Universidade de Aveiro Departamento de Química

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Ana Catarina da Silva e Sousa

Desenvolvimento de métodos rápidos para a deteção de microrganismos patogénicos, baseados na tecnologia de NAM-FISH

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Molecular, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Laura Isabel Macieira Cerqueira da empresa Biomode 2 S.A.

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agradecimentos

Finalizada uma das etapas mais importantes da minha vida, não posso deixar de agradecer a todas as pessoas que contribuíram direta ou indiretamente para a elaboração deste trabalho.

Antes de mais, um agradecimento à Biomode 2 S.A. por me ter acolhido como parte da sua equipa durante estes meses, na qual tive a oportunidade de viver uma experiência bastante enriquecedora.

Um agradecimento especial às minhas orientadoras, Doutora Adelaide Almeida e Doutora Laura Cerqueira, pela disponibilidade, apoio e simpatia que sempre demonstraram, bem como o tempo que despenderam com este trabalho.

Ao Mário, Ricardo, Rui, Nuno e Andreia, obrigada pela amizade, pela disponibilidade que sempre demonstraram em ajudar-me, pelas conversas, conselhos e por todos os momentos partilhados ao longo destes meses na Biomode, os quais não esquecerei.

À Doutora Carina Almeida e ao Ricardo Oliveira, do INIAV, obrigada pela colaboração na realização deste projeto, recebendo-me na instituição e transmitindo-me parte do vosso conhecimento.

Aos meus amigos, porque apesar da distância foram um apoio fundamental ao longo desta jornada. Obrigada por terem ouvido os meus desabafos e por todas as palavras de força e incentivo que me foram dando ao longo de todo o percurso!

E finalmente um muito obrigada à minha família, em especial aos meus pais e irmãs. Sem eles não seria possível ter chegado até aqui e este caminho teria sido mais duro. Obrigada por toda a paciência e compreensão, pelo apoio e incentivo que me deram ao longo de todo o percurso académico e por me terem chamado à razão quando foi preciso. Obrigada por tudo!

A todos vocês, o meu sincero agradecimento!

palavras-chave

NAM-FISH; *Helicobacter pylori*; Resistência antimicrobiana; Doenças transmitidas por alimentos; *Campylobacter* spp.; Enriquecimento; Autofluorescência; AOAC International

resumo

O acesso de microrganismos patogénicos ao corpo humano pode comprometer a saúde do indivíduo, provocando variadas manifestações clínicas. *Helicobacter pylori* e *Campylobacter* são dois importantes patógenos gastrointestinais. A infeção por *H. pylori* é uma das infeções humanas mais comuns, cujo tratamento inclui a administração de antibióticos, nomeadamente fluoroquinolonas (FQ). Contudo, tem-se verificado um aumento da resistência de *H. pylori* a FQ, o que pode resultar em falhas no tratamento, tornando-se importante não só a deteção da bactéria, mas também a definição do seu perfil de resistência. *Campylobacter* é atualmente considerada a principal causa de doenças transmitidas por alimentos, encontrando-se normalmente associada ao consumo de carne crua. A deteção de microrganismos patogénicos pode ser alcançada por técnicas de cultura convencionais ou por métodos moleculares, nomeadamente testes imunológicos ou de deteção de ácidos nucleicos. A Biomode é uma empresa inovadora que desenvolve e comercializa métodos de diagnóstico rápidos baseados na tecnologia de hibridação fluorescente *in situ* (FISH) de ácidos nucleicos mímicos (NAM), NAM-FISH, que possibilita a deteção rápida de microrganismos, através da hibridação de sondas fluorescentes complementares com sequências específicas presentes no microrganismo alvo. Neste contexto, o presente trabalho teve como foco duas aplicações do NAM-FISH. Na área clínica, o objetivo principal foi o desenvolvimento de um método para a deteção de *H. pylori* e da resistência a FQ. Para tal, procedeu-se ao desenho de sondas de *Peptide Nucleic Acid* (PNA) e *Locked Nucleic Acid* (LNA)/2'OMe para a deteção de mutações causadoras da resistência. De forma a cobrir as mutações mais prevalentes, bem como o fenótipo *wild-type*, foram selecionadas 5 sondas de LNA/2'OMe. Na área da segurança alimentar, foi objetivo a otimização de um método de PNA-FISH para a deteção de *Campylobacter* em amostras alimentares. Um ensaio preliminar da inclusividade/exclusividade da sonda *Campylobacter* resultou na deteção de dois microrganismos não-alvo, *H. cinaedi* e *H. pamatensis*. Para a otimização do procedimento, foram utilizadas amostras de carne de frango crua, inoculadas artificialmente com *C. jejuni*. Antes do PNA-FISH, foi introduzido um novo passo, no qual as amostras enriquecidas são sujeitas a uma centrifugação (10 000 g), seguida de ressuspensão em 0.1% Triton X-100, com o objetivo de reduzir a autofluorescência forte visualizada em amostras sem qualquer tratamento. Testou-se também a possibilidade de um enriquecimento das amostras em dois passos, no entanto, esta abordagem não demonstrou vantagens comparativamente ao procedimento em um passo. Efetuou-se ainda um teste de robustez, requerido pela AOAC International para a obtenção de certificação de produto, que revelou que a variação dos parâmetros do tempo e temperatura de hibridação influenciam a performance do método, pelo que as condições do PNA-FISH devem ser rigorosamente controladas. Os resultados obtidos neste estudo mostraram que o método PNA-FISH é adequado para a rápida deteção de *Campylobacter* em amostras alimentares. Com este trabalho conclui-se que, embora os dois métodos baseados em NAM-FISH sejam uma opção promissora para a deteção de *H. pylori* e *Campylobacter*, ambos necessitam de otimização futura.

keywords

NAM-FISH; *Helicobacter pylori*; Antimicrobial resistance; Foodborne ilnesses; *Campylobacter* spp.; Enrichment; Autofluorescence; AOAC international.

abstract

The access of pathogenic microorganisms to the human body can compromise the health of the individual, causing several clinical manifestations. *Helicobacter pylori* and *Campylobacter* are two important gastrointestinal pathogens. *H. pylori* infection is one of the most common human infections, whose treatment includes the administration of antibiotics, namely fluoroquinolones (FQ). However, there has been an increasing resistance of *H. pylori* to FQ, which can lead to treatment failures, making it important not only to detect the bacterium but also to define its resistance profile. *Campylobacter* is currently considered the leading cause of bacterial foodborne illnesses, usually associated with the consumption of raw meat. The detection of pathogenic microorganisms can be achieved by conventional culture techniques or by molecular methods, namely immunological tests or nucleic acid detection. Biomode is an innovative company that develops and commercializes rapid diagnostic methods based on Nucleic Acid Mimic (NAM) – fluorescence *in situ* hybridization (FISH) technology, which enables the rapid detection of microorganisms, through the hybridization of complementary fluorescent probes with specific sequences present in the target microorganism. In this context, the present work focused on two applications of NAM-FISH. In the clinical area, the main objective was the development of a method for the detection of *H. pylori* and its resistance to FQ. For this purpose, Peptide Nucleic Acid (PNA) and Locked Nucleic Acid (LNA)/2'OMe probes were designed for the detection of mutations that cause resistance. In order to cover the most prevalent mutations, as well as the wild-type phenotype, 5 LNA/2'OMe probes were selected. In the area of food safety, the objective was the optimization of a PNA-FISH method for *Campylobacter* detection in food samples. A preliminary testing of the inclusivity/exclusivity of the *Campylobacter* probe resulted in the detection of two non-target microorganisms, *H. cinaedi* and *H. pamatensis*. To optimize the procedure, samples of raw broiler meat inoculated artificially with *C. jejuni* were used. Prior to PNA -FISH, a new step was introduced in which the enriched samples are subjected to a centrifugation (10 000 g), followed by resuspension in 0.1% Triton X-100, in order to reduce the strong autofluorescence shown in samples without any treatment. The possibility of a two-step sample enrichment was also tested, however, this approach did not show advantages compared to the one-step procedure. Additionally, a robustness test, required by the AOAC International to obtain product certification, was performed, which showed that the variation of the parameters of the time and temperature of hybridization influence the performance of the method, showing that the PNA-FISH conditions must be strictly controlled. The results obtained in this study showed that the PNA-FISH method is suitable for the rapid detection of *Campylobacter* in food samples. With this work, it is concluded that although the two NAM-FISH based methods are a promising alternative for the detection of *H. pylori* and *Campylobacter*, they both require optimization.

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Glossary of acronyms and abbreviations

2'- F RNA	2'-deoxy-2'-fluoro-ß-D-ribonucleic acid
2'OMe	2'-O-Methyl RNA
BB	Bolton Broth
BLAST	Basic Local Alignment Search Tool
CBA	Columbia Blood Agar
CFU	Colony forming units
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
EUCAST	European Union Committee for Antimicrobial Susceptibility Testing
FISH	Fluorescence *in situ* hybridization
GBS	Guillain Barré Syndrome
IAC	Internal amplification control
IARC	International Agency for Research on Cancer
IMS	Immunomagnetic separation
ISH	*In situ* hybridization
ISO	International Organization for Standardization
LNA	Locked nucleic acid
mCCDA	modified charcoal cefoperazone deoxycholate agar
MIC	Minimum inhibitory concentration
NAM	Nucleic Acid Mimic
NCBI	National Centre for Biotechnology Information
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMQR	Plasmid-mediated quinolone resistance
PNA	Peptide nucleic acid
POD	Probability of detection
PPI	Proton-pump inhibitor
QRDR	Quinolone resistance - determining region
RNA	Ribonucleic acid
ROIs	Reactive oxygen intermediates
TSA	Tryptic soy agar
VBNC	Viable but non-culturable
WHO	World Health Organization

Part I – Introduction

1.1 Context

The human body is a vast and complex system that comprises not only human cells but also microbial cells that coexist in the same host. In fact, the number of microbial cells (10^{14}), which include protozoan, bacterial and fungal cells, exceeds the number of human cells (10^{13})[1]. These microbes constitute the normal flora, establishing symbiotic relationships with the host, important for several biological functions, and, usually, do not present health risks. However, other microbes can also gain access to the human body, causing several types of diseases. These pathogens are characterized by mechanisms that allow them to survive and multiply within the human body[1,2]. Some of the most prevailing human pathogens include *Helicobacter pylori* and bacteria belonging to *Campylobacter* spp., especially *Campylobacter jejuni*. They are closely related but distinct gastrointestinal pathogens, which, once inside the human body, can lead to clinical complications in individuals. They are both gram-negative, microaerophilic, fastidious and slow-growing bacteria that are widely distributed in the animal kingdom[3].

Having this is mind, it is becoming increasingly important to develop methods that allow the rapid detection of microorganisms. In both clinical and food field, it is important that pathogens can be detected as soon as possible, ensuring the health of the population. Although culture methods are still considered the *gold standard* in most microbiology laboratories, they present several disadvantages, especially the long time in obtaining results. Thus, rapid methods such as immunological and nucleic acids - based, namely PCR, have been developed to overcome the drawbacks of culture methods. However, these methods still have limitations, such as the need for DNA extraction and the complexity of the procedures for nucleic acids-based methods and the inability to distinguish between viable and non-viable microorganisms for both, nucleic acids-based and immunological methods. In this context, Fluorescence *in situ* hybridization (FISH) technology emerges as a simple, fast and effective alternative for the detection of microorganisms. The present work had as main objective the development of rapid methods based on FISH technology for the detection of *H. pylori* and *Campylobacter* spp. in clinical and food samples, respectively.

The experimental work of the dissertation was developed in Biomode 2, S.A, a biotechnology company with origin at the University of Minho and University of Porto. Biomode is an innovative company that develops and commercializes rapid diagnostic methods based on Nucleic Acid Mimics (NAM) – FISH technology, namely Peptide Nucleic Acid (PNA) - FISH, for the detection of foodborne and clinical pathogens. The focus of the company lies on the development of kits for a faster, more accurate and economic testing for both food industry and healthcare[4]. In clinical field, the company has already a product with CE marking, Probe4Pylori®, a kit for the rapid detection of *H. pylori* clarithromycin resistance in gastric biopsies. For food safety testing, Biomode 2, S.A. has three kits awarded with AOAC certification - Probe4Cronobacter®, Probe4Monocytogenes® and Probe4Salmonella®. In addition to the already developed kits, the company has several products under development, namely a method for the detection of *Campylobacter* spp. in food samples.

1.2 Structure of the thesis

This dissertation was divided into different sections. Initially, different methods of microbiological detection were discussed, with a special emphasis on NAM-FISH technology. Afterwards, two possible applications of the fluorescent technology were explored. In the clinical field, *H. pylori* infection and its increasing resistance to antibiotics, namely to fluoroquinolones, were discussed, including topics such as the characterization of the bacterium, mechanisms of fluoroquinolones resistance and methods for detecting *H. pylori* and its susceptibility profile to antibiotics. In the food safety area, foodborne diseases were explored, namely campylobacteriosis, as well as the characterization of the *Campylobacter* genus and methods for the *Campylobacter* spp. detection in food samples. Then, the proposed objectives and the materials and methods used during the practical work were presented, as well as the discussion of the results obtained. Finally, the conclusions of the work were presented, introducing some suggestions of future work.

Part II - Detection methods in Microbiology

Microbiological detection for pathogen control in both clinical and food field usually relies on standardized methods. These are usually culture-based, considered the *gold standard* in several laboratories and they are generally well accepted[5]. Although methods that involve the culture of microorganisms are cheap and simple to perform, they are labour-intensive, being characterized for the long time associated not only to the performance but also in obtaining the results[5]. This is a major drawback in both food industry and clinical diagnosis, where rapid results are essential, in order to protect public health. Besides, conventional culture methods may fail in detecting microorganisms in a viable but nonculturable (VBNC) state. This transformation can be induced by diverse stressful conditions, namely starvation, growth-inhibiting temperatures, non-optimal salinity or extreme pH values. Entering this state enables the bacteria to tolerate these hard conditions. When the surrounding environment become favorable, VBNC cells can leave the state of dormancy, and therefore become active and pathogenic within the hosts, including humans[6]. Since the transformed cells are not detected by the culture, the use of this method can lead to an underestimation of pathogen numbers in the sample, with the occurrence of false negatives that consequently lead to an incorrect diagnosis[6].

Therefore, alternative methods have been developed, characterized by rapid results, as well as the reduction of labor intensity, which may help revolutionize food and clinical microbiology. Currently, microbiological detection can already be performed by rapid tests such as the immunological interactions-based, nucleic acids-based methodologies or by hybridization techniques (Figure 1), which will be discussed below.

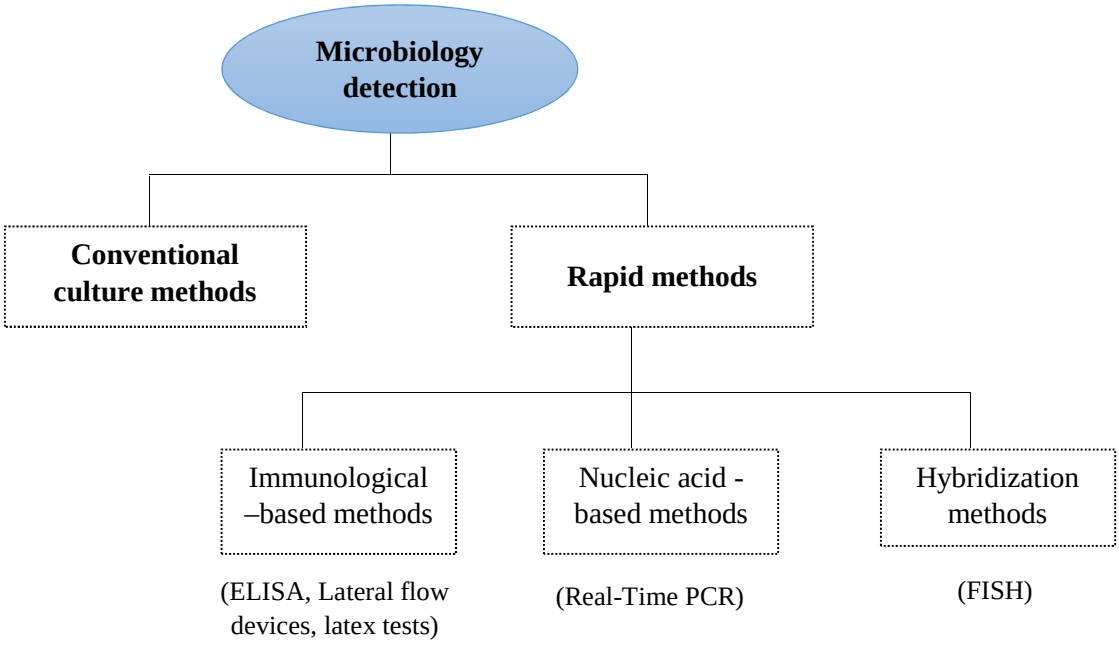


Figure 1 | Main methods used in the detection of microorganisms.

2.1 Immunological methods

Imunnological procedures are based in the specific binding between an antibody and an antigen, enabling the detection of the pathogen or their toxins in food or clinical samples[7]. The choice of the antibody is determinant for the performance of the immunoassay, namely for its specificity[5]. Although more expensive, monoclonal antibodies, which act against a specific antigen, are generally selected for such assays. In turn, polyclonal antibodies, which can act against multiple regions of the same or different targets, are a less expensive alternative but there is a high risk of cross reactivity[8].

ELISA (Enzyme-Linked Immunosorbent Assay) is one of the most frequently used immunological-based methods for detecting pathogens. It is usually performed through a "sandwich assay", a sensitive and robust format in which a primary antibody binds to the target antigen of the sample and an enzyme-conjugated secondary antibody binds to the antigen, forming a complex. The unbounded antibodies are then removed, and the detection is achieved by the addition of a colorless substrate, which is converted by the enzyme into a colored signal[9]. In lateral flow immunoassays, antibodies coupled to colloidal latex or gold particles will bind to a target microorganism present in a clinical or food sample. This antibody-target complex moves laterally to a zone with capture antibodies, with a consequent reaction that causes a visual signal, indicating a positive result. After sample pre-treatment,

the detection time can be simply about 5 - 10 min[8]. Latex agglutination tests are simple methods based in the agglutination of the target organism with latex particles covered with antibodies, induced by the reaction with proteins present in bacterial cell walls[10].

In general, immunological-based detection methods are an inexpensive alternative to culture methods, easy to perform and providing faster results[11]. The major drawbacks are the occurrence of false positives due to cross reactivity with non-target cells, as well as some of the methods may require trained people and specific equipment, which may not be accessible to all laboratories[9,12]. Besides, these methods do not allow the distinction between viable and non-viable microorganisms[13] and confirmation tests of the results obtained are usually required[5]. Finally, the antibody selection may increase the cost of the procedure, since the monoclonal antibodies, associated with a higher specificity, are a more expensive approach[8].

2.2 Nucleic acid – based methods

In recent years, nucleic acid-based methods have been revolutionizing routine analyzes for food and clinical control. They involve the complementary binding of a synthetic oligonucleotide (probe or primer) to a specific nucleic acid sequence in the microorganism, allowing a highly specific detection of target pathogens[7].

Polymerase Chain Reaction (PCR) is the most commonly used nucleic-acid based technology in microbiology laboratories, namely for pathogen detection. PCR involves the design of specific primers for the amplification of target genes in the pathogens. At the end of the process, there will be multiple copies of the target sequence, which can be further analyzed, for instance, through electrophoresis[11]. Conventional PCR subsequently evolved into a more robust technique, multiplex PCR, which allows the simultaneous detection of multiple pathogens, through the use of several sets of specific primers in the same assay[7]. The application of multiplex PCR assays in pathogen detection has led to saving time and reagent costs[14]. Nevertheless, both PCR techniques previous described require further analysis for the detection of PCR products, which makes the methodology more demanding in terms of time and effort[9].

More recently, the development of Real-time or quantitative PCR (qPCR) enabled both the detection and quantification of target microorganisms. In this PCR variant, there is no need of post-PCR steps since the amplification products are continuously monitored by generation of a fluorescence intensity proportional to the newly formed products. SYBR® green, TaqMan® probes or molecular beacons® are examples of systems that can be

incorporated in qPCR assays for fluorescence production. SYBR® green is a non-sequence specific dye whose fluorescence is higher when bound to double-stranded DNA (dsDNA)[9]. TaqMan® probes and molecular beacons® are both sequence-specific oligonucleotides that present a reporter fluorophore dye (at 5') and a quencher molecule (at 3'). With exposure to light, the fluorescence emission by the probe is naturally prevented by the proximity between the quencher and the reporter. The quencher has the capacity to absorb the emission of the reporter, however, during amplification, the two terminals are separated, whereby fluorescence emission occurs[8]. Among these reporters, SYBR® green is the simplest and cost-effective. The major drawback is the binding of the reporter to all dsDNA, including non-specific amplification products, and thus can lack specificity[9]. In turn, sequence-based TaqMan® probes and molecular beacons® confer a greater degree of specificity to the detection of PCR products[15], with the disadvantage of being more expensive alternatives[9].

The main advantages of nucleic-acid based methods are the high sensitivity and specificity, providing reliable results in a short time[9]. Besides, evolution of the PCR technique led to the development of methods that do not require further steps to visualize results[9]. However, in the specific case of PCR-based technologies, most of them require specialised people and expensive equipment[7], besides the fact that cell lysis and nucleic acid extraction are usually necessary procedures before the analysis, which implies a preceding step. In addition, the PCR reaction is very susceptible to cross-contamination and inhibition by components or competing DNA present in the samples, yielding false negatives. Similarly to immunological methods, PCR - based methods also lack the ability to distinguish viable cells from non-viable[11].

2.3 Hybridization methods - FISH technology

In situ hybridization (ISH) involves the detection of specific DNA or RNA sequences in cytological samples by hybridization with complementary nucleic acid probes. The subsequent inclusion of fluorophores in probes originated a new methodology, named Fluorescence *in situ* hybridization (FISH). The complementary binding between specific fluorescent probes, usually DNA probes, and nucleic acid sequences within the cell, tissue or organism under study yields a fluorescent signal, enabling the detection and identification of the target organism[16,17]. FISH applications range from basic microbiology studies[18,19] to clinical diagnosis[20,21].

The most common target of FISH in microbiology is the ribosomal RNA (rRNA), namely the 16S rRNA[16], although the 23S rRNA has been gaining importance. The latter is almost twice the size of the 16S rRNA, maintaining the ability to differentiate species, even the closely related strains which can not be distinguished through 16S rRNA detection[22]. Ribosomes, essential for normal cell physiology, are present in high numbers in bacterial cells[23], and there is a high conservation of the rRNA sequences, which allows a distinction between specific microorganisms[24]. The combination of both characteristics makes these molecules the target choice for the FISH procedure, where detection is achieved by simple addition of fluorescent elements to the probe, generally without the need for additional signal amplification mechanisms[23]. FISH studies have also been applied to the detection of mRNA[25] and viral DNA[26]. Table 1 summarizes some general advantages and disadvantages in the use of this methodology for the microbiological detection.

Table 1 | Advantages and disavantages of FISH method in microbiology

Fluorescence in situ hybridization (FISH)	
Advantages	• Offer results in a short period of time; • No need for culture, being applicable directly in samples such as clinical or environmental specimens; • Little complexity of required equipment, accessible to most laboratories; • Simultaneous detection of multiple sequences; • It is not affected by contamination, in contrast with other molecular techniques, as PCR; • Allows direct visualization of the microorganism, which can provide information about its location and distribution in the sample.
Disadvantages	• Occurrence of autofluorescence by both microorganisms themselves and other material present in samples; • Irreversible destruction of fluorophores, which lose the ability to emit fluorescence (*photobleaching);* • Inefficient hybridization due to low target content.

From: Moter and Göbel[16], Frickmann et al.[17], Petrich et al.[20], Wu et al.[21]

FISH technology typically comprises four main steps: fixation/permeabilization, hybridization, washing and detection (Figure 2).

Fixation step is essential for FISH performance, with the main purpose of maintaining the integrity of the cells, through structural stabilization, as well as ensuring that the target regions remain immobilized[24]. Beyond that, it is important to ensure permeabilization of the membrane, with the formation of pores that allow probe to enter the cell[17,27]. Though, the treatment should not be too aggressive, in order to avoid cell lysis[27]. The available fixing agents can be divided into two groups, the crosslinking agents, which include aldehydes such as formalin and paraformaldehyde, and precipitating agents such as

methanol and ethanol[23]. While some of the fixative compounds may contribute slightly to cell permeability, there are permeabilization agents, such as Triton X-100, Tween-20 or proteinase K[17]. Unfortunately, there is no standard procedure that can be applied to all types of microorganisms[24], making it a difficult optimization step[16].

Hybridization is a critical step for FISH efficacy, comprising the complementary binding of fluorescent oligonucleotides to the target sequence, creating specific hybrids[24]. The conditions under which the probe-target binding occurs must be strictly defined, particularly with respect to temperature, pH, ionic strength and formamide concentration[28]. Once hybridization occurs, washing the samples for the removal of the free probes must be carried out, in order to avoid false-positive results, and thus contribute to the specificity of the reaction[23].

Finally, the visualization of the hybridization results can be achieved by flow cytometry[29,30] or fluorescence microscopy[31,32]. The choice of the detection method should take into account the purpose of the analysis. Flow cytometry enables a quantitative analysis of a larger number of data, but it requires longer working time and the required equipment is expensive[23,29], which may be difficult to access for simpler laboratories. On the other hand, fluorescence microscopy, which usually carry out a qualitative analysis, appears as an appealing alternative due to its simplicity and ease of visualization of the samples[23].

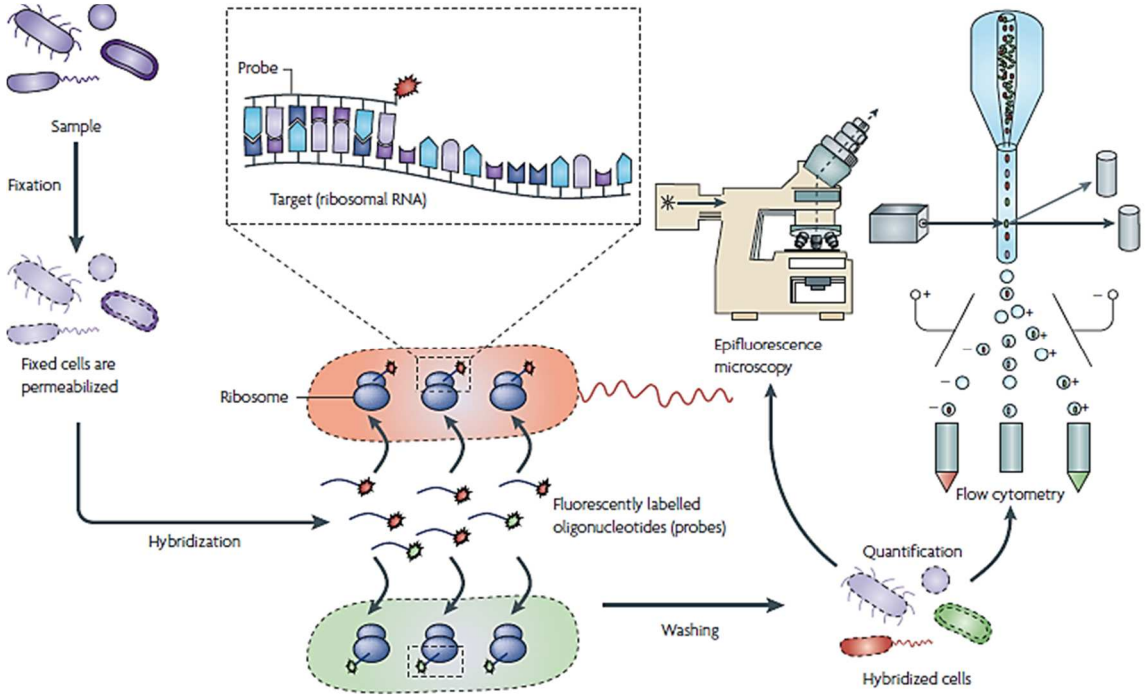


Figure 2 | Basic steps of FISH. 1 – The samples are fixed and permeabilized. 2 – The fluorescently labeled probe hybridizes with the target nucleic acid. 3 – Samples are washed to remove the free probes. 4 – Detection of fluorescence by microscopy or flow cytometry. Adapted from Amann and Fuchs (2008)[24]

2.3.1 Probes

The successful performance of FISH equally depends on the choice and correct design of the probes[17]. Probe selection should be made taking into account important factors such as sensitivity and specificity[16,28]. Probe sensitivity is related with ratio between the number of strains of the target microorganism detected and the total number of strains of the target microorganism present in the database[28]. In practical terms, is the ability of the probe to detect the target strain among a set of target and non-target microorganisms. Specificity is related with number of strains of the target microorganism detected and the total number of microbial strains detected ratio[28]. In practical terms, is the ability of the probe to bind only to the target for which it was designed, excluding the non-target microorganisms[23]. In addition, probe affinity, defined by the Gibbs free energy (ΔG), refers to its liability to bind to the target sequence under certain hybridization conditions[23]. Probes design should also consider the size and GC content, factors that influence the specificity of the hybridization reaction[17].

Fluorescein derivatives, such as Fluorescein-Isothiocyanate (FITC), rhodamine derivatives, such as Carboxytetramethylrhodamine (TAMRA) or Texas Red, cyanine dyes (Cy3 and Cy5) and Alexa Fluor are examples of fluorophores used in FISH for microbiology[16,23,28]. Additionally, the use of multiple fluorophores in the same assay allows visualizing multiple targets simultaneously. However, it is relevant to ensure that the fluorophores used have non-overlapping emission spectra, safeguarding the occurrence of inconclusive results[16].

2.3.2 Nucleic acid mimics (NAM) in FISH

FISH technology has commonly used DNA probes for target detection. However, such probes have several disadvantages, such as enzymatic degradation, low discrimination of single-base mismatches of target sequences[28] and inefficient hybridization due to lack of membrane permeability, which may bring to an insufficient access of probe[16,23,24]. Considering the problems associated with the DNA probes used in FISH, there was a need to investigate alternatives that overcome these limitations. As a result, molecules that mimic nucleic acids have been developed. Two of the most frequently used mimics in FISH for microbiological detection, Locked Nucleic Acid (LNA) and Peptide Nucleic Acid (PNA), as well as the identification of other existing mimics are described below.

a. *Locked Nucleic Acid (LNA)*

Locked Nucleic Acid (LNA) is a RNA mimic (Figure 3) that has several properties that make it feasible for microbial detection. Since it is a synthetic molecule, the LNA structure is not recognized by enzymes as substrate, remaining unaffected[28,33]. Also, DNA/RNA binding is performed by base complementarity according to Watson and Crick[33] and it is sensitive to the presence of mismatches, which means it has a greater ability to discriminate matched sequences from sequences with single nucleotide mismatch, in comparison with DNA oligonucleotides, demonstrating a higher affinity and specificity to the RNA/DNA target, enabling the reduction of the number of nucleotides of the probe[33,34]. LNA-DNA/RNA duplexes exhibit higher thermal stability, in comparison with DNA/DNA, causing an increase in melting temperature (Tm), which can be around 1 to 8 °C per LNA monomer introduced into DNA, or about 2 to 10 °C into RNA, depending on probe length and composition[35]. It should be noted that in this mimic the phosphate group is still present, and, as a result, the molecule has a negative charge, so electrostatic properties will be similar to those of the DNA/RNA, even presenting good water solubility characteristics similar to the original molecules[34,35]. One characteristic that may be useful is the possibility of combining LNA with different monomers as DNA, RNA or other mimics, due their similar synthesis[33].

LNA-FISH has been explored in different areas of knowledge, whether in the study of chromosomal alterations[36], or at the microbiological level[37]. This technology has thus been shown to be an effective method, regarding specificity and sensitivity[36,37].

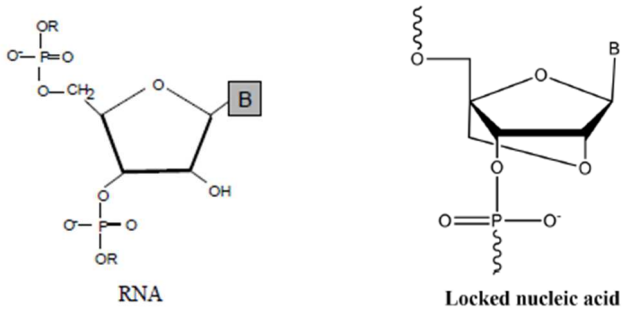


Figure 3 |Comparison between the chemical structures of RNA and the mimic Locked Nucleic Acid (LNA). Adapted from Cerqueira et al. (2008)[28] and Fontenete et al. (2002)[38]

b. Peptide Nucleic Acid (PNA)

Peptide Nucleic Acid (PNA), developed in 1991 by Nielsen et al. is a DNA mimic in which the structural column of deoxyribose and phosphate is replaced by a polyamide structure consisting of N-(2-aminoethyl) glycine repeat units[39], maintaining the DNA bases[40] (Figure 4).

PNA has the ability to establish interactions with both DNA (ssDNA or dsDNA) and RNA, which make possible broader applications[40,41]. Similarly to LNA, PNA also hybridizes with the target nucleic acid by complementarity base pairing according to Watson and Crick[41,42], has high affinity and resistance to enzymes[41,43]. In addition, this molecule has a structure which is neither too flexible nor too rigid, contributing to PNA being able to bind effectively to nucleic acids[44]. The main difference between LNA and PNA dwells in the absence of the phosphate group in PNA, which confers neutrality to the molecule[40]. As a result, there are no electrostatic repulsions between complementary strands, hence, when compared with DNA–DNA/RNA interactions, PNA-DNA/RNA duplexes present a higher thermal stability, resulting from a stronger bond between the molecules[45]. High stability implies a higher Tm than the observed for biological duplexes[41], enabling the design of shorter PNA probes with about 15 bp, comparing to DNA probes, which typically has between 20 - 24 bp[28]. As a result, a single mismatch can decrease the Tm about 8 to 20 ºC[45], significantly affecting hybridization, which demonstrates a high binding specificity[41,45]. The lack of electrostatic repulsions also contributes to the fact that Tm values are independent of saline concentration, so hybridization may occur under low salt concentration conditions[45]. Despite all characteristics described above, PNA applications may still be hampered by the low membrane permeability and consequent ineffectiveness in cellular uptake, willingness to self-aggregate and low solubility in aqueous media[44–46].

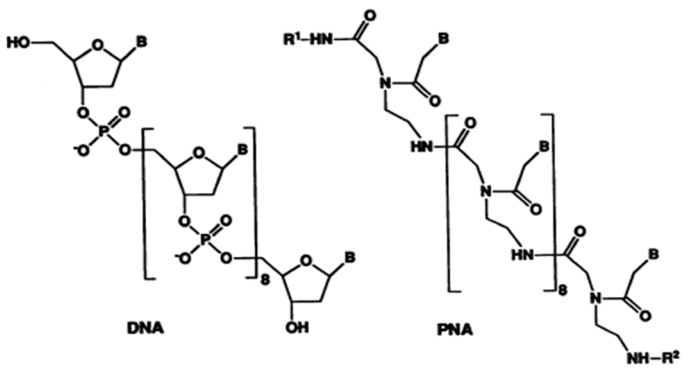


Figure 4 | Comparison between the chemical structures of DNA and PNA. Adapted from Nielsen et al. (1991)[39]

c. Other mimics

Besides PNA and LNA, there are other mimics, such as 2'-O-Methyl RNA (2'OMe) and 2'-deoxy-2'-fluoro-ß-D-ribonucleic acid (2'-F RNA)[28]. 2'-F RNA has a structure in which the 2'-hydroxyl group is replaced by a fluorine atom, exhibiting high affinity for RNA during hybridization[28,47]. This mimic has been explored mainly in antisense therapies[48]. 2'OMe oligoribonucleotides occur naturally in the RNA as a modification that gives rise to a methyl group at the oxygen in the position 2 of the ribose, forming a C3′-endo conformation[49] (Figure 5).

Although 2'-F RNA is not equally explored for the detection of microorganisms as LNA or PNA, possibly due to the complexity and cost of synthesis[28], the combined use of LNA + 2'OMe RNA probes has been applied to microbiology[50]. It is known that 2'OMe RNA probes bound to RNA with high affinity and high Tm values[49,51] and, like LNA and PNA, this mimic has also an increased resistance to enzymes[28]. Additionally, the conjugation between the mimics 2'-OMe RNA and LNA is known to promote greater stability of the formed duplexes, especially in the 2:1 ratio, thus contributing to an increase of sensitivity and specificity of the method[52,53]. In fact, it has been shown that the effects caused by LNA substitutions are more accentuated when LNA nucleotides are interspaced by at least one 2'OMe monomer[54]. Besides, LNA/2'OMe probes has a great ability to discriminate mismatches, combined with high flexibility in design, making them a future suitable alternative to be used in FISH technology[55].

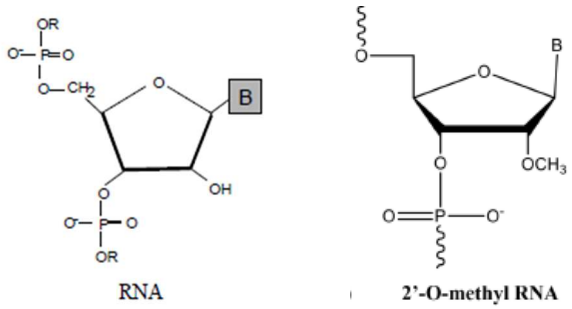


Figure 5 | Comparison between the chemical structures of RNA and the mimic 2'-O-methyl RNA (2'OMe RNA). Adapted from Cerqueira et al. (2008)[28] and Fontenete et al. (2002)[38]

In summary, the use of the NAM-FISH methodology for the detection of microorganisms provides several advantages over existing molecular methods. Firstly, the technology can be applied without the need for DNA extraction or sample processing, while maintaining specificity and sensitivity[4,56]. When used in association with the microscope, it allows the direct visualization of the bacteria, facilitating the interpretation of the results, and also the distinction between viable and dead cells, since only cells with stable ribosomal content are detectable[4,56]. Through NAM-FISH, even microorganisms in the VBNC state, unidentified by conventional culture methods, can be detected[22]. Additionally, the synthetic nature of mimics makes them resistant to enzymatic degradation, so the reaction is not compromised[4] and, similar to DNA and RNA, NAM probes can also be fluorescently labeled, enabling the target detection[41,57], with the advantage of overcoming the limitations of the probes originally used. Finally, regarding the application in microbiology laboratories, FISH technology has the additional advantage of being an economical technique in terms of investment costs, namely equipment and reagents, and consequently in the analysis per sample[58].

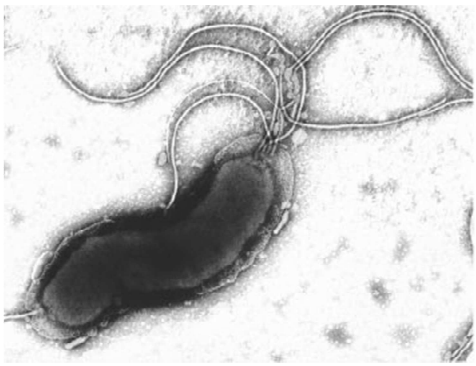
Part III - Development of a rapid method for the detection of fluoroquinolone resistance in *H. pylori*

3.1 *Helicobacter pylori*

H. pylori, formerly designated *Campylobacter pyloridis*, was cultivated and identified for the first time in 1982, by Barry J. Marshall and Robin Warren. *H. pylori* is a Gram-negative bacterium that colonizes the stomach, usually in the mucus layer in contact with the gastric epithelial cells[59,60]. This bacterium typically exhibits a curved or S conformation with dimensions around 3 μm x 0.5 μm, containing in one of its terminations some flagella with sheath (Figure 6-A). The flagellar structure allows microorganisms to have motility, however, it is possible to observe *H. pylori* that does not contain the typical flagella[59,61].

H. pylori is a fastidious bacterium, i.e., requires complex growth conditions. Firstly, it is a microaerophilic bacterium, growing only under reduced oxygen conditions. Secondly, its cultivation must be performed in rich medium, with nutrient-rich agars (for example, Columbia blood agar base - CBA), supplemented with blood from mammals (for instance horse, ox, or sheep). In addition, it is a slow growing bacterium, taking several days, usually at 37 ˚C and 5 - 10% CO_2, to form small, circular and non-pigmented colonies, visible through the blood agar medium[62], as can be seen in Figure 6-B.

A



B

Figure 6 | A – *H. pylori* typical morphology. Image by electron microscopy, in which it is possible to visualize the S-conformation and the presence of multiple flagella. Adapted from Worku et al.(1999)[63] B – *H. pylori* colonies, on Columbia Blood Agar. Adapted from Pokhrel (2015)[64].

H. pylori colonizes the stomach of more than half of the global population[65]. In Portugal, *H. pylori* infection affects a large proportion of the adult population (84.2%), presenting the highest prevalences among the regions belonging to Western Europe[66,67]. The majority of people infected with *H. pylori* remain asymptomatic during their lifetime[65], however, the persistence of the bacterium in the human stomach is associated with the development of multiple gastrointestinal diseases, such as gastritis, gastric and duodenal ulcers[59] and gastric cancer[68,69]. In fact, in 1994, *H. pylori* infection was considered by the *International Agency for Research on Cancer (IARC)* as a carcinogenic agent (Class 1) for humans[70], which was then reconfirmed in 2012[71].

Although the transmission of the infection is not yet fully understood, it is believed that it occurs mainly interpersonally, by oral-oral, fecal-oral or gastric-oral routes[72].

3.2 Fluoroquinolones resistance

The recommended universal treatment for the eradication of *H. pylori* infection includes the administration of a proton-pump inhibitor (PPI), such as omeprazole or lansoprazole + clarithromycin + amoxicillin or metronidazole, for 7 - 14 days, when resistance to clarithromycin is less than 15%[73,74]. However, the increasing antibiotic resistance of *H. pylori*, namely to clarithromycin, has led to treatment failures, with a consequent reduction in the infection eradication rate[73–75]. When first-line treatment with clarithromycin is not possible, or it fails, alternative regimens containing quinolones, namely fluoroquinolones are usually suggested by different consensuses[73,74]. However, because of the increased use in the hospital environment, there has also been observed increasing levels of fluoroquinolones resistance[72,74]. For this reason, it is important to know the underlying mechanisms related with resistance, so that detection methods, as well as new drugs that go beyond resistance mechanisms can be developed. The resistance of microorganisms to fluoroquinolones may be triggered by several mechanisms. Generally, the bacteria may exhibit resistance through more than one mechanism, which may create a stronger resistance system, or have a preference for a particular type of resistance mechanism[76].

The main responsible of high level bacterial resistance is the occurrence of specific point mutations in the genes encoding the main target enzymes of fluoroquinolones. These drugs naturally display two targets in most bacteria, DNA gyrase and topoisomerase IV, which play important roles in different cellular activities, such as DNA repair and

replication[77,78]. Mutations in the genes can change the amino acids present in the protein, which can modify its arrangement, and consequently, affect the binding of the drug to their targets[77,79]. A low or high level of resistance can be caused by transmission of resistance-associated genes, which are normally incorporated into plasmids, and are therefore called plasmid-mediated quinolone resistance *(PMQR)* genes[78]. Additionally, in order to face intracellular targets, fluoroquinolones have the ability to cross the bacterial membrane structure through the porins, and thereby exerting their action on the intracellular targets[80]. Hence, a reduction in the number of these proteins, as a result of mutations, can modify the membrane permeability, with a consequent decrease of the amount of fluoroquinolones that have access to the intracellular space[77,79]. Mutations that impair the normal activity of porins may also be the cause of the reduction of permeability, leading to resistance[80]. Cell efflux systems may also be involved in the resistance since they have the ability to actively remove the drug from the microbial cell, decreasing its concentration, and consequently action within the intracellular space. Mutations in regulatory systems of the microorganism may lead to an increased expression of these efflux systems, conferring resistance to drugs[77,79].

Regarding *H. pylori*, as in Gram-negative bacteria, in general, it is believed that fluoroquinolones inhibition is essentially on DNA gyrase[77]. In fact, as determined by genomic sequencing, *H. pylori* has a circular genome that does not contain the parC and parE genes corresponding to topoisomerase IV[81] and active elimination of antibiotics by bacterial efflux systems does not show a predominant role in resistance[82]. Therefore, the resistant profile of the bacterium to fluoroquinolones is essentially due to point mutations in the genes encoding DNA gyrase. The enzyme is a heterotetrameric structure consisting of two A subunits (gyrA) and two B subunits (gyrB), distinct from each other, encoded by the gyrA and gyrB genes, respectively[83].

Once within the cell, fluoroquinolones bind to the gyrase-DNA complex possibly through the gyrA subunit[78,84]. Even though resistance can be caused by changes in both genes encoding the enzyme, it is assumed that critical point of occurrence of mutations that confer a resistant phenotype is found in the gyrA gene[85], in a specific region called *quinolone resistance-determining region* (QRDR)[86]. Point mutations associated with resistance have been reported mostly at positions 87 and 91 of the QRDR, involving amino acid substitutions[85,87–100]. These positions are involved in the contact between the drug and the enzyme[101], thus, it is not surprising that changes in these positions modify the normal

establishment of interactions between structures, leading to the development of resistance. The major changes observed at critical positions 87 and 91 are represented in Table 2.

Table 2 | Summary of the main point mutations associated with fluoroquinolones resistance.

Position	Wild-type	Mutation	Base change
87	N87 (Aspargine)	N87K (Lysine)	C261A
			C261G
			T261G
			T261A
91	D91(Aspartate)	D91N (Aspargine)	G271A
		D91G (Glycine)	A272G
		D91Y (Tyrosine)	G271T

From: Chung et al.[90], Glocker et al.[91], Garcia et al.[96] and Nishizawa et al.[97]

Mutations in other positions of the gyrA gene[93,96,98] and in the gyrB gene[90,93,98,102,103] have also been reported. However, these are not particularly relevant to fluoroquinolones resistance, since they are usually less frequent, and commonly occur simultaneously with the mutations already mentioned in positions 87 and 91. Mutations in non-QRDR regions were also described[98,103], although with less frequency.

3.3 Testing for *H. pylori* antibiotic resistance

In face of increasing antibiotic resistance by *H. pylori*, it is important to determine the susceptibilities of the bacterium before starting treatment. Establishing the resistance profile of the microorganism avoids the prescribing of an incorrect or ineffective treatment, wherein the less time it takes to identify the susceptibility, the faster is the beginning of an appropriate therapy[104]. However, in many cases, the recommended triple therapy is prescribed without the knowledge of the microbial phenotype, and the susceptibility test is only performed after the first treatment failure. Considering that antibiotic treatments can be long, starting therapy with the appropriate antibiotic may contribute to the decrease of the days in the hospital, as well as the total costs associated with treatment[105]. Besides, in general, these drugs have several side effects, so it is important to prevent the patient from suffering more effects, by the use of inappropriate antibiotics, than those associated with the antibiotic that will effectively relieve the symptoms of infection. For all these reasons, it is essential to determine as soon as possible whether the bacterium causing the infection is

resistant to a given antibiotic before its administration, so that its prescription is carefully made.

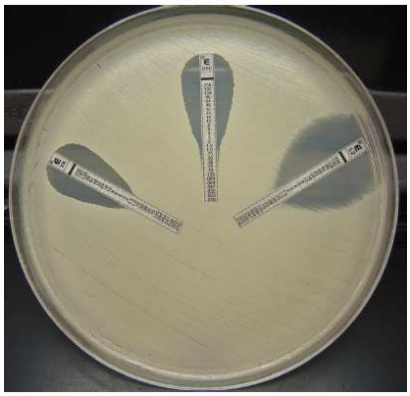
3.3.1 Histopathology methods

When a patient has symptoms of a bacterial gastric infection, it is common to perform an endoscopy, in which biopsies samples can be obtained. In most laboratories, the collected clinical specimens are initially subjected to staining, which can be performed with Gram stain, rapid Giemsa or fluorescent acridine orange and to microscopic visualization[106,107]. Through this procedure, the tissue damage can be directly observed and the bacterium can be detected. Also, it can help in the prescription of an empiric antibiotic treatment[108].

3.3.2 Culture methods

Biopsies specimens may also be prepared for culture[108], which can provide data about the susceptibility to antibiotics. To identify the resistance profile, two representative tests that use culture are E-test (*Epsilometer test)* and agar dilution, both allowing the determination of the Minimum Inhibitory Concentration (MIC) of the antibiotic. The E-test is based in a strip containing an increasing concentration gradient of the drug on the agar medium inoculated with the microorganism under analysis. After incubation, it is possible to observe a zone with no growth, which intersects the strip, allowing obtaining the MIC (Figure 7-A). The agar dilution method involves a serial dilution of the antibiotic in agar, resulting in different concentrations of the drug. After inoculation and incubation of the tested microorganism, the MIC is obtained through the lowest concentration of antibiotic that prevented visible microbial growth[109] (Figure 7-B).

A



B

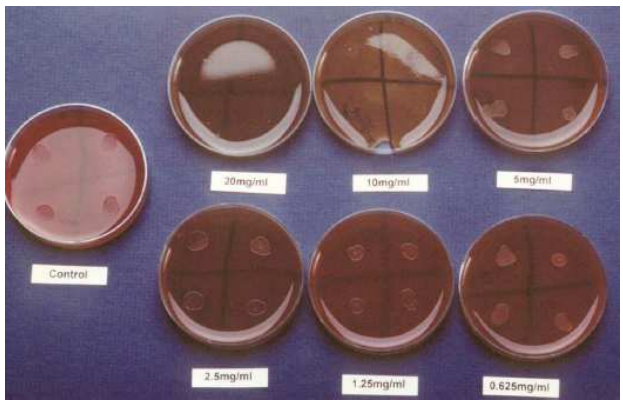


Figure 7 | Examples of culture based antibiotic susceptibility tests. A – E-test (Epsilometer test), performed in *Staphylococcus aureus*. Adapted from Jorgensen and Ferraro (2009)[110]. B – Agar dilution method, performed in *H. pylori*. Adapted from Huynh et al. (2004)[111]

The existence of patterns for the interpretation of results obtained by culture tests, established by committees such as the Clinical and Laboratory Standards Institute (CLSI) in the United States and the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) in Europe, guide therapeutic decisions of health professionals[58,112]. Nevertheless, the long time-to-result imply a delay in starting the appropriate treatment, which is the major disadvantage of this type of technique[58]. In fact, for the study of susceptible profile of fastidious and slow-growing microorganisms such as *H. pylori*, culture methods may not be the most suitable strategy, because of the difficulty in making them grow, which can take several days for having results[113]. Besides, another disadvantage of this method is associated with transporting the clinical sample to the microbiology laboratory. The time between the sample collection and its analysis is very important for the viability of the microorganisms. The longer this time, the more difficult is to detect these microorganisms by culture methods, as they may not grow, leading to incorrect results[107]. In addition, similar to other bacteria, *H. pylori* can, under adverse conditions, acquire a coccoidal form, being in a VBNC state, which can lead to an incorrect diagnosis by culture methods[114,115].

3.3.3 Rapid methods

PCR-based methods have proven to be a useful tool for the rapid detection of antibiotic resistance[58], and are usually reported as effective in detecting both fluoroquinolones and clarithromycin resistance by *H. pylori*[116]. More recently, Cambau et al. developed a new molecular test for the rapid detection of antibiotic resistance in *H. pylori*[117]. The test, named GenoType HelicoDR, is based on DNA strip technology, represented in Figure 8, which can be performed in material obtained from the treatment of gastric biopsies and enables the identification of both clarithromycin and fluoroquinolones resistance. The method development was based on known mutations associated with resistance, that is, *H. pylori* 23S rRNA gene mutations related to clarithromycin resistance, and mutations in the gyrA gene related to fluoroquinolones resistance. GenoType HelicoDR revealed great potential at identifying antibiotic resistance in *H. pylori*, but it requires DNA amplification[117].

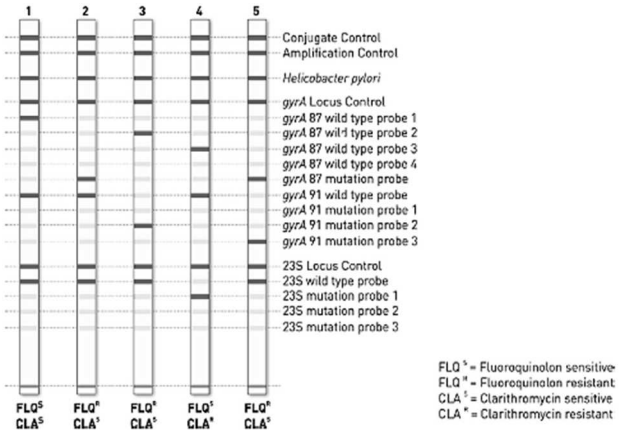


Figure 8 | Exemple of GenoType HelicoDR molecular test, based on DNA strip technology. Adapted from Cambau et al. (2009)[117]

FISH method has been explored for *H. pylori* detection, as well as in determination of its resistance to antibiotics. Russman et al. described the application of FISH in the detection of this bacterium and its resistance to clarithromycin, in a study conducted in cultures of *H. pylori*[118]. Trebesius et al. conducted a study with the same objective, but applying FISH methodology in gastric tissues[119]. These previous studies suggested that FISH has high sensitivity and efficacy in the detection of the bacterium and its resistance profile, in comparison with phenotypic methods. Thus, it was concluded that the technology will be viable for routine analyzes in microbiology laboratories[120].

In 2007, Guimarães et al. described the use of PNA-FISH for the *H. pylori* detection in gastric biopsies, showing very satisfying results for specificity and sensitivity[56]. More recently, the applicability of PNA-FISH technique in the identification of *H. pylori* and its clarithromycin resistance was explored. Cerqueira et al., in 2011, described a PNA-FISH based diagnostic test, which is based on the detection of 23S rRNA gene mutations, associated with resistance[121]. The test, applied directly in paraffin embedded gastric biopsies, was later validated, demonstrating rapid results obtaining, as well as high precision and specificity in the detection of the bacterium and its resistance profile[122].

Regarding LNA/2'OMe probes in FISH procedures, Azevedo et al., in 2015, studied the application of these probes in multiplex approaches within microbial biofilms, which demonstrated future potential of the technology in microbiology field[50]. Nonetheless, to the best of my knowledge, there is no reported studies about the application of LNA/2'OMe-FISH in the detection of fluoroquinolones resistance in *H. pylori.*

In summary, the use of sequence-based molecular tests, namely the FISH methodology, seems to be a better option for the detection of microorganisms and their antibiotic resistance, including fastidious microorganisms such as *H. pylori.* The strong association between known mutations and antibiotic resistance enables the development and application of faster and effective methods, which promise to rationalize the work in clinical microbiological laboratories, speeding up the process of establishing the most appropriate therapy to follow face to an infection. Regarding the resistance of *H. pylori* to fluoroquinolones specifically, the knowledge of the gyrA gene as a focus of resistance-associated mutations allows the development of rapid molecular tests based in the sequence of that region of the genome.

Part IV – Optimization of a procedure for the detection of *Campylobacter* spp.

4.1 Foodborne diseases: Campylobacteriosis

Foodborne diseases are defined as conditions caused by the consumption of food or water contaminated by pathogens, which gain access to the human body through the gastrointestinal tract, according to The European Food Safety Authority (EFSA)[123]. Foodborne diseases are currently a major cause of morbidity and mortality worldwide[124]. The World Health Organization (WHO) assumes that most people will suffer at least one episode of foodborne disease throughout their lives and report that almost 1 in 10 individuals suffer from these conditions every year, exposing the need to ensure food safety[125]. Besides, in addition to public health issues, an increase in the occurrence of foodborne diseases can have economic consequences, leading to high costs, particularly medical and legal costs, lost wages, among other indirect expenses[126].

By 1972, the importance of the genus *Campylobacter* members as a cause of foodborne diseases was recognized. Since then, *Campylobacter* spp. have been reported as a leading cause of bacterial foodborne diseases[127], with the global incidence of campylobacteriosis increasing in recent years[128]. In the European Union (EU), dissemination of data on campylobacteriosis is the responsibility of the EFSA, through annual reports[129]. Data from EFSA and the European Centre for Disease Prevention and Control (ECDC) report that, in 2016, human campylobacteriosis was the most commonly reported zoonotic disease in the EU, contributing to almost 70% of the reported cases, with 246 307 confirmed cases (Table 3). Despite the high number of cases, it should be noted that campylobacteriosis rarely causes death in infected individuals, with a fatality rate of only 0.03% in 2016, which has remained similar over the last 5 years[130].

Although *Campylobacter* infections are generally sporadic conditions[131], campylobacteriosis outbreaks are not rare events (Table 3), mainly associated with the consumption of unpasteurized milk, poultry products or water[128].

Table 3 | Human campylobacteriosis statistics, in the EU, during 2012-2016. Adapted from EFSA and ECDC report (2016)[130]

	2016	**2015**	**2014**	**2013**	**2012**	**Data source**
Total number of confirmed cases	246 307	232 134	236 818	214 710	214 300	ECDC[a]
Total number of confirmed cases/100 000 population (notification rates)	66.3	62.9	66.5	61.4	61.7	ECDC[a]
Number of reporting countries	27	27	26	26	26	ECDC[a]
Total number of food-borne outbreaks (including waterborne outbreaks)	461	399	454	417	503	EFSA[b]
Number of outbreak-related cases	4 606	1 488	2 082	1 836	1 555	EFSA[b]

[a] European Centre for Disease Prevention and Control
[b] European Food Safety Authority

4.2 *Campylobacter* spp.

The *Campylobacter* genus consists of a diverse group of bacteria, which currently includes 39 species and 16 subspecies[132]. *Campylobacter* spp. members are Gram-negative, non-spore forming and spiral, rod-shaped or curved bacteria, with dimensions around 0.2 - 0.8 μm in width and 0.5 - 5 μm in length[128] (Figure 9-A). In most *Campylobacter*, the presence of an unsheathed polar flagellum, located at one or both ends, provides motility, characterized by a rapid and corkscrew-shaped movement. Nevertheless, some species may lack the presence of flagellum (*C. gracilis*) or have multiple flagella (*C. showae*)[133,134]. When plated on blood agar, the bacteria usually appears as a beige or beige grey round, domed colonies with 1 - 2 mm dimensions in diameter[135] (Figure 9-B).

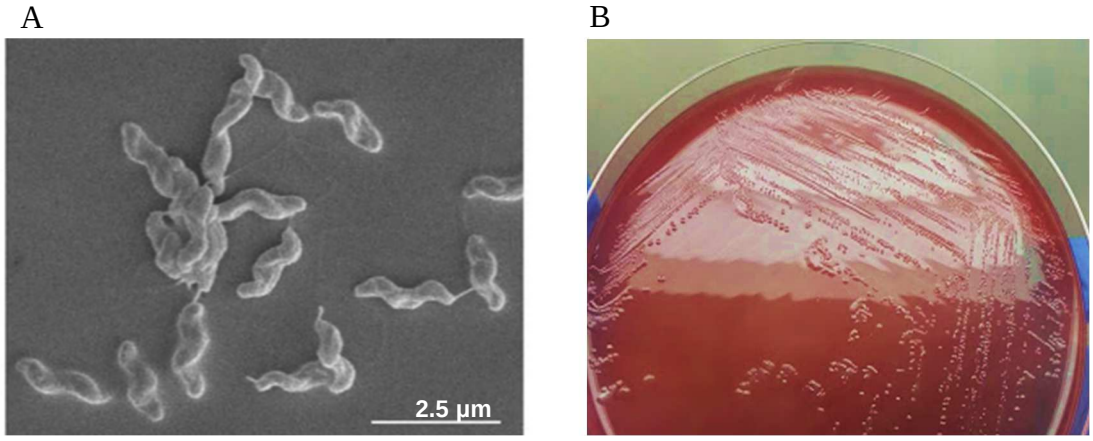


Figure 9 | *C. jejuni* typical morphology. A - Image obtained by electron microscopy, in which it is possible to visualize helical conformation of the bacteria. Adapted from Esson et al. (2017)[136] B – *C. jejuni* colonies on CBA plate.

The most studied members among *Campylobacter* spp. are *C. jejuni* and *C. coli*, which are closely related and the most important enteropathogens among the genus. Indeed, the majority of campylobacteriosis cases in humans are caused by *C. jejuni*, which may be involved in 90 to 95% of cases, and *C. coli*. Nevertheless, infections caused by other members of the genus, have also been reported, although less frequently[129,137] (Table 4).

Most of *Campylobacter* members have oxidase activity (with the exception of *C. gracilis*), reduce fumarate to succinate, reduce nitrate (with the exception of *C. jejuni* subsp. *Doylei)* and are indole negative[135]. All *Campylobacter* bacteria are fastidious and microaerophilic organisms, growing under strictly anaerobic or microaerobic conditions[138], with preference for an environment with low oxygen availability (5% O_2, 10% CO_2 and 85% N_2)[129]. In fact, *Campylobacter* spp. are sensitive to oxygen exposure and consequent formation of reactive oxygen intermediates (ROIs), such as superoxide, that can cause damage to cell nucleic acids, proteins and membranes if not eliminated[127]. Regarding metabolism, although there is still more to elucidate, the acquisition of nutrients seems to be a flexible process, allowing *Campylobacter* to survive outside its natural habitat, either in water, food or feces[139].

C. jejuni, C. coli, C. lari, and *C. upsaliensis* can be grouped into a close group of thermotolerant *Campylobacter*, since they can grow between 37 ºC and 42º C, with an optimal temperature of 41.5 ºC[126]. These thermotolerant strains are the main causative agents of human campylobacteriosis[140]. In turn, due to lack of cold shock protein, required for the

adaptation to low temperatures, these species are usually incapable of growing below 30 ^{o}C[126].

Table 4 | The *Campylobacter* strains most relevant to human health, with their respective clinical manifestation and sources. Adapted from Man (2011)[138]

***Campylobacter* spp.**	**Clinical manifestation**	**Sources**
C. coli	Gastroenteritis, septicaemia	Dogs, cattle and pigs
C. concisus	Gastroenteritis, periodontal disease, septicaemia; associated with inflammatory bowel disease, Barrett's oesophagus	Humans, dogs and cats
C. curvus	Abscess and gastroenteritis	Humans
C. fetus subsp. *fetus*	Meningitis, septicaemia, foetal loss and vascular infection	Cattle, dogs, sheep and turtles
C. fetus subsp. *venerealis*	Septicaemia	Cattle
C. gracilis	Abscess	Dogs and humans
C. hyointestinalis subsp. *hyointestinalis*	Gastroenteritis and septicaemia	Cattle, hamsters and pigs
C. insulaenigrae	Gastroenteritis and septicaemia	Porpoises and seals
C. jejuni subsp. *jejuni*	Gastroenteritis, septicaemia, foetal loss, mesenteric adenitis, colitis, myocarditis, reactive arthritis, Guillain–Barré syndrome and Miller Fisher syndrome	Cattle, dogs, poultry, sheep and wild birds
C. jejuni subsp. *doylei*	Gastroenteritis and septicaemia	Humans and dogs
C. lari subsp. *lari*	Gastroenteritis and septicaemia	Cats, dogs, chickens and seals
C. rectus	Abcess	Humans
C. showae	Septicaemia, cholangitis; associated with inflammatory bowel disease	Humans and dogs
C. sputorum biovar *sputorum*	Abscess	Humans, cattle, pigs and sheep
C. upsaliensis	Enteritis, septicaemia, abortion and abscesses	Cats, dogs, ducks and monkeys
C. ureolyticus	Associated with ulcerative colitis	Cattle
C. mucosalis	Gastroenteritis	Dogs

The consumption of poultry and related products is possibly responsible for 50% - 70% of the total cases of campylobacteriosis[139], which are usually colonized with *C. jejuni*. All commercial poultry species, such as chickens, ducks and turkeys, can be infected with

Campylobacter, however, the greater risk comes from the intake of contaminated chicken, which is generally consumed in large numbers[141] and present a high carriage rate of the bacterium[128]. Indeed, according to a study conducted by Bull et al., *C. jejuni* is present in up to 98% of the chicken meat retail in the US and in 60 - 80% in Europe[139,142]. Thus, the main risk for the transmission of campylobacteriosis is the handling and ingestion of raw or undercooked chicken meat[129].

The amount of microorganisms to cause the infection can be as low as 500 - 800 bacteria[143] and the infected individuals usually exhibit a moderate and self-limited gastroenteritis, although sometimes the infection may remain as an asymptomatic condition[133]. In more severe cases, the infection may be the precursor to the development of auto-immune conditions such as Guillain Barré Syndrome (GBS) or Miller Fischer Syndrome[139].

4.3 Detection of *Campylobacter* spp. in food products

The presence of foodborne pathogens in food should be an important topic with regard to public health. For this purpose, food industries need to implement methods that allow the detection of these microorganisms, ensuring that the food that comes to the consumer does not contain pathogenic microorganisms[9]. Nevertheless, microbiological analysis of food for pathogen detection is still a challenging process. The complex food matrices, the heterogeneous dispersion of pathogens, usually found in low numbers in foods, the stress to which microorganisms are exposed during food processing and the presence of other bacteria from the normal flora, which are usually present in high number, are some of the factors that can hamper the microbiological detection[144].

In general, a detection method should satisfy several requirements. High values of specificity, defined as the ability to discriminate a negative result, when the sample does not present the target pathogen, i.e, if the test only detect the target pathogen, excluding other microorganisms possibly present, and sensitivity, defined as the ability to provide a positive result when the pathogen is present in the sample, are important parameters when evaluating a test[8]. The sensitivity in particular, is a relevant requirement since, as previously described, pathogens are normally present in low numbers in food products. Therefore, detection tests should be sensitive enough to detect a single pathogen in food samples, since this may be sufficient to cause infection in the host[9]. The tests should also be accurate, ensuring the

minimal number of false-negatives and false-positives[145]. Short time-to-results, simplicity of procedure and equipment, and a reasonable price are other desired characteristics[11].

Conventional culture methods remain the *gold standard* of food microbiology. Several foodborne pathogens have an international reference method (i.e., an ISO standard) describing the procedure for their detection and identification, usually based on the microorganism culture. The need for rapid results led to an increasing development of alternative methods based on molecular techniques for the pathogen detection in food samples. Nevertheless, the industry often finds itself comfortable with the conventional techniques and is resistant to adopting alternative methods, which may delay their implementation in food microbiology[146].

The new methods are usually submitted to a validation process established by entities such as AOAC, the Association Français de Normalisation, France (AFNOR), the European Validation and Certification Organisation, Europe (MicroVal) and part of the Nordic Committee on Food Analysis, Norway (NordVal). These validated tests are often the methods adopted by the food industries, as they provide confidence by the analytical evaluation to which they have been subjected[146]. In Table 5 are presented some culture methods as well as immunological and molecular techniques currently available in the market for the detection and identification of *Campylobacter* spp. in food samples.

Table 5 | Test kits for the detection of *Campylobacter* spp. that have received validation by Independent Organizations (such as AOAC or AFNOR). Adapted from Oyarzabal & Fernández[10] and the Validated Test Kit table available at the website of the US Department of Agriculture.

Detection method	Example	Manufacturer	Validated matrices
Culture methods			
	CampyFood Agar (CFA)	BioMérieux	Fresh raw pork, raw chicken breast, processed chicken nuggets, chicken carcass rinse, turkey carcass sampled with sponge (25g)
	Brilliance™ CampyCount Agar	Oxoid Ltd	Poultry products
	RAPID' *Campylobacter* Agar	Bio-Rad	Meat products, and meat product and production environment samples
Immunological methods			
	VIDAS® *Campylobacter* (ELISA- based)	BioMérieux	Fresh raw pork, raw chicken breast, processed chicken nuggets, chicken carcass rinsate, turkey carcass sampled with sponge (25 g)
	Veriflow™ *Campylobacter* (Lateral Flow Imunnoassay)	Invisible Sentinel, Inc.	Chicken carcass rinse
	Singlepath® *Campylobacter* (Lateral Flow Imunnoassay)	Merck KGaA	Raw ground chicken, raw ground turkey (25 g), pasteurized milk
Nucleic acid – based methods			
	Campylobacter real-time PCR	Eurofins Genescan	Chicken raw meat, faeces on cloacae swabs, disposal shoe covers with chicken faeces
	iQ-Check™ *Campylobacter* Real-time PCR	Bio-Rad	Chicken carcass rinse, turkey carcass sponge, raw ground chicken breast (25 g)

4.3.1 Culture methods

In order to reduce the competitive microorganisms, culture methods for *Campylobacter* detection in food samples are usually based on the use of selective agents, namely antibiotics to which the *Campylobacter* bacteria present resistance, such as vancomycin, cefoperazone and cycloheximide, and a high incubation temperature (42 °C)

under a microaerophilic atmosphere[10]. In addition, due to *Campylobacter*'s sensitivity to oxygen and resulting oxidizing radicals, the selective media usually contain oxygen scavengers (for instance, blood)[126].

Food samples usually contain a low number of pathogens against a high background of competitive flora, and, besides, cells can be injured due to drying, heating processes, starvation, freezing or oxygen radicals[147]. For this reason, the isolation of *Campylobacter* spp. from food samples usually includes an enrichment procedure, which allows the recovery of damaged cells, increasing the number of target pathogens to detectable levels in the sample, facilitating their further detection[8]. Bolton Broth[147] and Preston Broth[148] are the commonly used enrichment media. Additionally, it has been suggested an initial incubation at 37 ºC for 3 - 4 h, to allow the optimal recovery of *Campylobacter* cells[10]. It should be noted, however, that the enrichment procedure can lead, at the same time, to an increase in the number of non-target bacteria also present in the sample[138]. Although essential, the enrichment step extend the total time of the detection process, since an enrichment time of 48 hours is usually used[10].

Following the enrichment procedure, the enriched suspension is plated on selective and differential medium, in order to observe typical colonies. The selective agars media can be divided into two groups: blood-containing and blood-free agars. The first group usually contains 5 - 7% (vol/vol) lysed horse blood, including the media Skirrow, Preston and Exeter. The blood-free media can be formulated with charcoal as an oxygen quencher, for instance the most commonly used mCCDA (modified Charcoal Cefoperazone, Deoxycholate agar) (Figure 10-A)[147].

More recently a new group of plate media called chromogenic agars have been applied to *Campylobacter* spp. detection. The principle of this technology is the presence of soluble colourless molecules (chromogens), which function as a substrate for a specific enzymatic activity in the target microorganism. After hydrolysis by the enzyme, a chromophore is released, exhibiting a distinctive colour. Due to low solubility, this compound forms a precipitate, which is expected to be localized on bacterial colonies, allowing their detection and visualization. Colored colonies are easily detected on the plate, allowing a better differentiation within the remaining flora[149,150]. The first chromogenic agar commercially available for the isolation of *Campylobacter* spp. was CampyFood ID agar (bioMerieux, Marcy l'Etoile, France) (Figure 10-B). Since then, other chromogenic agars

have been developed, all presenting equal sensitivity to conventional plates for the identification of the bacterium in food matrices[10].

Finally, to confirm the positive result, the colonies suspected to be the target pathogen are subjected to additional biochemical or serological tests, including microscopic examination, Gram stain technique and catalase and oxidase tests[8,147].

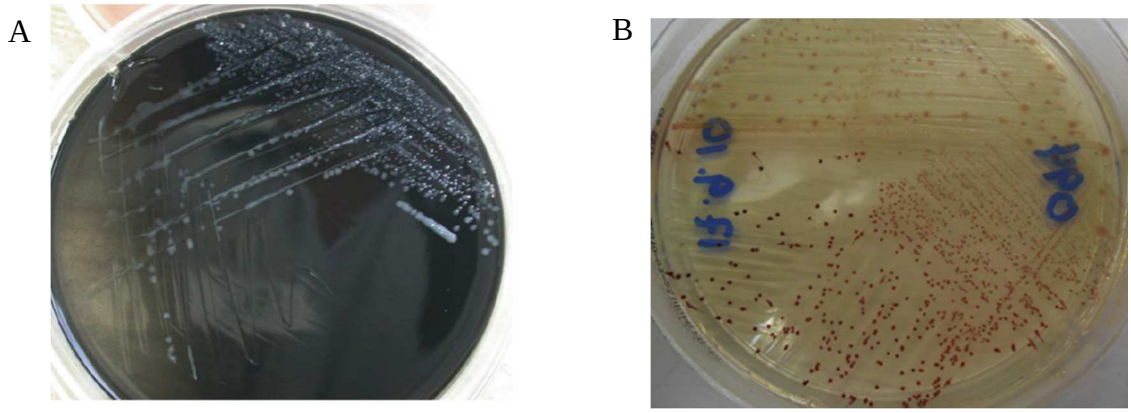


Figure 10 | Typical morphology of *Campylobacter* spp. in different selective media. A – mCCDA, with visible grey, moist colonies. B – CampyFood ID agar (bioMerieux), with visible red colored colonies. Adapted from Oyarzabal and Fernández (2016)[10].

As for other foodborne pathogens, there is an ISO standard for *Campylobacter* spp. detection, based on culture methods. The International Organization for Standardization (ISO) is a worldwide federation of national standards bodies that prepare International Standards through ISO technical committees. The purpose of ISO standards is to ensure the safety, reliability and good quality of products and services, covering several fields, from technology, to food safety, to agriculture and healthcare. Besides, they facilitate the access of companies to new markets and contribute for a free and fair global trade[151]. Despite the existence of different techniques for the detection of foodborne pathogens, ISO standards are considered the reference method. This is especially relevant for the entry of new detection methods on the market, which should be evaluated against the standardized method[5,151].

The actual standard method for detection and enumeration of *Campylobacter* spp. in food is the ISO 10272:2017. ISO 10272 is divided into two parts, namely, Part 1: Detection method and Part 2: Colony-count technique. The present work will be based on Part 1 (ISO 10272-1:2017), regarding to a horizontal method for the detection of *Campylobacter* spp. in food samples. The general procedure for the detection of *Campylobacter* spp. according to ISO standard is outlined in Figure 11.

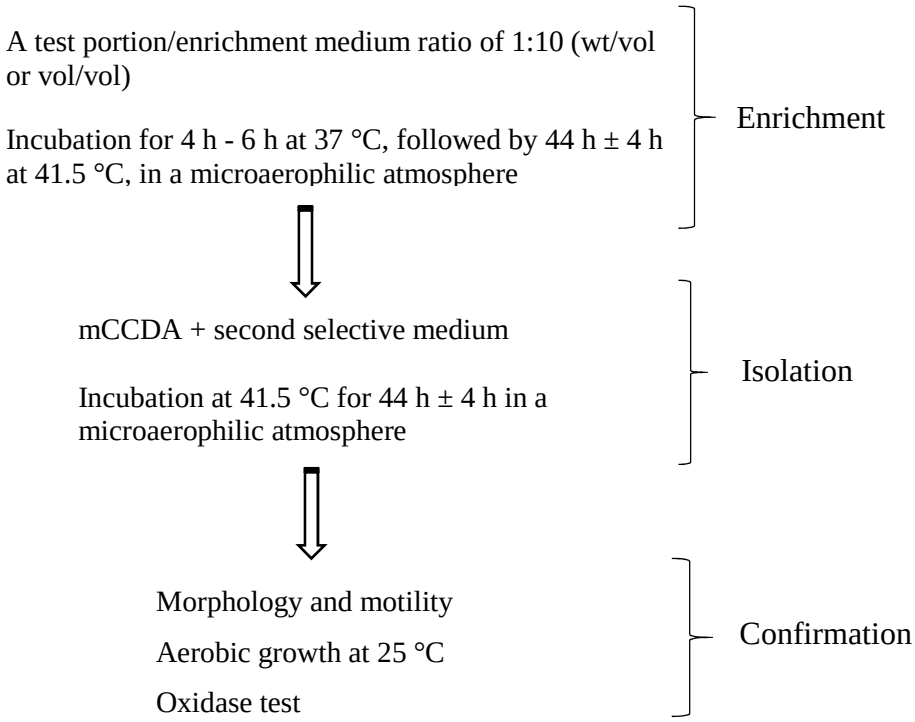


Figure 11 | Procedure defined by ISO 10272 for *Campylobacter* spp. detection. After the confirmation tests, it is concluded that *Campylobacter* was present in the initial sample if at least one colony exhibits a curved bacilli morphology with motility, absence of growth at 25 ºC and a positive result in oxidase test.

In conclusion, culture-based methods for *Campylobacter* spp. detection usually take 2 - 3 days for an initial detection, with the time being extended to up to a week or more for the confirmation tests[8]. Besides, the fastidious nature and the possible transformation to a VBNC state of Campylobacters are drawbacks for the industrial application of culture methods, namely to food industry[126]. In view of this, it is necessary the development and the application of methods that allow the rapid detection leading to faster results obtaining, while maintaining similar efficacy in detecting the pathogen.

4.3.2 Rapid methods

To overcome the limitations of the conventional culture, rapid methods for microbiological detection have been developed, ensuring the distribution of safe food, and thus protecting public health[7] (Table 5). It should be noted, however, that for any of the existing rapid methods, and similarly to culture methods, sample enrichment is required[8]. The advantage of the molecular methods is that, after the enrichment time, they provide results in a shorter period of time[144].

In general, immunological-based detection methods have a good performance in food matrices, with little interference from factors such as non-target cells, DNA, and proteins[7]. Both polyclonal and monoclonal antibodies have been developed for the detection of *Campylobacter* spp., namely *C. jejuni* and *C. coli*, leading to the development of several immunological methods for the detection of the bacterium[152] (Table 5).

More recently, nucleic acid-based methods have been revolutionizing routine analyzes for food control. Usually, for detecting *Campylobacter* spp. by PCR the target is a highly conserved region, such as the 16S or 23S rRNA, although more specific sites, for instance, the hippurate gene for *C. jejuni*, can be used for detecting specific strains[140]. Currently, PCR-based kits, namely based on Real-time PCR, are already commercially available, with validation for several food matrices (Table 5). The major drawback in PCR techniques applied to food samples are the possible failure of the reaction caused by inhibitory components or competing DNA sequences from the non-target microorganisms in the food matrix or enrichment media that can interfere with PCR reaction[11].

FISH has been increasingly explored in the field of food safety. Following the enrichment step, this fluorescence technology enables the rapid detection of foodborne pathogens, namely the most relevant pathogenic organisms, such as *Salmonella enterica*, *Campylobacter* spp. and *Listeria monocytogenes*. The enrichment of food samples is an important step for detecting small microorganisms, such as *Campylobacter* spp., by FISH methodology since it allows increasing the ribosomal content, producing stronger fluorescence signals. This brighter fluorescence helps in the distinction of the target bacteria from the high non-specific fluorescence that is generally visualized[22]. To be considered a viable alternative to other detection methods applied to food microbiology, namely the ISO standards, FISH has to reach similar limits of detection and exhibit similar performance. Since conventional methods should detect at least 1 CFU/test portion, FISH technology should also achieve the same goal[22].

FISH methodology has been described as a useful tool for the sensitive and specific detection of *Campylobacter* both in clinical diagnosis[153] and in water and sludge samples[154]. Regarding food safety, the use of FISH for rapid detection of the *Campylobacter* spp. in food samples has been subject to some studies. Schmid et al. and Moreno et al. evaluated the identification of thermolerant *Campylobacter* in chicken products by FISH, which were successfully achieved[155,156]. The use of PNA probes has also been shown to be advantageous

for the detection of *Campylobacter*, as evidenced by the study conducted by Lehtola et al.[157]. It was verified a greater accessibility of the mimic to rRNA target regions, as compared to DNA probes[157].

Even so, FISH technology is still not used in routine analysis for the detection of pathogens in food matrices, and thus an efficient FISH protocol in food samples has not yet been established. One factor that contribute for this is the complexity of the food matrices, which requires adaptations of the protocol, such as in the enrichment step[22,158].

Part V – Objetives

The main goal of this work was the development of methods for rapid detection of pathogenic microorganisms, based on NAM-FISH technology. The project was based on the detection of pathogens in clinical and food samples thus, the practical work presented in this document was divided in two main parts.

The first part was based on the product already developed by Biomode 2 S.A. in healthcare, Probe4Pylori®. This kit is designed for rapid detection of *H. pylori* clarithromycin resistance. However, resistance to other antibiotics used as an alternative, namely fluoroquinolones, has been observed. Since this resistance is also becoming a recurring problem, the aim of this part of the work was to initiate the development of a similar method for the detection of *H. pylori* resistance to fluoroquinolones in gastric biopsies, based in NAM-FISH technology.

The second part of the work focused on the optimization of a procedure for the detection of *Campylobacter* spp., a foodborne pathogen that is currently responsible for the vast majority of foodborne diseases. The company has previously developed a PNA-FISH method for detecting *Campylobacter* spp. in food samples. However, the procedure still needed to be optimized, which was the main purpose of this work.

Part VI - Materials and Methods

6.1 Bacterial species and growth conditions

The bacterial strains used in the assay were obtained from the internal culture collection of the Centre of Biological Engineering (CEB). *Campylobacter* strains were maintained on Columbia Blood Agar (CBA, Oxoid CM0331, UK), supplemented with 5% (volume/volume) defibrinated horse blood (Probiológica, Belas, Portugal), at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Fisher Scientific, EUA), under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). *Helicobacter* and *Arcobacter butzleri* strains were also maintained on CBA, under the same conditions as the *Campylobacter* strains, but at 37 °C. All strains were streaked onto fresh plates every 48 hours. *E. coli* strains were maintained in tryptic soy agar plates (TSA; Liofilchem, Italy) at 37 °C for 24 hours.

6.2 NAM probes design for the detection of fluoroquinolones resistance in *H. pylori*

To identify potentially useful oligonucleotides to use as probes, gyra gene sequences (wild-type and mutant) available at the National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/genbank/) were used. The probes were designed to specific target positions 87 and 91 of the gyra gene, which are associated with the modifications that cause resistance.

For this study, different NAMs were tested, namely Peptide Nucleic Acid (PNA) and Locked Nucleic Acid/2'O-Methyl (LNA/2'OMe). Several criteria were taking into account in the probes design, such as melting temperature (Tm), Gibbs free energy (ΔG) and sequence length (between 15 - 17 nucleotides). For LNA/2'OMe probes design, two additional important factors were taken into account. Probes were designed incorporating a LNA monomer at every three 2'OMe, as previously described on reported studies which demonstrated high values of sensitivity and specificity[52]. Additionally it was consider to place a triplet of LNA with the center base at the mismatch site, which is associated with a higher discrimination[159]. The final configuration of the drawn probes is exemplified in Figure 12. The NAM sequences were then screened *in silico* for non-specific cross-reactivity, using the Basic Local Alignment Search Tool (BLAST), available in https://blast.ncbi.nlm.nih.gov/Blast.cgi.

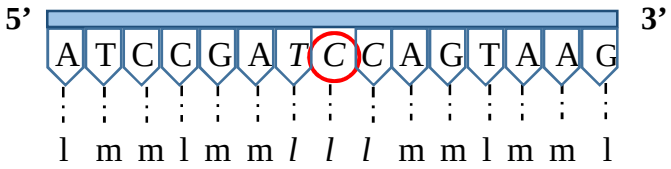


Figure 12 | Schematic design of LNA/2'OMe probes. A LNA monomer (represented by "l") was introduced at every three 2'OMe monomers (represented by "m") and a triplet of LNA was placed in the center base at the mutation site, which is surrounded by the red circle.

6.3 Detection of *Campylobacter* spp.

6.3.1 Real - time PCR (qPCR)

To perform the Real-time PCR assay, a loopful of biomass from 24-hours cultures was suspended in 250 µL of lysis solution, NZY Bacterial Cell Lysis Buffer (NZYTech, MB17801). The suspensions were then placed in a HLC Heating-ThermoMixer (DITABIS), where they remained under stirring at 98 °C for 15 min. The detection of *Campylobacter* spp, was achieved using a specific probe labeled with the FAM dye (λ_{max}/nm absorption - 494; λ_{max}/nm emission - 518). Additionally, each reaction contained an internal amplification control (IAC), a non-target DNA sequence from pUC 19, detected with a probe labeled with the ROX dye (λ_{max}/nm absorption - 575; λ_{max}/nm emission - 602). The presence of an IAC has the purpose of preventing the occurrence of false negatives that might be caused by the inhibition of PCR reaction[14]. The sequences of the primers and probe used in the assay are listed in Table 6.

The PCR mixture solution consisted of 185.5 µL of a master mix solution (SsoAdvanced™ Universal Probes Supermix, BioRad), containing dNTPs, Sso7d fusion polymerase, MgCl2, stabilizers and ROX normalization dyes, 7.4 µL of each primer (forward and reverse primers, 500 nM), 3.7 µL of each probe (200 nM) and 96.5 µL of ultrapure and sterilized water. All compounds must be DNAase/RNase free, in order to avoid the degradation of the target nucleic acids. The final mixture reaction submitted to the Real-time PCR run was prepared with 22.5 µL of the previously prepared PCR mixture solution and 2.5 µL of the sample lysate, for a final sample volume of 25 µL in each well. The PCR run was performed on a CFX96 Touch Deep Well Real-time PCR Detection System. Amplification of DNA was accomplished with 36 cycles, and PCR conditions consisted of a denaturation step at 95 °C for 10 seconds, followed by annealing at 58 °C for 20 seconds

and extension at 65 °C for 30 sec. In the first cycle, denaturation at 95 °C occurred for 3 min to allow all initial template molecules to be denatured. Finally, PCR run data were acquired and analyzed with CFX Manager™Software.

Table 6 | Primers and probe used in the Real-time PCR assay for the detection of *Campylobacter* spp.

Target	Primers/Probe	Sequence (5' – 3')
Campylobacter spp.	Reverse primer	TTCCTTAGGTACCCTCAGAA
	Forward primer	CTGCTTAACACAAGTTGAGTAGG
	Campylobacter probe	**FAM** - TGTCATCCTCCACGCGGCGT

FAM – fluorescein, fluorescent dye

6.3.2 Inoculum preparation

For direct analysis by PNA-FISH, a loopful of biomass from 24-hours cultures were harvested from the plate and suspended in 1 mL of dH_2O. For artificial contamination of food samples, the suspension was performed with autoclaved phosphate buffered saline (PBS 1x: NaCl 137 mM, KCl 2.7 mM, Na_2PO_4 10 mM, KH_2PO_4 1.8 mM), with the cell density to be subsequently assessed by optical density (OD) determination at 600 nm corresponding to approximately 1×10^8 cells/mL. The relationship between OD and total cell counts was previously accomplished by performing cell counts and OD readings at different cell dilutions, as described by Fernandes et al.[160]. From this initial suspension, successive dilutions of 1:10 were performed in order to obtain different concentrations of the bacterial suspension. Finally, a portion of the appropriate concentration was collected to obtain the desired inoculation levels: 1 CFU, 10 CFU and 100 CFU. To confirm the cell concentrations, the suspensions were plated in CBA and incubated under the same microaerophilic conditions used for culture maintenance. The plates were inspected after 24 h and colony counts were performed.

6.3.3 PNA-FISH procedure

For the PNA-FISH method performance, approximately 20 µL of the bacterial suspension or enriched sample were placed directly on 14 mm 3 well glass slides (Thermo Scientific, USA), which were allowed to dry. For permeabilization and fixation of bacteria, the smears were first immersed in 4% paraformaldehyde (Sigma-Aldrich-Aldrich, USA) and then in 50% (vol/vol) ethanol (Fisher Scientific, USA) for 10 min each, and then allowed to

air dry, at room temperature 20 °C ± 10 °C. After drying, fixed smears were covered with approximately 20 μL of hybridization solution which contains a mixture of 10% (wt/vol) dextran sulphate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) FICOLL, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100, 50 mM Tris–HCl (all from Sigma-Aldrich), 200 nM of *Campylobacter* probe (Panagene, South Korea) and 200 nM of blocker probe (Panagene) without fluorochrome. Blocker probe was added with the purpose of hybridizing specifically with a mismatch sequence of 3 nucleotides of *Escherichia* spp. and *Salmonella* spp., competing with the *Campylobacter* specific probe, increasing the specificity of the reaction. The smears were then covered with coverslips and incubated for 1 hour at 57 °C. After incubation, the coverslips were removed and the slides were placed in a pre-warmed (57 ºC) washing solution consisting of 5 mM Tris base, 15 mM NaCl, and 1% (vol/vol) Triton X (all from Sigma-Aldrich), for 30 minutes at 57 °C. The slides were then allowed to air dry, after which they were mounted with nonfluorescent mounting oil (Panreac AppliChem, Spain). Finally, the slides were observed using an inverted imaging system (EVOS® FL Imaging System) equipped with filters sensitive to the Alexa Fluor 594 molecule attached to the probe (Absorption max, 590 nm; Emission max, 617 nm). Visualization of samples was accomplished within less than 48 h after the experimental PNA-FISH procedure. The images were acquired through an integrated software with a Sony ICX445 monochrome CCD camera, using a magnification of x1000.

6.3.4 Enrichment procedure

The samples enrichment for subsequent detection of *Campylobacter* spp. by PNA-FISH was performed with Bolton Broth (BB) medium (Oxoid, CM0983), supplemented with Bolton Broth Selective Supplement (Oxoid, SR0183) and 5% (vol/vol) lysed horse blood (Probiológica, Belas, Portugal). The medium was maintained at 4 °C ± 2 ºC. The matrix used in the assay was fresh raw broiler meat, since it is the major transmission vector of *Campylobacter jejuni*, the main enteric agent. The samples were obtained from local retailers, in Braga, and maintained refrigerated at 4 °C ± 2 ºC.

For the food samples artificial contamination, 3 levels of inoculation were used: 1 CFU/10 g, 10 CFU/10 g and 100 CFU/10 g. For this purpose, 10 g of the fresh raw broiler meat were directly inoculated with a bacterial suspension of *C. jejuni* CNET 90, prepared as

described in section *6.3.2 - Inoculum preparation*, in stomacher bags with filters (VWR, USA). The bacterial amount to be inoculated was determined taking into account the decreased viability of the strain used after refrigerated storage, as determined in previous assays performed by Biomode's group, and it was controlled by plating on CBA medium and by colony count after 24 h. After the inoculation on the food matrix, the samples were maintained at 4 °C for 24 hours, in order to allow the bacteria to adapt to the new surrounding conditions. In addition, a non-inoculated sample was used in the assay, as negative control, ensuring that the original sample was not naturally contaminated.

To perform the enrichment step, the samples were then homogenized with 90 mL of BB in a stomacher (Eco Blender II) for 15 seconds and incubated at 37 °C for 4 hours followed by 44 hours at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation), under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). For the two-step enrichment assay, in addition to this initial procedure, 1 mL of each suspension was subsequently transferred to new medium, in the same initial proportion (1:10). The suspensions were then maintained for a further 24 h under the same microaerophilic incubation conditions, for a total of 48 h enrichment period.

After the enrichment step, the samples were subjected to the PNA-FISH procedure. For the confirmation of the PNA-FISH results, a loopful of the enriched samples was plated in the selective mediums mCCDA and Preston agar, as recommended by the ISO standards (ISO 10272-1:2017). The plates were incubated at 41.5 ºC under microaerophilic conditions. After 48 h of incubation, the plates were analyzed to detect the presence of *Campylobacter* colonies, according to the growth characteristics in each selective medium. All experiments were performed through independent assays, with three replicates for each level of inoculation.

6.3.5 Reduction of background autofluorescence

In previous reported studies of PNA-FISH in food samples, a high autofluorescence was observed, which may cause greater difficulty in the analysis of the results by microscopic visualization[161,162]. Thus, after the enrichment step, described in section *6.3.4 - Enrichment procedure*, the samples were submitted to different treatments, in an attempt to reduce the background fluorescence visualized. The tested treatments were: (1) 1 mL of enriched suspensions was centrifuged at 900 g for 1 minute to allow the sedimentation of

food particles, as suggested by Stevens and Jaykus[163]; (2) 1 mL of enriched suspensions was centrifuged at 10 000 g for 5 minutes and the pellet was resuspended with a 0.1% Triton X-100 (Sigma-Aldrich) solution to emulsify the fat particles, as suggested by Stevens and Jaykus[163] and Almeida et al.[162]; (3) 1 mL of enriched suspensions was centrifuged at 10 000 g for 5 minutes and the pellet was resuspended with dH_2O and (4) 1 mL enriched suspensions was centrifuged at 10 000 g for 5 minutes and the pellet was resuspended with a PBS solution. The protocols 3) and 4) were adapted from Stevens and Jaykus[163] and Almeida et al.[162]. After treatment of the samples, 20 µl were collected from each suspension and subjected to the same PNA-FISH procedure described in section *6.3.3 - PNA-FISH procedure*. In addition, 20 µl were directly collected from the enriched sample, for further comparison regarding the autofluorescence.

6.3.6 Robustness test

In order to obtain the product certification, the method should be subjected to validation studies. In this context, Biomode works in partnership with the AOAC International. AOAC is a globally recognized, independent and not-for-profit organization that provides technical guidelines for microbiological validation studies[164]. The robustness assay is one of the established tests to obtain AOAC certification, whose purpose is to evaluate if the developed method is affected by the variations that are expected to occur when it is performed by the end user. It is advisable to choose parameters that are most likely to affect the analytical performance and determine the range of variations that can occur without adversely affecting the performance of the method. Therefore, the following parameters were tested: hybridization time, hybridization temperature and time-to-result after mounting. The selected parameters were evaluated according to the range of variations shown in Table 7.

Table 7 | Selected parameters for the robustness test and the respective range of variations.

Robustness Test Parameter	**Low Value**	**Baseline value**	**High Value**
Hybridization time	30 mins ± 5 mins	60 mins ± 5 mins	45 mins ± 5 mins
Hybridization temperature	52 ± 1° C	57 ± 1° C	62 ± 1° C
Time-to-result after mounting	30 mins ± 5 mins	0 mins	90 mins ± 5 mins

For the robustness test performance, an initial sample of fresh raw broiler meat was divided into 20 portions of 10 g. Of these, 10 replicates were inoculated with the target microorganism, *C. jejuni* CNET 90, at a low inoculation level (1 CFU/10 g) and 10 replicates were inoculated with a non-target microorganism, *E. coli* CECT 515, at a higher inoculation level (10 CFU/10 g). After the artificial contamination, all samples were subjected to the same enrichment process in BB medium, involving an incubation period of 4 h at 37 ºC, followed by 44 h ± 4 h at 41.5 ºC. Thereafter, the samples were subjected to the previously optimized processing. Finally, the samples were analyzed by PNA-FISH, varying the parameters according to the factorial design of the experiment (Table 8). The probability of detection (POD) and confidence intervals (CI) were then determined according to the AOAC guideline (please see Annex I), and analyzed for the effects caused by changes in parameters.

Table 8 | Experimental design for the robustness test taking into account the selected parameters.

Combination	**Hybridization time**	**Hybridization temperature**	**Time-to-result after mounting**
1	30 mins	52 ºC	30 mins
2	30 mins	52 ºC	90 mins
3	30 mins	62 ºC	30 mins
4	30 mins	62 ºC	90 mins
5	45 mins	52 ºC	30 mins
6	45 mins	52 ºC	90 mins
7	45 mins	62 ºC	30 mins
8	45 mins	62 ºC	90 mins
9 (Baseline)	60 mins	57 ºC	0 mins

Part VII - Results and Discussion

7.1 Detection of fluoroquinolones resistance in *H. pylori*

The knowledge of the genomic sequence composition of the microorganisms has opened new possibilities regarding microbiological detection methods[108]. With respect to the resistance of pathogens to antibiotics, in particular, the discovery of the mutated sequences allowed the development of molecular methods for the detection of resistant phenotypes, which are faster and more specific than conventional culture methods[104]. Thus, the aim of the present work was the design of NAM probes that will allow the future development of a FISH method for the detection of *H. pylori* and its fluoroquinolone resistance profile in gastric samples.

For the probes design, an extensive analysis of the available bibliography regarding the mechanisms of resistance to fluoroquinolones was performed. As previously explored in the introduction, there are several mechanisms of action responsible for the resistance of the microorganisms to fluoroquinolones[77–79]. However, it is commonly reported that the main responsible for higher levels of resistance is the occurrence of specific point mutations in the genes encoding the target enzymes of fluoroquinolones (DNA gyrase, encoded by gyrA and gyrB genes; and topoisomerase IV, encoded by parC, and parE)[77]. In this study the gyrA gene was used because it was found to be the critical point of occurrence of mutations that confer a resistant phenotype in *H. pylori*[85]. The most common mutations are well characterized, with point mutations at positions 87 and 91 of the gyra gene to be critical for resistance[85,87–100]. Besides, genomic sequencing provided the information that *H. pylori* genome does not contain the parC and parE genes corresponding to topoisomerase IV[81]. According to Cambau et al., the most prevalent mutations in the gyrA gene are distributed with a prevalence of 41% for N87K mutation, 30% for D91N, 15% for D91Y and 11% for D91G[117]. Taking into account that these were also the most commonly reported mutations, found through an extensive bibliographic analysis[85,87–100], the surrounding regions were used to design the probes.

Probe4Pylori®, the kit already developed by Biomode 2 S.A. for the detection of *H. pylori* and its susceptibility to clarithromycin, contain a mixture of PNA probes that targets the rRNA of the bacterium, where point mutations in the peptidyltransferase region encoded

by the V domain of the 23S rRNA gene lead to resistance[121]. Conversely, in the product to be developed, the detection of the bacterium and its susceptibility to fluoroquinolones will be achieved by detecting the mRNA transcribed from the gyra gene. The mRNA molecule is usually present in low numbers in the cell, which may hinder detection of the fluorescence signal[23]. However, the gyra gene codes for a crucial bacterial enzyme, essential for the cells viability, which may be an advantage for this study. Given the essential role of the enzyme in several cellular processes[77,78], it is expected the existence of several copies of the corresponding mRNA within the bacterial cells, so it is possible that there will be no problem in detecting fluorescence. In fact, the sudy of an essential bacterial enzyme through its mRNA has already been described by Pernthaler and Amann[165]. Therefore, PNA probes for detecting gyra mRNA were designed, as well as probes corresponding to the wild-type phenotype in both critical positions. The resulting probes are presented in Table 9. Four probes were designed for position 87 (2 for the wild-type sequence and 2 for the mutations) and five probes were designed for position 91 (2 for the wild-type sequence and 3 for the mutations). The high number of probes is due not only to the need to cover the most prevalent mutations (N87K, D91G, D91N, D91Y), but also to the genetic variability present in the pathogen under study, observed in the sequences present in the database. In fact, *H. pylori* genome is generally characterized as highly variable. Even between *H. pylori* strains, phenomena as gene rearrangements, inversions and sequence variation can lead to a great genetic diversity[166,167]. In the analysis of different mutated and non-mutated sequences present in the GenBank database, it is possible to observe a nucleotide diversity of the gyra gene, in which the same amino acid may be represented by different codons. In view of this genetic heterogeneity, it was necessary to design more than one probe for the same mutation or wild-type sequence. Taking into account the most prevalent changes, the 9 total probes were reached. However, the number of probes was considered too high for what would be appropriate for the assay.

Table 9 | PNA probes designed for the detection of gyrA gene in *H. pylori* and their theoretical melting temperature, Gibbs free energy (ΔG) and GC content.

Target position	Codon variation	PNA probe (5' – 3')	Tm (ºC)[a]	%GC	ΔG (kcal mol^{-1})[a]
Asn (N87)	AA**T**	CCGCATTATCGCCATG	57.03	56.25	-19.87
Asn (N87)	AA**C**	CCGCGTTATCGCCAT	56.25	60.00	-19.90
Lys (N87K)	AA**A**	CCGCTTTATCGCCATG	55.57	56.25	-19.94
Lys (N87K)	AA**G**	CCGCCTTATCGCCAT	53.59	60.00	-19.31
Asp (D91)	**C**TA	CACTAGCGCATCATAAA	57.97	41.18	-18.88
Asp (D91)	**T**TA	CACTAACGCATCATAAA	56.10	35.29	-17.89
Gly (D91G)	G**G**T	CACTAGCGCATTATAAA	55.40	35.29	-17.66
Asn (D91N)	**A**AT	CTAGCGCACCATAAA	54.68	46.67	-17.08
Tyr (D91Y)	**T**AT	CACTAGCGCATAATAAA	56.93	35.29	-17.66
		Average value	**55.95 ± 1.32**	**47.36 ± 10. 92**	**-18.69 ± 1.13**

Shaded areas indicate the probes corresponding to mutations associated with fluoroquinolones resistance.
TM: melting temperature; ΔG: Gibbs free energy.
[a] Calculated according to SantaLucia (1998)[168], SantaLucia and Hicks (2004)[169]; and Owczarzy et al.(2011)[170]

In practical terms, it would be difficult to optimize the hybridization of such a large number of probes in the same multiplex assay, namely the establishment of hybridization conditions under which all the designed probes present similar levels of efficiency, as desired[171]. Additionally, the presence of several probes in the same hybridization solution involves evaluating the possibility of cross-hybridization between all the probes present[50]. In the perspective of the future product commercialization, the incorporation of the probes in a single kit would be complicated. In general, the synthesis of such molecules currently involves more costs than for the conventional DNA probes thus, a high number of probes would lead to an increase in the final price of the product. This could delay the commercialization of the product, since the cost is of considerable importance to the consumer[11]. Besides, for the method specificity not to be significantly affected, the conditions for the procedure would have to be carefully controlled, since a variation in the established conditions can compromise the performance of the method. To achieve the same optimal hybridization conditions for all the 9 probes would be very challenging and could imply rigidity on the method procedure conditions. This could translate for the method not to be executable in the day life of a laboratory, affecting the reproductibility of the method.

Thus, it was decided to select other NAM, the LNA/2'OMe. As previous mentioned, LNA probe design exhibits high flexibility, which allows the combination of LNA monomers with DNA, RNA or other mimics, due to their similar synthesis[33]. In addition, as reported by Fontenete et al., the design of probes with LNA and O-methyl in a 1:2 ratio also resulted in a good mismatch discrimination, with the advantage of allowing greater design flexibility, when compared to PNA probes[55]. The possibility of incorporating mixed bases (i.e. the possibility of having one, some or all of the bases in a same particular position of the probe) into LNA probes, allowing a decrease in the number of probes, was also an important factor for the selection of this mimic. Therefore, it was decided to design LNA/2'OMe probes, which are presented in Table 10. Two probes were designed for position 87 (1 for the wild-type sequence and 1 for the mutations), and three probes (1 for the wild-type sequence and 2 for the mutations) were designed for position 91. The incorporation of mixed bases in the probes allowed reducing the number of probes to be used to 5, which makes their application in the product to be developed more feasible, although fewer probes were desirable to incorporate in the same procedure. In addition, to achieve a greater discriminatory capacity it is advised to follow two conditions, as suggested by You et al.[159]. The first is to place the variation site close to the center position of the probe, which is associated with a maximum discriminatory power. However, in this study, this point was not possible to achieve, since the sites of the mutations are close to each other, which would lead to an overlapping of the probes. Nevertheless, the probes were designed to avoid the location of the variation site in the first 2 bases of the duplex ends, since the discrimination would be significantly affected. In fact, as reported by Owczarzy et al., to obtain a discriminatory power close to the maximum it is sufficient that the mismatch is located in the interior of the probe and away from the terminal, and is not therefore essential that the mismatch is exactly in the center of the duplex[170]. The second condition is to position the triplet of LNA with the center base at the single nucleotide variation site, which was followed in this study.

Table 10 | LNA/2'OMe probes designed for the detection of gyrA gene in *H. pylori* and their theoretical melting temperature, Gibbs free energy (ΔG) and GC content.

Target position	Codon variation	LNA/2'OMe probe (5' – 3')	Tm (°C)[a]	%GC[a]	ΔG[b] (kcal mol^{-1})
Asn (N87)	AA**T** AA**C**	lCmCmGlClRlTmTmAlTmCmGlCmCmAlT	76.85	56.65	-26.77
Lys (N87K)	AA**A** AA**G**	lCmCmGlClYlTmTmAlTmCmGlCmCmAlT	77.60	56.65	-28.02
Asp (D91)	**C**TA **T**TA	mClAmCmTlAlRlCmGmClAmTmClAmTmAlAmA	73.45	38.25	-26.97
Gly (D91G)	G**G**T	mClTmAmGlCmGmClAlClCmAmTlAmAmA	74.30	46.67	-27.78
Asn (D91N)	**A**AT	mClAmCmTlAmGmClGmCmAlTlWlAmTmAlAmA	72.30	35.30	-26.14
Tyr (D91Y)	**T**AT				
		Average value	**74.90 ±2.25**	**46.70 ± 9.99**	**-27.14 ± 0.77**

Shaded areas indicate the probes corresponding to mutations associated with fluoroquinolones resistance. TM: melting temperature; ΔG: Gibbs free energy. The mixed bases are shown in red on the probe, with the following pre-defined code: R - A or G; Y - C or T; W - A or T. LNA monomers are preceded by "l" and 2'OMe-RNA monomers are preceded by "m". [a] Obtained from https://eu.idtdna.com/calc/analyzer ; [b] Calculated according to Kierzek et al. (2005)[54]

The probes were designed and adjusted to have similar values of Tm and ΔG, since they are important parameters for the performance of the method. Besides, this is a multiplex study, where the probes must have equivalent performances under the same conditions. The Tm is defined as the temperature at which half of the nucleic acid are in the duplex form and the remaining are in the single-stranded form[55,172]. This temperature is commonly used as reference for the hybridization temperature (Th), although this parameter is influenced by biological and chemical factors related with the hybridization procedure[55]. Comparing the mean value of Tm between the two mimics, a significant increase in the temperature of the LNA probes (74.90 °C) was observed compared to the PNA probes (55.95 °C). This difference would be expected, since the incorporation of LNA monomers provides significantly increased melting temperatures[35]. The fact that the selected mimic (LNA/2'OMe probe) provides a higher value of Tm is advantageous, since a higher value of this temperature is associated with a higher thermal stability and affinity of the probe[173]. Nevertheless, as suggested by Fontenete et al., it is likely that, in general, Th is lower than

the Tm, which may be explained by components present in the hybridization solution, such as formamide, which acts as a denaturing agent[174].

The ΔG is also an important parameter, estimating whether the reaction will be thermodynamically favorable[174]. In general, a negative ΔG indicate that the reaction is favorable or spontaneous, with more negative values being associated with more favorable reactions[174]. For the LNA/2'OMe probes, ΔG values ranged from -26 to -28 kcal mol^{-1}, with an average value of -27.14 kcal mol^{-1}. For the PNA probes, ΔG values ranged from -17 to -20 kcal mol^{-1}, with an average value of -18.69 kcal mol^{-1}. Although for DNA probes the ΔG must be below -13.5 kcal mol^{-1}, as reported by Yilmaz et al.[175], a recommended ΔG value is not yet established for LNA/2'OMe probes[50]. Nevertheless, similar values of ΔG were obtained for each probe. Finally, regarding %GC level, for the PNA probes, GC content values ranged from 35.29% to 60.00% and for the LNA/2'OMe probes, GC content values ranged from 35.30% to 56.65%. Although for LNA/2'OMe probes there is no set value for %GC, the values obtained are within the range of 30 - 60% recommended for LNA probes[176].

All NAM probes designed were screened for non-specific cross-reactivity, using the BLAST tool (blastn program). As an example, in Figure 13 it is possible to observe a blastn analysis of a LNA/2'OMe wild-type probe for position 91, with significant alignments between the designed probe and the complementary sequences available at the NCBI website. In this particular example, it was observed that the designed probe produces significant alignments with several strains of *H. pylori*. In the 100 most significant alignments, the probe sequence aligned with 100 *H. pylori* strains, as desired. Additionally, both the query cover (i.e. the percentage of query covered by alignment to the database sequence) and identity (i.e. the highest percent identity between all query-subject alignments), show values around 100%. In this study, it is intended that the probes present a significant level of similarity with the *H. pylori* strains, so, this result suggests that this probe a good candidate. The same was observed for the other designed probes (data not shown).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Helicobacter pylori strain NCTC11637 genome assembly, chromosome: 1	34.2	34.2	100%	19	100%	LS483488.1
Helicobacter pylori strain FDAARGOS_298 chromosome, complete genome	34.2	34.2	100%	19	100%	CP028325.1
Helicobacter pylori strain 7.13_R1b chromosome, complete genome	34.2	34.2	100%	19	100%	CP024072.1
Helicobacter pylori isolate 7.13_D3c chromosome, complete genome	34.2	34.2	100%	19	100%	CP024023.1
Helicobacter pylori strain 7.13_D3b chromosome, complete genome	34.2	34.2	100%	19	100%	CP024022.1
Helicobacter pylori strain 7.13_D3a chromosome, complete genome	34.2	34.2	100%	19	100%	CP024021.1
Helicobacter pylori strain 7.13_D2c chromosome	34.2	34.2	100%	19	100%	CP024020.1
Helicobacter pylori strain 7.13_D2b chromosome	34.2	34.2	100%	19	100%	CP024019.1
Helicobacter pylori strain 7.13_D2a chromosome	34.2	34.2	100%	19	100%	CP024018.1
Helicobacter pylori strain 7.13_D1c chromosome, complete genome	34.2	34.2	100%	19	100%	CP024017.1

Figure 13 | Analysis of a LNA/2'OMe probe using the blastn program. The figure shows the first most significant alignments found between the probe and the sequences present in the database.

The effort to cover the nucleotide variations that most commonly arise is especially important in the clinical detection of the microorganism. In the same infected individual, colonization may occur through multiple strains, and modifications have also been observed during the same infection process of the host, occurring in the same strain[167]. Still, it is important to take into account the number of probes mixture within the hybridization solution, which as previously mentioned, should not be very high. With this in mind, the probes were designed to detect the most frequent variations in positions 87 e 91 of the gyrA gene. Mutations in these positions have been commonly reported to account for more than 90% of cases of resistance to fluoroquinolones in *H. pylori*[96,98,99,177]. Mutations in the gyrB gene have also been described in resistant strains, however, they are usually associated with mutations in the gyrA gene, so its role in resistance is not yet fully understood[98]. As previously explored, less frequently resistance may be caused by mutations in other positions of the genes, or other mechanisms not yet described[93,96,103].

The design of these probes has the ultimate purpose of allowing, at a clinical level, the rapid detection of *H. pylori* and its susceptibility to fluoroquinolones. The designed probes would be implemented in a kit, which would be applied directly to gastric biopsies to make the rapid diagnosis, allowing the prescription of the appropriate treatment[58]. It should also be noted that the analysis performed in this study involved preliminary tests, where the theoretical values of Tm and ΔG were calculated. In this study it was not consider other factors that may influence the method and performance of the probes, such as the

conditions used in the hybridization step[174], namely the composition of the hybridization solution. Therefore, this is a procedure that still needs to be optimized in the future in the laboratory. Initially, the probes should be experimentally tested and optimized, namely for the hybridization temperature of each probe, which should be similar between them to be applied in a multiplex method. After optimizing the procedure conditions, the practical specificity and sensitivity of the probes should be determined, through FISH experiments[121]. Then, the procedure should be validated in the real samples in which the method is to be applied, i.e. gastric biopsies[121,122].

7.2 Detection of *Campylobacter* spp. in food samples

7.2.1 Test of inclusivity and exclusivity of the probe

In order to perform the assays for the detection of *Campylobacter* spp., a PNA probe previously developed by the Biomode's group was used, although some further optimizations should be performed. The probe was initially tested for inclusivity and exclusivity, since these are important parameters to consider for the performance of the developed method. The inclusivity reflects the ability of the probe to detect as many strains of the same target species as possible. The exclusivity evaluates the discriminatory power of the probe between target and non-target microorganisms[178]. Thus, to evaluate the probe designed for the detection of *Campylobacter* spp. in terms of these parameters, different target and non-target strains were subjected to the developed PNA-FISH method. For this purpose, 8 different *Campylobacter* strains and 6 different non-C*ampylobacter* strains, obtained from the internal culture collection of the CEB, were used. The results of the assay are represented in Table 11.

Table 11 | *Campylobacter* and non-*Campylobacter* strains included in the exclusivity and inclusivity test of the probe, and their outcome with PNA-FISH procedure

	Target	Strain	PNA – FISH
Inclusivity test	*Campylobacter coli*	NCTC 11366	+
	Campylobacter coli	CNET 20	+
	Campylobacter jejuni	NCTC 11168	+
	Campylobacter jejuni	CNET 76	+
	Campylobacter jejuni	CNET 88	+
	Campylobacter jejuni	CNET 90	+
	Campylobacter jejuni	CNET 93	+
	Campylobacter jejuni	CNET 109	+
Exclusivity test	*Helicobacter pylori*	NCTC 11637	-
	Helicobacter muridarum	2A5	-
	Helicobacter cinaedi	33221 – 1.2	+
	Helicobacter pamatensis	CIP 104249	+
	Arcobacter butzleri	LCDC 11516	-
	Arcobacter butzleri	CCUG 30485	-

NCTC - National Collection of Type Cultures; CNET - *Campylobacter* collection hosted by DSM (Campynet); CIP - Collection of Institute Pasteur; LCDC - Laboratory Center for Disease Control (US); CCUG - Culture Collection, University of Gothenburg; (+) – positive; (-) - negative

From the 8 *Campylobacter* strains tested, all were correctly identified by the method. Contrarily, from the 6 non-*Campylobacter* strains tested, 4 were not detected by the method and 2 were detected: *H. cinaedi* 33221 – 1.2 and *H. pamatensis* CIP 104249. Since the developed probe intends to specifically detect *Campylobacter* spp., it was expected that all strains tested in the exclusivity test wouldn't be detected. However, the test of the two above-mentioned non-target strains resulted in fluorescence signals.

Performing this preliminary test was important since it allowed assessing whether the probe is working properly, i.e. if it detects only the species for which it was designated and excludes non-target microorganisms. In order to investigate whether the result obtained was not derived from a possible contamination or execution error during the procedure, leading to a false positive result, the test was repeated, under the same conditions. The same non-*Campylobacter* strains continued to show a fluorescence signal, confirming their detection by the PNA-FISH method. In this case, it is important to understand what is causing the detection of a non-target microorganism, in order to optimize the procedure. Two possible explanations arose: the false positive strains were misidentified (i.e. they are

not *Helicobacter* strains); or the non-specific detection can be probably related to the probe itself, which may have the ability to bind other microorganisms rather than *Campylobacter* spp., not having an optimal specificity level. A possible explanation may be the similarity of the specific sequence detected by the probe in *Campylobacter* and *Helicobacter* strains, leading to the detection of the latter. In this case, a redesign of the probe could be tested, in order to avoid the detection of *Helicobacter* strains. Thus, it is suggested a new search of conserved regions for the 16S and 23S rRNA[162] in *Campylobacter*, selecting a region of interest that is present in all *Campylobacter* strains, but is absent in *Helicobacter* strains.

In an effort to obtain more data to explain the detection of both strains by PNA-FISH, a simple bioinformatics analysis was performed using the BLAST tool. In short, the probe used in the developed PNA-FISH procedure was aligned with the nucleotide sequences for *H. cinaedi* and *H. pamatensis* present in the databases. The results of the alignment are represented in Figure 14. The comparison of sequences with *H. pamatensis* resulted in a total of 6 significant alignments (Figure 14-A), i.e. the probe sequence present similarity to 6 sequences of *H. pamatensis* deposited in the database used. Despite the 100% value for identity, it is possible to notice, through the query cover, that the percentage of query covered by alignment to the database sequence does not reach 100%, being the maximum value of 85%. Nevertheless, this similarity may be the explanation for the positive result obtained for this strain by the method designated for *Campylobacter* spp. detection. In turn, the blast analysis of *H. cinaedi* resulted in a greater number of significant alignments (Figure 14-B), in which several have 100% query cover and identity values. Thereby, it is possible that the homology between the probe and the sequences presented in the database explains the detection of both strains by PNA-FISH. However, this simple analysis does not guarantee that this is effectively what occurred with the strains under study. Thus, to evaluate this hypothesis and to obtain more data, the same strains were also tested with other detection method, a PCR-based method.

A

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Helicobacter pametensis strain ATCC 51478 23S ribosomal RNA gene, partial sequence	24.3	53.0	85%	0.002	100%	AY596230.1
Helicobacter pametensis strain Seymour B9A DNA gyrase A (gyrA) gene, partial cds	16.4	45.1	71%	0.59	100%	KM065466.1
Helicobacter pametensis strain B9A Seymour 16S ribosomal RNA gene, partial sequence	16.4	16.4	57%	0.59	100%	NR_028019.1
Helicobacter pametensis strain ATCC 51478 16S ribosomal RNA gene, partial sequence	16.4	16.4	57%	0.59	100%	NR_114586.1
Helicobacter pametensis partial hsp60 gene for heat shock protein 60, strain CCUG 29253	14.4	28.7	57%	2.3	100%	AJ558215.1
Helicobacter pametensis ATCC 51478 60 kDa chaperonin (cpn60) gene, partial cds	14.4	28.7	57%	2.3	100%	AY787945.1

B

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Helicobacter cinaedi P01D0000 DNA, complete genome	22.3	6596	100%	5.2	100%	AP018676.1
Helicobacter cinaedi P06D0798 DNA, complete genome	22.3	6474	100%	5.2	100%	AP018447.1
Helicobacter cinaedi P03D0629 DNA, complete genome	22.3	6492	100%	5.2	100%	AP018446.1
Helicobacter cinaedi isolate gHKSHhc_BC_20160917	22.3	6189	100%	5.2	100%	CP029337.1
Helicobacter cinaedi DNA, complete genome, strain: MRY08-1234	22.3	6414	100%	5.2	100%	AP017374.1
Helicobacter cinaedi CCUG 18818 = ATCC BAA-847 DNA, complete genome	22.3	6619	100%	5.2	100%	AP012492.1
Helicobacter cinaedi PAGU611 DNA, complete genome	22.3	6320	100%	5.2	100%	AP012344.1
Helicobacter cinaedi DNA, CRISPR2 region, strain: PAGU1922	18.3	18.3	64%	81	100%	LC217011.1
Helicobacter cinaedi DNA, CRISPR2 region, strain: PAGU1679	18.3	36.7	64%	81	100%	LC216999.1

Figure 14 | Results of the *Campylobacter* probe sequence alignment with the sequences of the non-*Campylobacter* strains detected by the developed FISH method. A – *H. pamatensis*; B – *H. cinaedi.* For *H. pamatensis* all the alignments originated are represented, whereas for *H. cinaedi* the first significant alignments are represented.

7.2.2 Real-time PCR (qPCR)

As noted above, *H. cinaedi* 33221 – 1.2 and *H. pamatensis* CIP 104249 strains resulted in a positive fluorescence signal with the developed PNA-FISH method. In view of that, a Real-time PCR assay was performed to assess whether those strains were also detected by a different molecular method also specifically designated for *Campylobacter* spp. As positive controls, two reference *Campylobacter* strains (*C. coli* NCTC 11366 and *C. jejuni* NCTC 11168) were included in the test. As negative control, a reference *Helicobacter* strain (*H. pylori* NCTC 11637) was included in the test. In addition, two *Campylobacter* and three non-*Campylobacter* strains, including *H. cinaedi* 33221 – 1.2 and *H. pamatensis* CIP 104249 strains, were tested. Although this methodology enables a quantitative analysis of the results, for the purpose of the present study, a qualitative analysis of the detection was sufficient. Thus, the results were evaluated in terms of presence/absence of detection. The performance

of the assay is represented in Table 12. All *Campylobacter* strains used in the assay obtained a positive result in the Real-time PCR test, which is consistent with the results observed in the developed PNA-FISH procedure. With respect to non-*Campylobacter* strains for which there had been fluorescent signals by the PNA-FISH method, *H. pamatensis* obtained a negative result by qPCR, while *H. cinaedi* resulted in a positive fluorescence signal. For the remaining non-*Campylobacter* strains, negative results were obtained.

Table 12 | PNA-FISH and Real-time PCR outcomes for *Campylobacter* and non-*Campylobacter* strains

Target	Strain	PNA-FISH	Real-time PCR
Campylobacter coli	NCTC 11366	+	+
Campylobacter coli	CNET 20	+	+
Campylobacter jejuni	NCTC 11168	+	+
Campylobacter jejuni	CNET 90	+	+
Helicobacter pylori	NCTC 11637	-	-
Helicobacter pamatensis	CIP 104249	+	-
Helicobacter cinaedi	33221 – 1.2	+	+
Helicobacter muridarium	2A5	-	-
Arcobacter butzeleri	CCUG 30485	-	-
Arcobacter butzeleri	LCDC 11516	-	-

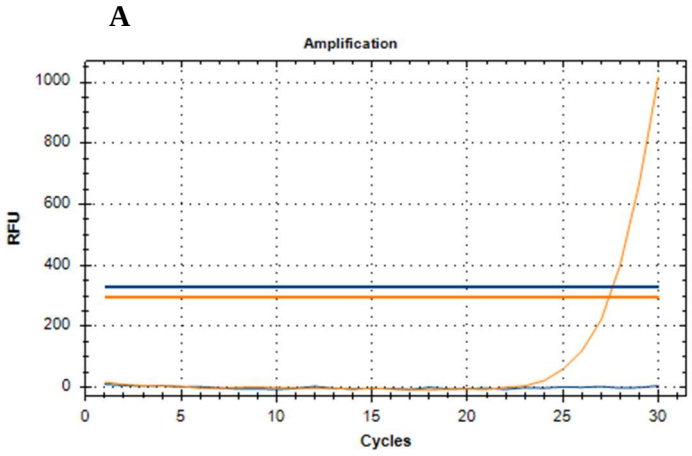
NCTC - National Collection of Type Cultures; CNET - *Campylobacter* collection hosted by DSM (Campynet); CIP - Collection of Institute Pasteur; LCDC - Laboratory Center for Disease Control (US); CCUG - Culture Collection, University of Gothenburg; (+) – positive; (-) - negative

Figure 15 shows the graphics representing visual result obtained for each of the two strains under analysis. As previously mentioned in the section *6.3.1 – Real - time PCR (qPCR)*, the use of an internal amplification control (IAC) is important to avoid an incorrect assumption of false negatives, since the IAC signal should always be detected in negative samples. Thus, two probes stained with two different fluorophore dyes were used in the assay. The probe stained with FAM (blue line on the graphic) indicates the presence of the pathogen, whereas the probe stained with ROX (orange line on the graphic) indicates a correct amplification reaction through the use of the IAC.

Regarding *H. pamatensis*, as shown in Figure 15-A, the generation of the blue curve associated with the probe designed for *Campylobacter* spp. detection did not occur. This result suggests that the bacterium was not present in the initial sample. Additionally, the IAC used has been detected, as can be seen by the orange curve of the same graphic. The generation of the curve for the IAC suggests that the non-detection of the pathogen was not caused by an inhibition of the PCR reaction, and therefore, this result is not a false negative. In view of these results, the positive outcome by PNA-FISH for *H. pamatensis* is probably

a false positive, which can be explained by the possible homology between the probe used in the fluorescent method and the strain sequence. In turn, the application of the qPCR test to *H. cinaedi* resulted in the appearance of the blue curve referring to the probe used for the detection of *Campylobacter* (Figure 15-B). Thus, similar to what happened in PNA-FISH, Real-time PCR also resulted in a positive detection for *H. cinaedi*. Again, the fact that *H. cinaedi* presented positive results in two different molecular methods may also be associated with the occurrence of false positives, or the possibility of the analyzed strain being in fact a *Campylobacter*.

Detection of *Helicobacter* strains by methods specifically designated for *Campylobacter* may also be due to the phylogenetic proximity between members of both genera, both belonging to *Epsilonproteobacteria* class and *Campylobacterales* order. As a matter of fact, some *Helicobacter* strains began to be initially attributed to the *Campylobacter* group due to the high similarity between the microorganisms[135]. *H. cinaedi*, first isolated in 1984, has been recognized as a pathogen of increasing importance for human health[179]. Although widespread in domestic animals such as dogs and hamsters[180], *H. cinaedi* has already been reported in broiler chicken[181]. As with other *Helicobacter* species, *H. cinaedi* may be erroneously classified as non-*jejuni*/*coli Campylobacter* species due to the various morphological and biochemical similarities. From the similarities in gram stain and colony appearance to the positive reactions in oxidase and catalase tests, and negative reactions in urease test, there are several similarities that make it difficult to distinguish between microorganisms of both genera[182]. As verified by the performed studies, a molecular analysis of the strains can also cause false positives.



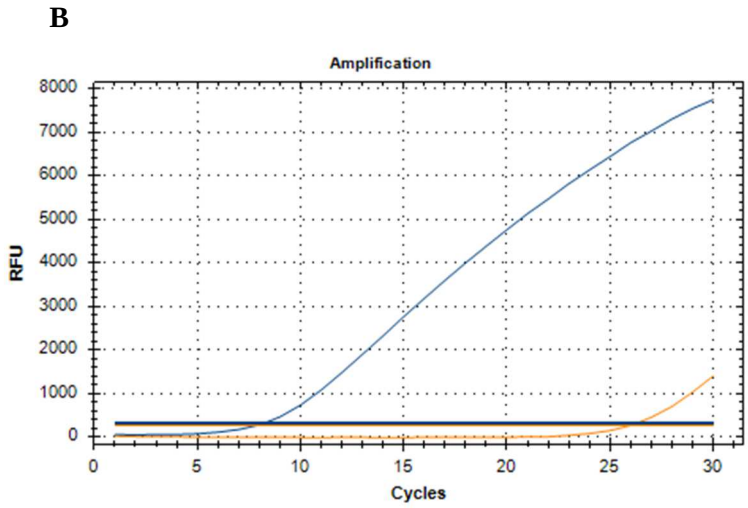


Figure 15 | Results obtained by the qPCR test for the two non-*Campylobacter* strains with positive outcome by PNA-FISH. The orange curve refers to the IAC, while the blue curve refers to the probe designated for the detection of *Campylobacter* spp.
A - *H. pamatensis*; B – *H. cinaedi*; RFU: Relative fluorescence units.

The aim of the method is the detection of *Campylobacter* spp. in meat samples, especially raw broiler meat. Some tests previously performed by Biomode's group suggested that probably there are still be some adjustments to be made in the developed procedure, since the detection of non-*Campylobacter* strains was observed, affecting the specificity of the method. Nevertheless, it is advisable to evaluate this strains in more detail. In view of the difficult distinction between the two genera through common biochemical tests, the genomic sequencing of the microorganisms is probably the best strategy to follow in order to obtain more data.

7.2.3 Reduction of background autofluorescence

Pathogens are usually present at low numbers in food samples and may be injured by processes such as heating, drying or freezing[147]. Thus, prior to the *Campylobacter* detection in food samples, an enrichment step is usually performed, to increase the number of target pathogens to detectable levels and to allow the recovery of damaged cells[8]. Although the enrichment step increases the amount of microorganisms, FISH detection may be affected by the strong autofluorescence derived from some food components[162]. Food matrices, whatever the origin, generally have a heterogeneous composition, which includes proteins, carbohydrates, fats and other compounds[183]. Thus, besides the possibility of interfering with the pathogen viability, the presence of these particles may hinder microbiological detection

by the generation of non-specific fluorescence, which may lead to inconclusive or incorrect results[183]. The background fluorescence may also result from the autofluorescence of the compounds present in the microorganisms, or from components present in the enrichment medium. Thus, one of the challenges of detection methods is the sample preparation for further analysis, which is crucial to the success[146]. One of the strategies for the background fluorescence decrease may be the separation of the target pathogens from the food particles. The separation process intends to isolate a particular bacterial population from a complex solution, such as the food matrix, discarding the food components[184]. However, with the demand for rapid pathogen detection by the food industry and the future application of the developed method in routine laboratories, where it is valued the simplicity and reduced time-to-analysis[11], it is desirable to avoid a too long and/or complex enrichment step. Centrifugation is one of the most commonly used separation techniques, as it is a rapid, economical and a simple method. The application of different gravitational forces enables different separation mechanisms. A low-speed centrifugation (less than 1000 g) allows the sedimentation of food components, while the microbial cells remain in the supernatant. With the increase of speed (more than 8000 g), the bacteria will sediment, as well as some matrix debris. Centrifugation may also be combined with other techniques in order to improve the separation efficiency[184]. One example is the use of enzymes, such as trypsin and collagenase, which act on the connective tissue of the meat, helping in the separation of bacteria from the food samples, as reported by Rodrigues-Szulc et al.[185].

Initially, a two-step enrichment assay was tested in order to evaluate if with the transfer of a portion of the sample to new medium, the commonly observed autofluorescence decreased. Additionally, it was also intended to evaluate the possibility of a greater growth capacity of the microorganisms when transferred to fresh, new medium, which could lead to the generation of a stronger fluorescence signal by the bacteria. Simultaneously with the two-step enrichment test, different types of treatment of the enriched samples were tested before the performance of the PNA-FISH. Thus, after enrichment, the samples tested were collected directly from the enriched suspension or subjected to the procedures 1 - 4 as described in the section *6.3.5 - Reduction of background autofluorescence*. At first, in addition to samples without any treatment, only two different treatments were tested: (1) low-speed centrifugation and (2) high-speed centrifugation followed by resuspension in 0.1% Triton X-100 solution. This was a preliminary test in order to verify the admissibility

of the treatments application to enriched poultry samples for the detection of *Campylobacter*. The results of this assay are shown in Figure 16. In samples without any treatment, a high background autofluorescence was visible, which hampered the visualization of bacteria (Figure 16-A_1). The difficult distinction between the microorganisms and the compounds in the food matrix present in the samples can lead to incorrect results, namely through false negatives. In fact, Buzatu et al. stated the same problem with regard to food samples analysis by flow cytometry[186]. In turn, Almeida et al. tested the reduction of autofluorescence derived from food particles, in order to reduce the interference in the visualization of the bacteria in food products by FISH[162]. In the present study, when the samples were submitted to the established treatments, some autofluorescence is still observed, but generally with lower intensity (Figures 16-B_1 and C_1), in comparison with the background present in the untreated samples. The decrease in non-specific fluorescence intensity was verified in both approaches, thus suggesting that before pathogen detection, a treatment should be applied to the samples. The results of this study are in agreement with previous studies that reported centrifugation as an additional effective step for the detection of microorganisms in food samples[162,187]. Fachmann et al. used centrifugation for the removal of food particles, facilitating the detection of *Salmonella enterica* in meat samples, by PCR[187]. Almeida et al. also verified the ability of centrifugation to decrease autofluorescence from food particles in the detection of *E. coli* O157 by PNA-FISH in ground beef and unpasteurized milk samples[162]. Thus, centrifugation is an effective treatment since it has the ability to reduce the autofluorescence generated by the food compounds, allowing a better visualization of the bacterial cells, if present.

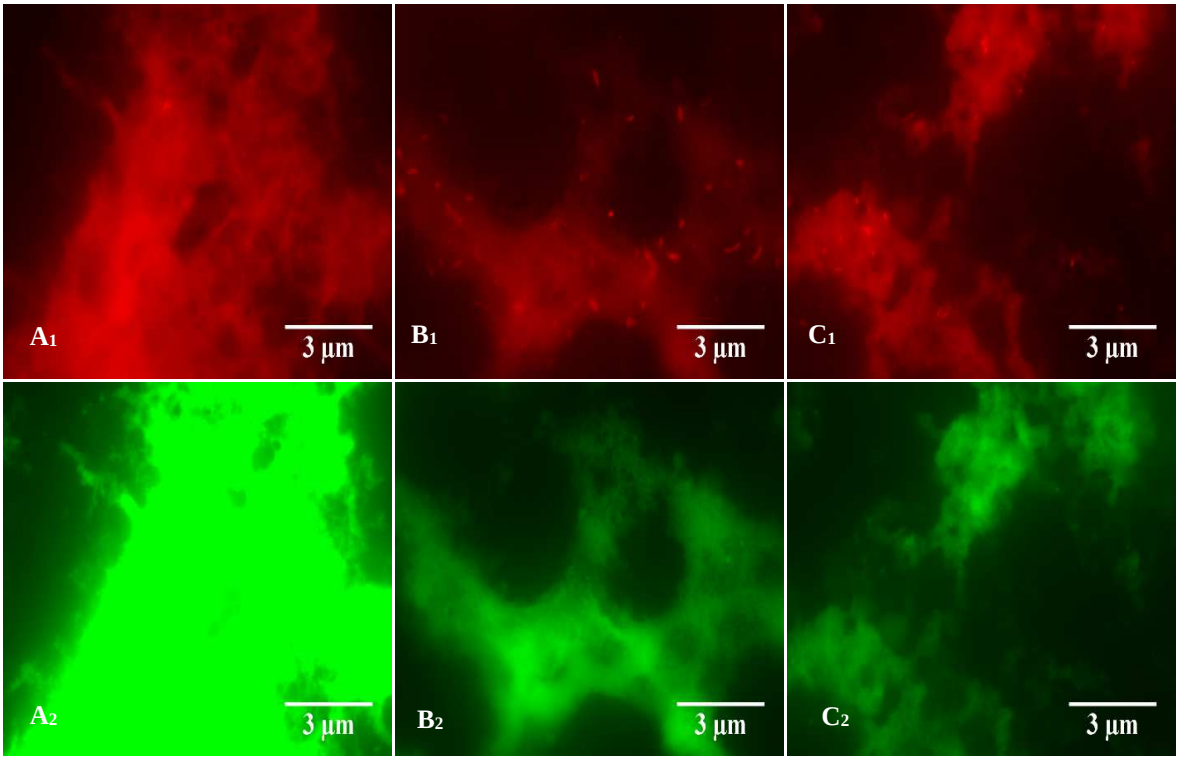


Figure 16 | PNA-FISH visualization of fresh raw broiler meat samples inoculated with *C. jejuni* CNET 90. Results obtained after the procedure performed to samples A - without any treatment, B - with a low-speed centrifugation and C – with a high-speed centrifugation and resuspension in 0.1% Triton X-100 solution. A_2 to C_2 - Visualization of the same microscopic field with the green channel as negative control of the *Campylobacter* probe. (Original magnification x 1000).

Besides assessing the reduction of the non-specific fluorescent signal, it is also important to assess whether the treatment applied does not reduce the effectiveness of the method. Thus, it should be ensured that the detection level of at least 1 CFU/10 g is maintained. The results regarding the detection of *Campylobacter* spp. in food samples are shown in Table 13. The total values of positive detections of 6/9 for both treatments suggest, at a first glance, that the treatments application to the samples does not significantly affect the detection. The lowest value for the number of positive results for the untreated samples (3/9) could be explained by the background intensity that "hides" the bacteria, or by the pipetting process itself, that is more difficult in the presence of food particles[188]. However, when the results are analyzed in terms of the different contamination levels, the results are not very consistent. Although in both approaches at least 1 CFU/10 g was detected at all levels, in general, more positive results were found at lower inoculation levels (1 and 10 CFU/10 g) than at the highest level (100 CFU/10 g), which was not expected. Since bacterial cells were detected at lower levels of inoculation, it would be expected that all samples inoculated with a high bacterial concentration would yield a positive detection. These results

could be explained by a failure in inoculation procedure or in the pipetting process of the highest level, or by the non-growth of *Campylobacter*, given its fastidious nature. The culture results reveal a greater number of positive detections, however, there are discrepancies in the results for the same level of contamination, when compared with the PNA-FISH results. For instance, for the 100 CFU/10 g inoculation level, the treatment with (2) high-speed centrifugation and resuspension in Triton X-100 yielded only 1 positive result in 3 samples, while more positive samples were detected with culture. Thus, these results are not satisfactory and do not allow obtaining a solid conclusion about the performance of the method.

Table 13 | PNA- FISH outcome with respect to the detection of positive samples for *C. jejuni CNET 90* in raw fresh broiler meat, after 48 h enrichment in BB medium, under microaerophilic conditions.

***C. jejuni* CNET 90 – Fresh raw broiler meat**						
Two-step enrichment						
Contamination level (CFU/10 g)	**Rep**	**PNA – FISH**			**Culture**	
		Standard (without further treatment)	Low-speed centrifug.	High-speed centrifug. + 0.1% Triton X-100	mCCDA	Preston
0	/	-	-	-	-	-
1	***1***	+	+	+	+	+
	2	-	-	+	+	+
	3	-	-	+	-	+
10	***1***	+	+	+	+	+
	2	+	+	+	+	+
	3	-	+	-	-	-
100	***1***	-	-	-	+	-
	2	-	+	+	+	+
	3	-	+	-	+	+
Total		**3/9**	**6/9**	**6/9**	**7/9**	**7/9**

Rep – Replica; (+) – positive; (-), shaded areas - negative

Considering the results obtained in the first test, and in order to acquire more data, the same assay was repeated, with the addition of two new treatments: (3) high-speed centrifugation followed by resuspension in dH_2O and (4) high-speed centrifugation followed by resuspension in PBS. These variations were added in view of the ultimate goal of the work, involving the application of the method in a context of routine analysis, where a simple method and with more accessible reagents is desired. The results of this second assay are presented in Figure 17 and Table 14. Similarly to the first test, an intense non-specific fluorescence was verified in samples without any treatment (Figure 17-A_1). The low-speed

centrifugation (1), although reducing autofluorescence (Figure 17-B_1), appeared to have a lower number of bacteria, compared to the remaining treatments. This can be explained by the fact that low-speed centrifugation essentially results in the deposition of food particles, which may have trapped bacteria[184]. Although high-speed centrifugation and (3) resuspension in dH_2O (Figure 17-D_1) and (4) resuspension in PBS (Figure 17-E_1) provided a decrease in background fluorescence compared to untreated samples, these were also the treatments where a greater presence of fluorescent clusters was found. For the resuspension in PBS, the fluorescence may be due to the presence of salts or the formation of precipitates after drying[189], which may interfere with the visualization of the bacteria. The (2) high-speed centrifugation followed by resuspension in 0.1% Triton X-100 was the treatment that obtained a better performance in the visualization of bacteria (Figure 17-C_1), indicating benefit in the use of detergent. In fact, bacteria have the ability to adhere to the food matrix through components of the bacterial cell wall, such as teichoic acids or proteins[190]. Thus, the combination of centrifugation with chemical methods, such as the use of detergents like Triton X-100, allowing the disruption of the established connections between the pathogen and the food matrix, can lead to an efficient separation[162,184]. This finding is in agreement with previous study by Almeida et al. which described the advantage of including a new step with Triton X-100 for the detection of pathogens in food samples, in order to reduce the autofluorescence signal of food particles[162].

Regarding the number of positive results obtained in *Campylobacter* detection (Table 14), for the lowest inoculation level, all approaches detected at least 1 CFU/10 g, however, one of the replicas was not detected in the standard condition and in treatments 1 and 3. For the inoculation level of 10 CFU/10 g, all approaches detected 2 positive results in 3 samples. By the analysis of Table 14, it is possible to verify that the negative result obtained corresponded to the same replica for all approaches. These results may have been caused by a failure in the inoculation of this sample, since the culture method also yield a negative result for this replicate. The highest inoculation level corresponding to 100 CFU/10 g was the only level where it was possible to detect the total positive samples (3/3) in all approaches, which was in agreement with the culture results. Thus, it is possible that the detection limit were affected by the two-step enrichment procedure, which can compromise the growth of bacteria, especially at inoculation levels where the concentration of bacteria is already low, resulting in more negative results compared to the highest levels of inoculation.

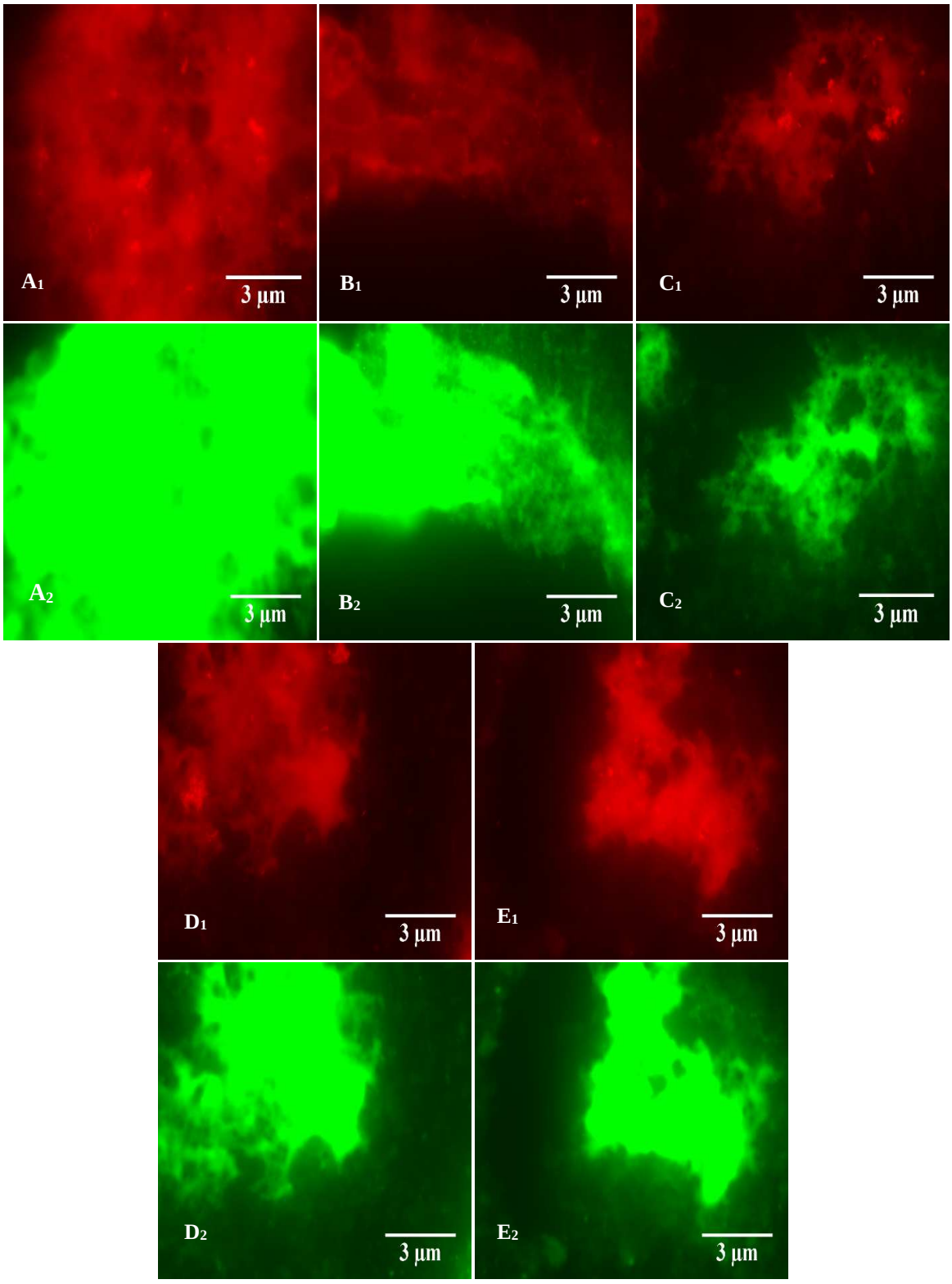


Figure 17 | PNA-FISH visualization of fresh raw broiler meat samples inoculated with *C. jejuni* CNET 90. In the figure are represented the results obtained after the procedure performed to samples A) without any treatment, B) with a low-speed centrifugation, C) with a high-speed centrifugation and resuspension in 0.1% Triton X-100 solution, D) with a high-speed centrifugation and resuspension in dH_2O and E) with a high-speed centrifugation and resuspension in PBS solution. A_2 to E_2 - Visualization of the same microscopic field with the green channel as negative control of the *Campylobacter* probe (Original magnification x 1000)

Table 14 | PNA- FISH outcome with respect to the detection of positive samples for *C. jejuni CNET 90* in raw fresh broiler meat, after 48 h enrichment in BB medium, under microaerophilic conditions. Results obtained after the direct application of the procedure to the samples and after the application of different treatments to reduce the background fluorescence.

***C. jejuni* CNET 90 – Fresh raw broiler meat**								
Two-step enrichment								
Contamination level (CFU/10 g)	**Rep**	**PNA – FISH**					**Culture**	
		Standard (without further treatment)	Low-speed centrifug.	High-speed centrifug. + 0.1% Triton X-100	High-speed centrifug. + dH_2O	High-speed centrifug. + PBS	mCCDA	Preston
0	/	-	-	-	-	-	-	-
1	***1***	+	+	+	+	+	+	+
	2	-	-	+	-	+	+	+
	3	+	+	+	+	+	-	+
10	***1***	+	+	+	+	+	+	+
	2	-	-	-	-	-	-	-
	3	+	+	+	+	+	+	+
100	***1***	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+
Total		**7/9**	**7/9**	**8/9**	**7/9**	**8/9**	**7/9**	**8/9**

Rep – Replica; (+) – positive; (-), shaded areas - negative

Additionally, one thing that it was possible to notice in these enrichment assays was a low number of bacteria in all samples comparing to the correspondent inoculation level. This probably may be associated with the inoculation procedures, since we are dealing with low range of concentrations, lack of growth of this fastidious bacterium, or even by the fact of increasing cellular manipulation in the two-step procedures. To investigate whether this was caused by manipulation of samples when using a two-step enrichment protocol, a one-step enrichment test assay was performed. In general, it was more difficult to find bacteria in the two-step enrichment assay due to the lower number of bacterial cells compared to the one-step assay where they were found in higher numbers, as exemplified in Figure 18. These results can be explained by the fact that in the two-step enrichment protocol, when the enrichment media was replaced, the bacteria have been exposed to the air oxygen. As previously described, *Campylobacter* spp. are sensitive to the presence of oxygen, requiring an O_2 concentration of about 5% for an optimal growth[127]. Thus, when exposed to the gas

concentrations found in the room air, their growth over the remaining enrichment time may be affected. This exposure to oxygen may also have provided the growth of other non-fastidious organisms present in the food matrix, which may exceed the growth of *Campylobacter* spp.

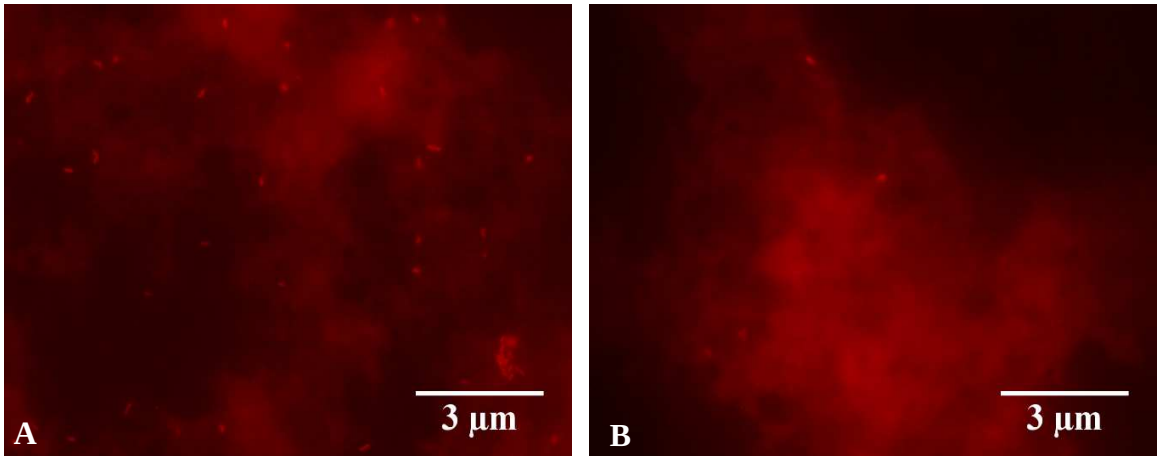


Figure 18 | Illustrative example of the difference found between the concentrations of bacteria found in samples subject to an one-step enrichment assay (A) and to a two-step enrichment assay (B). The images were obtained from the same level of inoculation (10 CFU/10 g). (Original magnification x 1000).

In addition, the same treatments evaluated in the two-step enrichment assays were tested in the one-step enrichment assay. With respect to the decrease of the background autofluorescence, the results obtained were similar to the previous tests, where it was possible to confirm the best visualization of bacteria to be achieved with (2) high-speed centrifugation and resuspension with 0.1% Triton X-100 solution, the two approaches already previously suggested by Almeida et al.[162]. As demonstrated in Table 15, the detection limit was not significantly affected in this assay, with all replicas to be detected at the lowest inoculation level 1 CFU/10 g in all approaches. Besides, a general analysis of the results shows that the one-step enrichment assay yields a greater number of positive results compared to the two-step enrichment procedure. Only one replica yield a negative result (replica 3 - 100CFU/10 g, standard condition), which, as previously mentioned, may be due to the difficult distinction of bacteria that may occur due to intense autofluorescence in untreated samples. Moreover, the results obtained by culture are generally in agreement with the PNA-FISH results. The exception is the replica 3 (10 CFU/10 g of the mCCDA), which may be a false negative, possibly explained by the VBNC status of the bacteria or due to the

difficulty in distinguishing the bacteria among the remaining high competitive flora, commonly visualized in the culture plates.

Another factor evaluated between the two-step and one-step enrichment assays was the fluorescence intensity observed in the bacteria. As can be seen in Figure 18, there was no significant increase in the fluorescent signal, with the bacteria being equally well detected in both assays.

Table 15 | PNA- FISH outcome with respect to the detection of positive samples for *C. jejuni* CNET 90 in raw fresh broiler meat, after 48 h enrichment in BB medium, under microaerophilic conditions. Results obtained after the direct application of the procedure to the samples and after the application of different treatments to reduce the background fluorescence.

***C. jejuni* CNET 90 – Fresh raw broiler meat**								
One-step enrichment								
Contamination level (CFU/10 g)	**Rep**	**PNA – FISH**					**Culture**	
		Standard	Low-speed centrifug.	High-speed centrifug. + 0.1% Triton X-100	High-speed centrifug. + dH_2O	High-speed centrifug. + PBS	mCCDA	Preston
0	**/**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
1	***1***	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+
10	***1***	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+
	3	+	+	+	+	+	**-**	+
100	***1***	**-**	+	+	+	+	+	+
	2	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+
Total		**8/9**	**9/9**	**9/9**	**9/9**	**8/9**	**8/9**	**9/9**

Rep – Replica; (+) – positive; (-), shaded areas - negative

These results allowed assuming that the two-step enrichment approach does not add advantages to the procedure when compared to the one-step procedure. Although the application of the treatments has resulted in a decrease in background fluorescence, the manipulation of the samples in a two-step enrichment appears to affect the detection limit of the method. In addition, it is important to keep in mind that the ultimate goal is to devise a simple and rapid procedure that microbiology laboratories can routinely use to detect pathogens. Having a second enrichment step makes the process more complex and less practical, since it requires that in the middle of the procedure, all the enriched suspensions

are transferred to a new medium. Therefore, the one-step enrichment procedure should be adopted. With respect to the treatment, it was also confirmed that the use of high-speed centrifugation with resuspension in 0.1% Triton X-100 solution is the approach that provides a better visualization of the bacterial cells, maintaining consistency in the detection of positive results. It should be noted that other types of treatment may be applied for the separation of pathogens from food matrix components, such as immunomagnetic separation (IMS)[184]. The IMS technique is based on the detection of pathogens by specific antibodies attached to magnetic spheres, and its application for the separation of *C. jejuni* from poultry meat samples has already been studied by Yu et al.[191]. Although it has demonstrated a high ability to remove food debris and to concentrate pathogen levels, this is a technique that can be expensive, and usually processes small sample amounts at a time[184]. Thus, this is not the most appropriate procedure for food microbiology method for application in routine laboratories. Regarding other molecular methods, Alves et al. described a multiplex PCR assay for the detection of *Campylobacter* spp. in raw chicken meat samples[14]. Bolton et al. reported a PCR-ELISA method for the detection of *C. jejuni* and *C. coli* in food samples[192]. Although both studies have demonstrated efficacy and specificity in the detection, the PCR-based techniques usually involve DNA extraction and are subject to contamination by inhibitory compounds present in food. Thus, the PNA-FISH method, as described in the present study, proves to be a simple, rapid and effective alternative in the detection of *Campylobacter* spp. in meat samples, withouth the need of DNA extraction and allowing the direct visualization of the bacteria.

7.2.4 Robustness test

The robustness assay is one of the tests established by AOAC International to obtain product certification. The purpose of the test is to evaluate whether the performance of the method under analysis is affected by small variations that may occur when it is used by the final consumer. The evaluation of the detection method by the robustness test is important given the possibility of variations in different parameters occurring in routine laboratories, which may affect the viability of the tests performed. Thus, it is important to ensure that regardless deviations, the method is reliable and robust, providing true results. Biomode 2 S.A. has as main focus the research, development and commercialization of rapid diagnostic kits based on FISH technology for microbial detection. It is also the company's concern to

ensure the safety of the method as well as its ability to provide robust and reliable results. In this context, Biomode works in partnership with the AOAC International, in order to obtain product certifications. Thereby, one of the steps for the commercialization of the test developed for the detection of *Campylobacter* spp. involves the validation of the method according to the technical guidelines provided by the AOAC, including the robustness test. Ideally, critical parameters should be chosen. Having this in mind, the hybridization temperature, hybridization time and time-to-result after mounting parameters were selected by the AOAC for the study. The results of the test and the analysis according to the guidelines defined by the AOAC are shown in Tables 16 and 17.

Table 16 | PNA-FISH results and POD, dPOD and 95% CI values for the robustness test applied to samples inoculated with 1 CFU/10 g of *C. jejuni* CNET 90 (target microorganism).

Target - *C. jejuni* CNET 90						
Treatment combination	**X[a]**	**N[b]**	**POD[c]**	**95% CI[d]**	**dPOD[e]**	**95% CI[d]**
1	5	10	0.50	(0.24; 0.76)	0.10	(-0.43; 0.63)
2	5	10	0.50	(0.24; 0.76)	0.10	(-0.43; 0.63)
3	2	10	0.20	(0.06; 0.51)	-0.20	(-0.65; 0.25)
4	2	10	0.20	(0.06; 0.51)	-0.20	(-0.65; 0.25)
5	6	10	0.60	(0.31; 0.83)	0.20	(-0.25; 0.65)
6	6	10	0.60	(0.31; 0.83)	0.20	(-0.25; 0.65)
7	5	10	0.50	(0.24; 0.76)	0.10	(-0.43; 0.63)
8	5	10	0.50	(0.24; 0.76)	0.10	(-0.43; 0.63)
9 (Baseline)	4	10	0.40	(0.17; 0.69)	-	-

[a]Number of positive tests
[b]Number of total tests
[c] Probability of detection; Calculated by the ratio between the number of positive tests and the total number of tests
[d] Range of POD/dPOD values with a 95% confidence level
[e] Differences in proportions between the condition tested and the baseline

Table 17 | PNA-FISH results and POD, dPOD and 95% CI values for the robustness test applied to samples inoculated with 10 CFU/10 g of *E. coli* CECT 515 (non - target microorganism).

Non-target - *E. coli* CECT 515						
Treatment combination	**X[a]**	**N[b]**	**POD[c]**	**95% CI[d]**	**dPOD[e]**	**95% CI[d]**
1	4	10	0.40	(0.17; 0.69)	0.40	(0.03; 0.77)
2	4	10	0.40	(0.17; 0.69)	0.40	(0.03; 0.77)
3	5	10	0.50	(0.24; 0.76)	0.50	(0.12; 0.88)
4	5	10	0.50	(0.24; 0.76)	0.50	(0.12; 0.88)
5	3	10	0.30	(0.11; 0.60)	0.30	(-0.05; 0.65)
6	3	10	0.30	(0.11; 0.60)	0.30	(-0.05; 0.65)
7	4	10	0.40	(0.17; 0.69)	0.40	(0.03; 0.77)
8	4	10	0.40	(0.17; 0.69)	0.40	(0.03; 0.77)
9 (Baseline)	0	10	0.00	(0.00; 0.28)	-	-

[a]Number of positive tests
[b]Number of total tests
[c] Probability of detection; Calculated by the ratio between the number of positive tests and the total number of tests
[d] Range of POD/dPOD values with a 95% confidence level
[e] Differences in proportions between the condition tested and the baseline

In order to perform the analysis, it was considered that the results obtained in the baseline conditions are the true values. For both tests (target and non-target), equal values of POD and dPOD were obtained for pairs of combinations 1 - 2, 3 - 4, 5 - 6 and 7 - 8. Since the only parameter that varies between each set is the time-to-result after mounting, it can be concluded that variations in this parameter does not significantly affect the performance of the method. Therefore, this parameter will not be a problem for the use of the method by the final consumer in the routine analyzes. In view of that, the results obtained were subsequently analyzed based on the two remaining selected parameters.

With regard to the target microorganism, POD values higher than baseline values were obtained in most combinations (Table 16), whose results may actually correspond to false positives. In turn, for combinations 3 and 4, the POD value is lower than the baseline value, with less positive samples being detected than in the baseline, which can be explained by the occurrence of false negatives. In these treatment combinations, a hybridization time of 30 mins and a hybridization temperature of 62 °C were evaluated. The false negatives may have been caused by the short hybridization time, which did not allow the probe to bind to all targets, or by the high hybridization temperature at which the probe may have lost sensitivity. Nevertheless, it is difficult to infer exactly which of the parameters affected the

detection, since in other combinations the same parameters resulted in more positive detections. In fact, the largest variations according to dPOD were verified in combinations 3 to 6, where the four parameter limits were tested (52 ºC and 62 °C for the hybridization temperature, and 30 mins and 45 mins for the hybridization time). Therefore, it is not possible to conclude exactly which parameter caused the variations in the positive detections. Neverthless, the confidence interval of the dPOD values showed that the differences obtained between the tested variations and the baseline conditions for the target are not statistically significant (the CI contains zero).

For the non-target strains, more discrepant values were verified. While at the baseline the number of positive results was, as expected, 0, in all combinations positive samples were detected (Table 17). Since baseline values are considered true values, the detected samples are possibly false positives. In fact, except for combinations 5 and 6, the confidence interval values obtained for all combinations do not include zero, thus concluding that the differences are statistically significant. These results may be related to the blocker probe used in the procedure. This probe was designed to detect essentially *Salmonella* spp. and *Escherichia* spp., which, although presenting a 3 nucleotide mismatch relative to *Campylobacter* probe, can yield a weak fluorescence signal. When bound to these strains, the blocker probe prevents such regions from being available for the binding of the *Campylobacter* probe, avoiding the generation of non-specific positive signal. The non-target microorganism used for the robustness test, *E. coli* CECT 515, is included in the species covered by the blocker probe. *E. coli* is a bacterium commonly found in the gastrointestinal tract of humans and warm-blooded animals. One of the common vehicles for the transmission of *E. coli* is precisely the retail meat[193], similarly to *Campylobacter*. For that reason, there was a need to include a blocker probe, in order to ensure that the signal emitted is effectively from *Campylobacter* spp. Hence, the results obtained in the robustness test may be related to a loss of blocker probe efficacy as a result of the variation in the method conditions. Consequently, the *Campylobacter* probe is allowed to bind to *E. coli* strains present in the samples. This can be a problem since the possible presence of *E. coli*. in meat samples can, under conditions other than those optimized, generate a non-specific fluorescence signal, leading to incorrect interpretations. This reflects not only the importance of a strict optimization of the hybridization conditions but also of the importance of the laboratories to follow the protocols established for the performance of the method inclosed in the kits.

Although these results do not allow concluding which of the parameters had more influence, it is known the critical role of the hybridization temperature for the probe function and, consequently, the performance of the method. Regarding factors that affect hybridization, the term stringency is commonly referred. The stringency represents the conditions under which the probe has access to the targets. Usually a high stringency involves a greater requirement in the binding between the target and the probe, i.e. it requires the sequences to be highly similar so that hybridization occurs. If the stringency is too high, the probe may not bind to the target. In turn, a low stringency does not require a perfect match, so if it is too low, the probe can bind to non-target microorganisms[194]. The stringency can then be a possible explanation for the results obtained. At higher temperatures, stringency increases, so probe binding to the target may not occur, which was found in combinations 3 and 4 (target). The non-target results, in turn, may have several explanations. While a low hybridization temperature decreases the stringency, which may cause probe binding to non-target microorganisms, the higher temperatures can affect the blocker probe, thus releasing non-target strains to the *Campylobacter* probe. Still, it is not possible to evaluate the effect of the hybridization time on the test performance.

In brief, the results obtained by the robustness test allowed to conclude that variations in two of the three parameters analyzed, the time and temperature of hybridization, affect the performance of the method, especially through the detection of non-target microorganisms. The time-to-result after mounting presented similar values within the range of variations tested, so variations in this parameter does not significantly affect the performance of the method. Therefore, it is important to realize whether method optimizations will be required so that the detection is not significantly affected by small variations in performance conditions. Additionally, it was not possible to conclude exactly which parameters significantly affected the detection. Thus, it would be advisable to complement the robustness test with an additional test, for instance, with a different experimental design, in which varied only one of the selected parameters. As a result, it would be possible to explore in more detail the effect that each variation in the parameter would have on the performance of the method. Nevertheless, considering the results obtained for the non-target microorganism, a blocker probe optimization could be tested. This would be achieved by the addition of one or two nucleotides to the blocker probe, which would increase the melting temperature, and, consequently, the hybridization temperature.

Part VIII - Conclusion and future perspectives

The present dissertation focused on the development of NAM-FISH methods for the application in different areas: clinical and food safety.

In the clinical area, the objective was to initiate the development of a method for the detection of *H. pylori* and its resistance to fluoroquinolones. It was concluded that the best mimic to use in the probes design is the conjugation of LNA/2'OMe. In addition to providing a good mismatch discrimination, which is essential for the detection of mutations associated with resistance, this type of mimic allows a greater flexibility in the design of the probes. A total of 5 probes were obtained, with similar values of Tm and ΔG. The *in silico* design of probes is a preliminary phase in the development of a new method, so this is still a procedure that requires optimization. Initially, the probes should be tested and optimized in the laboratory, followed by optimization of the procedure in the actual samples in which the method is to be applied, i.e. gastric biopsies. As a result, the detection of *H. pylori* and its susceptibility profile for fluoroquinolones would be achieved, which would reduce the time for analysis as well as the definition of the most appropriate treatment.

In the food area, the main objective was the optimization of a method for the detection of *Campylobacter* in broiler meat samples. In the initial tests to assess the exclusivity and inclusivity of the probe, all target strains and two non-target microorganisms, *H. cinaedi* and *H. pamatensis*, were detected. After Real-time PCR analysis, only *H. cinaedi* continued to present a positive result. In view of these results, it would be advisable to test the strains by different methods, such as sequencing. Obtaining more data is important to study the possibility of modifying either the *Campylobacter* probe or the blocker probe, in order to ensure a specificity level of 100%. For the optimization of PNA-FISH procedure, it was found that the introduction of a new step for processing the samples prior to application of the method is essential for the visualization of bacterial cells. Sample centrifugation (10 000 g) followed by ressuspension in 0.1% Triton X-100 was the treatment that showed the best results in reducing nonspecific fluorescence caused by matrix components, maintaining the desired detection limit of 1 CFU/10 g. In summary, it was concluded that for the detection of *Campylobacter*, samples should be submitted to a one-step enrichment in Bolton medium for 4 hours at 37 °C followed by 44 hours at 41.5 °C, under microaerophilic conditions. After enrichment, 1 ml of each suspension should be centrifuged at 10 000 g for 5 minutes and the

pellet resuspended in 0.1% Triton X-100 solution. Then, the suspension is analysed through PNA-FISH.

Finally, a robustness test required by AOAC International in future product certification was performed. The results showed that the variation of the parameters affect the performance of the method, essentially at the specificity level, since the detection of non-target microorganism was significantly affected, showing the importance of strictly following the conditions when executing the PNA-FISH procedure. A complementary future work would be the optimization of the blocker probe, in order to evaluate if the results obtained in the test had been caused by the loss of blocker probe efficacy. Another strategy would be to perform an additional test, with a different experimental design, for a better understanding of the results obtained in the robustness test.

In conclusion, NAM-FISH technology proves to be a simple and rapid alternative for microbiological detection. In 4 simple and easy to perform steps, the method allows to obtain the results in less than 3 h, after the enrichment. In comparison with other molecular methods, NAM-FISH does not involve DNA extraction, the probes are not susceptible to nucleases and proteases or other inhibitory compounds present in the sample and allows the direct visualization of the bacteria. Nevertheless, both procedures presented in this work are not fully developed, still requiring future work and optimization.

References

1. Alberts, B. *et al.* Introduction to Pathogens. *Molecular Biology of the Cell. 4th edition* (2002).
2. Viggiano, D. *et al.* Gut barrier in health and disease: focus on childhood. *European Review for Medical and Pharmacological Sciences* 1077–1085 (2015).
3. Perez-Perez, G. I. & Blaser, M. J. *Campylobacter* and *Helicobacter*. in *Medical Microbiology* (ed. Baron, S.) (University of Texas Medical Branch at Galveston, 1996).
4. Biomode SA - Biomolecular Determination. Available at: http://biomode-sa.com/. (Accessed: 13th August 2018)
5. Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A. & Uyttendaele, M. Alternative microbial methods: An overview and selection criteria. *Food Microbiology* **27**, 710–730 (2010).
6. Ayrapetyan, M. & Oliver, J. D. The viable but non-culturable state and its relevance in food safety. *Current Opinion in Food Science* **8**, 127–133 (2016).
7. Zhao, X., Lin, C.-W., Wang, J. & Oh, D. H. Advances in Rapid Detection Methods for Foodborne Pathogens. *Journal of Microbiology and Biotechnology* **24**, 297–312 (2014).
8. Dwivedi, H. P. & Jaykus, L.-A. Detection of pathogens in foods: the current state-of-the-art and future directions. *Critical Reviews in Microbiology* **37**, 40–63 (2011).
9. Law, J. W.-F., Ab Mutalib, N.-S., Chan, K.-G. & Lee, L.-H. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in Microbiology* **5**, 770 (2015).
10. Oyarzabal, O. A. & Fernández, H. Chapter 2. Isolation and Identification of *Campylobacter* spp. in Poultry. in *Campylobacter spp. and Related Organisms in Poultry* (eds. Fonseca, B. B., Fernandez, H. & Rossi, D. A.) 19–35 (Springer International Publishing, 2016). doi:10.1007/978-3-319-29907-5_2
11. Priyanka, B., Patil, R. K. & Dwarakanath, S. A review on detection methods used for foodborne pathogens. *Indian J Med Res* **144**, 327–338 (2016).
12. Adams, M. R. & Moss, M. O. *Food microbiology*. (RSC Publishing, 2008).
13. Leonard, P. *et al.* Advances in biosensors for detection of pathogens in food and water. *Enzyme and Microbial Technology* **32**, 3–13 (2003).
14. Alves, J., Hirooka, E. Y. & Oliveira, T. C. R. M. de. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. *LWT - Food Science and Technology* **72**, 175–181 (2016).
15. Hanna, S. E., Connor, C. J. & Wang, H. H. Real-time Polymerase Chain Reaction for the Food Microbiologist: Technologies, Applications, and Limitations. *Journal of Food Science* **70**, R49–R53 (2006).
16. Moter, A. & Göbel, U. B. Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. *Journal of Microbiological Methods* **41**, 85–112 (2000).

17. Frickmann, H. *et al.* Fluorescence *in situ* hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. *Critical Reviews in Microbiology* **43**, 263–293 (2017).
18. Amann, R. I., Krumholz, L. & Stahl, D. A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of bacteriology* **172**, 762–770 (1990).
19. DeLong, E. F., Wickham, G. S. & Pace, N. R. Phylogenetc Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. *Science* **243**, 1360–1363 (1989).
20. Petrich, A. *et al.* Fluorescence *in situ* hybridization for the identification of *Treponema pallidum* in tissue sections. *International Journal of Medical Microbiology* **305**, 709–718 (2015).
21. Wu, Q., Li, Y., Wang, M., Pan, X. P. & Tang, Y. F. Fluorescence *in situ* hybridization rapidly detects three different pathogenic bacteria in urinary tract infection samples. *Journal of Microbiological Methods* **83**, 175–178 (2010).
22. Rohde, A., Hammerl, J. A., Appel, B., Dieckmann, R. & Al Dahouk, S. FISHing for bacteria in food – A promising tool for the reliable detection of pathogenic bacteria? *Food Microbiology* **46**, 395–407 (2015).
23. Silverman, A. P. & Kool, E. T. Oligonucleotide Probes for RNA-Targeted Fluorescence *In Situ* Hybridization. in *Advances in Clinical Chemistry* **43**, 79–115 (Elsevier, 2007).
24. Amann, R. & Fuchs, B. M. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nature Reviews Microbiology* **6**, 339–348 (2008).
25. Coleman, J. R., Culley, D. E., Chrisler, W. B. & Brockman, F. J. mRNA-targeted fluorescent *in situ* hybridization (FISH) of Gram-negative bacteria without template amplification or tyramide signal amplification. *Journal of Microbiological Methods* **71**, 246–255 (2007).
26. Catez, F., Rousseau, A., Labetoulle, M. & Lomonte, P. Detection of the Genome and Transcripts of a Persistent DNA Virus in Neuronal Tissues by Fluorescent *In situ* Hybridization Combined with Immunostaining. *Journal of Visualized Experiments* e51091 (2014). doi:10.3791/51091
27. Horváth, A., Kristóf, K., Konkoly-Thege, M. & Nagy, K. Rapid identification of pathogens in blood culture with fluorescent *in situ* hybridization (FISH). *Acta Microbiologica et Immunologica Hungarica* **57**, 225–234 (2010).
28. Cerqueira, L. *et al.* DNA Mimics for the Rapid Identification of Microorganisms by Fluorescence *in situ* Hybridization (FISH). *International Journal of Molecular Sciences* **9**, 1944–1960 (2008).
29. Kempf, V. A. J., Mändle, T., Schumacher, U., Schäfer, A. & Autenrieth, I. B. Rapid detection and identification of pathogens in blood cultures by fluorescence *in situ* hybridization and flow cytometry. *International Journal of Medical Microbiology* **295**, 47–55 (2005).
30. Kalyuzhnaya, M. G. *et al.* Fluorescence *In Situ* Hybridization-Flow Cytometry-Cell Sorting-Based Method for Separation and Enrichment of Type I and Type II Methanotroph Populations. *Applied and Environmental Microbiology* **72**, 4293–4301 (2006).

31. Almeida, C., Azevedo, N. F., Fernandes, R. M., Keevil, C. W. & Vieira, M. J. Fluorescence *In Situ* Hybridization Method Using a Peptide Nucleic Acid Probe for Identification of *Salmonella* spp. in a Broad Spectrum of Samples. *Applied and Environmental Microbiology* **76**, 4476–4485 (2010).
32. Machado, A., Castro, J., Cereija, T., Almeida, C. & Cerca, N. Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence *in situ* hybridization method. *PeerJ* **3**, e780 (2015).
33. Vester, B. & Wengel, J. LNA (Locked Nucleic Acid): High-Affinity Targeting of Complementary RNA and DNA. *Biochemistry* **43**, 13233–13241 (2004).
34. Braasch, D. A. & Corey, D. R. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chemistry & Biology* **8**, 1–7 (2001).
35. Petersen, M. & Wengel, J. LNA: a versatile tool for therapeutics and genomics. *Trends in Biotechnology* **21**, 74–81 (2003).
36. Silahtaroglu, A., Pfundheller, H., Koshkin, A., Tommerup, N. & Kauppinen, S. LNA-modified oligonucleotides are highly efficient as FISH probes. *Cytogenetic and Genome Research* **107**, 32–37 (2004).
37. Robertson, K. L. & Vora, G. J. Locked Nucleic Acid and Flow Cytometry-Fluorescence *In Situ* Hybridization for the Detection of Bacterial Small Noncoding RNAs. *Applied and Environmental Microbiology* **78**, 14–20 (2012).
38. Fontenete, S. *et al.* Hybridization-Based Detection of *Helicobacter pylori* at Human Body Temperature Using Advanced Locked Nucleic Acid (LNA) Probes. *PLoS ONE* **8**, e81230 (2013).
39. Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. Sequence-Selective Recognition of DNA by Strand Displacement with a Thymine-Substituted Polyamide. *Science* **254**, 1497–1500 (1991).
40. Nielsen, P. E. & Egholm, M. An introduction to peptide nucleic acid. *Curr Issues Mol Biol* **1**, 89–104 (1999).
41. Pellestor, F., Paulasova, P. & Hamamah, S. Peptide nucleic acids (PNAs) as diagnostic devices for genetic and cytogenetic analysis. *Current pharmaceutical design* **14**, 2439–2444 (2008).
42. Egholm, M. *et al.* PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365**, 566–568 (1993).
43. Demidov, V. V. *et al.* Stability of peptide nucleic acids in human serum and cellular extracts. *Biochemical pharmacology* **48**, 1310–1313 (1994).
44. Corradini, R. *et al.* Peptide Nucleic Acids with a Structurally Biased Backbone. Updated Review and Emerging Challenges. *Current Topics in Medicinal Chemistry* **11**, 1535–1554 (2011).
45. Sharma, C. & Awasthi, S. K. Versatility of peptide nucleic acids (PNAs): role in chemical biology, drug discovery, and origins of life. *Chemical Biology & Drug Design* **89**, 16–37 (2017).
46. Shirude, P. S., Kumar, V. A. & Ganesh, K. N. (2S,5R/2R,5S)-Aminoethylpipecolyl aepip-aegPNA chimera: synthesis and duplex/triplex stability. *Tetrahedron* **60**, 9485–9491 (2004).
47. Yazbeck, D. R., Min, K.-L. & Damha, M. J. Molecular requirements for degradation of a modified sense RNA strand by *Escherichia coli* ribonuclease H1. *Nucleic Acids Res* **30**, 3015–3025 (2002).

48. Østergaard, M. E. *et al.* Comparison of Duplex Stabilizing Properties of 2′-Fluorinated Nucleic Acid Analogues with Furanose and Non-Furanose Sugar Rings. *The Journal of Organic Chemistry* **79**, 8877–8881 (2014).
49. Majlessi, M., Nelson, N. C. & Becker, M. M. Advantages of 2′-O-methyl oligoribonucleotide probes for detecting RNA targets. *Nucleic acids research* **26**, 2224–2229 (1998).
50. Azevedo, A. S. *et al.* Detection and discrimination of biofilm populations using locked nucleic acid/2′-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2′OMe-FISH). *Biochemical Engineering Journal* **104**, 64–73 (2015).
51. Tsourkas, A., Behlke, M. A. & Bao, G. Hybridization of 2′-O-methyl and 2′-deoxy molecular beacons to RNA and DNA targets. *Nucleic acids research* **30**, 5168–5174 (2002).
52. Søe, M. J., Møller, T., Dufva, M. & Holmstrøm, K. A sensitive alternative for microRNA *in situ* hybridizations using probes of 2′-O-methyl RNA + LNA. *Journal of Histochemistry & Cytochemistry* **59**, 661–672 (2011).
53. Maciaszek, A. *et al.* LNA units present in the (2′-OMe)-RNA strand stabilize parallel duplexes (2′-OMe)-RNA/[All-R $_{P}$ -PS]-DNA and parallel triplexes (2′-OMe)-RNA/[All-R $_{P}$ -PS]-DNA/RNA. An improved tool for the inhibition of reverse transcription. *Organic & Biomolecular Chemistry* **13**, 2375–2384 (2015).
54. Kierzek, E. The influence of locked nucleic acid residues on the thermodynamic properties of 2'-O-methyl RNA/RNA heteroduplexes. *Nucleic Acids Research* **33**, 5082–5093 (2005).
55. Fontenete, S. *et al.* Mismatch discrimination in fluorescent *in situ* hybridization using different types of nucleic acids. *Applied Microbiology and Biotechnology* **99**, 3961–3969 (2015).
56. Guimaraes, N., Azevedo, N. F., Figueiredo, C., Keevil, C. W. & Vieira, M. J. Development and Application of a Novel Peptide Nucleic Acid Probe for the Specific Detection of *Helicobacter pylori* in Gastric Biopsy Specimens. *Journal of Clinical Microbiology* **45**, 3089–3094 (2007).
57. Fontenete, S. *et al.* Application of locked nucleic acid-based probes in fluorescence *in situ* hybridization. *Applied Microbiology and Biotechnology* **100**, 5897–5906 (2016).
58. Frickmann, H., Masanta, W. O. & Zautner, A. E. Emerging Rapid Resistance Testing Methods for Clinical Microbiology Laboratories and Their Potential Impact on Patient Management. *BioMed Research International* **2014**, 375681 (2014).
59. Marshall, B. & Warren, J. R. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *The Lancet* **323**, 1311–1315 (1984).
60. Varbanova, M., Schulz, C. & Malfertheiner, P. *Helicobacter pylori* and Other Gastric Bacteria. *Digestive Diseases* **29**, 562–569 (2011).
61. Jones, D. M., Curry, A. & Fox, A. J. An Ultrastructural Study of the Gastric *Campylobacter*-like Organism '*Campylobacter pyloridis*'''. *Microbiology* **131**, 2335–2341 (1985).
62. Blanchard, T. G. & Nedrud, J. G. Laboratory Maintenance of *Helicobacter Species*. *Curr Protoc Microbiol* Unit8B.1 (2006). doi:10.1002/9780471729259.mc08b01s00

63. Worku, M. L., Sidebotham, R. L., Walker, M. M., Keshavarz, T. & Karim, Q. N. The relationship between *Helicobacter pylori motility*, morphology and phase of growth: implications for gastric colonization and pathology. *Microbiology* **145**, 2803–2811 (1999).
64. Pokhrel, P. Cultural Characteristics of *Helicobacter pylori*. *Microbiology Notes* (2015). Available at: http://www.microbiologynotes.com/cultural-characteristics-of-helicobacter-pylori/. (Accessed: 9th January 2018)
65. Wroblewski, L. E., Peek, R. M. & Wilson, K. T. *Helicobacter pylori* and Gastric Cancer: Factors That Modulate Disease Risk. *Clinical Microbiology Reviews* **23**, 713–739 (2010).
66. Bastos, J. *et al.* Sociodemographic Determinants of Prevalence and Incidence of *Helicobacter pylori* Infection in Portuguese Adults. *Helicobacter* **18**, 413–422 (2013).
67. Morais, S. *et al.* Trends in gastric cancer mortality and in the prevalence of *Helicobacter pylori* infection in Portugal: *European Journal of Cancer Prevention* **25**, 275–281 (2016).
68. Forman, D. *et al.* Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* **302**, 1302–1305 (1991).
69. Uemura, N. *et al. Helicobacter pylori* infection and the development of gastric cancer. *New England Journal of Medicine* **345**, 784–789 (2001).
70. *IARC monographs on the evaluation of carcinogenic risks to humans, volume 61. Schistosomes, Liver Flakes and Helicobacter pylori*. (World Health Organization, International Agency for Research on Cancer, 1994).
71. *IARC monographs on the evaluation of carcinogenic risks to humans, volume 100 B. Biological agents.* (World Health Organization, International Agency for Research on Cancer, 2012).
72. Thung, I. *et al.* Review article: the global emergence of *Helicobacter pylori* antibiotic resistance. *Alimentary Pharmacology & Therapeutics* **43**, 514–533 (2016).
73. Chey, W. D., Leontiadis, G. I., Howden, C. W. & Moss, S. F. ACG clinical guideline: treatment of *Helicobacter pylori* infection. *The American journal of gastroenterology* **112**, 212–239 (2017).
74. Malfertheiner, P. *et al.* Management of *Helicobacter pylori* infection—the Maastricht V/Florence Consensus Report. *Gut* **66**, 6–30 (2017).
75. Ghotaslou, R. Prevalence of antibiotic resistance in *Helicobacter pylori*: A recent literature review. *World Journal of Methodology* **5**, 164 (2015).
76. Munita, J. M. & Arias, C. A. Mechanisms of Antibiotic Resistance. in *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition* (eds. Kudva, I. T. et al.) 481–511 (American Society of Microbiology, 2016). doi:10.1128/microbiolspec.VMBF-0016-2015
77. Redgrave, L. S., Sutton, S. B., Webber, M. A. & Piddock, L. J. V. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends in Microbiology* **22**, 438–445 (2014).
78. Aldred, K. J., Kerns, R. J. & Osheroff, N. Mechanism of Quinolone Action and Resistance. *Biochemistry* **53**, 1565–1574 (2014).

79. Hooper, D. C. Emerging mechanisms of fluoroquinolone resistance. *Emerging infectious diseases* **7**, 337 (2001).
80. Galdiero, S. *et al.* Microbe-host interactions: structure and role of Gram-negative bacterial porins. *Current Protein and Peptide Science* **13**, 843–854 (2012).
81. Tomb, J.-F., White, O., Kerlavage, A. R., Clayton, R. A. & others. The complete genome sequence of the gastric pathogen *Helicobacter pylori. Nature* **388**, 539–552 (1997).
82. Bina, J. E., Alm, R. A., Uria-Nickelsen, M., Thomas, S. R. & Hancock, R. E. W. *Helicobacter pylori* uptake and efflux: basis for intrinsic susceptibility to antibiotics *in vitro*. *Antimicrobial agents and chemotherapy* **44**, 248–254 (2000).
83. Levine, C., Hiasa, H. & Marians, K. J. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* **1400**, 29–43 (1998).
84. Drlica, K., Malik, M., Kerns, R. J. & Zhao, X. Quinolone-Mediated Bacterial Death. *Antimicrobial Agents and Chemotherapy* **52**, 385–392 (2008).
85. Tankovic, J., Lascols, C., Sculo, Q., Petit, J.-C. & Soussy, C.-J. Single and Double Mutations in gyrA but Not in gyrB Are Associated with Low- and High-Level Fluoroquinolone Resistance in *Helicobacter pylori. Antimicrobial Agents and Chemotherapy* **47**, 3942–3944 (2003).
86. Yoshida, H., Bogaki, M., Nakamura, M. & Nakamura, S. Quinolone resistance-determining region in the DNA gyrase gyrA gene of *Escherichia coli*. *Antimicrobial agents and chemotherapy* **34**, 1271–1272 (1990).
87. Cattoir, V. *et al.* Update on fluoroquinolone resistance in *Helicobacter pylori*: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility. *International Journal of Antimicrobial Agents* **29**, 389–396 (2007).
88. Zerbetto De Palma, G. *et al.* Occurrence of Mutations in the Antimicrobial Target Genes Related to Levofloxacin, Clarithromycin, and Amoxicillin Resistance in *Helicobacter pylori* Isolates from Buenos Aires City. *Microbial Drug Resistance* **23**, 351–358 (2017).
89. Lee, C. C., Lee, V. W. Y., Chan, F. K. L. & Ling, T. K. W. Levofloxacin-Resistant *Helicobacter pylori* in Hong Kong. *Chemotherapy* **54**, 50–53 (2007).
90. Chung, J.-W. *et al.* Resistance of *Helicobacter pylori* strains to antibiotics in Korea with a focus on fluoroquinolone resistance: Antibiotic resistance of *H. pylori*. *Journal of Gastroenterology and Hepatology* **27**, 493–497 (2012).
91. Glocker, E., Stueger, H.-P. & Kist, M. Quinolone Resistance in *Helicobacter pylori* Isolates in Germany. *Antimicrobial Agents and Chemotherapy* **51**, 346–349 (2007).
92. Chisholm, S. A. & Owen, R. J. Frequency and molecular characteristics of ciprofloxacin- and rifampicin-resistant *Helicobacter pylori* from gastric infections in the UK. *Journal of Medical Microbiology* **58**, 1322–1328 (2009).

93. Miyachi, H. *et al.* Primary levofloxacin resistance and gyrA/B mutations among *Helicobacter pylori* in Japan. *Helicobacter* **11**, 243–249 (2006).
94. Bogaerts, P., Berhin, C., Nizet, H. & Glupczynski, Y. Prevalence and mechanisms of resistance to fluoroquinolones in *Helicobacter pylori* strains from patients living in Belgium. *Helicobacter* **11**, 441–445 (2006).
95. Ontsira Ngoyi, E. N. *et al.* Molecular Detection of *Helicobacter pylori* and its Antimicrobial Resistance in Brazzaville, Congo. *Helicobacter* **20**, 316–320 (2015).
96. Garcia, M., Raymond, J., Garnier, M., Cremniter, J. & Burucoa, C. Distribution of Spontaneous gyrA Mutations in 97 Fluoroquinolone-Resistant *Helicobacter pylori* Isolates Collected in France. *Antimicrobial Agents and Chemotherapy* **56**, 550–551 (2012).
97. Nishizawa, T. *et al.* Gatifloxacin Resistance and Mutations in gyrA after Unsuccessful *Helicobacter pylori* Eradication in Japan. *Antimicrobial Agents and Chemotherapy* **50**, 1538–1540 (2006).
98. Wang, L.-H. Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains. *World Journal of Gastroenterology* **16**, 2272–2277 (2010).
99. Navarro-Jarabo, J. M. *et al.* Prevalence of Primary Resistance of *Helicobacter pylori* to Clarithromycin and Levofloxacin in Southern Spain. *Digestion* **92**, 78–82 (2015).
100. Moore, R. A., Beckthold, B., Wong, S., Kureishi, A. & Bryan, L. E. Nucleotide sequence of the gyrA gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. *Antimicrobial agents and chemotherapy* **39**, 107–111 (1995).
101. Mustaev, A. *et al.* Fluoroquinolone-Gyrase-DNA Complexes: Two modes of drug binding. *Journal of Biological Chemistry* **289**, 12300–12312 (2014).
102. Miftahussurur, M. *et al.* Surveillance of *Helicobacter pylori* Antibiotic Susceptibility in Indonesia: Different Resistance Types among Regions and with Novel Genetic Mutations. *PLOS ONE* **11**, e0166199 (2016).
103. Rimbara, E., Noguchi, N., Kawai, T. & Sasatsu, M. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of GyrA on the level of resistance and identification of a resistance conferring mutation in GyrB. *Helicobacter* **17**, 36–42 (2012).
104. Woodford, N. & Sundsfjord, A. Molecular detection of antibiotic resistance: when and where? *Journal of Antimicrobial Chemotherapy* **56**, 259–261 (2005).
105. Galar, A. *et al.* Clinical and economic impact of rapid reporting of bacterial identification and antimicrobial susceptibility results of the most frequently processed specimen types. *European Journal of Clinical Microbiology & Infectious Diseases* **31**, 2445–2452 (2012).
106. Megraud, F. & Lehours, P. *Helicobacter pylori* Detection and Antimicrobial Susceptibility Testing. *Clinical Microbiology Reviews* **20**, 280–322 (2007).
107. van Belkum, A. *et al.* Rapid Clinical Bacteriology and Its Future Impact. *Annals of Laboratory Medicine* **33**, 14–27 (2013).

108. Fournier, P.-E. *et al.* Modern clinical microbiology: new challenges and solutions. *Nature Reviews Microbiology* **11**, 574–585 (2013).
109. Balouiri, M., Sadiki, M. & Ibnsouda, S. K. Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **6**, 71–79 (2016).
110. Jorgensen, J. H. & Ferraro, M. J. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases* **49**, 1749–1755 (2009).
111. Huynh, H. Q. *et al.* N-acetylcysteine, a novel treatment for *Helicobacter pylori* infection. *Digestive diseases and sciences* **49**, 1853–1861 (2004).
112. Doern, G. V. Antimicrobial Susceptibility Testing. *Journal of Clinical Microbiology* **49**, S4 (2011).
113. Redondo, J. J., Keller, P. M., Zbinden, R. & Wagner, K. A novel RT-PCR for the detection of *Helicobacter pylori* and identification of clarithromycin resistance mediated by mutations in the 23S rRNA gene. *Diagnostic Microbiology and Infectious Disease* **90**, 1–6 (2018).
114. Azevedo, N. F. *et al.* Coccoid Form of *Helicobacter pylori* as a Morphological Manifestation of Cell Adaptation to the Environment. *Applied and Environmental Microbiology* **73**, 3423–3427 (2007).
115. Ramamurthy, T., Ghosh, A., Pazhani, G. P. & Shinoda, S. Current Perspectives on Viable but Non-Culturable (VBNC) Pathogenic Bacteria. *Frontiers in Public Health* **2**, 103 (2014).
116. Trespalacios, A. A., Rimbara, E., Otero, W., Reddy, R. & Graham, D. Y. Improved allele-specific PCR assays for detection of clarithromycin and fluoroquinolone resistant of *Helicobacter pylori* in gastric biopsies: identification of N87I mutation in GyrA. *Diagnostic Microbiology and Infectious Disease* **81**, 251–255 (2015).
117. Cambau, E. *et al.* Evaluation of a New Test, GenoType HelicoDR, for Molecular Detection of Antibiotic Resistance in *Helicobacter pylori*. *Journal of Clinical Microbiology* **47**, 3600–3607 (2009).
118. Russmann, H. *et al.* Rapid and Accurate Determination of Genotypic Clarithromycin Resistance in Cultured *Helicobacter pylori* by Fluorescent *In Situ* Hybridization. *Journal of Clinical Microbiology* **39**, 4142–4144 (2001).
119. Trebesius, K. *et al.* Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent *in situ* hybridisation. *Gut* **46**, 608–614 (2000).
120. Russmann, H., Kempf, V. A. J., Koletzko, S., Heesemann, J. & Autenrieth, I. B. Comparison of Fluorescent *In Situ* Hybridization and Conventional Culturing for Detection of *Helicobacter pylori* in Gastric Biopsy Specimens. *Journal of Clinical Microbiology* **39**, 304–308 (2001).
121. Cerqueira, L. *et al.* PNA-FISH as a new diagnostic method for the determination of clarithromycin resistance of *Helicobacter pylori*. *BMC microbiology* **11**, 101 (2011).
122. Cerqueira, L. *et al.* Validation of a Fluorescence *In Situ* Hybridization Method Using Peptide Nucleic Acid Probes for Detection of *Helicobacter pylori* Clarithromycin Resistance in Gastric Biopsy Specimens. *Journal of Clinical Microbiology* **51**, 1887–1893 (2013).

123. Food-borne zoonotic diseases. *European Food Safety Authority* Available at: https://www.efsa.europa.eu/en/topics/topic/food-borne-zoonotic-diseases. (Accessed: 27th May 2018)
124. World Health Organization & Foodborne Disease Burden Epidemiology Reference Group. *WHO estimates of the global burden of foodborne diseases*. (2015).
125. Food safety. *World Health Organization* Available at: http://www.who.int/news-room/facts-in-pictures/detail/food-safety. (Accessed: 27th May 2018)
126. Silva, J. *et al. Campylobacter* spp. as a Foodborne Pathogen: A Review. *Frontiers in Microbiology* **2**, 200 (2011).
127. Park, S. F. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *International Journal of Food Microbiology* **74**, 177–188 (2002).
128. Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M. & Man, S. M. Global Epidemiology of *Campylobacter* Infection. *Clinical Microbiology Reviews* **28**, 687–720 (2015).
129. Facciolà, A. *et al. Campylobacter*: from microbiology to prevention. **58**, 79–92 (2017).
130. *The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016*. (European Food Safety Authority, European Centre for Disease Prevention and Control, 2017).
131. Fitzgerald, C. *Campylobacter*. *Clinics in Laboratory Medicine* **35**, 289–298 (2015).
132. *Campylobacter*. Available at: http://www.bacterio.net/campylobacter.html.
133. Bolton, D. J. *Campylobacter* virulence and survival factors. *Food Microbiology* **48**, 99–108 (2015).
134. Galate, D. L. & Bangde, S. *Campylobacter* – A Foodborne Pathogen. *International Journal of Science and Research (IJSR)* **4**, 1250–1259 (2013).
135. Lastovica, A. J., On, S. L. W. & Zhang, L. The Family Campylobacteraceae. in *The Prokaryotes* (eds. Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E. & Thompson, F.) 307–335 (Springer Berlin Heidelberg, 2014). doi:10.1007/978-3-642-39044-9_274
136. Esson, D. *et al.* Identification and initial characterisation of a protein involved in *Campylobacter jejuni* cell shape. *Microb Pathog* **104**, 202–211 (2017).
137. Pitkanen, T. & Hanninen, M.-L. Members of the family Campylobacteraceae: *Campylobacter jejuni, Campylobacter coli*. *Global Water Pathogens Project* 39
138. Man, S. M. The clinical importance of emerging *Campylobacter* species. *Nature Reviews Gastroenterology & Hepatology* **8**, 669–685 (2011).
139. Epps, S. *et al.* Foodborne *Campylobacter*: Infections, Metabolism, Pathogenesis and Reservoirs. *International Journal of Environmental Research and Public Health* **10**, 6292–6304 (2013).
140. Levin, R. E. *Campylobacter jejuni* : A Review of its Characteristics, Pathogenicity, Ecology, Distribution, Subspecies Characterization and Molecular Methods of Detection. *Food Biotechnology* **21**, 271–347 (2007).

141. Humphrey, T., O'Brien, S. & Madsen, M. Campylobacters as zoonotic pathogens: A food production perspective. *International Journal of Food Microbiology* **117**, 237–257 (2007).
142. Bull, S. A. *et al.* Sources of *Campylobacter* spp. Colonizing Housed Broiler Flocks during Rearing. *Applied and Environmental Microbiology* **72**, 645–652 (2006).
143. Young, K. T., Davis, L. M. & DiRita, V. J. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nature Reviews Microbiology* **5**, 665–679 (2007).
144. López-Campos, G., Martínez-Suárez, J. V., Aguado-Urda, M. & López-Alonso, V. Detection, Identification, and Analysis of Foodborne Pathogens. in *Microarray Detection and Characterization of Bacterial Foodborne Pathogens* 13–32 (Springer US, 2012). doi:10.1007/978-1-4614-3250-0_2
145. Mandal, P. K., Biswas, A. K., Choi, K. & Pal, U. K. Methods for Rapid Detection of Foodborne Pathogens: An Overview. *American Journal of Food Technology* **6**, 87–102 (2011).
146. *Molecular microbial diagnostic methods: pathways to implementation for the food and water industries*. (Elsevier/Academic Press, 2016).
147. Donnison, A. *Isolation of Thermotolerant Campylobacter – Review & Methods for New Zealand Laboratories. Client Report*. (2003).
148. Bolton, F. J. & Robertson, L. A selective medium for isolating *Campylobacter jejuni/coli*. *Journal of Clinical Pathology* **35**, 462–467 (1982).
149. Perry, J. D. & Freydière, A. M. The application of chromogenic media in clinical microbiology: Application of chromogenic media. *Journal of Applied Microbiology* **103**, 2046–2055 (2007).
150. CHROMagar Chromogenic Media. Available at: http://www.chromagar.com/. (Accessed: 10th August 2018)
151. International Organization for Standardization. Available at: https://www.iso.org/about-us.html. (Accessed: 28th July 2018)
152. Moore, J. E. *et al. Campylobacter*. *Veterinary Research* **36**, 351–382 (2005).
153. Poppert, S. *et al.* Identification of Thermotolerant *Campylobacter* Species by Fluorescence *In Situ* Hybridization. *Journal of Clinical Microbiology* **46**, 2133–2136 (2008).
154. Moreno, Y. *et al.* Specific Detection of *Arcobacter* and *Campylobacter* Strains in Water and Sewage by PCR and Fluorescent *In Situ* Hybridization. *Applied and Environmental Microbiology* **69**, 1181–1186 (2003).
155. Moreno, Y. *et al.* Direct detection of thermotolerant campylobacters in chicken products by PCR and *in situ* hybridization. *Research in Microbiology* **152**, 577–582 (2001).
156. Schmid, M., Lehner, A., Stephan, R., Schleifer, K. & Meier, H. Development and application of oligonucleotide probes for *in situ* detection of thermotolerant *Campylobacter* in chicken faecal and liver samples. *International Journal of Food Microbiology* **105**, 245–255 (2005).
157. Lehtola, M. J., Loades, C. J. & Keevil, C. W. Advantages of peptide nucleic acid oligonucleotides for sensitive site directed 16S rRNA fluorescence *in situ* hybridization (FISH) detection of

Campylobacter jejuni, *Campylobacter coli* and *Campylobacter lari*. *Journal of Microbiological Methods* **62**, 211–219 (2005).

158. Rohde, A., Hammerl, J. A. & Al Dahouk, S. Detection of foodborne bacterial zoonoses by fluorescence *in situ* hybridization. *Food Control* **69**, 297–305 (2016).
159. You, Y. Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Research* **34**, e60 (2006).
160. Fernandes, R. M. *et al.* Morphological transition of *Helicobacter pylori* adapted to water. *Future Microbiology* **12**, 1167–1179 (2017).
161. Almeida, C. *et al.* Development and Application of a Novel Peptide Nucleic Acid Probe for the Specific Detection of *Cronobacter Genomospecies (Enterobacter sakazakii)* in Powdered Infant Formula. *Applied and Environmental Microbiology* **75**, 2925–2930 (2009).
162. Almeida, C. *et al.* Detection of *Escherichia coli* O157 by Peptide Nucleic Acid Fluorescence *In Situ* Hybridization (PNA-FISH) and Comparison to a Standard Culture Method. *Applied and Environmental Microbiology* **79**, 6293–6300 (2013).
163. Stevens, K. A. & Jaykus, L.-A. Bacterial Separation and Concentration from Complex Sample Matrices: A Review. *Critical Reviews in Microbiology* **30**, 7–24 (2004).
164. AOAC International. (2018). Available at: https://www.aoac.org. (Accessed: 3rd August 2018)
165. Pernthaler, A. & Amann, R. Simultaneous Fluorescence *In Situ* Hybridization of mRNA and rRNA in Environmental Bacteria. *Appl. Environ. Microbiol.* **70**, 5426–5433 (2004).
166. Draper, J. L. *et al.* Fallacy of the Unique Genome: Sequence Diversity within Single *Helicobacter pylori* Strains. *mBio* **8**, e02321-16 (2017).
167. Suerbaum, S. & Josenhans, C. *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nature Reviews Microbiology* **5**, 441–452 (2007).
168. SantaLucia, J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proceedings of the National Academy of Sciences* **95**, 1460–1465 (1998).
169. SantaLucia, J. & Hicks, D. The Thermodynamics of DNA Structural Motifs. *Annual Review of Biophysics and Biomolecular Structure* **33**, 415–440 (2004).
170. Owczarzy, R., You, Y., Groth, C. L. & Tataurov, A. V. Stability and Mismatch Discrimination of Locked Nucleic Acid–DNA Duplexes. *Biochemistry* **50**, 9352–9367 (2011).
171. Valm, A. M., Welch, J. L. M. & Borisy, G. G. CLASI-FISH: Principles of Combinatorial Labeling and Spectral Imaging. *Syst Appl Microbiol* **35**, 496–502 (2012).
172. SantaLucia, J., Allawi, H. T. & Seneviratne, P. A. Improved Nearest-Neighbor Parameters for Predicting DNA Duplex Stability. *Biochemistry* **35**, 3555–3562 (1996).
173. Vilas Boas, D. *et al.* Discrimination of bacteriophage infected cells using locked nucleic acid fluorescent *in situ* hybridization (LNA-FISH). *Biofouling* **32**, 179–190 (2016).

174. Fontenete, S., Guimarães, N., Wengel, J. & Azevedo, N. F. Prediction of melting temperatures in fluorescence *in situ* hybridization (FISH) procedures using thermodynamic models. *Critical Reviews in Biotechnology* **36**, 566–577 (2015).
175. Yilmaz, L. Ş., Ökten, H. E. & Noguera, D. R. Making All Parts of the 16S rRNA of *Escherichia coli* Accessible *In Situ* to Single DNA Oligonucleotides. *Appl Environ Microbiol* **72**, 733–744 (2006).
176. LNA™ for RNA research - Exiqon. Available at: http://www.exiqon.com/. (Accessed: 7th August 2018)
177. Chang, W.-L. *et al.* Gemifloxacin can partially overcome quinolone resistance of *H. pylori* with gyrA mutation in Taiwan. *Helicobacter* **17**, 210–215 (2012).
178. AOAC International. Appendix J : AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces. Official Methods of Analysis of AOAC International. 1–21 (2012).
179. Mahon, C. R., Lehman, D. C. & Manuselis, G. *Textbook of Diagnostic Microbiology - E-Book*. (Elsevier Health Sciences, 2014).
180. Rimbara, E. *et al. Helicobacter cinaedi* and *Helicobacter fennelliae* Transmission in a Hospital from 2008 to 2012. *J. Clin. Microbiol.* **51**, 2439–2442 (2013).
181. Hamada, M. *et al. Helicobacter pylori* in a poultry slaughterhouse: Prevalence, genotyping and antibiotic resistance pattern. *Saudi Journal of Biological Sciences* **25**, 1072–1078 (2018).
182. Bateman, A. C. & Butler-Wu, S. M. The Brief Case: Bacteremia Caused by *Helicobacter cinaedi*. *J Clin Microbiol* **55**, 5–9 (2017).
183. Frederick, J. L., Walker, S. P., Crofcheck, C. L., Newman, M. & Payne, F. Evaluation of Chemical Additives for the Separation and Recovery of Bacteria from Food Matrices. *Biological Engineering Transactions* **6**, 105–115 (2013).
184. Stevens, K. A. & Jaykus, L.-A. Bacterial Separation and Concentration from Complex Sample Matrices: A Review. *Critical Reviews in Microbiology* **30**, 7–24 (2004).
185. Rodrigues-Szulc, U. M., Ventoura, G., Mackey, B. M. & Payne, M. J. Rapid physicochemical detachment, separation and concentration of bacteria from beef surfaces. *Journal of Applied Bacteriology* **80**, 673–681 (1996).
186. Buzatu, D. A. *et al.* An Integrated Flow Cytometry-Based System for Real-Time, High Sensitivity Bacterial Detection and Identification. *PLoS One* **9**, e94254 (2014).
187. Fachmann, M. S. R. *et al*. Detection of *Salmonella enterica* in Meat in Less than 5 Hours by a Low-Cost and Noncomplex Sample Preparation Method. *Appl. Environ. Microbiol.* **83**, e03151-16 (2017).
188. Entis, P. *Food Microbiology: The Laboratory*. (Phyllis Entis, 2002).
189. Held, P. Sample Preparation for Fluorescence Microscopy: An Introduction - Concepts and Tips for Better Fixed Sample Imaging Results. (2015). Available at:

https://www.biotek.com/resources/white-papers/sample-preparation-for-fluorescence-microscopy-an-introduction-concepts-and-tips-for-better-fixed-sample-imaging-results/. (Accessed: 6th August 2018)

190. Meylheuc, T., Giovannacci, I., Briandet, R. & Bellon-Fontaine, M.-N. Comparison of the cell surface properties and growth characteristics of *Listeria monocytogenes* and *Listeria innocua*. *J. Food Prot.* **65**, 786–793 (2002).
191. Yu, L. S. ., Uknalis, J. & Tu, S.-I. Immunomagnetic separation methods for the isolation of *Campylobacter jejuni* from ground poultry meats. *Journal of Immunological Methods* **256**, 11–18 (2001).
192. Bolton, F. J., Sails, A. D., Fox, A. J., Wareing, D. R. A. & Greenway, D. L. A. Detection of *Campylobacter jejuni* and *Campylobacter coli* in Foods by Enrichment Culture and Polymerase Chain Reaction Enzyme-Linked Immunosorbent Assay. *Journal of Food Protection* **65**, 760–767 (2002).
193. Eyi, A. & Arslan, S. Prevalence of *Escherichia coli* in retail poultry meat, ground beef and beef. *Med. Weter.* **68**, 237–240 (2012).
194. Noll, L. Hybridization Conditions and Melting Temperature. *Lablogatory* (2016). Available at: https://labmedicineblog.com/2016/06/06/hybridization-conditions-and-melting-temperature/. (Accessed: 21st July 2018)

Annexes

Annex I - POD analysis for the robustness test (AOAC International)

Calculation of POD and dPOD Values from Qualitative Method Single Laboratory Data

In general, four different probabilities detected (PODs) are to be calculated: POD_R (for the reference method), POD_C (for the confirmed candidate method), POD_{CP} (for the candidate presumptive method), and POD_{CC} (for the candidate confirmation method).

For each of these four cases, calculate the POD as the ratio of the number positive (x) to total number tested (N):

$$POD = \frac{x}{N}$$

where POD is POD_C, POD_R, etc.

The POD estimates and 95% confidence interval (LCL, UCL) estimates are given by:

(*1*) *For the case where x = 0.*

$$POD = 0$$

$$LCL = 0$$

$$UCL = 3.8415/(N + 3.8415)$$

(*2*) *For the case where x = N.*

$$POD = 1$$

$$LCL = N/(N + 3.8415)$$

$$UCL = 1$$

(*3*) *For the case where 0 < x < N.*

$$POD = x/N$$

$$LCL = \frac{x + 1.9207 - 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

$$UCL = \frac{x + 1.9207 + 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

dPOD for Paired Studies

If the replicates tested by the candidate and reference methods are paired (i.e., the enrichment conditions are the same, thus common test portions are analyzed by both methods), the associated 95% confidence interval (LCL, UCL) for the expected value of dPOD = $POD_1 - POD_2$ is estimated by the following:

Let

$$d_i = x_{1i} - x_{2i}$$

denote the numerical difference of the two method results on test portion i. Note that d_i must take on only the values –1, 0, or +1.

The recommended method for estimating dPOD is the mean of differences d_i:

$$d\text{POD} = \frac{\sum_{i=1}^{N} d_i}{N}$$

where N is the number of test portions.

The recommended approximate 95% confidence interval is the usual Student-*t* based interval, with the standard error of dPOD computed in the usual manner from the replicate differences:

$$s_d = \sqrt{\frac{\sum_{i=1}^{N} (d_i - d\text{POD})^2}{N-1}}$$

$$SE_{dPOD} = \frac{s_d}{\sqrt{N}}$$

and

$$LCL = d\text{POD} - t_\varepsilon \cdot SE_{dPOD}$$

$$UCL = d\text{POD} + t_\varepsilon \cdot SE_{dPOD}$$

where t_ε is the 97.5% quantile of the Student-*t* distribution for N-1 degrees of freedom, and the 95% confidence interval is (LCL, UCL).

Figure 19 | Equations used to calculate the POD and dPOD values, and respective 95% confidence intervals, according to the AOAC guidelines. From AOAC International (2012)[178]