (MLRM).

1.1-Objectives

The main objective of the present study was to detect and distinguish microorganisms that play an important role in the food and environmental sectors, namely *Escherichia coli*, *Enterococcus faecalis, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Although conventional methods such as smear techniques are still the most widely used, there is a need to develop rapid, easy-to-use, environmentally friendly and cost-effective screening detection methods. In this regard, the development of electrochemical sensors, including electronic nose (E-nose), has shown promise as bioinspired detection methods for screening microorganisms in recent decades. Therefore, the use of a laboratory fabricated electronic nose consisting of an array of nine metal oxide semiconductor gas sensors (MOS) was investigated to explore the advantages of these innovative (bio)sensors for the detection and discrimination of microorganisms.

2-Bibliographic review

To comprehend how an "electronic nose" (e-nose) functions, it is necessary to first define "smelling" and what defines an "odour," or fragrance. The basic features of odor molecules are as follows. The fact that they are light (relative molecular weights up to roughly 300 Da), tiny, polar, and typically hydrophobic are the most essential characteristics. The e-nose is a computerized version of the human olfactory system. The smell molecules are delivered to a test chamber housing the sensor array by a sampling mechanism. The interaction of the sensors with the volatile molecules causes a change in sensor response, which is subsequently analyzed by a pattern recognition system. To get the most out of e-nose technology, a neural network that works like human brain's memory may be installed, generating a library of sensor responses known as sensor profiles. The signals that make up a sensor array's output don't give a spectrum of smell components like a gas chromatogram, but rather information on the odour's attributes as defined by specific sensor response signatures. More recently, e-nose MS combinations have been able to offer not only an odour fingerprint but also the mass-to-charge ratio of its constituents. It is therefore feasible to offer both qualitative and quantitative information while looking for a certain molecule with a specific mass-to-charge ratio.

2.1-Microorganisms and foodborne disease

Microorganisms are extremely minute organisms that can only be seen under a microscope, but they play both beneficial and harmful functions. Microorganisms are everywhere, and they have an incredible capacity to adapt to new surroundings and reproduce in vast numbers in a short amount of time (Allen et al., 2015). They are found on soil surfaces, acidic hot springs, radioactive waste water, deep in the earth's crust, organic materials, and living bodies of flora and wildlife because of their capacity to adapt and reproduce on varied surfaces and in different situations (Balkwill et al., 1997).

Only a small number of different types of microorganisms, so-called pathogenic microorganisms, have the ability to cause foodborne disease. These can cause infections or food poisoning.

Pathogenic microorganisms and their foodborne toxins are the cause of numerous cases of foodborne illness worldwide (Hernández-Cortez et al., 2017).

Pathogens allowing FBDs to be easily transmitted have resulted from high population growth and food marketing, resulting in epidemics in many places, harming the morbidity, mortality, and economics of the affected population. FBDs are becoming more common in the United States, the United Kingdom, and Europe, and this will become a public health issue in the next years (Hernández-Cortez et al., 2017).

Campylobacter spp., enterotoxigenic *E. coli* (ETEC), enter pathogenic *E. coli (EPEC)*, *Salmonella spp*., *Shigella spp*., Shiga toxin-producing *E. coli (STEC)*, and *V. cholerae* are some of the genera usually linked with FBDs (Hernández-Cortez et al., 2017).

Bacteria are responsible for 66% of all foodborne illnesses. Botulism produced by *C. botulinum*, gastroenteritis caused by *E. coli* strains, *Salmonellosis*, and *Staphylococcal* poisoning are all common disorders. Furthermore, *B. cereus* and *V. cholerae* are bacteria that have been implicated in toxic infection caused by food (Hernández-Cortez et al., 2017).

Food poisoning produced by *S. aureus* is the most common in several countries; estimates suggest that *S. aureus* is responsible for up to 41% of food poisoning outbreaks. Although it may affect persons of any age, the most common age group is 20 to 49 years old, with up to 48 percent of cases occurring in this age group. Chicken and eggs, cakes, pastas, sauces, milk, and its derivatives are the principal food items linked to food poisoning caused by *S. aureus* (Hernández-Cortez et al., 2017).

2.2- Microbiological analysis of food & water

Microbiological analyzes of food, surfaces and handlers, as well as different types of water, are essential for control and quality, in order to avoid damage to human health. Since it is not feasible, in routine analyzes to search for all pathogens in food/water matrix, groups or species of microorganisms more easily determined are usually used, and of course whose presence in food between certain numerical limits, indicates exposure to conditions that may introduce and/or allow the proliferation of infectious or toxigenic microorganisms. When there is a positive link between the presence of an indicator microbe and the prevalence of a disease, microorganisms are widely employed to assess food quality and safety. *Escherichia coli* and *Staphylococcus aureus* are utilized as hygiene and safety indicators for a variety of food items across the world. The

Enterococci are often considered good indicators of fecal contamination, since they are highly resistant to adverse conditions such as freezing and drying.

Standard methods for the detection and enumeration of indicator bacteria and some pathogenic or opportunistic bacteria have been established by various organizations, namely the International Organization for Standardization (ISO), the European Committee for Standardization (CEN) and the American Public Health Association (APHA).

Conventional methods for detecting foodborne bacterial pathogens in foods/water matrix rely on the use of microbiological selective media to growth and enumerate bacteria. In some cases, it is necessary to carry out the biochemical characterization of the isolated colonies. These methods are sensitive, generally inexpensive, and provide both qualitative and quantitative results. However, the preparation of media and plates, as well as colony counting and subsequent biochemical characterization of isolated colonies makes this process time-consuming and laborious.

2.2.1-*Enterococcus faecalis*

E. faecalis is a Gram-positive coccus that lacks catalase (Opera and Zervos, 2007). Cells are the most common type of organism, and they can live individually, in pairs, or in short chains. They're facultative anaerobes that don't sporulate and can withstand high temperatures, salinity, and pH. As a result, they thrive in a 6.5 % NaCl broth with a pH of 9.6 and temperatures ranging from 10 to 45°C, with 35°C being the ideal temperature for development (Teixeira and Facklam, 2003).

Enterococci are found in the flora of both humans and animals. *Enterococcus faecalis* is a fecal contamination indication, but it is also found in the natural micro biota of many fermented foods (dairy, meat, and vegetables), where plays a significant role in the development of organoleptic features and safety of fermented goods. In food products, *E. faecalis* can also produce bacteriocin with antimicrobial action against foodborne pathogens such as *Listeria* and spoilage bacteria. Thus contributing to the safety of these foods (Franz, Holzapfel & Stiles., 1999).

Nevertheless, On the other hand, they are among the most common resistant hospital infections in the world, and they can act as a reservoir for virulence characteristics and antibiotic resistance (Giraffa., 2002; Andrighetto et al., 2001)

2.2.2-*Staphylococcus aureus*

S. aureus is a Gram-positive, spherical *staphylococci* arranged in clusters with diameters of 0.5 – 1.5 µm, 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; this microorganism can contaminate food products during their preparation and processing (Kadariya et *al*., 2014). It is a commensal and opportunistic pathogen that can cause wide 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 organism is well known for its ability to acquire resistance to various antibiotic classes (Kadariya et *al*., 2014).

2.2.3 –*Escherichia coli*

Escherichia 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 µm3 (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).

E. coli is a bacteria belonging to the group of fecal coliforms and therefore considered an indicator of fecal contamination of water and foodstuffs.

2.2.4-*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic rod bacterium of the *Pseudomonadaceae family* (a member of the Gamma proteo bacteria) with the measure of 0.5 to 0.8 μmby 1.5 to 3.0 μm. Almost all strains are motile by means of a single polar flagellum, and some strains have two orthreeflagella (Baron, 1996).

P. aeruginosa contains 12 other members in its family. Similar to other members of the genus, *P. aeruginosa* is commonly found in soil and water as well as in plants and humans.

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 an aerobically, 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. *Pseudomonas spp.* may be found in soil, water, plants, and foods (such as dairy and meat products), as well as on the surfaces of food processing plants, all of which are connected to their ability to create biofilms (Ahmed et al,2020). Furthermore, because these bacteria are psychotropic (i.e., they can thrive at low temperatures ranging from 0 to 7 degrees Celsius), they can colonize cold-stored foods and become the majority population (Stellato et al., 2017).

Serious infections in immune compromised people are caused by *P. aeruginosa*. Most *Pseudomonas spp*. are resistant to penicillin and similar -lactam antibiotics, which supported the identification of *P. aeruginosa* as a quality indicator microbe in water for human use. (Croughs et al, 2018).

2.3-Methods used to detect microorganisms

2.3.1- Conventional methods

To identify foodborne bacterial pathogens, present in food, traditional approaches rely on growing microorganisms on agar plates followed by routine biochemical identifications (Mandal et al., 2011). These procedures are often low-cost and straightforward, but they may be time-consuming since they rely on the microorganisms' capacity to grow in various culture media, such as pre-enrichment media, selective enrichment media, and selective plating media. Conventional procedures, in fact, take two to three days for preliminary identification and more than a week to confirm pathogen species (Zhao et al., 2014). They may also be time-consuming, since the preparation of culture medium, plate injection, and colonies counting all take time (Mandal et al., 2011).

Rapid approaches, on the other hand, are sensitive enough to identify infections in low levels in food. Because just one pathogen in food has the potential to cause illness, sensitivity is critical. These approaches are more efficient in terms of time and labor, as well as preventing human mistakes (Mandal et al., 2011). Rapid approaches, on the other hand, have benefits and drawbacks. Nucleic acid-based, biosensor-based, and immunological-based fast detection approaches are the most common (Zhao et al., 2014).

2.3.2- Immunological based methods

To identify the presence of bacteria, immunological tests rely on the interaction between the antigen and the antibody. Indeed, one of the most well-known immunological procedures is the enzyme-linked immunosorbent test (ELISA). Escherichia coli O157:H7, *Yersinia enterocolitica, Salmonella coli O157:H7, Yersinia enterocolitica, Salmonella typhimuriu*m, and *Listeria monocytogenes* have all been effectively detected using multiplexed immunoassays (Zhang, 2013).

2.4- Electrochemical techniques: E-nose

2.4.1-Electronic nose instrumentation

An electronic nose (E-nose) is a device that comprises a gas/smell sampling chamber, a sensing unit, a signal recording apparatus being the gathered signals preprocessed and analyzed using chemometric tools, delivering a olfactory fingerprint of the samples under study (Jia et al. 2018). The E-nose allows generating signal profiles when the sensors comprised in the device are exposed to the volatile chemical compounds that are released from of a specific sample. The signals are recorded using a data acquisition unit, controlled by a software, allowing transforming them into digital values, which are computed generating a unique volatile fingerprint of the sample under analysis, being then computed using chemometric tools, including unsupervised or supervised multivariate statistical techniques. Different feature extraction techniques can be applied aiming to establish the most informative dataset (Gila et al., 2020).

2.4.2-Electronic nose sensor types

The E-noses use several chemical sensors with different sensibilities and specificities that react to and respond to volatile organic chemicals found in gas samples (Jiang and Chen 2014; Zohora et al., 2013). Quartz crystal microbalance sensors, surface acoustic wave sensors, electrochemical sensors, optical sensors, and calorimetric sensors are among the many sensors that may be used. Wilson and Baietto (2009) provide a complete explanation of these sensors. For E-nose, chemical-based sensors were utilized (catalytic, semiconducting metal oxide, solid electrolyte, polymer, and field-effect transistor-based sensors). Researchers have typically employed metal oxide semiconductors (MOS) as sensing elements in the electronic nose because to its availability, high sensitivity, and capacity to respond to oxidizing and reducing substances. The MOS sensor works by causing a change in conductivity through the adsorption of gas molecules. The amount of volatile organic chemicals adsorbed is usually proportional to the change in conductivity.

2.4.3-Volatiles associated with the microbial growth

Volatile organic compounds (VOCs) are a class of carbon-based substances that are volatile at room temperature and detectable by smell. VOCs are easily volatilized because they have low molecular weights and high vapour pressures (Tait et al., 2014).

Microorganisms, including pathogenic and spoilage bacteria, grow in food substrates and release distinct VOCs. Thus, an electronic nose can, in principle, distinguish between numerous volatile compounds, which then can be related with the volatile substances produced during the growth and proliferation of the microorganisms (Avalos et al., 2018; Giungato et al., 2018). Some of these volatiles provide a distinct olfactory fingerprint, unique for each microorganism, allowing its identification and differentiation from other microorganism, avoiding the use of conventional time-consuming food analysis techniques. In fact, some bacteria are responsible for different odors (Balasubramanian et al., 2016; Berna et al., 2013):

- *E. coli* has an amino acid distinctive odor (indole gas).
- *Salmonella typhimurium* generates methyl ketenes, primary and secondary alcohols.

According to some researchers, it has been hypothesized that some of these VOCs are generated as signaling or defense responses of the microorganisms, and can be used for growth monitoring (Selim et al., 2017).

At the molecular level, VOC data offer fundamental information regarding activity of microorganisms and the supports the hypothesis that the olfactory profile can be used as an identification tool (Robin Michael Statham and John, 2012). Table 1 lists some relevant information regarding the VOCs that can be usually found for different microorganisms. The composition and amount of volatiles generated by a microorganism are affected by physiological parameters such as moisture content, oxygen, pH, and temperature. The carbon-energy sources available for the microorganism's growth also impact the content and diversity of the volatiles generated (Romoli et al., 2014). Primary metabolism (metabolites required for growth, and reproduction, such as DNA, amino acids, and fatty acid synthesis) and secondary metabolism are the major sources of VOCs generated by bacteria (organic metabolites not involved directly in the normal growth and reproduction and are intermediates of the primary metabolism). During the metabolic oxidation of glucose, fatty acids, acetic acid, keto acids, and amino acids function as precursors, culminating in the creation of certain Microbial Volatile Organic Compounds (MVOCs) (Selim et al., 2017)

Table 1: A summary of volatile organic compounds produced by foodborne pathogens

Foodborne pathogen	VOCs	References
Escherichia coli	Indole, 1-decene (E. coli O157:H7 in TSYA), Dimethyl disulfide, ethanol, 2-nonanone, 2-heptanone, indole, pentyl cyclopropane (E. coli in tryptone-yeast NaCl super-broth), 2,5-dimethyl tetrahydrofuran, dimethyl disulfide, 2-heptanone, 2 undecanone, indole, unknown, 2-tridecane, 2,5 dimethyl pyrazine, benzaldehyde, dimethyl trisulfide, 2-nonanone, nonanal, decanal (Escherichia coli O157:H7 and a nonpathogenic strain of E. coli)	Siripatrawan (2008a) and Senecal et al. (2002)
Listeria monocytogenes	Acetaldehyde, Ethanol, Acetone, 2-Methyl-propanal, 2,3-Butanedione, 2-Butanone Acetic acid, 1-Butanol,3-Methylbutanal, 2-Methyl-butanal,3-Methyl-3-buten-1-ol 3-Hydroxy-2-butanone, Dimethyl disulfide, Pyrazine, Pyrrole, Hexanal, Butyl ester acetic acid,3-Methyl-2-butenal, Methyl-pyrazine, Methoxy-phenyl-oxime 2,5-Dimethyl-pyrazine,4,6-Dimethylpyrimidine, D-Limonene, 6-Methyl-5-hepten-2-one, Octanal 2-Ethyl-1-hexanol, Benzaldehyde, 2-Ethyl-6-methylpyrazine,	Yu et al. (2014)
Salmonella	Primary alcohols (1-octanol, 1-decanol), secondary alcohols (2-undecanol, 2-tridecanol), methyl ketones (2-nonanone,2-undecanone), 3-methyl-1-butanol (S. typhimurium in tryptic soy yeast agar), Hydrogen sulfide, ethanol, carbon disulfide,dimethyl cyclopropane, 1-propanol (S. typhimurium intryptone-yeast NaCl super-broth), Dimethyl sulfide, carbon disulfide, heptane, acetic acid, ethyl	Senecal et al. (2002), Siripatrawan and Harte (2007) and Siripatrawan (2008a)

Staphylococcus aureus	Isovaleric acid, 2-methyl butyric acid, isobutyric acid, 1-hydroxy 2-propanone, 1-hydroxy 2-butanone, butyric acid, 4-methylhexanoic acid (S. aureus in blood agar)	Preti et al. (2009)
E. coli, S. sonnei, S. typhimurium, Bacillus cereus, L. monocytogenes, S. aureus	1 Octanol, 1-decanol, dodecanol, 2 undecanone, 2-tridecenone, indole (E. coli), 1 octanol, 1-decanol, dodecanol, 2-nonanone,1-undecene, 2-undecanone, 2-tridecanone (S. sonnei), 1 octanol,1-decanol, dodecanol (S. typhimurium), 2-undecanone,	Elgaali et al. (2002)

2.4.4-Application of electronic nose for foodborne pathogen detection

As described previously, several authors have reported the detection and measurements of VOCs associated with bacterial foodborne pathogens as well as other microorganisms, leading to different attempts to create a profile of microbial VOCs (MVOCs) for a particular pathogen. The application of E-nose for microorganism's detection is described in this section and summarized in Table 2.

Table 2: Recent studies on food pathogen detection by electronic nose

Pathogens	Matrix	Sensor types	Chemometric analysis	References
E. coli O157:H7, Salmonella typhimurium 857, Staphylococcus aureus 29213, Pseudomonas aeruginosa 27853	Beef	32-polymer sensor nose chip	-	Abdallah et al. (2013)
Salmonella typhimurium	Beef	8 MOS sensors	LDA, QDA	Balasubramanian et al. (2012)
E. coli, Salmonella typhimurium	Super broth	12 MOS sensors	PCA, BPNN	Siripatrawan (2008a)
E. coli, Listeria innocua.	Lysogeny broth, Brain–Heart Infusion media	12 MOS sensors	ULDA	Green et al. (2011)
E. coli DH5a, Listeria innocua, Enterococcus faecalis, E. coli Biotype I	Brain–heart Infusion	12 MOS sensors	PCA, ULDA	Green et al. (2014)
E. hormaechei and E. coli	Mixed vegetable soups	4 MOS sensors	LDA	Gobbi et al. (2015)
Escherichia coli	Processed Tomatoes	6 SMO sensors	PCA	Concina et al. (2009)
Staphylococcus. Salmonella, Shigella	Apple	6 SMO sensors	PCA, HCA	Ezhilan et al. (2018)
Escherichia coli, Listeria monocytogenes, Salmonella Typhimurium	Brain Heart Infusion	4 MOX thin film gas sensors and 2 MOX nanowires gas sensors	PCA	Sberveglieri et al. (2015)
Escherichia coli (ATCC 25922)	Alfalfa Sprouts	12 MOS sensors	ANN	Siripatrawan et al. (2006)
Enterococcus faecalis, Escherichia coli and Staphylococcus Aureus	Street foods	9 MOS sensors	SVM	Balbin et al. (2017)
Escherichia coli	Goat meat	32 polymer sensors	PCA	Ding et al. (2010)

Green et al. (2011) used an E-nose based on metal oxide semiconductors (MOS) sensors coupled with uncorrelated linear discriminate (ULDA) analysis to effectively identify and discriminate between *E. coli* and *Listeria innocua* in phosphate-buffered saline solution, with a classification accuracy of 92.4%. This approach was developed by analyzing the olfactory fingerprints of single bacterium colonies that were scraped from the agar medium's surface. Bacteria identification based on single colony E-nose responses provides quick findings and eliminates the need for culturing, serological, and biochemical testing. Green et al. (2014) also used individual colonies to test the reliability of employing an E-nose for bacterial identification at the genus level. This study used four non-pathogenic bacteria species (*E. coli* DH5a, *Listeria innocua*, *Enterococcus faecalis*, and *E. coli* Biotype I) and classification accuracy greater than 80% was achieved, with higher classification accuracy (96.7%) when the E-nose sampling was repeated for the same colony and all existing odor responses were used for sample characterization. Integrating E-nose data with chemometrics also allowed the fast identification of *L. monocytogenes*, *Staphylococcus lentus*, and *Bacillus cereus* (Yongxin and Zhao 2012). A research found that an E-nose together with cluster analysis and principal component analysis (PCA) allowed differentiating four distinct *Vibrio parahaemolyticus* strains, as well as discriminating four different *Pseudomonas* species. Xue et al.,2012 prosposed the application of an E-nose in conjunction with chemometrics to differentiate strains and species. Brain heart infusion broth was used to cultivate *L. monocytogenes*. The volatile compounds generated from nine strains of *Listeria monocytogenes* and four species of *Listeria* spp. and collected by the E-nose allowed a successful analysis based on PCA combined with ANN (artificial neural networks) for feature extraction.

Since discriminating between individual bacterial colonies at both the species and strain level was achievable, these findings demonstrated the E-nose has strong promise as an accurate early diagnostic screening technique for bacterial foodborne disease identification. Since virulence and pathogenicity are often linked with just a fraction of bacterial strains, it is critical for a method to be able to discriminate between pathogenic and nonpathogenic strains during a foodborne epidemic.

2.4.4.1- Microorganisms in food matrices detection

Meat products: Balasubramanian et al. (2008) achieved successful prediction of *Salmonella* typhimurium in contaminated beef using E-nose data and independent component analysis (ICA). A stepwise linear regression prediction (SLRP) model was built with the independent component (IC) and principal components (PC) with a prediction accuracy of 69.64% and 82.99%, and a root mean squared error (RMSE) of 1.358 and 0.803 for PCA and ICA respectively. The results showed that ICA performed better than PCA on the E-nose dataset, ICA which is higher-order statistical techniques can explore higher order information of the original inputs than PCA (Cao et al. 2003).

Balasubramanian et al. (2012) effectively screened *Salmonella typhimurium* in beef using two separate gas sensor-based artificial olfactory systems: conducting polymer-based and metal oxide-based sensors. For classifying ""No *Salmonella*" (counts < 0.7 $\log_{10}$ cfu/g) and "*Salmonella* inoculated" (counts < 0.7 $\log_{10}$ cfu/g) in meat samples stored at 10 °C, LDA and QDA classification models achieved varying levels of success for polymer E-nose (69%), metal oxide E-nose (>70%), and a fusion of the sensors (>80%). The utilization of just relevant sensors (as determined by Fisher Criteria Ranking of Sensors) and sensor fusion techniques were shown to be crucial in obtaining improved classification accuracy.

For the quick detection of *E. coli* in goat meat samples, Ding et al. (2010) used a Cyranose-320 E-nose based on 32 polymer sensors, with early findings indicating 18–77% detection accuracies for cultivated bacteria.

Due to overlapping or extremely near marking, there was no difference in PCA data collected for infected and uncontaminated meat samples, and the sensor was sensitive to lower bacteria concentrations.

Balbin et al. (2017) used SVM on E-nose signals to identify and classify *E. coli* and *Staphylococcus aureus* in street meals, which are a significant cause of foodborne diseases. The study's findings demonstrated that pathogens in street meals (Kwek–Kwek, pork barbeque, and isaw) could be detected before and after cooking, demonstrating the use of E-nose as an online tool for process monitoring during food preparations.

Abdallah et al. (2013) used an E-nose with a 32-sensor nose chip to identify *E. coli O157: H7*, *Salmonella typhimurium 857*, and *S. aureus 29213* in fresh and frozen beef. The study's findings revealed a substantial association ($p < 0.005$) between gas concentration before and after pathogen contamination.

Vegetables and fruits: E-nose was used by Concina et al. (2009) to identify microbial contamination in processed tomatoes. After 48 hours of inoculation, *E. coli* using both KNN pattern recognition methods showed high classification scores of 83%. The findings demonstrated that the metabolic kinetics of microorganisms have an impact on the headspace composition during microbial growth. Siripatrawan et al. (2006) used an E-nose with 12 metal oxide electronic sensors to capture volatile metabolites generated by *E. coli*. Using the data collected, an ANN model with a regression coefficient R^2=0.903 was used to predict *E. coli* counts in packed alfalfa sprouts. By merging E-nose data with PCA and BPNN models, Siripatrawan (2008a) established a fast approach for discriminating *E. coli* and *Salmonella typhimurium*. PCA was used to illustrate class separation among sample subgroups and for data exploration and dimensionality reduction. With a regression coefficient $R^2 = 0.96$ between actual and projected data, BPNN was able to make a good prediction.

Siripatrawan used a Self-organizing map method to classify *E. coli* bacteria in packed fresh vegetables (2008b). In the vegetable samples, the SOM algorithm paired with E-nose data correctly categorized *E. coli* over 10^5 cfu/g. Siripatrawan and Harte (2015) used the Kohonen network for data visualization of *Salmonella typhimurium* present in packed fresh alfalfa sprouts in a more recent investigation. On the self-organizing map, the Kohonen network could visually differentiate various amounts of *S. typhimurium* contamination (SOM).

The Kohonen network proved more useful and better at displaying multi-dimensional nonlinear data than a traditional linear principal component analysis (PCA) technique, and it demonstrated a far more perfect separation of distinct sample groups.

Gobbi et al. (2015) were able to quickly identify *E. coli* in vegetable soups. At a detection threshold of 8 and 3 cells/100 ml, E-nose with four metal oxide sensors and LDA analysis obtained a classification performance of 98% for *E. coli* contamination.

The starting and final microbial concentrations had no impact on the discrimination of bacterial contamination in this investigation.

Although the research demonstrated the ability to diagnose bacterial contamination throughout development, it should be highlighted that the production of VOCs from bacteria alters during their growth.

A trilayer technique based on a handmade E-nose was employed by Ezhilan et al. (2018) to investigate the prevalence of *Staphylococcus*, *Salmonella*, and *Shigella* bacteria in delicious royal apple at concentrations of zero, 10^2, 10^3 and 10^4cfu/mL. The samples were analyzed using voltage responses for E-nose sensors, as well as a PC and wards HCA. The created E-nose effectively evaluated the freshness or contamination levels of the apple samples by integrating data classification systems, bacterial culture investigation, and GC–MS analysis.

Others *Salmonella* enterica is a disease most often linked with chicken, and it is most commonly transmitted by manure contamination during processing. E-nose was used by Kizil et al. (2015) to identify the presence of *S. enteric* poultry manure, with the ANN model reaching a classification accuracy of 94% for both the training and validation sets. Sberveglieri et al. (2015) used six MOX gas sensors and PCA to examine the use of E-nose in food quality control for the identification of microorganisms in water and other food matrices. At a concentration of 9×10^8 bacteria/ml, *E. coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* were found.

2.4.4.2- Advantages and Potentialities of E-nose for bacterial pathogen detection

The E-noses used in the food sector have mostly been applied for food quality analysis, particularly for identification purposes. So, future prospects for using this technology for bacterial pathogen detection would be the possible extension to detect viable microorganisms. In contrast to various microbiological and molecular approaches, E-nose detection has limitations, including limited sensitivity and specificity. The identification of volatile chemicals is generally hampered by a complicated background combination of water vapour and carbon dioxide (Sanaeifar et al. 2017).

Other drawbacks include a high limit of detection (LOD), with Siripatrawan (2008b) reporting an E. coli detection limit of over 10^5 CFU/g and Gobbi et al. (2015) reporting E. coli sensitivity as low as 3 cfu per 100 ml. However, increasing the diagnostic specificity of VOCs by using enzyme substrates to release exogenous VOCs of foodborne bacterial pathogens might overcome this problem. This is accomplished by altering bacteria growth medium with substrates that, in response to the presence of enzyme activity shown by a target pathogen, release distinct VOCs via enzymatic metabolism. This approach has been successfully examined using traditional detection methods such as gas chromatography–ion mobility spectrometry (GC–IMS) and gas chromatography–mass spectrometry (GC–MS), and it may be enhanced with a noninvasive electronic nose application. 2-nitrophenyl-b-d-glucuronide (*E. coli*), 2-nitrophenyl-b-d-glucopyranoside (*Listeria spp.)* and 2-nitrophenyl-b-d-galactoside-6-phosphate are examples of substrates (*Staphylococcus aureus*).

Although, they are many advantages of E- nose like fast detection Non-invasive technique

Minimal sample pretreatment Green technique, not involving chemical reagent…

3. Materials and methods

3.1- Microorganisms growth

3.1.1 Bacterial strains and inoculum preparation

As target food-borne pathogens, three Gram positive spherical-shaped (*S. aureus ATCC653* and *E. faecalis ATCC29212*) and one Gram-negative rod-shaped (*P. aeruginosa ATCC15442*) bacteria were chosen. Since *E. coli ATCC29998* is a general indicator of feces and hygiene, it was also included in this investigation. The inocula were created by combining 700 µL of an overnight bacterial culture with 300 µL of glycerol (Sigma-Aldrich), as previously explained (Ghrissi et al., 2021). The growth was accelerated in the Brain Heart Infusion (BHI) (PanReac AppliChem, ITW Reagents) at 37 ℃ and 90 rpm (Orbital incubator S1500, Stuart). Prior to usage, each inoculum was cry preserved at 20 ℃.

Figure 1: Orbital incubator used in the study

3.1.2. Growth conditions

Each strain was aseptically inoculated into 250 mL Erlenmeyer flasks with 50 mL of BHI media. Until the stationary phase of the cells was attained, bacterial cultures were cultivated at 37°C with orbital agitation (90 rpm) for 24 hours. After that, a spectrophotometer was used to detect the optical density at 560 nm (UV-3100 PC Spectrophotometer, VWR). Preliminary tests (data not shown) revealed that overnight developed cultures were used to complete sets of pre-established serial dilutions of *E. faecalis* from 10-1 to 10-8 and *P. aeruginosa*, *S. aureus*, and *E. coli* from 10-1 to 10-9 using NaCl solutions (0.9% v/v). These colony forming units (CFU) were evaluated: 100 µL of each dilution was duplicated and plated on BHI agar. CFUs were counted following a 37°C overnight incubation period. Gram staining was used to confirm that there was no contamination of the culture, as stated by Gregersen.

Figure 2: Microorganisms growth

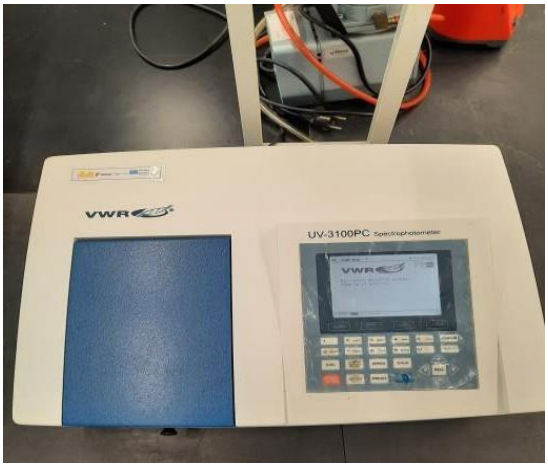

Figure 3: Spectrophotometer used in this study

3.1.3 HS-SPME-GC-MS evaluation of VOCs emitted by each bacteria

Through the use of headspace solid-phase micro extraction (HS-SPME) gas chromatography/mass spectrometry (GC/MS), the profiles of VOCs released by the four bacteria under investigation—each of which was cultured independently were determined. By examining the HS of glass vials (50 mL, Duran, Germany) containing 10 mL of BHI agar that had previously been infected with 100 µL of a diluted overnight culture, the volatile fraction was chromatographically analyzed (10-4 dilution for *E. faecalis* and 10-5 for the others bacterial strains). Polytetrafluroethylene/silicone screw caps were used to seal the vials, which were then incubated at 37°C for 24 hours. As a negative control, the HS of BHI culture medium that had not been infected was also examined.

The mass spectrometer used was a Shimadzu GC/MS-QP2010 SE detector with a Shimadzu GC-2010 Plus chromatograph. The internal standard (IS: α-pinene, 98% from Sigma Aldrich) methanolic solution, with a concentration of 0.50225 mg/mL, was accurately added to each vial after it had been inoculated with a predetermined amount of one of the four bacteria. The volatiles were adsorbed into an SPME fiber coated with divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS 50/30 μm, from Supelco, Bellefonte, USA). To allow the release of the VOCs, the vials were condition for 5 minutes at 37 degrees Celsius. Following this timeframe, the SPME fiber was exposed for 30 min at 37° C, allowing the volatile chemicals in the headspace to adsorb. The same method and the same amount of IS were used to study the volatile portion of the solid medium that had not been infected with any of the four bacteria For each bacterium,, the process was carried out twice using uninoculated media. As previously described (Marx et al., 2021), peaks' separation was accomplished on a TRB-5MS (30 m × 0.25 mm × 0.25 μm) column (Teknokroma, Spain).

The injector was set at 220 ºC and the manual injections were made in splitless mode.

The mobile phase consisted of helium (Praxair, Portugal) at a linear speed of 30 cm/s and a total flow of 24.4 mL/min. The oven gradient temperature was as follows: 40 ºC/1 min; 2 ºC/min until 220 ºC (30 min). The ionization source was maintained at 250ºC with ionization energy of 70 electron volts (eV) and an ionization current of 0.1 kilovolts (kV). All mass spectra were produced by electron ionization, and the individual spectra fragments were identified by comparing them to the free databases of PubChem and ChemSpider as well as the mass spectra in the NIST SRD-69 Library (National Institute of Standards and Technology, Gaithersburg, MD, USA).

For identification reasons, a minimum similarity of 85% was specified. The Kovat retention indices were also used to confirm the peak identification. By integrating the reconstructed chromatogram from the full scan chromatogram and utilizing the ion base (m/z intensity 100%) for each compound, the areas of the chromatographic peaks were identified. Without taking into account the response variables, the quantities of the discovered volatiles were determined by dividing the area of each base ion peak by the area of the internal standard base ion peak, and then converted to mass equivalents using the internal standard concentration.

3.2. E-nose setup

3.2.1. Apparatus

The E-nose used in this study was designed and built (Fig. 1) by the research team, as an all-in-one olfactory multi-sensor device. The device included a sampling heated unit (~30ºC), where up to 4 glass sampling vials (~ 25 mL) could be placed. Each vial was closed with a screw cap connected to a plastic gas valve allowing, when open, to collect and deliver the gas headspace to the sensing heated unit (~40 ºC) by means of a diaphragm vacuum mini pump. The vacuum was monitored using a mini Dial Air Vacuum Pressure Gauge Meter Digital Manometer. The sensing unit comprised 9 commercial MOS (Table 3) Figaro gas sensors (S1: TGS 2600 B00; S2: TGS 2602; S3: TGS 2610 C00; S4: TGS 2611 C00; S5: TGS 2610 D00; S6: TGS 2610 E00; S7: TGS 2612; S8: TGS 826 A00; TGS 823 C12N; specifications can be found at https://www.figarosensor.com/product/sensor/, accessed on 6 October 2022), which are sensitive towards alcohols, hydrocarbons, hydrogen, carbon monoxide, ammonia, hydrogen sulfide, among other gases.

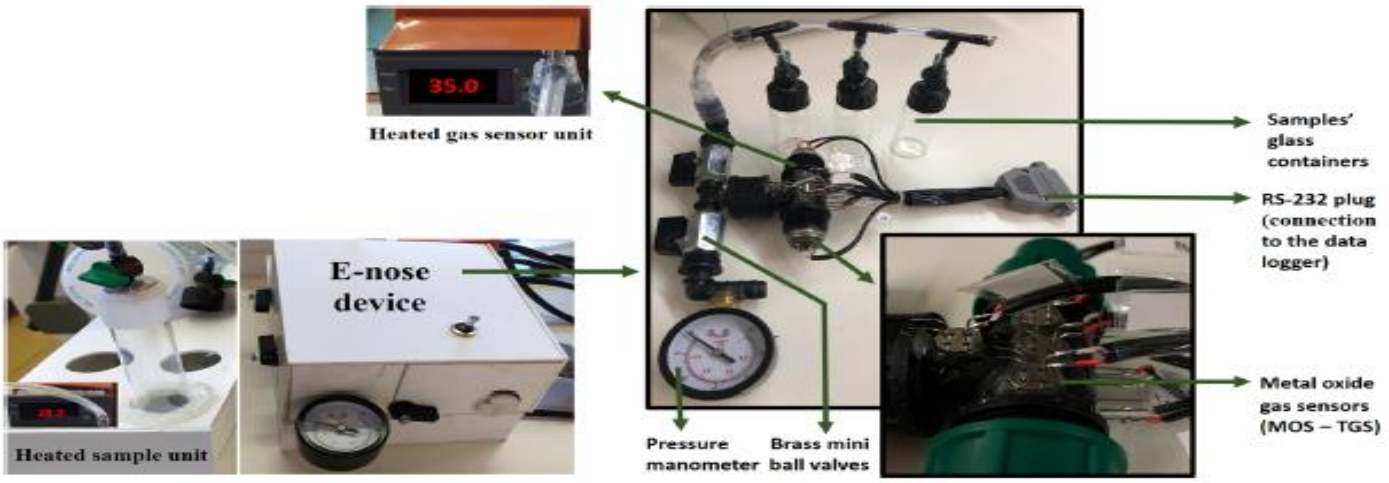

Figure 4: E-nose-MOS lab-made device comprising: sample heated unit, gas sensor heated unit and data logger interface.

Table 3: Metal oxide gas sensors (MOS) included on the lab-made E-nose device.

Sensor code	Commercial sensor code	Target gases
S1	TGS 2600 B00	General air contaminants
S2	TGS 2602	General air contaminants
S3	TGS 2610 C00	Butane, liquid petroleum gases
S4	TGS 2611 C00	Methane, natural gas
S5	TGS 2610 D00	Butane, liquid petroleum gases (carbon filter)
S6	TGS 2611 E00	Methane, natural gas (carbon filter)
S7	TGS 2612	Methane, propane, iso-butane
S8	TGS 826 A00	Ammonia
S9	TGS 823 C12 N	Organic solvent vapours

3.2.2. Sampling and analysis

During the first use, the MOS sensors' sensitive components were turned on for 48 hours, allowing them to attain the operating sensing temperature (> 200 ° C). All subsequent tests simply needed heating the sampling and sensing devices for about 30 minutes, followed by a thorough system clean using filtered air pumps. Before every study, a vacuum environment of 0.35 bar was also built up to eliminate any potential interfering chemicals that might be present in the external air flow and to encourage the cleaning of the sensitive sensor materials. After that, the gas static headspace of each vial (~ 25 mL) was collected by suction (vacuum pump) and delivered to the sensing unit, allowing the interaction, during 2.5 min, of the VOCs with the 9 MOS sensors, generating the respective resistive signal profiles, which were acquired each 4 s. Before pumping the sample's gas headspace, each glass vial, previously inoculated with a single strain and a known number of CFUs, was placed inside the sampling unit during 13 min to allow reaching the desired temperature (~30 ºC). The cleaning step was promoted during the samples heating time.

Overall two experimental designs were conducted. In the first, in order to evaluate the qualitative classification (i.e., discrimination) performance of the E-nose, glass vials (25 mL) with 7 mL of BHI agar medium, were inoculated with 100 µL of different diluted solutions obtained from an overnight culture of one of the four bacteria under study: dilutions of 10^{-1} to 10^{-7} for *E. faecalis*; and dilutions of 10^{-1} to 10^{-9} for the other three bacteria. In the second kind of assays, used for evaluating the quantitative performance of the E-nose to assess the number of CFUs of a single bacterium, sterile glass vials (25 mL) containing 7 mL of BHI agar medium were inoculated with 100 µL of different diluted solutions of an overnight grown bacteria culture with a known amount of CFUs. In this way, the vials were inoculated with dilutions of10^{-4} to 10^{-8} for *E. faecalis* (from 1.2 to 1.2 × 10^{6} CFUs), and from10^{-5} to 10^{-9} for the others bacteria (*E. coli*: 2.2 to 2.2 × 10^{8} CFUs; *P. aeruginosa*: 3.05 to 3.05 × 10^{5} CFUs; and, *S. aureus*: 1.2 to 1.2 × 10^{6} CFUs). After inoculation, the vials were closed with screw caps and incubated at 37ºC for 24h, being only analyzed with the E-nose those with visible bacterial growth.

3.2.3. Data acquisition, feature extraction and signal treatment

As previously described (Teixeira et al., 2021), the resistance signals (in ohms) generated due to the interaction between the VOCs emitted by each bacterium (*E. coli, P. aeruginosa, E. faecalis* or *S. aureus*), grown in solid medium, and the nine MOS sensors, were acquired by a data logger (Agilent 34970A) and then recorded by the Agilent Bench Link Data Logger software. For each analysis and sensor, a total of 37–38 resistance values were recorded (signals acquired during 2.5 min each 4 s).In total, seven feature extraction methods (Gregersen et al.,1978 Teixeira et al., 2021) were applied to obtain a representative E-nose fingerprint for the volatile profile of the compounds emitted during the growth of each of the four bacteria under study: last response point (LP) acquired; integral of each E-nose signal response curve (INT); maximum resistance response (MAX); minimum resistance response (MIN); sum of the response curve (SUM): sum of all the resistance signals recorded during the analysis time-period; average of the resistance signal curve (MEAN); and, standard deviation (SD) of the response curve. In this context, for each independent sample a response database comprising 9 sensors × 7 feature extraction method, is generated, being the basis for the statistical analysis.

3.3. Statistical analysis

Linear discriminate analysis (LDA) in combination with the simulated annealing (SA) algorithm was implemented as a supervised classification multivariate procedure to assess the E-nose predictive performance towards the discrimination of the four bacteria evaluated, based on the recorded resistance signals generated by the interaction between the sensors and the VOCs emitted by each bacterium, grown in solid medium. The classification potential was evaluated by determining the sensitivities (i.e., the correct classification percentages) for the leave-one-out cross-validation (LOO-CV) variant. Furthermore, for the training set, the classification was also checked graphically, by plotting the 2D graphs for the two most significant discriminate functions (DFs). Multiple linear regression (MLR) models, based on feature extraction parameters selected using the SA algorithm, were also developed. For this, the E-nose signals used were those generated when analyzing vials with solid medium, inoculated with pre-established known amounts (in CFU) of each of the four studied bacteria. The accuracy of the E-nose-MLRM was discussed based on the determination coefficients (R^2) and the root-mean-square errors (RMSEs) for the training and LOO-CV procedures. The accuracy of developed models was verified against the analytical conventional counting plate technique, being evaluated if the slope and intercept values of the regression line between the data for both approaches can be statistically assumed equal to those of a perfect line (i.e., 1 and 0, respectively) (Roig et al.,2003; Roig et al.,2021).

All statistical analyses were performed using the packages of the open-source statistical program R (R Studio 2021.09.0 Build 351), at a 5% significance level.

4. Results and Discussion

4.1. Evaluation of VOCs emitted during the bacterial growth by HS-SPME-GC-MS

The VOCs emitted during the bacterium growth depend on the bacterial metabolism that is influenced by the culture media composition and nutrient sources. Thus, many bacterial VOCs have been identified and their amounts and profiles greatly depend on the culture medium, incubation time, and bacterial species and strains used, being contradictory detection/non-detection data reported (Thorn et al., 2021 ; Bos et al.,2014; Tait et al.,2014). Also, background VOCs related to the growth media have been reported (Thorn et al., 2021).

Thus, as a first step, the VOCs emitted by the BHI un-inoculated medium were determined. Globally, 15 volatiles were identified (data not shown), being 10 of them also emitted by at least one of the four bacteria evaluated, namely two alcohols (1-butanol and 1-nonanol), three pyrazines (2-ethyl-6-methyl-pyrazine, 3-ethyl-2,5-dimethylpyrazine and trimethylpyrazine), three terpenes (camphene, D-limonene and β-pinene) and two other compounds (2,4-thujadiene and indole). The volatiles were identified in both un-inoculated and inoculated (with a 10^{-4} dilution for *E. faecalis* and 10^{-5} dilution for the other bacterial strains) solid culture medium (i.e., BHI agar medium), after overnight incubation. **Table 4** shows the amounts (in ng of each compound as IS equivalents) of the VOCs detected during the chromatographic analysis of the volatile head-space generated during the overnight growth of the two Gram-positive (*E. faecalis* and *S. aureus*) and the two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria, after subtracting the respective amounts quantified in the head-space of the un-inoculated culture medium.

Table 4: Identified volatile compounds and respective average amounts (ng of compoundas internal standard equivalents), for the *E. coli*, *P. aeruginosa*, *E. faecalis* or *S. aureus*, grown overnight in BHI solid medium after incubation with a 10^{-4} dilution for *E. faecalis* and 10^{-5} dilution for the other bacterial strains.

Volatile compounds	Gram-negative bacteria		Gram-positive bacteria	
	E. coli	***P. aeruginosa***	***E. faecalis***	***S. aureus***
Alcohols				
1-Butanol	33.82	nd	51.68	nd
1-Nonanol	5.42	nd	nd	nd
1-Pentanol	53.96	95.53	869.56	51.09
3-Chloro-2-methyl-2-pentanol	nd	nd	nd	11.11
Ethanol	nd	nd	695.93	nd
Phenylethyl alcohol	15.46	nd	23.30	nd
Aldehydes				
Phenol	6.19	nd	6.87	nd
Alkanes				
Isocetane	5.11	5.21	5.55	5.41
Alkenes				
(E)-1,4-Undecadiene	nd	70.09	nd	nd
1-Undecene	nd	32.00	23.71	43.71
Carboxylic acids				
2-Methylbutanoic acid	nd	nd	13.63	nd
Acetic acid	nd	nd	29.40	nd
Isovaleric acid	nd	43.40	37.17	nd
Undecane	9.89	11.45	16.63	7.22
Esters				
Methyl valerate	nd	70.17	nd	114.72
Ketones				
2-Tridecanone	7.92	nd	nd	nd
Phellandrenes				
α-Phellandrene	nd	nd	nd	6.81
Pyrazines				
2,5-Dimethylpyrazine	38.62	46.01	48.88	46.87
2-Ethyl-6-methyl-pyrazine	0.52	nd	0.79	nd
3-Ethyl-2,5-dimethylpyrazine	1.11	nd	nd	nd
Trimethylpyrazine	nd	nd	nd	0.59
Terpenes				
Camphene	nd	nd	nd	1.50
D-Limonene	0.74	nd	nd	nd
β-Pinene	2.56	4.78	4.71	3.08
Others				
2,4-Thujadiene	0.52	nd	1.47	0.59
E-7-Dodecen-1-ol acetate	nd	nd	11.67	nd
Indole	nd	2.97	nd	32.75
Methyl undecyl ether	nd	nd	19.82	nd

nd: not detected

As can be seen from **Table 4**, VOCs belonging to 10 chemical classes were identified, being alcohols the most abundantly emitted by *E. coli*, *E. faecalis* and *P. aeruginosa* during their growth in BHI solid medium, while esters were the predominant volatiles produced by *S. aureus* grown in the same culture medium. It should also be noticed that each bacterium had a specific profile of emitted VOCs, both in number of different volatiles identified as in the quantified amounts. In total, 17 VOCs were emitted during the growth of *E. faecalis*, followed by 14 VOCs for *E. coli*, 13 VOCs for *S. aureus* and only 10 VOCs for *P. aeruginosa*. As can also be inferred, some VOCs were specifically produced by only one of the four studied bacteria (*E. coli*: 1-nonanol, 2-tridecanone, 3-ethyl-2,5-dimethylpyrazine, and D-limonene; *P. aeruginosa*: (E)-1,4-undecadiene; *E. faecalis*: ethanol, 2-methylbutanoic acid, acetic acid, and methyl undecyl ether; *S. aureus*: 3-chloro-2-methyl-2-pentanol, α-phellandrene, trimethylpyrazine, and camphene), while others were common to two or more bacteria, although emitted, in general, in quite different amounts. These differences, in the emitted VOCs profiles and respective amounts generated by each bacterium growth, confirmed that VOCs can be used as bacterial identification biomarkers. However, it should be remarked that, for example, in this study, ethanol was only identified during the growth of *E. faecalis*, although according to the literature this alcohol is usually emitted by the four bacteria evaluated, being more abundant for *E. coli* and *S. aureus* compared to *E. faecalis* and *P. aeruginosa* (Bos et al.,2014).Also, indole is usually associated in the literature with *E. coli* Nieto-Arribas et al.,2021; Kladsomboon et al.,2018) but, in the present study, it was only identified for *P. aeruginosa* and *S. aureus*. On the other hand, (E)-1,4-undecadiene was only identified for *P. aeruginosa*, in-line with the literature data, according to which this alkene has been associated with *Pseudomonas* species, allowing its use as a biomarker for this species (Poveda et al.,2021). The observed differences may be tentatively attributed to the different bacterium strains, growth media and/or incubation conditions (time and temperature) used in the various studies as well as to the diverse chromatographic analysis conditions applied.

4.2. Bacterial species discrimination using the E-nose-MOS lab-made device

The dataset comprising the resistance signals based feature extraction data generated by the E-nose sensors (9 sensors × 7 feature extraction variables, for each vial inoculated with different pre-established CFU of a single bacterium) was used to evaluate the potential application of the lab-made E-nose for discriminating the four bacteria under study, which were grown in solid agar medium (BHI agar).

Figure 2 shows that an E-nose-MOS-LDA-SA model could be established, based on 25 feature extraction parameters selected by the SA algorithm (S6_LP, S1_INT, S5_INT, S8_INT, S9_INT, S1_MAX, S2_MAX, S4_MAX, S6_MAX, S2_MIN, S4_MIN, S5_MIN, S6_MIN, S2_SUM, S3_SUM, S5_SUM, S6_SUM, S8_SUM, S9_SUM, S2_MEAN, S5_MEAN, S1_SD, S2_SD, S3_SD, and S6_SD), which first two DFs explained 99.4% of the data. The model enabled the full discrimination of the four bacteria (sensitivity and specificity of 100%, for the training/original grouped data). Moreover, it should be highlighted that, according to the 1st DF, it was possible to successfully discriminate the two Gram-positive bacteria (*E. faecalis* and *S. aureus*) from the two Gram-negative bacteria (*E. coli* and *P. aeruginosa*), located in the positive and negative regions, respectively. The satisfactory classification performance of each single bacterial species as well as between Gram-positive and Gram-negative bacteria, could be attributed to the observed differences, in number and respective amount, of the VOCs emitted during the bacterial growth of each species (Table 3). As previously pointed out, the VOCs' profiles established in this study by HS-SPME-GC-MS clearly pointed out the existence of volatiles emitted by only one of the four bacteria, which would generate different signal responses during the E-nose analysis, justifying the discrimination power of this MOS sensor device. It should be highlighted that, although not confirmed in the present study, other VOCs have been reported in the literature, as bacterial species' biomarkers, namelyisovaleric acid, or 2-methyl-butanal for *S. aureus*; 1-undecene, 2,4-dimethyl-1-heptane, 2-butanone, 4-methyl-quinazoline, hydrogen cyanide, or methyl thiocyanide for *P. aeruginosa*; and, methanol, pentanol, ethyl acetate, or indole for *E. coli* (Bos et al.,2014).

In which concerns the predictive performance, an overall correct classification of 90% was achieved for the LOO-CV procedure, with a global specificity of 91%. Among the four bacteria, only *S. aureus* was not misclassified (sensitivity of 100%) but had the lowest specificity of (~83%). On the contrary, one of the 10 assays with *E. coli* was misclassified as *S. aureus* (sensitivity and specificity of 90%); one *E. faecalis* was misclassified as *P. aeruginosa* (sensitivity of 90% and specificity of 100%); and, finally, one sample of *P. aeruginosa* was incorrectly classified as *E. coli* and another as *S. aureus* (sensitivity of 80% and specificity of 89%).The misclassifications observed, although in a low number (4 in 40 independent bacteria samples) can be attributed to the fact that some VOCs are emitted by different bacterial species, which mitigate their use as unique chemical fingerprints. Indeed, the VOCs profiles established in this study showed that several

volatiles were emitted by two or more of the four bacteria under study, although in different amounts (e.g., 1-butanol, 1-pentanol, phenylethyl alcohol, phenol, isocetane, 1-undecene, isovaleric acid, undecane, methyl valerate, 2,5-dimethylpyrazine, 2-ethyl-6-methyl-pyrazine, β-pinene, 2,4-thujadiene or indole). Additionally, a systematic review performed by Bos et al. revealed that, for example, the four bacteria produce isopentanol, formaldehyde, methyl mercaptan, and trimethylamine, although not observed in the present study.

Nevertheless, it should be remarked that the discrimination rates (training and LOO-CV) are similar to the E-nose classification performances previously reported in the literature, which sensitivities varied from 84 to 100%, allowing differentiating/discriminating several bacterial species, including *E. coli*, *E. faecalis*, *P. aeruginosa* or *S. aureus*, among other pathogenic bacteria (Green et al.,2014; Bonah et al.,2019; Roda et al.,2016; Carrillo-Gómez et al.,2021).

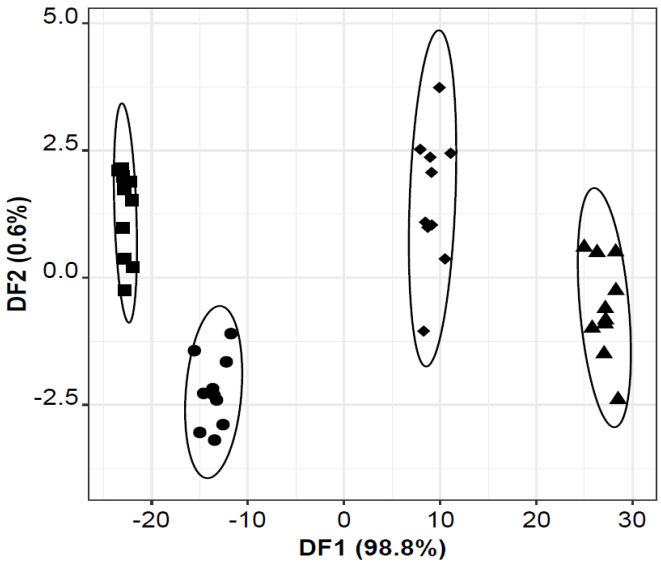

Figure 5: E-nose-MOS-LDA-SA model's discrimination of the four studied Gram-positive and Gram-negative bacteria grown in BHI agar medium: (▲) *E. faecalis*; (♦) *S. aureus*; (■) *E. coli*; and, (●) *P. aeruginosa*. Classification model based on 25 feature extraction MOS signal parameters (S6_LP, S1_INT, S5_INT, S8_INT, S9_INT, S1_MAX, S2_MAX, S4_MAX, S6_MAX, S2_MIN, S4_MIN, S5_MIN, S6_MIN, S2_SUM, S3_SUM, S5_SUM, S6_SUM, S8_SUM, S9_SUM, S2_MEAN, S5_MEAN, S1_SD, S2_SD, S3_SD, and S6_SD).

4.3. Quantification of bacteria CFUs using the E-nose-MOS lab-made device

As previously mentioned, few studies report the use of E-noses as semi-quantitative (Carrillo-Gómez et al., 2021; Carrillo-Gómez et al., 2019) or quantitative (Tonezzer et al., 2021; Barbri et al., 2009) tools to assess the levels of bacteria. In this sense, the present study also aimed

to evaluate the quantitative performance of the lab-made E-nose-MOS for determining the number of CFUs in BHI agar medium. For that, MLRM were developed relating the decimal logarithmic of the CFUs ($\log_{10}$(CFU)) of each one of the four bacteria as a multiple linear function of the selected feature extracted parameters derived from the E-nose-MOS response towards the VOCs emitted by each single bacterium grown in solid medium. The predictive models' performances were evaluated based on two goodness of fitting parameters (R^2 and RMSE) for the LOO-CV variant. Table 2 shows the goodness of fitting data as well as the information regarding the concentration range studied and the number of parameters included in each developed MLRM, which were selected by the SA algorithm.

The satisfactory predictive (LOO-CV) values of R^2 and RMSE ($0.943 \leq R^2 \leq 0.994$ and $0.158 \leq$ RMSE $\leq 0.602\log_{10}$ (CFU)), which are confirmed by the visualization of Figure 3, support the use of the lab-made E-nose-MOS for quantifying the number of CFU of each one of the four bacteria studied. Indeed, based on the RMSE values, the number of CFU sinitially inoculated in BHI agar medium, could be estimated after 24 h of incubation (at 37 ºC) with an accuracy as low as 1 to 4 CFUs, in-line with the accuracies previously reported in the literature for quantitative assessment of bacteria in food samples (Tonezzer et al., 2021; Barbri et al.,2009).

Table 5: Predictive performance (LOO-CV) of the MLRM developed based on selected feature extraction parameters derived from the interaction of the E-nose-MOS sensors with the VOCs emitted by each one of the four bacteria studied: *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus* (contents in $\log_{10}$(CFU)).

Microorganism		**Concentration range ($\log_{10}$(CFU))[a]**	**Selected extracted feature parameters[b]**	**Goodness of fitting parameters[c]**	
				R^2	**RMSE ($\log_{10}$(CFU))**
Gram-negative	*E. coli*	[0.342, 8.342]	S4_LP; S6_SUM; S9_MEAN	0.978	0.436
	P. aeruginosa	[0.079, 6.079]	S8_MAX; S9_MAX; S9_SD	0.995	0.174
Gram-positive	*E. faecalis*	[0.079, 8.079]	S9_INT; S9_MIN; S8_MEAN; S5_SD	0.943	0.602
	S. aureus	[0.484, 5.484]	S5_INT; S4_SUM; S4_MEAN	0.994	0.158

[a]Experimental concentration range in $\log_{10}$(CFU): 9 independent levels for *E. coli*; 7 independent levels for *P. aeruginosa*; 10 independent levels for *E. faecalis*; and, 6 independent levels for *S. aureus*.[b]Feature extracted parameters from the E-nose-MOS response, selected by the SA algorithm and included in each MLRM. [b]R^2: determination coefficient; RMSE: root mean square error.

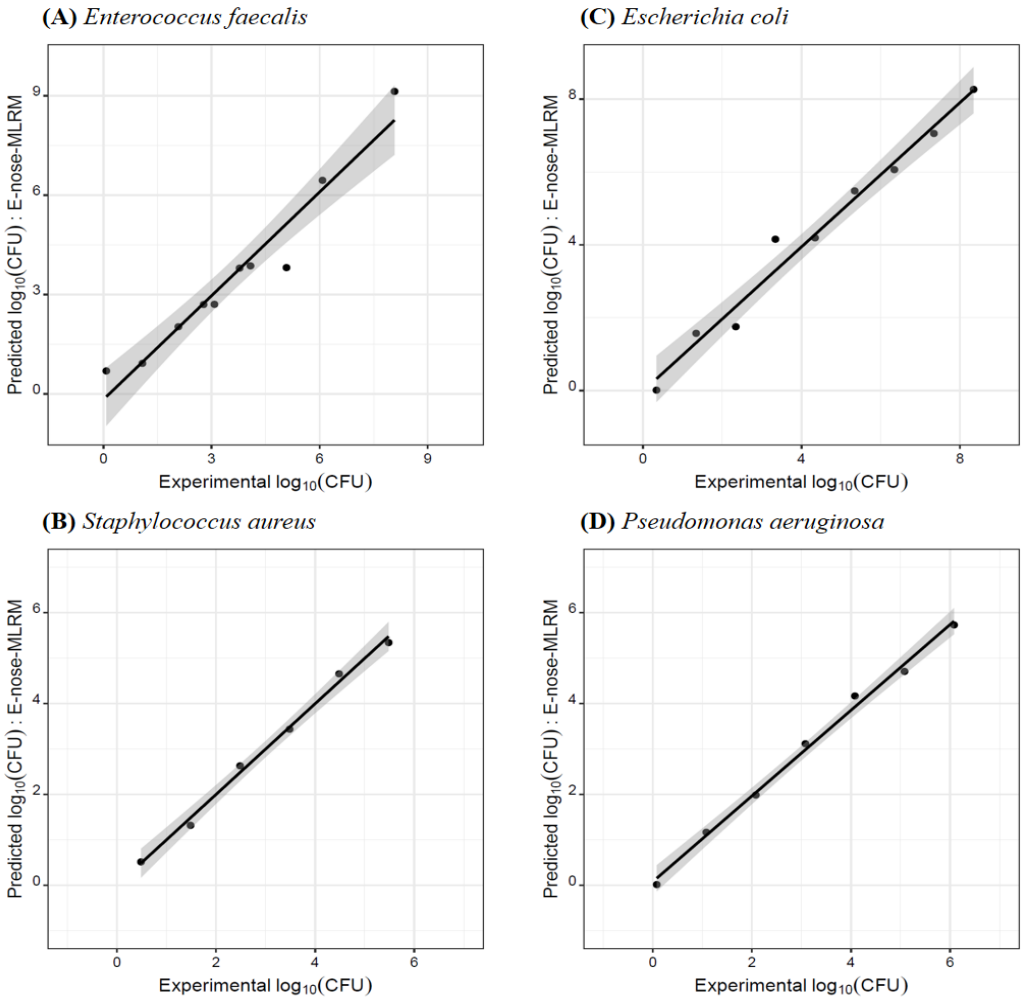

Figure 6: E-nose-MOS-MLR-SA model's predictions (leave-one-out cross-validation) of the decimal logarithmic of the colony forming units (CFU) for the studied Gram-positive and Gram-negative bacteria grown in BHI agar medium.

The possibility of using the E-nose-MOS as an alternative routine tool for estimating the number of CFU grown in a solid medium, was further checked following the methodology proposed by Roig and Thomas (Roig et al.,2003) ,which is based on the XPT 90-210 French standard (AFNOR,1997). For each bacterium, the decimal logarithmic of the CFU, predicted by the E-nose-MOS-MLRM were plotted versus the decimal logarithmic of the experimental number of CFU, determined by counting plate technique, and the respective parameters (slope and intercept values) of the single regression trend line were calculated. The regression analysis allowed verifying if the slope and intercept values were statistically equal to one and zero, respectively, which would correspond to a perfect linear fit.

Table 6 shows the parameters of the single linear regressions (R^2, slope and intercept values and the respective 95% confidence intervals, CI) for the LOO-CV procedure. The results clearly demonstrate that, at 5% significance level, the slope and intercept values were statistically equal to the expected theoretic values (i.e., the slope CI included the value 1; and, the intercept CI contained the value zero). Thus, it was confirmed that the lab-made E-nose-MOS device combined with MLRMs could be implemented as a fast, green and non-invasive tool to estimate the number of CFUs of each of the four bacteria studied inoculated separately in BHI agar medium, based on the sensors' response towards the VOCs emitted by each individual strain during a 24h growing-period in a solid synthetic medium.

Table 6: Parameters of the single linear regressions established between decimal logarithmic of the CFU predicted (LOO-CV) by the E-nose-MOS-MLM and the decimal logarithmic of the CFU experimentally determined by the conventional plate counting technique: coefficient of determination (R^2); slopes, intercept values and respective confidence intervals (CI) at 95%.

Microorganism		**R^2**	**Slope**	**Slope CI**	**Intercept (log_{10}(CFU))**	**Intercept CI (log_{10}(CFU))**
Gram-negative	*E. coli*	0.978	0.990	[0.857, 1.124]	-0.016	[-0.690, 0.658]
	P. aeruginosa	0.995	0.943	[0.863, 1.022]	0.082	[-0.211, 0.375]
Gram-positive	*E. faecalis*	0.943	1.044	[0.834, 1.254]	0.167	[-1.060, 0.726]
	S. aureus	0.994	0.998	[0.893, 1.103]	0.004	[-0.358, 0.366]

5. Conclusions

The research showed that a self-built electronic nose prototype with metal oxide semiconductor sensors may be used as a qualitative-quantitative tool for identifying and keeping track of four different bacteria. Gram-positive (*E. faecalis* and *S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria that had been cultured overnight in solid culture media could be distinguished by the proposed apparatus based on their distinct scents. Additionally, the olfactory-sensor device provided a quick, non-intrusive, and environmentally friendly alternative/complementary strategy to traditional analytical approaches by allowing the measurement of the number of colony-forming units of each of the four bacteria under study, grown separately in BHI solid culture medium.

Finally, although the impact of environmental factors and the impact resulting from the complexity and diversity of real sample matrices must be taken into consideration, the hypothesis of applying the proposed sensing-chemometric approach to the analysis of bacterial growth in solid food samples is strengthened by the promising and reliable classification and quantitative performances achieved with the lab-made electronic nose.

References

Abdallah SA, Al-Shatti LA, Alhajraf AF, Al-Hammad N, Al-AwadiB(2013) The detection ofFoodborne bacteria on beef: theapplication of the electronic nose. Springerplus 2:687.

Ahmed, A.B.H.; Abbassi, M.S.; Rojo-Bezares, B.; Ruiz-Roldán, L.;Dhahri, R.; Mehri, I.; Sáenz, Y.; Hassen(2020), A. Characterizationofpseudomonas aeruginosa isolated from various environmentalniches: New sts and occurrence of antibiotic susceptible "highriskclones". Int. Environ. Health Res. 30, 643–652.

Albin JR, Sese JT, Babaan CVR, Poblete DMM, Panganiban RP,Poblete JG (2017) Detection And classification of bacteria in common street foods using electronic nose and support vector Machine. In: 2017 7th IEEE international conference on control system, computing and Engineering (ICCSCE), 24–26 Nov 2017,pp 247–252.

Allen M, Bridle G, Briten E (2015)Life under the Microscope:Children's Ideas about Microbes. Primary Science: n136 p35–38.

Association Française de Normalisation (AFNOR). Recueil desNormes Françaises, Qualité de l'Eau, 2nd ed.; AssociationFrançaise de Normalisation (AFNOR): Paris, France, 1997;Volume 1, p. 293.

Astantri, P.F.; Prakoso, W.S.A.; Triyana, K.; Untari, T.; Airin,C.M.; Astudi, P. Lab-Made Electronic Nose for Fast Detection of *Listeria monocytogenes* and *Bacillus cereus*. *Vet. Sci.*2020, *7*, 20.

Avalos M, van Wezel GP, Raaijmakers JM, Garbeva P (2018)Healthy scents: microbial volatiles as new frontier in antibiotic research,Curropinmicrobiol 45:84–91.

Balasubramanian S, Amamcharla J, Panigrahi S, Logue CM,Marchello M, Sherwood JS(2012) Investigation of differentgas sensor-based artificial olfactory systems for Screening salmonella typhimurium contamination in beef. Food bioprocess technol 5:1206–1219.24

Balasubramanian S, Amamcharla J, Shin J-E (2016) Chapter 7Possibleapplication of Electronic nose systems for meat safety: an overview. In: Rodriguez Mendez ML (ed) ElectronicNoses andtongues in food science. Academic Press, San Diego, pp 59–71.

Balkwill D, Gwendolyn DR, Reeves RH, Fredrickson JK, WhiteDC, Ringelberg DB, (1997) Taxonomic study of aromatic-degrading bacteria from deep-terrestrial-subsurface sediments and description of *sphingomonasaromaticivorans* sp. Nov *subterranean* sp. Nov., and *Sphingomonasstygias*p.Nov.International Journal of Systematic and Evolutionary Microbiology.; 47(1):191–201.

Barbri, N.E.; Mirhisse, J.; Ionescu, R.; Bari, N.E.; Correig, X.;Bouchikhi, B.; Llobet, E. An electronic nose system based on amicro-machined gas sensor array to assess the freshness of sardines. Sens. Actuators B Chem.2009, *141(2)*, 538-543.

Berna Z, Webb CC, Erickson MC (2013) Electronic nose and fast gc for detection of volatiles From *Escherichia coli O157*:*H7,Escherichia* coli and Salmonella in lettuce. In: International Society For Horticultural Science (ISHS), Leuven, Belgium,pp 1255–1261.

Bonah, E.; Huang, X.; Aheto, J.H.; Osae, R. Application of electronic nose as a non-invasive technique for odor fingerprinting and detection of bacterial foodborne pathogens: a review. *J. FoodSci. Technol.*2020, *57(6)*, 1977–1990.

Bonah, E.; Huang, X.; Yi, R.; Aheto, J.H.; Osae, R.: Golly, M. Electronic nose classification and differentiation of bacterial foodborne pathogens based on support vector machine optimized with particle swarm optimization algorithm. *J. Food ProcessEng.*2019, *42(6)*, e13236.

Boots, A.W.; Smolinska, A.; Van Berkel, J.J.B.N.; Fijten, R.R.R.;Stobberingh, E.E.; Boumans, M.L.L.; Moonen, E.J.; Wouters,E.F.M.; Dallinga, J.W.; Van Schooten, F.J. Identification of microorganisms based on headspace analysis of volatile organic compounds by gas chromatography-mass spectrometry. *J. BreathRes.*2014, *8*, 027106.

Bordbar, M.M.; Tashkhourian, J.; Tavassoli, A.; Bahramali, E.;Hemmateenej, B. Ultrafast detection of infectious bacteria using optoelectronic nose based on metallic nanoparticles. *Sens.Actuators B Chem.*2020, *319*, 128262.

Bos, L.D.J.; Sterk, P.J.; Schultz, M.J. Volatile metabolites of pathogens: A systematic review. *Plos Pathog.*2013, *9*, e1003311.

Canhoto, O.; Magan, N. Electronic nose technology for the detection of microbial and chemical contamination of potable water. *Sens. Actuators B Chem.*2005, *106*, 3–6.

Cao LJ, Chua KS, Chong WK, Lee HP, Gu QM (2003) A comparison of PCA, KPCA and ICA For dimensionality reduction in support vector machine. Neurocomputing 55:321–336.25

Capuano, R.; Paba, E.; Mansi, A.; Marcelloni, A.M.; Chiominto,A.; Proietto, A.R.; Zampetti, E.; Macagnano, A.; Lvova, L.; Catini,A.; Paolesse, R.; Tranfo, G.; Di Natale, C. *Aspergillus* species discrimination using a gas sensor array. Sensors 2020, *20(14)*, 4004.

Carrillo, J.; Durán, C. Fast identification of Bacteria for Quality Control of Drinking Water through a Static Headspace Sampler Coupled to a Sensory Perception System. *Biosensors*2019, *9*, 23.

Carrillo-Gómez, J.; Durán-Acevedo, C.; García-Rico, R.Concentration detection of the E. Coli bacteria in drinking watertreatment plants through an E-nose and a volatiles extractionsystem (VES). *Water*2019, *11(4)*, 774.

Carrillo-Gómez, J.K.; Acevedo, C.M.D.; García-Rico, R.O.Detection of the bacteria concentration level in pasteurized milk byusing two different artificial multisensory methods. *Sens. Bio-Sens.Res.*2021, *33*, 100428.

Hernández-Cortez,Palma-Martínez, Gonzalez-Avila, guerrero mandujano, and Graciela Castro-Escarpulli G (2017)Food Poisoning Caused byBacteria (Food Toxins); chp3,37-38.

Croughs, P.D.; Klaassen, C.H.W.; van Rosmalen, J.; Maghdid,D.M.; Boers, S.A.; Hays, J.P.; Goessens ,W.H.F.(2018) Unexpected mechanisms of resistance in Dutch *Pseudomonas aeruginosa* isolates collected during 14 years of surveillance. Int. J.Antimicrob. Agents, 52, 407–410.

Dutta, R.; Das, A.; Stocks, N.G.; Morgan, D. Stochastic resonance-based electronic nose: A novel way to classify bacteria. *Sens.Actuators B Chem.*2006, *115*, 17–27.

Elgaali H, Hamilton-Kemp TR, Newman MC, Collins RW, YuK,Archbold DD (2002)Comparison of long-chain alcohols andother volatile compounds emitted from food-borne and Related gram positive and Gram negative bacteria. J Basic Microbiol Food Sci Techno l42:373–380.

Ezhilan M, Nesakumar N, jayanthbabu K, Srinandan CS,Rayappanjbb (2018) AnElectronic nose for royal delicious apple qualityassessment—a tri-layer approach. Food Res Int109:44–51.

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

Ghrissi, H.; Veloso, A.C.A.; Marx, Í.M.G.; Dias, T.; Peres, A.M. A potentiometric electronic tongue as a discrimination tool of water food indicator/contamination bacteria. *Chemosensors*2021, *9(6)*,143.

Giraffa, G., 2002. *Enterococci* from foods. FEMS Microbiol. Rev.26, 163–171.
Giungato P, Di Gilio A, Palmisani J, Marzocca A, MazzoneA,Brattoli M, Giua R, de Gennaro G (2018) Synergistic approachesfor odor active compounds monitoring and Identification: state of the art, integration, limits and potential ities of analytical andsensorial techniques. Trac Trends Anal Chem107:116–129.

Gobbi E, Falasconi M, Zambotti G, Sberveglieri V, Pulvirenti A,Sberveglieri G (2015) Rapid Diagnosis of *Enterobacteriaceae* invegetable soups by a metal oxide sensor based electronic nose.Sens Actuators B Chem 207:1104–1113.

Green GC, Chan ADC, Dan H, Lin M (2011) Using a metal oxide sensor (MOS)-based electronic nose for discrimination of bacteria based on individual colonies in suspension.Sens Actuators B Chem 152:21–28.

Green GC, Chan ADC, Lin M (2014) Robust identification of bacteria based on repeated odor measurements from individual bacteria colonies. Sens Actuators B Chem 190:16–24.

Gregersen, T. Rapid method for distinction of gram-negative from Gram-positive bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* 1978,*5*, 123–127.

Hanchi, H.; Mottawea,W.; Sebei, K.; Hammami, R(2018). The Genus *Enterococcus*: Between Probiotic Potential and Safety Concerns—an update. Front. Microbiol.9, 1791.
Ingledew, W.J., Poole, R.K., 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. 48, 222–271.

Jia W, Liang G, Wang Y, Wang J (2018) Electronic noses as a powerful tool for assessing meat quality: a mini review. Food Anal Methods 11:2916–2924.

Jiang H, Chen Q (2014) Development of electronic nose and near infrared spectroscopy analysis techniques to monitor the criticaltime in SSF process of feed protein. Sensors 14(10), 19441-19456.

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.

Kizil U¨, Genc¸ L, Genc¸ TT, Rahman S, Khaitsa ML (2015) Enose identification of Salmonella *enterica* in poultry manure. Br Poult Sci 56:149–156.

Kladsomboon, S.; Thippakorn, C.; Seesaard, T. Development of organic-inorganic hybrid optical gas sensors for the non-invasive monitoring of pathogenic bacteria. *Sensors* 2018, *18(10)*, 3189.
Le Loir, Y., Baron, F., Gautier, M., 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. GMR 2, 63–76.

Lowy, F.D., 1998. *Staphylococcus aureus* Infections. N. Engl. J.Med. 339, 520–532.
Martínez Gila, D.M., gámezgarcía, J., Bellincontro, A., Mencarelli,F., Gómez Ortega,J(2020), Fast tool based on electronic nose topredict olive fruit quality after harvest Postharvest Biology and Technology, 160, art. No. 111058.

Marx, Í.M.G.; Casal, S.; Rodrigues, N.; Cruz, R.; Peres, F.; Veloso,A.C.A.; Pereira, J.A.; Peres, A.M. Impact of fresh olive leavesaddition during the extraction of Arbequina virgin olive oils on thephenolic and volatile profiles. *Food Chem.*2022,*393*, 133327.

Nieto-Arribas, P.; Seseña, S.; Poveda, J.M.; Chicón, R.; Cabezas,L.; Palop, L. *Enterococcus* populations in artisanal Manchegocheese: Biodiversity, technological and safety aspects. FoodMicrobiol.2011, 28(5), 891-899.
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.

Pierson, M.; Zink, D.; Smoot, L(2007). Indicator Microorganismsand Microbiological Criteria. In Food Microbiology: Fundamentals and frontiers, 3rd ed.; Doyle, M., Beuchat, L., Eds.; ASM Press:Washington, DC, USA; pp. 69–85.

Poveda, J. Beneficial effects of microbial volatile organic compounds (mvocs) in plants. *Appl. Soil Ecol.*2021,*168*, 104118.

Prakoso, W.S.A.; Astantri, P.F.; Triyana, K.; Untari, T.; Airin,C.M.; A studi, P. Rapid Detection of *Escherichia coli* and*Salmonella Typhimurium* Using Lab-Made Electronic Nose Coupled with Chemometric Tools. *Int. J. Adv. Sci. Eng. Inf.Technol.*2021, *11(4)*, 1494-1500.

Preti G, Thaler E, Hanson CW, Troy M, Eades J, Gelperin A (2009) Volatile Compounds Characteristic of sinus-related bacteria andinfected sinus mucus: analysis by solid-phaseMicroextractionand gas chromatography–mass spectrometry. JChromatogr B877:2011–2018.

Robin Michael Statham T, John G (2012) Microbialvolatilecompounds in health and diseaseConditions. J BreathRes6:024001.

Roda, B.; Mirasoli, M.; Zattoni, A.; Casale, M.; Oliveri, P.; Bigi,A.; Reschiglian, P.; Simoni, P.; Roda, A. A new analytical platformbased on field-flow fractionation and olfactory sensor to improvethe detection of viable and non-viable bacteria in food. *Anal.Bioanal. Chem.*2016, *408*, 7367–7377.

Roig, B.; Thomas, O. Rapid estimation of global sugars by UV photo degradation and UV spectrophotometry. *Anal. Chim. Acta*2003, *477*, 325–329.
Roig, B.; Thomas, O. UV monitoring of sugars during winemaking. *Carbohydr. Res.*2003, *338*, 79–83.

Romoli R, Papaleo MC, De Pascale D, Tutino ML, Michaud L,logiudice A, Fani R,Bartolucci G (2014) GC–MS volatolomicapproach to study the antimicrobial activity of the Antarctic bacterium Pseudoalteromonas sp. TB41. Metabolomics10:42–51.

Sberveglieri V, Nunez Carmona E, Pulvirenti A (2015) Detection of microorganism in water and different food matrix by electronic nose. In: Mason A, Mukhopadhyay SC, Jayasundera KP(eds)Sensing technology: current status and future trends III.Springer, Cham, pp 243–258.

Seesaard, T.; Thippakorn, C.; Kerdcharoenc, T.; Kladsomboon, S.A hybrid electronic nose system for discrimination of pathogenic bacteria volatile compounds. *Anal. Methods*2020, *12*, 5671-5683.

Selim KA, El Ghwas DE, Selim RM, Abdelwahab Hassan MI(2017)Microbial volatile in Defense. In: Choudhary DK, Sharma AK,Agarwal P, Varma A, Tuteja N (eds) Volatiles and foodSecurity:role of volatiles in agro-ecosystems. Springer,Singapore,pp 135–170.

Senecal AG, Magnone J, Yeomans W, Powers EM (2002)Rapid detection of pathogenic bacteria by volatile organic compound(VOC) analysis. In: Environmental and industrial sensing,2002.SPIE, p 11.

Shauloff, N.; Morag, A.; Yaniv, K.; Singh, S.; Malishev, R.;Paz-Tal, O.; Rokach, L.; Jelinek R. Sniffing Bacteria with aCarbon-Dot Artificial Nose. *Nano-Micro Lett.*2021, 13, 112.

Shumeiko, V.; Zaken, Y.; Hidas, G.; Paltiel, Y.; Bisker, G.;Shoseyov, O. Peptide-Encapsulated Single-Wall Carbon Nanotube Based Near-Infrared Optical Nose for Bacteria Detection and Classification. *IEEE Sens. J.*2022, *22 (7)*, 6277-6287.

Siripatrawan U (2008) Rapid differentiation between *E. Coli* and *Salmonella typhimurium* usingMetal oxide sensors integrated with pattern recognition. Sens Actuators B Chem 133:414–419.23.

Siripatrawan U, Harte BR (2007) Solid phasemicroextraction/gaschromatography/mass spectrometry integrated with chemometricsfor detection of Salmonella typhimurium contamination in apackaged fresh vegetable. Anal Chim Acta581:63–70.

Siripatrawan U, Harte BR (2015) Data visualization of Salmonellatyphimurium contamination In packaged fresh alfalfa sproutsusing a Kohonen network. Talanta 136:128–135.

Stellato, G.; Utter, D.R.; Voorhis, A.; de Angelis, M.; Eren,A.M; Ercolini, D. (2017) A Few Pseudomonas Oligo types Dominatein the Meatand Dairy Processing Environment. Front. Microbiol, 2,264.

Tait E, Perry JD, Stanforth SP, Dean JR (2014) Use of volatile ecompounds as a diagnostic tool, for the detection of pathogenic bacteria. Trac Trends Anal Chem 53:117–125.

Tait, E.; Perry, J.D.; Stanforth, S.P.; Dean, J.R. Identification of volatile organic compounds produced by bacteria using HS-SPMEGC-MS. *J. Chromatogr. Sci.*2014, *52*, 363–373.

Teixeira, G.G.; Dias, L.G.; Rodrigues, N.; Marx, Í.M.G.; Veloso,A.C.A.; Pereira, J.A.; Peres, A.M. Application of a lab-made electronic nose for extra virgin olive oils commercial classification according to the perceived fruitiness intensity. *Talanta* 2021, *226*,122122.

Teixeira, G.G.; Peres, A.M.; Estevinho, L.; Geraldes, P.; GarciaCabezon, C.; Martin-Pedrosa, F.; Rodriguez-Mendez, M.L.; Dias,L.G. Enose lab made with vacuum sampling: quantitativeapplications. Chemosensors 2022,*10(7)*, 261.

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.

Thorn, R.M.S.; Reynolds, D.M.; Greenman, J. Multivariate analysisof bacterial volatile compound profiles for discrimination between selected species and strains in vitro. *J. Microbiol. Meth.*2011, *84*,258–264.

Tonezzer, M.; Thai, N.X.; Gasperi, F.; Duy, N.V.; Biasioli, F.Quantitative Assessment of Trout Fish Spoilage with a Single Nano wire Gas Sensor in a Thermal Gradient. *Nanomaterials*2021,*11*, 1604.

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, 232–254.21.

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

Wilson AD, Baietto M (2009) Applications and advances in electronic-nose technologies. sensors(Basel, Switzerland)9:5099–5148.

Xue C, Lin Y, Zhao Y, Xitao Z (2012) The identification of *Listeria monocytogene*s based on the electronic nose. In: 2012 International conference on computer science and information processing (CSIP), 24–26 Aug 2012. Pp 467–472.

Yongxin Y, Zhao Y (2012) Electronic nose integrated with chemometrics for rapid identification of foodborne pathogen. Intechopen.

Yu Y-x, Sun X-h, Liu Y, Pan Y-j, Zhao Y (2014) Odor fingerprinting of *Listeria monocytogenes* recognized by SPME–GC–MS ande-nose. Can J Microbiol 61:367–372.

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

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 bymultiplex PCR with a gexp analyzer. Food Control 32, 198–204.

Zohora SE, Khan AM, Hundewale N (2013) Chemical sensors employed in electronic noses: a review. In: Meghanathan N,Nagamalai D, Chaki N (eds) Advances in computing and information technology. Springer, Berlin, pp 177–18422.

Zscheppank, C.; Wiegand, H.L.; Lenzen, C.; Wingender, J.;Telgheder, U. Investigation of volatile metabolites during growth of *Escherichia coli* and *Pseudomonas aeruginosa* by needle trap-GCMS. Anal. Bioanal. Chem.2014, *406*, 6617–6628.