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ADVANCES IN METHODS TO DETECT, ISOLATE AND QUANTIFY FOODBORNE PATHOGENS

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ABSTRACT

Foodborne diseases impact human health and economies worldwide in terms of health care and productivity loss. Prevention is necessary and methods to detect, isolate and quantify foodborne pathogens play a fundamental role, changing continuously to face microorganisms and food production evolution.

Official methods are mainly based on microorganisms growth in different media and their isolation on selective agars followed by confirmation of presumptive colonies through biochemical and serological test. A complete identification requires form 7 to 10 days.

Over the last decades, new molecular techniques based on antibodies and nucleic acids allow a more accurate typing and a faster detection and quantification. The present thesis aims to apply molecular techniques to improve official methods performances regarding two pathogens: Shiga-like Toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes*.

In 2011, a new strain of STEC belonging to the serogroup O104 provoked a large outbreak. Therefore, the development of a method to detect and isolate STEC O104 is demanded.

The first objective of this work is the detection, isolation and identification of STEC O104 in sprouts artificially contaminated. Multiplex PCR assays and antibodies anti-O104 incorporated in reagents for immunomagnetic separation and latex agglutination were employed. Contamination levels of less than 1 CFU/g were detected. Multiplex PCR assays permitted a rapid screening of enriched food samples and identification of isolated colonies. Immunomagnetic separation and latex agglutination allowed a high sensitivity and rapid identification of O104 antigen, respectively.

The development of a rapid method to detect and quantify *Listeria monocytogenes*, a high-risk pathogen, is the second objective. Detection of 1 CFU/ml and quantification of 10–1,000 CFU/ml in raw milk were achieved by a sample pretreatment step and quantitative PCR in about 3h. *L. monocytogenes* growth in raw milk was also evaluated.

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ABBREVIATIONS

ACV	Acriflavin, Cefsulodin and Vancomycin
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
BHI	Brain Heart Infusion
CAC	Codex Alimentarius Commission
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
Conc.	Concentration
C_T	Threshold Cycle
CTRL	Negative Control
DAEC	Diffusely Adherent *E. coli*
DALY	Disability–Adjusted Life Year
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDA	Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network
FSIS	Food Safety and Inspection Service

HACCP	Hazard Analysis Critical Control Point
HS	HotSHOT
HUS	Haemolytic-Uraemic Syndrome
IMS	Immunomagnetic Separation
INSEE	Institut national de la statistique et des études économiques
ISO	International Organization for Standardization
L. monocytogenes	*Listeria monocytogenes*
mBPWp	modified Buffered Peptone Water with pyruvate
mRBA	modified Rainbow Agar O157
MPN	Most Probable Number
NACMCF	National Advisory Committee on Microbiological Criteria for Food
PBST	Phosphate Buffered Saline solution supplemented with Tween 20
PCR	Polymerase Chain Reaction
PS	Physiological Saline solution
SPC	Standard Plate Count
spp	various species
Std. Dev.	Standard Deviation
STEC	Shiga-like toxin producing *E. coli*
TESSy	The European Surveillance System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
USDA	United State Department of Agriculture

VTEC	Verocitotoxigenic *E. coli*
WHO	World Health Organization

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CHAPTER 1: General Introduction

Food is essential for living and despite the improvements in food hygiene and preserving technologies, foodborne outbreaks continue to occur worldwide. Globalization has led to a more intensive and centralized food production and a wider distribution of raw materials and food products promoting foodborne pathogens diffusion. Note that microorganisms continue evolution and changing in food habits have also contributed.

Illnesses associated to food and water consumption are frequent and even if the rate of hospitalization and deaths are low, the economic cost of health care and productivity losses are onerous. The estimation of burden of foodborne illnesses is complex because there are several pathogens, transmission routes are different and data are not homogeneous among the countries.

Foodborne pathogens are potentially everywhere and their infection is perpetuated by oral transmission associated with contaminated food consumption and distribution in the environment through feces. Consequently, it is necessary to control food products to prevent their contamination.

Competent authorities have defined microbiological criteria to establish the acceptability of food products and its production and distribution processes. Moreover, they provide official methods to detect and quantify specific microorganisms or their products. These methods are generally based on

microbiological cultures in several media to isolate single colonies on selective agars. Then, presumptive colonies are confirmed by biochemical and serological tests. It is widely accepted that official methods are time-consuming and demanding, in fact from 7 to 10 days are necessary for the complete identification of the target microorganism.

The advent of new molecular techniques based on antibodies or nucleic acids permitted a more accurate subtyping and a faster detection and quantification of microorganism or their products. These new techniques can support official methods and may substitute them in the future.

1.1 Objectives

The present thesis aims to improve methods for determination, isolation and quantification of foodborne pathogens. Two microorganisms were studied: 1) Shiga-like Toxin producing *Escherichia coli* (STEC), in particular the serogrup O104; and 2) *Listeria monocytogenes* (*L. monocytogenes*).

STEC O104 was considered a minor pathogen until 2011 when new recombinant strain provoked a large outbreak mainly located in Germany (Robert Koch institute, 2011). Official methods to identify this specific strain along with the serogroup O104 are under development. Regarding this pathogen, this work pursues to test the methods available for STEC, improve their sensitivity detecting the strain responsible of the German outbreak and other general STEC O104. Results will aid to define new official methods.

L. monocytogenes is a well-known pathogen, which has low incidence, but persistent, provoking severe symptoms that may lead to death. Therefore, it is very important to prevent its diffusion. The second objective of the thesis is to develop a rapid method for detection and quantification of *L. monocytogenes* in raw milk through quantitative Polymerase Chain Reaction (PCR).

The major impact of this objective relies in the ability to provide results very quickly, as it is possible to remove contaminated products before they enter in the market. Sensitivity and specificity have to be comparable to official methods. Furthermore, knowledge about level of *L. monocytogenes* contaminations is very useful to compare different farming methods or food processes.

CHAPTER 2: Literature Review

2.1 Emergency of foodborne pathogens

Foodborne illnesses appear to be a never-ending issue, which impact human health and economies worldwide. Food is fundamental for living; its increasing demand due to an expanding world population and globalization introduces new challenges to the food safety field. Over the last decades, new farming and cultivation methodologies were developed, achieving a more intensive and centralized food production. Moreover, new preserving and packing technologies allow for extended food shelf-life and distribution. Consequently, nowadays food production requires the use of machinery and more workers, which increases the chances for contamination. Raw materials are often imported from other countries allowing a worldwide diffusion of foodborne pathogens. Furthermore, people life-style changed during this period. For instance, the number of people eating in restaurants and the consumption of ready-to-eat products have increased, augmenting the risk to contract foodborne diseases (Nyachuba, 2010).

These diseases can be asymptomatic or provoke gastrointestinal symptoms that can also degenerate to serious life threatening forms or even death. Pathogens that can be transmitted with food include bacteria, viruses, fungi, parasites and prions. Together with chemicals, their appearance can potentially occur anytime, from food production to its consumption at home.

The quantification of the number of illnesses caused by foodborne pathogens is a very difficult task. Foodborne pathogens are a large and

heterogeneous group of causative agents that can also be transmitted by different routes, like direct contact with animals or indirect contact through a non-food vehicle. Surveillance is based on laboratory results. Thus, it means that a sick person has to look for medical care, a doctor has to send the specimen to analyze, the laboratory has to identify the pathogen, and then, the disease has to be reported to the competent health authority. Any interruptions of this chain of events result in an underestimation of the number of illnesses caused by foodborne pathogens due to underreporting. Additionally, certain countries do not monitor outbreaks properly; in developing countries, food dynamics and their role as transmission routes of foodborne diseases are not well understood (Flint et al., 2005).

In 2006, the World Health Organization (WHO) expressed the need of filling these gaps and proposed to estimate the global burden of foodborne disease in terms of incidence, morbidity and Disability–Adjusted Life Year (DALY); a parameter that includes the values of years spent in disability or lost due to premature death (WHO, 2006).

Despite the lacking of standard methods and the data heterogeneity among the foodborne pathogens spectrum, many studies were performed. In general, data from different surveillance programs were compared to calculate pathogens-specific multipliers in order to harmonize data derived from active, passive and outbreak surveillance reducing the number of under-diagnosed and under-reported cases. When laboratory-based surveillance was not available, usually the number of illnesses was evaluated from a scale down of the entire population through incidence data.

Table 2.1 – Evaluation of number of cases of domestically acquired foodborne illnesses

Country; Study Period	Population size[a]	Number of pathogens			Major pathogens (Cases per year)	Unspecified pathogens (Cases per year)	Hospitalizations (per year)	Deaths (per year)	Reference
		B	P	V					
Europe									
England and Wales; 1996–2000	52,700,000	18	3	5	885,171	839,144	99,597	602	Adak et al. (2005)
France; 1997–2000[b]	60,185,831	13	8	2	239,000–269,000[c]	N.A.	10,200–17,800[c]	228–691[c]	Vaillant et al. (2005)
Greece [d]; 1996–2006	N.A.	9	5	1	N.A.	369,305/million inhabitants	905/million inhabitants	3.1/million inhabitants	Gkogka et al. (2011)
Netherlands; 1998–2009	16,500,000	7	3	5	680,000	4,780,000	N.A.	78	Havelaar et al., (2012)
Oceania									
Australia; 2001–2002	18,972,350	11	2	3	1,480,000	N.A.	3,640	76	Hall et al. (2005)
New Zealand; 2000–2009	4,320,000	16	3	5	557,542	1,368,421	4,279	17	Cressey & Lake (2011)
North America									
Canada; 2000–2010	32,500,000	19	5	6	1,630,636	2,400,000	N.A.	N.A.	Thomas et al. (2013)
USA; 2000–2008	299,000,000	21	5	5	9,388,075	38,400,000	55,961	1,351	Scallan et al. (2011a; 2011b)

Note: B – Bacteria, P – Parasite, V – viruses; N.A. – Not Available

[a] Data were taken from the references. For England and Wales, France and Australia data were not available, therefore were obtained from their national office of statistic relative to the year 1999, 1999 and 2001, respectively (ABS, 2002; Horsfield, 2000; INSEE, no date)

[b] For rare diseases the period was extended by 5 years.

[c] Depending on the data sources, high and low estimates were evaluated and presented as “plausible interval”

[d] Data reported per million inhabitants. Information includes major and unspecified pathogens.

Table 2.1 summarizes the most up to date findings from studies that evaluated the number of cases of foodborne illnesses distributed by country. Care should be taken when doing comparisons because the information provided differs by source, methods of inference, period of time and number of foodborne pathogens considered. The annual number of cases from illnesses due to the known pathogens associated to food consumption ranged from 239,000 to 9,388,075 (Table 2.1). Greece was not considered because unspecified pathogens were also included (Gkogka et al., 2011). *Campylobacter* various species (spp) was always in the top three pathogens responsible for foodborne illnesses, except for Netherlands. England and Wales, Netherlands, New Zealand, Canada and USA provided also data on the number of domestic cases caused by undefined foodborne pathogens. Their amount ranged from 839,144 to 38,400,000, which is almost 1 to 7-fold higher than the reported cases for the major pathogens (Table 2.1). Considering population size, Greece reported the highest number of cases (369,305 cases per million of inhabitants), but is understandable since major and unspecified pathogens were considered together and gastroenteritis were not limited to foodborne pathogens.

Despite the high incidence, the number of hospitalizations and deaths are low. In general, less then 1% of cases were severe enough to require a hospital visit and less than 0.26 % lead to death. Only France and England and Wales, presented higher hospitalization rates, resulting in 4.3%–6.6% and 11%, respectively. Regarding undefined pathogens, hospitalization and death rates are similar than for known pathogens.

Among the studies collected in Table 2.1, Gkogka et al. (2011) and Havelaar et al. (2012) were the only ones to introduce the DALY parameter. DALY estimates a different aspect of the foodborne pathogens burden, because considers not only the frequency, but also the severity of the disease outcome. An example is the increase in importance for toxoplasmosis and listeriosis. This observation is far from surprising as, in the case of listeriosis, most of the studies showed the highest fatality rates, ranging from 15% to 35% (Adak et al., 2005; Cressey & Lake, 2011; Gkogka et al., 2011; Scallan et al., 2011a; Vaillant et al., 2005); excluding 5% in Netherlands (Havelaar et al., 2012).

Burden of foodborne illnesses can also be considered from an economic point of view. Robert Scharf (2012) estimated the losses of productivity, cost in medical care and illness-related mortality, for USA, using the data collected by Scallan et al. (2011a; 2011b). According to the author, USA estimated expenditure per year was 51.0 billion dollars resulting in 1,068 dollars per foodborne illness case. The annual cost rises up to 77.7 billion dollars, when loss of quality life is added to the model.

2.1.1 Europe situation

Although passive surveillance underestimates the burden of foodborne illnesses to the society, it is a powerful tool when doing risk management of these diseases and helping competent authorities to locate resources. The European Center for Disease Prevention and Control (ECDC) is the agency that coordinates the surveillance of foodborne diseases together with all the

other infective diseases in the European Union. Starting in 2007, ECDC reports in the European Surveillance System (TESSy), all the human cases of communicable disease for every state member including Norway and Iceland. Every year, according to the directive 2003/99/EC (European Commission, 2003), the European Food Safety Authority (EFSA) uses TESSy results to report about zoonosis, zoonotic agents and foodborne outbreaks. Switzerland reports its data directly to EFSA. The last published report estimated 5,648 food-borne outbreaks, including of 7,125 hospitalizations and 93 deaths, occurred in 2011 (EFSA & ECDC, 2013). Note that the previously stated values reported by EFSA are not cases but 'foodborne outbreaks', which comprise two or more cases of similar illness associated with the consumption of the same food to be regarded as an outbreak (European Commission, 2003). From 2008, the first reported year, until 2011 the number of food-borne outbreaks remained constant ranging from 5,276 to 5,648 (EFSA & ECDC, 2010; 2011; 2012; 2013).

Table 2. 2 – Human cases in Europe of the main foodborne pathogens in 2011 (EFSA & ECDC, 2013)

Foodborne pathogen	Europe[a]		Italy	
	reported	confirmed	reported	confirmed
Campylobacter spp[b]	235,836	231,301	468	468
Salmonella spp	100,532	98,183	3,344	3,344
STEC[c]	9,743	9,605	69	51
Yersinia spp[d]	7,128	7,077	15	15
Listeria spp[e]	1,553	1,545	83	83
Echinococcus spp[f]	786	784	-	-
Trichinella spp[g]	363	268	6	6
Brucella spp[h]	362	340	21	21
Micobacterium bovis[i]	147	147	11	11
Total	356,450	349,250	4,017	4,017

Note: spp – various species

[a] Twenty-seven European country members plus Iceland, Norway and Switzerland.

[b] No surveillance reports available for Greece and Portugal.

[c] No surveillance reports available for Portugal.

[d] No surveillance reports available for Iceland, Greece, Netherlands, Portugal and Switzerland.

[e] No surveillance reports available for Portugal.

[f] No surveillance reports available for Denmark, Iceland and Italy.

[g] No surveillance reports available for Denmark and Iceland.

[h] No surveillance reports available for Denmark.

[i] No surveillance reports available for France.

Table 2.2 lists all the human cases (including sporadic cases) occurred in Europe in 2011, divided by pathogen. Rabies was not considered because its transmission is not related to food or water consumption. A total number of 356,450 cases were reported, among them, 349,250 were confirmed and 4,017 were located in Italy. In 2011, *Campylobacter* spp and *Salmonella* spp resulted to be the main foodborne pathogens in Europe, being responsible of 94% of the human cases reported by EFSA (EFSA & ECDC, 2013). *Campylobacter* spp alone was 66% of the total reported cases and this number has increased since 2008, probably due to higher awareness for the pathogen and surveillance improvements. On the contrary, *Salmonella* spp human cases in 2011 are 5.4% less than in 2010; and a statistically significant negative trend was observed from 2008 to 2011. Probably the mandatory programs of control in egg poultry houses helped to achieve this result.

Campylobacter spp and *Salmonella* spp are gram-negative bacteria that provoke gastro-intestinal disease in humans. Their reservoirs are the alimentary tract of wild and domestic animals; hence contamination is not limited to animal produce, as it has been already seen in the case of vegetables (EFSA & ECDC, 2013).

Among the pathogens reported by EFSA in 2011, special attention should be given to STEC and *Listeria* spp, as they will be further examined during this thesis.

STEC is a group of *Escherichia coli* (*E. coli*) that produce at least one of the verocytotoxins also known as Shiga toxins. These toxins, together with other virulence factors, give the bacteria the ability to provoke enteric diseases such as bloody diarrhea that can degenerate in Haemolytic-Uraemic Syndrome (HUS). In 2011, the number of confirmed human cases resulted to be 159.4% more than the previous year due to a large outbreak caused by a recombinant *E. coli* belonging to the O104:H4 serogroup (EFSA & ECDC, 2013; Rasko et al., 2011; Rohde et al., 2011). Despite the spike on number of cases during 2011, human cases of STEC have a statistical significant positive trend from 2008 to 2011 (EFSA & ECDC, 2013).

All the cases of listeriosis are mainly provoked by the specie *monocytogenes*. Despite its low incidence and the lack of a specific trend during 2008 to 2011, this pathogen has the highest rate of fatality. In 2011, EFSA reported that 12.7% of the total of confirmed cases were deaths caused by listeriosis (134 out of 1,054 cases, when information about hospitalization and/or death was available). As a matter of fact, *L. monocytogenes* can provoke severe life-

threatening symptoms. Although direct and zoonotic transmissions are possible, the main cause of infection is the consumption of contaminated food.

Active monitoring of foodborne pathogens along with their studies, has strongly contributed to prevent and control foodborne illnesses. Even though before 1900, *Clostridium botulinum*, *Salmonella typhi* and toxigenic *Vibrio cholerae* were considered important foodborne pathogens, in 1997, they accounted for only 0.01% of the cases of foodborne diseases (Tauxe, 2002). Unfortunately, even if many advances in food safety were achieved, foodborne illnesses will continue to be a burden for society. Microorganisms can evolve very quickly and new pathogens will always emerge as noted by the outbreak of *E. coli* O104:H4 in Germany in 2011.

2.2 Techniques to detect, isolate and quantify foodborne pathogens

Diseases transmitted by food and water consumption are noteworthy and wide distributed all over the world. Foodborne pathogens are mainly zoonotic agents and they are potentially everywhere. The infection occurs by oral transmission and the host spreads the causative agents in the environment through its feces (Jay et al., 2005).

Domestic animals can infect one another in the farm. Contaminated feed, tools, operators and wild animals can also transmit the infection, being the last two both active and passive vehicles. These types of contamination may take place during any food production processes. In particular, the removal of the intestinal package during the slaughtering is a critical point (EFSA, 2013a).

Food producers, processors, packagers and distributors play an important role to avoid contaminations; hence, it is not enough to control just the final product. Therefore, these factors must be considered in order to prevent the spreading of foodborne diseases. This new concept of prevention was introduced in the late 1990s, when the Codex Alimentarius Commission (CAC) and the National Advisory Committee on Microbiological Criteria for Food (NACMCF) defined the principles for Hazard Analysis Critical Control Point (HACCP) (CAC, 1997; NACMCF, 1998). These principles guided food business operators to create an internal plan to find and monitor any critical point present in its production.

The HACCP system was used in 2004 by the European Commission (EC) to write the regulations 852, 853, 854 and 882 (European Commission, 2004a;

2004b; 2004c; 2004d) concerning hygiene of foodstuff; in particular for food of animal origins and its control. A year later, official microbiological criteria were established in the Regulation EC 2073 (European Commission, 2005) and modified over the years to integrate the HACCP system as powerful tools for control (European Commission, 2007; 2010; 2011; 2013a; 2013b). A microbiological criterion is defined as:

"*acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number of microorganisms, and/or on the quantity of their toxins/ metabolites, per unit(s) of mass, volume, area or batch"* (European Commission, 2005).

Microbiological criteria can be evaluated for pathogens or bacterial indicators of food quality during food product shelf-life or its production. Every criterion contains: 1) the target organism or its products; 2) the limit of tolerance that has to be verified; 3) the official method used for detection and/or quantification of the target organism or its products; 4) a sampling plan; and 5) the required number of compliant samples out of the total.

Official analytical methods are validated standard protocols (or they equivalents). In Europe, standards are provided by the International Organization for Standardization (ISO) and in the USA by the Food and Drug Administration (FDA) and the United State Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS).

2.2.1 Methods

During the history of food microbiology, different types of methods have been discovered and developed. Food safety requires fast detection and quantification techniques to achieve results before the food product enters in the market. Moreover, false-positive and false-negative results should be minimized and the cost of the control should not be overwhelming for the food business operator. Unfortunately, pathogens detection and quantification is very challenging and methods that accomplish all these requirements are still not available. Food matrixes have very different composition and background flora can be very high, especially in raw food. Moreover, pathogens can be stressed during food processing and their level of contamination be generally low and non-homogeneously distributed (Feng, 2007).

2.2.1.1 Conventional methods

Conventional cultural methods are based on cultivating certain foodborne pathogens using specific media. Basically, qualitative methods follow 4 steps: 1) food sample preparation by blending or homogenization; 2) pre-enrichment and/or selective enrichment; 3) isolation onto solid selective media; and 4) identification through several tests mainly of biochemical and serological nature. Quantitative cultural methods are also possible through Standard Plate Count (SPC) or Most Probable Number (MPN) techniques. In the first approach, serial dilutions are performed from prepared food sample and plated onto solid selective media. Then, Colony Forming Units (CFUs) are counted on one or more plates and the concentration is calculated considering the volume of inoculum and the dilution factor used. For MPN,

the sample is prepared in the same way as before, but more dilution replicates are done. Subsequently, each dilution tube undergoes enrichment, isolation and identification steps as done for qualitative methods. Ultimately, based on the combination of positive and negative tubes and their dilution factor, it is possible to calculate the most probable number of pathogen present in the sample (Anonymous, 2007; USDA, FSIS, 2014).

Biochemical tests for identification generally entail the cultivation of the isolated microorganism in different media. A variety of media types are available depending on the content of different substrates, such as carbohydrates or amino acids together with pH indicators. If the presumptive pathogen is able to grow, the pH would change modifying the media color. Other tests such as immunoagglutination and haemagglutination can be used to identify the serotype or toxins and confirm haemolysis ability (Jay et al., 2005).

Conventional cultural methods are widely used and they are the gold standard for detection and quantification of foodborne pathogens. These methods are characterized by laborious protocols and the need of bacterial growth, requiring approximately 7-10 days for pathogen identification. Moreover, quantitative methods may underestimate the number of pathogens as stress due to food processing or other environmental factors may cause damage making them not able to grow in selective media (Velusamy et al., 2010).

Other conventional methods like dye-reduction and microscope based enumeration methods are used on food. Their variability is very high due to dye distribution among single cells or clumps and dye metabolism from different types of cells. Due to their low specificity, they cannot be used for pathogens detection or quantification, but since they are fast simple and inexpensive, they are useful for microbial quality evaluation of raw milk and dairy product (Jay et al., 2005).

2.2.1.2 Advances in conventional cultural methods

In the last years, conventional cultural methods have been improved in order to achieve better performances, increase the work efficiency, and face the advent of new foodborne pathogen.

The demand to examine many samples at the same time encouraged industrials to produce instruments to automatize and improve many processes. Solid food samples can be pummeled by a stomacher or beaten by a pulsifier to avoid homogenization and facilitate the entrance of microorganisms into the initial suspension. Gravimetric-diluters and spiral plater apparatus are used for automatized dilution and plating, respectively. Also, software for image analysis have been developed to count CFUs on a plate pictures (Mandal et al., 2011).

Concentrations techniques such as filtration or Immunomagnetic Separation (IMS) can be applied before or after the enrichment step. Different kinds of

filters are commercially available and, depending of the pore size, they can trap or not bacteria.

IMS is a technique based on the association between magnetic beads and antibodies, which are able to bind specifically to one pathogen. Briefly, beads covered with specific antibodies are added to the enriched food sample in order to let the antibodies hybridize with antigens. Subsequently, the complexes beads–antibodies–pathogens are collected using a magnet, washed with saline solution and plated onto a solid selective media.

Filters and IMS are able to concentrate bacteria in a small volume, separating them from undesired substances present in the food matrixes or enrichment media. Therefore, these techniques increase the performance of cultural methods (Dwivedi & Jaykus, 2011).

Regarding pathogens isolation, the continue advent of new pathogens and advances in knowledge of bacteria metabolism permitted the development of new cultural media. For instance, the addition of specific substrates into the plates, which are metabolized in different ways by different microorganisms, lead to the formation of chromogenic, fluorogenic and luminogenic products. These compounds are visible on the plate facilitating pathogen identification (Orenga et al., 2009). Furthermore, ready-to-use kits based on SPC or MPN are available. Despite to the cost, they not require reagents preparation and are easier to perform (Jasson et al., 2010).

For the same advantages, commercial kits are also available for identification tests. Small biochemical kits containing a battery of different media provide

the confirmation of presumptive colonies in 16-24 h (Gracias & McKillip, 2004). Similarly, kits based on antigen-antibody reaction like latex agglutination provide the identification of a serogroup in real time.

Nucleic acids based techniques allow a more accurate microorganism typing and support conventional cultural methods. Although they are powerful tools for food outbreak investigations, these methods lack of standardized protocols (EFSA, 2013b).

2.2.1.3 Alternative methods for detection and quantification of foodborne pathogens

Among the immunological methods the most important is the Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is commonly used in diagnostic and biomedical research and it has been also applied for pathogens and toxins detection in food. ELISA uses antibodies to both capture specific antigens and bind enzymes (usually alkaline phosphatase or glucose oxidase) to them. When the substrate for the enzyme is provided, the product can be quantified through a spectrophotometer. If different dilutions of food samples are tested, ELISA can be quantitative (Jay et al., 2005). Besides its antibody specificity and the fast speed, ELISA lacks of sensitivity 10^3–10^5 CFU/ml for bacterial cells when used directly on food (Mandal et al., 2011).

Polymerase Chain Reaction (PCR) was developed in 1971 (Kleppe et al., 1971) and significantly improved in 1988 (Saiki et al., 1988; Stoflet et al., 1988). PCR is a technique that allows the amplification of a specific nucleic

acid sequence (template). The target sequence can be chosen arbitrarily designing specific oligomers (primers) that bind before and after it. A thermocycler changes repetitively the temperature up, to denaturate the double–stranded DNA, and down, to let the primers hybridize. When this happen, a thermo resistant DNA polymerase recognizes the primers and starts the replication of the template. Theoretically, this technique is able to recognize 1 single copy of the target sequence in the reaction mix and amplify it millions of times in a few hours, making the product visible in an agarose gel electrophoresis. Moreover, if the template sequence is specifically associated to determinate pathogens, it is possible to detect their presence in DNA extracted from food.

The potential of molecular techniques was already clear in the late 1990s but the presence of inhibitors in food matrixes made their application very difficult. Also, it is no possible to distinguish between the DNA extracted from dead or live cells (Feng, 1997). The addition of fluorogenic dyes into the reaction mix and the association of an optical detector to the thermocycler allowed to observe the amplification in real-time during all the cycles of the reaction. Therefore, real-time PCR assays do not necessitate of any sort of post-amplification analyses reducing the work time and the risk of contamination by previous amplified products. Using dyes that bind unspecifically DNA duplex, it is possible to run melting curves and check unwanted amplification. Fluorescent dyes can also be connected to an oligonucleotide that binds specifically to the template. In this way, more dyes can be used at the same time and a real-time multiplex PCR can be performed. Furthermore, these techniques allow for DNA quantification. In fact, the higher the amount of

DNA template in the reaction, the lower are the number of cycles (C_T) needed to produce enough fluorescence to exceed the threshold and becoming visible by the detector. Using the C_T values of DNA standards with known concentration, it is possible to create curves and quantify unknown samples. Real-time PCR is very sensitive, but its results depend on the quality of extracted DNA. As a matter of fact, it is very effective in food when it is preceded by an enrichment step (Hanna et al. 2005).

Biochemical methods based on measurement of impedance or adenosine triphosphate bioluminescence are able to enumerate microorganisms in food. However, considering their sensitivity and specificity, they are not appropriated for foodborne pathogens detection and quantification (Jasson et al., 2010).

Other techniques such as microarrays and biosensors are very interesting in terms of sensitivity and specificity. Potentially, they are powerful alternative methods, however the requirement of very expensive instrumentations limits their diffusion.

Microarray assays, through the hybridization of several DNA fragments incorporated into a chip, permit to detect a large number of target sequences simultaneously. This technique is generally used for typization of isolated microorganism, like *Salmonella* (Wattiau et al., 2008) or *E. coli* (Bugarel et al. 2010b); however, it is affected by all the relative issues of DNA extraction

from food matrixes. In the future, microarrays could be able to detect multiple pathogens at the same time (Jasson et al., 2010).

Biosensors are a new group of multidisciplinary techniques that use a bioreceptor to detect the target molecules and transduce them in a second signal. This signal is detected or quantified by an appropriate device. The most used bioreceptors are antibodies, enzymes and nucleic acids that produce optical, electrochemical or mass variations effects. Biosensors permit a rapid detection and could be the foundation to create compact devices for *in situ* analysis (Velusamy et al., 2010).

CHAPTER 3: Advances in method for Detecting and Isolating Shiga Toxin-Producing Escherichia coli O104 in sprouts

3.1 BACKGROUND

Escherichia coli are rod-shape, gram-negative bacteria, prevalent in the intestine flora of warm-blooded animals. Several *E. coli* are considered human pathogens, infecting the respiratory, urinary and intestinal tract. Special attention is given to diarrheagenic *E. coli* because they can be transmitted by consumption of contaminated food or water (CDC, 2012). These pathogens are divided in groups based on their mucosal colonization and pathogenic strategies: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004; Nataro & Kaper, 1998). Moreover, *E. coli* are usually identified by serotyping of the somatic antigens “O” and flagellar antigens “H” following the *Salmonella* scheme (Kauffmann, 1947).

EHEC is the most concerning group due to the severity of their outcome, provoking bloody diarrhea and hemolytic uremic syndrome (HUS), which may lead to death (Nataro & Kaper, 1998). EHEC is also called Verocitotoxigenic *E. coli* (VTEC) or Shiga-like Toxin producing *E. coli* (STEC) because it exhibits a cytotoxic effect in Vero cells as a consequence of Shiga toxin

production. For the purpose of this works, the author will continue using the name STEC.

E. coli O157:H7 is considered the major serotype involved in STEC outbreaks, but also many non-O157 serotype play an important role in human infections, requiring all STEC related diseases to be communicable (European Commission, 2003; Gould et al., 2013). During the following decade, the Foodborne Diseases Active Surveillance Network (FoodNet) collected data on STEC infections and in an analysis by Gould et al., (2013) it was reported that *E. coli* O157:H7 was isolated from 74% of the total 7695 human cases of STEC in USA. The remaining cases were mainly provoked by the serogroups O26, O45, O103, O111, O121 and O145 (Gould et al., 2013). Moreover, STEC O91, O113 and O104 have also been described as human pathogens (Bettelheim, 2007; Bielaszewska et al., 2011; Brooks et al., 2005; CDC, 1995).

In the past, the serogroup O104 was responsible for a small outbreak of 11 people in USA, 15 sporadic cases in Europe and 1 case in South Korea. Since the incidence rate was very low and HUS was described only in 4 cases, it was considered a minor STEC pathogen (ECDC & EFSA, 2011).
The serogroup O104 became relevant in 2011, when a strain of *E. coli* O104:H4 gave rise to a large outbreak mainly located in Germany. The infection was transmitted by sprouted fenugreek seeds and provoked bloody diarrhea in 3842 people, HUS in 855 and death in 50 (Robert Koch Institute, 2011). The pathogen was immediately characterized through multiplex real-time PCR assay, revealing that it possessed virulence genes associated to

both STEC (*stx*$_2$, *iha*, *lpf*$_{O26}$, *lpf*$_{O113}$) and EAEC (*aggA*, *aggR*, *set1*, *pic*, *aap*) groups (Bielaszewska et al., 2011). When the genome was sequenced, it was clear that *E. coli* O104:H4 is an EAEC which had acquired *stx*$_2$ gene and other antibiotic-resistance factors by a prophage-mediated transmission, becoming an enteroaggregative STEC (Rasko et al., 2011; Rohde et al., 2011).

Consumers diet changed among the last decades. Currently, eating fresh fruits and vegetables, including sprouts has become more common and the production of ready-to-eat food has augmented. This has exposed people to new risk of contracting foodborne illnesses (Berger et al., 2010; Taormina et al., 1999). The aggregative STEC O104:H4 outbreak could be an example of this trend. STEC are generally maintained in the environment through cattle reservoirs, which could contaminate the water that is used for cultivation (Gyles, 2007).

Seeds are full of nutrients and during the sprouting process they are incubated in a warm humid place. Consequently, this food matrix is particularly keen to bacterial growth; high level of background flora especially coliforms are troublesome during detection and isolation of STEC pathogens (Weagant & Bound, 2001). Weagant and Bound (Weagant & Bound, 2001) evaluated different selective media for sprouts enrichment and described that modified Buffered Peptone Water with pyruvate (mBPWp) supplemented with Acriflavin, Cefsulodin and Vancomycin (ACV) was very effective against sprouts background flora during the isolation of *E. coli* O157:H7.

Subsequently, FDA recommends, in the Bacteriological Analytical Manual the enrichment in mBPWp + ACV for detecting *E. coli* O157 (Feng et al., 2010). Moreover, Jinneman et al. (2012) successfully detected approximately 1 CFU/g of two STEC O104 strains in sprouts.

IMS can increase the sensitivity of cultural methods in difficult food matrixes (Weagant et al., 2011) and it is currently used in the ISO method to detect STEC O157:H7 (Anonymous, 2001).

In this work, novel IMS and latex agglutination reagents based on antibodies that recognize the antigen O104 were evaluated to isolate and identify two strains of STEC O104 in artificially contaminated sprouts. Furthermore, two multiplex real-time PCR assays with an internal positive control were developed based on the virulence genes profile of the enteroaggregative STEC O104 isolated during the German outbreak and a general STEC O104. Differently to *E. coli* O157:H7, STEC O104 strains do not present intimin (encoded by *eae* gene), but similarly to STEC O91 and O113 they usually produce enterohemolysin (encoded by *ehxA* gene) (Feng et al., 2001; Rump et al., 2012). The assay designed for enteroaggregative STEC O104 targeted stx_2 gene, responsible of the Shiga toxin 2 production, *aggR* gene, involved in the AAF fimbriae assembly, and wzy_{104}, which is associated to the O104 antigen. Meanwhile, STEC O104 real-time PCR assay targeted $stx_{1\text{-}2}$, *ehxA* (encoding for enterohemolysin), and wzx_{104}. The developed assays were tested for detection of enteroaggregative STEC O104 and STEC O104 in sprouts after enrichment and for confirmation of presumptive colonies isolated on selective agars.

3.2 METHODS AND MATERIALS

3.2.1 Bacterial strains and inoculum preparation

Two strains of STEC belonging to the serogroup O104 were used to artificially contaminate sprouts samples. Enteroaggregative STEC O104:H4 2011C-3493, provided by the Center for Disease Control and Prevention, was isolated from a U.S. traveler patient with HUS associated with the German outbreak in 2011. STEC O104:H7 RM9387 was isolated from cattle by Robert Mandrell (USDA, Agricultural Research Service, Western Regional Research Center in Albany California, USA).

STEC O104:H4 and O104:H7 were stocked in Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) with 20% glycerol at -80°C. The stock cultures, were scratched with a sterile loop to transfer part of the frozen culture in TSB, and incubated overnight at 37°C. Then, *E. coli* strains cultures were maintained in Tryptic Soy Agar (TSA; Becton, Dickinson and Company) plates.

The inoculum was prepared by picking a well-separated colony with a sterile loop and dissolving it into 10 ml of TSB. After an incubation at 37°C for 18 h, the grown culture was ten fold diluted with sterile 0.1% peptone water (Becton, Dickinson and Company). Volumes ranging from 1 to 3 ml of the 10^{-8} or 10^{-7} dilution were added to every sprouts sample in order contaminate them at two different levels of approximately 10 and 100 CFU in 25 g. A SPC

was performed to quantify the number of cells added to the samples, spreading the inoculum on TSA plates and incubating overnight at 37°C.

3.2.2 Enrichment and Immunomagnetic separation

Alfalfa and dill sprouts were obtained from a local market and stored at 4°C in their original package. In every experiment, samples of 25g were prepared and artificially contaminated as described above. Negative controls of uninoculated sprouts were also prepared. All the samples were stressed at 4°C for 48h and then BAM method enrichment was followed (Feng et al., 2010). First, a non selective pre-enrichment was performed adding 225 ml of mBPWp (Acumedia, Neogen Corporation, Lansing, Michigan), blending for 30s in a Stomacher Lab-Blender 400 (Seward Laboratory System) and incubating at 37°C for 5h. Second, a selective enrichment was carried out adding 10 mg/l of acriflavin hydrochloride (Sigma Aldrich, St. Louis, MO, USA), 10 mg/l of cefsulodin sodium salt (A. G. Scientific, Inc., San Diego, CA, USA) and 8 mg/l vancomycin hydrochloride (Sigma Aldrich) and incubating at 42°C for 18h. After the enrichment, samples were concentrated by IMS and screened by multiplex real-time PCR assay.

For IMS concentration, 1 ml of enriched sprouts sample broth was transferred to a 1.5 ml microtube and 20 µl of *E. coli* O104 IMS beads (Abraxis, Warminster, PA, USA) were added. The vial was incubated at room temperature for 10 min with continuous agitation by inversion and placed in a magnetic rack provided by Abraxis in order to trap all the beads. After 3 min, the supernatant was discarded and the beads were rinsed twice with 1 ml of

0.01 M Phosphate Buffered Saline solution supplemented with Tween 20 (PBST). Note that PBST solution was prepared by dissolving one tablet provided by Sigma Aldrich in 500 ml of autoclaved deionized water (0.138 M NaCl, 0.0027 M KCl, 0.05% Tween 20, and pH 7.4) and stored at 4°C.

Subsequently, the beads were resuspended in 100 µl of PBST, vortexed and placed on two solid selective media. A swab was used to spread the beads suspension (~50 µl) over one half of the plate and a sterile loop was used to streak the suspension over the last two quadrants to obtain isolated colonies. Samples contaminated with enteroaggregative STEC O104:H4 were plated onto modified Rainbow Agar O157 (mRBA) and CHROMagar STEC O104 (CHROMagar, Paris, France), while samples contaminated with STEC104:H7 were plated onto mRBA and CHROMagar STEC (CHROMagar). Rainbow Agar O157 (Biolog, Hayward, CA, USA) was modified according to (Tillman et al., 2012) adding 0.05 mg/l cefixime trihydrate (Sigma Aldrich), 5 mg/l novobiocin sodium salt (Sigma Aldrich) and 0.15 mg/l potassium tellurite hydrate (Sigma Aldrich). In order to let the bacteria grow, selective agars were placed in the incubator at 37°C for 24 h.

3.2.3 DNA extraction methods

DNA from enriched sprouts sample broth was extracted from 750 µl using PrepSEQ Rapid Spin Sample Preparation kit (Applied Biosystem, Foster City, CA, USA) following the manufacturer's instructions. The extracted DNA was used for the multiplex real-time PCR assay adding 2.9 µl in the PCR reaction mixture and storing the remaining DNA at -20 °C.

DNA extraction from presumptive colonies isolated on a solid selective agar, was performed by picking a well separated colony with a sterile loop, dissolving it in 100 µl of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA) and incubating it in a heat-block at 99°C for 10 min. After centrifugation at 12,000 g for 1 min, 1 µl of supernatant was used in the PCR reaction mixture.

3.2.4 Multiplex real-time PCR assays

Two multiplex real-time PCR assays were used to detect enteroaggregative STEC O104 and STEC O104 in enriched samples and to identify the presumptive isolated colonies. Both assays were performed with an Internal Positive Control by means of TaqMan® Exogenous Internal Positive Control Reagents (VIC Probe) kit (Life Technologies, Austin, TX, USA) and optimized with the 7500 Fast Dx Real-time PCR thermocycler (Applied Biosystem). Enteroaggregative STEC O104 assay was used to detect *E. coli* O104:H4, targeting the genes: stx_2, wzx_{104} and *aggR*. Additionally, STEC O104 assay was used to detect *E. coli* O104:H7, targeting the genes: $stx_{1\text{-}2}$, wzx_{104} and *ehxA*. Sequences and concentrations of primers and probes for both assays are described in Tables 3.1 and 3.2.

The PCR reaction mixture contained 1X TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 1X Exo IPC Mix, 1X Exo IPC DNA, and 2.9 µl of DNA template in 25 µl total volume. PCR cycling conditions included 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and 59°C for 1 min with standard temperature ramps.

Sensitivity of multiplex real-time PCR assay was evaluated as explained below. Complete enrichment of an uninoculated sprouts sample was performed and 675 µl of the enriched culture were transferred into 8 microtubes. Additionally, 8 microtubes containing 675 µl of clean mBPWp + ACV were prepared. For both strains, an overnight culture was decimally diluted in Peptone Water 0.1%. From each dilution tube, 75 µl were used to inoculate one microtube containing the enriched culture and one with mBPWp + ACV. Ultimately, microtubes of 10-fold increasing concentration (from 10^0 to 10^7 CFU/ml) for each strain were prepared.

DNA was extracted from the whole volume through PrepSEQ Rapid Spin Sample Preparation kit (Applied Biosystem) as explained previously and analyzed by multiplex real-time PCR.

Table 3. 1 – Primers and probes of enteroaggregative STEC O104 multiplex PCR assay

	Sequence[a]	**Conc. (µM)**	**Reference**
stx $_{1/2}$ F	5'-TTT GTY ACT GTS ACA GCW GAA GCY TTA CG-3'	0.1	
stx $_{1/2}$ R	5'-CCC CAG TTC ARW GTR AGR TCM ACR TC-3'	0.1	Wasilenko et al. (2012)
stx_2 P	6FAM/5'-TCG TCA GGC /ZEN/ ACT GTC TGA AAC TGC TCC -3'/IAbkFQ	0.2	
aggR-333 F	5'-CAG CGA TAC ATT AAG ACG CCT AAA G-3'	1	
aggR-448 R	5'-CGT CAG CAT CAG CTA CAA TTA TTC C-3'	1	Hidaka et al. (2009) dyes and probe modified
aggR P	6TAMN/5'-AGA TGC TTG CAG TTG TCC GAA TTG GTC[b]-3'/BHQ_2	0.2	
wzx$_{O104}$ F	5'-TGT CGC GCA AAG AAT TTC AAC-3'	1	
wzx$_{O104}$ R	5'-AAA ATC CTT TAA ACT ATA CGC CC-3'	1	Bugarel et al. (2010b) dyes modified
wzx$_{O104}$ P	Cy5/5'-TTG GTT TTT TTG TAT TAG CAA TAA GTG GTG TC-3'/BHQ_2	0.2	

Note: F – Forward primer, R – Reverse primer, P – Probes;

[a] Legend for degenerate nucleotides: Y (C,T), W (A,T), R (A,G), M (A,C), S (C,G).

[b] The underlined bases were added to the probe designed by Hidaka et al. (2009) in order to increase the melting temperature to be similar to other probes used in the multiplex PCR assay. Also the dyes were modified.

Table 3. 2 – Primers and probes of STEC O104 multiplex PCR assay

	Sequence[a]	Conc. (µM)	Reference
stx $_{1/2}$ F	5'-TTT GTY ACT GTS ACA GCW GAA GCY TTA CG-3'	0.1	Wasilenko et al., 2012
stx $_{1/2}$ R	5'-CCC CAG TTC ARW GTR AGR TCM ACR TC-3'	0.1	
stx_1 P	6FAM/5'-CTG GAT GAT /ZEN/ CTC AGT GGG CGT TCT TAT GTA A-3'/IAbkFQ	0.2	
stx_2 P	6FAM/5'-TCG TCA GGC /ZEN/ ACT GTC TGA AAC TGC TCC-3'/IAbkFQ	0.2	
ehxA F	5'-GTG TCA GTA GGG AAG CGA ACA-3'	1.25	Bugarel et al. (2010a) dyes modified
ehxA R	5'-ATC ATG TTT TCC GCC AAT G-3'	1.25	
ehxA P	6TAMN/ 5'-CGT GAT TTT GAA TTC AGA ACC GGT GG-3'/BHQ_2	0.2	
wzx_{O104} F	5'-TGT CGC GCA AAG AAT TTC AAC-3'	1.25	Bugarel et al. (2010b) dyes modified
wzx_{O104} R	5'-AAA ATC CTT TAA ACT ATA CGC CC-3'	1.25	
wzx_{O104} P	Cy5/5'-TTG GTT TTT TTG TAT TAG CAA TAA GTG GTG TC-3'/BHQ_2	0.2	

Note: F – Forward primer, R – Reverse primer, P – Probes;
[a] Legend for degenerate nucleotides: Y (C,T), W (A,T), R (A,G), M (A,C), S (C,G).

3.2.5 Identification of presumptive colonies

STEC forms mauve colonies in mRBA and both CHROMagar plates. Therefore, for every sprout sample a mauve colony was taken from each solid selective media and tested by multiplex real-time PCR and *E. coli* O104:H4 Latex Test Kit (Product No. 541060; Abraxis, Warminster, PA, USA) following manufacturer's instructions.

3.3 RESULTS AND DISCUSSION

The purpose of this work was to develop and evaluate tools using molecular techniques to improve the ability of detection an isolation of STEC O104. Abraxis LLC has recently created IMS and latex agglutination reagents based on antibodies that bind specifically to the antigen O104; they are used to increase the recovery and rapidly identify the serogroup O104 from isolated colonies, respectively. Moreover, two multiplex real-time PCR assays that detect a set of three genes associated with enteroaggregative STEC O104 and STEC O104, were developed and applied to screen the enriched sprouts samples and identify presumptive colonies.

Even though Jinneman et al. (2012) were able to detect STEC O104 using BPWp + ACV, they noticed that the high level of background flora present in sprouts interfered with pathogen detection. Hence, high sample dilution was necessary to recover well-separated colonies in most of the selective agar tested. Conversely, IMS was described to specifically concentrate STEC

O157:H7 from enriched sprouts (Weagant et al., 2011). Therefore, since IMS reagents for O104 are recently commercially available, a better isolation rate for STEC O104 was expected.

3.3.1 Screening by multiplex real-time PCR assay

Sprouts background flora was quantified before and after the stress treatment of 48h at 4°C by aerobic plate count using TSB or mBPWp for the initial suspension. Samples were diluted in 0.1% peptone water and plated on TSA. Level of flora after selective enrichment was also evaluated and results are listed in Table 5. The number of aerobic bacteria in sprouts was very high (approximately 10^7) and it was consistent after the cold treatment. Additionally, background flora was able to grow during the enrichment despite of the selective factors (Table 3.3).

Table 3. 3 – Sprouts background flora

Sample condition	**Initial suspension**	**Aerobic plate count**[a] (CFU/g)	
		Mean	**Std. Dev.**
None	TSB	1.10×10^7	3.97×10^6
Cold stressed (4°C for 48h)	mBPWp	1.08×10^7	2.22×10^6
Selective enrichment	mBPWp+ACV	1.38×10^8	7.37×10^7

Note: TSB – Tryptic Soy Broth; mBPWp – modified Buffered Peptone Water with pyruvate; ACV – Acriflavin, Cefsulodin and Vancomycin.
[a] Mean and standard deviation are calculated on four replicates.

Sprouts samples were artificially contaminated at ~10 CFU/25g and ~100 CFU/25g, cold stressed and subjected to enrichment. The sample enriched broth was screened by PCR assays and plated onto two selective agars after IMS.

As shown in Table 3.4, samples with high level of contamination (ranging from 43 to 160 CFU/25g) were detected by multiplex real-time PCR and mauve presumptive colonies were recovered. Samples with low level of contamination (below 24 CFU/25g) were also positive, except for a few inoculated samples, which resulted negative by multiplex real-time PCR and no presumptive colonies were isolated in all the selective agars tested (Table 6). Particularly, samples contaminated with enteroaggregative STEC O104:H4 at the levels of 14, 13 and 6 CFU/25g resulted negative in 1/5, 2/2 and 1/3 samples, respectively. Also, for sprouts inoculated with STEC O104:H7 at the levels of 22, 9 and 6 CFU/25g, only 3/4, 1/3 and 1/3 resulted negative, respectively.

The reason why these samples gave negative results is ambiguous since others with lower contamination levels such as 4 CFU/25g were screened positively and mauve colonies were isolated. Probably the cold stress may have damaged the cells of interest allowing the background flora to overgrown them during the enrichment. Moreover, some samples may have not received any cell from the inoculum since the level of pathogens was very low and pipetting errors may have also occurred.

A similar finding was reported by Tzschoppe et al. (2012) where 3 strains of STEC were not detected in salad samples with low level of contamination (1–

10 CFU/g) and stored at less than 6°C for 72h. Additionally, it was reported that salad background flora is able to grow at low temperature.

Multiplex real-time PCR assays included the internal positive control kit provided by Life Technologies, which resulted positive for all contaminated samples and controls. Therefore, negative results are not expected to occur due to possible presence of PCR inhibitors. The detection limit of the PCR assays for enteroaggregative STEC O104 and STEC O104 were $\leq 10^3$ CFU/ml and $\leq 10^4$ CFU/ml, respectively (Table 3.5). However, some genes were also amplified at lower dilutions. The presence of background flora in the enrichment did not seem to interfere with the sensitivity, obtaining similar performance. The author recommends repeating the experiment several times to better assess the detection limits. PCR assays designed by Fratamico et al. (2011) and Hidaka et al. (2009) have shown comparable detection limits ranging from 7×10^2 to 1.1×10^4 CFU/ml.

Moreover, Tzochoppe et al. (2012) designed a protocol for rapid detection of EHEC including enteroaggative STEC O104:H4. The method was tested on a ready-to-eat salad and entailed a 6h enrichment step followed by different real-time PCR assays. As the authors reported *E. coli* O104:H4 grew of up to 5.8×10^4, thus multiplex real-time PCR assay designed in this work could be applied after the short enrichment step (Tzschoppe et al., 2012).

Table 3. 4 – Positive sprouts samples to multiplex real-time screening and isolation of presumptive colonies

Contamination level		*E. coli* strain	Multiplex real-time PCR screening[a]	Isolation of presumptive colonies [a]
CFU/g	25 g			
3.6	90	Enteroaggregative STEC O104:H4	1/1	1/1
2.7	68		2/2	2/2
2.4	60		1/1	1/1
1.8	45		2/2	2/2
0.76	19		3/3	3/3
0.56	14		4/5	4/5
0.52	13		0/2	0/2
0.36	9		4/4	4/4
0.24	6		2/3	2/3
0.16	4		3/3	3/3
6.4	160	STEC O104:H7	1/1	1/1
4.4	110		2/2	2/2
3.8	95		2/2	2/2
3.2	80		1/1	1/1
1.72	43		3/3	3/3
0.96	24		5/5	5/5
0.88	22		1/4	1/4
0.52	13		2/2	2/2
0.36	9		2/3	2/3
0.24	6		2/3	2/3
0	0	CTRL	0/5	0/5

Note: CTRL – Negative Control

[a] Number of positive samples out of number of total sample enrichments tested for every experimental group

Table 3. 5 – Multiplex real-time PCR assays sensitivity

Enteroaggregative STEC O104		**Bacteria target concentration** (CFU/ml)							
Matrix	**Gene**	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
mBPWp+ACV	*stx_2*	-	-	-	+	+	+	+	+
	aggR	-	+	+	+	+	+	+	+
	wzx	-	-	+	+	+	+	+	+
sprouts[a]	*stx_2*	-	-	-	+	-	+	+	+
	aggR	-	+	+	+	+	+	+	+
	wzx	-	-	+	+	+	+	+	+
STEC O104									
Matrix	**Gene**								
mBPWp+ACV	*stx_{1-2}*	-	+	+	+	+	+	+	+
	ehxA	-	-	-	+	+	+	+	+
	wzx	-	-	+	+	+	+	+	+
sprouts[a]	*stx_{1-2}*	-	-	-	-	+	+	+	+
	ehxA	-	-	-	-	+	+	+	+
	wzx	-	-	-	+	+	+	+	+

Note: TSB – Tryptic Soy Broth; mBPWp – modified Buffered Peptone Water with pyruvate; ACV – Acriflavin, Cefsulodin and Vancomycin.

[a] Enrichment broth of an uninoculated sprouts sample.

3.3.2 Colonies identification

After IMS, enrichment broth of sprouts contaminated with enteroaggregative STEC O104:H4 was plated onto mRBA and CHROMagar STEC O104. In the same fashion, enriched sprouts inoculated with STEC O104:H7 were plated onto mRBA and CHROMagar STEC. Confirmation was done by picking two mauve colonies, one from each selective agar, and testing for latex agglutination and multiplex real-time PCR.

As stated previously, STEC form mauves colonies when they grow on mRBA and/or CHROMagar. Mauve colonies were recovered from every sample only when screened positively by PCR assay. Moreover, all the tested mauve colonies resulted positive by latex agglutination and multiplex real-time PCR. However, *E. coli* O104:H4 was not able to grow on CHROMagar STEC plates even if 100 µl of an overnight culture were plated (Table 3.6), thus the two presumptive colonies were picked solely from mRBA plates.

Similarly, Jinneman et al. (2012) described that CHROMagar O157 does not allow the growth of *E. coli* O104:H21. Since STEC strains that are not tellurite resistant, do not grow on CHROMagar O157 (Tzschoppe et al., 2012); probably the strains used in this work (*E. coli* O104:H7) and *E. coli* O104:H21 (Jinneman et al., 2012) lack of *terB* gene, associated to tellurite resistance.

E. coli O104:H7 grows easily on mRBA, therefore the modifications provided by Tillman et al. (2012) (reduction of tellurite and novobiocin concentration) are very effective. This statement is also supported by the results shown in Table 3.6, where almost no difference was observed when the same overnight culture is quantified through SPC in TSA and mRBA.

E. coli O104:H4 was successfully recovered in mRBA and CHROMagar STEC O104, but when its growth was tested on selective agar, a ~2 log difference was noted between TSA and CHROMagar STEC O104 (Table 3.6).

Table 3. 6 – Standard plate count of overnight culture in different selective agar

***E. coli* strain**	**Solid media**	**Concentration** (CFU/g)	
		no stress	**cold stress**[a]
Enteroaggregative STEC O104:H4	TSA	4.45×10^{8}	6.64×10^{8}
	mRBA	3.91×10^{8}	8.64×10^{8}
	CHROMagar STEC O104	7.27×10^{6}	2.73×10^{6}
STEC O104:H7	TSA	1.34×10^{9}	1.40×10^{9}
	mRBA	1.06×10^{9}	1.02×10^{9}
	CHROMagar STEC O104	0	0

Note: TSA – Tryptic Soy Agar; mRBA – modified Rainbow Agar.
[a] Overnight culture incubated at 4°C for 48h

3.4 CONCLUSIONS

STEC non-O157 are an heterogeneous group of emergent pathogens that like STEC O157:H7 can lead to severe foodborne illnesses. Since official methods are mainly based on the serogroup O157, it is necessary to broaden their detection spectra including other serogroups, because the advent of a novel dangerous strain can always occur.

The current study focused on STEC O104, which provoked a large outbreak in 2011 located mostly in Germany, and described a protocol able to detect less than 1 CFU/g. Modified BPWp supplemented with ACV and immunomagnetic separation were used to increase and concentrate the target microorganisms in artificially contaminated sprouts stressed at 4°C for 48h. Additionally, isolation on two different selective agars was evaluated. The enrichment and IMS resulted effective despite the high level of sprouts background flora and mRBA allowed an easy recovery of both enteroaggregative STEC and STEC O104 strains. The isolated presumptive colonies were tested by latex agglutination kit, which provided a rapid identification of the O104 antigen. Furthermore, two multiplex real-time PCR assays with an internal positive control were designed to detect three genes associated with enteroaggregative STEC and STEC O104. They were successfully used to screen sprout samples after the enrichment and to identify the isolated colonies.

CHAPTER 4: Rapid Method to Detect and Quantify *Listeria monocytogenes* in raw milk through real-time PCR

4.1 BACKGROUND

Listeria is a genus of gram-positive bacteria and the specie *monocytogenes* is basically the only responsible for listeriosis. However, human cases caused by the species *ivanovii* and *seeligeri* have been reported (McLauchlin et al., 2004). Although *Listeriae* are not sporigenic bacteria and it is still not clear who is their reservoir, they are widely distributed in the environment (Valderrama & Cutter, 2013). Moreover, they are able to grow in a wide range of temperatures (1°–45°C) and pH (4.1–9.6). Probably, listeria capacity to create biofilms contributes to its distribution and resistance (Jay et al., 2005). Cases of *L. monocytogenes* are not widespread, but they have a very high lethality rate as it was explained in Chapter 2. In fact, *L. monocytogenes* is an intracellular pathogen, which is able to reach the central nervous system by the hematogenous or retrograde neuronal route (respectively in human and animals) and provoke septicemias, meningitis, meningoencephalitis and rombhoencephalitis (Disson & Lecuit, 2012). People who have a weak immune defense system such as elderly, pregnant women and immunodepressed due to diseases or medical treatment, have higher risk to contract listeriosis (Muñoz et al., 2012; Ramaswamy et al.,

2007). Furthermore, *L. monocytogenes* is able to pass the placental blood barrier during pregnancy, spreading the infection to the fetus, which could degenerate into abortion (Mateus et al., 2013).

Milk is considered a good vehicle and it is very important to detect the contamination in time otherwise the pathogen can reach dangerous levels of 10^2–10^4 cells per g of food. Infective dose differs among *monocytogenes* specie and host susceptibility (Vazquez-Boland et al., 2001). *L. monocytogenes* has been isolated from 2.8% to 6.5% bulk tanks of raw milk taken from different producers across the USA (Jayarao & Henning, 2001; Jayarao et al., 2006; Van Kessel et al., 2004). Raw milk can be sold directly to consumers (Code of Federal Regulations, 2011; Bucchini, 2012) and it is used for cheese production. Even though pasteurization kills listeria, post contaminations can occur (Carminati et al., 2004; Lomonaco et al., 2009). Although it is not known if the milk was the contamination origin, the majority of *L. monocytogenes* outbreaks in USA between 2009 and 2011 were associated with fresh cheese consumption (CDC, 2013).

European Commission established two microbiological criteria for *L. monocytogenes*: 1) absence in 25 g for food designed for infants or medical purposes; and 2) a limit of 100 CFU/g for ready-to-eat food, where the pathogen is not able to grow. Furthermore, for ready-to-eat food that supports *L. monocytogenes* growth, absence in 25 g is required; however, if the producer can ensure that the pathogen will not exceed the upper limit of 100CFU/g during shelf-life, the second limit can be used. Note that this can

be applied only when the food product is placed in the market (European Commission, 2007; 2010; 2011; 2013a; 2013b). Similar criteria are used in USA, where FDA does not consider the product acceptable when *L. monocytogenes* is detected in 25g of sample (FDA, 2010). Only in ready-to-eat food that does not support the pathogen growth its presence is tolerated up to 100 CFU/g (FDA, 2008).

Standard methods to detect and quantify *L. monocytogenes* provided by ISO and FDA are based respectively on isolation or SPC techniques on chromogenic selective plate agars (Anonymous, 2004; Hitchins & Jinneman, 2010). As it was mention in Chapter 2, conventional cultural methods are time consuming and very demanding. Moreover, it has been described that background flora in food may not allow the growth of a low number of *L. monocytogenes* during the enrichment, leading to false negative results (Stessl et al., 2009).

Different real-time PCR assays have been developed to detect rapidly *L. monocytogenes*. However, they lose sensitivity when applied directly on heat treated milk (Dadkhah et al., 2012; Hein et al., 2001; Nogva et al., 2000; Rantsiou et al., 2008) because this food matrix contains many PCR inhibitors such as fats carbohydrates, ions and enzymes (Schrader et al., 2012). Many studies have avoided this problem adding an enrichment step before the real-time PCR assay (Oravcová et al., 2007; O' Grady et al., 2008; Rossmanith et al., 2006; Rossmanith et al., 2010; Schoder et al., 2012). Nevertheless, this extends the time of analysis and excludes pathogen quantification.

The aim of the present work is to evaluate *L. monocytogenes* growth in raw milk and develop a rapid method for detection and quantification.

Raw milk was artificially contaminated with *L. monocytogenes* at three different levels (1, 10 and 100 CFU/ml) and stored at 0°C, 2°C and 4°C. The pathogen concentration was estimated by SPC on ALOA during five days.

Paul et al., (2013) developed the first method able to detect 1 CFU/ml and quantify less than 10 CFU/ml of *E. coli* O157 in raw milk with quantitative PCR in 3 h. Their study introduced a pre-treatment for a 10 ml raw milk sample composed by different centrifugation steps to eliminate fat, collect bacteria in a volume of ~10 µl and wash them to remove PCR inhibitors. Since using a large sample size and an effective sample pre-treatment have significantly improved the method's sensitivity, the author decided to adapt and evaluate the above-mentioned method for *L. monocytogenes* analysis, considering its different dimensions and rate of sedimentation. Moreover, *L. monocytogenes* has shown a lower DNA yield compared to *E. coli* (Brewster & Paoli, 2013). Therefore, different DNA extraction procedures and sets of primers and probes will be tested to obtain a similar sensitivity as to the *E. coli* O157 method.

4.2 MATERIALS AND METHODS: Evaluation of *L. monocytogenes* growth in raw milk at low temperatures

Fresh bovine raw milk was purchased from an automatic vending device in a farm in Ozzano dell'Emila area and stored at 4°C.

4.2.1 Bacterial strains and inoculum preparation

L. monocytogenes ATCC 7644 and three strains isolated from food samples: 87-1771, 88-1777 and 115-1921 were used to evaluate the pathogen growth in raw milk. They were stocked at -20°C in TSB supplemented with 0.6% Yeast Extract (YE; Becton, Dickinson and company) and 20% Glycerol (Carlo Erba reagenti spa, Rodano, Italy).

Each strain was cultivated adding 100 µl of the stock solution into a tube containing 10 mL TSB-YE and incubated overnight at 37°C. Then, 100 µl of the grown culture were added again to a new tube with 10 ml of TSB-YE and incubated at 7°C for 96 h. The obtained cultures were diluted in a solution of 0.1% Tryptone (Oxoid Ltd, Basingstoke, England) and 0.85% NaCl (Oxoid Ltd, Basingstoke, England) and used to contaminate raw milk at level of 1, 10 and 100 CFU/ml. The concentrations of *L. monocytogenes* strains in TSB-YE were measured through SPC technique onto Trypticase Soy Agar supplemented with 0.6% of YE (TSA-YE, Becton, Dickinson and company).

4.2.2 Evaluation of *L. monocytogenes* growth in raw milk at low temperature

Three raw milk batches were tested for quantification of *L. monocytogenes* (Anonymous, 2004) and aerobic mesophilic bacteria (Anonymous, 2003).

Then, *L. monocytogenes* ATCC 7644, 87-1771, 88-1777 and 115-1921 were prepared as previously described and inoculated together in 500 ml raw milk samples. Three levels of *L. monocytogenes* (1, 10 and 100 CFU/ml) were performed in triplicates and samples were incubated at 4°C. *L. monocytogenes* concentrations were determinate after 3 h, 24 h, 48 h, 72 h and 96 h by SPC on Agar Listeria Ottaviani and Agosti (Biolife, Milan, Italy). The results were calculated and expressed according to ISO 7218 (Anonymous, 2007). The experiment was repeated changing the incubation temperature to 2°C and 0°C.

Least square linear regression of *L. monocytogenes* concentration (Log CFU/ml) versus time (h) was used to evaluate the pathogen growth in raw milk artificially contaminated at 100 CFU/ml and incubated at 0°C, 2°C and 4°C. Moreover, a Student's t-test was done to check the significance of the slopes. Welch one-way ANOVA followed by the Duncan-Waller post hoc test were used to compare line slopes. All data were analysed by means of R software version 2.12.2 (Copyright© 2011 The R Foundation for Statistical Computing).

4.3 MATERIALS AND METHODS: Rapid method to detect and quantify *L. monocytogenes* in raw milk

Fresh bovine raw milk was acquired from a store in Philadelphia area and stored at 4°C.

Chemical products utilized were of reagent grade and solutions were prepared with home made deionized water (Nanopure water treatment system, Barnstead, Dubuque, IA, USA).

4.3.1 Bacterial strain and inoculum preparation

L. monocytogenes ATCC 19115 was used to optimize the detection and quantification method by real-time PCR. *L. monocytogenes* was stocked in Brain Heart Infusion (BHI; Becton, Dickinson and Company) supplemented with 20% glycerol at -80°C. The stock cultures, were scratched with a sterile loop to transfer part of the frozen culture in BHI broth, and incubate it overnight at 37°C. Then, *L. monocytogenes* culture was maintained in BHI agar (BHIA; Becton, Dickinson and Company) plates.

Inocula were prepared picking a well separated colony with a sterile loop and dissolving it into 3 ml of BHI broth followed by incubation at 37°C, shaking at 250 rpm for 16–20 h. The grown culture was decimally diluted in physiological saline solution (PS; 0.85% NaCl; Fisher Scientific, Philadelphia, PA, USA) and 200–300 µl of the 10^{-7} dilution were plated on BHIA and incubated overnight at 37°C to enumerate *L. monocytogenes*. Artificial contaminations were performed with small volumes (20–200 µl) of the

appropriate dilution of *L. monocytogenes* ATCC 19115 overnight culture. Levels of contamination are specified in every experiment.

4.3.2 Raw milk pretreatment for molecular assays

Raw bovine milk was pretreated before DNA extraction according to Paul et al. (2013). In many steps, this procedure requires to discard fat layer or supernatants by aspiration. The aspirator is composed by a vacuum pump with a collection vessel connected through a tube to a 1 ml pipette with a micropipet tip in the other end. Aspiration was carried out under full vacuum (~500 torr) for larger volume samples and reduced vacuum (~100 torr) for smaller volume samples.

The pretreatment protocol is briefly described as follow: 10 ml of raw milk were placed in a 15 ml centrifuge tube made of polypropylene and 800 µl of 0.5 M EDTA (ethylenediaminetetraacetic acid; Integrated DNA Technologies). After an incubation at 40°C for 5 min in a water bath, *L. monocytogenes* (prepared as mentioned above) was added into the tube and mixed by inversion.

The contaminated raw milk tube was centrifuged in a swinging-bucket rotor at 4,696g for 15 min at room temperature, and the fat layer and supernatant were discarded. From the filtered sterilized lysis buffer was previously prepared containing 0.1% SDS (Fisher Scientific), 30 mM NaCl and 2 mM $MgCl_2$ (Sigma Aldrich), 450 µl were added. Pellet was resuspended by pipetting and transferred into a 0.6 ml microfuge tube containing 2 µl of 500 U/ml DNase I (Sigma Aldrich). Incubation at room temperature for 5 min followed by centrifugation in an angled rotor at 15,000 g for 2 min were done.

After removal of the supernatant, 100 µl of PS with 1 mM EDTA were added and the pellet was dissolved by pipetting. Then, 10 µg of Trypsin TCPK treated (Sigma Aldrich) were added. Room temperature incubation under vortex mixing every minute was done and, after 5 min, 400 µl of PS supplemented with 0.1% Tween-20 (Fisher Scientific) were added. The sample was then spun at 15,000 g for 2 min, and the supernatant was discarded by aspiration. Less than 5 µl remained in the microtube.

4.3.3 DNA extraction methods

Two DNA extraction methods were used: HotSHOT and QuickExtract™ kit.

According to Truett et al. (2000), extraction and neutralization solutions for HotSHOT protocol were prepared from 30x of 0.75 M NaOH, 0.75 M HCl, 6 mM disodium EDTA, and Tween-20 (0.3%) using autoclaved deionized water. HotSHOT (HS) and HS neutralizer reagents were designated as 2x, 30x etc. based the original composition of 25 mM NaOH and 0.2 mM EDTA, 25 mM HCl, respectively. Thus, 5x HS contained 125 mM NaOH and 1.0 mM EDTA. Pretreated milk sample was briefly centrifuged and 10 µl of 5x HS solution supplemented with 0.1% Tween-20 were added. After a vortex mixing, the sample was incubated at 95°C for 5 min in an MJ Research P-100 thermocycler (Waltham, MA, USA). Ten microliters of 5x HS neutralizer were added and followed by vortex mixing and brief centrifugation. Lastly, 9 µl of the mixed contents were added to the PCR reaction mixture.

QuickExtract™ Bacterial DNA Extraction kit (Epicentre Biotechnologies, Madison, WI, USA) was also tested to isolate DNA from pretreated milk samples following manufacturer's instructions. Ten microliters of QuickExtract Bacterial DNA Extraction Solution and 0.1 µL Ready-Lyse Lysozyme Solution were added to the vial followed by mixing by inversion and brief centrifugation. The sample was first incubated at room temperature for 15 min and second at 80°C for 2 min in a heat-block. Lastly, 9 µl of the extract were added to the PCR reaction mixture.

4.3.4 *L. monocytogenes* DNA standards preparations

Standards for *L. monocytogenes* were prepared by DNA purifications using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) or by DNA extractions using HotSHOT.

Preparation with Qiagen's kit: pretreatment for Gram-positive bacteria protocol and purification of total DNA from animal tissues spin-column protocol of the manufacture's handbook were done. Then, appropriate dilutions of the purified DNA were produced with nuclease-free water (Integrated DNA Technologies) and their concentrations in ng/µl were determined by an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Conversion of in copy number was estimated considering the *L. monocytogenes* genome size 2.23×10^6 bp.

Preparation with HotSHOT kit: a *L. monocytogenes* overnight culture in BHI was serially diluted in PS and an appropriated volume (10–100 µl) containing

~1,000 CFU was mixed with 400 µl of PS supplemented with 0.1% Tween-20 in a 0.6 ml tube. After centrifugation in an angled rotor at 15,000 g for 2 min, DNA was extracted from the pellet with HotSHOT as previously described. DNA standards were stored at -20°C.

4.3.5 Quantitative PCR assay

Two sets of primers and probes (*hlyA* and *hlyQ*) targeting listeriolysin O gene were tested to detect *L. monocytogenes* using StepOne real time thermocycler (Applied Biosystems). Sequences and references of both sets of primers and probes are shown in Table 4.1. The qPCR reaction mixture contained: 10 µl of TaqMan Fast Advanced Master Mix (Applied Biosystems), 1 µl of 20x primers/probe, and 9 µl of DNA template. Final concentrations of *hlyA* set and *hlyQ* set are shown in Table 4.1. Before run qPCR, samples were vortexed and spun at 1,500 g for 1 min.

PCR cycling conditions had fast temperature ramps. For *hlyA* set the conditions used were: 20s at 95°C, and 40 cycles of 1s at 95°C and 20s at 62°C. While for *hlyQ* set the conditions were: 20s at 95°C, and 40 cycles of 1s at 95°C and 20s at 65°C. Standard curves were prepared as descripted earlier and ran in triplicate.

Table 4. 1 – Sequences of *L. monocytoges* primers and probes

	Sequence	Conc.[a] (nM)	Reference
hlyA F	3'-TGC AAG TCC TAA GAC GCC A-5'	500	Nogva et al., (2000)
hlyA R	3'-CAC TGC ATC TCC GTG GTA TAC TAA-5'	500	
hlyA P	FAM/3'-CGA TTT CAT CC GCG TGT TTC TTT TCG-5'/BkFQ	250	
hlyQ F	3'-CAT GGC ACC ACC AGC ATC T-5'	250	Rodriguez-Lazaro et al. (2004)
hlyQ R	3'-ATC CGC GTG TTT CTT TTC GA-5'	250	
hlyQ P	FAM/3'-CGC CTG CAA GTC CTA AGA CGC CA-5'/BkFQ	125	

Note: F – Forward primer, R – Reverse primer, P – Probes;
[a] Sets primers and probe were purchased as PrimeTime® assay kits (Integrated DNA Technologies). Each kit contains 5 nmoles of both primers and 2.5 nmoles of probe which were resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 buffer (Integrated DNA Technologies).

4.4 RESULTS AND DISCUSSION: Evaluation of *L. monocytogenes* growth in raw milk at low temperatures

Raw milk was first tested for *L. monocytogenes* and aerobic mesophilic bacteria quantification by ISO 4833 (Anonymous, 2003) and ISO11290-2 (Anonymous, 2004), respectively. *L. monocytogenes* was not detected and background flora ranged from 2,337 to 3,204 CFU/ml.

Then, raw milk was aseptically aliquoted in 500 ml samples and artificially contaminated with four strains of *L. monocytogenes* cultivated at low temperatures. Uyttendaele et al. (2004) described that adapting the inoculum

at cold temperature reduces the lag time period of *L. monocytogenes* growth in the food matrix.

Contaminated raw milk samples were incubate at 4°C for 5 days and *L. monocytogenes* concentration was estimated every 24h. The experiment was repeated at temperature of 2°C and 0°C. Results are shown in Figure 4.1 and raw data are available in Appendix B.

Concentration of *L. monocytogenes* was under the detection limit (13 CFU/ml) in most of samples artificially contaminated at ~1 CFU/ml during 5 days of experimentation. Instead, the pathogen concentration was detectable, but below the quantification range in most samples inoculated at ~10 CFU/ml and incubated at 0 °C. Meanwhile, *L. monocytogenes* was quantified at a higher incubation temperature and it exceeded the criterion of 100 CFU/ml only in raw milk samples incubated at 4°C after 72 h (Figure 4.1). At the same temperature and initial concentration of ~100 CFU/ml, *L. monocytogenes* reached the level of 10^3 CFU/ml after 72h, which was associated with listeriosis (Vazquez-Boland et al., 2001).

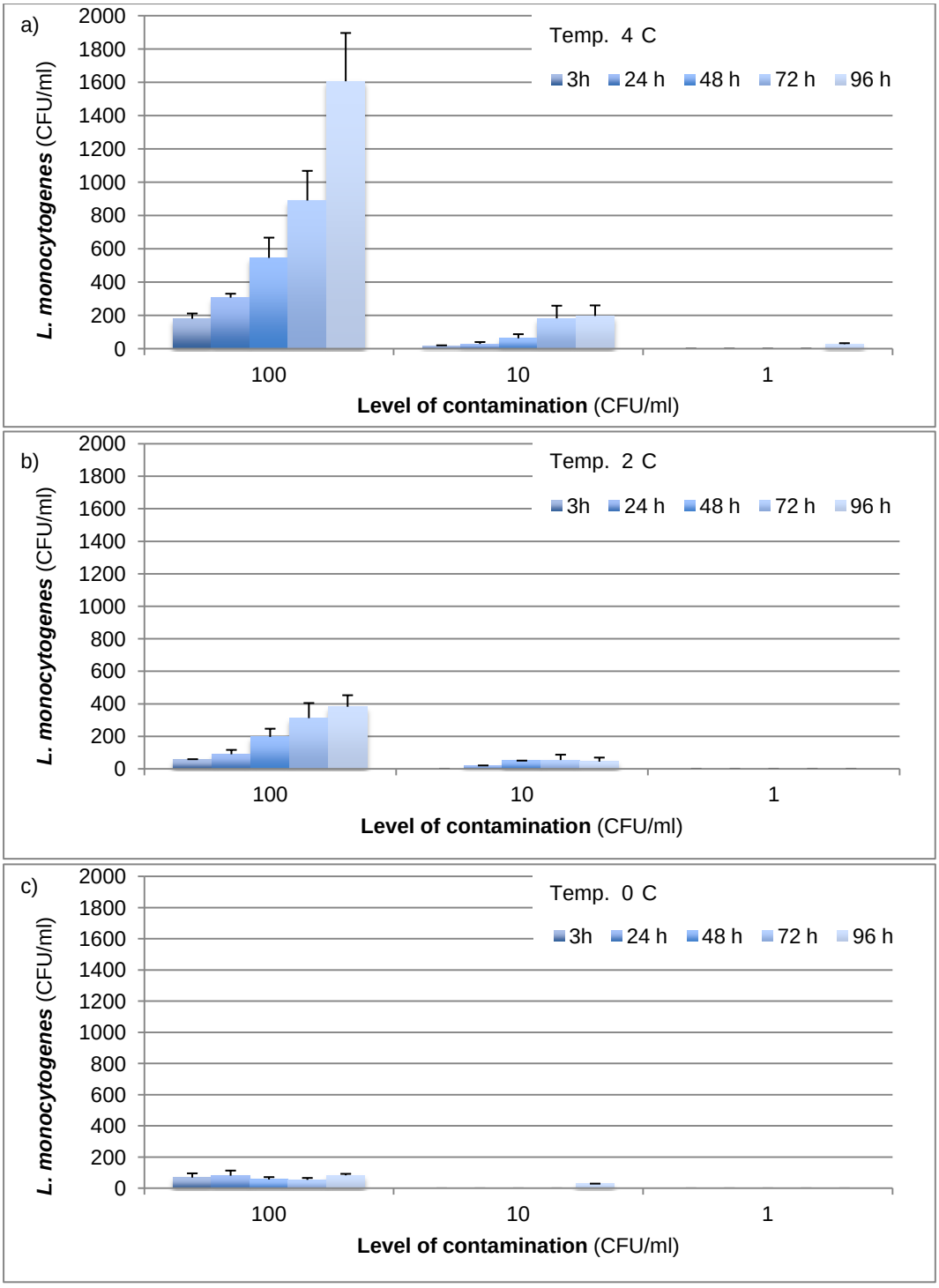

Figure 4. 1 – Concentration *L. monocytogenes* in raw milk after 3, 24, 48, 72 and 96 hours incubated at a) 4°C, b) 2°C and c) 0°C. Columns and error bars represent the average of three replicates and their standard deviation, respectively.

Data form raw milk samples contaminated at ~100 CFU/ml were used to evaluated *L. monocytogenes* growth rate by linear regression of bacterial concentration (Log CFU/ml) versus time (h). Lines for each replicate are graphically represented in Figure 4.2, and equations, slope significance and Pearson's coefficient of correlation (r) are reported in Table 4.2. The slopes were compared and values of samples incubated at 0°C were statistically different from those at 2°C and 4°C by Welch one-way ANOVA ($P < 0.01$) and Duncan-Waller post hoc test (slope difference > 0.0022 Log (CFU/ml)/h) (Table 4.2). Moreover, the null hypothesis (slope = 0) of Student's t test was not rejected for this group of slopes, therefore it can be presumed that *L. monocytogenes* growth was strongly decreased at a temperature of 0°C. In fact, Pearson's coefficient of correlation values of sample incubated at 0°C were < 0.518 and this did not occur for samples incubated at 2°C and 4°C (> 0.977, except for one replicate at 2°C).

Overnight cultures of *L. monocytogenes* strains used for the inocula were quantified by SPC on TSA and used for estimate the level of contamination in raw milk samples. Results are listed in Table 4.3. Samples incubated at 4°C showed levels of contaminations slightly higher than for the ones incubated at 2°C and 0°C. This could justify by the different values of intercepts. However, the latter observation did not affect the evaluation of growth rate since almost no difference was noticed between linear parameters of raw milk samples incubated at 2°C and 4°C.

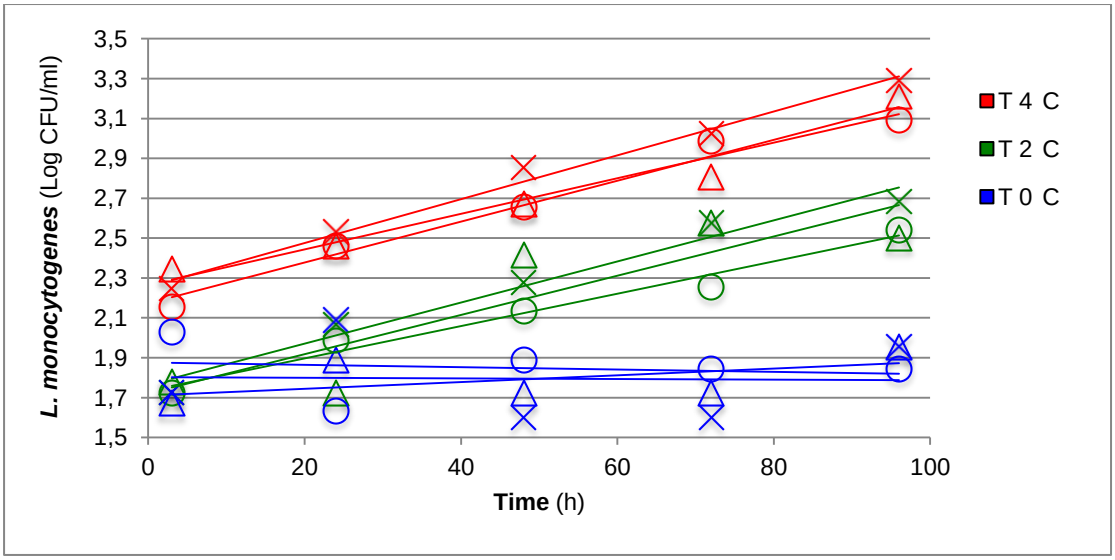

Figure 4. 2 – Linear trends of *L. monocytogenes* growth in raw milk artificially contaminated at ~100 CFU/ml incubated at 4°C (Red), 2°C (Green) and 0°C (Blue). Replicates were represented using three different symbols.

Table 4. 2 – Linear regression of concentration of *L. monocytogenes* (Log CFU/ml) versus time (h) under three temperature conditions. Parameters, r and significance of slopes are shown for each replicate.

Incubation temperature (°C)	**Line equations**	**Student's t test**[a] (P value)	**r**
4	Y = 0.0102 X + 2.173	0.0016*	0.988
	Y = 0.0109 X + 2.256	0.0006*	0.994
	Y = 0.0089 X + 2.265	0.0043*	0.977
2	Y = 0.0081 X + 1.735	0.0017*	0.987
	Y = 0.0103 X + 1.765	0.0024*	0.984
	Y = 0.0098 X + 1.721	0.0484*	0.881
0	Y = - 0.0006* X + 1.876	0.8053	-0.153
	Y = - 0.0002* X + 1.802	0.9654	-0.027
	Y = - 0.0017* X + 1.710	0.3718	0.518

Note: r – Pearson's coefficient of correlation; Y – *L. monocytogenes* (Log CFU/ml), X – Time (h).

[a] Student's t tests were performed with null hypothesis: Line slope = 0; which was rejected when P< 0.05.

The line slopes of samples incubated at 0°C were statistically different from those at 2°C and 4°C by Welch one-way ANOVA (P <0.01) and Duncan-Waller post hoc test (slope difference > 0.0022 Log (CFU/ml)/h).

Table 4. 3 – Contamination level of *L. monocytogenes* in raw milk based on overnight cultures concentration

***L. monocytogenes* concentration in raw milk** (CFU/ml)		
Temp. 4°C	Temp. 2°C	Temp. 0°C
195	60	51
19	6	5.1
1.9	0.6	0.5

4.5 RESULTS AND DISCUSSION: Rapid method for detect and quantify *L. monocytogenes* in raw milk

4.5.1 Real-time PCR: comparison of primers/probe sets

Nogva et al. (2000) and Rodriguez-Lazaro et al. (2004) have both designed a set of primers and probe (*hlyA* and *hlyQ*, respectively) that are able to detect *hly* gene in *L. monocytogenes*. These authors have also show the specificity of *hlyA* and *hlyQ* sets testing them on several *L. monocytogenes*, *L.* non-*monocytogenes* and non-*Listeria* strains. Both set were purchased from Integrated DNA Technologies as PrimeTime® assay kits and the manufacturer recommends using 500 nM of each primer and 250 nM of probe. In order to reduce possible non-specific amplification, lower concentrations and higher annealing temperatures compare to previously reported were evaluated. *L. monocytogenes* DNA purified with DNeasy Blood and Tissue kit was decimally diluted (1–1000 pg) and amplified in duplicates

by real-time PCR. Values of C_T and reactions efficiencies are shown in tables 4.4 and 4.5 for *hlyA* and *hlyQ*, respectively.

C_T values for *hlyQ* set are variable among annealing temperatures and primer concentrations and the highest C_T values was reported at 67°C. On the contrary, C_T values for *hlyA* set are more stable. Different combinations of concentrations of primers and annealing temperatures such as 250 nm at 62°C and 500 nm at 64°C for *hlyA* set and 250 nm at 63°C and 250 nm at 65°C for *hlyQ* set worked very well. Since the results of both sets are similar, the author decided to use *hlyA* set because it has been described to be effective in milk (Nogva et al., 2000). Additionally, the concentration of 250 nm was presumed to produce less non-specific amplifications.

Table 4. 4 – Real-time PCR assays with *hlyA* set of primers and probe at different concentrations and annealing temperatures.

DNA Template (pg)	Annealing temperature 60°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	29.4	0.02	99	31.7	0.04	91
10	25.9	0.07		28.0	0.00	
100	22.7	0.00		22.5	0.01	
1000	20.9	0.01		20.9	0.01	
	Annealing temperature 62°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	29.2	0.00	101	30.7	0.09	94
10	25.8	0.01		27.1	0.14	
100	22.5	0.01		23.9	0.01	
1000	20.9	0.00		20.2	0.01	
	Annealing temperature 64°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	28.7	0.18	97	30.8	0.14	89
10	25.9	0.07		27.2	0.39	
100	22.0	0.00		23.7	0.01	
1000	21.0	0.02		19.9	0.01	

Note: Conc. – concentration; C_T – Threshold Cycle; Std. Dev. – Standard Deviation

Table 4. 5 – Real-time PCR assays with *hlyQ* set of primers and probe at different concentrations and annealing temperatures.

DNA Template (pg)	Annealing temperature 63°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	28.7	0.01	85	29.7	0.02	95
10	24.8	0.02		26.0	0.02	
100	21.2	0.00		22.2	0.01	
1000	17.5	0.03		19.5	0.34	
	Annealing temperature 65°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	29.1	0.13	82	31.2	0.02	90
10	24.9	0.00		27.2	0.07	
100	21.3	0.04		23.2	0.00	
1000	17.5	0.02		20.6	0.03	
	Annealing temperature 67°C					
	Primer Conc. 500 nM			Primer Conc. 250 nM		
	C_T Mean	C_T Std. Dev.	Efficiency (%)	C_T Mean	C_T Std. Dev.	Efficiency (%)
1	33.0	0.00	58	39.3	0.37	69
10	26.9	0.07		33.9	0.00	
100	22.4	0.03		28.9	0.02	
1000	17.6	0.01		23.3	0.07	

Note: Conc. – concentration; C_T – Threshold Cycle; Std. Dev. – Standard Deviation

4.5.2 Selection of DNA extraction procedure

DNA extraction from gram-positive bacteria is more difficult compare to gram-negative ones due to the different composition of the cell's envelope. Additionally, if higher amount of extracted DNA is added to the PCR reaction mix, higher are the chances that a target sequence is recognized by the primers increasing the method's sensitivity (Brewster & Paoli, 2013). Therefore, a technique that is able to extract DNA from gram-positive cells in a small volume free of inhibitors is necessary. Two procedures were evaluated: the commercial kit QuickExtract™ Bacterial DNA Extraction by Epicentre Biotechnologies and HotSHOT DNA extraction (Truett et al., 2000). Epicentre Biotechnologies provides a protocol where pelleted cells are resuspended in QuickExtract™ Bacterial DNA Extraction solution, incubated at room temperature for 15 min followed by a second incubation at 80°C for 2 min. In order to evaluate the heating effect on DNA yield, 0, 1, 2 and 4 min at 80°C and the removal of the reagent were tested in triplicates by real-time PCR assay. Negative controls made of QuickExtract™ Bacterial DNA Extraction solution were also performed. The results from the extraction of 500 CFU sample are shown in Figure 4.3.

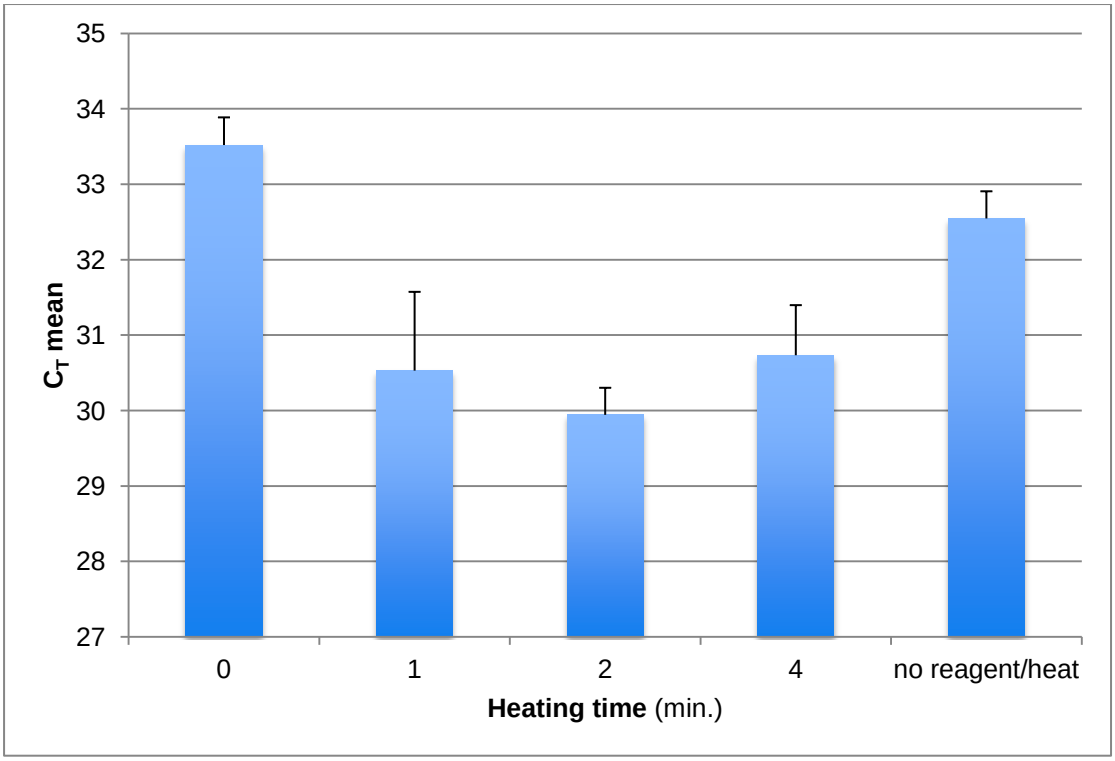

Figure 4. 3 – C_T values of real-time PCR assays of DNA extracted from 500 CFU by QuickExtract™ Bacterial DNA Extraction kit at different heating time. Columns and error bars represent the average of three replicates and their standard deviation, respectively. Negative controls were not detected by real-time PCR assays.

HotSHOT extraction techniques was used on *E. coli* cells by Paul et al. (2013) and no inhibition for PCR assay was observed. However, 2x HS is insufficient to extract DNA from *L. monocytogenes* and instead 5x HS is required (Brewster & Paoli, 2013). Consequently, different incubation times and temperatures (65°C/10 min, 65°C/20 min, 75°C/10 min, 85°C/10 min and 95°C/5 min) were evaluated with 5x HS reagent, extracting ~500 CFU of *L. monocytogenes* and running the DNA by PCR assay. The same amount of cells was also extracted by QuickExtract™ Bacterial DNA Extraction kit using 2 min of heating time at 80°C which gave the best performance. This

extracted DNA was amplified together with HotSHOT extracted samples to draw a comparison. The resulted C_T values are represented in Figure 4.4.

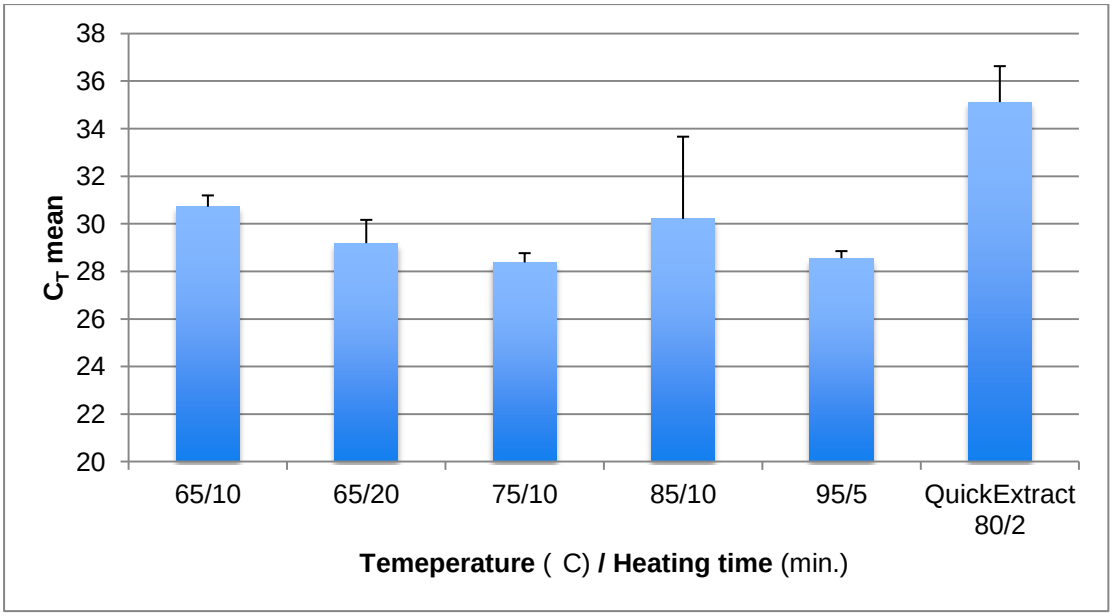

Figure 4. 4 – C_T values of real-time PCR assays of DNA extracted from 500 CFU by HotSHOT at different heating temperatures and times, and by QuickExtract™ Bacterial DNA Extraction kit. Columns and error bars represent the average of three replicates and their standard deviation, respectively.

4.5.3 L. monocytogenes quantification

Quantitative PCR assay was first tested for raw milk inhibition. Thus, DNA was extracted with HotSHOT from a *L. monocytogenes* pure culture and from raw milk contaminated right after the pretreatment step. Efficiency of the real-time PCR assay was calculated for both samples through four decimal dilutions of ~10,000 CFU/ml *L. monocytogenes* in triplicates. Resulted C_T values and efficiency of real-time PCR assay were reported in Table 4.6.

Similarly to Paul et al. (2013) work, the pretreatment step is able to clean effectively 10 ml of raw milk from PCR inhibitors. In fact, the efficiency of real-time PCR assay from pure culture was 100% while real-time PCR assay from contaminated pretreated raw milk was 99% (Table 4.6). Furthermore, C_T and *L. monocytogenes* concentration presented a linear relationship from 10 to 100,000 CFU, which is suitable for a standard curve. Less than 10 CFU were also detected.

Table 4. 6 – Real-time PCR amplification of DNA extracted from *L. monocytogenes* pure culture and raw milk contaminated after the pre-treatment enrichment step

L. monocytogenes[a] (CFU/ml)	**Pure culture**			**Artificially contaminated milk**		
	C_T Mean[b]	**C_T Std. Dev.**	**Efficiency** (%)	**C_T Mean**[b]	**C_T Std. Dev.**	**Efficiency** (%)
6	35.8	1.01	100	35.6	1.50	99
60	32.3	1.20		32.7	1.14	
600	28.1	0.97		28.7	0.94	
6000	24.5	0.74		24.6	0.92	

Note: C_T – Threshold Cycle; Std. Dev. – Standard Deviation

[a] *L. monocytogenes* concentration was calculated by SPC on BHIA

[b] Linear regression of *L. monocytogenes* concentration (Log CFU/ml) versus threshold cycles (C_T). For pure cultures: $C_T = -3.82 \times (\text{Log CFU/ml}) + 38.89$; $R^2 = 0.998$. For artificially contaminated milk: $C_T = -3.68 \times (\text{Log CFU/ml}) + 38.75$; $R^2 = 0.996$.

Since sample pretreatment, DNA extraction procedure and real-time PCR assay work, raw milk was contaminated with low levels of *L. monocytogenes* (1, 10 and 100 CFU/ml) and ran through the entire method to assess its sensitivity. The experiment was performed in triplicated and reproduced fourth times as represented in Figure 4.5. Uninoculated raw milk and 10 pg of DNA purified with DNeasy Blood and Tissue kit were used as negative and positive control, respectively. Amplifications with C_T values over 40 were considered false-positives. Raw data are also available in appendix C.

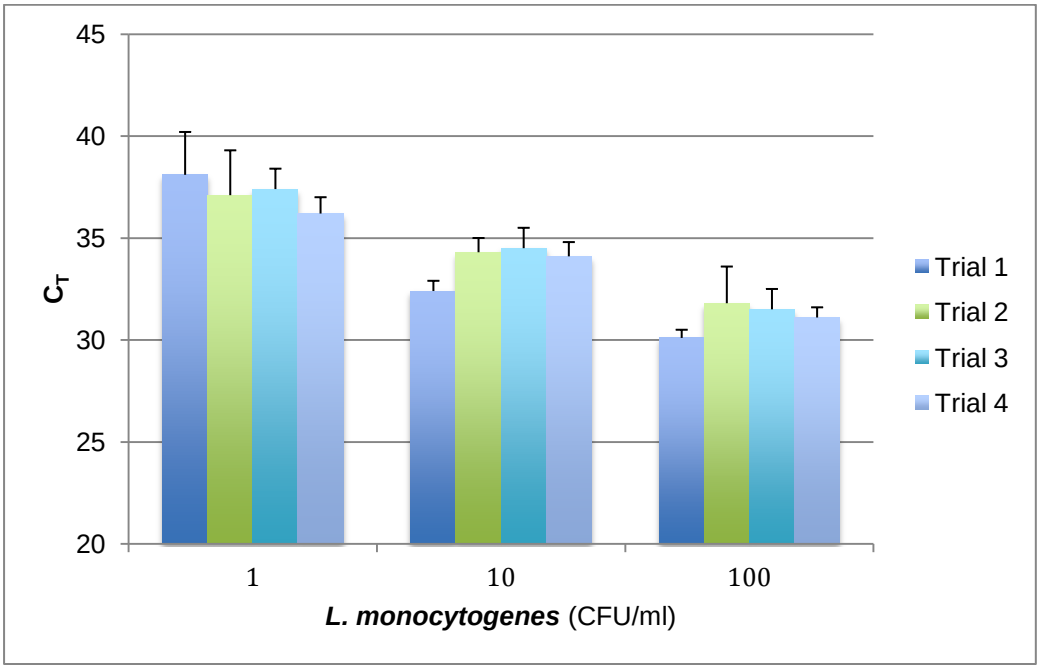

Figure 4. 5 – Threshold cycle values of real-time PCR of raw milk artificially contaminated at low levels. Columns and error bars represent the average of three replicates and their standard deviation, respectively.

Positive controls were always amplified with a C_T value of ~26. Regarding negative controls, 1 out 3 replicates of trials 1, 2 and 4 were amplified with a C_T values >40. However, one replicate of trials 1 and 4 gave false-positive results (C_T 39.1 and 37.8, respectively).

Contamination level of 1 CFU/ml was always detected except for one replicate in trial 2. Note that one replicate in trial 1 has a C_T value of 40.2, which is considered a false negative.

Despite the optimization of PCR assay parameters, it is not always possible to distinguish C_T values between uninoculated and inoculated samples at a level of 1 CFU/ml.

Furthermore, quantification was possible from level contamination of 10 CFU/ml and the reproducibility of C_T values was adequate.

4.6 CONCLUSIONS

The present work has evaluated *L. monocytogenes* growth in raw milk during 5 days. *L. monocytogenes* level was under the criterion of 100 CFU/ml in raw milk samples contaminated at 1 CFU/ml during four days at all incubation temperatures. Similarly, samples inoculated at 10 CFU/ml exceed that limit only when incubated at 4°C after 72h. Additionally, as shown by the slopes comparison *L. monocytogenes* grows slower when incubated at 0°C compared to 2°C and 4°C.

A new quantitative method for *L. monocytogenes* based on real-time PCR was also described, providing a rapid quantification of 10 CFU/ml in raw milk in about 3h.

Sample pretreatment protocol removed inhibitors for *L. monocytogenes* real-time PCR assay as for *E. coli* O157 assay (Paul et al., 2013) and the modified HotSHOT technique is able to extract DNA from gram-positive bacteria. Both procedures are powerful tools that can be used for other PCR assays. Since foodborne pathogens are distributed heterogeneously in food, it is important to use a sample volume large enough to represent the entire food matrix. In this work, 10 ml of raw milk were used in comparison with other methods that extract DNA from ~1 ml of milk (Dadkhah et al., 2012; Hein et al., 2001; Nogva et al., 2000).

Quantitative PCR is able to achieve results within a half-day and the limit of quantification is comparable to 4 CFU/ml of official SPC method (Anonymous, 2007). Note that the high-level background flora present in raw milk can be troublesome when 1 ml is spread in one plate. Consequently, in this study lower volumes of raw milk were spread in more plates achieving a detection limit of 13 CFU/ml.

CHAPTER 5: General Conclusion

Several foodborne illnesses are distributed worldwide. Food safety control from farm to fork is essential to seek sources of contaminations and prevent their diffusion. The presented work, developed new tools that can support detection and isolation of STEC 104, and quantification of *L. monocytogenes*. Potentially, these methods can be applied together to official methods.

Regarding Shiga-like toxin-producing *E. coli* O104, it has been demostrated that IMS helps to isolate these pathogens from a food matrix characterized by a high level of background flora such as sprouts. Multiplex real-time PCR assay was designed for enteroaggregative STEC O104 and other STEC O104. This approach demonstrated to be effective for screening samples after an enrichment step and for identifying presumptive isolated colonies. Moreover, the performances of commercially available latex agglutination kit that recognize the antigen O104 and two selective agars (mRBA and CHROMagar) were evaluated.

Several other serogroups of STEC non-O157 are emerging human pathogens and methods for their detection are needed. Currently, Abraxis is preparing new antibodies against antigens: O26, O45, O103, O111, O21, and O145, and producing reagents for IMS and latex agglutination. Our collaboration will continue by testing the specificity and sensitivity of those reagents. Then, protocols to isolate every serogroup will be optimized and, if possible, merged to design a single method able to detect all the major STEC pathogens at the same time.

L. monocytogenes was chosen because it provokes severe and lethal diseases, thus its presence in food has to be avoided.

As milk can be a good vehicle of *L. monocytogenes*, pathogen growth was first evaluated in fresh raw milk artificially contaminated at three levels under three temperature conditions. Moreover, a rapid quantitative method was developed.

Large raw milk samples of 10 ml were pretreated to concentrate the bacteria and remove PCR inhibitors. DNA extraction and real-time PCR protocols were optimized and a quantification range of 10 to 1000 CFU/ml was achieved. The designed quantitative PCR method has a similar sensitivity to SPC in raw milk and provides results in half a day, which is a great advantage compare to official conventional cultural methods. This is very important because it is possible to achieve results before raw milk is sold or used in further processes. Additionally, the raw milk pretreatment and DNA extraction with HotSHOT can be used for detection or quantification of other pathogens by real-time PCR.

Currently, the *L. monocytogenes* criterion of absence in 25 grams required by Europe and USA (European Commission, 2005; FDA, 2006) cannot be assessed directly with real-time PCR without a selective enrichment step. However, further studies can be done to enlarge the size of raw milk sample to 25 ml or 50 ml in order to improve the method sensitivity.

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APPENDICES

APPENDIX A: Publications

Publications derived from the Thesis's work:

Baranzoni G. M., Fratamico P. M., Rubio F., Glaze T., Bagi L. K. and Albonetti S. (2014). Detection and isolation of Shiga Toxin-Producing Escherichia coli (STEC) O104 from sprouts. *International Journal of Food Microbiology* 173, 99–104.

Paul M., **Baranzoni G. M.**, Albonetti S. and Brewster J. D. (September 10, 2013) Development of a qPCR direct detection method for *Listeria monocytogenes* in milk. *246th ACS National Meeting, Indianapolis, IN, USA*.

Albonetti S., **Baranzoni G. M.**, Paci A., Savigni F., Rosmini R. Valutazione della crescita di *Listeria monocytogenes* in latte crudo a temperature di refrigerazione. *Industrie Alimentari*, (accepted, 18-11-2013).

Other publications:

Guidi E., Lauriola A., Piumi M., Bartczak M. L., Serena F., Barontini F., Albonetti S., Rosmini R., **Baranzoni, G**. (2012). Applicazione dei prerequisiti nei piani di autocontrollo aziendali tra normativa cogente e volontaria. *Italian Journal of Food Safety*, *1*(4), 67–69.

Albonetti S., **Baranzoni G. M.**, Guidi E., Savigni F., Cuoghi G., Rosmini R. (2014). Sopravvivenza di *Listeria monocytogenes* in brodo di carne surgelato pronto. *Industrie Alimentari*, (IN PRESS).

APPENDIX B: Concentrations of *L. monocytogenes* in raw milk during 96h incubated at 4°C, 2°C and 0°C.

Table B. 1 – *L. monocytogenes* growth in raw milk at 4°C

Contamination level (CFU/ml)	***L. monocytogenes* concentration**[a] (CFU/ml)				
	3 h	24 h	48 h	72 h	96 h
100	143	290	453	970	1,243
	177	340	717	1,057	1,953
	220	290	467	643	1,623
10	20	40	97	77	220
	D	17	50	217	110
	17	D	40	253	260
1	ND	D	D	D	23
	D	ND	D	D	33
	ND	ND	ND	ND	ND

Note: D – Detected; ND – Non Detected.

[a] SPC was performed in three plates inoculated with 100 µl for each dilution factor. According to ISO 7218 (Anonymous, 2007), the limit of quantification was considered of 4 colonies counted in three plates (13 CFU/ml). When less than 4 colonies were counted, *L. monocytogenes* was considered detected but not quantified.

Table B. 2 – *L. monocytogenes* growth in raw milk at 2°C

Contamination level (CFU/ml)	***L. monocytogenes* concentration**[a] (CFU/ml)				
	3 h	24 h	48 h	72 h	96 h
100	53	97	137	180	347
	53	117	190	377	480
	60	53	260	377	317
10	D	20	50	33	77
	D	D	ND	27	37
	D	ND	ND	100	17
1	ND	D	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND

Note: D – Detected; ND – Non Detected.

[a] SPC was performed in three plates inoculated with 100 µl for each dilution factor. According to ISO 7218 (Anonymous, 2007), the limit of quantification was considered of 4 colonies counted in three plates (13 CFU/ml). When less than 4 colonies were counted, *L. monocytogenes* was considered detected but not quantified.

Table B. 3 – *L. monocytogenes* growth in raw milk at 0°C

Contamination level (CFU/ml)	***L. monocytogenes* concentration**[a] (CFU/ml)				
	3 h	24 h	48 h	72 h	96 h
100	107	43	77	70	70
	53	123	40	40	90
	47	77	53	53	90
10	D	D	D	ND	30
	D	D	D	D	ND
	D	D	D	D	D
1	ND	ND	D	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND

Note: D – Detected; ND – Non Detected.

[a] SPC was performed in three plates inoculated with 100 µl for each dilution factor. According to ISO 7218 (Anonymous, 2007), the limit of quantification was considered of 4 colonies counted in three plates (13 CFU/ml). When less than 4 colonies were counted, *L. monocytogenes* was considered detected but not quantified.

APPENDIX C: Reproducibility of real-time PCR on artificially contaminated raw milk

Table C. 1 – Threshold cycle values of real-time PCR of raw milk artificially contaminated at low levels.

L. monocytogenes (CFU/ml)	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	37.9	38.6	36.7	37.1
	40.2	35.6	38.1	35.6
	36.1	ND	37.4	36.0
10	31.8	33.8	33.9	33.5
	32.8	35.1	32.9	34.9
	32.5	34.1	36.8	33.8
100	30.6	31.1	31.3	33.5
	29.9	30.5	34.0	34.9
	29.9	34.1	29.2	33.8
Negative CTRL[a]	45.7	41.0	ND	37.8
	ND	ND	ND	ND
	39.1	ND	ND	41.2
Positive CTRL[b]	26.5	26.1	26.1	26.4
	27.1	26.1	25.9	25.9
	27.4	25.8	25.9	26.5

Note: ND – Non Detected

[a] Uninoculated raw milk sample

[b] 10 pg DNA purified with DNeasy Blood and Tissue kit