Universidade do Minho
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Ricardo Bruno da Costa Oliveira

Design and validation of an alternative molecular method using peptide nucleic acid fluorescence in situ hybridization (PNA FISH) for the detection of Campylobacter spp. in food samples

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Master's thesis

Master in Biotechnology

Work carried out under the direction of

Doctor Carina Almeida

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ABSTRACT

Design and validation of an alternative molecular method using peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) for the detection of *Campylobacter* spp. in food samples

Foodborne diseases are an important cause of morbidity and mortality, and a significant impediment to socioeconomic development worldwide. Among the foodborne bacterial pathogens, *Campylobacter* is recognized as the leading cause of foodborne illness. Several methods, from traditional culture to advanced molecular techniques, are currently used to prevent contaminated food from reaching consumers. Peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) is a new molecular technique with some application to food safety; but no method has been described for *Campylobacter* detection in foods. PNA FISH is a simple, fast and highly sensitive detection technique that uses fluorescence-labelled probes targeting specific regions of ribosomal RNA. Although the high reliability of this technique, a pre-enrichment procedure of food samples is still necessary to increase target pathogen concentration to detectable levels. In this way, the work presented in this thesis had two main objectives: to optimize an enrichment step for the detection of *Campylobacter* in food samples by PNA FISH; and then to evaluate the developed method according to the requirements necessary to obtain AOAC *Performance Tested Method* SM (PTM) certification. For this purpose, fresh raw broiler meat and fresh raw ground pork samples artificially contaminated with *Campylobacter jejuni* and *Campylobacter coli*, respectively, were analysed using different enrichment broths and different incubation times. After selecting the most suitable enrichment step for application in the PNA FISH methodology, the various tests required by the AOAC Research Institute for PTM certification were performed.

Initially, *Campylobacter* species were found to be severely affected by storage at low temperatures and, therefore, the reduction of cultivability in these conditions was estimated to adjust the pathogen concentration. Then, 48-hour enrichment in Bolton broth resulted in a higher number of positive samples and proved to be essential to achieve a detection limit of 1 CFU/25 g for both food matrices. In order to reduce the autofluorescence conferred by some compounds of food matrices, a new step involving centrifugation (10,000 g) and resuspension in 0.1% Tween-80, was introduced before the standard PNA FISH procedure. In the certification tests, the inclusivity and exclusivity assay revealed a sensitivity of 92.0% and a specificity of 96.9% for the developed method. In the food matrix comparison test, the PNA FISH method showed a similar performance to the ISO 10272-1:2017 reference method. The tests of product consistency and stability, and kit variation have also showed the reliability of the method. On the other hand, the ruggedness test has shown that PNA FISH conditions should be controlled very strictly as small variations can affected significantly the performance of the method.

The results presented in this work are, therefore, a confirmation that the developed PNA FISH method for the detection of *Campylobacter* spp. in food samples is suitable for PTM certification.

Keywords: Foodborne illnesses, *Campylobacter*, PNA FISH, Pre-enrichment, AOAC International

RESUMO

Design e validação de um método molecular alternativo usando hibridização *in situ* fluorescente de ácido nucleico peptídico (PNA FISH) para a deteção de *Campylobacter* spp. em amostras alimentares

As doenças transmitidas por alimentos são uma causa importante de morbidade e mortalidade, e um impedimento significativo para o desenvolvimento socioeconómico em todo o mundo. Entre os patógenos bacterianos transmitidos por alimentos, *Campylobacter* é reconhecida como a principal causa de doenças transmitidas por alimentos. Vários métodos, desde de técnicas de cultura tradicional até técnicas moleculares avançadas, são utilizados atualmente para evitar que alimentos contaminados cheguem aos consumidores. A hibridização *in situ* fluorescente de ácido nucleico peptídico (PNA FISH) é uma nova técnica molecular com alguma aplicação na segurança alimentar; mas nenhum método foi descrito para a deteção de *Campylobacter* em alimentos. PNA FISH é uma técnica de deteção simples, rápida e altamente sensível que usa sondas marcadas com fluorescência visando regiões específicas de RNA ribossómico. Apesar da alta credibilidade desta técnica, um procedimento de pré-enriquecimento das amostras alimentares ainda é necessário para aumentar a concentração de patógeno alvo até níveis detetáveis. Desta forma, o trabalho apresentado nesta tese teve dois objetivos principais: otimizar um passo de enriquecimento para a deteção de *Campylobacter* em amostras alimentares por PNA FISH; e depois avaliar o método desenvolvido de acordo com os requisitos necessários para obter a certificação AOAC *Performance Tested Method*℠ (PTM). Para o efeito, foram analisadas amostras frescas de carne de frango cru e carne de porco cru moída artificialmente contaminadas com *Campylobacter jejuni* e *Campylobacter coli*, respetivamente, utilizando diferentes meios de enriquecimento e diferentes tempos de incubação. Depois de selecionar o passo de enriquecimento mais adequado para aplicação na metodologia PNA FISH, foram realizados os vários testes exigidos pelo Instituto de Pesquisa AOAC para certificação PTM.

Inicialmente, verificou-se que as espécies de *Campylobacter* são severamente afetadas pelo armazenamento a baixas temperaturas e, portanto, a redução da cultivabilidade nessas condições foi estimada para ajustar a concentração de patógenos. Em seguida, o enriquecimento de 48 horas no caldo de Bolton resultou num maior número de amostras positivas e revelou-se essencial para atingir um limite de deteção de 1 CFU/25 g para ambas as matrizes alimentares. A fim de reduzir a autofluorescência conferida por alguns compostos das matrizes alimentares, foi introduzido um novo passo envolvendo uma centrifugação (10 000 g) e ressuspensão em 0.1% de Tween-80 antes do procedimento padrão PNA FISH. Nos testes de certificação, o ensaio de inclusividade e exclusividade revelou uma sensibilidade de 92.0% e uma especificidade de 96.9% para o método desenvolvido. No teste de comparação da matriz de alimentos, o método PNA FISH mostrou desempenho semelhante ao método de referência ISO 10272-1: 2017. Os testes de consistência e estabilidade do produto e variação do kit também mostraram a credibilidade do método. Por outro lado, o teste de robustez mostrou que as condições de PNA FISH devem ser controladas de forma muito estrita, pois pequenas variações podem afetar significativamente o desempenho do método.

Os resultados apresentados neste trabalho são, portanto, uma confirmação de que o método PNA FISH desenvolvido para a deteção de *Campylobacter* spp. em amostras alimentares é adequado para a certificação PTM.

Palavras-chave: Doenças transmitidas por alimentos, *Campylobacter*, PNA FISH, Pré-enriquecimento, AOAC International

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GLOSSARY OF ABBREVIATIONS

BB Bolton broth

cAMP Cyclic adenosine monophosphate

CBA Columbia blood agar

CDC Center for Disease Control and Prevention

CDT Cytolethal distending toxin

CFU Colony forming units

CJT *Campylobacter jejuni* toxin

CISH Chromogenic *in situ* hybridization

DNA Deoxyribonucleic acid

ECDC European Centre for Disease Prevention and Control

EDTA Ethylenediaminetetraacetic acid

EFSA European Food Safety Authority

ELISA Enzyme-linked immunosorbent assay

ESBL Extended spectrum beta-lactamase

EU European Union

FAO Food and Agriculture Organization

FISH Fluorescence *in situ* hybridization

GBS Guillain-Barré syndrome

ISH *in situ* hybridization

ISO International Organization for Standardisation

LPS Lipopolysaccharide

mCCDA modified Charcoal Cefoperazone Deoxycholate agar

MS Member states

MPN Most Probable Number

OD Optical density

PB Preston broth

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PNA Peptide nucleic acid

POD Probability of Detection

RNA Ribonucleic acid

rRNA Ribosomal RNA

TSA Tryptic soy agar

UK United Kingdom

US United States (of America)

USDA FSIS US Department of Agriculture, Food Safety Inspection Service

US FDA US, Food and Drug Administration

VBNC Viable but non-culturable cells

WHO World Health Organization

SECOND PRIZE IN A SCIENTIFIC POSTERS COMPETITION

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R. Oliveira, R. Rocha, M. Sousa, L. Cerqueira, Nuno F. Azevedo, C. Almeida. Development and validation of a peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) method for the detection of *Campylobacter* spp. in food samples.

CHAPTER I

INTRODUCTION

1.1. Outline of the dissertation

This dissertation is divided into 6 chapters. This first chapter consists of a brief contextualization of the topic addressed in this dissertation and defines the initial objectives proposed with this work.

In the second chapter, it is presented the state of the art regarding foodborne diseases as well as a review of the genus *Campylobacter*. Several aspects related to epidemiological data, transmission routes and detection methods for *Campylobacter* spp. are also presented.

The third chapter and fourth chapter addresses the main objectives proposed for this thesis. In this way, the main enrichment media used for the recovery and growth of *Campylobacter* in food samples, as well as the several tests that must be performed for an analytical method to fulfil the AOAC requirements and to obtain the corresponding certification are addressed. Then, the material and methods used to achieve both objective are presented, as well as the results obtained and a discussion of the main findings.

The fifth chapter contains the conclusions of all the results obtained, making an analysis of the selected pre-enrichment and the performance of the *Campylobacter* PNA FISH method in the validation tests. In addition, some proposals for future work are presented.

1.2. Context

Foodborne illnesses are a serious public health problem and a significant impediment to socio-economic development worldwide. For many years, the burden of foodborne diseases on public health and the economy has been underestimated due to underreporting and the difficulty of establishing causal relationships between food contamination and resulting disease or death. The World Health Organization (WHO) estimates that approximately 600 million foodborne illnesses and 420 000 deaths are reported every year, approximately 30 % among children under 5 years of age. Typically, they are infectious or toxic in nature and caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food or water. More than 250 different foodborne diseases have been described, while Norovirus, *Salmonella* spp. and *Campylobacter* spp. are among the most common foodborne pathogens.

As the world's population grows, the intensification and industrialization of agriculture and animal production to meet increasing demand for food created numerous challenges for food safety. Beyond the consequences to public health, the potential impact of a single event of a

foodborne disease on a food- related company can be devastating. Therefore, to protect consumers and food businesses, several integrated approaches to food safety from the farm to the fork have been adopted. However, the food industry is very complex and the contamination can occur at any point along the production chain - during production, processing, distribution, or preparation - which makes pathogen control a difficult task even with stringent safety programs. In this way, the key to the prevention of foodborne diseases appears to be the rapid identification of contaminated food in order to prevent them from reaching the final consumer. While traditional cultural methods are still the gold standard method, meeting high food safety requirements, they continue to be labour-intensive and time-consuming. Thus, with increased world trade and the need for rapid food transport, there is a growing need for rapid methods for the detection of contaminated food. Meanwhile, safe handling of raw meat and other raw food ingredients, thorough cooking and good kitchen hygiene can prevent or reduce the risk posed by contaminated food.

1.3. Objectives

Biomode® is an innovative biotechnology company whose core activities are focused on the research, development and commercialization of rapid diagnostic kits based on PNA FISH (peptide nucleic acid fluorescence *in situ* hybridization) technology for microbial detection in food and clinical samples. Up to now, Biomode® developed a wide range of FISH procedures with PNA probes aiming the health sector and, principally, the food safety market.

However, the company does not have a fully developed and optimized procedure for the detection of the microorganism currently responsible for the largest number of foodborne diseases worldwide – *Campylobacter* spp. Given the context described above, the main aim of this work was the development and validation of a complete PNA FISH method to detect *Campylobacter* spp. in food samples. Thus, the work carried out focused on the methodology development, aiming its applicability in real food samples and the certification with a recognized international entity. The objectives initially proposed were:

1 – To select and optimize an enrichment step that allows the detection of *Campylobacter* spp. in real food samples by the PNA FISH procedure;

2 – To evaluate the technical characteristics of the developed method (specificity, sensitivity, robustness, repeatability, etc.) according to the AOAC International requirements;

3 – To analyse and compile performance data in a final report.

CHAPTER II

STATE OF THE ART

2.1. Foodborne pathogens and illnesses

Foodborne illnesses have been a major issue for all societies since the beginning of humanity. Although the types and consequences of these illnesses have changed through the ages, they continue to be a growing public health problem worldwide and, consequently, a major concern of the international community (Capocefalo, Ridley, & Tranfield, 2016). Several factors such as: globalization that has led to the rapid and widespread international distribution of foods; the increase of immunosuppressed populations more susceptible to severe outcomes from foodborne diseases; microbial genomic diversification and emergence of new pathogens (Nyachuba, 2010); or, more recently, the migrant and refugees crisis that may expose communities to unfamiliar foodborne hazards in new environments (WHO, 2007); require the continuous adaption to a changing environment with improved methods to combat these threats.

Besides its obvious implication on human health, the potential impact of foodborne diseases outbreaks on a food business can be devastating and trigger a social alarm that could be very hard to control, resulting in unimaginable economic and reputation losses to the company (Hussain & Dawson, 2013). Although economic analysis of food safety-related costs showed that it is much cheaper for a producer to invest in preventing events of foodborne outbreaks than to deal with an event (Ribera et al., 2012); the huge pressure on the food companies to be competitive at a global level might sometimes result in a relaxed attitude toward food safety control.

For these reasons, several separate global agencies such as the World Health Organization and Food and Agriculture Organization (FAO), as well as regional agencies such as the European Food Safety Authority (EFSA), the European Centre for Disease Prevention and Control (ECDC), the US Food and Drug Administration (US FDA), and the US Department of Agriculture, Food Safety Inspection Service (USDA FSIS) were created to support food safety and risk analysis programs.

According to the WHO global estimate of foodborne diseases, thirty-one identified agents – bacteria, viruses, parasites, toxins and chemicals - cause approximately 600 million foodborne illnesses and 420,000 deaths every year. Almost one third (30%) of all deaths are in children under the age of 5 years. The most frequent causes of foodborne illness are diarrhoeal disease agents, particularly norovirus and *Campylobacter* spp. Consequently, diarrheal diseases are responsible for more than half of the global burden of foodborne diseases, causing 550 million people to fall ill and 230,000 deaths every year. Other major contributors to the global burden of foodborne diseases are typhoid fever, hepatitis A, *Taenia solium* (a tapeworm), and aflatoxin (produced by

mould on grain that is stored inappropriately) (WHO, 2015b). Most of foodborne diseases are caused by the consumption of food contaminated with some type of pathogenic microorganism; and the most common clinical presentation takes the form of gastrointestinal symptoms.

The risk of foodborne diseases is most severe in low- and middle-income countries, due to preparation of food with unsafe water; poor hygiene and inadequate conditions in food production and storage; lower levels of literacy and education; and insufficient food safety legislation or implementation of such legislation (WHO, 2015a). Even so, foodborne diseases are a serious public health threat in high-income countries. Although the European Region has the lowest estimated burden of foodborne diseases globally, over 320,000 human cases of foodborne illness are reported each year to the EFSA (EFSA & ECDC, 2013). However, the European authority believe that the real number is likely to be much higher because only a fraction of the people who become sick seek medical care. The WHO estimate that more than 23 million people in European Region fall ill from contaminated food every year, with the most common being Norovirus infections, causing an estimated 15 million cases, followed by *Campylobacter* infections, causing close to 5 million cases (WHO, 2015b).

According to official data from the EFSA and ECDC Community Zoonosis Report of 2015, *Campylobacter* is the most commonly reported gastrointestinal bacterial pathogen in humans since 2008. The number of reported cases of campylobacteriosis in 2015 was 229,213 with a notification rate of 65.5 per 100,000 population, resulting in 59 deaths (0.03% fatality rate). In the second place, *Salmonella* was responsible for a total of 94,625 confirmed salmonellosis cases and 126 fatal cases, resulting in a notification rate of 21.2 cases per 100,000 population and in a case fatality rate of 0.24%. Also, 7,202 confirmed cases of yersiniosis were reported in 2015, making it the third most commonly reported foodborne disease in the EU, corresponding to a notification rate of 2.20 cases per 100,000 population. However, no fatalities were reported among the confirmed yersiniosis cases. Furthermore, with a notification rate of 0.46 cases per 100,000 population, *Listeria* was responsible for the highest annual number of deaths in 2015 (270 human deaths), resulting in case fatality rate of 17.7% among the confirmed human cases **(Table 1)**.

In the last decade, with the introduction of mandatory monitoring and control programs, the number of infections caused by some of the most common bacterial pathogens, such as *Salmonella* and *Listeria monocytogenes*, has been declining. However, the number of infections caused by other foodborne pathogens, in particular, *Campylobacter* spp., has increased progressively and does not appear to tend to slow down.

Table 1. Reported hospitalisation and case fatality rates due to bacterial foodborne pathogens in confirmed human cases in the EU, 2015. NA - not applicable as the information is not collected for this disease. Adapted from (EFSA & ECDC, 2016)

Foodborne disease	No. of confirmed human cases	Reported hospitalised cases	Reported deaths	Fatality (%)
Campylobacteriosis	229,213	19 302	59	0.03
Salmonellosis	94,625	12 353	126	0.24
Yersiniosis	7,202	530	0	0.00
Shiga toxin-producing *E. coli*	5,901	853	8	0.24
Listeriosis	2,206	964	270	17.7
Tularaemia	1,079	89	0	0.00
Echinococcosis	872	107	1	0.49
Q fever	833	NA	3	0.36
Brucellosis	437	130	1	0.74
Trichinellosis	156	30	0	0.00
West Nile fever	127	54	2	1.57

Most member states (MS) of the EU reported increasing notification rates of *Campylobacter* cases in 2015 with almost half reporting significant increases since 2008. Of the reported cases of campylobacteriosis in the EU that provided information on the species, 81.0% were caused by *Campylobacter jejuni*, 8.4% by *C. coli*, 0.2% by *C. fetus*, 0.1% by *C. lari*, and 0.09% by *C. upsaliensis* (EFSA & ECDC, 2016). Other *Campylobacter* species accounted for the remaining 10.3%, but the large majority of those cases were reported at the national level as *"C. jejuni / C. coli / C. lari"* not differentiated.

Although the notification is required in most MS (Portugal reported campylobacteriosis data for the first time in 2015), there is currently no mandatory surveillance of *Campylobacter* in the EU for foodstuff. With this inadequate surveillance system, campylobacteriosis threat to become an even bigger problem for society if no measures are taken. Therefore, the constant food surveillance and testing shoul be a top-priority practice for most retailers, food-related companies or public health authorities in order to prevent the release and spread of contaminated products in the food market.

2.2. The genus *Campylobacter*

From a historical standpoint, the genus *Campylobacter* was first identified in 1906 by John McFadyean and Stewart Stockman when they described the presence of "large numbers of a peculiar organism" in the uterine mucus of a pregnant sheep (Skirrow, 2006). However, it is believed that the first report concerning these microorganisms was back in 1886 by Theodore Escherich. He observed and described non-culturable spiral-shaped bacteria which were found in the colon of children with an enteric disease called "cholera infantum" (Debruyne, Vandamme, & Gevers, 2008). Later on, in 1927, Theobold Smith and Marion Orcutt found a group of vibrio-like bacteria in the faeces of cattle with diarrhoea and, in 1931, Jones and coworkers showed a relationship between the microaerophilic vibrios and bovine dysentery, and the organism was eventually called *Vibrio jejuni* (Jones, Orcutt, & Little, 1931). In 1944, Doyle isolated a different *Vibrio* from the faeces of pigs with diarrhoea and classified it as *Vibrio coli* (L. P. Doyle, 1944). Only in 1963, Seabald and Vernon proposed the genus *Campylobacter* distinguishing them from the *Vibrio* genus based on their low GC (low guanine and cytosine) composition, non-fermentative metabolism and their microaerophilic growth requirements (On, 2001; Silva et al., 2011).

The family *Campylobacteraceae* presently contains three genera — *Campylobacter*, *Arcobacter*, and *Sulfurospirillum* (Lastovica, On, & Zhang, 2013); but *Campylobacter* is the type genus. The first well-documented incident of *Campylobacter* infection took place in Illinois in 1938, when 355 inmates in two adjacent state institutions were involved in a milk-borne outbreak of diarrhoea (Levy, 1946). Since its inception, the genus *Campylobacter* has experienced extensive changes and even some parts of the current taxonomic structure remain a matter of controversy. Some authors claim that the genus *Campylobacter* comprises 20 species and subspecies (Fernández, Vera, Villanueva, & García, 2008), while other authors have described 16 species with further six sub species (Foster et al., 2004; On, 2001). Debruyne et al. state that only 17 *Campylobacter* species have been validly described (Debruyne et al., 2008). More recently, 25 species with further six sub species have been described (Lastovica et al., 2013) **(Table 2)**. Despite all controversy with the taxonomic structure, it is unanimous that *Campylobacter jejuni subsp. jejuni* and *Campylobacter coli* are the most important enteropathogens among *Campylobacter* species.

Table 2. Species and subspecies of *Campylobacter* according to the classification of (Lastovica et al., 2013) as well as the recognized sources of each. NA - not applicable as no potential sources have been identified.

Species or subspecies	Recognised sources

Campylobacter avium	Chickens, turkeys
Campylobacter canadensis	Whooping and sandhill cranes
Campylobacter coli	Humans, dogs, cattle
Campylobacter concisus	Humans, dogs, cats
Campylobacter cuniculorum	Rabbits
Campylobacter curvus	Humans
Campylobacter fetus subsp. fetus	Cattle, sheep, dogs, turtles
Campylobacter fetus subsp. venerealis	Cattle
Campylobacter gracilis	Dogs, humans
Campylobacter helveticus	Dogs, cats
Campylobacter hominis	Humans
Campylobacter hyointestinalis subsp. hyointestinalis	Pigs, cattle, hamsters
Campylobacter hyointestinalis subsp. lawsonii	Pigs, poultry, birds
Campylobacter insulaenigrae	Seals, porpoises
Campylobacter jejuni subsp. jejuni	Humans, dogs, cattle, birds, poultry, cattle, sheep, milk, seafood, water
Campylobacter jejuni subsp. doylei	Humans, dogs
Campylobacter lanienae	Humans, cattle
Campylobacter lari subsp. lari	Cats, dogs, chickens, seals, mussels, oysters
Campylobacter lari subsp. concheus	NA
Campylobacter mucosalis	Pigs, dogs
Campylobacter peloridis	Humans, molluscs
Campylobacter rectus	Humans
Campylobacter showae	Humans, dogs
Campylobacter sputorum bv. paraureolyticus	Cattle, humans
Campylobacter sputorum bv. faecalis	Cattle
Campylobacter sputorum bv. sputorum	Humans, cattle, pigs, sheep
Campylobacter subantarcticus	Penguins, albatrosses
Campylobacter troglodytis	Chimpanzees
Campylobacter upsaliensis	Cats, dogs, ducks, monkeys
Campylobacter ureolyticus	Humans, milk, bovine feces
Campylobacter volucris	Black-headed gulls

Bacteria belonging to the genus *Campylobacter* are Gram-negative, slender and curved or helical rods, often displaying ''gull wing'' morphology **(Figure 1)**. They are between 0.2 – 0.9 μm wide and 0.5 – 5.0 μm long and are motile by means of a single polar unsheathed flagellum at one end or a flagellum at each end, except for *C. gracilis* and *C. hominis* which are non-motile and *C. showae* which has multiple flagella (Debruyne et al., 2008; Penner, 1988). Typically, *Campylobacter* bacteria have a low G+C content (29 – 47 mol%), reduce fumarate to succinate, are nonspore-forming, indole negative and oxidase positive (except for *C. gracilis*) (Debruyne et al., 2008; Lastovica et al., 2013). Most of them reduce nitrate, are catalase positive, urease negative and resistant to cephalothin and fluoroquinolones, normally used to treat human illness. In particular, *C. jejuni* and *C. coli* strains are capable of hydrolase indoxyl acetate, but *C. jejuni* is the only one capable of hydrolase hippurate (Galate & Bangde, 2015; Koenraad, Jacobs-Reitsma, Van der Laan, Beumer, & Rombouts, 1995). A classic example in *Campylobacter* is the use of the hippurate hydrolysis test to distinguish *C. jejuni* from other species. However, atypical (hippuricase-negative) *C. jejuni* strains exist and the recent description of a novel species, *C. avium*, that also hydrolyzes hippurate may complicate this feature a bit (Lastovica et al., 2013). In old cultures, or cultures exposed to unfavourable growth conditions, *Campylobacter* cells can change to spherical or coccoid forms and form viable but non-culturable cells (VBNC) (Lastovica et al., 2013).

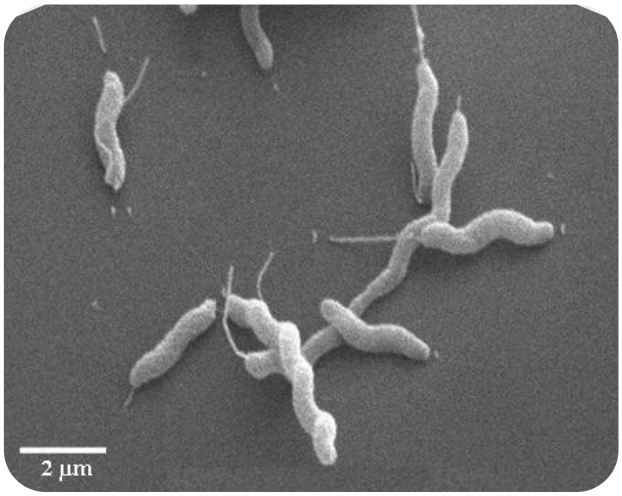

Figure 1. The electron micrograph of *Campylobacter jejuni*. Retrieved from (Lastovica et al., 2013)

In terms of metabolic processes, there is still more to learn about *Campylobacter* species. The process of nutrient acquisition has not been completely elucidated, yet their non-glycolytic nature was the main criterion used to discriminate them from the genus *Vibrio*. In fact, they do not ferment or oxidize carbohydrates. Instead, they obtain energy from amino acids, or tricarboxylic acid cycle intermediates (Hofreuter, Novik, & Galán, 2008). A further analysis of published genome

sequences revealed that *Campylobacter* spp. possess all glycolytic enzymes of the most common type of glycolysis, the *Embden-Meyerhof-Parnas* pathway, except the 6-phosphofructokinase, one of the most important regulatory enzymes (Velayudhan & Kelly, 2002). Furthermore, it is assumed that *Campylobacter* species can conserve energy through respiration and oxidation of hydrogen and formate to reduce electron acceptors such as fumarate, nitrate, sulphates and, if at low concentrations, oxygen, in order to generate proton motive force transport (Hoffman & Goodman, 1982; Laanbroek, Stal, & Veldkamp, 1978; Sellars, Hall, & Kelly, 2002; M. A. Smith, Mendz, Jorgensen, & Hazell, 1999). Nevertheless, recent studies have shown that some *Campylobacter* spp. may have the ability to metabolise glucose via another glycolytic pathway, the *Entner-Doudoroff* pathway, which eliminates the need for phosphofructokinase (Vorwerk et al., 2015).

2.2.1. Growth and survival characteristics

With respect to atmospheric conditions within the genus *Campylobacter,* there is a spectrum for optimum growth that extends from anaerobic requirements of some species to the natural tolerance to oxygen in another species (Neill, Campbell, O'Brien, Weatherup, & Ellis, 1985). However, most species are microaerophilic, unable to grow in air, growing best in an atmosphere of low oxygen tension (e.g. 5% O_2, 10% CO_2, and 85% N_2). *C. gracilis*, *C. hyointestinalis*, *C. showae*, and *C. sputorum* bv. *faecalis* will grow under anaerobic conditions, while *C. concisus*, *C. curvus*, *C. mucosalis*, *C. rectus*, and *C. gracilis* require H_2 or formate for growing (Lastovica et al., 2013).

Campylobacters have an optimum growth temperature ranging from 30 – 42 °C (Lastovica et al., 2013). However, most *Campylobacter* species, including *C. jejuni* and *C. coli*, grow between 37 °C and 42 °C and are therefore generally referred to as thermotolerant species. This group of *Campylobacter* have an optimum growth temperature of 41.5 °C and are unable to growth below 30°C due to the absence of cold shock protein genes which play a role in low-temperature adaptation in other foodborne pathogens such as *E. coli* and *Salmonella* (Silva et al., 2011).

Compared to other foodborne pathogens *Campylobacter* is sensitive to pH, do not survive below a pH of 4.9 and above pH 9.0 and grow optimally at pH 6.5 – 7.5 (Martin J Blaser, Hardesty, Powers, & Wang, 1980). Furthermore, these microorganisms are easily inactivated by heat treatments such as pasteurisation and conventional cooking processes. Freezing–thawing usually reduces significantly the population, with pure cultures being normally inactivated by frozen storage at −15 °C in as few as 3 days (Stern & Kotula, 1982). However, freezing may not eliminate completely the pathogen from contaminated foods which can still be isolated from frozen meats and poultry (Fernández & Pisón, 1996). Furthermore, although *Campylobacter* has mechanisms

for surviving exposure to toxic oxygen metabolites, it is very sensitive to oxidative stress. So, media containing substances which can neutralise the toxic effects of oxygen and its metabolites are normally used for culture. Also, *Campylobacter* spp. are less tolerant to osmotic stress than other foodborne pathogens, having a better growth in media containing 0.5% NaCl (M. P. Doyle & Roman, 1982).

In general, *Campylobacter* spp. are highly susceptible and less tolerant to environmental stresses than other foodborne pathogens, significantly reducing the number of viable cells when exposed to adverse conditions. All these characteristics reduce the ability of *Campylobacter* to proliferate outside of an animal host and in food during their processing and storage, making them fastidious and generally difficult to isolate (Park, 2002). Nonetheless, this fastidious behaviour contradicts the high prevalence values observed for this pathogen, which suggested that much remains to be understood regarding the properties and nutrient requirements of *Campylobacter.*

2.2.2. Pathogenesis

The mechanisms of virulence of *Campylobacter* species are not yet clearly elucidated. However, it is known that flagella-mediated motility, bacterial adhesion to the intestinal mucosa, invasive ability and the ability to produce toxins play an important role in the pathogenicity of these bacteria. The motility of *Campylobacter* is one of the key factors in colonisation and establishment of human disease. In a medical trial, only motile *C. jejuni* cells were recovered after passage in human volunteers tested with a mixture of motile and nonmotile *C. jejuni* strains (Black et al., 1988). The motility is driven by rotation of the flagella, controlled by a chemosensory system that allows the bacteria to move toward favourable environments and away from harmful conditions (Ottemann & Miller, 1997). Flagella give the ability to move in a viscous environment such as mucus, enabling the colonisation of the mucous membrane of the intestinal cell surface.

Bacterial adhesion is also a key factor in the colonization process. Although adhesion by bacterial pathogens is often mediated by fimbrial structures, it has been demonstrated that adherence of *Campylobacter* is not mediated by fimbria or pili, like in other Gram-negative bacteria (Nougayrède, Fernandes, & Donnenberg, 2003). Various others bacterial cell structures have been shown to contribute to the interaction of *Campylobacter* with host cells. Some of them were identified as true adhesins interacting directly with host cell receptors, such as the outer membrane proteins CadF, FlpA and JlpA (Flanagan, Neal-McKinney, Dhillon, Miller, & Konkel, 2009; Jin et al., 2001; Konkel, Garvis, Tipton, Anderson, Jr, & Cieplak, Jr, 1997). Other proteins, such as Cj1349, CapA , TlyA, PEB3, and even the flagellin, FlaA, have been suggested as possible adhesion-related

proteins, but insufficient and/or contradictory results do not allow to provide conclusive evidence on their function as true adhesins (Ashgar et al., 2007; Konkel et al., 1997; Pei, Ellison, & Blaser, 1991; Sałamaszyńska-Guz & Klimuszko, 2008; Yao et al., 1994).

Although colonization factors are extremely important for the virulence mechanism, toxin production is generally considered to be the major pathogenicity feature of bacteria. It has been proven that *Campylobacter* species produce both enterotoxins and cytotoxins (Wassenaar, 1997). Enterotoxin activity was first documented in isolates of *C. jejuni* in 1983 (Ruiz-Palacios et al., 1983). The *Campylobacter jejuni* toxin (CJT) was found to bind to a cellular receptor, enter the cell and elevate the intracellular cyclic adenosine monophosphate (cAMP) levels. This activity results in the deregulation of the intracellular adenylate cyclase regulatory system. In turn, this leads to an increase in chloride ion permeability of the apical membrane of intestinal mucosal cells, leakage into the lumen followed by sodium and water movement and production of watery type stools (Wassenaar, 1997). On the other hand, the best characterized cytotoxin produced by *Campylobacter* species is the cytolethal distending toxin (CDT). Widely distributed among Gram-negative bacteria, CDT has been described as an important virulence factor between these species (Asakura et al., 2008). This toxin cause eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and consequently leading to cell death (Z. Ge, Schauer, & Fox, 2008; Yamasaki et al., 2006; Zilbauer, Dorrell, Wren, & Bajaj-Elliott, 2008).

Several other bacterial constituents have also been shown important for the establishment of campylobacteriosis. For example, lipopolysaccharides present on the surface polysaccharide structures that constitute the outer membrane of Gram-negative bacteria has been shown to be involved in *Campylobacter* adhesion to host cells (McSweegan & Walker, 1986). LPS have also endotoxic properties, acting as a stimulant of the immune system (Levin, 2007; Naess & Hofstad, 1984). These surface polysaccharide structures of *Campylobacter* are sialyted and this modification is thought to be responsible for eliciting an immune response. Siglecs are a family of type I membrane proteins widely expressed on immune cells that have specificity for sialic acid-containing glycans, similar to those present on *Campylobacter* LPS (Crocker, 2005; van Kooyk & Rabinovich, 2008). Therefore, it is possible that *Campylobacter* species benefits from the expression of sialylated LPS to modulate the host innate immune response. Indeed, some authors have suggested that this characteristic is responsible for Guillain-Barre syndrome associated with immune disorders of the peripheral nervous system and experienced by individuals who have

recently undergone infections associated with the ingestion of food contaminated with *Campylobacter* (Nachamkin, Allos, & Ho, 1998).

2.2.3. Campylobacteriosis: epidemiology and pathophysiology

Based on the Community Zoonosis Reports of the EFSA and the ECDC, campylobacteriosis is the most commonly reported zoonosis since 2005 (EFSA & ECDC, 2016). In 2005, *Campylobacter* became the leading cause of zoonotic disease in humans, with 197,363 confirmed cases (EFSA & ECDC, 2006). However, only after 2008, the data began to worry the food authority institutions. In 2008, 190,566 human cases of campylobacteriosis were reported in the EU (EFSA & ECDC, 2010). When compared to the data from 2015 (229,213 confirmed cases) an increase of approximately 20% was observed in the number of reported cases and the rising trend does not seem to slow down. In the United States (US), *Campylobacter* is also the most common cause of bacterial foodborne illness. Active surveillance through FoodNet of the Center for Disease Control and Prevention (CDC) indicates that, in 2015, the number of reported infections and incidence per 100,000 population was 6,309 and 13, respectively (Huang et al., 2016).

However, it is expected that many more cases remain undiagnosed/unreported, underestimating the real number of campylobacteriosis cases worldwide. In the EU alone, it is estimated that the approximately 200,000 reported cases per year may be translated into not less than 2 million and possibly as high as 20 million cases per year (EFSA Panel on Biological Hazards, 2010), while in the USA it is estimated that this microorganism actually causes between 2.1 – 2.4 million cases of infection per year (Friedman, Neimann, Wegener, & Tauxe, 2000). In low- and middle-income countries, precise information is even more difficult to obtain, makes it even more difficult to assess the real incidence of gastroenteritis worldwide caused by *Campylobacter* spp. (WHO, 2012). Some authors claim that this increasing trend may be a result of the increase in chicken meat production in recent decades, from 58.5 million tonnes in 2000 to 95.5 million tonnes in 2014 (Skarp, Hänninen, & Rautelin, 2016). In any case, measures must be taken to prevent this growing trend from being a problem difficult to control.

Campylobacteriosis cases have no clear racial predilection, but some studies reveal that *Campylobacter* organisms are isolated more frequently from males than females (Strachan et al., 2008). Although infections can occur in all age groups, in developing countries, campylobacteriosis cases are more common in the first 5 years of life, while in industrialised countries, studies show a peak incidence in children younger than 1 year and in persons aged 15 – 29 years (Kaakoush, Castaño-Rodríguez, Mitchell, & Man, 2015). Also, it is interesting to note that data from between

2008 and 2015 show a clear seasonal variation of confirmed cases of campylobacteriosis reported in the EU with sharp peaks in the summer months (EFSA & ECDC, 2016). Although the reasons for *Campylobacter* seasonality are not well understood, factors such as increased potential reservoirs, human behaviour and climate are used as potential explanations (Skarp et al., 2016). Nonetheless, most cases occur as isolated, sporadic events, not as part of recognised outbreaks. Indeed, the reported foodborne outbreaks of campylobacteriosis are limited. In 2015, confirmed outbreaks in the EU constituted only 0.6% of all reported cases of campylobacteriosis (EFSA & ECDC, 2016). Even so, outbreak cases are important as they serve as a source of study for understanding various transmission mechanisms. **Table 3** shows examples of outbreaks that occurred worldwide in the recent years, mainly resulting from consumption of contaminated food products.

Table 3. Examples of *Campylobacter* outbreaks that have occurred worldwide in recent years. Adapted from (Kaakoush et al., 2015)

Year	Location	No. of cases	Sources	Reference
2015	California, US	6	Raw milk	(Marler Clark, 2015)
2013	Australia	56	Chicken liver pâté	(Moffatt et al., 2016)
2013	Pennsylvania, US	8	Raw milk	(Centers for Disease & Prevention, 2013)
2012	South Island of New Zealand	138	Tap water	(Bartholomew, Brunton, Mitchell, Williamson, & Gilpin, 2014)
2011	East of England, UK	49	Chicken liver pâté	(Edwards et al., 2014)
2010	Køge, Denmark	176	Tap water	(Gubbels et al., 2012)
2009	Incheon, South Korea	92	Undercooked chicken	(Yu et al., 2010)
2008	Washington, US	5	Raw milk	(Food Poison Journal, 2015)
2007	British Columbia, Canada	225	Ingestion of mud during a mountain bike race	(Stuart et al., 2010)

In pathological terms, not all *Campylobacter* infections cause the infected person to fall ill or to develop symptoms. The lack of symptoms can be the result of two things: the immunity state of the person infected; and the dose of organisms that reach the small intestines (Kaakoush et al., 2015). Only, when a patient is infected and develops symptoms, it is said that he presents campylobacteriosis. Symptoms begin after an incubation period of 1 day up to a week, and this timeframe is inversely related to the dose ingested (M. J. Blaser, Sazie, & Williams Jr., 1987). The infectious dose of *Campylobacter* is a controversial subject yet. Although most food institutions claim that 1,000 to 10,000 bacteria are necessary to cause infection, *Campylobacter* infections have been reported after ingestion of only 500 organisms (Black et al., 1988). Diarrhoea and abdominal pain are the most common symptoms. Other typical symptoms of *Campylobacter* infection include fever, nausea, vomiting, headache, and muscle pain (Griffiths & Park, 1990). Although most cases are self-limiting, some cases have a prolonged illness (longer than 1 week) or a relapse, and a small number may be followed by chronic sequelae (Kaakoush et al., 2015). One in 1,000 patients with *Campylobacter* infection develops Guillain-Barré syndrome (GBS), a rare disease in which the person's immune system attacks the body's nerves because they are chemically similar to *Campylobacter* LPS, resulting in paralysis that can last for several weeks or years (Kaakoush et al., 2015; Nachamkin et al., 1998). Other complications such as Miller Fisher syndrome and reactive arthritis are also common in patients with a history of *Campylobacter* infection. However, their severity in terms of reported case fatality is low. In 2015, the number of reported deaths attributed to campylobacteriosis was 59 resulting in an EU case fatality of 0.03% (EFSA & ECDC, 2016). Severe outcomes are more common when other diseases (e.g., cancer, liver disease, and immunodeficiency diseases) are present.

2.2.4. Prevalence in food and transmission routes

Campylobacter spp. are commensal organisms regularly associated with domesticated animals farmed for meat, such as cattle, sheep, swine, and poultry. Poultry, which includes chicken, turkey, duck and laying hens, are considered the most common hosts probably because of their higher body temperature (Skirrow, 1977). As a substantial source of a high-quality protein, essential amino acids, vitamin and minerals at a low price; broiler chickens are usually associated with a greater risk of carrying *Campylobacter* among commercial poultry due to the large quantities consumed in most countries (Humphrey, O'Brien, & Madsen, 2007).

Consequently, broiler meat is considered the most important source of human campylobacteriosis by all food authorities institutions (EFSA & ECDC, 2016; Silva et al., 2011).

According to European authorities, approximately 47% of the tested units of fresh broiler meat sampled at slaughter, processing and retail were found to be *Campylobacter*-positive (EFSA & ECDC, 2016). On the other hand, the main strain associated with food contamination by *Campylobacter* is *C. jejuni*, as mentioned before. In fact, a recent surveillance study reveals that *C. jejuni* is responsible for more than 12 times the number of cases of human campylobacteriosis compared to *C. coli* (Friedman et al., 2000). However, this data may vary from country to country. Still, *C. coli* is the second major cause of campylobacteriosis, although it is often associated with pork meat (Farzan, Friendship, Cook, & Pollari, 2010). In Ireland, more than 90% of retail pork samples that tested positive for *Campylobacter* were isolated as *C. coli* (Whyte et al., 2004).

From the primary production at rearing farms, transportation to slaughter, slaughtering and further processing of meat products, the sale of retail products until the handling and consumption at home; all these steps have a role in the transmission of *Campylobacter* from the farm to the fork (Skarp et al., 2016). The primary contamination site in the production chain is probably at the rearing farms, where usually 10,000 – 30,000 birds/100 – 200 swine cohabit per house. Horizontal transmission from the environment, including poultry sheds, water, waste, feed, fauna and footwear of farm workers, is considered to be the most likely source of *Campylobacter.* Once established, this microorganism spreads quickly, making it very difficult to control and eliminate (Jorgensen et al., 2011; Skarp et al., 2016). The processing step is probably the next step that further contributes to the spread of contaminated products. As the intestinal tract of chicken and pigs, especially the cecum and colon, can harbour a large number of *Campylobacter* species, leaking or rupturing the intestinal tract during processing can transfer the contents to the meat and/or the carcass (Berrang, Buhr, Cason, & Dickens, 2001). Transport and the following steps appear to have only a minor effect on the contamination of meat products.

Less common is the contamination of products after sale. However, this is still a possible source by cross-contamination of ready-to-eat foods from cutting boards, knives or hands during food preparation (Skarp et al., 2016). In addition to infections associated with meat consumption, cases of campylobacteriosis associated with the consumption of untreated water, rainwater and raw milk have already been described (M. J. Blaser et al., 1979; Eberhart-Phillips et al., 1997; Schorr et al., 1994). Although rare, non-food exposures, such as contact with animals, either domestic pets or farm animals, or with an environment contaminated by animal faeces, can also be a possible source of human infection (EFSA Panel on Biological Hazards, 2010).

Once the possible sources of contamination have been identified, it is important to take measures to prevent this phenomenon. Since the farm is the preliminary site of contamination, the major intervention strategies should be targeted at the farm level. It is not always easy to intervene at this level; but, with the continuous growth of meat production, pressure on industry and public health authorities will appear in order to reduce *Campylobacter* infections. Therefore, the development of rapid methods for the detection of *Campylobacter* is important to counter this growing trend, preventing the arrival of contaminated food to the market.

2.3. Isolation and identification of *Campylobacter* spp. from food products

As discussed in the previous section, the food industry is a complex industry so that food contamination can occur at any stage of the production process, from the breeding farm to the final consumption. This complexity makes control of foodborne pathogens a difficult task even with stringent safety programs. In this way, the key to the prevention of foodborne diseases appears to be the rapid identification of contaminated food in order to prevent them from reaching the final consumer (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). Over the past decades, the means by which foods are tested suffered a large shift, going from complete dependency on bacteriological protocols to the integration of advanced molecular methods (G. Zhang, 2013). Although this transition has had a profound impact on the food industry, dramatically improving food security; progress was uneven and foodborne outbreaks continue to be common in many countries (G. Zhang, 2013).

Traditionally, microbiological analysis of foods is based on the isolation of microorganisms by culture methods and confirmation by visual, biochemical, immunological, or genetic means, either before enrichment or after enrichment. Currently, there is already a wide range of methods for the detection of foodborne pathogens that can be separated into several categories: traditional culture methods, immunological methods, nucleic acid amplification-based methods and direct hybridization methods. **Table 4** shows some methods currently in the market for detecting *Campylobacter* spp. from foods that have received validation by AOAC International.

Table 4. Methods for detection of *Campylobacter* spp. from foods that have received validation by AOAC International [a]. The table has been modified from the Validated Test Kit table available at the website of the US Department of Agriculture.

Type of method	Method name	Manufacturer	Validated matrices
Culture-based methods	CampyFood Agar (CFA)	BioMérieux	Meat, poultry products, and production environmental samples
	Campylobacter Selective Agar®(CASA)		
	RAPID' *Campylobacter* agar	Bio-Rad Laboratories	Meat products and environment samples
	Brilliance™ CampyCount Agar	Oxoid Ltd	Poultry products
Nucleic acid-based methods	BAX® System Real-Time PCR Assay	DuPont Nutrition and Health Diagnostics	Faeces, ready-to-eat turkey and chicken products, or carcass rinses
	Campylobacter real-time PCR	Eurofins Genescan	Chicken raw meat and faeces samples
	iQ-Check™ *Campylobacter* real-time PCR	Bio-Rad Laboratories	Chicken carcass rinse, turkey carcass sponge, raw ground chicken
Immunological-based methods	VIDAS® *Campylobacter*	BioMérieux	Fresh raw pork, raw chicken breast, processed chicken nuggets, chicken and turkey carcass rinse
	Singlepath® *Campylobacter*	Merck KGaA	Raw ground chicken, raw ground turkey, pasteurized milk
	Veriflow™ *Campylobacter*	Invisible Sentinel, Inc	Chicken carcass rinse

a. AOAC International is a non-profit scientific organization commonly translated as "Association of Analytical Communities" which is dedicated to the validation of chemical and microbiological analyses.

In general, these methods can be sensitive, specific and give both qualitative information for the presence/absence of a pathogen or quantitative information about the bacterial load (López-Campos, Martínez-Suárez, Aguado-Urda, & López-Alonso, 2012). The major obstacles for virtually

all technologies continue to be the complexity of food matrices, the heterogeneous distribution of low levels of pathogens, the stress suffered by the microorganisms during the foods processing steps and the presence of competitive bacteria from the normal microbiota, especially in raw foods (Feng, 2007).

2.3.1. ISO reference method

The International Organization for Standardisation (ISO) is a worldwide federation of national standards bodies that prepare international standards. The aim of these standards is to aid in the creation of products and services that are safe, reliable and of good quality. Also, by enabling different products to be directly compared, they facilitate companies in entering new markets and assist in the development of global trade on a fair basis. Typically, different methods are used by different research groups for the isolation and identification of foodborne pathogens, however, standardized methods (i.e. ISO methods) are generally considered the analytical reference methods for official controls (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010).

Currently, the standard method for the detection and enumeration of *Campylobacter* spp. in food is ISO 10272:2017. The described method consists of two parts: Part 1: Detection method and Part 2: Colony-count technique. The method of detection is the most important for the study presented in this work since it will serve as an important comparison in the optimization of the new method of detection of PNA FISH. It describes a horizontal method for determination of the presence or absence of *Campylobacter*. It is applicable to products intended for human consumption or for the feeding of animals; for environmental samples in the area of food production and food handling; and for samples from the primary production stage such as animal faeces, dust, and swabs.

First, it is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Since *Campylobacter* spp. are very sensitive to freezing, but survive better in low temperatures; it is recommended that samples to be tested should not be frozen, but stored at 4 °C ± 2 °C and subjected to analysis as rapidly as possible. For the enrichment of food samples, the test portions should be inoculated into a liquid enrichment medium, homogenized and incubated microaerobically at 37 °C for (4 – 6) hours and then at 41.5 °C for 44 hours ± 4 hours. The volume of the enrichment medium should be nine times the quantity of the test portion (mass or volume), in order to obtain a test portion/enrichment medium ratio of 1:10 (mass/volume or volume/volume). For instance, a 25-g test portion is homogenized in 225 mL of enrichment medium. With respect to the enrichment medium, since

2006, the ISO recommends the use of Bolton broth for selective enrichment. However, in 2017, the ISO method was subjected to a technical review (ISO 10272-1:2017) and began to recommend the application of Bolton broth for foods with a low level of background microflora and/or stressed *Campylobacter*, and the use of Preston broth for the detection of *Campylobacter* in food with a high level of background microflora. According to the technical review, Preston broth was mainly added to overcome problems with background flora resistant to third generation ß-lactams, like cefoperazone in Bolton broth.

Then, the isolation and selection for confirmation of *Campylobacter* from the cultures obtained in the pre-enrichment is accomplished by inoculation in two selective solid media: mCCDA (modified Charcoal Cefoperazone Deoxycholate agar) and any other solid selective medium based on a principle different from that of mCCDA (such as Skirrow agar, Karmali agar or Preston agar). They should be incubated at 41.5 °C in a microaerobic atmosphere and inspected after 44 hours ± 4 hours to detect the presence of presumptive colonies based on the *Campylobacter* growth characteristics in the respective selective solid medium. Finally, for confirmation, the colonies presumed to be *Campylobacter* should be sub cultured on the non-selective Columbia blood agar (CBA) and incubated in a microaerobic atmosphere at 41.5 °C for 48 hours. Isolated colonies should then be confirmed by means of microscopic examination, appropriate biochemical and growth tests, which evaluate morphology, motility, aerobic growth at 25 °C and the presence of oxidase. Examination of morphology and motility is performed in 1 mL of Brucella broth using a microscope. The study of aerobic growth at 25 °C is performed in CBA plates, incubated at 25 °C in an aerobic atmosphere for 44 hours ± 4 hours. The detection of oxidase is performed using a well-isolated colony that is streak onto a filter paper moistened with the oxidase reagent. At the end, *Campylobacter* are present if at least one colony presents small curved bacilli morphology, motility, absence of growth at 25 °C in aerobic atmosphere and positive result in the oxidase test. Overall, the detection method recommended by ISO method requires 6 – 7 days to obtain definitive results of the presence or absence of *Campylobacter* in a test portion.

2.3.2. Selective growth media

There is an extensive scientific literature on the methods for isolation of *Campylobacter* spp., but traditional plating and cultivation is still the gold standard and most reliable method, meeting the high food safety requirements (B. Ge & Meng, 2009). Agar media for *Campylobacter* isolation can be divided into two groups: those that include blood (usually 5 – 7%): Skirrow, Campy Cefex, Butzler, Preston and Exeter media; and those that are blood-free but include charcoal as an

oxygen quencher: Karmali and mCCDA media (J. E. Corry, Post, Colin, & Laisney, 1995). All agar media have similar isolation efficiency for isolation of *Campylobacter*; however, some of them have features that allow an easier way to identify suspect colonies (Oyarzabal & Fernandez, 2016).

Among the selective agar media, mCCDA is the most commonly used plating medium for the isolation of *Campylobacter* because it is one of the most economical alternatives and *Campylobacter* colonies acquire unique characteristics, allowing an easy identification by trained personnel (Oyarzabal & Fernandez, 2016). The incubation time for mCCDA plates is normally 48 hours under microaerobic conditions at 37 °C. Typically, *C. jejuni* strains produce grey, moist flat spreading colonies, while *C. coli* strains tend to be creamy-grey in colour, moist, slightly raised and often produce discrete colonies. Some strains may have a green hue or a dry appearance, with a metallic sheen **(Figure 2b)**. In turn, Preston selective agar medium is probably the most commonly used blood-plating medium for the isolation of *Campylobacter*. Like CCDA, this medium was originally formulated by Bolton and Robertson (1982) and the components/antibiotics used are the same and at the same concentrations of those used in the respective enrichment broth (Bolton & Robertson, 1982). The incubation time for plates is normally 48 hours at 41.5 °C under microaerobic conditions. On Preston agar, *Campylobacter* species tend to form translucent colonies that may also appear as slightly pink, round, convex with a regular edge **(Figure 2a)**. These two selective agar media are considered the most reliable means and, therefore, recommended by ISO method for the isolation of *Campylobacter* from food matrices (Oyarzabal & Fernandez, 2016).

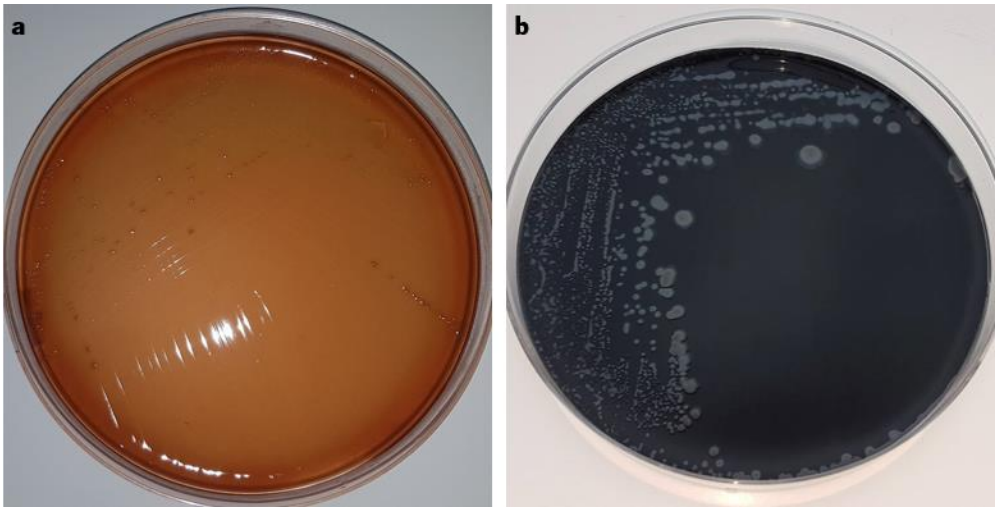

Figure 2. Typical characteristics of *Campylobacter* colonies in (a) Preston agar and (b) mCCDA.

In last decade, a newer group of plate media called chromogenic agars have appeared. This technology is based on soluble colourless molecules (called chromogens), composed of a

substrate (targeting a specific enzymatic activity) and a chromophore. When the target organism's enzyme cleaves the colourless chromogenic conjugate, the chromophore is released. In its unconjugated form, the chromophore exhibits its distinctive colour and, due to reduced solubility, forms a precipitate (López-Campos, Martínez-Suárez, Aguado-Urda, & López-Alonso, 2012). Nowadays, there are few chromogenic agar plates for the isolation and identification of *Campylobacter* spp. from food samples that are already commercially available **(Table 4)**. The first chromogenic agar that appeared in the market was CampyFood agar. All current chromogenic agars have been found to be equally sensitive to traditional plates (Ahmed, Leon-Velarde, & Odumeru, 2012). The principal disadvantage of this type of agar plates is that laboratories must buy premade media, increasing the cost of isolation substantially.

Overall, culture-based methods are simple, easy to implement, very practical, usually inexpensive and have a great sensitivity. However, they are normally labour-intensive, time-consuming, taking 2 – 3 days for initial presumptive results and up to more than 1 week for confirming the specific pathogenic microorganisms. Some of them may be also expensive (Velusamy et al., 2010). In addition, culture based techniques may be unable to identify viable but non-cultivable bacteria - in the VBNC state - which can subsequently lead to an underestimation of pathogen numbers or a failure to isolate a pathogen from a contaminated sample (Li, Mendis, Trigui, Oliver, & Faucher, 2014). Adding this possibility to the fastidious nature of this pathogen, culture and colony counting methods may be inadequate to prevent the rapid spread of infectious diseases, ensure the food safety, and thereby to protect public health. In this way, it is becoming increasingly necessary to develop detection methods that allow the results to be obtained faster, with similar sensitivity as the culture methods.

2.3.3. Immunological methods

In the last decade, immunological methods have emerged as an alternative to traditional methods of culture, making identification faster, more convenient, more sensitive and more specific (Zhao, Lin, Wang, & Oh, 2014). Although some of them are still used only to confirm presumptive colonies isolated by culture methods replacing biochemical tests, they can be used for the direct identification of pathogens after an enrichment step (López-Campos et al., 2012). Immunological methods such as latex tests, lateral flow devices and ELISA (enzyme-linked immunosorbent assay) are methods based on antigen–antibody binding (Zhao et al., 2014). In some cases, such as agglutination reactions, incubation times are usually very short and the antigen–antibody complex formed is directly measurable or even visible (López-Campos et al., 2012).

One of the most widely used immunological assays is the ELISA (Vernozy-Rozand, Mazuy-Cruchaudet, Bavai, & Richard, 2004). Several ELISA formats have been described but the most widely used is called the "sandwich" assay because the target antigen is bound between two primary antibodies: the capture antibody and the detection antibody. In this case, an antibody bound to a solid matrix is used to capture the antigen from enrichment cultures and a second antibody conjugated to an enzyme is used for detection. The enzyme is capable of generating a product detectable by a change in colour or in the case of enzyme-linked fluorescence assay, which allows for indirect measurement using fluorescent spectrophotometry (Capocefalo et al., 2016). The VIDAS® *Campylobacter* is the best example**,** an immuno-based test that is almost completely automated and that has been validated for several food matrices. The total time to results is 48 hours including enrichment time **(Table 4)** (Liu, Hussain, Miller, & Oyarzabal, 2009).

In turn, latex agglutination tests have been on the market for over 20 years and are even suggested for confirmation of isolates by some food safety authorities such as the US Department of Agriculture. They are based on the agglutination of microorganism cells with latex particles covered by the polyclonal antibodies that normally react with proteins present on the cell walls. An example is the Oxoid DrySpot® *Campylobacter* test, a latex agglutination test for the identification of enteropathogenic *Campylobacter* spp. from solid culture media **(Table 4)** (Oyarzabal & Fernandez, 2016).

More rare are lateral flow devices, based on an extremely simple to use and easy to interpret technique, does not require washing or manipulation and allows results to be obtained within 10 minutes after enrichment of the culture. They are typically comprised of a simple dipstick made of a porous membrane that contains coloured latex beads or colloidal gold particles coated with detection antibodies targeted toward a specific microorganism (Posthuma-Trumpie, Korf, & van Amerongen, 2009). For instance, the Veriflow® *Campylobacter* is an ultra-sensitive lateral flow technology for qualitative detection of *Campylobacter* from chicken carcass rinses, requiring an enrichment time of only 24 hours **(Table 4)**.

The main disadvantages associated with immunological techniques are related to false positives, frequently reported due to non-specific reactions in the sample or cross reactivity with a wide range of organisms (Peruski, Peruski, & Jr., 2003). Indeed, the suitability of these methods depends mainly on the antibodies' specificity and, consequently, monoclonal antibodies are often more useful than polyclonal antibodies for immunological detection of microbial contamination since they are more specific, sensitive, reproducible, and reliable (Jasson et al., 2010).

Furthermore, these methods are unable to indicate the viability of organisms and are usually associated with a high cost (Leonard et al., 2003).

2.3.4. Nucleic acid amplification-based methods

In this millennium, the introduction of nucleic acid-based molecular methods for the detection and identification of foodborne pathogens was the main responsible for the advance in food microbiology. Many assay formats were reported, but nucleic acid amplification techniques were most prominent for detecting foodborne pathogens. Practically all methods of nucleic acid amplification are based on the polymerase chain reaction (PCR). Despite being introduced later than immunoassays; nowadays, PCR is considered the principal molecular technique used in any microbiologic laboratory. The most common bacterial PCR methods use probes (primers) that recognize conserved DNA sequences of bacterial genes that encode ribosomal RNA (rRNA) (C. J. Smith & Osborn, 2009). This technique provided several advantages for the detection of pathogens compared to culture methods and other standard methods, such as specificity, sensitivity, rapidity, accuracy, ability to detect small amounts of target nucleic acid in a sample and automation (Toze, 1999).

Conventional PCR involves the amplification of the target gene(s) in a thermocycler, separation of PCR products by gel electrophoresis, followed by visualization and analysis of the resulting electrophoretic patterns, a process that can take a few hours. However, it is still able to detect foodborne pathogens in a shortest time than traditional methods of culture (Abubakar et al., 2007). More recently, the development of alternative PCR methods such as real-time PCR (sometimes also called qPCR or quantitative PCR) has enabled both the detection and quantification of microorganisms in real time. This is possible by the continuous measurement of a signal emitted by the amplified product using a fluorescent reporter. The fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (e.g. TaqMan® Probes) **(Figure 3)**. Results can be obtained in 2 hours or less which is considerably faster than conventional PCR (C. J. Smith & Osborn, 2009).

Most PCR technique for detecting foodborne pathogens normally require a pre-enrichment step that may go up to 48 hours, since most food samples are usually contaminated with few pathogen cells. The duration of this step is also related with the pathogen growth rate, the competing microflora and the qPCR limit of quantification typically around 10^3 –10^4 CFU/g (Jasson et al., 2010). Furthermore, PCR-based methods present some other disadvantages which cannot be ignored such as the complexity of the procedure (requiring specialised technicians); high

susceptibility to inhibitory substances that could provide false negative results; and incapacity to distinguish viable from non-viable cells (Maurer, 2011).

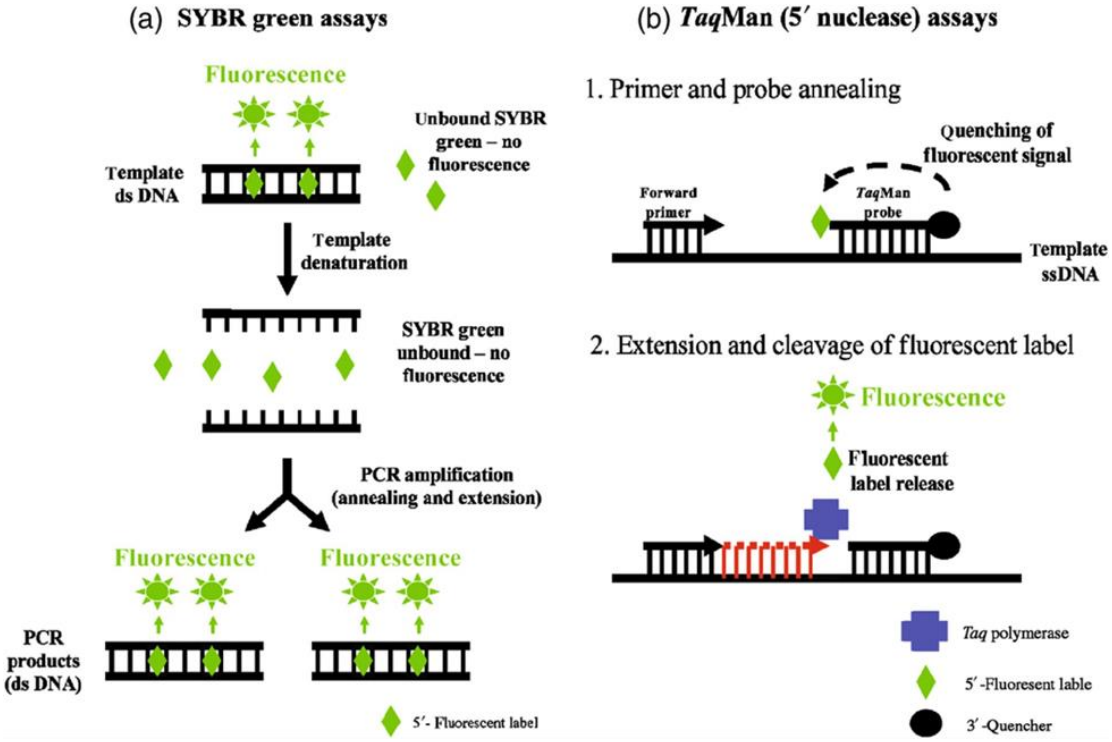

Figure 3. Mechanism of fluorescent reporter molecules commonly applied into real time-PCR techniques. (a) SYBR green detection. SYBR green binds to all double-stranded DNA and emits a fluorescent signal. In its unbound state, SYBR green does not fluoresce. Template amplification is therefore measured in each cycle by the corresponding increase in fluorescence. (b) TaqMan (5' nuclease) assay using TaqMan probes. During annealing, the TaqMan probe and primers bind to the template. When the TaqMan probe is intact, energy is transferred between the quencher and the reporter; as a result, no fluorescent signal is detected. As the new strand is synthesized by Taq polymerase, the 5' exonuclease activity of the enzyme cleaves the labelled 5' nucleotide of the probe, releasing the reporter from the probe. Once it is no longer near, the fluorescent signal from the probe is detected and template amplification is recorded by the corresponding increase in fluorescence. Retrieved from (C. J. Smith & Osborn, 2009).

2.3.5. Direct hybridization methods

As noted above, nucleic acid amplification techniques are not the only ones available for detecting foodborne pathogens, some other probe-based techniques have recently emerged as promising alternatives. There are currently many direct hybridization techniques described in the literature ready to be implemented in clinical and food microbiology. Among these, *in situ* hybridization (ISH) techniques are probably the most promising and useful technology for the rapid characterization of pathogens (Procop, 2007).

Traditionally, ISH procedures are based on the annealing of DNA or RNA probes to a specific target sequence within a cell and, then, on the detection of that annealing (also called hybridization) through one of the several possible strategies. Today there are mainly two basic ways to visualize the hybridization *in situ* – by means of fluorescence (FISH) or chromogenic (CISH) detection (Procop, 2007). Characteristics inherent in each method of detection have made FISH

and CISH useful for very distinct applications. Although both use a specific probe that hybridizes to the target, the procedure to visualise the samples is very different. CISH is a technique that combines the chromogenic signal detection method of immunohistochemistry techniques with *in situ* hybridization and is primarily used for gene expression and cytogenetics (Tanner et al., 2000). In turn, FISH is considered the standard *in situ* technique in the molecular pathology diagnostics and is based on nucleic acids attached to a fluorescent label which, upon hybridization, can be visualized using an epifluorescence microscope (Gupta, Middleton, Whitaker, & Abrams, 2003). In this thesis, a more detailed analysis of the FISH will be made, since this technique is powerful tool has been demonstrated to be useful for the detection of pathogens in food samples as well as in other clinical specimens. In general, FISH is a probe-based assay that belongs to the group of direct hybridization techniques and in an enzyme-independent manner may be used for the rapid and specific identification and characterization of pathogens (Rohde, Hammerl, Appel, Dieckmann, & Al Dahouk, 2015).

2.4. FISHing for *Campylobacter* in food

FISH was first described in 1989 by DeLong *et al*, but it was during the early 1990s that it gained increasing importance as a novel molecular technique for detection, identification and quantification of microorganisms based on hybridization of fluorescence labelled probes with complementary target sequences of nucleic acids (DeLong, Wickham, & Pace, 1989; Levsky & Singer, 2003). Initially applied in the medical and developmental biological domains, FISH is almost a 30-year-old technology that has evolved continuously and one of the most well-established molecular biology techniques. It has applications in a wide range of microbiology areas from the detection of pathogens in clinical samples (Guimaraes, Azevedo, Figueiredo, Keevil, & Vieira, 2007), identification of novel biomarkers for cancer progression (Bayani & Squire, 2007), characterization of communities' structure and diversity of natural habitats (Rogers, Moorman, & Ong, 2007), determination of genes presence and expression (Dmochowski & Tang, 2007) to even chromosomal stability in stem cell research (Catalina et al., 2007). In food microbiology, FISH is not yet a routinely used technique, in part due to the temporal advancement of culture methods and PCR-based techniques, however, it is a growing molecular technique with potential for identification and bacterial localization within samples (Rudolf Amann & Fuchs, 2008; Rudolf Amann, Glöckner, & Neef, 1997).

Since the first description of the FISH technique, traditional RNA and DNA probes are used in the procedures. However, due to serious limitations that affect the robustness of RNA FISH, most hybridization procedures employ DNA molecules (Cerqueira et al., 2008). Even so, it is possible to point out several limitations to DNA FISH. From the low permeability of cell membranes to DNA probes, the difficult accessibility of rRNA due to the ribosomal secondary structure, the degradation of the probe by proteases or endonucleases from living cells, to difficulties in discriminating sequences with a single base mismatch; it is no wonder that researchers started to search for alternatives to improve the robustness of this method (Cerqueira et al., 2008). The solution appears to have arrived in the form of nucleic acid analogues. Among the various types of nucleic acid mimics described so far, the main molecules described in FISH studies are peptide nucleic acid (PNA) (Nielsen, Egholm, Berg, & Buchardt, 1991), locked nucleic acid (LNA) (Obika et al., 1997) and 2'-O-methyl (2'-OMe) RNA (Cummins et al., 1995). Although all have advantages and disadvantages over others, the two main types of oligonucleotide probes currently used in FISH procedures are traditional DNA probes and PNA probes. Both types of probes are usually short ranging from 15 to 20 nucleotides, and are covalently linked to fluorescent labels or fluorophores that allow the visualisation of microorganisms after a correct ligation (Lehtola, Loades, & Keevil, 2005). Typical labels include cyanine, fluorescein or Alexa Fluors molecules (Cerqueira et al., 2008). Most published studies for detection of thermotolerant *Campylobacter* spp. by FISH use DNA probes, but it is already possible to find a study that uses a novel PNA probe for detection of the main species of *Campylobacter* (Lehtola et al., 2005).

In FISH, the rRNA is typically the main target for oligonucleotide probes because of its abundance in cells and because it presents regions of high variability as well as regions unusually conserved, making the characterization possible from distinguishing between related species up to comprising whole kingdoms (R. Amann & Kühl, 1998). Traditionally, the probes target a sequence of the 16S/23S rRNA in members of the Bacteria/Archaea domain. Ribosome numbers in a single cell range from 10^2 - 10^3 for *Mycobacterium tuberculosis* to 10^4 - 10^5 for *Escherichia coli*, which implies that the observed fluorescence intensity is related to ribosomal content (Cerqueira et al., 2008; DeLong et al., 1989). Furthermore, the efficacy of the technique and the signal intensity is often hindered by the choice of binding site on the target rRNA. DNA FISH methods generally target accessible binding sites in the rRNA, since DNA probes have limitations on displacing the complex tertiary structure of this target. On the other hand, PNA probes have proved that accessibility is not really issue. In fact, the only published PNA FISH method for the detection of *Campylobacter* spp.

uses a probe targeting a low-affinity DNA binding site on the 16S rRNA (Lehtola et al., 2005). This is only possible due to the advantages in permeability and accessibility that this type of probe brings to the FISH procedures.

Regarding the procedure, although protocols for FISH might differ significantly, the traditional procedure typically involves 4 steps: a fixation and permeabilization step of the sample; the probe hybridization with target sequence; the washing of unbound and excess probes; and finally the observation of cells **(Figure 4)** (Rudolf Amann & Fuchs, 2008).

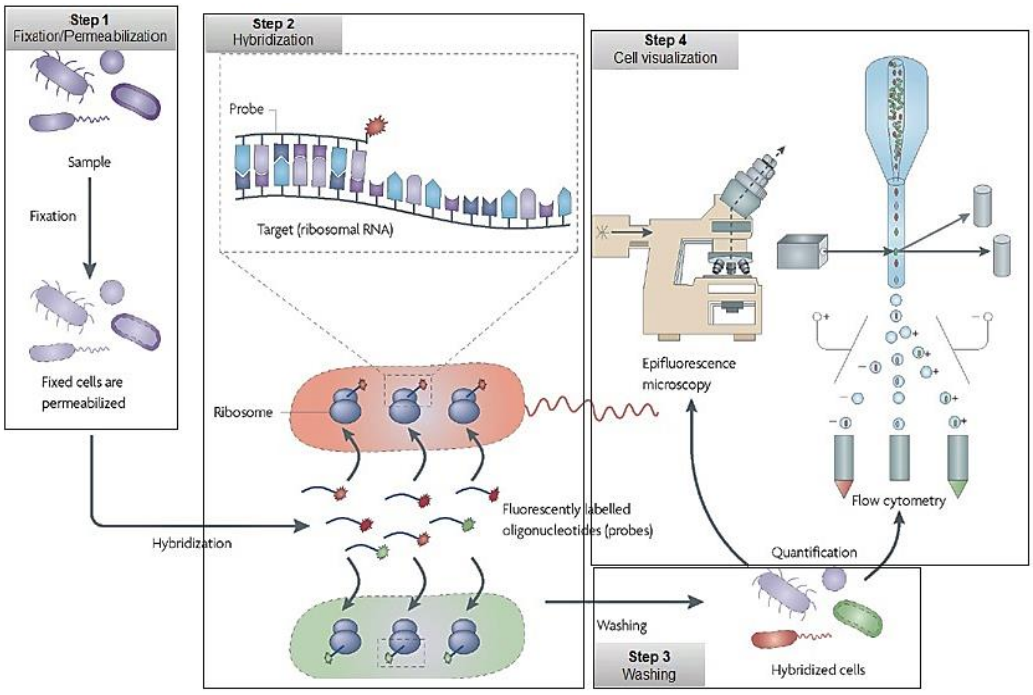

Figure 4. Basic steps of FISH. In the first step, the sample is fixed to stabilise the cells and permeabilize the cell membranes. In step 2, the labelled oligonucleotide probe is added to the cells and allowed to hybridise to its target, normally the ribosomal RNA. Then, the probe excess is washed away. And finally, the sample is ready for single-cell identification and quantification by either epifluorescence microscopy or flow cytometry. Adapted from (Rudolf Amann & Fuchs, 2008).

The fixation step aims to stabilise cell morphology, disable proteolytic enzymes, strengthen samples to withstand further processing and protect against microbial contamination and decomposition. For this purpose, it is generally used fixative agents and sometimes permeabilizing agents, such as paraformaldehyde and ethanol (Felix, 1982; Thavarajah, Mudimbaimannar, Elizabeth, Rao, & Ranganathan, 2012). The hybridization step is probably the most important and must be carried out under high stringent conditions to allow a correct connection between the target sequence and oligonucleotide probes. Several parameters, such as the salts and denaturant concentration (i.e. formamide), hybridization time and temperature, need to be well defined previously to guarantee that the probe accesses and hybridises with the target sequence (Azevedo, 2005). However, these parameters vary depending on the type of probe, the probe sequence and

even the target, which makes the optimization of this step a complex and time-consuming task. In turn, the washing step uses a mixture of detergents to ensure that all labelled or unbound probes are removed from the sample and will not interfere with the visualization of the samples. Finally, the observation of the cells may be done by microscopy or via flow cytometry (Rohde et al., 2015).

In food microbiology, additional steps are required to ensure the detection of pathogens by FISH, in particular a step of preparation and homogenization of the contaminated samples and a pre-enrichment to increase the pathogen load. The pre-enrichment, in particular, is essential to guarantee that pathogen load reaches the detection limit of the FISH technique (≈10^5 CFU/mL). This step not only increases the number of microorganisms to detectable levels but also recovers stressed cells and increases the number of ribosomes and, consequently, increases the fluorescence signal (Rohde et al., 2015). For organisms with a high ribosome content like *E. coli,* this might not be of major importance, but for more fastidious pathogens, such as *Campylobacter* spp., this step may be essential to allow detection (McCarthy, Saunders, & Milner, 2001). Another advantage of enrichment steps is the dilution of interfering food matrix components, which can heavily facilitate evaluation by fluorescence microscopy as well as by flow cytometry. In general, given a sufficient amount of time for the pre enrichment period, FISH is able to meet the ambitious goal of the conventional methods to detect 1 CFU per food portion (Rohde et al., 2015).

The main advantage of FISH over other molecular techniques is that by targeting abundant structures in living cells, this technique allows the visualization of cells with a stable ribosomal content, which is associated with an active metabolic state, thus allowing the differentiation of VBNC. The major challenge continues to be the critical influence of the food matrix in the procedure (Jasson et al., 2010), but the FISH procedures have been shown to be less influenced by this component than PCR-based methods, as no enzymes (that can be inhibited) are involved in hybridization process. Despite specificity, test velocity, and high throughput test potential, FISH is still not routinely used in food safety. However, this trend may be about to change as recent advances have demonstrated that the combined use of *in situ* hybridization and flow cytometry can bring several practical advantages to the microbiology laboratory. This would replace human interpretation with objective and quantitative measures that are performed by an automated instrument (Procop, 2007).

2.5. Peptide nucleic acid oligonucleotides

The probes properties are crucial for the success of the hybridization and, consequently, for the FISH procedure. As mentioned above, traditional DNA probes are used since the beginning for *in situ* hybridization. However, synthetic molecules are emerging as very promising alternatives, providing improved hybridization performance and making FISH procedures easier and more efficient than when using DNA probes (Cerqueira et al., 2008). Among the synthetic nucleic acid mimics published so far, PNAs are currently the main alternative to traditional DNA probes.

PNA molecules were firstly published in 1991 by Nielsen *et al.*, only two years after the emergence of the FISH technique (Nielsen et al., 1991). However, only in the late 1990s, these types of probes were introduced in FISH studies for the detection of microorganisms (H. Stender et al., 1999). PNAs are synthetic DNA mimics that do not occur naturally but can hybridise with nucleic acids according to the same rules of base pairing that apply to DNA, because of its chemical configuration similar to the natural DNA (Nielsen, 2001; Shakeel, Karim, & Ali, 2006). The most relevant difference between DNA and PNA probes is the "backbone" of the molecules. While the backbone of the DNA probes consists of repeating deoxyribose sugar and phosphate molecules that confer a net negative charge, PNAs probes are composed of a neutral polyamide backbone composed of N-(2-aminoethyl) glycine molecules **(Figure 5)** (Nielsen et al., 1991).

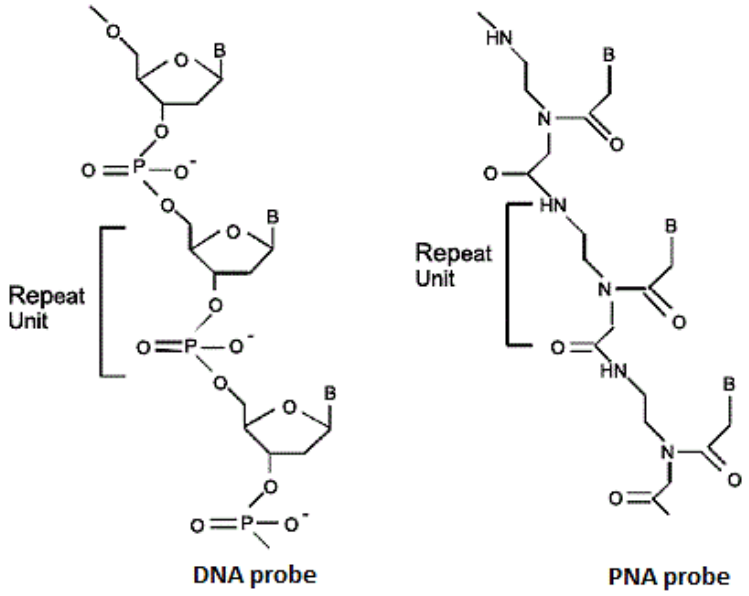

Figure 5. Chemical structure of DNA and PNA oligonucleotides. Retrieved from (Wang & Xu, 2004).

The lack of electrostatic repulsion, due to the non-charged nature of the PNA backbone is perhaps the main reason responsible for its advantages (Cerqueira et al., 2008). The lack of negative charge makes PNAs less susceptible to repulsive forces and contributes to a higher

thermal stability between PNA/DNA duplex when compared to DNA/DNA duplex, enabling PNA molecules to bind stronger to nucleic acids than DNA probes (Nielsen, 2001; Perry-O'Keefe et al., 2001). This characteristic implies that the melting temperature (T_m) for PNA/DNA duplexes is higher than for DNA/DNA, allowing the use of shorter PNA probes than most DNA probes and consequently a higher specificity for the sequence detection. In fact, sequences of approximately 15 bp have been found to be optimal for PNA probes whereas DNA probes typically need 20-24 bp (Cerqueira et al., 2008).

The neutral charge of PNA allows even its hybridization with nucleic acids in low salt concentrations and high temperatures (Orum et al., 1995). This ability is important when the target DNA or RNA possesses complex secondary structures like those found in rRNA. The low salt concentration and high temperatures will open easily the secondary structures, resulting in an improved access to target sequences and enabling the PNA binding to less accessible target sequences (Fuchs et al., 1998; Fuchs, Syutsubo, Ludwig, & Amann, 2001). Furthermore, as all other synthetic molecules, the PNAs also show greater resistance to the action of the nucleases/proteases, which increases the stability of the molecule (Henrik Stender, Fiandaca, Hyldig-Nielsen, & Coull, 2002; Wagner, Horn, & Daims, 2003), and the apolar characteristics increase penetration capacity of the PNA probes, resulting in an enhanced diffusion into the cell through cell membranes/walls or even into other naturally occurring micro structures such as the biofilm matrix (Drobniewski, More, & Harris, 2000). All these characteristics allow the hybridization time to be reduced while maintaining high hybridization efficiency.

In the last decade, a significant increase in the number of PNA probes published for application in food microbiology has been observed. For the detection of *Campylobacter* spp., only one PNA probe has been published so far, but has shown great efficacy in identifying specifically three species, *C. jejuni*, *C. coli* and *C. lari*, in drinking water samples. The published PNA probe was 15 bp-long and was designed to bind with high affinity to a previously reported low-affinity site on the 16S rRNA (Lehtola et al., 2005). The demonstrated efficacy was only possible because of the advantages that this type of probe offers to the FISH method, which would be almost impossible with a traditional DNA probe. **Table 5** shows some of the effective applications of PNA probes for the detection of relevant foodborne pathogens.

Table 5. FISH studies using PNA probes in food microbiology to detect foodborne bacteria. Adapted from (Rohde et al., 2015).

Pathogen (reference)	Target rRNA	Food matrix	FISH methodology, sensitivity and specificity[a]
E. coli (Henrik Stender, Oliveira, Rigby, Bargoot, & Coull, 2001)	16S	Milk	FISH-on-filter with formation of microcolonies
E. coli O157:H7 (Almeida, Sousa, et al., 2013)	23S	Ground beef, milk	Detection of 1 CFU/25 g in less than 24 hours - 100% specificity and 97% sensitivity
L. monocytogenes / Listeria spp. (X. Zhang et al., 2012)	16S	Raw and pasteurise milk, raw meat and seafood	Three PNAs with good specificity and sensitivity for rapid FISH identification of the genus *Listeria*
Salmonella spp. (Almeida, Cerqueira, Azevedo, & Vieira, 2013)	23S	Eggs, milk and mayonnaise	Detection of 1 CFU/25 g in 24 hours - 100% sensitivity and specificity
Campylobacter spp. (Lehtola et al., 2005)	16S	Drinking water samples	High affinity for detecting *C.* jejuni, *C.* coli and *C. lari* - 100% specificity and 91% sensitivity
Cronobacter spp. (Almeida et al., 2009)	16S	Powdered infant formula	Detection of 1 CFU/10 g in 12 hours - 100% sensitivity and specificity

a. Specificity is expressed as the percentage of the number of non-target strains present in the database that is not detected by the probe divided by the number of non-target strains present in the database. Sensitivity is calculated as the percentage of the number of strains of the microorganism of interest detected by the probe divided by the total number of strains of the microorganism of interest present in the database.

Overall, PNA FISH technique offer similar specificity, sensitivity and rapidity to other molecular methods. However, unlike PCR, is not so affected by the presence of matrix inhibitors and detects only organisms with stable ribosomal content which is an indicator of cell viability (Rohde et al., 2015). The main disadvantage in the application of PNA probes in FISH procedures is still the high cost of production.

CHAPTER III

OPTIMIZATION OF AN ENRICHMENT STEP FOR THE DETECTION OF *CAMPYLOBACTER* SPP.

3.1. Introduction

Isolation of most foodborne pathogens is often a difficult task due to the low number the pathogens of interest and the high levels of competitive microflora normally associated with food matrices. In the case of *Campylobacter* spp., the isolation is slightly more complex than most foodborne pathogens. This happen because of the fastidious growth of these bacteria (easily overgrown by other microorganims present in the food matrices) and also because of the well-known viable but nonculturable state not recovered by traditional culture techniques (Reilly & Gilliland, 2003). Furthermore, the complex requirements of growth and extra sensitivity to environmental stresses, resulting from the absence of the alternative factor RpoS (shown to produce greater stress resistance in stationary phase cells in a variety of foodborne pathogens such as *Salmonella* and *E. coli*), make the isolation of *Campylobacter* from food matrices even more difficult (Kelly, Park, Bovill, & Mackey, 2001).

Thereat, the detection of pathogens from food matrices, in particular *Campylobacter*, typically requires an enrichment step in selective medium prior to isolation, in order to suppress the growth of the competitive flora and ensure a level of target microorganism detectable by the detection method (Velusamy et al., 2010). Although this additional step is a limitation in terms of assay speed, it provides several essential benefits beyond those mentioned, such as the dilution of potential inhibitors; the differentiation of viable from nonviable cells; and the repair of cell stress or sub lethal-injury that bacteria may suffer when exposed to the harsh procedures used in food industry (Ray & Johnson, 1984).

The major advances in the isolation of *Campylobacter* began with the discovery of Skirrow's medium. Originally, this medium contained peptone as a source of nutrients, lysed horse blood to protect from the toxic effects of oxygen derivatives and antibiotics to prevent the growth of competitor microorganisms (Skirrow, 1977). Over the years, many enrichment media have been formulated based on these components and several have been modified to improve selectivity and sensitivity in the isolation of *Campylobacter* from food. Among the various enrichment media already described in the literature, there are currently four main ones that demonstrated better performance: Preston broth, Bolton broth, *Campylobacter* enrichment broth and Exeter selective enrichment broth.

Preston broth was proposed by Bolton and Robertson (1982) and can be used for either the broth, for pre-enrichment, or as an agar medium for isolation of *Campylobacter* (Bolton & Robertson, 1982). In its basic composition, it contains beef extract, peptone, sodium chloride and

5% (v/v) lysed horse blood. The antibiotics included are polymyxin B (5 IU/mL), rifampicin (10 μg/mL), trimethoprim (10 μg/mL) and cycloheximide (100 μg/mL). Later it was added to its composition sodium pyruvate, sodium metabisulfite and ferrous sulphate in order to assist in quenching the toxic oxygen metabolites (Bolton, Coates, & Hutchinson, 1984).

Bolton's broth was formulated in accordance with the recommendations of the ISO for the selective enrichment of *Campylobacter* spp. in foods. It contains peptone, yeast extract, α-ketoglutaric acid, sodium pyruvate, sodium metabisulfite and haemin. While sodium pyruvate and sodium metabisulfite assist in quenching the toxic oxygen metabolites; sodium carbonate is included to generate carbon dioxide during growth (Donnison, 2003). The complete medium also includes 5% (v/v) lysed horse blood, and cefoperazone (20 μg/mL), vancomycin (10 μg/mL), trimethoprim (10 μg/mL) and cycloheximide (50 μg/mL). *Campylobacter* enrichment broth has the same formulation as Bolton broth but is different in the substitution of cycloheximide for natamycin in the antibiotic supplements. The main reason for this difference is that natamycin is much safer to handle than cycloheximide, which has a number of toxicity effects described to the human health and the environment (Christian Pedersen, 1992).

The Exeter broth is a nutrient broth which originally contains beef extract, peptone and sodium chloride with 5% (v/v) lysed horse blood. It was later amended to include the oxygen quenchers sodium pyruvate, sodium metabisulfite and ferrous sulphate (Martin, Mason, McAlpine, & Humphrey, 1996). In addition, Exeter broth has incorporated antibiotics: cefoperazone (15 μg/mL), polymyxin B (5 IU/mL), trimethoprim (10 μg/mL), rifampicin (10 μg/mL), and amphotericin (2 μg/mL).

For the enrichment broths described, it is generally recommended an incubation in microaerophilic atmosphere, achieved using either using commercial *Campylobacter* gas packs or an incubator gassed with a flowing mixture of 5% O_2, 10% CO_2 and 85% N_2. Concerning the enrichment time, an initial resuscitation period (to help potentially injured cells to recover) of 4 hours at 37 °C is often specified. Following resuscitation, an incubation period of 44 hours at 41.5 °C is generally used to promote bacterial growth (FDA BAM, 1998).

In this way, the objective of the work presented in this chapter was to select the best enrichment procedure that guarantees the ambitious goal of detecting 1 CFU of *Campylobacter* per test portion by PNA FISH. For this, any feature in the enrichment stage that would lead to ambiguous results was investigated in order to obtain a procedure that would provide the confidence to proceed to the certification tests.

3.2. Material and methods

3.2.1. Bacterial species and culture maintenance

Within the Chapter III, two collection strains (*C. jejuni* NCTC 11168 and *C. coli* NCTC 11366) and two isolate strains (*C. jejuni* CNET 90 and *C. coli* CNET 20) were used for the different assays. The strains used were obtained from the internal culture collection of the Centre of Biological Engineering (CEB). All four strains were maintained into Columbia blood agar (CBA, Oxoid CM0331, UK) supplemented with 5% (volume/volume) defibrinated horse blood (Oxoid, SR0050) at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation, USA), set to 5% O_2, 10% CO_2, and 85% N_2, and streaked onto fresh plates every 48 hours.

3.2.2. Inoculum preparation

For all the tests carried out in the presented thesis, a loopful of biomass from 24-hours cultures were harvested from the respective plate, suspended in 1 mL of autoclaved phosphate buffered saline (PBS 1x: NaCl 137mM, KCl 2,7mM, Na_2PO_4 10mM, KH_2PO_4 1,8 mM) or distilled water (dH_2O) and subjected to a vortex for homogenization. For direct analysis by PNA FISH, the inoculums were prepared in dH_2O. For artificial contamination of food samples, the inoculums were prepared in PBS. The cell density was assessed by determining the optical density (OD) at 600 nm and the necessary volume of the initial inoculum was used to obtain a cell density corresponding to approximately 1×10^8 cells/mL. The relationship between OD and total cell counts was previously established by performing CFU counts and OD readings at several cell dilutions, as previously reported by (Fernandes et al., 2017). If necessary, cell suspensions were then diluted (1:10 dilutions) in PBS or dH_2O (according to the final application of the inoculum) to obtain the desired cell concentration. Cell concentrations were confirmed by plating the appropriate volume in the appropriate solid media and incubating under the same culture conditions used for culture maintenance.

3.2.3. ISO reference method

The various tests performed both in the optimization of the enrichment step and in the certification tests of the developed method, required a comparison of the results with a reference method. For *Campylobacter* spp., ISO 10272:2017 is the reference method commonly used by food safety institutions and, therefore, it was used as a basis for comparison.

For this purpose, Bolton broth (BB; Oxoid, CM0983), prepared according to the manufacturer's instructions with Bolton Broth Selective Supplement (Oxoid, SR0183) and 5% (volume/volume) lysed horse blood (Oxoid, SR0048), was used as enrichment media. For the analysis, test samples were homogenized into BB, in a test portion/enrichment medium ratio of 1:10 (mass/volume), with a stomacher (Eco Blender II, VWR pbi, Italy) for 30 seconds and then incubated in a microaerobic atmosphere at 37 °C for 4 hours plus 44 hours at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation, USA), set to 5% O_2, 10% CO_2, and 85% N_2.

At the end of enrichment, the isolation of *Campylobacter* was accomplished by inoculation in two selective solid media: mCCDA (Oxoid, CM0739 with CCDA Selective Supplement, Oxoid, SR0155) and Preston agar (prepared with Nutrient Broth No. 2 (Oxoid, CM0067), Preston *Campylobacter* Selective Supplement (Oxoid, SR0117) and *Campylobacter* Growth Supplement (Oxoid, SR0232), 5% (volume/volume) defibrinated horse blood (Oxoid) and 15 g/L agar). For this, a loopful of enriched food suspensions were plated on the two selective solid media and incubated at 41.5 °C under the same microaerophilic conditions used for sample enrichment. After 48 hours, the plates were inspected to detect the presence of presumptive *Campylobacter* colonies based on the characteristics they develop in each selective solid medium.

Finally, for confirmation of *Campylobacter* species, one colony, suspected as being *Campylobacter,* was taken from each selective solid medium and sub cultured on CBA at 41.5 °C for 48 hours under the same microaerophilic conditions defined above. Then, well isolated colonies were confirmed by: microscopic examination (based on morphology); aerobic growth at 25 °C; and the presence of oxidase. The morphology examination was performed under a microscope, taking into account the morphological characteristics of *Campylobacter* bacteria. The study of aerobic growth at 25 °C was performed by plating a suspected colony on a CBA plate at 25 °C for 48 hours in aerobic atmosphere. The detection of oxidase was performed using a well-isolated colony that was streaked onto a filter paper of the BD BBL DrySlide Oxidase test kit (Becton, Dickinson and Company, USA). The appearance of a mauve, violet or deep blue colour within 10 seconds indicated a positive reaction. *Campylobacter* was present in the initial food sample if at least one colony presents small curved bacilli morphology, absence of growth at 25 °C in aerobic atmosphere and positive result in the oxidase test.

3.2.4. PNA FISH protocol

For the PNA FISH procedure, 20 µL samples were collected and placed on coated glass slides. After drying, smears were first fixed by immersion in 4% (mass/volume) paraformaldehyde

(Sigma-Aldrich-Aldrich, USA) and then in 50% (vol/vol) ethanol (Fisher Scientific, USA) for 10 min each and incubated at room temperature 20 °C ± 10 °C. Smears were then be covered with 20 µL of hybridization solution containing 10% (mass/volume) dextran sulphate (Sigma-Aldrich), 10 mM NaCl (Sigma-Aldrich), 30% (volume/volume) formamide (Sigma-Aldrich), 0.1% (mass/volume) sodium pyrophosphate (Sigma-Aldrich), 0.2% (mass/volume) polyvinylpyrrolidone (Sigma-Aldrich), 0.2% (mass/volume) Ficol (Sigma-Aldrich), 5 mM disodium EDTA (Sigma-Aldrich), 0.1% (volume/volume) Triton X-100 (Sigma-Aldrich), 50 mM Tris–HCl (pH 7.5; Sigma-Aldrich), 200 nM of *Campylobacter* probe (Panagene, South Korea) and 200 nM of blocker probe (Panagene) without fluorochrome. Blocker probe was designed to hybridise specifically with a mismatch sequence of 3 nucleotides of *E. coli* and *Salmonella* strains. Samples were covered with coverslips, placed in moist chambers, and incubated for approximately 60 minutes at 57 °C. Subsequently, the coverslips were removed, and the slides submerged in a pre-warmed (57 °C) washing solution containing 5 mM Tris base (Sigma-Aldrich), 15 mM NaCl (Sigma-Aldrich), and 1% (volume/volume) Triton X (pH 10; Sigma-Aldrich). Washing was performed for 30 minutes, and the slides were allowed to air dry. The smears were then mounted with one drop of non-fluorescent immersion oil (Panreac AppliChem, Spain) and covered with coverslips. Finally, visualization was performed using a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments, Japan) equipped with one filter sensitive to the Alexa Fluor 594 molecule attached to the PNA probe (excitation, 530 to 550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). Other filters present in the microscope were used in order to confirm that cells did not autofluorescence. All images were acquired using the NIS elements BR software with a magnification of 600×. In the thesis presented, there is no reference to the sequence or location of the probes used for reasons of company confidentiality.

3.2.5. Evaluation of the cultivability during storage at low temperature

In order to evaluate the effect of refrigeration at low temperatures on the recovery and growth capacity of *Campylobacter*, the four strains were exposed to storage at 4 °C for 3 days. These conditions were chosen to simulate the conditions to which food products are normally exposed during processing and subsequent commercial storage. In this way, three cell concentrations of each strain of *Campylobacter* (approx. 10^5 CFU/mL, 10^4 CFU/mL and 10^3 CFU/mL) were prepared as described above in 3.2.2 and, then, were stored in a refrigerator at 4 °C. Initial cell concentrations were confirmed by plating the appropriate dilution on CBA and incubating at 41.5 °C for 48 hours. To determine the cultivability of the strains, samples of each cell concentration were collected after 24 hours, 48 hours and 72 hours of storage. Then, triplicates

of the appropriate dilution (1:10 dilutions in PBS) were plated in CBA and incubated under the same culture conditions used for culture maintenance. Finally, the number of CFUs were counted and the cultivability decrease after refrigeration was evaluated taking into account the initial concentrations of each cell suspension. Each experiment was carried out at least twice.

3.2.6. Enrichment step optimization

For the optimization of the enrichment step, the two enrichment broths were selected: BB (prepared as described above) and Preston Broth (PB) prepared with Nutrient Broth No. 2 (Oxoid, CM0067), Preston *Campylobacter* Selective Supplement (Oxoid, SR0117) and *Campylobacter* Growth Supplement (Oxoid, SR0232). All contained 5% (volume/volume) lysed horse blood (Oxoid, SR0048) and prepared according to the manufacturer's instructions. Furthermore, in order to simulate real conditions, the assays were performed using the two main food matrices associated with *Campylobacter* infections: fresh raw broiler meat and fresh raw ground pork. All food matrices were obtained from local retailers in Braga, stored at 4 °C ± 2 °C and subjected to analysis as rapidly as possible. Prior to starting, all food matrices were pre-screened for natural contamination. Only if natural contamination were not found, the product was used for the next steps.

For the artificial contamination, 25 g samples of each matrix were directly inoculated in stomacher bags with filters (VWR, USA) with the appropriate amount of cells of the respective target species, so that on the day of experiment initiation there are three inoculation levels: 1 CFU/25 g, 10 CFU/25 g and 100 CFU/25 g. *C. jejuni* CNET 90 and *C. coli* CNET 20 inoculums, prepared as described above in 3.2.2, were used to artificially contaminate broiler meat samples and pork samples, respectively. The inoculum levels of the suspensions were confirmed by the standard spread plate technique on CBA plates. Only the isolates (CNET strains) were used in the tests for selection of the enrichment procedure, since they were more sensitive to cold storage and, therefore, the best model to ensure that the procedure is reliable. A non-inoculated food sample was included in each experiment to ensure that samples were *Campylobacter*-free.

After inoculation, microorganisms were allowed to equilibrate in the matrix at 4 °C in the refrigerator for 24 hours. The amount of cells inoculated into the food samples took into account the expected decrease in cultivability determined previously. Then, the 25 g test portions were mixed with 225 mL of BB or PP, homogenized in a stomacher (Eco Blender II) for 30 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), set to 5% O_2, 10% CO_2, and 85% N_2.

After each enrichment, 20 µL samples were placed directly on a microscope slide and the PNA FISH procedure was performed as described above. At the same time, confirmation of the PNA FISH outcome was achieved by culture method as previously described in the ISO method (see 3.2.3). For this purpose, a loopful of enriched suspensions was plated in mCCDA and Preston agar and incubated at 41.5 °C under the same microaerophilic conditions used in sample enrichment. After 48 hours, the plates were inspected to detect the presence of *Campylobacter* colonies based on the growth characteristics in the respective selective solid medium (see Selective growth media).

3.2.7. Reduction of autofluorescence signal

In the enrichment step optimization assays, a strong autofluorescence was observed in some samples after the PNA FISH procedure, making it difficult to observe and confirm the results under the microscope. As such, the artificially inoculated food samples were homogenized in 225 mL of BB and incubated as described above; and then subjected to different tentative autofluorescence-reduction assays. As, some studies suggest that red blood cells may confer autofluorescence to samples analysed by FISH (Almeida, Azevedo, Fernandes, Keevil, & Vieira, 2010); a BB enrichment without the addition of lysed horse blood was tested in an independent assay. Alternatively, some additional treatment steps were introduced before the PNA FISH procedure to try to remove some autofluorescence food particles. Briefly, (1) 15 µL of enriched suspensions were mixed with 15 µL of a 1% Triton X-100 (Sigma-Aldrich) solution directly on the microscope slides to emulsify the fatty compounds, as previously reported by Almeida, Sousa, et al. (2013); (2) 1 mL of enriched suspensions was centrifuged at 900 g for 1 minute to sediment food particles, as suggested by Stevens & Jaykus (2004); (3) 1 mL of enriched suspensions was diluted (1:2 dilution) in dH_2O to dilute autofluorescent food particles; (4) 1 mL enriched suspensions was centrifuged at 10,000 g for 5 minutes and the pellet was then resuspended with a 0.1% Tween-80 (Sigma-Aldrich) solution to emulsify the fat compounds and disrupt possible hydrophobic and electrostatic interactions between the target organism and the food particles, as suggested by Stevens & Jaykus (2004).

After each enrichment, 20 µL of the enriched suspensions were placed on microscope slides and the PNA FISH procedure was performed as described above in 3.2.4. Finally, the PNA FISH results of the different treatments were compared with no-treated samples to select the technique that allowed the best reduction of autofluorescence without compromising the performance of the previously selected enrichment.

3.3. Results and discussion

3.3.1. Preliminary results

Ideally, the enrichment step should provide high concentration of the target pathogens in a short period of time. Besides increasing the concentration of target pathogens into detectable numbers, enrichment broths should also be able to recover stressed or injured cells and limit the growth of background microflora improving detection efficiency (Velusamy et al., 2010). For rapid technologies applied to the detection of foodborne pathogens, the aim is always to try to reduce the enrichment period or, at least, not to exceed the time used for traditional methods (Zhao et al., 2014). However, as mentioned before, the isolation of *Campylobacter* is more complex than most foodborne pathogens due to their complex growth requirements, and lower tolerance to environmental stresses (Reilly & Gilliland, 2003). All these characteristics contribute to a slower growth of *Campylobacter* species compared to the microflora normally associated with food samples.

In this way, it was initially tested the enrichment step recommended in the ISO reference method in food samples. At that time, artificial contamination of fresh raw broiler meat and fresh raw ground pork was performed without taking into account any possible reduction in cells viability after stabilization at 4 °C. As such, after a stabilization period of 72 hours under refrigerated conditions, samples were taken, subjected to enrichment and analysed by PNA FISH and by culture method, as described in 3.2.4 and 3.2.6 **(Table 6)**.

Table 6. PNA FISH and culture (ISO 10272-1:2017) results for the detection of *C. jejuni* and *C. coli* in different food matrices after enrichment with Bolton broth. Food samples were artificially inoculated and subjected to a refrigerated storage period of 72 hours. The results presented comprise two independent assays with three replicates of each inoculation level.

Contamination level (CFU/25 g) [a]	***C. jejuni* CNET 90 Fresh raw broiler meat**		***C. coli* CNET 20 Fresh raw ground pork**	
	PNA FISH	Culture	PNA FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	- (0/6)	- (0/6)	- (0/6)	- (0/6)
10	- (0/6)	- (0/6)	+ (3/6)	+ (2/6)
100	+ (1/6)	+ (1/6)	+ (5/6)	+ (5/6)

a. Real concentration of bacteria (CFU/25 g): 1st assay – *C. jejuni:* 1.3 ± 0.6| 16.0 ± 4.6| 98.0 ± 4.0|; *C. coli:* 1.2 ± 0.1| 11.2 ± 0.5| 102.3 ± 8.1|; 2nd assay – *C. jejuni:* 2.0 ± 1.2| 11.7 ± 2.5| 90.0 ± 4.4|; *C. coli:* 1.0 ± 0.0| 11.0 ± 3.0| 99.3 ± 3.2|;

The results obtained were, in part, unexpected. All low level inoculated samples of both food matrices were negative by the PNA FISH method and by the culture method. Regarding the inoculation level of 10 CFU/25 g, all broiler meat samples inoculated with *C. jejuni* were also negative by both detection methods, but 3 samples of pork were positive by the PNA FISH method and 2 samples were positive by culture method in a total of 6 samples tested. For the broiler meat, positive samples were obtained only for the high level of inoculation (1 in 6 by both methods of detection). For the pork samples, at the high inoculation level, 5 positive samples were obtained in a total of 6 samples by the FISH PNA method and by the culture method.

The large number of negative results seems to suggest an inefficiency of the enrichment step; since the problem does not appear to be related with the PNA FISH method itself, because the results of both methods are negative. However, an enrichment step suggested by a reference ISO method should not fail the recovery and growth of *Campylobacter*, leading to such a large number of negative results, particularly, at high inoculation level (10 - 100 CFU/25 g).

So, if the problem was not related with the detection methods, it could be related with the inoculation or preparation of the food samples. As the counting controls for the bacterial suspensions confirmed the initial inoculation of the samples at the desired levels, the stabilization/storage at refrigeration conditions (for the inoculated food samples) was pointed out as the possible source of problems. Given the extreme sensitivity of *Campylobacter* strains to stress conditions, it could be possible that cold exposure is causing such negative results. This step is essential and required in the certification process of new detection methods that involve an artificial contamination of food samples, as a way of simulating the real conditions. As such, it cannot be simply eliminated. Indeed, as noted, *Campylobacter* bacteria are characterized by extreme sensitivity not only to exposure to air but also to low temperatures and freezing. Some studies have shown that counts of *Campylobacter* in food samples decrease significantly during refrigeration at 4 °C and that surviving cells suffer sub-lethal injury that lead to an extra sensitivity to antibiotics and to high incubation temperature (Maziero & de Oliveira, 2010; Ray & Johnson, 1984). Therefore, it is possible that even by inoculating the correct amount of cells, some die during cold storage and those that survive suffer sub-lethal injury that lead to an extra sensitivity to antibiotics present in the enrichment medium, resulting in an inability to grow and multiply. There are no studies that reveal the proportion of cultivability reduction and whether this effect is similar for different *Campylobacter* species. Thus, the cultivability reduction of *Campylobacter* inoculum should be studied to confirm the suspicion and, thus, correct the amount of inoculated cells so

that, at the beginning of the incubation of the samples, the desired inoculation levels are guaranteed.

3.3.2. Evaluation of the cultivability during storage at low temperature

In the food chain, during processing, transport, retail, and storage, products are normally stored at refrigeration or frozen temperatures for different time periods. Refrigerated storage (1 – 4 °C) is the simplest method of preserving fresh products for a short period of time and, because of that, it is widely used to preserve perishable foods. This type of treatment aims to avoid the decomposition of food products by micro-organisms, typically bacteria. There are two completely different groups of bacteria that normally contaminate food: pathogenic bacteria, the kind that cause foodborne illness, such as *Campylobacter*; and spoilage bacteria, the kind of bacteria that cause foods to deteriorate and develop unpleasant odours, tastes, and textures (USDA FSIS, 2010). The exact effect of refrigeration on the survival of bacteria present in food is not entirely clear. It is assumed that refrigeration does not kill all bacteria nor prevent them from multiplying, but simply slows bacterial growth. However, there are several contradictory evidences on the effect of refrigeration on bacteria present in food. In fact, some authors argue that bacterial cells experience high death rate at refrigeration temperatures even higher than in frozen storage (Pradhan et al., 2012).

Thus, the effect of refrigeration on bacteria is not as simple as previously thought; in fact, in recent years several studies have shown that the response to low temperatures varies according to the bacteria (Ray & Johnson, 1984; USDA FSIS, 2010). Typically, pathogenic bacteria are characterized by a rapid multiplication in a temperature range between (8 – 60) °C, but by a decrease of the metabolic activity and cultivability at low temperatures; while spoilage bacteria can grow normally even at low temperatures (USDA FSIS, 2010). But this is not completely correct. Within pathogenic bacteria there is also great variation in the ability to survive and grow at low temperatures. For example, *L. monocytogenes*, one of the most dangerous foodborne pathogens, are well adapted to survive and multiply at low temperatures. On the other hand, *Campylobacter* species are less well adapted for survival outside the digestive tract of animals and are therefore particularly affected by low temperatures (Del Río, Capita, Prieto, & Alonso-Calleja, 2006). Even so, several studies show that *Campylobacter* can survive at low temperatures even without growing, and, because of this, they continue being the main bacterial contaminant in food products.

Therefore, to facilitate developing proper intervention strategies to control *Campylobacter*, there is a significant need to understand the change in bacterial numbers during refrigerated

storage. For that, cell suspensions of the four *Campylobacter* strains used in this chapter were prepared and stored at 4 °C for 72 hours as previously described in 3.2.5. **Figure 6** shows the decrease in cultivability of *Campylobacter* strains observed during storage at low temperature.

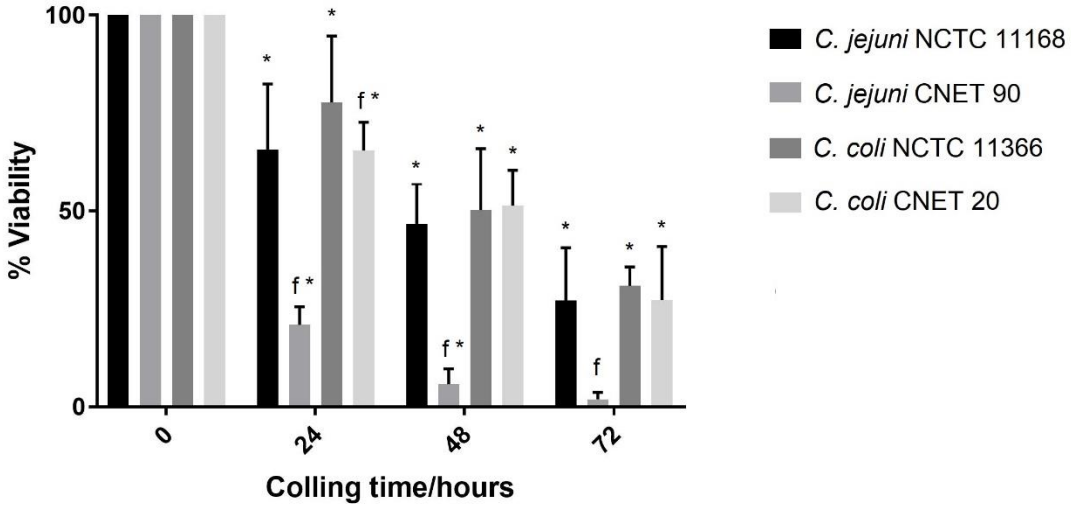

Figure 6. Decrease in cultivability of bacterial suspensions of *C. jejuni* NCTC 11168, *C. jejuni* CNET 90, *C. coli* NCTC 11366 and *C. coli* CNET 20 during storage at 4 °C. Samples collected from the bacterial suspensions were plated on CBA plates and incubated at 41.5 °C for 48 hours prior to CFUs count of survivors. The percentage of survivors (cultivable cells) was determined from the CFU difference between the initial suspensions and the samples collected at each time point. Each bar represents average CFU counts of two independents experiments ± standard deviations. Symbols indicate statistically different values ($p<0.05$) between different refrigeration times and the "0" timepoint for the same strain (*), and between strains for the same refrigeration time (*f*).

From the analysis of the figure, it is possible to observe a significant reduction in cultivability of *Campylobacter* strains during refrigerated storage. Although predictable due to the extra sensitivity to unfavourable conditions that characterize *Campylobacter* bacteria, this reduction was greater than expected. *C. jejuni* CNET 90 was by far the most sensitive strain to low temperature. In fact, only 20.93% ± 4.58% of the *C. jejuni* CNET 90 cells remained cultivable after 24 hours, almost losing total cultivability after 72 hours of refrigerated storage. All other strains were able to maintain superior levels of cultivability throughout the refrigerated storage, but, even so, the decrease in the number of cultivable cells is significant.

These results seem to contradict the assumption of some food safety institutions that refrigeration does not kill most bacteria but only slows down the rate at which they multiply. Nonetheless, caution should be taking regarding such statement, since *Campylobacter* is known for its VBNC state, which cannot be excluded here (Li et al., 2014). Even so, culture remains the gold standard for comparison and certification purposes, and, thus, any evaluation protocol should sill focus on the cultivability data. Concerning the percentage of cultivable cells for *C. jejuni* NCTC 11168, *C. jejuni* CNET 90, *C. coli* NCTC 11366 and *C. coli* CNET 20 after 72 hours of refrigeration;

values were 27.12% ± 13.51%, 1.91% ± 1.78%, 30.87% ± 4.83% and 27.27% ± 13.66%, respectively. These results reveal a large decrease in the cultivability of *Campylobacter* strains after 72 hours of refrigerated storage, showing that refrigeration actually has a significant effect on bacterial numbers.

Interestingly, these results also show that the decrease in cultivability after refrigerated storage is different within the genus *Campylobacter*, even for strains of the same species. In fact, within the same species, individual strains can be very different, presenting different responses to stressors, different rates of growth and multiplication, and differ on many other characteristics (Baron, 1996). The two strains of *C. coli* and the strain *C. jejuni* NCTC 11168 had statistically similar results during refrigerated storage. But, the strain *C. jejuni* CNET 90 had a more marked cultivability reduction than the other strains. Fernández et al. have suggested that *C. coli* strains are more resistant than *C. jejuni* strains to exposure to low temperatures (Fernández & Pisón, 1996). This is in part supported by our data. In fact, *C. jejuni* CNET 90 was far more sensitive to low temperatures than the two *C. coli* strains tested. Thus, it is possible to say that *C. jejuni* strains appear to be more sensitive to refrigeration than *C. coli* strains. Even so, since a limited number of strains have been tested, a more comprehensive study would be necessary to validate this information.

Regarding the explanation for the high number of negative samples in the preliminary tests; where suspensions of *C. jejuni* CNET 90 and *C. coli* CNET 20 were used to artificially contaminate samples of fresh raw meat (with concentrations between 1 and 100 CFU per 25 g), it becomes clear that storage at 4 °C for 72 hours has decreased the inoculation levels, since the inoculation did not take into account the decrease in the number of bacteria during refrigerated storage. If we apply the reduction percentages observed after 72 hours of refrigeration (Figure 5), only about 2% of *C. jejuni* cells and 27% of *C. coli* cells would be cultivable at this point. Theoretically, if 1 CFU, 10 CFU or 100 CFU of *C. jejuni* CNET 90 was initially inoculated, after 72 hours of refrigerated storage, only 0.02 CFU, 0.2 CFU or 2 CFU would remain, respectively. If we inoculated 1 CFU, 10 CFU or 100 CFU of *C. coli* CNET 20, after 72 hours of refrigerated storage 0.27 CFU, 2.7 CFU or 27 CFU would remain, respectively. Therefore, in view of these results, the large number of negative results obtained in the preliminary tests is not surprising. Thus, the following tests have already taken into account the expected decrease of *Campylobacter* cells during refrigerated storage so that, the desired levels of inoculation were warranted at the beginning of the enrichment.

3.3.3. Enrichment step optimization

Enrichment techniques for foodborne pathogens will always be dependent upon the compromise between selectivity and the inhibition of competitor organisms, and the recovery and growth of the target organism to detectable levels (Baylis, MacPhee, Martin, Humphrey, & Betts, 2000). Typically, the amount of pathogens present in food is low and the amount of background microflora is high. Furthermore, foods are generally subjected to harsh processes and conditions which, as shown above, induce the death of a large number of the bacterial cells and sub-lethal lesions in surviving cells. These cells, although injured, remain viable and can recover if they find adequate environmental conditions as in the human intestine. Thus, on the one hand, the enrichment step should ensure maximum growth of the target organism and inhibit the growth of the competitive microflora in order to guarantee the detection limit of the method used (which in the case of PNA FISH is approximately 10^5 CFU/mL); and, on the other, it should also be able to recover stressed cells, since the infectious dose is generally low for foodborne pathogens (≈ 500 cells for *Campylobacter* spp.) (Almeida et al., 2009; Hara-Kudo & Takatori, 2011).

As reviewed in the introduction to this chapter, there are several enrichment media described in the literature for the isolation of *Campylobacter* spp. from food. Regarding the time and conditions of incubation, due to the complex growth requirements that characterize the bacteria belonging to the genus *Campylobacter*, there is no great variation in the procedures found. Even so, the unpredictability and specific growth requirements, as well as the proportion of acquired sub lethal lesions, make the selection of the best enrichment procedure a difficult task, especially when the aim is to detect 1 CFU per test portion.

For this purpose, two enrichment broths were initially tested: BB and PB, both currently recommended by ISO 10272-1:2017. The incubation conditions used were also those recommended by ISO method: 4 hours at 37 °C plus 44 hours at 41.5 °C in microaerophilia. Fresh raw broiler meat and fresh raw ground pork samples were artificially contaminated and subjected to a refrigerated storage period as previously described in 3.2.6. Typically, a refrigerated storage period of 48 – 72 hours is used in food microbiology studies. However, since only the isolates (CNET strains) were used in these tests, it was decided to use a refrigerated storage period of only 24 hours due to high sensibility of these strains to low temperatures as observed in 3.3.2. In addition, to obtain reliable results, two independents assays with three replicates of each inoculation level were performed. After enrichment, the presence or absence of *Campylobacter* was determined by culture method and PNA FISH as described in 3.2.3 and 3.2.4 **(Table 7)**.

Table 7. PNA FISH and culture (ISO 10272-1:2017) results for the detection of *C. jejuni* and *C. coli* in different food matrices after enrichment with BB and PB. Food samples were artificially inoculated and subjected to a refrigerated storage period of 24 hours. The results presented comprise the two independent assays.

Contamination level (CFU/25 g) [a]	Bolton broth			
	***C. jejuni* CNET 90** Fresh raw broiler meat		***C. coli* CNET 20** Fresh raw ground pork	
	PNA FISH	Culture	PNA FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	+ (4/6)	+ (4/6)	+ (5/6)	+ (5/6)
10	+(6/6)	+ (6/6)	+ (6/6)	+ (6/6)
100	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)
Contamination level (CFU/25 g) [a]	**Preston broth**			
	***C. jejuni* CNET 90** Fresh raw broiler meat		***C. coli* CNET 20** Fresh raw ground pork	
	PNA FISH	Culture	PNA FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	+ (2/6)	+ (2/6)	+ (2/6)	+ (2/6)
10	+ (3/6)	+ (3/6)	+ (4/6)	+ (4/6)
100	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)

a. Real concentration of bacteria (CFU/25 g): 1st assay – *C. jejuni:* 2.0 ± 0.6| 9.0 ± 2.0| 88 ± 3.1; *C. coli:* 2.0 ± 0.0| 31.0 ± 11.7| 168.0 ± 21.4; 2nd assay – *C. jejuni:* 1.3 ± 0.6| 9.0 ± 2.0| 90.0 ± 4.4; *C. coli:* 1.0 ± 0.0| 11.0 ± 3.0| 99.3 ± 3.2;

As observed in **Table 7**, the samples enriched in Bolton broth resulted in more *Campylobacter* positive samples than in Preston broth. In total, *Campylobacter* was detected by both detection method in 91.7% (33/36) of inoculated samples enriched with Bolton broth, while only 63.9% (23/36) of inoculated samples enriched with PB were *Campylobacter*-positive. More specifically, enrichment in BB resulted in a positive detection of 5 out of 6 samples from both food matrices inoculated with 1 CFU/25g of *C. jejuni/C. coli*, while enrichment in PB resulted in a positive detection of only 2 out of 6 samples from both food matrices inoculated with 1 CFU/25 g of *C. jejuni/C. coli*. Enrichment in the Bolton broth also resulted in a positive detection of all samples from the other two levels of inoculation of both food matrices (10 CFU/25 g and 100 CFU/25 g), but the enrichment in the Preston broth resulted only in a positive detection of all samples of the highest inoculation level (100 CFU/25 g). Furthermore, all results were concordant either using BB/PB and subsequent culture (ISO 10272-1:2017) or using BB/PB and subsequent PNA FISH, indicating that the PNA FISH method has a sensitivity (limit of detection) similar to the traditional culture method. **Figure 7** shows examples of PNA FISH results obtained for the inoculation levels of both *Campylobacter* strains analysed.

These results reveal that enrichment in BB provide a higher recovery rate of *Campylobacter* than enrichment in PB for both *C. jejuni* and *C. coli* in the respective food matrices. This conclusion are in agreement with results presented by (Baylis et al., 2000; Seliwiorstow et al., 2016), who reported that significantly less *Campylobacter* positive samples were detected when PB was applied. The main reason for these results seems to be associated with the composition of selective agents that each medium present. Unlike PB, BB does not contain polymyxin and rifampicin, which can inhibit the growth of stressed *Campylobacter* cells (Baylis et al., 2000; J. Corry, Atabay, Forsythe, & Mansfield, 2003). In fact, the ISO 10272-1:2017 protocol recommends the application of Bolton broth for foods with a low level of background microflora and/or stressed *Campylobacter*, while the use of Preston broth is recommended for the detection of *Campylobacter* in food with a high level of background microflora. As previously noted in the cultivability study, refrigeration has a more significant effect on *Campylobacter* bacteria than previously thought, resulting in stressed cells among the survivors and sensitivity to selective agents to which they are normally resistant. In samples contaminated with few *Campylobacter*, it is normal that the proportion of stressed cells (number of stressed cells/total number of cells) during the preparation and refrigeration processes is higher than in samples contaminated with higher numbers of *Campylobacter*. That is why the samples inoculated with 1 CFU/25 g and 10 CFU/25 g of *C.jejuni/C.coli* resulted in less positive detections after enrichment in PB than in BB. Thus, since the goal is to develop a method capable of detecting 1 CFU per test portion, BB is the most appropriate enrichment medium to ensure the detection of *Campylobacter*.

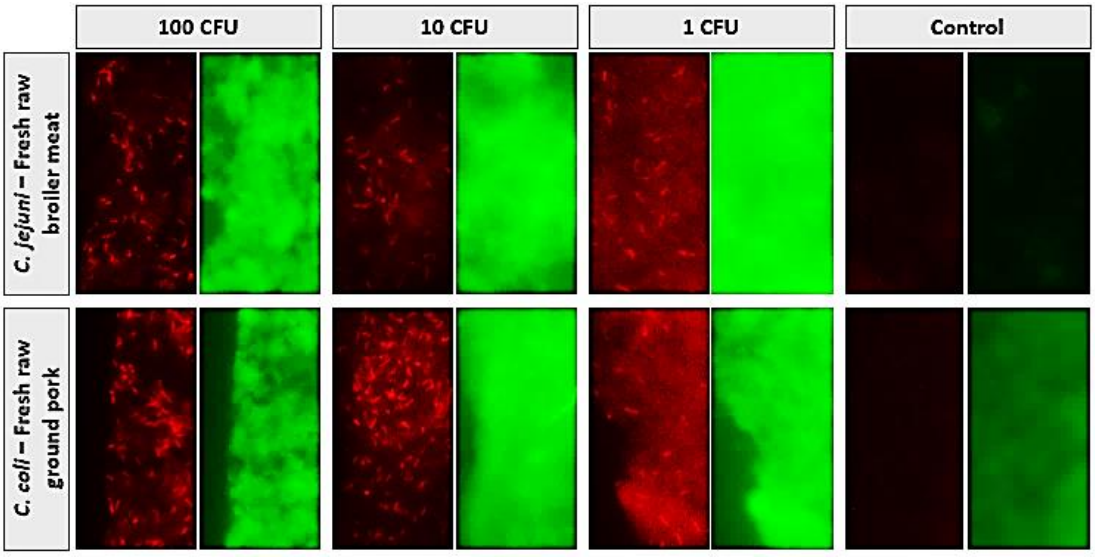

Figure 7. PNA FISH outcome of both food matrices artificially inoculated with 100, 10, 1 CFU of *C. jejuni* CNET 90 and *C. coli* CNET 20 per 25 g of food sample. At the lowest inoculation level (1 CFU/25 g) of both food matrices, samples with autofluorescence can be observed.

While the tests described above were performed using a 48-hour incubation time, as recommended by the ISO method, in food microbiology, the lower the enrichment time, the greater the probability of preventing contaminated foods from reaching the final consumer. Thus, 24 hours of enrichment in BB (4 hours at 37 °C plus 20 hours at 41.5 °C) was tested and compared with the enrichment of 48 hours in order to verify if it guarantees the level of detection established. For this purpose, fresh raw broiler meat and fresh raw ground pork were artificially contaminated and subjected to a refrigerated storage period of 24 hours as previously described in 3.2.6. After a 24h-enrichment, the presence or absence of *Campylobacter* was determined by culture and PNA FISH as described in 3.2.3 and 3.2.4 **(Table 8)**.

Table 8. PNA FISH and culture (ISO 10272-1:2017) results for the detection of *C. jejuni* and *C. coli* in different food matrices after enrichment with BB during two different enrichment times (24 hours and 48 hours). Food samples were artificially inoculated and subjected to a refrigerated storage period of 24 hours.

Contamination level (CFU/25 g) [a]	***C. jejuni* CNET 90 Fresh raw broiler meat**			
	24 hours		**48 hours**	
	PNA FISH	Culture	PNA FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	- (0/6)	+ (3/6)	+ (3/6)	+ (3/6)
10	+ (2/6)	+ (5/6)	+ (6/6)	+ (6/6)
100	+ (4/6)	+ (5/6)	+ (6/6)	+ (6/6)
Contamination level (CFU/25 g) [a]	***C. coli* CNET 20 Fresh raw ground pork**			
	24 hours		**48 hours**	
	PNA FISH	Culture	PNA FISH	Culture
0	- (0/2)	- (0/2)	- (0/2)	- (0/2)
1	+ (1/6)	+ (4/6)	+ (5/6)	+ (5/6)
10	+ (1/6)	+ (3/6)	+ (6/6)	+ (6/6)
100	+(3/6)	+(6/6)	+ (6/6)	+ (6/6)

a. Real concentrations of bacteria (CFU/25 g): 1st assay: *C. jejuni:* 1.7 ± 1.5| 15.0 ± 5.3| 106.3 ± 18.9; *C. coli:* 1.3 ± 0.6| 9.0 ± 1.7| 102.7 ± 21.6; 2nd assay – *C. jejuni*: 1.3 ± 0.6| 9.0 ± 2.0| 90.0 ± 4.4; *C. coli*: 1.0 ± 0.0| 11.0 ± 3.0| 99.3 ± 3.2;

As observed in the previous table, the samples incubated for 48 hours resulted in more *Campylobacter* positive samples than the samples incubated for 24 hours. In total, *Campylobacter* was detected in 32 out of 36 samples incubated for 48 hours, while only 11 out of 36 samples incubated for 24 hours were *Campylobacter*-positive. In addition, the 24-hour enrichment in Bolton Broth only detected a sample of pork of the lowest inoculation level, while the enrichment of 48

hours in Bolton broth detected 8 out of 12 samples of the lowest inoculation level from both food matrices. The enrichment of 48 hours in BB demonstrated similar performance to the previous test and the comparison with ISO method revealed a complete agreement. These results demonstrate that 48 hours enrichment is required to achieve the desired detection limit by PNA FISH using BB for the detection of *Campylobacter*. For the culture method, 24 hours of enrichment appears to be sufficient for the detection of *Campylobacter* in food, since the results obtained for the two enrichment times were similar. In fact, several studies show that the difference of positive samples by the culture method after 48 hours and 24 hours of enrichment is not significant, suggesting that 24 hours of enrichment are sufficient to detect *Campylobacter* by culture (Habib, Uyttendaele, & De Zutter, 2011; Seliwiorstow et al., 2016). This enrichment time difference for the culture method and PNA FISH method may be related to the detection limit of each method. In fact, the detection limit of the PNA FISH method is approximately 10^5 CFU/mL; while for the culture method, if 1 CFU grows in the plate after isolation with a 10 μL loop, the corresponding detection limit would be 100 CFU/mL. Thus, the PNA FISH method would require a longer enrichment to assure an adequate *Campylobacter* load. To ensure the detection of 1 CFU of *Campylobacter* in food samples by PNA FISH, Bolton broth should be used for 4 hours at 37 °C plus 44 hours at 41.5 °C under microaerophilic conditions (i.e., 5% O_2, 10% CO_2, and 85% N_2).

In addition to the results presented above, it is important to notice that the culture method revealed a high growth of competitive microflora in some plates (less frequent after enrichment in Preston broth and in samples plated on Preston agar) which interfered with the isolation and confirmation of presumptive colonies of *Campylobacter*. Although the presence of competitive microflora did not interfere directly with the performance of the PNA FISH method, as only the target cells became fluorescent (probe specificity), it must be delaying the growth of *Campylobacter* during sample enrichment. This fact may in future be problematic in food samples with a higher amount of competitive microflora, masking the presence of *Campylobacter* and, consequently, influencing the PNA FISH outcome. The emergence of antimicrobial resistance and especially the increasing prevalence of ESBL *E. coli* in food, especially in broiler meat, might be the reason. ESBL *E. coli* hydrolyse sodium cefoperazone, the principal selective agent in Bolton broth and mCCDA plates, using enzymes called beta-lactamases and allowing an abundant growth (Depoorter et al., 2012). Therefore, the use of β-lactamase inhibitors added to the enrichment broth to prevent the growth of competitive microflora should be analysed in an attempt to restore the selectivity of enrichment media. Inclusion of these compounds in the enrichment broth will allow faster growth

of *Campylobacter* (due to the absence of competitive microflora) and, perhaps, allow a decrease in the enrichment time required for the detection of the levels desired by PNA FISH.

Furthermore, it is also important to note that some samples of the PNA FISH method showed strong autofluorescence in the spectrum of the probe fluorescent marker, which in some cases made it difficult to determine the result, as can be seen in **Figure 7**. This phenomenon directly affected the visualization of the PNA FISH result and, as such, has become a priority in the optimization of the method. In this way, some techniques were selected and tested in order to eliminate, or at least reduce, this phenomenon. This issue is covered in more detail in the following chapter.

3.3.4. Reduction of autofluorescence signal

Optimal sensitivity of FISH requires bright signals and low background fluorescence (Szöllösi, Lockett, Balázs, & Waldman, 1995). However, background fluorescence is a common problem when using FISH techniques for the detection of pathogens from food samples. This phenomenon can arise from exogenous and/or endogenous sources, such as autofluorescence of bacterial components, food matrix particles or even components of the enrichment media. Autofluorescence may make it impossible to distinguish the specific probe signal from the non-specific background, especially for laboratory technicians not familiar with this phenomenon (Duffy, George, Love, & Zhang, 2012).

As mentioned previously in the optimization of the enrichment step, a strong background fluorescence was observed in some samples, which affected the visualization and confirmation of the PNA FISH outcome **(Figure 7)**. Initially, it was thought that the background fluorescence could result from the autofluorescence of red blood cells as previously reported by Almeida et al. (2010). Thus, an enrichment step using BB without addition of lysed horse blood was tested. However, this phenomenon is also often associated with particles of the food matrices, such as fatty compounds, which exhibit fluorescence in the observed spectrum range (Almeida, Sousa, et al., 2013); therefore, four different pre-hybridization approaches were tested in the enriched samples: (1) the use of a 1% Triton X-100 solution directly in enriched samples on the microscope slides to emulsify the fatty compounds in the samples; (2) a centrifugation at low-speed to remove autofluorescent food particles by sedimentation; (3) a 1:2 dilution of the enriched samples to dilute the autofluorescence particles; (4) a centrifugation at high-speed and resuspension of the pellet with a 0.1% Tween-80 solution to emulsify the fat compounds and disrupt possible hydrophobic and electrostatic interactions between the target organism and the food particles.

For this purpose, samples of fresh raw broiler meat and fresh raw ground pork were artificially contaminated, subjected to a refrigerated storage period of 24 hours, enriched for 48 hours, and then analysed directly without further treatment or submitted to the additional treatments as described in 3.2.7. The different treatments were performed from the same enriched samples (except the enrichment with BB without lysed horse blood that was tested with a different set of inoculated samples). Then, the presence or absence of *Campylobacter,* as well as the autofluorescence of the samples, was determined by PNA FISH as previously described in 3.2.4 **(Table 9)**.

Table 9. PNA FISH results for the detection of *C. jejuni* and *C. coli* in different food matrices after 48 hours enrichment with BB. Results were obtained using a direct hybridization protocol after enrichment with BB (with and without addition of lysed horse blood) and using different autofluorescence reducing-steps before the PNA FISH procedure. (+) Indicates condition

***C. jejuni* CNET 90** **Fresh raw broiler meat**						
Contamination level (CFU/25 g) [a]	**Treatment**					
	BB (standard condition)	BB without blood	1% Triton X-100	Low-speed centrifugation	1:2 Dilution	High-speed centrifugation + 0.1% Tween-80
0	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)
1	+ (2/3)	+ (1/3)	- (0/3)	+ (1/3)	- (0/3)	+ (1/3)
10	+ (3/3)	+ (1/3)	+ (1/3)	+ (3/3)	+ (2/3)	+ (3/3)
100	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
***C. coli* CNET 20** **Fresh raw ground pork**						
Contamination level (CFU/25 g) [a]	**Treatment**					
	BB (standard condition)	BB without blood	1% Triton X-100	Low-speed centrifugation	1:2 Dilution	High-speed centrifugation + 0.1% Tween-80
0	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)
1	+ (2/3)	- (0/3)	+ (1/3)	+ (1/3)	+ (1/3)	+ (2/3)
10	+ (3/3)	+ (2/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (3/3)
100	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)

a. Real concentrations of bacteria (CFU/25 g): 1ª assay: *C. jejuni:* 2.3 ± 1.5| 31.0 ± 6.0| 112.3 ± 9.9; *C. coli:* 1.7 ± 1.5| 9.0 ± 2.6| 93.3 ± 4.7;

More important than eliminating or reducing autofluorescence, it is essential that the treatments used maintain the limit of detection established in previous tests for the PNA FISH method. As observed in the previous table, not all techniques allowed to maintain the limit of

detection achieved in enrichment optimization for both *Campylobacter* strains. Standard enrichment in BB without further treatment resulted in the positive detection of 16 samples of both food matrices inoculated with *C. jejuni* CNET 90 and *C. coli* CNET 20. However, it continues to reveal a strong background fluorescence **(Figure 8)** which in some cases made it difficult to confirm the result of PNA FISH. Regarding the techniques used to reduce autofluorescence, enrichment in BB without addition of lysed horse blood resulted in the positive detection of 10 samples from both food matrices, detecting only one sample of the lowest inoculation level of *C. jejuni* CNET 90. Treatment of samples enriched with 1% Triton X-100 directly on the slides resulted in a positive detection of 11 samples from both food matrices, also detecting only one sample of the lowest inoculation level of *C. coli* CNET 20. In turn, the low speed centrifugation of the enriched samples resulted in a positive detection of 14 enriched samples from both food matrices. The 1:2 dilution in dH_20 of the enriched samples resulted in a positive detection of 10 samples from both food matrixes. Finally, high-speed centrifugation of the enriched samples followed by resuspension of the bacterial pellet with a 0.1% Tween-80 solution resulted in a positive detection of 15 samples from both food matrices.

The enrichment in BB without lysed horse blood and the 1:2 dilution of enriched samples were the two techniques tested which resulted in the highest number of negative results compared to standard enrichment with direct analysis by PNA FISH. In addition, the samples analysed by these two techniques showed little or no decrease in autofluorescence **(Figure 8)**. These results demonstrate that the addition of lysed horse blood to the enrichment broth is essential to ensure the desired level of detection (1 CFU/test portion) and the red blood cells were not responsible for the observed autofluorescence. Dilution of the samples also appears to dilute the number of *Campylobacter* cells to levels not detectable by the PNA FISH method, reducing the limit of detection desired.

The two techniques that involve sample centrifugation and the technique using Triton X-100 were those that demonstrated a better performance in reducing the autofluorescence observed **(Figure 8)**. However, the treatment of the enriched samples with Triton X-100 showed more negative results in comparison with the standard enrichment than the other two treatments. The explanation found for this was that the use of the detergent directly on the slides should interfere with the fixation of the cells to the slides and lead to the detachment/loss of these during washing procedures in the PNA FISH method. In turn, low speed centrifugation resulted in two more negative results than the direct analysis of the enriched samples, whereas high speed

centrifugation and pellet resuspension with a 0.1% Tween-80 solution resulted in only one more negative result. These two techniques were the ones that both demonstrated a better performance in the reduction of autofluorescence and allowed to maintain a similar limit of detection by PNA FISH **(Figure 8)**.

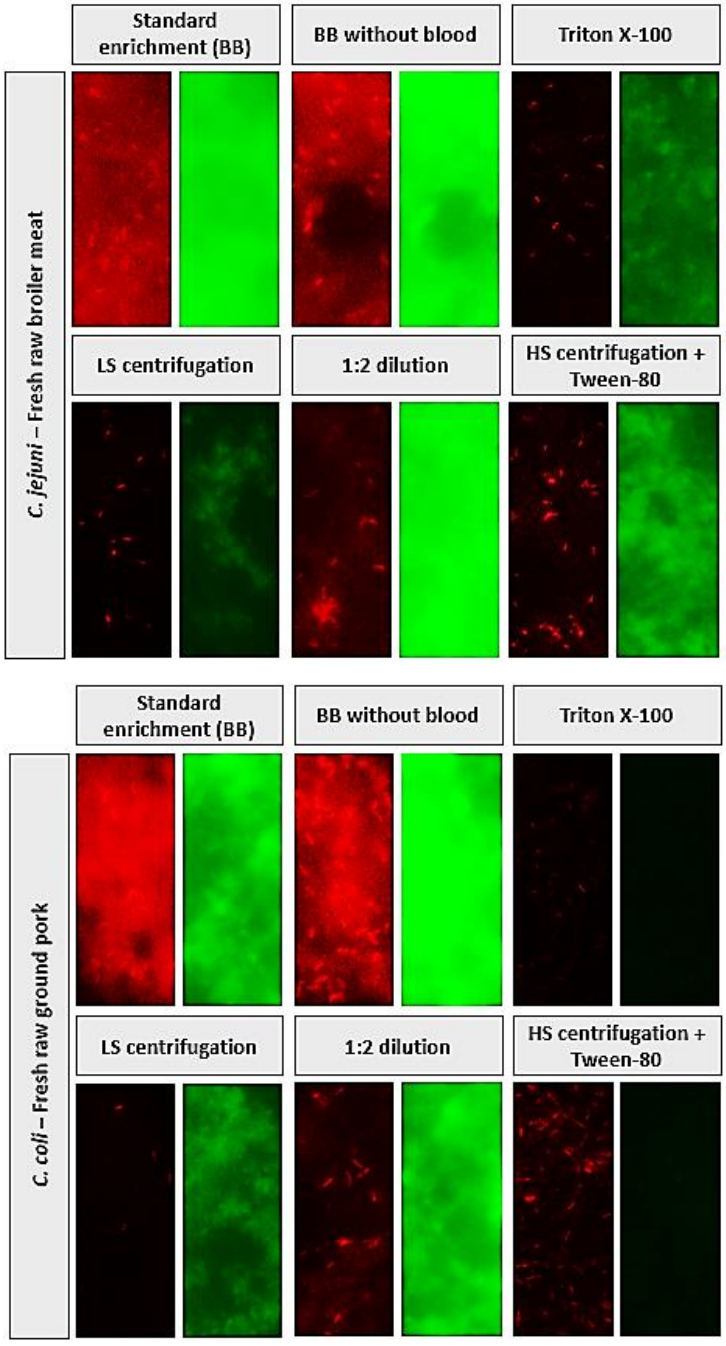

Figure 8. PNA FISH outcome for both food matrices artificially inoculated with 100 CFU/25 g of *C. jejuni* CNET 90 and *C. coli* CNET 20. Results were obtained using a PNA FISH protocol without additional treatment of BB enriched samples (with and without addition of lysed horse blood) or using one of the additional treatments selected before the PNA FISH procedure. It is possible to observe a more pronounced reduction of the autofluorescence intensity in the red and green channels in the treatments with Triton X-100, low speed centrifugation (LS) and high speed (HS) centrifugation with Tween-80 pellet resuspension.

However, a more careful analysis found that the amount of cells observed under the microscope was lower after the low-speed centrifugation of the samples than the high-speed centrifugation with resuspension in Tween-80 **(Figure 8)**. An analysis by PNA FISH of pellets discarded after the low speed centrifugation resulted in a positive detection for *Campylobacter* in most samples. This revealed that this centrifugation technique, even at low speed, led to the deposition of bacteria together with the food particles. Thus, the technique that showed a better ability to reduce the autofluorescence of the enriched samples without compromising the effectiveness of the PNA FISH method was the high-speed centrifugation with subsequent resuspension of the pellet with Tween-80.

Finally, the optimized enrichment step for the detection of *Campylobacter* in food samples by PNA FISH involves an incubation of the samples in BB for 4 hours at 37 °C plus 44 hours at 41.5 °C in a CO_2 incubator. Following the incubation, 1 mL of enriched suspension should be centrifuged at 10,000 g for 5 minutes and then the pellet resuspended with a 0.1% Tween-80 solution. Thereafter, 20 µL of the suspension should be placed on a coated glass slide to undergo the PNA FISH procedure. The PNA FISH method was thus prepared for the assessment of its performance in accordance with the requirements of AOAC International. For this purpose, several lots of *Campylobacter* test kits were produced.

CHAPTER IV

EVALUATION OF THE PNA FISH METHOD ACCORDING TO THE AOAC INTERNATIONAL REQUIREMENTS

4.1. Introduction

Direct hybridization methods are emerging as a suitable alternative to traditional culture-based techniques. Biomode, as an innovative biotechnology company focused on the commercialization of rapid diagnostic kits based on PNA FISH technology for the food industry, always seeks to meet the requirements imposed by food safety institutions to ensure customer confidence in their products. In this way, the implementation of new detection methods in the market depends mainly on the certification processes that assure to the clients the conformity of the method with the international standards. Those processes are ruled by international entities that provided guidance, assistance and, ultimately, certify the product's compliance. For food safety, two entities are recognised worldwide: AOAC International (US) and AFNOR (France).

Biomode normally works in partnership with the AOAC International. This entity is a globally recognised, independent and not-for-profit association founded in 1884 as the Association of Official Agricultural Chemists by the United States Department of Agriculture, to establish uniform chemical analysis methods for analysing fertilizers. Later in 1965, the AOAC's name changed to Association of Official Analytical Chemists to accurately reflect its scope beyond agriculture. The designation AOAC International emerged in 1991 to reflect the international scope of the organization's work and its international membership. Currently, AOAC is no longer an acronym and has no legal meaning, but can be interpreted as Association of Analytical Communities (AOAC International, 2017).

The AOAC Research Institute is responsible for AOAC conformity assessment programs including the AOAC Performance Tested Methods (PTM) program, which evaluates the technical characteristics of new alternative methods. Briefly, the PTM program is a certification program that ensures that certified methods meet the claims documented by the manufacturer and therefore are used throughout the global market and within the regulatory area as a quality assurance. The PTM program includes 5 steps to obtain a final certification.

Step 1: Application process - consists in submit a completed PTM Review Application Package to the AOAC Research Institute. Method developers are required to collect and submit data as a manuscript using the appropriate AOAC format that supports the product performance claims of the test kit. The required documents for submission include a completed PTM Review Application; Method Package, Method User Instructions, or Method User Guide(s); Method Packaging Labels; Manufacturing Quality Assurance/Quality Control Synopsis; Method Developer Validation Study Report; and Proposal for Independent Laboratory Testing Protocol. In the validation

study, the method developer needs to perform a set of tests which include an inclusivity test (to ensure that the method can identify specifically many different strains within the target group); an exclusivity test (to determine the ability of the test method to discriminate target organism from non-target organisms); a food matrix comparison test (the most important validation test, where the candidate method is compared with the appropriate reference method which is performed in different food matrices), a product consistency and stability test (to examine the lot-to-lot variability and the product stability over the time); a kit variation test (to examine the variation of some test kits of the same lot in the detection of the organism); and a robustness test (to evaluate the ability of the method to remain unaffected by small variations in method parameters that might be expected to occur when the method is performed by the end user).

Step 2: Independent Laboratory Testing Process - consists in a verification of the evaluated performance claims and method applicability. It requires an agreement between AOAC Research Institute and an independent laboratory. Only AOAC Research Institute communicates with the independent laboratory. The independent laboratory will reproduce part of the internal validation study performed by the method developer described above.

Step 3: Expert Peer Review of Data - the method developer compiles the method developer study (single laboratory validation or SLV) and the independent laboratory study into a formatted manuscript. Then a set reviewers examine the independent data and the SLV data. Reviewers also evaluate the data against the proposed method applicability and the package insert information.

Step 4: AOAC Performance Tested℠ Certification - if the experts concur on their recommendation to approve, then the Research Institute grants AOAC Performance Tested℠ certification for the method. Certification involves signing a Certification Mark License Agreement.

Finally, step 5: Annual Review and Certification Renewal - the certifications should be annually reviewed and renewed and if no changes, a new certificate is granted for the next year.

In this way, this chapter had as objective the accomplishment of all the tests required by AOAC International to obtain certification of the developed method. For this, the complete PNA FISH method developed and optimized in this dissertation was tested according to the AOAC guidelines as impartially as possible to ensure that it is ready for moving on to the certification process.

4.2. Material and methods

4.2.1. Bacterial species and culture maintenance

Several bacterial strains were used in the different tests within the Chapter IV **(Table 14 and 15)**. The strains used were obtained from the internal culture collection of the CEB. *Campylobacter* strains were maintained into CBA plates (Oxoid) supplemented with 5% (volume/volume) defibrinated horse blood (Oxoid) at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation), set to 5% O_2, 10% CO_2, and 85% N_2, and streaked onto fresh plates every 48 hours. *Helicobacter* strains and *Arcobacter butzleri* strains were also maintained in CBA plates (Oxoid) supplemented with 5% (volume/volume) defibrinated horse blood (Oxoid) under the same culture conditions as the *Campylobacter* strains, but at 37 °C. *Salmonella* strains, *Escherichia coli* strains, *Listeria monocytogenes* strains, *Citrobacter freudii* and *Klebsiella pneumoniae* were maintained into tryptic soy agar plates (TSA; Liofilchem, Italy) at 37 °C for 24 hours and streaked onto fresh plates every 48 hours. *Wolinella succinogenes* were maintained into CBA plates (Oxoid) supplemented with 5% (volume/volume) defibrinated horse blood (Oxoid) at 37 °C in anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) using AnaeroGen Compact™ gas generation kits (Oxoid), and streaked onto fresh plates every 48 hours.

4.2.2. Inclusivity and exclusivity test

The purpose of inclusivity testing is to ensure that the candidate test can identify many different strains of the target species, while the exclusivity test is to determine the ability of the candidate test to discriminate target organisms from non-target organisms (AOAC International, 2012). For the inclusivity test, 50 *Campylobacter* strains were used; while for the exclusivity test, 32 related non-*Campylobacter* strains were used. All strains used as well as their source/origin are documented in **Table 14 and 15**. Inclusivity and exclusivity evaluations were performed together as one study. For this purpose, a small loopful of biomass from 24-hours cultures of each bacterial strain was suspended directly in 3 mL of BB. The cell suspensions were then incubated at 37 °C for 4 hours plus 44 hours at 41.5 °C in a CO_2 incubator (HERAcell 150i), set to 5% O_2, 10% CO_2, and 85% N_2. Following incubation, 1 mL of enriched suspensions was centrifuged at 10 000 g for 5 minutes and the pellet was resuspended with a 0.1% Tween-80 solution. Thereafter, the inclusivity and exclusivity test samples were blind coded and intermingled, and then 20 µL of each treated enriched suspension were placed on a microscope slide and tested in duplicate following the PNA FISH procedure previously described in 3.2.4. In the end, the results were decoded and

analysed. If any result was unexpected, the strain in question was tested once more by the same procedure. Finally, the experimental values of sensitivity and specificity of the PNA FISH method were calculated using the Clinical Calculator 1 software, available in http://vassarstats.net/.

4.2.3. Food matrix comparison test

The certification process requires the comparison of the candidate method with a reference method. For *Campylobacter* spp., the commonly used reference method is ISO 10272-1: 2017. At this stage, AOAC International allows validation of the candidate method for a range of specific food matrices that are selected by the method developer. The final certification will only recognize the validity of the method for the matrices claimed in the matrix comparison study (AOAC International, 2012). In our case, the PNA FISH method was tested for the two food matrices considered the main transmission vehicles of *Campylobacter:* fresh raw broiler meat and fresh raw ground pork. All food matrices were obtained from local retailers in Braga, stored at 4 °C ± 2 °C and subjected to analysis as rapidly as possible.

Prior to starting, all matrices were pre-screened for natural contamination. Only if natural contamination were not found, the product was used for the next steps. Following the screening, each matrix batch was divided into three portions. Two portions of each matrix were inoculated with the appropriate amount of cells of the respective target species, so that on the day of initiation of pre-enrichment there are two inoculation levels: low level (approx. 0.2 – 2 CFU/25 g); and high level (approx. 2 – 10 CFU/25 g). For this purpose, *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 inoculums were prepared as previously described in 3.2.2 to simulate the natural contamination of the broiler meat and pork meat samples, respectively, during the production and storage processes. The other portion served as the uncontaminated level.

After the artificial contamination, all food samples were mixed well by kneading, with extreme care to achieve as close as possible a homogeneous distribution of microorganisms and, then, the microorganisms were allowed to equilibrate in the matrix at 4 °C ± 2 °C for 48 hours, as required by the AOAC guidelines. The amount of cells inoculated into the samples took into account the decrease in cultivability during the refrigerated storage of each strain previously determined in 3.3.2. After that, the low-level inoculated samples were divided into 20 replicate test portions of 25 g and the high-level inoculated samples were divided into 5 replicate test portions of 25 g, and placed in sealed stomacher bags with filters. In addition, 5 replicates of the uncontaminated samples from each matrix were also placed in sealed stomacher bags. **Table 10** shows the details of the matrices, contamination levels and replicates required for this type of test.

Table 10. Food matrix comparison test design with the details for the matrices, target strains, contamination levels and replicates.

Group A: Meat Products			
Food Matrix	**Strain**	**Contamination Level**	**Number of Replicates**
Fresh raw broiler meat	*C. jejuni* NCTC 11168	0 CFU/25 g	5
		0.2 – 2.0 CFU/25 g	20
		2.0 - 10 CFU/25 g	5
Fresh raw ground pork	*C. coli* NCTC 11366	0 CFU/25 g	5
		0.2 – 2.0 CFU/25 g	20
		2.0 - 10 CFU/25 g	5

After the preparation of the test portions, the PNA FISH method and the ISO method were performed. As both method share a common enrichment procedure, the same set of inoculated samples were used for both procedures (paired study). For this purpose, the 25 g test portions were homogenized in 225 mL of BB in a stomacher (Eco Blender II) for 30 seconds. After that, they were incubated at 37 °C for 4 hours plus 44 hours at 41.5 °C in a CO_2 incubator (HERAcell 150i, Thermo Electron Corporation), set to 5% O_2, 10% CO_2, and 85% N_2. Following incubation, 1 mL of enriched suspensions was centrifuged at 10,000 g for 5 minutes and the pellet was resuspended with a 0.1 % Tween-80 solution. Then, 20 µL of each treated enriched suspension was placed on a microscope slide and the PNA FISH procedure was performed as previously described in 3.2.4. At the same time, a direct loopful of each enriched suspension was plated in mCCDA and Preston agar for isolation and confirmation of the presence of *Campylobacter*, according to the ISO method previously described in 3.2.3. Finally, the data obtained were analysed by Probability of Detection (POD) statistics for each food matrix.

4.2.4. Most probable number (MPN) analysis

The MPN procedure was also performed in conjunction with the food matrix comparison test in order to estimate the real number of organisms inoculated in food samples. According to the AOAC guidelines, it is necessary to perform a 3-level MPN for the low and the high inoculation levels of each food matrix. For that, 5 additional test portions of 50 g and 5 additional test portions of 10 g were taken from the low-level inoculated samples of each matrix and prepared as described for the samples from the matrix comparison study. For the high-level inoculated samples, 5 additional test portions of 10 g and 5 additional test portions of 5 g of each food matrix were also taken and prepared as described for the samples from the matrix comparison study. Then, each test portion was analysed following the ISO reference method as previously described in 3.2.3. The

MPN analysis scheme also make use of the reference method replicates from the matrix comparison study. So, the results of reference method of the 20 test portions of 25 g of low-level inoculated samples and the 5 test portions of 25 g of high-level inoculated samples of both matrices, analysed in matrix comparison study described above, were used as the third level for the MPN **(Table 11)**. Finally, the number of positives from the 3 MPN levels of each food matrix were used to determine the MPN values and the 95% confidence intervals using the MPN calculator available at http://www.lcfltd.com/customer/LCFMPNCalculator.exe.

Table 11. MPN summary table. Reference method replicates are the low-level and high-level inoculated test portions from both food matrices analysed in the matrix comparison study.

Contamination Level	Test portion	Number of Replicates
0.2 – 2.0 CFU/Test portion	50 g	5
	25 g	20[a]
	10 g	5
2.0 - 10 CFU/Test portion	25 g	5[a]
	10 g	5
	5 g	5

a. Replicates of the reference method used in the food matrix comparison test described above.

4.2.5. Product consistency and stability test

The product consistency (lot-to-lot) and stability study is a study required for PTM certification to ensure that the performance of the product is consistent from lot-to-lot and over time under normal storage conditions for the shelf life of the product. The final certification requires an accelerated stability test and a real-time stability test (AOAC International, 2012). For a matter of time, only the accelerated stability test was performed in the work presented here. For this purpose, three candidate test kits from different lots were stored at room temperature and tested at specific time points **(Table 12)**.

At each time point, each kit from different lots was tested with a pure suspension of a target strain (*C. jejuni* NCTC 11168) and a pure suspension of a non-target strain (*E. coli* CECT 515), prepared as previously described in 3.2.2. *C. jejuni* strain was diluted to 10 times the limit of detection of the PNA FISH method (≈10^6 CFU/mL) and *E. coli* strain was diluted to 100 times the limit of detection of the FISH method (≈10^7 CFU/mL). Each assay was performed with 10 replicates of diluted target strain and 5 replicates of diluted of non-target strain, placed directly in coated glass slides in a randomized blind coded mode and following the PNA FISH procedure previously described in 3.2.4. At the end, the results were decoded and POD values with confidence intervals were calculated and analysed for variable detection between lots and over time.

Table 12. Storage temperature and time points for analysis of candidate test kits by the two methods required in the AOAC guidelines.

Candidate Test Kit	Storage Temperature	Time Points (from the date of production)
Accelerated	25 °C ± 2 °C	1 days, 30 days, 60 days, 90 days
Real time	5 °C ± 2 °C	3 months, 6 months, 9 months, 12 months, 18 months

4.2.6. Kit variation test

This study, required for PTM certification, is designed to ensure that the performance of the method is consistent from test kit to test kit (AOAC International, 2012). In this way, three test kits from a single lot of candidate test kits were examined to verify that there is no statistical difference in detection between test kits. For this, a pure suspension of *C. jejuni* NCTC 11168 and a pure suspension of *E. coli* CECT 515 were prepared as previously described in 3.2.2. The target strain was diluted to the limit of detection of the FISH method (≈10^5 CFU/mL) while the non-target strain was used without dilution. Then, each kit was tested with 10 replicates of the target strain and 5 replicates of undiluted non-target strain, placed directly in coated glass slides in a randomized blind coded mode and following the PNA FISH procedure previously described in 3.2.4. At the end, the results were decoded and POD values with confidence intervals were calculated and analysed for variable detection between kits.

4.2.7. Ruggedness test

The ruggedness test evaluates the ability of the method to remain unaffected by small variations in method parameters that might be expected to occur when the method is performed by an end user. It is important to know that statistically significant findings in this experiment are not indicative of a faulty method, and the discovery of significance is not a barrier to successful method validation. Normally, at least 3 parameters were chosen to vary and the test may utilize pure cultures or may require the inoculation of food matrices.

For the evaluation of the *Campylobacter* test kits, it was defined that this test should be performed in a food matrix since the enrichment step and the matrix components can influence the PNA FISH outcome. Three parameters were selected to vary in this test: hybridization time,

hybridization temperature; and time to result after mounting. In addition, an experiment factorial design was used to obtain a more comprehensive analysis of the selected parameters **(Table 13)**.

Table 13. Factorial design of the test taking into account the selected parameters. For each parameter, a lower value and a higher value than the normal value was tested (except for the hybridization time which was tested two lower values).

Treatment Combination	Hybridization time	Hybridization temperature	Time to result after mounting
1	30 mins	52 °C	30 mins
2	30 mins	52 °C	90 mins
3	30 mins	62 °C	30 mins
4	30 mins	62 °C	90 mins
5	45 mins	52 °C	30 mins
6	45 mins	52 °C	90 mins
7	45 mins	62 °C	30 mins
8	45 mins	62 °C	90 mins
9 (Nominal Values)	60 mins	57 °C	0 mins

For this purpose, fresh raw broiler meat was obtained from local retailers and pre-screened for natural contamination as described above in 4.2.3. Thereafter, the matrix was divided into 20 replicates of 25 g of test portion and placed directly into stomacher bags with filter. Of these, 10 test portions were artificially contaminated with approximately 1 CFU/25 g of *C. jejuni* NCTC 11168 and 10 test portions were artificially contaminated with 10 CFU/25 g of *E. coli* CECT 515. The inoculation of the samples was performed with inoculums of both bacteria previously prepared as described in 3.2.2. Then, the contaminated test portions were homogenized and incubated with BB as described above in 4.2.3. Following incubation, 1 mL of the enriched suspensions were taken and processed as described above in 4.2.3, but varying the selected parameters according to the **Table 13**. Finally, POD values with confidence intervals were calculated and analysed for variable detection due to changes in parameter settings.

4.3. Results and discussion

4.3.1. Inclusivity and exclusivity test

As noted in the introduction, the validation study is intended to determine the performance characteristics of the candidate method by evaluating several key parameters. The purpose of the inclusivity test is to ensure that the method developed identifies as many different strains of the

target species as possible; while the purpose of the exclusivity test is to determine the ability of the method to discriminate target organisms from non-target organisms. The inclusivity is therefore associated with the sensitivity of the method and the exclusivity is associated with the specificity of the method. In order to evaluate these parameters, 50 different *Campylobacter* strains and 32 non-*Campylobacter* species were collected from the internal culture collection of the CEB and Biomode. The inclusivity strains were target strains that the method should detect while the exclusivity strains were non-target strains that should not be detected but which are potentially cross-reactive because of their phylogenetic proximity or association with the same transmission vehicles (i.e. other foodborne pathogens). Since the candidate method includes an enrichment step that influences the growth of all bacteria, this assay was performed using pure cultures from each species/strain grown in enrichment medium (BB) as described above in 4.2.2. Each bacterium was initially tested in duplicate. If any unexpected result was detected, the analysis was repeated once again for the species in question. The performance of the developed method is presented in **Table 14 and 15**.

Table 14. *Campylobacter* strains included in the inclusivity test and their outcome with the PNA FISH method optimized in the previous chapter. HP - Partial hybridization (usually associated with a weak fluorescence signal).

Target species	Strain[a]	Source (Origin)	PNA FISH outcome
Campylobacter coli	NCTC 11366	Culture collection	+
C. coli	4133	Pig isolate (Denmark)	+
C. coli	A2	Pig isolate (DK)	+
C. coli	CNET 19	Chicken outbreak (Netherlands)	+
C. coli	CNET 20	Chicken outbreak (NL)	+
C. coli	CNET 51	Human isolate (France)	+
C. coli	CNET 62	Chicken isolate (NL)	+
C. coli	CNET 64	Chicken isolate (DK)	+
C. coli	CNET 69	Pig isolate (NIUK)	+
C. coli	CNET 70	Pig isolate (DK)	+
C. coli	CNET 72	Pig isolate (DK)	+
C. coli	CNET 82	Chicken isolate (United Kingdom)	+
Campylobacter jejuni subsp. jejuni	NCTC 11168	Culture collection	+
C. jejuni	2140	Chicken isolate (NL)	+
C. jejuni	CNET 22	Chicken outbreak (DK)	+
C. jejuni	CNET 23	Chicken outbreak (DK)	+
C. jejuni	CNET 31	Human isolate (NL)	+
C. jejuni	CNET 73	Cattle isolate (NL)	+

C. jejuni	CNET 75	Cattle isolate (UK)	+
C. jejuni	CNET 76	Chicken isolate (NL)	+
C. jejuni	CNET 77	Chicken isolate (NL)	+
C. jejuni	CNET 83	Chicken isolate (UK)	+
C. jejuni	CNET 85	Chicken isolate (UK)	+
C. jejuni	CNET 86	Chicken isolate (UK)	+
C. jejuni	CNET 87	Chicken isolate (UK)	+
C. jejuni	CNET 88	Chicken isolate (UK)	+
C. jejuni	CNET 90	Chicken isolate (UK)	+
C. jejuni	CNET 91	Chicken isolate (DK)	+
C. jejuni	CNET 92	Chicken isolate (DK)	+
C. jejuni	CNET 93	Chicken isolate (DK)	+
C. jejuni	CNET 94	Chicken isolate (DK)	+
C. jejuni	CNET 95	Chicken isolate (DK)	+
C. jejuni	CNET 96	Chicken isolate (DK)	+
C. jejuni	CNET 100	Human isolate (UK)	+
C. jejuni	CNET 101	Human isolate (UK)	+
C. jejuni	CNET 103	Water isolate (UK)	+
C. jejuni	CNET 105	Ovine isolate (UK)	+
C. jejuni	CNET 106	Ovine isolate (UK)	+
C. jejuni	CNET 107	Ovine isolate (UK)	+
C. jejuni	CNET 109	Canine isolate (Sweden)	+
C. jejuni	CNET 110	Canine isolate (SE)	-
C. jejuni	CNET 111	Canine isolate (SE)	+
C. jejuni	CNET 112	Canine isolate (SE)	+
C. jejuni	CNET 113	Canine isolate (SE)	+
Campylobacter hyointestinalis	DSM 19053	Culture collection	+
Campylobacter upsaliensis	DSM 5365	Culture collection	-
Campylobacter fetus subsp. fetus	DSM 5361	Culture collection	+
Campylobacter sputorum subsp. sputorum	DSM 5363	Culture collection	HP
Campylobacter lari subsp. lari	DSM 11375	Culture collection	+
Campylobacter mucosalis	DSM 21682	Culture collection	-

a. DSM, German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen); CNET, *Campylobacter* collection hosted by DSM (Campynet); NCTC, National Collection of Type Cultures;

Table 15. *Campylobacter* strains included in the exclusivity test and their outcome with the PNA FISH method optimized in the previous chapter. HP - Partial hybridization (usually associated with a weak fluorescence signal). NI - It was not possible to obtain information on the specific strain or source.

Non-target species	Strain[a]	Source (Origin)	PNA-FISH outcome
Wolinella succinogenes	DSM 1740	Culture collection	-
Arcobacter butzleri	LCDC 11516	Culture collection	-
A. butzleri	CCUG 30485	Culture collection	-
Helicobacter pylori	NCTC 11637	Culture collection	-
H. pylori	166	Human isolate (Germany)	-
H. pylori	169	Human isolate (DE)	-
H. pylori	1198	Human isolate (DE)	-
H. pylori	2191	Human isolate (Portugal)	-
H. pylori	2538	Human isolate (PT)	-
H. pylori	2768	Human isolate (PT)	-
H. pylori	3991	Human isolate (PT)	-
H. pylori	9156	Human isolate (PT)	-
H. pylori	9159	Human isolate (PT)	-
Helicobacter canis	CIP 104753	Culture collection	-
Helicobacter pomatensis	CIP 104249	Culture collection	HP
Helicobacter bilis	NI	NI	-
Helicobacter salomonis	CIP 105607	Culture collection	-
Helicobacter canadensis	CCUG 47163	Culture collection	-
Helicobacter muridarum	2A5	Human isolate (United States)	-
Helicobacter cinaedii	33221-1.2	Human isolate (US)	-
Escherichia coli	CECT 434	Culture collection	-
E. coli	CECT 515	Culture collection	-
E. coli	CECT 533	Culture collection	-
Escherichia hermannii	ATCC 33650	Culture collection	-
Citrobacter freudii	SGSC 5345	Culture collection	-
Klebsiella pneumoniae	ATCC 11269	Culture collection	-
Salmonella entritidis	ATCC 13076	Culture collection	-
S. entritidis	SGSC 2474	Culture collection	-
Salmonella tiphymurium	SGSC 1423	Culture collection	-
S. tiphymurium	SGSC 2243	Culture collection	-
Listeria monocytogenes	CECT 933	Culture collection	-
L. monocytogenes	CECT 5873	Culture collection	-

a. DSM, Deutsche Sammlung von Mikroorganismen; SGSC, Salmonella Genetic Stock Centre; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CECT, Spanish Type Culture Collection; CIP, Collection of Institute Pasteur; LCDC, Laboratory Center for Disease Control (US)

Of the 50 specific inclusivity strains tested, 46 were detected and 4 were not detected. Those strains not detected were the following: *C. jejuni* CNET 110, *C. upsaliensis* DSM 5365, *C. sputorum subsp. bubulus* DSM 5363 and *C. mucosalis* DSM 21682 **(Table 14)**. In turn, of the 30 specific exclusivity strains tested, 29 were not detected and one was detected: *H. pomatensis* CIP 104249 **(Table 15)**.

These results correspond to a sensitivity rate of 92.0% (95% CI: 79.9% - 97.4%) and a specificity rate of 96.9% (95% CI: 82.0% - 99.8%). Compared with the theoretical values of sensitivity and specificity calculated for the PNA probe designed to detect *Campylobacter* spp. (99.8%, and 100% respectively – Appendix 1), a small difference, more significant for the sensitivity value, between the theoretical values and the practical values obtained is verifiable. This may, in part, be explained by the fact that the theoretical values were calculated based only on the probe sequence and on the genomes of microorganisms present in the database, while the practical values were calculated based on the results of the complete PNA FISH method which includes the enrichment step which influences the growth of all microorganisms. Thus, it is important to analyse the unexpected results.

Of the four *Campylobacter* strains not detected by the PNA FISH procedure after enrichment, three did not show any fluorescence signal in the replicates performed, whereas one (*C. sputorum subsp. bubulus*) revealed a weak fluorescence signal but was considered negative since it can be problematic for unexperienced lab technicians **(Table 14)**. In order to find an explanation for these results, a direct analysis of these fourth *Campylobacter* strains by PNA FISH was performed. In addition, the identity of these strains was evaluated using a latex agglutination test specific for the identification of enteropathogenic *Campylobacter* spp. (Oxoid). The results were positive for all of them, revealing that these bacteria are actually *Campylobacter* and that the PNA FISH protocol allow the detection of these strains (data not provide). Thus, the only explanation for the negative results should be related with the enrichment step. After the investigation in the DSM database, it was verified that *C. upsaliensis* DSM 5365 requires incubation in microaerophilia with H_2 (i.e. 6% O_2, 7,5% H_2, 4% CO_2, 82.5% N_2); *C. sputorum subsp. bubulus* DSM 5363 reveals difficulty in growing in liquid medium containing blood; and *C. mucosalis* DSM 21682 requires anaerobic incubation. The negative results of these three strains may therefore be explained by the alternative growth requirements they require and which are not met by the enrichment step followed. Regarding *C. jejuni* CNET 110, there is no obvious explanation for the negative detection. However, within the same species, genetic variability may be large enough to occur different growth

behaviours. This *C. jejuni* strain, although detectable by the PNA probe, may exhibit slow growth and thus not reach a level detectable by the detection method.

Regarding the exclusivity test **(Table 15)**, *H. pomatensis* CIP 104249 resulted in a weak fluorescence signal which may be confusing to unexperienced laboratory technicians. A direct analysis by PNA FISH revealed the same weak fluorescent signal and the latex agglutination test specific for the identification of enteropathogenic *Campylobacter* spp. (Oxoid) resulted also in a weak positive for this *Helicobacter* strain. In fact, the latex agglutination test instructions state obtaining possible cross-reactivity with this particular species. This may be related to the phylogenetic proximity between bacteria of the genera *Helicobacter* and *Campylobacter*. Both are part of the same order *Campylobacterales* and, therefore, there is a genetic proximity that may result in similar phenotypic characteristics and growth behaviours (Lastovica et al., 2013). Thus, it is possible that this specific *Helicobacter* strain is phylogenetically closer to the bacteria of the genus *Campylobacter* than the other bacteria of its genus, and therefore survive the selective effect of the enrichment step and result in the weak fluorescence signal observed. On the other hand, since the cross-reactivity of this *Helicobacter* strain is already known in other molecular methods, it is possible that it is poorly classified. A more in-depth analysis would be needed to prove this hypothesis.

4.3.2. Food matrix comparison test

The food matrix comparison test is the central study in the certification process of an alternative method for the detection of foodborne pathogens. In this test, the candidate method is compared to a cultural reference method, and must demonstrate a better or similar performance to obtain alternative method certification. AOAC International recognizes claims for the range of specific food matrices successfully validated. The number of different matrices required for testing depends on the applicability of the method (AOAC International, 2012). For C*ampylobacter* spp., it makes sense to evaluate the performance of the PNA FISH method for at least the two main food matrices associated with contamination: broiler meat and pork. The method comparison study consisted on evaluating a total of 30 paired sample replicates of each food matrix by both detection methods. Within the sample sets, there were 5 uninoculated samples (0 CFU/25 g), 20 low level inoculated samples (0,2-2 CFU/25 g), and 5 high level inoculated samples (2 - 10 CFU/25 g).

For this purpose, *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 inoculums were prepared and used to artificially contaminate fresh raw broiler meat and fresh raw ground pork, respectively. After preparation, all test portions were analysed by the PNA FISH method and the ISO reference

method. Furthermore, the real concentration of *Campylobacter* in the low-level inoculum and high-level inoculum was determined by MPN technique. A summary of the method comparison results is presented in **Table 16**.

Table 16. Comparative results for the detection of *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 in fresh raw broiler meat and fresh raw ground pork, respectively, by the PNA FISH method and the ISO 10272-1:2017 reference culture method.

Fresh raw broiler meat ***C. jejuni* NCTC 11168**[1]			**Fresh raw ground pork** ***C. coli* NCTC 11366**[1]		
MPN determination (CFU/25 g)	**PNA FISH**	**ISO 10272-1:2017**	**MPN determination** (CFU/25 g)	**PNA FISH**	**ISO 10272-1:2017**
Control	0/5	0/5	Control	0/5	0/5
0.85 (0.51 – 1.40)	12/20	12/20	0.84 (0.49 – 1.40)	11/20	11/20
6.4 (2.9 – 14.4)	5/5	5/5	5,6 (2.6 – 11.8)	5/5	5/5

1. NCTC, National Collection of Type Cultures

Firstly, it is important to note that the contamination levels of the inoculated samples were fulfilled as confirmed by the MPN determination. A summary of the MPN results is presented in Appendix 2. In addition, as required by the AOAC guidelines, fractional positive results (25 - 75% positive) were also fulfilled for the low-level inoculated samples of both food matrices. For the low inoculation level, there were 12 positive results in 20 samples of fresh raw broiler meat and 11 positive results in 20 samples of fresh raw ground pork, for both the PNA FISH method and the culture reference method. Similarly, for the high inoculation level, there were 5 positive results in 5 samples of fresh raw broiler meat and 5 positive results in 5 samples of fresh raw ground pork, for both the PNA FISH method and the culture reference method. These results demonstrate a similar performance between the two methods, proving that the candidate method guarantees the same level of detection as the reference method.

Nevertheless, an analysis of POD statistics was performed as required in the AOAC guidelines. The POD values and respective 95% confidence intervals (LCL, UCL) for the PNA FISH method and the reference method were calculated as the number of positive outcomes divided by the total number of trials, and, subsequently, the difference in the performance of the PNA FISH method and the reference method, dPOD (C, R), and respective 95% confidence intervals (LCL, UCL) were calculated according the Appendix J of the AOAC Official Methods of Analysis Manual for paired studies (AOAC International, 2012). As expected, equal POD values and dPOD (C, R)

values of 0.00 (95% CI: 0.00;0.00) were obtained for both food matrices. Thus, the POD analysis confirms that there is no statistically significant difference at the 5% level between the PNA FISH method and the reference method for the detection of *Campylobacter* in fresh raw broiler meat and fresh raw ground pork. Detailed results of the POD analyses are presented in Appendix 3.

4.3.3. Product consistency and stability test

The product consistency and stability test is a study designed to ensure that the performance of the product is consistent from lot-to-lot and over time under normal storage conditions (AOAC International, 2012). For the lot-to-lot consistency study, three lots of product must be tested and show consistent results. For product stability, either a real-time and an accelerated stability study must be carried out to support the shelf life of the product.

For this purpose, three different *Campylobacter* test kits from different lots were compared for the assessment of the stability and consistency of the method. In this report only the accelerated stability data is presented. Accelerated stability study was performed based on the Arrhenius model (assuming E_a=20 kcal, 1 year at 5 °C ≈ 32 days at 25 °C) to evaluate the shelf life of the kit at 2–8 °C. The three test kits were stored at 25 °C and tested at specific time points after the date of production. For this, 10 replicates of *C. jejuni* NCTC 11168 and 5 replicates of *E. coli* CECT 515 were used to evaluate each kit at each time point as described above in 4.2.5 **(Table 17)**.

Table 17. Results of the consistency and accelerated stability test and the respective POD values for the three test kits from different lots.

Time points (days)		*C. jejuni* NCTC 11168				*E. coli* CECT 515			
		N[a]	X[b]	POD_T[c]	95% CI[e]	N[a]	X[b]	POD_{NT}[d]	95% CI[e]
1	**Kit 1**	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 2	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 3	10	10	1	0.72; 1	5	0	0	0.00; 0.43
30	**Kit 1**	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 2	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 3	10	10	1	0.72; 1	5	0	0	0.00; 0.43
60	**Kit 1**	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 2	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 3	10	10	1	0.72; 1	5	0	0	0.00; 0.43
90	**Kit 1**	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 2	10	10	1	0.72; 1	5	0	0	0.00; 0.43
	Kit 3	10	10	1	0.72; 1	5	0	0	0.00; 0.43

a. N = Number of tests.
b. X = Number of positive tests.
c. POD_T = PNA FISH positive outcomes divided by the total number of trials with target *C. jejuni* NCTC 11168.
d. POD_{NT} = PNA FISH positive outcomes divided by the total number of trials with non-target *E. coli* CECT 515.
e. 95% CI = Range of POD values with a 95% confidence level.

As can be seen in the previous table, the POD values calculated for the accelerated stability assay have not shown any differences on the performance of *Campylobacter* kits from different lots over time. The 10 replicates of the target strain were detected successfully at all time points by the three kits analysed, while the 5 replicates of the non-target strain did not show any fluorescence signal for the same analysis. These results of product consistency and stability show the reliability of the method from lot-to-lot and over time for the analysed period. In addition, based on these results and the Arrhenius model, it is possible to claim a shelf life for the *Campylobacter* test kits that ensure the perfect performance of the developed method.

4.3.4. Kit variation test

The kit variation study is designed to ensure that the performance of the method is consistent from test kit to test kit. This test guarantees repeatability of the method, i.e. ensures that equivalent results are obtained for different kits with the same method for equivalent samples by the same operator using the same equipment in short time intervals (AOAC International, 2012).

For this purpose, three different *Campylobacter* test kits of the same lot were compared for the assessment of the variations amongst kits. The strains used in this study were the same as those used in product consistency and stability study, *C. jejuni* NCTC 11168 and *E. coli* CECT 515. For each test kit, 10 replicates of target strain and 5 replicates of non-target strain were used to evaluate the performance of each kit as previously described in 4.2.6. The performance of the three *Campylobacter* test kits is shown in the **Table 18**.

Table 18. Results of the kit variation study and the respective POD values for the three test kits from the same lot.

Test kit	*C. jejuni* NCTC 11168				*E. coli* CECT 515			
	N[a]	X[b]	POD_T[c]	95% CI[e]	N[a]	X[b]	POD_{NT}[d]	95% CI[e]
Kit 1	10	7	0.7	0.40; 0.89	5	0	0	0.00; 0.43
Kit 2	10	8	0.8	0.49; 0.94	5	0	0	0.00; 0.43
Kit 3	10	7	0.7	0.40; 0.89	5	0	0	0.00; 0.43

a. N = Number of tests.
b. X = Number of positive tests.
c. POD_T = PNA FISH positive outcomes divided by the total number of trials with target *C. jejuni* NCTC 11168.
d. POD_{NT} = PNA FISH positive outcomes divided by the total number of trials with non-target *E. coli* CECT 515.
e. 95% CI = Range of POD values with a 95% confidence level.

Firstly, it is important to note that fractional positive results (25 – 75% positive) were fulfilled for the target species in the tests performed for the three test kits, as required in AOAC guidelines (AOAC International, 2012). Regarding the analysis of the results, as observed in Table 17, no significant differences were observed among the three kits. The 5 replicates of the non-target strain

were negative for the three test kits. For the target strain, not all 10 replicates were positive for the three test kits, but this result is not surprising, since *C. jejuni* NCTC 11168 was diluted to a level that yields fractional recovery. Thus, these results demonstrate that there is no variation in the performance of the different kits of the same lot, ensuring the repeatability of the method.

4.3.5. Ruggedness test

The ruggedness study is probably the most laborious certification test due to the number of samples analysed. This test intends to evaluate the effect of small variations in the method parameters that may occur while using the kit. Typically, it is made a good faith effort to choose parameters that are most likely to affect the analytical performance and consequently determine the range of variations that can occur without adversely affecting analytical results. In this way, the hybridization temperature, hybridization time and time to result after mounting were chosen to vary and, consequently, to analyse its effects on the performance of the developed PNA FISH method. In addition, a factorial design of the experiment was followed for a more comprehensive analysis of the effect of the variation of these three parameters.

The test involved the inoculation of ten 25 g portions of fresh raw broiler meat with 0.5 to 1 CFU/test portion of *C. jejuni* NCTC 11168 (a level that yields fractional positives) and another ten 25 g portions of fresh raw broiler meat with 10 CFU/test portion of *E. coli* CECT 515. All test portions were incubated with BB for 4 hours at 37 °C plus 44 hours at 41.5 °C in microaerophilia and then analysed by the PNA FISH method, varying the three selected parameters as described in 4.2.7. The PNA FISH results and respective POD analysis are presented in **Table 19**.

Table 19. PNA FISH results and POD statistics analysis for the ruggedness study in fresh raw broiler meat artificially contaminated with *C. jejuni* NCTC 11168 and *E. coli* CECT 515. In addition, the accuracy of the results obtained by the various combinations of parameters tested in relation to the normal combination of these parameters was calculated.

	***C. jejuni* NCTC 11168**						
Combination	**N[a]**	**X[b]**	**POD_T[c]**	**95% CI[e]**	**$dPOD_{CB}$[f]**	**95% CI[e]**	**Accuracy[g]**
1	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
2	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
3	10	9	0.90	0.60; 0.83	0.40	0.28; 0.52	60%
4	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
5	10	6	0.60	0.31; 0.83	0.10	-0.08; 0.28	50%
6	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
7	10	10	1.00	0.72; 1.00	0.50	0.37; 0.63	50%
8	10	6	0.60	0.31; 0.83	0.10	-0.11; 0.31	30%
9 (Baseline)	10	5	0.50	0.24; 0.76	/	/	100%

E. coli CECT 515							
Combination	**N[a]**	**X[b]**	**POD_{NT}[d]**	**95% CI[e]**	**$dPOD_{CB}$[f]**	**95% CI[e]**	**Accuracy[g]**
1	10	9	0.90	0.60; 0.83	0.90	0.82; 0.98	10%
2	10	10	1.00	0.72; 1.00	1.00	1.00; 1.00	0%
3	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
4	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
5	10	8	0.30	0.11; 0.60	0.80	0.70; 0.90	20%
6	10	10	1.00	0.72; 1.00	1.00	1.00; 1.00	0%
7	10	9	0.90	0.60; 0.83	0.90	0.82; 0.98	10%
8	10	6	0.60	0.31; 0.83	0.60	0.48; 0.72	40%
9 (Baseline)	10	0	0.00	0.00; 0.28	/	/	100%

a. N = Number of tests;
b. X = Number of positive tests;
c. POD_T = Positive outcomes divided by the total number of trials with target *C. jejuni* NCTC 11168 for the correspondent condition;
d. POD_{NT} = Positive outcomes divided by the total number of trials with non-target *E. coli* CECT 515 for the correspondent condition;
e. 95% CI = Range of POD/dPOD values with a 95% confidence level;
f. $dPOD_{CB}$ = Difference between the condition analysed (C) and the baseline (B) POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level;
g. Accuracy = Percentage of PNA FISH results (either positive or negative) that corresponds to true results according to the baseline.

First, it is important to note that it is mandatory to consider that the results obtained for the normal values of the parameters (baseline) are the true results and the reference to draw conclusions from the ruggedness test. In this way, the analysis of the robustness study shows that the variation of the selected parameters significantly affects the performance of the PNA FISH method. The POD values for all parameter combinations were higher than the baseline POD value for both the target and non-target strains. Although this result seems to suggest that the combinations tested are better than the baseline values, this is not true. POD values greater than baseline POD values suggest that we are dealing with false positive results that are overestimating these values. The best example confirming this hypothesis is the POD values obtained for the combinations tested with the non-target strain. While 0 would be the expected result for all conditions tested in the non-target species, this was only observed for the baseline condition. All other POD values were greater than 0. Since the developed PNA FISH method had already been shown to be *Campylobacter* specific and capable of discriminating *E. coli* strains under normal conditions, the POD obtained at the baseline are in line with the previous results. However, the increased POD values observed at the other conditions show that parameter variation can result in false positive results. Furthermore, the confidence interval of the $dPOD_{CB}$ values also showed significant differences in the performance of the PNA FISH method between the combinations tested and baseline conditions (the confidence interval of the dPOD values does not contain zero), except for combinations 5 and 8 of the target strain.

Since POD statistical analysis suggested by the AOAC guidelines can at some extent difficult the results interpretation (as some would expect that the higher the POD the better), an additional parameter was estimated to simplify the data analysis. In this way, an accuracy analysis was included since it compares the number of correct results (either positive or negative) obtained for the different combinations, using the baseline results as reference. Thus, as can be seen in the **Table 19**, accuracy values calculated were all low for the combinations tested, including for combinations 5 and 8 of the target strain which previously have not shown dPOD significant differences from the baseline. This shows that the POD analysis alone can be limited, as accuracy values clearly show that all the combinations tested have a marked negative effect in the performance of the PNA FISH method. We conclude that the difference observed between POD analysis and accuracy analysis is a consequence of false positive and false negative results for each combination tested in comparison with the baseline.

In general, the results do not allow to conclude which of the parameters tested has a more significant effect on the performance of the method, as the factorial design used implies the simultaneous variation of three parameter. However, we believe that the hybridization time and the hybridization temperature are the two main parameters responsible for the significant difference in the performance of the PNA FISH method. Tests carried out previously under the normal conditions of the parameters of the PNA FISH method (baseline), but in which the result time after mounting ranged from hours to weeks, did not reveal unexpected results as obtained in this study. On the other hand, short hybridization times and low hybridization temperatures typically favour non-specific binding of the probes to non-target sequences leading to false positives, which explain the higher POD values and the statistically significant difference for the tested combinations involving these kind of conditions (Noll, 2016). Higher hybridization temperatures than optimal value do not favour the binding of the probes to their target, leading typically to a decrease in the number of true positive results and, consequently, resulting in false negatives (Noll, 2016). Nevertheless, the conditions tested in this study involving a higher hybridization temperature appear to have favoured both false negatives and false positives, as explained above.

The most plausible explanation seems to be associated with the blocker probe which together with the *Campylobacter*-specific probe is present in the hybridization solution. The blocker probe was designed to prevent cross-hybridization of the detecting-probe to non-specific sequences of *E. coli* and *Salmonella* strains. These two species present a three-nucleotide mismatch; but, in fact, previous optimization experiments of the PNA FISH procedure have shown a weak fluorescent

signal with both species, even under optimum conditions for hybridization with the *Campylobacter* sequence. This was the reason why a blocker prober was included in the hybridization reaction. This blocker probe has a perfect match with *E. coli* and *Salmonella*, thus preventing the binding of the mismatched probe. Precisely, the non-target strain used in the ruggedness test was an *E. coli* strain, which, in addition, is naturally associated with the food matrix used. Thus, we believe that, with the increase of the hybridization temperature, the blocker probe loses the blocking effect that prevents nonspecific binding of the *Campylobacter* probe. Consequently, the detecting probe will gain access to the mismatched sequences in the *E. coli*. It is thus assumed that high hybridization temperatures are affecting the binding of the specific probe to the *Campylobacter* sequence (false negatives) and compromises the effect of the blocker probe on the non-target strains; but, it still allows partial binding of the specific probe to non-target sequences which the blocker probe prevented under normal conditions (resulting in false positives as well). One way to prove this hypothesis and to solve this problem would be to increase the affinity of the blocker probe for the *E. coli* and *Salmonella* strains sequences. By increasing the size of the blocker probe in 1 or 2 nucleotides, the blocker probe affinity will increase. Thus, the melting temperature of the probe increases and, consequently, the effect of the probe on preventing non-specific binding of the *Campylobacter* probe is maintained even at higher temperatures.

Nevertheless, this statistically significant findings are not indicative of a faulty method and is not a barrier to successful method validation, as mentioned before (AOAC International, 2012). In fact, this findings should be seen as indications of a well-designed experiment and be used to modify method kit instructions, emphasize areas of caution, or even possibly to narrow specific parameter options (AOAC International, 2012).

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

5. Conclusions and future perspectives

As discussed initially, this master's thesis had two main objectives. The first involved the optimization of an enrichment step that allow the detection of *Campylobacter* in food samples by PNA FISH. The second objective involved the evaluation of the developed PNA FISH method, taking into account the requirements of AOAC International to obtain PTM certification.

From the initial tests, it is possible to conclude that *Campylobacter* bacteria are more affected by refrigerated storage (2 - 8 °C) than most foodborne pathogens, revealing a marked decrease in cultivability after 72 hours. However, the decreasing rate observed at refrigerated conditions might be dependent on the strain. Any study involving artificial contamination of food samples with *Campylobacter* and requiring the storage of samples at low temperatures should consider the decrease in cultivability to guarantee desired levels of pathogen for the tests.

Regarding the optimization of the enrichment step, it was concluded that Bolton broth allowed the best growth conditions and, consequently, provided the detection of 1 CFU/25 g of *C. jejuni* and *C. coli* inoculated in fresh raw broiler meat and fresh raw ground pork samples, respectively. Concerning the incubation time, it has been shown that 48 hours are essential to allow the growth of *Campylobacter* to levels detectable by PNA FISH. However, the presence of competitive microflora, even after selective enrichment, may be affecting the growth and multiplication of *Campylobacter*. In the work presented, the background microflora level seems not to have affected significantly, however in the future this factor may be decisive for the detection of *Campylobacter* in food samples with a high level of competitive microflora. In addition, it was found that some components of food matrices confer autofluorescence to the samples, making it difficult to discriminate the signal from the target cells of the background signal. This effect was particularly noticed for samples with low concentrations of target pathogens.

At the end, it was concluded that the best enrichment step comprises: an incubation with Bolton broth for 4 hours at 37 °C plus 44 hours at 41.5 °C in microaerophilia (i.e. 5% O_2, 10% CO_2, and 85% N_2). After incubation, 1 mL of the enriched suspension should be centrifuged at 10 000 g for 5 minutes and the pellet resuspended in a 0.1% Tween-80 solution to decrease the autofluorescence conferred by the matrix compounds. Thereafter, 20 µL of the treated suspension can be placed on a coated glass slide to follow the PNA FISH procedure developed by Biomode.

Regarding the second objective, it can be concluded that the PNA FISH method shows a performance similar to the ISO 10272-1:2017 reference method for the two claimed food matrices, fresh raw broiler meat and fresh raw pork. The inclusivity and exclusivity study revealed a sensitivity

of 92.0% and a specificity of 96.9% for the PNA FISH method. In addition, the studies of stability, consistency and kit variation have also showed the reliability of the method. On the other hand, it can be concluded from the ruggedness test that the parameters and variations tested affect significantly the performance of the PNA FISH procedure. However, as previously reported, these findings are not indicative of a faulty method and are not a barrier to successful method validation. Nonetheless, those findings should be better investigated and used to improve the product leaflet.

In general, the developed PNA FISH method for detection of *Campylobacter* spp. in food samples is provided in a ready-to-use format kit with 4 short and easy-to-do steps, providing results in less than 3 hours after a 48-hours enrichment, while the ISO 10272-1:2017 method requires at least another 48 hours to obtain results. Unlike other molecular detection methods, PNA FISH does not involve DNA extraction or amplification, is not susceptible to inhibitory compounds and only detects bacterial cells with stable rRNA content.

Nevertheless, since the certification process involves a great effort at economic and resource level of the company, it is important to evaluate some previously described evidences that may influence the performance of the PNA FISH method. First, the abundant growth of competitive microflora evidenced by the culture method may in the future represent a barrier to the detection of *Campylobacter* in food samples with a high level of background microflora, as mentioned above. Then, the inclusivity and exclusivity test revealed that the developed PNA FISH method is not 100% specific and sensitive for *Campylobacter* spp, as would be ideal. Also, although it is not an obstacle to the final certification, the ruggedness test demonstrated that the accuracy of the method cannot be ensured if the hybridization conditions are not maintained very strictly. In this way, some suggestions of future and complementary work are presented:

- Since the high level of competitive microflora should be related to the increased prevalence of ESBL *E. coli* in food products, the use of β-lactamase inhibitors (such as potassium clavulanate and triclosan), added to the enrichment broth, should be tested to restore the selectivity of enrichment media. In this way, it will be possible to assure with greater confidence that the enrichment step guarantees the level of detection desired even in food samples with a high level of competitive microflora;
- To increase the sensitivity of the method, the possibility of suggesting an optional enrichment step in the PNA FISH method for the detection of the rarer/atypical *Campylobacter* strains (that have different growth requirements) should be analysed. Also, the signal obtained for

the *H. pomatensis* should be investigated and further genomic studies can be done to evaluate any possible misclassification of this species;

- Finally, a deep kinetic study involving a longer blocker probe should be performed in order to understand the complex results obtained in the robustness test; to confirm the hypothesis presented in this thesis; and, finally, to provide quantitative information on the blocking effect of the probe for a more efficient design of the probe-blocking pairs.

CHAPTER VI

REFERENCES

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REFERENCES

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CHAPTER VI

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APPENDIX

Appendix 1 - Theoretical evaluation of the PNA probe performance

The *Campylobacter* probe used was designed a few years ago, and, as such, it was required to re-evaluate the intrinsic characteristics of the probe since the genomic information deposited in public databases has evolved greatly in recent years. Thus, its performance was evaluated to determine the theoretical values for sensitivity and specificity using the ProbeCheck software, available in the ARB Silva database (http://www.arb-silva.de/). Specificity was calculated as the number of non-target sequences present in the database that do not match with probe divided by the total number of non-target sequences present in the database. Sensitivity was calculated as the number of target sequences that match with the probe divided by the total number of target sequences present in the database. For this theoretical estimate, the PNA probe sequence was aligned with all sequences present in the large-subunit ([LSU]; 23/28S) ARB Silva database and only good quality sequences with at least 1,900 bp were considered.

Table 20. Calculation of the theoretical values of specificity and sensitivity of the PNA probe used in the work presented.

Total number of sequences present in the database (TSs)	**Total number of *Campylobacter* sequences present in database (TCs)**	**Number of non-*Campylobacter* sequences matched to the probe (nCs)**	**Number of *Campylobacter* sequences matched to the probe (Cs)**
154,297	1,640	7	1,636
Specificity		**Sensitivity**	
100.0%		99.8%	

The specificity and sensitivity values were calculated using the software present in https://www.arb-silva.de/search/testprobe/ for the ARB SILVA database: LSU 128 REF (0 Mismatches). Specificity was calculated as [(TSs – TCs) – nCs]/(TSs - TCs) × 100; Sensitivity was calculated as (Cs/TCs) × 100;

The probe was matched to all Campylobacter sequences present in the database, except for 4 sequences from rare and poorly documented *Campylobacter* strains. On the other hand, the probe also had correspondence for 7 sequences of *Sulfurimonas gotlandica,* chemoautotrophic and psychrotolerant epsilonproteobacterium found in deep water.

Appendix 2 - MPN summary table for the matrix comparison study (AOAC test)

Table 21. MPN summary table for fresh raw broiler meat contaminated with *C. jejuni* NCTC 11168.

Fresh raw broiler meat *C. jejuni* NCTC 11168	
Low Level Inoculum (0.2-2 MPN/Test Portion)	
5 x 50 g	4/5
20 x 25 g (Reference Samples)	12/20
5 x 10 g	1/5
MPN/Test portion	0.85
Low Conf. Limit MPN/Test Portion	0.51
High Conf. Limit MPN/Test Portion	1.40
High Level Inoculum (5-10 MPN/Test Portion)	
5 x 25 g (Reference Samples)	5/5
5 x 15 g	5/5
5 x 5 g	3/5
MPN/Test portion	6.4
Low Conf. Limit MPN/Test Portion	2.9
High Conf. Limit MPN/Test Portion	14.4

[a]MPN were calculated for both inoculation levels using the results of the culture method of the fresh raw broiler meat samples and the fresh raw ground pork samples and using the LCF MPN Calculator version 1.6 provided by AOAC-RI **http://www.lcfltd.com/customer/LCFMPNCalculator**

Table 22. MPN summary table for fresh raw ground pork contaminated with *C. coli* NCTC 11366.

Fresh raw ground pork *C. coli* NCTC 11366	
Low Level Inoculum (0.2-2 MPN/Test Portion)	
5 x 50 g	4/5
20 x 25 g (Reference Samples)	11/20
5 x 10 g	2/5
MPN/Test portion	0.84
Low Conf. Limit MPN/Test Portion	0.49
High Conf. Limit MPN/Test Portion	1.40
High Level Inoculum (5-10 MPN/Test Portion)	
5 x 25 g (Reference Samples)	5/5
5 x 15 g	4/5
5 x 5 g	4/5
MPN/Test portion	5.6
Low Conf. Limit MPN/Test Portion	2.6
High Conf. Limit MPN/Test Portion	11.8

[a]MPN were calculated for both inoculation levels using the results of the culture method of the fresh raw broiler meat samples and the fresh raw ground pork samples and using the LCF MPN Calculator version 1.6 provided by AOAC-RI **http://www.lcfltd.com/customer/LCFMPNCalculator**

Appendix 3 – POD analysis for the matrix comparison study (AOAC test)

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):

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

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 $x = 0$.

$$\mathrm{POD} = 0$$

$$\mathrm{LCL} = 0$$

$$\mathrm{UCL} = 3.8415/(N + 3.8415)$$

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

$$\mathrm{POD} = 1$$

$$\mathrm{LCL} = N/(N + 3.8415)$$

$$\mathrm{UCL} = 1$$

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

$$\mathrm{POD} = x/N$$

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

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

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:

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

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:

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

$$\mathrm{SE}_{d\mathrm{POD}} = \frac{s_d}{\sqrt{N}}$$

and

$$LCL = d\mathrm{POD} - t_c \cdot \mathrm{SE}_{d\mathrm{POD}}$$

$$UCL = d\mathrm{POD} + t_c \cdot \mathrm{SE}_{d\mathrm{POD}}$$

where t_c 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 9. Equations necessary to calculate the POD values, dPOD values and respective 95% confidence intervals for paired studies.

Table 23. Intermediate calculations required for the POD analysis of the comparative study of the PNA FISH method and ISO 10272-1:2017 reference method.

Replicate	Fresh raw broiler meat *C. jejuni* NCTC 11168				Fresh raw ground pork *C. coli* NCTC 11366			
	PNA FISH	ISO 10272-1:2017	d_i	$(d_i - dPOD)^2$	PNA FISH	ISO 10272-1:2017	d_i	$(d_i - dPOD)^2$
Uninoculated samples					**Uninoculated samples**			
Rep1	0	0	0	0	0	0	0	0
Rep2	0	0	0	0	0	0	0	0
Rep3	0	0	0	0	0	0	0	0
Rep4	0	0	0	0	0	0	0	0
Rep5	0	0	0	0	0	0	0	0
Low level inoculated samples					**Low level inoculated samples**			
Rep1	1	1	0	0	1	1	0	0
Rep2	1	1	0	0	0	0	0	0
Rep3	1	1	0	0	1	1	0	0
Rep4	1	1	0	0	1	1	0	0
Rep5	0	0	0	0	0	0	0	0
Rep6	1	1	0	0	1	1	0	0
Rep7	0	0	0	0	1	1	0	0
Rep8	0	0	0	0	0	0	0	0
Rep9	1	1	0	0	0	0	0	0
Rep10	0	0	0	0	0	0	0	0
Rep11	1	1	0	0	1	1	0	0
Rep12	1	1	0	0	0	0	0	0
Rep13	1	1	0	0	0	0	0	0
Rep14	1	1	0	0	1	1	0	0
Rep15	1	1	0	0	1	1	0	0
Rep16	0	0	0	0	1	1	0	0
Rep17	0	0	0	0	0	0	0	0
Rep18	1	1	0	0	1	1	0	0
Rep19	0	0	0	0	0	0	0	0
Rep20	0	0	0	0	1	1	0	0
High Level inoculated samples					**High Level inoculated samples**			
Rep1	1	1	0	0	1	1	0	0
Rep2	1	1	0	0	1	1	0	0
Rep3	1	1	0	0	1	1	0	0
Rep4	1	1	0	0	1	1	0	0
Rep5	1	1	0	0	1	1	0	0

Table 24. Results of the POD analysis for the PNA FISH method vs ISO 10272-1:2017 reference method in fresh raw broiler meat contaminated with *C. jejuni* NCTC 11168.

Inoculation level	Concentration CFU/25 g (MPN)	PNA FISH method (C)				ISO 10272-1:2017 (R)				C vs R	
		N[a]	X[b]	POD (C)[c]	95% CI[e]	N[a]	X[b]	POD (R)[c]	95% CI[e]	dPOD (C,R)[d]	95% CI[e]
Control	0 (0;0)	5	0	0.00	0.00; 0.43	5	0	0.00	0.00; 0.43	0.00	0.00; 0.00
Low level	0.85 (0.51; 1.40)	20	12	0.60	0.39; 0.78	20	11	0.60	0.39; 0.78	0.00	0.00; 0.00
High level	6,4 (2.9; 14.4)	5	5	1.00	0.57; 1.00	5	5	1.00	0.57; 1.00	0.00	0.00; 0.00

a. N = Number of test portions
b. x = Number of positive test portions
c. POD = PNA FISH method (C)/ISO method (R) positive outcomes divided by the total number of trials
d. dPOD (C,R) = Difference between the candidate method presumptive result and candidate method confirmed result POD values
e. 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level

Table 25. Results of the POD analysis for the PNA FISH method vs ISO 10272-1:2017 reference method in fresh raw ground pork contaminated with *C. coli* NCTC 11366.

Inoculation level	Concentration CFU/25 g (MPN)	PNA FISH method (C)				ISO 10272-1:2017 (R)				C vs R	
		N[a]	X[b]	POD (C)[c]	95% CI[e]	N[a]	X[b]	POD (R)[c]	95% CI[e]	dPOD (C,R)[d]	95% CI[e]
Control	0 (0;0)	5	0	0.00	0.00; 0.43	5	0	0.00	0.00; 0.43	0.00	0.00; 0.00
Low level	0.84 (0.49; 1.40)	20	11	0.55	0.34; 0.74	20	11	0.55	0.34; 0.74	0.00	0.00; 0.00
High level	5,6 (2.6; 11.8)	5	5	1.00	0.57; 1.00	5	5	1.00	0.57; 1.00	0.00	0.00; 0.00

a. N = Number of test portions
b. x = Number of positive test portions
c. POD = PNA FISH method (C)/ISO method (R) positive outcomes divided by the total number of trials
d. dPOD (C,R) = Difference between the candidate method presumptive result and candidate method confirmed result POD values
e. 95% CI = 95% confidence interval for POD/dPOD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level