Optimization of Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) for the identification of microorganisms in food matrices.

A Dissertation presented to the University of Porto for the degree of Doctor in Chemical and Biological Engineering by

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Abstract

Foodborne illnesses remain until today as a major health problem. They result from the consumption of contaminated food with bacteria, viruses, fungi, parasites, toxins or chemicals, causing an array of more than 250 different known diseases. Overall, the World Health Organization estimates the occurrence of more than 600 million food-related illnesses in a single year and over 420,000 deaths. One in every 10 people fall ill as a result of consumption of contaminated food. The high impact of foodborne diseases has raised awareness in consumers, companies and national/international organizations for the production and distribution of safe food products. As such, over the years an improvement in the monitoring and cleaning methods has been observed for controlling agents responsible for causing illnesses. However, the monitoring of illness-causing agents in food products can be challenging. The detection of microbial pathogens can be achieved with several methodologies, including traditional culture plating techniques, immunological methods, methods based on nucleic acid amplification (PCR and variants) or hybridization (*e.g.* microarrays), biosensors, among others. Of those, peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) has, over the last few years, gained popularity as a reliable tool for pathogen detection, due to their superior performance characteristics when compared to standard FISH (using traditional DNA or RNA as probes) and other commonly employed methods.

Listeria monocytogenes is one of the most important foodborne pathogens. Despite the lower incidence levels in comparison to other agents, the illnesses caused by *L. monocytogenes* have one of the highest hospitalization and mortality rates among foodborne pathogens. In Chapter 2, a PNA-FISH method for the detection of *L. monocytogenes* is described and validated in a variety of food matrices, namely ground beef, ground pork, milk, lettuce and cooked shrimp. The here described method presents a detection performance similar to the traditional culture method commonly employed for the detection of the pathogen, ISO 11290 and a detection limit of 0.5 CFU/25 g or mL of food sample. Moreover, the results are obtained in two days, which represents a time reduction of more than 50% in comparison to the ISO 11290.

The origin of the PNA molecule dates to the yearly 90's and was rapidly employed for the detection of bacteria in FISH. Despite its wide application in FISH, there is still a lack of information regarding the influence of the several parameters and their interplay on the fluorescence outcome of the technique. As such, in Chapter 3, was performed a

systematic evaluation of the parameters pH, probe and dextran sulfate concentration on the hybridization solution using response surface methodology for protocol optimization in Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive species (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). The results showed that a probe concentration higher than 300 nM is favorable for both groups of bacteria. Regarding pH and dextran sulfate however, a clear distinction between the two groups was found. While Gram-negative had the best outcome under a high pH (approx. 10), combined with low dextran sulfate concentration (approx. 2% [wt/vol]), Gram-positive species, on the other hand hybridized better with a near-neutral pH (approx. 8) and higher dextran sulfate concentrations (approx. 10% [wt/vol]). This behavior seems to result from the ability of pH and dextran sulfate to influence probe diffusion towards the rRNA target.

Similarly to the hybridization step, the fixation/permeabilization step is also crucial for a positive PNA-FISH outcome. In order to assess its influence, an evaluation of different fixation/permeabilization strategies, that consisted on the use of an organic solvent (ethanol), detergent (triton X-100) and an enzyme-based protocol with lysozyme in conjugation with paraformaldehyde, were performed in the same set of Gram-negative and Gram-positive species of Chapter 3. In general, Gram-positive species required harsher fixation/permeabilization conditions in comparison to Gram-negative species, with the exception of *B. cereus* in triton X-100 and lysozyme. Ultimately, the fixation/permeabilization step recurring to paraformaldehyde and ethanol proved to have a significantly superior performance for all tested species, especially for Gram-positive species ($p<0.05$). (Chapter 4).

In summary, a complete understanding and optimization of a PNA-FISH procedure, as illustrated with the development of a detection method for *L. monocytogenes* (Chapter 2), is required in order to have a reliable and robust method. The here disclosed optimal parameters (Chapters 3 and 4) found differences between Gram-positive and Gram-negative bacteria that arise from their inherent differences in terms of cell envelope structures, namely their content in peptidoglycan. These protocol variances means that a unified optimal protocol for all microorganisms is unlikely to be obtained. Nonetheless, this optimization effort will greatly assist in the development of new detection methods based on PNA-FISH for other microorganisms. Furthermore, the extension of the optimization scope to the remaining steps and components of PNA-FISH procedure, is crucial to increase the knowledge necessary in order to have a complete

understanding of the technique. Also, the expansion to other bacteria, especially with different cell envelope structures, and also other nucleic acid mimics is also of great relevance.

Resumo

As doenças com origem alimentar constituem até aos dias de hoje um grave problema de saúde pública. Estas resultam do consumo de alimentos contaminados com bactérias, fungos, parasitas, toxinas ou químicos, capazes de causar um universo de mais de 250 doenças diferentes. De um modo geral, a Organização Mundial de Saúde estima uma incidência anual de mais de 600 milhões de casos e mais de 420 000 mortes causadas por doenças relacionadas com a alimentação. De facto, uma em cada 10 pessoas adoece em resultado do consumo de alimentos contaminados. O elevado impacto das doenças relacionadas com a alimentação, levou a uma consciencialização por parte dos consumidores, empresas e organizações nacionais/internacionais para a necessidade de produzir e distribuir produtos alimentares seguros. Nesse sentido, ao longo dos anos, tem-se verificado uma melhoria ao nível dos procedimentos de monitorização e higienização por forma a assegurar um controlo eficaz dos agentes causadores de doenças. No entanto, a monitorização destes agentes pode se tornar problemática. Na detecção de agentes patogénicos microbianos podem ser empregues diversas metodologias, nomeadamente os tradicionais métodos de cultura, métodos imunológicos, métodos baseados na amplificação (PCR e variantes) ou hibridação (por exemplo *microarrays*) de ácidos nucleicos, biossensores, entre outros. De entre estes, a utilização da metodologia de ácido péptido nucleico por hibridação fluorescente *in situ* (PNA-FISH na sigla em Inglês), tem nos últimos anos ganho popularidade como uma metodologia fiável para a detecção de agentes patogénicos. Isto advém das particularidades da metodologia, que possui características de desempenho superiores, quando comparados com o FISH tradicional (que usa sondas de DNA ou RNA) e outros métodos comummente utilizados.

Listeria monocytogenes é um dos agentes patogénicos alimentares com maior relevância. Embora apresente valores de incidência relativamente baixos, as doenças provocadas por infeções de *L. monocytogenes*, possuem umas das mais altas taxas de hospitalização e mortalidade, entre os agentes patogénicos alimentares. No Capítulo 2, é descrita e validada uma metodologia de PNA-FISH para a detecção de *L. monocytogenes* num grupo alargado de matrizes alimentares, nomeadamente em carne de vaca crua picada, carne de porco crua picada, leite, alface e camarão cozido. O método desenvolvido apresenta uma performance similar ao método de cultura utilizado na identificação de *L. monocytogenes*, ISO 11290 com um limite de detecção de 0,5 CFU/25 g ou mL de

amostra alimentar. Não obstante, a detecção de *L. monocytogenes* é realizada em dois dias, o que representa uma redução de 50% quando comparado com a ISO 11290.

A molécula de PNA teve a sua origem no início dos anos 90 e devido às suas características foi rapidamente utilizada na detecção de bactérias em FISH. Porém, apesar da sua vasta utilização, subsiste uma deficiência de conhecimento ao nível da influência que os vários parâmetros metodológicos e a sua interação têm na fluorescência observada com a aplicação da metodologia. Nesse sentido, no Capítulo 3, foi realizada uma avaliação sistemática dos parâmetros pH, concentração de sonda e de sulfato de dextrano presentes na solução de hibridação, recorrendo à metodologia de *response surface methodology*, para a optimização do protocolo em espécies Gram-negativas (*Escherichia coli* and *Pseudomonas fluorescens*) e Gram-positivas (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). Os resultados demonstraram que para ambos os grupos uma concentração de sonda de 300 nM é a condição que se traduz numa maior intensidade de fluorescência. No que diz respeito ao pH e à concentração de sulfato de dextrano, foi observada uma clara distinção entre os dois grupos. No caso das espécies Gram-negativas, a maior intensidade de fluorescência foi obtida combinando um valor de pH elevado (aproximadamente 10) com uma concentração baixa de sulfato de dextrano (aproximadamente 2% [m/vol]). Por outro lado, para espécies Gram-positivas, os valores óptimos de fluorescência foram obtidos combinando um pH próximo do valor neutral (aproximadamente 8) com uma alta concentração de sulfato de dextrano (aproximadamente 10% [m/vol]). Estes resultados resultam aparentemente da influência que o pH e o sulfato de dextrano têm na difusão da sonda de encontro ao rRNA alvo.

O passo de fixação/permeabilização, tal como o passo de hibridação, é essencial para a obtenção de um resultado positivo em PNA-FISH. De forma a aferir a sua influência, foram avaliadas diversas estratégias de fixação/permeabilização, sendo testados procedimentos combinando paraformaldeído com um solvente orgânico (etanol), um detergente (triton X-100) ou um procedimento enzimático com lisozima e executados no mesmo grupo de espécies Gram-positivas e Gram-negativas utilizadas no Capítulo 3. De uma maneira geral, as espécies Gram-positivas necessitaram de condições de fixação/permeabilização mais agressivas quando comparadas com as condições optimizadas para as espécies Gram-negativas, com excepção de *B. cereus* para os procedimentos com recurso ao triton X-100 e à lisozima. O passo de fixação/permeabilização, combinando paraformaldeído e etanol, demonstrou ter uma

performance superior em todas as espécies testadas, com particular ênfase nas espécies Gram-positivas (p<0,05). (Capítulo 4).

Em suma, um conhecimento alargado e uma correcta optimização do procedimento de PNA-FISH, como demonstrado com o desenvolvimento do método para a detecção de *L. monocytogenes* (Capítulo 2), é requisito essencial para se obter uma metodologia fiável e robusta. As optimizações dos diversos parâmetros avaliados neste trabalho (Capítulo 3 e 4), permitiram verificar que as diferenças encontradas entre os procedimentos das espécies Gram-positivas e Gram-negativas resultam das variações em termos da composição da parede celular, nomeadamente do conteúdo em peptidoglicano. Esta observação implica que o desenvolvimento de um procedimento universal óptimo para todos os microrganismos seja uma opção remota. Não obstante, este trabalho de optimização será uma mais valia futura, nomeadamente para o desenvolvimento de novas metodologias de detecção de microrganismos baseadas em PNA-FISH. No entanto, será crucial abordar os parâmetros que ainda não foram alvo de estudo, por forma a completar e compreender na globalidade o funcionamento da metodologia de PNA-FISH. Por fim, a extensão do âmbito das optimizações a outras bactérias, com especial ênfase a espécies com estruturas de parede celular diversas e a análogos de ácidos nucleicos será pertinente.

Aims and thesis structure

The work developed throughout the PhD scholarship, whose main results are presented in this thesis, had as the main aims the development and optimization of Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) as a tool for the detection and identification of pathogenic bacteria in food matrices.

This thesis is organized into five different chapters. In **Chapter 1**, a general introduction to the diverse themes addressed in the thesis are presented. It starts by presenting the topic of food safety and its importance worldwide. An overview of the more commonly used methods for the detection of foodborne pathogens is provided and the importance of fast detection methods is discussed. Finally, the practicability of using fluorescence *is situ* hybridization as a tool for foodborne pathogen detection is addressed and the major parameters that affect the technique are object of discussion.

Listeria monocytogenes is one of the most important foodborne pathogens worldwide, responsible for a high morbidity and mortality rates. Hence, **Chapter 2** described the work undertaken for the development of PNA-FISH method for the specific detection of *L. monocytogenes*. To that end, an assessment of previously described probes for the identification of *L. monocytogenes* was performed and the best probe selected. Then the hybridization conditions were optimized in order to achieve the required high degree of sensitivity and specificity, which included the design and application of a blocker probe. Finally, the enrichment procedure was also matter of optimization and the overall procedure was validated against a standard method in real scenarios of *L. monocytogenes* contamination.

The development of the PNA-FISH method for the detection of *L. monocytogenes*, highlighted the lack or little understanding of the variables and variable interplay that dictate the successes of failure of PNA-FISH procedures. Thus **Chapter 3** evaluated the influence of the hybridization solution pH, dextran sulfate and probe concentration in the fluorescent outcome of PNA-FISH. To that end, a previously developed approach combining Response Surface Methodology (RSM) and flow cytometry, which had successfully disclosed the influence of temperature, time and formamide content in PNA-FISH, was employed.

In **Chapter 4**, another step is taken towards the more complete understanding of the variables that affect PNA-FISH. This time, the effect and duration of different

fixation/permeabilization protocols on the fluorescence outcome of PNA-FISH, was evaluated. The previously optimized hybridization conditions defined in Chapter 3, were used as a starting point for the evaluation of the fixation/permeabilization conditions.

Finally, in **Chapter 5**, the main conclusions of the work described throughout this thesis are summarized. Future research perspectives on FISH optimization are also foreseen from a purely scientific point of view and also from a market-oriented perspective.

The present thesis reports the work performed at Biomode S. A., at the BEL group at LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy), Faculty of Engineering of the University of Porto and at the LIBRO laboratory (Laboratório de Investigação em Biofilmes Rosário Oliveira) at the Center of Biological Engineering, Department of Biological Engineering of the University of Minho.

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Abbreviations

°C	Celsius degrees
ε	Extinction coefficient (M.cm)
ΔG°	Gibbs free energy change (Kcal/mol)
λmax	Maximum absorption wavelength (nm)
λem	Maximum emission wavelength (nm)
µg	Microgram
µL	Microliter
%	Percent
Φ	Quantum yield
η	Viscosity (mPa.s)
A	Adenine
ACM-FISH	Alpha (centromere), Classical (1q12) and Midi (1p36.3) - Fluorescence *in situ* Hybridization
AEEA	8-amino-3,6-dioxa octanoic acid
AFLP	Amplified Restriction-Length Polymorphism
AHB	Abeyta Hunt Bark Agar
ALOA	Agar Listeria According to Ottaviani and Agostini
AMV-RT	Avian myeloblastosis virus reverse transcriptase
ANOVA	One-Way analysis of variance
ATCC	American Type Culture Collection
a.u.	Arbitrary fluorescence units
bp	Base pair
BHI	Brain Heart Infusion
BLEB	Buffered Listeria Enrichment Broth Base
BPW	Buffered Peptone Water
BS	Bismuth Sulfite
Bst	*Bacillus stearothermophilus*
C	Cytosine
CARD-FISH	Catalyzed Reporter Deposition - Fluorescence *in situ* Hybridization
catFISH	Cellular Compartment Analysis of Temporal Activity - Fluorescence *in situ* Hybridization
CB-FISH	Cytochalasin B - Fluorescence *in situ* Hybridization
CC	Cellobiose Colistin agar
CCD	Central Composite Design
CCI	Chromogenic Cronobacter Isolation Agar
cDNA	Complementary Deoxyribonucleic acid
CECT	Spanish Type Culture Collection
CFU	Colony-forming units
CI	Confidence Internal
CLASI-FISH	Combinatorial Labeling and Spectral Imaging - Fluorescence *in situ* Hybridization
CO_2	Carbon dioxide
CO-FISH	Chromosome Orientation - Fluorescence *in situ* Hybridization
COBRA-FISH	Combined Binary Ration - Fluorescence *in situ* Hybridization
COD-FISH	Chromosome Orientation and Direction - Fluorescence *in situ* Hybridization

	Concomitant Oncoprotein Detection - Fluorescence *in situ* Hybridization
	Combined CaCO3 Optical Detection - Fluorescence *in situ* Hybridization
COMBO-FISH	Combinatorial Oligonucleotide - Fluorescence *in situ* Hybridization
CSB	Cronobacter Screening Broth
CT-SMAC	MacConkey Agar with Sorbitol, Cefixime and Tellurite
Cyx	Cyanine; the x stands for the number of carbons in the polymethine chain
D-FISH	Double Fusion - Fluorescence *in situ* Hybridization
DALYS	Disability Adjusted Life Years
DBD-FISH	DNA Breakage Detection - Fluorescence *in situ* Hybridization
DEFT	Direct Epifluorescence Filter Technique
DFB	Demi-Fraser Broth
DFI	Brilliance Enterobacter sakazakii Agar
DGGE	Denaturant Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DPOD	Difference Probability of Detection
DOPE-FISH	Doubly Labeled Oligonucleotide Probe - Fluorescence *in situ* Hybridization
ds	Double strand
DS	Dextran sulfate
DVC-FISH	Direct Viable Count - Fluorescence *in situ* Hybridization
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
e.g.	(*exempli gratia*) for example
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular polymeric substance
Et	Ethanol
et al	(*et alli*) and others
etc.	Et cetera
FAM	fluorescein diacetate and carboxyfluorescein
FAO	Food and Agriculture Organization of the United Nations
FB	Fraser Broth
FDA-BAM	United States Food and Drug Administration - Bacteriological Analytical Manual
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein isothiocyanate
FS	Forward angle light scatter
g	Gram
g	Relative centrifugal force
G	Guanine
GC	Guanine-cytosine content
h	Hour
HACCP	Hazard Analysis and Critical Control Points
HE	Hektoen Enteric
HL	High Level

ISH	*In situ* Hybridization
ISO	International Organization for Standardization
K_2HPO_4	Potassium Phosphate Dibasic
KCl	Potassium chloride
kDa	KiloDalton
KH_2PO_4	Potassium Dihydrogenphosphate
Kg	Kilogram
LCR	Ligase Chain Reaction
LAA	Latex Agglutination Assay
LAMP	Loop-mediated isothermal Amplification
LFI	Lateral Flow Immunoassay
LL	Low Level
Lm	*L. monocytogenes* strains
LNA	Locked Nucleic Acid
LNA-FISH	Locked Nucleic Acid - Fluorescence *in situ* Hybridization
LPS	Lipopolysaccharide
LSU	rRNA large subunit
Lyz	Lysozyme
M	Molar (mol/L)
M-FISH	Multiplex - Fluorescence *in situ* Hybridization
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-Of-Flight
MC	MacConkey Agar
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
mCPC	Modified Cellobiose Polymyxin B Colistin
mg	Milligram
MgSO4.7H2O	Magnesium sulfate heptahydrate
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin Broth
mL	Milliliter
ML-FISH	Multi-locus - Fluorescence *in situ* Hybridization
min	Minutes
mM	Millimolar
$MnSO_4.4H_2O$	Manganese sulfate tetrahydrate
mPCR	Multiplex Polymerase Chain Reaction
MPN	Most Probable Number
MRS	de Man, Rogosa and Sharpe agar
mTSB	Modified Tryptone Soya Broth
MW	Molecular weight
N	Novobiocin
NA	Not available
NaCl	Sodium chloride
NAG	N-acetylglucosamine
$Na_2HPO_4.2H_2O$	Sodium hydrogen phosphate dihydrate
NAM	N-acetylmuramic acid
NaOH	Sodium hydroxide
NASBA	Nucleic Acid Sequence-Based Amplification
NE	Not evaluated
nLm	Non-*L. monocytogenes* strains
nm	Nanometer
nM	Nanomolar
NPCR	Nested Polymerase Chain Reaction

O_2	Oxygen
OBL	One Broth Listeria
OECD	Organization for Economic Co-operation and Development
p	Statistical significance level
P	Permeabilizant
PCC-FISH	Premature Chromosome Condensation - Fluorescence *in situ* Hybridization
PCR	Polymerase Chain Reaction
Pf	Paraformaldehyde
PFGE	Pulse Field Gel Electrophoresis
PFU	Plaque-forming unit
pH	Potential hydrogen
PNA	Peptide Nucleic Acid
PNA-FISH	Peptide Nucleic Acid - Fluorescence *in situ* Hybridization
POD	Probability of Detection
Q-FISH	Quantitative - Fluorescence *in situ* Hybridization
QD-FISH	Quantum Dots - Fluorescence *in situ* Hybridization
qPCR	Quantitative real-time Polymerase Chain Reaction
R^2	Coefficient of determination
RAPD	Randomly Amplified Polymorphic DNA
RCA-FISH	Rolling Circle Amplification - Fluorescence *in situ* Hybridization
Red-FISH	Replicative Detargeting - Fluorescence *in situ* Hybridization
R&F	Enterobacter sakazakii chromogenic plating agar
RFLP	Restriction Fragment Length Polymorphism
RING-FISH	Recognition of Individual Genes - Fluorescence *in situ* Hybridization
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSM	Response Surface Methodology
RSV	Rappaport-Vassiliadis Soya Peptone Broth
RT-LAMP	Reverse Transcription Loop-mediated isothermal Amplification
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
s	Seconds
SPC	Solid Phase Cytometry
spp.	Plural of specie
ss	Single strand
SS	Side angle light scatter
SSU	rRNA small subunit
T	Timine
T-FISH	Tissue - Fluorescence *in situ* Hybridization Telomere - Fluorescence *in situ* Hybridization
TAMRA	Carboxytetramethylrhodamine
TCBS	Thiosulfate Citrate Bile Salts Sucrose Agar
TLm	Total *L. monocytogenes* strains
T_m	Melting temperature (ºC)
TMR	Tetramethylrhodamine
TnLm	Total non-*L. monocytogenes* strains
TRIS-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
TRIS-Base	2-Amino-2-hydroxymethyl-propane-1,3-diol base

TRITC	Tetramethylrhodamine isothiocyanate
TSA	Tryptic Soy Agar
TSA-FISH	Tyramide Signal Amplification - Fluorescence *in situ* Hybridization
TSB	Tryptic Soy Broth
TT	Tetrathionate Broth
Tx	Triton X-100
U	Uracil
UPB	Universal Preenrichment Broth
USD	United States Dollar
USDA-FSIS	United States Department of Agriculture - Food Safety and Inspection Service
UVM	University of Vermont
vol	Volume (L)
WHO	World Health Organization
wt	Weight (g)
XLD	Xylose Lysine Deoxycholate Agar
YLDS	Years Lived with Disability
YLLS	Years of Life Lost
Zoo-FISH	Cross Species Chromosome Painting - Fluorescence *in situ* Hybridization

Scientific outputs

Papers in peer reviewed journals:

Rui Rocha, José M. Sousa, Laura Cerqueira, Maria J. Vieira, Carina Almeida and Nuno F. Azevedo. 2016. Development and application of Peptide Nucleic Acid Fluorescence *in situ* Hybridization for the specific detection of *Listeria monocytogenes*. (publication on-hold due to possible patent submission) (Chapter 2)

Rui Rocha, Rita S. Santos, Pedro Madureira, Carina Almeida and Nuno F. Azevedo. 2016. Optimization of peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) for the detection of bacteria: the effect of pH, dextran sulfate and probe concentration. Journal of Biotechnology, 226:1-7. DOI:10.1016/j.jbiotec.2016.03.047. (Chapter 3)

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Oral communications:

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Poster communications:

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Chapter 1

General Introduction

This chapter presents a general overview of the topics that will be discussed on this thesis. It starts by addressing the emergence, transmission and burden of foodborne pathogens in the World with a special focus at the European level. It then discusses the risk management strategies, and regulatory and legislative practices applied at the moment. Additionally, the most widely-used methods for the detection of foodborne pathogens are listed and their advantages and disadvantages in comparison to the other methods described. In the previous topic, the importance of the introduction of fast methods for the detection of foodborne pathogens to the population in general, government entities and the food industry is also a matter of discussion. The use of fluorescence *is situ* hybridization (FISH) as a methodology for foodborne pathogen detection is then explored in more detail, starting by providing an historical perspective of the technique, the various labelling strategies and types of fluorophores that are usually employed. Afterwards, the standard FISH procedure is presented, the several variables that affect its fluorescent outcome are addressed and their major limitations are referred. Finally, the peptide nucleic acid probes are introduced as an improvement for FISH procedures (PNA-FISH) and their application to pathogen detection explored.

1.1. Pressure on global food production

The World's population is increasing at a remarkable rate, reaching in 2017 7.5 billion and is expected to reach 10 billion in 2056[1]. This increase alongside with economic development and environmental change imposes an unprecedented pressure on the planet resources (Schneider *et al*., 2011). Today, the amount of land required to produce all renewable resources used by mankind, the so called human footprint, is 1.6 Earths[2]. A substantial part of it is assigned to fulfill the demand of the global food commodity's market in the form of croplands, grazing lands, forests and fishing grounds. As such, it is not surprising that the resources for food production in all world countries have reached substantial proportions (Schneider *et al*., 2011). In order to meet the increasing global demand, OECD-FAO estimates that food production expansion will be satisfied mainly through improvements in efficiency rather than by increasing farming areas (OECD-FAO, 2016). Generally, an increase in food items output is expected from 2015 to 2016 (Table 1.1). Cereal was the most harvested food product, with over 2 billion tons, followed by vegetables with over 1 billion tons.

Table 1.1 - Estimative and forecast production (in million tons), for each type of food product for the years 2015 and 2016, respectively. Also present is the forecast for the volume involved in trade (adapted from OECD/FAO, 2016 and www.fao.org/faostat/en/).

	Food	2015 Production (Estimative)	2016 Production (Forecast)	2015 – 2016 Variation (%)	Trade 2016 (Forecast)
Cereal	Maize	1006.0	1029.3	2.3	384.8
	Wheat	733.8	742.4	1.2	
	Rice	491.5	497.8	1.3	
	Barley	147.4	142.5	-3.4	
	Sorghum	63.1	64.5	2.2	
	Millet Rye Oats Other Grains	88.9	92.5	3.9	
	Cassava	281.1	288.4	2.6	28.2
Oilcrops	Soybeans	314.4	329.5	4.8	159.8
	Rapeseed	69.9	67.4	-3.5	
	Cottonseed	38.2	40.3	5.4	
	Groundnuts	37.6	40.5	7.7	
	Sunflower seed	42.2	45.9	8.7	
	Palm kernels	14.7	15.8	7.5	
	Copra	5.4	5.8	8.2	
	Other	11.7	11.7	0.0	

[1] www.un.org
[2] www.worldwildlife.org/

Table 1.1 (continuation)

	Fruit	689.9*	NA	NA	NA
	Vegetable	1169.4*	NA	NA	NA
Meat and Meat products	Bovine	67.6	67.8	0.3	31.1
	Poultry	114.8	115.8	0.9	
	Pig	117.2	116.5	-0.6	
	Ovine	14.0	14.1	0.6	
	Others	5.6	5.6	0.0	
Milk and Milk Products		808.7	817.2	1.1	72.3
Fish and Fish Products		171.0	174.1	1.8	60.0

[a] data for 2014;
NA - data not available.

1.2. Impact of food production, distribution and consumer demand in food safety - major transmission routes

The last few decades revealed a rapid increase in terms of volume of food items traded globally, well beyond the observed increase in food production. The global trade market of food products is valued today in about 1 trillion dollars (USD) with tendency to increase in the coming decades (OECD-FAO, 2016). This arises from regional asymmetries in food production and balance of supply and demand, resulting in imports and exports of food items according to regional needs. Another fact is an ever evolving consumption pattern, demanding for fresh, healthier and minimally processed food products (OECD-FAO, 2016; Yeni *et al*., 2014; Wang and Salazar, 2016). As result, food production and the corresponding distribution chain has become more complex, creating potential food safety problems, either by the quantity produced and/or transportation issues, or by the differences in food safety practices of each producing country (Mangal *et al*., 2016; Yeni *et al*., 2014). Aware of the importance of this problem, over the last decades, an effort in order to comprehend the mechanisms that govern the occurrence and emergence of pathogens in the food chain was made. However to this date, a reliable prediction for the occurrence of these events is still not possible (Hoorfar, 2011). Exposure can occur in a variety of forms, including water and air, contact with soil and fertilizers, in the food processing environment, from the raw material to the finished product, in the conservation and preparation stages, among others (Figure 1.1) (Wang and Salazar, 2016; WHO, 2015).

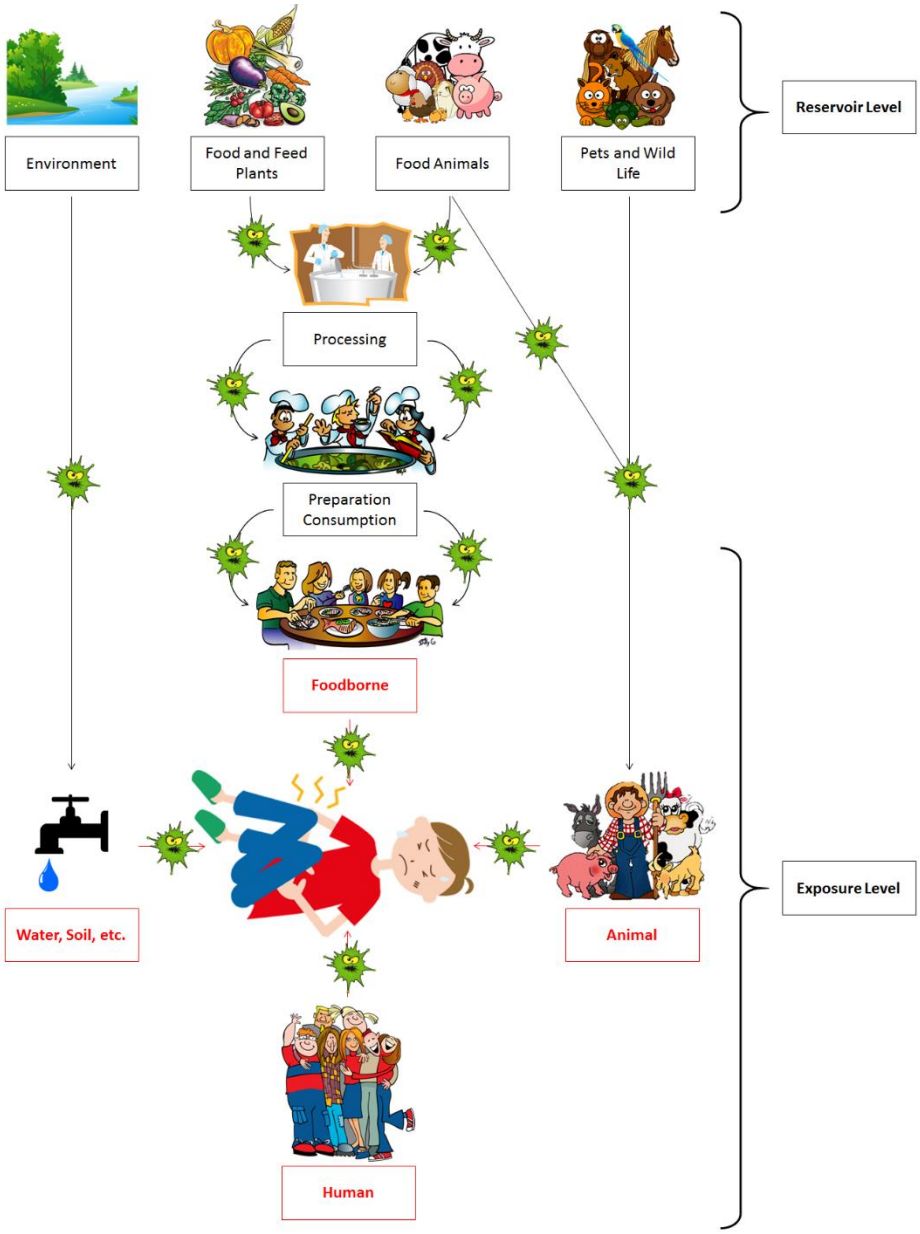

Figure 1.1 - Major transmission routes and pathways of human foodborne diseases from the reservoir level - origin of the disease agent - to human exposure (adapted from WHO, 2015).

1.3. Accessing Global and European burden of foodborne diseases

In recent years, a high number of foodborne outbreaks caused by contaminated food has been observed all over the world (Yeni *et al*., 2014). These outbreaks resulted in a high morbidity and mortality, and hence remain a major global public health problem. There are over 30 agents recognized as foodborne hazards, ranging from bacteria, viruses, fungi, parasites, toxins to chemicals, causing more than 250 different known foodborne diseases (Mangal *et al*., 2016; WHO, 2015). However, the full extent of the problem is hard to quantify. To this end, the World Health Organization (WHO) launched an

initiative in order to, for the first time, accurately estimate the global extent and burden of foodborne diseases for the year of 2010 (Table 1.2).

Table 1.2 - Estimative of the global number of foodborne illnesses, deaths and burden for the year of 2010 (from WHO, 2015).

Hazard	Cases	Deaths	YLDS[a]	YLLS[b]	DALYS[c]
Total	600 652 361	418 608	5 580 028	27 201 701	32 841 428
Diarrheal disease agents	548 595 679	230 111	839 463	16 821 418	17 659 226
Viruses					
Norovirus	124 803 946	34 929	91 357	2 403 107	2 496 078
Bacteria	349 405 380	187 285	685 212	13 795 606	14 490 808
Campylobacter spp.	95 613 970	21 374	442 075	1 689 291	2 141 926
Enterotoxigenic *E. coli*	86 502 735	26 170	70 567	2 011 635	2 084 229
Non-typhoidal *Salmonella* spp.	78 707 591	59 153	78 306	3 976 386	4 067 929
Shigella spp.	51 014 050	15 156	51 163	1 181 231	1 237 103
Enteropathogenic *E. coli*	23 797 284	37 077	22 977	2 908 551	2 938 407
Shiga toxin-producing *E. coli*	1 176 854	128	3 486	9 454	12 953
Vibrio cholerae	763 451	24 649	2 721	1 719 381	1 722 312
Protozoa	67 182 645	5 558	57 536	432 316	492 354
Giardia spp.	28 236 123	0	26 270	0	26 270
Entamoeba histolytica	28 023 571	1 470	20 851	115 740	138 863
Cryptosporidium spp.	8 584 805	3 759	8 155	287 690	296 156
Invasive infectious disease agents	35 770 163	117 223	1 098 675	6 960 656	8 065 581
Viruses					
Hepatitis A virus	13 709 836	27 731	85 885	1 258 812	1 353 767
Bacteria	10 352 042	85 269	225 792	5 472 374	5 697 913
Salmonella typhi	7 570 087	52 472	117 334	3 604 940	3 720 565
Salmonella paratyphi A	1 741 120	12 069	26 987	829 136	855 730
Brucella spp.	393 239	1 957	13 324	110 971	124 884
Mycobacterium bovis	121 268	10 545	50 733	556 998	607 775
Listeria monocytogenes	14 169	3 175	2 255	116 109	118 340
Protozoa					
Toxoplasma gondii	10 280 089	684	763 326	62 899	829 071
Helminths	12 928 944	45 226	3 367 987	2 428 929	5 810 589
Nematodes	12 285 286	1 012	518 451	80 021	605 738
Ascaris spp.	12 280 767	1 008	518 096	79 800	605 278
Trichinella spp.	4 472	4	342	210	550
Cestodes	430 864	36 500	1 220 578	1 932 154	3 158 826
Taenia solium	370 710	28 114	1 192 236	1 586 288	2 788 426
Echinococcus granulosus	43 076	482	12 121	27 626	39 950
Echinococcus multilocularis	8 375	7 771	8 749	303 039	312 461
Trematodes	218 569	7 533	1 616 785	403 884	2 024 592
Paragonimus spp.	139 238	250	1 033 097	15 535	1 048 937
Clonorchis sinensis	31 620	5 770	219 637	302 160	522 863
Intestinal flukes[d]	18 924	0	155 165	0	155 165
Opisthorchis spp.	16 315	1 498	102 705	85 364	188 346
Fasciola spp.	10 635	0	90 041	0	90 041
Chemicals and toxins	217 632	19 712	247 920	650 157	908 356
Dioxin	193 447	0	240 056	0	240 056
Aflatoxin	21 757	19 455	3 945	623 901	636 869
Cassava cyanide	1 066	227	2 521	15 694	18 203

[a] Years Lived with Disability;
[b] Years of Life Lost;
[c] Disability Adjusted Life Years;
[d] Includes selected species of the families *Echinostomatidae*, *Fasciolidae*, *Gymnophallidae*, *Heterophyidae*, *Nanophyetidae*, *Neodiplostomidae* and *Plagiorchiidae*.

Worldwide, the WHO estimated the occurrence of more than 600 million illnesses, resulting in almost 420 thousand deaths, more than 5 million years lived with disability and more than 27 million life years lost. This results in more than 32 million disability adjusted life years (WHO, 2015). Diarrheal disease agents were responsible for most of the aforementioned diseases and deaths, followed by invasive infectious disease agents, helminths and finally chemicals and toxins. From the diarrheal disease agents, Norovirus and *Campylobacter* spp. were responsible for the most illnesses, over 120 and 95 million, respectively; while *Salmonella* spp. (typhoid and non-typhoid) was responsible for most of the deaths - over 120 thousand. Although the total number of illnesses caused by invasive infectious disease agents was less than 6% of the total cases, it accounts for more or less 25% of total deaths, 20% of the total years lived with disability and 25% of the life years lost, as a result of the severity of the diseases caused by this group of agents.

This report also points out that there is a considerable difference between regions of low and high income regarding the burden of foodborne diseases. This fact is known and pointed out throughout various reports, suggesting that these differences arise from underlying food safety problems and because of that the current burden is avoidable (WHO, 2015; Zhao *et al*., 2014).

In the specific case of the Europe, a close monitoring regarding the occurrence of zoonosis is performed by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). With the collected information, every year a report of the trends, sources and agents of zoonosis in the European Union and 4 other non-member states is released. In 2015, it is possible to see that *Campylobacter* spp. is the main reported foodborne agent with almost 230 thousand cases, followed by *Salmonella* spp. with almost 95 thousand cases (Table 1.3). *Campylobacter* spp. and *Salmonella* spp. were also the principal cause of hospitalization, however *Listeria monocytogenes* was reported as having the highest number of fatalities, 270, followed by *Salmonella* spp. with 126 and *Campylobacter* spp. with 59.

Table 1.3 - Number of reported zoonosis, hospitalization and case fatality rates in the European Union and 4 non-member states in 2015. Only foodborne pathogens with over 1000 reported cases are listed (adapted from EFSA/ECDC, 2016).

Hazard	Cases	Hospitalization	Deaths
Campylobacter spp.	229 213	19 302	59
Salmonella spp.	94 625	12 353	126
Yersinia spp.	7 202	530	0
Shiga toxin-producing *E. coli*	5 901	853	8
Listeria monocytogenes	2 206	964	270

1.4. Legislation and practices to ensure food safety

As shown in the previous section, foodborne diseases have a tremendous impact worldwide. This fact, over the years, led to an increased awareness and focus of consumers, companies and national/international organizations for the need to distribute safe food products (Barlow *et al*., 2015; Hoorfar, 2011; Mangal *et al*., 2016; Yeni *et al*., 2014). Currently, food safety legislation relies in a risk assessment/quality assurance system, the Hazard Analysis and Critical Control Points (HACCP). The application of HACCP ensures food safety by preventing or minimizing food contamination along the food chain (Hoorfar, 2011; Mangal *et al*., 2016; Yeni *et al*., 2014). To that end, it employs a mix of both hazard and risk-based approaches. In hazard-based approaches, the detection of potentially harmful agents is used as a basis for action (*e.g.* acute and potent hazards, genotoxic substances, allergenic ingredients, etc.). On the other hand, risk-based approaches employ a risk assessment process that tries to calculate or estimate acceptable or tolerable guidance values of exposure to a given harmful agent (*e.g.* chemical contamination) (Barlow *et al*., 2015). In the specific case of microbial contamination, the screening was originally only performed to the finished products (Hoorfar, 2011; Yeni *et al*., 2014). However, this approach was faulty due to logistical sampling complexities and heterogeneous contaminant distribution (Hoorfar, 2011; Mangal *et al*., 2016; Yeni *et al*., 2014). In HACCP, microbiological testing remains a critical tool in monitoring process control, quality control, surveillance and providing inputs to risk assessment (Hoorfar, 2011). Raw materials, processes and/or food products are now inspected for the presence or absence of microorganisms per unit(s) of mass, volume, area or batch in a mix of hazard and risk based approaches. Hazard based approaches are effective, useful and efficient, however they are mainly applicable to zero-tolerance pathogens due to their stringency.

If not the case, a risk assessment approach is more appropriate, such is the case of *Campylobacter* spp. (Barlow *et al*., 2015).

1.5. Methods employed in foodborne pathogen detection

As discussed in the previous section, microbial monitoring remain a critical tool in food industries and as such is with no surprise that the food safety testing market have been gaining importance over the years (Mangal *et al*., 2016). In fact, in Europe alone this sector is expected to represent a business volume over 3 billion euros in 2017 and grow at a 7.35% rate between 2016 - 2021, according to a recent market report from Market Data Forecast[3].

Pathogen monitoring in foods can be performed using several different detection techniques, from traditional culture plating techniques, immunological methods, biosensors, methods based on nucleic acid amplification (PCR and variants) or hybridization (*e.g.* microarrays), among others (Hoorfar, 2011; Law *et al*., 2015; Mangal *et al*., 2016; Priyanka *et al*., 2016; Rohde *et al*., 2015; Wang and Salazar, 2016; Yeni *et al*., 2014; Zhao *et al*., 2014). However in order to be successful, these techniques must overcome several challenges namely, the inherent complexity of the food matrices to be tested, the low prevalence of pathogens and its heterogeneous distribution in the food matrix, the presence of normal microflora (raging from absent to over log 7 CFU/g), the presence of assay interference compounds and the occurrence of stress-injured bacteria, due to heat, cold, acid, osmotic shock, among other stress conditions, during food processing (Hoorfar, 2011; Wang and Salazar, 2016).

1.5.1. Culture plating techniques

Culture plating techniques have been used for many years in food testing programs and for that reason they are often mentioned as traditional techniques (Hoorfar, 2011). These methodologies are versatile, allowing the detection and enumeration of pathogens in food samples. Furthermore, they are still considered the gold standard and the method of choice for the detection of pathogens (Figure 1.2) (Mangal *et al*., 2016; Rohde *et al*., 2015; Yeni *et al*., 2014).

[3] http://www.marketdataforecast.com/market-reports/europe-food-safety-testing-market-759/

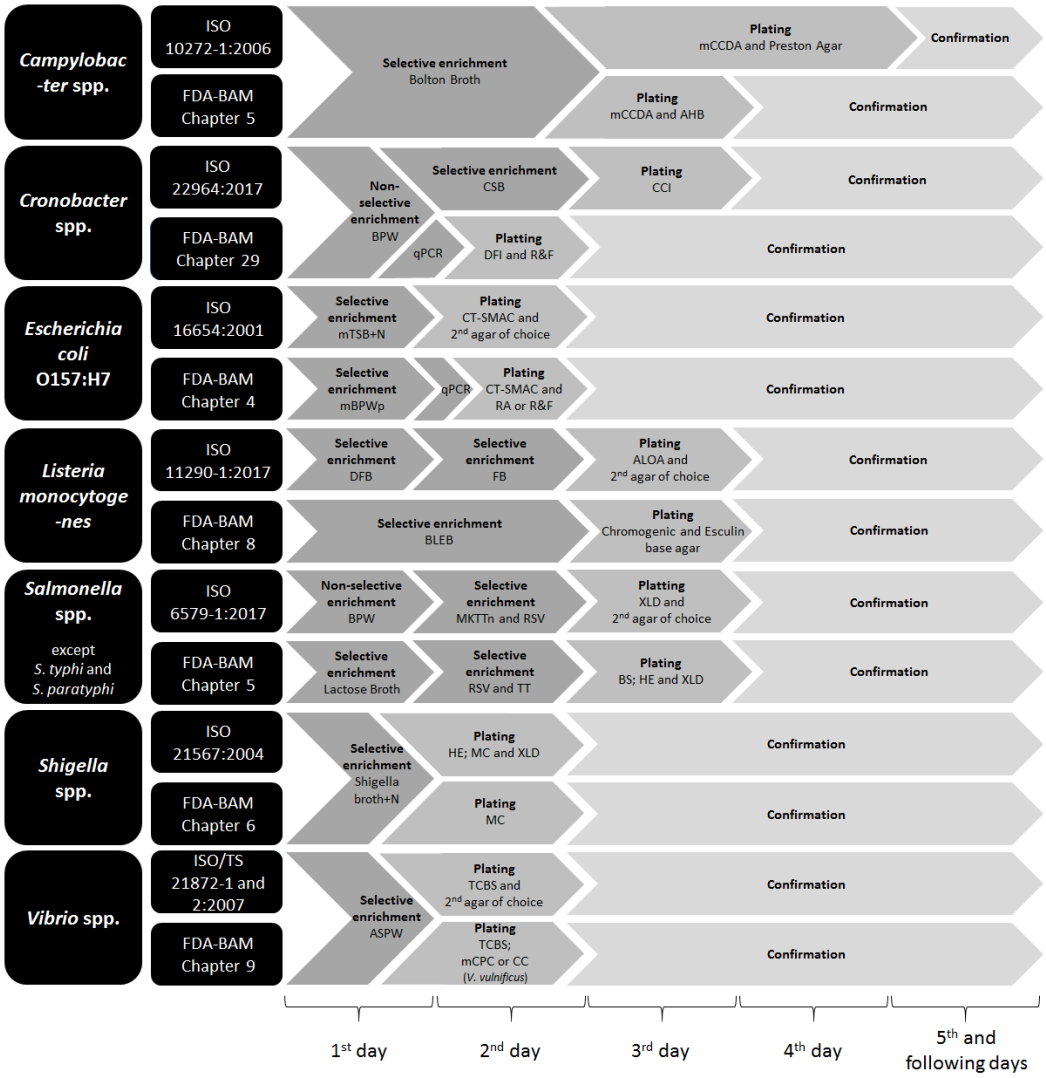

Figure 1.2 - Timeline of standardized culture plating techniques for the detection and identification of common foodborne bacteria in food samples. The image compares the methods from the International Organization for Standardization (ISO) and the US Food and Drug Administration - Bacteriological Analytical Manual (FDA-BAM).

Abbreviations: AHB - Abeyta Hunt Bark Agar; ALOA - Agar Listeria According to Ottaviani and Agostini; BLEB - Buffered Listeria Enrichment Broth Base; BPW - Buffered Peptone Water; BS - Bismuth Sulfite; CC - Cellobiose Colistin agar; CCI - Chromogenic Cronobacter Isolation Agar; CSB - Cronobacter Screening Broth; CT- SMAC - MacConkey Agar with Sorbitol, Cefixime and Tellurite; DFB - Demi-Fraser Broth; DFI - Brilliance Enterobacter sakazakii Agar; FB - Fraser Broth; HE - Hektoen Enteric; MC - MacConkey Agar; mCCDA - Modified Charcoal Cefoperazone Deoxycholate Agar; mCPC - Modified Cellobiose Polymyxin B Colistin; MKTTn - Muller-Kauffmann Tetrathionate-Novobiocin Broth; mTSB - Modified Tryptone Soya Broth; N - Novobiocin; R&F - Enterobacter sakazakii chromogenic plating agar; RSV - Rappaport-Vassiliadis Soya Peptone Broth; TCBS - Thiosulfate Citrate Bile Salts Sucrose Agar; TT - Tetrathionate Broth; XLD - Xylose Lysine Deoxycholate Agar.

A general procedure involves the use of liquid and solid culture media in several stages: (i) Pre-enrichment - which dilutes inhibitory compounds, rehydrate the bacterial cells and allows the recovery of injured bacteria; (ii) Selective enrichment - which suppress background microflora and increases the number of cells of the target pathogen;

(iii) selective plating - isolation of the target pathogen; (iv) confirmation - biochemical identification and serological confirmation of the results (Mangal *et al*., 2016; López-Campos *et al*., 2012; Välimaa *et al*., 2015; Wang and Salazar, 2016). These methodologies have been broadly accepted and standardized protocols are reliable, efficient, sensitive, cost-effective, of simple execution and applicable to a wide range of food matrices (Priyanka *et al*., 2016; Wang and Salazar, 2016; Yeni *et al*., 2014). However, they are tedious, laborious and time-consuming, taking several days to provide the results, in some cases up to 10 days (Mangal *et al*., 2016). These drawbacks make culture plating techniques inadequate for the modern food industry, considering the speed and amount of food products available in the modern agro food markets (Yeni *et al*., 2014; Zhao *et al*., 2014). As such, in order to prevent the spread of infectious diseases, ensure the food safety and protect public health, there is an ever-increasing demand for more rapid methods to be routinely used in screening practices of foodborne pathogens (Yeni *et al*., 2014; Zhao *et al*., 2014). These methods are especially required in an initial fast sample screening, where most of the test results are expected to be negative, leading to faster product release for sale (Hoorfar, 2011). Also, positive results obtained from these rapid techniques are only regarded as presumptive, requiring further confirmation using standard methods (Yeni *et al*., 2014).

1.5.2. Rapid Methods

1.5.2.1. Immunoassays

Immunoassays are currently widely used for the detection and identification of pathogens and microbial toxins in food samples (Zhao *et al*., 2014). These methodologies rely on antigen-antibody interactions, where a particular antibody bind to a specific antigen even in the presence of other molecules (Law *et al*., 2015; Mangal *et al*., 2016; Yeni *et al*., 2014; Zhao *et al*., 2014). As such, the antigen-antibody complex depends mainly on the antibody specificity (Mangal *et al*., 2016; Zhao *et al*., 2014). A variety of antibodies have been employed in immunological assays, from conventional, heavy chain, polyclonal, monoclonal to recombinant antibodies (Yeni *et al*., 2014). Polyclonal antibodies can be easily derived from rabbit or goat serum and because of that are faster and relatively inexpensive to produce in comparison to other alternatives. However, they pose challenges in their purification due to their different cellular origin and suffer from lack of specificity and sensibility due to their polyvalence, reacting with several antigens

(Priyanka *et al*., 2016; Yeni *et al*., 2014; Zhao *et al*., 2014). The emergence of monoclonal antibodies shifts the course of immunological assays, conferring them more specificity, sensitivity, reproducibility and reliability. This arises from the monovalence characteristics of the monoclonal antibodies which react to a single antigen (Priyanka *et al*., 2016; Zhao *et al*., 2014). The downside of monoclonal antibodies is that they are more expensive than their polyclonal counterparts (Yeni *et al*., 2014).

There are several immunoassays with application in food safety, the latex agglutination assay (LAA), the Enzyme-Linked Immunosorbent Assay (ELISA) and the lateral flow immunoassay (LFI) are the ones most commonly employed (Mangal *et al*., 2016; Zhao *et al*., 2014). Among the immunoassays, the LAA were the first to be applied in food safety protocols, for the confirmation step of culture plating techniques. The procedure makes use of latex beads coated with antibodies that agglutinate in the presence of the target antigen (bacteria) forming a visible precipitate (Mangal *et al*., 2016).

ELISA is the most popular and the most widely-used method in the immunological assays category for the detection of foodborne pathogens (Mangal *et al*., 2016; Wang and Salazar, 2016; Zhao *et al*., 2014). A general ELISA procedure makes use of reporter molecules and substrates that produce observable color changes in the presence of the antigen (Zhao *et al*., 2014). Several variants of an ELISA procedure have been developed, however the most powerful format is called the "sandwich" assay. The name arises from the fact that the target antigen, if present, becomes trapped between a capture antibody, immobilized in the support of reaction, and a detection enzyme-conjugated antibody. The detection is then achieved by the addition of a colorless substrate that will be enzymatically converted, ultimately resulting in a visible color change of the solution (Law *et al*., 2015; Zhao *et al*., 2014).

Generally, the capture antibody is immobilized on the walls of microtiter plates, however, other supports such as dipsticks, paddles, membranes, pipet tips, and other solid matrices have also been successfully used (Zhao *et al*., 2014). There are also different types of enzymes that are commonly used in ELISA procedures, including horseradish peroxidase, alkaline phosphatase and beta-galactosidase (Mangal *et al*., 2016; Yeni *et al*.,2014). Several other variants of ELISA methods have been developed bringing higher sensitivity, shorter detection time and the capability of detecting multiple targets simultaneously in one assay - multiplexing. One of them is the use of fluorescent and

chemiluminescent labels instead of the traditional color change indication, called the enzyme-linked fluorescent immunoassay (ELFA) (Wang and Salazar, 2016).

The LFI is mainly an on-site immunological technique, developed in various forms, namely as dipstick, immunochromatography and immunofiltration (Law *et al*., 2015; Wang and Salazar, 2016; Zhao *et al*., 2014). Basically, the sample is placed and flows along a solid substrate via capillary action. On its path encounters and mixes with a color reagent, that can be an antibody or antigen labelled by colloidal latex or gold particles, and moves forward until it reaches the test zone, that is pretreated with an antibody or antigen. In case of a positive sample the target antigen and the color reagent become trapped in the test zone originating a positive visual outcome. The LFI are powerful tools for on-site detection, less expensive, present short detection times (from 2 to 10 minutes) and, unlike the ELISA, do not require skilled technicians. The downside of LFI is the higher rate of false-positive results obtained (Law *et al*., 2015; Zhao *et al*., 2014).

Generally, the immunoassays are specific, sensitive, rapid, robust and present the possibility of procedure automation. Is also a versatile technique, able to detect from bacterial cells and spores to viruses with a detection limit of 10^4-10^5 CFU or PFU/mL and toxins up to 1 µg/Kg. Overall, immunoassays are less specific and sensitive than the nucleic acid-based assays due to cross reactivity of the antibody with antigens of other non-pathogenic bacteria and/or food particles (Law *et al*., 2015; Mangal *et al*., 2016; Priyanka *et al*., 2016; Yeni *et al*.,2014; Zhao *et al*., 2014). Finally, choosing the target antigen can be problematic and should be matter of attention, as their expression can be influenced by temperature, preservatives, acids, salts, or other chemicals found and applied in food processing (Mangal *et al*., 2016; Zhao *et al*., 2014).

1.5.2.2. Nucleic acid-based assays

Nucleic acid-based assays are nowadays, widely used for the detection and monitoring of foodborne pathogens. These types of assays rely on the specific detection of nucleic acid sequences, DNA or RNA, within the target organism (Mangal *et al*., 2016; Wang and Salazar, 2016; Zhao *et al*., 2014). The development of a new nucleic acid-based assay starts with the definition of the target, directing the detection system to a gene or region that is conserved throughout a particular species or genus or in the identification of a particular gene. In order to develop a successful assay, the target should be present

at a relatively high copy number and present heterology at the sequence level. Common targets are genomic DNA, rRNA, genes coding for toxins or virulence factors and genes involved in cellular metabolism (Mangal *et al*., 2016). Nucleic acid-based assays consist of two main methodologies, the nucleic acid amplification techniques, namely Polymerase Chain Reaction (PCR) or related techniques, and nucleic acid hybridization, such as Fluorescence *in situ* Hybridization (FISH) or related techniques (Wang and Salazar, 2016).

1.5.2.2.1. Nucleic acid amplification techniques

PCR was first described by Mullis *et al* in 1986 as a method to amplify DNA *in vitro*, enabling exponential amplification of a certain sequence in a short period of time (Mangal *et al*., 2016). PCR is even considered one of the milestone discoveries in recombinant DNA technology (Priyanka *et al*., 2016). Currently, PCR is the most well-known and the most commonly-used nucleic acid amplification technique for the detection of foodborne pathogens (Wang and Salazar, 2016; Zhao *et al*., 2014). The amplification is accomplished in 3 steps, denaturation, annealing and elongation. In the denaturation step, the double stranded (ds) DNA is denatured into single strands (ss), then, in the annealing step, two complementary primers bind specifically to each of the ss target sequences. In the elongation step, the thermostable DNA polymerase makes a strand that is complementary to the template in the presence of free deoxynucleoside triphosphates (dNTPs). These steps are repeated, 20 to 40 times, doubling the number of target sequences with each cycle. The amplification product can then be visualized in a ethidium-bromide stained electrophoresis gel. The original PCR technique can be extensively modified and several formats of PCR-based methodologies are now available for the detection of food pathogens (Table 1.4) (Zhao *et al*., 2014; Mangal *et al*., 2016). The most relevant of these novel formats will now be discussed in more detail.

Table 1.4 - Nucleic acid amplification techniques developed for the detection of foodborne pathogens subdivided by methodology.

Detection Method	Target Organism	Reference
PCR	*Escherichia coli* spp.	Schmidt *et al*., 1995; Tsai *et al*., 1993.
	Listeria monocytogenes	Simon *et al*., 1996.
	Salmonella spp.	Kumar *et al*., 2008; Malorny *et al*., 2003; Stark and Made, 2007.
	Shigella spp.	Frankel *et al*., 1990.
	Staphylococcus aureus	Riyaz-Ul-Hassan *et al*., 2008; Wilson *et al*., 1991.
	Vibrio parahaemolyticus	Tada *et al*., 1992.
	Vibrio vulnificus	Brauns *et al*., 1991.
	Yersinia enterocolitica	Ibrahim *et al*., 1992; Nakajima *et al*., 1992.
NPCR	*Listeria monocytogenes*	Herman *et al*., 1995.
	Salmonella spp.	Saroj *et al*., 2008.
qPCR	*Bacillus cereus*	Martínez-Blanch *et al*., 2009; Messelhäusser *et al*., 2007.
	Campylobacter jejuni	Rantsiou *et al*., 2010; Ronner and Lindmark, 2007.
	Escherichia coli O157:H7	Ibekwe *et al*., 2006; Singh *et al*., 2009.
	Listeria monocytogenes	Berrada *et al*., 2006; Guibaud *et al*., 2005; Oravcová *et al*., 2007; Rantsiou *et al*., 2008; Rodríguez-Lázaro *et al*., 2004.
	Salmonella spp.	Farrel *et al*., 2005; Liming and Bhagwat, 2004.
	Yersinia enterocolitica	Iliev *et al*., 2008; Lambertz *et al*., 2008.
RT-PCR	*Escherichia coli* O157:H7	Yaron and Matthews, 2002.
	Salmonella spp.	Choi and Lee, 2004.
mPCR	Enterohemorrhagic *Escherichia coli*	Gannon *et al*., 1997.
	Listeria spp.	Chen and Knabel, 2007.
	Listeria monocytogenes + *Salmonella* spp.	Jothikumar *et al*., 2003.
	Escherichia coli O157:H7 + *Listeria monocytogenes*	Mukhopadhyay and Mukhopadhyay, 2007.
	Escherichia coli O157:H7 + *Listeria monocytogenes* + *Salmonella* spp.	Kawasaki *et al*., 2011.
	Escherichia coli O157:H7 + *Listeria monocytogenes* + *Salmonella* spp. + *Staphylococcus aureus* + *Vibrio parahaemolyticus*	Kim *et al*., 2007.
mRT-PCR	*Escherichia coli* O157:H7 + *Listeria monocytogenes* + *Salmonella typhi* + *Shigella* spp. + *Staphylococcus aureus* + *Streptococcus pyogenes* + *Vibrio cholerae* + *Vibrio parahaemolyticus*	Huang *et al*., 2007a.
LCR	*Listeria monocytogenes*	Wiedmann *et al*., 1992; Wiedmann *et al*., 1993.
NASBA	*Listeria monocytogenes*	Blais *et al*., 1997; Nadal *et al*., 2007; Uyttendaele *et al*., 1995.
	Campylobacter spp.	Uyttendaele *et al*., 1996.
LAMP	*Bacillus anthracis*	Qiao *et al*., 2007.
	Escherichia coli O157	Zhao *et al*., 2010a.
	Listeria monocytogenes	Wang *et al*., 2011.
	Pseudomonas aeruginosa	Zhao *et al*., 2011.
	Salmonella spp.	Chen *et al*., 2011; Ye *et al*., 2011; Zhao *et al*., 2010b.
	Vibrio parahaemolyticus	Nemoto *et al*., 2011; Yamazaki, *et al*., 2008; Zhao *et al*., 2010c.
	Shigella spp. + enteroinvasive *Escherichia coli*	Song *et al*., 2005.

The nested polymerase chain reaction (NPCR) employs two sets of primers, used in two successive runs of PCR. The first set of primers are used to amplify a pre-determined region and the second set is specific for an internal region of the first amplicon. This means that the secondary amplification only occurs if the primary amplification is successful and specific. NPCR is therefore more sensitive and specific than conventional PCR. (Fan *et al*., 2009).

Real-time polymerase chain reaction, also called quantitative real-time polymerase chain reaction (qPCR), is a technique that offers simultaneous amplification and monitoring/quantification of the target DNA molecule. The monitoring is performed recurring to nonspecific binding fluorescent dyes, such as SYBR-green or ethidium bromide or specific oligonucleotides probes labelled with a fluorescent reporter, such as TaqMan, molecular beacons, Scorpion Probes, etc. The quantification of the amplification products is performed at every cycle, dispensing post-amplification analysis (Navarro *et al*., 2015).

Reverse transcription polymerase chain reaction (RT-PCR) is a technique that uses RNA instead of DNA as starting material in the reaction. The RNA is first converted into a complementary DNA (cDNA) using a reverse transcriptase and specific primers. Then the previously formed cDNA is used as a template for exponential amplification as in a general PCR assay. Like PCR, RT-PCR can also be adapted to give quantitative results - RT-qPCR (Bustin, 2000).

Multiplex polymerase chain reaction (mPCR) is a PCR variant where it is possible to simultaneously detect multiple targets. This is accomplished by the use of multiple sets of primers, each pair for a specific target organism gene, gene variant or genomic marker (Wang and Salazar, 2016).

Ligase chain reaction (LCR) is an amplification technique rather different than PCR. The amplification is accomplished by the establishment of a phosphodiester bound, catalyzed by DNA ligase, between two oligonucleotide probes adjacent to each other and complementary to the target sequence. The repetition of the process allows the amplification of the target sequence. This amplification technique is very sensitive, since it allows the discrimination of single mismatch target sequences (Wiedmann *et al*., 1994).

Nucleic acid sequence-based amplification (NASBA) is an amplification technique that is carried under isothermal temperatures, unlike PCR or PCR variants. The procedure uses 3 viral enzymes, avian myeloblastosis virus reverse transcriptase (AMV-

RT), T7 DNA-dependent RNA polymerase, RNase H and two primers, a forward specific for the RNA target and with a promoter region for the T7 RNA polymerase and a reverse. Initially the AMV-RT recognizes the forward primer bonded to the RNA target sequence and converts it into cDNA. Subsequently, the RNase H hydrolyzes the RNA sequences in the reaction mixture and then the reverse primer anneals leading to the extension of the ss cDNA into ds cDNA by the AMV-RT. Finally, the T7 RNA polymerase binds to its promoter region on the ds cDNA, transcribing it into RNA. The RNA copies are then used as a template and the procedure restarts, resulting in exponential amplification of the target product. The amplified products are generally visualized using post-amplification methods, although real time analysis using molecular beacons was reported (Fakruddin *et al*., 2013).

Loop-mediated isothermal amplification (LAMP) is a one-step DNA amplification technique performed like NASBA, under isothermal temperatures. The technique requires the use of a DNA polymerase with strand-displacement activity, generally from *Bacillus stearothermophilus* (Bst), two inner primers and two outer primers which recognize six separate regions within a target DNA. Is even possible to accelerate the reaction with the addition of two more primers, the loop primers. The procedure is performed in 3 different steps: an initial step, a cycling amplification step and an elongation step. In the initial step the 3' inner primer anneals with the target region and the Bst DNA polymerase synthetizes a complementary sequence. Then the 3' outer primer anneals with the target region and Bst DNA polymerase synthetizes a complementary sequence releasing the one previously formed with the inner primer. The process is repeated this time for the 5' of the target region. In the end, the target sequences are present in the characteristic form of loop structures. In the cycling amplification and elongation steps the activity of the Bst DNA polymerase increases the numbers of target sequences which are comprised by a mixture of amplicons with different sizes and structures. Detection in LAMP is visible to the naked eye without requiring the use of post-amplification methods. LAMP, like NASBA, is adaptable for real time detection and the addition of reverse transcriptase makes possible to use RNA as starting material (RT-LAMP) (Sahoo *et al*., 2016).

Molecular methods based on nucleic acid amplification techniques are rapid, specific and sensitive. However, they require nucleic acid extraction and post analysis steps; present changes in sensitivity depending on food matrix type; are susceptible to

inhibitors; prone to cross-contamination; and can amplify DNA from non-viable cells (or even naked DNA), resulting in the appearance of both false negative and false positive results. Mitigation of these limitations was achieved by improvements at procedure level, with optimization of the extraction procedures and inclusion of internal controls, and with the development of PCR variants using RNA as a starting material (RT-PCR, NASBA, etc.) and real-time detection (Mangal *et al*., 2016; Wang and Salazar, 2016; Yeni *et al*., 2014; Zhao *et al*., 2014).

1.5.2.2.2. Nucleic acid hybridization techniques

Pathogen detection based on nucleic acid hybridization techniques, unlike the ones described in the previous section, do not required amplification of genetic material for detection, but rather rely in the hybridization of beforehand designed probe(s) and the genetic material of the pathogen of interest (Table 1.5). Among the nucleic acid hybridization techniques for detection of foodborne pathogens, FISH is the most commonly employed method (López-Campos *et al*., 2012). It was in the late 1980s with the works of DeLong *et al* (1989) that FISH was first applied for the detection and identification of microorganisms targeting the rRNA of the cells. A standard FISH procedure is performed in 4 different steps: fixation/permeabilization, hybridization, washing and visualization/detection (Amann and Fuchs, 2008; Rohde *et al*., 2015). The specifics of the FISH procedure and overall technique will be more comprehensively address later on this chapter.

Over the years several improvements were performed to FISH, that includes the use of nucleic acid mimic molecules as probes, such as Peptide Nucleic Acid (PNA-FISH) or Locked Nucleic Acid (LNA-FISH), the detection of low copy number targets through signal enhancement using probes labelled with reporter enzymes (CARD-FISH), double-labeled probes (DOPE-FISH) or target different regions simultaneously (ML-FISH) (Cerqueira *et al*., 2008; Rohde *et al*., 2015).

Microarrays, like FISH, rely on probe-target hybridization for the detection of the genetic material of interest. Generally, microarrays are a small, high-throughput platform where multiple spots of previously-synthetized short ss DNA probes are covalently bound to a glass or silicon surface. A standard procedure then involves the extraction of DNA or RNA from the samples and labelling them with fluorescent dyes, silver, among others. The labelled extracted DNA or RNA is then denatured and placed on the spots where it

binds to their corresponding complementary probes on the array. The detection is accomplished through visualization of the reporting signal when double-stranded DNA is formed (Trevino *et al*., 2007).

Table 1.5 - Nucleic acid hybridization techniques developed for the detection of foodborne pathogens subdivided by methodology.

Detection Method	Target Organism	Reference
FISH	*Escherichia coli*	Stender *et al*., 2001a; Tortorello and Reineke, 2000.
	Listeria spp.	Brehm-Stecher *et al*., 2005; Fuchizawa *et al*., 2008; Schmid *et al*., 2003; Stephan *et al*., 2003.
	Listeria monocytogenes	Almeida *et al*., 2011; Fuchizawa *et al*., 2009; Moreno *et al*., 2011; Oliveira *et al*., 2003; Zhang *et al*., 2012.
	Salmonella spp.	Almeida *et al*., 2010; Bisha and Brehm-Stecher, 2009; Bisha and Brehm-Stecher, 2010; Fang *et al*., 2003; Oliveira *et al*., 2012; Vieira-Pinto *et al*., 2005; Vieira-Pinto *et al*., 2007.
	Campylobacter spp.	Moreno *et al*., 2001; Schmid *et al*., 2005.
	Pseudomonas spp.	Gunasekera *et al*., 2003, Kitaguchi *et al*., 2005.
	B. cereus	Laflamme *et al*., 2009.
	Cronobacter spp.	Almeida *et al*., 2009.
	Clostridium perfringens	Shimizu *et al*., 2009.
	Enterobacteriaceae	Ootsubo *et al*., 2003.
mFISH	*Listeria monocytogenes* + *Salmonella* spp.	Oliveira *et al*., 2004.
	Pseudomonas spp. + *Enterobacteriaceae*	Yamaguchi *et al*., 2012.
Microarray	*Staphylococcus* spp.	Sergeev *et al*., 2004.
	Escherichia coli + *Shigella* spp.	Li *et al*., 2006.
	Escherichia coli + *Salmonella* spp. + *Shigella* spp.	Loy *et al*., 2005.
	Vibrio cholerae + *Vibrio parahaemolyticus* + *Vibrio vulnificus*	Panicker *et al*., 2004.
	Campylobacter coli + *Campylobacter jejuni* + *Campylobacter lari* + *Campylobacter upsaliensis*	Volokhov *et al*., 2003.
	Campylobacter jejuni + *Escherichia coli* O157:H7 + *Listeria monocytogenes* + *Salmonella enterica*	Suo *et al*., 2010.
	Listeria grayi + *Listeria innocua* + *Listeria ivanovii* + *Listeria monocytogenes* + *Listeria seeligeri* + *Listeria welshimeri*	Volokhov *et al*., 2002.
	Listeria monocytogenes + Proteus mirabilis + Proteus vulgaris + *Salmonella* spp. + *Shigella* spp. + *Staphylococcus aureus* + *Streptococcus pyogenes* + *Vibrio cholerae* + *Vibrio parahaemolyticus* + *Vibrio vulnificus* + *Yersinia enterocolitica*	Cao *et al*., 2011.

Table 1.5 (continuation)

	Bacillus anthracis + *Brucella abortus* + *Clostridium botulinum* + *Clostridium perfringens* + *Coxiella burnetii* + *Francisella tularensi* + *Rickettsia prowazekii* + *Staphylococcus aureus* + *Vibrio alginolyticus* + *Vibrio cholerae* + *Yersinia pestis*	Wilson *et al*., 2002.
Microarray	*Aeromonas hydrophila* + *Bacillus cereus* + *Campylobacter jejuni* + *Clostridium botulinum* + *Clostridium perfringens* + *Clostridium tetani* + *Enterococcus faecalis* + *Legionella pneumophila* + *Listeria monocytogenes* + *Micobacterium tuberculosis* + *Proteus spp.* + *Pseudomonas aeruginosa* + *Pseudomonas cocovenenans subsp.*, *Farinofermentans* + *Salmonella* spp. + *Shigella* spp. + *Staphylococcu aureus* + *Staphylococcus haemolyticus* + *Vibrio cholerae* + *Vibrio fluvialis* + *Vibrio parahaemolyticus* + *Yersinia enterocolitica*	Wang *et al*., 2007.

Nucleic acid-based assays based on hybridization techniques generally present a detection limit of 10^3-10^6 CFU/mL. The major advantages, like for the amplification techniques, rely on their specificity, sensitivity and suitability for simultaneous detection of pathogens, such as multiplex-FISH (M-FISH) and especially in microarrays. However, these techniques can be expensive, namely in the production of the microarray chips that alongside with lack of standardization, automation and unavailability of high-throughput systems impair their practicability and broad implementation in food safety laboratories (López-Campos *et al*., 2012; Mangal *et al*., 2016; Rohde *et al*., 2015).

1.5.2.3. Biosensors

Biosensors are one of the latest developed technologies used in food safety for pathogen detection (Priyanka *et al*., 2016). According to Zhao *et al* (2014), a biosensor can be defined as an "analytical device incorporating a biological (*e.g.* tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived (recombinant antibodies, engineered proteins, aptamers, etc.) or a biomimic material (synthetic catalyst, combinatorial ligands and imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem". Basically, it is comprised of 3 different elements, the capturing material (that binds to a specific target), the transducer mechanism and a data output system (Priyanka *et al*., 2016; Zhao *et al*., 2014). Ultimately, this means that is possible to adapt previous developed mechanisms of detection, such

antibodies and probes for the construction of a biosensor. The categorization of the biosensors is based on the different strategies of transducing mechanisms. There are the optical, electrochemical, mass-based, thermometric, micromechanical and magnetic mechanisms (Law *et al*., 2015; Wang and Salazar, 2016). From these the ones most commonly applied for the detection of foodborne pathogens are the optical, electrochemical and mass-based type biosensors (Table 1.6) (Law *et al*., 2015; Zhao *et al*., 2014).

Table 1.6 - Biosensors developed for the detection of foodborne pathogens subdivided according to their respective classification.

Biosensor Type	Target Organism	Reference
Optical	*Campylobacter jejuni*	Wei *et al*., 2007.
	Cronobacter sakazakii	Rodriguez-Emmenegger *et al*., 2011.
	Escherichia coli	Linman *et al*., 2010.
	Escherichia coli O157:H7	Meeusen *et al*., 2005; Si *et al*., 2011; Wang *et al*., 2013a; Waswa *et al*., 2007; You *et al*., 2011.
	Salmonella enteritidis	Son *et al*., 2007; Song *et al*., 2014.
	Salmonella typhimurium	Ko and Grant, 2006; Lan *et al*., 2008; Seo *et al*., 1999.
	Escherichia coli + *Salmonella enteritidis*	Waswa *et al*., 2006.
	Escherichia coli + *Staphylococcus aureus*	Pires *et al*., 2011.
	Campylobacter jejuni + *Escherichia coli* O157:H7 + *Listeria monocytogenes* + *Salmonella typhimurium*	Taylor *et al*., 2006.
Electrochemical	*Bacillus cereus*	Pal *et al*., 2008.
	Escherichia coli O157:H7	Gehring and Tu, 2005; Joung *et al*., 2013; Li *et al*., 2011; Lin *et al*., 2008; Varshney and Li, 2007; Wang *et al*., 2013b.
	Listeria innocua	Tolba *et al*., 2012.
	Salmonella spp.	Afonso *et al*., 2013.
	Salmonella typhi	Rao *et al*., 2005.
	Salmonella typhimurium	Dong *et al*., 2013.
	Campylobacter spp. + *Escherichia coli* O157:H7 + *Salmonella* spp.	Viswanathan *et al*., 2012.
Mass-based	*Bacillus anthracis*	Campbell and Mutharasan, 2006.
	Escherichia coli	Yilmaz *et al*., 2015.
	Escherichia coli O157:H7	Chen *et al*., 2008; Wu *et al*., 2007. Maraldo and Mutharasan, 2007.
	Listeria monocytogenes	Sharma and Mutharasan, 2013.
	Pseudomonas aeruginosa	Tokonami *et al*., 2013.

The optical biosensors are the most reported class of biosensors and consequently the most widely used in foodborne pathogen detection (Wang and Salazar, 2016). In these, the interaction of the capturing material with the target modifies the characteristics of the optical transducer inducing a response. There are several mechanisms of

transduction detection and they are also used to subcategorize the optical biosensors, namely light absorbance, reflection, refraction, Raman, infrared, chemiluminescence, dispersion, fluorescence and phosphorescence (Zhao *et al*., 2014).

Electrochemical biosensors like the optical biosensors can be further subcategorized according to the type of analyzed response. These can be amperometric, impedimetric, potentiometric and conductometric, measuring changes in current, impedance, voltage and conductance, respectively (Law *et al*., 2015; Zhao *et al*., 2014).

Finally, mass-based, piezoelectric or even mass-sensitive biosensors work, as the name points out, by detecting changes in mass. The mass-based biosensors use piezoelectric crystals that present a specific vibratory frequency. Alterations to that vibration can occur when the target molecule is bound to the capturing material, leading to detection. There are two major types, bulk acoustic wave resonators or quartz crystal microbalance and surface acoustic wave resonators. These applications, however possess lower detection performance characteristics than the optical and electrochemical biosensors (Law *et al*., 2015).

Overall biosensor technology is rapid, specific, sensitive and allow real time analysis, however is not cost effective, requires specific instrumentation for analysis, presents poor in-field performance and reproducibility deficiencies. As such, still requires significant methodological improvements in order to ensure reliability, stability of biomaterials used and compatibility to in-field tests. (Priyanka *et al*., 2016; Wang and Salazar, 2016; Yeni *et al*., 2014).

1.5.2.4. Other techniques

The above-mentioned techniques are the most commonly employed methods for the detection of foodborne pathogens. However, over the years several others methodologies have been or are being developed, although with less expression/applicability in the market, namely Direct Epifluorescence Filter Technique (DEFT) (Pettipher and Rodrigues, 1982), Solid Phase Cytometry (SPC) (López-Campos *et al*., 2012), Randomly Amplified Polymorphic DNA (RAPD) (Boughattas and Salehi, 2014; Adzitey *et al*., 2013), Amplified Restriction-Length Polymorphism (AFLP) (Adzitey *et al*., 2013), Denaturing Gradient Gel Electrophoresis (DGGE) (Anderson *et al*., 2010), MALDI-TOF mass spectrometry (Singhal *et al*., 2015), Pulse Field Gel Electrophoresis (PFGE) (Adzitey *et al*., 2013; Boughattas and Salehi, 2014), Restriction

fragment length polymorphism (RFLP) (Adzitey *et al*., 2013), bacteriophage and endolysin based detection (Bai *et al*., 2016), etc.

1.6. Fluorescence *in situ* Hybridization - FISH

1.6.1. Origin, diversity and applications of *in situ* hybridization techniques

In situ hybridization (ISH) includes an array of methodologies that ultimately allow the specific detection of nucleic acid sequences in biological samples. The first reported ISH experiments were done independently by Pardue and Gall (1969) and Jonh *et al* (1969). At that time radioisotopes were the only labels available for nucleic acids and autoradiography was the only mean of detection of hybridized sequences. Soon after the first reports, radioactive labels were quickly replaced by fluorescent labels as reporter molecules due to their higher safety, stability and ease of detection (Rudkin and Stollar, 1977). Hence the emergence of the term Fluorescence *in situ* Hybridization (FISH). The first application of FISH used 3' end fluorescent labeled RNA probes for the specific detection of virus, parasitic and insect DNA sequences by Bauman *et al* (1980). The application of FISH for the detection of microorganisms was first described in the late 80's by DeLong *et al* (1989), targeting the ribosomal RNA (rRNA) with oligonucleotide probes. Since then, FISH became a tool widely used in the field of microbiology (Amann and Fuchs, 2008), namely in the identification, quantification and characterization of phylogenetically defined microbial populations in complex environments (Wagner *et al*., 2003). Furthermore, in these almost 40 years of existence, FISH applications expanded to several fields of research, including cellular genomics, chromosome biology, clinical genetics, comparative genomics, evolutionary biology, microbial ecology, neuroscience, reproductive medicine, toxicology, among others (Frickmann *et al*., 2017; Volpi and Bridger, 2008). This results from a diversification of FISH-based diagnostic assays that ultimately lead to the improvement in sensitivity, specificity and resolution of the technique (Table 1.7).

Table 1.7 - Overview of the diversity of FISH-based applications. The most commonly used FISH variants are seen in the table, together with a small description of their intended use and differentiation in comparison to standard FISH procedures.

Abbreviation	Denomination	Description	Reference
-	3D-FISH	FISH variant for the evaluation of the positioning and relative organization of chromosomes and subchromosomal regions within the nucleus;	Cremer *et al*., 1993; Zirbel *et al.*, 1993; Maalouf *et al.*, 2010.
ACM-FISH	Alpha (centromere), Classical (1q12) and Midi (1p36.3)-FISH	A color FISH variant for simultaneous detection and enumeration of abnormalities in chromosome 1 of sperm cells;	Sloter *et al*., 2000; Volpi and Bridger, 2008.
armFISH	-	A multiplex-FISH designed for the detection of abnormalities in the p- and q-arms of human chromosomes;	Karhu *et al.*, 2001.
CARD-FISH	Catalyzed Reporter Deposition-FISH	Signal amplification FISH assay, similar to TSA-FISH;	Kerstens *et al.*, 1995; Raap *et al.*, 1995; Kubota *et al.*, 2013.
catFISH	Cellular Compartment Analysis of Temporal Activity-FISH	FISH variant for the analysis of gene expression patterns in the brain through the quantification and discrimination of activated neuronal populations;	Guzowski *et al.*, 1999; Guzowski *et al.*, 2001.
CB-FISH	Cytochalasin-B-FISH	FISH variant developed for cytological analysis of micronucleation and aneuploidy events through blockage of cytokinesis (using Cytochalasin-B);	Kirsch-Volders *et al.*, 1996; Surrallés *et al*., 1996.
CLASI-FISH	Combinatorial Labeling and Spectral Imaging-FISH	FISH variant for simultaneous identification of dozens of targets through fluorophore combination;	Valm *et al.*, 2011; Valm *et al.*, 2012; Welch *et al.*, 2016.
CO-FISH	Chromosome Orientation-FISH	FISH variant for determination of relative chromosomal orientation;	Goodwin.and Meyne, 1993; Bailey *et al.*, 2010.
COBRA-FISH	Combined Binary Ration-FISH	FISH variant for simultaneous identification of up to 48 different targets through fluorophore combination;	Tanke *et al*., 1999; Raap and Tanke, 2006.
COD-FISH	Chromosome Orientation and Direction-FISH	Similar to CO-FISH, however also combines information about the directional organization of telomeric sequences;	Meyne and Goodwin, 1995; Bailey *et al.*, 2010.
	Concomitant Oncoprotein Detection-FISH	FISH variant for the detection of oncogenes and their respective protein products;	Tubbs *et al.*, 2000; Tubbs *et al.*, 2002.
	Combined $CaCO_3$ Optical Detection-FISH	Detection of calcifying microorganisms;	Frada *et al*., 2006.
COMBO-FISH	Combinatorial Oligonucleotide-FISH	FISH variant for specific detection of genomic sites without DNA denaturation using homopurine/homopyrimidine oligonucleotide probes;	Hausmann *et al*., 2003.
-	comet-FISH	A combination of comet assay and FISH, for evaluation of DNA damage within the cells;	Santos *et al*., 1997; Hovhannisyan and Aroutiounian, 2016.

Table 1.7 (continuation)

-	Cryo-FISH	FISH variant for the study of nuclear genome organization using ultrathin cryosections of well-fixed, sucrose-embedded cells;	Branco and Pombo, 2006.
D-FISH	Double Fusion-FISH	Evolution of Fusion Signal-FISH (below), using two differentially labeled probes for the two translocation breakpoints;	Volpi and Bridger, 2008.
DBD-FISH	DNA Breakage Detection-FISH	FISH variant for the analysis of DNA damage/breaks;	Fernández *et al*., 1998; Fernández and Gosálvez, 2002.
DOPE-FISH	Doubly Labeled Oligonucleotide Probe-FISH	Signal amplification FISH technique using oligonucleotide probes labeled both at the 3' and 5';	Stoecker *et al*., 2010.
DVC-FISH	Direct Viable Count-FISH	Enumeration FISH technique of viable cells through inhibition of DNA-gyrase;	Moreno *et al*., 2012.
-	Fiber-FISH	FISH variant used for gene and chromosomal mapping on fibers of chromatin or DNA. Allows the assessment of gaps and overlaps in contigs and analysis of segmental duplications and copy number variants;	Heng *et al*., 1992; Ye and Heng, 2017.
-	Flow-FISH	Adaptation of FISH to flow cytometry;	Rufer *et al*., 1998; Volpi and Bridger, 2008.
-	Fusion Signal-FISH	Detection of recurring chromosomal translocations in hematological malignancies;	Dongen *et al*., 2005.
-	Halo-FISH	FISH variant performed on DNA/chromatin previously permeabilized and extracted recurring to high salt concentration solutions, forming a halo around the nucleus;	Wiegant *et al*., 1992; Gerdes *et al*., 1994; Volpi and Bridger, 2008.
-	Harlequin-FISH	FISH variant for precise quantification of lymphocyte chromosomal damage in biodosimetry analysis;	Kulka *et al*., 1995; Jordan *et al*., 1999; Volpi and Bridger, 2008.
-	Immuno-FISH	Combination of FISH with and immunofluorescence. This allows the analysis of both DNA and protein content in the sample;	Brown *et al*., 1997; Volpi and Bridger, 2008.
LNA-FISH	Locked Nucleic Acid-FISH	Use of locked nucleic acid probes in FISH;	Koshkin *et al*., 1998; Cerqueira *et al*., 2008.
M-FISH	Multiplex-FISH	Use of a variety of differently labelled probes for the simultaneous detection of several targets in a sample;	Speicher *et al*., 1996; Anderson, 2010; Rohde *et al*., 2015.
ML-FISH	Multi-locus-FISH	FISH assay for the analysis of chromosomal abnormalities using multiple locus specific probes;	Lee *et al*., 1993; Ligon *et al*., 1997; Rohde *et al*., 2015.
PCC-FISH	Premature Chromosome Condensation-FISH	FISH assay for analysis of chromosomal damage after irradiation;	Brown and Evans, 1992; Brown *et al*., 1992; Volpi and Bridger, 2008.
PNA-FISH	Peptide Nucleic Acid-FISH	Use of peptide nucleic acid probes in FISH;	Cerqueira *et al*., 2008; Frickmann *et al*., 2017.

Table 1.7 (continuation)

Q-FISH	Quantitative-FISH	Methodology that allows the quantification of probe signal intensity;	Martens *et al.*, 1998; Iourov *et al.*, 2017.
QD-FISH	Quantum Dots-FISH	Utilization of probes conjugated with quantum dots;	Pathak *et al.*, 2001; Müller *et al.*, 2009.
-	Rainbow-FISH	Advanced digital imaging procedure for simultaneous discrimination and quantification of up to seven different strains and or phylogenetic groups;	Sunamura and Maruyama, 2006.
-	Raman-FISH	Combination of FISH with Raman microspectroscopy;	Huang *et al.*, 2007b.
RCA-FISH	Rolling Circle Amplification-FISH	Signal amplification assay recurring to *in situ* target amplification;	Mezger *et al.*, 2016.
Red-FISH	Replicative Detargeting-FISH	Similar to CO-FISH, however allows the determination of replication time;	Zou *et al.*, 2004; Bailey *et al.*, 2010.
-	Reverse-FISH	Use of previously extracted genetic material of interest as a probe for *in situ* hybridization;	Lichter *et al.*, 1990; Volpi and Bridger, 2008.
RING-FISH	Recognition of Individual Genes-FISH	Signal amplification FISH assay;	Zwirglmaier *et al.*, 2004; Zwirglmaier *et al.*, 2005.
-	Rx-FISH	Color banding technique, that relies on sequence homologies to produce specific banding pattern of human metaphase chromosomes;	Müller and Wienberg, 2001.
-	Split-Signal-FISH	Dual-color assay for the detection of frequently occurring chromosome translocations;	Dongen *et al.*, 2005.
T-FISH	Tissue-FISH	Method for the use of tissues samples in FISH;	Nomura *et al.*, 2003.
	Telomere-FISH	Use of telomere probes;	Lansdorp *et al.*, 1996; Vera and Blasco, 2012.
TSA-FISH	Tyramide Signal Amplification-FISH	Signal amplification FISH assay, similar to CARD-FISH;	Raap *et al.*, 1995; Kubota *et al.*, 2013.
Zoo-FISH	Cross Species Chromosome Painting-FISH	FISH assay for the identification of similar chromosomal regions between species.	Scherthan *et al.*, 1994; Rubes *et al.*, 2009.

1.6.2. Fluorescent dyes as reporter molecules

In order to have a visible signal in FISH, the probes must be attached to a reporter molecule, either by direct or indirect labelling (Figure 1.3). In the case of direct labelling, the fluorescent molecule is directly bound to the probe by chemical conjugation. This means that probe-target hybrids can be visualized immediately after the hybridization reaction (Figure 1.3a and 1.3b). In the case of indirect labelling a non-fluorescent molecule is used as reporter molecule, for instance a chemically-conjugated biotin or hapten or a conjugated enzyme (Figure 1.3c, 1.3d and 1.3e). After hybridization and washing, the detection results from the addition of a fluorescent labelled avidin or antibody, in the case of the biotin or hapten conjugates, respectively. Another possibility is the addition of a specific substrate for the conjugated enzyme, that is converted to a fluorescent precipitate or a highly reactive fluorescent compound (Bottari *et al*., 2006; Morrison *et al*., 2003). Indirect labeling has the advantage of signal amplification that could be useful in low target content microorganisms. However, it presents the disadvantage of requiring additional incubation steps in order to bind the antibody and avidin reagents and it may also increase background fluorescence by nonspecific binding (Morrison *et al*., 2003). Direct labelling is hence the easiest, fastest and cheapest method because it does not require any further detection step(s) after hybridization, being the most commonly used probe labeling procedure in FISH (Bottari *et al*., 2006; Moter and Gobel, 2000).

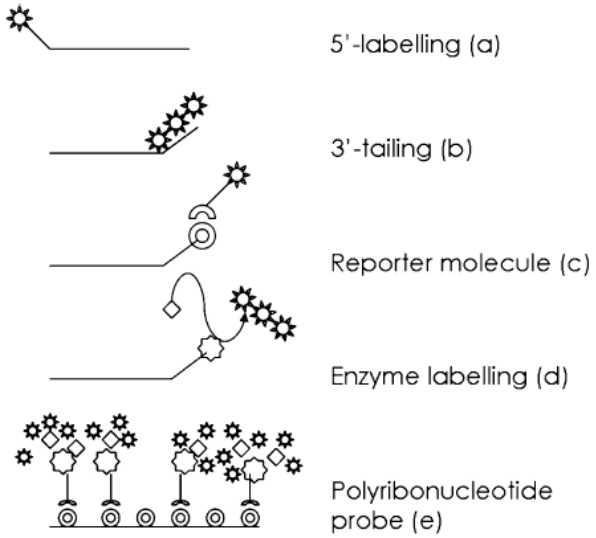

Figure 1.3 - Direct (a and b) and indirect (c, d and e) probe labeling methodologies on *in situ* hybridization protocols (from Bottari *et al*., 2006).

1.6.2.1. Types of fluorophores

The study of fluorescence is several centuries old and in modern day-life fluorescent compounds have multiple applications, especially in biological sciences. These can be used as probes (in the study of physical and structural parameters, etc.), as indicators (for the estimation of molecular concentrations, among others) and/or as labels/tracers (for visualization and localization of biomolecules, etc.) (Nishi *et al*., 2015; Valeur and Berberan-Santos, 2013). The development of fluorophores is a very active research field, with new molecules being continuously developed and perfected according to the specifications of each application (Nishi *et al*., 2015). At the moment, it is possible to categorize the existing fluorophores in three different classes, the ones with a defined structure that includes the organic dyes, the metal-ligand complexes (*e.g.* lanthanide chelates, etc.) and the fluorophores of biological origin (*e.g.* proteins); the size-dependent nanocrystal fluorophores that includes the quantum dots (made from semiconductors, carbon and silicon nanoparticles) and self-luminescent organic nanoparticles and the size independent nanometer-micrometer particles (Resh-Genger *et al*., 2008).

1.6.2.2. Development and choice of a suitable fluorophore

The development and choice of a suitable fluorophore must take into consideration several characteristics: (i) convenient excitation range, on one hand should be detectable by conventional instrumentation and on the other hand move away from wavelengths that promote the excitation of the biological matrix; (ii) intense brightness, fluorophores with high quantum yield (Φ) and high extinction coefficients (ε); (iii) soluble in relevant buffers, cell culture media or body fluids; (iv) stable under procedural conditions; (v) have functional groups for site-specific labeling; (vi) have published data about its photophysics and (vi) similar performance under mass production. Depending on the application, other additional considerations may include: (vii) steric and size-related effects; (ix) possibility of delivery inside the cells; (x) toxicity; (xi) suitability for multiplexing and (xii) compatibility for signal-amplification strategies (Resh-Genger *et al*., 2008). From the previous list of fluorophores, the organic molecules are the dyes that present higher versatility. This fact made them reference dyes in life science applications, namely in *in vitro* assays and *in vivo* imaging (Resh-Genger *et al*., 2008).

1.6.2.2.1. Organic dyes as the preferable choice for FISH

Organic dyes are generally composed by polyaromatic or heterocyclic hydrocarbons. In order to fluoresce they undergo a three-stage process, excitation, excited-state lifetime and fluorescence emission (Nishi *et al*., 2015). The fluorescent dyes are characterized by key properties, such maximum absorption wavelength (λmax), maximum emission wavelength (λem), extinction coefficient and fluorescence quantum yield (Nishi *et al*., 2015).

In the specific case of *in situ* hybridization probes, a wide variety of labeling fluorophores are available with emission spectra ranging from ultraviolet to the near infrared spectrum. The most frequently used fluorophores for *in situ* hybridization procedures belong to several common chemical classes, such coumarins, fluoresceins, rhodamines and cyanines (Figure 1.4).

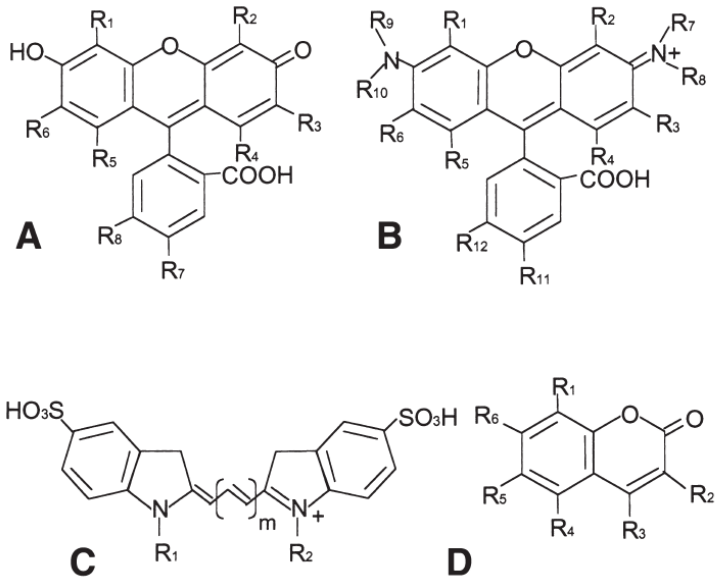

Figure 1.4 - Chemical structures of the four most commonly used organic dyes in FISH. A - fluoresceins; B - rhodamines; C - cyanines (Cy3, Cy5 and Cy7 only); D - coumarins. The addition of different chemical substituents in the R's groups generate variants within each family (from Morrison *et al*., 2003).

Coumarin dyes are composed of different functional groups added to the natural aromatic lactone structure. These dyes emit light only in the blue-green range. Fluorescein is composed by two parts of xanthene, the chromophore and benzene. Other dyes belonging to the fluorescein family are Oregon Green, fluorescein isothiocyanate (FITC), fluorescein diacetate and carboxyfluorescein (FAM). Rhodamine dye is an isolog of fluorescein differing due to the presence of two amino groups one of which positively charged. Also belonging to the rhodamine family are rhodamine B, lissamine rhodamine

B, sulforhodamine B, Texas Red, carboxytetramethylrhodamine (TAMRA), tetramethylrhodamine (TMR) and tetramethylrhodamine isothiocyanate (TRITC). Cyanines dyes are composed of two quaternized heteroaromatic bases joined by a polymethine chain. The color of the dye depends on the number of carbons in the polymethine chain. The cyanine family includes Cy2, Cy3, Cy3.5, Cy5, Cy5.5 and Cy7 dyes. Finally, Alexa Fluor dyes are synthesized through sulfonation of groups present in the family dyes referred before. These modifications confer higher photostability and brightness as well as lower pH sensitivity in comparison to the derived dyes (Morrison *et al*., 2003; Nishi *et al*., 2015; Resh-Genger *et al*., 2008).

1.6.3. Targeting rRNA in FISH

Since the works of DeLong *et al* (1989), the rRNA of both the small (SSU) and large (LSU) ribosomal subunits remains the preferable target in FISH. This is due to the presence of these structures in high numbers, that can reach up to 100 000 per cell, which allows direct detection without the need for signal amplification (Delong *et al*., 1989; Amann and Fuchs, 2008). Furthermore, in a recent past, the categorization of microorganisms shifted from morphology or physiology to domains, phyla and classes, where comparative rRNA sequence analysis is a well-established method playing a fundamental role in microbial identification (Amann and Fuchs, 2008). In fact, Bergey's Manual of Systematic Bacteriology was revised based on the 16S rRNA sequence information (Stender, 2003). Hence, the use of rRNA allows not only the development of probes for higher taxonomic entities, such as phyla, classes or orders, but also for lower taxonomic entities, such genera and at a species level, respectively (Amann and Fuchs, 2008; Stender, 2003).

1.6.4. FISH procedure

As mentioned above, a standard FISH protocol for rRNA-targeted oligonucleotide probes consists of four different steps: fixation/permeabilization, hybridization, washing and visualization or detection of labeled cells by microscopy or flow cytometry (Figure 1.5) (Amann and Fuchs, 2008; Rohde *et al*., 2015).

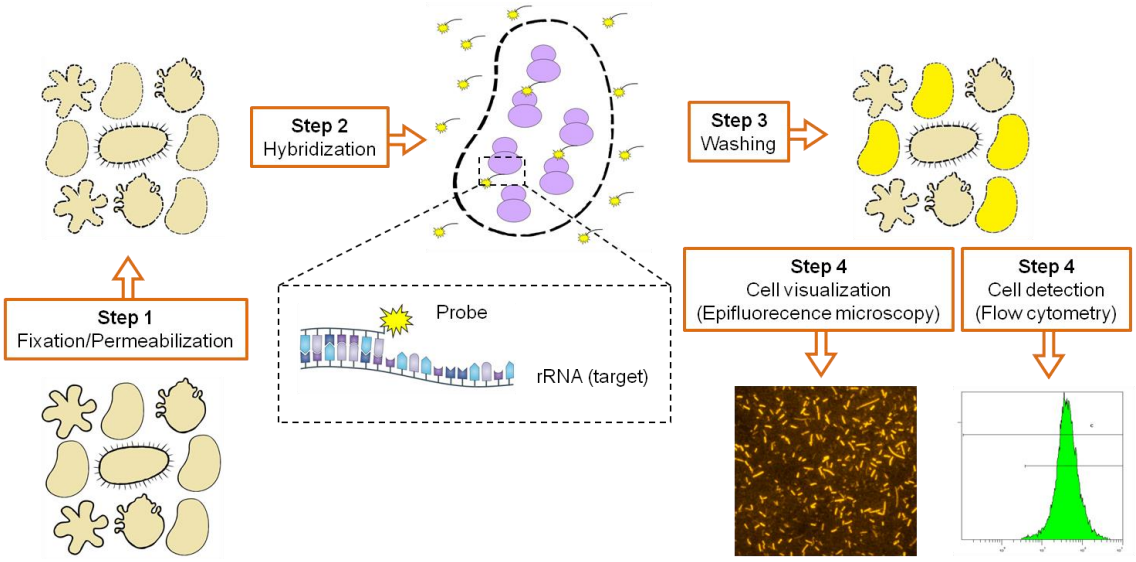

Figure 1.5 - Standard four steps of Fluorescent *in situ* Hybridization procedure using rRNA target oligonucleotide probes.

1.6.4.1. Fixation/permeabilization step

Fixation/permeabilization is the first step of a standard FISH procedure and is crucial to a successful outcome. It must prepare the samples to (i) preserve cell shape, (ii) maintain the rRNA integrity by avoiding the attack of endogenous RNAses and (iii) prevent cell loss through lysis. On the other hand, this step also must allow the permeabilization of as many cells as possible in order to allow the diffusion of the labeled probes into the cell to their rRNA target molecules (Amann and Fuchs, 2008; Moter and Gobel, 2000). Fixation/permeabilization steps in FISH, usually involves the use of one or more fixative compounds and sometimes permeabilizing agents.

Fixation compounds for in situ analysis can be divided in four major groups, aldehydes, oxidizing agents, alcohols and metallic fixatives (Thavarajah *et al*., 2012). Aldehyde fixatives includes formaldehyde, glutaraldehyde, choral hydrate and glyoxal molecules (Rhodes, 2012). Fixation is accomplished through macromolecular cross-linking, creating a mesh type structure within the cell (Thavarajah *et al*., 2012). Oxidizing agents includes osmium tetraoxide, potassium permanganate, potassium dichromate and chromium trioxide (Rhodes, 2012; Thavarajah *et al*., 2012). Similarly to aldehydes, fixation is accomplished through macromolecular cross-linking (Rhodes, 2012). Methanol and ethanol are the most used alcohol fixatives. Fixation occurs trough dehydratation of the samples, leading to denaturation and precipitation of proteins (Thavarajah *et al*., 2012). Metallic fixatives include metals such copper, lead, cobalt,

chromium, silver, zinc, barium, mercury and uranium and a non-metal compound, picric acid. Fixation is accomplished by the formation of insoluble metallic precipitates (Thavarajah *et al*., 2012). Moreover, sample fixation can also be accomplished with physical methods, such as heat, microwaving and freeze-drying (Rhodes, 2012).

Permeabilization methods are used with the purpose of cause physical damage on the organized structure of the cell envelope of the microorganisms, from where the probes can access the interior of the cell. This can be achieved through three main strategies, physical, chemical and enzymatic treatments (Felix, 1982). Physical treatments include ultrasonic treatments, osmotic and temperature shocks. Chemical treatments comprise the application of organic solvents (including benzene, n-butanol, chloroform, dimethylsulfoxide, ether, methanol, ethanol, phenethyl alcohol and toluene), antibiotics (including N-acetylcandidin, amphotericin B, aureofungin A and B, candicidin, chainin, dermostatin, filipin, hamycin A and B, nigericin, nystatin, pimaricin and polymyxin), macromolecules (including thionins, ribonuclease, chitosan and lysolecithin), detergents (including Brij 58, N-Cetyl-N,N,N-trimethylammonium bromide, diethylaminoethyl dextran, Nonidet P-40, sarkosyl, sodium deoxycholate, sodium dodecyl sulfate, Triton X-100 and Tween 80) and chelating agents (EDTA) (Felix, 1982). Enzymatic treatments refer to the use of lytic enzymes such glycosidases (lysozymes and glucosaminidases), endopeptidases and amidases in *Eubacteria* samples (Salazar and Asenjo, 2007); proteases or pseudomurein endopeptidase in *Archaea* samples (Kubota, 2013, Lloyd *et al*., 2013); glucanases, proteases, mannanases and chitinases in *Eukarya*/*Fungi* samples (Salazar and Asenjo, 2007).

Ultimately, the selection of a particular fixation/permeabilization procedure will depend on the characteristics of the microorganism(s) cell envelope to be detected (Felix, 1982).

1.6.4.2. Hybridization step

Hybridization is the second step of a standard FISH protocol. Is characterized by the addition of a buffer containing the labeled oligonucleotide probe to the sample. The probe will then anneal with the specific target sequence inside de the cell, if present (Amann and Fuchs, 2008; Bottari *et al*., 2006; Moter and Gobel, 2000). The buffer composition can included several components besides the detecting probe(s), such blocker and helper probes, inert polymers (most commonly dextran sulfate and others such polyethylene glycol, gelatin or bovine serum), organic solvents (most commonly

formamide, urea or ethylene carbonate), salts (most commonly NaCl, but also sodium citrate, among others), detergents (Triton X-100 or SDS), Denhardt's solution (sodium pyrophosphate, polyvinylpyrrolidone and Ficoll), quelating agents (most commonly EDTA), pH buffering compounds (most commonly Tris-HCl, among others) (Almeida *et al*., 2010; Kessler, 2000; Lawson *et al*., 2011; Matthiesen and Hansen, 2012; Perry-O'Keefe *et al*., 2001; Swiger and Tucker, 1996). A successful FISH procedure results from the optimization of several conditions of hybridization, such time, temperature, ionic strength, among others, which translate into a wide variety of FISH procedures described in the literature (Santos *et al*., 2014).

1.6.4.3. Washing step

In the third step of a standard FISH procedure, the sample is washed, by placing it in a pre-warmed aqueous solution, that will remove all the unbound and nonspecific hybridized probes. To that end several variables that affects duplex stability, such temperature, ionic strength and pH are modulated and optimized in order to achieve a high degree of specificity and sensibility of each procedure. The hybridization together with the washing steps confer the observed specificity of FISH procedures (Amann and Fuchs, 2008; Bottari *et al*., 2006; Moter and Gobel, 2000).

1.6.4.4. Visualization/detection step

The fourth and final step of a FISH procedure is the visualization or detection of the labeled cells by microscopy or flow cytometry, respectively. The first approach allows the direct visualization of the sample, hence providing information about the morphology and spatial distribution of the microorganism, while the second allows a more automatic quantification of the microorganisms in the sample and also the quantification of the fluorescent signal of each individual cell (Amann and Fuchs, 2008; Bottari *et al*., 2006; Moter and Gobel, 2000).

1.6.5. Variables affecting FISH outcome

FISH has a long history and widespread applicability, however its use is not yet straightforward. A successful hybridization is the culmination of a wide variety of variables and their interplay. This means that modifications in one factor have the ability to influence the impact of several others (Figure 1.6). Consequently, the implementation

of a FISH procedure requires *in silico* design, optimization tests and sometimes a trial-and-error approach in order to develop a FISH procedure that provides specific, sensitive and bright outcomes (Bottari *et al.*, 2006; Bouvier and Del Giorgio, 2003; Herzer and Englert, 2001; Santos *et al.*, 2014). It is possible to distinguish two types of variables with significant influence in FISH, the biotic and abiotic variables (Bouvier and Del Giorgio, 2003).

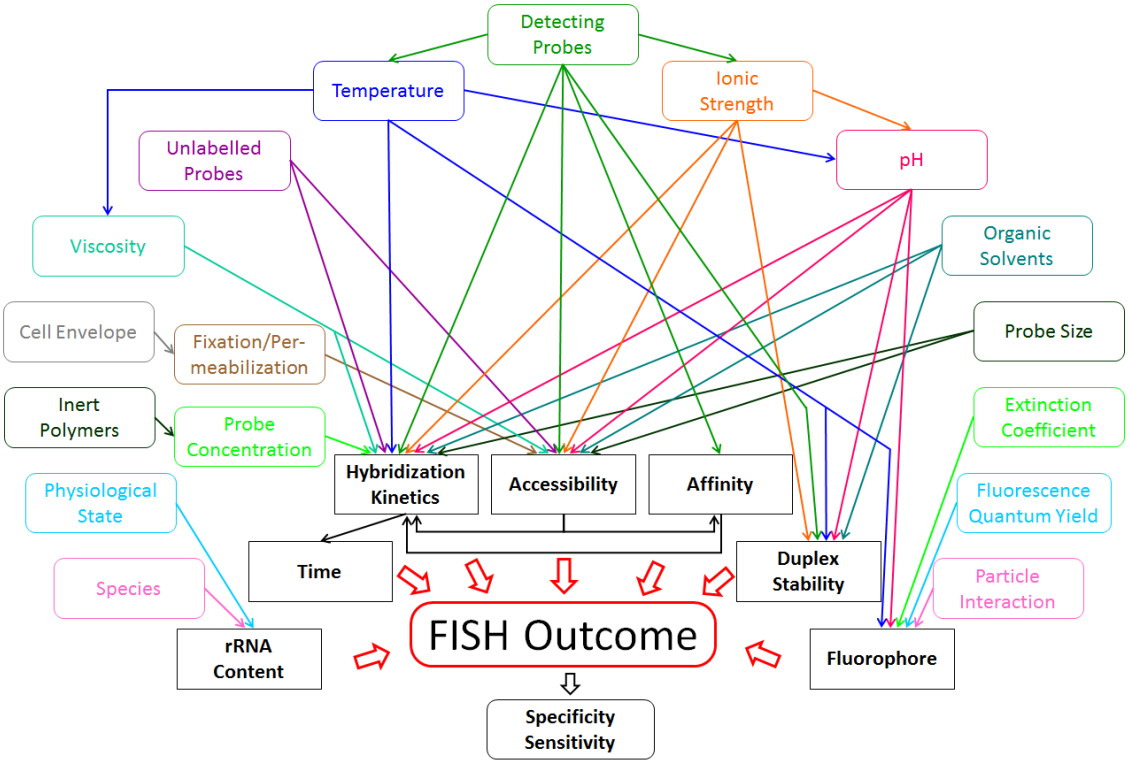

Figure 1.6 - Biotic and abiotic variables that influence the outcome of FISH procedures. The arrows show the interplay between the different variables.

1.6.5.1. Biotic variables

The influence of the biotic variables on FISH outcome are attributed mainly to the rRNA content, physiological state and cell envelope of the microorganisms. Detection of rRNA is appointed as one of the advantages of FISH due to their high abundance in bacterial cells. However, the number of copies varies considerably between species, ranging from a few hundred to around hundred thousand. For example, *Sphingomonas* spp. possess a maximum of 2,000 ribosomes wile in *E. coli* the number can reach up to 72,000 (Amann and Fuchs, 2008; Fegatella *et al.*, 1998; Moter and Gobel, 2000). The rRNA content is also dependent on the physiological state of bacteria, especially the

growth rate. Bacteria that is experiencing starvation and growing slowly or under non-steady-state conditions, generally present lower ribosomal content. Interestingly, the physiological history of the cell also affects rRNA content, as the decrease in rRNA content is slower in bacteria grown under nutrient limitation than non-limited bacteria (Oda *et al*., 2000).

Regarding the cell envelope, this structure is present in most microorganisms, from *Eubacteria*, *Archaea* to *Eukarya* (fungi and algae) cells. It plays a critical role, acting as an exoskeleton, maintaining cell shape and protecting against injury, damage and osmotic lysis (Willey *et al*., 2008). Distinctive cell envelope architectures are found among the three domains (Albers and Meyer, 2011; Bowman and Free, 2006; Madigan *et al*., 2011; Pommerville, 2010; Willey *et al*., 2008).

In the specific case of *Eubacteria*, the principal characteristic of their cell envelope is its composition in peptidoglycan. Peptidoglycan is a polymer composed by the alternation of two sugar derivates, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) through β-1,4-glycosidic bonds. NAM monosaccharides have a side chain attached, composed of four amino acids involved in the establishment of peptide cross-bridges, directly between side chains or through the use of linkers, ultimately conferring rigidity to the peptidoglycan structure (Willey *et al*., 2008; Pommerville, 2010; Madigan *et al*., 2011). There are described more than 100 different peptidoglycan structures, mainly regarding the types of peptide cross-links and side-chains (Schleifer and Kandler, 1972). In *Eubacteria* is possible to distinguish two major forms of cell envelope architecture, Gram-positive and Gram-negative (Figure 1.7). Gram-positive cell envelope is composed of a very thick and rigid peptidoglycan structure, also containing teichoic and lipoteichoic acids intertwined with peptidoglycan. Gram-negative, however, present a small layer of peptidoglycan between the cell membrane and an outer membrane, whose inner half have a similar composition to cell membranes and the outer half mainly composed by lipopolysaccharide (LPS), polysaccharides attached to lipid A molecules (Willey *et al*., 2008; Pommerville, 2010; Madigan *et al*., 2011).

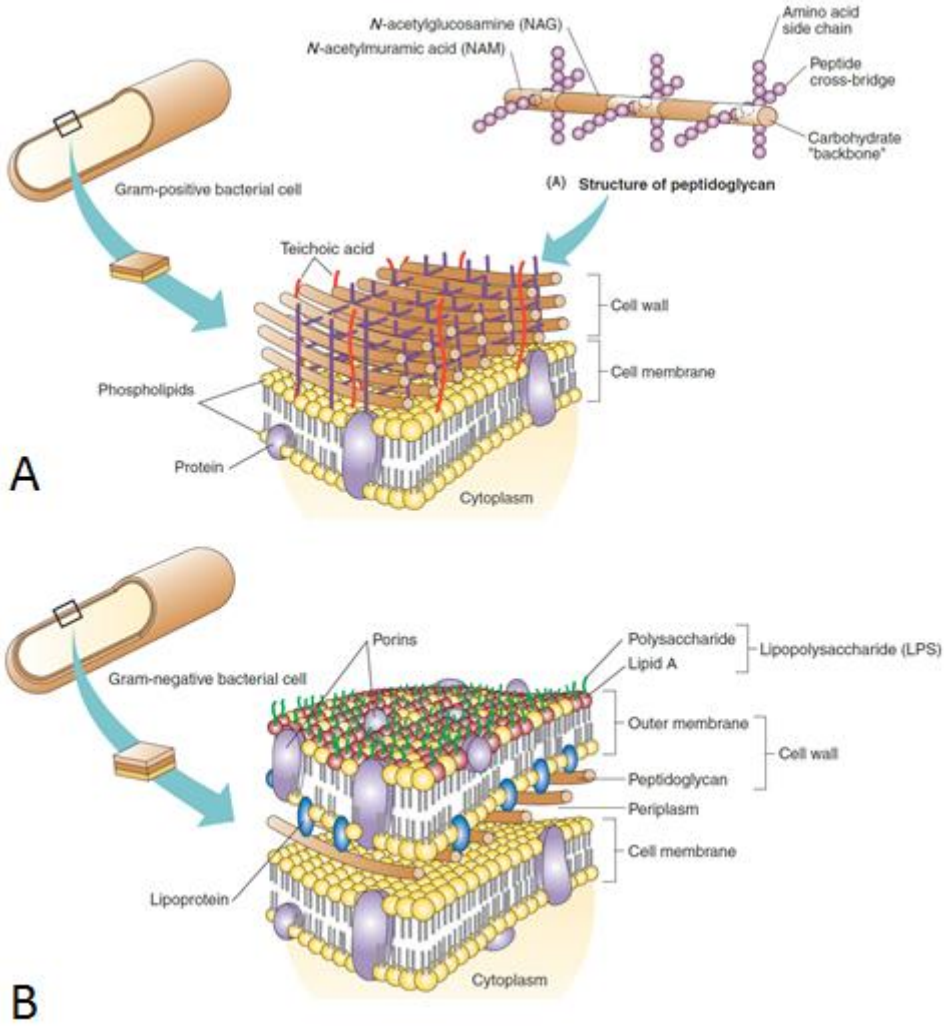

Figure 1.7 - Schematic structure of *Eubacteria* cell envelope: A - Gram-positive; B - Gram-negative (from Pommerville, 2010).

Due to their inherent differences regarding the cell envelope architecture, Gram-positive and Gram-negative bacteria present different susceptibilities to fixation/permeabilization protocols. Thus, the type and duration of the fixation/permeabilization procedure translates into differences in terms of FISH outcome. This, ultimately translates into a mandatory protocol optimization in order to assess the conditions that provide the best results (Moter and Gobel, 2000). Nonetheless, a fixation/permeabilization protocol using 3 - 4% (wt/vol) formaldehyde or paraformaldehyde solution followed by ethanol 50% (vol/vol) step is generally sufficient for a successful FISH outcome in most Gram-negative and Gram-positive bacteria. When that is not true, other and/or additional steps including the use of enzymes, solvents, detergents or even hydrochloric acid may resolve the problem (Amann and Fuchs, 2008; Frickmann *et al*., 2017).

1.6.5.2. Abiotic variables

The abiotic variables influence FISH by ultimately interfering with the ability of the probe to reach and recognize its target and/or influence the brightness of the signal. In the next paragraphs the interference of each variable on FISH is addressed.

The hybridization time is intrinsically related to the kinetics of the process and the accessibility of the probe from the extracellular space until it reaches and binds to its target (Bottari *et al*., 2006; Bruns *et al*., 2007; Santos *et al*., 2014; Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004). The hybridization of two complementary sequences, or in this specific case of a probe binding to its target, is the result of two events, the nucleation reaction and the rapid zippering. The nucleation reaction initiates the hybridization process, being characterized by the hybridization of a small number of base pairs. From the formation of this structure, a rapid zippering of the remaining nucleotides happens, originating a stable hybrid. Due to its nature, the nucleation reaction is the rate limiting step in nucleic acid hybridization (Bruns *et al*., 2007). Several parameters, described in this section, have the ability to influence the kinetics of nucleic acid hybridization and therefore able to influence, lowering or increasing, the time required to have a successful FISH outcome (Bruns *et al*., 2007; Kessler, 2000; Nakamura, 1990; Swiger and Tucker, 1996; Wetmur, 1991).

Accessibility is other parameter that can influence the time required to a have a successful FISH outcome. It is a function of two major rate limiting factors, probe diffusion through the cell envelope and probe diffusion within the structure of the ribosome to reach the target site (Bottari *et al*., 2006; Yilmaz *et al*., 2006). The effect of the envelope permeabilization, as previously discussed, is easily understood. A poor permeabilization will hinder the access of the probe inside the cell, and as a consequence longer hybridization times will be required in order to have a visible result. Regarding the accessibility within the ribosomal structure, it results from the three-dimensional organization of the ribosome, mainly from the secondary rRNA-rRNA interactions. However, the influence of tertiary rRNA-rRNA and protein-rRNA interactions cannot be fully discarded (Bottari *et al*., 2006; Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004). In fact, the first systematic studies addressing the issue of ribosomal accessibility within the small and large subunits introduced the concept of easy accessible in opposition to inaccessible regions, in the absence or presence of the previously referred interactions, respectively (Behrens *et al*., 2003; Fuchs *et al*., 1998; Fuchs *et al*., 2001). However, later

studies refuted this idea, and found that these regions of highly rRNA-rRNA and rRNA-protein interactions, for example in the 16S rRNA 3' major domain, are not inaccessible, but instead require longer hybridization times (from 3h to 96h using DNA probes) in order to have a positive outcome (Yilmaz *et al*., 2006).

As seen before, the FISH outcome is a function of the accessibility of the probe to the target site (Bottari *et al*., 2006; Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004). However, the dependence on the type of organism, on the exact location of the target site and on the type of fluorophore used, raised the question if accessibility was the only non-methodological factor affecting hybridization outcome. Further studies confirm those suspicions and the notion of affinity was introduced. Affinity is a thermodynamic parameter that results from the proposed hybridization mechanism reported for FISH by Mathews *et al* (1999). The affinity model states that the hybridization efficiency is not only the result of the accessibility to the target site, rRNA-rRNA interactions, but also from probe-probe interactions and probe-target interactions (Figure 1.8) (Bottari *et al*., 2006; Yilmaz and Noguera, 2004; Yilmaz and Noguera, 2006).

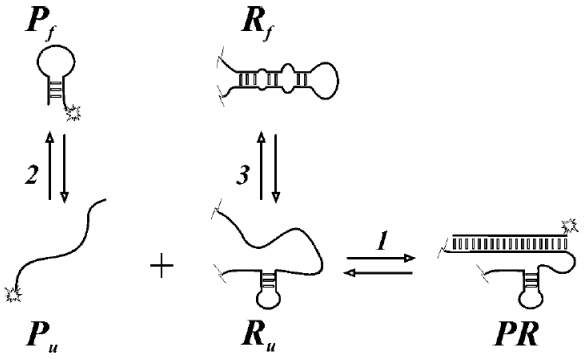

Figure 1.8 - Model representing the hybridization mechanism as proposed by Mathews *et al* (1999), between a probe (P) and an rRNA target (R) to form a probe/target complex (PR). Subscripts f and u indicate folded and unfolded states, respectively (from Yilmaz and Noguera, 2007).

The model assumes that in order to allow the binding of the probe to the complementary target, reaction 1, the target region must undergo conformational changes, from the folded to unfolded state - reversible unfolding - represented in reaction 3, while similarly, the probe itself needs to undergo unfolding in case of self-complementarity, represented in reaction 2. Therefore, affinity can be estimated as an overall Gibbs free energy change ($\Delta G°$overall), obtained from the estimation of free energy change for the three individual reactions, $\Delta G°_1$, $\Delta G°_2$ and $\Delta G°_3$ (Yilmaz and

Noguera, 2004). A more negative ΔG°overall represents a greater concentration of the hybrid and, thus, greater brightness of the sample (Yilmaz and Noguera, 2004). In order to maximize hybridization efficiency, a theoretical threshold of −13.0 Kcal/mol should be targeted (Yilmaz *et al*., 2006).

Temperature is possibly one of the most important methodological factors determining the success or failure of any FISH procedure. Temperature significantly affects the hybridization kinetics, hybrid stability and drives the specificity and sensibility of a given FISH procedure (Kessler, 2000; Nakamura, 1990; Swiger and Tucker, 1996; Wetmur, 1991). The formation and stability of the duplex between two complementary sequences arises from the establishment of hydrogen bonds between complementary base pairs, A-T/U and C-G and through electrostatic as well as hydrophobic interactions (Kessler, 2000; Nakamura, 1990). High temperatures will either prevent annealing of complementary strands, or lead to duplex dissociation, due to disruption of nucleic acid bonding forces, mainly by impairing hydrogen bond formation or through the disruption of the established hydrogen bonds, respectively (Marky *et al*., 2010; Nakamura, 1990). Therefore, temperature must be a compromise between specific hybridization and signal outcome (Figure 1.9). High temperatures will disable the annealing of the probe to the target sequence and low temperatures will allow the annealing of the probe to mismatch sequences leading to false positive results. To this moment, an accurate model prediction of the optimal hybridization temperature for a given oligonucleotide probe in FISH procedures is yet out of reach (Fontenete *et al*., 2016).

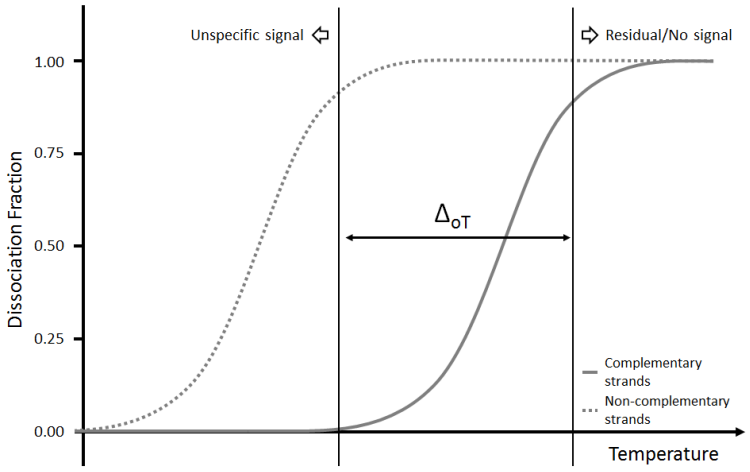

Figure 1.9 - Dissociation fraction curves for complementary strands (full line) and for strands with one mismatch (dotted line). Optimal hybridization temperature range (Δ_{oT}) to be optimized in order to avoid loss of signal (Temperature > Δ_{oT}) and nonspecific hybridization (Temperature < Δ_{oT}).

Ionic strength, like temperature, significantly affects hybridization kinetics and hybrid stability (Kessler, 2000; Wetmur, 1991). Monovalent cations, namely sodium and potassium ions (Herzer and Englert, 2001), neutralize electrostatic repulsive forces between the negatively-charged phosphate groups on opposing strands, allowing their approximation and consequently the occurrence of the nucleation reaction, that would not be favorable otherwise. However, high salt concentrations, above 1M, have also the ability to destabilize the duplexes. This arises from the solvation of the hydrophobic nucleic acid bases, favoring the ss rather than the ds conformation. A similar process happens with nucleic acids exposed to organic solvents, as addressed below (Doktycz, 1997). Increasing ionic strength also leads to the strengthening of the hydrophilic interactions due to a decrease in the solubility of the nucleotide bases (Kessler, 2000; Nakamura, 1990; Swiger and Tucker, 1996; Wetmur, 1991).

The use of organic solvents in the hybridization solution, such as formamide, urea or ethylene carbonate have a significant impact on the hybridization kinetics and hybrid stability (Bouvier and Del Giorgio, 2003; Kessler, 2000; Nakamura, 1990; Swiger and Tucker, 1996). Their effect is applied at a higher or minor extent by two different mechanisms. On the one hand, organic solvents act by impairing hydrogen bonding between complementary base pairs; and, on the other hand, by reducing the hydrophobic interactions between the duplex, contributing to their destabilization (Matthiesen and Hansen, 2012; Priyakumar *et al*., 2009). The first mechanism is in nature similar to what happens by increasing hybridization temperatures and ionic strength above 1M (Yilmaz and Noguera 2007). In fact, the introduction of organic solvents in the hybridization solution results in a decrease in the thermal stability of nucleic acids, implying that hybridization will be performed at lower temperature (Yilmaz and Noguera 2007). As expected, the organic solvent destabilization effect is more evident in A-T/U, rather than C-G rich sequences (Swiger and Tucker, 1996; Yilmaz and Noguera 2007). Furthermore, the introduction of organic solvents has the disadvantage of longer hybridization steps being required for a successful hybridization (Swiger and Tucker, 1996). The use of organic solvents, namely formamide, in the hybridization solution is also suggested to have a denaturing effect on potentially rate-limiting higher-order structures of ribosomes, such as in their secondary and tertiary interactions (Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004). Moreover, Santos et al (2014), have recently noticed a damaging effect of formamide on the cell envelope of bacteria.

The viscosity of the solvent in the hybridization solution negatively impacts the hybridization kinetics (Kessler, 2000; Wetmur, 1991). This effect is due to the fact that the constant for duplex formation, a function of the nucleation reaction, is inversely proportional to viscosity (Wetmur, 1991). With increased viscosity a decrease in molecular diffusion is observed, and that will be translated into slower hybridization rates (Kessler, 2000; Kosar and Phillips, 1995; Zustiak *et al*., 2011).

The pH has little or no effect on the hybridization kinetics and hybrid stability of the probe target duplexes, as long as the assay is performed between pH 5 to 9. In fact, in this interval the nucleotide bases are uncharged and hybridization occurs without interference. However, at higher pH, guanine, uracil and thymine bases become deprotonated (pKa 9.2 - 9.7), while at lower pH, adenine and cytosine bases become protonated (pKa 3.5 and 4.2). This ultimately disfavors pairing, through an increase in electrostatic repulsion destabilizing hydrogen bonding (Blackburn *et al*., 2006; Kessler, 2000; Swiger and Tucker, 1996; Vieregg, 2010; Wetmur, 1991).

Inert polymers are generally used in the hybridization solution in order to accelerate the hybridization reaction. The acceleration is accomplished by the decrease of the free space in the hybridization reaction and thus the volume of solvent available for the probe, which is translated into an apparent increase in probe concentration (Kessler, 2000; Nakamura, 1990; Swiger and Tucker, 1996; Wetmur, 1991). Polymers such as dextran sulfate, polyethylene glycol, gelatin or bovine serum albumin can be employed to this end. Dextran sulfate is the most frequently used polymer due to its higher hydrophilic behavior in comparison to DNA or RNA molecules and due to the fact that is strongly hydrated in aqueous solutions (Kessler, 2000; Swiger and Tucker, 1996).

As mentioned above, much of the success of a FISH procedure relies on the choice of a suitable fluorophore. Dyes with high extinction coefficients, greater than 10,000/M·cm and high fluorescence quantum yields, higher than 0.2, are recommended (Morrison *et al*., 2003). Besides their spectral properties, the stability of the dyes under working conditions (pH, temperature, etc.), interaction with cellular components, cellular debris, extracellular matrix and slide surface, must also be taken into consideration as they can lead to loss of signal and/or background staining (Bouvier and Giorgio, 2003; Morrison *et al*., 2003; Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004).

Currently, there are a diverse array of oligonucleotides that can be used as probes in FISH, from conventional DNA and RNA to synthetic molecules such peptide nucleic

acid (PNA) or locked nucleic acid (LNA) (Cerqueira *et al*., 2008). Possibly the most important characteristic to be evaluated when selecting the type of probe relies in the stability of duplex formation (Nakamura, 1990). RNA-DNA duplexes are the least stable, followed by RNA-RNA and finally the synthetic duplexes, PNA-RNA and LNA-RNA, (Cerqueira *et al*., 2008; Kessler, 2000; Nakamura, 1990). This arises either from the lack of repulsion between backbones, in the case of PNA; or a more efficient base stacking of the duplexes, in the case of LNA (Bhattacharyya *et al*., 2011; Cerqueira *et al*., 2008). Other characteristic that may influence the choice of probe type is the ability of the probe to diffuse across the cell envelope, that is easier in the case of PNA than DNA, RNA or LNA, due to its neutral backbone (Cerqueira *et al*., 2008; Lefmann *et al*., 2006). The resistance to degradation by nucleases and proteases is other important characteristic, being far superior for PNA and LNA due to their synthetic nature, than for RNA or DNA (Cerqueira *et al*., 2008). Finally, mismatch discrimination also varies among oligonucleotides. As an example, PNA and LNA have superior capacity for mismatch discrimination than DNA probes (Fontenete *et al*., 2015).

Probe size, either in terms of probe-reporter molecule complex and number of nucleotides that composes it, impacts FISH outcome at several levels. Namely, on probe accessibility to the target, hybridization kinetics and the ability to discriminate mismatches (Bottari *et al*., 2006; Bruns *et al*., 2007; Kessler, 2000; Muro, 2005; Nakamura, 1990; Yilmaz and Noguera 2004). In terms of accessibility, increasing the size of the probe complex leads to higher difficulty in accessing to their target, either by impairment at the cell envelope or at the target site (Bottari *et al*., 2006; Yilmaz and Noguera 2004). Probe length is also a factor that is associated with the nucleation reaction, as for a given probe concentration, the hybridization rate is inversely proportional to its length (Bruns *et al*., 2007; Kessler, 2000; Muro, 2005; Nakamura, 1990). Finally, probe length also has a direct impact on mismatch discrimination and therefore in the sensitivity and specificity of the method. Shorter probes are able to more easily distinguish targets with mismatch(es) than longer probes. This observation arises from the fact that longer probes dilute the impact of the mismatch(es) between a higher number of nucleotides, than in shorter probes (Bottari *et al*., 2006; Nakamura, 1990).

Probe concentration has a significant impact in hybridization kinetics (Bruns *et al*., 2007; Kessler, 2000; Wetmur, 1991). The hybridization of complementary sequences follows a second order kinetics if in solution the concentration of the hybrid strands is

similar. In this case, higher concentration of hybrid strands in solution translates into higher hybridization rates (Bruns *et al*., 2007). However, FISH protocols usually use probe concentration in excess relatively to the number of target sequence(s) (Yilmaz and Noguera, 2004), so a pseudo-first order kinetics is applied (Bruns *et al*., 2007). Thus, hybridization depends only on the concentration of the target, however, the hybridization rate is proportional to probe concentration (Bruns *et al*., 2007; Kessler, 2000; Wetmur, 1991).

Besides the use of probes attached to a reporter molecule, the detection probes, other unlabelled probes are sometimes included in the hybridization solution in order to increase specificity or sensibility of the FISH procedure, namely blocker and helper probes, respectively (Bottari *et al*., 2006; Stender *et al*., 2001b; Swiger and Tucker, 1996). Blocker probes are used to suppress the binding of the detecting probe to unwanted target sequences. In this way a specific signal is achieved without the need to use more stringent hybridization conditions that could decrease the signal-to-noise ratio (Stender *et al*., 2001b). Helper probes are designed to bind adjacently to the detecting probe. Their function aims to unfold the structure of the nucleic acid near to the target site, thus facilitating the accessibility of the probe to its target (Bottari *et al*., 2006; Swiger and Tucker, 1996).

In a FISH procedure, the most important compound in the hybridization solution is the labeled oligonucleotide probe, the detection probe, which will hopefully, bind to the desired target inside de the cell. In order to have a successful FISH protocol, careful attention must be paid to the design of this probe. Generally, the designing or selection of the probe should consider specificity, sensitivity, ease of tissue and cell penetration and affinity to its target (Bottari *et al*., 2006). However, other characteristics can be fundamental (Amann, 1995; Cerqueira *et al*., 2008; Muro, 2005; Nakamura, 1990; Stahl and Amann, 1991; Yilmaz *et al*., 2006):

- The type of nucleic acid or nucleic acid mimic should complement perfectly the intention of the work; all the options have advantages and disadvantages that make them good or bad candidates for any specific assay;
- The design of a good probe is as good as the database accessed;
- Sensibility (the ability to detect the target organisms) and specificity (ability to discriminate non-target organisms) of a probe should be well balanced;

- In order to achieve a good degree of specificity, a probe should be designed with the highest number and more centrally located mismatches as possible;
- Maximize mismatch discrimination by the use of short rather than longer probes;
- Avoid self-complementary sequences within probe design, especially in nucleic acid mimic probes, as these will lead to the formation of hairpin structures, that will be translated into low/absent fluorescence outcomes due to non-dissociation of the probe-probe duplexes;
- The design of the probe should have a GC content from 40 to 60%, since higher values have higher probability of nonspecific binding;
- Avoid sequences containing more than four single base repetitions;
- Design probes with an affinity value of at least -13.0 Kcal/mol.

1.6.6. FISH limitations

Despite its importance and wide application in microbiology, standard FISH procedure presents some limitations that affect its robustness, leading to the presentation of false positive or false negative results (Moter and Gobel, 2000; Wagner *et al*., 2003). Sample autofluorescence for example, can be intrinsic of some microorganisms. It has been observed in molds, yeasts, cyanobacteria, members of *Pseudomonas*, *Legionella* and *Rodospirillum* genera for example. It can also be due to the material surrounding the bacteria, namely, organic and inorganic debris (Moter and Gobel, 2000). This phenomenon ultimately leads to erroneous results, decreasing signal-to-noise ratio and masking the specific fluorescent signal. As previously addressed, other important limitation is the insufficient probe accessibility to the target site, either by impairment at the cell envelope, or due to the three-dimensional structure of the ribosome (Bottari *et al*., 2006; Moter and Gobel, 2000; Wagner *et al*., 2003; Yilmaz *et al*., 2006; Yilmaz and Noguera, 2004). Furthermore, the use of rRNA-targeted oligonucleotide probes, which are covalently mono-labelled with fluorescent dyes, limits the sensitivity of the method and is especially problematic when analyzing microorganisms with low ribosome content (Moter and Gobel, 2000; Wagner *et al*., 2003). Another drawback of FISH is that specific hybridization and washing conditions cannot be accurately determined for uncultured microorganisms (Wagner *et al*., 2003). In addition, quantitative analyses in complex samples (*e.g.* densely colonized biofilms) requires time-consuming manual microscopic

counting which leads to low accuracy in their quantification and impair the analysis of relatively high sample numbers (Wagner *et al*., 2003).

Other limitations arise from exploiting rRNA as the target molecule for the design of the probes. The differentiation of closed related microorganisms through the design of a probe for a single target site can be laborious or even an impossible task. However, the contrary is also frequent, namely in the detection of a diverse number of strains or species with a single probe. (Moter and Gobel, 2000; Wagner *et al*., 2003). Furthermore, FISH does not provide information on the ecophysiology of the identified microorganisms and even the general physiological activity of a cell cannot always be inferred from the cellular rRNA content (Morgenroth *et al*., 2000; Oda *et al*., 2000; Wagner *et al*., 1995).

As previously stated, significant methodological improvements of FISH and their combination with other methods have been reported, which allow to overcome some of the limitations described above, increasing the robustness of FISH technique (Table 1.8).

1.7. PNA-FISH as a robust tool for de detection of foodborne pathogens

Peptide nucleic acid (PNA) is a nucleic acid mimic were the negatively charged sugar-phosphate backbone of DNA is replaced by a neutral polyamide backbone composed of N-(2-aminoethyl) glycine units (Figure 1.10) (Nielsen *et al*., 1991). Although PNA has a different backbone composition, the chemical configurations of the nucleobases are positioned practically in the same place and within the same distance as it occurs to the natural DNA. This allows PNA to hybridize with complementary DNA or RNA sequences (Nielsen, 2001; Shakeel *et al*., 2006).

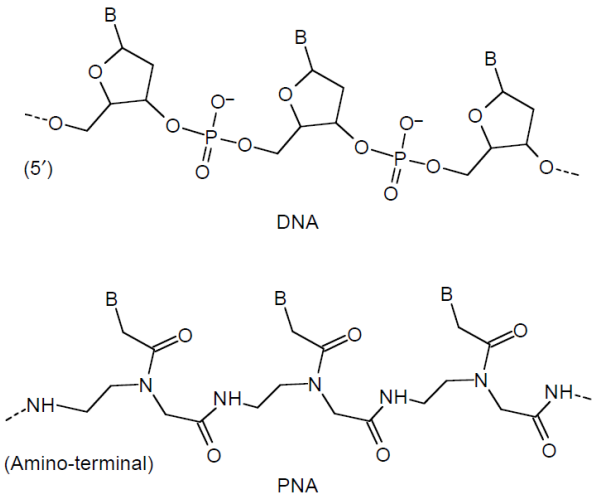

Figure 1.10 - Chemical structure of DNA and DNA mimic PNA (from Nielsen, 2001).

PNA probes were first introduced in FISH studies for the detection of microorganisms during the late 1990's (Drobniewski *et al*., 2000; Prescott and Fricker, 1999; Stender *et al*., 1999; Stender *et al*., 2000) and since then several PNA probes have been described for the detection of a diverse array of microorganisms (Cerqueira *et al*., 2008). The use of PNA probes allowed the construction of more robust, more specific and faster FISH procedures. It also brings several advantages over DNA probes, namely the lack of electrostatic repulsion, due to the non-charged nature of the PNA backbone and higher thermal stability in comparison to with DNA/DNA duplexes (Perry-O'Keefe *et al*., 2001; Nielsen, 2001). This implies higher melting temperatures (T_m) and higher binding affinities for PNA/DNA than for DNA/DNA duplexes (Cerqueira *et al*., 2008; Schwarz *et al*., 1999). This increased T_m allows the design of shorter PNA probes, approximately 15 base pairs (bp), than most DNA probes that go up to 30bp (Bottari *et al*., 2006; Cerqueira *et al*., 2008). The effect on the T_m of a single-base mismatch in a duplex has a higher impact in the PNA/DNA hybridization than in a DNA/DNA hybridization, bringing higher specificity to PNA over DNA probes (Fontenete *et al*., 2015; Lomakin and Frank-Kamenetskii, 1998). In addition, hybridization can be efficiently performed under low salt concentrations, which promotes the destabilization of rRNA-rRNA interactions (Orum *et al*., 1998), thus improving accessibility to target sites out of reach with standard DNA or RNA probes (Fuchs *et al*., 1998; Fuchs *et al*., 2001; Yilmaz *et al*., 2006). As with other synthetic molecules, PNA presents an increased resistance to nucleases and proteases (Demidov *et al*., 1994; Stender *et al*., 2002; Wagner *et al*., 2003). Finally, diffusion through the cell envelope and naturally occurring microstructures might be facilitated, even in Gram-positive species, due to the hydrophobic character of PNA in comparison to DNA (Drobniewski *et al*., 2000; Lefmann *et al*., 2006).

The increased robustness and performance characteristics of PNA-FISH in comparison to conventional DNA-FISH procedures, allowed PNA-FISH to become an important tool for the specific and fast detection of several clinical relevant microorganisms on a broad range of samples (Cerqueira *et al*., 2008; Frickmann *et al*., 2017). Of these, food safety applications, namely in the detection of foodborne pathogens, as risen in the last years. Consequently, several pathogens have been successfully detected in a variety of food matrices (Almeida *et al*., 2009; Almeida *et al*., 2010; Almeida *et al*., 2013; Machado *et al*., 2013; Zhang *et al*., 2012; Zhang *et al*., 2015).

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Chapter 2

Development and application of Peptide Nucleic Acid Fluorescence *in situ* Hybridization for the specific detection of *Listeria monocytogenes*.

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(publication on-hold due to possible patent submission)

Abstract

Listeria monocytogenes is one of the most important foodborne pathogens due to the high hospitalization and mortality rates associated to an outbreak. Several new molecular methods that accelerate the identification of *L. monocytogenes* are continuously being developed, however conventional culture-based methods still remain the gold standard. In this work we developed a novel Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) method for the specific detection of *L. monocytogenes*. The method was based on an already existing PNA probe, LmPNA1253, coupled with a novel blocker probe in a 1:2 ratio. The method was optimized for the detection of *L. monocytogenes* in food samples through an evaluation of several rich and selective enrichment broths. The best outcome was achieved using One Broth Listeria in a two-step enrichment of 24h plus 18h. For validation in food samples, ground beef, ground pork, milk, lettuce and cooked shrimp were artificially contaminated with two ranges of inoculum: a Low Level (0.2 - 2 CFU/25g or mL) and a High Level (2 - 10 CFU/25g or mL). Samples were then enriched and analyzed by both PNA-FISH and the traditional method, ISO 11290-1. The PNA-FISH method performed well in all types of food matrices to a detection limit as low as 0.5 CFU/25 g or mL of food sample. Results indicate that PNA-FISH performed similarly to the traditional culture method and can reduce the procedure time for up to two days.

Keywords

Listeria monocytogenes, PNA-FISH, Blocker Probe, Enrichment Procedure.

2.1. Introduction

Listeria spp. are Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria with a low G+C content (Ponniah *et al*., 2010). The *Listeria* genus is composed of seventeen species and among them, *Listeria monocytogenes* is a primary human pathogen. Nonetheless, there have been rare reports of illnesses caused *by Listeria seeligeri, Listeria ivanovii* and *Listeria innocua* (Gasanov *et al*., 2005; Guillet *et al*., 2010; Perrin *et al*., 2003; Weller *et al*., 2015).

L. monocytogenes is recognized worldwide as an important foodborne pathogen due to high morbidity, hospitalization (over 90%) and mortality (25 to 30%) rates in vulnerable populations (pregnant, neonates, elderly and immunocompromised people) (Zunabovic *et al*., 2011). Symptoms of listeriosis (illnesses associated with *Listeria* spp. infections) range from flu-like illness to severe complications including meningitis, septicemia and spontaneous abortion (FAO/WHO, 2004). In 2015 the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported 2,206 confirmed human cases of listeriosis in the 28 European Member States (0.46 cases per 100,000 population), 97.4% of which needed hospitalization. More importantly, 270 of these cases were fatal. EFSA/ECDC also reported an increasing trend of listeriosis since 2008, but the number of cases has stabilized from 2014 onwards (EFSA/ECDC 2015; 2016). Infection with *L. monocytogenes* in humans occurs in 99% of the cases as a result of consumption of contaminated ready-to-eat and raw food products such as vegetables, milk, soft cheese, meat, poultry, seafood and dairy products (Mead *et al*., 1999; Schlech and Acheson, 2000; Volokhov *et al*., 2002).

The detection, differentiation and identification of *Listeria* spp. usually depends on phenotypic, biochemical and immunological assays as well as genotypic methodologies (Zunabovic *et al*., 2011). Conventional culture-based methods for detecting *Listeria* spp. in foods, ISO 11290-1:1996 (Horizontal method for the detection and enumeration of *Listeria monocytogenes*), are simple to perform, but they are also time-consuming and take too long to deliver the results. Consequently, culture-independent approaches, such as Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) techniques, have become important tools for the specific, reliable and fast detection of human pathogens (Rohde *et al*., 2015).

Several 16S or 23S rRNA probes have been developed for the detection of *Listeria* spp. by FISH methods (Almeida *et al*., 2011; Brehm-Stecher *et al*., 2005; Fuchizawa *et al*., 2008, Fuchizawa *et al*., 2009; Moreno *et al*., 2011; Schmid *et al*., 2003; Wang *et al*., 1991; Zhang *et al*., 2012) but only a few of them are able to specifically detect *L. monocytogenes* (Almeida *et al*., 2011; Fuchizawa *et al*., 2009; Moreno *et al*., 2011; Wang *et al*., 1991; Zhang *et al*., 2012). These methods have been described as being highly specific and sensitive but there is no comparison between the probes. Additionally, due to the advent of genome sequencing technologies, public databases are now much more updated and accurate and thus theoretical estimation is more realistic.

In this study we started by performing a theoretical evaluation of the published probes for the specific detection of *L. monocytogenes*. The most promising probe was further evaluated in a representative set of bacterial strains and the addition of a blocker probe was assessed to increase method specificity. Finally, the enrichment step was optimized for the detection of *L. monocytogenes* in food matrices through PNA-FISH and its performance compared to ISO 11290-1:1996.

2.2. Materials and methods

2.2.1. Bacterial strains and culture maintenance

A total of 67 bacterial strains from both the genus *Listeria* and other related genera were included in this study (Table 2.1). The *Streptococcus* strain was maintained on Columbia agar (Oxoid, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (Probiológica, Portugal) and incubated at 37°C in a CO2 incubator (HERAcell 150; Thermo Electron Corporation, United States of America) set to 10% (vol/vol) CO2 and 5% (vol/vol) O2. Single colonies were streaked onto fresh plates every 2 or 3 days. *Gemella morbillorum* was grown in Brain Heart Infusion (BHI) (Liofilchem, Italy) supplemented with 5% bovine calf serum; *Brochothrix thermosphacta* was maintained in Corynebacterium agar (1% casein peptone; 0.5% yeast extract; 0.5% Glucose; 0.5% NaCl and 1.5% agar) (Liofilchem); *Lactobacillus paracasei* was maintained in MRS agar (Liofilchem) and *Lactococcus lactis* was maintained in YGLPB medium (1% peptone; 0.3% yeast extract; 0.5% glucose; 0.5% lactose; 0.8% beef extract; 0.25% KH2PO4; 0.25% K2HPO4; 0.02% MgSO4.7H2O and 0.005% MnSO4.4H2O) (Liofilchem). *Bacillus thurigiensis* and *Bacillus thermosphacta* strains were grown at

26ºC, while *L. paracasei* and *G. morbillorum* strains were grown under anaerobic conditions. All remaining bacterial species were maintained on BHI at 37 ºC and streaked onto fresh plates every 24 hours.

2.2.2. Theoretical evaluation of the probes

The theoretical specificity and sensitivity of the probes described for *L. monocytogenes* were evaluated using the TestProbe analysis software at SILVA database (Quast *et al*., 2013). The probes were aligned with a total of 1922213 sequences present in the SILVA 16SREF database (last accession in September 2017). They were also tested against the large subunit (23S/28S, LSU) database, to evaluate the existence of possible cross-hybridization. Specificity was calculated as nLm/(TnLm)x100, where nLm stands for the number of non-*L. monocytogenes* strains that did not react with the probe and TnLm for total of non-*L. monocytogenes* strains examined. Sensitivity was calculated as Lm/(TLm)x100, where Lm stands for the number of *L. monocytogenes* strains detected by the probe and TLm for the total number of *L. monocytogenes* strains existent in the databases (Almeida *et al*., 2010).

The selected sequence, 5'- GACCCTTTGTACTAT -3' (Almeida *et al*., 2011), was synthesized (PANAGENE, South Korea) and the oligonucleotide N terminus attached to Alexa Fluor 568 via a double AEEA linker (-8-amino-3,6-dioxa octanoic acid).

2.2.3. Blocker probe design

A blocker probe suppresses the binding of the detecting probe to an unwanted target sequence (Stender *et al*., 2001). In this work a blocker probe was designed to block non-specific binding to non-*L. monocytogenes Listeria* species. The LmPNA1253 probe was aligned with the 16S rRNA sequences from both *L. monocytogenes* and other *Listeria* species. Sequences were obtained from SILVA database and the alignments were performed using the Clustal Omega program available at the EBI website (http://www.ebi.ac.uk/). Both blocker and detection probes were evaluated regarding their melting temperatures and free energy (Giesen *et al*., 1998; Yilmaz and Noguera, 2004) to ensure a similar affinity to the corresponding target sequences. The blocker probe was also synthesized as described above but no linker or fluorochrome were added to the probe.

2.2.4. Application of the PNA-FISH procedure

The hybridization procedure was performed as previously reported in Almeida *et al*. (2010) with some modifications. Smears of each strain were prepared by standard procedures, immersed in 4% (wt/vol) paraformaldehyde (Sigma) followed by 50% (vol/vol) ethanol (Fisher Scientific) for 10 minutes each and allowed to air dry. The smears were then covered with 20 μL of hybridization solution containing 10% (wt/vol) dextran sulfate (6,500 - 10,000 Molecular Weight - Sigma), 10 mM NaCl (Sigma), 5.5% (vol/vol) formamide (Sigma), 0.1% (wt/vol) sodium pyrophosphate (Sigma), 0.2% (wt/vol) polyvinylpyrrolidone (average 10,000 Molecular Weight - Sigma), 0.2% (wt/vol) Ficoll (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (vol/vol) Triton X-100 (Sigma), 50 mM Tris-HCl (pH 7.5; Sigma), 200 nM PNA probe and 400 nM of blocker probe. Samples were covered with coverslips, placed in moist chambers and incubated for 60 minutes at 60ºC. Subsequently, the coverslips were removed and the slides were submerged in a pre-warmed (60ºC) washing solution containing 15 mM NaCl (Sigma), 0.1% (vol/vol) Triton X (Sigma) and 5 mM Tris Base (pH 10; Sigma). Washing was performed at 60ºC for 30 minutes and the slides were subsequently air-dried. During protocol optimization, the hybridization was performed at different hybridization and washing temperatures (55 to 65ºC), hybridization times (45 to 90 minutes), formamide concentrations (5.5%, 30% and 50% [vol/vol]) and blocker and detection probe concentrations (1:1 and 2:1 ratio). The above described procedure was the one that allowed a better discrimination between *L. monocytogenes* and non-*L. monocytogenes* strains and used throughout the rest of the work.

2.2.5. Optimization of an enrichment step for *L. monocytogenes* detection in food samples

After PNA-FISH optimization, several enrichment broths were tested in order to obtain a positive PNA-FISH output for artificially contaminated samples with concentrations as low as 1 CFU/25 g or mL of food. Several time points, from 8 to 48h at 30ºC or 37ºC were also tested. Both universal and selective enrichment broths were evaluated. Universal broths included: BHI, Tryptic Soy Broth (TSB) (Liofilchem), Buffered Peptone Water (BPW) (Liofilchem) and Universal Preenrichment Broth (UPB) (Becton Dickinson). Selective enrichment broths for Listeria spp. and L. monocytogenes tested were: University of Vermont (UVM) (Liofilchem), Demi-Fraser Broth (DFB)

(Liofilchem), Fraser Broth (FB) (Liofilchem), Buffered Listeria Enrichment Broth (BLEB) (Liofilchem) and One Broth Listeria (OBL) (Oxoid). Two-step enrichment protocols were also tested using selective broths, as follows: UVM-UVM, UVM-BLEB, UVM-FB, UVM-OBL, OBL-OBL, OBL-UVM, OBL-BLEB, OBL-FB. The first medium was always used to dilute the matrix using 225 mL of broth + 25 g or mL of food sample in a stomacher bag; while the second medium was used in a 9 mL-tube, inoculated with 1 mL of the previous enrichment.

At specific time points, 8, 12, 18, 24, 36 and 48h of the enrichment step, 20 µL of the sample were placed directly in the microscope slide, dried in the incubator and then hybridization was performed as described above.

2.2.6. Detection of *L. monocytogenes* in artificially contaminated food matrices

To assess the performance of the pre-enrichment step in the detection of *L. monocytogenes* by PNA-FISH, five different food matrices from a local retailer (Pingo Doce, Portugal) were tested: ground beef, ground pork, milk, lettuce and cooked shrimp. These matrices were selected to evaluate the suitability of method in a diverse array of matrices including meats, seafood, vegetables and dairy products, known for a recurrent prevalence of *L. monocytogenes* and/or frequently associated with listeriosis outbreaks (Adzitey and Huda, 2010; Larivière-Gauthier *et al*., 2014; Navratilova *et al*., 2004; Paranjpye *et al*., 2008; Rebagliati *et al*., 2009; Ryser and Marth, 2007; Shantha and Gopal, 2014; Smith *et al*., 2011; Thévenot *et al*., 2006; Wan Norhana *et al*., 2010; Zhu *et al*., 2017).

Three bulk batches for each matrix were prepared, one non-inoculated (NI) used to check for the presence of *L. monocytogenes* (≈300 g of matrix for each NI batch), a low level (LL) inoculum batch with 0.2 - 2 CFU/25 g or mL of sample (≈1400 g of matrix for each LL batch) and a high level (HL) inoculum batch with 2 - 10 CFU/25 g or mL of sample (≈400g of matrix for each HL batch). The inoculum was prepared using overnight grown colonies of *L. monocytogenes* diluted in phosphate-buffered saline (137mM NaCl; 2.7mM KCl; 10mM Na2HPO4.2H2O and 1.8mM KH2PO4 [Sigma]) and subsequently spread and mixed into the matrix bulk batches to achieve the desired concentration. For the cooked shrimp, before matrix inoculation, the inoculum was placed at 50ºC for 10 minutes to mimic the stress that natural microflora passed during the cooking. Then, the

prepared bulk batches were placed at 4ºC for 48 to 72h to allow the stabilization of the inoculum and simulate refrigerated conditions.

For all matrixes, the test design included 5 control samples, 20 low level samples and 5 high level samples, each containing a portion of 25 g or mL retrieved from the correspondent bulk batch prepared as described above in duplicate in an unpaired design. One was used to run PNA-FISH procedure and the other was subjected to ISO 11290 for comparison purposes (Figure S2.1 of supplemental material). Test portions were diluted in 225 mL of OBL and DFB for the PNA-FISH and the ISO 11290-1:1996 method, respectively, and homogenized in a stomacher (VWR/PBI, Italy) for 15s at high speed. Sample pre-enrichment was performed in the optimal conditions obtained in the present study. More precisely, a two-step procedure in OBL (incubation for 24h followed by a 1/10 dilution [1 mL of pre-enriched sample + 9 mL OBL] and a second incubation step for 18h), was used. For ISO 11290-1:1996 the samples in DFB were incubated for 24h at 30ºC. From this, a 100 µL sample of the DFB enrichment was placed in 10 mL of FB and incubated for 48h at 37ºC. To confirm the presence of *L. monocytogenes*, the secondary enriched media was plated on ALOA agar (Biomerieux) and Oxford agar (Liofilchem). When presumptive positive colonies appeared, a biochemical characterization was performed according to the ISO 11290-1:1996. Biochemical characterization included xylose and rhamnose sugar fermentation (Liofilchem), hemolysis and CAMP test profiles.

2.2.7. Probability of Detection and Difference Probability of Detection estimation

Due to the unpaired nature of the study and to compare the performance of PNA-FISH procedure with ISO 11290-1:1996, the Probability of Detection (POD) and the Difference Probability of Detection (DPOD) parameters were calculated (Wehling *et al*., 2011). The estimation of POD is based on the assumption that the test result (y) arise from the sum of three components, overall mean expected response (m), laboratory bias (b) and random error (e), equation 1:

$$(1)\quad y = m + b + e$$

In a qualitative procedure such as this, the test result is limited to the values 0 and 1. More precisely, 0 is used for samples tested negative for *L. monocytogenes* and 1 for

samples tested positive for *L. monocytogenes*. Because of that, the three variables of equation 1 are constraint for each replicate:

$$0 \leq m \leq 1;\ -1 \leq b \leq 1 \text{ and } -1 \leq e \leq 1.$$

For quantitative methods the m is either the concentration of analyte or derived from a calibration function, however in qualitative binary methods the m is only obtained under replication and because of that is assumed as the probability of a positive response at a given concentration tested: m = POD = P(+/c). As such, the POD can be considered to be the qualitative analog of the mean parameter of a quantitative method. The POD was calculated as the number of positive samples for each method in each level of contamination (Control; Low Level and High Level) divided by the total number of samples of that level. For method performance comparison the DPOD parameter is used as an analog to the quantitative parameter of bias between methods, and is calculated as the difference between the obtained POD for PNA-FISH and the respective POD for ISO 11290-1:1996 (Wehling *et al*., 2011).

2.2.8. Most Probable Number estimation

For the estimation of the inoculation level in the low and high level matrix samples, a Most Probable Number (MPN) evaluation was performed following the ISO 11290-1:1996 protocol. For low level estimation, samples of 50 (x5 replicates) and 10 (x5 replicates) g or mL each were taken from the corresponding bulk batch, prepared as described above, and mixed with 450 and 90 mL of DFB, respectively. For high level estimation, samples of 10 (x5 replicates) and 5 (x5 replicates) g or mL each were taken from the corresponding bulk batch and mixed with 90 and 45 mL of DFB, respectively. The samples were then processed as described earlier in this section. The number of positive samples obtained for each level were used to estimate real contamination levels (LaBudde, 2008).

2.2.9. Microscopy visualization

This step was performed using an OLYMPUS BX51 (OLYMPUS Portugal SA, Portugal) epifluorescence microscope equipped with one filter sensitive to the Alexa Fluor 568 molecule attached to PNA probe (Excitation 530 to 550 nm; Barrier 570 nm; Emission LP 591 nm). Other filters present in the microscope were used to confirm that cells did not autofluoresce. For every experiment, a negative control was performed

simultaneously, where all the steps described above were carried out, but where no probes were added during the hybridization procedure. The outcome of a PNA-FISH sample was only assessed after the analysis of the entire area of the glass slide well where the 20 µL sample was present. A positive outcome was determined when at least 10 fluorescent cells were visualized/microscopic field, which implies a concentration of ≈2.0x105 cell/mL (this calculation considered a microscopic field area of 0.1364 mm^2 and well area of 50.27 mm^2). All images were acquired using the Olympus CellB software with equal exposure time.

2.3. Results and Discussion

Extensive research has been carried out to develop molecular methods that could accelerate identification of *L. monocytogenes* on both food and clinical settings (Ponniah *et al*., 2010; Välimaa *et al*., 2015; Zunabovic *et al*., 2011). The optimal test for routine procedure should be simple to perform even by non-specialized technicians, sensitive enough to detect an inoculum level as low as 1 CFU/sample of food product, and fast (providing results within a few hours). Most researchers focused on PCR-based procedures, however, it is well known that PCR is susceptible to inhibitors, cross-contamination and can amplify DNA from non-viable cells (or even naked DNA), resulting in the appearance of both false negative and false positive results (Adzitey *et al*., 2013; Oikarinen *et al*., 2009; Rådström *et al*., 2008; Singer *et al*., 2006). Additionally, it requires specialized personnel and involves more complex procedures than the traditional culture methods. Some of these limitations have been solved in the meantime by improving the DNA extraction protocols, including internal controls and use RNA instead of DNA as template (Mangal *et al*., 2016; Marlony *et al*., 2008; Rådström *et al*., 2008).

Further research has been focusing in the development of alternative molecular technologies that are not susceptible to the previously stated factors. FISH is an alternative molecular method used to identify and quantify microbial populations (Costa *et al*., 2017). The combination of this method with peptide nucleic acid (PNA) probes has shown to have many advantages, including higher robustness, increased specificity and faster procedure, when compared to conventional DNA-FISH procedures. The use of PNA probes allowed a standardization of FISH procedures and this methodology has

already been applied for the detection of several clinical relevant microorganisms on a broad range of samples (Cerqueira *et al*., 2008; Rohde *et al*., 2015).

2.3.1. Evaluation of the *L. monocytogenes* probes described in literature

There are already FISH procedures developed for *Listeria* spp. detection, but only a few probes are specific for *L. monocytogenes* (Almeida *et al*., 2011; Fuchizawa *et al*., 2009; Moreno *et al*., 2011; Wang *et al*., 1991; Zhang *et al*., 2012) (Table S2.1 of supplementary material). Most of the existing probes are not simultaneously specific and sensitive because of the high number of non-target strains or the limited coverage of the target strains. Only two probes, LmPNA1253 and Lmon-16S-2, present adequate theoretical values considering the following thresholds: Number of non-target strains detected <10, Specificity >99.9% and Sensitivity >95%. These probes are nearly identical, as probe Lm-16S-2 shifted by only one nucleotide in relation to the 16S target sequence when comparing to the LmPNA1253. Therefore, these two probes detect both *L. monocytogenes*, *L. marthii* and also one *L. welshimeri* sequence (out of the 1922213 sequences available at the database). *L. marthii* is a relatively new species that has, so far, only been isolated in a specific area of the New York State in the USA (Orsi and Wiedmann, 2016).

Analyzing the target sequences for those probes, some closely related species (*Listeria* and *Bacillus* spp.) differ by only one nucleotide, which can hinder the discrimination between these species. For both probes, mismatches are placed near the probes 5' or 3' ends which can difficult even more the discrimination. However, since discrimination from closely-related *Listeria* species is usually a major challenge, as discussed in more detail in the next section (2.3.2.), LmPNA1253 was selected for further tests as, in this case, the mismatch with other *Listeria* species is placed at the probe third position (Table S2.2 of supplementary material).

2.3.2. Improving the *L. monocytogenes* PNA-FISH procedure specificity by including a blocker probe

Laboratory testing on representative strains have shown that the best hybridization conditions for LmPNA1253 were achieved using hybridization solution containing 5.5% (vol/vol) of formamide for 1 hour, from 55 to 60ºC. However, LmPNA1253 still detected a few strains of *Listeria* non-monocytogenes, even after increasing the hybridization

temperature (Table S2.3 of supplementary material). Increasing the temperature above 60°C improved hybridization specificity but a decrease in fluorescence intensity of the target species was also noticed.

While PNA has been described as highly effective for the discrimination of single-base mismatch sequences (Fontenete *et al*., 2015; Lefmann *et al*., 2006), the fact is that effective discrimination might also be dependent on the mismatch position. Those at the center are usually associated with an easier discrimination, while those near the 5' or 3' ends, which is the case in here, are reported as less effective for discrimination purposes (Amann, 1995; Lefmann *et al*., 2006). In fact, our results have shown some cross-reactivity with *L. innocua*, *L. welshimeri* and *L. ivanovii* (Table S2.3 of supplementary material). In order to block non-specific hybridization and hence increase robustness, an unlabeled blocker probe was used. PNA probes are particularly efficient blocker probes due to their particular thermodynamic properties (Fiandaca *et al*., 1999; Stender *et al*., 2001). The alignment with closely related strains, as stated before, have shown two positions potentially important for ensuring the method specificity and robustness (Table S2.2 of supplementary material). Blocking position 3 would bring important advantages on preventing cross-hybridization with other *Listeria* spp., while blocking position 15 would avoid cross-hybridization with *Bacillus* spp. A more detailed evaluation of these two possibilities have shown that the most relevant *Bacillus* species do not present this risk (Table S2.4 of supplementary material). On the other hand, the other *Listeria* spp. are widespread in nature and can be present in food and food processing plants (Ryser and Marth, 2007; Sauders *et al*., 2012). The most prevalent in this type of environments are usually *L. monocytogenes* and *L. innocua* (MacGowan *et al*., 1994) and several researchers have observed that *L. innocua* can outcompete *L. monocytogenes* if the two species are cultivated together in commonly used enrichment media (Carvalheira *et al*., 2010). Consequently, the non-*L. monocytogenes Listeria* species represent an increased risk of cross-hybridization in detection methods for *L. monocytogenes*. The addition of a blocker probe (in a 2:1 ratio), was able to effectively block the cross-hybridization for this set of strains. This effect was more efficient at 60ºC (Figure 2.1 and Table S2.3 of supplementary material).

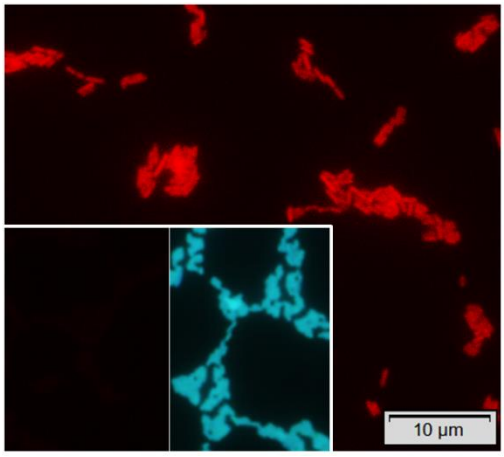

Figure 2.1 - Hybridization results for the LmPNA1253/blocking probes combination with a pure culture smear of *Listeria monocytogenes* CECT 933. In the white box a pure culture smear of *Listeriainnocua* CECT 910 exhibiting absence of signal on the left and DAPI stained on the right. The experiments were performed simultaneously and images were obtained with equal exposure times.

Subsequently, the final protocol was tested in 67 strains (including 50 *Listeria* strains). The results showed 100% agreement between PNA-FISH and strain identification (Table 2.1). Based on this, a specificity value of 100% (95% Confidence Internal [CI], 85.4 - 100) and a sensitivity value of 100% (95% CI, 88.6 - 100), were obtained for this detection protocol.

Table 2.1 - Specificity and sensitivity test for PNA-FISH including simultaneously both LmPNA1253 and *Listeria* blocker probe at a 1:2 ratio at 60ºC. The PNA-FISH test was repeated three times for each strain with similar outcomes.

Microorganism	Serotype	PNA-FISH outcome
L. monocytogenes CECT 5873	1/2a	+
L. monocytogenes CECT 5725	4c	+
L. monocytogenes CECT 938	3c	+
L. monocytogenes CECT 911	1/2c	+
L. monocytogenes CECT 933	3a	+
L. monocytogenes CECT 934	4a	+
L. monocytogenes CECT 937	3b	+
L. monocytogenes CECT 936	1/2b	+
L. monocytogenes CECT 4031T	1a	+
L. monocytogenes 747[a]	1/2b	+
L. monocytogenes 812[a]	1/2b	+
L. monocytogenes 832[a]	1/2b	+
L. monocytogenes 924[a]	1/2b	+
L. monocytogenes 925[a]	1/2b	+
L. monocytogenes 930[a]	1/2b	+
L. monocytogenes 994[a]	4ab	+
L. monocytogenes 1559[a]	1/2b	+
L. monocytogenes 1562A[a]	4b	+
L. monocytogenes 1014[a]	1/2a	+
L. monocytogenes 1360[a]	4b	+
L. monocytogenes 2241[a]	4b	+
L. monocytogenes 2020[a]	1/2c	+

Table 2.1 (continuation)

L. monocytogenes 1809[a]	1/2a	+
L. monocytogenes 2723[a]	-	+
L. monocytogenes L1B1[b]	-	+
L. monocytogenes L1D1[b]	-	+
L. monocytogenes L1F3[b]	-	+
L. monocytogenes L1L1[b]	-	+
L. monocytogenes L1L12[b]	-	+
L. innocua CECT 910	6a	-
L. innocua CECT 5376	-	-
L. innocua CECT 4030	-	-
L. innocua 1325[a]	-	-
L. innocua 2110[a]	-	-
L. innocua 1141[a]	-	-
L. ivanovii CECT 913	5	-
L. ivanovii londoniensis CECT 5375	-	-
L. ivanovii ivanovii CECT 5368	5	-
L. ivanovii londoniensis CECT 5374	-	-
L. ivanovii ivanovii CECT 5369	-	-
L. ivanovii 1326[a]	-	-
L. seeligeri CECT 917	1/2b	-
L. seeligeri CECT 5340	-	-
L. seeligeri CECT 5339	6b	-
L. seeligeri 2136[a]	-	-
L. welshimeri CECT 919	6a	-
L. welshimeri CECT 5370	1/2b	-
L. welshimeri CECT 5380	-	-
L. welshimeri CECT 5371	6a	-
L. grayi CECT 942	-	-
L. grayi CECT 931	-	-
Brochothrix thermosphacta CECT 847	-	-
Bacillus cereus[c]	-	-
Bacillus thuringiensis CECT 197	-	-
Enterococcus faecalis CECT 183	-	-
Enterococcus faecium CECT 410	-	-
Lactococcus lactis CECT 188	-	-
Lactobacillus paracasei CECT 277	-	-
Gemella morbillorum CECT 991	-	-
Staphylococcus aureus CECT 259	-	-
Staphylococcus aureus[c]	-	-
Staphylococcus epidermidis CECT 4184	-	-
Staphylococcus epidermidis CECT 231	-	-
Streptococcus mutans[c]	-	-
Escherichia coli CECT 533	-	-
Escherichia coli CECT 515	-	-
Salmonella dublin SGSC 2470	-	-

[a] Isolated strain provided by Professor Paula Teixeira from the Catholic University, Porto - Portugal;
[b] Isolated strain provided by Professor Marta Cabo from the Institute of Marine Research, Vigo - Spain;
[c] Isolated strain from our group.

2.3.3. Optimization of an enrichment step for *Listeria monocytogenes* detection in food samples.

Single cell pathogen detection directly in the food samples is still a goal rather than a reality for old and newly developed methods (López-Campos *et al*., 2012). As such, pathogen enrichment in an enrichment medium before analysis is required. In

traditional bacteriological methods, this is generally achieved by applying a two-step enrichment process. Typically, the first comprises a non-or semi-selective medium to recover injured organisms, dilute the inhibitory compounds and rehydrate the bacterial cells. The second is generally a selective medium that suppresses the background flora and increases the target pathogen, enabling its isolation and detection (Välimaa *et al*., 2015).

PNA-FISH, like most other molecular and culture-based methods, requires an enrichment step to successfully detect as low as 1 CFU of the pathogen in a sample (López-Campos *et al*., 2012). Without the inclusion of a filtration step, a typical PNA-FISH procedure has a detection limit of 10^5 cells/mL (Almeida *et al*., 2009). In order to reach that concentration (ideally $>10^6$ cells/mL), different enrichment broths were tested (Table S2.5 of supplementary material), starting with frequently-used rich enrichment broths, such BHI, TSB, BPW and UPB. Ground beef was used on these experiments due to the high load of background microflora found in this food matrix that potentially hinders *L. monocytogenes* growth (Gill and McGinnis, 1993). Initial experiments using non-selective enrichment broths were not able to detect *L. monocytogenes* even with an inoculum of up to 500 CFUs in 10 g of ground beef. This is in agreement with previous reports that indicated that after 24h, the concentration of *L. monocytogenes* in meat matrices was approx. 10^4/mL, a value below the PNA-FISH detection limit (Duffy *et al*., 2001; Gehring *et al*., 2012).

Subsequently, a set of commonly used selective enrichment broths used for the detection of *L. monocytogenes*, namely, FB and DFB (ISO 11290-1:1996), BLEB (FDA) and UVM (USDA-FSIS) and a more recent commercial enrichment broth (OBL), were tested. From this set of enrichment broths, only UVM and OBL were able to recover *L. monocytogenes* at a concentration that met the detection requirements (Table S2.5 of supplementary material). However, a low fluorescence intensity of the bacteria was obtained, probably arising from the low metabolic state/decreased rRNA levels of the cells as the cultures enter the stationary phase. The lack of a positive outcome observed for BLEB, DFB and FB could originate from the competing microflora present in meat (similarly to the non-selective enrichment broths) or from increased lag phases as a result of bacteria adaptation to these media. In fact, similarly to non-selective enrichment broths, previous reports indicate that at 24h *L. monocytogenes* levels only reaches around 10^3 - 10^4 CFU/mL (Gehring *et al*., 2012; Vlaemynck *et al*., 2000).

As none of the above-described strategies was successful, a two-step enrichment procedure of 18h plus 8h was tested, using in the first and second steps OBL and UVM. Two other selective broths, FB and BLEB, were also tested in the second step. All tested combinations were able to retrieve *L. monocytogenes* above the defined threshold of detection (Table S2.5 of supplementary material) using a 200 CFU/10g *L. monocytogenes* inoculum. Combinations including FB were excluded due to the presence of autofluorescing microflora observed in the green channel. Combinations including BLEB were excluded due to the low fluorescence intensity and low numbers of *L. monocytogenes*. The OBL + OBL was preferred over UVM combinations due to the consistently higher concentrations of *L. monocytogenes* cells observed in the microscope after the PNA-FISH procedure.

Since the two-step enrichment with OBL provided the best results, further tests were performed with lower concentrations to confirm if the desired detection level of 1 CFU/25 g or mL of sample was achieved. Results have shown that, for low inoculation levels, a centrifugation step was needed to concentrate the cells. To avoid centrifugation, the time of both enrichment steps was extended from 18 to 24 and from 8 to 18h. This modification allowed the detection of 1 CFU of *L. monocytogenes* in 25 g or mL of sample by PNA-FISH without the need for a centrifugation step in ground beef, milk and lettuce matrices (Figure 2.2 and Table 2.2).

Table 2.2 - List of assays performed to successfully detect *L. monocytogenes* in food matrices to 1 CFU/25 g or mL of sample. Optimizations were conducted in ground beef and subsequently validated in milk and lettuce matrices. All OBL enrichments steps were performed at 30ºC. Three replicates of each assay were performed with two different *L. monocytogenes* strains - CECT 938 and CECT 5873, with an inoculation level of 2.9 ± 2.0 CFU/25 g or mL of sample.

Matrix		Ground Beef		Milk	Lettuce
Enrichment Procedure	OBL 18h + OBL 8h.	OBL 18h + OBL 8h; 5 min 10 000*g* centrifugation.	OBL 24h + OBL 18h.	OBL 24h + OBL 18h.	OBL 24h + OBL 18h.
***L. monocytogenes* PNA-FISH outcome**	-	+	++	++	++

- No presence of *L. monocytogenes*; + Presence of *L. monocytogenes* near the defined detection limit; ++ Presence of *L. monocytogenes* above the defined detection limit.

An interesting observation was that *L. monocytogenes* cells grown in OBL do not present the typical small rod-shaped bacilli (Ponniah *et al*., 2010). They are present in a chain-like elongated form (filamentous) (Figure 2.2). We theorize that this morphology is due to the components present in the OBL broth. In fact, this behavior was already

reported in *L. monocytogenes* strains in the presence of some antimicrobial agents (Giotis *et al*., 2007; Hazeleger *et al*., 2006).

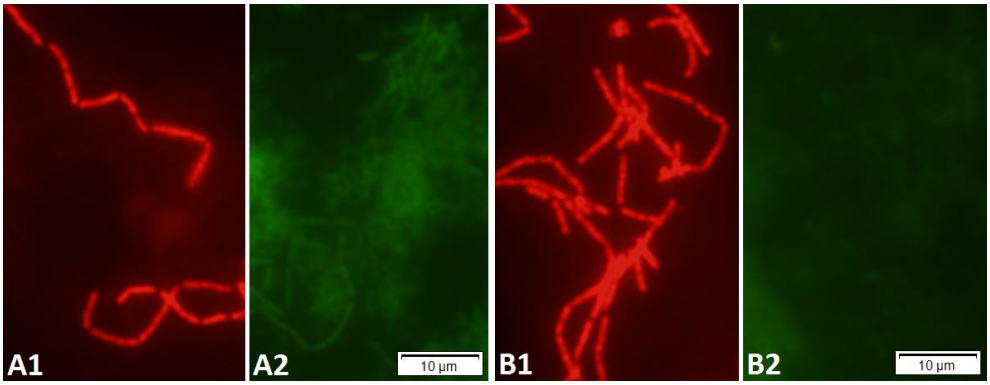

Figure 2.2 - Detection of *L. monocytogenes* in ground beef artificially inoculated with ≈1 CFU/25 g of sample, using LmPNA1253 probe attached to Alexa Fluor 568. A - Sample taken after 8h in the second enrichment step with OBL and a 5min centrifugation step; B - Sample taken after 18h in the second enrichment step with OBL. Cells of *L. monocytogenes* visible at the red channel (1), while the green channel (2) was used to check for the absence of autofluorescence.

Finally, a validation assay was performed to verify the applicability and specificity of the PNA-FISH protocol for the detection of *L. monocytogenes* in real scenarios of contamination. To that end, food samples were contaminated with two levels of inoculum, a low (0.2-2 CFU/sample) and high (2-10 CFU/sample) level. Different *L. monocytogenes* strains were selected for each food matrix (Table S2.6 of supplementary material*). L. monocytogenes* was detected in all tested matrices in levels as low as 0.5 CFU/25 g or mL of sample. For all 150 samples, only 2 false negative results were encountered, one in low level ground beef and the other in high level cooked shrimp assays.

Comparing PNA-FISH and ISO 11290-1:1996 performance for the detection of *L. monocytogenes* across all matrixes tested is challenging, due to the inapplicability of standard statistical methodologies to unpaired designs. However, McNemar's x2, sensitivity, specificity, false positive and false negative parameters could be used to that end. Furthermore, the Probability of Detection (POD) combines almost all the parameters referred above, while presenting other advantages such as simplicity, comparison over all ranges of concentration, graphical representation of results with error bars and performance comparison of methodologies between studies through estimation of the difference of PODs (DPOD) (Wehling *et al*., 2011). As such, in this study the performance comparison of PNA-FISH and ISO 11290-1:1996, was achieved through the estimation of POD and DPOD values (Table 2.3). As expected, we encountered 3

divergent POD values between the 2 methodologies in ground beef, lettuce and cooked shrimp. However, according to the obtained DPOD values, these differences do not present statistical significance (indicated by the inclusion of 0 in the 95% confidence interval). Taking into consideration the here obtained results is possible to claim that the PNA-FISH procedure for the detection of *L. monocytogenes* have a performance similar to the one obtained by the standard protocol, ISO 11290-1:1996.

Table 2.3 - Probability of Detection (POD) and Difference of Probability of Detection (DPOD) obtained for the 5 matrices with three inoculum levels, Control - no inoculation, Low Level (LL) - 0.2 - 2 CFU/25 g or mL of sample and High Level (HL) - 2 - 10 CFU/25 g or mL of sample, used to validate PNA-FISH procedure for the detection of *L. monocytogenes* against ISO 11290-1:1996.

Matrix	**Inoculum Level**	**POD PNA-FISH** (CI 95%)	**POD ISO 11290-1** (CI 95%)	**DPOD (CI 95%)**
Ground Beef	Control	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.4)	0.0 (-0.4 - 0.4)
	LL	0.6 (0.4 - 0.8)	0.5 (0.3 - 0.7)	0.1 (-0.2 - 0.5)
	HL	0.8 (0.4 - 1.0)	0.8 (0.4 - 1.0)	0.0 (-0.5 - 0.5)
Ground Pork	Control	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.4)	0.0 (-0.4 - 0.4)
	LL	0.7 (0.4 - 0.8)	0.7 (0.4 - 0.8)	0.0 (-0.3 - 0.3)
	HL	1.0 (0.6 - 1.0)	1.0 (0.6 - 1.0)	0.0 (-0.4 - 0.4)
Milk	Control	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.4)	0.0 (-0.4 - 0.4)
	LL	0.5 (0.3 - 0.7)	0.5 (0.3 - 0.7)	0.0 (-0.3 - 0.3)
	HL	1.0 (0.6 - 1.0)	1.0 (0.6 - 1.0)	0.0 (-0.4 - 0.4)
Lettuce	Control	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.4)	0.0 (-0.4 - 0.4)
	LL	0.9 (0.7 - 1.0)	0.8 (0.6 - 0.9)	0.1 (-0.1 - 0.3)
	HL	1.0 (0.6 - 1.0)	1.0 (0.6 - 1.0)	0.0 (-0.4 - 0.4)
Cooked Shrimp	Control	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.4)	0.0 (-0.4 - 0.4)
	LL	0.8 (0.5 - 0.9)	0.9 (0.6 - 1.0)	-0.1 (-0.3 - 0.1)
	HL	0.8 (0.4 - 1.0)	0.8 (0.4 - 1.0)	0.0 (-0.5 - 0.5)

2.4. Conclusions

This work describes the development of a new detection method for *L. monocytogenes* in food matrices. The method is based on a PNA-FISH procedure that combines the use of a previously described probe by Almeida *et al*. (2011), LmPNA1253, with a blocker probe resulting in an overall 100% specificity and sensitivity values. In order to be able to detect 1 CFU of *L. monocytogenes* in 25 g or mL of sample, several selective and non-selective enrichment broths were evaluated. Overall, a two-step enrichment procedure in One Broth Listeria, provided the most reliable results at the desired limit of detection. The total time-to-result of the method is 29h, if a centrifugation step is included, or a 45h without a centrifugation step. A validation assay in five different food matrices showed that the method present performance characteristics similar to the gold standard ISO 11290-1. The method high specificity, sensibility, robust performance and faster time-to-result makes it a good candidate for routine application in food safety laboratories.

The development of a PNA-FISH based method for the identification of microorganisms, is often hindered by the optimization of methodological parameters in order to adjust the hybridization efficiency (Santos *et al*., 2014). A complete understanding of the influence of each parameter on the fluorescence outcome of PNA-FISH procedure is lacking and their disclosure would greatly reduce the time required to developed new detection methods.

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2.6. Supplemental material

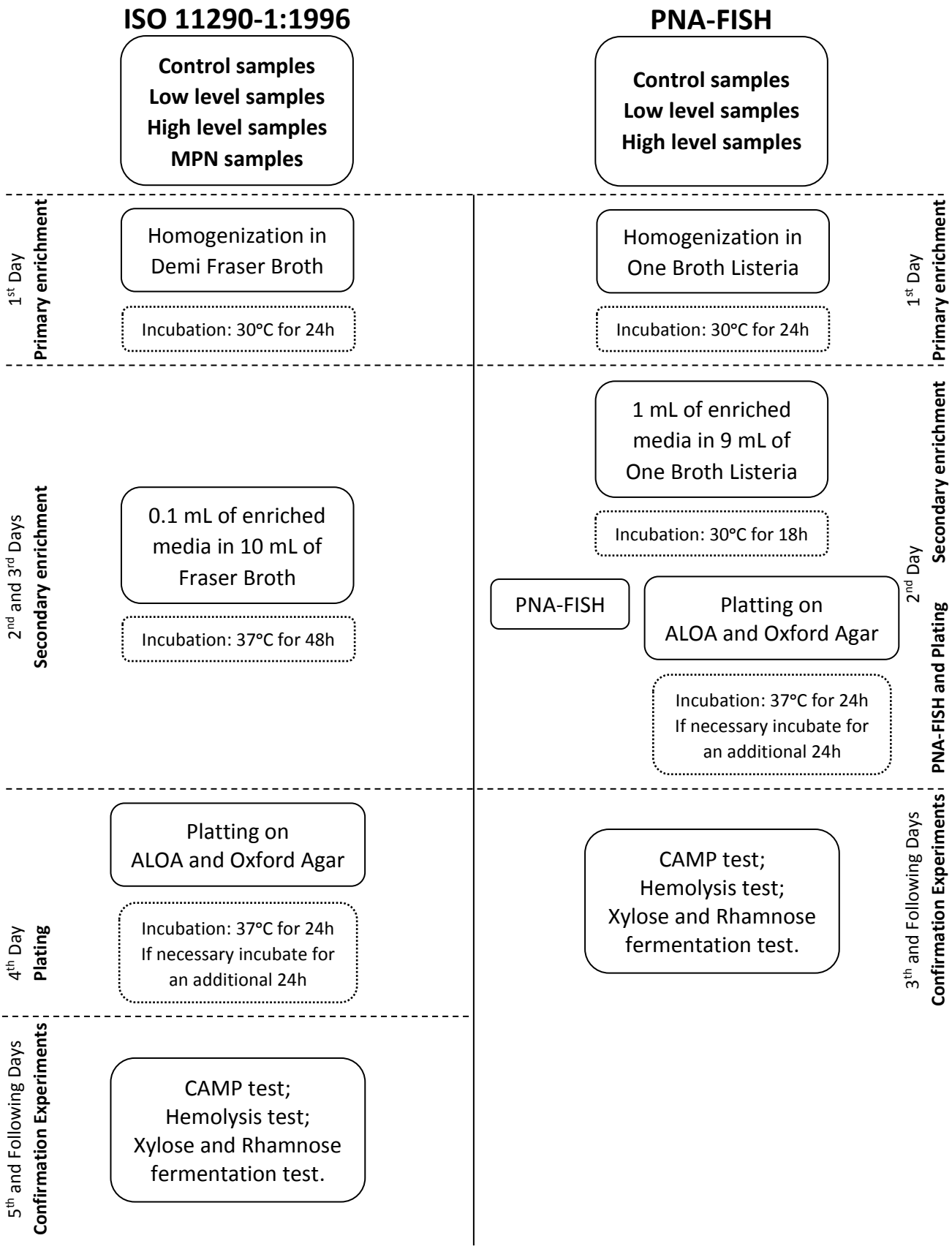

Figure S2.1 - Timeline for ISO 11290-1:1996 and PNA-FISH procedures performed in the validation assays for the detection and identification of *L. monocytogenes* in five artificially contaminated food matrices.

Table S2.1 - Theoretical evaluation of the existing probes for *L. monocytogenes* detection (last accession - September 2017).

Probe	Probe Sequence (5' - 3')	rRNA Subunit	Probe Starting Position (*E. coli* numbering)	Nº of strains detected[a]	Nº of non-target strains detected	Specificity (%)[b]	Sensitivity (%)	Method	Reference
RL-1	CTTTGTACTATCCATTGTA	16S	1234	777	119	>99.9	98.4	Dot blot hybridization	Wang *et al.*, 1991
RL-2	ATAGTTTTATGGGATTAGC	16S	1275	770	96	>99.9	97.5	Dot blot hybridization	Wang *et al.*, 1991
mRL-2	AGAATAGTTTTATGGGATTAGCTCCACC	16S	1269	764	96	>99.9	96.7	FISH-based method	Fuchizawa *et al.*, 2009
Lmon	CTATCCATTGTAGCACGTG	16S	1227	778	293	>99.9	98.5	FISH	Moreno *et al.*, 2011
LmPNA1253	GACCCTTTGTACTAT	16S	1242	772	5	>99.9	97.7	PNA-FISH	Almeida *et al.*, 2011
Lm-16S-2	CGACCCTTTGTACTA	16S	1243	772	5	>99.9	97.7	PNA-FISH	Zhang *et al.*, 2012
Lm-16S-3	ACACTTTATCATTCG	16S	175	647	2	>99.9	81.9	PNA-FISH	Zhang *et al.*, 2012
Lm-16S-4	AAAGCGTGGCATGCG	16S	185	713	3	>99.9	90.3	PNA-FISH	Zhang *et al.*, 2012

[a] *L. monocytogenes* strains detected in a total of 790 *L. monocytogenes* sequences present in the SILVA 16SREF database;
[b] Determined considering a total of 1922213 rRNA sequences present in the SILVA 16SREF database.

Table S2.2 - 16S rRNA sequence alignment of *Listeria* genus and other closely related species. Region targeted by LmPNA1253 are shown in grey and the mismatches found in this region to LmPNA1253 highlighted in black.

Species	Collection Number	Sequence/mismatches
Listeria monocytogenes	ATCC 19117	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2372	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2375	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2376	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2378	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2379	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2540	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 2755	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 5850	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria monocytogenes	SLCC 7179	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Listeria marthii	DSM 23813	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Bacillus acidiceler	DSM 18954	UACAAUGGAUAGUACAAAGGGUUGCAAGACC
Bacillus luciferensis	DSM 18845	UACAAUGGAUAGUACAAAGGGUUGCAAGACC
Listeria innocua	SLCC 3423	UACAAUGGAUGGUACAAAGGGUCGCGAAGCC
Listeria ivanovii ivanovii	ATCC BAA 678	UACAAUGGAUGGUACAAAGGGUCGCGAAGCC
Listeria welshimeri	SLCC 5334	UACAAUGGAUGGUACAAAGGGUCGCGAAGCC
Listeria floridensis	DSM 26687	UACAAUGGAUGGUACAAAGGGUAGCGAAGCC
Listeria seeligeri	SLCC 3954	UACAAUGGAUGGUACAAAGGGUAGCGAAGCC
Brochothrix thermosphacta	DSM 20171	UACAAUGGAUAAUACAAAGGGUCGCGAAGCC
Listeria booriae	DSM 28860	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria cornellensis	DSM 26689	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria grandensis	DSM 26688	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria newyorkensis	DSM 28861	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria riparia	DSM 26685	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria rocourtiae	FSL F6-920	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria weihenstephanensis	DSM 24698	UACAAUGGAUAAUACAAAGGGUUGCCAAACC
Listeria grayi	SLCC 332/64	UACAAUGGAUGAUACAAAGGGUCGCGAAGCC
Listeria fleischmannii	FSL S10-1203	UACAAUGGAUGGUACAAAGGGCAGCGAAGCC
Listeria aquatica	DSM 26686	UACAAUGGAUGGAACAAAGGGYAGCGAAGCC

ATCC - American Type Culture Collection; SLCC - Special Listeria Culture Collection; DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen; FSL - Food Safety Laboratory collection at Cornell University.

Table S2.3 - Initial probe specificity test for a selected group of reference strains from *Listeria* genus. The probe was tested at different hybridization temperatures and in the presence of an unlabeled blocker probe (2:1 blocker/detecting probes ratio) to hamper non-specific binding to non-*L. monocytogenes* strains.

Microorganism	Serotype	Probe			Probe + Blocker Probe		
		57ºC	59ºC	60ºC	57ºC	59ºC	60ºC
L. monocytogenes CECT 911	1/2c	+	+	+	+	+	+
L. monocytogenes CECT 933	3a	+	+	+	+	+	+
L. monocytogenes CECT 934	4a	+	+	+	+	+	+
L. monocytogenes CECT 938	3c	+	+	+	+	+	+
L. monocytogenes CECT 5725	4c	+	+	+	+	+	+
L. monocytogenes CECT 5873	1/2a	+	+	+	+	+	+
L. innocua CECT 910	6a	+	+	+	+	+[a]	-
L. innocua CECT 4030	-	+	+	+	+	+[a]	-
L. innocua CECT 5376	-	+	+	+	+	+[a]	-
L. ivanovii ivanovii CECT 5368	5	-	-	-	-	-	-
L. ivanovii ivanovii CECT 5369	-	+	+	+	+	-	-
L. ivanovii londoniensis CECT 5375	-	-	-	-	-	-	-
L. seeligeri CECT 5339	6b	-	-	-	-	-	-
L. seeligeri CECT 5340	-	-	-	-	-	-	-
L. welshimeri CECT 5371	6a	+	+	+	+	+[a]	-
L. welshimeri CECT 5380	-	+	+	-	+	-	-
L. grayi CECT 931	-	+	-	-	-	-	-
L. grayi CECT 942	-	-	-	-	-	-	-

[a] Low intensity fluorescent outcome

Table S2.4 - 16S rRNA sequence alignment of some species of *Bacillus* genus and *L. monocytogenes*. Region targeted by LmPNA1253 are shown in grey and the mismatches found in this region to LmPNA1253 highlighted in black.

Species	Collection Number	Sequence/mismatches
Listeria monocytogenes	ATCC 19117	UACAAUGGAUAGUACAAAGGGUCGCGAAGCC
Bacillus acidiceler	DSM 18954	UACAAUGGAUAGUACAAAGGGUUGCAAGACC
Bacillus luciferensis	DSM 18845	UACAAUGGAUAGUACAAAGGGUUGCAAGACC
Bacillus megaterium	DSM 319	UACAAUGGAUGGUACAAAGGGCUGCAAGACC
Bacillus pumilus	ATCC 7061	UACAAUGGACAGAACAAAGGGCUGCGAGACC
Bacillus subtilis	ATCC 6633	UACAAUGGACAGAACAAAGGGCAGCGAAACC
Bacillus anthracis	ATCC 14578	UACAAUGGACGGUACAAAGAGCUGCAAGACC
Bacillus cereus	ATCC 10987	UACAAUGGACGGUACAAAGAGCUGCAAGACC
Bacillus thuringiensis	ATCC 35646	UACAAUGGACGGUACAAAGAGCUGCAAGACC
Bacillus licheniformis	DSM 13	UACAAUGGGCAGAACAAAGGGCAGCGAAGCC

ATCC - American Type Culture Collection; DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Table S2.5 - List of assays concerning different enrichment mediums and conditions for the detection of *L. monocytogenes* in ground beef.

Enrichment medium	Incubation conditions	Inoculum concentration (CFU/10g)	*L. monocytogenes* PNA-FISH outcome	Observations
BHI	– Up to 48h; – 30 and 37ºC.	± 500	Absent	• Competing microflora.
TSB	– Up to 48h; – 30 and 37ºC.	± 500	Absent	• Competing microflora.
BPW	– Up to 48h; – 30 and 37ºC.	± 500	Absent	• Competing microflora.
UPB	– Up to 48h; – 30 and 37ºC.	± 500	Absent	• Competing microflora.
DFB	– Up to 48h; – 30ºC.	± 500	Absent	• Competing microflora; • Autofluorescence microflora green channel.
FB	– Up to 48h; – 30ºC.	± 500	Absent	• Competing microflora; • Autofluorescence microflora green channel.
BLEB	– Up to 48h; – 30ºC.	± 500	Absent	• Competing microflora.
UVM	– 24h; – 30ºC.	± 500	Present	• Low fluorescence signal.
OBL	– 24h; – 30ºC.	± 500	Present	• Low fluorescence signal.
Double enrichment steps				
UVM	– UVM 18h + UVM 8h – 30ºC.	± 200	Present	• Good fluorescence intensity.
UVM and BLEB	– UVM 18h + BLEB 8h; – 30ºC.	± 200	Present	• Low number of cells; • Low fluorescence intensity.
UVM and FB	– UVM 18h + FB 8h; – 30ºC.	± 200	Present	• Autofluoresncence microflora green channel.
UVM and OBL	– UVM 18h + OBL 8h; – 30ºC.	± 200	Present	• Good fluorescence intensity.
OBL	– **OBL 18h + OBL 8h;** – **30ºC.**	± **200**	**Present**	• **Good fluorescence intensity and high numbers of *L. monocytogenes*.**
OBL and UVM	– OBL 18h + UVM 8h; – 30ºC.	± 200	Present	• Good fluorescence intensity.
OBL and FB	– OBL 18h + FB 8h; – 30ºC.	± 200	Present	• Autofluoresncence microflora green channel.
OBL and BLEB	– OBL 18h + BLEB 8h; – 30ºC.	± 200	Present	• Low number of cells; • Low fluorescence intensity.

Table S2.3 - PNA-FISH and confirmation, as well ISO 11290-1:1996 results, obtained for the detection of *L. monocytogenes* on different food matrices inoculated with Low Level and High Level ranges in 25 g or mL samples.

Matrix	Ground Beef				Ground Pork				Milk				Lettuce				Cooked Shrimp			
Strain	*L. monocytogenes* L1F3				*L. monocytogenes* 2723				*L. monocytogenes* 812				*L. monocytogenes* 1360				*L. monocytogenes* L1D1			
Replicate	MPN (CI 95%)	PNA-FISH	Confir-mation	ISO 11290	MPN (CI 95%)	PNA-FISH	Confir-mation	ISO 11290	MPN (CI 95%)	PNA-FISH	Confir-mation	ISO 11290	MPN (CI 95%)	PNA-FISH	Confir-mation	ISO 11290	MPN (CI 95%)	PNA-FISH	Confir-mation	ISO 11290
C1		-	-	-		-	-	-		-	-	-		-	-	-		-	-	-
C2		-	-	-		-	-	-		-	-	-		-	-	-		-	-	-
C3	/	-	-	-	/	-	-	-	/	-	-	-	/	-	-	-	/	-	-	-
C4		-	-	-		-	-	-		-	-	-		-	-	-		-	-	-
C5		-	-	-		-	-	-		-	-	-		-	-	-		-	-	-
LL1		+	+	+		-	-	+		-	-	+		+	+	+		+	+	+
LL2		+	+	-		-	-	+		'	-	+		+	+	+		+	+	+
LL3		+	+	+		-	-	-		-	-	-		+	+	-		-	-	+
LL4		+	+	+		+	+	+		+	+	+		+	+	+		+	+	+
LL5		+	+	-		+	+	+		-	-	-		-	-	+		+	+	+
LL6		+	+	-		+	+	-		+	+	-		+	+	+		-	-	+
LL7		-	-	+		+	+	+		+	+	+		+	+	+		+	+	-
LL8		+	+	-		+	+	+		-	-	-		+	+	+		+	+	+
LL9		+	+	+		+	+	+		+	+	-		+	+	-		+	+	+
LL10	0.5 (0.2 - 0.8)	-	-	+	0.7 (0.4 - 1.2)	+	+	+	0.8 (0.4 - 1.3)	+	+	+	1.6 (1.0 - 2.9)	+	+	+	1.8 (1.2 - 3.2)	-	-	-
LL11		+	+	-		+	+	+		-	-	-		-	-	+		+	+	+
LL12		-	+	-		-	-	+		+	+	+		+	+	+		+	+	+
LL13		+	+	+		+	+	+		-	-	+		+	+	+		+	+	+
LL14		-	-	+		+	+	-		+	+	+		+	+	-		+	+	+
LL15		-	-	-		-	-	-		+	+	-		+	+	+		+	+	+
LL16		-	-	+		-	-	-		-	-	-		+	+	+		-	-	+
LL17		-	-	-		+	+	-		-	-	-		+	+	-		-	-	-
LL18		+	+	-		+	+	+		+	+	-		+	+	+		+	+	+
LL19		-	-	-		+	+	+		+	+	-		+	+	+		+	+	+
LL20		-	-	-		-	-	-		-	-	+		+	+	+		+	+	+
HL1		+	+	+		+	+	+		+	+	+		+	+	+		+	+	+
HL2	2.0 (1.0 - 4.1)	+	+	+	2.3 (1.1 - 4.7)	+	+	+	6.4 (3.8 - 14.5)	+	+	+	5.6 (2.6 - 11.8)	+	+	+	2.3 (1.2 - 4.6)	+	+	+
HL3		+	+	+		+	+	+		+	+	+		+	+	+		+	+	-
HL4		-	-	+		+	+	+		+	+	+		+	+	+		-	+	+
HL5		+	+	-		+	+	+		+	+	+		+	+	+		+	+	+

Chapter 3

Optimization of Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) for the detection of bacteria: the effect of pH, dextran sulfate and probe concentration.

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Abstract

Fluorescence *in situ* hybridization (FISH) is a molecular technique widely used for the detection and characterization of microbial populations. FISH is affected by a wide variety of abiotic and biotic variables and the way they interact with each other. This is translated into a wide variability of FISH procedures found in the literature. The aim of this work is to systematically study the effects of pH, dextran sulfate and probe concentration in the FISH protocol, using a general peptide nucleic acid (PNA) probe for the *Eubacteria* domain. For this, response surface methodology was used to optimize these 3 PNA-FISH parameters for Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive species (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). The obtained results show that a probe concentration higher than 300 nM is favorable for both groups. Interestingly, a clear distinction between the two groups regarding the optimal pH and dextran sulfate concentration was found: a high pH (approx. 10), combined with lower dextran sulfate concentration (approx. 2% [wt/vol]) for Gram-negative species and near-neutral pH (approx. 8), together with higher dextran sulfate concentrations (approx. 10% [wt/vol]) for Gram-positive species. This behavior seems to result from an interplay between pH and dextran sulfate and their ability to influence probe concentration and diffusion towards the rRNA target. This study shows that, for an optimum hybridization protocol, dextran sulfate and pH should be adjusted according to the target bacteria.

Keywords

PNA-FISH, *Eubacteria*, dextran sulfate, pH, PNA EUB338.

3.1. Introduction

In situ hybridization (ISH) consists of an array of methodologies that ultimately allow the specific detection of nucleic acid sequences in biological samples (Jin and Lloyd, 1997). At the present moment, most ISH techniques use fluorescent dyes as reporter molecules, in a process called Fluorescence *in situ* Hybridization (FISH) (Speicher and Carter, 2005; Trask, 2002). On its original form, FISH consists essentially on hybridizing an oligonucleotide probe to its complementary sequence in previously fixed samples, obeying to the Watson-Crick hydrogen-bonding rules (Cerqueira *et al*., 2008; Volpi and Bridger, 2008). FISH is widely used in the field of microbiology (Amann and Fuchs, 2008), namely in the identification, quantification and characterization of phylogenetically defined microbial populations in complex environments (Wagner *et al*., 2003).

Since the first application of FISH to microorganisms by DeLong *et al* (1989), diverse FISH-based diagnostic assays have been developed (see review from Volpi and Bridger, 2008). These result from combinations of FISH with other techniques or improvements at the FISH procedure level, such as the use of other molecules, rather than standard DNA or RNA, as probes. A good example of this is the application of peptide nucleic acid (PNA), a nucleic acid mimic with recognized superior hybridization features (Cerqueira *et al*., 2008; Stender *et al*., 2000; Stender *et al*., 1999). PNA is comprised of a neutral polyamide backbone (Nielsen *et al*., 1991) with an identical chemical configuration to the DNA molecules that allows PNA to hybridize with complementary DNA or RNA sequences (Nielsen, 2001; Shakeel *et al*., 2006). Its superior hybridization features arise from the lack of electrostatic repulsion between the non-charged polyamide backbone and the charged DNA/RNA phosphodiester backbone. This is translated into an improved thermal stability of the duplex (Nielsen, 2001; Perry-O'Keefe *et al*., 2001) and allows the hybridization step to be performed under low salt concentrations (Orum *et al*., 1998), a condition that destabilizes the rRNA secondary structures and results in an improved access to target sequences (Azevedo *et al*., 2003; Fuchs *et al*., 1998; Yilmaz *et al*., 2006). The neutrally-charged PNA also diffuses well through the bacterial membrane (Drobniewski *et al*., 2000) and its synthetic nature leads to an increased resistance to nucleases and proteases (Demidov *et al*., 1994; Stender *et al*., 2002; Wagner *et al*., 2003).

In spite of PNA-FISH robustness, there is considerable variability between the procedures described in the literature and its implementation usually requires an initial optimization to adjust the hybridization efficiency (Herzer and Englert, 2001), currently performed as a trial-and-error approach. This is a laborious and time-consuming step that could be greatly shortened if knowledge on how to develop a novel PNA-FISH method was at hand. In fact, variables such as type of fixative used (aldehyde or alcohol-based fixation), hybridization time, temperature, pH, concentration of probe, dextran sulfate (DS) and formamide, among others, are known to affect hybridization efficiency. Santos *et al*. (2014) recently assessed the effects of formamide, temperature and time on the hybridization efficiency, while successfully establishing an approach for FISH optimization, applying response surface methodology (RSM).

The present work aimed to understand the effect of hybridization pH, DS and probe concentration (and their interplay) on PNA-FISH efficiency for different bacteria. To this end, RSM was used to model the hybridization of an universal *Eubacteria* PNA probe (EUB338) (Amann *et al*., 1990; Santos *et al*., 2014), and signal quantification was assessed by flow cytometry.

3.2. Materials and methods

3.3.1. Bacterial strains

The bacterial strains selected for this study were *Escherichia coli* CECT 434, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* CECT 910, *Staphylococcus epidermidis* RP61A and *Bacillus cereus* isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões *et al.*, 2007). *E. coli* and *L. innocua* were grown on tryptic soy agar (TSA) [3% (wt/vol) tryptic soy broth and 1.5% (wt/vol) agar] (Oxoid, Basingstoke, England and Merck, Darmstadt, Germany). *B. cereus*, *P. fluorescens* and *S. epidermidis* were grown in plate count agar (Merck). All cultures were grown overnight at 30ºC and streaked onto fresh plates every 2 or 3 days.

3.3.2. PNA-FISH method

In order to evaluate the influence of pH, DS and probe concentration in the fluorescent signal outcome, a PNA-FISH protocol similar to the one described by Santos *et al* (2014) was implemented, followed by signal quantification using flow cytometry. A

universal PNA probe EUB338 (5'-TGCCTCCCGTAGGA-3'), based on the work of Amann *et al* (1990), which recognizes a conserved region of the 16S rRNA in the domain *Eubacteria*, was used. The probe was synthesized and labelled at the N terminus with AlexaFluor488 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (Panagene, Daejeon, South Korea).

Bacterial cells were harvested from plates and suspended in sterile water to a final concentration of 10^8 to 10^9 cells/mL. For sample fixation, the cell suspension was pelleted by centrifugation at 10,000 × *g* for 5 min, resuspended in 400 µL of 4% (wt/vol) paraformaldehyde (Acros Organics, New Jersey, USA) and incubated for 1 h at room temperature. After centrifugation at 10,000 × *g* for 5 min, the pellet was resuspended in 500 µL of 50% (vol/vol) ethanol and incubated at -20°C for at least 30 min. For hybridization, 100 µL of the fixed-cell aliquot were pelleted by centrifugation (10,000 × *g* for 5 min) and resuspended in 100 µL of hybridization solution. With the exception of the parameters under study, the composition of the hybridization solution was the same as the one reported by Santos *et al*. (2014), with the optimum formamide concentration obtained on that study. Consequently, formamide (Acros Organics) at 5.5% (vol/vol) was used for *E. coli*, *P. fluorescens, L. innocua* and *S. epidermidis* and at 49.5% (vol/vol) for *B. cereus.* Regarding the 3 parameters under study, the ranges selected are presented in Table 3.1. The conditions for assay 1 were selected to cover the values commonly described in the literature (Table S3.1 of supplemental material). Based on the results obtained in assay 1, new ranges were selected for assay 2, to achieve a suitable model for *E. coli* and *P. fluorescens*. Ranges defined in assay 3 and 4 were used to further evaluate the influence of DS molecular weight (MW) and pH on the signal outcome of Gram-positive bacteria. Different buffers were used at a concentration of 50 mM to control the pH of the hybridization solution, specifically citrate-phosphate (for pH 4 to 6); Tris-HCl (pH 7 to 8); Glycine-NaOH (pH 9 to 10); Sodium bicarbonate-NaOH (pH 11.2 and 11.3) and potassium chloride-NaOH (for pH above 12). Samples were hybridized at 60ºC for 55 min, except for *B. cereus* samples that were incubated for 110 min, based on the optimum conditions found by Santos *et al*. (2014). Negative controls were prepared using the same conditions stated previously and resuspended in the hybridization solution without probe. After hybridization, cells were centrifuged (10,000 × *g* for 5 min), resuspended in 500 µL of washing solution containing 5 mM Tris base (pH 10; Fisher Scientific, New Jersey, USA), 15 mM NaCl (Panreac, Barcelona, Spain) and 0.1%

(vol/vol) Triton X-100 (Panreac) and incubated for 30 min at 60ºC. After centrifugation (10,000 × *g* for 5 min), the pellet was resuspended in 700 µL sterile saline solution, 0.9% (wt/vol) NaCl (Panreac). Each experiment was performed in triplicate.

Table 3.1 - Experimental levels for the variables used in the optimization of the PNA-FISH hybridization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus* species.

Assay		Variables	Range and level				
			$-\alpha$	-1	0	$+1$	$+\alpha$
1[a]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 500 kDa] % (wt/vol)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
2[b]	x_1	pH	5.9	7.3	9.3	11.3	12.6
	x_2	[DS 500 kDa] % (wt/vol)	0.0	1.0	2.5	3.9	5.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
3[c]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 10 kDa] % (wt/vol)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
4[d]	x_1	pH	6.5	7.3	9.3	11.2	12.0
	x_2	[DS 0.5 kDa] % (wt/vol)	1.9	5.0	12.5	20.0	23.1

[a] Experimental levels set in the optimization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus*.
[b] Experimental levels set in the optimization protocol for *E. coli* and *P. fluorescens*.
[c] Experimental levels set in the optimization protocol for *L. innocua*, *S. epidermidis* and *B. cereus*.
[d] Experimental levels set in the optimization protocol for *L. innocua*. Probe concentration at 200 nM.

3.3.3. Flow cytometry analysis

The fluorescence intensity of hybridized samples and negative controls was quantified by an Epics XL flow cytometer (Beckman Coulter, Florida, USA) equipped with a 488 nm argon ion laser. Forward angle light scatter (FS), side angle light scatter (SS), and green fluorescence (FL1) were detected at logarithmic scale. A minimum of 20,000 events falling into the bacterial gate defined on the FS-SS plot were acquired per sample. The data was analyzed with the Expo32 software (Beckman Coulter), and the average fluorescence intensity was determined for each triplicate experiment.

3.3.4. Response surface methodology (RSM)

In order to model the effect of pH, DS and probe concentration in the hybridization of PNA EUB338 probe in bacteria, RSM was employed according to the procedure applied by Santos *et al* (2014). The average fluorescence intensity obtained after PNA-FISH was used as the dependent variable.

Central composite designs (CCD) were set up for *E. coli, B. cereus, P. fluorescens, L. innocua* and *S. epidermidis*, using the statistical software Design Expert® 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model. The range and levels of all variables were defined according to previous studies (Table S3.1 of supplemental material) and the results obtained within this study. Each CCD for assays 1, 2 and 3 included 2^3 factorial points (coded at ± 1), 6 axial points (coded as ± α) that represent extreme values used for the estimation of the model curvature and 6 center points (all factors at coded level 0) repeated to take into account the experimental error (Myers and Montgomery, 1995; Silva *et al*., 2011). Therefore, each design matrix consisted of 20 PNA-FISH experiments. For the assay number 4 the CCD included 2^2 factorial points (coded as ± 1), 4 axial points (coded as ± α) and 5 center points (all factors at coded level 0). Therefore, this design matrix consisted of 13 PNA-FISH experiments.

3.3.5. Viscometer analysis

Viscosity measurements of DS 500 kDa solutions at pH 6, pH 9 and pH 12 were performed using a Cannon-Fenske viscometer size 100 (Hipex, Portugal). Different buffers were used at a concentration of 50 mM to control de pH of the DS solutions, specifically citrate-phosphate for pH 6, Glycine-NaOH for pH 9 and potassium chloride-NaOH for pH 12. The viscometer was placed in a water bath at a constant temperature of 25 ± 1ºC. The viscosity of DS solutions at different pH was determined by the comparison of the flow time of DS solutions against the flow time of distilled water in triplicate.

3.3.6. Statistical analysis

In order to find the optimum hybridization conditions for all five species in the study, the average intensity fluorescence values obtained by flow cytometry were introduced in the software Design Expert® 8.0.7.1 to fit a quadratic model and each obtained model was analyzed using analysis of variance (ANOVA). The interaction of the three independent variables and their effect on the fluorescence intensity was inspected by constructing the response surface and contour plots. The optimization function of the software was then used to estimate the optimum conditions within the experimental range that maximized the fluorescence intensity. A confirmation experiment of the predicted optimum point was performed for each bacterium in triplicate.

3.3. Results and Discussion

3.3.1. PNA-FISH optimization in bacteria: pH, DS and probe concentration

In this work, the effect of three parameters (pH, DS and probe concentration) on the hybridization efficiency of PNA-FISH was studied. To model their effect, RSM was applied to the hybridization data obtained from 3 Gram-positive (*L. innocua*, *S. epidermidis* and *B. cereus*) and 2 Gram-negative species (*E. coli* and *P. fluorescens*).

The first range of pH, DS and probe concentrations tested in the CCD were based on the values typically described in the literature for PNA-FISH methods (Table 3.1, assay 1 and Table S3.1 of supplemental material). After performing the CCD set of experiments, significant quadratic models (p-value <0.05), a non-significant lack of fit (p-value >0.05) and a satisfactory coefficient of determination (R^2) combined with an optimum on the response surface plots were obtained for all three Gram-positive species tested (Figure 3.1). However, for the Gram-negative species, an optimum value from the response surface plots was not obtained, although a general tendency for lower DS concentrations and higher pH values was observed (Figure 3.1).

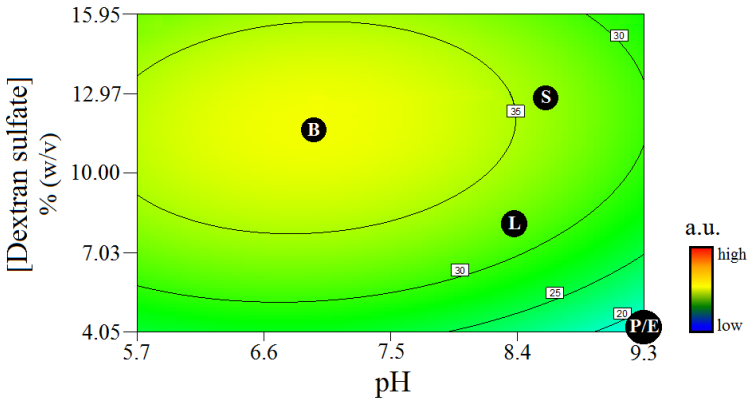

Figure 3.1 - Contour plot of *B. cereus* showing the effect of pH and DS (500 kDa) concentration on the fluorescence intensity (with probe concentration at the optimum of 300 nM). The fluorescence values (in arbitrary units) of the contour lines are the ones obtained for *B. cereus*. The optimum points predicted by the software for *E. coli*, *P. fluorescens*, *L. innocua*, *B. cereus* and *S. epidermidis* are represented in black circles with its respective initial letter. For *E. coli* and *P. fluorescens* no optimum value was obtained, but the overall behavior observed indicates that lower DS concentrations and higher pH values should be preferred to redefine the testing concentrations.

In order to obtain a satisfactory model for the Gram-negative species, the range of pH and DS concentration on the CCD were redesigned for higher pH values and lower DS concentrations (Table 3.1, assay 2), while maintaining the probe concentration level.

Using those designs for Gram-positive (Table 3.1, assay 1) and Gram-negative species (Table 3.1, assay 2), significant quadratic models were obtained for all five species tested (Table S3.2 and S3.3 of supplemental material). The successful modelling of the three studied parameters (pH, DS and probe concentration) allowed the determination of the optimal conditions for the maximum fluorescence (Figure 3.2).

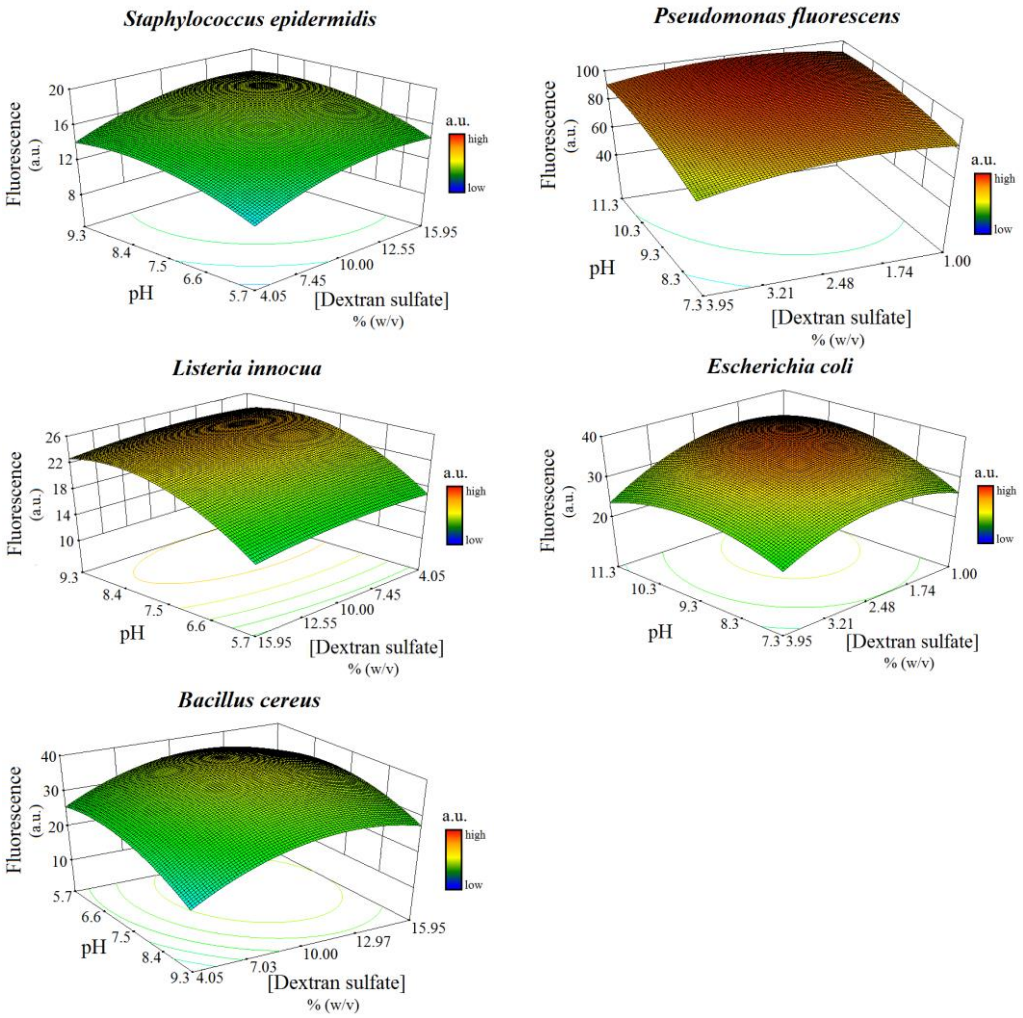

Figure 3.2 - Surface response plots representing the interaction effect of pH and DS on the fluorescence response of *S. epidermidis*, *L. innocua*, *B. cereus*, *E. coli* and *P. fluorescens*. The optimal PNA EUB338 probe concentration was 300 nM for all 5 strains. Fluorescence values are presented in arbitrary units (a.u.).

Moreover, the confirmatory experiment showed an agreement between experimental and predicted values (Table 3.2). The average fluorescence for negative controls was equal or lower than 1 a.u. (data not shown), while for positive samples the values ranged from 7 to 150 a.u., depending on the microorganism and the conditions tested (Figure 3.2).

Table 3.2 - Optimum hybridization pH, DS and probe concentration predicted through the RSM models for the tested species. Predicted, average negative control and obtained fluorescence with error values (standard deviation) in those conditions are shown.

Bacteria	Optimum conditions			Predicted Fluorescence (a.u.)	Obtained Fluorescence (a.u.)	Negative Control (a.u.)
	pH	DS (% wt/vol)	Probe (nM)			
E. coli	9.87	1.93	300	37.1	38 ± 2	0.6 ± 0.1
P. fluorescens	10.83	2.32	300	98.2	172 ± 8	0.9 ± 0.1
L. innocua	8.36	7.94	300	24.9	21.6 ± 0.2	0.6 ± 0.1
B. cereus	6.92	11.70	300	37.9	31 ± 1	0.5 ± 0.1
S. epidermidis	8.56	12.84	300	18.0	17 ± 2	0.4 ± 0.1

Analyzing Table 3.2 is also possible to observe that the optimal probe concentration for all species was the maximum tested and considered by the model, 300 nM (+1 factor). This was expected, since the probe concentration is a key factor for the nucleation reaction and the time needed for hybridization (Bruns *et al*., 2007). The nucleation reaction is the rate-limiting step in the hybridization of nucleic acids, being characterized by the formation of a small number of base pairs that initiate the hybridization, proceeding then as a rapid zippering of the remaining nucleotides (Bruns *et al*., 2007). If the concentration of hybrid strands in solution is similar, the hybridization follows a second order kinetics, meaning that the higher the concentration of hybrid strands in solution, the higher the annealing rate will be (Bruns *et al*., 2007). However, as FISH protocols usually use probe concentration in excess relatively to the number of target sequence(s) (Yilmaz and Noguera, 2004) a pseudo-first order kinetics is applied (Bruns *et al*., 2007), and in this case the hybridization depends only on the concentration of the target. However, the time required to hybridize the probe to the target remains inversely proportional to the probe concentration (Bruns *et al*., 2007). Other variables such as target accessibility, probe length and complexity have also an impact on the hybridization (Bruns *et al*., 2007), but these were not of concern since the same probe (PNA EUB338) was used throughout this work.

Interestingly, analyzing the results of the optimal pH and DS concentration (Table 3.2), it is possible to distinguish 2 different behaviors. A higher pH, approx. 10, combined with lower DS concentration, approx. 2% (wt/vol), were found to be favorable for Gram-negative species (*E. coli* and *P. fluorescens*), while near-neutral pH, approx. 8, together with higher DS concentrations, approx. 10% (wt/vol), favored Gram-positive species (*L. innocua*, *S. epidermidis* and *B. cereus*).

The application of DS in the hybridization solution has two main effects in FISH. On the one hand, higher concentrations of DS should be favorable to FISH as they cause

an apparent increase in probe concentration (Azevedo, 2005; Cmarko and Koberna, 2007). On the other hand, it is well known that DS increases the viscosity of a solution, hence decreasing molecular diffusion (Kosar and Phillips, 1995; Zustiak *et al*., 2011). In order to understand why DS affected differently Gram-positive and Gram-negative bacteria, we considered that the access of the probe to the target rRNA occurs in three steps: 1) diffusion on the suspension, 2) diffusion through the cell envelop (including the cell wall) and 3) diffusion in the cytoplasm. For the Gram-positive, the limiting step is possibly 2), considering that they possess a peptidoglycan layer much thicker than Gram-negative bacteria (Franks *et al*., 1998; Roller *et al*., 1994) and as such, a higher probe concentration gradient is needed. For the Gram-negative the limiting diffusion step is 1), so the increase in viscosity might be more relevant.

In order to explore this hypothesis of the interplay between viscosity and optimum DS concentration needed for the probe to overcome the thick cell wall of Gram-positive bacteria, we further tested different MW DS (besides the previously used 500 kDa in Table 3.1, assays 3 and 4), as the viscosity of DS molecules in solution decreases with lower MW DS (Joosse *et al*., 2007). At 30ºC 10% (wt/vol) DS of 500 kDa presents a viscosity of ≈35 mPa.s (Demetriades and McClements, 1998), whereas 10% (wt/vol) DS of 10 kDa presents ≈2 mPa.s (Algotsson *et al*., 2013). So, using lower MW DS we would expect to observe an increase in the optimum DS concentration values, due to the lower viscosity of the hybridization solution obtained. The results presented in Table 3.3 confirmed the anticipated outcome stated above.

Table 3.3 - Optimum pH and DS concentration, for 500, 10 and 0.5 kDa MW molecules, in hybridization solution predicted through RSM models for Gram-positive species in study.

Dextran sulfate	**Species**					
	S. epidermidis		*L. innocua*		*B. cereus*	
(MW)	pH	[DS] (% wt/vol)	pH	[DS] (% wt/vol)	pH	[DS] (% wt/vol)
500 kDa	8.56	12.84	8.36	7.94	6.92	10.70
10 kDa	9.30	15.43	9.14	10.52	8.09	12.16
0.5 kDa	NE	NE	9.76	12.66	NE	NE

NE - Not Evaluated.

Lastly, the pH of the hybridization solution may also impact FISH in 2 different ways. On one side, it affects the ionization of nucleotides (Blackburn *et al*., 2006; Vieregg, 2010). In fact, from pH 5 to 9 all bases are uncharged so hybridization occurs

without interference. At higher pH, guanine, uracil and thymine bases become deprotonated (pK_a 9.2-9.7), while at lower pH, adenine and cytosine bases become protonated (pK_a 3.5 and 4.2). This ultimately disfavors pairing, through an increase in electrostatic repulsion at high pHs and destabilization of hydrogen bonding (Blackburn *et al*., 2006; Vieregg, 2010). On the other side, pH ionizes DS molecules, which affects its viscosity (Katchalsky, 1964). This was actually confirmed by viscosity measurements at 25 ± 1ºC of DS 500kDa 10% (wt/vol) solutions at pH 6, pH 9 and pH 12, having respectively 57.20 ± 0.01 mPa.s, 60.38 ± 0.02 mPa.s and 55.25 ± 0.03 mPa.s (Figure S3.1 of supplemental material).

Taking into account the viscosity measurements we could argue a limiting effect of increased viscosity with pH allied to a high content in DS. Nonetheless, viscosity readings show a rather small impact on this parameter when compared with the variance in viscosity of DS with different MW. Still, when using lower MW DS (Table 3.1, assay 3 and 4), that produces a far less viscous hybridization solution than the one using 500 kDa, we observe a higher optimum pH for Gram-positive (Table 3.3) close to the ones obtained for Gram-negative species with a DS of 500 kDa.

Taking into account the results obtained we were able to reach to an optimized PNA-FISH procedure for bacteria in terms of hybridization pH, DS and probe concentration. These results can be added to previous optimization disclosed by Santos *et al*. (2015) to greatly improve the efficiency of the hybridization protocols used. In fact, putting all this information together, a more optimized PNA-FISH hybridization procedure can be obtained in accordance to the properties of the target bacteria (Table 3.4).

Table 3.4 - Optimized hybridization variables for PNA-FISH in 5 Gram-positive and Gram-negative species, by RSM, obtained in this work and reported in Santos *et al* (2014).

	Variable	Time (minutes)	Temperature (ºC)	Formamide (% vol/vol)	pH	DS (% wt/vol)	Probe (nM)
Bacteria	*E. coli* *P. fluorescens* *L. innocua*	55	60	5.5	10	2	≥ 300
	S. epidermidis				8	10	
	B. cereus	120		49.5			

3.4. Conclusions

While optimum values/concentrations were obtained for the three parameters under study, an important observation of the present work was how pH and dextran sulfate interplay, affecting the probe gradient and consequently the hybridization efficiency. In Gram-positive, differently from Gram-negative species, a compromise between pH and DS concentration should be taken into consideration in order to maximize the hybridization efficiency (Figure 3.3).

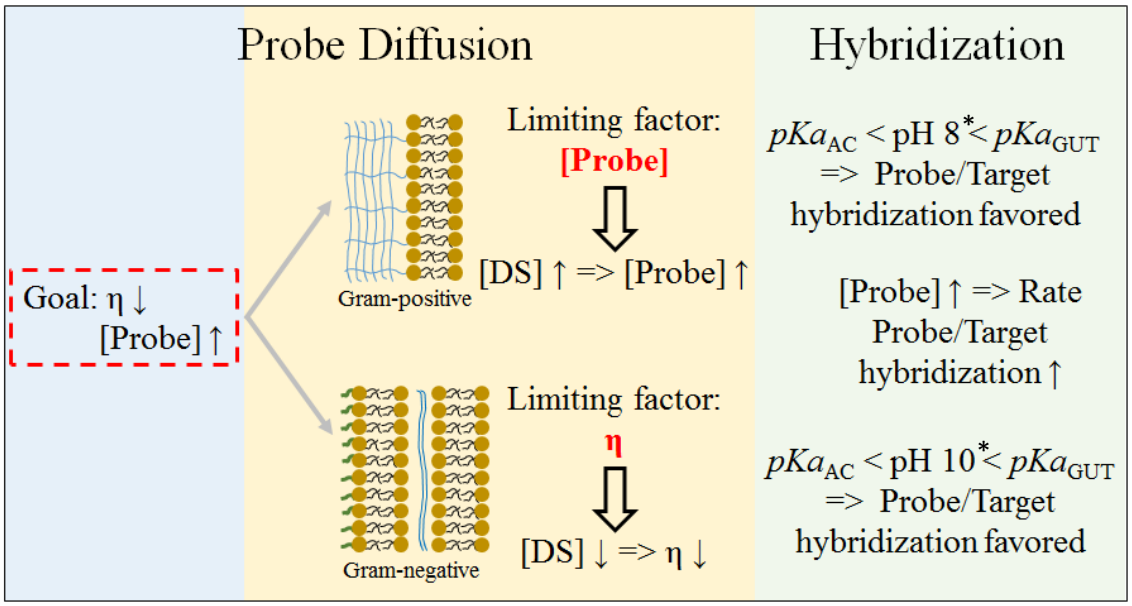

Figure 3.3 - Schematic illustration showing the influence of pH, DS and probe concentration in PNA-FISH for Gram-positive and Gram-negative species. The identification of the limiting factor for Gram-positive and Gram-negative bacteria regarding probe diffusion inside the cell and the adjustment in terms of [DS] needed in order to maximize it. The η stand for viscosity and the A; C; G; U and T in front of *pKa* stand for the Watson-Crick nucleotide bases. The * represent pH values measured at room temperature 20 ± 2ºC.

Bacteria with thick peptidoglycan cell walls are harder to permeabilize (Roller *et al.*, 1994), so a higher probe gradient between the extracellular environment and the cell cytoplasm is necessary to improve probe diffusion through the cell wall. This is accomplished using high concentrations of probe, 300 nM, and DS. The concentration of DS is, however, limited by the viscosity conferred by this molecule to the hybridization solution and in some extent by the pH. If the viscosity is too high, the diffusion of the probe in solution will be the limiting step, if it is too low, the probe gradient driving its diffusion across the cell envelope will be the limiting step for hybridization. So, a balance of DS and pH should always be considered for an efficient hybridization and this work

might be used as a guideline according to the bacteria properties. Future work can expand the scope of this optimization to other steps of the FISH procedures, to a broader range of microorganisms, including species from the other two Domains, *Archaea* and *Eukarya* and eventually, to a set of different nucleic acid mimic probes.

3.5. References

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3.6. Supplemental material

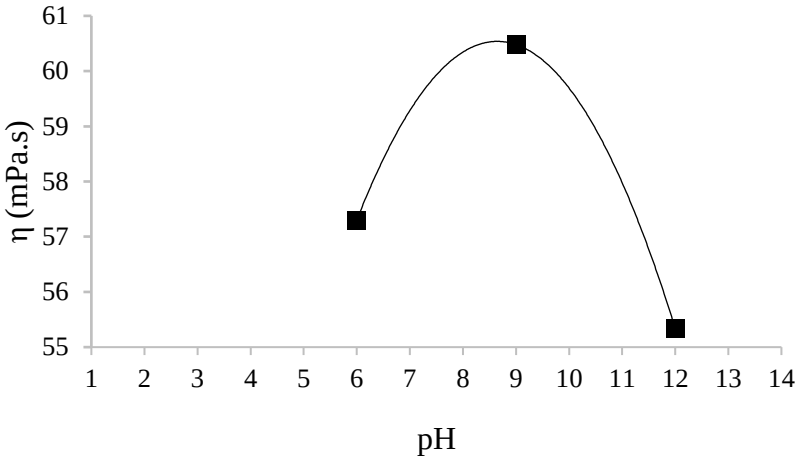

Figure S3.1 - Viscosity measurements of 10% (wt/vol) DS 500 kDa at pH 6, pH 9 and pH 12, in a Cannon-Fenske viscometer at 25 ± 1ºC.

Table S3.1 - pH, DS and probe concentration commonly used in the hybridization solution of previously described PNA-FISH protocols.

Reference	pH	Dextran (% w/v)	Probe (nM)	Application
Stender *et al*., 1999	7.5	10	25/100	Glass slide
Oliveira *et al*., 2001	7.5	10	100/500	Glass slide
Perry-O'Keefe *et al*., 2001	9.0	0	50-300	Suspension
Brehm-Stecher *et al*., 2005	9.0	0	100/300	Suspension
Almeida *et al*., 2010	7.5	10	200	Suspension/Glass slide
Cerqueira *et al*., 2011	7.5	10	200	Glass slide/Biopsy
Zhang *et al*., 2012	7.5	10	300	Glass slide

Table S3.2 - Adjusted quadratic models for the different bacteria in study, in terms of coded values, considering the effect of hybridization pH (x_1), DS concentration (x_2) and probe concentration in the hybridization solution (x_3) and their interactions on the predicted fluorescence intensity (Y).

	Model
B. cereus	$\frac{1}{\sqrt{Y}} = 30.71 - 1.01x_1 + 3.24x_2 + 8.27x_3 + 0.95x_1x_2 - 2.12x_1x_3 + 0.87x_2x_3 - 4.42x_1^2 - 6.62x_2^2 - 2.14x_3^2$
E. coli	$\frac{1}{\sqrt{Y}} = 32.19 + 1.20x_1 - 1.04x_2 + 6.84x_3 - 1.51x_1x_2 + 0.77x_1x_3 - 2.23x_2x_3 - 4.47x_1^2 - 5.03x_2^2 - 2.84x_3^2$
P. fluorescens	$\frac{1}{\sqrt{Y}} = 76.24 + 6.45x_1 + 22.64x_2 + 2.22x_3 - 0.048x_1x_2 + 1.81x_1x_3 - 4.97x_2x_3 - 4.06x_1^2 - 3.22x_2^2 - 6.50x_3^2$
L. innocua	$\frac{1}{\sqrt{Y}} = 21.05 + 2.46x_1 + 0.85x_2 + 4.73x_3 + 0.13x_1x_2 + 0.95x_1x_3 - 1.42x_2x_3 - 3.52x_1^2 - 0.73x_2^2 - 1.76x_3^2$
S. epidermidis	$\frac{1}{\sqrt{Y}} = 14.68 + 0.71x_1 + 1.47x_2 + 2.56x_3 - 0.025x_1x_2 + 0.93x_1x_3 - 0.050x_2x_3 - 1.38x_1^2 - 1.47x_2^2 - 0.045x_3^2$

Table S3.3 - Analysis of variance (ANOVA) for each second-order model.

	B. cereus	*E. coli*	*P. fluorescens*	*L. innocua*	*S. epidermidis*
Model F-value	5.82	4.36	5.36	4.39	4.94
Model *p*-value	0.0055	0.0155	0.0075	0.0152	0.0100
Lack-of-fit *p*-value	0.1088	0.1082	0.1190	0.3824	0.0504
Model R^2	0.8397	0.7968	0.8283	0.7979	0.8163

Chapter 4

Influence of the fixation/permeabilization step on Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) for the detection of bacteria.

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(submitted)

Abstract

Fluorescence *in situ* Hybridization (FISH) is a versatile, widespread and widely-used technique in microbiology. The first step of FISH - fixation/permeabilization - is crucial to the outcome of the method. This work aimed to systematically evaluate fixation/permeabilization protocols employing ethanol, triton X-100 and lysozyme in conjugation with paraformaldehyde for Peptide Nucleic Acid (PNA)-FISH. Response surface methodology was used to optimize these protocols for Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive species (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). In general, the optimal PNA-FISH fluorescent outcome in Gram-positive bacteria was obtained employing harsher permeabilization conditions when compared to Gram-negative optimal protocols. The observed differences arise from the intrinsic cell envelope properties of each species and the ability of the fixation/permeabilization compounds to effectively increase the permeability of these structures while maintaining structural integrity. Ultimately, the combination of paraformaldehyde and ethanol proved to have significantly superior performance for all tested bacteria, especially for Gram-positive species ($p<0.05$).

Keywords

PNA-FISH, *Eubacteria*, PNA EUB338, Fixation, Permeabilization, Paraformaldehyde, Ethanol, Triton-X100, Lysozyme.

4.1. Introduction

Fluorescence *in situ* Hybridization (FISH) is a widely used technique in the field of microbiology (Amann and Fuchs, 2008). Since the first application to microorganisms by DeLong *et al* (1989), FISH progressed into a versatile technique allowing the identification, quantification and characterization of phylogenetically defined microbial populations in complex environments (Wagner *et al*., 2003).

A standard FISH protocol targeting the rRNA, involves 4 different steps: fixation/permeabilization, hybridization, washing and visualization (Cerqueira *et al*., 2008; Frickmann *et al*., 2017). The fixation/permeabilization step is crucial in FISH. On the one hand, it must preserve the integrity of rRNA, cell shape and prevent cell loss through lysis; on the other hand, it must permeabilize the cells in order to allow the diffusion of the probes through the cell envelope (Amann and Fuchs, 2008; Moter and Gobel, 2000). Fixation of bacteria usually employs 4% (wt/vol) paraformaldehyde, an aldehyde that cross-links cellular macromolecules, namely proteins, ultimately creating a mesh type structure within the cell (Amann and Fuchs, 2008; Moter and Gobel, 2000; Thavarajah *et al*., 2012). The use of paraformaldehyde for most Gram-negative bacteria is sufficient to have a successful FISH outcome. However, some Gram-negative and many Gram-positive species require the use of permeabilization agents such as enzymes, solvents, detergents or even hydrochloric acid (Amann and Fuchs, 2008; Frickmann *et al*., 2017). These will cause physical damage on the organized structure of the cell envelope in the form of pores, from where the probes can access the interior of the cell. The choice of the permeabilization procedure to be employed will depend on the characteristics of the microorganism(s) and their cell envelope composition (Felix, 1982), ultimately requiring a pre-optimization stage in order to assess the conditions that provide the best results (Beimfohr *et al*., 1993; Moter and Gobel, 2000; Roller *et al*., 1994; Wagner *et al*., 1998).

Improvements at a procedure level, or as a result of combination with other techniques, allowed the emergence of a diverse array of FISH-based assays (Volpi and Bridger, 2008). One example of this is the application of peptide nucleic acid (PNA) as probes. PNA is a DNA mimic composed by a neutral polyamide backbone with recognized superior hybridization features, such improved thermal stability of the duplexes (Perry-O'Keefe *et al*., 2001; Nielsen, 2001), easier diffusion through the

bacterial envelope (Lefmann *et al*., 2006) and increased resistance to nucleases and proteases (Demidov *et al*., 1994; Stender *et al*., 2002; Wagner *et al*., 2003).

Even though improvements to FISH are noticeable, its outcome is still influenced by a wide variety of abiotic and biotic variables and the way they interplay with each other (Chapter 3; Bouvier and Del Giorgio, 2003; Tang *et al*., 2005). While biotic variation is mainly attributed to the physiological state of microorganisms, abiotic variation is mainly associated to protocol implementation, such as the type of fixative used (aldehyde or alcohol-based fixation), composition of the hybridization solution, hybridization time and temperature. Recent works have successfully disclosed the effect of temperature, time, pH, formamide, probe and dextran sulfate concentration in PNA-FISH through the application of response surface methodology (RSM) (Chapter 3; Santos *et al*., 2014). However, a systematic study addressing the effects of the type of fixation/permeabilization protocol in PNA-FISH is lacking.

This work aimed to disclose the effect (and interplay) of different strategies in the fixation/permeabilization step on PNA-FISH efficiency for bacteria. To this end, three different permeabilization compounds, ethanol, triton X-100 and lysozyme were combined with paraformaldehyde in a series of fixation/permeabilization protocols. Response surface methodology was then used to model the hybridization of a universal *Eubacteria* PNA probe (EUB338) (Amann *et al*., 1990; Santos *et al*., 2014) and signal quantification was assessed by flow cytometry.

4.2. Materials and methods

4.2.1. Bacterial strains

The bacterial strains used in this study were the ones selected in previous works of PNA-FISH modelling and optimization (Chapter 3; Santos *et al*., 2014), including *Pseudomonas fluorescens* ATCC 13525, *Escherichia coli* CECT 434, *Staphylococcus epidermidis* RP61A, *Listeria innocua* CECT 910 and *Bacillus cereus*. All strains were grown on tryptic soy agar (TSA) [3% (wt/vol) tryptic soy broth and 1.5% (wt/vol) agar] (Liofilchem, Italy) at 30ºC and streaked onto fresh plates every 2 or 3 days.

4.2.2. PNA-FISH method

To evaluate the influence of the type of fixation/permeabilization step in the fluorescent signal outcome, a PNA-FISH protocol similar to the one described in Chapter 3 and Santos *et al* (2014) was implemented, followed by signal quantification using flow cytometry. A universal PNA probe EUB338 (5'-TGCCTCCCGTAGGA-3'), based on the work of Amann *et al* (1990), which recognizes a conserved region of the 16S rRNA in the domain *Eubacteria*, was used. The probe was synthesized and labelled at the N terminus with AlexaFluor488 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (Panagene, South Korea).

Overnight grown bacterial cells were harvested from plates and suspended in phosphate-buffered saline (PBS) (137mM NaCl [Sigma, USA]; 2.7mM KCl [Sigma]; 10mM $Na_2HPO_4.2H_2O$ [Sigma] and 1.8mM KH_2PO_4 [Sigma]) to a final concentration of 10^8 to 10^9 cells/mL. For sample fixation/permeabilization, three strategies were evaluated, in representation of different classes of permeabilizers: organic solvents (ethanol), detergents (triton X-100) and enzymes (lysozyme). The ranges selected are presented in Table 4.1. The conditions were selected to cover the normally used procedures described in the literature (Beimfohr *et al*., 1993; Fischer *et al*., 2008; Mohapatra and Duc, 2012; Pernthaler *et al*., 2002; Perry-O'Keefe *et al*., 2001; Roller *et al*., 1994; Wagner *et al*., 1998).

One mL of previously prepared cell suspensions were pelleted by centrifugation at 10,000 × *g* for 5 min, resuspended in 400 µL of 4% (wt/vol) paraformaldehyde (Sigma) and incubated at room temperature according to the experimental design. After centrifugation at 10,000 × *g* for 5 min, the pellet was resuspended in 500 µL of ethanol (Fisher Scientific, USA), triton X-100 (Sigma) or lysozyme (from chicken egg white, ~70000 U/mg - Sigma) and incubated at -20°C, room temperature or 37ºC, respectively, according to the experimental design. For hybridization, 100 µL of the previously fixed bacteria cells were pelleted by centrifugation at 10,000 × *g* for 5 min and resuspended in 100 µL of hybridization solution. The composition of the hybridization solution used took into consideration the optimum conditions already evaluated in previous studies (Chapter 3; Santos *et al*., 2014) with the exception of probe concentration that was kept at 200 nM. Briefly, hybridization solution for *E. coli* and *P. fluorescens* contained 2% (wt/vol) dextran sulfate (average 500,000 Molecular Weight - Sigma), 0.1% (vol/vol) triton X-100, 5.5% (vol/vol) formamide (Sigma) and 50 mM Tris-base (pH 10; Sigma). For *L.*

innocua and *S. epidermidis* it contained 10% (wt/vol) dextran sulfate, 0.1% (vol/vol) triton X-100, 5.5% (vol/vol) formamide and 50 mM Tris-HCl (pH 8). Finally, for *B. cereus* the solution had the same composition as the one used for *L. innocua* and *S. epidermidis* except for the formamide concentration, which was of 49.5% (vol/vol). Samples were hybridized at 60ºC for 55 min, except for *B. cereus* samples that were incubated for 110 min. Negative controls were prepared using the same conditions stated previously and resuspended in hybridization solution without probe. After hybridization, cells were centrifuged, at 10,000 × *g* for 5 min, resuspended in 500 µL of washing solution containing 5 mM Tris base (pH 10; Sigma), 15 mM NaCl (Sigma) and 0.1% (vol/vol) triton X-100 and incubated for 30 min at 60ºC. After centrifugation, at 10,000 × *g* for 5 min, the pellet was resuspended in 500 µL sterile saline solution, 0.9% (wt/vol) NaCl. Each experiment was performed in triplicate.

Table 4.1 - Central composite design levels for the variables used to evaluate the influence of the type of fixation/permeabilization protocol in PNA-FISH for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus* species.

Assay		Variables	Range and level				
			$-\alpha$	-1	0	$+1$	$+\alpha$
1[a]	x_1	Time in Paraformaldehyde 4% (wt/vol) (min)	9.6	30.0	60.0	90.0	110.5
	x_2	[Ethanol] % (vol/vol)	8.0	25.0	50.0	75.0	92.0
	x_3	Time in Ethanol (min)	4.8	15.0	30.0	45.0	55.2
1[b]	x_1	Time in Paraformaldehyde 4% (wt/vol) (min)	9.6	30.0	60.0	90.0	110.5
	x_2	[Triton X-100] % (vol/vol)	0.1	0.6	1.3	2.0	2.5
	x_3	Time in Triton X-100 (min)	4.8	15.0	30.0	45.0	55.2
1[c]	x_1	Time in Paraformaldehyde 4% (wt/vol) (min)	9.6	30.0	60.0	90.0	110.5
	x_2	[Lysozyme] (mg/mL)	0.1	1.1	2.6	4.0	5.0
	x_3	Time in Lysozyme (min)	4.8	15.0	30.0	45.0	55.2

[a] Experimental levels set for Paraformaldehyde-Ethanol fixation/permeabilization studies. Ethanol solutions were prepared in deionized H_2O.
[b] Experimental levels set for Paraformaldehyde-Triton X-100 fixation/permeabilization studies. Ethanol solutions were prepared in deionized H_2O.
[c] Experimental levels set for Paraformaldehyde-Lysozyme fixation/permeabilization studies. Lysozyme solutions were prepared in PBS.

4.2.3. Flow cytometry analysis

The fluorescence intensity of hybridized samples and negative controls was quantified by a Sony EC800 flow cytometer (Sony Biotechnology Inc., San Jose, USA) equipped with a 488 nm argon ion laser. Forward angle light scatter (FS), side angle light scatter (SS) and green fluorescence (FL1) were detected at logarithmic scale. A minimum of 40,000 events falling into the bacterial gate defined on the FS-SS plot were acquired per sample at a flow rate of 20 µL/min. The data was analyzed with Sony analysis

software (Sony Biotechnology Inc), and the average fluorescence intensity was determined for each triplicate experiment.

4.2.4. Response surface methodology (RSM)

The evaluation of the impact of each type of fixation/permeabilization step in the fluorescent signal outcome of bacteria was accessed recurring to RSM, accordingly to the procedure applied by Santos *et al* (2014). The average fluorescence intensity obtained after PNA-FISH was used as the dependent variable.

Central composite designs (CCD) were set up for *P. fluorescens*, *E. coli*, *S. epidermidis*, *L. innocua* and *B. cereus* using the statistical software Design Expert® 10.0.5.0 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model. The range and levels of all variables were defined according to previous studies (Beimfohr *et al*., 1993; Fischer *et al*., 2008; Mohapatra and Duc, 2012; Pernthaler *et al*., 2002; Perry-O'Keefe *et al*., 2001; Roller *et al*., 1994; Wagner *et al*., 1998). Each CCD included 2^3 factorial points (coded at ± 1), 6 axial points (coded as ± α) that represent extreme values used for the estimation of the model curvature and 6 center points (all factors at coded level 0) repeated to take into account the experimental error (Myers and Montgomery, 1995; Silva *et al*., 2011). Therefore, each design matrix consisted of 20 PNA-FISH experiments (Table S4.1, S4.2 and S4.3 of supplemental material).

4.2.5. Statistical analysis

In order infer the best fixation/permeabilization procedure for all five species, the average fluorescence intensity values obtained by flow cytometry were introduced in Design Expert® 10.0.5.0 software to fit a quadratic model and each obtained model was analyzed using analysis of variance (ANOVA). The interaction of the three independent variables and their effect on the fluorescence intensity was inspected by constructing the response surface and contour plots. The optimization function of the software was then used to estimate the optimum conditions within the experimental range that maximized the fluorescence intensity.

A confirmation experiment of the predicted optimum points for the 3 fixation/permeabilization protocols was performed simultaneously for each species in triplicate. The fluorescence intensity obtained in the confirmation experiments was evaluated using a one-way ANOVA followed by Tukey´s test to assess the significance

between the different fixation/permeabilization protocols for each species. The ANOVA and Tukey´s test analysis were performed using the software GraphPad Prism 5 (GraphPad Software, Inc., USA).

4.3. Results and Discussion

This work intended to study and model the effect of different fixation/permeabilization strategies of bacteria during a PNA-FISH procedure. This step is of the utmost importance in FISH, since it can dictate the success or failure of the whole procedure. To model its effect, RSM was applied to the hybridization data obtained from 3 Gram-positive (*S. epidermidis, L. innocua* and *B. cereus*) and 2 Gram-negative species (*P. fluorescens* and *E. coli*). These species were selected in order to include bacteria with different cell wall thicknesses (from thin, e.g. Gram-negative *P. fluorescens* [2.41 ± 0.54 nm, values for *P. aeruginosa* excluding the outer membrane], to thick cell walls, e.g. Gram-positive *B. cereus* [33.3 ± 4.7 nm, values for *B. subtillis*] [Vollmer and Seligman, 2010]) and as a follow up of previous modelling and optimization works (Chapter 3; Santos *et al*., 2014).

Initial CCD designs were based on the values typically described in the literature for FISH fixation/permeabilization protocols (Beimfohr *et al*., 1993; Fischer *et al*., 2008; Mohapatra and Duc, 2012; Pernthaler *et al*., 2002; Perry-O'Keefe *et al*., 2001; Roller *et al*., 1994; Wagner *et al*., 1998). It should be noticed that paraformaldehyde at a concentration of 4% (wt/vol) is a common step to most of the procedures (as this is a preferential compound for fixative purposes) and the main procedural differences are related to the type of permeabilization agent used, as well as the concentration and exposure periods (Amann and Fuchs, 2008; Moter and Gobel, 2000). The application of the fixation/permeabilization protocols according to the CCD designs and their application to PNA-FISH for the five different species under study was successful, since significant quadratic models (for at least one of the test conditions at each fixation/permeabilization combination) (p-value <0.05) and satisfactory coefficients of determination (R^2) were obtained (Tables S4.4 and S4.5 of supplemental material). This allowed to determine the optimal conditions for maximum fluorescence (Table 4.2).

Table 4.2 - Optimum PNA-FISH fixation/permeabilization protocols predicted through RSM models for each species. Fixation/permeabilization combinations included: paraformaldehyde and ethanol, paraformaldehyde and triton X-100 and paraformaldehyde and lysozyme. Negative control, predicted and average obtained fluorescence with error values (standard deviation) under optimum conditions are presented.

Bacteria	**Fixation/Permeabilization Protocol**	**Optimum conditions**			**Confirmation Experiments**		
		Time in Paraformaldehyde 4% (wt/vol) (min)	[Permeabilization Agent] % (vol/vol) or (mg/mL)	Time in Permeabilization Agent (min)	Predicted Fluorescence (a.u.)	Obtained Fluorescence (a.u.)	Negative Control (a.u.)
P. fluorescens	Paraformaldehyde + Ethanol	53.1	25.0	15	215.3	370 ± 30	7.6
	Paraformaldehyde + Triton X-100	70.0	2.0	15	344.0	420 ± 70	16.0
	Paraformaldehyde + Lysozyme	90.0	1.1	15	348.5	350 ± 40	8.7
E. coli	Paraformaldehyde + Ethanol	89.9	25.1	15	205.8	290 ±10[a]	11.4
	Paraformaldehyde + Triton X-100	82.9	2.0	15	179.3	278 ± 4[a]	11.7
	Paraformaldehyde + Lysozyme	90.0	1.1	15	160.6	151 ± 7	8.9
S. epidermidis	Paraformaldehyde + Ethanol	30.0	51.3	15	105.2	102 ± 1[b]	8.8
	Paraformaldehyde + Triton X-100	90.0	2.0	45	67.2	75.2 ± 0.6[b]	7.5
	Paraformaldehyde + Lysozyme	90.0	4.0	15	28.9	38 ± 2[b]	7.5
L. innocua	Paraformaldehyde + Ethanol	30.0	25.0	45	126.5	160 ± 10	12.6
	Paraformaldehyde + Triton X-100	35.2	2.0	45	146.4	210 ± 30	7.5
	Paraformaldehyde + Lysozyme	90.0	1.5	45	163.4	180 ± 40	7.6
B. cereus	Paraformaldehyde + Ethanol	90.0	75.0	15	2136.8	1700 ± 200[c]	21.9
	Paraformaldehyde + Triton X-100	88.8	0.6	15	1861.0	1000 ± 300	21.6
	Paraformaldehyde + Lysozyme	86.0	1.1	15	1062.7	1200 ± 100	18.0

[a] Indicates significant differences among the fixation/permeabilization protocol and the one using lysozyme, $p < 0.05$;
[b] Indicates significant differences among all the fixation/permeabilization protocols, $p < 0.05$;
[c] Indicates significant differences among the fixation/permeabilization protocol using ethanol and the ones using triton X-100 and lysozyme, $p < 0.05$.

As in previous optimization studies, differences in fluorescence intensity values between species are observed. The fluorescence signal for positive samples ranged from 17.4 to 2449.0 a.u., depending on the microorganism and condition tested. Overall, higher values were obtained for Gram-negative over Gram-positive species, except for *B. cereus*. These variations are known to be, at a certain level, intrinsic to the target RNA content and probe accessibility (Chapter 3; Santos *et al*., 2014). As significant variations in the fluorescence intensity can be found between species when applying different fixation/permeabilization protocols for each bacteria (see Table 4.2 and Fig 4.1D).

For each fixation/permeabilization method, the results in Table 4.2 were transposed to level factors and plotted into a radar chart for further analysis (Figure 4.1).

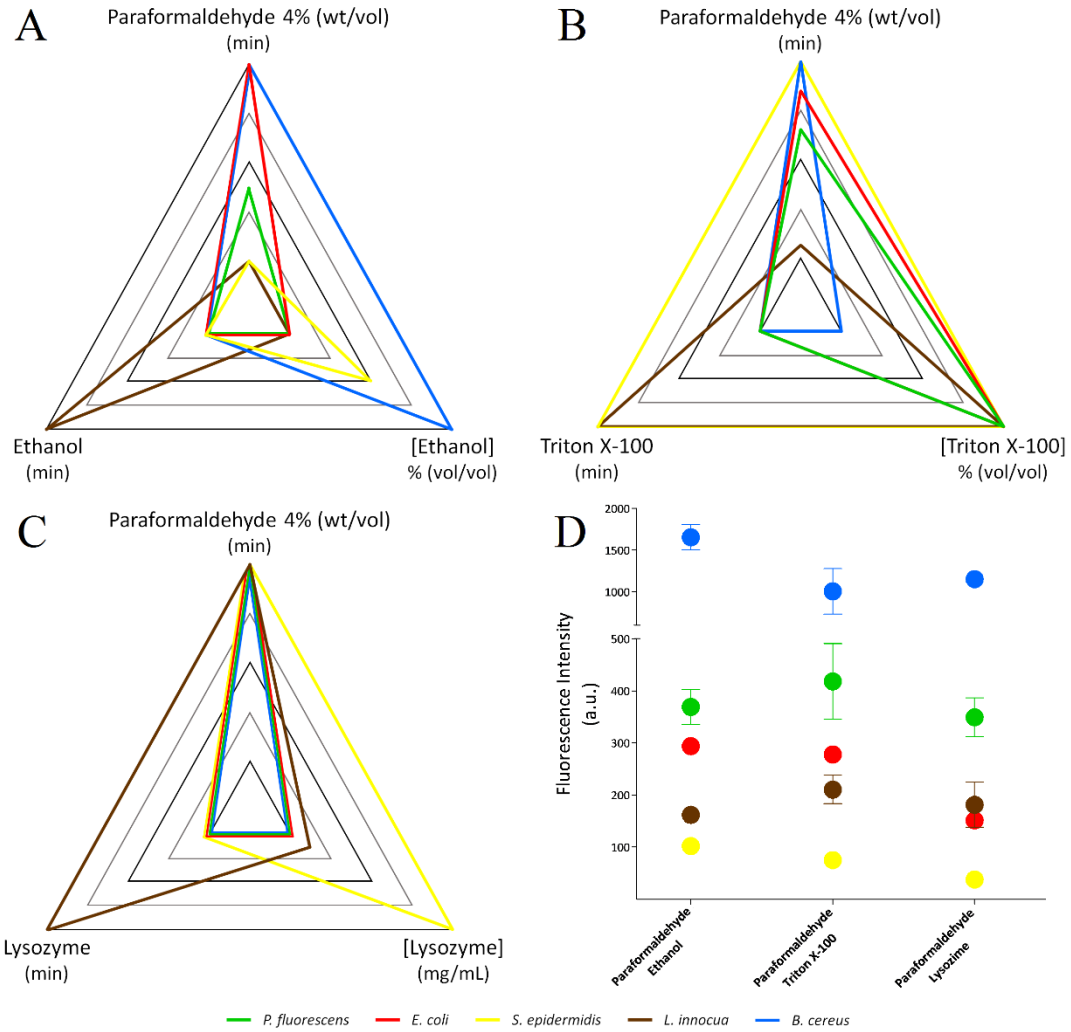

Figure 4.1 - Optimum PNA-FISH fixation/permeabilization protocol and fluorescence intensity outcome obtained for each species. Radar chart representation in terms of level factors (-1 [inner vertices] to 1 [outer vertices]): A - Paraformaldehyde and ethanol; B - Paraformaldehyde and triton X-100; C - Paraformaldehyde and lysozyme. D - Average fluorescence intensity and error bars (standard deviation) of the confirmation experiment for the optimum fixation/permeabilization protocol for each species (*P. fluorescens* - green; *E. coli* - red; *S. epidermidis* - yellow; *L. innocua* - brown and *B. cereus* - blue).

4.3.1. Treatment with paraformaldehyde and ethanol

Ethanol is used in FISH procedure as a fixative and as a permeabilization agent (Perry-O'Keefe *et al*., 2001; Thavarajah *et al*., 2012; Wagner *et al*., 1998). Ethanol fixative capability, similarly to other alcohols, arises from the coagulation, precipitation and denaturation of proteins, through the interference with their hydration cloud (Rhodes, 2012; Thavarajah *et al*., 2012). On the other hand, permeabilization is accomplished by promoting the solubilization of cell envelope components (Felix, 1982).

Analyzing Figure 4.1A, a Gram-specific behavior is observed in the optimal fixation/permeabilization protocol found for each species. Gram-negative *P. fluorescens* and *E. coli* required longer paraformaldehyde steps (above 50 minutes) combined with low ethanol concentrations for short periods (25% [vol/vol] for 15 minutes). These findings are not surprising, since previous reports using DNA probes, stated that hybridization of Gram-negative species can be successfully achieved using only paraformaldehyde as a fixation/permeabilization agent (Frickmann *et al*., 2017; Roller *et al*., 1994; Wagner *et al*., 1998). This arises from the fact that aldehyde fixatives present also a weak detergent-like activity (Frickmann *et al*., 2017). On the other side, Gram-positive *S. epidermidis* and *B. cereus* required exposure to higher ethanol concentrations (50% and 75% [vol/vol], respectively) or, as found for *L. innocua*, a longer ethanol step (45 minutes at 25% [vol/vol]) for an effective permeabilization. Again, this was an anticipated result since Gram-positive bacteria are known to be harder to permeabilize (Roller *et al*., 1994). Overall, these optimizations are directly connected with the specific cell envelope architecture, while Gram-positive species cell wall is mainly composed of thick and rigid peptidoglycan structure intertwined with teichoic and lipoteichoic acids, Gram-negative species present a small layer of peptidoglycan between the cell membrane and an outer membrane (Madigan *et al*., 2011; Pommerville *et al*., 2010; Willey *et al*., 2008).

Furthermore, it is also possible to observed that short paraformaldehyde steps of 30 minutes, are preferred for Gram-positive species, excluding *B. cereus*. These results are in line with previous reports stating detrimental effects of cross-linking agents in terms of fluorescent outcome on whole cell hybridization of Gram-positive species (Braun-Howland *et al*., 1992; Nettman *et al*., 2013).

4.3.2. Treatment with paraformaldehyde and triton X-100

Triton X-100 is a nonionic chemical surfactant used in FISH in the fixation/permeabilization step or/and as part of the hybridization solution (Almeida *et al*., 2010; Ficher *et al*., 2008; Mohapatra and Duc, 2012; Pernthaler *et al*., 2002). It is a very effective detergent in the solubilization of phospholipids, due to the high binding affinity to hydrophobic molecules. Permeabilization arises from a channel-forming effect that results from two main events: interaction and substitution of cell envelope lipid molecules and conformational changes in cell envelope proteins (Felix, 1982; Hettwer and Wang, 1988).

Analyzing paraformaldehyde and triton X-100 testing results, Figure 4.1B, is possible to observe a Gram-specific pattern, as with previous paraformaldehyde/ethanol optimizations. Overall, Gram-negative species, *P. fluorescens* and *E. coli*, required less exposure to triton X-100 (15 minutes) in order to achieve the highest fluorescence intensity than Gram-positive species, *L. innocua* and *S. epidermidis* (45 minutes).

An interesting finding regarding *B. cereus* optimal protocol was encountered, since the optimal triton X-100 concentration and exposure time were considerably lower (0.6% [vol/vol] for 15 minutes) than those found for the other Gram-positive species. This could result from the use of a relatively high formamide content in the hybridization solution (resulting from previous optimizations - 49.5% [vol/vol]), which is known to have a damaging effect on the integrity of the cell wall and thus might present a synergetic effect with triton X-100 treatment (Santos *et al*., 2014).

4.3.3. Treatment with paraformaldehyde and lysozyme

Lysozyme is a lytic enzyme that hydrolyses the β-1,4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan (Kubota, 2013). Since peptidoglycan is a common component of the cell wall of Eubacteria, especially in Gram-positive species, this enzyme is typically used for permeabilization of bacteria in FISH procedures (Beimfohr *et al*., 1993; Kubota, 2013; Mohapatra and Duc, 2012; Wagner *et al*., 1998). As with other lytic enzymes, lysozyme has a narrow applicability spectrum when compared to chemical permeabilization. This results from the specificity of the enzyme-target reaction and loss of activity if somehow their action site is inaccessible and/or modified (Kubota, 2013).

Analyzing paraformaldehyde and lysozyme results (Fig 4.1C), a Gram-specific pattern is observed again. Gram-negative species present a higher fluorescent outcome with a fixation/permeabilization step with long exposures to paraformaldehyde (90 minutes) and short exposures to lysozyme (15 minutes) at low concentrations (1.1 mg/mL). Generally, in Gram-negative species the outer membrane precludes the access to lytic enzymes; thus, membrane removal by detergents or chelating agents is usually required for a successful permeabilization (Salazar and Asenjo, 2007). However, the compromised membranes of fixed cells assure the enzyme access to the peptidoglycan. In fact, an extended exposure to lysozyme could result on cell lysis even before Gram-positive cells became permeable (Beimfohr *et al*., 1993; Kubota, 2013). The results obtained here seem to confirm this last observation, since higher exposure to lysozyme would induce a lower PNA-FISH fluorescence outcome in Gram-negative bacteria, likely due to extensive damage in the cell envelope.

In Gram-positive species, *B. cereus* presents a behavior similar to the one observed for Gram-negative species. The optimal protocol for *L. innocua* required an higher lysozyme exposure (45 minutes), while *S. epidermidis* required an higher lysozyme concentration (4.0 mg/mL). This species-specific behavior could be related to lysozyme sensitivity/resistance of each species, the degree of cross-linking, type and content of glycan modifications in the peptidoglycan, which are characteristics that can affect lysozyme activity (Loskill *et al*., 2014; Pucciarelli *et al*., 2007; Vollmer, 2008; Boneca *et al*., 2007; Nawrocki *et al*., 2014). One clear example of this is the observed low fluorescence outcome of S. epidermidis (Table 4.2). In fact, the *Staphylococcus* genus is known to have a peptidoglycan insensitive to lysozyme activity, resulting mainly from modifications (O-acetylation) of peptidoglycan monomers (Bera *et al*., 2005). As such, *S. epidermidis*, was expected to be poorly permeabilized by paraformaldehyde/lysozyme protocols.

4.3.4. Towards a fully optimized PNA-FISH procedure

Following fixation/permeabilization protocol optimization for each species, the predictions made by the different models were confirmed experimentally. From the confirmation experiments, a general agreement between the predicted and the obtained fluorescence values was observed (Table 4.2). Furthermore, these assays also enable a comparison between the different fixation/permeabilization protocols (Table 4.2 and

Figure 4.1D). For *B. cereus* the fixation/permeabilization protocol using ethanol performed significantly better than the other two tested ($p<0.05$). Regarding *E. coli* and *S. epidermidis* both ethanol and triton X-100 protocols worked significantly better than the one using lysozyme ($p<0.05$). For *P. fluorescens* and *L. innocua* all tested fixation/permeabilization protocols provided similar PNA-FISH outcomes ($p>0.05$). Based on the above, paraformaldehyde and ethanol was the fixation/permeabilization PNA-FISH protocol which allowed an overall higher fluorescence outcome in all five Eubacteria species tested.

The results obtained here can be combined with previous optimizations and subsequently be used for the development of new PNA-FISH methodologies for microbial detection. In fact, putting all this information together, an almost fully optimized PNA-FISH procedure can be obtained in accordance to the properties of the target bacteria (Table 4.3).

Although the results point towards a species-specific optimal fixation/permeabilization protocol, a compromise between protocol and fluorescence outcome in PNA-FISH is possible. This arises from the observation that highly fluorescent species, such *B. cereus* and *P. fluorescens*, will exhibit a similar or higher fluorescence outcome when compared other low fluorescent-species, such *S. epidermidis*, *E. coli* and *L. innocua*, even when protocol conditions are very different from their optimal procedures. Moreover, all species showed a positive outcome, in some cases even coinciding with their optimum procedure, using the shortest fixation/permeabilization protocol, 30 minutes in paraformaldehyde followed by 15 minutes of permeabilization (Figures S4.1, S4.2 and S4.3 of supplemental material). These observations have particular significance in applications where time to result is an important factor, such the food safety and the medical diagnostics area and also for multiplex applications where several species can be targeted in a single assay (Frickmann *et al*., 2017; Rohde *et al*., 2015). Namely, the species with lower rRNA content (thus weaker basal fluorescence signal) might be favored in terms of hybridization and permeabilization protocols, so the population's signals can be balanced.

Table 4.3 - Optimized methodological variables for PNA-FISH. Conditions for 5 Gram-positive and Gram-negative species, obtained by RSM in this work and in previous studies (Chapter 3; Santos *et al.*, 2014).

Variable		Fixation/Permeabilization			Hybridization					
		Paraformaldehyde 4% (wt/vol) (min)	Ethanol % (vol/vol)	Ethanol (min)	Time (min)	Temperature (ºC)	Formamide (% vol/vol)	pH	DS % (wt/vol)	Probe $\overline{\overline{\text{}}}$ (nM)
Bacteria	*P. fluorescens*	50	25	15	55	60	5.5	10	2	≥ 300
	E. coli	90		45						
	L. innocua	30	50	15	120		49.5	8	10	
	S. epidermidis	90	75							
	B. cereus									

Finally, it is possible that the optimized conditions for the fixation/permeabilization protocols can be applicable to other microorganisms when PNA probes are used. Nonetheless, adjustments to the optimum conditions described in this work cannot be excluded, especially for target species with very different cell envelope compositions. It is also important to notice that the optimizations described above are likely not applicable to DNA, RNA and other nucleic acid mimics probes such as LNA or 2'OMe RNA, as their molecular structure differs markedly from PNA oligonucleotides (Cerqueira *et al*., 2008).

4.4. Conclusions

In this work we have shown that paraformaldehyde fixation followed by organic solvent (ethanol), detergent (triton X-100) or enzymatic (lysozyme) permeabilization are suitable strategies for PNA-FISH procedures targeting Eubacteria. However, a unique optimal protocol was not found for all tested species. Despite this, of the three tested strategies, paraformaldehyde followed by ethanol has proven to be the best fixation/permeabilization protocol for PNA-FISH procedures. The differences between optimal protocols obtained were mainly attributed to the inherent differences in the cell envelope, more precisely in terms of peptidoglycan thickness. As such, for Gram-negative species with a thinner peptidoglycan cell wall structure, the combination of a short step with low concentration of permeabilizant provided the best PNA-FISH outcomes. On the contrary, Gram-positive species with a thicker peptidoglycan cell wall structure, a longer step and/or higher permeabilizant concentrations were required for an optimal PNA-FISH outcome. Additionally, the duration of the paraformaldehyde step was identified as another driving factor for Gram-positive species, especially for ethanol procedures. Prolonged exposure proved to have a detrimental effect on the fluorescence outcome and as such, short procedures were generally preferred (Figure 4.2).

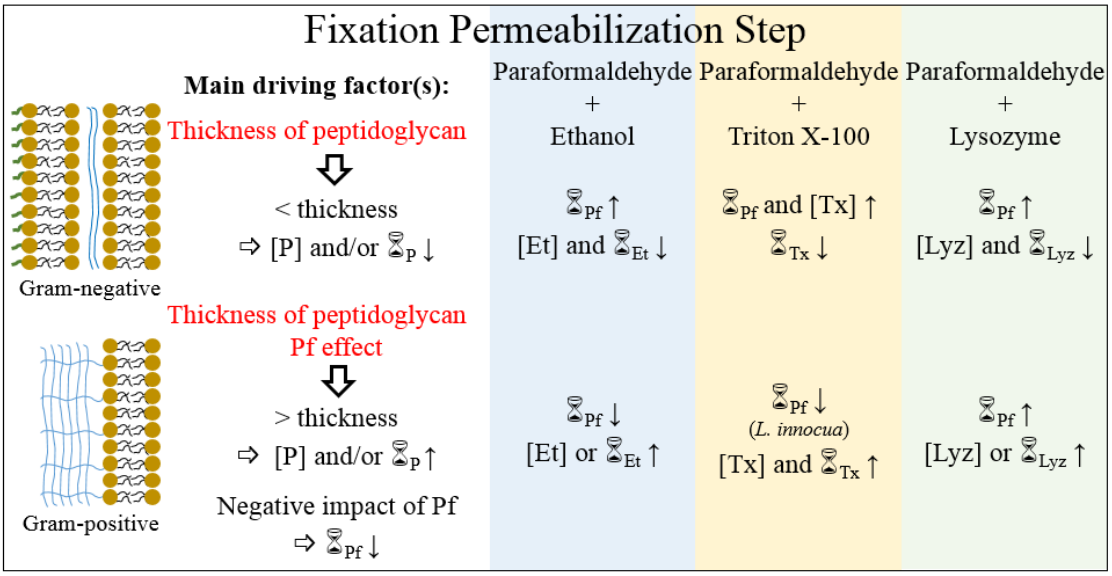

Figure 4.2 - Identification of the driving factors that influence each fixation/permeabilization protocol in PNA-FISH for Gram-positive and Gram-negative species (except *B. cereus*). P - Permeabilizant; Pf - Paraformaldehyde; Et - Ethanol; Tx - Triton X-100; Lyz - Lysozyme; [Xx] - Concentration of permeabilizant X; ⌛X - Duration of substance X application.

Further research, could focus in the expansion of the scope of this optimization to a broader range permeabilization compounds, microorganisms, including species from the other two Domains, Archaea and Eukarya, and eventually, to a set of different nucleic acid mimic probes.

4.5. References

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4.6. Supplemental material

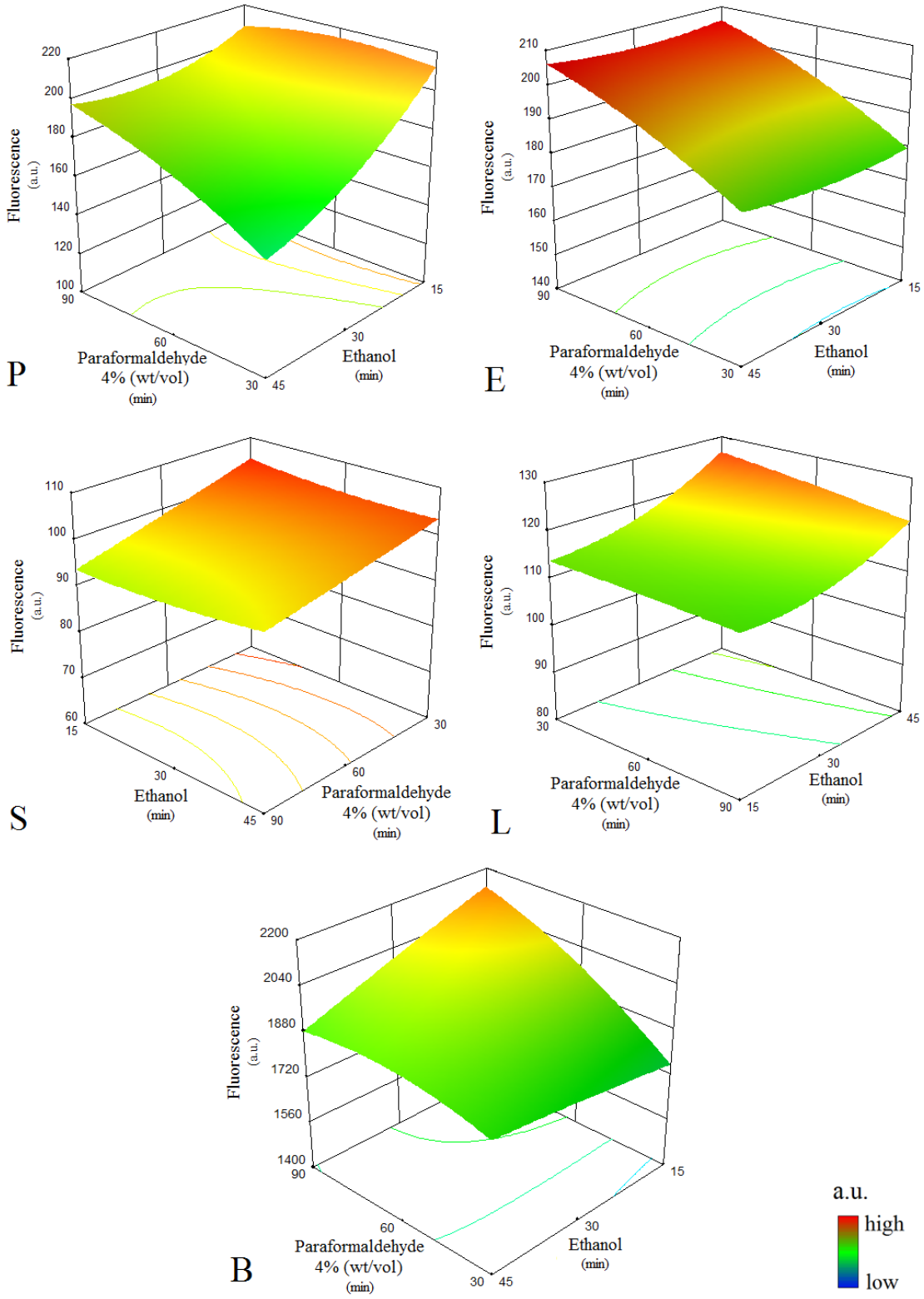

Figure S4.1 - Surface response plots for the fluorescence response of *P. fluorescens* (P), *E. coli* (E), *S. epidermidis* (S), *L. innocua* (L) and *B. cereus* (B), regarding the fixation/permeabilization protocol using paraformaldehyde and ethanol. The permeabilizant concentration was kept at their optimum value in each graph. Fluorescence values are presented in arbitrary units (a.u.).

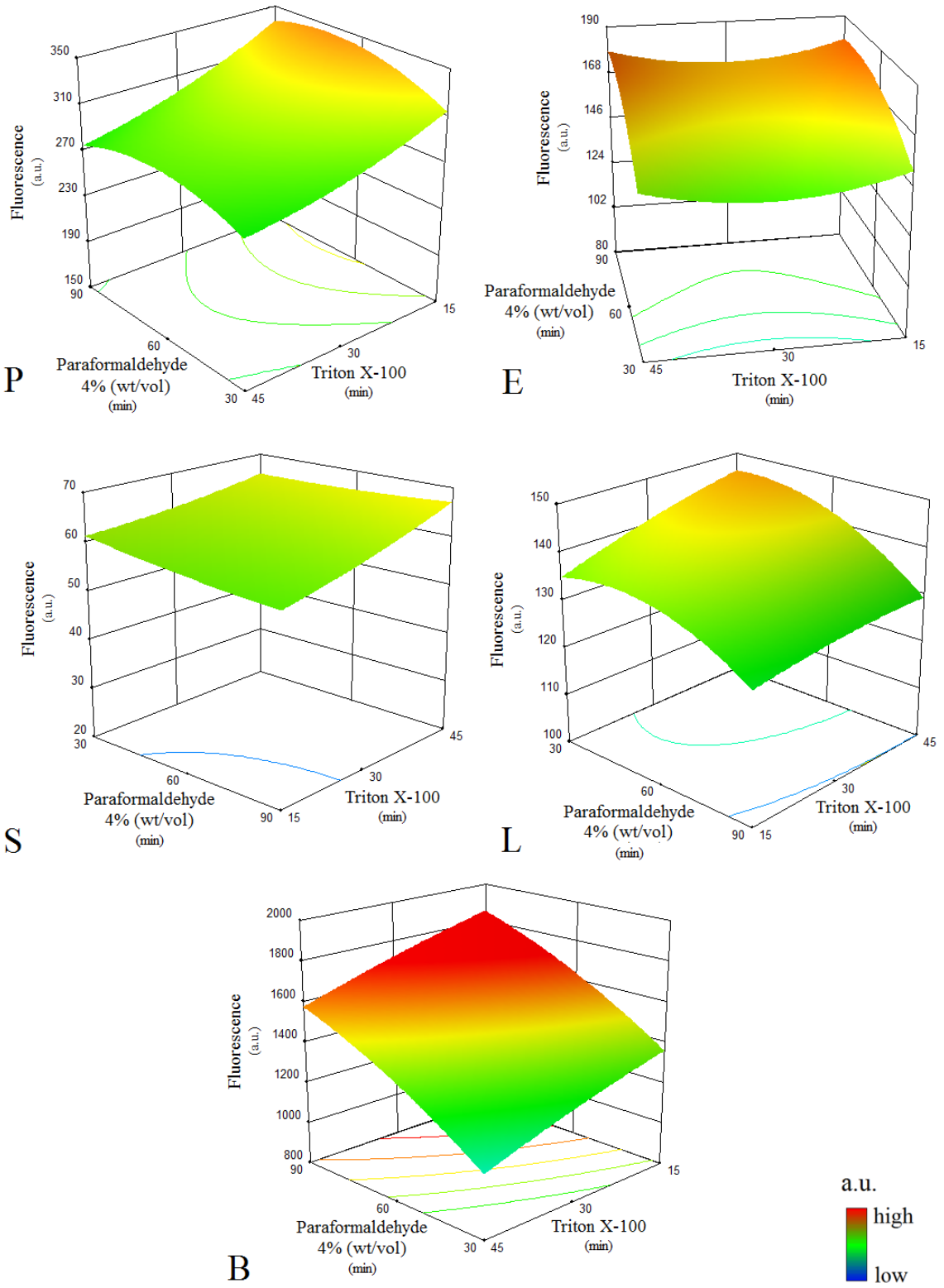

Figure S4.2 - Surface response plots for the fluorescence response of *P. fluorescens* (P), *E. coli* (E), *S. epidermidis* (S), *L. innocua* (L) and *B. cereus* (B), regarding the fixation/permeabilization protocol using paraformaldehyde and triton X-100. The permeabilizant concentration was kept at their optimum value in each graph. Fluorescence values are presented in arbitrary units (a.u.).

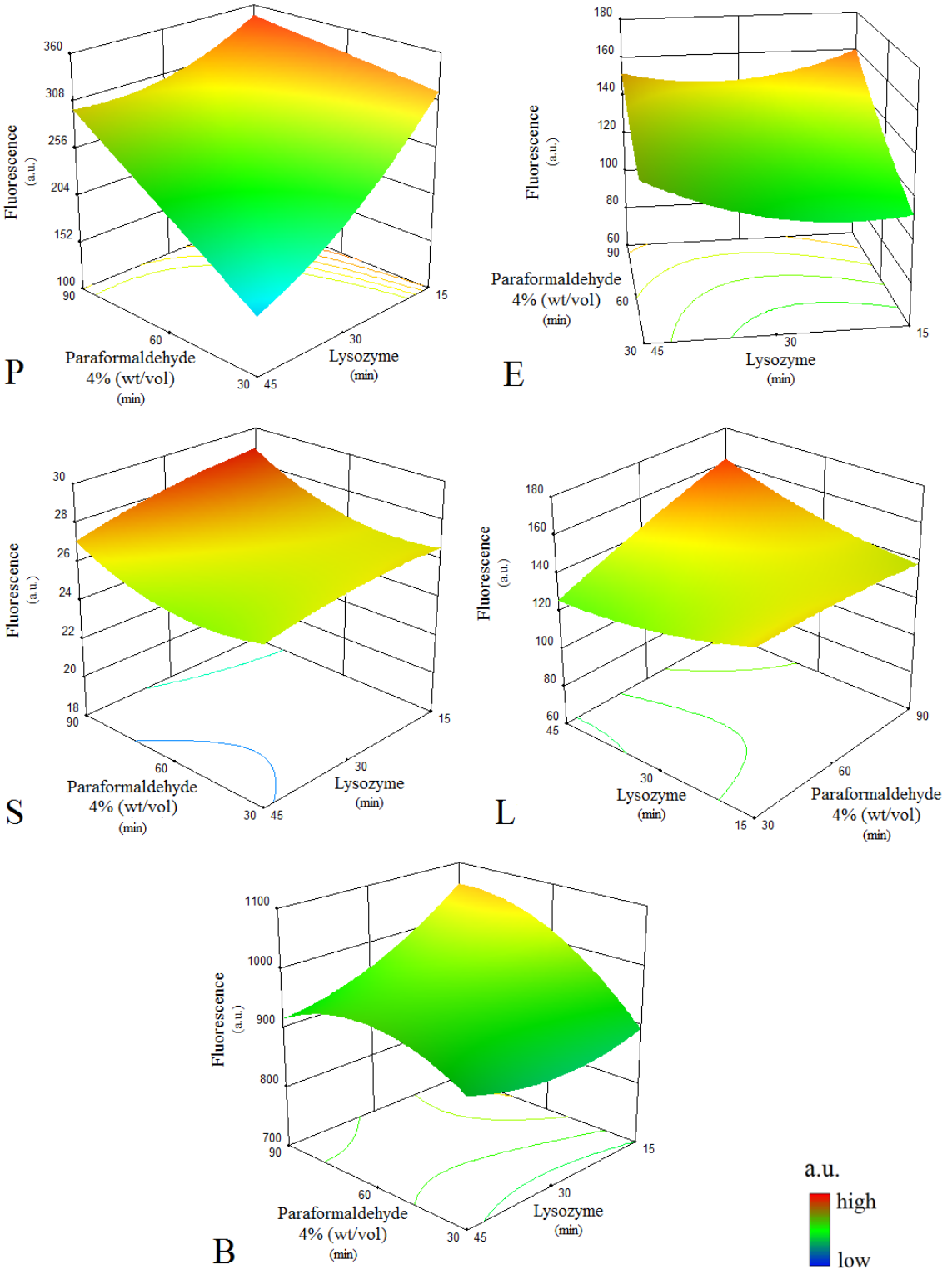

Figure S4.3 - Surface response plots for the fluorescence response of *P. fluorescens* (P), *E. coli* (E), *S. epidermidis* (S), *L. innocua* (L) and *B. cereus* (B), regarding the fixation/permeabilization protocol using paraformaldehyde and lysozyme. The permeabilizant concentration was kept at their optimum value in each graph. Fluorescence values are presented in arbitrary units (a.u.).

Table S4.1 - Central composite designs with the list of experiments performed for the optimization of a fixation/permeabilization PNA-FISH procedure using paraformaldehyde 4% (wt/vol) combined with ethanol for *P. fluorescens*, *E. coli*, *S. epidermidis*, *L. innocua* and *B. cereus*.

Experiment	**Variables**		
	Paraformaldehyde 4% (wt/vol) (min)	Ethanol % (vol/vol)	Ethanol (min)
1	30.00	25.00	15.00
2	90.00	25.00	15.00
3	30.00	75.00	15.00
4	90.00	75.00	15.00
5	30.00	25.00	45.00
6	90.00	25.00	45.00
7	30.00	75.00	45.00
8	90.00	75.00	45.00
9	9.55	50.00	30.00
10	110.45	50.00	30.00
11	60.00	7.96	30.00
12	60.00	92.04	30.00
13	60.00	50.00	4.77
14	60.00	50.00	55.23
15	60.00	50.00	30.00
16	60.00	50.00	30.00
17	60.00	50.00	30.00
18	60.00	50.00	30.00
19	60.00	50.00	30.00
20	60.00	50.00	30.00

Table S4.2 - Central composite designs with the list of experiments performed for the optimization of a fixation/permeabilization PNA-FISH procedure using paraformaldehyde 4% (wt/vol) combined with triton X-100 for *P. fluorescens*, *E. coli*, *S. epidermidis*, *L. innocua* and *B. cereus*.

Experiment	**Variables**		
	Paraformaldehyde 4% (wt/vol) (min)	Triton X-100 % (vol/vol)	Triton X-100 (min)
1	30.00	0.59	15.00
2	90.00	0.59	15.00
3	30.00	2.01	15.00
4	90.00	2.01	15.00
5	30.00	0.59	45.00
6	90.00	0.59	45.00
7	30.00	2.01	45.00
8	90.00	2.01	45.00
9	9.55	1.30	30.00
10	110.45	1.30	30.00
11	60.00	0.10	30.00
12	60.00	2.50	30.00
13	60.00	1.30	4.77
14	60.00	1.30	55.23
15	60.00	1.30	30.00
16	60.00	1.30	30.00
17	60.00	1.30	30.00
18	60.00	1.30	30.00
19	60.00	1.30	30.00
20	60.00	1.30	30.00

Table S4.3 - Central composite designs with the list of experiments performed for the optimization of a fixation/permeabilization PNA-FISH procedure using paraformaldehyde 4% (wt/vol) combined with lysozyme for *P. fluorescens, E. coli, S. epidermidis, L. innocua* and *B. cereus*.

Experiment	**Variables**		
	Paraformaldehyde 4% (wt/vol) (min)	Lysozyme (mg/mL)	Lysozyme (min)
1	30.00	1.09	15.00
2	90.00	1.09	15.00
3	30.00	4.01	15.00
4	90.00	4.01	15.00
5	30.00	1.09	45.00
6	90.00	1.09	45.00
7	30.00	4.01	45.00
8	90.00	4.01	45.00
9	9.55	2.55	30.00
10	110.45	2.55	30.00
11	60.00	0.10	30.00
12	60.00	5.00	30.00
13	60.00	2.55	4.77
14	60.00	2.55	55.23
15	60.00	2.55	30.00
16	60.00	2.55	30.00
17	60.00	2.55	30.00
18	60.00	2.55	30.00
19	60.00	2.55	30.00
20	60.00	2.55	30.00

Table S4.4 - Analysis of variance (ANOVA) for each second-order model and each individual factor (x_1 - Time in Paraformaldehyde 4% [wt/vol] [min]; x_2 - [Permeabilizant] [% vol/vol for Ethanol and Triton X-100 and mg/mL for Lysozyme]; x_3 - Time in Permeabilizant [min]).

	P. fluorescens			*E. coli*			*S. epidermidis*			*L. innocua*			*B. cereus*		
	Pf/Et	Pf/Tx	Pf/Lyz	Pf/Et	Pf/Tx	Pf/Lyz	Pf/Et	Pf/Tx	Pf/Lyz	Pf/Et	Pf/Tx	Pf/Lyz	Pf/Et	Pf/Tx	Pf/Lyz
Model F-value	1.98	1.71	2.62	4.97	4.52	1.60	3.60	9.89	6.09	1.82	10.70	15.22	3.51	5.31	3.89
Model *p*-value	0.1507	0.2076	0.0750	0.0098	0.0136	0.2372	0.0292	0.0007	0.0046	0.1810	0.0005	0.0001	0.0317	0.0077	0.0227
Lack-of-fit F-value	2.03	2.82	1.58	2.00	17.10	8.47	2.03	1.24	1.43	3.61	0.28	1.49	1.68	3.54	1.95
Lack-of-fit *p*-value	0.2273	0.1397	0.3145	0.2318	0.0037	0.0175	0.2282	0.4100	0.3518	0.0924	0.9060	0.3358	0.2914	0.0958	0.2408
Model R^2	0.6407	0.6061	0.7020	0.8174	0.8028	0.5901	0.7642	0.8990	0.8457	0.6216	0.9059	0.9320	0.7595	0.8271	0.7780
x_1 F-value	2.73	2.96	10.99	13.17	9.61	7.87	5.97	0.46	2.23	5.68	35.29	2.23	4.49	9.52	2.91
x_1 p-value	0.1296	0.1158	0.0078	0.0046	0.0113	0.0186	0.0347	0.5119	0.1663	0.0384	0.0001	0.1666	0.0601	0.0115	0.1190
x_2 F-value	3.06	3.07	0.0017	28.92	21.01	0.22	0.022	74.05	22.08	3.69	12.17	59.39	10.92	10.74	3.63
x_2 p-value	0.1107	0.1102	0.9684	0.0003	0.0010	0.6510	0.8862	<0.0001	0.0008	0.0838	0.0058	<0.0001	0.0079	0.0083	0.0858
x_3 F-value	6.98	5.10	7.40	0.11	0.37	0.52	0.0085	0.79	5.07	1.42	0.77	2.98	0.32	4.82	13.94
x_3 p-value	0.0247	0.0474	0.0216	0.7451	0.5561	0.4865	0.9284	0.3961	0.0480	0.2613	0.3999	0.1151	0.5864	0.0529	0.0039
x_1x_2 F-value	0.018	0.83	0.028	0.99	0.0002	0.0005	0.57	0.082	6.80	1.41	1.00	5.17	0.61	7.19	0.19
x_1x_2 p-value	0.8955	0.3828	0.8715	0.3429	0.9876	0.9818	0.4667	0.7803	0.0261	0.2627	0.3403	0.0464	0.4533	0.0231	0.6713
x_1x_3 F-value	1.96	0.10	2.36	0.0063	0.0031	1.08	0.17	0.22	0.16	0.12	1.37	13.85	3.77	0.0014	2.35
x_1x_3 p-value	0.1921	0.7549	0.1553	0.9066	0.9568	0.3230	0.6851	0.6466	0.6937	0.7352	0.2691	0.0040	0.0807	0.9704	0.1562
x_2x_3 F-value	0.0004	0.0002	0.64	0.014	0.27	0.0056	0.24	0.23	7.64	1.03	7.26	1.37	1.83	1.31	2.43
x_2x_3 p-value	0.9845	0.9880	0.4408	0.3796	0.6171	0.9419	0.6319	0.6384	0.0200	0.3348	0.0225	0.2685	0.2054	0.2793	0.1504
x_1^2 F-value	0.61	2.14	0.0006	0.85	4.19	0.24	0.0052	0.039	4.42	0.013	14.75	0.46	1.63	1.48	6.43
x_1^2 p-value	0.4515	0.1738	0.9809	0.3796	0.0678	0.6353	0.9538	0.8465	0.0619	0.9103	0.0033	0.5146	0.2301	0.2513	0.0296
x_2^2 F-value	0.52	0.48	0.22	0.0014	0.090	1.97	24.28	13.16	4.99	1.25	19.39	44.77	8.59	13.52	0.22
x_2^2 p-value	0.4872	0.5053	0.6463	0.9708	0.7707	0.1905	0.0006	0.0046	0.0495	0.2888	0.0013	<0.0001	0.0150	0.0043	0.6473
x_3^2 F-value	1.78	0.36	1.98	0.55	4.25	3.13	0.21	0.089	0.41	1.99	0.48	4.00	0.022	0.14	2.03
x_3^2 p-value	0.2113	0.5605	0.1893	0.4746	0.0662	0.1071	0.6552	0.7711	0.5383	0.1883	0.5062	0.0735	0.8854	0.7169	0.1850

Pf - Paraformaldehyde; Et - Ethanol; Tx - Triton X-100; Lyz - Lysozyme.

Table S4.5 - Adjusted quadratic models for the different bacteria in study, in terms of coded values, considering the effect of time in Paraformaldehyde 4% (wt/vol) (min) (x_1), [Permeabilizant] (% vol/vol for Ethanol and Triton X-100 and mg/mL for Lysozyme) (x_2) and time in Permeabilizant (min) (x_3) and their interactions on the predicted fluorescence intensity (Y).

Species	Fixation/Permabilization Protocol	Model
P. fluorescens	Paraformaldehyde + Ethanol	$\frac{1}{\sqrt{Y}} = 175.72 + 10.11x_1 - 10.71x_2 - 16.17x_3 + 1.08x_1x_2 + 11.19x_1x_3 + 0.16x_2x_3 - 4.67x_1^2 + 4.30x_2^2 + 7.96x_3^2$
	Paraformaldehyde + Triton X-100	$\frac{1}{\sqrt{Y}} = 282.80 + 19.50x_1 + 19.84x_2 - 25.58x_3 - 13.50x_1x_2 - 4.75x_1x_3 + 0.23x_2x_3 - 16.14x_1^2 + 7.62x_2^2 + 6.64x_3^2$
	Paraformaldehyde + Lysozyme	$\frac{1}{\sqrt{Y}} = 259.42 + 44.55x_1 + 0.55x_2 - 36.547x_3 + 2.91x_1x_2 + 26.99x_1x_3 + 14.09x_2x_3 - 0.32x_1^2 + 6.19x_2^2 + 18.43x_3^2$
E. coli	Paraformaldehyde + Ethanol	$\frac{1}{\sqrt{Y}} = 180.64 + 9.15x_1 - 13.56x_2 + 0.84x_3 - 3.28x_1x_2 - 0.26x_1x_3 + 0.40x_2x_3 - 2.26x_1^2 - 0.092x_2^2 + 1.82x_3^2$
	Paraformaldehyde + Triton X-100	$\frac{1}{\sqrt{Y}} = 148.67 + 12.62x_1 + 18.66x_2 + 2.48x_3 + 0.084x_1x_2 + 0.30x_1x_3 - 2.74x_2x_3 - 8.12x_1^2 - 1.19x_2^2 + 8.17x_3^2$
	Paraformaldehyde + Lysozyme	$\frac{1}{\sqrt{Y}} = 117.78 + 15.82x_1 - 2.63x_2 + 4.07x_3 - 0.17x_1x_2 - 7.66x_1x_3 + 0.55x_2x_3 + 2.68x_1^2 + 7.71x_2^2 + 9.72x_3^2$
S. epidermidis	Paraformaldehyde + Ethanol	$\frac{1}{\sqrt{Y}} = 98.95 - 4.45x_1 + 0.274x_2 + 0.17x_3 - 1.80x_1x_2 + 0.99x_1x_3 + 1.18x_2x_3 + 0.13x_1^2 - 8.74x_2^2 + 0.82x_3^2$
	Paraformaldehyde + Triton X-100	$\frac{1}{\sqrt{Y}} = 33.46 - 1.59x_1 + 20.09x_2 + 2.07x_3 + 0.87x_1x_2 + 1.44x_1x_3 + 1.48x_2x_3 + 0.45x_1^2 + 8.24x_2^2 + 0.68x_3^2$
	Paraformaldehyde + Lysozyme	$\frac{1}{\sqrt{Y}} = 25.35 - 0.69x_1 + 2.16x_2 + 1.04x_3 + 1.57x_1x_2 - 0.24x_1x_3 - 1.66x_2x_3 + 0.94x_1^2 - 1.00x_2^2 - 0.294x_3^2$
L. innocua	Paraformaldehyde + Ethanol	$\frac{1}{\sqrt{Y}} = 109.77 - 4.94x_1 - 3.98x_2 + 2.47x_3 - 3.22x_1x_2 - 0.94x_1x_3 - 2.75x_2x_3 - 0.23x_1^2 + 2.26x_2^2 + 2.85x_3^2$
	Paraformaldehyde + Triton X-100	$\frac{1}{\sqrt{Y}} = 130.18 - 7.86x_1 + 4.62x_2 - 1.16x_3 + 1.73x_1x_2 - 2.02x_1x_3 + 4.66x_2x_3 - 4.95x_1^2 + 5.68x_2^2 - 0.89x_3^2$
	Paraformaldehyde + Lysozyme	$\frac{1}{\sqrt{Y}} = 136.76 + 3.16x_1 - 16.33x_2 + 3.66x_3 - 6.29x_1x_2 + 10.31x_1x_3 + 3.24x_2x_3 - 1.39x_1^2 - 13.81x_2^2 + 4.12x_3^2$
B. cereus	Paraformaldehyde + Ethanol	$\frac{1}{\sqrt{Y}} = 1943.81 + 68.79x_1 + 107.27x_2 + 18.24x_3 + 33.09x_1x_2 - 82.37x_1x_3 - 57.43x_2x_3 - 40.38x_1^2 - 92.62x_2^2 - 4.67x_3^2$
	Paraformaldehyde + Triton X-100	$\frac{1}{\sqrt{Y}} = 1549.49 + 113.40x_1 - 120.44x_2 - 80.70x_3 - 128.74x_1x_2 - 1.83x_1x_3 + 54.94x_2x_3 - 43.57x_1^2 - 131.56x_2^2 - 13.35x_3^2$
	Paraformaldehyde + Lysozyme	$\frac{1}{\sqrt{Y}} = 922.97 + 32.62x_1 - 36.45x_2 - 71.42x_3 - 10.92x_1x_2 - 38.32x_1x_3 - 38.93x_2x_3 - 47.23x_1^2 + 8.78x_2^2 + 26.51x_3^2$

Chapter 5

Concluding remarks and Future perspectives

5.1. Concluding remarks

The main objectives of this thesis were to develop new methods for the detection and identification of pathogenic bacteria in food matrices based on PNA-FISH. It also aimed to do a comprehensive study and optimization of several methodological variables of PNA-FISH, that would assist in the development of new products. Foodborne diseases, as seen in Chapter 1, currently present a high health and economic impact worldwide, with high morbidity and mortality (WHO, 2015). As such, it is a topic under the spotlight of legislators and regulatory agencies, in order to enhance safety and preventive practices that ultimately lead to a decrease in infection numbers. In fact, it has been estimated that the main burden of foodborne diseases could be avoidable (WHO, 2015; Zhao *et al*., 2014). Pathogen detection along the food chain plays a central role in food safety and there are a wide variety of methodologies to choose from, with advantages and disadvantages against each other (Mangal *et al*., 2016; Yeni *et al*., 2014; Wang and Salazar, 2016; Rohde *et al*., 2015; Priyanka *et al*., 2016; Zhao *et al*., 2014; Law *et al*., 2015). Over the last decade, PNA-FISH has proven to have superior performance characteristics then standard FISH in the detection of microorganisms both in clinical and environmental areas (Amann and Fuchs, 2008; Cerqueira *et al*., 2008; Frickmann *et al*., 2017; Rohde *et al*., 2015). Taking into consideration the above-mentioned statement, PNA-FISH application to food safety procedures, can be seen as a natural evolution (Almeida *et al*., 2009; Almeida *et al*., 2010; Zhang *et al*., 2015; Zhang *et al*., 2012; Machado *et al*., 2013).

In Chapter 2, the optimization of a PNA-FISH procedure for the specific detection of *L. monocytogenes* is described. The developed procedure allows the detection of *L. monocytogenes* in a variety of relevant food matrices in concentrations as low as 0.5 CFU/25 g or mL of sample with the same performance characteristics of the standard cultured plating method, ISO 11290, while reducing the time to results in more than 50%. However, the development of the final procedure was no easy task, regarding two different factors, optimization of the enrichment procedure and the optimization of the hybridization conditions. Regarding the enrichment procedures, the processes was hindered by the low growth rate of *L. monocytogenes* when compared against background flora (Carvalheira *et al*., 2010) in order to achieve a concentration detectable by PNA-FISH. Secondly, the optimization of the hybridization conditions, in order to have a

sensitive and specific method, were a challenge. In fact, the probe used in the development of the PNA-FISH method for the detection of *L. monocytogenes* had been matter of optimization in previous works (Fontão, 2012; Sousa, 2013). However, the developed methods lacked robustness and reproducibility. A turning point occurred after the work of Santos *et al* (2014) stating that a low formamide content in the hybridization solution would be beneficial to PNA-FISH procedures. Taking into consideration this information it was possible to achieve a reliable and reproducible PNA-FISH method for the detection of *L. monocytogenes*, as intended. From this, also arises the notion that the understanding of the different variables that affect the signal outcome of PNA-FISH was lacking and require further investigation.

In order to address the lack of information identified above, in Chapter 3, the effect of pH, dextran sulfate and probe concentration was disclosed. It was proposed that the access of the probe to the target rRNA occurs in three steps: 1) diffusion on the suspension, 2) diffusion through the cell envelope and 3) diffusion in the cytoplasm. It was found that the cell envelope plays a central role for probe diffusion. For Gram-negative bacteria with thin peptidoglycan cell walls, the limiting step for probe access depends on the diffusion of the probe in suspension. However, for Gram-positive bacteria with thicker peptidoglycan cell wall, it lays in the diffusion through the cell envelope. Accordingly, in order to have a successful outcome, a higher probe gradient between the outside and cell cytoplasm is required. This is accomplished using higher concentrations of dextran sulfate, a volume excluder molecule, that will artificially increase the probe concentration. Nonetheless, dextran sulfate concentration and in some extent the pH are limited by the viscosity of the hybridization solution produced. At high concentrations, the diffusion of the probe in solution will be limited and at low concentrations, the probe gradient driving its diffusion across the cell envelope will be insufficient. In conclusion, for an efficient hybridization a balance of dextran sulfate and pH should be taken into consideration.

In Chapter 4, the optimization efforts continued, this time with the evaluation of the effects of different fixation/permeabilization protocols in PNA-FISH for bacteria. As in the previous optimization, the differences between the tested species in terms of cell envelope structure, namely in their peptidoglycan thickness, were the main drivers of the optimized protocols. On top of that, the susceptibility of the target species to the permeabilizing agent also played an important role. The permeabilization of Gram-

negative species, with a thin peptidoglycan layer, was found to be optimal under a combination of low permeabilizant concentration with short duration of the permeabilization step, except in triton X-100. Regarding the permeabilization of Gram-positive bacteria, their thicker peptidoglycan layer resulted in optimal protocols with high concentration of permeabilizant agent in combination to long periods of permeabilizant application, except *B. cereus*, where the above-mentioned situation was only verified for the paraformaldehyde-ethanol protocol. The duration of the paraformaldehyde step was found to be species- and protocol-specific. Shorter steps were optimal for Gram-positive species under paraformaldehyde-ethanol protocol with the exception of *B. cereus* and *L. innocua* in triton X-100. Furthermore, all protocols were able to yield fluorescence outcomes above their respective negative protocol in the shortest fixation/permeabilization step, 30 minutes in paraformaldehyde and 15 minutes in permeabilizant agent. This is an important observation for applications where is possible a tradeoff between fluorescent outcome and protocol duration. Moreover, in multiplex applications is possible to favor protocol settings of species with weaker basal signals. This translates into a more balanced fluorescent outcome of the targeted species, simplifying sample analysis. Overall, the best PNA-FISH outcome was achieved under paraformaldehyde-ethanol fixation/permeabilization steps for all bacteria.

The optimizations performed for PNA-FISH procedures, described in the Chapters 3 and 4, as already referred, will greatly assist in the development of new methodologies of microbial detection. In the specific case of the PNA-FISH method described in Chapter 2 for the detection of *L. monocytogenes*, is possible to see that both the pH and the dextran sulfate concentration are used near to the optimum conditions, 7.5 vs 8 and the 10 vs 10% (wt/vol), for the *L. monocytogenes* procedure and *L. innocua* optimization, respectively. The major variation concerns probe concentration, 200 nM vs 300 nM, respectively. However, the introduction of the optimized concentration into the *L. monocytogenes* procedure would translate into a 50% increase manufacturing cost for the probe component and consequently an increase in the final price of the kit. This would decrease market penetration, since price competiveness is one of the most important factors for overall product dissemination. Moreover, the introduction of the optimized concentration into the *L. monocytogenes* procedure would change the ratio of detection/blocker probe, with possible implications in method specificity. This would at least imply a new optimization of the probes ratio. Regarding the

fixation/permeabilization optimizations, the *L. monocytogenes* method uses a 10 minutes step in paraformaldehyde 4% (wt/vol) and a second 10 minutes step in 50% (vol/vol) ethanol, while in the optimization for *L. innocua* uses 30 min in paraformaldehyde 4% (wt/vol) and 25% (vol/vol) ethanol for 45 minutes. Again, as with the optimization of the probe concentration, the optimization variables of the fixation/permeabilization step will most likely not be introduced in the *L. monocytogenes* procedure. First of all, the *L. monocytogenes* procedure is performed in glass slides while the optimizations were performed in suspension. Consequently, the samples for the *L. monocytogenes* procedure pass through a heat fixation step before the application of the fixation/permeabilization compounds. Therefore, the extension of the paraformaldehyde step would probably be redundant. Regarding the ethanol step, the extension from 10 to 45 minutes would result in an overall duration increase of the procedure for a relatively marginal gain in fluorescence. On top of that it would confuse the technician in a scenario where different Biomode S. A. products are used, each with different fixation/permeabilization steps.

To summarize, PNA-FISH is a reliable tool for foodborne pathogen detection, however in order to have a reliable and reproducible procedure, the variables that influence the fluorescent signal outcome must be correctly adjusted. In this thesis the optimization results of the fixation/permeabilization step and hybridization solution (namely pH, dextran sulfate and probe concentration) are described for bacteria. Adding this information to previous works (Table 4.3), an almost complete optimized PNA-FISH procedure can be found enabling protocol adaptation specifically to the target organism. However, a universal PNA-FISH protocol optimal for all species is not possible to attain. This results from the inherent differences in terms of cell envelope of the different species tested and their effect on probe permeation and fixation/permeabilization efficiency.

5.2. Future perspectives

This thesis had an ambivalent orientation, in one hand the development and optimization of new products based on PNA-FISH methodology with interest in the food safety area. On the other hand, the enlightenment and comprehension of the effect of each parameter and interplay on the PNA-FISH fluorescent outcome of bacteria. As such the work to be developed in the future, can also have two main focus: 1) a scientific and 2) a commercial point of view.

From a scientific perspective, although fundamental aspects of the PNA-FISH procedure were already disclosed in this thesis and by Santos *et al* (2014), some parameters are still not fully revealed and therefore require addressing. Namely the evaluation of the effects of the Denhardt's solution on the hybridization solution, commonly used as blocking reagents preventing the unspecific binding of nucleic acids to nitrocellulose or nylon membranes in hybridization procedures (Denhardt, 1966) and also the use of detergents (Azevedo, 2005), have yet to be addressed. Furthermore, an optimization of the washing step has never been fully performed. The expansion of the scope of optimization in the fixation/permeabilization step with the inclusion of other compounds commonly used in *in situ* Hybridization (ISH) applications: fixatives such glutaraldehyde, choral hydrate, glyoxal, oxidizing agents, acetone, among others; permeabilization compounds such methanol, thionins, poly-L-lysine, acids, bases, other detergents, lytic enzymes among others (Felix, 1982; Rhodes, 2012; Thavarajah *et al*., 2012), would be desirable. Also, would be important to do an expansion of the scope of optimization to other bacteria and microorganisms belonging to the *Archaea* and *Eukarya* domains, with diverse cell envelope compositions, since this structure plays a central role in the choice of the optimal procedure, as seen before. Finally, the extension of the optimization scope to other nucleic acid mimics, such locked nucleic acid, 2' O-Methyl RNA, among others, due to the increasing interest of using these molecules as probes for ISH applications (Fontenete *et al*., 2015) and structural differences in comparison to PNA probes (Cerqueira *et al*., 2008), should also be performed.

In order to become a player in the worldwide food safety market testing, FISH-based companies have to address two main challenges: 1) the diversification of product portfolio based on fast and reliable FISH methods and 2) the development of an automation procedure for high-throughput sample analysis. The Portuguese company Biomode S. A. have up to this date developed (and undergoing AOAC certification) two products for the detection and identification of the most relevant foodborne pathogens, *Salmonella* spp. - Probe4Salmonella - and *Listeria monocytogenes* - Probe4Monocytogenes (Chapter 2); and a third one for *Cronobacter* spp. - Probe4Cronobacter. However, in order to gather the interest of food safety laboratories, it is not enough to have superior performance products. The company should provide alternatives for the detection of other less important foodborne pathogens (and with lower

market share expression), such *Campylobacter* spp., *Vibrio* spp., pathogenic *E. coli*, *Yersinia* spp., *Shigella* spp., among others (Zhao *et al*., 2014).

The ideal foodborne pathogen detection method, should be inexpensive, simple, sensitive, reliable, rapid, versatile, portable, automated and allow high-throughput, on-site and real-time testing (Fusco and Quiero, 2014). Of those characteristics, the PNA-FISH technique is sensitive, reliable, rapid and versatile, however, as many other nucleic acid hybridizations detection methods, lack automation and do not present high-throughput on-site and real-time testing systems (López-Campos *et al*., 2012; Mangal *et al*., 2016; Rohde *et al*., 2015). Although the strong points are sufficient for small scale food safety testing laboratories; a broader implementation, to the large-scale laboratories becomes hindered from the lack of automation. In order to address this issue, 3 means of procedure automation and high-throughput analysis can be identified: 1) flow cytometry; 2) FISH automation and 3) lab-on-a-chip devices - micro fluidics (Frickmann *et al*., 2017; Fusco and Quiero, 2014; Liu *et al*., 2012; Meagher and Wu, 2016; Rohde *et al*., 2015). Flow cytometry analysis is perhaps the fastest way of developing a high-throughput analysis for PNA-FISH technique, however it presents the disadvantage of hands-on time increase due to step multiplication as a result of the centrifugation steps that have to precede the application of each kit component. Although the hands-on time could be partly or almost fully decreased using sample processing systems, the multiplication of centrifugation steps will ultimately lead to an irreversible loss of some target cells, which ultimately can decrease the limit of detection of the method (Liu *et al*., 2011).

FISH automation would be achievable using a modular system apparatus that will independently perform all protocol procedures of the FISH technique, namely sample processing, comprising the fixation, permeabilization, hybridization and washing steps and sample read-out (Rohde *et al*., 2015). While slide processing devices are already available and could be relatively "easily" converted to the specification of FISH procedure, the construction of the sample read-out module will require further optimization. Contrary to what is possibly the general opinion, the identification of the target bacteria in a food sample analysis is not simply limited to the observation of a fluorescent spot under the microscope. Sample background noise, can appear as a result of the presence of biological particles and other debris, masking the signal of the hybridized target (Moter and Gobel, 2000). As such the read-out module would have to be capable of distinguishing the target bacteria from artifacts. Nonetheless, some reports

have presented encouraging results in this regard (Evans *et al*., 2006, Wauters *et al*., 2007) and a German FISH company, Miacom GmbH have already developed a modular system for FISH procedure and analysis.

Lastly, lab-on-a-chip devices are probably the most promising technology for the development high-throughput on-site and real-time testing FISH systems (Meagher and Wu, 2016). In fact, an enormous research effort, over the last years, has been done for the miniaturization of laboratorial procedures, including FISH, into microfluidic platforms (Fusco and Quiero, 2014; Liu *et al*., 2011). The processes of miniaturization take advantage of the emerging micro and nanofabrication technologies and their conjugation with electromechanical systems (Fusco and Quiero, 2014). Adding to the previously referred advantages, these systems are designed to bring improved performance, reduce cost, portability and reduced reagent consumption. Although being an active research field, the development of a fully integrated lab-on-a-chip device for the identification of bacteria, to the extent of my knowledge, was solely described by Liu *et al* (2011). The construction of these kind of devices will truly revolutionize the food safety and the clinical industry, launching FISH-based companies as a serious competitor to market established organizations.

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