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Postgraduate thesis

Rapid methods for foodborne pathogens detection and quantification: a review

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«Ταχείες μέθοδοι για την ανίχνευση και ποσοτικοποίηση τροφιμογενών παθογόνων: μια ανασκόπηση»

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Rapid methods for foodborne pathogens detection and quantification: a review

MSc: Food Science and Technology
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Abstract

Foodborne pathogens cause millions of illnesses each year, therefore their early detection and quantification is an issue of major importance. The purpose of this thesis was the review of almost all rapid methods that could be implemented in food microbiology. The methods were categorized as: i.) Nucleic acid-based techniques, ii.) Spectroscopic techniques, iii.) Spectral imaging techniques, iv.) Biosensors, v.) Metabolomics and vi.) Other instrumental techniques. For each technique, the theoretical background was described along with recent applications in microbiology. Nucleic-acid based techniques were considered as one of the most sensitive and specific methods that could be invaluable in food microbiology with the well-established Polymerase Chain Reaction (PCR) variants and with the more recent approach of Next Generation Sequencing (NGS). Spectroscopic and spectral imaging techniques, even not widely implemented for microbiological purposes, seemed to have great potential with the evolvement of data analysis tools due to their non-destructive approach. Biosensors appeared to address portability, easy-of-use and low cost although their in-field applications were limited. Metabolomics along with chromatographic and –omics techniques share the same holistic approach that is closely related to phenotype, although the complexity of instrumentation, analysis procedure and data analysis maybe was the main cause of limited applications. Flow cytometry "addressed" some limitations of the past that were reducing its sensitivity and specificity although the high cost still remained a significant obstacle of wide-scale implementation. Impedance microbiology seemed to have better results when integrated in biosensors although such applications were still limited. Immunological-based techniques have begun to be more prevalent in food microbiology laboratories although the high cost of some approaches remained a limiting factor. In conclusion, the most important problem of almost all of the rapid methods included in this review had to do with the need of preliminary steps of sample processing

that increased the theoretical total time of analysis, yet not in levels of conventional methods.

Scientific area: Food microbiology

Key words: Rapid methods, foodborne pathogens, nucleic-acid based techniques, spectroscopic techniques, spectral imaging techniques, biosensors, metabolomics

Ταχείες μέθοδοι για την ανίχνευση και ποσοτικοποίηση τροφιμογενών παθογόνων: μια ανασκόπηση

ΠΜΣ: Επιστήμη και Τεχνολογία Τροφίμων
Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου

Περίληψη

Τα τροφιμογενή παθογόνα προκαλούν εκατομμύρια ασθένειες κάθε χρόνο, επομένως η έγκαιρη ανίχνευση και ποσοτικοποίησή τους είναι ένα ζήτημα μείζονος σημασίας. Ο σκοπός αυτής της διατριβής ήταν η βιβλιογραφική ανασκόπηση σχεδόν όλων των ταχείων μεθόδων που θα μπορούσαν να αξιοποιηθουν στη μικροβιολογία τροφίμων. Οι μέθοδοι κατηγοριοποιήθηκαν ως: i.) Τεχνικές που σχετίζονται με τα νουκλεϊκά οξέα, ii.) Τεχνικές φασματοσκοπίας, iii.) Τεχνικές φασματικής απεικόνισης, iv.) Βιοαισθητήρες, v.) Μεταβολομική (metabolomics) και vi.) Άλλες τεχνικές ενόργανης ανάλυσης.

Για κάθε τεχνική, αναλύθηκε το θεωρητικό της υπόβαθρο μαζί με πρόσφατες εφαρμογές στη μικροβιολογία. Οι τεχνικές που σχετίζονται με τα νουκλεϊκά οξέα κατατάχθηκαν ανάμεσα στις μεθόδους με την υψηλότερη ευαισθησία (sensitivity) και εξειδίκευση (specificity) και συνεπώς θα μπορούσαν να είναι πολύτιμες στη μικροβιολογία τροφίμων, κυρίως, μέσω της Αλυσιδωτής Αντίδρασης Πολυμεράσης (PCR) και των διάφορων παραλλαγών της καθώς και μέσω της πιο πρόσφατης προσέγγισης του Next Generation Sequencing (NGS). Οι φασματοσκοπικές τεχνικές και οι τεχνικές φασματικής απεικόνισης, αν και δεν έχουν εφαρμοστεί σε ευρεία κλίμακα για μικροβιολογικούς σκοπούς, φάνηκαν να έχουν σημαντικές προοπτικές με την ανάπτυξη σύγχρονων εργαλείων ανάλυσης δεδομένων, λόγω της μη καταστρεπτικής προσέγγισής τους. Οι βιοαισθητήρες φάνηκαν να ικανοποιούν τις απαιτήσεις που σχετίζονται με τη φορητότητα, την ευκολία στη χρήση και το χαμηλό κόστος, παρόλο που οι ρεαλιστικές εφαρμογές τους στην πράξη ήταν περιορισμένες. Η μεταβολομική (metabolomics) μαζί με τις τεχνικές χρωματογραφίας και άλλες –omics προσεγγίσεις μοιράζονται την ίδια ολιστική προσέγγιση που είναι άμεσα συνδεδεμένη με τον φαινότυπο, ωστόσο η πολυπλοκότητα των μηχανημάτων, της διαδικασίας ανάλυσης καθώς και της ανάλυσης των δεδομένων ήταν ίσως η κύρια αιτία των περιορισμένων εφαρμογών. Η

κυτταρομετρία ροής «αντιμετώπισε» ορισμένα προβλήματα του παρελθόντος που περιόριζαν την ευαισθησία και την ειδικότητά της, ωστόσο το υψηλό κόστος εξακολουθεί να αποτελεί σημαντικό εμπόδιο στην ευρεία εφαρμογή της. Η μικροβιολογία σύνθετης αντίστασης (impedance microbiology) φάνηκε να παρουσιάζει καλύτερα αποτελέσματα όταν ενσωματώθηκε σε βιοαισθητήρες αν και τέτοιου είδους εφαρμογές ήταν ακόμη περιορισμένες. Οι ανοσολογικές τεχνικές έχουν αρχίσει να είναι πιο διαδεδομένες στα εργαστήρια μικροβιολογίας τροφίμων, αν και το υψηλό κόστος ορισμένων προσεγγίσεων παρέμεινε περιοριστικός παράγοντας. Συμπερασματικά, το πιο σημαντικό πρόβλημα σχεδόν όλων των ταχείων μεθόδων που περιλαμβάνονται σε αυτήν την ανασκόπηση είχε να κάνει με την ανάγκη προκαταρκτικών βημάτων επεξεργασίας των δειγμάτων που αύξαναν τον συνολικό, θεωρητικό χρόνο ανάλυσης, όμως όχι στα επίπεδα των συμβατικών μεθόδων.

Επιστημονική περιοχή: Μικροβιολογία τροφίμων

Λέξεις κλειδιά: Ταχείες μέθοδοι, τροφιμογενή παθογόνα, τεχνικές νουκλεϊκού οξέος, φασματοσκοπικές τεχνικές, τεχνικές φασματικής απεικόνισης, βιοαισθητήρες, μεταβολομική

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This thesis was part of my postgraduate studies and had begun as an experimental thesis in the laboratory of food microbiology of the Institute of Technology of Agricultural Products, Hellenic Agricultural Organization - DIMITRA, although was finished with a literature review due to implications of COVID-19.

So, at first I would like to begin with the institute and thank my supervisor and research director of the institute Dr. Chryssoula Tassou for the given opportunity to obtain useful, practical knowledge about various, novel and rapid methods of microbiology and their real-time comparison with the conventional ones. Also I would like to thank the post-doc Athena Grounta who guided me through conventional (ISO) methods of foodborne pathogens' detection so that there is a measure of comparison. Lastly, I would like to thank the researcher Dr. Agapi Doulgeraki for her insights about nucleic acid-based techniques.

Moving to Agricultural University of Athens where my thesis was continued, I would like to thank the post-doc Anastasia Lytou for her invaluable guidance in the field of Metabolomics and other -omics techniques and for her precious help with the layout of the presented methods which made the review more legible.

Last but not least, I would like to thank my supervisor, Professor George-John Nychas for the countless of opportunities he gave me during our cooperation that equipped me with experience, knowledge and a different mindset. It should not be overlooked the fact, that he has been always by my side, counseling and making things simpler.

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List of abbreviations

2-DE: Two-dimensional gel electrophoresis

3D-SERS: Three-Dimensional Surface-Enhanced Raman Scattering

Au@AgNPs: Gold and Silver Nanoparticles

BLAST: Basic Local Alignment Search Tool which finds regions of local similarity between sequences

CE: Capillary Electrophoresis

Cq: Cycle of quantification

CuNPs: Copper Nanoparticles

DALYs: Disability-Adjusted Life Years

dNTPs: Deoxynucleoside triphosphates

ECDC: European Centre for Disease Prevention and Control

EFSA: European Food Safety Authority

ELFA: Enzyme Linked Fluorescent Assay)

ELISA: Enzyme-Linked Immunosorbent Assay

EPEC: Enteropathogenic Escherichia coli

ET-LIBS: Elemental-Tags Laser-Induced Breakdown Spectroscopy

FDA: Food and Drug Administration of United States

FERG: Foodborne Disease Burden Epidemiology Reference Group

GA: Genetic Algorithm

GC-MS: Gas Chromatography-Mass Spectrometry

HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry

HSI: Hyperspectral Imaging

IMS: Immunomagnetic Separation Assay

IRIV: Informative Variables

KNN: k-nearest neighbors algorithm

LIBS: Laser-Induced Breakdown Spectroscopy

LOD: Limit Of Detection

LOQ: Limit Of Quantification

LTRS: Laser Tweezers Raman Spectroscopy

MALDI-ToF MS: Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

MCR-ALS: Multivariate Curve Resolution with Alternating Least-Squares

mPCR: Multiplex Polymerase Chain Reaction

MSI: Multispectral Imaging

NGS: Next Generation Sequencing

NMR: Nuclear Magnetic Resonance

PCA: Principal Components Analysis

PCR: Polymerase Chain Reaction

PFGE: Pulsed Field Gel Electrophoresis

PLS-R: Partial Least Squares-Regression

PMA: Propidium Monoazide

RAPD: Random Amplified Polymorphic DNA

REP: Repetitive Extragenic Palindrome sequence

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SDO: Deoxycholate

SIFT-MS: Selective Ion Flow Tube Mass Spectrometry

SiNWs: Silicon nanowires (elemental tags)

SNV: Standard Normal Variate

SR-FTIR: Synchrotron Radiation based Fourier-Transform Infrared microspectroscopy

STEC: Shiga Toxin-Producing Escherichia Coli

SVM: Support Vector Machine algorithm

SWCNT: Single Walled Carbon Nanotube based multi-junction sensor

Thz: Terahertz

THz-TDS: Terahertz Time-Domain Spectroscopy

Tm: Primers' melting point

TVC: Total Viable Counts

UPLC: Ultra Performance Liquid Chromatography

US CDC: United States Centers for Disease Control and Prevention

VBNC: Viable But Not Culturable

VCPA: Variable Combination Population Analysis

Vis-NIR: Visible-Near Infrared

VOC: Volatile Organic Compounds

WGS: Whole Genome Sequencing

WHO: World Health Organization

WKL: White-Kauffmann-Le Minor scheme for Salmonella serotyping

1. Introduction

Foodborne diseases still remain an unsolved problem with adverse effects primarily on consumer health and secondarily on economy. This is a multidimensional issue that, according to the report: "WHO estimates the global burden of foodborne diseases", cause illness to 600 million people and lead to 420,000 deaths, annually (as estimated in 2010, **Table 1.**). According to WHO (2015), 31 global hazards were responsible for all foodborne diseases from 2007 to 2015 (WHO, 2015). The most important causes were diarrheal disease agents i.e. 1 virus, 7 bacteria, 3 protozoa (**Table 1.**). In terms of frequency, Norovirus and *Campylobacter* spp. were responsible for most cases, whereas the largest disease burden was resulted by diarrheal disease agents (230,111 deaths) and mainly by non-typhoidal *Salmonella enterica* with 59,153 deaths, worldwide (**Table 1.).** It should be mentioned that foodborne diseases also resulted to 33 million Disability-Adjusted Life Years (DALYs) which are defined as: "the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability". Of these, diarrheal disease agents, at their own, accounted for 18 million DALYs and 40% of all DALYs were among children under 5 years of age (WHO, 2015).

Table 1. Median global number of foodborne illnesses, deaths and Disability Adjusted Life Years (DALYs), 2010 (WHO, 2015)

HAZARD	FOODBORNE ILLNESSES	FOODBORNE DEATHS	FOODBORNE DALYS
TOTAL	600,652,361	418,608	32,841,428
Diarrheal disease agents	548,595,679	230,111	17,659,226
Viruses	124,803,946	34,929	2,496,078
Norovirus	124,803,946	34,929	2,496,078
Bacteria	349,405,380	187,285	14,490,808
Campylobacter spp.	95,613,970	21,374	2,141,926
Enteropathogenic *E. coli*- EPEC	23,797,284	37,077	2,938,407
Enterotoxigenic *E. coli*- ETEC	86,502,735	26,170	2,084,229
Shiga toxin-producing *E. coli*- STEC	1,176,854	128	12,953
Non-typhoidal *S. enterica*	78,707,591	59,153	4,067,929
Shigella spp.	51,014,050	15,156	1,237,103
Vibrio cholerae	763,451	24,649	1,722,312
Protozoa	67,182,645	5,558	492,354
Cryptosporidium spp.	8,584,805	3,759	296,156
Entamoeba histolytica	28,023,571	1470	138,863
Giardia spp.	28,236,123	0	26,270

Similar data about foodborne diseases can be found from Centers for Disease Control and Prevention in United States, where in a relevant study (in relevant time period) it was estimated that every year occur 9.4 million cases of foodborne illness, 55,961 hospitalizations and 1,351 deaths (Scallan et al., 2011). In United States, Norovirus was also the most frequent cause of foodborne illness (58% of total foodborne illnesses) but followed by non-typhoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10%) and *Campylobacter* spp. (9%). The largest disease burden was also caused by non-typhoidal *Salmonella* spp. (28% of total foodborne deaths) but instead followed by *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), and Norovirus (11%) (Scallan et al., 2011).

As far as Europe is concerned, European Food Safety Authority and European Centre for Disease Prevention and Control recently published a report about zoonoses and food-borne outbreaks within the EU in 2019 (EFSA and ECDC, 2021). In this report, *Campylobacter* spp. has been identified as the most frequent cause (220,682 confirmed cases, EU notification rate: 59.7 cases per 100.000 population) of foodborne disease for 2019 but also for any year since 2005 (**Table 2.**). The second most frequent cause (87,923 confirmed cases, EU notification rate: 20.0 cases per 100.000 population) was *Salmonella* spp. (with no discrimination between typhoidal and non-typhoidal species) followed by Shiga toxin-producing *E. coli*- STEC (7,775 confirmed cases, EU notification rate: 2.2 cases per 100.000 population) (**Table 2.**). It should be noted that cases of foodborne illess in European Union are far less in comparison with the aforementioned data that were reported by WHO (2015) and US CDC (2011) even when the differences in population size are considered (Scallan et al., 2011; WHO, 2015). This was only because the data published by EFSA and ECDC (2021) were only about the confirmed cases, whereas US CDC (2011) and WHO (2015) made also estimations regarding unregistered foodborne illnesses (EFSA and ECDC, 2021; Scallan et al., 2011; WHO, 2015). In terms of disease burden, the most foodborne deaths (300) were caused by *Listeria* spp. although the associated cases were far less (2,621 cases, EU notification rate: 0.46) in comparison with the above pathogens (**Table 2.**). These results about *Listeria* spp. and foodborne deaths are in contrast with US CDC and WHO data which highlighted non-typhoidal *Salmonella*

spp. as the most deadly foodborne disease and it should be noted that Norovirus cases were not included in this EU report. The Case Fatality (%) for *Campylobacter* spp., Shiga toxin-producing *E. coli*- STEC, *Salmonella* spp. and *Listeria* spp. were calculated 0.03%, 0.21%, 0.22% and 17.6%, respectively (**Table 2.**) (EFSA and ECDC, 2021).

Table 2. Reported hospitalizations, deaths and case fatalities due to five most frequent foodborne diseases in confirmed human cases in the EU, 2019 (EFSA and ECDC, 2021)

Disease	Number of confirmed human cases	Reported hospitalized cases	Reported deaths	Case fatality (%)
Campylobacteriosis	220,682	20,432	47	0.03
Salmonellosis	87,923	16,628	140	0.22
STEC infections	7,775	1,100	10	0.21
Yersiniosis	6,961	648	2	0.05
Listeriosis	2,621	1,234	300	17.6

As it might seems obvious, the early detection of foodborne pathogens could improve food safety and prevent some of the aforementioned cases of foodborne illness. However, the current, "gold-standard" methods for detecting foodborne pathogens in food samples are time consuming, therefore limiting food business operators, in many cases, to first release their products to market and then having the full microbiological data about a specific batch (especially in fresh products) (Foddai and Grant, 2020; Nychas et al., 2016).

These (conventional) methods are based on culturing microorganisms on agar plates and for the pathogens in trace concentrations there is also a need for pre-enrichment and/or enrichment in selective broth media followed by biochemical, agglutination and/or molecular testing. This is a laborious approach which results in low time-effectiveness with a duration of 5 or more days for the identification of pathogens like *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. (Law et al., 2014; Lee et al., 2015). In addition, they might provide lower sensitivity than other methods (e.g. nucleic acid-based methods) and there is always a probability of false-negative results due to Viable But Not

Culturable (VBNC) pathogens (Law et al., 2014). Given the disadvantages, conventional methods are standardized with proven, high enough, specificity and sensitivity and are widely accepted for their reliability and for the high ease of use combined with the low cost. Cost is a key factor that still hinders the widespread application of molecular microbiology on a routine basis (Aggarwal et al., 2020). The above, combined with current legislation, may explain the persistence of many food microbiology laboratories in them while the familiarity of current microbiologists with conventional methods should not be overlooked (Gracias and McKillip, 2004; Lee et al., 2015).

In 2005 (effective since 2006), European Commission with Commission Regulation (EC) No 2073/2005 first mentioned rapid methods as "analytical methods other than the reference methods that food business operators should have the possibility to use as long as they provide equivalent results". This regulation created increased needs for microbial testing for specific pathogens and products e.g. *Listeria* spp. in RTE (ready-to-eat) products and opened new avenues in rapid detection of microorganisms (European Commission, 2005; Koutsoumanis and Angelidis, 2007).

Nowadays there are many different approaches for detecting pathogens like *Salmonella* spp. and *Campylobacter* spp. in food samples which differ in terms of ease of use, reliability of results, cost and time effectiveness etc. Although rapid methods such as impedance microbiology, immunological-based techniques, instrumental techniques, biosensors, nucleic acid-based techniques and spectroscopic techniques have emerged more than a decade ago, there are still some challenges to fully replace or even substitute conventional methods in detecting pathogens (Gracias and McKillip, 2004; Lee et al., 2015).

2. Purpose and thesis outline

2.1. Purpose

As it was mentioned above, the early detection of foodborne pathogens is crucial for the prevention of foodborne diseases but also for the appropriate and immediate treatment of patients in case the disease could not be avoided. To my knowledge, there is no review in international literature that is up-to-date and includes a broad range of

different methods. The purpose of this review was to summarize the recent progresses in rapid methods of foodborne pathogens' detection and quantification. An extensive analysis has been carried out to include the majority of alternative methods that can be utilized in microbiology. In this context, it should be noted that some applications that have been mentioned below may refer to microorganisms other than foodborne pathogens. However these methods will still be mentioned due to their potential to be used in foodborne pathogens in the future.

2.2. Thesis outline

The thesis is consisted of six main sections i.e. i.) Nucleic acid-based techniques, ii.) Spectroscopic techniques, iii.) Spectral imaging techniques, iv.) Biosensors, v) Metabolomics and vi.) Other instrumental techniques. The most sections are also subdivided in order to describe properly the individual "representatives" within the same approach. The theoretical background of each method was described and special mention was made about their applications in microbiology. A special effort has been made in order to discuss the practical limitations and future challenges of each method in a separate section, which was followed by the conclusions for future research.

3. Nucleic acid-based techniques

Nowadays, there are various approaches in microbiology based on the nucleic acid-based concept including amplification, hybridization, microarrays and biochips (Zhao et al., 2014). Also, recent progress in technology and the reduction of cost led Next Generation Sequencing (NGS) and its applications in Whole Genome Sequencing (WGS) to gain ground on conventional microbiology methods (Fanning et al., 2017). However, the main representative of nucleic acid-based techniques still remains Polymerase Chain Reaction (PCR) on its standard, multiplex or quantitative variant (Law et al., 2014).

3.1. PCR

3.1.1. Basic Principles

Briefly, all variants share the same "PCR core idea" which is the amplification of a predefined, double-stranded DNA target region into hundreds of millions by using two

proper DNA fragments (single-stranded sequences). The predefined sequence act as template and each of the primers (DNA fragments) use it as a starting point and hybridization occurs between the two primers and each strand of the DNA template (parallel and anti-parallel). After the hybridization, DNA polymerase find the 3'-hydroxyl terminus of each hybridized strand and start the synthesis of 2 new DNA sequences, therefore creating two copies of the original double-stranded DNA target region. This process occurs many times and that's how the hundred millions of the DNA target are produced. The amplified DNA target region is often verified and visualized with agarose gel electrophoresis. Briefly, this visualization is possible with the use of dyes and a DNA ladder (acts as marker for molecular weight of DNA fragments) which is also added to agarose gel for comparison. Due to the fact the process relies on specific hybridizations, which cannot be done if full complementation is missing, it is widely accepted that when the primers are well designed and all the steps (including preliminary steps like DNA extraction) are performed correctly, PCR provide results with high sensitivity and specificity (Van Pelt-Verkuil et al., 2008).

In detail, the standard PCR procedure consists of 3 main, repetitive steps (denaturation, annealing, elongation) and includes 5 main components (target DNA, primers, DNA polymerase, dNTPs, buffer) (Clark et al., 2019).

3.1.2. Components

1. Target DNA: It seems obvious that the first component to be discussed is the target DNA region and it should be mentioned that it can take part in the reaction even if is present in a very low quantity. The target DNA region may be pure after culture-based separation of the target microorganism combined with a DNA extraction protocol, or may be a mixture of different organisms' genome and may be even the exact sample to be analyzed, minimally or not processed at all, omitting the DNA extraction step (direct PCR)(Chin et al., 2017; Clark et al., 2019).

2. Primers: As mentioned above, two predefined primers that are complementary to the 3' end of each target DNA strand are essential for hybridization to occur and give the starting point to DNA polymerase.

3. DNA Polymerase: It is a heat-resistant enzyme responsible for doubling the DNA region in every PCR cycle. The main representative of this PCR component is Taq polymerase because is able to withstand the high temperatures of the denaturation step.

4. Deoxynucleoside triphosphates (dNTPs): These are the building blocks of DNA, the ingredients that DNA polymerase will use in order to create the copies of DNA.

5. Buffer: A solution that provide the optimal ionic environment for DNA polymerase functionality (Clark et al., 2019).

3.1.3. Procedure (Figure 1.)

Step 1. Denaturation: It is the first part of the typical PCR cycle and consists of heating at temperatures above 90 ^{o}C so that the hydrogen bonds between complementary bases of the DNA target region break and the double helix unfold (Jalali et al., 2017).

Step 2. Annealing: In this step, the temperature is lowered to 45-65 ^{o}C for about 20 seconds so that primers bind (with hydrogen bonds) complementary to the 3' end of the antiparallel strand of the DNA target (hybridization). The selection of the appropriate temperature is crucial and can determine the efficiency and specificity of the method. Illustratively, if the temperature is too low the primer may mismatch whereas too high temperatures may block the binding at all. For this reason the optimal temperature is usually defined at 3-5 ^{o}C lower of the primers' melting point (T_m) which depends on the amount of guanine-cytosine and on their length (Caetano-Anollés, 2013; Jalali et al., 2017; Kalendar et al., 2009; Porta and Enners, 2012).

Step 3. Elongation or extension: The temperature is set close to the optimum of DNA polymerase (72 ^{o}C for Taq polymerase). DNA polymerase adds complementary nucleotides sourced from dNTPs (dATP, dGTP, dCTP, dTTP) to the 3' hydroxyl group of each primer (with 5' → 3' direction for both strands, **Figure 1.**). The new complementary strand at such conditions is synthesized at a rate of about 1000 bases min^{-1}, while the total time of the elongation step depends on the length of the DNA target and typically ranges between 20 seconds and 2 minutes. This step marks the end of the first PCR cycle in which, under optimal conditions, the DNA target region is doubled. In the second PCR cycle (denaturation-annealing-elongation) that follow afterwards, the original DNA target

region plus the new one will be doubled again, and so goes the third, fourth cycle etc. indicating an exponential rate of the synthesis (**Figure 1**.). The whole procedure is typically consisted of 30 cycles, therefore leading to 2^{30} or 1,073,741,824 copies (a.k.a. PCR products or PCR amplicons) of the original DNA target region (Caetano-Anollés, 2013; Clark et al., 2019).

It should be noted that the aforementioned procedure, is sometimes enriched with extra steps and some authors refer to some sub-procedures as separate steps. These steps are: initialization, final elongation and final hold.

Initialization (a.k.a. "hot-start) is a step that takes place only on the first cycle, just before the denaturation but this affect only DNA polymerases that need a heat activation before the main procedure. The initialization usually last up to 10 minutes at a temperature of about 95 ^{o}C.

Final elongation is an optional step and as its name implies, takes place only in the last PCR cycle after the main elongation and applies a temperature of 72 ^{o}C for about 10 minutes. Final elongation's purpose is to ensure that any remaining single-stranded DNA is elongated and therefore not affecting the PCR results.

Final hold is the last step of the PCR procedure and it is nothing else but the short-term storage of the PCR products at cooling conditions (e.g. 4 ^{o}C), so that they do not deteriorate (Caetano-Anollés, 2013).

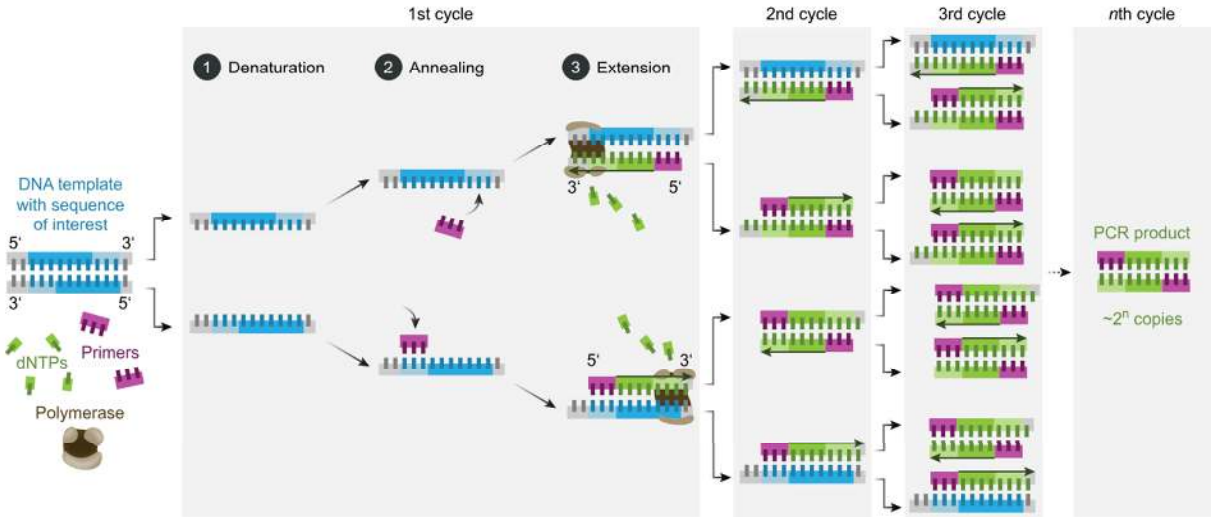

Figure 1. Schematic illustration of PCR procedure (Enzoklop, 2020)

3.2. Multiplex PCR

Multiplex PCR (mPCR) shares the same perspective with standard PCR for which special mention was made above, with the only difference of using more than one set of parallel and antiparallel primers. This difference provides a more rapid detection because the amplicons produced, contain more than one DNA target regions, thus enabling the simultaneous detection of multiple pathogens at one PCR procedure (Law et al., 2014). This setup, although offers many advantages, has some limitations that have to do with the interactions that may occur between the different primer sets. This is an issue that can lead to false or no results and is related also to primers' quantities, apart from their design. Also, as obvious as it seems, all primers must have about the same melting temperature for the annealing step and for proper visualization through agarose gel eletrophoresis there should be differences either on the amplicon's size or in the dyes used during the primers' design (Zhao et al., 2014). These limitations along with the low number of species that can be distinguished simultaneously have bounded the applications of mPCR in food analysis at previous years, whilst recent applications overcome this obstacles by using universal primers able to reduce these issues significantly (W. Liu et al., 2019).

3.3. Quantitative PCR or Real-time PCR

3.3.1. Introduction

Quantitative PCR (qPCR) or Real-time PCR is a more complex form of PCR that took about a decade to develop since the first PCR invented by Karry Mullis and coworkers in 1983. Until today, standard PCR provide reliably only qualitative results for the DNA target and only after the end of the procedure. One of the main problems during the first attempts of quantification of the target sequence was the high sensitivity of PCR which, although desirable, created problems since even a small contamination could alter the results (Fairfax and Salimnia, 2010; Hoy, 2013).

3.3.2. Basic Principles

Quantitative PCR differentiates enough from standard PCR and provides results in real-time, introduces fluorescence in the method and skips the subsequent electrophoresis that was previously mandatory for results' visualization. It can be described as a PCR method that uses sensors to measure fluorescence changes (in real time, usually after each PCR cycle) which come as a result of adding fluorescents in a manner that when each PCR cycle ends and the DNA target is doubled, the fluorescence is also increased linearly therefore giving the quantification capability. These fluorescents are either dyes that can intercalate between any double-stranded DNA in a non-specific manner to fluoresce at a defined wavelength, or DNA probes that are coupled with fluorescent reporters and can bind specifically to the DNA target. In the first case (DNA-binding dyes, e.g. SYBR green) there is a need for the extra step of melting curve analysis so as to ensure high specificity. Melting curve analysis is a procedure in which samples are treated with a thermal protocol which is based on the specific amplified DNA fragment's melting temperature (the temperature in which the 50% of DNA is denatured, thus the dye can intercalate better at this segment) while fluorescence is measured at the same time. There are two ways in order to associate fluorescence with DNA quantity: absolute quantification and relative quantification (Fairfax and Salimnia, 2010; Löfström et al., 2015; Maddocks and Jenkins, 2017).

3.3.3. Quantifications methods

3.3.3.1. Absolute quantification

Absolute quantification can be performed either by standard curve method or by digital PCR. In standard curve method, serial dilutions of different concentrations of a gene (e.g. DNA target region or its RNA transcript if gene expression is studied) or an organism (e.g. foodborne pathogen) are created and processed with real-time PCR in order to produce the so-called standard curve which includes the fluorescence values of each cycle and form the basis of quantification. Afterwards, follows the comparison between the Cq (Cycle of quantification, refers to the number of cycles required for the fluorescent signal to cross a threshold value) of unknown samples and the standard curve (Löfström et al., 2015).

Digital PCR is a PCR method that includes a first step of separation of the sample, as much as possible, (e.g. 10 million droplets) so as to provide discrete signals based on each sample partition independent reaction. These discrete signals are either positive or negative in fluorescence (based on DNA probes). It is assumed that these signals follow Poisson distribution and so the probabilities of given droplets to contain one or more target partition-molecules are estimated and taken into account to quantify the number of DNA molecules in the original sample (Pabinger et al., 2014).

3.3.3.2. Relative quantification

Relative quantification is mostly used when gene expression amounts are studied which is also a real-time PCR application of utmost importance. Gene expression is quantified via copies of the RNA target which is first reverse-transcribed to complementary DNA (cDNA). In relative quantification, a control sample with known gene expression and fluorescence (after Reverse Transcription (RT) Quantitative PCR) is used as a reference and is compared with the unknown sample. This comparison determines a test/control ratio after proper normalization of the results is made with housekeeping genes (predefined genes with, as much as possible, same gene expression and fluorescence as the RNA target) (Löfström et al., 2015).

3.4. PCR applications in food microbiology

In recent years, food microbiology has used the PCR variants that were mentioned above in several applications. These applications range from the detection of spoilage microorganisms and fermentation monitoring to foodborne pathogens' detection and serotyping (**Table 3.**).

3.4.1. Detection of spoilage microorganisms

Moschonas et al. (2021) studied the spoilage potential of *Bacillus subtilis* in a neutral-pH dairy dessert and concluded that *Bacillus subtilis* subsp. *subtilis* was the dominant spoilage microorganism during preservation at 12 ^{o}C and 15 ^{o}C (**Table 3.**). For the *Bacillus* species and subspecies determination a repetitive extragenic palindrome (rep)-PCR method was performed, followed by PCR amplification of the V1-V3 variable region of the 16S rRNA. Briefly, rep-PCR is an application of standard PCR for fingerprinting bacterial genomes by comparing REP sequences between the suspect samples (after selective isolation) to find the ones that differ. Then, these samples were followed by PCR amplification of the V1–V3 variable region of the *16S rRNA* gene which is a bacterial fingerprint up to subspecies level. The suspect samples found to share the same pattern in PFGE (after 16S rRNA gene PCR) with a predetermined *Bacillus subtilis* subsp. *subtilis* strain and the results were subsequently verified through sequencing and compared with an open-access database (Moschonas et al., 2021).

3.4.2. Study of gene expression with Real time PCR and relative quantification

Doulgeraki et al. (2016) studied gene expression of *Salmonella enterica* in biofilm formation on rocket leaves and extracts by using Reverse Transcription (RT) Quantitative PCR with relative quantification (**Table 3.**). In this study SYBR green was used as a non-specific DNA-binding dye and also the extra step of melting curve analysis was performed as explained in previous section (Section **3.3.2. Basic Principles**). In short, 7 specific genes that were previously associated with stress related mechanisms, were amplified (after RNA to cDNA conversion) and compared with planktonic cells on LB broth whereas rrsG gene was selected as the reference for *Salmonella enterica*

housekeeping gene (a gene like 16S rRNA, which is present in all bacteria but with specific regions different in each species). Housekeeping gene is a form of verification that the sample studied and therefore the results obtained, were referring to only a specific microorganism. The above comparison was made in order to determine a relative gene expression under different conditions (planktonic or biofilm state, temperature of 10 ^{o}C or 20 ^{o}C, extract or leaves etc.). One of the observed results was an overexpression of specific genes under stress conditions (Doulgeraki et al., 2016).

3.4.3. Strain specific detection and fermentation monitoring through RAPD and multiplex PCR

Saxami et al. (2016) developed a molecular method based on RAPD (Random Amplified Polymorphic DNA) analysis and multiplex PCR to detect two potential probiotic lactobacilli strains which were then added as complementary starters in yogurt production. RAPD analysis is a PCR assay which uses short, random sequence primers which are strain specific and can be used as DNA fingerprints for different strains and visualized with the subsequent agar gel electrophoresis (Butler, 2012). Saxami et al. used RAPD analysis in order first to determine 123 arbitary primers and then the primers that were most important for the separation of the two strains were selected among others. RAPD analysis showed that there were 32 primers for *L. pentosus* B281 and 24 for *L. plantarum* B282 that produced more than 5 scorable bands, respectively. Among these RAPD-derived primers, only two had the capability to separate the two strains. These two primers were combined with a universal set of primers for the 16S rRNA gene to create a multiplex PCR method able to detect these lactobacilli strains and therefore to monitor the survival of the two strains during fermentation and preservation of yogurt (Saxami et al., 2016).

3.4.4. Direct quantification of foodborne pathogens using Real-time PCR

Zhou et al. (2017) developed a qPCR-based molecular method for the direct and simultaneous quantification of *Salmonella* spp. and *E. coli* O157:H7 in artificially spiked milk (**Table 3.**). This study used multiplex Real-time PCR with specific DNA probes

(coupled with fluorescent reporters) and absolute quantification (more details at Sections **3.3.2. Basic Principles** and **3.3.3. Quantifications methods**). The genes used to design primers and probes where *inv*A and *fliC* for *Salmonella* spp. and *E. coli* O157:H7, respectively. In order to separate viable from injured/dead cells they used propidium monoazide (PMA) as a DNA-intercalating dye, partly able to avoid intercalating with dead cells. They also used sodium deoxycholate (SDO) as a detergent that can enhance this ability. They standardized the PMA-SDO optimal concentration and created standard curves based on multiplex Real-time PCR results for serial 10-fold dilutions of DNA extracts of pure bacteria and artificially spiked milk. The results determined linear relationships for each standard curve and a limit of detection (LOD) of 10^2 CFU/ml for spiked milk (same as pure bacteria) for both *Salmonella* spp. and *E. coli* O157:H7. They also reported high specificity against other microorganisms and with almost none interaction of the results in the presence of dead/injured cells (Zhou et al., 2017).

At the same wavelength, Liu et al. (2019) developed a TaqMan Real-time PCR assay for the direct, simultaneous quantification of 12 foodborne pathogens (**Table 3.**). Primers and TaqMan probes were designed based on pathogens' specific virulent genes. They also reported high specificity and sensitivity with a LOD of 10^4 CFU/g in meat samples for almost all foodborne pathogens (Liu et al., 2019).

3.5. Serotyping *Salmonella* using PCR

3.5.1. *Salmonella* nomenclature and serotyping background

Salmonella was selected among other foodborne pathogens because of its important role in foodborne diseases as described in introduction (Chapter **1. Introduction**) but also because of its complex nomenclature and the big number of involved serotypes. *Salmonella* belongs to *Enterobacteriaceae* family and according to CDC nomenclature consists of two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* includes six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*. According to White-Kauffmann-Le Minor (WKL) scheme, *Salmonella bongori*, was previously considered as

the V subspecies of *Salmonella enterica* but then gained a species status (Brenner et al., 2000). The traditional serotyping method is based on the expression of somatic (O) and flagella (H) antigens which if present will agglutinate when in contact with proper antisera. The combination of 46 O and 114 H antigens has already provided more than 2,600 different serovars (or serotypes) (Diep et al., 2019). This can be chaotic and lab-intensive because the routine method needs more than 150 different antisera-tests for each sample and also implies some issues in cases of non-specific agglutination, auto-agglutination and loss of antigens expression (Agbaje et al., 2011; Diep et al., 2019).

3.5.2 Salmonella serotyping through PCR-based methods

Kim et al. (2006) developed a method, consisting of two multiplex PCR assays, able to detect 30 serotypes of *Salmonella enterica* subsp. *enterica*. This method was not applied directly to samples but required the preliminary steps of selective isolation of *Salmonella* among other microorganisms. The first multiplex PCR assay included five different sets of primers which were selected after whole genome sequencing (more details at: Section **3.6. Next Generation Sequencing (NGS) and Whole Genome Sequencing (WGS))** and comparative genomic hybridization of specific *Salmonella* serovars. All five sets where designed for five loci in the *S. enterica* serovar Typhimurium genome. The second multiplex PCR assay also included five different sets of primers, four for *S. enterica* serovar Typhi and one for Typhimurium genome (different from the five used in the first assay). These sets of primers that were mentioned above were combined with two additional sets of primers (for some cases in which patterns were overlapping in PFGE) and succeeded to discriminate 30 different common serotypes. The discrimination was based on the PFGE patterns of each amplicon compared to the patterns of pre-determined serotypes. The practical advantages of this method that should be considered are the simplicity of equipment-procedure and the low cost per sample especially when set side by side with other approaches such as DNA sequencing, Real-time PCR, microarrays, mass spectrometry etc. The main disadvantage of this method is the possibility of false-positive results if a rare serotype comes in light and happen to share the same PFGE patterns with the ones that were determined. However, the

probability of such an event will be able to be controlled more and more as new, different serotypes are added to the method's database (Kim et al., 2006).

Beaubrun et al. (2012) followed the Kim et al. methodology mentioned above but with some modifications in PCR mastermix and parameters, in order to evaluate its serotyping performance when applied on reference and other various samples (either after selective isolation of *Salmonella* spp. or directly from pre-enrichment broths) (**Table 3.**). Based on FDA reference collection apart from evaluating the method, Beaubrun et al. also proposed modified banding patterns in PFGE and tested the method with proficiency ("blind") test samples. The PCR assay consisted of two five-plex and one two-plex reactions with primers that were previously selected by Kim et al. For easier interpretation of the results Beuaubrun et al. created something like an alphabet (A-L) for different patterns, so as every pattern correspond to a letter and provide a unique result based on the 3 reactions e.g. BCE banding pattern in PFGE indicates *Salmonella enterica* susbp. *enterica* serovar Hadar. The results showed similarities on serotyping patterns with Kim et al. but also differences in some serotyping patterns (in 8 out of 30 serovars) therefore indicating the need for a universal database of multiple copies of each serovar's patterns that would act as reference. Beaubrun et al. also added new patterns for other serovars that were not previously included in the method and more importantly they determined the method's good enough sensitivity and specificity even when background microbiota (non-Salmoneallae) was present (pre-enrichment broths from food samples) for four different serotypes. Proficiency tests were also successful and this study was able to detect the presence and serotype of Salmonellae in lettuce samples within 24 hours of receiving samples. This was determined as the first study that didn't require pure colonies for *Salmonella* serotyping (Beaubrun et al., 2012).

Table 3. The most representative applications of PCR variants in food microbiology

Method	Microorganisms	Purpose	Performance indicators	References
Standard PCR	*B. subtilis*	Detection in neutral-pH dairy dessert	Successful detection after selective isolation	(Moschonas et al., 2021)
RT-qPCR	*S. enterica*	Gene expression in biofilm formation	Successful detection of stress-related genes	(Doulgeraki et al., 2016)
RAPD and multiplex PCR	*L. pentosus* B281, *L. plantarum* B282	Detection	Successful detection among other strains	(Saxami et al., 2016)
Multiplex qPCR	*Salmonella* spp., *E. coli* O157:H7	Direct, simultaneous quantification in milk	LOD = 10^2 CFU/ml	(Zhou et al., 2017)
TaqMan qPCR	12 common foodborne	Direct, simultaneous	LOD ≈ 10^4 CFU/g	(Y. Liu et al., 2019)

	pathogens	quantification in meat		
Multiplex PCR	30 serotypes of *Salmonella enterica*	Direct serotyping in lettuce (pre-enrichment broths)	Succesful discrimination, verified with proficiency tests	(Jean-Gilles Beaubrun et al., 2012)

Other researchers followed the multiplex PCR-PFGE concept but did not follow the primers that were determined by Kim et al. (2006) and used pan genome analysis to determine new serovar-specific target genes for serovars in *Salmonella* B, C1 and E serogroups (Shang et al., 2020; Ye et al., 2021a, 2021b).

Bugarel et al. (2017) developed a two-step real-time PCR method for the detection of five important *Salmonella enterica* subsp. *enterica* serovars (i.e. Typhimurium, Enteritidis, Heidelberg, Newport, and Hadar). The primers used where designed with information provided by *in silico* comparisons of genomes with BLAST and sequences available on NCBI GenBank. The results showed high specificity and sensitivity for both isolated colonies and enrichment broths of ground beef and also the possibility of using the mRNA conversion of the determined target genes (i.e. RT-qPCR) in order to discriminate dead from alive bacteria (Bugarel et al., 2017).

3.6. Next Generation Sequencing (NGS) and Whole Genome Sequencing (WGS)

3.6.1. Background

Next Generation Sequencing (NGS) or massive parallel sequencing has no strict definition and usually is considered as all the methods developed after Sanger sequencing that can produce large volumes of sequence data in short time and in relative low cost. There are four primary NGS methods: cyclic reversible termination, sequencing by ligation, pyrosequencing and real-time sequencing (Metzker, 2010). These methods although different, share the same basic concept and involve a direct DNA extraction of the sample which is then followed by sequencing of millions small fragments of DNA in

parallel and in a single run. These sequences will be then analyzed and mapped with bioinformatics approaches in order to be compared with a reference library (Behjati and Tarpey, 2013). Next generation sequencing unfolds a whole new set of applications that is wider from one's imagination, in a manner that is comparable with the early days of PCR history (Metzker, 2010). One of these applications is the use of Whole Genome Sequencing (WGS) by public health authorities for real-time surveillance of foodborne bacterial pathogens. WGS, as its name pronounces, is the sequencing of the entire bacterial genome in a timely and cost efficient manner (through NGS) and tends to become the "gold-standard" method for outbreaks surveillance due to greater discrimination capability and cluster resolution in phylogenetic analysis against traditional methods (Brown et al., 2019; WHO, 2018).

3.6.2. Applications in microbiology

Dourou et al. (2021) explored the bacterial communities of chicken breast and thigh fillets at different temperatures (0 ^{o}C, 5 ^{o}C and 10 ^{o}C) during 5 days of preservation through conventional methods and NGS. NGS was applied for 16S rRNA gene amplicon sequencing and the results were compared with the conventional methods. NGS showed a great capability of monitoring in relevance, the bacterial communities of chicken breast and thigh fillets for quality and safety purposes. Also, revealed bacterial families and genera (e.g. *Acinetobacter* and *Photobacterium*) that were previously ignored with conventional methods and may contribute to spoilage (Dourou et al., 2021b).

Lewis et al. (2020) applied NGS as a screening method for foodborne pathogens in fresh produce samples. The methodology was as follows: Lettuce samples were artificially spiked with serial dilutions of *Salmonella* and phage MS2 (a Norovirus surrogate). Then the DNA extracted from the samples and got sequenced with 3 different methods of NGS while the results were also processed with different bioinformatics methods to investigate how they interrogate with the limit of detection (LOD). The results showed great potential of NGS in detecting *Salmonella* and phage MS2 in short time and without pre-enrichment or cultivation procedures. This was promising for detecting VBNC bacteria but also pathogens outside of the "list of common foodborne pathogens",

a “list” in which conventional and PCR methods are aiming. However, even the most sensitive combination of NGS and bioinformatics methods, was far less sensitive than conventional methods with a LOD of 10^4 CFU reaction^{-1} for *Salmonella* and 10^5 PFU reaction^{-1} (Plaque Forming Units) for phage MS2 (Lewis et al., 2020).

4. Spectroscopic techniques

Spectroscopic techniques employ electromagnetic radiation to a sample in order to obtain useful information based on the interaction between them. The basic categorization of spectroscopic techniques differs depending on the wavelength region of the electromagnetic spectrum (i.e. gamma, x-ray, ultra-violet, visible, infrared, microwave and radiowave), the type of the interaction: electromagnetic radiation-sample (i.e. absorption, emission, refraction, scattering, resonance etc.) and the type of the material (i.e. atoms, molecules, nuclei etc.) (Ball, 2009; Penner, 2010). The most common spectroscopic techniques used in food microbiology are: Fluorescence spectroscopy, Terahertz (time□domain) spectroscopy, Laser-induced breakdown spectroscopy, Raman spectroscopy and Fourier-transform infrared spectroscopy (Hameed et al., 2018; Saravanan et al., 2020; Wang et al., 2018a; Zhao et al., 2018).

4.1. Fluorescence spectroscopy

4.1.1. Background

Fluorescence spectroscopy can be considered as a type of emission spectroscopy. In emission spectroscopy, treatments such as electromagnetic radiation bring atoms or molecules in the excited state and then follows a transition of electrons from excited to lower energy states which cause the emission spectrum (the spectrum of frequencies of the emitted electromagnetic radiation). The discrimination capability resides mainly on involving molecules (typically aromatic molecules) which in their excited states (prior to the emission phenomenon) emit fluorescence (Lakowicz, 2006; Misra, 2019). The standard setup used is a spectrofluorometer which is consisted of: i) a light source, ii) a monochromator and/or filter for selecting the emission wavelengths, iii) photosensitive

detectors and iv) a data analysis platform. The samples with known concentrations of a substance or a microorganism are placed along with the unknown samples in the spectrofluorometer. In short, as light passes through the sample the detected fluorescence signal is converted to digital values which construct a graph. This graph is then used to predict the concentrations of the unknown samples through data analysis (Karoui, 2018).

4.1.2. Applications in microbiology

Pu et al. (2013) applied fluorescent spectroscopy in order to predict meat spoilage. This study developed fluorescent "fingerprints" (at an excitation wavelength of 340 nm) of meat samples stored at 4 ^{o}C and 15 ^{o}C. The results were then processed with Multivariate Curve Resolution with Alternating Least-Squares (MCR-ALS) to provide quantitative results that were correlated to fluorescence changes and were attributed mainly to NADH content (Pu et al., 2013).

Yoshimura et al. (2014) also investigated the potential of fluorescence fingerprint spectroscopy in order to predict the total viable counts (TVC) in the surface of beef samples stored aerobically at 15 ^{o}C. Fluorescent fingerprints of beef samples were collected in the spectrum range of 200-900 nm along with conventional enumeration of total viable counts. The results were processed with Partial Least Squares Regression (PLS-R) and provided a prediction range from 1.7 to 7.8 log CFU/cm^2 with a prediction error of 0.752 log CFU/cm^2. The main molecules to which the fluorescence emission was attributed, were tryptophan, NAD(P)H, vitamin A, porphyrins, and flavins. Their changes were interpreted as results of bacterial metabolic processes (Yoshimura et al., 2014).

Sohn et al. (2009) developed a rapid method able to detect *Escherichia coli*, *Salmonella* and *Campylobacter* based on fluorescence spectroscopy and data analysis. The method was developed on liquid forms of isolated pathogens (in saline 0.85% saline solution) at different, predetermined concentrations ranging from 10^3 to 10^7 CFU/ml. Samples were analyzed with a fluorescence spectrometer and the optimum excitation and emission wavelengths were determined at 225 nm and 280 nm for the excitation and at 345 nm for the emission, respectively. The results that were processed with principal component analysis (PCA) to classify the 3 bacteria were encouraging, however the

method was not validated when background microbiota is present therefore indicated the need of further research (Sohn et al., 2009).

4.2. Terahertz spectroscopy

4.2.1. Background

Terahertz spectroscopy employs THz radiation to a matter in order to obtain useful information about its properties based on the interaction THz radiation-matter. The THz radiation is a non-ionizing electromagnetic wave that refers to the region between microwaves and far-IR (THz gap) and is often defined with a band of frequencies ranging from 0.3 to 3 THz or wider from 0.1 to 10 THz (i.e. wavelengths ranging from 3.3 to 333.6 cm^{-1}) (Wang et al., 2018b). Due to the fact that THz radiation is non-ionizing, it can be employed in food or microorganisms without causing unwanted complications or damages, unlike other forms of radiation such as X-rays or microwaves radiation. The interaction THz radiation-matter can act as a fingerprint and reveal differences between molecules due to the vibrational and rotational modes of many molecules in the THz region (Ren et al., 2019). Terahertz technology bases its detection capabilities on the excitation of low frequency molecular vibrations during THz radiation. It detects mainly weak, intermolecular interactions like hydrogen bonds, Van der Waals forces and hydrophobic interactions. The most important advantages of Terahertz against other regions (e.g. infrared region) is the better penetrating capacity against cell tissues and the lower absorption of water molecules (compared to far infrared). (Globus et al., 2012).

4.2.2. Applications in microbiology

Terahertz time-domain spectroscopy (THz-TDS) is a recently developed tool which uses Terahertz technology and has great potential in food microbiology (Ren et al., 2019; Wang et al., 2018b; Yang et al., 2016a). In simple terms, Terahertz time-domain spectroscopy utilizes, in addition to weak interactions, the time factor to reveal time-resolved dynamics which may hide unique structural and dynamic properties which in the case of other types of spectroscopy are overlooked (Yang et al., 2016a).

Yang et al. (2016) demonstrated the potential of THz-TDS to detect *S. aureus, E. coli, P. aeruginosa* and *A. baumanii* (**Table 4.**). The process included only samples of pure colonies and thermal treatment for some samples in order to be assessed if the method could discriminate dead and alive cells. It also included freeze-drying of some samples because water seemed a significant factor that can interfere with the discrimination capability. The results, after Fourier-Transformation of spectra signals, showed that absorption coefficients differed between various intracellular water contents and the ratio between bulk and hydration water. These attributes, according to authors, could be used as indicators of metabolic statements and therefore to be utilized as discrimination factors for alive/dead cells and for bacterial species identification. Yang et al. although claimed the potential of THz-TDS for a label-free, cost-effective and rapid detection of common pathogens, they also indicated some critical gaps that need to be filled in the future (Yang et al., 2016a).

Hindle et al. (2018) employed Terahertz spectroscopy to develop a rapid method of microbiological quality assessment of refrigerated salmon fillets packed under protective atmosphere (100% N_2). The method used hydrogen sulfide (H_2S) of headspace gases as a spoilage indicator that was analyzed by both Terahertz spectroscopy and Selective Ion Flow Tube Mass Spectrometry (SIFT-MS). SIFT-MS was used as a validation method and as a basis for the development of the quantitative prediction model. The results showed a promising capability of THz spectroscopy to detect hydrogen sulfide as a spoilage indicator with a limit of detection (LOD) of 220 ppb (at 400-500 ppb of H_2S spoilage could be easily detected in a sensory evaluation). Authors highlighted that this method could be also applied to other Volatile Organic Compounds (VOCs) for a more holistic approach. Finally, for a future perspective it was proposed that this technology could be integrated to packages by constructing sensors that use silicon-based fabrication processes (Hindle et al., 2018).

Zeng et al. (2019) developed a Terahertz spectroscopy method for the identification of 16 common pathogens (incl. *Cronobacter sakazakii* and *Salmonella* Enteritidis) by using Principal Components Analysis (PCA) and Siamese neural networks. They obtained representative spectral data of each pathogen with Terahertz spectroscopy

and then they proceeded with data analysis. Firstly, the obtained spectra were smoothed with Savitzky-Golay filter which is a digital filter able to reduce some noise and fluorescence background of the spectra. Afterwards, the spectral data were normalized for better model performance and then PCA algorithm was applied to select the 3 principal components against a total of 140. Finally, siamese neural networks were selected among other traditional machine-learning algorithms such as SVM, KNN, Logistic regression etc. In simple terms, Siamese neural network discriminates pairs of spectral data to similar and non-similar based on predetermined spectral data of a specific pathogen and therefore identifies the most probable pathogens within the training set. This approach is very close to how face-recognition applications are working. The final results claimed a recognition rate of 97.34%, however the need of more samples to build a better database and reduce the similarity of mismatched spectra was highlighted. (Zeng et al., 2019).

4.3. Laser-Induced Breakdown Spectroscopy (LIBS)

4.3.1. Background

Laser-Induced Breakdown Spectroscopy (LIBS) is an atomic emission technique able to detect (theoretically) spectral fingerprints of all periodic table's elements by analyzing the UV, Visible and IR emissions in laser-generated sparks (Singh and Thakur, 2020). The process include high-power, laser-generated sparks as excitation source, which when come in contact with the sample (gas, liquid or solid targets) their energy is partly converted into heat and the temperature of the sample increases to form a high-temperature plasma that vaporizes a small amount of material. The produced plasma excites the sample's constituents and emits radiation before it finally decays to emit an element-specific radiation. A part of this emission (spectral fingerprint) is collected with detectors such as a modified optical fiber bundle and is analyzed to predict the relative abundance of (theoretically) all elements in the sample (Musazzi and Perini, 2014; Noll, 2012; Rendón Sauz et al., 2017; Singh et al., 2018).

4.3.2. Applications in microbiology

Marcos-Martinez et al. (2011) developed a method for the identification of *Pseudomonas aeroginosa*, *Escherichia coli* and *Salmonella* Typhimurium isolates, based on Laser-Induced Breakdown Spectroscopy (LIBS) and Neural Networks (NNs). Initially, a reference spectral database was created for the aforementioned microorganisms. The samples (isolates) were defrosted and cultured directly in 3 different agar media at 37 ^{o}C for 18 hours. The spectra was collected with LIBS in the range of 200-1000 nm. Then, a three-layer Neural Network model was created by using a back-propagation (BP) algorithm for the learning process (the process that optimizes the weight of the output of a previous layer of the Neural Network to the next), tested with common metrics such as sensitivity and specificity and was externally validated. The method demonstrated 100% correct (and independent of the culture media) identification for both known and unknown samples, however the small sample size used for the development of the method was highlighted (Marcos-Martinez et al., 2011).

Liao et al. (2018) developed a method for qualitative and quantitative detection of four types of bacteria (incl. *Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli*) based on the combination of Three-Dimensional Surface-Enhanced Raman Scattering (3D SERS) and Laser-Induced Breakdown Spectroscopy (LIBS) (**Table 4.**). 3D SERS is a spectroscopic method which is based on the capture of the inelastic scattering of molecules that are located near metal nanostructures (more details about Raman Scattering at: Section **4.4. Raman spectroscopy**). The 3D SERS method was used for qualitative detection whereas LIBS was used for quantification. The methodology included *in situ* synthesis of settled and free Ag nanoparticles (the nanostructures mentioned above) to form a colloidal bacterial suspension and to create effective nanogaps between them that are necessary in order to enhance the electromagnetic signal. The spectral data were obtained from the natural evaporation of a droplet of the aforementioned suspension and the identification of bacteria through 3D SERS was attributed to the different spectral properties of cell walls. The spectral data were analyzed with Principal Components Analysis followed by Hierarchy Cluster

Analysis and provided correct classification in all cases. The LIBS method was then applied for the spectral region of 200-800 nm and the most intense atomic emission line was selected for quantification, which was at 279.5 nm and corresponded to the intracellular magnesium ions. For this method, the spectral data were analyzed by fitting the emission lines to Voigt profile (a probability distribution) to reduce noise and then by applying log-log regression between spectral data and bacterial concentrations to make quantitative predictions. The correlation coefficient R^2 was determined > 0.97 and the limit of quantification (LOQ) was estimated at about $5x10^3$ CFU/ml. It is notable that the method was also applied in various water samples with an approximate total duration of 30 minutes and provided also reliable results (relative standard deviation < 14.9%) within the range of $5.8x10^3$ CFU/ml to $5.8x10^7$ CFU/ml (Liao et al., 2018).

Yang et al. (2020) developed a method for the identification and quantification of *Salmonella* Typhimurium based on Elemental-Tags Laser-Induced Breakdown Spectroscopy (ETLIBS) (**Table 4.**). The procedure included DNA aptamers (more details about aptamers at: Section **8.1. Flow cytometry**) that were used as elemental tags, silicon nanowires (SiNWs) modified by metal nanoparticles to form a substrate and Laser-Induced Breakdown Spectroscopy for the analysis. For the modification of the silicon nanowires (SiNWs), a mixture of gold and silver (Au@Ag) nanoparticles (Au@AgNPs) was added and the SiNWs-Au@Ag substrate was created. The elemental-tags were created by assembling copper nanoparticles (CuNPs) with ssDNA oligonucleotides (aptamer for capture and poly-T sequences as template for CuNPs) and were then incubated with *Salmonella* Typhimurium (*S.ty*) suspensions for 30 minutes to achieve labeling. The labeled samples were then added to the SiNWs-Au@Ag substrate to create a complex of SiNWs-Au@Ag/*S.ty*/CuNPs that was used for the LIBS analysis which lasted about 5 mins. After the complex was created, the point was to follow Cu peaks in order to obtain information about the sample. The use of Cu as indicator for *Salmonella* Typhimurium prevalence and quantification was evaluated and established. Subsequently, the spectral data (emission lines) were first fitted to Voigt profile to reduce noise and then a log-log regression between spectral data and bacterial suspensions, with a correlation coefficient $R^2 = 0.978$, was made to form the quantitative model. The limit of detection

(LOD) was estimated at 61 CFU/ml at a range of detection from 10^2 to 10^6 CFU/ml. The capability of this method was tested with suspensions of spiked samples with background microbiota present and by comparison with qPCR. The method achieved *Salmonella* Typhimurium recoveries > 87% and relative standard deviations (RSD) < 11.56% at spiked samples, in all cases. In conclusion, it should be mentioned that this method could possibly integrate other pathogens with different aptamers for simultaneous identification and quantification, however the method is not yet tested with "real samples" that were naturally containing *Salmonella* Typhimurium but with food samples' dilutions (made with ultra pure water for 12 hours) that were artificially spiked (Yang et al., 2020).

4.4. Raman spectroscopy

4.4.1. Background

Raman spectroscopy is a vibrational spectroscopy method that may take part as a useful tool in food microbiology since it is non-destructive, requires minimal preparation of samples, can be integrated in a portable device and can provide information about different molecules, simultaneously (Argyri et al., 2013).

Raman spectroscopy source its name from the Raman scattering or Raman effect that was experimentally observed by Raman and Krishnan in 1928, nevertheless the starting point of the method was after 1960 with the evolution of technology (Chauvet et al., 2017). In short terms, in Raman spectroscopy, a sample is targeted and exposed at a monochromatic light which leads more to the transmission of light through sample, less to the scattering of light with the same wavelength and even less to inelastic scattering of light with a different wavelength. The last mentioned effect is known as Raman scattering and, although the least in terms of scattering photons (about 1 inelastic-scattered photon out of 10^6-10^8 incident photons) it is the most important. This is because these photons, when fall in the sample, cause some molecules to move at a different rotational/vibrational state and also the scattered photons will move at a different state (Raman shift). The energy difference between the two states of photons will be equal to the energy difference between the two vibrational states of the molecules and can

therefore provide useful information about the molecules in the sample (Chauvet et al., 2017; Lorenz et al., 2017; Lu et al., 2011).

Biological samples like foods or microorganisms' suspensions contain a lot of different molecules, so the Raman spectrum arises mainly from the spectral superposition of the molecules within the monochromatic light and serves as a spectral fingerprint i.e. a reference for microbiological interpretation of spectral results after proper analysis (Lorenz et al., 2017; Lu et al., 2020).

4.4.2. Applications in microbiology

Argyri et al. (2013) demonstrated for the first time that Raman spectroscopy can become a useful tool for rapid assessment of meat spoilage. The method was based on implementing direct analysis of meat samples with Raman spectroscopy along with simultaneous, conventional microbiological analysis for the development of a quantitative model after proper data analysis for the correlation of the results. At the same time, the pH changes in meat samples as well as their organoleptic acceptance during preservation were also monitored, and a qualitative (semi-quantitative) model with 3 classifications (fresh, semi-fresh, spoiled) was developed. Data analysis was conducted with advanced multivariate statistical methods and machine learning methods and the developed models were validated with half-out cross validation. In conclusion, the quantitative results were promising regarding the time needed for the direct analysis with Raman Spectroscopy. Specifically, for Raman models, SVRR and SVRP gave acceptable predictions ($\%\ PE = \left(\frac{PEin}{PEtotal}\right) x\ 100 > 70\%$) for all of the counts. In the case of the Raman spectra, the GA-ANN gave better results considering the fact that no fresh sample was misclassified as spoiled and vice versa (Argyri et al., 2013).

Díaz-Amaya et al. (2019) developed an aptamer based Surface-Enhanced Raman Spectroscopy (SERS) for the detection of *E. coli* O157:H7 (**Table 4.**). SERS is a surface-sensitive, Raman spectroscopy variant which was developed in order to enhance the Raman scattering effect to detect single molecules, by using metallic nanoparticles which can act as signal enhancing substrates. Diaz-Amaya et al. combined SERS with gold nanoparticles (Au-NPs) conjugated DNA aptamers specialized for *E. coli* O157:H7

detection. The procedure involved creating the AuNPs-Raman reporter complexes and their proper mixture: i) with serial dilutions of the pathogen (pure cultures) and ii) with dilutes of ground beef samples spiked with predetermined concentrations of the pathogen. Afterwards, spectral data of the samples were collected with SERS and analyzed by One-way ANOVA test, accompanied by means comparison using Dunnett's method. The results determined the ability of detection and quantification of *E. coli* O157:H7 with an LOD of 10^1 CFU/ml and 10^2 CFU/ml for pure cultures and ground beef samples, respectively (Díaz-Amaya et al., 2019).

Lu et al. (2020) developed a label-free method that combined artificial intelligence and Laser Tweezers Raman Spectroscopy (LTRS) for the identification of 14 microbial species (Lu et al., 2020). Briefly, LTRS is a Raman spectroscopy variant that is used to analyze single cells and biological particles suspended in an aqueous environment (Navas-Moreno and Chan, 2018). Lu et al. (2020) used a Raman Tweezers System that combined an optical trap, a confocal microscope, a Raman spectroscope and other optical elements in order to analyze single cells (as discriminated and determined by the optical trap and the confocal microscope) at different growth states of 14 microbial species. The full Raman spectra (defined as ramanome) of each microbial cell was collected and analyzed with a convolutional neural network (ConVet). For better investigation of the ConVet results, they developed the Occlusion-Based Raman Spectra Feature Extraction (ORSFE) tool which provided the ability to visualize the Raman features that contributed most at the classification. The results determined correct classifications of the 14 microbial species with an average accuracy of classification at 95.64 ± 5.46%, although it should be mentioned that the current, high cost of the confocal microscopy instrumentation may limit the applications, especially if this method is modified to find food microbiology-related applications (Lu et al., 2020).

4.5. Fourier-Transform Infrared Spectroscopy (FTIR)

4.5.1. Background

Fourier-Transform Infrared Spectroscopy (FTIR) is an analytical, vibrational spectroscopy technique that is able to obtain information about the rotational and

vibrational transitions of molecules based on their infrared spectrum of absorption and emission. In the FTIR, a full spectrum beam of IR radiation is passed through the Michelson interferometer. Michelson interferometer is an array of mirrors, one of which is moved by a motor. As this mirror moves, some wavelengths of light in the beam are periodically emitted while others are blocked, providing different recombinations of the initial beam each time (Griffiths and de Haseth, 2007; Mohamed et al., 2017; Shepherd, 2003). Every recombination contains multiple, predetermined wavelengths of light and the emitted spectrum is targeted to the sample where absorption and emission are calculated and indicate some vibrations within the molecules (changes at bond lengths (stretching) or changes at the bond angles (bending)). The process is repeated multiple times and the collected data consist the interferogram. These data show the absorption for each movement of the mirror and not the absorption for each wavelength, therefore needing a conversion, which is made with the Fourier-transform algorithm (Griffiths and de Haseth, 2007; Ismail et al., 1997; Mohamed Shameer and Mohamed Nishath, 2019; Moraes et al., 2008).

The above features were utilized in several applications in food microbiology where FTIR was used as a biochemical fingerprint technique which when combined with proper data analysis could provide useful insights related to food safety and quality (Dourou et al., 2021a). The main point of this approach is that when bacteria are growing in food or other substrates are producing certain metabolites (biochemical fingerprint) that can be detected with FTIR and correlated with microbial populations to provide qualitative or quantitative assumptions (Argyri et al., 2010).

4.5.2. Applications in microbiology

Fengou et al. (2019) evaluated FTIR for its capability of estimating fish microbiological quality. The methodology was based on the approach of Argyri et al. (biochemical fingerprinting) that was mentioned above and included FTIR measurements of fish samples during preservation at different temperatures and at different time points (Argyri et al., 2010; Fengou et al., 2019a). FTIR results were then correlated with TVC

(as determined by conventional microbiology methods) to provide quantitative predictions through PLS-R models. The FTIR spectra that were mainly utilized were in the range of 3100 to 2700 and 1800 to 900 cm^{-1} and the developed model was validated with leave-one-out cross-validation. The results showed that FTIR could be a useful tool for predictions of TVC in fish samples (both whole and fillets) and it is indicatively mentioned as a metric that root mean square error (RMSE) of the developed model was estimated at 0.717 log CFU/g (Fengou et al., 2019a).

Under the same point of view, Spyrelli et al. (2021) evaluated FTIR as a method for assessing spoilage on the surface of chicken breast fillets. The method involved the analysis of samples with FTIR in various temperatures and time points until spoilage. Simultaneously, the samples were analyzed with conventional microbiology methods for TVC and *Pseudomonas* spp. enumeration. After FTIR analysis, spectral data were first smoothed with Savitzky-Golay filter for noise reduction of spectra and then the range of 900-2000 cm^{-1} was utilized for correlation with TVC and *Pseudomonas* spp. populations through PLS-R models. A second approach for data analysis was also implemented with the testing of 9 machine learning algorithms in a wide spectra of 800 to 4000 cm^{-1}. In both cases the developed models were validated and metrics for predictions' performance such as correlation coefficient I and RMSE were calculated. The results claimed reliable quantitative predictions for TVC and *Pseudomonas* spp. at chicken breast fillets through FTIR with PLS-R model but also with some machine learning algorithms. It is indicatively stated that the least-angle regression (lars) model achieved quantitative predictions of TVC in samples of independent batches with a RMSE of 0.851 log CFU/cm^2 (Spyrelli et al., 2021).

For the detection of foodborne pathogens, Wang et al. (2017) combined synchrotron radiation based FTIR (SR-FTIR) microspectroscopy to develop a method able to identify and discriminate ten foodborne bacteria (including *Salmonella* spp. and *Vibrio* spp.) (**Table 4.**). SR-FTIR microspectroscopy is an FTIR variant that can analyze samples at the micron level therefore providing higher signal-to-noise, precise results in a manner of detecting differences between bacteria at single cell level. Wang et al. utilized the advantages of SR-FTIR in the analysis of bacterial suspensions. They created a

database of spectra (both full spectra and subdivided in regions) for isolated bacterial suspensions of the ten pathogens and then employed PCA to develop the identification and discrimination pattern. The results showed that full spectra could provide better identification and discrimination of foodborne pathogens and in fact it could even discriminate species between the same genus. However it should be mentioned that this method was not tested against background microbiota of real samples or mixtures and also involved preliminary steps that were therefore increasing the total time of the analysis (Wang et al., 2017).

Table 4. Most representative applications of spectroscopic techniques in foodborne pathogens' detection and quantification

Technique	Microorganisms	Purpose	Performance indicators	References
Fluorescence spectroscopy	*E. coli*, *Salmonella*, *Campylobacter*	Rapid (< 10 mins) differentiation of pathogens in liquids	LOD ≈ 10^3-10^4 CFU/ml (artificial samples)	(Sohn et al., 2009)
THz spectroscopy	16 common pathogens	Detection	Recognition rate = 97.34% but substrate was not mentioned	(Zeng et al., 2019)
THz-TDS	*S. aureus, E. coli, P. aeruginosa*, *A. baumanii*.	Identification and alive/dead cells discrimination	Successful in pure colonies	(Yang et al., 2016a)
LIBS	*P. aeroginosa*, *E. coli*, *Salmonella* Typhimurium	Identification after culture in agar media	Succesful identification of isolates	(Marcos-Martinez et al., 2011)

Technique	Microorganisms	Purpose	Performance indicators	References
			cultured in various agar media in less than 1 day total	
3D SERS and LIBS	*S. aureus, Salmonella* Typhimurium*, E. coli*	Direct quantification in water	Range: 5 x 10^3 – 5 x 10^5 CFU/ml in 30 mins total	(Liao et al., 2018)
ETLIBS	*Salmonella* Typhimurium	Quantification in bacterial suspensions	LOD = 61 CFU/ml Range: 10^2 to 10^6 CFU/ml in 65 mins (+12 hours for food samples)	(Yang et al., 2020)
SERS	*E. coli* O157:H7	Quantification	LOD = 10^1 CFU/ml (pure cultures); 10^2 CFU/g (ground beef)	(Díaz-Amaya et al., 2019)
LTRS	14 microbial species	Identification of single cells	Accuracy of classification = 95.64%	(Lu et al., 2020)
SR-FTIR microspectroscopy	10 foodborne bacteria	Identification in bacterial suspensions	Successful discrimination (artificial samples)	(Wang et al., 2017)

5. Spectral imaging techniques

The term spectral imaging techniques can be used to describe all the methods that combine spectroscopy and imaging. Spectroscopy was well discussed above, while imaging, in short terms, is about obtaining spatial and temporal data information from objects through various methods (X-rays, MRI, optical methods etc.) of which optical ones are the most relevant in our case. When the two methods are combined can provide dual information about spectra and pixels and specifically the spectrum at each pixel (**Figure 2.**) (Garini et al., 2006). Spectral images can be two dimensional with (2-D) with a spectral and a spatial dimension or three dimensional (3-D) with two spatial dimensions and one spectral dimension (Bian et al., 2016; Qin et al., 2013). Spectral imaging uses wavelengths that are sometimes not limited in the visible spectrum like images from a conventional camera capture but are extending with more details in the wider electromagnetic spectrum of UV and IR. The difference between multispectral imaging and hyperspectral imaging is that the former uses a low number (usually < 20) of specific bands (multiband imaging) in the electromagnetic spectrum while the latter uses a more continuous approach with often hundreds of different spectral bands (Qin et al., 2013; Wang et al., 2018b).

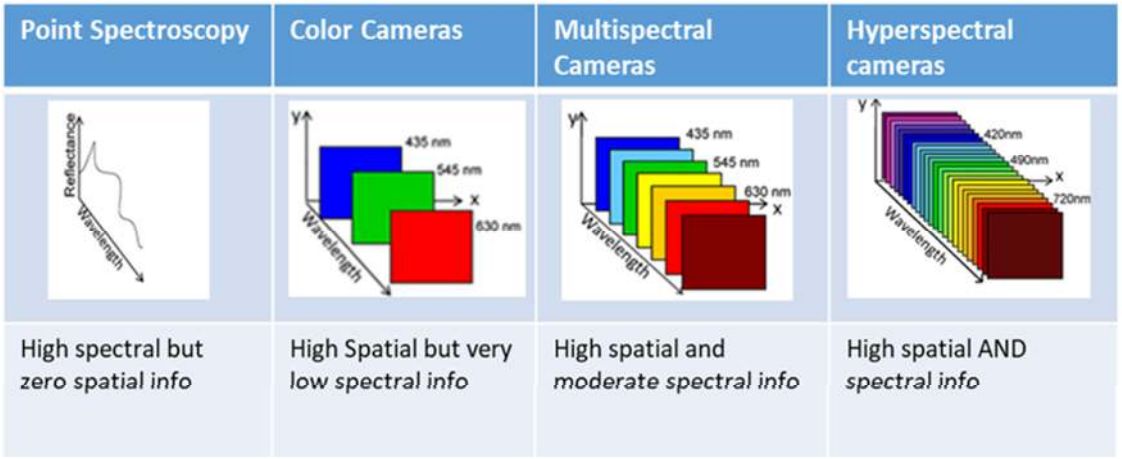

Figure 2. Main differences between MSI, HSI, color camera imaging and point spectroscopy (Boas et al., 2016)

5.1. Multispectral imaging (MSI)

5.1.1. Background

Multispectral imaging (MSI) is a form of spectral imaging that is usually performed through wavelength dispersive devices or narrow band filters to separate lights of different spectral bands while an array detector is used to capture them (Bian et al., 2016). So, the main components of a multispectral imaging system are: a light source, a dispersive device, and a detector. Briefly, the light is emitted from the light source, it is separated in spectral bands and then is targeted to the sample. One part of the incident light is transmitted through the sample and another one is reflected, so the detection capability of the method relies on these two attributes (light transmission and light reflection) and computer vision technologies (e.g. image registration) (Ortega et al., 2020).

5.1.2. Applications in microbiology

Manthou et al. (2020) utilized MSI to develop a rapid method for estimation of TVC and sensory attributes in ready-to-eat pineapple samples. The methodology involved MSI analysis of samples that were preserved at different temperature conditions until spoilage, while at the same time, conventional methods of microbiology for enumeration

of TVC and sensory analysis were applied. After MSI analysis, spectral data were associated with TVC with 3 different ways: linear regression, Unscrambler (a commercial software product for multivariate data analysis) and SorfML (an automated ranking platform for machine learning regression models) to develop quantitative models but Unscrambler and SorfML were also used for qualitative models based on sensory analysis. Although coefficient of determination (R^2) was low in quantitative models, RMSE values were under 1 log CFU/g which were defined as acceptable, regarding the total time of the direct analysis. For the sensory features (qualitative models), the results showed a potential although further investigation with more balanced data was needed for more reliable conclusions (Manthou et al., 2020).

At the same concept, Spyrelli et al. (2020) developed a method for rapid quantification of *Pseudomonas* spp. and TVC in different chicken products, through MSI to estimate the "time from slaughter". The procedure was as described above and for the development of the quantitative model, Partial Least Squares Regression (PLS-R) was used. The results showed that the developed models could effectively predict *Pseudomonas* spp. and TVC in any chicken product (and therefore the "time from slaughter") and is indicatively mentioned that the developed model for the chicken thigh achieved a RMSE value of 0.160 and a correlation coefficient I of 0.859 (Spyrelli et al., 2020).

Regarding the detection of foodborne pathogens with Multispectral imaging, so far there are no relevant research works available.

5.2. Hyperspectral imaging (HSI)

5.2.1. Background

As mentioned above, Hyperspectral imaging (HSI) shares the same principles with MSI and the difference lies in the number of bands involved. MSI is characterized by a few bands, while HSI provides higher spectral resolution and lower spatial resolution as a result of the hundred(s) of bands (continuous approach) measured (Feng et al., 2020). The higher number of bands can provide more in-depth details and accurate fingerprints of

samples, however the extra bands may reduce intensity and signal-to-noise ratio. The above characteristics of HSI, as it will be discussed in the next section, may provide the capability of foodborne pathogens' detection, although the increased complexity of data processing may limit the applications in food industry (Amigo, 2020; Ropodi et al., 2016).

5.2.2. Applications in microbiology

Michael et al. (2019) developed a method for rapid differentiation of previously isolated *Cronobacter sakazakii*, *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* through HSI. The method involved the selective isolation of different strains of the aforementioned bacteria and the immobilization of them in glass slides which were then analyzed with HSI for the development of a database. The wavelength range of 425.57 to 753.84 nm was selected and then PCA and kNN (k□nearest neighbor) classifier modeling were applied. The developed model was cross-validated and the results were determined as acceptable for some strains, and not acceptable for others. The authors proposed the use of the method at presumptive levels for foodborne pathogens' detection but it was mentioned that it cannot yet replace conventional microbiology methods (Michael et al., 2019).

Bonah et al. (2020) compared variable selection algorithms for quantitative monitoring of *Escherichia coli* O157:H7 and *Staphylococcus aureus* in pork samples through visible near-infrared (Vis-NIR) hyperspectral imaging. The methodology involved the inoculation of pork samples with the pathogens at predetermined, different levels of colonies before the acquisition of the spectra with Vis-NIR HIS. The spectral data were preliminary processed with "noise-reducing algorithms", including Savitzky–Golay smoothing, 2nd derivatives, and Standard Normal Variate (SNV). Afterwards, for the determination of representative variables they compared six different wavelength selection algorithms and their combinations for predictions' optimization. The algorithms' predictions were evaluated through various metrics including root mean square errors of: i) calibration, ii) cross validation and iii) prediction on the prediction set. The results determined that the combination of Variable Combination Population Analysis (VCPA),

informative variables (IRIV) and Genetic Algorithm (GA), may be a suitable set of algorithms for quantitative monitoring of foodborne pathogens in food samples through HSI (Bonah et al., 2020).

6. Biosensors

6.1. Background

Some of the above methods, especially instrumental methods, are often combined with/or categorized as sensors/biosensors. In this study, biosensors are considered as methods, which convert easily measurable properties such as optical, electrochemical, magnetic, piezoelectric, electrochemical etc. to microbiological predictions. In that manner, the typical components of a biosensor are: a detector (interacts with possible antibodies, nucleic acids, metabolites, proteins etc. and provides a signal), a transducer (converts this signal to an electronic output i.e. the easily measurable properties that were mentioned above) and a display layout (provides the microbiological prediction) (Sharma and Mutharasan, 2013). There are several types of biosensors and also there is not just one categorization method. Briefly, the types with the most applications are electrochemical, optical and mass-sensitive biosensors.

Electrochemical biosensors (amperometric, potentiometric, conductometric, impedimetric) measure changes at electrons or ions, as a result of different reactions between the bio-recognition element (core component) and the captured molecules of the analyte (Mehrotra, 2016; Zhang et al., 2019).

Optical biosensors are devices that use optical transduction and therefore their assumptions are based on changes of light emission/absorption and usually changes of the refractive index as a result of the core component-analyte binding (Zanchetta et al., 2017). Apart from the refraction, other “light-based” properties can also act as indicators, thus creating sub-categories such as reflection, resonance, Raman scattering, fluorescence, chemiluminescence biosensors etc. (Mehrotra, 2016).

Mass-sensitive biosensors, also known as piezoelectric biosensors, “sense” minute changes in mass by featuring materials of fixed mass that accumulate piezoelectricity and vibrate in a specific frequency at a predetermined alternating current. When the mass

changes as a result of the core component-analyte binding, so does the vibration of the material and consequently the output signal (Mehrotra, 2016). The most common biosensors of this category are surface acoustic wave biosensors, resonant-mode piezoelectric sensors and quartz crystal microbalance sensors (Masdor et al., 2016; Sharma and Mutharasan, 2013).

6.2. Applications in microbiology

Yamada et al. (2016) designed a single walled carbon nanotube- (SWCNT) based multi-junction sensor and tested it against *S. aureus* and *E. coli*. The assumption was based on the linear regression between microorganisms and sensor results, providing a detection range of 10^2-10^5 CFU/mL (Yamada et al., 2016). Another new biosensor was made by Huang et al. (2018) and combined: i) double-layer capillary based high gradient immunomagnetic separation, ii) invertase-nanocluster based signal amplification and iii) glucose meter based signal detection to create a new biosensor able to detect *E. coli* O157:H7, an assumption that was based on the linearity between the signal and a pathogen, with a LOD of 79 CFU/mL (Huang et al., 2018). Lately, Wang et al. (2019) combined immunomagnetic separation, fluorescence labeling and smartphone video processing to create a microfluidic biosensor for *Salmonella* Typhimurium detection with a LOD (under given conditions) of 58 CFU/mL (Wang et al., 2019).

7. Metabolomics

7.1. Background

Metabolomics can be defined as an holistic approach which includes the comprehensive measurement of all metabolites and low molecular weight molecules (usually < 1500 Da) in a biological system, simultaneously (Clish, 2015). Metabolites are the most downstream end products of genes and proteins expression and therefore can provide closely related information with the phenotype (Manchester and Anand, 2017). This is also a significant reason why metabolomics could find various applications in the complex food matrices either in terms of physicochemical analyses or in food

microbiology. If this information is combined with multivariate data analysis can provide also insights about the metabolic mechanisms of a biological sample (Manchester and Anand, 2017). There are two types of metabolomics: untargeted metabolomics and targeted metabolomics. Untargeted metabolomics consist of the measurement of all metabolites in a sample without predetermined knowledge about its metabolome. This approach results in complex data which are highly affected by the analytical method and need computational tools for the metabolites identification. In contrast, targeted metabolomics consist of the measurement of metabolites in a sample within a specific range that has been defined in previous studies and is strongly associated with the specific sample. In this case, the analytical methods are already optimized for the metabolites of interest which gives the analysis higher sensitivity and selectivity although it may compromises a bit the entirety of the approach (Johnson et al., 2016).

When applied in microbiology, the typical approach to metabolomics, briefly, includes the selective isolation of the microorganism and then a metabolism quenching method (e.g. 60% aqueous methanol at -48 ^{o}C) followed by the extraction of the intracellular metabolites of the microorganism (e.g. with 100% methanol at -48^{o}C). As in every metabolomics application, for the identification of metabolites, the data should be first processed with bioinformatics and statistical tools specifically designed for metabolomics data mining (Braga and Adamec, 2018) (**Figure 2.**). In food microbiology, as mentioned in Section **1. Introduction** the time of analysis is a crucial factor that will determine the decisions of food business operators. Consequently, the first step of a metabolomics workflow (**Figure 3.**) is usually modified and instead of selective isolation the analysis is conducted directly on food samples with minimal preparation (e.g. centrifugation before metabolites extraction etc.) while the others steps remain the same. Whether the purpose is food quality or food safety monitoring, the approach is often the same and is summarized in the metabolome comparison between the spoiled or contaminated sample with the fresh or non-contaminated sample, respectively (Li et al., 2020). However, it should be noted that for foodborne pathogens' detection the selective isolation step usually cannot be bypassed because the microorganisms involved may make

a very small percentage of food microflora and therefore their metabolites may be in trace concentrations (Oyedeji et al., 2021).

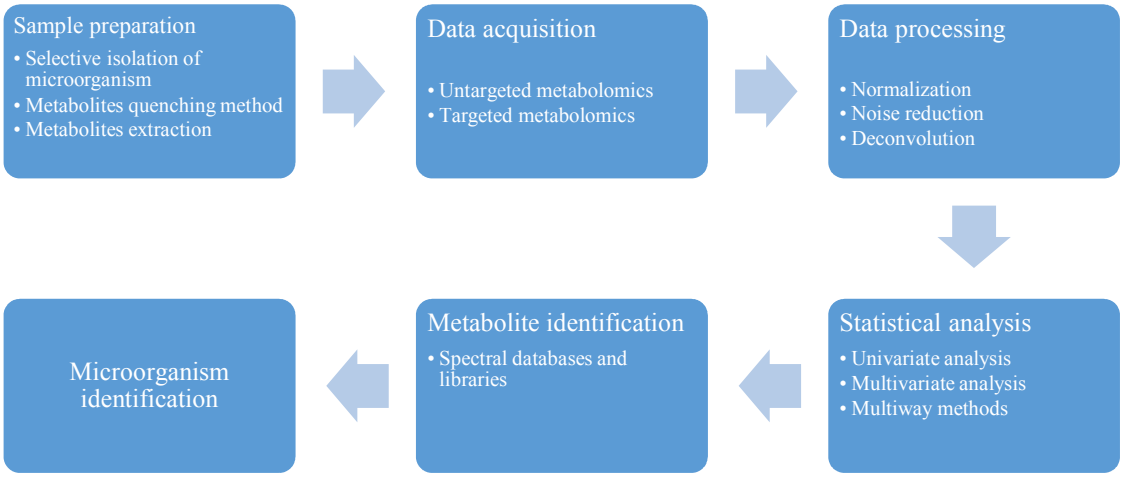

Figure 3. A typical metabolomics workflow for applications in microbiology (Karaman, 2017)

7.2. Applications in microbiology

Cevallos-Cevallos et al. (2011) was one of the first studies that tried to use metabolomics for foodborne pathogens detection using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS), however its approach was not so close to industrial applications. The approach involved the enrichment of *E. coli* O157:H7, *Salmonella* Hartford, *Salmonella* Typhimurium, and *Salmonella* Muenchen in a non-selective broth (Tryptic Soy Broth) at 37 ^{o}C for 24 hours with a metabolome analysis occurring every 2 hours. These metabolome profiles were compared with other microorganisms like *E. coli* K12, *Pseudomonas aeruginosa* and their mixtures. Principal Components Analysis (PCA) was performed for the discrimination of the microorganisms. The developed model was claimed to detect *E. coli* O157:H7 and *Salmonella* spp. in ground beef and chicken at

levels of approximately 7 ± 2 cfu/25 g (these levels were before 18h of enrichment) (Cevallos-Cevallos et al., 2011).

Jadhav et al. (2018) used GC-MS to develop a metabolomics approach and to establish putative biomarkers that are associated with spiked meat samples with *Listeria monocytogenes*, *Salmonella enterica* and *E. coli* O157:H7. The procedure included the selective enrichment of samples for 24h, 18h and 12h, respectively. In this study, the results were analyzed also with PCA but also followed by Partially Least Squares Discriminant Analysis (PLS-DA) which is closely related to PCA but in a supervised manner. In addition, for the quicker identification of the statistically significant metabolites in the discrimination process, volcano plots were also constructed to show better insights into the comparative magnitude of metabolites (Jadhav et al., 2018a).

8. Other instrumental techniques

In this section, instrumental techniques will be considered the analytical chemistry techniques that find applications to foodborne pathogens detection apart from the spectroscopic techniques for which special mention was made above (Chapter **4. Spectroscopic techniques**). The most widespread instrumental techniques with microbiology-related applications are: flow cytometry, electronic nose, gas chromatography, liquid chromatography, capillary electrophoresis, mass spectrometry, nuclear magnetic resonance, impedance and immunological-based techniques (Hameed et al., 2018; Oms-Oliu et al., 2013; Saravanan et al., 2020).

8.1. Flow cytometry

Flow cytometry, in short terms, is a technique used to detect light-scattering and fluorescence of cells or molecules by forcing them to flow, as separate as possible, through one or more laser beams in order to obtain information about their sorting, characteristics, functionality etc. (Duan et al., 2013). In food industries this technique seems useful in detecting microbial contamination (concentration and physiological state) (Hameed et al., 2018). Usually the light-scattering attributes, as mentioned above, are combined with fluorescence by attaching dyes to antibodies, as so when the antibody

binds to the antigen, fluoresces as a result, providing assumptions about the microbial population (Buzatu et al., 2014). However, this setup has some limitations because antigen-antibody binding can lead to lower sensitivity if antigens used for bacterial recognition are not expressed on a single cell manner. Consequently, this creates the need for more than one antibodies for accurate assumptions leading to higher cost and complexity (Duan et al., 2013). Current developments regarding flow cytometry for foodborne pathogens' detection use aptamers in lieu of antibodies combined with quantum dots as probes. Aptamers are oligonucleotide or peptide molecules that bind to specific targets and are preferred because they are cost efficient, stable (between different batches and between temperature changes), high-penetrating to tissues and can be engineered completely in a test tube. Quantum dots are used instead of organic fluorophores or fluorescent proteins because they provide broader excitation spectra and narrower emission spectra, therefore giving flow cytometry the ability of detecting 2 or more pathogens simultaneously, in the same sample (Duan et al., 2013).

8.3. Chromatographic and "–omics techniques"

Gas chromatography (GC), liquid chromatography (LC), electrophoresis (mainly capillary electrophoresis (CE) and two-dimensional gel electrophoresis (2-DE)), mass spectrometry (MS), Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and some of their combinations are all methods that lately found applications in food microbiology in the context of metabolomics and proteomics. In brief, as it was discussed in Section **7. Metabolomics**, metabolomics is a holistic approach for studying small molecules (< 1500 Da) within a living system at one time in order to acquire insights into multiple aspects of living organisms (Liu and Locasale, 2017; Markley et al., 2017). Proteomics shares the same perspective but instead of metabolites, studies the proteome (proteins and peptides) of a biological system at one time (Ortea et al., 2016). Usually GC and LC (in its Ultra Performance (UPLC) or High performance (HPLC) form), CE and 2-DE are used for the separation of metabolites and proteins whereas MS and NMR are used for the detection of them. UPLC and HPLC are widespread in modern laboratories and offer a strong potential on separating almost all metabolites, however GC and CE are more commonly used on separating metabolites due

to their high resolution. When all devices available, usually NMR is used for highly polar metabolites, whereas MS is used for non-polar or semi-polar metabolites (Oms-Oliu et al., 2013). It should be noted that lately FTIR is also gaining ground on –omics approaches due to its low cost (instrumentation and analysis), its simple analysis procedure and its time effectiveness even though is less robust and specific when compared to the aforementioned methods (Oyedeji et al., 2021). Currently, GC-MS seems the most common system when it comes to foodborne pathogens' detection because is less complex than LC-MS and allows the analysis of volatile organic compounds (VOC), the importance of which as biomarkers in bacterial growth is highlighted. There are several studies that, in addition to the aforementioned methods, refer to the value of volatilomics, as studied by simpler systems like an electronic nose. These studies mainly aim in predicting directly from food samples the spoilage microflora and several other properties (Lytou et al., 2019; Pavlidis et al., 2019) and fewer but still existing aim in determining the presence of common pathogens (Hu et al., 2020; Núñez-Carmona et al., 2019a). The typical approach to "translating" the –omics to microbiology, referring all of the above methods, is comparing the metabolome, proteome or a volatilome of a contaminated and a non-contaminated food with statistical tools (e.g. prediction models with PLS regressions, PCA analyses and SVMs) followed by proper validation (Pinu, 2016).

As for proteomics, the most common, automated device for foodborne pathogens' detection using this approach is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) which detects high abundant proteins (spectral fingerprint) in order to determine indirectly the presence or the absence of a specific pathogen such as *Listeria, Salmonella* and *E. coli* O157:H7. The assumption is made by comparing the fingerprint to an already well-determined spectral database with whatever limitation this entails (e.g. new pathogen species). In addition, with the existing knowledge, if population of the suspect pathogen is low there is a need for pre-enrichment (in one or two stages) and often the selection of a single colony in order to have accurate results, therefore degrading the time-effectiveness of the approach (Jadhav et al., 2018b; Pavlovic et al., 2013).

8.4. Impedance microbiology

Impedance microbiology is the method of monitoring bacterial growth through electric measurable changes in the growth medium that occur because of metabolic processes. More specifically, while bacteria grow, the resistance to flow of an alternating current that passes through a conducting growth media (impedance) decreases and therefore the conductance of the media increases (Bancalari et al., 2016). This happens because during metabolic processes high molecular weight nutrients break down into smaller, charged ionic components. The method includes either directly measuring the impedance of the growth media with a pair of electrodes at a predetermined frequency or indirectly by measuring carbon dioxide as a metabolic product (Bancalari et al., 2016; Furst and Francis, 2019; Gracias and McKillip, 2004).

8.5. Immunological-based techniques

Immunological-based techniques determine their capability to detect pathogens by antibody-antigen interactions. There are a lot approaches that share the same perspective from a simple latex agglutination assay which also serves as a confirmation test at some ISO standard methods for pathogens detection (e.g. *Salmonella*) to more complicated methods like Immunomagnetic Separation Assay (IMS) and Enzyme-Linked Immunosorbent Assay (ELISA) (Zhao et al., 2014).

Latex agglutination assay is the simplest immunology-based assay and employs latex beads with embedded antibodies which may react with surface antigens of a specific pathogen (suspected colony needs to be isolated). If the pathogen is present, the latex beads will clump (agglutinate) together (Lee et al., 2015).

Another simple method is Lateral Flow Immunoassay which consists of simple test kits (e.g. dipsticks and immunochromatographic strips) that provide a qualitative result based on antigen-antibody binding, fast (2-10 mins) and on-site. The positive result of the test is usually visualized by 2 lines one in test zone and one in control zone, however there is a need for pre-enrichment otherwise the detection levels might be rather high ($> 10^3$ CFU/g) (Law et al., 2014; Zhao et al., 2014).

Immunomagnetic Separation Assay (IMS) is a method in which antibodies are immobilized at magnetic beads and therefore they can capture via magnetism specific antigens from various food matrices (Park et al., 2020).

ELISA is a more complicated, yet accurate and sensitive method which require specialized equipment and well-trained personnel. The most widely used is ELISA format is the "sandwich" assay, because the antigen (if present) binds between 2 antibodies (capture and detection). The primary (capture) antibody binds with the antigen and after that the second (detection) antibody which carry an enzyme is added. Then the unbound molecules are removed and a colorless substrate is added so it will convert into a colored one as an indicator of antigen-antibody complex (Law et al., 2014; Zhao et al., 2014). The indirect quantification of the antigen is also possible by measuring the change in color with a spectrometer although it should be mentioned that also in this case the detection limits (without pre-enrichment) are rather high ($> 10^3$ CFU/g) (Chunglok et al., 2011; Gracias and McKillip, 2004). ELISA has also been used for the development of vaccines (e.g. for *Salmonella*) but the purpose there, was about detecting the antibodies instead of antigens (Lee et al., 2015; Meenakshi et al., 1999).

The current immunological-based applications include automated systems of ELISA and the closely related "Enzyme Linked Fluorescent Assay" (ELFA) (e.g. Vitek Immuno Diagnostic Assay System (VIDAS®) (bioMérieux, Marcy l'Etoile, France)) which provides high sensitivity and specificity but needs a pre-enrichment step. (Gracias and McKillip, 2004; Law et al., 2014; Lee et al., 2015; Park et al., 2020; Saravanan et al., 2020; Zhao et al., 2014).

9. Discussion

Conventional methods, while still implemented in a wide range of laboratories, lack in terms of time effectiveness by giving retrospective results and consist a lab-intensive approach on foodborne pathogens' detection and quantification (Jadhav et al., 2018b; Li and Zhu, 2017). This review was differentiated from others in order to include almost all current technologies that can be utilized for microbiological purposes. A

comprehensive summary of each technique's advantages, limitations and future challenges can be found in **Table 5.**

Nucleic-acid based techniques are well known and widely implemented in laboratories of food microbiology the recent years, with PCR and its variants being the main representatives. PCR's high sensitivity and specificity is taken for granted nowadays however there are still some issues associated with PCR inhibitors, primers' dimers, primers' mismatches etc. (**Table 5.**). The most significant problems concerning standard PCR, is the time effectiveness due to the need of pure colonies, while the real-time PCR is rapid but also more expensive for routine analysis in food industry. Also real-time PCR involves complex processing steps and requires predetermined sequence data of the specific target gene (Jayan et al., 2020). It should be noted that in terms of serotyping of *Salmonella* spp., multiplex PCR is far more cost and time efficient than the traditional, phenotypic method based on the WKL scheme, yet not so widely implemented (Jean-Gilles Beaubrun et al., 2012). The most recent and important addition in nucleic-acid based techniques is Next Generation Sequencing (NGS). NGS opened new dimensions in food microbiology with its ability to explore the bacterial communities (and its proportions) in a food sample, in a single analysis but still with unaffordable cost for routine analysis in food industry. NGS also features the Whole Genome Sequencing (WGS) approach which can be invaluable in foodborne outbreaks' surveillance. As for foodborne pathogens' detection the LOD of the existing applications remained rather high ($\approx 10^4$), without pre-enrichment (**3.6. Next Generation Sequencing (NGS) and Whole Genome Sequencing (WGS)**).

Spectroscopic techniques showed great potential on detecting and quantifying foodborne pathogens even though they did not offer the sensitivity and specificity of nucleic-acid based techniques. While not as precise in microbiological applications, they could provide an alternative, non-invasive, sensitive-enough approach with low or zero cost of analysis, rapid results and simple steps of analysis (**Table 5).** It should be mentioned that the spectroscopic techniques that were described in this thesis vary a lot in terms of sensitivity. THz, Fluorescence and FTIR, although simple and cost efficient appeared to lack behind in robust foodborne pathogens' applications, however the

existing applications were still mentioned (**4. Spectroscopic techniques**). From the above, FTIR already proved its efficiency on detecting and estimating spoilage microorganisms (Argyri et al., 2010; Dourou et al., 2021a; Fengou et al., 2019a; Spyrelli et al., 2021). Laser-Induced Breakdown Spectroscopy (LIBS) and Raman spectroscopy i.e. SERS and LTRS seemed to show more robust applications on foodborne pathogens especially when combined with other approaches (e.g. LIBS with elemental tags, SERS with aptamers and LIBS with 3D-SERS) (**4. Spectroscopic techniques**). Even so, both of these techniques when implemented in food samples, needed pre-enrichment in the most cases of low levels of contamination ($<10^3$ CFU/g) (**Table 5.**).

Spectral imaging techniques are also non-destructive with low or zero cost of analysis and even more rapid (measurement in a few seconds) than spectroscopic approaches. However, they seemed to be less sensitive than spectroscopic techniques and although showed great potential on detecting spoilage microorganisms in various food matrices and on-line (Fengou et al., 2019a; Manthou et al., 2020; Ropodi et al., 2016), their applications on foodborne pathogens detection remain limited (**5. Spectral imaging techniques**).

Biosensors evolved since previous reviews and now seem to provide great potential on foodborne pathogens' detection and to be really close to in-field applications, yet not validated there. They claimed strengths like easy-to-use, portability and low cost which could be invaluable in food industries. However, their main weakness had to do with the limit of detection (LOD) which showed to be rather high ($>10^3$) without pre-enrichment or when not in combination with other techniques (**Table 5.**).

Metabolomics differentiate from other techniques due to their holistic approach that is closely related to phenotype. This approach though, comes with complex instruments and many difficulties in data analysis. The applications on foodborne pathogens' detection are still at their infancy, but when utilized they could provide simultaneous results about different microorganisms with a pre-enrichment step (**Table 5.**).

Flow cutometry, chromatographic and –omics techniques, impedance microbiology, and immunological-based techniques were all categorized as other instrumental approaches that could be utilized in food microbiology. Flow cytometry addressed some limitations from the past by featuring aptamers in lieu of antibodies and quantum dots instead of fluorophores which seemed to provide better sensitivity and the possibility of detecting more than one pathogens, simultaneously within only 6.5 hours of pre-enrichment (in milk samples) (Duan et al., 2013; S. Liu et al., 2019) . However, the main problems associated with flow cytometry lie on the need of a cell sorter and the high cost (**Table 5.)**. Chromatographic and –omics techniques were described as other approaches in addition to metabolomics i.e. proteomics and volatilomics. Volatilomics seemed to be a simple approach (in terms of instrumentation and analysis procedure i.e. electronic nose) which was successfully implemented to predict the spoilage microflora (Lytou et al., 2019; Pavlidis et al., 2019) while showing also potential for foodborne pathogens' detection (Hu et al., 2020; Núñez-Carmona et al., 2019b). Proteomics applications where mainly represented from MALDI-ToF MS which could detect high abundant proteins (spectral fingerprint) in order to determine indirectly the presence or the absence of a specific pathogen after pre-enrichment (**8.3. Chromatographic and "–omics techniques"**). Impedance microbiology could return to the forefront with its combination with novel approaches like biosensors and nanochips to provide better sensitivity, portability and lower cost (**Table 5.).** Immunological-based techniques could be either in the forms of Latex Agglutination or Lateral Flow Immunoassays (like SARS-CoV-2 rapid tests) or in the form of automated immunoanalyzers (e.g. ELISA, ELFA etc.). In all of these cases the pre-enrichment consisted a necessary step when the levels of contamination were lower than 10^3-10^5 CFU/g with the former methods providing ease of use and low cost without instrumental needs and the latter methods providing better sensitivity and specificity (especially ELFA i.e. VIDAS®) (**8.5. Immunological-based techniques**).

Table 5. A comprehensive summary of each technique's advantages, limitations and future challenges

Techniques		Advantages	Limitations	Future challenges	References
Nucleic acid-based	Standard PCR	• High sensitivity and specificity • Moderate cost	• Pure colonies • Qualitative results • Possibility of PCR inhibitors	• Total time reduction • Alternative visualization methods	(Law et al., 2014; Priyanka et al., 2016)
	Multiplex PCR	• Serotyping capability • Simultaneous detection of multiple microorganisms	• Pure colonies or enrichment broths • Qualitative results • Higher probability of primer dimers or primer mismatches	• Primers design and cost	(Chen et al., 2012; Tao et al., 2020)

Techniques		Advantages	Limitations	Future challenges	References
	qPCR	• Direct identification and quantification • VBNC	• Cost • DNA isolates, not completely free of food matrix inhibitors • LOD ≥ 10^2 and LOQ ≥ 10^3-10^4 CFU/g, for direct analysis, strongly dependent on food matrix	• Cost reduction • DNA isolation methods	(Chapela et al., 2015; Cremonesi et al., 2014; Kim and Oh, 2021)
	NGS	• Holistic approach in food microflora • Direct identification • Real-time surveillance of foodborne pathogens through WGS with relatively low cost	• Cost (for routine analysis in food industry) • LOD ≥ 10^4 CFU/g (without pre-enrichment)	• Cost reduction • Detection of low-level contamination	(Desdouits et al., 2020; Ferrario et al., 2017; Lewis et al., 2020; Taylor et al., 2019)

Techniques		Advantages	Limitations	Future challenges	References
Spectroscopic	Fluorescence	• May provides quantitative estimations • Non-invasive • Rapid analysis in a few minutes • Low or zero cost of analysis	• Limited applications on foodborne pathogens • Needs pre-enrichment and possibly other steps also for foodborne pathogens	• Utilization of data analysis • Improvement of specificity and sensitivity	(Pu et al., 2013; Sohn et al., 2009; Yoshimura et al., 2014)
	THz	• Label-free • Non-invasive • Possible discrimination of dead or alive cells • Lower absorption of water molecules compared to far IR	• Limited applications on foodborne pahogens • Needs pre-enrichment and possibly other steps also for foodborne pathogens • Limited sensitivity • Overall less advantages than Raman spectroscopy	• Integration in biosensors will address some of the limitations and provide portability	(Globus et al., 2012; Yang et al., 2016b)

Techniques		Advantages	Limitations	Future challenges	References
	LIBS	• Higher sensitivity and specificity, than most spectroscopic techniques • Great potential on dead/alive cells discrimination • Can be combined with SERS and elemental tags for better results • Can provide quantitative assumptions and simultaneous detection of different pathogens	• Needs signal enhancement methods • Needs pre-enrichment • LOD ≈ 10^3-10^5	• Further research on combinations with other methods • Cost reduction • Improvements on sensitivity and specificity compared to PCR and qPCR	(Barnett et al., 2011; Liao et al., 2018; Multari et al., 2013; Sezer et al., 2017; Yang et al., 2020)

Techniques		Advantages	Limitations	Future challenges	References
	Raman	• SERS and LTRS have great potential on foodborne pathogens' detection • Can be combined with aptamers for more reliable results • High sensitivity and specificity compared to other spectroscopic techniques • Can provide quantitative assumptions	• Not all variants of Raman spectroscopy share high sensitivity and specificity • Cost of machinery • Needs pre-enrichment in most cases	• Combination with other technologies • Development of a universal database with "Raman fingerprints" • "Real-world " applications	(Díaz-Amaya et al., 2019; Lu et al., 2020; Stöckel et al., 2016; Zhao et al., 2018)

Techniques		Advantages	Limitations	Future challenges	References
	FTIR	• A simpler approach to metabolomics • Successful quantification of spoilage microorganisms • Easy to use • Low or zero cost of analysis	• Limited applications on foodborne pathogens which include pre-enrichment and other steps like lipopolysaccharides (LPS) extraction • Specific variants of FTIR e.g. SR-FTIR are more precise but also more complex • Complex data analysis • Initial cost of specific variants of FTIR	• Utilization of data analysis • Improvement of specificity and sensitivity • Further research on foodborne pathogens	(Fengou et al., 2019b; Kim et al., 2005; S. Kim et al., 2006; Oyedeji et al., 2021; Spyrelli et al., 2021)

Techniques		Advantages	Limitations	Future challenges	References
Spectral imaging	MSI	• Non-invasive, direct analysis of food samples • Only a few seconds analysis • Zero cost of analysis • Acceptable quantitative estimations of spoilage bacteria	• No available applications on foodborne pathogens' detection • Sensitivity and specificity • Initial cost of machinery	• Further research on foodborne pathogens • Utilization of data analysis • Improvement on sensitivity and specificity	(Fengou et al., 2019a; Manthou et al., 2020; Spyrelli et al., 2020)
	HSI	• More precise analysis than MSI • Non-invasive, on-line	• More complex data than MSI • Initial cost of machinery • Limited applications on detecting foodborne pathogens, especially directly from food samples	• Utilization and time reduction of data analysis • Improvement of sensitivity and specificity	(Bonah et al., 2019)

Techniques	Advantages	Limitations	Future challenges	References
Biosensors	• Theoretically, on-site monitoring • Can be combined with other approaches for more reliable results • Low-cost • Easy to use • Capability of miniaturization	• Usually need pre-enrichment or other preparation steps such as separation, filtration DNA isolation etc., to achieve a LOD < 10^3CFU/g, especially in solid samples	• Progress in materials and biorecognition systems (e.g. aptamers) • Further research for combined approaches of biosensors • In-field applications	(Ligaj et al., 2014; Sharma and Mutharasan, 2013; Shen et al., 2021; Wang et al., 2019)
Metabolomics	• Holistic approach • Closely related with phenotype • Low cost of analysis • Results within 24h even with pre-enrichment	• Limited applications on foodborne pathogens • Usually needs pre-enrichment • Complex devices and equipment that need highly-trained personnel • Complexity of the food matrix and the acquired data	• Cost reduction • Utilization of data analysis	(Li et al., 2020; Oyedeji et al., 2021; Xu, 2017)

Techniques		Advantages	Limitations	Future challenges	References
Other instrumental techniques	Flow cytometry	• Single cell sensitivity • Pre-enrichment of only 6.5 hours (in milk) • Simultaneous detection of 2 or more pathogens	• Needs pre-enrichment • Requires dilution method or flowcytometric cell sorter for single cells • Qualitative results • High cost	• Utilization of aptamers and quantum dots for higher sensitivity and specificity • Cost reduction	(Afari and Hung, 2018; Buzatu et al., 2014; Duan et al., 2013; S. Liu et al., 2019)
	Chromatographic and -omics	• Holistic approach • Limited applications on foodborne pathogens • Closely related with the phenotype	• Usually need pre-enrichment • Complex devices and equipment that need highly-trained personnel • Initial cost of the machinery	• Cost reduction • Utilization of data analysis	(Vieira et al., 2021)vi

Techniques		Advantages	Limitations	Future challenges	References
	Impedance	• Rapid ≈ 30 mins (without pre-enrichment) • Can be integrated in novel approaches like biosensors, microchips	• Low sensitivity and LOD when not combined with other approaches • Needs pre-enrichment • Limited applications	• Cost reduction • Further research for impedance biosensors	(Flint et al., 2015)
	Immunological-based	• Rapid ≤ 15 mins • Also available in single-use tests • Low cost • Devices with high sensitivity and specificity e.g. Vitek Immuno Diagnostic Assay System (VIDAS®)	• LOD ≥ 10^3 - 10^5 CFU/g (without pre-enrichment) • Surface antigens are not always expressed • Cross-reactivity issues • Qualitative results	• Improvement on sensitivity and specificity • Cost reduction	(Buzatu et al., 2014)

10. Conclusions

As was determined in previous reviews (Hameed et al., 2018; Law et al., 2014; Priyanka et al., 2016; Zhao et al., 2014) the need for rapid methods development, able to detect and quantify foodborne pathogens was increased and so were the proposed alternative methods. These approaches although, did not come without limitations (Hameed et al., 2018; Law et al., 2014; Priyanka et al., 2016; Zhao et al., 2014). This review differentiated from the past in terms of including methods that were previously omitted or overlooked because their potential and applicability were not yet established (e.g. NGS, LIBS etc.). In addition, the methods that were previously examined were again described along with recent applications in order to provide up-to-date insights.

Nucleic acid-based techniques although seemed robust in terms of sensitivity and specificity, a future challenge that should be mentioned is the further cost reduction in order to be widely implemented (especially referring to real-time PCR and NGS). Also a future challenge that would be useful in rapid detection (and quantification) of foodborne pathogens is the development of better DNA extraction protocols (for all PCR variants) and alternative visualization methods other than gel electrophoresis (standard and Multiplex PCR) (**Table 5.**).

Spectroscopic and spectral imaging techniques, as discussed above, share the same strengths as they are both non-destructive and have low or zero cost of analysis (**9. Discussion).** Spectroscopic techniques seemed to be more precise than spectral imaging although spectral imaging could give easier the possibility of on-line monitoring. For the spectroscopic techniques it would be interesting to carry out more applications with combination of the two techniques simultaneously (e.g. SERS with LIBS) or the combination of spectroscopy and biosensors (e.g. THz biosensors) since these combinations seemed to provide more precise results on foodborne pathogens' detection and quantitative estimation (**Table 4.** and **Table 5.**). For spectral imaging the improvement of sensitivity and specificity would be an important factor for future applications. For both spectroscopic and spectral imaging techniques a future challenge

would be the utilization of data analysis (e.g. with multivariate and machine learning approaches) (Manthou et al., 2020).

As a future perspective regarding biosensors, the development of materials and biorecognition systems (e.g. aptamers) should be mentioned. Moreover, the cost reduction and the validation of biosensors with in–field applications could attract food industries' interest (**Table 5.).**

Metabolomics, chromatographic and –omics techniques appeared to be still in their first steps referring foodborne pathogens detection. So, more applications would provide more accurate insights in the future about the capability of the approach. At the present time, the main challenge has to do with the cost of the instruments that can perform metabolomics approaches and with the complex data analysis of the results (**Table 5.).**

Flow cytometry, even though showed great potential on foodborne pathogens detection, with the use of aptamers and quantum dots, there still appears to be space on improving the properties and reducing the cost of the instrumentation (**Table 5.).** For impedance microbiology a future challenge would be the integration of the approach in biosensors and the reduction of cost. (**Table 5.).** Finally, immunological-based techniques, especially in the form of ELFA and ELISA, began to consist a common approach in laboratories of food microbiology yet the cost reduction of such instrumentation and the improvement of specificity and sensitivity would be a major challenge (**Table 5.).**

In summary, not every of the aforementioned methods seemed to be in the same distance from "real" applications in common laboratories or food industries. Some of them appeared to be closer to wide-scale implementation like NGS, biosensors and immunological based techniques (e.g. ELFA), others appeared to be still at their infancy like Spectral imaging techniques and Metabolomics, while spectroscopic techniques appeared to be in an intermediate state. A significant problem of some of these methods was the increased cost (instrumentation or analysis cost) for industrial applications, however the main problem of almost all of the methods had to do with the unavoidable

step of pre-enrichment or other preliminary steps. Pre-enrichment increased the total time of analysis to at least 1 day after receiving a sample and even more if other preliminary steps were necessary. This duration was longer than the theoretical (few minutes or hours) some methods claimed but also far shorter than the 5 or 7 days of the conventional (ISO) methods for certain foodborne pathogens (e.g. *Listeria* spp., *Salmonella* spp.). The capability of some methods to provide also quantitative estimations or to detect simultaneously more than one foodborne pathogens, should not be overlooked (**Table 5.).**

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