Traditional and Molecular Methods vs Biosensors for the Detection of Pathogens in Poultry Meat

a Ph.D. dissertation presented by

Marco Fontanot

to the

University of Udine

for the degree of Ph.D. in the subject of

Food Science (Cycle XXVI)

Department of Food Science

UNIVERSITY OF UDINE

Italy

March 2014

Coordinator: Mara Lucia Stecchini, professor

Department of Food Science

University of Udine, Italy

Supervisor: Marisa Manzano, professor

Department of Food Science

University of Udine, Italy

Reviewers: Robert S. Marks, professor

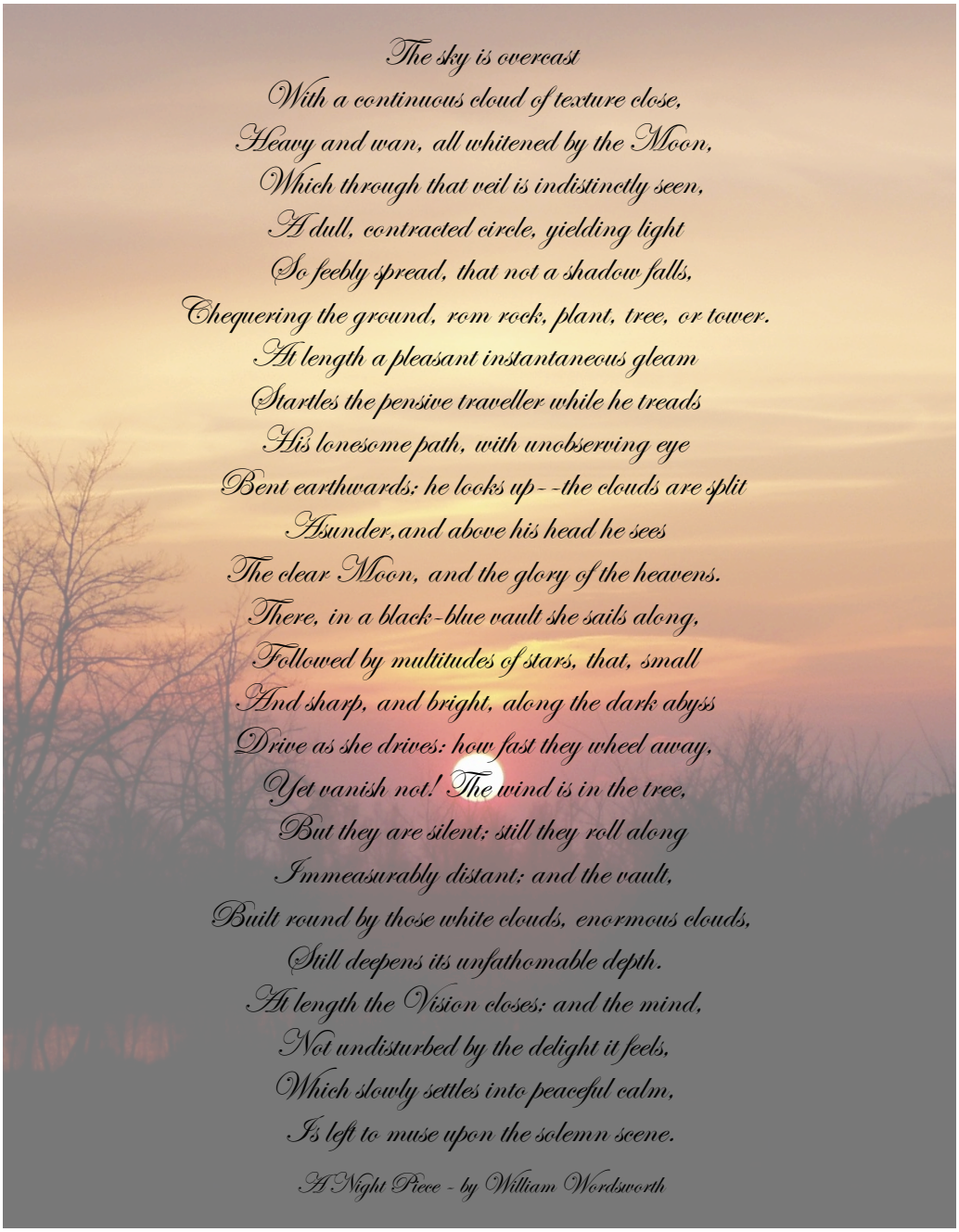
Department of Biotechnology Engineering

Ben Gurion University of the Negev, Israel

Anna Reale, Ph.D.

Department of Food and Microbiology Science and Technology

University of Molise, Italy



The sky is overcast
With a continuous cloud of texture close,
Heavy and wan, all whitened by the Moon,
Which through that veil is indistinctly seen,
A dull, contracted circle, yielding light
So feebly spread, that not a shadow falls,
Chequering the ground, rom rock, plant, tree, or tower.
At length a pleasant instantaneous gleam
Startles the pensive traveller while he treads
His lonesome path, with unobserving eye
Bent earthwards; he looks up--the clouds are split
Asunder,and above his head he sees
The clear Moon, and the glory of the heavens.
There, in a black-blue vault she sails along,
Followed by multitudes of stars, that, small
And sharp, and bright, along the dark abyss
Drive as she drives: how fast they wheel away,
Yet vanish not! The wind is in the tree,
But they are silent; still they roll along
Immeasurably distant; and the vault,
Built round by those white clouds, enormous clouds,
Still deepens its unfathomable depth.
At length the Vision closes; and the mind,
Not undisturbed by the delight it feels,
Which slowly settles into peaceful calm,
Is left to muse upon the solemn scene.

A Night Piece - by William Wordsworth

ABSTRACT

The research of pathogenic microorganisms in foods has seen an intensification of the efforts to develop more specific and sensitive methods able to ascertain the presence of any virus or bacteria potentially harmful for health before marketing the products. This thesis was developed on the basis of these considerations. The purpose was to optimize molecular biology techniques that allow to reduce the time, often very long, required by traditional microbiology and to develop new based-biosensor protocols to get a fast and precise result. This is a very important aspect for food industries that need answers in a short time to avoid or limit economic losses.

Campylobacter's *por*A gene, 16S rRNA gene and 16S-ITS-23S operon (Chapter 2) were used for the direct detection of the pathogens found in poultry meat through the utilization of primers and probes annealing specific DNA sequences. The great genetic variability into the *por*A gene of different *Campylobacter* species was used for the design of four specie-specific sets of primers able to distinguish between the four most important pathogenic species in humans and animals. On the contrary, 16S rDNA gene (that codes for the rRNA component of the ribosomes) is a conserved section of prokaryotic DNA found in all bacteria and Archaea and it is a useful tool for studying bacterial communities. For this reason it has been choosen to design two probes for the quick identification at genus level with Dot Blot and OLED biochip. The sequence of the internal transcribed spacer (ITS) region between the 16S and 23S rRNA, variable in size depending from the species considered, was used to design a unique couple of primers that allows to differentiate, in a single PCR assay, between the three *Campylobacter* species mainly involved in foodborne disease.

Diagnosis of *Listeria monocytogenes* (Chapter 3) is normally made through the use of internationally certified (ISO) methods which need the selective enrichment and the streak on selective and differential media. For this reason, as for *Campylobacter*, the aim of the work was the direct detection of the pathogen found in poultry meat through the utilization of primers and probes annealing specific DNA sequences. For the obtainement of the results, the probes and primers used were designed within a gene own of the genus *Listeria*, the *iap* gene, selecting portions of the sequence that ensure reliable identification for *L. monocytogenes*, as the only species pathogenic for humans. PCR and Dot Blot, used to confirm and optimize the identification obtained through the use of traditional ISO methods, and a preliminary study applying magnetic beads and LSPR biosensor protocols were made to verifiy the possibility of diagnosis of this pathogen through the use of these biosensors.

Table of contents

PREFACE

Food represents a significant risk for the transmission of foodborne pathogens, although other infection ways exist, such as environment, water and contact with farm animals and pets. Microorganisms are influenced not only by environmental parameters, but also by the interactions between the organisms present in the food. In most cases, the food ecosystems are neither homogeneous nor static, so they change with the time. The microflora in food could be useful, thanks to their biochemical activity, to transform the raw material in final products with a longer shelf-life and with a better taste. However, there are other undesired microorganisms that can survive in the food matrix: pathogens and spoilage. Meat industry is one of the most important economic food sector and it is well recognised that all the farm animals are reservoires of many pathogens. In particular, broiler and turkey are sources for pathogenic *Campylobacter* and *Listeria* strains and the meat, contaminated with these pathogens during processing, is responsible to be a cause of zoonoses with high incidence for human health in terms of morbidity and mortality. Campylobacteriosis was the most frequently reported zoonotic disease in humans in most of the Member States of European Union with 200.507 confirmed cases (Fig. 1) and bacteria were isolated from 26% of fresh poultry (*Gallus gallus* and *Meleagris gallopavo*) meat analysed. In particular, *Campylobacter* spp. possesses a high morbidity and is the pathogen more often implicated and isolated in patients with gastroenteritis. On the contrary, number of listeriosis cases in humans are not increased but a high fatality was reported among the cases in fact, *L. monocytogenes* possesses a high mortality. For this reason, this Ph.D. thesis compares different techniques for the identification of the four principal *Campylobacter* species involved in gastrointestinal diseases and bacteremia in humans (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) and *L. monocytogenes* which is related to many cases of gastroenteritis, invasive diseases and abortion. During the last years, the approach to food microbiology changed and all the techniques for pathogen

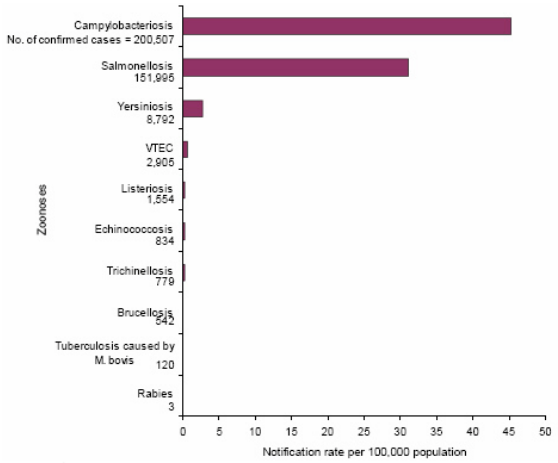


Figure. 1: Reported zoonoses rates in confirmed human cases in the EU, 2007 (EFSA, 2009).
http://www.thebeefsite.com/articles/1845/zoonoses-in-the-eu-trends-and-sources

diagnostic are projected to be rapid, sensitive, cheaper and able to identifiy the pathogen also analysing complex food matrices. All these requirements are needed for early disease diagnosis and treatment. Moreover, traditional microbiology techniques that need enrichment and plating procedures (that could produce negative results for microorganisms forming viable but non culturable cells) can often show negative results. The use of molecular techniques and the new field of biosensors allow the detection of pathogens avoiding all the bias of the tradional methods. For this reason, identification following ISO (International Organization for Standardization) procedures, molecular biology techniques such as PCR and Dot Blot and analysis using biosensor (OLED biochip, magnetic beads and LSPR) were performed to evaluate specificity and sensitivity, advantages and problematics.

CHAPTER 1.

1. Introduction

1.1 Why analyse foods?

One of the most important reasons for analyzing foods is to ensure their safety. The control of the entire production process of a food is a critical point in a modern industrial environment because most of foods can easily undergo drastic changes that will modify the organoleptic properties. All food products require analysis as part of a quality management program throughout the development process, through production, and after a product is in the market. The characteristics of foods are used to answer specific requests for regulatory purposes and typical quality control (Suzanne, 2010). In fact, each food has to meet legal and labeling standards of quality and safety, hygienic aspects, nutritional adequacy and genuineness in order to ensure high quality standards and to minimize the risks for the consumer. It would be economically disastrous, as well as being rather unpleasant to consumers, if a food manufacturer sold a product that was harmful or toxic. It is therefore important that food manufacturers do everything they can to ensure that these harmful substances are not present, or that they are effectively eliminated before the food is consumed. This can be achieved by following good manufacturing practice**,** regulations specified by the government for specific food products and by having analytical techniques that are capable of detecting harmful substances. In many situations it is important to use analytical techniques that have a high sensitivity, *i.e.,* that can reliably detect low levels of harmful material and organisms. The characterization of the food includes the nutritional composition (proteins, carbohydrates, lipids and vitamins), food additives (eg. colorants), chemical contaminants (pesticides or residues of veterinary drugs or pesticides), extraneous matter (e.g., glass, wood, metal, insect matter) and microbiological contaminants (mycotoxins, *C. botulinum* toxin). Food can also be a source of microbiological contamination in any stage of process, such as preparation and handling, packaging and storage. Appear to be particularly dangerous, contamination by bacteria (*Salmonella enterica*, *Campylobacter* spp., *Listeria monocytogenes*, *Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*), viruses (rotavirus, norovirus, astrovirus, hepatitis A virus), fungi (e.g. *Aspergillus* spp. and *Claviceps purpurea*) and parasites (*Giardia intestinalis*, *Cryptosporidium parvum*, *Taenia solium* and *Taenia saginata*) resulting in an economic loss to the industry. The analysis and research of pathogens in foods by following traditional methods, often takes a long time to arrive at a diagnostic response, for this reason other

techniques, such as molecular assays and biosensors were developed during the last years.

1.2 Introduction to molecular techniques

Molecular techhniques and immunological methods are becoming increasingly important in food and feed analysis because the food industry and government agencies often require determination of food composition and characteristics. Traditional methods for microbial enumeration, identification and characterization, such as enrichment and plating methods, followed by biochemical analysis, are insufficient for monitoring specific strains in complex microbial communities and often fail in the identification of the microorganism present in lesser amount. Moreover, one of the most critical problems during the detection of microorganisms by traditional methods is represented by "viable but non-colturable (VBNC) cells" often induced by environmental stresses, such as low temperature, high osmolarity, and nutrient starvation (Nicolò and Guglielmino, 2012). In the last decade, due to the use of molecular methods, the knowledge about the microbial diversity of microbial ecosystems has increased and new and highly performing culture-independent and culture-dependent molecular techniques are now available to study food-associated microbial communities. Molecular approaches to study the evolution of microbial flora could be useful to better comprehend the microbiological processes involved in food processing and ripening, improve microbiological safety by monitoring *in situ* pathogenic bacteria, and evaluate the effective composition of the microbial populations (Giraffa and Carminati, 2008). Compared to traditional culturing, culture independent methods aim to obtain a picture of a microbial population without the need to isolate and culture single components but only obtaining representative genomic DNA from food samples.

One of the most important discoveries of the last century was the PCR assay which has revolutionized microbial ecology, resulting in the development of several techniques of microbial community fingerprinting. PCR is a rapid and simple alternative method to bacteriological culture as it provides more rapid identification of species than conventional methods (Yamazaki-Matsune et al., 2007; Estibaliz et al., 2005). However, detection of bacteria with PCR in contaminated samples can be complicated by several factors such as inhibitors (Kreader, 1996; Park et al., 2006). Molecular techniques should yield the reproducibility of the results (this means that the assay should ensure the same result when it is repeated several times) and be adaptable to any type of pathogen with some modifications of the protocol. Other practical aspects include ease of execution and interpretation of results, as well as cost and availability of reagents and equipment.

1.2.1 Nucleic acid hybridization: methods and principle

The nucleic acid hybridization is the process where two DNA or RNA in a single stranded state reconstruct the double strand configuration, based on nucleotide complementarity, resulting in DNA-DNA, RNA-RNA or DNA-RNA hybrids. Hybridization occurs with a process called "nucleation" whereby the two separate nucleic acid strands come into close proximity of each other. A duplex region is formed where a minum of three bases of one strand complements to those on the second strand. If the remaining parts of the strains are complementary, the strands will anneal together very quickly. The main purpose of the hybridization techniques is the recognition of specific sequences of oligonucleotides within target genes for the identification of genus or species or for the diagnosis of diseases. Presence or absence of microorganisms can be detected by using PCR, which is the most sensitive of the existing rapid methods to detect microbial pathogens, in particular when pathogens are difficult to growth (Yamamoto, 2002). Pathogens can be detected also using genus or species-specific probes by chemiluminescent or colorimetric detection with blotting techniques (Joseph, 2008). The use of DNA probes offers a great potential in microbial identification. The detection via blotting techniques needs the target molecule (protein, DNA, RNA) and the probe molecule which identifies the target by hybridization. Hybridization that is conducted on a solid carrier is divided in three categories: in southern blotting DNA molecules are identified using DNA or RNA probes; in northern blotting RNA molecules are identified using RNA or DNA probes and in western blotting proteic sequences are identified using specific antibodies. Oligonucleotide probes need to be long enough to allow a specific hybridization with the target avoiding the non-specific interaction with other nucleic acid sequences and not hybridize with itself (possible if probe shows intracatenary complementary areas).

PCR assay

PCR (Polymerase Chain Reaction, Fig. 1.1) is an *in vitro* method to generate copies of a defined DNA sequence. In principle, a specific DNA fragment, flanked by two oligonucleotides serving as primers for the reaction, is amplified by a thermo-stable polymerase. The reaction consists of three functional steps per cycle of amplification. The first, denaturing, takes place at high temperatures (92-95°C), and it is necessary for the separation of the double strand into two single strands. The second, annealing, allow the hybridization of the primers to their complementary sequence present in the template DNA. The annealing temperature is very important as it determines the specificity (stringency) of the reaction. The primer annealing temperature range is ogten 37-55°C, it cannot be too low, to avoid non-specific binding of the primers to

DNA sequence that do not match, leading to amplification of DNA other than the intended sequence. The third, extension, is the polymerization by means of a thermo-stable DNA polymerase (Taq DNA polymerase) that requires the presence of Mg^{2+} ions for its activity, it consists on the extension of the primers using the four deoxyribonucleotide triphosphates (dNTPs), and resulting in the duplication of the region of interest.

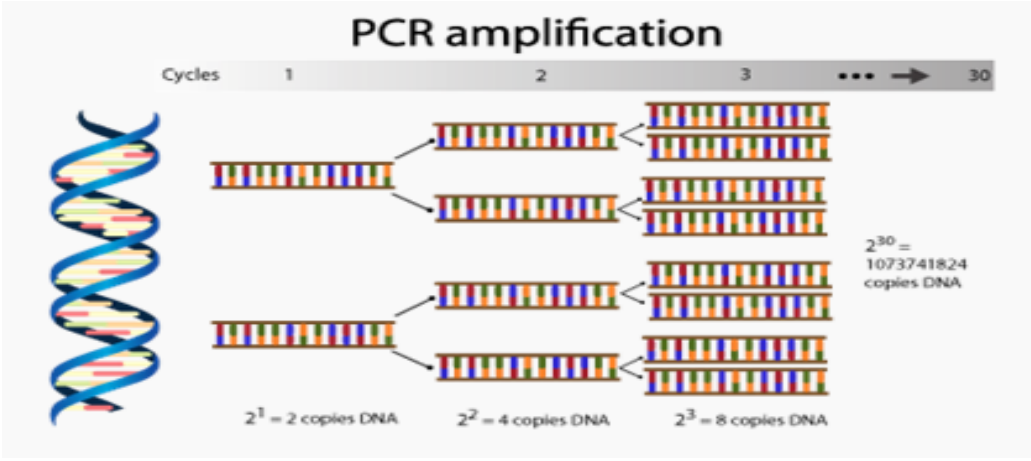


Figure 1.1: PCR scheme. The reaction consists in three functional steps per cycle of amplification: *denaturation* (heat briefly to separate DNA strands); *annealing* (cool to allow primers to form hydrogen bond with the target sequence) and *extension* (DNA polymerase adds nucleotides to the 3' end of each primer).
http://www.gmotesting.com/Testing-Options/Genetic-analysis.aspx

Each complete cycle takes about 2 minutes and doubles the quantity of DNA produced in the preceding cycles. For an efficient amplification of DNA, 20 to 40 cycles are necessary (Trinidade et al., 2003). In this way, DNA target is amplified approximately a billion fold.
Primers are short single-stranded DNA molecules, usually 18 to 35 bases in length, designed to bind selectively to the complementary sequences of the target DNA segment. During primer annealing one primer has to bind in forward and the other one in a defined distance in reverse orientation to the separated DNA strands. Designing PCR primers is a critical step because they need high specificity. In other words, the best choice would be a DNA sequences characteristic and unique for the organism to be identified.
When applied to food analysis, PCR requires the following steps:

- ✓ isolation of DNA from the food;
- ✓ amplification of the target sequences by PCR;
- ✓ visualization of the amplicons by agarose gel electrophoresis; using a fluorescent dye;
- ✓ estimation of their fragment size by comparison with a DNA molecular weight marker.

Dot blot

Dot blot is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. Digoxigenin (DIG) which is commonly used to label RNA and DNA molecules, is a small plant-derived molecule, extracted from foxglow (*Digitalis purpurea*, Fig. 1.2 and *Digitalis lanata*) not found in animals. Foxgloves are poisonous because they contain two cardiac glycosides, digoxin and digitoxin, which are found in all parts of the foxglove plant but are most concentrated in the leaves. Apart from its cardiac glycosides, *Digitalis* also contains a steroid, digoxigenin (DIG). Digoxigenin (Fig. 1.3) is a fairly small little molecule that can be coupled to the nucleotides that makeup DNA or RNA. Therefore, potential background issues can be avoided when DIG is used to label nucleic acid probes for *in situ* hybridization in mammalian tissues. The DIG-probes are monitored by anti-DIG antibodies conjugated with alkaline phosphatase.

Figure 1.2: *Digitalis purpurea* http://www.johnsonscreek.co.uk/Digitalis%20purpurea26-05-04.jpg

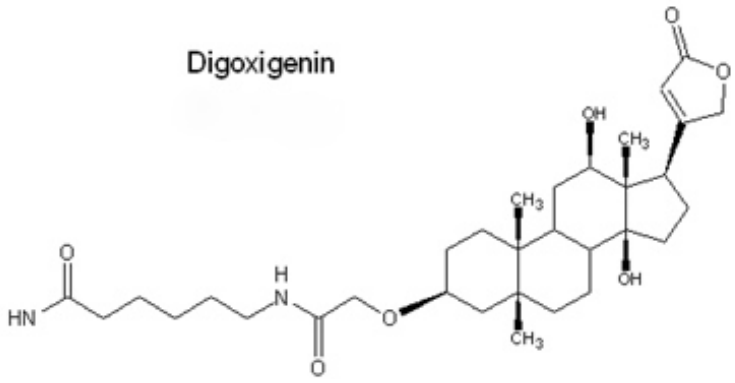


Figure 1.3: Molecular structure of digoxigenin (http://www.genelink.com/newsite/products/mod_detail.asp?modid=6)

In the Dot Blot method, DNA denatured samples are spotted onto a membrane (solid carrier) and exposed to UV light to cross-links nucleic acids to the carrier structure. After cross-linking, molecular hybridization consists in the immersion of the membrane (with the bond DNA) in an appropriate buffer containing the labelled single-strand probe for 14 – 16 hours. The probes can be labelled radioactively and non-radioactively. The first one is carried out using ^{32}P or ^{35}S. The method has the advantage that very small amounts of nucleic acids can be detected but some disadvantages are mentioned: risk of handling radioactive

materials, imbalance of isotopes and long exposure times (Walker et al., 1996). Non-radioactive labelling uses reporter molecules that are directly or indirectly detectable. Such reporter molecules are: fluorochromes, biotin coupled with avidin or with streptavidin or digoxigenin (DIG-labelled DNA probe) coupled with antibodies-antidigoxigenin (Gösseringer et al., 1997, Monedero et al., 1997). After hybridization, the non-hybridized probe is removed by serial washing steps. Probe-target hybrids are usually detected with an alkaline-phosphatase-conjugated antibody either by a colour reaction or by a chemiluminescence reaction. NBT/BCIP (Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) is usually used for colour reactions and CDP-Star (Disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate) or CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) for chemiluminescence reactions (Barbu, 2007). BCIP and NBT, together, yield an intense, insoluble purple precipitate when they react with Alkaline Phosphatase. The NBT/BCIP reaction is illustrated in Fig. 1.4.

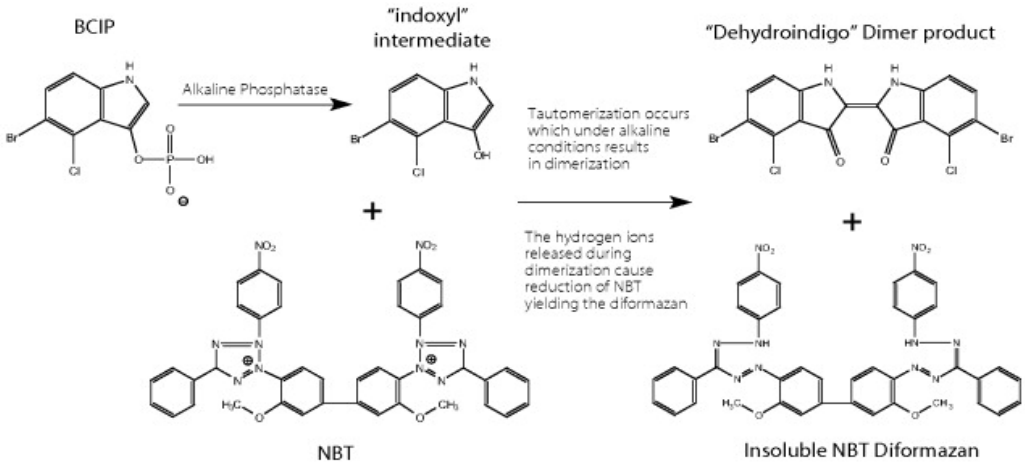


Figure 1.4: NBT/BCIP reaction scheme (http://www.mpbio.com/product.php?pid=0898077)

BCIP is hydrolysed by alkaline phosphatase (conjugated to the antibody) to form an intermediate that undergoes dimerization to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by the dimerization (Stuyver et al., 2003).

1.3 Foods and biosensors

Biosensors are an important option in food sector to existing diagnostic methods to control production processes. In the context of microbiological analysis of food, for example, the use of biosensors is of great interest to ensure the quality, safety, and health of food, taking into account the needs of consumers, the food industry and national and international regulations. The adoption of the various methods used to detect the microorganisms of interest must be taken in consideration the food matrix on which they will operate and consider some parameters, e.g. precision, accuracy, specificity, sensitivity, and economic impact of the method, focusing on most economical, reliable and highly specific. The nano-biosensors in particular, can perform all the steps traditionally carried out by a laboratory in a space of a few centimetres, and limit the use of chemical reagents reducing the environmental impact analysis. Given the miniaturized dimensions, it is possible to make kits for the analysis directly in the field, with important applications, e.g. for traceability of GMOs, water analysis or for zootechnic investigations. Biosensors can also be useful in the implementation of hazard analysis critical control point (HACCP) plans by verifying process developments. In particular for the detection of pathogenic microorganisms, biosensor can provide an advantageous tool, especially for those bacteria responsible for foodborne diseases that cause important economic losses in food industry and cause high hospitalization, such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp. and *Escherichia coli*. Microbiological analyses during processing and on final products must ensure the absence or the presence within the range of legality of these pathogens. Since traditional methods often requires long response time and involving high identification costs (Meng and Doyle, 2002), biosensors offer a valid alternative to control biological hazards due to the potential for miniaturization, rapidity, specificity, and sensitivity (Pathirana et al., 2000; Ropkins and Beck, 2000). Biosensors have a high potential for automation and allow the construction of simple and portable equipment for fast analysis. These properties will open up many new applications within quality and process control, including fermentation, quality and safety control of raw materials. Rapid feedback of information could help the food or drinks manufacturer to both reduce wastage from poorly controlled processes and increase productivity (Schnerr, 2007). Recent advances in biosensor technology promise sensitive and specific results in shorter times. Optical sensors (Passaro et al., 2007), acoustic sensors (Jia et al., 2012), microwire sensors (Lu and Jun, 2012) and electrochemical biosensors (Marks et al., 2007) proposed highly sensitive, and rapid devices that can be used for rapid screening of foods for the detection of foodborne pathogens prior to distribution. The biosensors currently in use are capable of providing a limit of pathogens detection from 1-2 log CFU/g or mL (Chen et al,

2008; Setterington and Alocilja, 2011). In response to these demands, many researchers have developed various biosensor detection systems in order to decrease these limits. E.g. Guo et al. (2012), using a piezoelectric biosensor (Quartz Crystal Microbalance, QCM) for the capture and enrichment of viable cells of *E. coli* O157: H7 reaching a detection limit of 0-1 log CFU/mL, demonstrating the great potential of this system for microbiological analysis of food samples; Badana et al. (2009) used an optical biosensor based on the "light scattering sensor" technology (LSS) for the detection of *L. monocytogenes*, *E. coli* O157: H7 and *Salmonella* spp. in inoculated vegetable and meat samples. The limit of detection was 1 cell in 25 g of sample, even in the presence of the natural contaminant microbial population. Current technology, such as polymerase chain reaction (PCR) leads toward such tests and devices, but nanotechnology is expanding the options currently available, which will result in greater sensitivity and far better efficiency and economy (Fakruddin et al., 2012).

1.4 The interdisciplinary nature of nanobiotechnology

The enormous potential of nanobiotechnology derives from its interdisciplinary nature, spanning across all fields of science, engineering and technology (Sanner et al., 2005). Especially biotechnology and nanotechnology are two of the 21st century's most promising technologies. Nanotechnology (sometimes referred to as nanotech) is defined as the "design, development and application of materials and devices whose least functional make up is on a nanometer scale (Emerich and Thanos, 2003; Sahoo and Labhasetwar, 2003). Generally, nanotechnology deals with developing materials, devices, or other structures possessing at least one dimension sized from 1 to 100 nanometers. Meanwhile, biotechnology deals with metabolic and other physiological processes of biological subjects including microorganisms. Association of these two technologies, i.e. nanobiotechnology can play a vital role in developing and implementing many useful tools in the study of life. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale to investigating whether we can directly control matters on/in the atomic scale/level. This idea entails the application of fields of science as diverse as surface science, organic chemistry, molecular biology, semiconductor physics, microfabrication, etc. (Fakruddin et al., 2012). Living organisms are built of cells that are typically 10 μm across. However, the cell parts are much smaller and are in the sub-micron size domain. Even smaller are the proteins with a typical size of just 5 nm, which is comparable with the dimensions of smallest manmade nanoparticles (Taton, 2002). Understanding of biological processes on the nanoscale level is a strong

driving force behind development of nanotechnology (Whitesides, 2003). A list of some of the applications of nanomaterials to biology or medicine is given below:

- ✓ Fluorescent biological labels (Bruchez et al., 1998; Wang et al., 2002)
- ✓ Drug and gene delivery (Mah et al., 2000; Panatarotto et al., 2003)
- ✓ Bio-detection of pathogens (Nam et al., 2003)
- ✓ Probing of DNA structure (Mahtab et al., 1995)
- ✓ Tissue engineering (Ma et al., 2003; de la Isla et al., 2003)
- ✓ Tumor destruction via heating (hyperthermia) (Yoshida and Kobayashi, 1999)
- ✓ Separation and purification of biological molecules and cells (Molday and MacKenzie, 1982)
- ✓ Magnetic Resonance Imaging (MRI) contrast enhancement (Weissleder et al., 1990)
- ✓ Phagokinetic studies (Parak et al., 2002)

Current diagnostic methods for most diseases depend on the manifestation of visible symptoms before medical professionals can recognize that the patient suffers from a specific illness. But by the time those symptoms have appeared, treatment may have a decreased chance of being effective. Optimally, diseases should be diagnosed and cured before symptoms even manifest themselves. Nucleic acid diagnostics will play a crucial role in that process, as they allow the detection of pathogens and diseases/diseased cells at such an early symptomless the age of disease progression that effective treatment is more feasible.

1.5 Biosensors and biochips

IUPAC defines a biosensor (Fig. 1.5) as *"a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element"* (Thevenot et al., 2001).

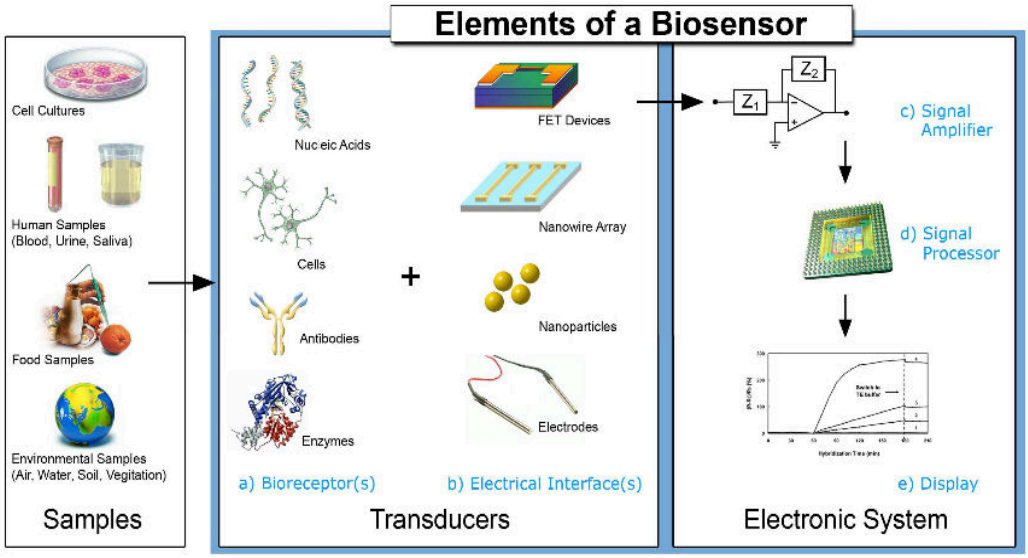


Figure 1.5: Elements of a biosensor: a biosensor usually contains two parts; the biological sensing element, and the transducer that converts the biochemical information into measurable signals (Turner, 2000).
(http://dou-demo.blogspot.it/2013/09/the-biosensor-system.html)

Based on the biological component, biosensors are:

- ✓ Biocatalitic biosensors;
- ✓ Affinity-based biosensors: - immunosensors;
  - chemosensors;
  - DNA and RNA sensors;
  - Aptamers-based sensors.

From the point of view of signal transducers, biosensors are divided into:

- ✓ Electrochemical biosensors: - amperometric;
  - potenziometric;
  - conductometric.
- ✓ Optical biosensors (photodiodes, optical fibers, SPR, LSPR);
- ✓ Acoustic biosensors (piezoelectric crystals);
- ✓ Calorimetric biosensors.

Biosensors can be used for the construction of biochips, which can be defined as biosensors that integrate the construction techniques and data recording own of microelectronics. Tipically, a biochip's surface area is no larger than a fingernail. The biochips that allow simultaneously detection of multiple analytes are defined microarrays, these chips are like miniaturized laboratories that can perform hundreds or thousand of simoultaneous biochemical reactions (Rao et al, 2012).

Biological recognition elements

The last generation of sensors incorporate various recognition elements and complex methods of detection. These include antigens, antibodies, nucleic acids, whole cells, and proteins (Lei et al., 2006). The changes in these elements upon sensing a signal are detected via optical, electrochemical, calorimetric, acoustic, piezoelectric, magnetic and micromechanical transducers (Nayak et al., 2009).

Biosensors, according to the biomolecules involved, can be classified as immunosensors (interaction between antibody and antigen), genosensors (affinity between complementary oligonucleotides) and biosensors based on a lingand immobilized on the surface of biosensor that interacts with specific biological receptor.

In the immunosensors, the antibodies or antigens (cells, proteins, haptens) can be immobilized directly on the surface of the biosensor (direct immunosensor), where the bond that is to be established between the two components will result in a change of the property that will be converted into a measurable signal (this kind of sensor does'nt need label biomolecule). Most suitable transduction mechanisms for this technique are those potentiometric, piezoelectric and surface plasmon resonance. In the case of indirect immunosensors (sandwich assay), the steps and time required to get the diagnostic response are longer but they offer the advantage of being able to use methods of transduction most performant from the point of view of the signal acquisition. After the interaction of the antigen with the antibody immobilized on the surface of the biosensor, must be used a secondary labeled monoclonal antibody. This technique can only be used in the presence of antigens quite large and containing at least two epitopes for the two different monoclonal antibodies but offers the advantage of being able to be used with any optical system (with a properly labelled secondary antibody) and increases the sensitivity in the case of use with gravimetric biosensors (Ahluwalia, 2004).

A great challenge in the field of DNA detection is the development of methods that do not require PCR. Traditional DNA sensing technologies rely on a

combination of target DNA sequence amplification (PCR) and optical detection based on the use of fluorophore labels. However, several major drawbacks still remain due to the need for expensive reagents and pitfalls resulting from contamination or matrix effects (Shi et al., 2008). DNA biomolecules are particularly suitable for applications in nanobiotechnology (Zhang and Seeling, 2011; Krishnan and Simmel, 2011). In particular, DNA is a biological element useful for the creation of genosensors (DNA-biosensors) (Cecchini et al., 2012) which allow rapid monitoring of hybridization with the target DNAs. The DNA biosensors are based on the ability of the single chain DNA or RNA to hybridize with complementary sequences immobilized on the surface of the biosensor (probes) highly specific and sensitive for the target. The DNA sensors play an important role in the identification of bacterial and viral pathogen species. Very important in the field of food safety, environmental monitoring and public health management, the main problems associated with these devices are the sensitivity and specificity of the receptor and the transducer. These problems can be solved with the appropriate choice of the sensing element concentration and the kind of transducer. The transduction mechanisms to detect DNA hybridization include electrochemical systems, piezoelectric and optical devices. Traditional DNA sensing technologies rely on a combination of target DNA sequence amplification by polymerase chain reaction (PCR) and optical detection based on the use of fluorophore labels but PCR involves time-consuming thermal cycles and is subject to severe contamination problems, and for these reasons a major challenge for rapid and reliable DNA analysis appears to be the development of ultrasensitive rapid and multiplexed methods that are able to perform PCR-free DNA analyses (Zanoli et al. 2012).

Transduction elements

The transduction element is an essential component of the biosensor and is crucial because its influence on the overall performances of the biosensor, both sensitivity and specificity. The transducer converts the biochemical interaction between the biomolecule and markers in a measurable electronic signal (Turner, 2000). The transducers most commonly used are electrochemical, electro-optical, acoustic and mechanical. From an operative point of view they can be divided into direct and indirect (Battisti and Solaro, 2003). In direct detection, the biological interaction is measured in real time using non-catalytic elements such as cell receptors, antibodies and genetic material. To this category belong the plasmon resonance sensors, piezoelectric sensors and optomechanical (microcantilever). In indirect detection, is necessary to use a secondary element that often possesses catalytic activity, such as an enzyme. To this category belong electrochemical biosensors that are based on the biotransformation of substrates which, through redox reactions catalyzed by enzymes, determine a

flow of electrons detectable thanks to an electrochemical transducer which converts it into an electronic output signal related to the concentration of analyte in exam. The signal, properly processed and amplified, is then displayed. The electrodes (signal transducers) normally used to assemble electrochemical biosensors are amperometric and potentiometric.

1.5.1 Optical sensing techniques

During the last years, a rapid growth in the development of optical biosensors was done. The application fields range from medical diagnosis and monitoring of disease, drug discovery and detection of environmental pollutants and microorganisms (Turner, 2000). Optical sensing techniques are based on different sensing transduction mechanisms, e.g. chemiluminescence (Chan and Nie, 1998; Chan et al. 2002), fluorescence (Mucic et al., 1998), light absorption and scattering (Malinsky et al, 2001; McFarland and Van Duyne, 2003), reflectance (Hicks et al., 2005), surface plasmon resonance (SPR) (Mrksich et al., 1995; Berger et al., 1998; Yonzon et al., 2004) and Raman scattering (Nie and Emory, 1997; Zhang et al., 2005; Jiang et al., 2003).

1.5.1.1 Organic Light Emitting Diode (OLED)

A typical OLED structure (Fig. 1.6) consists of a thin film of organic material (electron-transport and hole-injection layers) between two electrodes. Light is produced by recombination of holes and electrons which have to be injected at the electrodes. The bottom electrode (anode) is a transparent indium tin oxide (ITO) layer and the top electrode (cathode) is usually a metallic thin film with high reflectivity and electrical conductivity. The thickness of the organic layer is between 100 and 150 nm (Geffroy et al., 2006). When an appropriate voltage (typically between 2 and 10 volts) is applied to the cell, charges are injected in the organic material, holes from the anode and electrons from the cathode. Then, the charges move inside the organic material and recombine to form excitons. After diffusion, the excitons recombine and a photon is emitted to produce light (electro luminescence) (Geffroy et al., 2006). The structure of the organic layers and the

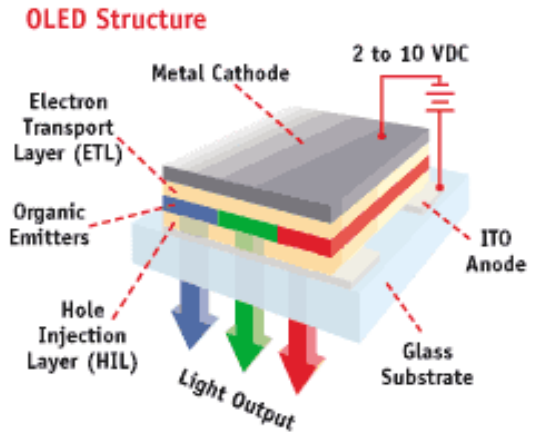


Figure 1.6: OLED stucture. (http://www.electroschematics.com/5178/organic-led-the-exciting-display-device/)

choice of anode and cathode are designed to maximize the recombination process in the emissive layer, thus maximizing the light output from the OLED device. The Spectral emission optimized OLED is then used as the excitation source to stimulate the emission of a fluorophore that can be bind to a biological receptor. Currently, for the fuorophore extitation, the most used excitation source is a laser beam that scans, e.g. a micro-array surface being deflected by a system of mirrors, and the weak fluorescence signal is detected by a photomultiplier or CCD camera. This optical configuration allows the detection of very low fluorescence signals with high sensitivity but also some drawbacks. (Marcello et al., 2013). The application of OLED to the biological and chemical fields started several years ago with Ruth and Josef Shinar from Ames Laboratory-USDOE Iowa State University and the David Bradley group from Imperial College London in the UK, that investigated the use of OLED as integrated light source in different kinds of biological sensor based in fluorescence detection of tagged probes (Banerjee et al., 2010; Nakajima et al., 2011; Pais et al., 2008; Ren et al., 2007; Yao eta l., 2005). It is possible to build a biochip using a DNA micro-array integrated with an OLED source that can be used in pathogens detection. For the fluorescent detection, Alexa Fluor succinimidyl esters are particularly suitable for the labelling with oligonucleotides, and probes modificated by addiction of an amino group can be bond on the surface of glasses through modification of the glass by 3-aminopropyltriethoxysilane (APTES). This molecule is an aminosilane used in the process of silanization for the functionalization of surfaces. In particular, the amine-reactive Alexa Fluor 430 (Fig. 1.7) carboxylic acid, succinimidyl ester and its conjugates exhibit the rare combination of absorption between 400 nm and 450 nm and fluorescence emission beyond 500 nm (excitation/emission maxima ~434/539 nm) and it can be used for the label of oligonucleotides for the detection of pathogens' DNA.

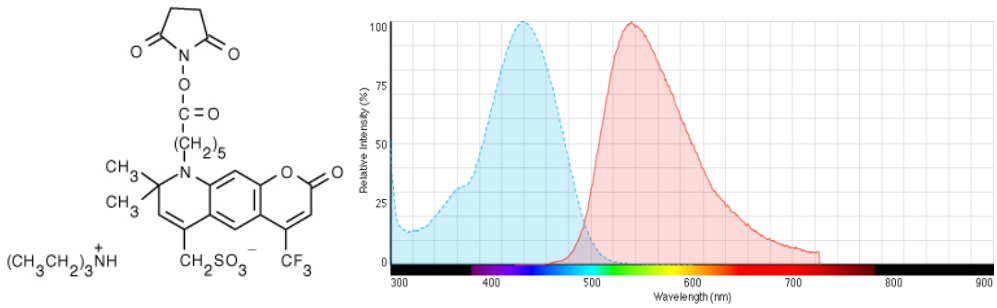


Figure 1.7: molecular structure of Alexa Fluor 430 (left) and its absorption and emission waveleghts (right). (http://www.lifetechnologies.com/order/catalog/product/A10169)

Detection of a signal by a CCD camera

Film and cooled charged-coupled device (CCD) cameras are very useful tools in laboratories for the quantification of the chemiluminescent and fluorescent signals generated with various techniques, although CCD-based cameras are considered the most robust technology. They show a larger dynamic range and greater linearity but also a lower sensitivity that often requires longer exposure times (Fournier et al., 2003). Benefits of utilizing a CCD detector include rapid data digitization and more accurate quantitation of signals compared to film-based densitometry owing to the significantly greater dynamic range (Martin and Bronstein, 2005). CCD sensors are made of silica and its role is to convert photons into electrons by photoelectric effect which will be collected through the creation of a potential well. The charges are then transferred varying the potential of the electrodes and extracted through the output circuit, converted in numeric values at 8, 16 or 32 bit and transferred to a pc for the elaboration of images. The most important properties of CCD camera are: quantum efficiency "QE" (the percentage of generated electronic charges by the incoming photons), light sensitivity "S" (is the measure of the detectibility of a CCD imaging system), dark current (is important for the sensitivity of camera, and it results from the temperature-dependent thermal generation of electrons), full well capacity "FWC" (is the maximum number of electrons that one pixel can contain before saturation), linearity "Lin" (mean that the digital signal should be proportional to the number of incoming photons), and binning (is the combination of intensities of adjacent pixels into an image).

1.5.1.2 Magnetic Beads

In the last decades magnetism and magnets have found a growing field of application in biotechnology and medical technology. Combining the forces of magnetism with micro and nanotechnology has further miniaturized the modes of application (Pamme, 2006). Applications range from biosensors and biochips (Graham et al., 2004; Miller et al., 2001) to visualization of biological events (Stahl et al., 2007; Pettersson et al., 2009). The force on the magnetic particle is dependent from various parameters; the diameter of the particle and the strength of the magnet as well as the magnetic susceptibility of the material. In biological field, magnetic particles (Fig. 1.8) are used for several purposes such as transporting, carrying or collecting biomolecules in solution (Pettersson et al., 2013). The detection of food-borne pathogen could be made in very rapid way if the target pathogens were separated, concentrated and purified from the matrix before detection (Stevens and Jakus, 2004). The immobilization of antibodies to magnetic beads for the immunomagnetic separation (IMS) has

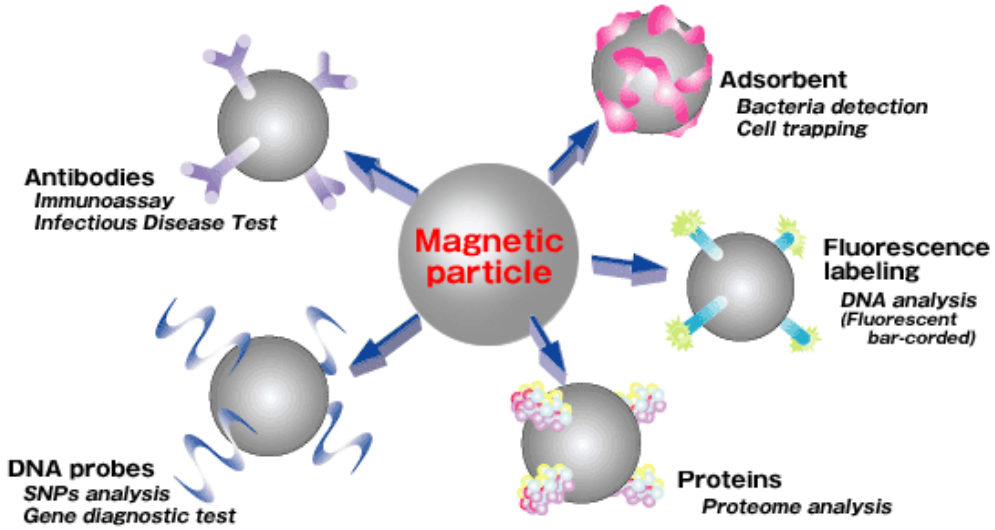


Figure 1.8: Magnetic beads possess large surface area that promotes efficient and fast reactions for the quantitative capture of targets (DNA probes, antibodies, proteins, etc.) and an efficient separation can be done with a powerful magnet. (http://www.gibthai.com/services/technical_detail.php?ID=13)

been widely used for the separation of target microorganisms from complex matrix (Dwivedi and Jakus, 2011). But antibodies, commonly used like ligands, are difficult and expensive to produce, have short shelf-lives, and have varying degrees of target specificity and avidity, with implications during capture efficiency and successive analytical detection sensitivity (Suh and Jaykus, 2013). Other ligands for pathogen capture and detection are nucleic acid aptamers (20-80 mer), single stranded DNA and RNA molecules that specifically interact to their target through their 3-dimensional structure and intermolecular hydrogen bond (Jayasena, 1999). These molecules are more advantageous then antibody-based affinity molecules for their production and stability, due to the chemical properties of nucleic acids versus amino acids. (Bunka and Stocjley, 2006; Khati, 2010). Detection of DNA hybridization to a surface by using magnetic particles is possible using advanced sensor technologies (Graham et al., 2005; Martins et al., 2009). There are several studies which confirm that flow-through hybridization of DNA onto probe-conjugated microbeads was successfully performed and the theyr use as solid supports for capture probes is advantageous because there is an easy modification of the beads surface and also an enhanced hybridization efficiency due to the effective mixing of solutions (Seong and Crooks, 2002). Moreover, chemiluminescent detection method provide high sensitivity while using simple instrumentation, it has become a powerful tool for a wide range of applications in fields such as molecular biotechnology, pharmacology and biomedical chemistry (Fan et al., 2005) as demosntrate by Haigang and Zhike (2009) using a magnetic bead-based DNA hybridization assay that allowed the detection of

H1N1 virus at levels as low as 10 amol. Luminescence is the emission of light, which occurs when a molecule in an excited state relaxes to its ground state. In chemiluminescence, the energy is produced by a chemical reaction without an outer radiation and the energy produced is in sufficient amount (approximately 300 KJ/mol for blue light emission and 150 KJ/mol for red light emission) to induce the transition of an electron from its ground state to an excited electronic state and returns to the ground state with emission of a photon. The reaction of chemiluminescence (Fig. 1.9) is triggered by a biocatalyst molecule, like Horseradish-peroxidase (HRP) providing high specific activity, steady-state kinetics and proportionate to the label's concentration. This glycoprotein, when incubated with a proper substrate (e.g. luminol), generates an enhanced chemiluminescence reaction, detectable and quantifiable. The outcome of such chemiluminescence reaction is light emission in the visible region (λ = 430 nm) (Hermann and Marks, 2007; Marks et al., 1997).

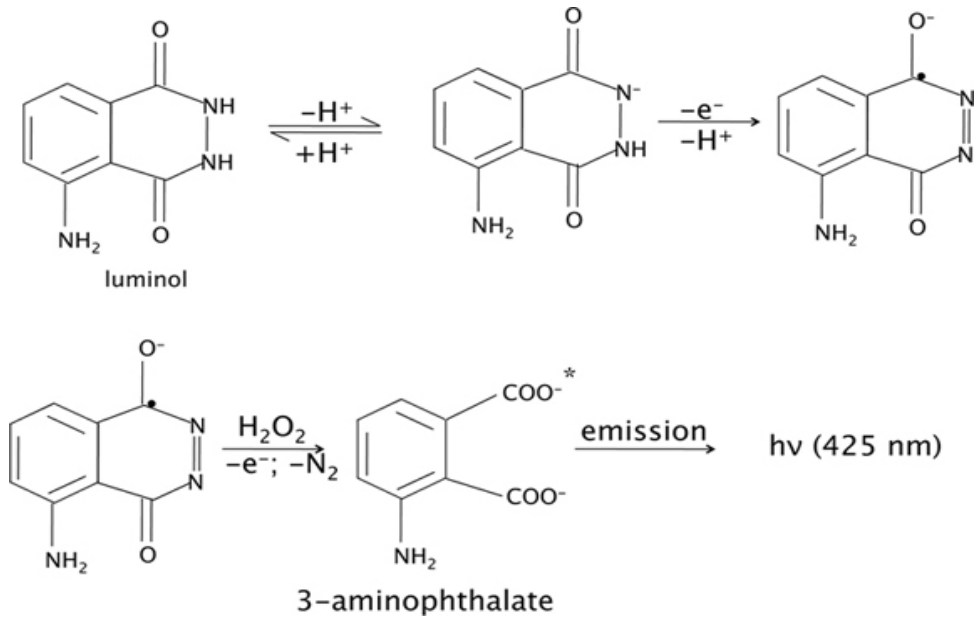


Figure 1.9: The mechanism of ECL reaction of luminol in the presence of H_2O_2 as the co-reactant. Chemiluminescent reaction catalysed by horseradish-peroxidase (HRP) breaks hydrogen peroxidase into oxygen radicals that oxidizes luminol to yield aminophtalic acid. The excited part of the oxidized molecules of luminol emits the quanta of light. (http://www.bioscience.org/2011/V16/af/3737/fulltext.php?bframe=figures.htm)

Detection of a signal by photon-counting system

The function of a photomultiplier (Fig. 1.10) is to convert the received optical signal (usually referred to as RLU, Relative Light Unit) into an electrical signal which is then amplified by emission of secondary electrons. This device consists of the following elements: a photocatode for the conversion of light flux into electron flux, an electron-optical input system which focuses and accellerates the electron flux, an electron multiplier that consists of a series of secondary-emission electrodes (dynodes) and an anode which collects the electron flux from the multiplier and supplies the output signal. The two fundamental phenomena of a photomultiplier are photoemission and secondary emission. Currently, there are many kinds of photodetectors with availability of integrated commercial products incorporating analysis software and the efficiency, previously reviewed (Buzhan et al., 2003; Cova et al., 2004; Hermann and Marks, 2007; Michalet et al., 2006).

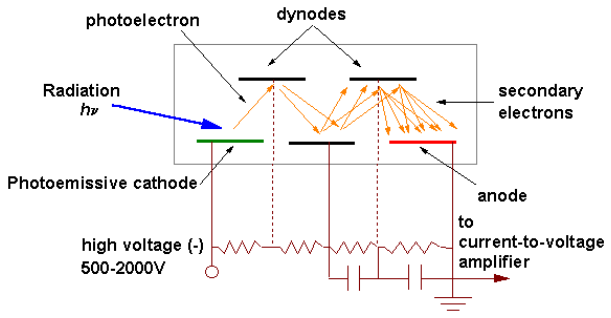


Figure 1.10: scheme of a Photomultiplier tube (PMT)
(http://www.files.chem.vt.edu/chem-ed/optics/detector/pmt.html)

In choosing a photodetector, it is important to define what is required from it therefore, it is important to notice some operating characteristics: photocatode spectral sensitivity (operating waveleght), high reliability of the received signal, large electrical response to the received optical signal, time and frequency response, minimal noise or background signals, performance stability in different measurement conditions, detector size and low cost.

Photo Multiplier Tube (PMT) is an efficient and accurate instrument for light detection. The size of the required photosensitive area of the detector varies depending on the application. The surface material of the area where photon incident in the photo-cathode plays a key role in the functioning of a photo-emissive light detector, and the wavelength range of sensitivity is dictated primarily by the absorption band of the cathode material and to some extent the work function of the surface. Such devices have very low noise and presently, photon counting using PMT still seems to be the most cost effective way to detect very low light levels (Hermann and Marks, 2007).

1.5.1.3 Localized Surface Plasmon Resonance (LSPR)

LSPR is one of the optical properties of noble metal nanoparticles. The scattering and absorption of visible light from noble metal nanoparticles is the source of some of the most beautiful colours in stained glass windows; these nanoparticles exhibit a strong UV-vis absorption band that is not present in the spectrum of the bulk metal (Haynes and Van Duyne, 2001; Hulteen et al., 1999). Different technologies to prepare well organized (in size and shape) plasmonic nanostructures with controlled morphology are developed, such as "top-down" physical nanofabrication (electron beam lithography) and "bottom-up" chemical synthesis or templated assisted assembling (Fu et al., 2003). When a gold nanostructure encounters electromagnetic radiation of an appropriate wavelength, the delocalized conduction electrons of the metal will begin to oscillate collectively relative to the lattice of positive nuclei with the frequency of the incoming light. This process can be divided into two types of interactions: scattering, in which the incoming light is re-radiated at the same wavelength in all directions, and absorption, in which the energy is transferred into vibrations of the lattice, typically observed as heat. Together, these processes are referred to as extinction (extinction = absorption + scattering) (Cobley et al., 2011). Nanoparticles are the bases for the sensing principle relies on the LSPR spectral shifts, that are caused by the surrounding dielectric environmental change in a binding event when excited by incident light. In LSPR (Fig. 1.11), the plasmon surface is localized in nanoparticles that were studied because of their size-dependent physical and chemical properties (Murray et al., 2000).

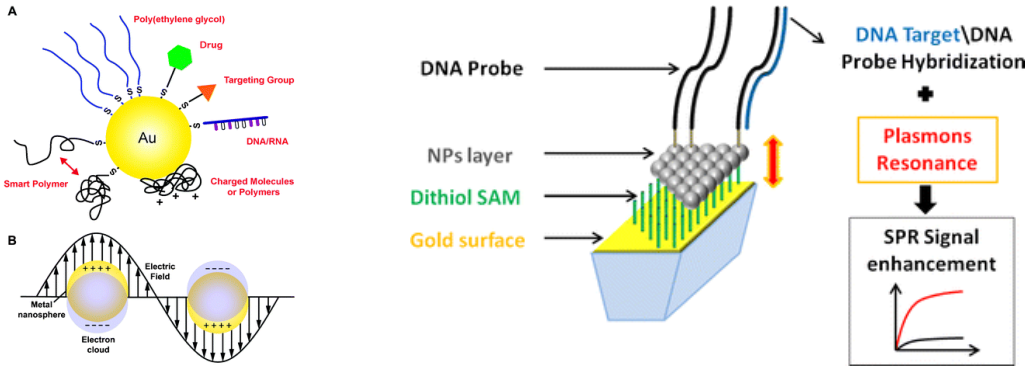


Figure 1.11 (A) Gold nanostructures can be conjugated with a wide variety of functional moieties, both through the gold–thiol bond and by passive adsorption. (B) Localized surface plasmon resonance (LSPR) is another critical property of gold nanostructures that results from the collective oscillation of delocalized electrons in response to an external electric field (Cobley et al., 2011)**.**

The LSPR properties are dependent on the geometric parameters (size, shape and interparticle distance) and local dielectric environment of nanostructures (Park et al., 2009). By using noble nanostructures of different morphology, a large plasmonic tunability ranging from visible to near-infrared wavelength can be readily obtained (Barchiesi et al., 2013). Nanoparticle usually forms the core of nano-biomaterial. It can be used as a convenient surface for molecular assembly, and may be composed of inorganic or polymeric materials. The shape is more often spherical but cylindrical, plate-like and other shapes are possible and also the size and size distribution might be important in some cases. Organic molecules that are adsorbed or chemisorbed on the surface of the particle are used but more often an additional layer of linker molecules is required to proceed with further functionalization. This linear linker molecule has reactive groups at both ends. One group is aimed at attaching the linker to the nanoparticle surface and the other is used to bind various moieties depending on the function required by the application (Salata, 2004). Nano gold particles studded with short segments of DNA form the basis of the easy-to-read test for the presence of any given genetic sequence. The DNA or oligonucleotides have been widely used because DNA can be synthesized using PCR techniques, and various functional groups can be integrated to allow the interaction with Au NPs. This technology allows/facilitates the detection of pathogenic microorganisms giving much higher sensitivity than tests that are currently being used (Nanosphere Inc., 2004). Other kinds of biological structures such as proteins, peptides, microorganisms (virus and bacteria) can be used to realize the complex Au assembly (McMillan et al., 2002; Sun et al., 2007). Nanostructure fabricated by these ways can be used as a substrates for immobilization of capture DNA and subsequent detection of target DNA without labelling because the LSPR signals of the nanostructures are affected by the interaction between biomolecules and the substrate (Haes et al., 2005; Kanda et al., 2004).

Detection of LSPR spectra

The LSPR extinction maximum (λ_{max}) of metal nanoparticles can be recorded and measured with UV-visible extinction spectroscopy using optical instruments, in solution phase or solid state, where the plasmonic properties including resonant wavelength, maximum optical density and peak shape can be easily resolved (Kelly et al, 2003). Generally, there are two detection protocols in optical biosensing: fluorescence-based detection (target or biorecognition molecules are labelled with fluorescent dye) and label free detection (target molecules are not labelled and are detected in their natural form). Label free detection normally is much easier performed, when compared to the fluorescent base detection that can be affected by problems like

quenching effect. In LSPR biosensors, plasmonic properties are dependent from refractive index of local medium that surrounds the nanoparticles and the biochemical reaction used during different assays contribute to an increasing of local refractive index, which corresponds to a red-shift of resonant wavelength and/or increasing of maximum optical density (OD) value (Sepùlveda et al., 2009). Metal nanoparticles are therefore used as transducers in the LSPR biosensors, from which the specific biochemical biding events have been converted into quantifiable optical signals. After exposition to the target molecules, the refractive index of the surrounding environment changes due to the binding to the receptors, inducing a shift in the nanoparticles. This wavelength shift is monitored by UV-visible spectroscopy (Zhao et al., 2006).

AIM OF THE PROJECT

The aim of this project was to designe new sets of primer and probes (for molecular analysis and for the development of new based biosensor protocols) and validate them. They were used on thirty-one samples of chicken and turkey meat and eight clinical samples collected from *Campylobacter* infected patients for the genus-specific and specie-specific detection of *Campylobacter* species and *Listeria monocytogenes.* Each molecular and based biosensor analysis was preceded by the standard procedures for identification of pathogens in foods according to ISO methods in order to ensure a perfect match between the internationally certified methods and the new identification procedures proposed.

CHAPTER 2.

2. *Campylobacter* spp.

2.1 *Campylobacter* spp., this elusive foodborne pathogen

The genus *Campylobacter* consists of 25 species recognized to date, including *Campylobacter jejuni* (Fig. 2.1)*, C. coli, C. fetus, C. lari* (previously *C. laridis*), *C. upsaliensis*, and *C. hyointestinalis* (Lynch et al., 2011) important pathogens in humans. All these species are foodborne pathogens that cause diarrheal diseases and gastroenteritis, accounting for 400 million cases in adults and children worldwide each year (Man et al., 2010). The bacteria pertaining at genus *Campylobacter* (the name derives from the Greek "καμπυλος", meaning curved) are Gram negative, oxidase and catalase positive and urease negative, usually curved or spiral rod-shaped, 0.2-0.5 μm wide and 0.5-8 μm long. Most species posses a polar flagellum, at one or both ends which gives them a very characteristic "cork-screw" motility (EFSA Journal, 2005). Compared with other important foodborne pathogens, such as *Salmonella* species, *Campylobacter* spp. seem ill-equipped to survive outside an animal host. They require a microaerobic atmosphere (3-5% oxygen and 10% carbon dioxide) and cannot multiply below about 30°C. However, even at 4°C, low-level metabolic activity can be detected, suggesting that cell integrity is maintained (Park, 2002). The temperature range for growth of the thermophilic *Campylobacter* species is 34-44°C, with an optimal temperature of 42°C, which probably reflects an adaptation to the intestines of warm-blooded birds (van Vliet and Ketley, 2001). They are usually found close to the intestinal wall, where the oxygen level is close to their optimum, and their mode of motility enables them to move through the viscous intestinal mucus layer (EFSA Journal, 2005). *Campylobacter* spp., normally spiral-shaped, have been reported to change into coccoid forms on exposure to atmospheric oxygen levels or other stresses. These coccoid forms have been coined viable non-culturable (VBNC) (Portner et al., 2007), and this form has been suggested to be a dormant state required for survival under conditions not supporting growth of *Campylobacter*, e.g. during transmission or storage (Rollins and Colwell, 1986).

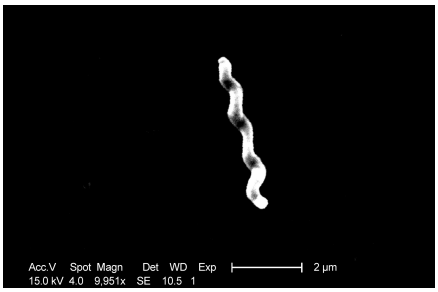


Figure 2.1: Scanning electron micrograph of *Campylobacter jejuni* (from CDC Public Healt Image Library).

The most important species of *Campylobacter* are the thermophilic species: *C. jejuni* ssp. *jejuni*, *C. co*li and *C. lari* (formerly known as "nalidixic acid resistant thermophilic *Campylobacter* spp. – NARTC"). Other species which are known to cause human illness are *C. upsaliensis*, *C. fetus* ssp. *fetus* and *C. jejuni* ssp. *doylei*. Most physiological, biochemical, epidemiological and survival information concerns *C. jejuni*, as this species is the most frequently recovered on human disease. Urease positive thermophilic *Campylobacter* (UPTC) are variants of *C. lari* often associated with seawater environments and consequently found in shellfish and sea birds (Megraud et al., 1988; Jones, 2001; On, 2001).

C. upsaliensis is frequently found in both cats and dogs, regardless of whether the animals are sick or healthy (prevalence, 5%-66% in cats vs. 5%-48% in dogs) (Hald and Matsen, 1997; Moreno et al., 1993; Burnens and Nicolet, 1992). It has been postulated that these animals are the main source of *C. upsaliensis* that produces disease in humans (Goossens et al., 1990; Patton et al., 1989), while poultry probably has a low rate of *C. upsaliensis* colonization. However, a definitive link has not been established (Goossens et al., 1991; De Silva et al., 1992; Stanley et al., 1994).

Campylobacter spp. survive poorly in dry or acid conditions, and in sodium chloride above 2%. Survival in foods is better at chill temperatures than higher (e.g., ambient), and freezing inactivates many, but not all those bacteria present. *Campylobacter* spp. are relatively sensitive to heat and irradiation, and so can readily be inactivated during cooking (ICMSF, 1996). Their sensitivity to environmental stresses seems to be confirmed by their lack of genes analogous to those in other bacteria, enabling physiological adaptation to adverse environments – e.g., oxidative stress, osmoregulation, starvation/stationary phase, heat and cold shock (Park, 2002).

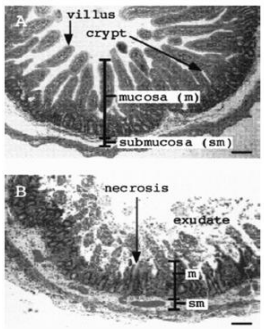


Figure 2.2: Hematoxylin and eosin stained sections of the small intestines infected by *E.coli* (A) and *C. jejuni* (B).

Campylobacter spp. is the main cause of bacterial gastrointestinal illness in the developed world and this genus is common gastrointestinal microbiota of poultry, wild birds and other animals. *C. jejuni* is the species most regulary isolated from food samples, and is the most frequently identified etiological agent in human cases of *Campylobacter*-associated gastroenteritis (Fig. 2.2), followed by *C. coli* (Vandamme, 2000), *C. upsaliensis* (Labarca et al., 2002), and *C. lari* (He et al., 2010; Prasad et al., 2001). Although host factors may also play a role in the manifestation of the type of diarrhoea, it is believed that CTLT (cholera toxin-like toxin) may

contribute to watery diarrhoea, and cytotoxin to inflammatory diarrhoea (Albert, 2011; Wassenaar, 1997). In *Campylobacter* infection, plays an importan role the biding and entry in host cells and CDT (cytolethal distending toxin) causes modifications in cells that become slowly distended, which progress into cell death. The invasion of epithelial cells, dependent on motility and flagellar expression, and production of toxins, can lead to the mucosal damage and inflammation (van Vliet and Ketley, 2001). A relatively small infective-dose is required to cause illness; as few as 800 bacteria can produce disease in healthy persons. Illness generally occurs following a 2-4 days of incubation period when the bacteria multiply in the intestine, reaching numbers similar to *Salmonella* and *Shigella* infections (10^6-10^9 per gram of feces). Symptoms resemble an acute enteritis with fever, diarrhea, nausea, vomit and abdominal pain. These symptoms persist from one to seven days, but in 20% of cases may exceed the week. Most *C. jejuni*-related enteric infections are self-limiting and immunocompetent patients rarely require clinical treatment (Altekruse et al., 1999; Deckert et al., 2010). More invasive manifestations may occur in elderly or very young individuals, and are responsible for important economic losses. *C. jejuni* has also been associated with a wide range of extra-intestinal infections including, Guillan-Barré syndrome, meningitis, myocarditis, hepatitis, myelitis, pancreatitis and haemolytic-uraemia syndrome (Baar et al., 2007; Braun et al., 2008; Burch at al., 1999; Carter and Cimolai, 1996; Cunningham and Lee, 2003; Kandula et al., 2006; Ropper, 1992). Infection with *C. jejuni* is the most common antecedent of the peripheral neuropathies Guillain–Barré (GBS) and Miller Fisher Syndromes (FS), certain *C. jejuni* strains specifically trigger GBS and that others specifically trigger FS (Takahashi et al., 2005), but not all strains of *C. jejuni* are capable of causing these sequelae. Campylobacter infection may also lead to reactive arthritis. Symptoms include inflammation of the joints, eyes, or reproductive or urinary organs. On average, symptoms appear 18 days after infection. Recent studies also suggest that the infection by *C. jejuni* can be one of the factors implicated in the development of intestinal Crohn's disease (Lamhonwah et al., 2005). In the treatment of campylobacteriosis is fundamental the rehydration of lost body fluids because of watery diarrhea and are subjected to antibiotic treatment (with erythromycin, tetracycline or fluoroquinolone), only the more severe infections. Animal infection is usually asymptomatic, it provides sources of human infection via contaminated food products or untreated water (Hopkins et al., 1984). Campylobacteriosis is estimated to affect over 2.4 millions people every year. In the last 10 years, the spread of this disease increased representing an extensive public health issue which causes high hospitalization and therefore important economic losses. EFSA (2012) reported 212,064 cases of campylobacteriosis in 2010 in the European Union, a tendency in light increase in comparison to the 198,682 cases of 2009. In the UK it has been reported that

82% of people admitted to hospital with a diagnosis of 'food poisoning' were suffering from *Campylobacter* infection (Adak et al., 2002). Raw poultry products are well-documented sources of *Campylobacter* (EFSA Journal, 2010; Sammarco et al., 2010); in particular the consumption of undercooked chicken (Mori et al., 2008; O'Leary et al., 2009), pigs and cattle (Singh et al., 2011) products or the cross-contamination of ready-to-eat food products by raw poultry (Goodman et al., 1983; Luber et al., 2006) have been associated with some outbreaks.

The risk factors for contracting the pathogen can be identified in the handling and consumption of raw or undercooked poultry meat (Friedman et al., 2008), unpasteurized milk (Doyle et al., 1984; EFSA Journal, 2006; Arun, 2008) and raw foods that have undergone possible cross-contamination with poultry already contaminated by the bacteria. To these food-borne causes, we may add the consumption of water contaminated by the organism through waste resulting from slaughterhouses of poultry and infected animals (Blaser et al., 1984; Rosef et al., 2001) or faeces derived from wild birds. Several environmental reservoirs can lead to human infection by *C. jejuni*. It colonizes the chicken gastrointestinal tract in high numbers, primarily in the mucosal layer, and is passed between chicks within a flock through the faecal–oral route. *C. jejuni* can enter the water supply, where it can associate with protozoans, such as freshwater amoebae, and possibly form biofilms. *C. jejuni* can infect humans directly through the drinking water or through the consumption of contaminated animal products, such as unpasteurized milk or meat, particularly poultry (Fig. 2.3) (Young et al., 2007).

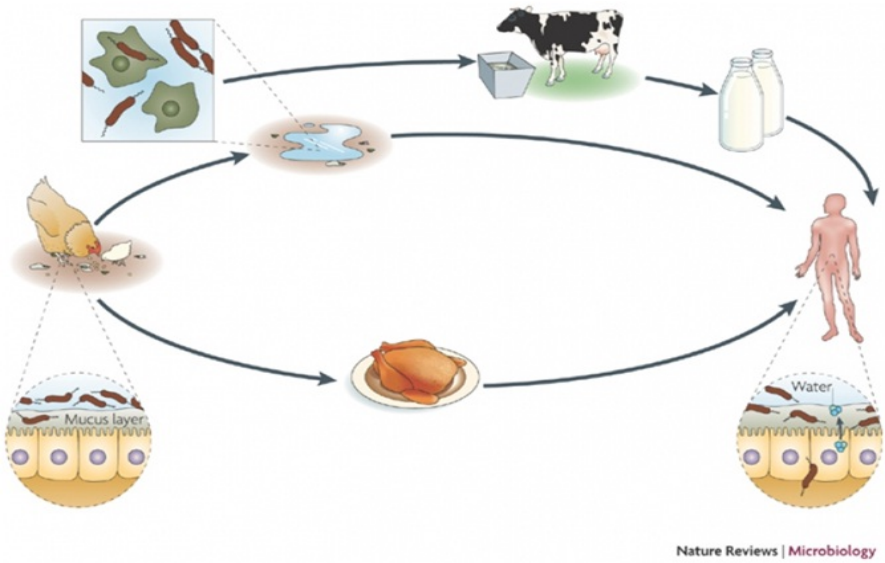


Figure 2.3: Transmission pathway of *Campylobacter* spp. (Young et al. (2007) Nature Review Microbiology).

The thermophilic *Campylobacter* species are commonly found as commensals of the gastrointestinal tract of wild species, such as ducks and gulls; farmed species, such as cattle, small ruminants and pigs, pet species, such as cats and dogs, and in all the poultry species (Danborg et al., 2004; Bae et al., 2005; Acik et al., 2006; EFSA Journal, 2011). Recent studies have demonstrated the absence of clinical or pathological signs in animals resulted positive to the isolation of the bacterium, confirming the commensal role of this microorganism (Dhillon et al., 2006). Therefore, in addition to the consumption of food contaminated by the bacteria, another possible reason of infection in humans, is represented by the contact with animals which act as a reservoir (Blaser et al., 1984). The entry of the bacterium in the livestock is still a matter of discussion, but the main sources are: environment, feed, farm-technicians that can act as a vehicle for the pathogen, wild birds, pets that may be present in the livestock and even insects such as flies (Shane et al., 1985; Newell and Farnely, 2003; Bates et al., 2004; Hald et al., 2004). As confirmation of the last transmission way, some studies have shown that the seasonal insect prevalence may be associated with an increased risk of contamination of poultry products. Moreover, the environmental control of flies, through the use of barriers in the ventilation system of the livestock, showed a delay and a reduction of the colonization of poultry by *Campylobacter* (Hald et al., 2007). When an outbreak is present in the livestock, the high bacterial load excreted by faeces and coprophagia by healthy subjects becomes a way for the rapid spread of the bacteria. The transmission is then horizontal, between infected subject and healthy subject. The main critical points during slaughter, for the carcass contamination have been identified in plucking, as the skin follicles remain open and are a great place for contamination by bacteria, evisceration and in the final wash of the carcass, with a variation on the prevalence of the bacterium depending on the operation. The scalding of carcasses, implemented with water at temperatures higher than 60° C, is a good method to reduce the bacterial load, which increases during the operations of plucking causing cross contamination (Guerin et al., 2010). The bacteria load increases with evisceration due to leakage of intestinal contents rich in *Campylobacter* in positive animals (Rosequist et al., 2006; Guerin et al., 2010). After gutting, another method to reduce the microbial load is represented by the washing of the carcass which could also cause an entrapment of the pathogen at the level of the abdominal cavity or feather follicles (Wempe et al., 1983; Guerin et al., 2010). The contamination of the slaughterhouses where animals positive for *Campylobacter* are processed, cannot therefore be avoided (Herman et al., 2003). In another study, bacteria carried by airborne particles and micro-drops during the various processes have been identified, even for animals resulted negative for the presence of the microorganism (Allen et al., 2007). The

bacteria can also resists to the cleaning and disinfection operations adopted in the slaughterhouse, remaining vital and contaminating subsequent carcasses, or remain on the surface of the carcass, facilitated by the formation of a biofilm, providing an additional source of contamination (Perko - Makela et al., 2009). The high number of animals kept in confined areas and the use of industrial feed, favored the uncontrolled distribution of saprophytic microorganisms, especially opportunists and potential pathogens in the farms, slaughterhouses and poultry industry. Poultry meat has a rich indigenous microbial flora or derived from feces, feed, water or air by which they come in contact (farms, slaughterhouses, equipment) (De Felip, 2001). The microbial contamination of the skin is greatly influenced by the environmental and hygienic conditions and the tendency, in general, is to increase with the progression of meat processing. Initially the live bird microbial flora consists of Gram-positive bacteria of environmental origin, after slaughtering there is a predominance of Gram-negative bacteria from both faecal and environmental origin. The main microorganisms present on poultry meat are *Pseudomonas* spp., *Micrococcus* spp., *Staphylococcus* spp., *Flavobacterium* spp., *Acinetobacter* spp., *Moraxella* spp., *E. coli* and other coliforms, *Listeria* spp., *Salmonella* spp., *Campylobacter* spp., Group D *Streptococcus*, *Clostridium perfringens*, yeasts and molds (De Felip, 2001). Most *Campylobacter* isolated from poultry products belong to the species *C. jejuni*, followed by *C. coli* and rarely from other species of little epidemiological interest (Acuff et al., 1986; Logue et al., 2003; Atanassova et al., 2007; Perko - Makela et al., 2009). Some studies have reported conflicting data on a high prevalence of *C. coli* compared to the species *C. jejuni* (Pezzotti et al., 2003; Smith et al., 2004). The prevalence of either species can be induced by different antimicrobial treatment implemented during the farming, able to encourage the colonization of the host by the species with greater resistance (Logue et al., 2010). There are some very interesting studies that have found a seasonal variation in the incidence of infection with *Campylobacter* in Turkey. (Doyle 1984; Logue et al. 2003) which showed a higher incidence of infection during the winter and spring compared to the summer months, while Blaser et al. (1984) and Willis and Murray (1997) found data in stark contrast, with a major impact during the summer period (prevalence of 7-33 % in the winter months vs. 87-97 % in the summer). These differences in the prevalence of the different microorganism, linked to seasonal variations, may be associated with different geographical location in which the samples were collected (Logue et al., 2003).

2.2 Methods to detect *Campylobacter* spp.

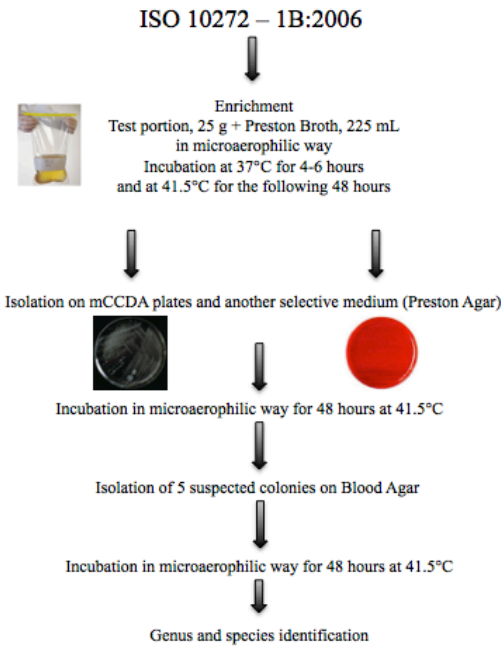
Campylobacter spp. can be detected through both plating and biochemical assays; it forms typical colonies on agar selective media when it is incubated in microaerophilic conditions at 41.5°C and it possesses a very characteristic motility and biochemical properties. Rosenquist et al. (2007) asserted that direct plating on mCCDA is a good method to enumerate the thermo-tolerant *Campylobacter* in poultry meat. Moreover, mCCDA is the recommended ISO (International Organization for Standardization) medium for enumeration of thermo-tolerant *Campylobacter* in foods (ISO 10272-1-2006).

This standard method include three fundamental steps:

- ✓ Enrichment and purification;
- ✓ Genus confirmation;
- ✓ Species identification (facultative).

In 2006, The ISO standard method (Fig. 2.4) propose the utilization of Bolton broth instead of Preston Broth for the selective enrichment step but in 2010 a further revision suggest to utilize one of the two different selective enrichment broths depending on the matrix. Bolton broth was suggested for the detection of *Campylobacter* in foods with low background of non-*Campylobacter* (cooked or frozen products) and/or with stressed campylobacters (ISO 10272-1A), and Preston broth for the detection of *Campylobacter* in foods with high background of non-*Campylobacter* (raw meats and raw milk) (ISO 10272-1B). The use of Preston broth instead of Bolton broth was necessary after noticing the lack of recovery of *C. jejuni* using the method ISO 10272-1A in the presence of ESBL (Extended-Spectrum-Beta-Lactamase) *E. coli,* normally well represented in meat food samples (Jasson et al., 2009). Bolton broth in fact, contains cefoperazone that pertains to the beta-lactamase antibiotics group. In Preston broth, polymyxin B (instead of cefoperazone) is active against Gram negative bacteria, rifampicin (against both gram positive and negative), trimethoprim for the inhibition of *Proteus* species and cycloheximide as fungal agent. The adding of lysed horse blood and *Campylobacter* growth supplement (sodium pyruvate, sodium metabisulphite and ferrous sulphate) is made to enhanced growth and aerotolerance of *Campylobacter*. Both the methods include microaerobic incubation for 4-6 hours at 37°C and 40-48 hours at 41.5°C and the isolation on mCCDA and 2nd medium. Genus confirmation needs also the growth of *Campylobacter* suspected colonies in aerobic conditions at 25°C on blood agar, and their morphology and motility examination directly from blood agar or mCCDA under microscopy, and the oxidase test (Revision ISO 10272, 2010). Biochemical assays and antibiotic

sensitivity can discriminate between different species although some strains show increased resistance for these antibiotics.



GENUS IDENTIFICATION

MORPHOLOGY	Small, curved bacillus
MOTILITY	Caracteristic
GROWTH AT 25°C (MICROAEROPHILIC)	-
GROWTH AT 41.5°C (AEROBICALLY)	-
OXIDASE	+

SPECIES IDENTIFICATION

	C. jejuni	*C. coli*	*C. lari*	*C. upsaliensis*
CATALASE	+	+	+	-/+
NALIDIXIC ACID	S	S	R/S	S
CEPHALOTHIN	R	R	R	S
HIPPURATE hydrol.	+	-	-	-
ACETATE hydrol.	+	+	-	+

Figure 2.4: Standard ISO method for the detection and isolation of *Campylobacter* spp.

The different characteristics of antibiotic - resistance and risk for development of disease in humans, make the precise identification of the bacterial species necessary (Gillespie et al., 2002) but biochemical-based assays for *Campylobacter* species identification are time-consuming, and laborious, due to the fastidious growth requirements, and the paucity of informative biochemical characteristic. Therefore, several diagnostic methods were developed, such as Real Time PCR (Sails et al., 2003; Yang et al., 2003; Lund et al. 2004) and numerous PCR based assays able to identify *Campylobacter* species (Comi et al., 1995; Comi et al., 1996; Linton et al., 1996; Inglis and Kalischuk, 2003; Leblanc et al., 2011; O'Mahony et al., 2011). PCR is often affected by some problems due to the presence of contaminants into the DNA extraxted from food matrix, for this reason, to overcome the problem, rapid methods have been developed for detecting and confirming *Campylobacter* spp., such as fluorescence *in situ* hybridization (FISH) (Lehtola et al., 2006) and oligonucleotide- based microarray (Suo et al., 2010). *Campylobacter jejuni* and *C. coli* have a genome of approximately 1600-1700 kilobases (kb), which is relatively small compared with that of enteropathogens such as *Escherichia coli*, which have a genome of approximately 4500 kb (Chang and Taylor, 1990, Nuijtien, 1990). Into the entire genomes of these bacteria, various DNA

sequences have been chosen for the rapid detection of *Campylobacter* species during the last twenty years, from 16S rDNA, 23S rDNA, *fla*A and *fla*B genes, *cad*F virulence gene, *hip*O hyppuricase gene, to *ceu*E, and *por*A. The 16S rRNA gene has been extensively utilized for rapid detection and identification of *Campylobacter* species (Kulkarni et al. 2002, Linton et al., 1996, Linton et al. 1997, Maher et al., 2003), due to the considerable length (approximately 1.500 bp), and presence in members of the *Campylobacter* genus and almost all bacteria (Clarridge, 2004; Woese, 1987). 16S rRNA gene is relatively short at 1.5 Kb, to allow easy sequencing, but at the same time long enough to be informative. Nevertheless the high similarity observed in 16S rDNA between members of the *Campylobacter* genus makes it difficult to differentiate between species such as *C. jejuni* and *C. coli* (Harrington and On, 1999; On, 2001). Although currently there is relatively little sequence data available on the internal transcribed spacer (ITS) region that lies between the 16S and 23S rRNA genes (within the 16S-ITS-23S operon), comparative sequence analyses of the 16S and 23S rRNA genes and the ITS region of members of the *Campylobacter* genus revealed that the most discriminatory region for species and strain differentiation was the ITS region (Man et al., 2010; Van Camp et al., 1993; Meinersmann et al., 2009). Moreover, the ITS region in *C. jejuni* and *C. coli* has been reported to be highly variable in size and/or sequence composition (Christensen et al., 1999; Khan and Edge, 2007). Another gene that can be chosen for the specie-specific identification of *Campylobacter*, is the *por*A gene. The *por*A gene encodes for the major outer-membrane protein (MOMP) of *Campylobacter*, it is extremely genetically diverse, and it consists of seven highly variable regions interspersed among conserved sequences. Deduced amino acid sequences comprise long irregular external loops connected by 18 b-strands and short periplasmic turns (Zhang et al., 2000). MOMP are pore proteins that have a number of roles including regulation of cell membrane permeability to small molecules, adherence to host cells and antibiotic resistance (Buchanan, 1999; Cowan et al., 1992; Jeanteur et al., 1991; Kervella et al., 1992; Moser et al., 1997; Page et al., 1989; Schroder and Moser, 1997). Conformational MOMP epitopes are important in host immunity, and variation in surface-exposed regions probably occurs as a result of positive immune selection during infection. Therefore, they are sites of interaction of the bacterial cell with the environment and may play an important role in the adaptation of *Campylobacter* to various hosts. Moreover, *por*A complexed to a high molecular weight carbohydrate was reported to exhibit cytotoxicity in vitro and in vivo. The active moiety of the toxin was characterised as the *por*A protein (Bacon et al., 1999) and Parkhill et al. (2000) reported thet the N-terminal amino acid sequence of the cytotoxic protein matched that of the porin protein in the sequenced strain.

2.3 *por*A specific primers for the identification of *Campylobacter* species in food and clinical samples

Raw poultry meat samples were analysed both by plate count method, and using four couple of species specific primers for *C. jejuni*, *C. coli*, *C. upsaliensis* and *C. lari* designed bridging the *por*A gene. The produced primers showed high sensitivity and specificity, and were useful for the analyses of poultry meat samples both after direct plating onto isolates from mCCDA, and after DNA extraction from 48 h in Preston enrichment broth. The couples of primers showed good performances also when used for species identification in human isolates. The aim of this study is to propose a sensitive, cheap and fast method for the detection and/or identification of *Campylobacter* species, with particular emphasis toward *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, which are the four most frequent species isolated in foods and hospitalized humans.

2.3.1 Materials and methods

2.3.1.1 Bacterial strains and culture media

The reference *Campylobacter* strains used in this work, and reported in Table 2.1a were grown in appropriate medium, Brain Heart Infusion Broth (Oxoid, Milan, Italy) and grown in Blood Agar Base N° 2 (Oxoid) supplemented with 7% Laked Horse Blood (Oxoid) in 6% O_2, 7% CO_2, 7% H_2, 80% N_2 microaerophilic atmosphere (CampyGen, Oxoid) at 37 °C for 48 hours, according to the supplier's specifications. These strains were used to check the specificity and the sensitivity of the primers. Eight *Campylobacter* spp. strains from hospitalized patients with diarrhoea (clinical samples 1, 2, 3, 4, 5, 6, 7 and 8) were obtained from Azienda Servizi Sanitari n. 2 Ospedale San Polo (Monfalcone, Italy). Two *Campylobacter* spp. strains from pig intestine (pig sample 1 and 2) were gathered from the Veterinary Section of the Department of Food Science (Udine, Italy). *Campylobacter* strains from clinical and veterinary swabs were isolated on two different selective media: mCCD Agar (Oxoid) supplemented with CCDA Selective Supplement (Oxoid) and *Campylobacter* Selective Preston Agar (Oxoid) supplemented with Modified Preston Selective Supplement (Oxoid) and Laked Horse Blood (Oxoid) in microaerophilic atmosphere at 41.5 °C for 48 hours. The typical colonies were purified on Blood Agar Base N° 2 supplemented with 7% Laked Horse Blood, and confirmed as *Campylobacter* based on their characteristic cell morphology, motility, catalase and oxidase test.

Table 2.1a: Reference *Campylobacter* strains used for the optimisation of the PCR protocol.

N°	Microorganism	Source
1	*Campylobacter jejuni* subsp. *jejuni*	*DSM 4688
2	*C. jejuni* subsp. *jejuni*	§ATCC BAA-1153
3	*C. jejuni* subsp. *jejuni*	§ATCC 49943
4	*C. coli*	*DSM 24155
5	*C. coli*	*DSM 24128
6	*C. coli*	§ATCC 43478
7	*C. lari* subsp. *lari*	*DSM 11375
8	*C. upsaliensis*	*DSM 5365

§ATCC: American Type Culture Collection (Manassas, VA, USA); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany)

In order to check the specificity of the PCR protocols, all the primers have been tested also using the no-*Campylobacter* strains reported in Table 2.1b.

Table 2.1b: Reference no-*Campylobacter* strains used for the optimisation of the PCR protocol.

N°	Microorganism	Source
1	*Weissella cibaria*	*DSM 14295
2	*Vibrio* spp.	*DSM 14379
3	*Escherichia coli*	°DISTAM
4	*Pseudomonas aeruginosa*	°DISTAM
5	*P. migulae*	°DISTAM
6	*P. fluorescens*	°DISTAM
7	*P. brennerii*	°DISTAM
8	*Bacillus coagulans*	*DSM 2308
9	*B. subtilis*	*DSM 1029
10	*B. cereus*	*DSM 2301
11	*Proteus vulgaris*	°DISTAM
12	*Yersinia enterocolitica*	°DISTAM
13	*Morganella morganii*	°DISTAM
14	*Salmonella enteritidis*	*DSM 4883
15	*Listeria monocytogenes*	§ATCC 7644
16	*Citrobacter freundii*	*DSM 15979
17	*Enterobacter cloacae*	*DSM 30054
18	*Aeromonas sobria*	*DSM 19176
19	*Lactobacillus plantarum*	*DSM 20174
20	*Pediococcus pentosaceus*	*DSM 20336
21	*Leuconostoc lactis*	#CECT 4173
22	*Saccharomyces cerevisiae*	§ATCC 36024

§ATCC: American Type Culture Collection (Manassas, VA, USA); °DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany); #CECT: Colección Española de Cultivos Tipo (University of Valencia, Spain.

2.3.1.2 Samples

24 fresh chicken meat samples (thighs, wings and breast), and 7 fresh turkey meat samples (thighs, wings and breast) were collected from local supermarket. Totally 31 samples were analysed according to ISO 10272 - 1B: 2006 procedures for enrichment and enumeration of *Campylobacter*. Two different selective media, mCCD Agar (Oxoid, Milan, Italy) supplemented with CCDA Selective Supplement (Oxoid, Milan, Italy), and *Campylobacter* Selective Preston Agar (Oxoid) supplemented with Modified Preston Selective Supplement (Oxoid) and Laked Horse Blood (Oxoid) were used in microaerophilic atmosphere at 41.5°C for 48 hours. In order to isolate *Campylobacter* strains, five colonies were picked from mCCD agar plates when possible, purified on Blood Agar Base No 2 supplemented with 7% Laked Horse Blood, and subjected to cell morphology analyses using an optical microscope (Carl Zeiss, Oberkochen, Germany) at a 1,000X final magnification prior of being subjected to motility, catalase, oxidase test, and growth at 25°C. The positive strains were subjected to PCR protocol for identification. Ten ml from the enrichment broth after 48 h were used for DNA extraction and subjected to PCR.

Samples were also analysed for the presence of *Salmonella* spp. according to ISO 6579:2004, *Listeria* spp. according to ISO 11290:2005, for the aerobic bacterial count, *Enterobacteriaceae*, and coliforms. 25 g of skin from chicken and turkey, were transferred into a sterile stomacher bag, added with 100 ml of saline-peptone water (8 g/l NaCl, 1 g/l bacteriological peptone, Oxoid), and mixed for 1.5 min in a Stomacher machine (PBI, Milan, Italy). From this homogenate, three 25 ml aliquots were used for the enrichment protocols as shown in Fig. 2.5. One ml aliquots were used for serial decimal dilutions to inoculate VRBG (Violet Red Bile Glucose Agar, Oxoid) (37°C for 24 h) for the enumeration of *Enterobacteriaceae*, and VRBL (Violet Red Bile Agar, Oxoid) (37°C for 24 h) for the evaluation of total coliforms, and 0.1 ml from each dilution was spread onto Plate Count agar (PCA, Oxoid) plates for the enumeration of the total aerobic count. For the detection of *Campylobacter* spp. one ml was spread onto 5 plates of mCCD agar (0.2 ml each) and incubated at 41.5°C for 48 h under microaerophilic conditions.

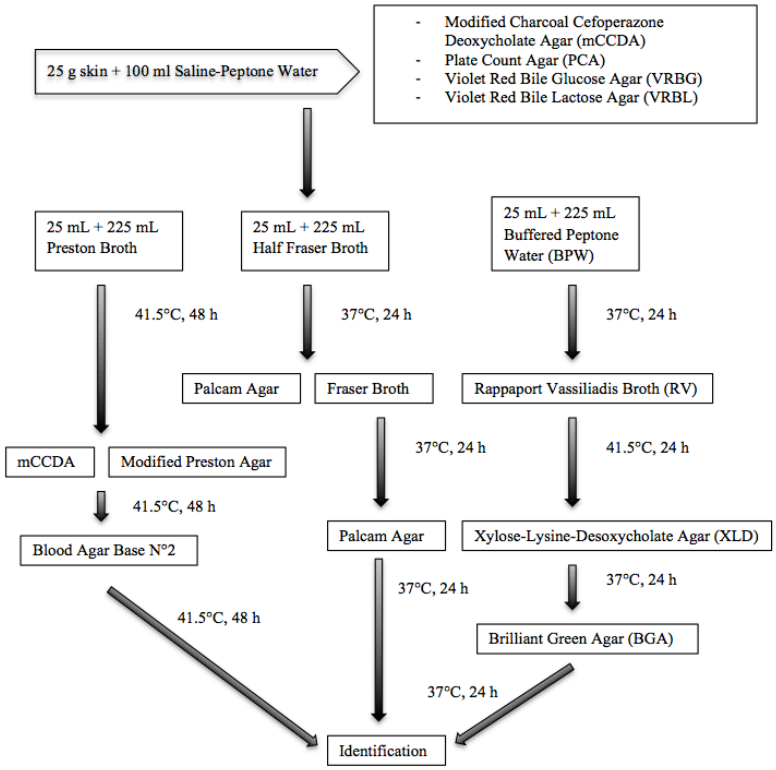


Figure 2.5: enrichment protocols.

2.3.1.3 DNA extraction from pure cultures and poultry samples

In order to obtain DNA from isolated colonies, one ml of overnight cultures (Cecchini et al. 2012) was used for the DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy) according to the manufacturer's instructions. Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.) was used for the standardisation of the DNA at the final concentration of 100 ng/µL with sterile DNA-free Milli-Q water. For the DNA extraction from enrichment broth, bacterial pellet was obtained from 10 ml of 48 h Preston broth enrichment at 41.5°C. After centrifugation at 14.000 x g for 5 min, the pellet was resuspended in 300 µL of breaking buffer [2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris (pH 8), 1mM EDTA (pH 8)] and transferred into a screw cap tube containing 0.3 g of glass beads with a diameter of 0.1 mm and subsequently 300 µL of phenol/chlorophorm/isoamyl alcohol (25:24:1, pH 6.7; Sigma) were added and the cells were homogenized in a bead beater (Fast Prep 24, Bio 101, Vista, CA)

three times each for 30 s, with an interval of 15 s between each treatment. 300 µL of TE buffer (10 mM Trizma base, 5 mM EDTA, pH 8) were added and the tubes were centrifuged at 14,000 x g for 10 min. The aqueous phase was collected and precipitated with 1 mL of ice-cold absolute ethanol. The DNA was collected with a centrifugation at 14,000 x g for 10 min and the pellet was dried under vacuum at room temperature. 50 µL of DNase-free sterile water (Sigma) were added and a 30 min period at 45 °C was used to facilitate the nucleic acid solubilisation. 1 µL of DNase-free RNase (Roche Diagnostics, Milan, Italy) was added to digest RNA with incubation at 37 °C for 1 h (Iacumin et al., 2009).

2.3.1.4 Primer design for C. jejuni, C. coli, C. lari, and C. upsaliensis

All the *Campylobacter* sequences of *por*A gene were retrieved from GenBank and aligned using "Multiple sequence alignment with hierarchical clustering" (Fig. 2.6) (Corpet, 1988). Sequences accession numbers: *Campylobacter jejuni* DQ341255.1, *Campylobacter coli* FJ946222.1, *Campylobacter lari* AB569421.2, *Campylobacter upsaliensis* DQ868939.1. Four couple of primers (Table 3.2), JejuniF-JejuniR, ColiF-ColiR, LariF-LariR, UpsF-UpsR, were designed within the *por*A gene sequence for the detection of *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* respectively. Primer sequences are reported in Table 2.2. All the primers were tested using AmplifX 1.6.2 (Jullien, 2013) and synthesized by MWG-Biotech and Sigma-Aldrich Co.

Table 2.2: Sequences of the primers used, target gene, and amplicon length expressed in base pair (bp).

Primer Name	Primer Sequences	Target Gene	Amplicon Length
JejuniF	5'-TTCAGCAGCTAACGCTACT- 3'	*por*A	
JeiuniR	5'-AGCGATTACACTTGTAGCA- 3'	*por*A	340 bp
ColiF	5'-TGGTTGGGATGCAACTCTT- 3'	*por*A	
ColiR	5'-GCCTACACGAACTGTTTCGTTG- 3'	*por*A	211 bp
LariF	5'-CAATACTTAGGAAATAGCTTAGAC- 3'	*por*A	
LariR	5'-GCTTGTTTAGATTTACCACCGA -3'	*por*A	359 bp
UpsF	5'-TGGAATGGCTTTGACGCT - 3'	*por*A	
UpsR	5'-GGTATAACCAGCAGTTAGG- 3'	*por*A	192 bp

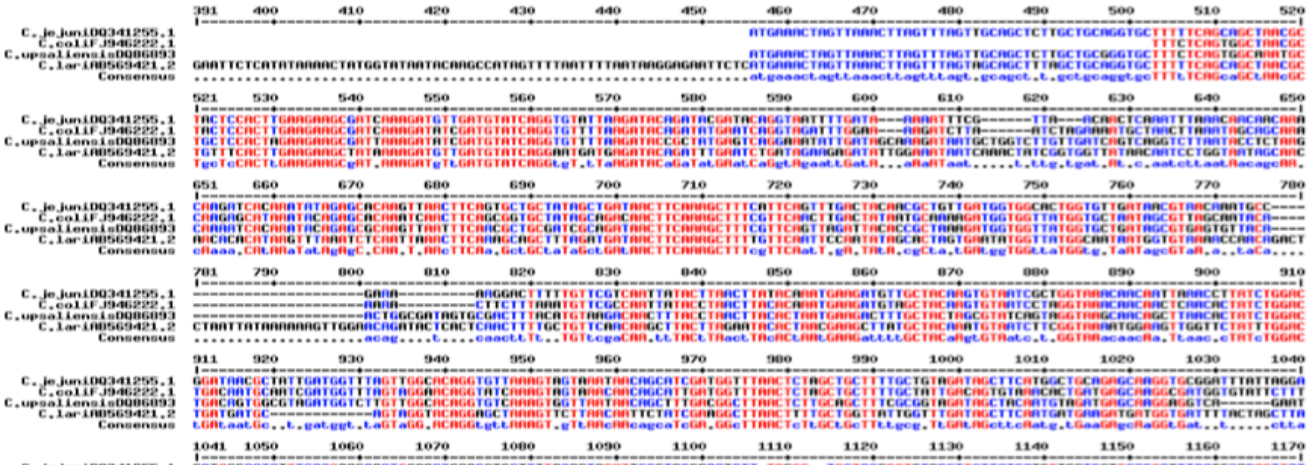


Figure 2.6: Alignment among the *por*A sequence of *Campylobacter* species. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used.

2.3.1.5 PCR protocols

For each couple of primer a specific annealing temperature was optimized. PCR assays were carried out in reaction mixtures containing GoTaq® Buffer 1x (Promega, Milan, Italy), 200 mmoL/L of each dNTP, 200 μmoL/L of each primer, 1.5 mmoL/L $MgCl_2$, 1.25 U of GoTaq® DNA Polymerase (Promega), and sterile water to reach the volume of 49 μL. 1 μL of DNA (100 ng/μL) was added to reach a final volume of 50 μL. Thermal cycler (PTC220 DNA Engine DYAD MJ Research Celbio, Italy) conditions consisted of 95°C denaturation for 5 min, followed by 35 cycles of 95°C for 1 min, annealing for 30 s at 56°C for *C. jejuni*, 59°C for *C. coli*, 52°C for *C. lari* and *C. upsaliensis*, extension at 72°C for 30 s, and a final step at 72°C for 7 min. The sensitivity of the PCR assays was tested using serial dilutions from 100 ng/μL to 0.01 ng/μL of the corresponding DNA from the *C. jejuni, C. coli, C. lari and C. upsaliensis* reference strains.

2.3.1.6 Electrophoresis

Five μL aliquots of the PCR products were resolved electrophoretically in a 1.5 % agarose gel (Sigma, Milan, Italy) in 0.5x TBE (2 mM EDTA, 80 mM Tris-acetate, pH 8.0) stained with GelRed from Biotium (Hayward, CA) and compared with 100 bp DNA Ladder (Promega). The results were examined under UV light and processed using BioImaging System GeneGenius (SynGene, UK).

2.3.2 Results

2.3.2.1 Plate count bacterial enumeration of poultry samples

The aerobic bacterial count, as well as *Enterobacteriaceae*, total Coliforms, *Campylobacter* spp., enumeration, and the presence or absence of *Salmonella, Listeria* and *Campylobacter* obtained for the 31 samples reported in Table 2.3 showed the following results: nineteen out of the 31 samples analysed were positive for *Salmonella* spp. and 25 resulted positive for *Listeria* spp. presence*;* seventeen samples (15 chicken and 2 turkey) were positive for the presence of *Campylobacter* spp., and 14 samples resulted below the detection limit of the protocol adopted (5 cfu/g). Direct correlation was found between the enrichment in Preston broth, and the direct plate count method. In fact the samples positive for *Campylobacter* spp. gave the same results using both procedures apart for the turkey thighs (FS 25) which resulted negative by mCCDA direct plating and were positive after enrichment. The isolates from mCCDA and enrichment broth of the 31 food samples were subjected to the optimized PCR protocols for the confirmation, and *Campylobacter* species

identification demonstrating the high specificity of the primers designed within the *por*A gene.

Table 2.3: Plate count data in Colony Forming Unit (CFU)/g and presence (+) or absence (-) for *Campylobacter* spp., *Listeria* spp. and *Salmonella* spp. after ISO protocol. * detection limit

Sample	Aerobic bacterial count	*Enterobacteriaceae*	Coliforms	*Campylobacter* spp. on mCCDA	*Campylobacter* spp. ISO 10272-1B:2006	*Salmonella* spp. ISO 6579:2004	*Listeria* spp. ISO 11290:2005
1 chicken wings	7.8×10^7	2.0×10^4	1.5×10^2	0.5×10	+	+	+
2 chicken thighs	3.6×10^6	1.8×10^3	3.0×10^2	< 5 *	-	+	+
3 chicken thighs	3.7×10^7	3.9×10^5	4.5×10^4	< 5*	-	+	+
4 chicken thighs	5.9×10^8	3.3×10^7	4.3×10^5	< 5*	-	+	+
5 turkey wings	3.9×10^7	3.7×10^6	3.0×10^4	0.5×10	+	+	+
6 chicken thighs	1.4×10^4	7.0×10^2	3.8×10^2	2.0×10	+	+	+
7 chicken thighs	6.5×10^7	6.0×10^5	7.5×10^4	1.5×10	+	+	+
8 turkey wings	2.9×10^8	1.8×10^5	1.7×10^4	< 5*	-	+	+
9 turkey wings	3.1×10^8	7.0×10^5	1.6×10^4	3.6×10^3	+	+	+
10 chicken thighs	8.3×10^5	6.2×10^3	1.8×10^2	< 5*	-	-	-
11 chicken thighs	1.7×10^5	8.8×10^3	3.3×10^2	< 5*	-	+	+
12 chicken wings	3.0×10^4	2.7×10^3	1.5×10^2	1.6×10^3	+	+	+
13 chicken thighs	3.3×10^6	6.6×10^3	1.9×10^2	< 5*	-	-	-
14 chicken breast	5.8×10^6	5.0×10^4	3.5×10^3	4.0×10^2	+	-	-
15 chicken thighs	2.3×10^4	6.8×10^3	2.6×10^2	< 5*	-	+	+
16 chicken thighs	4.0×10^4	5.3×10^3	4.0×10^2	1.5×10	+	+	+
17 chicken thighs	3.8×10^5	2.5×10^3	9.5×10^2	1.5×10	+	+	+

18 chicken thighs	2.5×10^7	3.4×10^4	2.4×10^2	< 5*	-	+	+
19 turkey breast	5.2×10^7	1.6×10^4	5.3×10^2	< 5*	-	+	+
20 chicken thighs	3.3×10^7	3.7×10^5	3.2×10^3	4.5×10^2	+	-	+
21 chicken wings	2.2×10^7	7.0×10^3	2.3×10^3	2.0×10	+	+	-
22 chicken thighs	1.2×10^6	2.9×10^2	2.5×10^2	1.5×10^2	+	-	+
23 chicken thighs	6.8×10^5	4.7×10^3	2.1×10^2	$3.5x\ 10^2$	+	+	+
24 chicken thighs	1.8×10^5	3.1×10^3	1.0×10^3	3.6×10	+	-	+
25 turkey thighs	1.4×10^9	2.1×10^5	9.2×10^4	< 5*	+	-	+
26 chicken thighs	5.4×10^5	6.4×10^3	1.7×10^3	< 5*	-	+	+
27 chicken thighs	2.3×10^6	2.8×10^3	2.3×10^2	3.2×10	+	-	-
28 turkey thighs	1.1×10^8	4.8×10^4	3.2×10^4	< 5*	-	-	+
29 chicken thighs	3.3×10^7	8.0×10^2	1.2×10^4	4.0×10	+	-	-
30 turkey thighs	1.4×10^7	6.8×10^3	3.6×10^3	< 5*	-	-	+
31 chicken thighs	4.7×10^7	2.0×10^4	2.4×10^4	8	+	-	+

2.3.2.2 PCR

Only *Campylobacter* strains gave the expected PCR amplicons confirming the specificity of the designed primers. The sensitivity (Fig. 2.7 A and B) varied, in fact, primers ColiF-ColiR, LariF-LariR, and UpsF-UpsR produced a clear amplicon starting from 0.01 ng/µL DNA, while JejuniF-JejuniR primers needed a DNA concentration of 0.5 ng/µL.

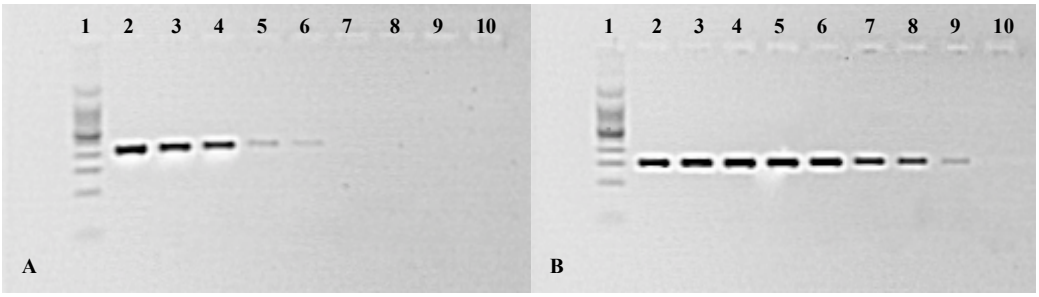


Figure 2.7: Sensitivity test with JejuniF-JejuniR and ColiF-ColiR primers
A - Sensitivity test with JejuniF-JejuniR primers. Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); *Campylobacter jejuni* ATCC BAA-1153 (line 2-9), line 2: 100 ng/µL DNA; line 3: 10 ng/µL DNA; line 4: 5 ng/µL DNA; line 5: 1 ng/µL DNA; line 6: 0.5 ng/µL DNA; line 7: 0.1 ng/µL DNA; line 8: 0.05 ng/µL DNA; line 9: 0.01 ng/µL DNA; line 10: negative control.
B - Sensitivity test with ColiF-ColiR primers Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); *Campylobacter coli* DSM 24128 (line 2-9), line 2: 100 ng/µL DNA; line 3: 10 ng/µL DNA; line 4: 5 ng/µL DNA; line 5: 1 ng/µL DNA; line 6: 0.5 ng/µL DNA; line 7: 0.1 ng/µL DNA; line 8: 0.05 ng/µL DNA; line 9: 0.01 ng/µL DNA; line 10: negative control.

The designed specific primers were used for the identification of the clinical samples (CS), food samples (FS) and for the two strains isolated from pig intestine (PIS). Four of the eight *Campylobacter* strains isolated from hospitalized patients (CS) were identified as *C. coli* (Fig. 2.8 A, lines 2, 5, 6 and 9), and four as *C. jejuni* (Fig. 2.8 B, lines 3, 4, 7 and 8). Each isolates produced the expected amplicon only for one couple of primers confirming the specificity of the primers.

Samples from pig intestine were identified as *C. coli*, based on the presence of the expected amplicon of 211 bp (Fig. 2.8 A, lines 10 and 11). The absence of a PCR product for these two samples, using the JejuniF-JejuniR primers, confirmed the correct identification of the samples as *C. coli.*

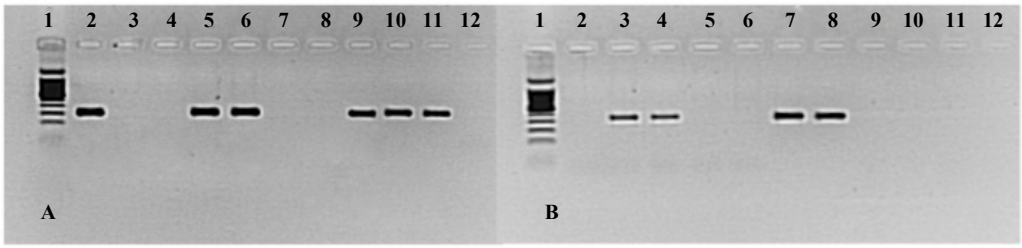


Figure 2.8: Clinical samples (CS), and Pig intestine samples (PIS) PCR.
A - Specie-specific PCR using ColiF-ColiR primers. Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); line 2: CS1; line 3: CS2; line 4: CS3; line 5: CS4; line 6: CS5; line 7: CS6; line 8: CS7; line 9: CS8; line 10: PIS1; line 11: PIS2; line 12: negative control.
B - Specie-specific PCR using JejuniF-JejuniR primers. Line 1: 100 bp DNA Ladder (Promega, Milan, Italy; line 2: CS1; line 3: CS2; line 4: CS3; line 5: CS4; line 6: CS5; line 7: CS6; line 8: CS7; line 9: CS8; line 10: PIS1; line 11: PIS2; line 12: negative control.

No amplification products were obtained from clinical samples and pig intestine samples when amplified with the specific primers (LariF-LariR, and UpsF-UpsR) designed for the detection of *C. lari* and *C. upsaliensis*.

Fifty-five colonies of *Campylobacter* were isolated from mCCDA from 18 out of the 31 food samples of chicken and turkey meat (FS number 1, 5, 6, 7, 9, 12, 14, 16, 17, 20, 21, 22, 23, 24, 25, 27, 29, and 31) (Table 2.3). No amplification products were obtained for isolates from food samples when amplified using the specific primers LariF-LariR and UpsF-UpsR, indicating the absence of *C. lari* and *C. upsaliensis* in the analysed food samples.

Twenty amplicons were obtained from food samples (FS) isolates using the couple ColiF-ColiR, and 35 using the primers JejuniF-JejuniR (Fig. 2.9 shows results from some samples). Considering the 18 food samples contaminated by *Campylobacter* spp., 8 were spoiled by *C. jejuni* (FS number 6, 7, 12, 14, 16, 20, 21 and 22), 5 by *C. coli* (FS number 1, 5, 9, 23 and 25), and 5 samples by both *C. coli* and *C. jejuni* (FS number 17, 24, 27, 29 and 31). *C. jejuni* was the prevalent species isolated from food samples with 35 isolates vs 20 *C. coli* isolates. *C. coli* and *C. jejuni* were detected also using the designed primers on DNA extracted from the enrichment broth after 48 h demonstrating the possibility to use PCR directly after enrichment, without the need of strain isolation. In fact, sample 25 that was negative on mCCDA, gave an amplicon using DNA extracted from the Preston broth enrichment at 48 h. This sample showed a contamination due to *C. coli*. The identification obtained on the isolates was confirmed also on the DNA extracted after enrichment. No amplicons were obtained using the specific primers UpsF-UpsR and LariF-LariR for isolates and DNA samples after enrichment.

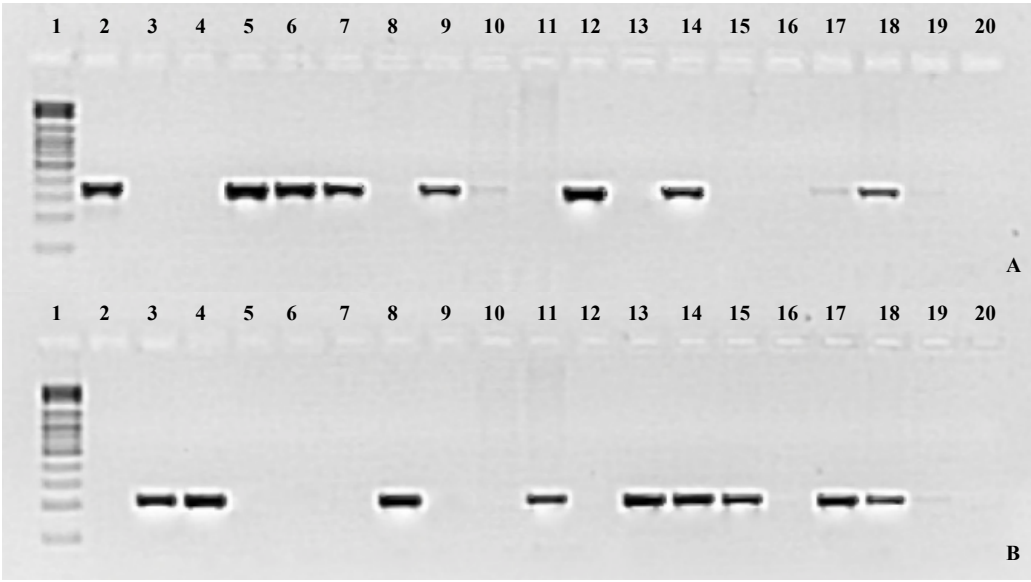


Figure 2.9: PCRs on Isolates and broths from Food Samples.
A - Specie-specific PCR using JejuniF-JejuniR primers Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); line 2: *C. jejuni* ATCC-BAA 1153; line 3: *C. coli* DSM 24128; line 4: FS1; line 5: FS6; line 6: FS14; line 7: FS17; line 8: FS17; line 9: FS20; line 10: FS31; line 11: FS31; line 12: FS22 broth; line 13: FS23 broth; line 14: FS24 broth; line 15: FS25 broth; line 16: FS26 broth; line 17: FS27 broth; line 18: FS29 broth; line 19: FS31 broth; line 20: negative control.
B - Specie-specific PCR using ColiF-ColiR primers; Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); line 2: *C. jejuni* ATCC-BAA 1153; line 3: *C. coli* DSM 24128; line 4: FS1; line 5: FS6; line 6: FS14; line 7: FS17; line 8: FS17; line 9: FS20; line 10: FS31; line 11: FS31; line 12: FS22 broth; line 13: FS23 broth; line 14: FS24 broth; line 15: FS25 broth; line 16: FS26 broth; line 17: FS27 broth; line 18: FS29 broth; line 19: FS31 broth; line 20: negative control.

2.3.3 Discussion

A good differentiation among the most common *Campylobacter* species responsible for campilobacteriosis in humans is difficult also using molecular methods, especially between *C. jejuni* and *C. coli* due to the high similarity of the genomes. *por*A gene showed a variability that allowed to differentiate among the more common strains responsible for human campilobacteriosis. Although *C. upsaliensis* is the second most frequently isolated species from individuals (Labarca et al. 2002), it is not often identified, as usually laboratories focus their attention on the two major species found in food, *C. jejuni* and *C. coli*, that cause illness in humans. The primers developed in this work are able to distinguish among isolates without any doubt, and they show high sensitivity (0.01 ng/μL DNA) also for the detection of *C. upsaliensis*, 100 times the sensitivity showed by the primer developed by Linton et al. (1996) that was 10 ng/μL for the 16S rRNA gene. They allow also the identification of *C. lari* with the same sensitivity and, although the clinical significance of these

species is well documented, reports of their occurrence in chicken meat are rare (Lynch et al. 2011; Moran et al., 2009; Krause et al., 2002; Werno et al., 2002). The reason could be the use of thermo tolerant conditions for the enrichment broths, followed by plate count methods, which not allow a wider recovery of *Campylobacter* genus. To overcome the reduced detection of *Campylobacter* species Lynch et al. (2010) proposed a modified protocol which uses lower incubation temperatures, but laboratories still need the application of procedures from International Standard Organization: ISO 10272-1B:2006 for the detection of *Campylobacter* spp. in products intended for human consumption. Our work demonstrates the possibility to identify *Campylobacter* strains with a direct plate count method followed by a specific PCR using *por*A as target gene in food samples and in human isolates, and the possibility to use these sensitive and specific primers on DNA extracted from enrichment broth at 48 h allowing the obtainment of a quick result according with ISO 10272-1B:2006.

This molecular method has the advantage to be able to detect also viable but non-cultivable cells of *Campylobacter* that can be present on meat samples, but not detected by classical plate methods (Silva et al. 2011).

As the consumption of poultry products has been implicated over the recent years in a large number of outbreaks of acute campylobacteriosis in human populations worldwide (Silva et al. 2011) causing economic losses in terms of hospitalization, a useful method for meat products surveillance is needed.

The advantage of this protocol is that it doesn't require expensive devices, or high level technicians, it is cheap, fast, sensitive, useful in the food laboratory to screen or confirm the presence of *Campylobacter* strains still maintain a good level of efficiency and the capability to give results in a short time. It could be useful also for epidemiological purposes if applied on isolates from hospitalized humans.

2.4 Quick *Campylobacter* spp. detection on poultry samples by Dot Blot and PCR

This work proposes two molecular methods, Dot Blot and PCR. Dot blot obtained a sensitivity of 25 ng/μL on DNA extracted from pure culture using a digoxigenin labelled probe that results also able to hybridize the target DNA extracted from the enrichment broth at 24 h. PCR used a couple of primers, sensitive and specific, able to differentiate *C. jejuni*, *C. coli* and *C. lari*. Primers were also used for the detection of *Campylobacter* spp. starting from real samples contaminated by 5 x 10 cfu/g and 1.5 x 10^2 cfu/g (which means that the number of cells present in the enrichment broth at 0 h was 1 and 3 cell/g at 24 h enrichment in Preston broth) and in an artificial inoculum of *C. jejuni* and *C. coli* in Preston broth using a concentration of 10 cell/mL. Due to the recognition that *Campylobacter* is the leading cause of bacterial foodborne diarrheal disease worldwide, and that in the last 5 years, campylobacteriosis has been the most commonly reported zoonosis in the EU (Silva et al., 2011), the aim of this study was to produce fast and simple protocols for *Campylobacter* detection using Dot Blot, for a quick screening of food samples, and PCR to differentiate between the three most important pathogenic *Campylobacter* contaminating food.

2.4.1 Material and Methods

2.4.1.1 Bacterial strains

Eight reference *Campylobacter* strains from international culture collections (Table 2.4a) and twenty-two non-Campylobacter strains were used for the optimisation of the PCR and dot-blot assays (Table 2.4b).

Table 2.4a: Reference *Campylobacter* strains used for the optimisation of the PCR and Dot-blot protocol.

N°	Microorganism	Source
1	*Campylobacter jejuni* subsp. *jejuni*	*DSM 4688
2	*C. jejuni* subsp. *jejuni*	§ATCC BAA-1153
3	*C. jejuni* subsp. *jejuni*	§ATCC 49943
4	*C. coli*	*DSM 24155
5	*C. coli*	*DSM 24128
6	*C. coli*	§ATCC 43478
7	*C. lari* subsp. *lari*	*DSM 11375
8	*C. upsaliensis*	*DSM 5365

§ATCC: American Type Culture Collection (Manassas, VA, USA); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany).

Table 2.4b: Reference no-*Campylobacter* strains used for the optimisation of the PCR and Dot blot protocol.

N°	Microorganism	Source
1	*Weissella cibaria*	*DSM 14295
2	*Vibrio* spp.	*DSM 14379
3	*Escherichia coli*	°DISTAM
4	*Pseudomonas aeruginosa*	°DISTAM
5	*P. migulae*	°DISTAM
6	*P. fluorescens*	°DISTAM
7	*P. brennerii*	°DISTAM

8	*Bacillus coagulans*	*DSM 2308
9	*B. subtilis*	*DSM 1029
10	*B. cereus*	*DSM 2301
11	*Proteus vulgaris*	°DISTAM
12	*Yersinia enterocolitica*	°DISTAM
13	*Morganella morganii*	°DISTAM
14	*Salmonella enteritidis*	*DSM 4883
15	*Listeria monocytogenes*	§ATCC 7644
16	*Citrobacter freundii*	*DSM 15979
17	*Enterobacter cloacae*	*DSM 30054
18	*Aeromonas sobria*	*DSM 19176
19	*Lactobacillus plantarum*	*DSM 20174
20	*Pediococcus pentosaceus*	*DSM 20336
21	*Leuconostoc lactis*	#CECT 4173
22	*Saccharomyces cerevisiae*	§ATCC 36024

§ATCC: American Type Culture Collection (Manassas, VA, USA); °DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany); #CECT: Colección Española de Cultivos Tipo (University of Valencia, Spain)

2.4.1.2 Food samples

29 poultry samples collected from local markets were analysed for the detection of *Campylobacter* spp. according to Revision EN ISO 10272-1:2006 & ISO/TS 10272-2:2006 (Voedsel en Warent Autoriteit, 2010). From that proposal, the ISO 10272-1B: 2006 has been chosen. 25 g of skin from the poultry samples were transferred into a filter sterile stomacher bag, added with 100 ml of saline-peptone water (8 g/l NaCl, 1 g /l bacteriological peptone, Oxoid, Milan, Italy), and mixed for 1.5 min in a Stomacher (PBI, Milan, Italy) and used for the aerobic bacterial count, *Enterobacteriaceae*, and coliforms. Up to three colonies from mCCD agar plates (after 48 h enrichment) were picked when possible, and purified on Blood Agar Base No 2 (Oxoid) supplemented with 7% Laked Horse Blood (Oxoid). Isolates were subjected to cell morphology analyses using an optical microscope (Carl Zeiss, Arese, Italy) at a 1,000X final magnification. The colonies suspected to be *Campylobacter* spp. after being confirmed based on motility, Gram staining, oxidase and catalase activity, growth at 25°C, were

subjected to Dot Blot and PCR. DNA of samples 3CW and 17CT (analysed according to ISO 10272 - 1B: 2006) were extracted at 0, 6, 12, 24 and 48 hours enrichment in Preston broth. At the same time the two samples were analysed also by streaking onto mCCDA (Oxoid) and Modified Preston Agar (Oxoid).

2.4.1.3 Artificial inoculum of Campylobacter in Preston broth

For the inoculum, two suspensions of *C. jejuni* ATCC BAA-1153 and *C. coli* ATCC 43478 were prepared separately in physiological solution using colonies grown on BHI agar plates. After the measure of the absorbance and the standardization of the suspensions at 0.1 OD with Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.), the amount of suspension suitable to inoculate 100 mL of Preston broth was taken in order to obtain a concentration of 10 cell/mL. The first sample of Preston broth was inoculated with 10 cell/mL of *C. jejuni* ATCC BAA-1153, the second one with 10 cell/mL of *C. coli* ATCC 43478 and the third one with 10 cell/mL of both the species. Following the ISO procedure for the enrichment and isolation of *Campylobacter* species from food samples, all the three samples of Preston broth samples were incubated for 6 hours at 37°C and then at 41.5°C for the following 48 hours in microaerophilic conditions. The DNA was extracted from 10 mL of each broth at 0, 6, 12, 24 and 48 hours of enrichment procedure with the Phenol-Chloroform method. At the end, DNAs were treated with RNase and used for the PCR and Dot blot assays.

2.4.1.4 DNA extraction from pure cultures and enrichment broth

One ml broth from overnight culture (Cecchini et al., 2012) for each strain reported previously were used for the extraction and purification of DNA using the Wizard® Genomic DNA Purification Kit (Promega). Ten ml from Preston enrichment broth cultures at 0, 6, 12, 24, 48 h and ten ml saline-peptone water from the filter sterile stomacher bags, were used for the extraction and purification of DNA according to Manzano et al. (2003). Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.) was used to obtain the DNA concentration. For the DNA extraction from enrichment broth and saline-peptone water, bacterial pellet was obtained from 10 ml after centrifugation at 14,000 x g for 5 min. The pellet was resuspended in 300 µL of breaking buffer [2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris (pH 8), 1mM EDTA (pH 8)] and transferred into a screw cap tube containing 0.3 g of glass beads with a diameter of 0.1 mm and subsequently 300 µL of phenol/chlorophorm/isoamyl alcohol (25:24:1, pH 6.7; Sigma) were added and the cells were homogenized in a bead beater (Fast Prep 24, Bio 101, Vista, CA) three times each for 30 s, with an interval of 15 s

between each treatment. 300 µL of TE buffer (10 mM Trizma base, 5 mM EDTA, pH 8) were added and the tubes were centrifuged at 14,000 x g for 10 min. The aqueous phase was collected and precipitated with 1 mL of ice-cold absolute ethanol. The DNA was collected with a centrifugation at 14,000 x g for 10 min and the pellet was dried under vacuum at room temperature. 50 µL of DNase-free sterile water (Sigma) were added and a 30 min period at 45 °C was used to facilitate the nucleic acid solubilisation. 1 µL of DNase-free RNase (Roche Diagnostics, Milan, Italy) was added to digest RNA with incubation at 37 °C for 1 h (Iacumin et al., 2009).

2.4.1.5 Probes design

A 45 bp DNA probe named CampyDig: 5'- Dig-GGG AGA GGC AGA TGG AAT TGG TGG TGT AGG GGT AAA ATC CGT AGA - Dig-3', was designed after alignment of the sequence retrieved from GeneBank (accession numbers: HM007568.1, DQ174142.1, DQ174141.1for *C. jejuni*, HM007569.1, AB542728.1, JX912505.1 for *C. coli*, GQ167657.1, AF550634.1 for *C. lari* and DQ174157.1, AF497805.1, GQ167658.1 for *C. upsaliensis*) using "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988), and tested with Blast (http://blast.ncbi.nlm.gov/Blast.cgi). The probe labelled with digoxigenin at the 5' and 3' end for the immunological detection, annealed into the 16S rRNA gene (Fig. 2.10). To test the efficiency of the blotting protocol, a ssDNA sequence complementary to the labelled probe was synthesised: 5' - TCT ACG GAT TTT ACC CCT ACA CCA CCA ATT CCA TCT GCC TCT CCC -3' as a positive control (AntiDig).

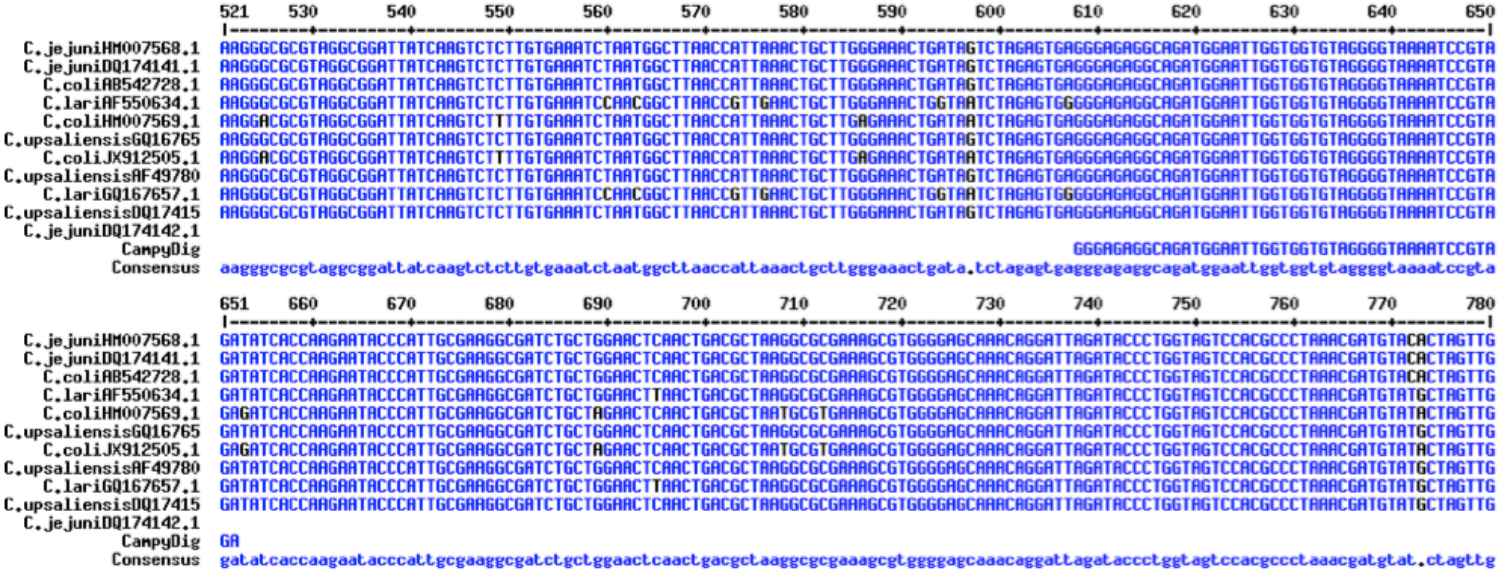


Figure 2.10: Alignment among the 16S rRNA gene of *Campylobacter* species. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used.

2.4.1.6 Samples preparation for Dot Blot Assay

In Dot Blot experiments, the following samples were used: 1 μL at various concentrations of the ssDNA AntiDig probe, to test the sensitivity of the method; 1 μL of DNA (100 ng/μL) extracted from each strain reported in Material and Methods, *Bacterial Strain* section as positive or negative controls; 1 μL of DNA (100 ng/μL) extracted from each strain isolated on mCCDA; 1 μl of DNA extracted from saline-peptone water; 1 μL of DNA extracted from Preston enrichment broths at 0, 6, 12, 24, and 48 h.

2.4.1.7 Dot Blot protocol

The probe was first tested using synthetic ssDNA AntiDig probe (complementary to the digoxigenin-labelled probe CampyDig) to evaluate the potential sensitivity of the specific DNA probe and to establish the correct concentration of the labelled probe used during the blotting experiments. One microliter aliquots from 100 ng/μL to 0.1 ng/μL of the synthetic probe after 5 min at 95°C, were spotted onto the nylon membrane (Sigma–Aldrich, Milan, Italy) and hybridised with the CampyDig probe at 20 pmol/ml (considered the best concentration after testing concentrations from 20 pmol/mL to 10 pmoL/mL). CampyDig was then tested for sensitivity using *Campylobacter* DNAs from 100 ng/μL to 0.1 ng/μL. After denaturation for 10 min at 95°C, one μL DNAs were spotted onto the positively charged nylon membrane, and cross-linked to the air-dried membranes by exposure to UV light for 10 min. After a prehybridisation in a pre-warmed Dig Easy Hyb buffer (Roche Diagnostic, Mannheim, Germany) for 30 min at 63°C, the membrane was hybridised in the same buffer supplemented with 20 pmol/ml of the digoxigenin-labelled probe kept at 63°C overnight, washed twice with 2X SSC (Promega) with 0.1% (w/v) SDS for 5 min at room temperature, and twice with 0.5X SSC with 0.1% (w/v) SDS for 15 min. After a wash with 1X washing buffer (Roche) for 5 min on a shaker, the membrane was incubated in blocking solution 1X (obtained by tenfold dilution of 10X blocking solution with 1X Maleic Acid buffer, Roche) for 30 min with shaking, and subsequently, an antibody solution (anti-digoxygenin-AP diluted in blocking solution 1:5,000, Roche) was added for 30 min while shaking. The membrane was washed twice with washing buffer 1X on a shaker for 15 min. Detection buffer 1X (Roche) for 5 min was used to neutralise the membrane that was then incubated with a colour solution (NBT/BCIP in 1X Detection Buffer, Roche) in the dark without shaking, until the reaction was complete, and stopped by rinsing the membrane for 5 min in sterile water.

2.4.1.8 Oligonucleotides design

The newly designed primers were based on the variable regions, identified by aligning ITS sequences of the *Campylobacter* strains. *Campylobacter* sequences (accession numbers: GQ167702.1, GQ167721.1, GQ167719.1 for *C. jejuni*, GQ167720.1, DQ871247.1, GQ167717.1 for *C. coli* and AB644222.1, AB602361.1, GQ167703.1 for *C. lari*) were retrieved from GeneBank and aligned using "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988). All the primers were tested using AmplifX 1.6.2 (Jullien, 2013), and were synthesized by MWG-Biotech and Sigma-Aldrich Co. Primers CampyForw (5'- CTG ATA AGG GTG AGG TCA CAA GT -3') (from 414 to 436) and CampyRev (5'- CTT GCT TGT GAC TCT TAA CAA TG - 3') (from 687 to 709) were designed within the ITS (intergenic spacer 16S - 23S) gene sequence for the detection of *Campylobacter* spp., and with the aim to differentiate *C. jejuni*, *C. coli* and *C. lari* (Fig. 2.11). The expected amplicons were of 295 bp for *C. jejuni*, 207 bp for *C. coli*, and 253 bp for *C. lari.*

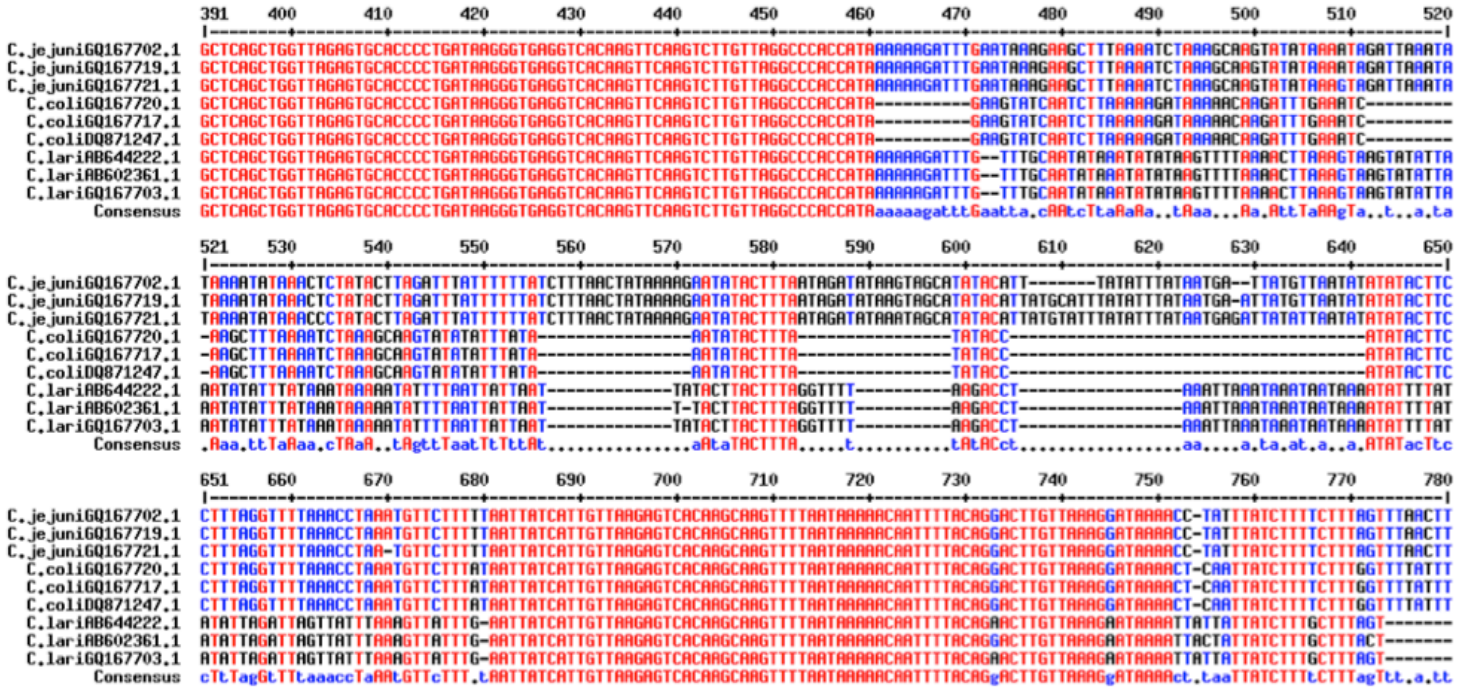


Figure 2.11: Alignment among the 16S-23S ITS sequence of *Campylobacter* species. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used

2.4.1.9 PCR protocol

Genus specific-PCR was carried out using the primers CampyForw - CampyRev in the reaction mixture containing the following reagents: 10 µL GoTaq® Buffer 5x (Promega, Milan, Italy), 1 µL PCR Nucleotide Mix, 10 mM each (Promega); 1 µL of each primer (CampyForw-CampyRev at 10 mM); 0.25 µL GoTaq® DNA Polymerase-5 units/µL (Promega) and 1 µL of DNA (100 ng/µL). Thermal cycler conditions consisted of 95°C denaturation for 5 min, 35 cycles of 95°C for 1 min, 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for 7 min in a Thermal Cycler (PTC220 DNA Engine DYAD MJ Research Celbio, Italia).

2.4.1.10 Electrophoresis

Five µL aliquots of the PCR products were resolved electrophoretically in a 1.5 % agarose gel (Sigma, Milan, Italy) in 0.5x TBE (2 mM EDTA, 80 mM Tris-acetate, pH 8.0) stained with GelRed from Biotium (Hayward, CA) and compared with 100 bp DNA Ladder (Promega). The DNA amplicons obtained by the primers CampyForw and CampyRev were sent to MWG sequencing centre.

2.4.2 Results

2.4.2.1 Plate count method

Plate count method results for the enumeration of the total aerobic mesophilic microflora, *Enterobacteriaceae*, coliforms and the presence of *Campylobacter* spp. on mCCDA and Modified Preston Selective Agar after 48 h enrichment in Preston broth are reported in Table 2.5. Total bacterial contamination of poultry meat varied from 2.3 $x10^4$ to 1.4 $x10^9$ CFU/g, coliforms from 1.5 $x10^2$ to 4.3 $x10^5$ CFU/g, and *Enterobacteriaceae* from 2.9 $x10^2$ to 3.3 $x10^7$ CFU/g. No correlation between the bacterial contamination level and the *Campylobacter* presence was found. Seventeen samples were positive for *Campylobacter* out of 29 samples tested by streaking onto mCCDA after 24, and 48 h enrichment in Preston broth according to Revision EN ISO 10272-1B:2006. Isolates were purified onto Blood Agar N°2 agar and subjected to Dot Blot and PCR.

Table 2.5: Plate count data of the poultry samples in Colony Forming Unit (CFU)/g.

Sample	Aerobic bacterial count	*Enterobacteriaceae*	Coliforms	*Campylobacter* spp. ISO 10272-1B:2006
Chicken				
1 CW*	7.8×10^7	2.0×10^4	1.5×10^2	+
2 CW	3.0×10^4	2.7×10^3	1.5×10^2	+
3 CW	2.2×10^7	7.0×10^3	2.3×10^3	+
4 CW	3.7×10^7	3.9×10^5	4.5×10^4	-
5 CT**	5.9×10^8	3.3×10^7	4.3×10^5	-
6 CT	1.4×10^4	7.0×10^2	3.8×10^2	+
7 CT	6.5×10^7	7.0×10^5	7.5×10^4	+
8 CT	8.3×10^5	6.2×10^3	1.8×10^2	-
9 CT	1.7×10^5	8.8×10^3	3.3×10^2	-
10 CT	3.3×10^6	6.6×10^3	1.9×10^2	-
11 CT	2.3×10^4	6.8×10^3	2.6×10^2	-
12 CT	4.0×10^4	5.3×10^3	4.0×10^2	+
13 CT	3.8×10^5	2.5×10^3	9.5×10^2	+
14 CT	2.5×10^7	3.4×10^4	2.4×10^2	-
15 CT	3.6×10^6	1.8×10^3	3.0×10^2	-
16 CT	3.3×10^7	3.7×10^5	3.2×10^3	+
17 CT	1.2×10^6	2.9×10^2	2.5×10^2	+
18 CT	6.8×10^5	4.7×10^3	2.1×10^2	+
19 CT	4.7×10^7	2.0×10^4	2.4×10^4	+
20 CT	1.8×10^5	3.1×10^3	1.0×10^3	+
21 CT	5.4×10^5	6.4×10^3	1.7×10^3	-
22 CT	2.3×10^6	2.8×10^3	2.3×10^2	+
23 CT	3.3×10^5	8.0×10^3	1.2×10^3	+
Turkey				
1 TW§	3.9×10^7	3.7×10^6	3.0×10^4	+
2 TW	2.9×10^8	1.8×10^5	1.7×10^4	-
3 TW	3.1×10^8	7.0×10^5	1.6×10^4	+
4 TT§§	1.4×10^9	2.1×10^5	9.2×10^4	+
5 TT	1.1×10^8	4.8×10^4	3.2×10^4	-
6 TT	1.4×10^7	6.8×10^3	3.6×10^3	-

*: Chicken Wings; ** Chicken Thighs; §: Turkey Wings; §§: Turkey Thighs

2.4.2.2 Dot Blot

Specificity and sensitivity

The sensitivity of the digoxigenin-labelled CampyDig probe at the concentration of 20 pmoL/mL was 1 ng/µL using the AntiDig probe as target. Visible spots (positive) were obtained at the concentrations of 100 ng/µL, 50 ng/µL, 25 ng/µL, 10 ng/µL, 5 ng/µL, and 1 ng/µL (Fig. 2.12, spots from 1 to 6); the light spot obtained for 0.5 ng/µL (spot 7) and the not visible spot achieved for the 0.1 ng/µl concentration (spot 8) were considered negative.

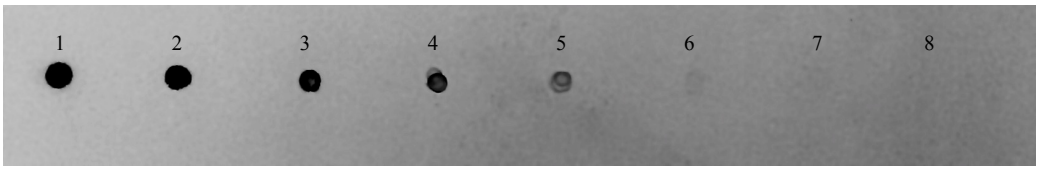


Figure 2.12: Dot Blot sensitivity of the CampyDig probe using ssDNA complementary probe as template at various concentrations. 1: 100 ng/µl; 2: 50 ng/µl; 3: 25 ng/µl; 4: 10 ng/µl; 5: 5 ng/µl; 6: 1 ng/µl; 7: 0.5 ng/µl; 8: 0.1 ng/µl.

The specificity of the protocol is reported in Fig. 2.13. The blue spots of the positive controls used (*C. jejuni* ATCC BAA-1153, *C. jejuni* ATCC 49943, *C. jejuni* DSM 4688; *C. coli* DSM 24155; *C. coli* ATCC 43478; *C. coli* DSM 24128: *C. lari* DSM 11375; *C. upsaliensis* DSM 5365) are visible in Row B, whereas no spots for the negative controls spotted in Row A, as expected, demonstrating the specificity of the designed CampyDig probe.

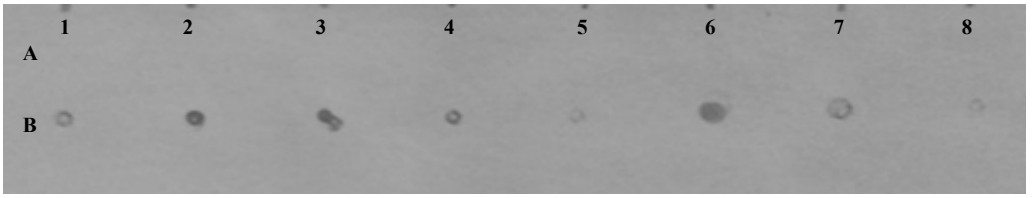


Figure 2.13: Dot-blot specificity on some strains used as negative control and on *Campylobacter* strains used as positive controls (1 µl DNA at 100 ng/µl per spot) (CampyDig probe at 200 ng/µl). Row A, A1: *Vibrio* spp. 14379 (DSM); A2: *Escherichia coli (*DISTAM), A3: *Pseudomonas aeruginosa* (DISTAM), A4: *Bacillus cereus* 2301 (DSM), A5: *Yersinia enterocolitica* (DISTAM), A6: *Aeromonas sobria* 19176 (DSM), A7: *Salmonella enteritidis* 4883 (DSM), A8: *Enterobacter cloacae* 30054 (DSM); Row B, B1: *C. jejuni* subsp. *jejuni* ATCC BAA-1153; B2: *C. jejuni* subsp. *jejuni* ATCC 49943; B3: *C. jejuni* subsp. *jejuni* DSM 4688; B4: *C. coli* DSM 24155; B5: *C. coli* ATCC 43478; B6: *C. coli* DSM 24128: B7: *C. lari* DSM 11375; B8: *C. upsaliensis* DSM 5365.

The same digoxigenin-labelled CampyDig probe, used on the DNA extracted from the *Campylobacter* reference strains, gave a sensitivity of 25 ng/µL.

Dot Blot gave positive results also on artificially inoculated Preston broths after 24 h and 48 h enrichment (spots 4, 5, 9, 10, 14, 15), results are reported in Fig. 2.14. No positivity was found at 0, 6 and 12 h (spots 1 to 3, 6 to 8 and 1 to 13).

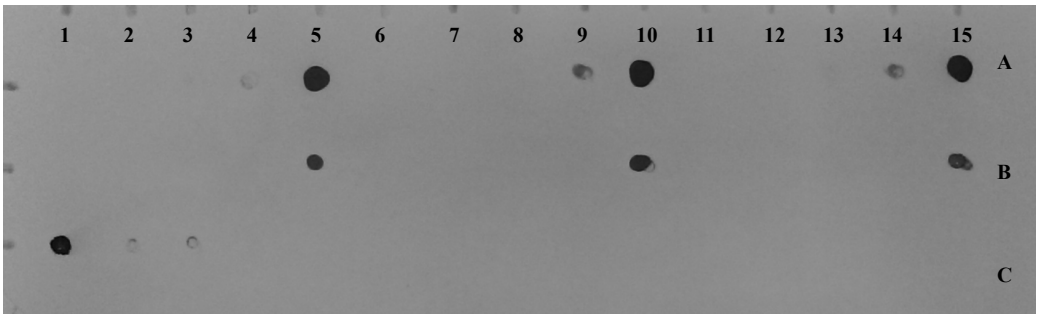


Figure 2.14 Row A - Dot blot on DNAs extracted from Preston broth. Line 1 to 5: total DNA extracted from Preston enrichment broth inoculated with 10 cell/mL of *C. jejuni* ATCC-BAA 1153 at 0, 6, 12, 24 and 48 hours; line 6 to 10: total DNA extracted from Preston enrichment broth inoculated with 10 cell/mL of *C. coli* ATCC 43478 at 0, 6, 12, 24 and 48 hours; line 11 to 15: total DNA extracted from Preston enrichment broth inoculated with10 cell/mL of *C. jejuni* ATCC-BAA 1153 and 10 cell/mL of *C. coli* ATCC 43478 at 0, 6, 12, 24 and 48 hours.
Row B - Dot blot on DNAs extracted from Preston broth Line 1 to 15: tenfold dilutions of the same DNA used on line A.
Row C – Line 1: complementary sequence at 100 ng/µL; line 2: *C. jejuni* subsp. *jejuni* ATCC BAA-1153; line 3: *C. coli* DSM 24128.

Dot Blot samples analysis

The DNAs extracted from saline-peptone water gave negative results (Fig. 2.15, Row A), while the CampyDig probe gave positive results on DNAs extracted from Preston broth after 48 h according to EN ISO 10272-1B:2006 method (Fig. 3.6, Row B, lines 1, 2, 3, 4, 6, 8, and 10). The presence of the spots indicate the positive hybridisation reaction between the *Campylobacter* DNAs present in the poultry samples spotted onto the membrane and the CampyDig labelled probe. On the contrary, lines 5, 7 and 9 indicate the absence of *Campylobacter* in the samples. In Row C and D are visible the spots of *C. jejuni* ATCC BAA-1153, *C. coli* ATCC 43478 and the complementary sequence to the CampyDig probe respectively. Seventeen of the twenty-nine samples were positive for *Campylobacter* using the specific CampyDig probe on the DNAs extracted from Preston broths at 24 and 48 h (EN ISO 10272-1B:2006). 3CW and 17CT were positive only at 24 h and 48 h enrichment in Preston broth like the artificially contaminated broths. These data are in agreement with the results obtained by testing isolates from mCCDA. The twelve samples negative by Dot Blot were negative also on mCCDA as reported in Table 2.5.

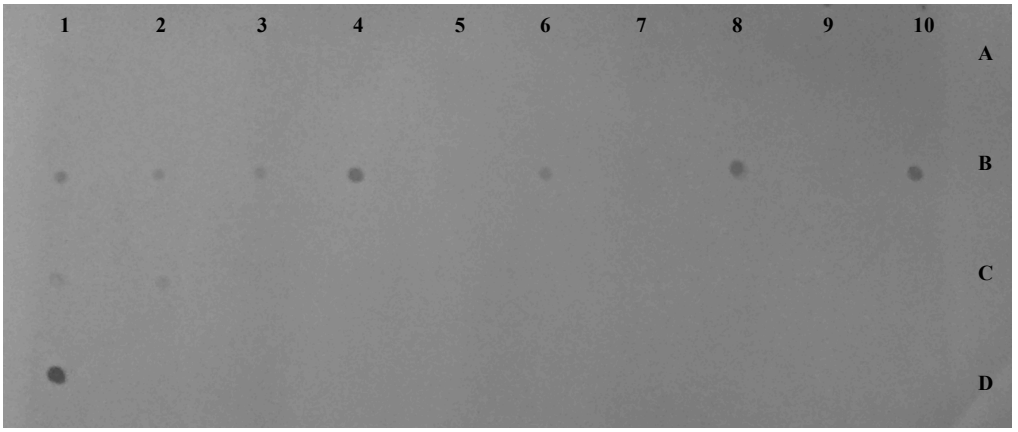


Figure 2.15: Dot blot on some DNAs extracted from saline-peptone water (spw) (Row A), Preston broths (b) (Row B) and positive controls (Row C and D). Row A. A1: 17CT$_{spw}$; A2: 18CT$_{spw}$; A3: 16CT$_{spw}$; A4: 4TT$_{spw}$; A5: 21CT$_{spw}$; A6: 22CT$_{spw}$; A7: 5TT$_{spw}$; A8: 23CT$_{spw}$; A9: 6TT$_{spw}$; A10: 19CT$_{spw}$. Row B. B1: 17CT$_{b}$; B2: 18CT$_{b}$; B3: 20CT$_{b}$; B4: 4TT$_{b}$; B5: 21CT$_{b}$; B6: 22CT$_{b}$; B7: 5TT$_{b}$; B8: 23CT$_{b}$; B9: 6TT$_{b}$; B10: 19CT$_{b}$. Row C. C1: *Campylobacter jejuni* (ATCC-BAA 1153) (100 ng/µL); C2: *Campylobacter coli* (DSM 24128) (100 ng/µL); Row D: D1: ssDNA complementary to labelled probe (100 ng/µL).

2.4.2.3 PCR

Specificity and sensitivity

CampyForw-CampyRev primers showed the expected specificity as no amplicons were obtained for the microorganisms listed in Material and Methods, *Bacterial Strains* section, apart from *Campylobacter* species. The amplicons of *C. jejuni*, *C. coli* and *C. lari* produced the expected different weights allowing the identification of the species (Fig. 2.16). The sensitivity of the primers tested using serial dilutions of the corresponding DNAs was 1 ng/μL.

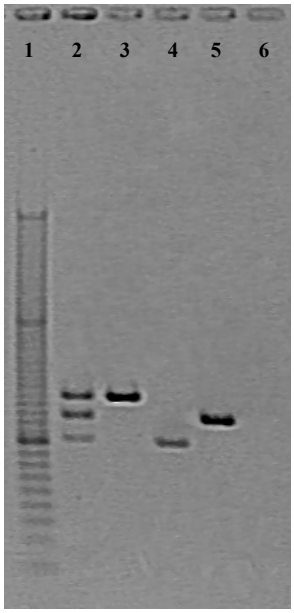


Figure 2.16: PCR with CampyForw – CampyRev primers. Line 1: 20 bp Low Ladder (Sigma, Milan, Italy); line 2: mix of *C. jejuni* ATCC BAA-1153; *C. coli* DSM 24128 and *C. lari* DSM 11375 DNAs (100 ng/μL); line 3: *C. jejuni* ATCC BAA-1153 (100 ng/μL); line 4: *C. coli* DSM 24128 (100 ng/μL); line 5: *C. lari* DSM 11375 (100 ng/μL); line 6: negative control.

Enrichment protocol carried out inoculating both the two species found on poultry samples, didn't allow their differentiation. Enrichment step seems to promote *C. jejuni* species growth, although the band pertaining to *C. coli* was present using DNA extracted from enrichment broth inoculated with this species. In fact, only the band corresponding to *C. jejuni* is present when 10 cell/mL of *C. jejuni* and 10 cell/mL *C. coli* were inoculated in the same tube. For both the species, PCR protocol proposed in this work allows the detection of *C. jejuni* and *C. coli* on DNA extracted after 24 h and 48 h of enrichment (lines 7, 8, 12, 13, 17, 18, Fig. 2.17).

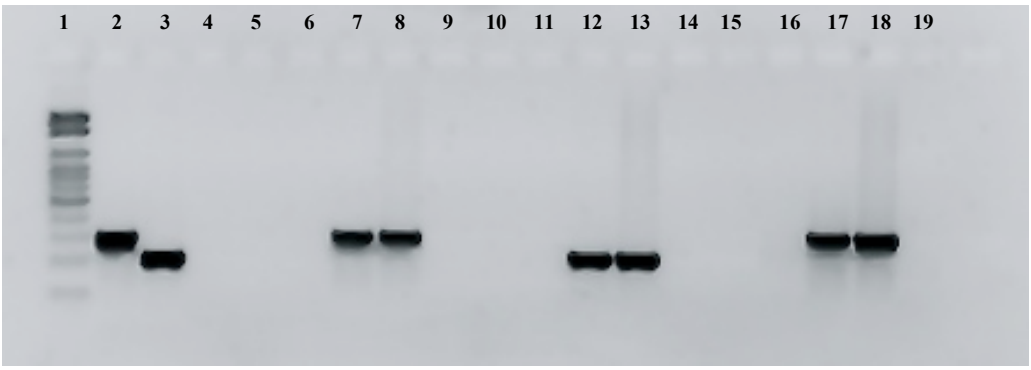


Figure 2.17: PCR with CampyForw - CampyRev primers using DNA extracted from Preston broth at 0, 6, 12, 24 and 48 hours after an artificially inoculum of 10 cell/mL. Line 1: 100 bp DNA Ladder (Promega); line 2: *C. jejuni* ATCC-BAA 1153; line 3: *C. coli* ATCC 43478; line 4: DNA of *C. jejuni* ATCC-BAA 1153 at 0 h; line 5: DNA of *C. jejuni* ATCC-BAA 1153 at 6 h; line 6: DNA of *C. jejuni* ATCC-BAA 1153 at 12 h; line 7: DNA of *C. jejuni* ATCC-BAA 1153 at 24 h; line 8: DNA of *C. jejuni* ATCC-BAA 1153 at 48 h; line 9: DNA of *C. coli* ATCC 43478 at 0 h; line 10: DNA of *C. coli* ATCC 43478 at 6 h; line 11: DNA of *C. coli* ATCC 43478 at 12 h; line 12: DNA of *C. coli* ATCC 43478 at 24 h; line 13: DNA of *C. coli* ATCC 43478 at 48 h; line 14: DNA of both *C. jejuni* ATCC-BAA 1153 and *C. coli* ATCC 43478 at 0 h; line 15: DNA of both *C. jejuni* ATCC-BAA 1153 and *C. coli* ATCC 43478 at 6 h; line 16: DNA of both *C. jejuni* ATCC-BAA 1153 and *C. coli* ATCC 43478 at 12 h; line 17: DNA of both *C. jejuni* ATCC-BAA 1153 and *C. coli* ATCC 43478 at 24 h; line 18: DNA of both *C. jejuni* ATCC-BAA 1153 and *C. coli* ATCC 43478 at 48 h; line 19: negative control.

PCR samples analysis

DNAs extracted from Preston broth at 48 h and the isolates from mCCDA plates after 48 h enrichment in Preston broth which showed *Campylobacter* characteristics, were confirmed *Campylobacter* by PCR using the couple of primers CampyForw - CampyRev. It was possible to differentiate the isolates as *C. coli* or *C. jejuni* based on the weight of the amplicons (Fig. 2.18).

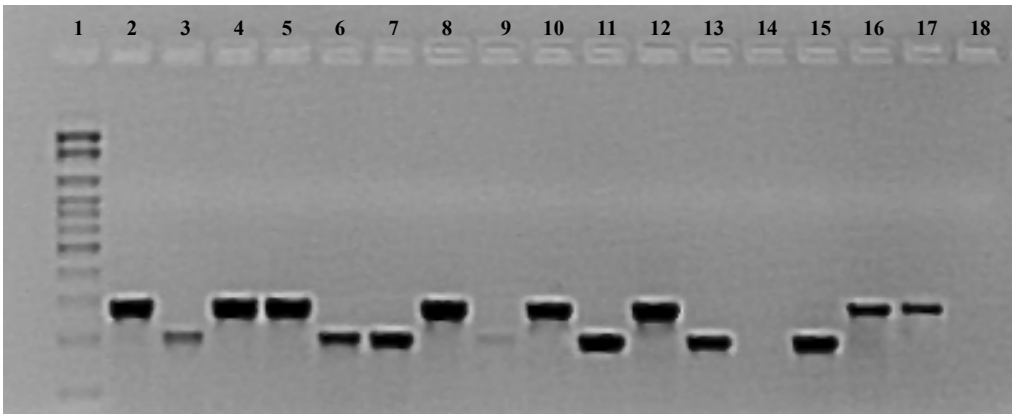


Figure 2.18: PCR with CampyForw - CampyRev primers using DNA extracted from isolates (i) after 48 h enrichment in Preston broth (4 - 9) and DNAs from the enrichment broth at 48 h (b) (10 - 17). Line 1: molecular weight marker (100 bp, Promega); line 2: *Campylobacter jejuni* subsp. *jejuni* ATCC-BAA 1153; line 3: *Campylobacter coli* DSM 24128; line 4: 17_1CT_i; line 5: 17_2CT_i; line 6: 4_1TT_i; line 7: 4_2TT_i; line 8: $23CT_i$; line 9: $22CT_i$; line 10: $18CT_b$; line 11: $3CW_b$; line 12: $17CT_b$; line 13: $4TT_b$; line 14: $21CT_b$; line 15: $22CT_b$; line 16: $23CT_b$; line 17: $19CT_b$; line 18: negative control.

The species identification obtained for isolates was confirmed by the results obtained using DNAs extracted directly from broths. Lines 4 and 5 in Fig. 2.18 show the identification of the isolates from sample 17CT (*C. jejuni*), and line 12 confirm the identification obtained with the DNA extracted from Preston broth at 48 h. Similarly, lines 6 and 7 show the identification of the isolates from sample 4TT (*C. coli*), and line 13 confirm the identification obtained from Preston broth at 48 h. CampyForw - CampyRev primers gave positive results also using the DNAs extracted from the two samples 3CW and 17CT at 24 h enrichment in Preston broth as shown in Fig. 2.19, line 16 and 17, although the band is less strong then the one at 48 h (line 18 and 19).

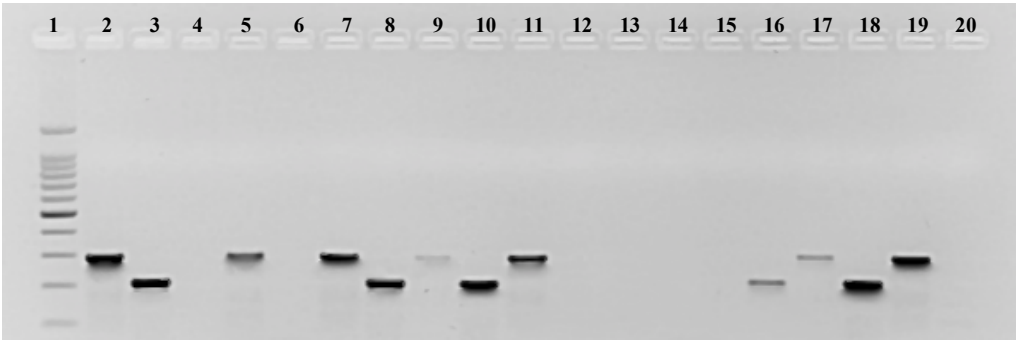


Figure 2.19: PCR with CampyForw - CampyRev primers using DNA extracted from isolates (i) on mCCDA after 48 h enrichment in Preston broth (lines 4 - 11) and DNAs from the enrichment broth at different times (b) (lines 12 - 19). Line 1: molecular weight marker (100 bp, Promega); line 2: *Campylobacter jejuni* subsp. *jejuni* ATCC-BAA 1153; line 3: *Campylobacter coli* DSM 24128; line 4: $3CW_i$ at 0 h; line 5: $17CT_i$ at 0 h; line 6: $3CW_i$ at 6 h; line 7: $17CT_i$ at 6 h; line 8: $3CW_i$ at 24 h; line 9: $17CT_i$ at 24 h; line 10: $3CW_i$ at 48 h; line 11: $17CT_i$ at 48 h; line 12: $3CW_b$ at 0 h; line 13; $17CT_b$ at 0 h; line 14: $3CW_b$ at 6 h; line 15: $17CT_b$ at 6 h; line 16: $3CW_b$ at 24 h; line 17: $17CT_b$ at 24 h; line 18: $3CW_b$ at 48 h; line 19: $17CT_b$ at 48 h; line 20: negative control.

Eight of the 17 positive samples resulted contaminated by *C. coli* (1CW, 1TW, 3TW, 3CW, 4TT, 13CT, 20CT, 22CT) and nine by *C. jejuni* (2CW, 6CT, 7CT, 12CT, 16CT, 17CT, 18CT, 19CT, 23CT) by both PCRs on DNAs from isolates and from the broths. *C. jejuni* was the prevalent species isolated in the poultry meat samples analysed. The amplicons sent to MWG for sequencing matched 100% using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) the sequences retrieved in GenBank confirming the species identification obtained by PCR (Fig. 2.20).

Campylobacter jejuni 4031 genome sequence	276	828	99%	4e-71	100%	HG428754.1
Campylobacter jejuni subsp. jejuni M1, complete genome	276	828	99%	4e-71	100%	CP001900.1
Campylobacter jejuni subsp. jejuni 81116, complete genome	276	828	99%	4e-71	100%	CP000814.1
Campylobacter jejuni strain KJ7 tRNA-Ala and tRNA-Ile genes, complete sequence	276	276	99%	4e-71	100%	AF074830.1
Campylobacter coli strain ATCC 33559 16S-23S ribosomal RNA intergenic spacer, partial sequence	267	267	94%	3e-68	100%	GQ167720.1
Campylobacter coli strain X3 16S-23S ribosomal RNA intergenic spacer, partial sequence >gb|GQ1(	267	267	94%	3e-68	100%	GQ167717.1
Campylobacter coli strain X7 16S-23S ribosomal RNA intergenic spacer, partial sequence	267	267	94%	3e-68	100%	GQ167716.1
Campylobacter coli strain ATCC BAA-371 16S-23S ribosomal RNA intergenic spacer, partial sequer	267	267	94%	3e-68	100%	DQ871247.1
Campylobacter coli 16S ribosomal RNA gene, partial sequence; tRNA-Ala and tRNA-Ile genes, com	267	267	94%	3e-68	100%	AF146727.1

Figure 2.20: results obtained after sequencing and alignment with Blast of the amplicons sent to MWG.

2.4.3 Discussion

The detection and identification of *Campylobacter* spp. contaminating poultry meat samples have been conducted for many years using culturing techniques, which are laborious and time consuming. Moreover, *Campylobacter* spp. can survive as viable but non cultivable (VBNC) forms which may not grow on selective media. Molecular methods allowed an improvement in microbial detection as they are faster and more reliable (Silva et al., 2011). Dot Blot on DNAs extracted from isolates and Preston broths at 24 and 48 h was useful to verify the contamination of poultry meat by *Campylobacter* spp. The method reached a good sensitivity, in fact, 25 ng *Campylobacter* DNA of the reference strains produced visible blue spots when hybridized with the specific CampyDig probe.

The analysis on artificially contaminated broths inoculated with the same amount of *C. coli* and *C. jejuni* (10 cells/mL each, alone or in mixture) demonstrated the sensitivity of the method which allowed the detection of *Campylobacter* spp. in the broths at 24 h reducing the time needed.

The same seventeen samples positive by Dot Blot were positive by PCR (using CampyForw - CampyRev primers) and also by plate count method. The choice of the variable sequence, ITS, allowed the utilization of PCR both for a quick screening of food samples and for the identification of *C. jejuni C. coli* and *C. lari.* In fact, these primers were able to produce amplicons of different sizes (295 bp for *C. jejuni*, 207 bp for *C. coli* and 253 bp for *C. lari*) that allowed the differentiation between the different species, reducing the time for the identification of the species contaminating meat samples. These results suggest the possible utilization of these primers for epidemiological application on human and veterinary isolates. The positive results obtained for the two samples 3CW and 17CT, at 24 h enrichment, both from enrichment broths and isolates, demonstrated the validity of the proposed primers, in detecting *Campylobacter* in meat samples as the time required to get the results was 24 h shorter. Also the two samples 3CW and 17CT were positive by Dot Blot at 24 h and 48 h. The number of *Campylobacter* cells evaluated by mCCDA plate count method at 0 h for the two samples was 5 x 10 CFU/g for sample 3CW and 1.5 x 10^2 CFU/g for sample 17CT. Taking into consideration the dilutions that the samples were subjected to (1:50) the initial number of cells in the Preston enrichment broths was 1 cell/mL and 3 cells/mL respectively. 24 h enrichment were sufficient to allow PCR the detection of *Campylobacter* using CampyForw - CampyRev primers as shown in Fig. 2.19, lines 16 and 17, confirming the sensitivity of the protocol. The data from the Preston broth step being evidence of *C. jejuni* species growth is favoured in respect of *C. coli*,

although both the two species can be detected by these primers if present in the same sample as shown in Fig. 2.16. The data obtained by the culture plate method applied validates the data obtained by the two molecular methods used in this work confirming the validity of the protocols.

Sample was considered positive when positive results were found for at least two methods (cultural and/or molecular). Both the molecular methods proposed in this work and able to detect *Campylobacter* in poultry meat, are cost-effective, fast and reliable. 16S rDNA sequence is useful for the detection of *Campylobacter* spp. by the CampyDig probe annealing all the species of *Campylobacter* responsible for human gastroenteritis after 24 h enrichment in Preston broth. The variability of the ITS region is good for the differentiation between *C. coli* and *C. jejuni* detected in food samples using PCR both on isolates and broths at 24 h. Both molecular methods proposed in this work show good sensitivity and specificity reducing time for results. The choice of the method can be based on laboratory routine, scope of the analyses and ability of personnel involved.

2.5 OLED-based biochip for *Campylobacter* spp. detection in poultry meat

The utilization of OLED (organic light emitting device) source to make a biochip has been demonstrated by Marcello et al., (2013) on proteins. In this work we apply the OLED light source to a DNA-biochip to detect *Campylobacter* in poultry meat samples to demonstrate the applicability of this system for a rapid, simple detection of one of the most important pathogens responsible for human gastroenteritis, which is still causing big economic losses worldwide.

2.5.1 Materials and methods

2.5.1.1 Bacterial strains

The strains listed in Table 2.6 were used to test the specificity and sensitivity of the DNA probes used. DNA of reference strains was extracted and purified from one ml overnight culture broth using the Wizard® Genomic DNA Purification Kit (Promega) (Cecchini et al., 2012). Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.) was used for the evaluation of the DNA concentrations.

Table 2.6: Reference strains used for the optimisation of the Dot blot protocol.

N°	Microorganism	Source
1	*Campylobacter jejuni* subsp. *jejuni*	*DSM 4688
2	*C. jejuni* subsp. *jejuni*	§ATCC BAA-1153
3	*C. jejuni* subsp. *jejuni*	§ATCC 49943
4	*C. coli*	*DSM 24155
5	*C. coli*	*DSM 24128
6	*C. coli*	§ATCC 43478
7	*C. lari* subsp. *lari*	*DSM 11375
8	*C. upsaliensis*	*DSM 5365
9	*Weissella cibaria*	*DSM 14295
10	*Vibrio* spp.	*DSM 14379
11	*Escherichia coli*	°DISTAM
12	*Pseudomonas aeruginosa*	°DISTAM
13	*P. migulae*	°DISTAM
14	*P. fluorescens*	°DISTAM
15	*P. brennerii*	°DISTAM
16	*Bacillus coagulans*	*DSM 2308
17	*B. subtilis*	*DSM 1029

18	*B. cereus*	*DSM 2301
19	*Proteus vulgaris*	°DISTAM
20	*Yersinia enterocolitica*	°DISTAM
21	*Morganella morganii*	°DISTAM
22	*Salmonella enteritidis*	*DSM 4883
23	*Listeria monocytogenes*	§ATCC 7644
24	*Citrobacter freundii*	*DSM 15979
25	*Enterobacter cloacae*	*DSM 30054
26	*Aeromonas sobria*	*DSM 19176
27	*Lactobacillus plantarum*	*DSM 20174
28	*Pediococcus pentosaceus*	*DSM 20336
29	*Leuconostoc lactis*	#CECT 4173
30	*Saccharomyces cerevisiae*	§ATCC 36024

§ATCC: American Type Culture Collection (Manassas, VA, USA); °DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany); #CECT: Colección Española de Cultivos Tipo (University of Valencia, Spain).

2.5.1.2 Probes design

Two DNA probes, a 45 bp capture probe (CampyCapt) (5'-GGGAGAGGCAGATGGAATTGGTGGTGTAGGGGTAAAATCCGTAGA-3') and a 55 bp detection probe (CampyDet) (5'-CACTTTTCGGAGCGTAAACTCCTTTTCTTAGGGAAGAATTCTGACGG TACCTAAG - 3') specific for 16S rRNA gene of *Campylobacter* spp., were designed using "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988) and tested in silico with Blast (http://blast.ncbi.nlm.gov/Blast.cgi). The alignment of the sequences retrieved from GenBank (accession numbers: HM007568.1, DQ174142.1, DQ174141.1for *C. jejuni*, HM007569.1, AB542728.1, JX912505.1 for *C. coli*, GQ167657.1, AF550634.1 for *C. lari* and DQ174157.1, AF497805.1, GQ167658.1 for *C. upsaliensis*) is reported in Fig. 2.21. After a 5' end and 3' end labelling with digoxigenin, both the probes (Dig-CampyCapt and Dig-

CampyDet) were tested by Dot Blot technique on ssDNA sequences complementary to the probes to establish the sensitivity of the method. DNAs extracted from the strains reported in Table 2.6 were used to test the specificity of the probes using Dot Blot following the protocol reported in Chapter 2, Section 2.4.1.7.

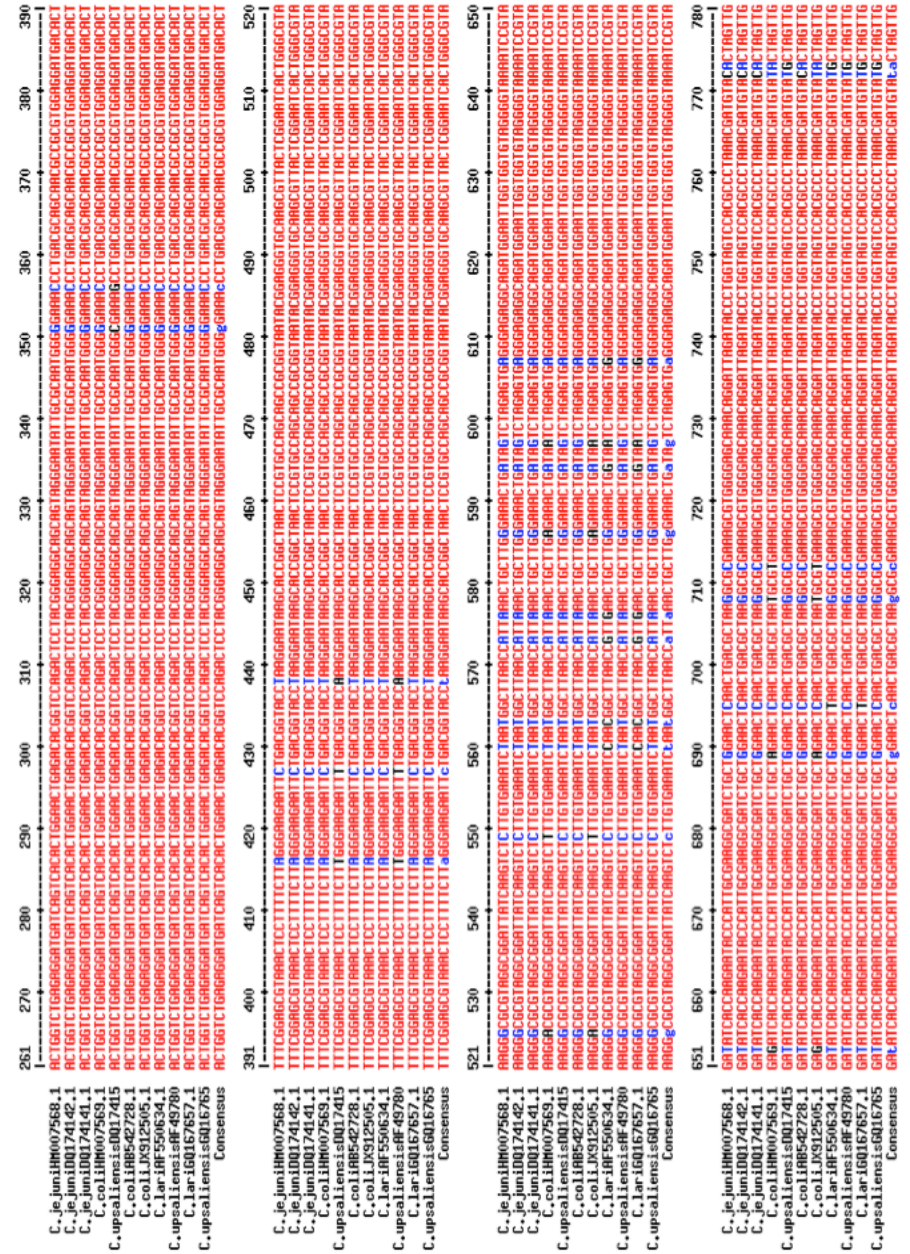


Figure 2.21: Alignment among the 16S rRNA sequence of *Campylobacter* species. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used.

2.5.1.3 Silanization of the glass slides and capture-probe binding

As support of the biochip, microscope cover glasses (28 x 19 mm) were used. Silanization was performed following the protocol described by Marcello et al. (2013) with some modifications. The cover glasses were treated with 10% NaOH (2.5 mM, Sigma, Italy) at room temperature for 1h, rinsed with deionized water and treated with 0.1 N HCl for 15 min. After a washing step with deionized water, the glass slides were rinsed in acetone and dried at 50°C for few minutes, and immersed in a 0.5 % APTES (3-aminopropyltriethoxysilane, Fluka) solution in deionized water for 30 min at room temperature. Slides were then rinsed three times in deionized water followed by once washing for 10 min shaking, dried at 160°C for 1 h and cooled at room temperature for 30 min. The CampyCapt probe was modified by the addition of an amino group at 5' end (amino-CampyCapt probe) and used for binding to the silanized glass slides.
1 µL of the amino-CampyCapt probe at 100 ng/µL, in triplicate, were drop off on each glass slide and incubated at 4°C overnight to bind the silanized glass surface.

2.5.1.4 Labelling of the detection probe by Alexa Fluor® 430

250 µg of Alexa Fluor® 430 (Invitrogen, Monza, Italy) were mixed with 14 µL of DMSO (Sigma, Italy), 7 µL of water, 75 µL of sodium tetraborohydrate 0.1M (pH 8.5) and 4 µL of the CampyDet probe. The tube was incubated overnight. After incubation, precipitation of the labelled oligonucleotide was permormed adding one-tenth volume of NaCl 3 M and 2.5 volumes of cold absolute ethanol. Solution was mixed in the tube and incubated at -20°C for 30 min. The tube was then centrifuged at 12,000 x g for 30 min and the pellet resuspended in 200 µL of 50% formamide. 20 µL of the probe with 5 µL of Gel Loading Buffer were loaded into an agarose gel at 2% for purification, eluted, collected in a tube and maintained at -20°C till utilization.

2.5.1.5 Poultry meat samples and DNA extraction

Eight poultry samples (thighs and wings) were collected from local markets and analysed for the presence of *Campylobacter* spp. according to ISO 10272-1B: 2006 (Voedsel en Warent Autoriteit, 2010). Moreover, 25 g of skin from each poultry sample were transferred into a filter sterile stomacher bag, added with 100 mL of saline-peptone water (8 g/L NaCl, 1 g /L bacteriological peptone, Oxoid, Milan, Italy), and mixed for 1.5 min in a filter Stomacher bag (PBI, Milan, Italy) and used for the aerobic bacterial count on Plate Count Agar

(PCA, Oxoid), *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) Agar and *Campylobacter* spp. on Modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA, Oxoid). The colonies suspected to be *Campylobacter* species were confirmed based on motility, Gram staining, oxidase and catalase activity, growth at 25°C, and subjected to PCR using the protocol proposed in Chapter 3, Section, 3.1.9. Amplicons were sent to MWG for DNA sequencing. DNAs from samples were extracted from 10 mL saline-peptone water after centrifugation at 14,000 xg for 5 min. The pellet was resuspended in 300 µL of breaking buffer [2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris (pH 8), 1mM EDTA (pH 8)] and transferred into a screw cap tube containing 0.3 g glass beads 0.1 mm diameter and subsequently 300 µL of phenol/chlorophorm/isoamyl alcohol (25:24:1, pH 6.7; Sigma) were added. Cells were homogenized in a bead beater (Fast Prep 24, Bio 101, Vista, CA) three times each for 30 s, with an interval of 15 s between each treatment. 300 µL of TE buffer (10 mM Trizma base, 5 mM EDTA, pH 8) were added and the tubes were centrifuged at 14,000 xg for 10 min. The aqueous phase was collected and precipitated with 1 mL of ice-cold absolute ethanol. The DNA was collected with a centrifugation at 14,000 xg for 10 min and the pellet was dried under vacuum at room temperature. 50 µL of DNase-free sterile water (Sigma) were added and a 30 min period at 45 °C was used to facilitate the nucleic acid solubilisation. 1 µL of DNase-free RNase (Roche Diagnostics, Milan, Italy) was added to digest RNA with incubation at 37 °C for 1 h (Iacumin et al., 2009).

2.5.1.6 OLED biochip utilization and signal detection

In order to verifiy the specificity of the designed probes with OLED biochip, reference strains of *C. jejuni* subsp. *jejuni* ATCC 49943 and *Enterobacter cloacae* DSM 30054 were used for the DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy) according to the manufacturer's instructions. Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.) was used for the standardisation of the DNA at the final work concentrations with sterile DNA-free Milli-Q water.
0.5 µL of the DNAs extracted both from reference strains and poultry samples were spotted (in triplicate) onto a silanized glass slide bound with the amino-probe, and used with the Alexa-CampyDet probe (Alexa Fluor® 430 labelled) at 10 pmol/mL. The two probes, amino-CampyCapt probe and Alexa-CampyDet, were used on the OLED (organic light-emitting diode) biochip system. The glass slides bound with the amino-CampyCapt probe were washed in deionized water prior utilization. Then on each slide were spotted 0.5 µL of the DNA samples and 0.5 µL of Alexa-CampyDet probe. A sensitivity curve was

performed using different concentrations of DNA from *C. jejuni* subsp. *jejuni* ATCC 49943 (25 ng/µL, 12.5 ng/µL, 6.25 ng/µL, 3.125 ng/µL, 1.5 ng/µL and 0.75 ng/µL) and various concentrations (100 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL, 3.12 ng ng/µL, and 1.5 ng/µL) of the Alexa-CampyDet probe. Various silanized glasses were used as negative controls: without *C. jejuni* subsp. *jejuni* ATCC 49943 DNA at 25 ng and 12.5 ng/µL, with *Enterobacter cloacae* DSM 30054 DNA at 25 ng and 12.5 ng/µL, without amino-CampyCapt, without Alexa-CampyDet probe. The glass slides were incubated at 63°C for 1.5 h in a Petri dish to prevent evaporation, and washed two times in sterile deionized water to eliminate the unbound DNA and the CampyDet probe. The glass slides were put on the OLED biochip (OR-EL d.o.o., Kobarid, Slovenjia) and fluorescence was measured, based on the mean of pixels' intensity minus the background of surrounded area, by a CCD camera (Hamamatsu Orca R2).

2.5.2 Results

2.5.2.1 Specificity and sensitivity of the probes

The sensitivity of both the digoxigenin labelled probes (Dig-CampyCapt and Dig-CampyDet) tested by Dot Blot at 20 pmol/mL, was 1 ng/µL using as target the ssDNA oligonucleotide sequence complementary to the probe. The sensitivity was 25 ng/µL using as target the genomic DNA extracted from the *Campylobacter* reference strains reported in Table 2.6. The probes showed high specificity at the conditions proposed in the described protocol as they both annealed only *Campylobacter* strains. The OLED biochip showed good results when tested with various concentrations (0.75, 1.5, 3.125, 6.25, 12.5 and 25 ng/µL) of DNA from pure culture strains of *C. jejuni* subsp. *jejuni* ATCC 49943 and Alexa-CampyDet probe at the concentration of 10 pmol/mL. A linear regression analysis obtained by the biochip system was made ($R^2 = 0.99$). Values for each concentration, tested in triplicate, are expressed in Intensity (A.U.) in a range scale of grey from 0 (black) to 4095 (white). The results show how comparing the fluorescence intensity from the lowest concentration (0.75 ng/µL=104 A.E.) to the highest concentration (25 ng/µL=3290 A.E.) we can observe a constant and linear increment with intermediates values of 142 A.E (1.5 ng/µL), 250 A.E (3.125 ng/µL), 578 A.E. (6.25 ng/µL), 1570 A.E. (12.5 ng/µL). The OLED biochip, thus including both the amino-CampyCapt probe and the Alexa-CampyDet probe, showed a sensitivity of 1.5 ng/µL using *C. jejuni* subsp. *jejuni* ATCC 49943 DNA as target (Fig. 2.22 shows the results for the concentration of 25, 12.5 and 6.25 ng/µL). No signals were obtained from the negative controls used.

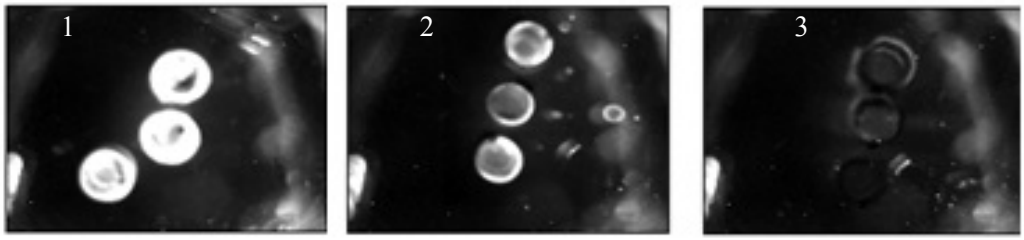


Figure 2.22: OLED response: 1) *C. jejuni* subsp. *Jejuni* ATCC 49943 DNA at 25 ng/µL, 2) 12.5 ng/µL, 3) 6.25 ng/µL.

2.5.2.2 Poultry Meat Samples

The results of the plate count method are reported in Table 2.7. The total bacterial contamination varied from 3.0 x10^4 to 5.9 x 10^8 CFU/g, and *Enterobacteriaceae* from 1.8 x 10^3 to 3.3 x 10^7 CFU/g. *Campylobacter* spp. was detected in five of the eight chicken samples analysed by direct plating onto mCCDA and varied from 50 CFU/g to 3.6 x 10^3 CFU/g.

Table 2.7: Plate count data of the poultry samples, values expressed in Colony Forming Unit (CFU)/g.

Sample	mCCDA	PCA	VRBG	Preston broth
CW1*	0.5 x 10	7.8 x 10^7	2.0 x 10^4	+
CT2**	< 5	3.6 x 10^6	1.8 x 10^3	-
CT3	< 5	3.7 x 10^7	3.9 x 10^5	-
CT4	< 5	5.9 x 10^8	3.3 x 10^7	-
CT5	1.5 x 10	6.5 x 10^7	6.0 x 10^5	+
TW6	3.6 x 10^3	3.1 x 10^8	7.0 x 10^5	+
CW7	1.6 x 10^3	3.0 x 10^4	2.7 x 10^3	+
CT8	1.5 x 10	4.0 x 10^4	5.3 x 10^3	+

*Chicken Wings; **Chicken Thights

Samples CW1, CT5, TW6, CW7 and CT8, analysed by ISO 10272-1B: 2006 were positive for the presence of *Campylobacter* spp. after 48 h enrichment in Preston broth, as showed by the typical *Campylobacter* colonies present on mCCDA plates. Isolates from mCCDA plates after 48 h enrichment were confirmed *Campylobacter* spp. both by motility, Gram staining, oxidase and

catalase activity, growth at 25°C and by PCR using the primers designed within 16S-23S ITS region, following the protocol proposed in chapter 2, section 2.4.1.9 of this thesis. The amplicons obtained by the PCR protocol applied and sent to MWG for sequencing, matched 100% the *Campylobacter* sequences retrieved from GenBank using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirming the identification obtained by PCR. OLED biochip system applied to the analysis of poultry samples allowed the detection of *Campylobacter* in samples CW1, CT5, TW6, CW7 and CT8 directly from DNA extracted from phisiologycal solution confirming the data obtained by ISO 10272-1B: 2006. Samples CT2, CT3 and CT4 were negative by ISO 10272-1B: 2006, by PCR, as no amplicons were obtained, and by the OLED biochip system.

2.5.3 Discussion

Classic methods for *Campylobacter* identification in food samples rely on broth enrichment and colony growth on selective agar plate taking at least five days (ISO 10272-1B: 2006). The food companies demand for specific, sensitive, rapid, simple, cheaper testing methods for food pathogen, based on DNA hybridization, overtaking the DNA polymerase inhibition problem affecting PCR, has encouraged research in the field of DNA sensors or genosensors. In this work, we proposed an OLED-based DNA biochip for the detection of *Campylobacter* spp. in food samples. In order to verify the applicability of the two probes used, samples were before tested with traditional ISO 10172-1B:2006 method and Dot blot assay. Showing good specificity and sensitivity, probes were then used on reference strains and real samples by OLED biochip, showing great specificity and sensitivity, both on reference strains and real sample.

The OLED biochip tested in this work showed applicability, specificity and sensitivity. The possibility to make portable devices will be the next goal to obtain a useful tool for the rapid and simply diagnosis of this pathogen.

CHAPTER 3.

3. *Listeria monocytogenes*

3.1 *Listeria monocytogenes* and its role in foodborne diseases

Listeria monocytogenes (Fig. 3.1) has been reported for the first time in 1924 by Murray, who called it *Bacterium monocytogenes*. The name *Listeria* was assigned by Pirie in 1940 and the first know case of epidemic listeriosis dates back to 1949, in German infants. *L. monocytogenes* is a rare cause of illness in the general population. However, in some groups, including neonates, pregnant women elderly person, and those with impaired cell-mediated immunity due to underlying disease or immunosuppressive therapy, it is an important cause of life-threatening bacteraemia and meningoencephalitis (Lorber 1997; Bucholz and Mascola 2001; Wing and Gregory 2002), with fatality rates of 20–30% (Jeyaletchumi et al. 2010). In Europe, 55.6% of cases of listeriosis were reported in patients older than 65 years (EFSA, 2007). *L. monocytogenes* is a small, facultatively anaerobic, non-sporulating, catalase-positive, oxidase-negative, gram-positive bacterium that grows readily on blood agar, producing incomplete β-haemolysis (Farber and Peterkin 1991; Bille et al. 2003) and the temperature range for growth of the *Listeria* species is 30-37°C. Increasing interest in this organism has resulted from food-borne outbreaks, concerns about food safety and the recognition that food-borne infection may result in self-limited febrile gastroenteritis as well as invasive disease. *L. monocytogenes* is isolated from foods such as milk, dairy products, vegetables, sliced vacuum-packaged meat products, chicken and meat (Destro et al. 1991; Sakate et al., 2003). Vegetables are contaminated by soil where they growth and from manure used as fertilizer. Infected animals are often asymptomatic and they are vehicle for the bacteria that can contaminate food, such as uncooked or undercooked meat, raw milk and soft cheeses (Nicolò and Guglielmino, 2012). Risk behaviors that lead to a possible infection are: consumption of raw foods (dairy products, meat and fish), improper storage of refrigerated foods and food preparation practices by favoring the cross-contamination. *L. monocytogenes* is a ubiquitous microorganism and is estimated that from 2% to 10% of the human population is an asymptomatic carrier of the bacteria in the intestine. Due to these characteristics, is very difficult to remove it completely and the only valid prevention system is in accordance with the rules of good working practice and hygiene during the various steps of the food production process. Tests on raw and seasoned meat

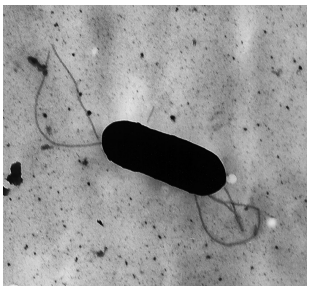


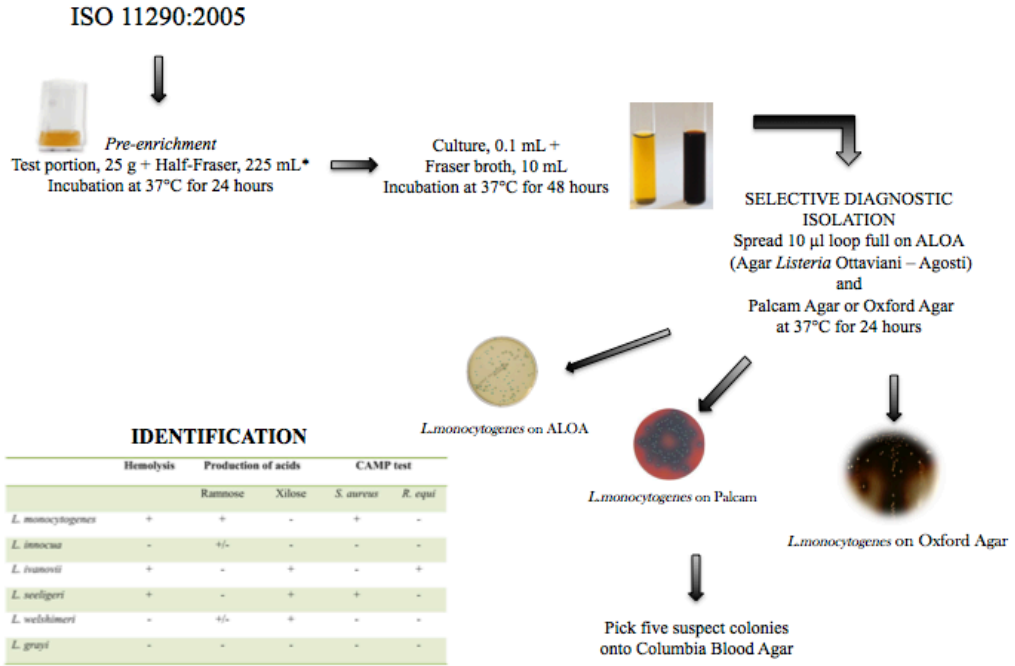
Figure 3.1: ***L. monocytogenes*** (from CDC Public Healt Image Library).

products found a positivity of 58.88% in food preparation to be consumed after cooking (chicken burgers, sausages and pork), 34.78% in chicken and turkey raw meat and 30.6% in raw pork meat (Marzadori, 2004). Of the six *Listeria* species (*L. monocytigenes, L. seeligeri, L. welshimeri, L. innocua, L. ivanovii* and *L. grayi*), only *L. monocytigenes* is pathogenic for humans. There are at least 13 serotypes of *L. monocytigenes* based on cellular O and flagellar H antigens (Schuchat et al. 1991; Bucholz and Mascola 2001). Difference in virulence between strains may also influence infection and clinical outcome. Serotypes 1/2a, 1/2b and 1/2c are the types most frequently isolated from food or from the food production environment. However, more than 95% of infections in humans are caused by the three serotypes 1/2a, 1/2b and 4b. A majority of listeriosis outbreaks are caused by strains of serotype 4b (Swaminathan and Gerner-Smidt, 2007). The incubation period for Listeria gastroenteritis is substantially shorter than the incubation period for invasive disease (Ooi and Lorber, 2005; Dalton et al., 1997; Linnan et al., 1988). For gastroenteritis, the mean incubation period is 24 hours (range 6 hours to 10 days) (Ooi and Lorber, 2005), whereas for invasive listeriosis the median incubation period is 35 days (range 1 to 91 days) (Linnan et al., 1988). The major clinical symptoms of *L. monocytogenes* infections in humans are abortions, septicaemia and meningitis (Gandhi and Chikindas, 2007). Human listeriosis is tipically acquired through ingestion of contaminated food and another way of transmission occurs from mother to child transplacentally or through an infected birth canal, but except for this type of vertical transmission and the rare cases of cross-infection in neonatal nursery, human-to-human infection has not been documented (Farber et al., 1992; Colodner et al., 2003). Infection in fact, occurs after ingestion of contaminated foods and 100 cells/g are enough to produce the clinical manifestations. Several virulence factors have benn associated to the capacity of *L. monocytogenes* to be intracellular microorganism. The virulence of the bacterium is conditioned by a number of factors, some of them own of the microorganism, the others consisting of the different nature of the food substrate and the condition of health of the host in which the bacterium penetrates (Hof and Rocourt, 1992). *L. monocytogenes* and *L. ivanovii* can penetrate and survive not only in the cells responsible for the destruction of bacteria, macrophages, but also in other cell types (epithelial cells, hepatocytes, endothelial cells of serous such as pleura, peritoneum, and meninges). The bacterium possesses the cell surface protein internalin (InlA InlB, with the respective genes that encode them, and *inl*A *inl*B) that interacts with E-cadherin, a receptor on macrophages and intestinal lining cells, to induce its own ingestion (Farber and Peterkin, 1991; Mengard et al., 1996; Cossart and Sansonetti, 2004) and three phospholipase (PlcA, PlcB and PlcC with the respective virulence genes *plc*A, *plc*B and *plc*C), thanks to which the bacteria, penetrated in the cell cytoplasm, is not destroyed by lysosomal enzymes , but

lisa the wall of the phagosome and becomes free in the cytoplasm. Once penetrated within a single cell, thank to ActA protein (encoded by the gene *act*A), *L. monocytogenes* possesses a very specific intracellular cycle. Using the fibrils of the cytoskeleton of the host cell, *L. monocytogenes* pressed against the membrane of the host cell towards the adjacent hepatocyte membrane and penetrates into the cytoplasm of the second cell. When the bridge of cytoplasm stops, *L. monocytogenes* is free into cytoplasm of another cell and the cycle resumes. This behavior enables it to invade slowly, but progressively, the whole organ without ever coming into contact with the antibodies that flow in the blood and in the lymph, to the outside of cells (Giaccone and Bertoja, 2003). Another virulence factor appears to be the ability to scavenge iron, in fact this element enhances organism growth and the clinical associations of literial infection with hemochromatosis and of outbreaks with transfusion-induced iron overload attes the importance of iron acquisition during infection (Nieman and Lorber, 1980; Mossey and Sondheimer, 1985). The mechanism of pathogenicity in *L. monocytigenes* is also related with the presence of invasion associated protein p60 encoded by the *iap* gene. The region encoding a central domain of protein p60 and is characterized by the presence of a tandem repeated sequence (TRS) of ACAAAT, which correspond to the amino acid threonine and asparagine (TN) (de Mello et al., 2007). Protein p60 is encoded by an open reading frame of 1,452 bp, which gives rise to a protein with a theoretical molecular size of 50.34 kDa and a theoretical pI of 9.75. The protein has a typical N-terminal signal sequence which is removed during secretion, and it is characterized by the presence of a series of threonine-asparagine repeats (Köhler et al. 1991). The protein shows a clear domain structure, with two highly conserved regions at the N and C termini covering roughly 100 and 120 amino acids, respectively. The central part of the protein is constituted by a highly variable region including the threonine-asparagine repeats (Bubert et al. 1992). An SH3 domain has been identified in the highly conserved N-terminal region of p60 (Whisstock and Lesk, 1999); however, it is still unclear whether this domain is functional in any sense. The C-terminal region is homologous to a number of hydrolytic enzymes and hence is thought to confer the hydrolytic activity of p60 (Wuenscher et al. 1993). Proteins highly related to p60 have been found in all six *Listeria* species (Bubert et al. 1992). The treatment in the case of confirmed listeriosis consists in the association of penicillin G or ampicillin with aminoglycosides or trimethoprim-sulfametaxazolo and should be continued for several weeks.

3.2 Methods to detect Listeria monocytogenes

Standard methods for detecting *L. monocytogenes* and the other *Listeria* species in food and environmental samples needs cultural enrichment and selective/differential plating following by biochemical identification (Hudson et al., 2001; Skjerve et al., 1990; Uyttendaele et al., 2000). The introduction of chromogenic media has efficiently improved the isolation of *L. monocytogenes,* but also molecular techniques have greatly contributed to the detection of this pathogen. The ISO standard method 11290:2005 (Fig. 3.2) establishes to use Half Fraser broth and Fraser broth for the selective enrichment of *Listeria* spp. in food samples. Both broths allow the detection of the β-D-glucosidase activity of *Listeria* species thank to the presence of esculin and ammonium iron (III) citrate. The glucose esculin is cleaved by β-D-glucosidase into esculetine and glucose that forms a black complex with the iron (III) ions. The black colour observed during enrichment indicates that *Listeria* is present into the sample analysed. The growth of background bacteria is inhibited by lithium chloride, nalidixic acid and acriflavine hydrochloride. Then, a loop full of enrichment culture is used for the selective growth on Palcam agar and Agar Listeria Ottaviani-Agosti (ALOA).



	Hemolysis	Production of acids		CAMP test	
		Ramnose	Xilose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	-	+	-
L. innocua	-	+/-	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	+	-	+	+	-
L. welshimeri	-	+/-	+	-	-
L. grayi	-	-	-	-	-

Figure 3.2: Standard ISO method for the detection and isolation of *Listeria* spp.

For the species identification, several tests can be made. Conventional biochemical analysis, growth on blood agar for the verification of β - haemolisis, API® gallery to verifiy the utilization of carbohydrates and CAMP-test are traditional assays that can be made for the specie-specific identification, but they are time consuming and often the final result can show incongruity. Moreover, for CAMP-test, Vasquez-Boland et al. (1990) demonstrate that this characterization method appeared to be unreliable for two reasons: first, a positive CAMP test with *Rodococcus equi* is not specific for *Listeria ivanovii,* as *Listeria monocytogenes* (and *Listeria seeligeri*) give also a clear positive reaction; second, doubtful reactions could be observed with *Staphylococcus aureus* when assaying haemolytic and non-haemolytic *Listeria* strains (possibility of false negative and false positive results; subjectivity of the interpretation).

Listeria monocytogenes has a genome of approximately 3 megabases (Mb) (He and Luchansky, 1997; von Both et al., 1999) and several DNA or RNA-based assays can be performed for the rapid genus or specie-specific identification of *Listeria*, in particular with PCR methods (Manzano et al., 1997; Doumith et al., 2005, Amagliani et al., 2007), PCR-based assay combined with microplate capture hybridization technique using the *iap* gene (Cocolin et al., 1997) and northern and dot blot using RNA-based oligomer probes (Wang et al., 1991). Enzyme-linked-immunosorbet assays (ELISA) (Kim et al., 2005) was also used for the detection of *L. monocytogenes* using antibody against flagellar epitopes. Although these approaches are relatively rapid, sensitive and reproducible, they still require multiple reaction steps, long reaction time, and well-equipped facilities (Hearty et al., 2006). For this reason other methods such as fiber-optic biosensor via antibody-aptamer functionalization (Ohk et al., 2009), optical biosensor based on the "light scattering sensor" technology (LSS) (Badana et al. 2009), quartz crystal microbalance (QCM) immunosensor (Vaughan et al. 2001) and immunomagnetic separation coupled with fiber optic immunosensor (Mendonca et al., 2012) were developed.

3.3 Detection of *Listeria monocytogenes* DNA through ISO 11290:2005, PCR, Dot Blot and magnetic beads

In this work we propose four different methods to detect *L. monocytogenes*, including classical ISO 11290:2005, PCR, Dot Blot and a novel approach with biosensing using magnetic beads. Molecular methods and biosensors can decrease the detection time required for the identification at species level; PCR and Dot Blot have been able to detect the presence of *L. monocytogenes* using DNA directly from enrichment broth. Magnetic beads assay promise to be very specific and sensitive. In this protocol, a sandwich DNA hybridization is performed by mixing the target DNA with the magnetic bead-captured probe and the biotinylated detection probe, followed through the biotin–avidin reaction with conjugated HRP, and then the conjugated HRP is determined by the chemiluminescent system.

3.3.1 Material and methods

3.3.1.1 Bacterial strains and culture media

L. monocytogenes DSM 19094 (Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Braunschweigh, Germany) as positive control and *Escherichia coli* HD5a1 (DISTAM) and *L. innocua* DSM 20649, as negative controls were used to check the specificity and sensitivity of the DNA probe used with the Magnetic Beads protocol. *L. monocytogenes* DSM 19094 were used to check the sensitivity of Mar1-Mar2 primers (Manzano et al., 1997) and the species listed in Table 3.1a and 3.2b for the specificity and sensitivity of the Dot Blot assay using Listm-capt-probe and Listm-det-probe. Primers and probe were then used to confirm the presence of *L. monocytogenes* in eight food samples using DNA extracted directly from Fraser enrichment broth and from isolates. *Listeria* species listed in Table 3.1a and the colonies isolate from food samples were also used on TSS (Trypticase Soy Agar + 5% sheep blood, Biomérieux) and ALOA (Agar *Listeria* Ottaviani-Agosti, Biolife) to verifiy the β-haemolysis, presents in all the strains of *L. monocytogens*, *L. seeligeri* and *L. ivanovii*, and the presence of phospholipase C (PLC), tipycal in all the strains of *L. monocytogenes* and in some strains of *L. ivanovii*.

Table 3.1a: Reference *Listeria* strains used for the protocols optimisation proposed in this work.

N°	Microorganism	Source
1	*Listeria monocytogenes*	*DSM 19094
2	*L. innocua*	*DSM 20649
3	*L. seeligeri*	*DSM 20751
4	*L. ivanovii*	*DSM 12491
5	*L. welshimeri*	*DSM 20650
6	*L. grayi*	*DSM 20596

*DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH

All the *Listeria* strains and *E. coli* strain were rehydrated in Brain Heart Infusion Broth (Oxoid, Milan, Italy) and then grown on Brain Heart Infusion agar (Oxoid) at 37 °C for 24 h in aerobic conditions.

Table 3.1b: Reference no-*Listeria* strains used for the optimisation of the Dot Blot protocol.

N°	Microorganism	Source
1	*Weissella cibaria*	*DSM 14295
2	*Vibrio* spp.	*DSM 14379
3	*Escherichia coli*	°DISTAM
4	*Pseudomonas aeruginosa*	°DISTAM
5	*P. migulae*	°DISTAM
6	*P. fluorescens*	°DISTAM
7	*P. brennerii*	°DISTAM
8	*Bacillus coagulans*	*DSM 2308
9	*B. subtilis*	*DSM 1029
10	*B. cereus*	*DSM 2301
11	*Proteus vulgaris*	°DISTAM
12	*Yersinia enterocolitica*	°DISTAM
13	*Morganella morganii*	°DISTAM
14	*Salmonella enteritidis*	*DSM 4883
15	*Campylobacter jejuni* subsp. *jejuni*	§ATCC BAA-1153
16	*Citrobacter freundii*	*DSM 15979
17	*Enterobacter cloacae*	*DSM 30054
18	*Aeromonas sobria*	*DSM 19176
19	*Lactobacillus plantarum*	*DSM 20174
20	*Pediococcus pentosaceus*	*DSM 20336
21	*Leuconostoc lactis*	#CECT 4173
22	*Saccharomyces cerevisiae*	§ATCC 36024

§ATCC: American Type Culture Collection (Manassas, VA, USA); °DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy); *DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH

3.3.1.2 Samples

Eight fresh chicken meat (thighs) were analysed for the presence of *Listeria* spp., according to ISO 11290:2005 25 g of skin were transferred into a sterile stomacher bag, added with 100 mL of saline-peptone water (8 g/L NaCl, 1 g/L bacteriological peptone, Oxoid), and mixed for 1.5 min in a Stomacher machine (PBI, Milan, Italy). From this homogenate, 25 mL aliquots were used for the pre-enrichment protocols in 225 mL of Half Fraser Broth at 37°C. After 24 h of incubation, 1 mL of pre-enrichment broth was added to 9 mL of Fraser Broth and kept for 24 h more at 37°C. Growth of typical colony of *Listeria* spp. were verified by streaking on Palcam Agar at 24 and 48 h, followed by streaking on TSS + 5% sheep blood and ALOA. Samples were also analysed also for the presence of *Salmonella* spp. according to ISO 6579:2004, *Campylobacter* spp. according to ISO 10271-1B:2006, and for the aerobic bacterial count, Enterobacteriaceae and coliforms. From the samples resulted positive for *Listeria* spp., three colonies were picked up and subjected to Gram staining and DNA extraction.

3.3.1.3 DNA Extraction from pure cultures and poultry samples

One mL of broth pure culture at 24 h for all the *Listeria* species and *E. coli*, were used for the DNA extraction and purification using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy) according to the manufacturer's instruction for DNA purification from cell samples. DNA concentration was measured using Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE 19810 U.S.A.).

Bacterial pellet was obtained using 10 mL Fraser Broth enrichment by centrifugation at 6,000 x g. Pellet was resuspended in 2 mL of sterile water and subjected to DNA extraction extracted with the Phenol-Chloroform method. After a second centrifuge at 14,000 x g for 5 minutes, the pellet was resuspended in300 µL of breaking buffer [2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris (pH 8), 1mM EDTA (pH 8)] and transferred into a screwcap tube containing 0.3 g of glass beads with a diameter of 0.1 mm. Subsequently, 300 µL of phenol/chlorophorm/isoamyl alcohol (25:24:1, pH 6.7; Sigma) were added and the cells were homogenized in a bead beater instrument (Fast Prep 24, Bio 101, Vista, CA) three times each for 30 s, with an interval of 15 s between each treatment. 300 µL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8) were added and the tubes were centrifuged at 14,000 x g for 10 min. The aqueous phase was collected and precipitated with 1 mL of ice-cold absolute ethanol. The DNA was collected with a centrifugation at 14,000 x g for 10 min and the pellet was dried under vacuum at room temperature. 50 µL of DNase-free sterile water (Sigma) were added and a 30

min period at 45 °C was used to facilitate the nucleic acid solubilisation. 1 µL of DNase-free RNase (Roche Diagnostics, Milan, Italy) was added to digest RNA with incubation at 37 °C for 1 h.

3.3.1.4 Probes design

The probes for the specie-specific detection of *Listeria monocytogenes*, targeting the *iap* gene, were designed and synthesized by MWG-Biotech (Germany). All the sequences were retrieved from GenBank and aligned using the "Multiple sequence alignment with hierarchical clustering" algorithm (Corpet, 1988); the specificity was tested with Blast (http://blast.ncbi.nlm.gov/Blast.cgi). The sequence of 34 bp degenerate capture probe (Listm-capt-probe) was: 5'-TAAAAATACCAATACTAAYWCAAACTCYAATACG -3' and annealed the *iap* gene. Also the sequence of the 32 bp detection probe (Listm-det-probe) which sequence is 5'-GCTAATCAAGGTTCTTCTAACAATAACAGCAA-3' annealed the *iap* gene (Fig. 3.3). The two probes were labelled with digoxigenin at 3' and 5' for the immunological detection in order to check the sensitivity and the specificity of the oligonucleotides.

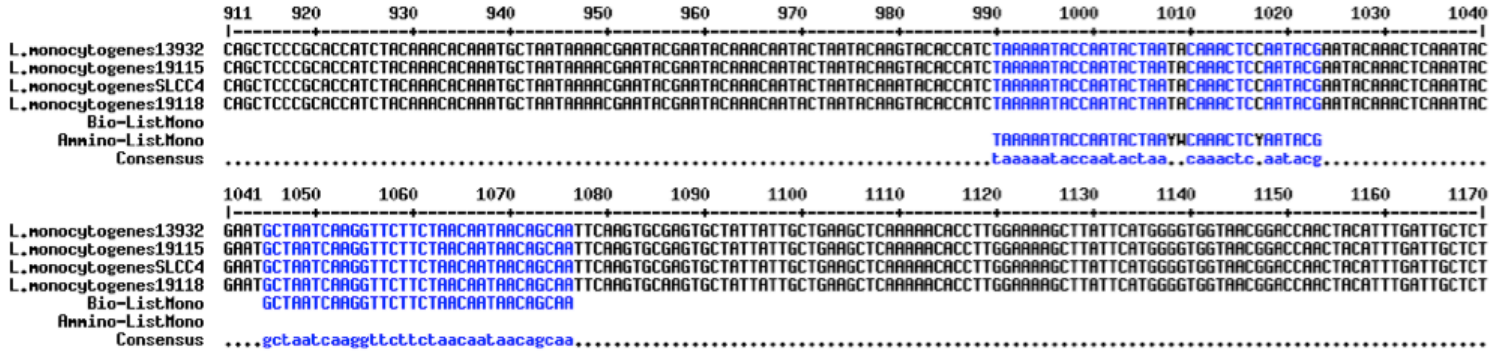


Figure 3.3: Alignment among the *iap* gene sequence of *L. monocytogenes*. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used.

3.3.1.5 PCR protocol

Specie specific-PCR was carried out using the primers Mar1-Mar2 in the reaction mixture containing the following reagents: 5 µL AmpliTaq® Buffer 10x (Life Technologies), 1 µL $MgCl_2$, 25 mM (Life Technologies), 1 µL PCR Nucleotide Mix, 10 mM each (Life Technologies); 1 µL of each primer (Mar1-Mar2 at 10 mM); 0.25 µL AmpliTaq® DNA Polymerase-5 units/µL (Life Technologies) and 1 µL of DNA (100 ng/µL). Thermal cycler conditions consisted of 95°C denaturation for 5 min, 35 cycles of 95°C for 1 min, 46°C for 45 s, 72°C for 30 s and a final extension at 72°C for 7 min in a Thermal Cycler (PTC220 DNA Engine DYAD MJ Research Celbio, Italia).

3.3.1.6 Electrophoresis

Five µL aliquots of the PCR products were resolved electrophoretically in a 1.5 % agarose gel (Sigma, Milan, Italy) in 0.5x TBE (2 mM EDTA, 80 mM Tris-acetate, pH 8.0) stained with GelRed from Biotium (Hayward, CA) and compared with 100 bp DNA Ladder (Promega).

3.3.1.7 Dot Blot protocol

Listm-capt-probe and Listm-det-probe probes after modification at 5' of the sequence by labelling with a digoxigenin (Listm-Dig-capt-probe and Listm-Dig-det-probe) were tested for sensitivity using synthetic ssDNA complementary to the probes sequences. For each probe 1 µL aliquots from 100 ng/µL to 1 ng/µL of the ssDNAs, after 5 min at 95°C, were spotted onto a nylon membrane (Sigma–Aldrich, Milan, Italy) and cross-linked to the air-dried membranes by exposure to UV light for 10 min. After a prehybridisation in pre-warmed Dig Easy Hyb buffers (Roche Diagnostic, Mannheim, Germany) for 30 min at 41°C, the membranes were hybridised in the same buffer supplemented with 10 pmol/ml (considered the best concentration after testing concentrations from 20 pmol/mL to 10 pmol/mL) of the digoxigenin-labelled oligonucleotide probes, at 41°C overnight, washed twice with 2X SSC (Promega) with 0.1% (w/v) SDS for 5 min at room temperature, and twice with 0.5X SSC with 0.1% (w/v) SDS for 15 min.

After washing with 1X washing buffer (Roche) for 5 min on a shaker, the membranes were incubated in blocking solution (1X Maleic Acid buffer and 10X blocking solution, Roche, Milan, Italy) for 30 min with shaking, and subsequently, an antibody solution (anti-digoxygenin-AP diluted in blocking solution 1:5.000, Roche) was added for 30 min while shaking. The membranes were washed twice with 1X washing buffer on a shaker for 15 min. Detection buffer 1X (Roche) for 5 min was used to neutralise the membrane that was then

incubated with a colour solution (NBT/BCIP in 1X Detection Buffer, Roche) in the dark without shaking, until the reaction was complete, and stopped by rinsing the membrane for 5 min in sterile water.

3.3.1.8 Activation of magnetic beads

The Listm-capt-probe was modified by addition of an amino-group at the 5' end (Listm-amino-probe) 5'NH_2-TAAAAATACCAATACTAAYWCAAACTCYAATACG -3' to allow the chemical bond with magnetic beads (Abnova, Taiwan). The Listm-det-probe was labelled with biotin at 3' end (Listm-biotin-probe) 5'-GCTAATCAAGGTTCTTCTAACAATAACAGCAA- Bio3' to allow the reaction for light production used for detection and measure. 20 μL of beads suspended in phosphate buffered saline, were transferred in a tube and immobilized to tube wall by placing the tube on a magnetic stand twice and subjected to Abnova protocol for activation (Fig. 3.4). This kit consists of magnetic beads made of single crystal of Fe_3O_4 sphere core and dextran coating layer. Through chemical modification of dextran, functional groups –COOH are joined with a covalent bond to the magnetic beads trough a short hydrophilic linker. Then, the capture probe modified with amino group could be conjugated to the magnetic beads through the activation with 1-(3-dimethylaminopropyl)-3-ethylcarbo-diimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the manufacturer instructions.

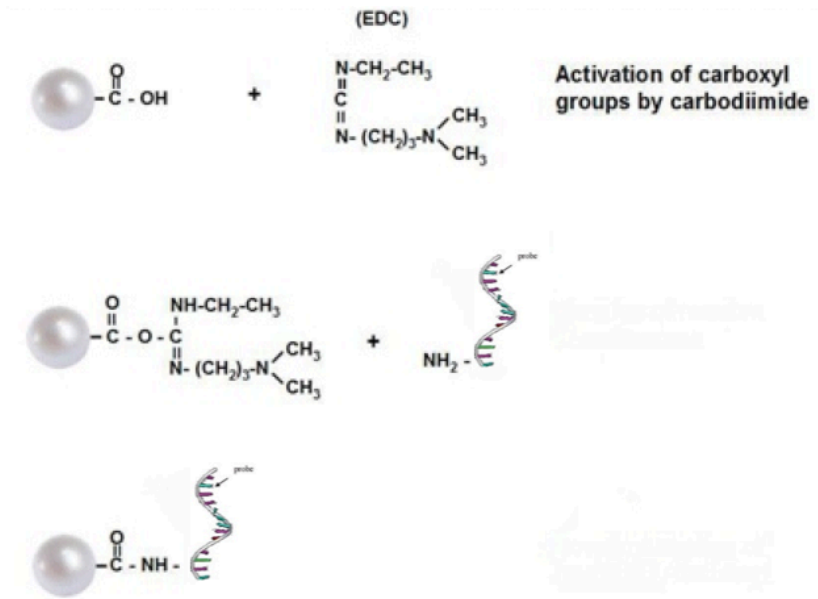


Figure 3.4: Reaction scheme for the activation of magnetic beads.

3.3.1.9 Binding of the List-amino-probe to magnetic beads

Beads with surface -COOH group for conjugation of DNA were conjugated to the 34 bp Listm-amino-probe in MES buffer (100 mM MES and 500 mM NaCl, pH 6.0) containing the List-amino-probe at the concentration of 100 ng/µL, by incubation with tilt rotation at 4°C overnight. Before immobilisation the probe was denaturated at 95°C for 5 minutes and then put immediately on ice for 5 minutes to avoid the secondary structure of the oligonucleotide. After washing in MES buffer, the beads conjugated with the probe, were placed on the magnetic stand for 60 s, and after removal from magnetic stand, added of 500 µL of Quench Buffer 1X (Tris-Buffered Saline, TBS pH 8.0) to stop the reaction incubating the beads with tilt rotation for 30 minutes at room temperature. After two washing with Quench Buffer for few seconds, the beads were washed twice with 500 µL of PBS 1X (Phosphate Buffered Saline, pH 7.2), and added with 100 µL PBST-10% Skim Milk, incubate for 1 hour in tilt rotation at 37^0C, and placed on the magnetic stand for 30-60 s to immobilize the beads at tube wall. After washing twice with 100 µL 0.05% PBST 1X (Phosphate Buffered Saline Tween, pH 7.2) and immobilization of the beads at tube wall, the beads with amino-probe are ready for the hybridization with the DNA.

3.3.1.10 Magnetic Beads hybridization

DNA of *L. monocytogenes* DSM 19094 at 40, 12.5 and 6 ng/µL were used as positive controls. *L. innocua* DSM 20649 at 40 ng/µL and *E. coli* at 40 ng/µL were used as negative controls. Additional samples were used as negative controls to check the system: no amino (beads without the addition of the Listm-amino-probe), no DNA (no DNA samples added), no biotin (without the Listm-biotin-probe), no AV-HRP (without conjugated avidin-HRP) and adding only magnetic beads.

DNAs extracted from 10 mL Fraser enrichment broth of samples CT3 and CT4 were standardized at 40, 12.5 and 6 ng/µL. The DNAs (from the pure culture strains or from samples) and the Listm-biotin-probe at 50 ng/µL were added in one step and the mixture was incubated for 1.5 h with 5% PBST- 5% Skim Milk at 41°C while shaking. After immobilization of the magnetic beads to the tube wall 100 µL PBST- 5% Skim Milk containing diluted AV-HRP 1:500 were added and the samples incubated in tilt rotation at room temperature for 1 h, and washed twice with 100 µL 0.05% PBS by quick vortexing. 100 µL of the beads solution were split in 3 tubes, 33 µL in each. 33 µL Immun-Star™ HRP Peroxide Buffer (Biorad) were added to each tube before placing into the dark chamber (sensor box). Then 33 µL Immun-Star™ HRP Luminol/Enhancer

(Biorad) were added and the samples were subjected to analyses (Fig. 3.5: reaction scheme of the process).

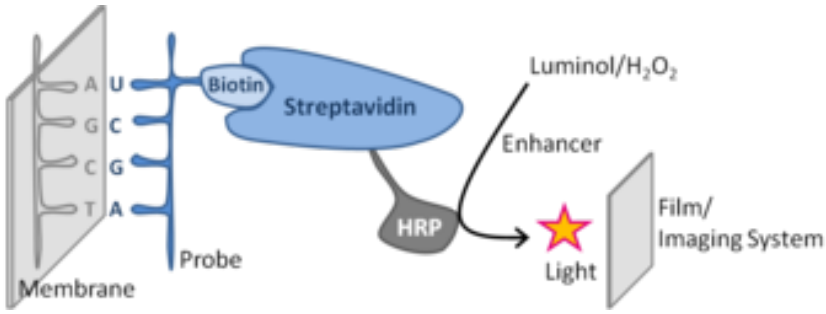


Figure 3.5: Glycoprotein avidin contains four binding sites with a high affinity for the small vitamin biotin (bonds with nucleic acids or proteins). Reaction between biotin-conjugated probe, avidin and horseradish peroxidase (HRP), luminol and H_2O_2 products chemiluminescence (Lin et al., 2008). (http://www.applichem.com/produkte/biochemica/western-blot-elisa/)

3.3.1.11 Chemiluminescence detection

Measurements were conducted using Hamamatsu HC135-01 Photo Multiplier Tube (PMT) Sensor Module, combining the sensitivity of a photomultiplier tube with the intelligence of a microcontroller. The detector is optimized to the blue light region and includes a 21 mm diameter active area convenient to gather light radiation without any optical focusing elements. The instrument was setup placed in a light-tight box equipped with a manual shutter (71430, Oriel-Newport Corporation) placed in front of the detector and a workshop made lever placed outside the box to move the slide shutter of the photon counting unit within. Each measurement was taken as the mean value of photon counts in 20 s, and each measurement point was presented as the mean calculation and standard deviation obtained from a single magnetic beads set (3 tubes); the Avidin-HRP conjugated reacts with peroxidase and Luminol (5-ammino-2,3-diidro-1,4-ftalazindione) and the measurement is a light signal expressed in Relative Light Unit (RLU).

3.3.2. Results

3.3.2.1 Plate count and species identification

Plate count method results for the enumeration of the total aerobic mesophilic microflora, *Enterobacteriaceae*, coliforms and the presence of *Listeria* spp., *Salmonella* spp. and *Campylobacter* spp. are showed in table 3.2. *Listeria* spp. was present in six of the eight sample analysed (CT1, CT2, CT3, CT5, CT7 and CT8), *Salmonella* spp. was detected only on sample CT3 while *Campylobacter* spp. was found on sample CT1, CT2, CT4, CT6 and CT8. Total bacterial contamination of poultry meat ranged from 1.8 x 10^5 to 1.4 x10^9 CFU/g,

Enterobacteriaceae from 8.0 x10^2 to 2.1 x 10^5 CFU/g, and coliforms from 2.3 x 10^2 to 9.2 x10^4 CFU/g.

Table 3.2: Plate count data of the poultry samples in Colony Forming Unit (CFU)/g

Sample	Aerobic bacterial count	Enterobacteriaceae	Coliforms	*Campylobacter* spp. ISO 10272-1B:2006	*Salmonella* spp. ISO 6579:2004	*Listeria* spp. ISO 11290:2005
CT1 chicken thighs	1.8 x 10^5	3.1 x 10^3	1.0 x 10^3	+	-	+
CT2 turkey thighs	1.4 x 10^9	2.1 x 10^5	9.2 x 10^4	+	-	+
CT3 chicken thighs	5.4 x 10^5	6.4 x 10^3	1.7 x 10^3	-	+	+
CT4 chicken thighs	2.3 x 10^6	2.8 x 10^3	2.3 x 10^2	+	-	-
CT5 turkey thighs	1.1 x 10^8	4.8 x 10^4	3.2 x 10^4	-	-	+
CT6 chicken thighs	3.3 x 10^7	8.0 x 10^2	1.2 x 10^4	+	-	-
CT7 turkey thighs	1.4 x 10^7	6.8 x 10^3	3.6 x 10^3	-	-	+
CT8 chicken thighs	4.7 x 10^7	2.0 x 10^4	2.4 x 10^4	+	-	+

Eighteen colonies of supposed *L. monocytogenes*, were isolated from Palcam agar after plating at 48 h from the 6 food samples enrichments. All the colonies were streaked on TSS (for the detection of β-haemolytic reaction) and on ALOA (for the detection of the strains positive for the presence of PLC); Fig. 3.6.

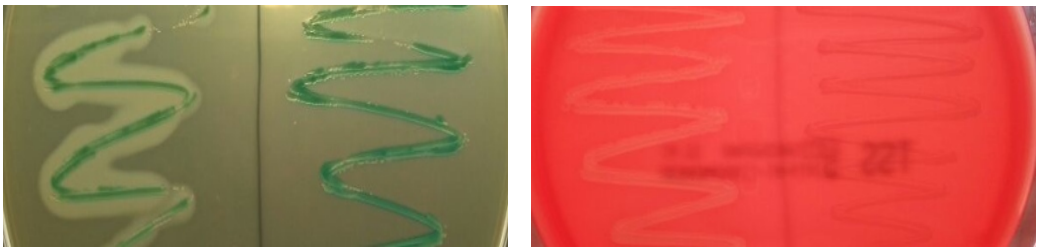


Figure 3.6: on the left are visible the results obtained on ALOA. Strains that possesses PLC product a visible opaque halo. On the right results obtained on TSS. β-Haemolytic activity products a transparent area due to the haemolysis of red blood cells.

Eight colonies out of the eighteen isolated were positive for both the tests (CT2, CT3, CT5, CT8), wherease all the colonies isolated from samples CT1 and CT7 were negative. Cross-comparison of the positivity found on the samples CT2, CT3, CT5 and CT8 allows to identifiy the strains as *L. monocytogenes* or *L. ivanovii*, while the negativity found on the samples CT1, CT7 and in four colonies isolated from samples CT3 and CT8, allows to identifiy the strains as *L. innocua*, *L. welshimeri* or *L. grayi*. In order to get an unmistakable result, DNAs extracted from the eighteen colonies and from the enrichment broth were subjected to PCR using specie-specific primers for *L. monocytogenes*.

3.3.2.2 PCR

Specificity and sensitivity

Mar1-Mar2 primers showed the expected specificity using the DNA of *L. monocytogenes* DSM 19094 producing the expected amplicon of 453 bp as no amplicons were obtained for the other *Listeria* species. The sensitivity of the primers tested using serial dilutions of the corresponding DNAs was 1 ng/µL (Fig. 3.7).

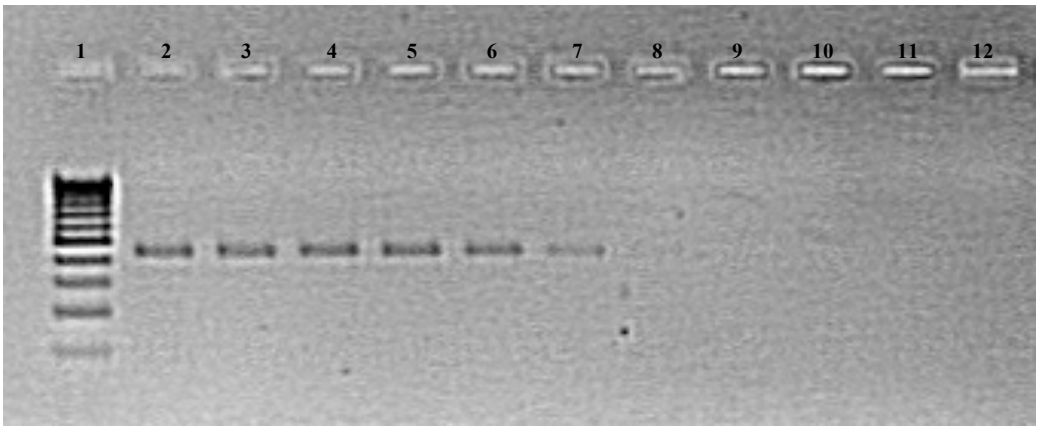


Figure 3.7: Sensitivity test with Mar1-Mar2 primers
Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); *L. monocytogenes* DSM 19094 (line 2-11), line 2: 100 ng/µL DNA; line 3: 50 ng/µL DNA; line 4: 25 ng/µL DNA; line 5: 10 ng/µL DNA; line 6: 5 ng/µL DNA; line 7: 1 ng/µL DNA; line 8: 0.5 ng/µL DNA; line 9: 0.1 ng/µL DNA; line 10: 0.05 ng/µL DNA; line 11: 0.01 ng/µL DNA; line 12: negative control.

Samples analysis

The results obtained using Mar1-Mar2 primers on DNAs extracted from Fraser broth at 48 h are reported in Fig. 3.8. Four of the six samples already positive for *Listeria* spp. using ISO 11290:2005 method were confirmed as *L. monocytogenes* (CT2, CT3, CT5, CT8) using the PCR protocol proposed. Samples CT1 and CT7 were negative by direct PCR.

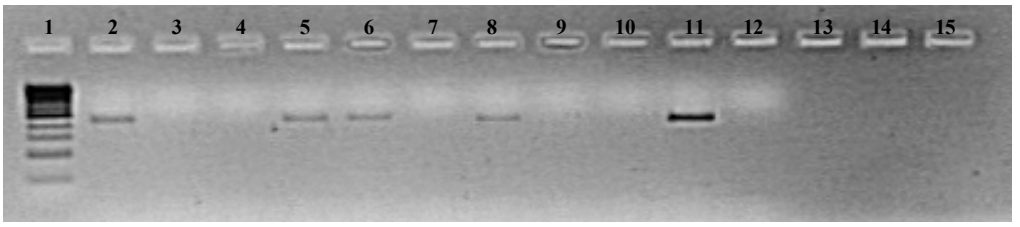


Figure 3.8: PCR with Mar1-Mar2 primers using DNA extracted from Fraser broth at 24 h enrichment. Line 1: 100 bp DNA Ladder (Promega); line 2: *L. monocytogenes* DSM 19094; line 3: *L. innocua* DSM 20649; line 4: CT1; line 5: CT2; line 6: CT3; line 7: CT4; line 8: CT5; line 9: CT6; line 10: CT7; line 11: CT8; line 12: negative control.

Colonies already tested by growth on TSS and ALOA agar were used in PCR to confirm their identification as *L. monocytogenes* using Mar1-Mar2 primers. Eight colonies out of the eighteen isolated were positive after amplification with the specific primers as shown in Fig. 3.9.

Samples CT2, CT3, CT5 and CT8 were positive, whereas no amplicons were obtained on isolates from samples CT1 and CT7, confirming the results obtained using DNA from Fraser enrichment broth and validating the results obtained by plating on TSS and ALOA and allowing also the discrimination among *L. monocytogenes* and *L. ivanovii*, not still possible using ALOA. This PCR protocol shows a high specificity as it allows an immediate discrimination between the samples containing *L. monocytogenes* from the samples containing other *Listeria* species.

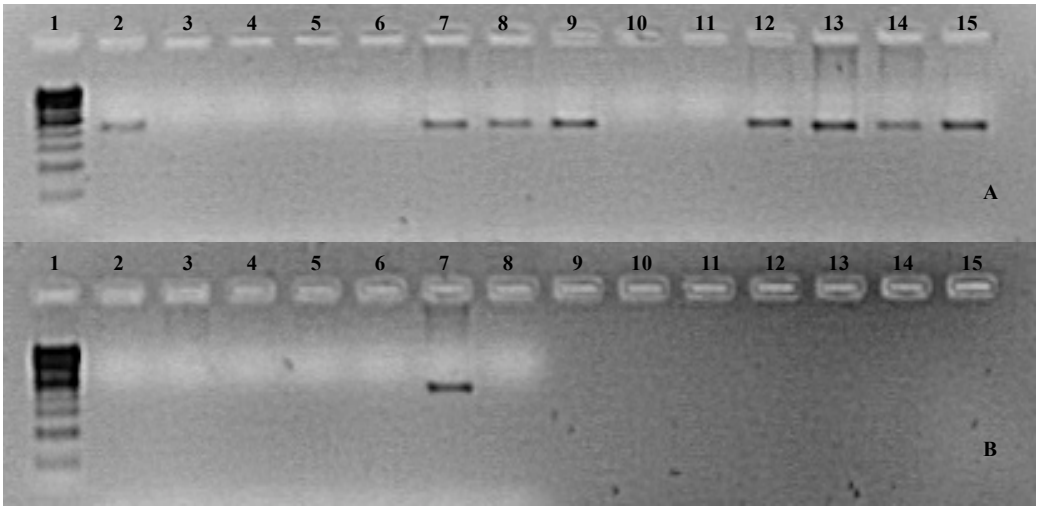


Figure 3.9: PCR on Isolates Food Samples using Mar1-Mar2 primers
A - Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); line 2: *L. monocytogenes* DSM 19094; line 3: *L. innocua* DSM 20649; line 4 to 6: isolates from CT1; line 7 to 9: isolates from CT2; line 10 to 12: isolates from CT3; line 13 to 15: isolates from CT5.
B - Line 1: 100 bp DNA Ladder (Promega, Milan, Italy); line 2 to 4: isolates from CT7; line 5 to 7: isolates from CT8; line 8: negative control.

3.3.2.3 Dot Blot

Specificity and Sensitivity

The sensitivity of both the Listm-Dig-capt-probe and Listm-Dig-det-probe at the concentration of 10 pmol/mL was 25 ng/μL using the synthetic ssDNA oligonucleotides complementary to the probes as target. Visible spots (positive) were obtained at the concentrations of 100 ng/μL, 50 ng/μL and 25 ng/μL (spots 1 to 3, Fig. 3.10), whereas 10 ng/μL, 5 ng/μL, and 1 ng/μL gave negative results (spots 4 to 6). The DNAs extracted from *L. monocytogenes* reference

strains showed a sensitivity of 100 ng/μL, while at the same conditions DNAs from other species of *Listeria* (Table 3.1a) and on non-*Listeria* strains (Table 3.1b) were negative.

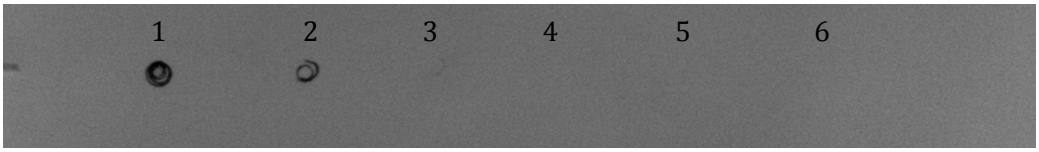


Figure 3.10: Dot Blot sensitivity of the Listm-det-probe using ssDNA complementary probe as template at various concentrations. 1: 100 ng/μL; 2: 50 ng/μL; 3: 25 ng/μL; 4: 10 ng/μL; 5: 5 ng/μl; 6: 1 ng/μL.

Sample analysis

The DNAs extracted from the enrichment broths of the samples at 48 h, according to ISO 11290:2005, gave positive results using both probes. The spots present in Fig. 3.11 using the Listm-det-probe (Row A, lines 2, 3, 5 and 8) indicate the positive hybridisation reaction between the *L. monocytigenes* DNAs present in the DNA samples spotted onto the membrane and Listm-det-probe. The absence of the spots in Lines 1, 4, 6 and 7 indicate the absence of *L. monocytogenes* in those food samples. The visible spots present in Fig. 3.11 Row B correspond to the ssDNA sequence complementary to the Listm-det-probe (Line 1) and to the DNA of *L. monocytogenes* DSM 19094 used as positive controls (Line 2). These data are in agreement with the results obtained by plating on Palcam agar, TSS and ALOA, PCR after enrichment in Fraser broth, and PCR on isolates from Palcam agar.

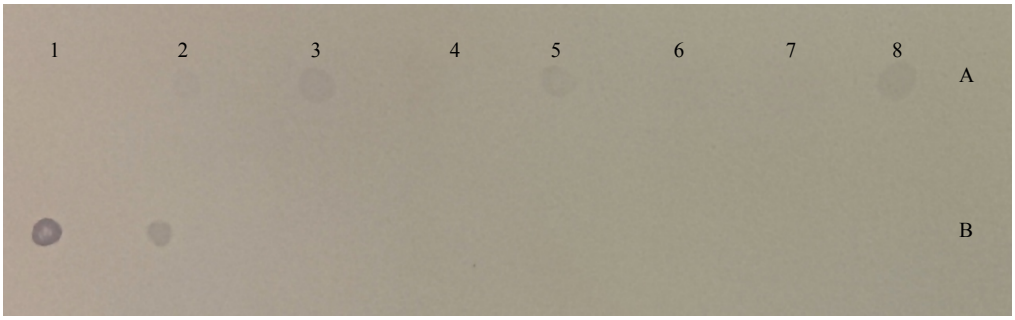


Figure 3.11: Row A - Dot blot on DNAs extracted from Fraser broth Line 1: CT1; line 2: CT2; line 3: CT3; line4: CT4; line 5: CT5; line 6: CT6; line 7: CT7; line 8: CT8.
Row B – Line 1: ssDNA complementary probe (100 ng/μL); line 2: *L. monocytogenes* DSM 19094 (100 ng/μL).

3.3.2.4 Magnetic Beads System

Sensitivity of the system

In order to verify the sensitivity of the method, three different concentrations of DNA (40 ng/µL, 12.5 ng/µL and 6 ng/µL) pertaining to *L. monocytogenes* DSM 19094 pure culture were used during the hybridization step on magnetic beads. The same hybridization protocol was repeted for the DNAs from the poultry samples CT3 and CT4. The results obtained and reported in Fig. 3.12 were consistent, as in both cases it was found an inverse proportional tendency between the increment of light emission and the DNA concentration. In fact, the concentration of 40 ng/µL of DNA from *L. monocytogenes* DSM 19094 (Fig. 3.12, bar 1) showed the lowest value, 604883 RLU, while the concentration of 6 ng/µL the highest value, 2001409 RLU (Fig. 3.12, bar 3); DNAs from sample CT3 showed the same trend as 1195332 RLU were obtained with 40 ng DNA/µL (Fig. 3.12, bar 4) and 11342591 RLU with 6 ng DNA/µL Fig. 3.12, bar 6). Values close to 0 RLU were obtained for the control (no DNA addition, bar 8) and for the sample CT4, used as negative sample as it was negative by PCR, Dot Blot and plating methods.

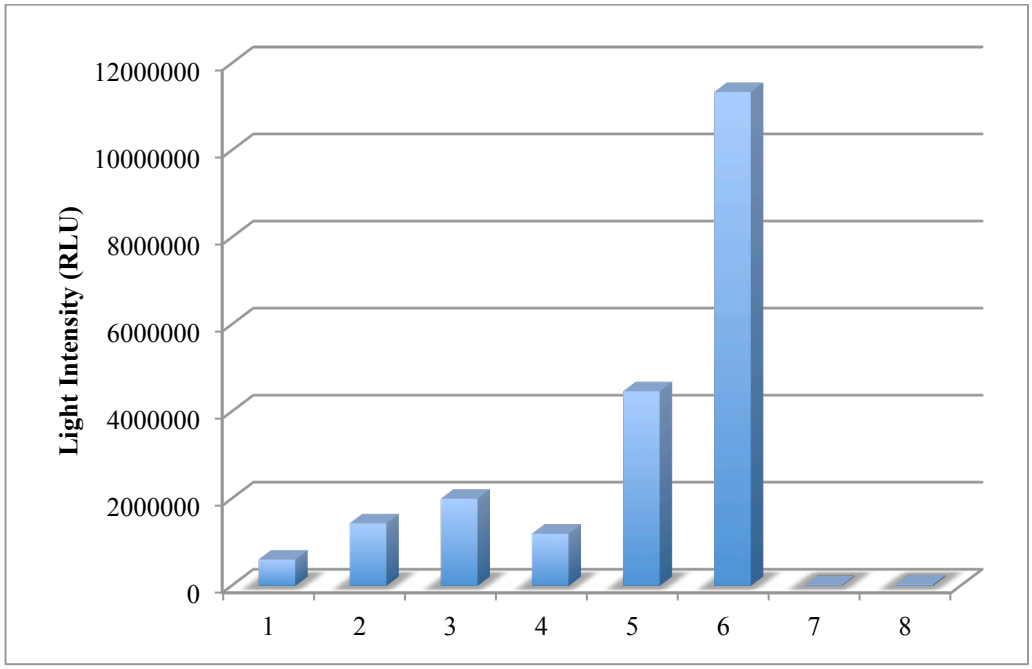


Figure 3.12: Samples analyses. Values of the light intensity are expressed as RLU (Relative Light Units). Positive control: (bar1 to 3) *L. monocytogenes* DSM 19094 DNA at 40 ng/µL, 12.5 ng/µl and 6 ng/µL. Samples CT3 DNA at 40 ng/µL, 12.5 ng/µL and 6 ng/µl (bar 4 to 6). Samples CT4 DNA at 40 ng/µL (bar 7). Negative control: NO DNA (omission of the DNA) (bar 8).

Specificity of the System

In order to check the specificity of the system, DNAs of *L. monocytogenes* DSM 19094, *L. innocua* DSM 20649 and *E. coli* were used (Fig. 3.13). The RLU value of 604883 obtained using DNA of *L. monocytogenes* was higher than the values found for *L. innocua* (352859 RLU) and *E. coli* (172454 RLU) using the same concentration of DNA (40 ng/µL). The negative control used to verify the correct evolution of the experiment, showed the lowest value of 35080 RLU.

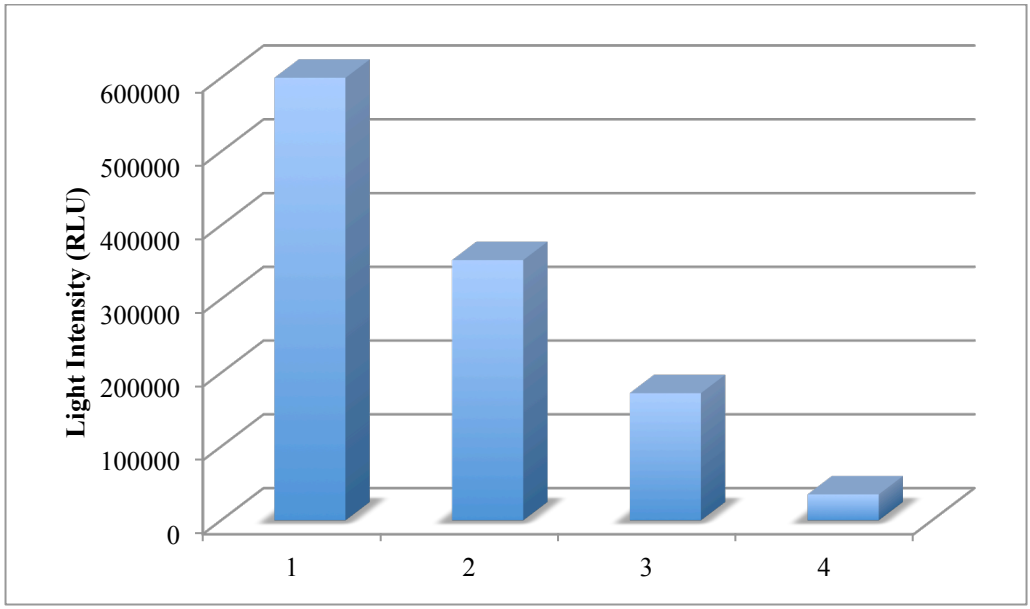


Figure 3.13. Specificity of the probe. Values of the light intensity are expressed as RLU (Relative Light Units). Positive control: *L. monocytogenes* DSM 19094 DNA at 40 ng/µL (bar 1). *L. innocua* DSM 20649 at 40 ng/µL (bar 2), *Escherichia coli* at 40 ng/µL (bar 3). Negative control: NO DNA: omission of the DNA (bar 4).

Optimization of the System

Creating an histogram with all the data obtained using DNA of *L. monocytogenes* DSM 19094, *L. innocua* DSM 20649, no amino (without the addition of the amino-labelled capture probe), no biotin (without the biotin-labelled secondary probe), no AV-HRP (without conjugated avidin-HRP), no DNA (with no DNA added) and only magnetic beads, it is possible to establish a cut off value over which samples can be considered positive (Fig. 3.14). In this system the background value corresponds to 352859 RLU, obtained for *L. innocua* DSM 20649, thus samples proucing RLU values higher than 352859, are considered positive for the presence of *L. monocytogenes*.

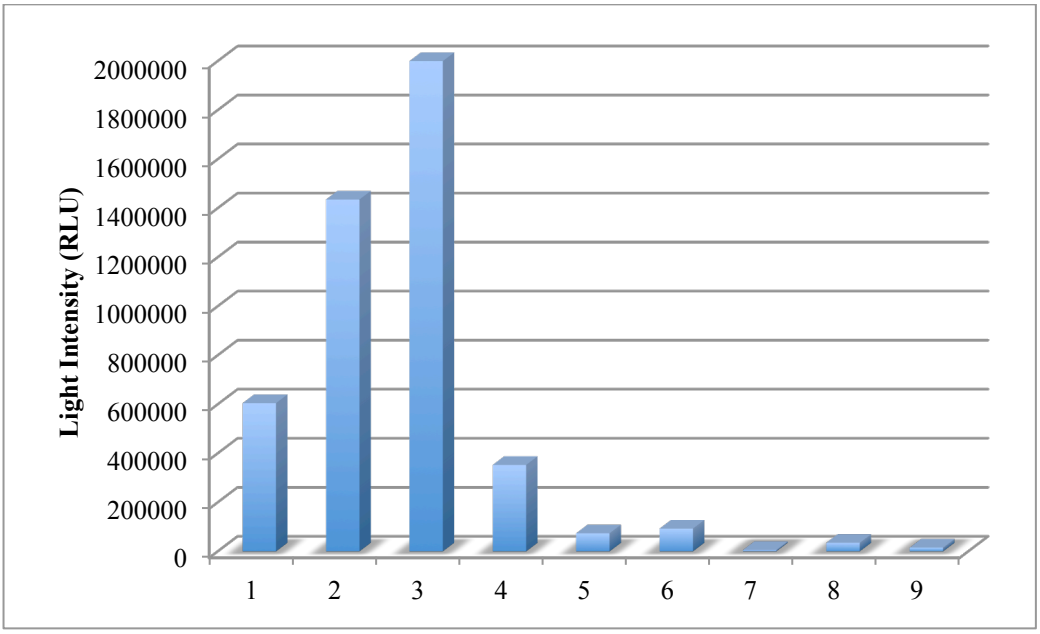


Figure 3.14: Optimization of the system. Values of the light intensity are expressed as RLU (Relative Light Units). Positive control: *L. monocytogenes* DSM 19094 DNA at 40 ng/µL, 12.5 ng/µL and 6 ng/µL (bar 1 to 3). Negative controls: *L. innocua* DSM 20649 (bar 4); No amino-probe: omission of List-amino-capture probe (bar 5); NO biotin-probe: omission of the biotin-probe (bar 6); NO AV-HRP: omission of the avidin- HRP conjugate (bar 7); NO DNA: omission of the DNA (bar 8); only Magnetic Beads (bar 9).

3.3.3 Discussion

The hybridization reactions are very important for the improvement of the performance of the DNA biosensor, thus before testing the system that uses magnetic beads, two different assays were performed PCR and Dot blot. PCR was specific, like reported by Manzano et al. (1997) and sensitive, allowing the detection of 1 ng/µL DNA. Moreover, PCR used in this work allowed a fast discrimination between *L. monocytogenes* and the other species of *Listeria*. In fact, only four of the six samples that where positive for *Listeria* spp. with the ISO standard method, were confirmed *L. monocytogenes* by both PCR and Dot blot, demonstrating the rapidity and specificity of the molecular techniques.
Dot blot showed the high specificity of both the proposed probes as they produced visible spots only in the presence of DNA of *L. monocytogenes,* allowing the detection of the pathogen also using heterogeneous DNAs extracted from enrichment Fraser broths. The sensitivity was 25 ng/µL on the ssDNA sequence complementary to the labelled probe, and 100 ng/µL on *L. monocytogenes* reference strains. In this work, a 5'-amino-labelled oligonucleotide probe, specific for *L. monocytogenes*, was immobilized on the surface of magnetic beads and used for the detection of *L. monocytogenes* using a secondary 3'-biotin labelled probe via an avidin bridge. The establishment of

a cut off value allowed the discrimination between positive and negative food samples. The developed system was able to detect the presence of *L. monocytogenes* even when heterogeneous DNA was used indicating high specificity and sensitivity. The development of a biosensor based on the utilization of magnetic beads and able to detect the presence of *L. monocytogenes* using DNAs extracted from enrichment broths, avoiding the fastidious and time consuming step of isolation from selective medium is ongoing. This system could replace PCR that can be affected by factors such as DNA polymerase inhibitors present in the samples (Kreader, 1996; Park et al., 2006) and producing false negative. The inverse proportional tendency between the increment of light emission and the concentration of DNA can be explained with the phenomenon of quenching due to high concentrations of DNA. In fact, the interaction of fluorescent dyes with nucleotides has been studied for various molecules during fluorescence quenching experiments (Doose et al., 2009; Torimura et al., 2001). Nucleoside guanosine has the lowest oxidation potential among the four DNA bases, and can quench many fluorophores via photoinduced electron transfer (Dunn et al., 1991; Yao et al., 1977; Jovanovic and Simic, 1986). Ranjit and Levitus (2012) reported that interaction between fluorescent dyes and dNMPs (N=A, T, G, C) produce a decrease in the apparent diffusion coefficient of the dye. In another study, Chu et al. (2010) found that the quenching effect of dsDNA on ECL of luminol was related to the pH of the media. Moreover, using different electrodes modified with ssDNA and dsDNA and compared with a CNTs/AuNPS/GC reference electrode, they found that ds DNA quenched 92.8% of ECL intensity and ssDNA only 30.7%. These data can therefore explain the results obtained in our experiments, the lowest value obtained using the higher DNA concentration (40 ng/μL) could be related to the quenching effect of the DNA on the luminol, effect that results obviously lower using the concentration of 6 ng/μL, allowing the obtainment of a high RLU value. The identification of the maximum DNA concentration to use with this system could solve the quenching problem. However, more experiments must be done to solve this problem due to the quenching effect of DNA on luminol, in order to obtain a usable method to detect *L. monocytogenes* in foods avoiding the time-consuming step of isolation and identification of this pathogen from the traditional selective media. The specificity of the systems adopted in this work was demonstrated by the absence of amplicons and spots on negative reference strains and negative food samples using PCR and Dot Blot, and by the results obtained with magnetic beads.

3.4 Use of LSPR technology for the detection of genomic DNA of *Listeria monocytogenes*

This work is focused on the detection of *Listeria monocytogenes* DNA using the technique of Localized Surface Plasmon Resonance (LSPR). A specie-specific probe was designed into the Invasion Associated Protein (*iap*) gene and functionalized with thiol-group for the chemical bond on the surface of the gold nanostructures. After the DNA extraction, hybridisation was induced by exposing DNA to thiol-probe and after the hybrid was placed on the glass modified with gold nanoparticles (NPs). Upon hybridization of target DNA, a dose-dependent LSPR spectra red-shift is detected. LSPR for the detection of genomic DNA of *L. monocytogenes* demonstrates to be very sensitive and concentration of 0.1 ng/μL is detectable.

3.4.1 Material and methods

3.4.1.1 Bacterial strains

To check the specificity and the sensitivity of the probe used in the LSPR protocols, one reference strain of *Listeria monocytogenes* from international culture collections (DSM 15675) was used as a reference strain. As negative controls *Salmonella enteritidis* BH4 was used.

3.4.1.2 Culture conditions

Listeria monocytogenes and *Salmonella enteritidis* strains were rehydrated in Brain Heart Infusion Broth (Oxoid, Milan, Italy) and then grown on Brain Heart Infusion agar (Oxoid, Milan, Italy) at 37 °C for 24 h under aerobic conditions.

3.4.1.3 DNA extraction from pure cultures

One mL of 24 h broth culture of *L. monocytogenes* and *S. enteritidis* were used for the DNA extraction and purification using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy) according to the manufacturer's instruction for DNA purification from cell samples. DNA concentration was measured using Varian Cary 100 UV-Vis spectrophotometer (Agilent Technologies).

3.4.1.4 Probe design to detect Listeria monocytogenes

The probe for the specie-specific detection of *L. monocytogenes*, targeting the *iap* gene, was manually designed and synthesized by MWG-Biotech (Germany). All the sequences were retrieved from GenBank and aligned using the "Multiple sequence alignment with hierarchical clustering" algorithm (Corpet, 1988); the specificity was tested with Blast (http://blast.ncbi.nlm.gov/Blast.cgi). The 32 bp probe was labelled with thiol at the 5' end to allow for the chemical bond with gold. The sequence of the probe was: 5'ThiC6- GCTAATCAAGGTTCTTCTAACAATAACAGCAA-3' and annealed at position 1045-1076 of the *iap* gene as shown in Fig. 3.15. Before the use with LSPR technique, the specificity and sensitivity of the probe were tested with dot-blot assay, as reported in chapter 3, section 3.3.1.7.

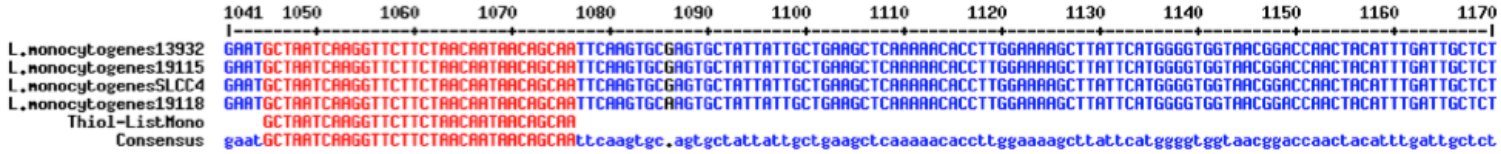


Figure 3.15: Alignment among the *iap* gene sequence of *L. monocytogenes*. For the alignment Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/) was used.

3.4.1.5 Equipment for plasmonic measurement

For the LSPR measurement, an homemade optical setup (Fig 3.16) based on transmission UV-vis-NIR spectroscopy which contains a white light source (DH-2000-BAC, Ocean Optics), two optic fibers (diameter of 600 μm for illumination beam and 150 μm for transmission beam), and a portable photospectrometer (QE65000, Ocean Optics) with a wavelength ranging from 200 nm to 1100 nm, a wavelength sensitivity of 1 nm and a data integration time of 100 ms were used.

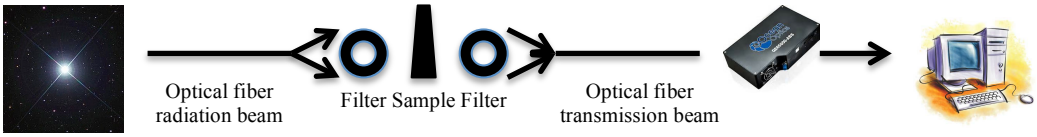


Figure 3.16: LSPR setup.

3.4.1.6 Treatment of the glass substrates

Classical microscope glass slides (Carl Roth GmbH+Co.KG, Germany) were cut in rectangles of 25x8 mm with a diamond tip and subsequently, used as substrates for gold film deposition. Before the evaporation, all the glass substrates were placed in a plastic sample holder and washed in a mixture of detergent (Decon 90) and deionized water (2:8 v/v ratio) in an ultrasonic water bath (Elmasonic S30H, Elma®, Germany) at 50°C for 15 min. Therefore, the resulting samples were rinsed with excess amount of deionized water, dried by air flow and subjected to another ultrasonication washing in deionized water at 50°C for 5 min. Finally, the glass substrates were rinsed three times with deionized water and dried in an oven at 100°C for 10 min. After washing, in each glass substrate, were fixed the TEM grids (Fig. 3.17) (Strata Tek™ Double Folding Grids, Ted Pella, Inc.) as a mask for the identification of test area; each grids consists of a pattern of 100 or 200 wells. Then, the glasses were ready for gold evaporation.

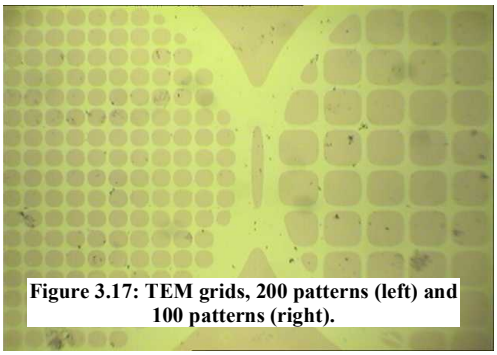


Figure 3.17: TEM grids, 200 patterns (left) and 100 patterns (right).

3.4.1.7 Preparation of gold NPs on glass substrate

The evaporation was conducted in an evaporator (MEB 400, PLASSYS, France, Fig 3.18) using the electron beam evaporation mode at ambient temperature under a high vacuum (pressure was around $1.0x10^{-6}$ Torr). The evaporation rate was adjusted around 0.08 nm/s by slowly changing the current intensity. The gold film thickness was monitored by a build-in quartz crystal sensor and the process was automatically stopped at the thickness of 6 nm. After evaporation, the TEM mask were removed and the modified glass samples were transferred in a high temperature oven (Naberthem, Germany) for 8 h at the annealing temperature of 500°C in order to obtain well organized gold nanoparticles from gold film (Fig 3.19). After annealing, the glass samples were firstly cleaned and stabilized by washing with acetone-ethanol 1:1 in an ultrasonic water bath for 30 min, then other two washings with deionized water and ethanol and a drying step were performed.

Figure 3.18: Evaporator PLASSYS MEB 400 http://www.clipproteomic.fr/Equipement.php?menu=menubar_equipement.html

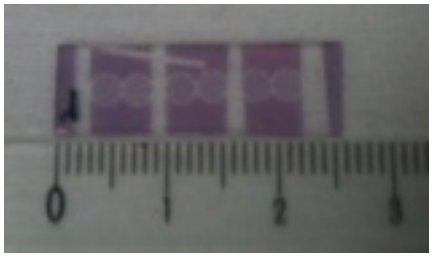


Figure 3.19: Glass with Gold NPs.

3.4.1.8 Hybridization of the DNA with thiol probe

This step was set up testing three different buffers, as SSPE 1X (20×SSPE: 3M NaCl, 175.3 g/L; 230 mM NaH_2PO_4 anhydrous, 27.6 g/L; 25 mM EDTAx2 H_2O, 9.4 g/L; pH 7.2), PBS 1X (10X PBS: 1.5M NaCl, 87.6 g/L; 81 mM Na_2HPO_4 anhydrous, 11.5 g/L; 19 mM NaH_2PO_4 anhydrous, 2.3 g/L; pH 7.2), and 1X TRIS-HCl buffer (10X TRIS-HCl: 0.5 M Tris-HCl, 79 g/L; pH 8.0). Sterilization and removal of all the suspended particles are carried out by passing through 0.2 μm filter (Millipore, USA). The work solution was

obtained by dilution to 1X with Milli-Q-water. The probe and the DNA were denaturated at 95°C for 5 min and then put immediately in ice for 5 min in order to avoid the reassembling of the double strand DNA and the formation of secondary structure of the probe. Then 15 μL of denaturated DNA and 15 μL of denaturated probe (100 ng/μL) for each sample were used for hybridization at 48°C for 14 h. Two types of experiments were performed. The first experiment was set up testing three concentrations of DNA (10, 25, 100 ng/μL) while the second experiment was set up with four concentrations of DNA (0.1, 1, 10 and 100 ng/μL).

3.4.1.9 Biofunctionalization of gold NPs with thiol-probe

The LSPR spectra of the clean gold nanoparticles were firstly recorded as plasmonic references. Successively**,** to verify the amount of thiol-probe that can be bond on the gold NPs, 1 μL of 100 ng/μL denaturated probe were put on the gold-nanostructures in order to allow for the bond of the thiol with gold, each sample in a different grid. After incubation, the LSPR spectra of biofunctionalized gold nanoparticles were recorded after 1-2-6 and 10 hours of immobilization of the thiol probe at 4°C. To avoid fast liquid evaporation, the glasses with the gold NPs were placed into a humid Petri dish. After washing with deionized water to remove the excess of unbound probe, the LSPR spectra of biofunctionalized gold nanoparticles were recorded, and then compared with those of the clean gold nanoparticles. At the end, all the data were processed with software Origin Pro 8.5.

3.4.1.10 Biofunctionalization of gold NPs with DNA

Gold nanoparticles were also used to check the specificity and the sensitivity of the probe using the DNA of *L. monocytogenes* and *S. enteritidis* at the concentration and the conditions previously described. 1 μL of DNA-probe hybrid were put on the Gold NPs and after 1 h of immobilization at 4°C the glasses were washed with dd H_2O, allowed to air dry and then the LSPR spectra were measured and compared with those ones of clean NPs.

3.4.2 Results

3.4.2.1 Biofunctionalization of gold NPs with thiol-probe

Gold nanoparticles obtained by annealing the samples with 4 nm evaporated gold film thickness at 500°C for 8 h were used to verify the optimized time for the chemical bond of Thiol-probe with gold nanoparticles. Different LSPR spectra correspond to different immobilization time of the Thiol-ListMono probe with Au-NPs (Fig. 3.20).

The plasmonic peak after 1 h and 2 h of immobilization were very close, showing a value of 567 nm with optical density of 0.307 and 572 nm with optical density of 0.312. Comparing the values with those one of clean gold nanoparticles (556 nm and 0.274 OD) it is possible to notice a large red-shift at 1 h immobilization (11 nm) with an increment of only 5 nm after 2 h immobilization. The curves at 6 h and 10 h showed a larger and identical red-shift (compared to clean gold nanoparticles) of 23 nm with a value of 579 nm and a small increment in extinction moving from 0.323 OD to 0.330 OD. To obtain a diagnostic response in a shorter time, and taking in account the initial shift of 11 nm, 1 h of immobilization was chosen like optimal time to perform the experiments.

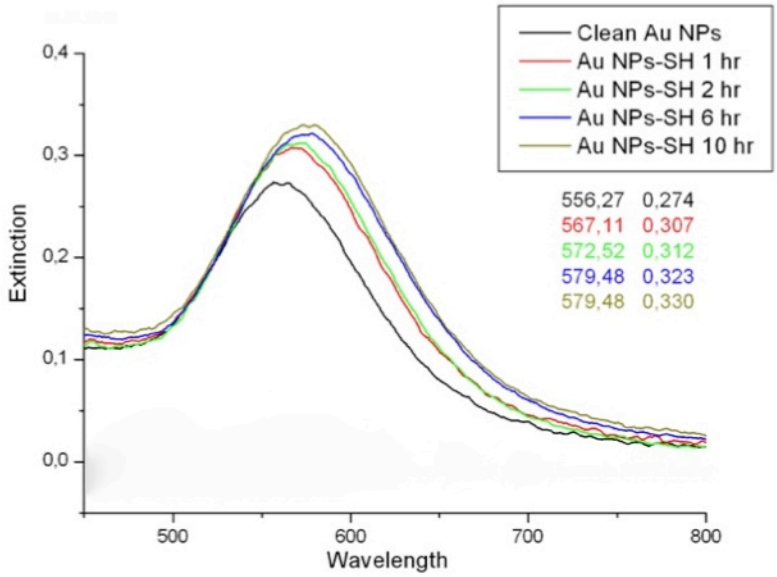


Figure 3.20: Thiol-probe immobilization time optimization.

3.4.2.2 Biofunctionalization of gold NPs with DNA-probe hybrid

Clean Au NPs with reproducible plasmonic spectra is a very important requirement to obtain comparable results. For this reason, several measurements were recorded only on the pattern that showed the same extinction and wavelength. Biofunctionalization of gold nanoparticles with probe-DNA hybrid was made using three different buffers (SSPE, PBS and TRIS-HCl). The best result was obtained using SSPE buffer (Fig. 3.21), performing an hybridization step between thiol-probe and DNA for 14 h at 48°C and a following

immobilization step of hybrid at 4°C for 1 h in a humid Petri-dish. The results show how comparing the wavelengths between the clean gold nanoparticles (554 nm) with the wavelengths of probe-DNA modified gold nanoparticles, a wavelength red-shift was observed increasing the DNA concentration, from a minimum of 15 nm for the concentration of 10 ng/μL (value of 569 nm) to a maximum of 28 nm for the concentration of 100 ng/μL (value of 582 nm), the shift for the concentration of 25 ng/μL was only 19 nm, very close to those one of 10 ng/μL. Also the optical density showed an increase from lowest to highest concentration with values of 0.288 OD, 0.305 OD and 0.357 OD.

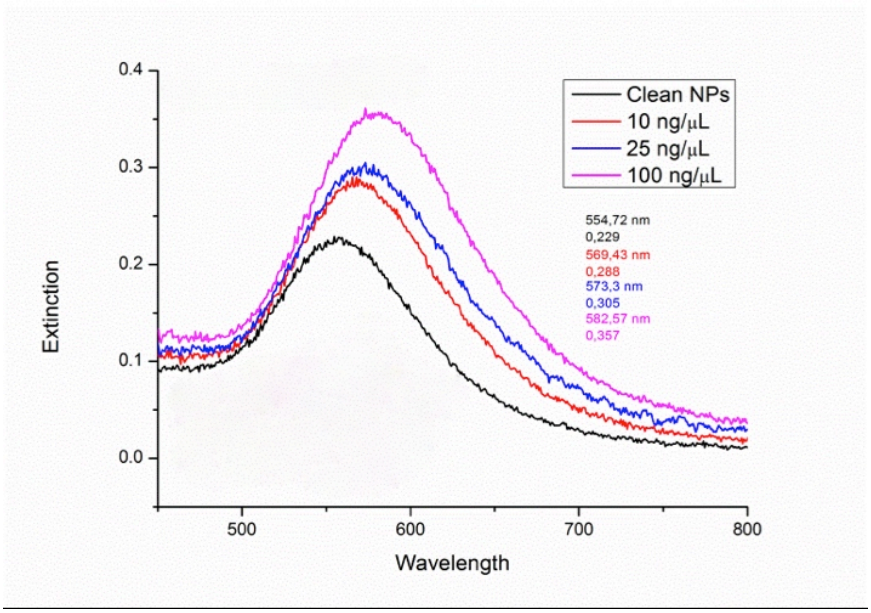


Figure 3.21: Experiment performed in SSPE buffer using 10 ng/μL, 25 ng/μL and 100 ng/μL DNA of *L. monocytogenes.*

The LSPR spectra obtained comparing the three different buffers (SSPE, PBS and TRIS-HCl) are shown in Fig. 3.22. Data show how the hybridization and immobilization steps conducted in SSPE buffer were the most performing. In fact, the plasmonic peak for the concentration of 10 ng/μL increase using TRIS-HCl, PBS and SSPE respectively with values of 542 nm, 543 and 569 nm as well the optical density increase from 0.267 to 0.288. For the concentration of 100 ng/μL it is possible to observe a similar tendency with a larger red-shift using the different buffers. The plasmonic peak increase red-shift using TRIS-HCl, PBS and SSPE respectively with values of 560 nm, 573 nm and 582 nm with a similar tendency for the optical density, moving from 0.282 to 0.357.

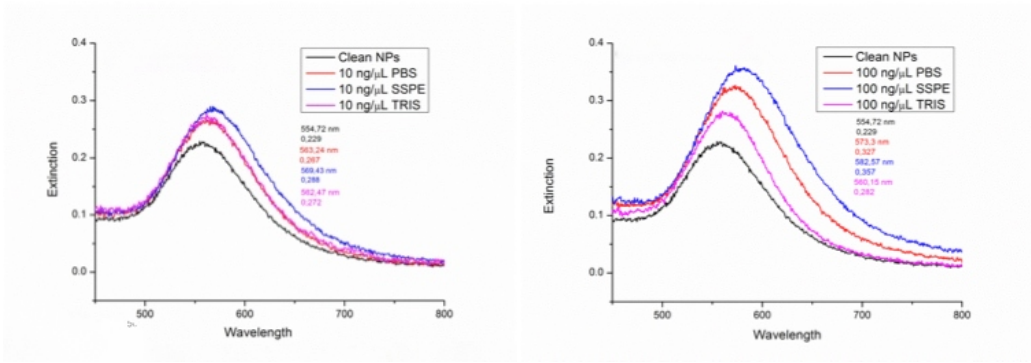


Figure 3.22: Comparison between the values obtained with 10 ng/µL (left) and 100 ng/µL (right) using the three different buffers tested.

One more experiment was performed to verify the sensitivity of the system, using the DNA of *L. monocytigenes* at the concentration of 0.1 ng/µL, 1 ng/µL, 10 ng/µL and 100 ng/µL (Fig. 3.23). The data obtained were comparable and demonstrated the sensitivity of the system, making it possible the detection of *L. monocytogenes* DNA also at concentrations of 1 ng/µL and 0.1 ng/µL. Comparing the LSPR spectra, the different concentration of DNA of *L. monocytogenes* can be easily distinguished valuating the red-shift of resonant wavelength and concurrent increase of optical density. An increasing of concentration from 0.1 ng/µL to 100 ng/µL corresponds to a red-shift from 562 nm to 574 nm with a concurrent optical density increased from 0.240 to 0.343.

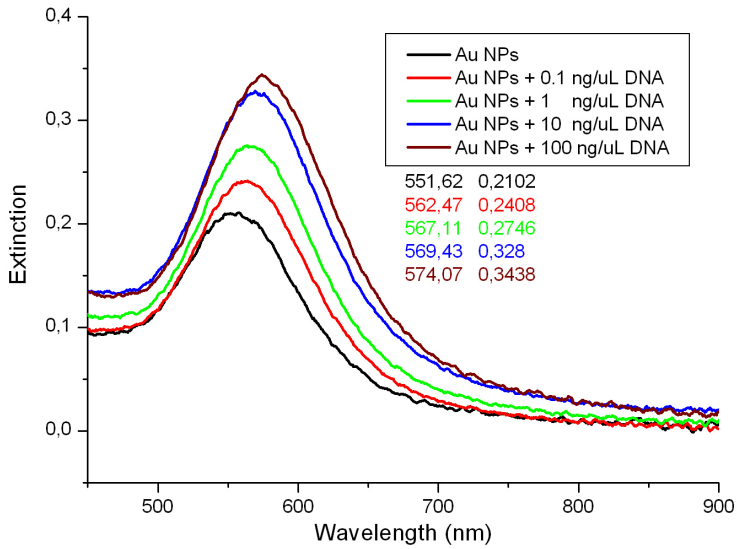


Figure 3.23: Experiment performed in SSPE buffer using 0.1 ng/µL, 1 ng/µL, 10 ng/µL, and 100 ng/µL DNA of *L. monocytogenes*.

On the contrary, experiment performed immobilizing DNA of *S. enteritidis* BH4 (Fig. 3.24) at the same concentrations and conditions, no significant plasmonic shifts were observed, demonstrating the specificity of the system.

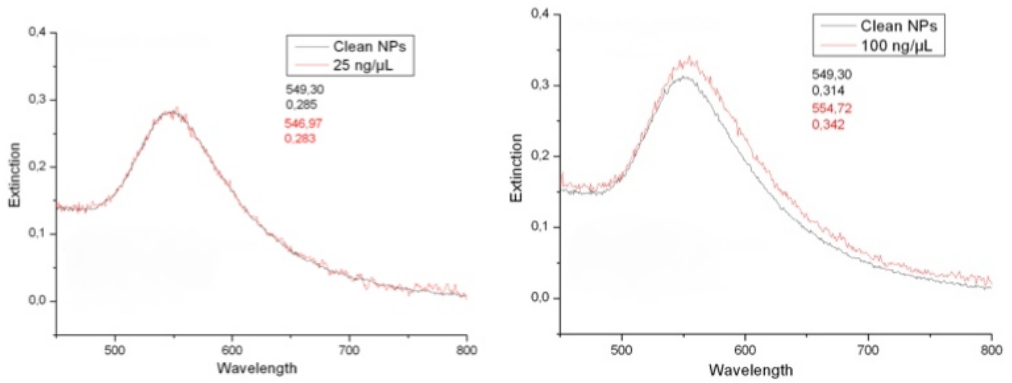


Figure 3.24: Experiment performed in SSPE buffer using 25 ng/µL and 100 ng/µL DNA of *S. enteritidis*.

As reported in Fig. 3.24, only for the concentration of 100 ng/µL a small red-shift of 5 nm is detectable, with a concurrent optical density increased from 0.314 to 0.342, lesser than the red-shift of 11 nm and the increment optical density reported for the concentration of 0.1 ng/µL using DNA of *L. monocytogenes*. Starting from the concentration of 25 ng/µL, variations in wavelength or optical density are not observable.

3.4.3 Discussion

In this study we developed an LSPR based biosensor, specific and sensitive, for the detection of genomic DNA of *Listeria monocitogenes*. Under the optimized conditions, the sensitivity of the annealed gold nanostructure modified with the specie-specific thiol-probe, evaluated with LSPR assay, was 0.1 ng/µL. However, more detailed studies should be carried out considering the optimization of AuNPs dimension and stability. In fact, Jia et al. (2013) reported that thickness of gold film and annealing temperature can play an important role on the LSPR properties and particles morphology evolution. All these parameters can influence the bond of the thiol-modified probe on the surface of gold nanoparticles. It is easy to understand how the dimension of AuNPs can establish the number of thiol that can be bond on the surface. The quasi-covalent biding of thiol groups to Au surface allows the immobilization of several hundred thiol-modified oligonucleotides on a single AuNP (Zanoli et al., 2012) influencing the quantity of DNA that can hybridize with the specific probe with an obvious different final result in terms of red-shift and optical

density. Moreover, it is very important to emphasize the role of surface biomodification in the future experiments considering that the orientation of ssDNA thiol probe on the surface of gold nanoparticles should be precisely controlled by appropriate surface biomodification, concerning the ultra-sensitive plasmonic responses in the nanoscale and other experiments testing food samples (containing heterogeneous DNA derived from different microorganism) should be done to test the applicability of the protocol to real samples. This protocol still requires complex devices but promises to be useful for the creation of nanoparticles-based biochip to screen or confirm the presence of *Listeria* and other pathogens with a very good efficiency and sensitivity.

CONCLUSIONS AND FUTURE PERSPECTIVES

With the introduction of HACCP system during the entire production process of a food, it has been need to develop some online systems that allow the monitoring of the entire production cycle. Microbiological analysis of food, considering trends and demands of consumers, food industry and national and international regulations, is an essential step to guarantee the quality, safety, nutritional and hygienic aspects of food supply. Validation of each method, able to detect the interested microorganism and dependent on the specific food matrix, should consider some parameters: precision, accuracy, specificity, sensitivity and economic impact. For this reason, highly specific, cost-effective and reliable methods are increasingly needed.

In this PhD thesis the comparison among different diagnostic methods, resulted in great performances and several limits for each method. Traditional microbiological assays, such as enrichment and plating on selective media following international procedures proved to be able to provide a diagnostic response although the required times are often long. The steps to get the final result are hard-working and the staff involved in phenotypic identification of microorganisms needs to have high competences.

Molecular biology techniques have been able to shorten the time in obtaining a diagnostic response, in fact, by extracting the DNA from the complex food matrix is was possible to obtain an identification of the microorganism of interest in a few hours against several days required using traditional methods.

The new field of biosensors is very promising and interesting, and their use allows the obtainment of accurate results with a high sensitivity and specificity. Pretreatment of the sample is not necessary, the amount of sample needed could be reduced, there is the possibility for repeated use and it is possible to build portable devices although the costs for their implementation are still high and a qualified staff is necessary. Actually, biosensors are tested more often with proteins and cells and the detection of the target is made by using antibody. The few works proposing DNA based detection are still using a PCR product as target. For this reason would be very interesting to increase the knowledge in this field using genomic DNA from pure cultures and DNA extracted directly from complex food matrices, like proposed in this thesis. Moreover, one of the most important future perspectives will be the obtainement of a multiplexed sensing chip to minimize the number of required sensors. The benefits would be enormous, including rapid and parallel detection of multiple microorganisms on a single chip, lower costs, reduced sample volume and short response time.

REFERENCES

Acik M.N., Cetinkaya B. (2006). Heterogeneity of *Campylobacter jejuni* and *Campylobacter coli* strains from healthy sheep. *Veterinary Microbiology* 115: 370-375.

Acuff G.R., Vanderzant C., Hanna M.O., Ehlers J.G., Golan F.A., Gardner F.A. (1986). Prevalence of *Campylobacter jejuni* in turkey carcass processing and further processing of turkey product. *Journal of Food Protection* 49, 712-717.

Adak, G.K., Long, S.M., O'Brien, S.J. (2002). Trends in indigenous food borne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51, 832 – 841.

Ahluwalia A. (2004). Biosensori. In "Sensori per misure biomediche" ed. Patron. De Rossi D., Ahluwalia A., Mazzoldi A., Pede D., Scilingo E.P. (2004), pp. 175-224.

Albert M.J. (2011). Cross-reactivity of outer membrane proteins of *Campylobacter* species with cholera toxin. *Indian Journal of Medical Research* 133(2), 207-211.

Allen V.M., Bull S.A., Corry J.E., Domingue G., Jorgensen F., Frost J.A., Whyte R., Gonzalez A., Elviss N., Humphrey T.J. (2007). *Campylobacter spp*. contamination of chicken carcasses during processing in relation to flock colonisation. Int *Journal of Food Microbiology*. 113, 54-61.

Altekruse S.F., Stern N.J., Fields P.I. and Swerdlow D.L. (1999). *Campylobacter jejuni*-an emerging foodborne pathogen. *Emerging Infectious Diseases* 5, 28-35.

Amagliani G., Giammarini C., Omiccioli E., Brandi G., Magnani M. (2007). Detection of Listeria monocytogenes using a commercial PCR kit and different DNA extraction methods. *Food Control* 18, 1137-1142.

Arun K.B. (2008) In: Foodborne Microbial Pathogens: mechanisms and pathogenesis of *Campylobacter* and *Arcobacter,* pp 217-226. Springer Publisher, New York.

Atanassova V., Reich F., Beckmann L., Klein G. (2007). Prevalence of *Campylobacter spp*. in turkey meat from a slaughterhouse and in turkey meat retail products. FEMS *Medical Microbiology and Immunology* 49, 141-145.

Baar I., Jacobs B.C., Govers N., Jorens P.G., Parizel P.M. and Cras P. (2007). *Campylobacter jejuni*-induced acute transverse myelitis. *Spinal Cord* 45, 690-694.

Bacon D.J., Johnson W.M., Rodgers F.G. (1999). Identification and characterisation of a cytotoxic porin-lipopolysaccharide complex from *Campylobacter jejuni*. *Journal of Medical Microbiology*, 48 139–148.

Badana P.P., Huff K., Bae E., Rajwa B., Aroonnual A., Bayraktar B., Adil A., Robinson J.P., Hirleman E.D., Bhunia A.K. (2009). Label-free detection of multiple bacterial pathogens using light-scattering sensor. *Biosensors and Bioelectronics* 26, 1685-1692.

Bae W., Kaya K.N., Hancock D.D., Call D.R., Park Y.H., Besser T.E. (2005). Prevalence and antimicrobial resistance of thermopilic *Campylobacter* spp. from cattle farms in Washington State. *Applied and Environmental Microbiology* 71, 169-74.

Banerjee A., Shuai Y., Dixit R., Papautsky I., Klotzkin D. (2010). Concentration dependence of fluorescence signal in a microfluidic fluorescence detector. *Journal of Luminescence* 130, 1095-1100.

Barbu V., Molecular hybridization techniques of nucleic acids (2007). *Innovative Romanian Food Biotechnology* 1 (30): p. 1-12.

Barchiesi D., Kessentini S., Guillot N., Lamy de la Chapelle M., Grosges T. (2013). Localized surface plasmon resonance in arrays of nano-gold cylinders: inverse problem and propagation of uncertainties. *Optical Express* 21 (2), 2245-2261.

Bates C., Hiett K.L., Stern N.J. (2004). Relationship of *Campylobacter* isolated from poultry and from darkling beetles in New Zealend. *Avian Diseases* 48, 138-47.

Battisti A. and Solaro R. (2003). Biosensori, biochip e microarray. Principi ed applicazioni nel settore biomedico. pp. 14-23.

Berger C.E.H., Beumer T.A.M., Kooyman R.P.H., Greve J. (1998). Surface plasmon resonance multisensing. *Analytical Chemistry* 70, 703-706.

Bille J., Rocourt J., Swaminathan B. (2003). *Listeria* and *Erysipelothrix*. In: Murray P.R., Baron E.J., Jorgensen J.H. et al. (eds) Manual of clinical microbiology, 8th edn. ASM, Washington, DC, pp. 461-471. In: Godfine H., Shen H. (eds) *Listeria monocytigenes*: Pathogenesis and Host response; 2007 Springer Science+Bussiness Media, LLC. pp. 13.

Blaser M.J., Taylor D.N., Feldam R.A. (1984). Epidemiology of *Campylobacter* infections. In: Butzler J.P. (Eds), *Campylobacter* Infection in Man and Animals, pp 143-161. CRC Press Boca Raton, FL.

Braun K.P., Theissig F., Ernst H., May M. and Krülls-Münch J. (2008). *Campylobacter jejuni*-associated hepatitis and myocardial injury. *Mekizinische Klinik* 103, 346-348.

Bruchez M., Moronne M., Gin P., Weiss S., Alivisatos A.P. (1998). Semiconductor Nanocrystals as Fluorescent Biological Labels. *Science* 281, 2013-2016.

Bubert, A., Kuhn M., Goebel W., and Köhler S. (1992). Structural and functional properties of the p60 proteins from different *Listeria* species. *Journal of Bacteriology* 174, 8166-8171.

Buchanan, S. K. (1999). Beta-barrel proteins from bacterial outer membranes: structure, function and refolding. *Current Opinion in Structural Biology* 9, 455–461.

Bucholz U., Mascola L. (2001). Transmission, pathogenesis and epidemiology of *Listeria monocytogenes*. *Infectious Diseases in Clinical Practices* 10, 34-41. In: Godfine H., Shen H. (eds) *Listeria monocytigenes*: Pathogenesis and Host response; 2007 Springer Science+Bussiness Media, LLC. pp. 13-14.

Bunka D.H.J., Stockley P.G. (2006). Aptamers come of age-at last. *Nature Reviews Microbiology* 4, 588-596.

Burch K.L., Saaed K., Sails A.D., Wright P.A. (1999). Succesful treatment by Meropenem of *Campylobacter jejuni* meningitis in a chronic alcoholic following neurosurgery. *Journal of Infection* 39, 241-243.

Burnens A.P., Nicolet J. (1992). Detection of *Campylobacter upsaliensis* in diarrheic dogs and cats, using a selective medium with cefoperazone. *American Journal of Veterinary Research* 53, 48-51.

Buzhan P., Dolgoshein B., Filatov L., Ilyin A., Kantzerov V., Kaplin V., Karakash A., Kayumov F., Klemin S., Popova E. and Smirnov S. (2003). Silicon photomultiplier and its possible applications. *Nuclear Instruments and Methods in Physics Research*. A: p. 48-52.

Carter J.E., Cimolai N. (1996). Hemolytic-uremic syndrome associated with acute *Campylobacter upsaliensis* gastroenteritis. *Nephron Journal* 74, 489.

Cecchini F., Manzano M., Mandabi Y., Perelman E., Marks R.S. (2012). Chemiluminescent DNA optical fibre sensor for *Brettanomyces bruxellensis* detection. *Journal of Biotechnology* 157, 25-30.

Chan W.C.W. and Nie S.M.. (1998). Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* 281, 2016-2018.

Chan W.C.W., Maxwell D.J., Gao X.H., Bailey R.E., Han M.Y., Nie S.M. (2002). Luminescent quantum dots for multiplexed biological detection and imaging. *Current Opinion in Biotechnology* 13, 40-46.

Chang N. and Taylor D.E. (1990) Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a SalI map of *Campylobacter jejuni* UA580. *Journal of Bacteriology* 172, 5211-5217.

Chen S.H., Wu V.C.H., Chuang Y.C., Lin C.S. (2008). Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor. *Journal of Microbiological Methods* 73, 7-17.

Christensen H., Jorgensen K., Olsen J. E. (1999). Differentiation of *Campylobacter coli* and *C. jejuni* by length and DNA sequence of the 16S-23S rRNA internal spacer region. *Microbiology* 145, 99–105.

Chu H.H., Yan J.L., Tu Y.F. (2010). Study of a luminol-based electrochemiluminescent sensor for label-free DNA sensing. *Sensors* 10, 9481-9492.

Clarridge J. E. III. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Review* 17, 840–862.

Cobley C., Chen J., Cho E. C:, Wang L.V., Xia Y. (2011). Gold nanostructures: a class of multifunctional materials for biomedical applications. *Chemical Society Rewievs* 40, 44-56.

Cocolin L., Manzano M., Cantoni C., Comi G. (1997). A PCR-microplate capture hybridization method to detect *Listeria monocytogenes* in blood. *Molecular and Cellular Probes* 11, 453-455.

Colodner R., Sakran W., Miron D. (2003). *Listeria monocytogenes* cross-contamination in a nursery. *American Journal of Infection Control* 31, 322-324.

Comi G., Ferroni P., Cocolin L., Cantoni C., Manzano, M. (1995). Detection and identification of *Campylobacter coli* and *Campylobacter jejuni* by two-step polymerase chain reaction. *Molecular Biotechnology* 3, 266-268.

Comi G., Pipan C., Botta G., Cocolin L., Cantoni C., Manzano M., (1996). A combined polymerase chain reaction and restriction endonuclease enzyme assay for discriminating between *Campylobacter coli* and *Campylobacter jejuni. FEMS Immunology Medical Microbiology* 16, 45-49.

Cossart P., Sansonetti P.J. (2004). Bacterial invasion: the paradigm of enteroinvasive pathogens. *Science* 304, 242-248.

Cova S., Ghioni M., Loitto A., Rech I., Zappa F. (2004) Evolution and prospects for single-photon avalanche diodes and quenching circuits. *Journal of Modern Optics* 51, 1267-1288.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N., Rosenbusch, J.P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* 358, 727–733.

Cunningham C., Lee C.H. (2003). Myocarditis related to *Campylobacter jejuni* infection: a case report. *BMC Infectious Diseases* 17, 16.

Dalton C.B., Austin C.C., Sobel J. (1997). An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *The New England Journal of Medicine* 336, 100.

Damborg P., Olsen K.E.P., Nielson E.M., Guardabassi L. (2004). Occurrence of *Campylobacter jejuni* in pets living with human patients infected with *C. jejuni*. *Journal of Clinical Microbiology* 42, 1363-1364.

De Felip G. (2001). Recenti sviluppi di igiene e microbiologia degli alimenti. Pp 227-228.

de la Isla A., Brostow W., Bujard B., Estevez M., Rodriguez J.R., Vargas S., Castano V.M. (2003). Nanohybrid scratch resistant coating for teeth and bone viscoelasticity manifested in tribology. *Material Research Innovation* 7, 110-114.

de Mello J.F., Einsfeldt K., Guedes Frazzon A.P., da Costa M., Frazzon J. (2007). Molecular analysis of the *iap* gene of *Listeria monocytogenes* isolated from cheeses in Rio Grande do Sul, Brazil. *Brazilian Lournal of Microbiology* 39, 169-172.

De Silva Tatley F.M., Lastovica A.J. and Steyn L.M. (1992). Plasmid profiles of *Campylobacter upsaliensis* isolated from blood cultures and stools of paediatric patients. *Journal of Medical Microbiology* 37, 8-14.

Deckert A., Valdivieso-Garcia A., Reid-Smith R., Tamblyn S., Seliske P., Irwin R., Dewey C., Boerlin P. and McEwen S.A. (2010). Prevalence and antimicrobial resistance in *Campylobacter* spp. isolated from retail chicken in two healt units in Ontario. *Journal of Food Protection* 73, 1317-1324.

Destro M.T., Serrano A.M., Kabuk D.Y. (1991). Isolation of *Listeria* species from some Brazilian meat and dairy products. *Food Control* 2 (2), 110-112.

Dhillon A.S., Shuvaprasad H.L., Schaberg D., Wier F., Weber S., Bandli D. (2006). *Campylobacter jejuni* infection in broiler chickens. *Avian Diseases* 50, 55-58.

Doose S., Neuweiler H., Sauer M. (2009). Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules. *ChemPhysChem* 10, 1389-1398.

Doumith M., Jacquest C., Gerner-Smidt P., Graves L.M., Loncarevic S., Mathisen T., Morvan A., Salcedo C., Torpdahl M., Vazquez J.A., Martin P. (2005). Multicenter validation of a multiplex PCR assay for differentiating the major *Listeria monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b: toward an international standard. *Journal of Food Protection* 68, 2648-2650.

Doyle M.P. (1984). Association of *Campylobacter jejuni* with laying hens and eggs. *Applied and Environmental Microbiology* 47, 533-536.

Dunn D.A., Lin V.H., Kochevar I.E. (1991). The role of ground-state complexation in the electron-transfer quenching of methylene-blue fluorescence by purine nucleotides. *Photochemistry and Photobiology* 53, 47-56.

Dwivedi H.P., Jaykus L.A. (2011). Detection of pathogens in foods: the current state of-the art and future directions. *Critical Reviews in Microbiology* 37, 40-63.

EFSA Journal (2005) Scientific report on *Campylobacter* in animals and foodstuffs. 173, 1-105.

EFSA Journal (2006). The community summary report on trends and sources of zoonosis, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2005. *EFSA Journal* 94, 84-288.

EFSA Journal (2007). Request for updating the former SCVPH opinion on *Listeria monocytogenes* risk related to ready-to-eat foods and scientific advice on different levels of *Listeria monocytogenes* in ready to eat foods and the related risk for human illness. *EFSA Journal* 599, 1-42.

EFSA Journal (2009). Trends and sources of zoonoses and zoonotic agents in the European Union in 2007. *EFSA Journal*, 223.

EFSA Journal (2011). Scientific report of EFSA and ECDC. The European Union summary report on antimicrobial resistance and indicator bacteria from humans, animals and food in the European Union in 2009. *EFSA Journal* 9, 2154-2475.

EFSA Journal (2012). SCIENTIFIC REPORT OF EFSA AND ECDC. The European Union summary report on trends and sources of zoonosis, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal* 10 (3), 2597.

EFSA report. (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* on broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA Journal* 8, 1503.

Emerich D.F., Thanos C.G. (2003). Nanotechnology and medicine. Expert *Opinion in Biological Therapy* 3, 665-663.

Estibaliz M., Cárcamo J., Urquijo M., Perales I., Fernández-Astorga A. (2005). Evaluation of a PCR assay for the detection and identification of *Campylobacter jejuni* and *Campylobacter coli* in retail poultry products. *Research in Microbiology* 156, 568–574.

Fakruddin M., Hossain Z., Afroz H. (2012). Prospects and applications oh nanobiotechnology: a medical perspective. *Journal of Nanobiotechnology* 10, 31.

Fan A., Lau C., Lu J. (2005). Magnetic bead-based chemiluminescent metal immunoassay with a colloidal gold label. *Analytical Chemistry* 77 (10), 3238-42.

Farber J.M., Peterkin P.I. (1991). *Listeria monocytogenes*, a food-born pathogen. *Microbiological Reviews* 55, 476-511.

Farber J.M., Peterkin P.I., Carte A.O. (1992). Neonatal listeriosis due to cross-infection confirmed by isoenzyme typing and DNA fingerprinting. *Journal of Infecteous Diseases* 163, 927-928.

Fournier C., Brons S., Taucher-Scholz G. (2003). Quantification of chemiluminescent signals using photon-sensitive films or a CCD camera. *BioTechniques* 35, 284-900.

Friendman C., Neimann J., Wegener H., Tauxe R. (2008). Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin I., Szymanski C.M., Blaser M.J. (Eds), *Campylobacter*, 4th Ed, pp. 645-665. ASM Press, Washington, DC.

Fu G.H., Cai C.X:, Li C.C., Zhang L. (2003). Controllable optical properties of Au/SiO_2 nanocomposite induced by ultrasonic irradiation and thermal annealing. *Applied Physics Letters* 83, 36.

Gandhi M., Chikindas M.L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *International Journal of Food Microbiology* 113, 1-15.

Geffroy B., le Roy P., Prat C. (2006). Review. Organic light-emitting diode (OLED) technology: materials, devices and display technologies. *Polymer International* 55, 572-582.

Giaccone V., Bertoja G. (2003) "Considerazioni tecniche sulla pericolosità, per la salute umana, di *Listeria monocytogenes*". *Il Progresso veterinario* 12, 571-578.

Gillespie I.A., O'Brien S.J., Frost J.A., Adak G.K., Horby P., Swan A.V., Painter M.J., Neal K.R. (2002). A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. *Campylobacter* sentinel surveillance scheme collaborators. *Emerging Infectious Diseases* 8, 937-942.

Giraffa G. and Carminati D. (2008). Ed. Molecular techniques in food fermentation: principles and applications. 1.

Goodman L.J., Harris A.A., Sokalski S.J., Kellie S., Barret J.E., Ruthe A., Finn A., Kaplan R.L. (1983). A restaurant-associated *Campylobacter* outbreak. *European Journal Clinical Microbiology* 2, 394 -395.

Goossens H., Vlaes L., Butzler J., et al. (1991). *Campylobacter upsaliensis* enteritis associated with canine infections. *Lancet* 337, 1486-7.

Goossens H., Vlaes L., De Boeck M. et al. (1990). Is *Campylobacter upsaliensis* an unrecognised cause of human diarrhoea? *Lancet* 335, 584-6.

Gösseringer R., Küster E., Galinier A., Deutscher J. and Hillen W. (1997). Cooperative and noncooperative DNA binding modes of catabolic control protein CcpA from *Bacillus megaterium* result from sensing two different signals. *Journal of Molecular Biology* 266, 665-676.

Graham D.L., Ferreira H.A., Felicianoa N, Freitas P.P., Clarke L., et al. (2005). Magnetic-field assisted DNA hybridisation and simultaneous detection using micron-sized spin-valve sensors and magnetic nanoparticles. *Sensors and Actuators B: Chemical* 107, 936-944.

Graham D.L., Ferreira H.A., Freitas P.P. (2004). Magnetoresistive-based biosensors and biochips. *Trends in Biotechnology* 22, 455-462.

Guerin M.T., Sir C., Sargeant J.M., Waddell L., O'Connor A.M., Wills R.W., Bailey R.H., Byrd J.A. (2010). The change in prevalence of *Campylobacter* on chicken carcasses during processing: a systematic review. *Poultry Science* 89, 1070-1084.

Guo X., Lin C.S., Chen S.H., Ye R., Wu V.C.H. (2012). A piezoelectric immunosensor for specific capture and enrichment of viable pathogens by quartz crystal microbalance sensor, followed by detection with antibody-functionalized gold nanoparticles. *Biosensensors and Bioelectronics* 38(1):177-183.

Haes A.J., Chang L., Klein W.L., Van Duyne R.P. (2005). Detection of a Biomarker for Alzheimer's disease from synthetic and clinical samples using a nanoscale optical biosensor. *Journal of the American Chemical Society* 127, 2264-2271.

Haiang L., Zhike H. (2009). Magnetic bead-based DNA hybridization assay with chemiluminescence and chemiluminescent imaging detection. *Analyst* 134, 800-804.

Hald B. and Madsen M. (1997). Healthy puppies and kittens as carrier of *Campylobacter* spp., with special reference to *Campylobacter upsaliensis*. *Journal of Clinical Microbiology* 35, 3351-2.

Hald B., Skovgard H., Bang D.D., Pedersen K., Dybdahl J., Jespersen J.B., Madsen M. (2004). Flies and *Campylobacter* infection of broiler flocks. *Emerging Infectious Diseases* 10, 1490-1492.

Hald B., Sommer H.M., Skovgard H. (2007). Use of fly screens to reduce *Campylobacter spp*. introduction in broiler houses. *Emerging Infectious Diseases* 13, 1951-1953.

Harrington C.S., On, S.L. (1999). Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *International Journal Systematic Bacteriol*ogy 49, 1171–1175.

Haynes C.L., Van Duyne R.P. (2001). Nanosphere lithography: a versatile nanofrabication tool for studies of size-dependent nanoparticle optics. *Journal of Physical Chemistry* 105, 5599-5611.

He W., Luchansky J.B. (1997). Construction of the temperature-sensitive vectors pLUCH80 and pLUCH88 for delivery of Tn917: NotI/SmaI and use of these vectors to derive a circular map of *Listeria monocytogenes* Scott A, a serotype 4b isolate. *Applied and Environmental Microbiology* 63, 3480-3487.

He Y., Yao X., Gunther IV N.W., Xie Y., Tu S.I., Shi X. (2010). Simultaneous detection and differentiation of *Campylobacter jejuni*, *C. coli*, and *C. lari* in chickens using a multiplex real-time PCR assay. *Food Analitical Methods*, DOI 10.1007/s12161-010-9136-6.

Hearty S., Leonard P., Kennedy R. (2006). Production, characterization and potential application of a novel monoclonal antibody for rapid identification of virulent *Listeria monocytogenes*. Journal *of Microbiological Methods* 66, 294-312.

Herman L., Heyndrickx M., Grijspeerdt K., Vandekerchove D., Rollier I., De Zutter L. (2003). Routes for *Campylobacter* contamination of poultry meat: epidemiological study from hatchery slaughterhouse. *Epidemiology and Infection* 131, 1169-1180.

Hermann S., Marks R. (2007). Chemiluminescent optical fiber immunosensors. Wiley Editor.

Hicks E.M., Zhang X.Y., Zou S.L. (2005). Plasmonic properties of film over nanowell surfaces fabricated by nanosphere lithography. *Journal of Physical Chemistry B* 109, 22351-22358.

Hof H., Rocourt J. (1992). Is any strain of *Listeria monocytogenes* detected in food a health risk? *International Journal of Food Microbiology* 16, 173–182.

Hopkins R.S., Olmsted R., Istre,G.R. (1984). Endemic *Campylobacter jejuni* infection in Colorado: identified risk factors. *American Journal Public Health* 74, 249–250.

Hudson J., Lake R., Savill M:, Scholes P., McCormick R. (2001). Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *Journal of Applied Microbiology* 90, 614-621.

Hulteen J.C., Treichel D.A., Smith M.T., Duval M.L., Jensen T.R., Van Duyne R.P. (1999). Nanosphere lithography: size-tunable silver nanoparticle and surface cluster arrays. *Journal of Physical Chemistry* 103, 3854-3863.

Iacumin L., Cecchini F., Manzano M., Osualdini M., Boscolo D., Orlic S., Comi G. (2009) Description of the Microflora of Sourdoughs by Culture-Dependent and Culture-Independent Methods, *Food Microbiology* 26, 128-135.

ICMSF (1996) Microorganisms in foods 5. Characteristics of microbial pathogens. Blackie Academic & Professional, London, pp. 45-65.

Inglis G.D., Kalischuk L.D. (2003). Use of PCR for direct detection of *Campylobacter species* in bovine faeces. *Applied Environmental Microbiology* 69, 3435–3447.

Jasson V., Sampers I., Botteldoorn N., Lopez-Galvez F, Baert L., Denayer S., Rajkovic A., Habib I., De Zutter L., Debevere J., Uyttendale M. (2009). Characterization of *Escherichia coli* from raw poultry in Belgium and impact on the detection of *Campylobacter jejuni* using Bolton broth. *International Journal of Food Microbiology* 135, 248-253.

Jayasena S.D. (1999). Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clinical Chemistry* 45, 1628.

Jeanteur, D., Lakey, J.H., Pattus, F. (1991). The bacterial porin superfamily: sequence alignment and structure prediction. *Molecular Microbiology* 5, 2153–2164.

Jeyaletchumi P., Tunung R., Margaret S.P., Son R., Farinazleen M.G., Cheah Y.K (2010). Detection of *Listeria monocytogenes* in foods, *International Food Research Journal* 17: 1-11.

Jia K., Bijeon J.L., Adam P.M., Ionescu R.E. (2013). Large scale fabrication of gold nano-structured substrates via high temperature annealing and their direct use for the LSPR detection of atrazine. *Plasmonics* 8, 143-151.

Jia K., Toury, T., Ionescu R.E. (2012). Fabrication of an atrazine acoustic immunosensor based on a drop-deposition procedure. *IEEE Transaction Ultrasonics, Ferroelectrics and Frequency Control* 59, 9, 2015-21.

Jiang J., Bosnik K., Maillard M., Brus L. (2003). Single molecule Raman spectroscopy at the junctions of large Ag nanocrystals. *Journal of Physical Chemistry B* 107, 9964-9972.

Jones K. (2001) Campylobacters in water, sewage and the environment. *Journal of Applied Microbiology* 90, 68S-79S.

Joseph I. (2008). Marine microbial diversity and its application in biotechnology. In *Microbial Biotechnology*. Ed. Delhi, p. 180.

Jovanovic S.V., Simic M.G. (1986). One-electron redox potentials of purines and pyrimidines. *Journal of Physical Chemistry* 90, 974-978.

Kanda V., Kariuki J.K., Harrison D.J., McDermott M.T. (2004). Label-free reading of microarray-based immunoassays with surface plasmon resonance imaging. *Analytical Chemistry* 76 (24), 7257-7262.

Kandula L., Khan S., Whitcomb D.C. and Lowe M.E. (2006). Acute pancreatitis in association with *Campylobacter jejuni*-associated diarrhea in a 15-year-old with CFTR mutations: Is there a link? *Journal of the Pancreas* 7, 482-485.

Kelly K.L:, Coronado E., Zhao L.L., Schatz G.C. (2003). The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. *The Journal of Physical Chemistry B* 107 (3), 668-677.

Kervella, M., Fauchere, J.L., Fourel, D., Pages, J.M. (1992). Immunological cross-reactivity between outer membrane pore proteins of *Campylobacter jejuni* and *Escherichia coli*. *FEMS Microbiology Letters* 78, 281–285.

Khan I. U., and Edge T. A. (2007). Development of a novel triplex PCR assay for the detection and differentiation of thermophilic species of *Campylobacter* using 16S-23S rDNA internal transcribed spacer (ITS) region. *Journal of Applied Microbiology* 103, 2561–2569.

Khati M. (2010). The future of aptamers in medicine. *Journal of Clinical Pathology* 63, 480-487.

Kim S.H., Park M.H., Kim J.Y., Chuong P.D., Lee Y.S., Yoon B.S., Hwang K.K., Lim Y.K. (2005). Development of a sandwich ELISA for the detection of *Listeria* sp. Usong specific flagella antibodies. *Journal of Veterinary Science* 6(1), 41-46.

Köhler S., Bubert A., Vogel M., and Goebel W. (1991). Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. *Journal of Bacteriology* 173, 4668-4674.

Krause R., Ramschak-Schwarzer S., Gorkiewicz G., Schnedl W.J., Feierl G., Wenisch C., Reisinger E.C. (2002). Recurrent septicaemia due to *Campylobacter fetus* and *Campylobacter lari* in an immunocompetent patient. *Infection* 30, 171–174.

Kreader C.A. (1996). Relief of amplification in PCR with bovine serum albumin or T4 gene 32 protein. *Applied and Environmental Microbiology* 62, 1102-1106.

Krishnan Y., Simmel F.C. (2011) Nucleic acid based molecular devices. *Angewandte Chemie International Edition* 50, 3124-3156.

Kulkarni S.P., Lever S., Logan J.M., Lawson A.J., Stanley J., Shafi M.S. (2002). Detection of *Campylobacter* species: a comparison of culture and polymerase chain reaction based methods. *Journal Clinical Patholology* 55, 749–753.

Labarca J.A., Sturgeon J., Borenstein L., Salem N., Harvey S.M., Lehnkering E., Reporter R., Mascola L. (2002). *Campylobacter upsaliensis*: another pathogen for consideration in the United States. *Clinical Infection Dis*ease 34, 59–60.

Lamhonwah A., Ackerley C., Onizuka R., Tiups A., Lamhonwah D., Chung C. et al. (2005). Epitope shared by functional variant of organic cation/carnitine transporter, OCTN1, *Campylobacter jejuni* and *Mycobacterium paratubercolosis* may underlie susceptibility to Crohon's disease at 5q31. *Biochemical and Biophysical Research Communications* 337, 1165-1175.

Leblanc-Maridor M., Beaudeau F., Seegers H., Denis M., Belloc C. (2011) Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiology* 11, 113.

Lehtola, M.J., Pitkanen, T., Miebach, L. and Miettinen, I.T. (2006). Survival of *Campylobacter jejuni* in potable water biofilms: a comparative study with different detection methods. *Water Science Technology* 54, 57–61.

Lei Y., Chen W., Mulchandani A., (2006). Microbial biosensors. *Analytica Chimica Acta* 568, 200–210.

Lin Z., Wang X., Li Z.J., Ren S.Q., Chen G.N., Ying X.T., Lin J.M. (2008). Development of a sensitive, rapid, biotin-streptavidin based chemiluminescent enzyme immunoassay for human thyroid stimulating hormone. *Science Direct* 75, 965-972.

Linnan M.J., Mascola L., Lou X.D. (1988). Epidemic listeriosis associated with Mexican-style cheese. *The New England Journal of Medicine* 319, 823.

Linton, D., Owen, R.J., & Stanley, J., (1996). Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter species* enteropathogenic for man and animals. *Research Microbiology* 147, 707–718.

Linton, D.A., Lawson, J., Owen, R.J., and Stanley, J., (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *Journal of Clinical Microbiology* 35, 2568–2572.

Logue C.M., Danzeisen G.T., Sherwood J.S., Thorsness J.L., Mercier B.M., Axtman J.E. (2010). Repeated therapeutic dosing selects macrolide-resistant *Campylobacter spp*. in a turkey facility. *Journal of Applied Microbiology* 109, 1379-1388.

Logue S.M., Sherwood J.S., Elijah L.M., Dockter M.R., Dockter O. (2003). The incidence of *Campylobacter spp*. on processed turkey from processing plants in the Midwestern United States. *Journal of Applied Microbiology* 95, 234-241.

Lorber B. (1997). Listeriosis. Clin. Infect. Dis. 24:1-11. In: Godfine H., Shen H. (eds) *Listeria monocytigenes*: *Pathogenesis and Host response*; 2007 Springer Science+Bussiness Media, LLC. pp.13.

Lu L., and Jun S. (2012). Evaluation of a microwire sensor functionalized to detect *Escherichia coli* bacterial cells. *Biosensors and Bioelectronics* 36, 257–261.

Luber P., Brynestad S., Topsch D., Scherer K., Bartelt E. (2006). Quantification of *Campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. *Applied and Environmental Microbiology* 72 (1), 66-70.

Lund, M., Steen , S., Pedersen, K., and Madsen, M. (2004). Detection of *Campylobacter* spp. in chicken faecal samples by Real-Time PCR. *Journal of Clinical Microbiology* 41 (11), 5125–5132.

Lynch O.A., Cagney C., McDowell D.A., Duffy G. (2010). A method for the growth and recovery of 17 species of Campylobacter and its subsequent application to inoculated beef. *Journal of Microbiological Methods* 83, 1–7.

Lynch O.A., Cagney C., McDowell D.A., Duffy G. (2011). Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol. *International Journal Food Microbiology* 150, 171–177.

Ma J., Wong H., Kong L.B., Peng K.W. (2003). Biomimetic procesing of nanocrystallite bioactive apatite coating on titanium. *Nanotechnology* 14, 619-623.

Mah C., Zolotukhin I., Fraites T.J., Dobson J., Batich C., Byrne B.J. (2000). Microsphere-mediated delivery of recombinant AAV vectors *in vitro* and *in vivo*. *Md Therapy* 1, S239.

Maher M., Finnegan C., Collins E., Ward B., Carroll C., Cormican M. (2003). Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of faeces. *Journal Clinical Microbiology* 41, 2980–2986.

Mahtab R., Rogers J.P., Murphy C.J. (1995). Protein-sized quantum dot luminescence can distinguish between “straight”, “bent”, and “linked” oligonucleotides. *Journal of the American Chemical Society* 117, 9099-9100.

Malinsky M.D., Kelly K.L., Schatz G.C., Van Duyne R.P. (2001). Chain length dependence and sensing capabilities of the localized surface plasmon resonance of silver nanoparticles chemically modified with alkanethiol self-assembled monolayers. *Journal of American Chemical Society* 123, 1471-1482.

Man S. M., Kaakoush N.O., Octavia S., Mitchell H. (2010). The Internal Transcribed Spacer Region, a New Tool for Use in Species Differentiation and Delineation of Systematic Relationships within the *Campylobacter* Genus. *Applied Environmental Microbiology* 76 (10), 3071-3081.

Manzano M., Cocolin L., Cantoni C., Comi G. (1997) Detection and identification of Listeria monocytogenes from milk and cheese by a single step PCR. *Molecular Biotechnology* 7, 85-88 ISSN: 1073-6085.

Manzano M., Cocolin L., Cantoni C., Comi G. (2003). *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* differentiation using a PCR-RE technique, *International Journal Food Microbiol*ogy 81, 249-254.

Marcello A., Sblattero D., Cioarec C., Maiuri P., Melpignano P. (2013). A deep-blue OLED-based biochip for protein microarray fluorescence detection. *Biosensors and Bioelectronics* 46, 44-47.

Marks R.S., Bassis E., Bychenko A., Levine M. (1997). Chemiluminescent optical fiber immunosensor for detecting cholera antitoxin. *Optical Engineering* 36, 3258-3264.

Marks R.S., Cullen D. Lowe C., Weetall H.H., Karube I.. *Handbook of Biosensors and Biochips*. John Wiley & Sons Ltd. 5 October 2007. chapter 2, pp. 23-39. ISBN: 978-0-470-01905-4.

Martin C.S., Bronstein I. (2005). Imaging of chemiluminescent signals with cooled CCD camera systems. *Luminescence* 9, 145-153.

Martins V.C., Cardoso F.A., Germano J., Cardoso S., Sousa L., et al. (2009). Femtomolar limit of detection with a magnetoresistive biochip. *Biosensors and Bioelectronics* 24, 2690-2695.

Marzadori F. (2004). Isolamento di *Listeria monocytogenes* in prodotti alimentari nella provincia di Ravenna. Periodo gennaio 2002 - giugno 2004. *Workshop-I Forum Interlaboratorio su Listeria monocytogenes.* 11-12 ottobre 2004.

McFarland A.D., Van Duyne R.P. (2003). Single silver nanoparticles as real-time optical sensors with zeptomole sensitivity. *Nano Letters* 3, 1057-1062.

McMillan R.A., Paavola C.D., Howard J., Chan S.L., Zaluzec N.J., Trent J.D. (2002). Ordered nanoparticle arrays formed ob engineered chaperonin protein templates. *Nature Materials* 1(4), 247-252.

Megraud F., Chevrier D., Desplaces N., Sedallian A. and Guesdon J.L. (1988) Urease-positive thermophilic *Campylobacter* (*Campylobacter laridis* variant) isolated from an appendix and from human feces. *Journal of Clinical Microbiology* 26 1050-1051.

Meinersmann, R. J., Phillips, R. W., and Ladely, S. R. (2009). Inter- and intra-genomic heterogeneity of the intervening sequence in the 23S ribosomal RNA gene of *Campylobacter jejuni* and *Campylobacter coli*. *Systematic Applied Microbiology* 32, 91–100.

Mendonca M., Conrad N.L., Conceicao F.R., Moreira A.N., da Silva W.P., Aleixo J.A.G., Bhunia A.K. (2012). Highly specific fiver optic immunosensor coupled with immunomagnetic separation for detection of low levels of *Listeria monocytogenes* and *L. ivanovii*. *BMC Microbiology* 12, 275.

Meng J. and Doyle M. (2002). Introduction. Microbiological food safety. *Microbes and Infection* 4, 395-397.

Mengard J., Ohayon H., Gounon P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* 84, 923-932.

Michalet X., Siegund O.H.W., Vallerga J.V., Jelinsky P., Millaud J.E., Weiss S. (2006) Detectors for single-molecule fluorescence imaging and spectroscopy. *Journal of Modern Optics* 54, 239-281.

Miller M.M., Sheehan P.E., Edelstein R.L., Tamanaha C.R., Zhong L., et al. (2001). A DNA array sensor utilizing magnetic microbeads and magnetoelectronic detection. *Journal of Magnetism and Magnetic Materials* 225, 238.

Molday R.S., MacKenzie D. (1982). Immunospecific ferromagnetic iron dextran reagents for the labeling and magnetic separation of cells. *Journal of Immunological Methods* 52, 352-367.

Monedero V., Gosalbes M.J., Perez-Martinez G. (1997). Catabolite repression in *Lactobacillus casei* ATCC 393 is mediated by CcpA. *Journal of Bacteriology* 179 (21), 6657-6664.

Moran L., Scates P., Madden R.H. (2009). Prevalence of *Campylobacter* spp. in raw retail poultry on sale in Northern Ireland. *Journal of Food Protection,* 72, 1830–1835.

Moreno G.S., Griffiths P.L., Connerton I.F. and Park R.W.A. (1993). Occurrence of *Campylobacter* in small domestic and laboratory animals. *Journal of Applied Bacteriolgy* 75, 49-54.

Mori M., Koga M., Yuki N., Hattori T., Kuwabara S. (2008). Bickerstaff's brainstern encephalitis after an outbreak of *Campylobacter jejuni* enteritis. *Journal of Neuroimmunology* 196, 143 – 146.

Moser, I., Schroeder, W., & Salnikow, J. (1997). *Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. *FEMS Microbiology Letters* 157, 233–238.

Mossey R.T. and Sondheimer J. (1985). Listeriosis in patients with long-term hemodialysis and transfusional iron overload. *American Journal of Medicine* 79, 397-400.

Mrksich M., Grunwell J.R., Whitesides G.M. (1995). Biospecific adsorption of carbonic-anhydrase to self-assembled monolayers of alkanethiolates that present benzenesulfonamide groups on gold. *Journal of American Chemical Society* 117, 12009-12010.

Mucic R.C., Storhoff J.J., Mirkin C.A., Letsinger R.L. (1998). DNA-direct synthesis of binary nanoparticle network materials. *Journal of American Chemical Society* 120, 12674-12675.

Murray C.B., Kagan C.R., Bawendi M.G. (2000). Synthesis and characterization of monodisperse nanocrystals and close-packed nanocrystal assemblies. *Annual Review of Materials Science* 30, 545-610.

Nakajima H., Okuma Y., Morioka K., Miyake M., Hemmi A., Tobita T., Yahiro M., Yokoyama D., Adachi C., Soh N., Nakano K., Xue S., Zeng H., Uchiyama K., Imato T. (2011). An integrated enzyme-linked immunosorbent assay system with an organic light-emitting diode and a charge-coupled device for fluorescence detection. *Journal of Separation Science* 34 (20), 2906-2912.

Nam J.M., Thaxton C.C., Mirkin C.A. (2003). Nanoparticles-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 301, 1884-1886.

Nayak M., Kotian A., Marathe S., Chakravortty D. (2009). Detection of microorganism using biosensors, a smarter way towards detection techniques. *Biosensors and Bioelectronics* 25, 661-667.

Newell D.G., Fearnley C. (2003). Sources of *Campylobacter* colonization in broiler chickens. *Applied and Environmental Microbiology* 69, 4343-4351.

Nicolò M.S., Guglielmino S.P.P. (2012). Viable but nonculturable bacteria in food. In *Public Health – Methodology, Environmental and Systems Issues*. Ed. By prof. Jay Maddock.

Nie S., Emory S.R. (1997). Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science* 275, 1102-1106.

Nieman R.E., Lorber B. (1980). Listeriosis in adults: a changing pattern. Report of eight cases and review of the literature, 1968-1978. *Reviews od Infectious Diseases* 2, 207-227.

Nuijten P.J.M., Bartels C., Bleumink-Pluym N.M.C., Gastra W., van der Zeijst B.A.M. (1990). Size and physical map of the *Campylobacter jejuni* chromosome. *Nucleic Acid Research* 18, 6211-6214.

O'Mahony, E., Buckley, J.F., Bolton, D., Whyte, P., Fanning, S. (2011). Molecular epidemiology of Campylobacter isolates from poultry production units in Southern Ireland, *Plos one* 6 (12), 1-9, e28490.

O'Leary, M.C., Harding, O., Fisher, L., Cowden, J., (2009). A continuous common-source outbreak of campylobacteriosis associated with changes to the preparation of chicken liver paté. *Epidemiology Infections* 137, 383 – 388.

Ohk S.H., Koo O.K., Sen T., Yamamoto C.M., Bhunia A.K. (2009). Antibody-aptamer functionalized fiber-optic biosensor for specific detection of *Listeria monocytogenes* from food. *Applied Microbiology* 109, 808-817.

On S.L.W. (2001) Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. *Journal of Applied Microbiology* 90, 1S-15S.

Ooi S.T., Lorber B. (2005). Gastroenteritis due to *Listeria monocytogenes*. *Clinical Infectious diseases* 40, 1327.

Page, W. J., Huyer, G., Huyer, M., Worobec, E. A. (1989). Characterization of the porins of *Campylobacter jejuni* and *Campylobacter coli* and implications for antibiotic susceptibility. *Antimicrobial Agents Chemotherapy* 33, 297–303.

Pais A., Banerjee A., Klotzkin D., Papautsky I. (2008). High-sensitivity, disposable lab-on-a-chip with thin-film organic electronics for fluorescence detection. *Lab on a Chip* 8 (5), 794-800.

Pamme N. (2006). Magnetism and microfluidics. *Lab on a Chip* 6, 24-38.

Panatarotto D., Prtidos C.D., Hoebeke J., Brown F., Kramer E., Briand J.P., Muller S., Prato M., Bianco A. (2003). Immunization with peptide-functionalized carbon nanotubes enhances virus. Specific neutralizing antibody responses. *Chemistry and Biology* 10, 961-966.

Parak W.J., Boudreau R., Gros M.L., Gerion D., Zanchet D., Micheel C.M., Williams S.C., Alvisatos A.P., Larabell C.A. (2002). Cell motility and metastatic potential studies based on quantum dot imaging of phagokinetic tracks. *Advanced Materials* 14, 882-885.

Park K.H., Yang S., Park H.G. (2009). Detection of DNA immobilization and hybridization on gold/silver nanostructures using Localized Surface Plasmon Resonance. *Journal of Nanoscience and Nanotechnology* 9, 1374-1378.

Park S.F. (2002) The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *International Journal of Food Microbiology* 74, 177-188.

Park Y.S., Lee S.R., Kim Y.J. (2006). Detection of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogens* in kimchi by multiplex polymerase chain reaction. *Journal of Microbiology* 44, 92-97.

Parkhill J., Wren B. W., Mungall K., Ketley J. M., Churcher C., Basham D., Chillingworth T., Davies R. M., Feltwell T., Holroyd S., Jagels K., Karlyshev A. V., Moule S., Pallen M. J., Penn C. W., Quail M. A., Rajandream M. A., Rutherford K. M., van Vliet A. H., Whitehead S., Barrell B. G. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature*, 403 (2000) 665–668.

Passaro, V. M. N., Dell'Olio, F., Casamassima,B., and De Leonardis, F. (2007). Guided-wave optical biosensors. *Sensors,* ISSN 1424-8220, 7, 508-536.

Pathirana S., Barbaree J., Chin B., Hartell M., Neely W., Vodyanoy V. (2000). Rapid and sensitive biosensor for *Salmonella*. *Biosensors and Bioelectronics* 15, 135-141.

Patton C.M., Shaffer N., Edmonds P., et al. (1989). Human disease associated with *Campylobacter upsaliensis* (catalase-negative or weakly positive *Campylobacter* species) in the United States. *Journal of Clinical Microbiology* 27, 66-73.

Perko-Makela P., Isohanni P., Katzav M., Lund M., Hanninen M.L., Lyhs U. (2009). A longitudinal study of *Campylobacter* distribution in turkey production chain. *Acta Veterinaria Scandinavica* 51, 18-28.

Pettersson E., Ahmadian A., Stahl P.L. (2013) A novel method for rapid hybridization of DNA to a solid support. *Plos One* 8.

Pettersson E., Stahl P.L., Mahdessian H., Käller M., Lundenberg J., et al. (2009). Visual DNA as a diagnostic tool. *Electrophoresis* 30, 3691-3695.

Pezzotti G., Serafin A., Luzzi I., Mioni R., Milan M., Perin R. (2003). Occurrence and resistance to antibiotics of *Campylobacter jejuni* and *Campylobacter coli* in animals and meat in north-eastern Italy. *International Journal of Food Microbiology* 82, 281-287.

Portner, D.C., Leuschner, R.G.K. and Murray, B.S. (2007). Optimising the viability during storage of freeze-dried cell preparations of *Campylobacter jejuni*. *Cryobiology* 54, 265–270.

Prasad, K.N., Dixit, A.K., and Ayagari, A., (2001). *Campylobacter* species associated with diarrhoea in patients from a tertiary care centre of north India. *Indian Jounal Medical Research* 114, 12–17.

Ranjit S., Levitus M. (2012). Probing the interaction between fluorophores and DNA nucleotides by fluorescence correlation spectroscopy and fluorescence quenching. *Photochemistry and Photobiology* 88, 782-791.

Rao T.V.N., Sai Sukruthi G., Raj G. (2012). Biochip Technology-A gigantic Innovation. *International Journal of Emerging Technology and Advanced Engineering* 2, 129-135.

Ren K., Liang Q., Yao B., Luo G., Wamg L., Gao Y., Wang Y., Qiu Y. (2007). Whole column fluorescence imaging on a microchip by using a programmed organic light emitting diode array as a spatial-scanning light source and a single photomultiplier tube as detector. *Lab on a Chip* 7 (11), 1574-1580.

Rollins D.M. and Colwell R.R. (1986) Viable but non culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology* 52, 531-538.

Ropkins K., Beck A. (2000). Evaluation of worldwide approaches to the use of HACCP to control food safety. *Trends in Food Science and Technology* 11, 10-21.

Ropper A.H. (1992). The Guillain-Barré syndrome. *The new England Journal of Medicine* 326, 1130-1136.

Rosef O., Rettedal G., Lageide L. (2001). Thermophilic *Campylobacters* in surface water: a potential risk of *Campylobacter*. *Internation Journal of Environmental Health Research* 11, 321-327.

Rosenquist H., Bengtsson A., Hansen T.B. (2007). A collaborative study on a Nordic standard protocol for detection and enumeration of thermotolerant *Campylobacter* in food (NMKL 119, 3. Ed., 2007). *International Journal of Food Microbiology* 118, 201-213.

Rosenquist H., Sommer H.M., Nielsen N.L., Christensen B.B. (2006). The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *International Journal of Food Microbiology* 108, 226-232.

Sahoo K.S., Labhasetwar V. (2003). Nanotech approaches to drug delivery and imaging. *DDT* 8 (24), 1112-1120.

Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A., Greenway, D. L. A. (2003). A Real-Time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Applied and Environmental Microbiology* 69, 1383-1390.

Sakate R.I., Aragon L.C., Raghiante F., Landgraf M., Franco B.D.G.M., Destro M.T. (2003). Quantificação de *Listeria monocytogenes* em salames fatiados embalados a vácuo. *Archivos Latinoamericanos de Nutritiòn* 53(2), 184-187.

Salata O.V. (2004). Applications of nanoparticles in biology and medicine. *Journal of Nanobiotechnology*.

Sammarco, M.L., Ripabelli, G., Fanelli, I., Grasso, G.M., Tamburro, M. (2010). Prevalence and biomolecular characterisation of *Campylobacter* spp. isolated from retail meat. *Journal Food Protection,* 73, 720 – 728.

Sanner M.F., Stolz M., Burkhard P., Kong X.P., Min G., Sun T.T., Driamov S., Aebi U., Stoffler D. (2005). Visualizing nature at work from the nano to the macro scale. *Nanobiotechnology* 1, 7-21.

Schnerr H.G. Food and beverage applications of biosensors technologies. In *Handbook of Biosensors and Biochips*, Marks R., Cullen D.C., Karube I., Lowe C.R., Weetall H.H., Editors, 2007. p. 1191-1193.

Schroder, W., Moser, I. (1997). Primary structure analysis and adhesion studies on the major outer membrane protein of *Campylobacter jejuni*. *FEMS Microbiology Letters* 150, 141–147.

Schuchat A., Swaminathan B., Broome C.V. (1991). Epidemiology of human listeriosis. Clinical Microbiology Rev. 1991; 4:169-183. In: Godfine H., Shen H. (eds) *Listeria monocytigenes*: Pathogenesis and Host response; 2007 Springer Science+Bussiness Media, LLC. pp. 14.*of Physical Chemistry* 100, 5541-5553.

Seong G.H., Crooks R.M. (2002). Efficient mixing and reactions within microfluidic channels using microbead-supported catalyst. *Journal of the American Chemical Society* 124, 13360.

Sepùlveda B., Angelomé P.C., Lechuga L.M:, Liz-Marzàn L.M. (2009). LSPR based nanobiosensors. *Nano Today* 4 (3), 244-251.

Setterington E., Alocilja E. (2011). Rapid electrochemical detection of polyaniline-labeled *Escherichia coli* O157:H7. *Biosensors and Bioelectronics* 26, 2208-2214.

Shane S.M., Montrose M.S. (1985). The occurrence and significance of *Campylobacter jejuni* in man and animals. *Veterinary Research Communications* 9, 167- 198.

Shi L., Perkins R.G., Fang H., Tong W. (2008). Reprodicible and reliable microarray results through quality control: good laboratory proficiency and appropriate data analysis practices are essentials. *Current Opinion in Biotechnology* 19, 10-18.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs P.A., and Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Frontiers in Microbiology, Food Microbiology* 2, 200, 1-12.

Singh, H., Rathore, R.S., Singh, S., Singh Cheema, P. (2011). Comparative analysis of cultural isolation and PCR based assay for detection of *Campylobacter jejuni* in food and faecal samples, *Brazilian Journal of Microbiology* 42, 181-186.

Skjerve E., Rorvik L.M., Olsvik O. (1990). Detection of *Listeria monocytogenes* in foods by immunomagnetic separation. *Applied and Environmental Microbiology* 56, 3478-3481.

Smith, K., N. Reimers, H. J. Barnes, B. C. Lee, R. Siletzky, and S. Kathariou, (2004). *Campylobacter* colonization of sibling turkey flocks reared under different management conditions. *Journal of Food Protection* 67, 1463–1468.

Stahl P.L., Gantelius J., Natanaelsson C., Ahmadian A., Andersson-Svahn H., et al. (2007). Visual DNA-identification of DNA sequence variations by bead trapping. *Genomics* 90, 741-745.

Stanley J., Jones C., Burnens A. and Owen R.J. (1994). Distinct genotypes of human and canine isolates of *Campylobacter upsaliensis* determined by 16S rRNA gene typing and plasmid profiling. *Journal of Clinical Microbiology* 32, 1788-94.

Stevens K.A., Jaykus L.A. (2004). Bacterial separation and concentration from complex sample matrices: a review. *Critical Reviews in Microbiology* 30, 7-24.

Stuyver L.V., Whitaker T., McBrayer T.R., Hernandez-Santiago B.I., Lostia S., Tharnish P.M., Ramesh M., Chu C.K., Jordan R., Shi J., Rachakonda S., Watanabe K.A., Otto M.J. and Schinazi R.F. (2003). Ribonucleoside analogue that blocks replication of bovine viral diarrhea and hepatitis C viruses in culture. *Antimicrobial Agents and Chemioterapy* 47(1), 244-254.

Suh S.H., Jaykus L.A. (2013). Nucleic acid aptamers for capture and detection of *Listeria* spp. *Journal of Biotechnology* 167, 454-461.

Sun J., DuFort C., Daniel M.C., Murali A., Chen C., Gopinath K., Stein B., De M., Rotello V.M., Holzenburg A. (2007). Core-controlled polymorphism in virus-like particles. *Proceedings of the National Accademy of Sciences* 104(4), 1354-1359.

Suo B., He Y., Paoli G., Gehring A., Tu S., Shi X. (2010). Development of an oligonucleotide-based microarray to detect multiple foodborne pathogens, *Molecular and Cellular Probes* 24, 77-86.

Suzanne S., *Food Analysis* (2010). Suzanne S., Editor. p. 5.

Swaminathan B., Gerner-Smidt P. (2007). The epidemioloy of human listeriosis. *Microbes and infection* 9, 1236-1243.

Takahashi M., Koga M., Yokoyama K., Yuki N. (2005). Epidemiology of *Campylobacter jejuni* isolated from patients with Guillain – Barré and Fisher syndromes in Japan. *Jounal Clinical Microbiology* 43, 335 – 339.

Taton T.A. (2002). Nanostructures as tailored biological probes. *Trends in Biotechnology* 20, 277-279.

Thevenot D.R., Toth K., Durst R.A., Wilson G.S. (2001). Electrochemical biosensors: recommended definitions and classifications. *Biosensors and Bioelectronics* 16, 121-131.

Torimura M., Kurata S., Yamada K., Yokomaku T., Kamagata Y., Kanagawa T., Kurane R. (2001). Fluorescence-quenching phenomenon by pohotoinduced electron transfer between a fluorescent dye and a nucleotide base. *Analytical Sciences*17, 155-160.

Trindade P.A., McCulloch J.A., Oliveira G.A. and Mamizuka E.M. (2003). Molecular techniques for MRSA typing: current issues and perspectives. *The Brazilian Journal of Infectious Diseases* 7 (1), 32-43.

Turner A.P. (2000). Biosensors: sense and sensitivity. *Science* 290, 1315-1317.

Uyttendaele M., Van Hoorde I., Debevere J. (2000). The use of immune-magnetic separation (IMS) as a tool in a sample preparation method for direct detection of *L. monocytogenes* in cheese. *Internation Journal of food Microbiology* 54, 205-212.

Van Camp, G., Van de Peer, Y., Nicolai, S., Neefs, J.-M., Vandamme, P. and De Wachter, R. (1993). Structure of 16S and 23S ribosomal RNA genes in *Campylobacter* species: phylogenetic analysis of the genus *Campylobacter* and presence of internal transcribed spacers. *Systematic Applied Microbiology* 16, 361–368.

van Vliet A.H.M. and Ketley J.M. (2001) Pathogenesis of enteric *Campylobacter* infection. *Journal of Applied Microbiology* 90, 45S-56S.

Vandamme, P. (2000). Taxonomy of the family Campylobacteriaceae. 3–27. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*. American Society for Microbiology Press, Washington, D.C.

Vasquez-Boland J.A., Dominguez L., Fernandez J.F., Rodriguez-Ferri E.F., Briones V., Blanco M., Suarez G. (1990). Revision of the validity of CAMP tests for *Listeria* identification. Proposal of an alternative method for the determination of haemolytic activity by *Listeria* strains. *Acta Microbiologica Hungarica* 37(2), 201-206.

Vaughan R.D., O'Sullivan C.K., Guibault G.G. (2001). Development of a quartz crystal microbalance (QCM) immunosensor for the detection of *Listeria monocytogenes*. *Enzyme and Microbial technology* 29, 635-638.

Voedsel en Warent Autoriteit, Ministerie van Landbouw, Natur en Voedselkwaliteit. 2010. Revision ISO 10272 parts 1 and 2. Ad Hoc Group *Campylobacter* ISO/CEN, Uppsala, October 2010.

von Both U., Otten S., Darbouche A., Domann E., Chakraborty T. (1999). Physical and genetic map of *Listeria monocytogenes* EGD serotype 1/2a chromosome. *FEMS Microbiology Letters* 175, 281-289.

Walker D.C., Aoyama K., Klaenhammer T.R. (1996). Electrotransformation of *Lactobacillus acidophilus* group A1. *FEMS Letters* 138, 233-237.

Wang R.F., Cao W.W., Johnson M.G. (1991). Development of a 16S rRNA-based oligomer probe specific for *Listeria monocytogenes*. *Applied and Environmental Microbiology* 57(12), 3666-3670.

Wang S., Mamedova N., Kotov N.A., Chen W., Studer J. (2002). Antigen/antibody immunocomplex from CdTe nanoparticle bioconjugates. *Nano Letters* 2, 817-822.

Wassenaar T. M. (1997). Toxin production by *Campylobacter* spp. *Clinical Microbiology Reviews*, 10 466-476.

Weissleder R., Elizondo G., Wittenburg J., Rabito C.A., Bengelr H.H., Josephson L. (1990). Ultrasmall supermagnetic iron oxide: characterization of a new class of contrast agents for MR imaging. *Radiology* 175, 489-493.

Wempe J.M, Genigeorgis C.A., Farver T.B., Yusufu H.L. (1983). Prevalence of *Campylobacter jejuni* in two California chicken processing plants. *Applied and Environmental Microbiology* 45, 355-359.

Werno A.M., Klena J.D., Shaw G.M., Murdoch D.R.. (2002). Fatal case of *Campylobacter lari* prosthetic knee infection and bacteraemia in an immunocompetent patient. *Journal Clinical Microbiology* 40, 1053–1055.

Whisstock, J.C., and. Lesk A.M. (1999). SH3 domains in prokaryotes. *Trends in Biochemical Science* 24:132-133.

Whitesides G.M. (2003). The "Right" Size in Nanobiotechnology. *Nature Biotechnology* 21, 1161-1165.

Willis W.L., Murray C. (1997). *Campylobacter jejuni* seasonal recovery observations of retail market broilers. *Poultry Science* 76, 314-317.

Wing E.J., Gregory S.H. (2002). *Listeria monocytogenes*: Clinical and experimental update. J. Infect. Dis. 185 (Suppl.): S18-S24. In: Godfine H., Shen H. (eds.) *Listeria monocytigenes*: Pathogenesis and Host response; 2007 Springer Science+Bussiness Media, LLC. pp. 13.

Woese, C. R. (1987). Bacterial evolution. *Microbiology Review*, 51, 221–271.

Wuenscher, M.D., Köhler S., Bubert A., Gerike U., and Goebel W. (1993). The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. *Journal of Bacteriology* 175, 3491-3501.

Yamamoto Y. (2002). PCR in diagnosis of infection. Detection of bacteria in cerebrospinal fluids. *Clinical and Diagnostic Laboratory Immunology* 9(3), 508-514.

Yamazaki-Matsune W., Taguchi M., Seto K., Kawahara R., Kawatsu K., Kumeda Y., Kitazato M., Nukina M., Misawa N. and Tsukamoto T. (2007). Development of a multiplex PCR assay for identification of *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis*. *Journal of Medical Microbiology* 56, 1467–1473.

Yang C., Jiang Y., Huang K., Zhu C., Yin Y. (2003). Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS mmunology and Medical Microbiology* 38, 265-271.

Yao B., Luo G., Wamg L., Gao Y., Lei G., Ren K., Chen L., Wang Y., Hu Y., Qiu Y. (2005). A microfluidic device using a green organic light emitting diode as an integrated excitation source. *Lab on a Chip* 5 (10), 1041-1047.

Yao T., Wasa T., Musha S. (1977). Linear-sweep voltammetry and simultaneous determination of purine-bases and their nucleosides in glassy carbon electrode. *Bulletin of the Chemical Society of Japan* 50, 2917-2920.

Yonzon C.R., Jeoung E., Zou S., Schatz G.C., Mrkisch M., Van Duyne R.P. (2004). A comparative analysis of localized and propagating surface plasmon resonance sensors: the biding of concanavalin A to a monosaccharide functionalized self-assembled monolayer. *Journal of American Chemical Society* 126, 12669-12676.

Yoshida J., Kobayashi T. (1999). Intracellular hyperthermia for cancer using magnetite cationic liposomes. *Journal of Magnetism and Magnetic Materials* 194, 176-184.

Young K., Davis L.M., Di Rita J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nature Review Microbiology* 5, 665-679.

Zanoli L.M., D'Agata R., Spoto G. (2012). Functionalized gold nanoparticles for ultrasensitive DNA detection. *Analytical and Bioanalytical Chemistry* 402, 1759-1771.

Zhang D.Y., Seelig G. (2011). Dynamic DNA nanotechnology using strand-displacement reactions. *Nature Chemistry* 3, 103-113.

Zhang Q., Meitzler J.C., Huang S., Morishita T. (2000). Sequence polymorphism, predicted secondary structures, and surface-exposed conformational epitopes of *Campylobacter* major outer membrane protein. *Infection and Immunity* 68, 5679-5689.

Zhang X.Y., Young M.A., Lyandres O., Van Duyne R.P. (2005). Rapid detection of an anthrax biomarker by surfaces-enhanced Raman spectroscopy. *Journal of American Chemical Society* 127, 4484-4489.

Zhao J., Zhang X., Yonzon C.R., H A.J., Van Duyne R.P. (2006). Localized surface plasmon resonance biosensors. *Nanomedicine* 1(2), 219-228.

Software

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acid Research* 16 (22), 10881–10890.

Jullien, N., AmplifX 1.6.2; CNRS, Aix-Marseille Université (2013) http://crn2m.univ-mrs.fr/pub/amplifx-dist.

WEB Sites

http://blast.ncbi.nlm.gov/Blast.cgi

Nanosphere Inc. (2004). Available at http://www.nanosphere-inc.com

Acknowledgements

Four years, including three as a doctoral student … it seemed a very long time, and yet I'm already writing the thanks on the last page of my thesis, it seems incredible. In these years I worked and shared my life with great people, friends, family and colleagues.

So…at the end of my third universitary adventure, the first person that I would like to thank is my tutor, prof. Marisa Manzano, who gave me the opportunity to work and learn from her. She helped me a lot organizing my projects and with the final draft of my Ph.D. thesis. I think that fate has decided that I had to work with her…same day of birth…same degree in Natural Sciencies. I had no choice.

I would like to tank prof. Elena Rodica Ionescu; she introduced me in the (for me completely new) world of biosensors and for the period of 5 months she gave me the chance to perform all my LSPR experiments in the laboratory of Nanotechnology and Instrumental Optics (LNIO) at the "Université de Technologie de Troyes" (UTT) in France.

Thank you to dr. Patrizia Melpignano for the help and for making available the laboratory of "Organska Elektronika d.o.o." (OR-EL) at the Primorski Tehnoloski Park (PTP) in Vrtojba (Slovenia) to perform the OLED experiments.

A special thanks to dr. Francesco Fontana (director of the microbioly section of Azienda Servizi Sanitari n. 2, Ospedale San Polo, Monfalcone, Italy) for introducing me to the world of microbiology five years ago and for having kindly donated all the Campylobacter strains from patients with campylobacteriosis and the media to perform some analysis.

And…prof. Giuseppe Comi and dr. Lucilla Iacumin (Lucilla)…a dear thought to you also, in those years we have not had the opportunity to work together very often, but I'll keep always a wonderful memory of you.

Thanks to the reviewers, prof. Robert S. Marks and dr. Anna Reale for all the suggestions to improve my Ph.D. dissertation.

A special thanks to Kun (now Ph.D. Kun Jia) for the friendship and for the patience in explaining me all the new techniques that I used for my experiments in France. Even…I will not ever forget all the fantastic dinners that we made all together (you, Priya, Nan, and I), Hot Pot and Sicchuan pepper will always be in my mind. And…Priya, how to forget all breakfasts, dinners and night walks to downtown during all those months in Troyes? Thanks a lot for the friendship.

Thanks to my parents, Rosa Maria (Maria Rosa) and Giuliano (Giuly), they supported me throughout all those years and especially you, mum, what would we do without your delicious cakes?

A special place in my life is reserved to my "pregiati" colleagues, my lab (now called "boratorio", right Vi?) family: Marco (Vi or Moccou), Romina (Romini or Rumenia), Federica (Ginalda or Fredrica), Francesca (Franceschi), Debbie and…the newcomers Jelena and Lucia. There are too many wonderful memories of those four years together (within and outside the walls of the laboratory, all of you aren't only collegues but also wonderful friends.

And now…Sara (Divina), Alessandro (Ale), Fabio (Hg), Claudia and…Marco (Vi) again… you are not only part of my life but you are my life…how many adventures and how many nights spent together doing all the things that make us inseparable friends. I really thank you for all.

I wish all the best to all the person that I mentioned here, without you all my life wouldn't be the same.

Marco (To)