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Thesis submitted in fulfilment of the requirements for the degree of
PhD in agronomical sciences and bio-engineering

A system for detection and identification of
foodborne pathogenic bacteria based on a
“Combinatory qPCR” technology

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Preamble

This PhD thesis was supported by an Ylief grant from the Belgian Federal Public Service: Public Health Food Chain Safety and Environment. The project entitled CoSYPS Path Food was a collaboration between Prof. Jacques Mahillon of the Laboratory of Food and Environmental Microbiology of the “Université catholique de Louvain” (UCL) and two teams of the “Institut Scientifique de Santé Publique” (WIV-ISP) located in Brussels. These two teams are the Food Pathogens service with Dr. Nadine Botteldoorn as co-promoter of this thesis and the Platform Molecular Biology and Biotechnology (PBB) with Dr. Nancy Roosens, reviewer and functional chief of the PhD student.

The objective of this thesis was the development of a combinatory SYBR®Green real-time PCR platform for rapid and simultaneous detection of living *Salmonella* spp. and *Listeria monocytogenes* in carcass samples.

First, a multi-genera, multi-levels and multi-targets qPCR screening system for the simultaneous detection of *Salmonella* spp. and *Listeria* spp was developed and validated. All assays were designed to be run simultaneously on the same plate and all were validated using the same strategy. Afterwards, all steps from food sample to strain isolation and confirmation were added to get the complete CoSYPS Path Food workflow. This complete workflow was then applied and validated on beef carcass swab samples, spiked with *Salmonella* and/or *Listeria*.

This work is presented into three main parts. The first part introduces the subject and gives the objectives of the thesis. The second part displays the results of the thesis in three chapters: i) the development of the CoSYPS Path Food system, ii) the discrimination of dead bacteria using molecular methods and iii) the complete CoSYPS Path Food workflow validation on beef carcass swab samples. Finally, the third part of this thesis discusses the obtained results and formulates the conclusion and future perspectives. This thesis led to six publications (four published and two submitted).

Included Publications

Published papers:

1. Barbau-Piednoir, E., Mahillon, J., Roosens, N. H., Botteldoorn, N. (2014) **Detection of *Listeria* spp. and *Listeria monocytogenes* in food and feed products.** In Hambrick E.C. (ed.) "*Listeria monocytogenes*: food sources, prevalence and management strategies". Nova Sciences Publishers, USA, pp. 147-166.
2. Barbau-Piednoir, E., Botteldoorn, N., Yde, M., Mahillon, J., Roosens, N. H. (2013) **Development and validation of qualitative SYBR®Green Real-Time PCR for detection and discrimination of *Listeria* spp. and *Listeria monocytogenes*.** Appl. Microbiol. Biotechnol. 97: 4021-4037. doi: [10.1007/s00253-012-4477-2](https://doi.org/10.1007/s00253-012-4477-2)
3. Barbau-Piednoir, E., Roosens, N. H., Bertrand, S., Mahillon, J., Botteldoorn, N. (2013) **SYBR®Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels.** Appl. Microbiol. Biotechnol. 97: 9811-9824. doi: [10.1007/s00253-013-5234-x](https://doi.org/10.1007/s00253-013-5234-x)
4. Barbau-Piednoir E, Bertrand S, Roosens NH, De Keersmaecker SCJ. 2014. **Genome sequence of the *Salmonella enterica* subsp. *enterica* serovar Namur strain 05-2929, lacking the *Salmonella* atypical fimbrial operon.** Genome Announc. 2(2):e00299-14. doi: [10.1128/genomeA.00299-14](https://doi.org/10.1128/genomeA.00299-14)

In press paper:

5. Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Roosens, N. H., Botteldoorn, N. **Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis.** In press in J. Microbiol. Meth. doi: [10.1016/j.mimet.2014.06.003](https://doi.org/10.1016/j.mimet.2014.06.003)

Submitted paper:

6. Barbau-Piednoir, E., Botteldoorn, N., Mahillon, J., Dierick, K., Roosens, N. H., **Fast and discriminative CoSYPS detection system of viable *Salmonella* spp. and *Listeria* spp. in carcass swab samples** Submitted to Int. J. Food Microbiol.

Abstract

Foodborne outbreaks are important issues worldwide. Two of the most important foodborne pathogens are *Salmonella* spp. and *Listeria monocytogenes*. The reference methods to detect foodborne bacteria are international standard operating procedures, ISO methods, which are recognized in the whole world. These methods are mostly culture-based methods that are time consuming (several days) and labor intensive. In order to identify faster the source of foodborne outbreaks, to better manage food-related crises and to allow a rapid batch release during own checks by the food business operators, tools for rapid and reliable detection and identification of pathogens in foodstuffs have been developed. The most promising alternative approaches are based on PCR or Real-Time PCR (qPCR) methods. However, several major drawbacks remain: i) the already developed qPCR detection systems mostly target only one foodborne pathogen (single-genus) and are therefore unable to simultaneously detect several foodborne pathogens; ii) these PCR or qPCR foodborne detection assays use a single primer-pair (single-target) and this can potentially lead to false negative results in case of mutation or deletion of the target sequence; iii) these assays are not harmonized and use distinct chemistries (e.g. TaqMan® or SYBR®Green) and different PCR programs and therefore cannot be used altogether as multi-genera detection system and iv) no official guideline with specific acceptance criteria are available to validate food microbiology qPCR assays.

The CoSYPS Path Food project intends to overcome these drawbacks by developing and validating a multi-genera, multi-levels and multi-targets screening system for simultaneous detection of living *Salmonella* spp. and *Listeria* spp. in food samples. The name of the developed system is CoSYPS Path Food detection system for Combinatory SYBR®Green qPCR Screening system for pathogens detection in food samples. This detection system is composed of 11 SYBR®Green qPCR assays for the detection of *Salmonella* and *Listeria* genera and their discrimination at species and sub-species levels. Each level of discrimination (genus, species and subspecies) is composed of at least two assays in order to increase the probability to detect atypical strains which may be deleted or mutated for one of the targets. Thus, this multi-target system reduces the probability of false negative results compared to the current mono-target (q)PCR detection systems. All qPCR assays developed for the CoSYPS Path Food detection system were designed to be run simultaneously on the

same plate and were all validated based on two available guidelines: one for food-microbiology PCR assays validation (ISO 22118: 2011) and the other for the validation of qPCR assays for GMO detection where qPCR is the gold standard (ENGL guidelines).

In order to further facilitate the CoSYPS Path Food detection system analysis, a support tool has been developed. This tool provides all the information about the 11 SYBR®Green qPCR assays (e.g. primer sequences and primer concentration) as well as an automatic calculation of each PCR mix and a decision support system (DSS) to obtain a harmonized analysis of the qPCR results. This automated interpretation of the qPCR results guarantees the same conclusion regardless of the analyst. To apply the developed CoSYPS Path Food detection system to food matrices containing both dead and viable bacteria, a discrimination study on the dead *versus* viable bacteria using viability-qPCR was performed. This study demonstrated the limits of the viability-qPCR and concluded that an enrichment step before the qPCR analysis remains preferable to a viability-qPCR. From these results, the complete CoSYPS Path Food workflow was developed. It contains all steps, from food sample enrichment to strain isolation and confirmation, and was then validated on beef carcass swab samples. This type of sample was chosen as a food matrix model since meat is one of the most important vehicles of foodborne bacteria and "swabbing", which is a surface sampling, was retained as generally recognized as the most appropriate technique in performing microbiological control of carcasses at the slaughterhouse. The complete CoSYPS Path Food workflow was thus validated on beef carcass swab samples spiked with one or both food pathogens: *Salmonella* and *Listeria*, at different concentrations. The validation was performed by comparing, according to the ISO 16140:2003, the results of the complete CoSYPS Path Food workflow with the results of the conventional culture-based methods.

To conclude, due to its multi-targets strategy, the CoSYPS Path Food system is reliable because a lower number of false negative results is expected compared to mono-target qPCR detection systems. Moreover, the CoSYPS Path Food system gives additional information on the detected strains (species, subspecies) thanks to its multi-levels strategy. This may facilitate the isolation and recovery of the detected strains in the sample. Furthermore, the complete CoSYPS Path Food workflow is also a fast alternative to conventional culture-based methods, as target strains are detected in two days whereas four to five days are required with the conventional ISO methods. Likewise, the complete CoSYPS Path Food workflow is as efficient as

the conventional methods since the same limit of detection is obtained, i.e. 4 to 16 CFU¹ *Listeria*/swab and 2 to 11 CFU *Salmonella*/swab, even when both pathogens are present in samples containing a high level of background microflora. Moreover, the complete CoSYPS Path Food workflow is modular as it can be adapted to different food matrices and/or target pathogens. The complete CoSYPS Path Food is therefore a good alternative to the conventional culture-based methods for the detection of foodborne pathogenic bacteria.

¹ CFU: Colony Forming Unit

Résumé

Les épidémies d'origine alimentaire sont une problématique importante à travers le monde. Deux agents pathogènes alimentaires parmi les plus importants sont *Salmonella* spp. et *Listeria monocytogenes*. Les méthodes de référence pour détecter ces bactéries sont des procédures internationales normalisées : méthodes ISO, qui sont reconnues dans le monde entier. Ces méthodes sont essentiellement basées sur des méthodes de culture longues (plusieurs jours) et assez laborieuses. Afin d'identifier rapidement la source des épidémies d'origine alimentaire, de mieux gérer les crises liées à l'alimentation et de permettre une libération rapide des lots autocontrôlés par les industriels du secteur alimentaire, des outils rapides et fiables pour la détection et l'identification des agents pathogènes dans les denrées alimentaires ont été développés. Les méthodes alternatives les plus prometteuses sont basées sur la PCR ou PCR en temps réel (qPCR). Cependant, plusieurs inconvénients majeurs demeurent: i) les systèmes de détection qPCR déjà développés ne ciblent, en général, qu'un agent pathogène (mono-genre) et ne permettent donc pas d'en détecter plusieurs simultanément; ii) ces systèmes de détection n'utilisent en général qu'une seule paire d'amorces (mono-cible) et ceci peut potentiellement conduire à des faux-négatifs quand la séquence cible est mutée ou délétée ; iii) ces différents systèmes de détection ne sont pas harmonisés et utilisent différentes technologies (par exemple TaqMan® ou SYBR®Green) et des programmes PCR différents. Ils ne peuvent donc pas être utilisés ensemble dans un système multi-cibles et iv) aucune directive officielle, comprenant des critères spécifiques d'acceptation, n'est disponible pour valider des analyses qPCR pour la microbiologie alimentaire.

Le projet *CoSYPS Path Food* tend à remédier à ces inconvénients en développant et validant un système multi-genres, multi-cibles et multi-niveaux pour la détection simultanée dans les matrices alimentaires, des *Salmonella* spp. et des *Listeria* spp. vivantes. Le système développé dans ce projet s'appelle le système de détection *CoSYPS Path Food* pour "Combinatory SYBR®Green qPCR Screening system for pathogens detection in food samples". Il consiste en 11 tests qPCR pour la détection des genres *Salmonella* et *Listeria* et leur discrimination au niveau du genre, de l'espèce et de la sous-espèce. Chaque niveau de discrimination (genre, espèce et sous-espèce) est constitué d'au moins deux cibles afin d'augmenter la probabilité de

détecter des souches atypiques qui auraient une délétion ou une mutation dans une des cibles. Ce système multi-cibles réduit donc la probabilité de faux-négatifs par rapport aux systèmes (q)PCR mono-cibles actuels. Tous les tests qPCR développés pour le système de détection *CoSYPS Path Food* ont été conçus pour être exécutés simultanément sur la même plaque et ont tous été validés sur la base de deux directives existantes: une pour la validation des tests de PCR en microbiologie alimentaire (ISO 22118:2011) et l'autre pour la validation des analyses qPCR pour la détection des OGM, domaine dans lequel la qPCR est la méthode de détection de référence (lignes directrices ENGL).

Pour faciliter l'analyse et l'interprétation des résultats, un outil informatique de soutien a été développé. Cet outil contient toutes les informations sur les 11 tests qPCR (séquences des amorces, concentrations de chaque amorce...) ainsi qu'un calcul automatique des mixes PCR pour chaque test et un système support de décision (SSD) afin d'obtenir une analyse uniforme des résultats. Cette interprétation automatique des résultats de la qPCR garantit d'obtenir la même conclusion quel que soit l'analyste. Ensuite, afin d'appliquer ce système sur des matrices alimentaires qui peuvent contenir des bactéries mortes ou vivantes, une étude sur la discrimination de bactéries mortes et vivantes à l'aide de la « *viability-qPCR* » a été effectuée. Cette étude a montré les limites de la « *viability-qPCR* » et a permis de conclure qu'une étape d'enrichissement avant la qPCR était préférable à l'utilisation de la « *viability-qPCR* ». Au regard de ces résultats, la méthode complète *CoSYPS Path Food* a pu être développée. Elle comprend toutes les étapes de l'enrichissement de l'échantillon alimentaire jusqu'à l'isolement et la confirmation de la souche et a ensuite été validée, sur des prélèvements externes (éponge) de carcasse de bœuf. Ce type d'échantillon a été choisi comme modèle parce que la viande est une des sources les plus importantes de toxi-infection alimentaire et l'éponge de carcasse de bœuf, qui est un échantillonnage de la surface, est reconnue comme étant la technique d'échantillonnage la plus appropriée pour le contrôle microbiologique de la viande dans les abattoirs. La méthode complète *CoSYPS Path Food* a donc été validée sur ces éponges de carcasse de bœuf artificiellement contaminées avec un ou deux pathogènes alimentaires : *Salmonella* et *Listeria*, à différentes concentrations. La validation a été effectuée en comparant, selon la norme ISO 16140:2003, les résultats obtenus avec la méthode complète *CoSYPS Path Food* et ceux obtenus avec les méthodes conventionnelles de culture.

Pour conclure, grâce à sa stratégie multi-cibles, le système *CoSYPS Path Food* est un système fiable car un nombre inférieur de faux-négatifs est attendu par rapport aux systèmes de détection qPCR mono-cible. En outre, grâce à sa stratégie multi-niveaux, le système *CoSYPS Path Food* donne des informations supplémentaires sur les souches détectées (espèces, sous-espèces), qui peuvent orienter l'isolement et faciliter la récupération de la souche détectée dans l'échantillon. La méthode complète *CoSYPS Path Food* est aussi une alternative rapide aux méthodes conventionnelles basées sur la culture puisque les souches cibles sont détectées en deux jours alors que quatre à cinq jours sont nécessaires avec les méthodes classiques de culture. En outre, la méthode complète *CoSYPS Path Food* est aussi efficace que les méthodes conventionnelles puisque leurs limites de détection sont les mêmes, c'est-à-dire 4 à 16 UFC *Listeria*/éponge et de 2 à 11 UFC² *Salmonella*/éponge, même si les deux pathogènes sont présents en même temps dans des échantillons contenant beaucoup de microflore annexe. Par ailleurs, la méthode complète *CoSYPS Path Food* est modulaire et peut être adaptée à différentes matrices alimentaires et/ou d'autres pathogènes. La méthode complète *CoSYPS Path Food* est donc une bonne alternative aux méthodes de culture conventionnelles de détection des bactéries pathogènes dans l'alimentation.

² UFC: Unité Formant Colonie

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List of Acronyms

ABI:	A ppplied B iosystems
AFNOR:	A ssociation F rançaise de N ormalisation
AC:	Relative Accuracy
Acc. Num.:	Accession Number
ALC:	A ctive-labile nucleic acid-modifying compound
ALOA:	A gar <i>L</i> isteria according to O ttaviani and A gosti
ATCC:	A merican T ype C ulture C ollection
BDC:	B uoyant D ensity gradient C entrifugation
BHI:	B rain- H eart I nfusion liquid medium
BilE:	B ile exclusion system
BPW:	B uffered P eptone W ater
BSH:	B ile salt hydrolase
C.:	<i>Campylobacter</i>
CAMP:	C hristie, A tkins, M unch- P etersen test
cDNA:	complementary DNA .
CFU:	C olony F orming U nit
CoSYPS:	C ombinatory S YBR®Green q P CR S creening
CTD:	C alf T hymus DNA
Cq:	q uantification C ycle
DH:	D irect H ybridization
DNA:	D eoxyribonucleic acid
DSS:	D ecision S upport S ystem
E.:	<i>Escherichia</i>
E:	PCR efficiency
EFSA:	E uropean F ood S afety A uthority
EIC:	E xtraction and I nhibition C ontrol
ELFA:	E nzyme L inked F luorescent A ssay
ELISA:	E nzyme- L inked I mmunosorbent A ssay
EMA:	E thidium monoazide
ENGL:	E uropean N etwork of G MO L aboratories
EU:	E uropean U nion
FAE:	F ollicle-associated epithelium
FASFC:	F ederal A gency for the S afety of the F ood C hain

FRET:	F luorescent R esonance E nergy T ransfer
GAD:	G lutamate d eshydrogenase
gDNA:	g enomic DNA
GMO:	G enetically M odified O rganism
GPP:	G ödel P rime number P roduct
HACCP:	H azard A nalysis C ritical C ontrol P oints
<i>hlyA</i> :	Listeriolysin O encoding gene
IAC:	I nternal A mplification C ontrol
<i>iap</i> :	I nvasion a ssociated p rotein
IMS:	I mmunomagnetic s eparation
<i>inl</i> :	Internalin gene
<i>invA</i> :	Invasine A gene
IPH :	Institute of P ublic H ealth
<i>iroB</i> :	C-glycosyltransferase gene
ISO:	I nternational O rganization for S tandardization
<i>L.</i> :	<i>Listeria</i>
LAMP:	L oop-mediated isothermal a mplification
LDA:	L igation D ependent A mplification
<i>ldh</i> :	L actate d ehydrogenase gene
LLO:	L isteriolysin O
LOD:	L imit o f D etection
MIQE:	M inimum standard for the provision of information for q PCR experiments
MKTTn:	M uller- K auffman tetrathionate- n ovobiocine broth
NCBI:	N ational C enter for B iotechnology I nformation
NGS:	N ext- G eneration S equencing
NTC:	N o T emplate C ontrol
OpuC/	osmolyte transport system
PBB:	P latform B iotechnology and M olecular B iology
PCR:	P olymerase c hain r eaction
PI-PLC:	P hosphatidylinositol-specific p hospholipase C
<i>plc</i> :	Phosphatidylinositol-specific p hospholipase C gene
PMA:	P ropidium m onoazide
PP:	P eyer's P atch
<i>prfA</i> :	P ositive r egulator A gene
<i>prs</i> :	P hosphoribosyl pyrophosphate synthetase gene

qPCR:	Real-Time PCR
r:	Correlation coefficient
R ² :	Coefficient of determination
RASFF:	R apid A lert S ystem for F ood and F eed
RNA:	R ibonucleic acid
<i>rpoD</i> :	R NA P olymerase Sigma-70 factor gene
RSD _r :	R elative S tandard D eviation of repeatability
RSD _R :	R elative S tandard D eviation of R eproducibility
RT:	R everse T ranscriptase enzyme
RT-PCR:	R everse T ranscriptase PCR
RVS:	R appaport- V assiliadis S oja broth
S.:	<i>Salmonella</i>
<i>saf</i> :	<i>Salmonella</i> atypical fimbriae
<i>safC</i> :	Outer membrane usher protein gene
SCV:	S almonella-containing V acuole
SPI:	S almonella P athogenicity I sland
spp.:	species
SPR®:	S olid P hase R eceptacle® (Biomérieux)
STEC:	S higa toxin-producing <i>Escherichia coli</i>
subsp.:	subspecies
T3SS:	T ype III S ecretion S ystem
T _m :	m elting T emperature
Ttr:	T etrathionate reductase
U:	expanded uncertainty
U.V.:	U ltra V iolet
VIDAS®:	V itek I mmuno D iagnostic A ssay S ystem assays ® (Biomérieux)
YOPI	Y oung, O ld, P regnant and I mmunodepressed
WTO:	W orld T rade O rganization
XLD:	X ylose- L ysine- D eoxycholate medium

Part I: Introduction and Objectives

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Chapter I: Introduction

I. Foodborne pathogenic microorganisms

Food products contain microorganisms that can be divided in three groups, the beneficial, the spoilage and the disease-causing microorganisms. The last group is also called the foodborne pathogens. The beneficial microorganisms are those implicated in the food process such as fermentation leading to e.g. cheeses, yogurts. The spoilage microorganisms are those which alter the organoleptic characteristics of food products (odor, flavor, taste or appearance) but do not induce disease. The foodborne pathogens are microorganisms responsible for food intoxication (ingestion of preformed toxin), toxicoinfection (toxin produced into the host) or foodborne infection (ingestion of infective pathogens) (1).

A pathogen is able to replicate into its host, to breach or destroy cellular or humoral barriers, to establish itself into the host tissues and cause cellular damages by expressing virulent determinants and to be transmitted to a new susceptible host. The cellular damages result in clinical symptoms and can lead to morbidity (being diseased or unhealthy) or mortality (death). Pathogens are classified by their transmission pattern and movement among different hosts and vectors. They can be zoonotic, geonotic or anthroponotic. Zoonotic pathogens are characterized by transmission of infective agents from animals to humans; geonotic pathogens are transmitted by soil, water or decaying plant origin while the anthroponotic pathogens are transmitted from human-to-human, or from human to animal (rare) (1).

Although foodborne pathogenic microorganisms can belong to the three categories, zoonotic pathogens such as *Salmonella* spp. and *Listeria monocytogenes* are those responsible for most of the public health problems (2).

A. *Salmonella*

Salmonella was firstly isolated from a pig in 1885 by Theobald Smith, an assistant of Daniel E. Salmon. The genus *Salmonella* was therefore named after him (3).

1) Biology

Salmonella is a group of Gram-negative, rod shape, motile (peritrichous ciliature) (except Pullorum and Gallinarum serovars), and facultative anaerobes bacteria. They can grow between 5 and 45°C with an optimum temperature between 35 and 37°C.

They are able to grow at low pH and are generally sensitive to high salt concentrations (4). The different species of *Salmonella* have different biochemical characteristics, as listed in Table 1.

Table 1: Differential characters of *Salmonella* species and subspecies. Reprinted from Grimont et Weill, 2007 (5)

Species	<i>S. enterica</i>						<i>S. bongori</i>
Subspecies	<i>enterica</i>	<i>salamae</i>	<i>arizonae</i>	<i>diarizonae</i>	<i>houtenae</i>	<i>indica</i>	
Dulcitol	+	+	-	-	-	V	+
ONPG (2 h)	-	-	+	+	-	V	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth with KCN	-	-	-	-	+	-	+
L(+)-tartrate(a)	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-glutamyltransferase	+(*)	+	-	+	+	+	+
β-glucuronidase	V	V	-	+	-	V	-
Mucate	+	+	+	- (70%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	- (75%)	+	-	V	-
Lysed by phage O1	+	+	-	+	-	+	V
Usual habitat	Warm-blooded animals		Cold-blooded animals and environment				

(a) d-tartrate; (*) except Typhimurium: d and Dublin: -; +:90 % or more positive reactions; -: 90 % or more negative reactions; v: different reactions given by different serovars.

2) Classification

Salmonella belongs to the *Proteobacteria* phylum, the *Gamma-proteobacteria* class, the *Enterobacteriales* order and the *Enterobacteriaceae* family (6). The genus *Salmonella* includes 2 species namely *S. enterica* and *S. bongori*, which are both pathogenic for human (7). *Salmonella enterica* is divided in 6 subspecies: *S. enterica* subsp. *enterica* (subsp. I), *salamae* (subsp. II), *arizonae* (subsp. IIIa), *diarizonae* (subsp. IIIb), *houtenae* (subsp. IV) and *indica* (subsp. VI) (8). To date, the *Salmonella* genus is composed of 2,610 serovars unequally distributed since 1,547 belong to *S. enterica* subsp. *enterica* (7). All serovars can be grouped, in the White-Kauffman-Le Minor classification scheme, into 45 O-groups based on their somatic (O) antigenic pattern (5). In this classification, each serovar is further characterized by its flagellar 1 (H1) and flagellar 2 (H2) antigenic patterns. The antigenic formula of each serovar is displayed as: O antigen : H1 antigen : H2 antigen.

3) Salmonellosis

Salmonella-related human diseases can be divided into three groups with regard to their clinical patterns, namely, nontyphoidal salmonellosis (gastroenteritis), typhoid and paratyphoid fever (enteric fever) and bacteremia (9).

a) Nontyphoidal salmonellosis (gastroenteritis)

Nontyphoidal salmonellosis is caused by at least 150 serovars of *S. enterica enterica* (9) from which Typhimurium and Enteritidis are the most frequent (2;9). The incubation period depends on the dose of bacteria. Symptoms usually begin 6 to 24 hours after ingestion of contaminated food or water (10). In healthy humans, the nontyphoidal salmonellosis symptoms are: moderate fever, nausea, vomiting, headache, chills, bloody or non-bloody diarrhea, abdominal pain (resembling appendicitis), muscular weakness, muscle pain, and faintness. These symptoms are self-resolving in healthy individuals within 3-4 days (4). In children and immunocompromised individuals, the disease is more serious with a possible systemic infection. The infectious dose varies widely, from 1 to 10^7 CFU/g depending on the host (age, medication and immune status), the *Salmonella* serovar and the type of food. The infection dose also decreases in liquid products that traverse stomach rapidly and with food that neutralize stomach acidity e.g. cheese or milk (4).

b) Typhoid and paratyphoid fever (enteric fever)

S. enterica enterica Typhi and Paratyphi A, B and C cause typhoid and paratyphoid fevers, respectively. These serovars are not transmitted by food but mainly by drinking water tainted by urine and feces of infected individuals (human origin pathogens) (9).

c) Bacteremia

Around 8% of untreated nontyphoidal salmonellosis result in bacteremia, which is the presence of a bacteria into the bloodstream. Bacteremia is associated with highly invasive serovars (e.g. Choleraesuis or Dublin) which enter the bloodstream after passing through the intestinal barrier. Salmonellosis bacteremia causes fever (9).

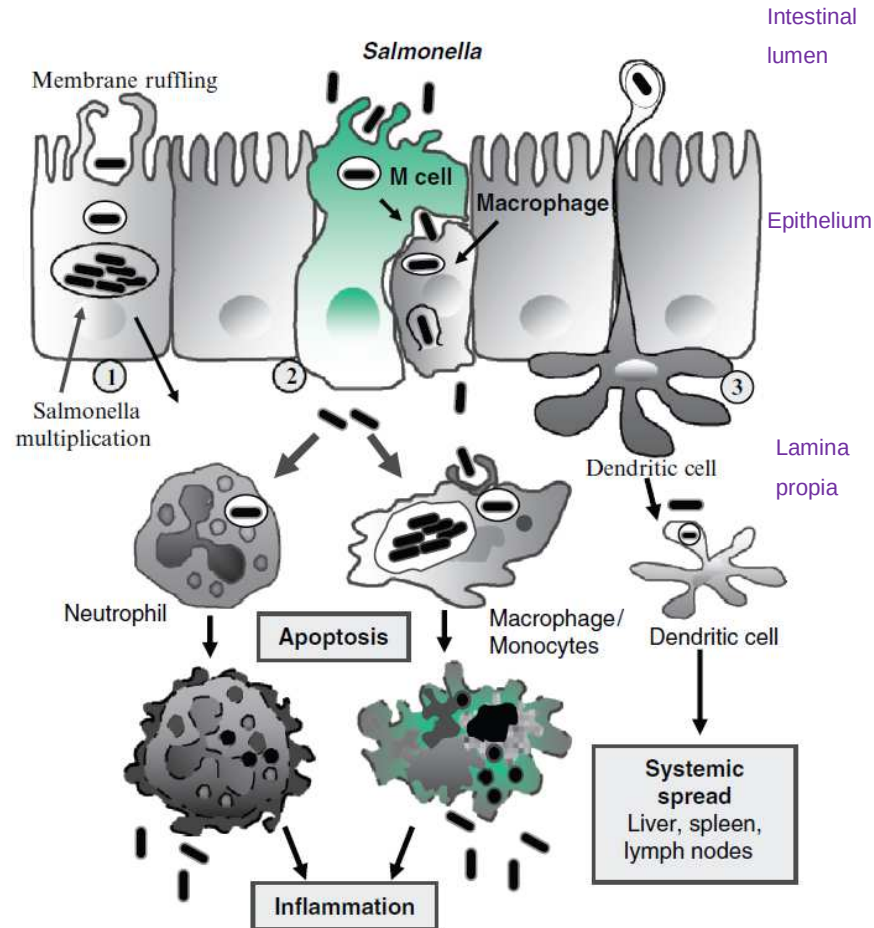


Figure 1: *Salmonella* spp. invasion through host mucosal membrane.

Salmonella spp. translocate by three different pathways (1) they induce membrane ruffling by triggering mechanism to allow their own uptake and multiply inside the vacuole; (2) they translocate through M-cells to reach subcellular location; and (3) they are taken up by dendritic cells from the lumen and transported to subcellular location. They also multiply inside phagocytic cells such as macrophages, neutrophils and dendritic cells and induce apoptosis and promote inflammation. Dendritic cells are responsible for systemic dissemination of bacteria to lymph nodes, liver and spleen. Reprinted and modified from Bhunia, 2008 (4).

4) Pathogenic mechanisms

Salmonella spp. are facultative intracellular pathogens. They can invade macrophages, dendritic and epithelial cells. They also adhere to intestinal epithelium cells or M-cells via fimbrial adhesion. The bacterium uses its type III secretion system (T3SS) to induce its uptake (11).

M-cells constitute a small subset of the highly specialized follicle-associated epithelium (FAE) overlying the Peyer's patches (PP). The PPs (or aggregated lymphoid nodules) are organized lymphoid nodules found especially in the lowest portion of the small intestine i.e. the ileum.

PPs are responsible for the immune surveillance of the intestinal lumen and generate the immune response within the mucosa. M-cells and dendritic cells ingest luminal microbiota to maintain intestinal homeostasis. This process allows *Salmonella* spp. to cross the epithelial barrier. In the lamina propria, they are engulfed by resident dendritic cells or macrophages and replicates inside host cells or induce apoptosis (Figure 1) (4). During infection, *Salmonella* spp. are also inducing the increase of the number of M-cells into the FAE to promote host colonization and invasion (12).

Salmonella spp. contain an arsenal to establish a successful infection of the host. This arsenal is encoded by virulence genes that are clustered in large regions of the bacterial chromosome (so far 21) called *Salmonella* pathogenicity islands (SPI). (13). These pathogenicity island (PAI), common in Gram-negative bacteria, have generally a GC content lower than the rest of the bacterial chromosome (14). The most important genes are located on 12 SPIs (Table 2) (4). While some SPIs are conserved through the genus, some SPIs are thought to be acquired by horizontal gene transfer and some are specific of certain serovars. The known virulence genes responsible for intestinal infection are located on SPI-1 and SPI-2. The SPI-2 and the other SPI are responsible for systemic infection, intracellular survival, fimbrial expression and $MgCl_2$ or iron uptake (4). SPI-1 and SPI-2 are of particular importance since mutations into these SPIs impair the bacteria's ability to induce infection (15).

a) SPI-1

SPI-1 contains 31 genes and is present in all serovars of *S. enterica* and *S. bongori* (16). SPI-1 is important for intestinal phase of *Salmonella* infection by encoding a specialized secretion system, the Type III Secretion System-1 (T3SS-1) (15). T3SS-1

is also called molecular syringe and permit contact-dependent secretion or delivery of virulence (effector) proteins into the host cell. This apparatus is present in *Salmonella* spp. as well as in *Shigella* spp. and *Escherichia coli*. The T3SS-1 is formed on the bacterial envelope and contains four parts: the needle, the outer rings, the neck and the inner rings (4) (see structure in Figure 2). The majority of the SPI-1 genes are expressed under intestinal environment conditions and repressed when the bacterium reaches the intracellular compartment (17). SPI-1 effector proteins also influence several host cell functions such as cytoskeletal rearrangement (e.g. ruffling), that mediate bacterial uptake by epithelial cells (15), apoptosis of macrophage (18) and neutrophil recruitment (19).

Table 2: Description of *Salmonella* spp. pathogenicity islands (SPI) (reprinted and modified from Bhunia, 2008 (4))

Islands	<i>Salmonella</i> serovars	Length (kb)	GC (%)	Function
SPI-1	<i>Salmonella enterica</i> and <i>S. bongori</i>	43	47	T3SS-1 (15), invasion, iron uptake
SPI-2	<i>S. enterica</i>	40	44.6	T3SS-2, invasion, systemic infection (20)
SPI-3	<i>S. enterica</i> and <i>S. bongori</i>	17	39.8-49.3	Mg ²⁺ uptake: macrophage survival (21), T5SS (10)
SPI-4	<i>S. enterica</i> and <i>S. bongori</i>	27	37-54	Non fimbrial adhesion, type 1 secretion system (22), Macrophage survival
SPI-5	<i>S. enterica</i> and <i>S. bongori</i>	7.6	43.6	Enteropathogenicity
SPI-6	<i>S. enterica</i> subspecies <i>enterica</i> serovars	59	51.5	Fimbriae (23)
SPI-7	Serovars Typhi, Dublin, Paratyphi	133	49.7	Vi antigen
SPI-8	Serovar Typhi	6.8	38.1	Unknown
SPI-9	<i>S. enterica</i> and <i>S. bongori</i>	16.3	56.7	Type I secretion system (24) and RTX-like toxin
SPI-10	Serovars Typhi and Enteritidis	32.8	46.6	Sef fimbriae
HPI	<i>S. enterica</i> subspecies IIIa, IIIb, IV	?	?	High affinity iron uptake, septicemia

SPI: *Salmonella* Pathogenicity Island; HPI: High Pathogenicity Island; T3SS: Type III secretion system.

b) SPI-2

SPI-2 is essential for intracellular survival and intracellular replication of *Salmonella* spp. The expression of SPI-2 is activated once the bacterium is inside the host cell. SPI-2 contains 44 genes distributed into two distinct regions (25). The first one is only present in *S. enterica* and encodes a second T3SS (T3SS-2) used to deliver the virulence effector from the *Salmonella*-containing vacuole (SCV) to the cytoplasm of the host cells (Figure 2). The second part of the SPI-2 is present in *S. enterica* and *S. bongori*. It encodes the tetrathionate reductase (Ttr) required for anaerobic respiration (20).

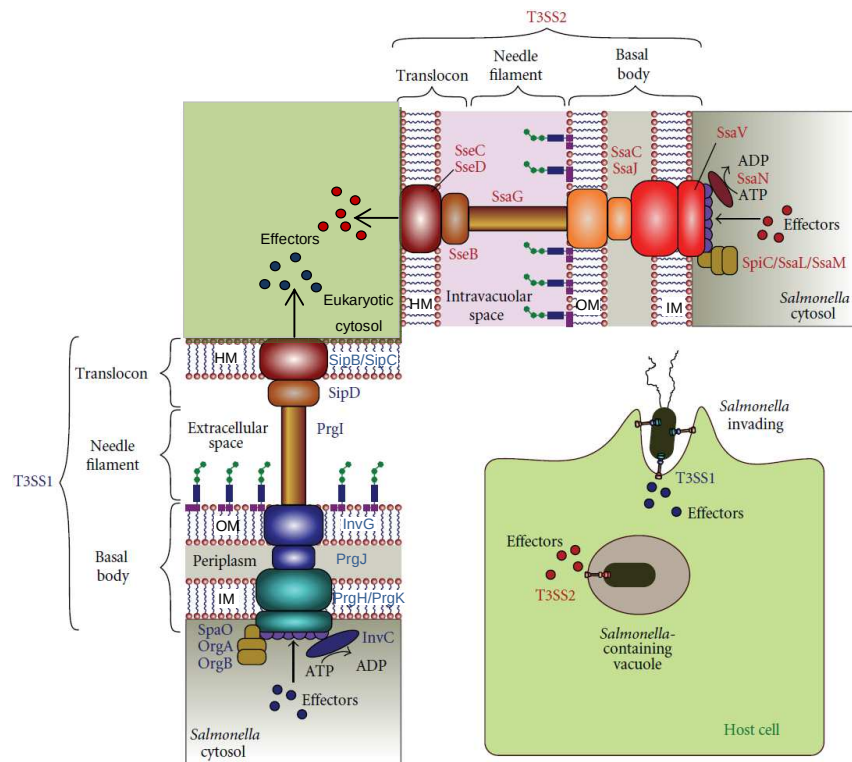


Figure 2 : Schematic representation of *Salmonella* T3SS-1 and T3SS-2. Reprinted and modified from Ramos-Morales, 2012 (26).

Virulence proteins (effectors) are translocated through T3SS-1 or T3SS-2 at the different stage of invasion. T3SS-1 forming proteins are encoded by SPI-1 for host cell invasion, SPI-1 genes expression is induced under intestinal environment condition. T3SS-2 forming proteins are encoded by SPI-2 for secretion of the effectors from the *Salmonella*-containing vacuole to the cytosol of the eukaryotic host cell.

HM: host plasma membrane; VM: vacuolar membrane; OM: outer membrane; IM: inner membrane. Inv: Invasion protein; Org: Oxygen-regulated protein; Prg: PhoP-repressed protein; Sic: *Salmonella* invasion chaperone; Sip: *Salmonella* invasion protein; Spa: Surface presentation of antigen; Spi: *Salmonella* putative pathogenicity island 2 secreted effector protein; Ssa: Secretion system apparatus protein; Sse: Secretion system effector protein.

5) Reservoirs and contamination routes

Salmonella is found in the environment and the gastrointestinal tract of wild and farmed animals. Animals may become infected by *Salmonella* through environment, other animals or contaminated feed. Both animals and humans can be *Salmonella* reservoirs (27). The subspecies *enterica* (I) is the most often found in humans and farmed animals, mainly poultry, but also cattle, swine and sheep (9). Infected cattle, sheep and horses may present fever, diarrhea and abortion whereas pigs, goats and poultry usually show no obvious sign of infection (2). *Salmonella bongori* and *Salmonella enterica* subsp II, IIIa, IIIb, IV, VI and VII are rarely found in Salmonellosis cases since they are associated with cold-blood invertebrates (28;29). Transmission to human is caused by the consumption of contaminated food such as meat, raw eggs and cheese, and in a less extent, waterborne transmission and numerous environmental, animal exposure and person-to-person transmission (28). Patients recovering from non-typhoidal salmonellosis may serve as chronic carriers and shed *Salmonella* for 6 weeks to 3 months depending on the serovar. Only 0.1% shed it in stool samples for more than 1 year (9).

6) Sporadic cases and outbreaks

Worldwide there are 16 million of annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths due to *Salmonella* (4). In Europe, *Salmonella* is the first cause of notification of foodborne contamination due to microorganisms into the Rapid Alert System for Food and Feed (30) and the first detected causative agent in reported foodborne outbreaks, representing ca. 30 % of the total number of reported outbreaks and caused almost 100,000 confirmed human cases in Europe in 2012 (2). Typhimurium and Enteritidis are the most often involved serovars. Eggs and egg products are the most important food vehicles of *Salmonella* responsible for 45.2 % of outbreaks due to *Salmonella* (2). Outbreaks caused by Enteritidis serovar are mainly occurring via eggs and egg products (62.6%) whereas outbreaks caused by Typhimurium are mainly occurring via pig meat and product thereof (24.5%) (2). In Belgium, during summer 2005, a *Salmonella* outbreak involving Ohio serovar occurred. The outbreak concerned 60 people from all age groups and caused self-limiting gastroenteritis. Pork meat was identified as the source of the contamination (31).

B. *Listeria*

Listeria monocytogenes was firstly isolated as *Bacterium monocytogenes* in 1924 by E.G.D. Murray, from an outbreak of sudden death of laboratory young rabbits (32). In 1940, the genus was renamed *Listeria* in the honor Dr. Joseph Lister (33). It is only in 1981, in Halifax, Canada that *Listeria monocytogenes* was recognized as a foodborne pathogen with an outbreak, linked to coleslaw consumption, involving pregnant women and neonates. The cabbage was fertilized with *Listeria monocytogenes*-contaminated sheep manure (34).

1) Biology

Listeria spp. is a group of Gram-positive, small bacilli, ubiquitous, non-spore forming, and facultative anaerobic bacteria. These psychrophilic bacteria grow between -2 to 50°C with an optimal growth between 30 and 37°C (35;36). Thanks to their peritrichous flagella, they display a tumbling motility. Flagella expression is temperature dependent i.e. maximum expression between 4 and 30°C and minimum at 37°C (37). *Listeria* spp. are ubiquitous in nature and survive in extreme conditions such as broad pH (4.1-9.6), high salts (10 %) and in presence of antimicrobial agents (37). The different *Listeria* species display distinct biochemical characteristics, as reported in Table 3.

Table 3 : Biochemical characteristics of *Listeria* species. Reprinted and modified from ISO 11290-1:1996 (38).

	<i>L. monocytogenes</i>	<i>L. innocua</i>	<i>L. ivanovii</i>	<i>L. seeligeri</i>	<i>L. welshimeri</i>	<i>L. grayi</i>
Motile	+	+	+	+	+	+
Haemolysin	+	-	+	(+)	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-SS
L-Rhamnose	+	+/-	-	-	+/-	+/-
D-Xylose	-	-	+	+	+	-
Mannitol	-	-	-	-	-	+
CAMP test on <i>S. aureus</i>	+	-	-	(+)	-	-
CAMP test on <i>R. equi</i>	-	-	+	-	-	-

+: >90% positive; -: no reaction; (+): weak reaction; +/-: variable reaction.

2) Classification

The genus *Listeria* belongs to the *Firmicute* phylum, the *Bacilli* class, the *Bacilliales* order and the *Listeriaceae* family (6). The genus *Listeria* officially include 6 species *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (6). Four other species, *L. marthii* (close to *L. monocytogenes* and *L. innocua* (39)), *L. rocourtiae*, *L. fleischmannii* and *L. weihenstephanensis* (the last three close to *L. grayi* (39)) have been described recently (40-43). These four new species have not yet been introduced in the official classification. Human listeriosis are almost exclusively caused by *L. monocytogenes* (2), which contains 13 distinct somatic (O)-antigenic types (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7) and 5 flagellar (H)-antigenic types (A, B, C, D and E). *L. monocytogenes* are also grouped in three lineages based on their genomic fingerprint patterns and their association with outbreaks as described in Table 4. The serotypes involved in human listeriosis are mainly 1/2a and 4b, representing ca. 90% followed by 1/2b ca. 8% and, to a lesser extent, 1/2c and 3a (2). Serotypes 1/2a, 1/2b and 1/2c are adapted to food processing plants and are frequently isolated from these environments (44).

Table 4: Classification of *L. monocytogenes* based on genomic fingerprint patterns and association with epidemic outbreaks. Reprinted from Bhunia, 2008 (37).

Groups	Outbreaks	Pathogenic potential	Subgroups	Predominant serotypes
Lineage I	Epidemic clones and Responsible for most outbreaks	High	-	1/2b, 3b, 4b, 4d, 4e
Lineage II	Sporadic listeriosis cases	Medium	-	1/2a, 1/2c, 3c, 3a
Lineage III	Rarely cause human disease	Low	IIIA (Rham +ve)	4a (avirulent) and 4c (virulent)
			IIIB (Rham -ve)	Virulent nonserotype 4a and nonserotype 4c; Serotype ??
			IIIC (Rham -ve)	Virulent 4c

Rham rhamnose fermentation property.

3) Listeriosis

Among these species, *L. monocytogenes* is from far the most pathogenic for human (45) (listeriosis), even though few cases of illness have also been attributed to *L. ivanovii* (46-48), *L. innocua* (49), *L. seeligeri* (50) and *L. grayi* (51;52).

Worldwide, foodborne listeriosis is a relatively rare but serious disease. About 1,500 cases per year are recorded in Europe and 2,500 per year in US (2;37). However, because of its high fatality rate (between 20 and 30 %), listeriosis ranks among the

most frequent cause of human death due to foodborne illnesses in USA and in Europe (2;53;54). Foodborne listeriosis mainly affects a specific group of the population with increased susceptibility, the YOPI (young, old, pregnant and immunodepressed) (36). Lethality in immunodepressed, elderly and among patients suffering from central nervous system infections is higher i.e. 38 to 45% (45;55). Since these populations, mainly elderly, are in constant increase in our countries, the control of *Listeria* spp. is an important issue. In case of infection, it is difficult to identify the source since *Listeria* spp. has a long latency period of 1 to 91 days regarding the immune status of the host and the number of cell ingested (36). The infective dose is unknown but estimated to be between 100 and 10^6 cells depending on the immune status of the host (37). Listeriosis can take three different forms with regard to the host: i) gastrointestinal listeriosis, ii) systemic listeriosis and iii) abortion and neonatal listeriosis.

a) Gastrointestinal listeriosis

The gastrointestinal listeriosis targets more the adults than the children. It affects healthy adults who have ingested a large number of cells or people receiving gastric acid suppressive medication (37). The symptoms are fever, headache, nausea, vomiting, abdominal pain and watery diarrhea (37).

b) Systemic listeriosis

The systemic listeriosis is a rare but often fatal disease. It is affecting YOPI people, which have a weaker immune system. *L. monocytogenes* colonizes the intestine for a short period of time and reaches the blood circulation and the lymphatic circulation by passing the intestinal barrier. The symptoms of systemic listeriosis are: fever, headache, malaise, septicemia, meningitides, brain stem encephalitis, ataxia, bacteremia and liver abscess (37). Twenty-four hours after ingestion, *L. monocytogenes* is mainly targeting the liver (90%) and to a lesser extent the spleen (10%), the gall bladder and the lymph nodes region. After a brief bacteremic phase, *L. monocytogenes* crosses the brain barrier and infects the brain causing meningitis and encephalitis (37).

c) Abortion and neonatal listeriosis.

Pregnancy is associated with suppression of cell-mediated immunity to protect the fetus from rejection which renders pregnant women highly susceptible to listeriosis. The first symptoms are flu-like and gradually progress to a severe headache.

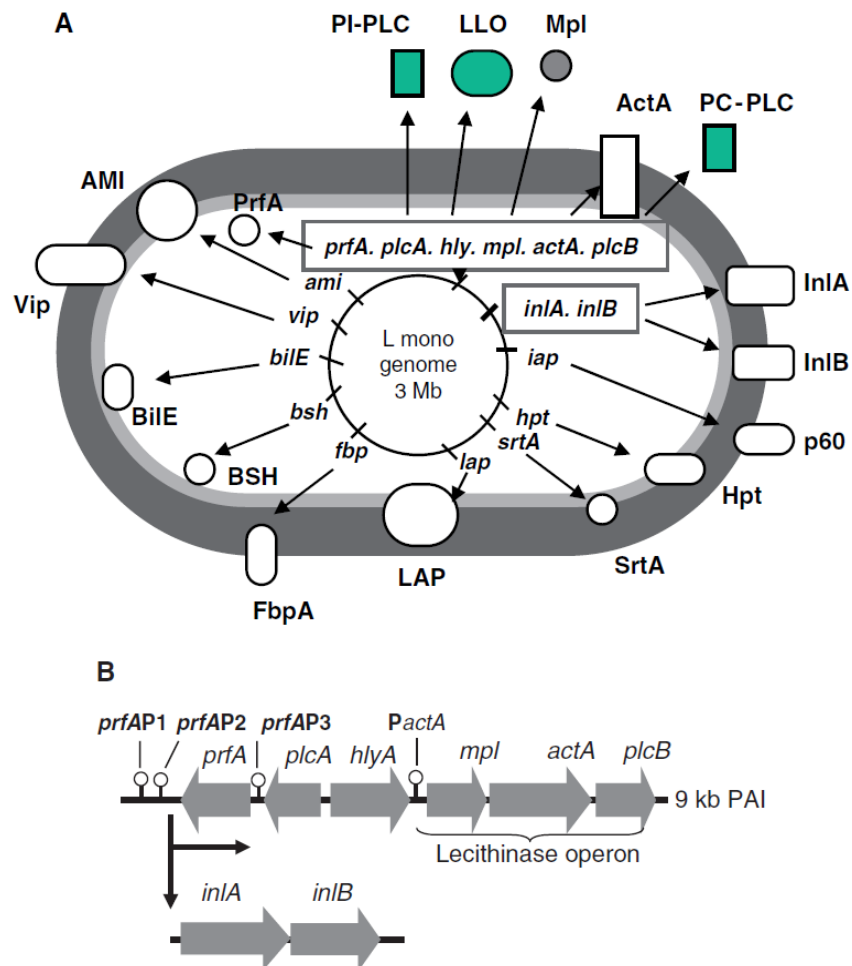


Figure 3: Schematic representation of the virulence genes and location of their products involved in *L. monocytogenes* infection. Reprinted from Bhunia, 2008 (37). **A.** Approximate location of *L. monocytogenes* virulence genes on the chromosome and the location of gene products in the extracellular milieu, cell wall (dark shade) and cytoplasmic membrane (light shade). **B.** *L. monocytogenes* Pathogenicity Island representation (upper scheme) and internalin operon (lower scheme). Open circles represent promoters.

Intrauterine infection is often occurring in systemic listeriosis of pregnant women. Moreover, *L. monocytogenes* is able to cross the placental barrier and infect the fetus. In the early stages of pregnancy, listeriosis usually leads to spontaneous abortion. In the following stage of pregnancy, *L. monocytogenes* infection can lead to miscarriage, stillbirth, premature delivery, or infection of the newborn (37).

4) Pathogenic mechanisms

L. monocytogenes is an opportunistic intracellular pathogen. It produces numerous virulence factors that ensure its survival in its host (4). *L. monocytogenes* is pathogenic for both humans and animals without showing significant host specificity. The majority of virulence genes are clustered on the *L. monocytogenes* pathogenicity island 1 (LIPI-1) (56). This LIPI-1 is a 9 kb fragment containing 6 genes (*prfA-plcA-hlyA-mpl-actA-plcB*) (Figure 3). The first gene of the LIPI-1, namely positive regulator A (*prfA*), is regulating other virulence genes located on chromosomal region distinct from LIPI-1, i.e. internalin genes (*inlA* and *inlB*), *hpt* and *bilE* (37).

Most of the infections by *L. monocytogenes* are foodborne related. After ingestion, *L. monocytogenes* passes through the stomach and reaches the small intestine. *L. monocytogenes* has a set of protective tools: the glutamate deshydrogenase (GAD) system protecting it from gastric acid, the bile salt hydrolase (BSH) and the bile exclusion system (BilE) protecting it against bile salts and the osmolyte transport system (OpuC) providing it an osmotolerance (37). The cellular mechanism of *L. monocytogenes* pathogenicity involves four steps: i) attachment and entry of the host cells; ii) survival into vacuole (phagosome) and vacuole lysis; iii) intracellular replication and iv) cell-to-cell spread (37).

a) Attachment and entry of the host cells

After passing the stomach, *L. monocytogenes* reaches the small intestine, pierces through the mucosal layer and breaches the intestinal barrier by three possible pathways: i) through M-cells overlying Peyer's Patches (PP), ii) through epithelial cells and iii) through dendritic cells sampling the lumen intestine. As M-cells and dendritic cells are phagocytosing *L. monocytogenes*, it is a passive process while, invasion of epithelial cell is an active process. Listerial adhesion factors i.e. internalin A and B (IntA and IntB) are the surface proteins which are involved in the invasion of host cells. IntA interacts with epithelial cadherin (E-cadherin) to promote invasion of intestinal and uteroplacental epithelial cells (maybe also brain barrier) (37).

E-cadherin is a transmembrane glycoprotein located at the junction or at the basolateral face of epithelial cells. It is thought that E-cadherins are exposed to IntA during renewal of intestinal villous epithelial cells (37). IntB binds to the Met receptors of the host cells. This binding induces a series of enzymatic reaction (see Figure 4) leading to bacterial entry by zipper mechanism. IntB induces invasion of hepatocytes and endothelial cells.

The *L. monocytogenes* adhesion protein (LAP) expression is enhanced by nutrient-limiting and anaerobic environment. LAP promotes translocation during intestinal phase of infection by interacting with eukaryotic receptor Hsp60 (37). The autolysin amidase (Ami) is an autolytic enzyme, which serves as adhesion protein. Ami has an autolytic action (37).

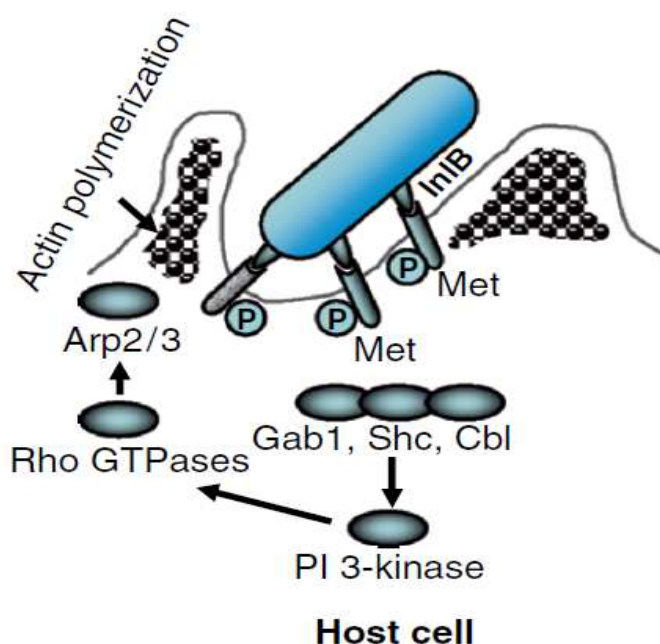


Figure 4: Schematic drawing of *L. monocytogenes*- induced entry into cell by zipper mechanism. Reprinted from Bhunia, 2008 (10).

In zipper mechanism, listerial IntB interacts with Met receptors, which autophosphorylates and recruits protein adapters: Gab1, Cbl, and Shc. These proteins activate phosphatidylinositol 3-kinase (PI 3 kinase) and small Rho GTPase kinase, which in turn promotes actin polymerization via Arp2/3.

The p60 is a major extracellular protein encoded by the invasion-associated protein (*iap*) gene and p60 is present in all *Listeria* spp., but heterogeneous among species (37). The *iap* expression is not regulated by *prfA*.

The virulence protein (Vip) is a surface protein responsible for the invasion of host cells during intestinal and later phases of infection. Vip binds to the cellular receptor Gp96 of the endoplasmic reticulum (37). Five fibronectin-binding proteins (Fbp) bind to fibronectin present in plasma, in extracellular fluids and cell surface. Fbp contributes to the adhesion to human epithelium cells (37).

Lipoteichoic acid (LTA), a structural component of Gram-positive cell wall, is also involved into *L. monocytogenes* adhesion and virulence (37). Lipoprotein promoting entry (LpeA) is a listerial cell surface-exposed lipoprotein required for invasion of nonprofessional phagocytic cells (37).

b) Survival into vacuole (phagosome) and vacuole lysis

After entry the host cell, *L. monocytogenes* is trapped into a vacuole (phagosome), from which is able to escape using the listeriolysin O (LLO) encoded by *hlyA* gene and the phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme encoded by *plcA* gene. Both *hlyA* and *plcA* genes belonging to LIPI-1 are regulated by *prfA* (37). PI-PLC and LLO works synergically to destroy the lipid bilayer membrane of the phagosome allowing *Listeria* to escape (37) (Figure 5).

*c) Intracellular replication of *L. monocytogenes**

After escaping the phagosome, *L. monocytogenes* is multiplying before enhancing the cell-to-cell spread.

d) Cell-to-cell spread

Actin polymerization protein (ActA) initiates actin polymerization creating the actin-comet tail of *Listeria monocytogenes* (57). ActA is the only bacterial protein required for actin-based movement of *Listeria monocytogenes* (37). The *actA* gene belongs to LIPI-1 and is under the regulation of *prfA*. *actA*-mutants are defective in infection of the adjacent cells (37). The comet tail induce movement of *Listeria* both within the cytoplasm of the host cells and from one host cell to the next (57). When the bacterium reaches the host cell membrane, a pseudopod-like protruding structure is formed. Little is known about the mechanism of *Listeria* cell-to-cell spread.

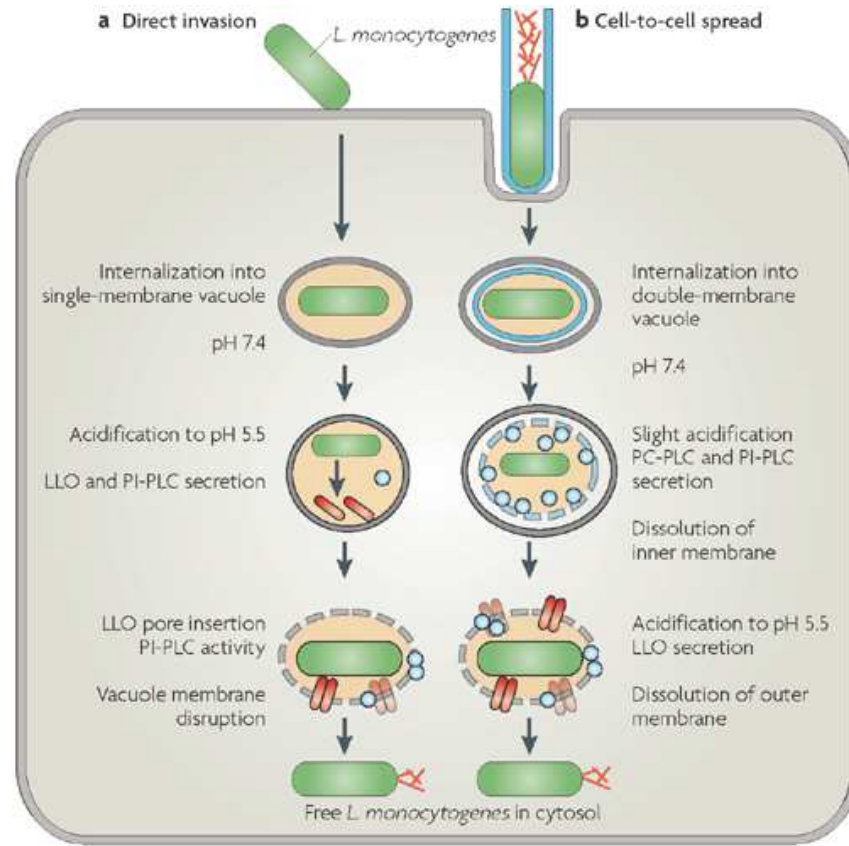


Figure 5: *L. monocytogenes* vacuole escape into cytosol of the host cell under the two different invasion conditions. Reprinted from Hybiske and Stephens, 2008 (58).

a. After direct invasion of host cells, *L. monocytogenes* is stuck into a monomembrane vacuole. The acidification of the vacuole to a pH of 5.5 induces secretion of the listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) by *L. monocytogenes*. LLO inserts into the vacuole membrane giving access of PI-PLC to the membrane leaflets leading to membrane destruction and the escape of *L. monocytogenes* into the cytosol of the host cell. **b.** After invasion via cell-to-cell spread, *L. monocytogenes* is stuck into a bi-membrane vacuole. Slight acidification of the vacuole induces secretion of phosphatidylcholine-specific phospholipase (PC-PLC) and PI-PLC dissolving the inner membrane of the vacuole. The vacuole acidification to a pH of 5.5 induces secretion of LLO. LLO and PI-PLC induce outer membrane disruption as in the direct invasion strategy.

It is thought that bacteria take advantage of the normal paracytophagic behavior (engulfment of portions of neighboring cells) to induce cell-to-cell spread (59). Into the neighboring cell, the engulfed *Listeria* forms a bimembrane vacuole. The outer membrane is formed from the newly infected cell and the inner membrane from the previously infected cell.

Listeria is able to escape from this cell-to-cell spread forming vacuole by secreting the phosphatidylcholine-specific phospholipase C (PC-PLC), encoded by *plcB* gene, and the PI-PLC which enhance dissolution of the inner membrane. Then, LLO is secreted and with PI-PLC they induce dissolution of the outer membrane. This whole mechanism permit *Listeria* to escape from the cell-to-cell spread forming vacuole (58) (Figure 5).

5) Reservoirs and contamination routes

L. monocytogenes is a ubiquitous bacterium, found in soil, forage, water, plants and intestinal tract of domestic animals (cattle, sheep, goats, etc.) (37). The main route of transmission to humans is consumption of contaminated food (e.g. meat, vegetables, fish, etc.) (2). *Listeria* spp. are not thermo-resistant. Moreover, the characteristic of *Listeria* spp. to grow at low temperature (until -2°C (34)) increases the risk when the contaminated food is conserved at low temperature. Most of listeriosis cases are associated with the consumption of unprocessed food product such as raw milk, vegetables (60), fish (61), seafood (55;62), raw meat (62;63), ready-to-eat food (63) and post-processing contaminated food (64-66). One to five percent of healthy humans are reported as carriers able to transmit *L. monocytogenes* to other humans via fecal-oral route, however, this contamination route is expected to be really rare due to the low transmitted dose.

6) Sporadic cases and outbreaks

Since *Listeria* is an opportunistic pathogen (affecting sensitive peoples) sporadic cases are more often occurring than outbreaks. Around 1,500 cases per year of listeriosis are recorded in EU. In 2012, only five outbreaks related to *Listeria* have been reported in EU counting 55 patients (47 hospitalization and 9 deaths) (2). However, the high level of hospitalization (91.6 %) and mortality (17.6%) makes *L. monocytogenes* one of the most important foodborne pathogen (2). In Belgium, a *Listeria* outbreak related to hard cheese made with pasteurized milk occurred in 2011 involving 12 human cases, with 100% hospitalization and four fatalities (33%) (67).

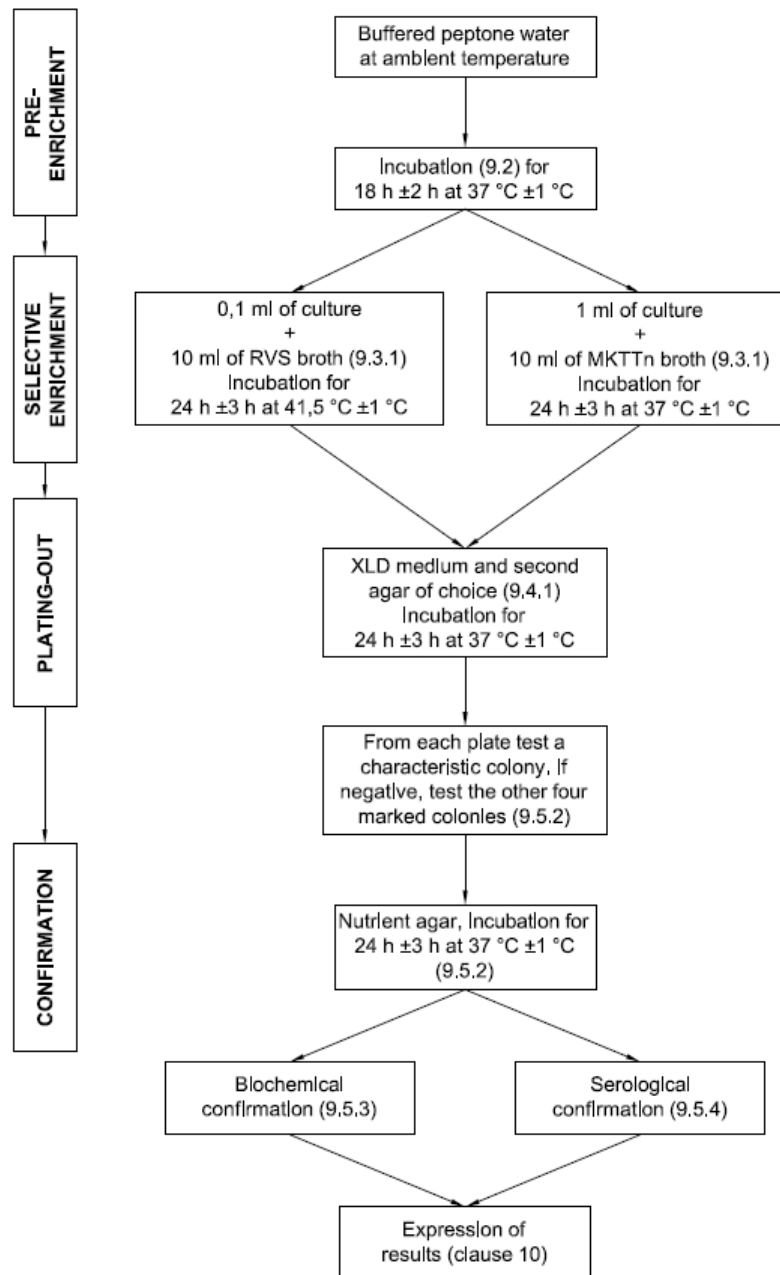


Figure 6: Flow diagram of the ISO 6579 procedure for detection of *Salmonella* spp. in food and feed products. Reprinted from ISO 6579:2002 (68).
 RVS: Rappaport-Vassiliadis; MKTTn: Muller-Kauffman Tetrathionate-novobiocine; XLD: Xylose-Lysine-Deoxycholate. See text for details.

II. Detection strategies for foodborne *Salmonella* and *Listeria* spp.

In order to be able to protect the consumer against *Salmonella* and *Listeria* infections due to ingestion of contaminated foodstuffs, surveillance of the food market is performed (see part III for regulation details) using target specific detection assays. To be considered as contaminated, the pathogen presence should be detected and the strains should be isolated and confirmed biochemically (and serologically). Beside the standard culture-based method for *L. monocytogenes* and *Salmonella* spp. detection in food and feed (38;68), several alternative strategies for a faster detection have been developed. In this section, the past, present and future detection technologies are presented.

A. Culture-based detection methods

The culture-based methods are the reference approaches (gold standards) and have to be used by the National Reference Laboratories (NRL) for foodstuff control. They are relatively easy to implement and cheap in consumables but they are time consuming and labor intensive. The standard culture method ISO 6579:2002 [*Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Salmonella spp*] (68) and ISO 11290-1:1996 [*Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of L. monocytogenes - Part 1: Detection method*] (38;69) are horizontal methods, meaning that they can, theoretically, be applied to all food categories and feed matrices. All culture-based methods followed the same successive steps which take around one day each:

1. Primary enrichment in moderately or non-selective liquid medium,
2. Secondary enrichment in fully selective liquid medium,
3. Isolation of strains on selective plates and identification,
4. Biochemical (and serological) confirmation of the suspected strains.

1) Culture-based *Salmonella* spp. detection method

The entire flow of the ISO 6579:2002 for detection and confirmation of *Salmonella* spp. into food and feed products procedure is presented in the diagram displays in Figure 6. In brief, a subsample of the tested sample (max 25g) is diluted ten times in buffered peptone water (BPW) and incubated for around 18h. This pre-enrichment allows stressed bacteria to recuperate. Then, aliquots of this non selective pre-enrichment are inoculated into selective broths, namely Rappaport-Vassiliadis Soja

(RVS) and Müller-Kauffman tetrathionate-novobiocine (MKTn) for the specific outgrowth of *Salmonella*. MKTn broth contains i) bile salts and brilliant green inhibiting Gram-positive bacteria development, ii) tetrathionate inhibiting coliform bacteria and most of intestinal bacteria except *Salmonella* spp. containing tetrathionate reductase on SPI-2 (see I.A.4.b), iii) novobiocin inhibiting *Proteus* genus and iv) calcium carbonate neutralizing the sulfuric acid produced during tetrathionate reduction by *Salmonella* spp. and maintain optimum pH. RVS broth, with an incubation at 41.5°C, exploits the four characteristics of *Salmonella* spp. over other *Enterobacteriaceae*: i) the relatively high osmotic pressures ability to survive, using sodium chloride for osmotic balance and magnesium chloride to raise the osmotic pressure in the broth, ii) relatively low pH tolerance, iii) relatively more resistant to malachite green, iv) relatively less demanding nutritional requirements.

After this selective enrichment, isolation is performed on Xylose-Lysine-Deoxycholate (XLD) agar medium and a second solid medium of choice which is based on other biochemical characteristics than XLD medium. XLD is a selective medium for *Salmonella* spp. and *Shigella* spp. On XLD plates, typical *Salmonella* spp. display red colonies or red colonies with black center. XLD contains i) sodium deoxycholate inhibiting the Gram-positive bacteria development; ii) xylose which is fermented by *Salmonella* spp. and produces acid decreasing the pH, thus the pH indicator, namely phenol red, induces yellow turning color. *Shigella* colonies cannot ferment xylose therefore they remain red; iii) lysine which is decarboxylated by *Salmonella* spp. colonies, increasing the pH to alkaline and mimicking the red *Shigella* colonies; iv) lactose and sucrose which are not fermented by *Salmonella* spp. (Table 1) can be fermented by others lysine-positive coliforms; this fermentation produces acid in excess preventing alkaline reversion, thus the colonies formed by these non-specific bacteria remain yellow, v) complex sodium thiosulfate-ferric ammonium citrate which is metabolized by *Salmonella* spp. to produce hydrogen sulfide (H₂S), resulting in the formation of colonies with black centers and allowing them to be differentiated from the similarly colored *Shigella* colonies. It is important to note that atypical *Salmonella* can display on XLD plates, pink colonies with dark pink center or yellow colonies without the blackening of the center.

One typical colony on each type of selective plates is selected, biochemically and serologically confirmed. If this colony is negative, four other typical colonies are tested. To perform biochemical and serological confirmation, the suspected colonies are streaked onto nutrient agar plates to obtain well-isolated colonies. The

biochemical confirmation can be performed using biochemical identification galleries such as API 20E (Biomérieux). The biochemical confirmation tests are: glucose, lactose and sucrose fermentation, gas formation by glucose fermentation, H₂S formation e.g. using Kligler-Hajna medium, urea hydrolysis, L-lysine decarboxylation, β -galactosidase reaction, Voges-Proskauer (acetoin production from glucose) reaction and indol production (68). Strains that have been identified as *Salmonella* by the biochemical tests are serologically confirmed. The serological confirmation consists of agglutination tests using monovalent, polyvalent serum or anti-serum to identify the O-, Vi- and H- antigens (68).

2) Reference detection method for *Listeria* spp.

The entire flow of the ISO 11290-1 for detection and confirmation of *Listeria* spp. into food and feed products procedure is presented in the diagram displays in Figure 7. In brief, the primary and secondary enrichments are performed in Half-Fraser and Fraser media, respectively. These selective media for *Listeria* spp. contain, in addition to growth components, aesculin, ferric ions, lithium chloride, nalidixic and acriflavine (70). All *Listeria* spp. hydrolyze aesculin to aesculetin, which reacts with ferric ions and results in the blackening of the medium. The blackening of the medium is therefore a presumptive evidence for the presence of *Listeria*. However, the non-blackening cultures can contain *Listeria*. Ferric ions enhance the growth of *L. monocytogenes* (71) and lithium chloride is included in the medium to inhibit the growth of enterococci that can also hydrolyze aesculin. Nalidixic acid inhibits growth of Gram-negative organisms while acriflavine inhibits many Gram-positive bacteria. The two last compounds are present at half concentration in the Half-Fraser medium.

From the primary and the secondary enrichments, dilution and plating out are made on two selective agar media to get well-separated colonies. According to the Amendment 1 of ISO 11290-1 (69), the selective media to use are Agar *Listeria* according to Ottaviani and Agosti (ALOA) and another selective medium chosen by the laboratory (e.g. Oxford, PALCAM or MOX). The selectivity of all of these media is obtained by antibiotics anti-Gram-negative bacteria. The chromogenic differentiation of *Listeria* spp. from the other bacteria is based on the β -D-glucosidase activity present in all *Listeria* species.

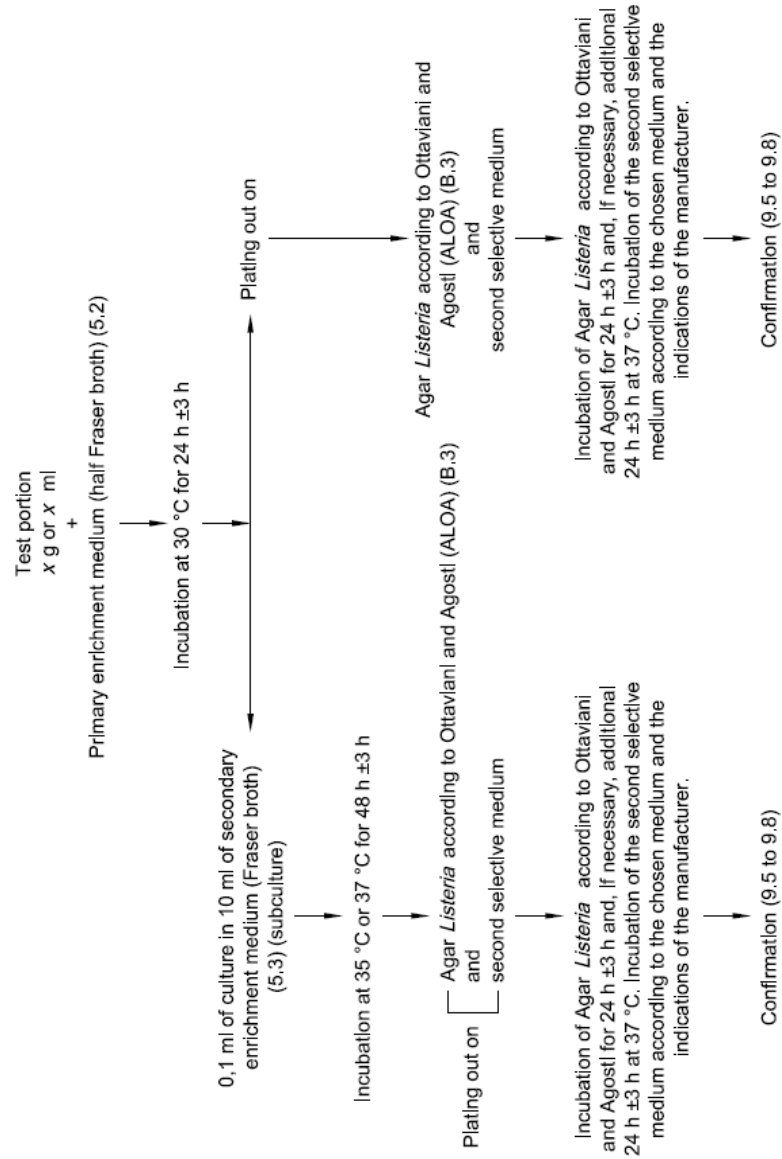


Figure 7 : Flow diagram for the detection of *L. monocytogenes* using the ISO 11290-1. Reprinted from ISO 11290-1/A1:2005 (69). See details in the text.

The second discrimination is done via the phosphatidylinositol-specific phospholipase C (PI-PLC) activity, which is present in *L. monocytogenes* and *L. ivanovii*. This virulence factor is involved in vacuole escape (see part I.B.4.b). Up to now, it was difficult to have a media able to discriminate *L. monocytogenes* from *L. ivanovii*. Only the Rapid *L'mono* from Bio-Rad allows this discrimination, *L. monocytogenes* and *L. ivanovii* are discriminated from each other by the xylose fermentation activity, which is present in *L. ivanovii*, but not in *L. monocytogenes*. However, with these Rapid *L'mono* selective plates, some false positive have been recorded with other bacterial genera.

These chromogenic media have the advantages of being simple to use, affordable, simple to interpret and allows the isolation of the bacterial strain. However, although these selective/chromogenic plates are robust for common strains, atypical strains lacking one of the enzymatic activity used for the chromogenic discrimination, will give rise to false negative results.

After the isolation on selective plates, typical colonies should be confirmed to be *L. monocytogenes* via morphological, physiological and biochemical tests (38;69): catalase reaction, Gram staining, motility test, Christie, Atkins, Munch-Petersen (CAMP) test, hemolytic activity and fermentation of xylose/rhamnose. So far, all these tests have to be performed and to give the features of *L. monocytogenes* (Table 3). In the future, the confirmation step will be simplified, through a current ISO 11290-1 revision.

B. Immunological detection methods

Immunological methods, based on the antibody-antigen affinity, are popular because they are simple, sensitive, accurate and can be carried out directly on enrichments from food or feed samples without cumbersome sample preparation. However, due to the antigenic heterogeneity of microorganisms, the specificity of these immunological assays is not always acceptable. To solve this drawback, alternative assays have been developed using bacteriophage proteins binding domain instead of antibodies (72-74). Many immunological methods are commercially available and are AOAC- or AFNOR-certified. Positive results obtained with immunoassays should always be confirmed by isolation from the enrichment broth on selective plating media since both dead and viable bacteria can be detected by immunological tests. Once isolated, confirmation of the bacteria is also mandatory.

1) Enzyme-linked immunosorbent assay (ELISA)

ELISA methods used immobilized antibodies in a microtitre well (plate) or on a nitrocellulose membrane as a single line (strip). When present in the enriched sample, the targeted bacteria will bind to their specific antibodies. For the plate test, the sample is added into the well. Then unbound sample is removed by washing. A second antibody coupled with an enzyme (or other label) is added, followed by another washing step to remove unbound antibodies. The staining is done by the addition of a substrate, which displays color after an enzymatic reaction. After a final washing step, this color signal is measured for each sample.

For the strip test, the ELISA is performed on a nitrocellulose membrane (the stationary phase). The loading extremity of the strip is immersed into an aliquot of the enrich medium (the mobile phase). Gold colloid labeled anti-targeted bacteria antibodies, free on the membrane, are dissolved into the mobile phase and can form complexes with the targeted bacteria, if present. These complexes migrate across the membrane by capillarity and are trapped by anti-targeted bacteria antibodies fixed at the test line position to develop a reddish purple line by the gold colloid. Thus, whether the sample solution contains the targeted bacteria or not can be determined by visually inspecting the test line. Irrespective of the presence/absence of the targeted bacteria in the sample, the excess gold colloid labeled antibodies further migrates across the membrane and are then trapped by specific antibodies of the free antibodies, fixed at the control line position to develop a reddish purple line. It can be confirmed that the sample solution has migrated across the membrane by the development of the control line.

The Enzyme Linked Fluorescent Assay (ELFA), which combines the ELISA method with a final fluorescence reading, is mainly used in Vitek Immuno Diagnostic Assay System assays (VIDAS®, Biomérieux). It is based on the same principle than the ELISA described above except that the antibodies are coated on a Solid Phase Receptacle® (SPR®) that serves as a solid phase and pipetting device. The second antibody is conjugated with alkaline phosphatase and binds to the targeted bacteria captured on the wall of the SPR®. Finally, the enzyme catalyzes the conversion of 4-methyl-umbelliferyl phosphate into the fluorescent compound 4-methyl-umbelliferone phosphate (75). The optical scanner of the instrument measures the intensity of fluorescence in each sample. VIDAS® systems are commercially available and are AFNOR/ISO16140-validated (7). VIDAS® has several advantages: it is fast, automated and uses a general protocol for all food products. However, the VIDAS®

system has also a drawback: the apparatus is not versatile and can only be used with the VIDAS® detection kits. Several kits exist for food matrices analysis: the specific detection of *L. monocytogenes*, the detection of *Salmonella* spp., the discrimination between *L. monocytogenes* and *Listeria* spp. and for a general detection of *Listeria* spp. As these VIDAS® detection kits are AOAC- and AFNOR-validated, they are extensively used as alternatives to the culture-based methods, especially in routine quality control laboratories which perform analysis for food business operators in the frame of own checks to guaranty the microbiological quality of the food products.

2) Immunomagnetic separation (IMS)

The immunomagnetic separation is based on antibodies immobilized on magnetic beads. The beads are mixed with the enriched sample. If the targeted bacteria are present, they will bind to the anti-targeted bacteria antibodies. A magnetic separation will allow upconcentration of the targeted bacteria from background microflora and PCR inhibitors. Consequently, downstream isolation on selective plates, immunoassays or nucleic acid based assays are simpler, faster and give less false negative results.

C. Nucleic Acid-based detection systems

The detection of foodborne bacteria from food sample via molecular methods is gaining recognition since it is also fast, accurate, specific and sensitive. Moreover, unlike biochemical and RNA-based detection methods, the DNA-based detection methods are not influenced by the expression of specific biochemical features. As for the immunological assays, positive results obtained with molecular assays should always be confirmed by isolation from the enrichment broth on selective plating media. Once isolated, confirmation is also required.

A critical pre-analytical step for nucleic acid-based assays is the nucleic acid extraction. It is important to use an extraction protocol appropriate to the tested matrix to get the best yield and the less PCR inhibitors (76-78). Indeed, some matrices with high background microflora could inhibit the growth of the target bacteria. In this case, extraction methods giving high yield will be needed. For matrices containing high level of PCR inhibitors, a highly selective extraction method will be needed. However, more selective and more efficient extraction methods are also more expensive and time-consuming.

This nucleic acid extraction consists in lysis of the cell to free RNA and DNA, which are then separated from the background sample by affinity membranes, organic extraction, filter membranes or magnetic particles. After this separation, RNA and DNA are eluted into an appropriate buffer. If only RNA or DNA have to be isolated, DNase or RNase step will be included, respectively.

1) DNA or RNA hybridization

DNA or RNA hybridization is the simplest molecular method for detection of foodborne bacteria in food samples. However, it is an old-fashioned technique which is almost not used anymore. The presence of the target is detected using a single stranded oligonucleotide probe complementary to the RNA or DNA target sequence. Target specific probes are immobilized in a well and are labeled on one or both ends by a non-specific detection system like radioactive isotopes, biotin, digoxigenin or fluorescent dyes. DNA or RNA hybridization could be used directly on enriched samples, on isolated colonies or after PCR amplification (79). The reading is done according to the selected dye.

2) Reverse transcriptase

The reverse transcriptase is a step performed before the Polymerase Chain Reaction (PCR) when the target is RNA. This step consists in the reverse transcription of RNA to a complementary DNA (cDNA). This is done using the reverse transcriptase (RT) enzyme. This step is necessary since RNA cannot be used as a PCR reaction template and is highly sensitive to degradation by RNases. When reverse transcription is performed in the same tube than the PCR amplification, this is called a one-step RT-PCR for Reverse Transcriptase-PCR. This technique which is the gold standard for foodborne RNA virus detection is almost not used for foodborne bacteria detection. It is more suitable for gene expression studies than for a detection system (80).

3) Polymerase Chain reaction

PCR have been developed in the 80's by Mullis *et al.* (81). Prior to a PCR analysis, the DNA should be extracted from the enriched sample. PCR is an enzymatic reaction amplifying *in vitro* a specific DNA region thanks to a thermostable DNA polymerase and specific oligonucleotides complementary to the target sequence used as primers for polymerization. PCR is a cycled thermal process: DNA denaturation (90-95°C), hybridization of oligonucleotides (annealing: 50-65°C) and

DNA polymerase elongation (synthesis of DNA). This reaction is performed in a thermocycler. Usually PCR reaction is made of ca. 35 cycles, each cycle leading to the synthesis of one copy of the target DNA region. It is an exponential amplification that leads to billions of copies of the DNA target at the end of the reaction. This DNA amplification is visualized on agarose gel with ethidium bromide staining. Although PCR is somehow an “old-fashioned” detection technique, it remains a good option as it is easiest and cheapest to perform compared with the real-time PCR.

4) Real-Time PCR

The real-time or quantitative PCR (qPCR), developed by Heid *et al.* (82) in the 90's, is an evolution of the classical PCR technology. In qPCR assays, the DNA polymerization is not measured at post-amplification, as in classical PCR, but all along the process (hence “real-time”). At each cycle of amplification, the amount of polymerized DNA is measured via fluorescent dyes. The result of a qPCR assay is expressed as a threshold cycle (Ct), crossing point (Cp), take-off point (TOF) or quantification cycle (Cq) value, all different names of the same measure. Note that the Cq abbreviation has now been recommended (83). The Cq-value represents the time-point at which the PCR amplification reach the prior-set threshold level for the reaction. The threshold level takes into consideration the background level during early cycle and the start of measurable amplification (84;85). As such, the lack of measurable Cq value in a qPCR assay is to be interpreted as the absence of amplification above the background level.

qPCR is more sensitive than PCR and has been widely accepted as a faster alternative to culture-based methods for the detection of pathogenic bacteria in food (80). Numerous commercial qPCR kits have been validated AOAC or AFNOR, which guarantees their efficiency. To perform a qPCR detection, DNA from the enriched sample should be extracted, most commercially available qPCR detection system provide/advice a specific DNA extraction kit. Two main qPCR technologies exist: i) the probe-based qPCR assays and ii) the intercalating agent-based qPCR assays.

a) Probe-based qPCR assays

In the probe-based qPCR assays, in addition to the primers, one or two labeled probe(s) is/are used. The advantages of the probe-based assay are the extra specificity provided by the probe, and the possibility of multiplex assay.

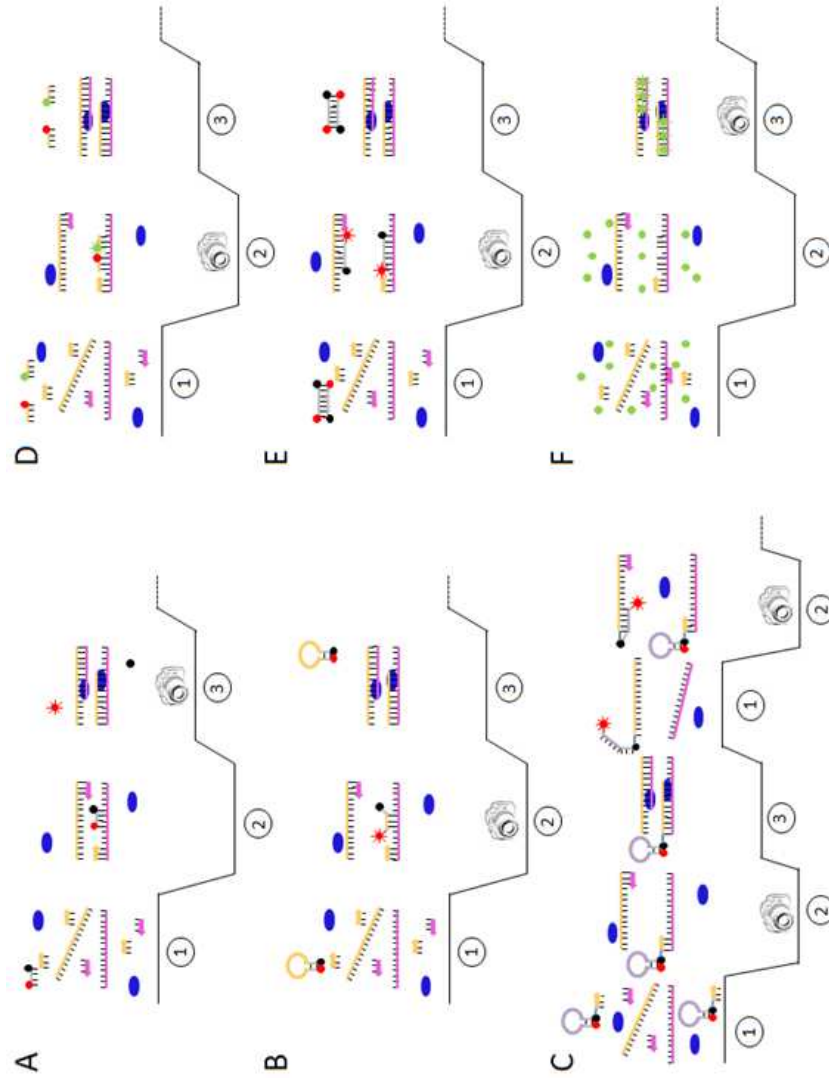


Figure 8: Schemes of the different qPCR technologies mechanisms.

A. 5' nuclease probe (TaqMan®), B. Molecular beacons, C. Scorpion, D. Fluorescent resonance energy transfer, E. Double-stranded probes, F. Intercalating agent (SYBR®Green). 1. Denaturation, 2. Annealing and 3. Elongation. Taq-polymerase, : Forward and reverse primers, fluorescent dyes, probe. See main text for details.

However, the drawbacks reside in the risk of false-negative when the target sequence is polymorph, and the high price of the labeled probes. Five different probe-based technologies are available: i) the 5'-nuclease probe (TaqMan®), ii) the molecular beacons, iii) the Scorpion, iv) the fluorescent resonance energy transfer (FRET) and v) the double-stranded probes.

5'-nuclease probe (TaqMan®)

The 5'-nuclease probe technology, also named TaqMan® technology, was the first qPCR technology to be developed (82) and is the mostly used technology for qPCR analysis. Most available commercial kits are using the TaqMan® chemistry (e.g. GeneDisc® System from PALL®). A TaqMan® probe is a short oligonucleotide containing a 5'-fluorescent dye and 3'-quenching dye which inhibits the fluorescence of the dye when they are both close to each other. To generate a signal, the fluorescent dye should be separated from the quenching dye by hydrolysis of the probe by the DNA polymerase (5'-3' exonuclease activity) during the elongation step. The fluorescence signal is then measured at the elongation step of the qPCR reaction (Figure 8A). However, since the probe is hydrolyzed during the elongation step no melting curve analysis is possible.

Molecular beacons

Molecular beacons have been described for the first time in 1996 (86). This technique is based on one oligonucleotide hybridization probe that is hairpin-shaped thanks to complementary regions at both extremities. The loop is 18-30 base region complementary to the target sequence, while the dsDNA stem corresponds to the two hybridized ends. This probe is 5'-end labeled with a reporter fluorescent dye and 3'-end labeled by a suppressor fluorescent dye (quencher). No fluorescence is emitted from the probe at hairpin shape stage. During denaturation, the probe is linearized. During the following annealing step, it either returns to its original hairpin shape if no complementary sequence is present in the sample (no fluorescence), or it hybridizes to its complementary sequence of the target DNA (emission of fluorescence as quencher and reporter are separated). Contrary to TaqMan® probes, this probe will not be hydrolyzed by the DNA polymerase, and the fluorescence will be measured at the annealing step (Figure 8B). As the probe is not hydrolyzed during the elongation step, melting curve analysis is possible.

Scorpion

The scorpion technology is a variant of the molecular beacons probes. The hairpin structure is dyed at both ends as a molecular beacons probe. The main difference is that the hairpin probe is coupled with one of the primer. The primer is linked to the hairpin structure via the PCR blocker hexaethylene glycol phosphoramidite (HEG). The polymerization of the target DNA occurs as in classical PCR reaction, but the PCR blocker stops the polymerization to avoid the probe to be extended. At the next denaturation step, the probe is linearized (giving the scorpion tail shape) then during annealing step the probe is able to hybridized to the polymerized DNA from last amplification by a turnaround, this allows the reporter dye to emit fluorescent by distancing from the quencher dye. The fluorescence at each cycle is measured during the annealing step (Figure 8C).

Fluorescent resonance energy transfer: FRET

The FRET technology uses two probes, named FRET hybridization probes or LightCycler® probes. One probes is 3'-end labeled with a fluorescent dye, the other is 5'-end labeled by an acceptor dye. Probes are designed to hybridize on the target sequence, separated by 1 to 5 bases. When alone, the fluorescent dye emits only a background signal. When both dyes are at very small distance, the proximity allows the fluorescent resonance energy transfer from the donor fluorescent dye to the acceptor dye that emits a strong fluorescent signal. The fluorescence is measured at annealing step, during elongation step the probes are not hydrolyzed by the polymerase but only detached (Figure 8D). The two probes encompass a region of 40 to 50 bases, providing a larger specificity than other probe-based qPCR technology. As the probes are not hydrolyzed during elongation step, a melting curve analysis is possible.

Double-stranded probes

The double-stranded (ds) probes technology is specific to the iQ-check test kits of Bio-Rad® (87). Ds-probes are labeled on both strands in 5'-end with a fluorescent dye and in 3'-ends with a quencher. When the probes are double-stranded, no fluorescence is emitted since the quencher inhibits the fluorescence of the dye. When the probes are hybridized to their specific targets, the fluorescent dye can emit and be measured (Figure 8E). This technology has the advantage compared to the other probe-based technologies to double the fluorescent signal per target as the probe is labeled on both strands.

b) Intercalating agent-based qPCR assays

Intercalating agent-based qPCR uses intercalating dyes to monitor the polymerization of the target sequence in real-time. This technology was firstly described in the beginning of the 90's by Higuchi *et al.* using ethidium bromide (88). However, ethidium bromide is known to be carcinogen and, at the end of the 90's, it has been replaced by SYBR®Green (89), an asymmetric cyanine dye (90) intercalating dsDNA (91). In SYBR®Green qPCR assay, the reaction is performed as a classical PCR reaction, except that SYBR®Green is added into the mix. SYBR®Green is very lightly fluorescent when it is free and highly fluorescent when bound into dsDNA. During annealing and elongation step, SYBR®Green is intercalated into the dsDNA and gives a fluorescent signal. At each cycle the amount of polymerized DNA increases, as for the fluorescence intensity of the SYBR®Green (Figure 8F). This fluorescent signal is reversible as SYBR®Green is freed at each denaturation step. This property allows a post amplification check of the end product (amplicon) specificity. So SYBR®Green qPCR assay, in addition to the Cq-value, gives a second value: the melting temperature of the amplicon (T_m). This T_m-value represents the temperature at which 50% of the SYBR®Green fluorescence is lost due to denaturation (separation of the strands) of the amplicon. The T_m-value is a physical parameter specific and inherent to the sequence of the amplified product (especially GC content) and is influenced by chemical factors affecting the dsDNA stability (e.g. salt concentration or DMSO) (92). The other advantages of SYBR®Green technology are the easiness of implementation and the low cost, since no probes are need. The drawback of this technique is that multiplexing is less easy and effective than with probe-based qPCR assay. Indeed, several amplicons obtained from a SYBR®Green qPCR assay would give a common Cq-value since SYBR®Green is not sequence specific and the discrimination of each amplicon would be done based on T_m-values observed. Thus, multiplex with more than two targets seem difficult to perform.

c) Important controls to perform with qPCR detection systems

As with every detection method, controls are needed to validate the results with qPCR analysis. With qPCR detection assays, in addition to the classical positive and negative controls, an inhibition control is advised (93). These controls avoid occurrence of false negative or false positive results due to technical problems. They assess the efficiency of the PCR reaction (positive control), the non-contamination of the PCR mix (negative control) and the quality of the extract DNA (inhibition control). The inhibition control is crucial in a foodborne qPCR detection system because of the

presence of potential inhibitory substances in food matrices (94). Common inhibitors are numerous and include various constituents of bacterial cells, non-target DNA and contaminants, food constituents (e.g., organic and phenolic compounds, glycogen, cellulose, fats, and calcium), components of enrichment media and DNA extraction kits (e.g. detergents, lysozyme, NaOH or alcohols), components of body fluids (e.g., hemoglobin, urea, and heparin), environmental compounds (e.g., pollen, phenolic compounds, humic acids, and heavy metals), and laboratory items (e.g. glove powder, and laboratory plastic ware) (76;95;96). The choice of a DNA extraction kit appropriate to the tested food matrices/enrichment media is important in reducing the presence of inhibitors into the extracted DNA. The presence of inhibitors into a sample could be tested with an external (not performed in the same well as the detection reaction) or an internal (performed in the same well as the detection reaction) inhibition control usually called internal amplification control (IAC). Most commercial kits include an IAC. The IAC can be an housekeeping gene (gene present in the sample) detected by another pair of primers, an heterologous gene (addition of a target such as plasmid containing a target gene different from the detection gene) or homologous gene (addition of a target such as plasmid containing a target gene amplified by the same primers than the detection gene but with a different internal sequence (annealing of the probe)). However, by definition an IAC is performed in the same well as the detection assay, as a multiplex assay. This could lead to competition between assays and could lead to false negative results of the detection assay if the IAC was not sufficiently well-developed. Moreover, the use of an IAC is inappropriate in SYBR®Green qPCR detection system since the dye is not specific to one target but to all amplified DNA. An external amplification control, i.e. performed on the same sample but in an other well, would be a better choice.

5) Luminex

Luminex assays are immunological, enzymatic, receptor-ligand and nucleic acid assays. Although the Luminex technology has not yet been used for foodborne pathogens detection in food matrices, immunological (97) and nucleic acid-based (98-105) Luminex assays have already been developed for the genetic characterization of isolated strains or for pathogens detection in human stool samples for clinical purposes. The Luminex technology is a multiplex detection system, from 50-plex (MagPix®) up to 500-plex (FlexMap® 3D). It uses beads with a unique spectral address consisting of a mix of red and infra-red color. The nucleic

acid-based Luminex technology can use direct hybridization (DH) or Ligation dependent amplification (LDA) technology (106).

6) Next-Generation Sequencing

The Next-Generation Sequencing (NGS) is not yet used as a detection system in foodstuff microbiological control as the price is still too high and the microbiological control laboratory are not yet ready to use this technology. However, during the spring 2011, this type of technology played a key role in the characterization of the strain of *E. coli* responsible for a large outbreak in Europe (107). Moreover, in the recent years, several new sequencing technologies have emerged inducing a drastic drop of the price which is predicted to continue to decrease. Therefore, this technology may become an interesting option in the future.

The Sanger sequencing technology was amongst the first sequencing technology to be developed to sequence DNA and remained the gold standard for many years (108). The Next Generation Sequencing technologies are, as indicated in the name, a new generation of technologies that has been recently developed. They are not using the chain-terminator technology used in the Sanger technology but different technologies such as sequencing-by-synthesis approach which rapidly produce a million of bases of data (108).

D. Comparison of the different detection technologies

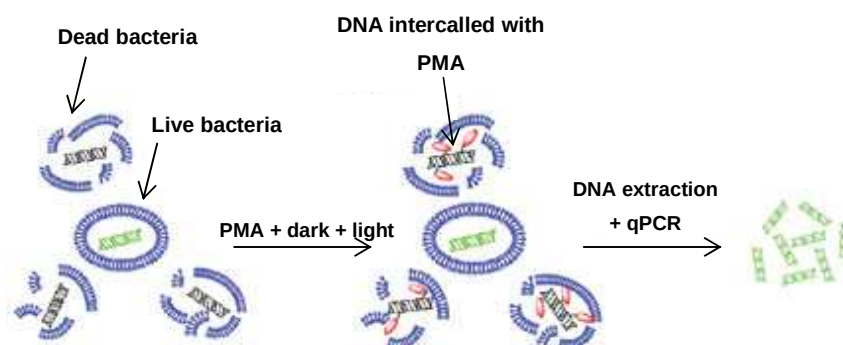
Each method presents advantages and drawbacks that are presented in 5.

E. Viable and dead problematic

In food samples, only live bacteria are of interest since dead bacteria can not cause an infection. Therefore it is very important that detection methods can discriminate between live and dead bacteria. Indeed, dead bacteria are non-infectious microorganisms but remain detectable by PCR and qPCR since their DNA remains after death (109-111). Two techniques can be used to discriminate dead vs. viable/live bacteria. The first one is reverse-transcriptase (RT) (q)PCR which is based on RNA detection (112;113). However, this detection technique requires expression of the targeted gene, which can vary under stress conditions. Furthermore, RNA is very sensitive to degradation in complex matrices such as food. The use of RT-(q)PCR technology is more adapted for gene expression studies than for detection systems (see review Postollec *et al.*, 2011 (80)).

Table 5 : Advantages and drawbacks of the different technologies for the detection of foodborne bacteria in food and feed products.

	Bacteriological method	Immunological tests	Nucleic acid based tests
Advantages	Specific Easy to perform Sensitive (depends on the matrix) Cheap An isolate is available for further characterizations	Fast Specific (often false positive results) Sensitive Not dependent of expression of biochemical characteristics Low influence of background microflora	Fast Specific Sensitive Not dependent of expression of biochemical characteristics Low influence of background microflora and analyzed matrix
Drawbacks	Time-consuming Dependent of expression of biochemical characteristics Large influence of analyzed matrix Large influence of background microflora	Need of specific apparatus Cost Influence of the matrix Isolation has to be performed after positive result	Need of specific apparatus Cost Risk of contamination (false positive results) Isolation has to be performed after positive result Influence of inhibitors (false negative results)

**Figure 9 :** Mechanism of v-qPCR to amplify only viable bacteria. Reprinted and modified from Biotium website (114).

Propidium monoazide (PMA) is added to a sample. During dark incubation, PMA is able to reach DNA from altered-membrane bacteria (dead cells) as PMA is not able to go through intact membrane (viable cells). A light treatment covalently binds PMA to the DNA. Finally, after DNA extraction, the DNA from viable bacteria is the only one involved into the qPCR amplification.

The second available technique is the viability PCR or qPCR (v-PCR or v-qPCR) which is based on DNA detection. In v-(q)PCR, before (q)PCR amplification, a viability discrimination step is performed. This step uses a dye that intercalate in the double helix of the DNA every 4-5 nucleotides (115). Two molecules can be used: ethidium monoazide (EMA) and propidium monoazide (PMA), derivatives from ethidium bromide and propidium iodide, respectively. EMA and PMA are positively charged molecules which are excluded by intact, negatively charged, bacterial cell-walls but can enter bacteria with damaged cell-wall/membrane (116). EMA was firstly reported to be useful to quantify viable bacteria by v-PCR (117;118) but was found to be able to penetrate and being toxic for viable bacteria. Afterwards, PMA was used as a non-toxic alternative (116;119) able of covalent cross-linkage with DNA under light exposure (120). The precise action mode of PMA remains unclear: DNA charge change (118) or DNA cleavage (121)), leading to reduce extractability or amplificability of DNA, respectively. Since PMA is not able to penetrate intact cell-wall/membranes of live bacteria, only DNA from bacteria with compromised cell-wall/membranes is bound and is thus not amplifiable (116;122) (Figure 9). PMA was recently coupled with PCR (123-125) or qPCR (126-135) in order to detect only viable bacteria. Originally presented to fully remove the signal of dead bacteria in v-PCR (116) and v-qPCR analysis (126), the limit of the technique rapidly came out. Indeed, PMA treatment is not always giving complete removal of the qPCR signal of dead bacteria (see review in Fittipaldi *et al.*, 2010 (136)). In particular, recent studies have shown that PMA treatment do not fully remove the signal from dead bacteria if the amplicon size of the qPCR assay is short (137-140), if the target bacteria is at high concentration (137;141-143), if the concentration of Mg^{2+} in the PCR reaction is not adapted (116), if the fat content of food sample is high (144), and may also vary according the death treatment (144-147).

III. Food safety regulations

A. Food safety laws

The commission of the *Codex Alimentarius* defines international standards, directives and recommendations to guaranty the food safety. The *Codex Alimentarius* includes risk analysis, integrated food chain approach and the notorious Hazard Analysis Critical Control Points (HACCP). These international guidelines are the bases of the European Legislation.

In Europe, the different food crises of the 90's, e.g. Bovine Spongiform Encephalopathy and dioxin, altered the citizen confidence about food products and encouraged the lawmakers to look for more effective means to protect consumers. In 1997, the European Commission opened a public consultation with the green paper on the general principles of food law (148). In 2000, the White Paper on Food Safety (149) gave a detailed proposition of law. Moreover, in 2000, the European Food Safety Authority (EFSA), the independent risk assessment body of the European Union (EU), was created. Finally in 2002, the Food law was published: Regulation (EC) n°178/2002 [laying down the general principles and requirements of food law, establishing the EFSA and laying down procedures in matters of food safety] (150). This law describes the overall and integrated food safety approach throughout the food chain. The responsibility of the food chain security (production and inspection) is shared between the food business operators which have the primary responsibility to ensure food safety, the national relevant authorities, who should enforce food law, and perform controls all along the food chain, and finally the European Commission which should control that the national relevant authorities are correctly performing their controls. This regulation (EC) n°178/2002 is also initiating the European Rapid Alert System for Food and Feed (RASFF), a web portal where Member States can introduce notifications about serious risks detected in relation to food or feed. This exchange of information helps to act more rapidly and in a coordinated manner in response to a health threat caused by food or feed.

In 2004, the EC regulation n°882/2004 (151), which describes the execution of the official controls was published. It consists of controls at all production stages and in all sectors. The EC regulation n°854/2004 (152) on official controls on products of animal origin is completing the previous regulation. Finally, the EC regulation n°853/2004 (153) lays down the rules for hygiene of foodstuffs. In 2005, the EC regulation n°1831/2003 on food additives (154), laid down the

microbiological criteria for certain micro-organisms in different types of food categories and the implementing rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to Article 4 of EC regulation n°852/2004 (153).

The exact procedures for risk assessment differ considerably in the different member-states. In Belgium, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) is responsible for the food chain safety. To guaranty it, FASFC has developed an integrated official control program to check compliance with the EU regulations, see review in Maudoux *et al.*, 2006 (155).

Salmonella spp. specific laws

To protect human health against zoonotic salmonellosis, the EU regulation n°2160/2003 (156), (amended or implemented by EC regulations n°1003/2005 (157), n°646/2007 (158), n°584/2008 (159), n°213/2009 (160), n°200/2010 (161) and n°517/2011 (162)) obliges and gives rules to the European member states for national control of *Salmonella* serovars in poultry and pigs deemed to be of particular importance for public health. Moreover, the EC regulation n°1237/2007 (163), gives specific rules for the table-eggs to reduce the risks of table-eggs outbreaks due to *Salmonella* spp.

B. Food sampling

The regulation n°2073/2005 on microbiological criteria for foodstuffs (154), also gives instructions about sampling plan and sampling rules. The sampling procedure are described for each type of food matrices in ISO norms 6887 from 1 to 6 (164-169) and a specific ISO norm 17604 (170) describes the swabbing sampling.

C. Acceptance limit

1) *Salmonella* spp.

The EC regulation n°2073/2005 (154), amended by EC regulations n°1441/2007 (171) and n°1086/2011 (172) set limits and analytical reference methods to detect the presence of *Salmonella* spp. in specific food categories (Table 6).

Table 6: Food safety criteria for *Salmonella* applied to product placed on the market. Reprinted from Commission regulation (EC) No 2073/2005 (154).

Food category	Limit	Analytical reference method	Stage where the criterion applies
1.4 Minced meat and meat preparations intended to be eaten raw	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.5 Minced meat and meat preparations made from poultry meat intended to be eaten cooked	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.6 Minced meat and meat preparations made from other species than poultry intended to be eaten cooked	<i>Salmonella</i> spp.: absence in 10 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.7 Mechanically separated meat (MSM) ⁽¹⁾	<i>Salmonella</i> spp.: absence in 10 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.8 Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the <i>Salmonella</i> risk	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.9 Meat products made from poultry meat intended to be eaten cooked	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.10 Gelatin and collagen	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.11 Cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurization ⁽²⁾	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.12 Milk powder and whey powder	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.13 Ice cream ⁽³⁾ , excluding products where the manufacturing process or the composition of the product will eliminate the <i>Salmonella</i> spp. risk	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.14 Egg products, excluding products where the manufacturing process or the composition of the product will eliminate the <i>Salmonella</i> spp. risk	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.15 Ready-to-eat foods containing raw egg, excluding products where the manufacturing process or the composition of the product will eliminate the <i>Salmonella</i> spp. risk.	<i>Salmonella</i> spp.: absence in 25 g or ml	EN/ISO 6579	Products placed on the market during their shelf-life
1.16 Cooked crustaceans and molluscan shellfish	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.17 Live bivalve molluscs and live echinoderms, tunicates and gastropods	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life

Table 6 (continued)

Food category	Limit	Analytical reference method	Stage where the criterion applies
1.18 Sprouted seeds (ready-to-eat) ⁽⁴⁾	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.19 Precut fruit and vegetables (ready-to-eat)	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.20 Unpasteurized fruit and vegetable juices (ready-to-eat)	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.21 Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria in Chapter 2.2 of Annex of regulation EC 2073/2005	Not detected in 25 g	European Screening method of the CRL for Milk ⁽⁵⁾	Products placed on the market during their shelf-life
1.22 Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the Enterobacteriaceae criterion in Chapter 2.2 of Annex of EC regulation 2073/2005	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.23 Dried follow-on formulae	<i>Salmonella</i> spp.: absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
1.28 Fresh poultry meat ⁽⁶⁾	<i>Salmonella</i> Typhimurium ⁽⁷⁾ and Enteritidis: absence in 25 g	EN/ISO 6579 (for detection) White-Kaufmann-Le Minor scheme (for serotyping)	Products placed on the market during their shelf-life
2.1.3 Carcasses of cattle, sheep, goats and horses	<i>Salmonella</i> spp.: absence in the area tested per carcass	EN/ISO 6579	Carcasses after dressing but before chilling
2.1.4 Carcasses of pigs <i>Salmonella</i>	<i>Salmonella</i> spp.: absence in the area tested per carcass	EN/ISO 6579	Carcasses after dressing but before chilling
2.1.5 Poultry carcasses of broilers and turkeys	<i>Salmonella</i> spp. ⁽⁸⁾ : absence in 25 g of a pooled sample of neck skin	EN/ISO 6579	Carcasses after chilling

(1) This criterion shall apply to mechanically separated meat (MSM) produced with the techniques referred to in paragraph 3 of Chapter III of Section V of Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council. (2) Excluding products when the manufacturer can demonstrate to the satisfaction of the competent authorities that, due to the ripening time and a_w of the product where appropriate, there is no *Salmonella* spp. risk. (3) Only ice creams containing milk ingredients. (4) Preliminary testing of the batch of seeds before starting the sprouting process or the sampling must be carried out at the stage where the highest probability of finding *Salmonella* spp. is expected. (5) Reference: Community reference laboratory for coagulase positive staphylococci. European screening method for the detection of staphylococcal enterotoxins in milk and milk products. (6) This criterion shall apply to fresh meat from breeding flocks of *Gallus gallus*, laying hens, broilers and breeding and fattening flocks of turkeys. (7) As regards with monophasic *Salmonella* Typhimurium only 1,4,[5],12:i:- is included. (8) Where *Salmonella* spp. is found, the isolates shall be further serotyped for *Salmonella* Typhimurium and Enteritidis in order to verify compliance with the microbiological criterion set out in row 1.28.

2) *Listeria*

A wide range of different foodstuffs can be contaminated with *L. monocytogenes*. For a healthy human population, foods not exceeding the level of 100 CFU/g are considered to pose a negligible risk (2). EC Regulation No 2073/2005 (154) (amended by EC regulation n°365/2010 (173)) covers primarily ready-to-eat food products, and requires to keep the concentration of *L. monocytogenes* in food below 100 CFU/g all along the shelf-life. More stringent rules are applied for ready-to-eat foods intended for infants and for special medical purposes since they should not contain any *Listeria monocytogenes* in 25 g of product all along their shelf-life (Table 7). Consequently, the food business operators, responsible for the manufacture of the product, must conduct studies in order to investigate compliance with these criteria and in particular ready-to-eat food products that are able to support the growth of *L. monocytogenes* and may therefore be a risk for public health.

Table 7: Food safety criteria for *L. monocytogenes* applied to product placed on the market. Reprinted from Commission regulation (EC) No 2073/2005 (154).

Food category	Limit	Analytical reference method	Stage where the criterion applies
1.1 Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes ⁽¹⁾	<i>L. monocytogenes</i> : absence in 25 g	EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2 Ready-to-eat foods able to support the growth of <i>L. monocytogenes</i> , other than those intended for infants and for special medical purposes	<i>L. monocytogenes</i> : ≤100 cfu/g ⁽²⁾	EN/ISO 11290-2 ⁽³⁾	Products placed on the market during their shelf-life
	<i>L. monocytogenes</i> : absence in 25 g ⁽⁴⁾	EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3 Ready-to-eat foods unable to support the growth of <i>L. monocytogenes</i> , other than those intended for infants and for special medical purposes (1) (5)	<i>L. monocytogenes</i> : ≤100 cfu/g ⁽²⁾	EN/ISO 11290-2 ⁽³⁾	Products placed on the market during their shelf-life

(1) Regular testing against the criterion is not required in normal circumstances for the following ready-to-eat foods: i) those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (for example, products heat treated in their final package); ii) fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds; iii) bread, biscuits and similar products; iv) bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products; v) sugar, honey and confectionery, including cocoa and chocolate products; vi) live bivalve molluscs; vii) food grade salt. (2) This criterion shall apply if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 CFU/g throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 CFU/g is not exceeded at the end of shelf-life. (3) 1 ml of inoculum is plated on a Petri dish of 140 mm diameter or on three Petri dishes of 90 mm diameter. (4) This criterion shall apply to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout the shelf-life. (5) Products with pH ≤ 4,4 or a_w ≤ 0,92, products with pH ≤ 5,0 and a_w ≤ 0,94, products with a shelf-life of less than five days shall be automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific justification.

IV. Validation detection methods

In order to be used under accreditation in food control laboratory, detection methods should be validated. Several guidelines are available. The International Organization for Standardization (ISO) organism is a recognized organism displaying norms on a wide range of activity. The “Microbiology of food and animal feeding stuffs” is one of the ISO categories. So far (February 2014), 58 norms are available in this category. Among them are presented all the reference methods for foodborne detection such as *Salmonella* spp. (68), *L. monocytogenes* (38), *Campylobacter* spp. (174), Shiga toxin-producing *E. coli* (175) and also several guidelines for validating PCR or qPCR methods for foodborne pathogens detection.

A. Validation of PCR assay for detection of foodborne pathogens

For the validation of PCR assays for the detection of foodborne pathogens, five ISO norms are available: i) ISO 22174:2005 which gives general requirements and definitions (176); ii) ISO/TS 20836: 2005 which gives performance testing for thermal cyclers (177); iii) ISO 20837:2006 which gives requirements for sample preparation (178) ; iv) ISO 20838:2006 which gives requirements for amplification (179) and v) ISO 22118:2011 which gives performance characteristics (180). In these norms, definitions and procedures are given to evaluate PCR for foodborne detection validation criteria and their acceptance limits such as selectivity, sensitivity, trueness and robustness.

1) Selectivity

The selectivity is a measure of the inclusivity and exclusivity (180). Inclusivity is the detection of positive samples. It should be tested on at least 50 positive strains, if possible. Exclusivity is the non-detection of negative sample. Exclusivity should be tested on at least 30 negative strains containing non-target micro-organism naturally present in food and feed stuffs. The list of non-target organisms should contain around 90% of bacteria and 10 % of yeast, molds or viruses. A minimum list of microorganism to test in exclusivity analysis is given in the ISO 22118 (180).

2) Specificity

The specificity of a PCR assay is the capacity to exclusively recognize the target, distinguishing it from similar non-target organisms and impurities (180).

3) Sensitivity

The sensitivity is a measure of the lowest number of cells, particles or molecules which can be detected in a single reaction (180). The sensitivity test consists of testing different concentrations of the target in five different food categories. From the sensitivity test it is possible to identify the limit of detection (LOD) of a PCR assay. This LOD is expressed as the lowest concentration of analyte or content of target organism per defined amount of matrix that gives a positive result with 95% of probability (180). The LOD should be comprised between 1 and 10 CFU/assay for bacteria and 10 to 100 particles or genomes equivalent for viruses.

4) Trueness

The trueness is the closeness agreement between the measured data and the true value (180).

5) Precision

The precision is the closeness agreement between independent test/measurement obtained under stipulated conditions (180).

6) Robustness

The robustness is the influence on the method performance when the analysis is subjected to small procedural or environmental factors variations (180).

B. Validation of qPCR assays for detection of foodborne pathogens

Since the use of qPCR assays for detection of foodborne pathogens is recent, only one ISO norm is currently available. The ISO 22119:2011 gives the general requirements and definitions of qPCR assays used in food microbiology analysis (181). No ISO norms are available for the validation of qPCR assays applied to food microbiology. However, general guidelines of qPCR were given by Bustin and colleagues with the MIQE: Minimum standard for the provision of Information for qPCR Experiments (83;182-185). Moreover, in the field of Genetically Modified Organisms (GMO) where qPCR is the detection method of reference, guidelines were delivered by the European Network of GMO Laboratories (ENGL) (186). In these guidelines, specific qPCR validation characteristic and their acceptance criteria are given such as the dynamic range, the PCR efficiency (E), the coefficient of correlation (R^2), the relative standard deviation of repeatability (RSD_r), and the relative standard deviation of reproducibility (RSD_R),

1) Dynamic Range

The dynamic range of a qPCR assay is the concentration range where it performs in a linear manner. The dynamic range should cover from 1/10 to 5 times the legislative requirement of the target (186).

2) PCR efficiency

The PCR efficiency (E) is the efficiency of the PCR reaction to double the number of targets at each cycle during the exponential phase. E should be between 89.6 and 110.2% (186) and can be calculated from the slope of the dynamic range curve (slope) (187): $E = 10^{(-1/\text{slope})} - 1$.

3) Coefficient of correlation

Coefficient of correlation (R^2) is an indicator of the correlation of data regarding the linear regression curve. It should be higher than 0.98 (186).

4) Relative Standard Deviation of repeatability

The Relative Standard Deviation of repeatability (RSD_r) is the deviation of the results observed under repeatable conditions. Repeatable conditions are conditions where the results are obtained with the same method, on identical samples, in the same laboratory, by the same operator using the same equipment but within short intervals of time. The RSD_r is expressed in percent. It should be inferior to 25% (186) and is obtained by multiplying the repeatability standard deviation (s_r) by 100 and dividing this product by the repeatability median (MED_r): $RSD_r = 100 s_r / MED_r$ (188).

5) Relative Standard Deviation of reproducibility

The Relative Standard Deviation of reproducibility (RSD_R) is the deviation of the results observed under reproducible conditions. Reproducible conditions are conditions where the results are obtained with the same method, on identical samples, in different laboratories, by different operators using different equipments. RSD_R is expressed in percent. It should be inferior to 35% and is obtained by multiplying the reproducibility standard deviation (s_R) by 100 and dividing this product by the reproducibility median (MED_R): $RSD_R = 100 s_R / MED_R$ (188).

C. Validation of an alternative method

Once a PCR or qPCR detection assay (the alternative method), has been validated, it can be validated against the reference method to check if it is as efficient. The

reference method should be internationally recognized and widely accepted. The ISO norm 16140:2003 (188) explains this validation process and the protocol to follow in detail. Such validation comprises two phases: i) a method comparison study against the reference method carried out in the organizing laboratory and ii) an interlaboratory study of each of the two methods. The first phase would be enough to use the alternative method instead of the reference method under the laboratory quality system. The second step of the validation is required if the system has to be transferred to other laboratories or to be commercialized. For commercialisation, the inter-laboratory study should be performed by an expert laboratory which is nominated by a certification organisation such as AFNOR-certification, NordVal, MicroVal or AOAC-validation.

In the first phase of the validation, four criteria have to be assessed (188): i) the relative accuracy; ii) the relative sensitivity; iii) the relative specificity; and iv) the relative detection level.

1) The relative accuracy, relative sensitivity and relative specificity

The relative accuracy (AC) is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (188).

The relative sensitivity (SE) is the ability of the alternative method to detect the analyte when it is detected by the reference method (188).

The relative specificity (SP) is the ability of the alternative method to not detect the analyte when it is not detected by the reference method (188).

They are all expressed in percent and are calculated using these formulas (188):

$$SE = \frac{PA \times 100}{PA+ND}$$

$$SP = \frac{NA \times 100}{NA+PD}$$

$$AC = \frac{(PA+NA) \times 100}{PA+NA+PD+ND}$$

PA is the positive agreement: number of positive results by both reference and alternative methods. **NA** is the negative agreement: number of negative results by both reference and alternative methods. **PD** is the positive deviation: number of negative results with the reference method and positive results with the alternative method. **ND** is the negative deviation: number of positive results with the reference method and negative results with the alternative results.

2) The relative detection level

The relative detection level is the smallest number of culturable microorganisms that can be detected in the sample in 50% of the occasions by the alternative and by the reference methods (188). It is expressed in number of CFU/sample.

V. Weaknesses of the available *Salmonella* and *Listeria* detection methods

Currently, the official methods for the detection of *Salmonella* and *Listeria* in food samples are ISO norms that are culture-based methods. Although reliable, they are specific for a single pathogen (single-genus or single species), time consuming and labor intensive (e.g. at least 5 days for *Salmonella* detection (68)).

Foodborne diseases cause enormous economic burden due to sick leaves, treatments, hospitalizations and mortality (189) and also economic losses for farmers and the food industry. The most recent large outbreak of the *E. coli* O104:H4 serotype in Germany was a dramatic illustration of all the consequences of a severe outbreak (190). Therefore, there is a growing need for the development of faster alternative methods to quickly identify the source of foodborne outbreaks, to better manage food-related crises and to allow a rapid batch release during own checks by the food business operators.

Tools for rapid and reliable detection of foodborne pathogens such as immunological or nucleic-based assays have been developed. Nucleic acid-based methods, and particularly real-time PCR (qPCR) assays, are increasingly accepted as good alternatives since they are fast, sensitive and specific (80). Moreover, they can be quickly implemented in food microbiology control laboratories since they are already equipped with qPCR apparatus, they have qualified analysts and the expertise of qPCR as this technology is already used in these laboratories for further analysis such as characterisation of strains.

Several detection qPCR assays for *Salmonella* spp. (191-202) and *L. monocytogenes* (60;117;203-208) in food products are already available. However, these assays are either single-genus (only *Salmonella* spp.) or single-species (only *L. monocytogenes* species) detection assays. Moreover, they target a single gene within a single assay, i.e. one primer-pair (single-target). These single-target assays could lead to false negative results with atypical strains mutated or deleted in the target sequence (209). To mitigate these inconveniences, approaches targeting two genes for *Salmonella* spp. detection (multi-targets) (210) or targeting several bacteria at the same time (multi-genera) with a single-target (211-215) have been developed. No system combining a multi-genera and multi-targets approach is available, albeit the multi-genera detection approach would further accelerate the screening as it

V. Weaknesses of the available *Salmonella* and *Listeria* detection methods

would enable to screen the presence or absence of several foodborne pathogens in a single analysis and the multi-target strategy would also allow a more effective system regarding the detection of atypical strains.

To create such detection system, it is however not possible to use the already developed assays since they use distinct chemistries (e.g. TaqMan® or SYBR®Green), use different PCR programs and were validated using different processes since no official guidelines with specific acceptance criteria are available to specifically validate food microbiology qPCR assays.

Few years ago, in the field of Genetically Modified Organisms (GMO) detection, a system named CoSYPS (for Combinatory SYBR®Green qPCR Screening) was developed (216;217). This system is associated with a decision support system (DSS) that automatically compares the results of the samples with the combination of positive markers present in each known GMO. This DSS gives the list of GMO potentially present in the tested sample. This system provides a fast and multi-targets GMO detection approach. Its ability to detect several targets in a same screening system using a set of simplex qPCR assays (Combinatory qPCR) would be of great interest to overcome the drawbacks identified in the foodborne pathogens detection problematic. Moreover, the use of the SYBR®Green technology will allow to easily implement the system in food microbiology control laboratories as it is runnable in every qPCR apparatus and is relatively affordable compared with other qPCR technologies.

Chapter II: Objectives of the study

Salmonella spp. and *L. monocytogenes* are major foodborne pathogens. Conventional culture-based methods to detect them in food products are time consuming and labor intensive. Available PCR or qPCR alternative methods are much faster but present several major drawbacks: i) already developed qPCR assays mostly target only one foodborne pathogen (single-genus) and are therefore unable to simultaneously detect several foodborne pathogens; ii) these detection assays use a single primer-pair (single-target) and this can potentially lead to false negative results in case of mutation or deletion of the target sequence; iii) these assays are not harmonized: use distinct chemistries, different PCR programs and therefore can't be used altogether as multi-genera detection system and iv) no official guideline with specific acceptance criteria are available to validate food microbiology qPCR assays.

The CoSYPS Path Food project (*Combinatory SYBR®Green qPCR Screening system for pathogens detection in food samples*) tends to overcome these drawbacks by developing and validating a multi-genera, multi-levels and multi-targets SYBR®Green qPCR screening system for simultaneous detection of living *Salmonella* spp. and *Listeria* spp in food samples and to validate the complete workflow (containing enrichment, qPCR detection, isolation and confirmation of the detected strain) on carcass swab samples. Aforementioned drawbacks were taken into consideration in applying the following strategies. First, to reduce the potentiality of false-negatives due to atypical strains which may be deleted or mutated for one of the targets each discrimination level is composed of at least two qPCR assays (Chap III). In order to get a system that is economical, easily implementable in routine lab and capable to display good selectivity, the SYBR®Green chemistry was chosen (Chap III). Then to create a user friendly system, all assays were designed to be run simultaneously on a single-plate (Chap III). Afterwards, to validate our qPCR system taking into consideration food-microbiology specificity, an adapted validation process and acceptance criteria were applied (Chap III). Next, to get a harmonized results-analysis, a decision support system, containing all assays, was designed (Chap III). To consider the viability problematic in qPCR detection, a study using viability-qPCR was performed (Chap IV) and finally to get a system applicable on beef carcass swab samples, all steps from food sample to strain isolation and confirmation were added and ISO 16140:2003 validated by comparison to the reference methods (Chap V).

Part II: Results

"Luck helps sometimes, work always."

Hindu proverb

Divided in three chapters:

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Chapter III: Development of the CoSYPS Path Food detection system

Section 1: Development and validation of *Listeria* qPCR assays

The first phase of the CoSYPS Path Food detection system development was the development of SYBR®Green qPCR assays for the detection of all *Listeria* spp. and the discrimination of the most important species of this genus i.e. *Listeria monocytogenes*. The qPCR assays constituting this system should all be run with the same PCR program to permit their run on a single plate. Moreover, they were all assessed for a number parameters taking into account the specificity of the qPCR technology and the detection of pathogenic bacteria in food samples.

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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Development and validation of qualitative
SYBR®Green Real-Time PCR for detection and
discrimination of *Listeria* spp. and *Listeria*
monocytogenes

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Abstract

A combination of four qualitative SYBR®Green qPCR screening assays targeting two levels of discrimination: *Listeria* genus (except *Listeria grayi*) and *Listeria monocytogenes*, is presented. These assays have been developed to be run simultaneously using the same polymerase chain reaction (PCR) program. The paper also proposes a new validation procedure to specifically validate qPCR assays applied to food microbiology according to two guidelines: the ISO 22118 norm and the “Definition of minimum performance requirements for analytical methods of GMO testing”. The developed assays target the *iap*, *prs* and *hlyA* genes that belong to or neighbour the virulence cluster of *Listeria* spp. The selected primers were designed to amplify short fragments (60 to 103 bp) in order to obtain optimal PCR efficiency (between 97 and 107% efficiency). The limit of detection of the SYBR®Green qPCR assays is two to five copies of target genes per qPCR reaction. These assays are highly accurate (98.08 and 100% accuracy for the *Listeria* spp. and *L. monocytogenes* assays, respectively).

Keywords

Real-time PCR, SYBR®Green, Foodborne pathogens, Detection, *Listeria*, qPCR validation

I. Introduction

Listeria are small Gram-positive bacilli, ubiquitous, non-sporeforming, facultative anaerobic bacteria that grow between –2 and 50°C, with optimal growth between 30 and 37°C (Bajard *et al.* 1996; Farber and Peterkin 1991). The *Listeria* genus officially includes six species: *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria grayi* (Garrity *et al.* 2004). Two other species, *Listeria marthii* (close to *L. monocytogenes* and *L. innocua*) and *Listeria rocourtiae* (close to *L. grayi*), have recently been described (Graves *et al.* 2010 and Leclercq *et al.* 2010) but have not yet been introduced into the official classification (Garrity *et al.* 2004). Among those, *L. monocytogenes* is the most reported as pathogenic for humans (listeriosis) (McLauchlin *et al.* 2004). However, some cases of listeriosis have also been attributed to *L. ivanovii* (Cummins *et al.* 1994; Guillet *et al.* 2010; Lessing *et al.* 1994), *L. innocua* (Perrin *et al.* 2003) and *L. seeligeri* (Rocourt *et al.* 1986).

L. monocytogenes has a low annual incidence in Europe, with about 1,500 cases per year (Anonymous 2011a), but with its high fatality rate listeriosis ranks among the most frequent cause of human death due to foodborne illnesses (Cardoen *et al.* 2009; de Valk 2005). Foodborne listeriosis mainly affects a specific group of the population with increased susceptibility: the YOPI (young, old, pregnant and immunodepressed) (Anonymous 2001). Hence, due to ageing of the population, control of *Listeria* spp. is becoming an increasingly important issue.

Listeriosis cases are associated with the consumption of raw food products (Berrada *et al.* 2006; Tham *et al.* 2000; Inoue *et al.* 2000; Rocourt *et al.* 2000; de Valk 2001; Inoue *et al.* 2000), ready-to-eat food (de Valk 2001) and post-processing contaminated food (Majjala *et al.* 2001; Makino *et al.* 2005; Norrung *et al.* 1999). The ability of *Listeria* spp. to grow at low temperatures increases the risk of infection. International standards exist for the detection and enumeration of *L. monocytogenes* in food and feed (Anonymous 1996a; Anonymous 1996b). These methods are time consuming (at least 5 days) and labour intensive. In order to quickly identify the source of foodborne outbreaks or for a faster commercial batch release, tools for rapid detection and identification of pathogens in food have been developed. The majority of these assays are limited to the specific detection of *L. monocytogenes*. They are classical PCR assays (Amagliani *et al.* 2004; Jung *et al.* 2003; Li *et al.* 2000; Liu *et al.* 2004; Mukhopadhyay and Mukhopadhyay 2007; Winters *et al.* 1999), reverse-transcription PCR (RT-PCR) (Klein and Juneja 1997) and, more recently, real-time PCR (qPCR) using mainly the TaqMan® technology (Hough *et al.* 2002; O'Grady *et al.* 2008; Oravcova *et al.* 2007; Rossmanith *et al.* 2006; Rudi *et al.* 2005). They are targeting genes such as *iap*, *prfA* and *hlyA* involved in *L. monocytogenes* pathogenicity (Dussurget *et al.* 2004) and are therefore specific for this species.

In this paper, we propose a new detection system that facilitates a rational detection of pathogenic bacteria using as study case the *Listeria* genus, considered as one of the most important of foodborne pathogens transmitted by food and water (Cardoen *et al.* 2009; Anonymous 2011b). The Combinatory SYBR®Green qPCR screening for foodborne pathogens (CoSYPS Path Food) is based on two detection levels. A first set of generic assays allows the detection of the presence of all bacteria belonging to the *Listeria* genus. A second set of assays allows the specific detection of *L. monocytogenes*. The four primer pairs were chosen in order to perform at the same PCR conditions, allowing the different assays to be performed as four simplex assays simultaneously, on the same plate. Moreover, as there is no official method to

validate a qPCR assay applied to food microbiology, a guideline to validate food microbiology qPCR assay based on the ISO 22118 norm (Anonymous 2011b) and the “Definition of minimum performance requirements for analytical methods of GMO testing” (Anonymous 2008a) is also proposed. The four SYBR®Green qPCR assays developed in this study were evaluated for selectivity, sensitivity, dynamic range, PCR efficiencies, repeatability and reproducibility. The advantages of the CoSYS Path Food screening strategy and the validation guideline are discussed.

II. Materials and methods

A. Bacterial strains

The bacterial strains used in this study are listed in Table 8. A panel of 128 bacterial⁴, two moulds, two yeast and two virus strains has been used. The strains were obtained from the National Reference Centres and Laboratories.

B. Bacterial growth conditions, DNA extraction and DNA quantification

Overnight cultures of each bacterial strain were grown in liquid brain–heart infusion or Bolton liquid medium (for *Campylobacter*) at adequate temperature and oxygen conditions. The total DNA from each strain was extracted with the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (gDNA) from yeast and fungal were extracted using the Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH) and ZR Fungal/Bacterial gDNA extraction (Zymo Research), respectively. Viral RNA was extracted using the RNeasy mini kit (Qiagen) and cDNA was obtained by reverse transcription using the Transcriptor high-fidelity cDNA synthesis kit (Roche). cDNA amplification was checked with a specific PCR amplification. All kits were used according to the manufacturer’s recommendations. DNA quality was controlled on agarose gel and DNA concentration was measured using a Nanodrop® 2000 device according to the manufacturer’s recommendations.

C. Development and *in silico* assessment of primer pairs

A uniform primer design approach was applied in the development of all primer pairs. The first step consisted of collecting a set of genes of potential interest, either genus or species specific (Glaser *et al.* 2001; Hough *et al.* 2002; Kerouanton *et al.* 2009; Liu *et al.* 2004; McLauchlin *et al.* 2004; O’Grady *et al.* 2008; Oravcova *et al.* 2007; Pan and

⁴ Strains previously characterized by the laboratory providing them.

Breidt 2007). The second step included the collection of DNA sequences relevant for the selected targets from the NCBI public database (<http://www.ncbi.nlm.nih.gov/sites/entrez>). The primer pairs were designed, preferentially within conserved regions, using the "Primer 3" program (<http://frodo.wi.mit.edu/primer3/>) (Rozen and Skaletsky 2000) with the "product size range" specification set at "60 to 120 bp" and "primer size" optimal set at "22 bases". An *in silico* test of the primer pairs' selectivity was then performed. This test consisted of a bioinformatical analysis carried out with the "wprimersearch" software (<https://wembooss.uio.no/wEMBOSS/>) (Rice *et al.* 2000; Sarachu and Colet 2005), which mimics the PCR amplification of the tested primers on a database of bacterial genome sequences from NCBI of 217 bacteria, representing 103 species belonging to 61 genera. Only primer pairs that gave the expected *in silico* amplification were retained for the *in situ* test. When mismatches between the primers and one of the targets were observed, degenerate nucleotides were introduced into the primer sequence. However, primer pairs with no degenerate nucleotides were always preferred.

Table 8 Selectivity assessment of the four SYBR®Green qPCR assays: "*iap*-50-deg", "*prs*-2-deg", "*hlyA*-177" and "*hlyA*-146-deg-tronc"

Genus	Species	Serogroup	GRAM	Origin	Reference	<i>iap</i> -50-deg	<i>prs</i> -2-deg	<i>hlyA</i> -177	<i>hlyA</i> -146-deg-tronc
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/13	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/14	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/15	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/20	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/21	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/22	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	10/23	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2a	+	List-NRC	ATCC 51772	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	ATCC 51777	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	10/2	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	10/28	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	10/50	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	10/68	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2b	+	List-NRC	10/109	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/48	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/16	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/49	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/58	nt	nt	+	+

Part II: Results, Chapter III: Development of the CoSYS Path Food detection system
Section 1: Development and validation of *Listeria* qPCR assays

Table 8 (continued)

Genus	Species	Serogroup	GRAM	Origin	Reference	iap-50-deg	prs-2-deg	hlyA-177	hlyA-146-deg-tronc
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/153	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/160	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/184	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	1/2c	+	List-NRC	10/192	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	10/29	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	10/202	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	10/237	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	9/109	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	9/181	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3a	+	List-NRC	8/171	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3b	+	List-NRC	8/115	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3b	+	List-NRC	Würzburg	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3c	+	List-NRC	6/64	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3c	+	List-NRC	6/125	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3c	+	List-NRC	6/137	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3c	+	List-NRC	6/275	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	3c	+	List-NRC	6/301	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4a	+	List-NRC	10/118	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4a	+	List-NRC	ATCC 19114	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4b	+	List-NRC	10/24	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4b	+	List-NRC	ATCC 51780	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4b	+	List-NRC	10/7	+	+	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4b	+	List-NRC	10/1	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4b	+	List-NRC	10/3	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4d	+	List-NRC	7/89	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4d	+	List-NRC	7/114	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4d	+	List-NRC	5/163	nt	nt	+	+
<i>Listeria</i>	<i>monocytogenes</i>	4e	+	List-NRC	10/35	+	+	+	+
<i>Listeria</i>	<i>ivanovii</i>		+	List-NRC	CIP 7842	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	ILVO	LMG11388	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	List-NRC	06/124	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	List-NRC	06/129	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	EU-RL List	TQA237	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	EU-RL List	TQA238	+	+	-	-
<i>Listeria</i>	<i>ivanovii</i>		+	EU-RL List	00CHPL02	+	+	-	-
<i>Listeria</i>	<i>seeligeri</i>		+	List-NRC	ATCC 35967	+	+	-	-
<i>Listeria</i>	<i>seeligeri</i>		+	ILVO	MB43:	+	+	-	-
					LMG16764				
<i>Listeria</i>	<i>seeligeri</i>		+	EU-RL List	TQA231	+	+	-	-
<i>Listeria</i>	<i>seeligeri</i>		+	EU-RL List	TQA232	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	ATCC 35897	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	06/102	+	+	-	-

Table 8 (continued)

Genus	Species	Serogroup	GRAM	Origin	Reference	iap-50- deg	prs-2- deg	hlyA- 177	hlyA-146- deg-tronc
<i>Listeria</i>	<i>seeligeri</i>		+	EU-RL List	TQA232	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	ATCC 35897	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	06/102	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	06/229	+	+	nt	nt
<i>Listeria</i>	<i>welshimeri</i>		+	List-NRC	04/341	+	+	nt	nt
<i>Listeria</i>	<i>welshimeri</i>		+	EU-RL List	02CHPL153	+	+	nt	nt
<i>Listeria</i>	<i>welshimeri</i>		+	EU-RL List	02CHPL154	+	+	nt	nt
<i>Listeria</i>	<i>welshimeri</i>		+	EU-RL List	03CHPL91	+	+	nt	nt
<i>Listeria</i>	<i>welshimeri</i>		+	EU-RL List	TQA230	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	EU-RL List	03CHPL98	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	CIP 8011	+	+	-	-
<i>Listeria</i>	<i>innocua</i>		+	ILVO	MB176(T) FML2011	+	+	-	-
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	10/85	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	10/101	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	10/122	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/158	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/221	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/291	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	10/101	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	10/122	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/158	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/221	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	09/291	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	08/147	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	07/92	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	List-NRC	06/237	+	+	nt	nt
<i>Listeria</i>	<i>innocua</i>		+	IPH-FP	TIAC 706	+	+	nt	nt
<i>Listeria</i>	<i>grayi</i>		+	List-NRC	ATCC 25401	-	-	-	-
<i>Listeria</i>	<i>grayi</i>		+	ILVO	LMG16490	-	-	-	-
<i>Aeromonas</i>	<i>hydrophila</i>		-	IPH-CB	6688 (M/2862) (EEQ 2003/2))	-	-	-	-
<i>Bacillus</i>	<i>cereus</i>		+	IPH-FP	ATCC 14579	-	-	-	-
<i>Bacillus</i>	<i>circulans</i>		+	IPH-FP	TIAC 100	-	-	-	-
<i>Bacillus</i>	<i>lentus</i>		+	IPH-FP	TIAC 101	-	-	-	-
<i>Bacillus</i>	<i>licheniformis</i>		+	IPH-FP	TIAC 102	-	-	-	-
<i>Bacillus</i>	<i>mycoides</i>		+	IPH-FP	TIAC 97	-	-	-	-
<i>Bacillus</i>	<i>sphaericus</i>		+	IPH-FP	TIAC 104	-	-	-	-
<i>Bacillus</i>	<i>subtilis</i>		+	IPH-FP	TIAC 103	-	-	-	-
<i>Bacillus</i>	<i>thuringiensis</i>		+	IPH-FP	TIAC 96	-	-	-	-
<i>Brevibacillus</i>	<i>borstelensis</i>		+	IPH-FP	TIAC 099	-	-	-	-

Part II: Results, Chapter III: Development of the CoSYS Path Food detection system
Section 1: Development and validation of *Listeria* qPCR assays

Table 8 (continued)

Genus	Species	Serogroup	GRAM	Origin	Reference	iap-50-deg	prs-2-deg	hlyA-177	hlyA-146-deg-tronc
<i>Bacillus</i>	<i>subtilis</i>		+	IPH-FP	TIAC 103	-	-	-	-
<i>Bacillus</i>	<i>thuringiensis</i>		+	IPH-FP	TIAC 96	-	-	-	-
<i>Brevibacillus</i>	<i>borstelensis</i>		+	IPH-FP	TIAC 099	-	-	-	-
<i>Brochothrix</i>	<i>thermosphacta</i>		+	IPH-FP	TIAC 400	-	-	-	-
<i>Campylobacter</i>	<i>coli</i>		-	IPH-FP	ATCC 33559 T	-	-	-	-
<i>Campylobacter</i>	<i>jejuni</i>		-	IPH-FP	ATCC 33291	-	-	-	-
<i>Campylobacter</i>	<i>lari</i>		-	IPH-FP	TIAC 542	-	-	-	-
<i>Carnobacterium</i>	<i>divergens</i>		+	IPH-FP	Argentijns vlees B21	-	-	-	-
<i>Citrobacter</i>	<i>freundii</i>		-	IPH-FP	TIAC 554	-	-	-	-
<i>Clostridium</i>	<i>perfringens</i>		+	IPH-FP	ATCC 13124 T	-	-	-	-
<i>Carnobacterium</i>	<i>divergens</i>		+	IPH-FP	Argentijns vlees B21	-	-	-	-
<i>Citrobacter</i>	<i>freundii</i>		-	IPH-FP	TIAC 554	-	-	-	-
<i>Clostridium</i>	<i>perfringens</i>		+	IPH-FP	ATCC 13124 T	-	-	-	-
<i>Enterobacter</i>	<i>cloacae</i>		-	IPH-FP	TIAC 445	-	-	-	-
<i>Enterococcus</i>	<i>faecalis</i>		+	IPH-CB	ATCC 29212	-	-	-	-
<i>Escherichia</i>	<i>coli</i>		-	IPH-FP	ATCC 25922	-	-	-	-
<i>Escherichia</i>	<i>coli</i>	O157	-	IPH-FP	EH 630	-	-	-	-
<i>Hafnia</i>	<i>alvei</i>		-	IPH-CB	7186	-	-	-	-
<i>Klebsiella</i>	<i>pneumoniae</i>		-	IPH-FP	TIAC 446	-	-	-	-
<i>Lactobacillus</i>	<i>acidophilus</i>		+	IPH-FP	Argentijns vlees A19	-	-	-	-
<i>Lactobacillus</i>	<i>brevis</i>		+	IPH-FP	Argentijns vlees A53	-	-	-	-
<i>Lactobacillus</i>	<i>curvatus</i>		+	IPH-FP	Argentijns vlees A1	-	-	-	-
<i>Lactobacillus</i>	<i>delbrucki</i>		+	IPH-FP	Argentijns vlees B17	-	-	-	-
<i>Lactobacillus</i>	<i>plantarum</i>		+	IPH-FP	Argentijns vlees B34	-	-	-	-
<i>Lactococcus</i>	<i>lactis lactis</i>		+	IPH-FP	Argentijns vlees A31	-	-	-	-
<i>Leuconostoc</i>	<i>citreum</i>		+	IPH-FP	Argentijns vlees B24	-	-	-	-
<i>Leuconostoc</i>	<i>mesenteroides</i>		+	IPH-FP	Argentijns vlees B6	-	-	-	-
<i>Paenibacillus</i>	<i>polymyxa</i>		+	IPH-FP	TIAC 105	-	-	-	-
<i>Proteus</i>	<i>vulgaris</i>		-	IPH-CB	6223 (M/654 (EEQ 1996/3))	-	-	-	-

Table 8 (continued)

Genus	Species	Serogroups	GRAM	Origin	Reference	<i>iap</i> -50- deg	<i>prs</i> -2- deg	<i>hlyA</i> - 177	<i>hlyA</i> -146- deg-tronc
<i>Leuconostoc</i>	<i>citreum</i>		+	IPH-FP	Argentijns vlees B24	-	-	-	-
<i>Leuconostoc</i>	<i>mesenteroides</i>		+	IPH-FP	Argentijns vlees B6	-	-	-	-
<i>Paenibacillus</i>	<i>polymyxa</i>		+	IPH-FP	TIAC 105	-	-	-	-
<i>Proteus</i>	<i>vulgaris</i>		-	IPH-CB	6223	-	-	-	-
<i>Pseudomonas</i>	<i>aeruginosa</i>		-	IPH-FP	LMG 6395	-	-	-	-
<i>Salmonella</i>	<i>enterica enterica</i>	Enteritidis	-	Salm-NRC	H,VI,6,32	-	-	-	-
<i>Salmonella</i>	<i>enterica enterica</i>	Typhimurium	-	Salm-NRC	H,II,32,32	-	-	-	-
<i>Serratia</i>	<i>marcescens</i>		-	IPH-CB	7015	-	-	-	-
<i>Shigella</i>	<i>sonnei</i>		-	Salm-NRC	10-03865	-	-	-	-
<i>Staphylococcus</i>	<i>aureus</i>		+	IPH-FP	ATCC 25923	-	-	-	-
<i>Staphylococcus</i>	<i>epidermidis</i>		+	IPH-FP	TIAC 367	-	-	-	-
<i>Staphylococcus</i>	<i>pisciferm</i>		+	IPH-FP	TIAC 364	-	-	-	-
<i>Streptococcus</i>	<i>faecales</i>		+	IPH-FP	TIAC 300	-	-	-	-
<i>Vibrio</i>	<i>parahaemoliticus</i>		-	IPH-FP	TIAC 610	-	-	-	-
<i>Yersinia</i>	<i>enterocolitica</i>		-	IPH-FP	LMG 15558	-	-	-	-
<i>Aspergillus</i>	<i>fumigatus</i>		na	IPH-MA	BCCM/IHE M 19436	-	-	-	-
<i>Cladosporium</i>	<i>sphaerospermum</i>		na	IPH-MA	BCCM/IHE M 24474	-	-	-	-
<i>Saccharomyces</i>	<i>cerevisiae</i>		na	IPH-MA	BCCM/IHE M 3961	-	-	-	-
<i>Candida</i>	<i>parapsilosis</i>		na	IPH-MA	BCCM/IHE M 6478	-	-	-	-
Hepatitis A Virus			na	IPH-FP	27 (WZ)	-	-	-	-
Norovirus			na	IPH-FP	2593	-	-	-	-
No template controle			na	na		-	-	-	-

+ there is an amplification and a T_m value similar for all corresponding strains, - no amplification, List-NRC Belgian *Listeria* National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium, EU-RL List EU-RL *Listeria monocytogenes*, 23 avenue du Général de Gaulle, 94706 Maisons-Alfort cedex, France, ILVO Instituut voor Landbouw en Visserijonderzoek, Technology & Food Science Unit, Food Safety, Product Quality and Innovation, and Business Unit and Service Centre, Brusselsesteenweg 370, 9090 Melle, Belgium, IPH-FP Scientific Institute of Public Health, Food Pathogens Laboratory, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium, IPH-CB Scientific Institute of Public Health, Clinical Biology, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium, Salm-NRC Belgian *Salmonella* and *Shigella* National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium, IPH-MA Mycology and Aerobiology, Scientific Institute of Public Health, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium, nt not tested, na not applicable

D. Qualitative SYBR®Green qPCR assay

All qPCR assays were performed in accordance with the general requirements from the ISO norm 22119 (Anonymous 2011c) except those specific for the TaqMan® chemistry since the SYBR®Green was used. All qPCR assays were performed on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) with MicroAmp® Optical 96-Well Reaction Plate closed with the MicroAmp® Optical 8-Cap Strip (Applied Biosystems). The reaction was performed in a final volume of 25 µl containing 5 µl of the appropriate template (10^4 copies of gDNA for the selectivity test or serial dilution of gDNA for the sensitivity test), 1X SYBR®Green PCR Mastermix (Diagenode) and the appropriate concentration of each primer (Table 9). The following thermal program was applied: a single cycle of DNA polymerase activation for 10 min at 95°C followed by 40 amplification cycles of 15 s at 95°C (denaturing step) and 1 min at 60°C (annealing & extension step). Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60 to 95°C in 20 min ($\pm 0.6^\circ\text{C}/20\text{s}$). The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit setting was performed in automatic mode, according to the ABI Sequence Detection Software version 1.4 (Applied Biosystems). “No template” controls (NTC) using DNase and RNase free water (Acros) were included in each reaction to assess primer dimer formation or non-specific amplification.

For the interpretation of a SYBR®Green qPCR assay, two criteria were taken into consideration: the quantification cycle (Cq) value and the melting temperature of the amplicon (Tm). The Cq value represents the fractional cycle at which PCR amplification reaches the threshold level for the reaction (Bustin 2000). Since it is a screening assay, a qualitative response is required. To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level, with a single peak upon melting analysis giving a unique Tm value. A signal was considered as negative when no Cq value was obtained.

E. Selectivity test and accuracy calculation

Primer pairs that passed the *in silico* evaluation were tested *in situ*. The latter selectivity test consisted of two steps:

1. A preliminary selectivity test involving few target strains (closely relative families) and few non-target strains (most important pathogenic bacteria) was performed. Primer pairs amplifying only the target strains were tested for full selectivity.

2. The full selectivity test allows testing the inclusivity and exclusivity of each developed assay. This experimental design follows the ISO 22118 norm (Anonymous 2011b) as it involves 50 target strains and at least 52 non-target strains representing 53 species belonging to 29 genera and a NTC (Table 8) (Anonymous 2011b). The non-target relevant microorganisms to test the exclusivity were chosen among taxonomically closely related and not closely related (pathogenic or not) bacteria that can be present in the food matrices (Anonymous 2011b).

The qPCR reactions were performed with approximately 10^4 copies of genomic DNA calculated according to the genome size of each targeted bacteria using the following formula:

$$Cn = \frac{m \times Ac}{Mw \times Gs}$$

where Cn = copy number, m = amount of gDNA (grams), Ac = Avogadro's constant (Mohr *et al.* 2008) $0.602214179 \times 10^{23} \text{ mol}^{-1}$, Mw = base pair mean molecular weight = 649 Da and Gs = Genome size (in base pairs).

The accuracy of the assay can be calculated from the selectivity test. The accuracy represents the closeness of agreement between a test result and the accepted reference value (Anonymous 1993). Its formula is found in Anonymous (2003). Five criteria were set to define a "specific signal" generated in the selectivity of a SYBR®Green qPCR analysis (Barbau-Piednoir *et al.* 2010): (1) an (exponential) amplification above the threshold level should be obtained with template DNA for the positive strains, while negative controls (NTC and gDNA from negative strains) should not yield such amplification, (2) with positive strain template DNA, the obtained PCR product(s) should present a single peak upon melting analysis with a unique T_m value, while no specific peak should be detectable in the negative strains and negative controls, (3) positive reactions should display a single band on agarose gel analysis with (4) a size corresponding to the one predicted ($SD \pm 10$ bp) and (5) the sequence of the amplicon, verified by sequencing, should be correct as to guarantee that the amplified fragment is indeed the target.

F. Amplicon cloning and sequencing

All PCR reactions for cloning and sequencing were performed on an iCycler PCR System (Biorad) in 25- μ l reaction volume containing 10^6 copies of gDNA, 1 U Pfu DNA Polymerase (Fermentas), 1X DNA polymerase buffer, 0.2 mM of each dNTP and 250 nM of each primer (Table 9; T7 forward and M13 reverse primer). The following thermal programme was applied: a single cycle of initial denaturation for 2 min at 95°C followed by 30 amplification cycles of 15 s at 95°C (denaturing step), 15 s at 60°C (annealing step) and 1 min at 72°C (extension step). A subsequent final elongation step of the amplification products was performed using 72°C for 10 min. PCR fragments obtained by “classical” PCR amplification using *L. monocytogenes* 1/2a strain ATCC 51772 as template were cloned into a TOPO pCR®2.1 plasmid (Invitrogen) according to the manufacturer’s recommendations. The plasmids containing the different amplicons (TOPO pCR® 2.1-amplicon) were transformed into TOP10F’ competent cells (Invitrogen) according to the manufacturer’s recommendations. The “TOPO pCR 2.1-amplicon” was checked for insert length by PCR reaction using T7 forward and M13 reverse primers. Agarose gel electrophoresis was performed using 1 or 3% precast gels (Biorad) and 1x TBE (89 mM TRIS-borate, 2 mM EDTA) at 100 V for 15 min, including a 100-bp–2-kb Molecular Marker (BioRad). Plasmids that gave the expected size on agarose gel were purified with the Plasmid Mini kit (Qiagen). The inserts were then sequenced with the T7 forward and M13 reverse primers using a dideoxy sequence analysis on an ABI3130xl Genetic Analyzer apparatus (Applied Biosystems) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer’s recommendations. Forward and reverse sequences of a “TOPO pCR2.1-amplicon” plasmid were aligned by ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The amplicon sequence was compared to NCBI sequences database (MEGABLAST) to confirm that it corresponds to the target gene (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

G. Determination of the optimal primer concentration

The optimal concentration of the selected primer pairs was determined by testing different concentrations of each primer between 250 and 1,000 nM. The concentrations giving the lowest Cq value without inducing the formation of a high level of primer dimers were selected. The primer dimer dissociation peak should not be higher than the dissociation peak from the positive samples from high concentrations until the limit of detection (LOD).

Table 9: Primer pair sequence, concentration and amplicon size and T_m value for each SYBR®Green qPCR assay.

Targeted genus	Targeted species	Targeted gene	Primer pair name	Primer Sequence 5'→3'	Primer concentration (nM)	Product size (bp)	Tm amplicon	Reference
<i>Listeria</i>	all (except <i>L. grayi</i>)	invasion associated protein (<i>iap</i>)	<i>iap</i> -50-deg	<i>iap</i> -31deg-F CAYCCGCWAGCACWGTAGTAGT	1000	78	75.5-76°C <i>L. monocytogenes</i> , <i>L. seeligeri</i> , <i>L. welshimeri</i> and <i>L. ivanovi</i> ; 77°C <i>L. innocua</i> .	This study
			<i>iap</i> -50-deg-R	GCGTCRACAGTWTGCGH TT	1000			
		Phosphoribosyl pyrophosphate synthetase (<i>prs</i>)	<i>prs</i> -2-deg	<i>prs</i> -2-F ATTTTCTCGCTAAATCTAA TCGTG	250	60	71.6°C	This study
			<i>prs</i> -2-R-deg	CAATACCACTTCTTTTCGCAATCTC	250			
			<i>hlyA</i> -177-F	TGCAAGTCCTAA GACGCCA	250	112	74°C	Nogva et al., 2000
<i>Listeria</i>	<i>monocytogenes</i>	Listeriolysin O (<i>hlyA</i>)	<i>hlyA</i> -177-R	CACTGCATCTCCGTGGTAACTACTAA	250			
			<i>hlyA</i> -146-deg-front-F	AAATCTGTCTCA GGYGATGT	1000	103	73.5-74°C for <i>Listeria monocytogenes</i> 12a, 12c, 3a, 3c, 4a, 4d and 4e and 75°C for <i>Listeria monocytogenes</i> 12b, 3b, 4b and 4c.	Adapted from Hough et al., 2002
			<i>hlyA</i> -146-deg-front-R	CGATGATTTGAACTTCATCTTTTC	1000			

In italics are degenerated nucleotides: R=A or G, Y=C or T, W=A or T, S=C or G, H=A or C or T

H. Dynamic range and calculation of the PCR efficiency

The dynamic range of an assay is the concentration range where it performs in a linear manner. The SYBR®Green qPCR assays dynamic range was assessed by the analysis in duplicate of a serial dilution in a carrier DNA (4 ng/µl calf thymus DNA (Invitrogen)) of pure strain DNA (1,000 to 0.01 theoretical genomic copies) of *L. monocytogenes* 1/2a ATCC 51772 and *L. ivanovii* LMG 11388 for *Listeria* spp. assays and *L. monocytogenes* 1/2a ATCC 51772 and *L. monocytogenes* 4b ATCC 51777 for *L. monocytogenes* assays. The carrier DNA is meant to avoid the dilution problem associated with low gDNA concentration. These analyses allow the assessment of the coefficient of determination (R^2) and the PCR efficiency (E) for each SYBR®Green qPCR assay. R^2 is an indicator of the correlation of data regarding the linear regression curve. R^2 of a dynamic range curve should be above 0.98 (Anonymous 2008a). E should be between 89.6 and 110.2% (Anonymous 2008a) and can be calculated according to the formula described in Rutledge and Cote (2003).

I. Sensitivity test

Primer pairs passing the selectivity test were subsequently examined for their sensitivity. Using serial dilution, the SYBR®Green qPCR assays were tested to evaluate their LOD, which is defined as the concentration of an analyte that gives a positive result with a probability of 95% (Anonymous 2008). The strains used were *L. monocytogenes* 1/2a ATCC51772 and *L. ivanovii* LMG 11388 for *Listeria* spp. assays and *L. monocytogenes* 1/2a ATCC 51772 and *L. monocytogenes* 4b ATCC 51777 for *L. monocytogenes* assays. The calculation of the target genomic copy numbers for each dilution point was done according to the equation given in the selectivity part, considering that the gDNA size of *L. monocytogenes* and *L. ivanovii* are 2,976,163 bp (accession # CP002816) and 2,928,879 bp (accession # FR687253), respectively. To determine LOD, a range of copy number between 10 and 0.1 theoretical copies was tested (i.e. 10, 5, 2, 1, 0.5, 0.2 and 0.1). Each dilution was tested in six replicates per plate for both strains. Moreover, the analysis was performed three independent times under repeatable conditions, resulting in 36 repeats for each dilution point. The dilution series continued after the theoretical single copy to assess the dilution series correctness. Indeed it is statistically impossible to get amplification in all the reactions with the dilutions below 1 theoretical copy. If this is the case, the template concentration has to be checked and the dilution series have to be redone.

J. Repeatability calculation

To evaluate the repeatability of the assays, the data from the independent tests performed for the sensitivity test, with the same protocol, with the same samples and by the same operator using the same apparatus within a short interval of time (Anonymous, 1993) were used. The repeatability limit (r) is the maximal difference between two test results, obtained under repeatable conditions, expected with a probability of 95% (Anonymous, 1993). In future analyses, if the difference between values obtained under repeatable conditions exceeds r , the values should be considered suspicious. The repeatability limit is obtained with the formula found in Anonymous (2003).

The relative standard deviation of repeatability (RSD_r) represents the absolute value of the coefficient of variation. It is expressed in percent and is obtained by multiplying the repeatability standard deviation by 100 and dividing this product by the repeatability median (Anonymous 2003).

According to the guidelines of Anonymous (2008a), RSD_r should be $\leq 25\%$ for all the dilutions above LOD. The RSD_r and r values of the C_q values have been calculated at each dilution point. The RSD_r and r values of the T_m values have been calculated with all the T_m values coupled with amplification ($C_q \neq 40$).

K. Reproducibility study and calculation

To evaluate the reproducibility of the assays (Anonymous 1993), independent tests were performed with the same protocol, using the same eight samples, in two different laboratories by two different operators using two different apparatus (ABI7300 and Bio-Rad iQ5). The eight samples tested were all gDNA from *L. monocytogenes* strains at different concentrations between 200 and 5 genomic copies per reaction. Each sample was analysed in duplicate by each operator.

Two reproducibility measures can be calculated from these results: the relative standard deviation of reproducibility (RSD_R) and the uncertainty (U). RSD_R represents the absolute value of the coefficient of variation. It is expressed in percent and is obtained by multiplying the reproducibility standard deviation by 100 and dividing this product by the reproducibility median (Anonymous 2003).

According to the guidelines of ENGL (Anonymous 2008a), RSD_R should be $\leq 35\%$ for all samples tested. The RSD_R of the C_q and T_m values are calculated for the eight samples tested.

“The uncertainty is the parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand” (Anonymous 2008b). The uncertainty of the C_q and T_m values of each SYBR®Green qPCR assay could be calculated from the results of the reproducibility test. The uncertainty can be expressed by the expanded uncertainty (U) which is the quantity defining “an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand” (Anonymous 2008b).

The expanded uncertainty U is obtained by multiplying the combined standard uncertainty by a coverage factor (Anonymous 2008b).

L. Combination of the four SYBR®Green qPCR detection assays

The four assays have been run, on the same plate with the same PCR programme (as described previously), using the appropriate concentration for each primer (Table 9) on 10⁴ copies of four gDNA extractions from pure culture of *L. monocytogenes* 4b (ATCC 51780), *L. ivanovii* (CIP 7848), *L. seeligeri* (ATCC 25401) and *L. grayi* (ATCC 25401). All of these strains were collected from the Belgian National Reference Centre of *L. monocytogenes*.

Table 10 : Limit of detection determination for all *Listeria* SYBR®Green qPCR assays.

The results are based on 36 repetitions with genomic targets as DNA template. Average, standard deviation and percentage of positive signal of Cq values obtained at each dilution point

		Mean Cq value $\pm$ SD (% positive)			
		<i>Listeria</i> spp. except <i>L. grayi</i> assays		<i>Listeria monocytogenes</i> assays	
		<i>iap</i> -50-deg	<i>prs</i> -2-deg	<i>hlyA</i> -177	<i>hlyA</i> -146-deg-tronc
Serial dilution points	Theoretical copy number/ assay				
10	10	32.96 $\pm$ 1.02 (100)	31.18 $\pm$ 0.74 (100)	32.85 $\pm$ 1.12 (100)	33.23 $\pm$ 0.87 (100)
	5	34.11 $\pm$ 1.45 (97.2)	32.19 $\pm$ 0.92 (100)	33.79 $\pm$ 1.31 (100)	33.93 $\pm$ 0.79 (97.2)
	2	34.75 $\pm$ 1.01 (80.6)	33.43 $\pm$ 0.70 (72.2)	35.2 $\pm$ 1.13 (75)	34.96 $\pm$ 1.2 (75)
	1	35.44 $\pm$ 1.02 (75)	34.23 $\pm$ 1.08 (55.6)	35.63 $\pm$ 0.97 (61.1)	34.18 $\pm$ 3.78 (50)
	0,5	35.51 $\pm$ 0.86 (44.4)	34.67 $\pm$ 0.52 (44.4)	36.04 $\pm$ 1.20 (36.1)	35.57 $\pm$ 1.20 (44.4)
	0,2	35.99 $\pm$ 1.05 (19.4)	34.61 $\pm$ 0.69 (27.8)	36.08 $\pm$ 1.09 (22.2)	35.62 $\pm$ 1.69 (22.2)
	0,1	35.85 $\pm$ 0.98 (22.2)	34.59 $\pm$ 0.48 (16.7)	36.46 $\pm$ 0.21 (8.3)	35.64 $\pm$ 1.62 (11.1)
Negative controls	Dilution buffer control	Below LOD	Below LOD	Below LOD	Below LOD
	Water control	Below LOD	Below LOD	Below LOD	Below LOD

LOD limit of detection

III. Results

A. Development and selection of qPCR SYBR®Green assays

1) Selection of the targeted genes

First, a survey of available genetic and genomic data was performed in order to select the *Listeria* genus and *L. monocytogenes* species-specific genes. For the *Listeria* genus, three genes were retained: *iap*, belonging to the known virulence genes of *Listeria* (Kuhn and Goebel 1989), and *ldh* and *prs* that are not involved in the virulence but directly flank a virulence gene cluster (Schmid *et al.* 2005). These three genes are present in all *Listeria* species (Schmid *et al.* 2005). For the specific detection of *L. monocytogenes*, the chosen gene was *hlyA*. This gene is involved, and is crucial, in the virulence of *L. monocytogenes* (Dussurget *et al.* 2004). The four selected genes (*iap*, *prs*, *ldh* and *hlyA*) are present in a single copy on *Listeria* chromosomes (Dussurget *et al.* 2004; Schmid *et al.* 2005).

2) Selection of the primer pairs

For the detection of the *Listeria* genus, 50 primer pairs were designed on *iap*, *prs* and *ldh* genes and tested together with another previously described *iap*-based primer pair (Klein and Juneja 1997). For the detection of *L. monocytogenes*, three *hlyA*-based primer pairs were selected from previous works (Hough *et al.* 2002; Nogva *et al.* 2000; Thulin Hedberg *et al.* 2009). After *in silico* evaluation of all these primer pairs and degeneration of some nucleotides (when necessary), 19 primer pairs remained. These 19 primer pairs were tested *in situ* with the preliminary selectivity test (data not shown) and resulted in only six pairs: *iap*-55-deg, *iap*-50-deg, *ldh*-2 and *prs*-2-deg for the *Listeria* spp. detection and *hlyA*-177 and *hlyA*-146-deg-tronc for the *L. monocytogenes* detection. These six pairs were then tested to determine the optimal concentration to be used in the SYBR®Green qPCR amplification (Table 9). Following this test, the *iap*-55-deg pair was discarded because of the formation of a high level of primer dimers.

B. Determination of SYBR®Green qPCR assays selectivity

Table 8 gives an overview of the strains tested for the selectivity test of each qPCR assay. For *iap*-50-deg and *prs*-2-deg *Listeria* spp. detection assays, a specific amplification was observed for 50 of the 52 target strains. The two *L. grayi* strains showed no amplification. As expected, no amplification was observed with the 52

non-target strains and the NTC (Table 8). Thus, these first two assays are 98.08% accurate for their target (*Listeria* spp.), giving 0% of false positive and 3.85% of false negative (two strains of *L. grayi*) results. The *ldh*-2 primer pair assay was discarded during the sensitivity study because of a high level of primer dimers at low target concentrations (data not shown). It has to be mentioned that *L. marthii* (accession # NZ_CM001047.1), which is close to *L. innocua* and *L. monocytogenes* (Graves *et al.* 2010) can be amplified, *in silico*, by the *Listeria* spp. assays (data not shown) and not by the *L. monocytogenes* assays (data not shown). *L. rocourtiae*, which is closely related to *L. grayi* (Leclercq *et al.* 2010), will probably be amplified by none of the developed SYBR®Green qPCR assays.

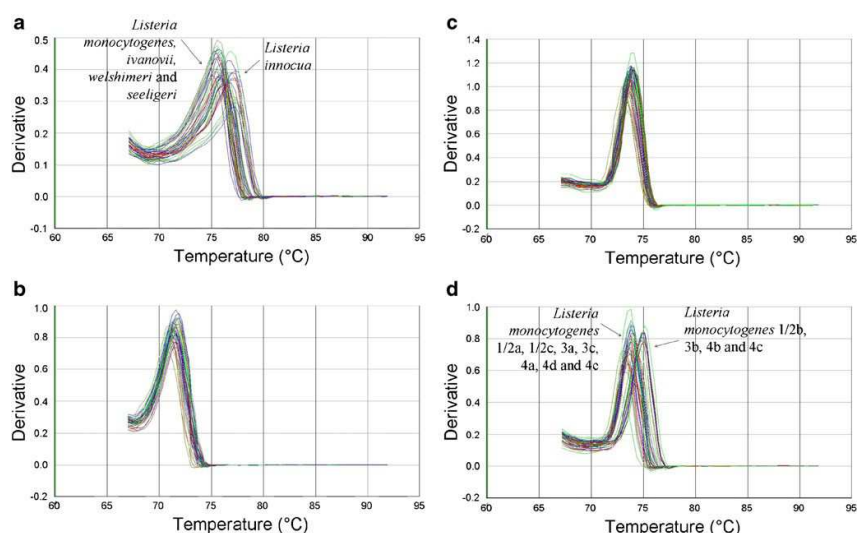


Figure 10 : Melting curves obtained by SYBR®Green qPCR analysis of the positive pure strains listed in Table 8.

The different qPCR assays are *iap*-50-deg (a), *prs*-2-deg (b), *hlyA*-177 (c) and *hlyA*-146-deg-tronc (d). The temperature is plotted on the X-axis versus the inverse of the first derivate of the best-fitted curve of the measured fluorescence decrease on the Y-axis

For *hlyA*-177 (Nogva *et al.* 2000) and *hlyA*-146-deg-tronc (adapted from Hough *et al.* 2002) *L. monocytogenes* detection strategy, a specific amplification was observed with the 50 target strains and no amplification was observed with the 65 non-target strains and the NTC (Table 8). These two approaches are 100% accurate for their targets (*L. monocytogenes*), giving 0% of false positive and 0% of false negative results.

These four assays always gave rise to a unique band of the expected size and the sequence of each amplicon corresponded with the one expected of *L. monocytogenes* (data not shown). In addition, all four SYBR®Green qPCR assays gave a unique melting peak for each target with different T_m values (Figure 10 and Table 9).

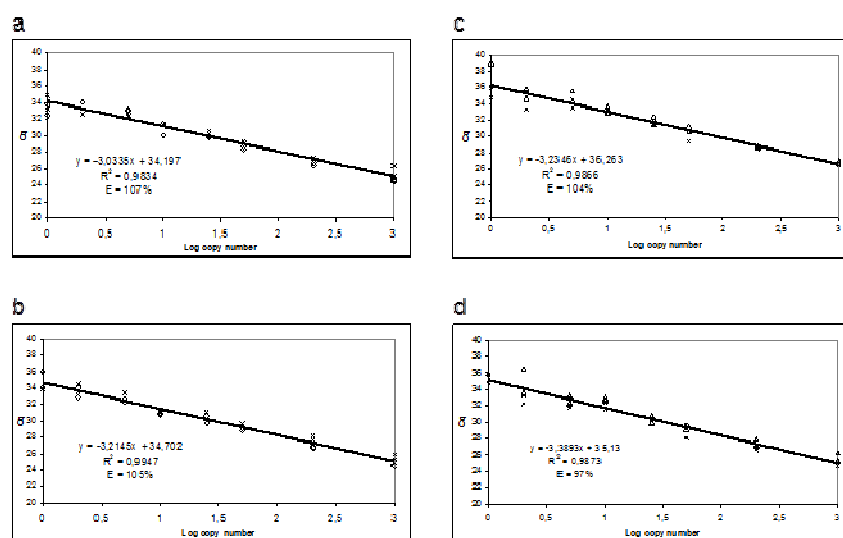


Figure 11 : Dynamic range, coefficient of determination and PCR efficiency of the four *Listeria* SYBR®Green qPCR assays.

Curves were obtained from two replicates for each concentration (expressed in copy number) from two different strains. a) *iap*-50-deg, b) *prs*-2-deg, c) *hlyA*-177, d) *hlyA*-146-deg-tronc. Multiplication symbol *L. monocytogenes* 1/2a (ATCC 51772), open diamond *L. ivanovii* (LMG 11388), open triangle *L. monocytogenes* 4b (ATCC 51777)

Table 11: Repeatability of the Cq values at the LOD for all *Listeria* SYBR®Green qPCR assays.

The results are based on 36 repetitions with genomic targets as DNA template

	Repeatability calculation on Cq values at the LOD					
	<i>L. monocytogenes</i> 1/2a (ATCC 51772)		<i>L. monocytogenes</i> 4b (ATCC 51777)		<i>Listeria ivanovii</i> (LMG 11388)	
	RSDr (%)	r	RSDr (%)	r	RSDr (%)	r
<i>iap</i> -50-deg	4.9	4.8	na	na	2	1.9
<i>prs</i> -2-deg	2.4	2.2	na	na	2.3	2.1
<i>hlyA</i> -177	3.8	3.6	4.1	3.8	na	na
<i>hlyA</i> -146-deg-tronc	1.6	1.5	2.9	2.8	na	na

na not applicable, LOD limit of detection

Table 12 : Repeatability of the Tm values for all *Listeria* SYBR®Green qPCR assays. The results are based on all Tm values coupled with amplification (Cq#40)

	Repeatability calculation on Tm-value								
	<i>L. monocytogenes</i> 1/2a (ATCC 51772)			<i>L. monocytogenes</i> 4b (ATCC 51777)			<i>Listeria ivanovii</i> (LMG 11388)		
	Tm-value ± SD (°C)	RSDr (%)	r	Tm-value ± SD (°C)	RSDr (%)	r	Tm-value ± SD (°C)	RSDr (%)	r
<i>iap</i> -50-deg	76.8 ± 0.4	0.6	1.2	nt	na	na	75.9 ± 0.5	0.6	1.3
<i>prs</i> -2-deg	71.3 ± 0.2	0.2	0.5	nt	na	na	71.1 ± 0.3	0.4	0.7
<i>hlyA</i> -177	74.3 ± 0.2	0.3	0.6	73.7 ± 0.3	0.4	0.9	nt	na	na
<i>hlyA</i> -146-deg-tronc	74 ± 0.4	0.6	1.2	74.5 ± 0.3	0.4	0.9	nt	na	na

nt not tested, na not applicable

C. Determination of dynamic range and PCR efficiency of SYBR®Green qPCR assays

All of the assays performed in a linear manner between 1,000 and 1 copy of the targeted gene since the R^2 values of the four assays, *iap*-50-deg, *prs*-2-deg, *hlyA*-177 and *hlyA*-146-deg-tronc, ranged between 0.9834 and 0.9947 (Figure 11). The results from the dynamic range analyses allowed the determination of the PCR efficiency (E) of each of the four developed SYBR®Green qPCR assays. The four assays displayed a PCR efficiency of 107, 105, 104 and 97% for *iap*-50-deg, *prs*-2-deg, *hlyA*-177 and *hlyA*-146-deg-tronc, respectively (Figure 11). The R^2 and E values of the developed SYBR®Green qPCR comply with the acceptance limits.

D. Determination of sensitivity and repeatability of SYBR®Green qPCR assays

LOD was identified to be between two to five copies for the four SYBR®Green qPCR assays: *iap*-50-deg, *prs*-2-deg, *hlyA*-177 and *hlyA*-146-deg-tronc (Table 10). The r value at LOD of the Cq values ranges between 1.5 to 4.8 Cq (Table 11). The r value of the Tm values ranges between 0.5 to 1.3°C (Table 12). The RSDr value at LOD of the Cq values was below 5% for the four developed assays ranging between 1.6 and 4.9%. The RSDr value of the Tm values was below 1% for the four developed assays ranging between 0.2 and 0.6% (Table 12). The LOD and RSDr values of the developed SYBR®Green qPCR comply with the acceptance limits.

E. Determination of reproducibility of SYBR®Green qPCR assays

The RSDR was calculated for each sample on the Cq and the Tm values (Table 13). For each SYBR®Green qPCR assay, this RSDR was between 0.05 and 0.95% for the Tm values and was between 0.23 and 6.19% for the Cq values. The RSDR values of the developed SYBR®Green qPCR comply with the acceptance limits. The expanded uncertainty at 99% of confidence has also been calculated from the reproducibility data from the eight samples. U was ranging between 0.46 and 1.42°C for the Tm values and between 0.95 and 3.72 Cq for the Cq values (Table 13).

F. Combination of the four SYBR®Green qPCR detection assays

Since the CoSYPS Path Food system is a screening (qualitative) system, the detection and discrimination of several *Listeria* species have been examined. The four assays have been run, on the same plate with the same PCR programme (as described previously), using the appropriate concentration of each primer (Table 9)

on four gDNA extractions from pure cultures of *L. monocytogenes* serotype 4b, *L. ivanovii*, *L. seeligeri* and *L. grayi*. The four SYBR®Green qPCR assays amplified *L. monocytogenes* with a Cq value which is comparable between assays (Table 14 and Table 15). The species *L. ivanovii* and *L. seeligeri* were amplified by the two assays amplifying the *Listeria* spp. The species *L. grayi* was amplified by none of the four assays (Table 14 and Table 15).

IV. Discussion

L. monocytogenes is an important foodborne pathogen and is widely tested in food, environmental and clinical samples (Gasarov *et al.* 2005). The detection of *L. monocytogenes* is traditionally performed with culture methods using selective enrichment and plating, followed by characterization based on colony morphology and biochemical properties (Anonymous 1996a). Such methods are labour intensive and time consuming. Therefore, in the last decade, the need arose for the development of rapid detection methods (Postollec *et al.* 2011), especially in the case of bio-emergency. The development of PCR or qPCR assays in the field of microbiology has increased markedly and they are now generally accepted as a faster alternative to the conventional microbiological methods (Postollec *et al.* 2011). However, the success of a PCR or qPCR assays is based on the primer pair design and its efficient evaluation.

In this study, four qualitative SYBR®Green qPCR assays have been successfully developed to detect the presence of *Listeria* genus bacteria, as well as the specific identification of *L. monocytogenes*. The four SYBR®Green qPCR assays targets the *iap* and *hlyA* virulence genes and *prs*, a gene flanking a virulence cluster. The SYBR®Green qPCR strategy described is based on two detection levels. A first set of generic assays allows the detection of the presence of all bacteria belonging to the *Listeria* genus (except *L. grayi*). A second set of assays allows the specific detection of *L. monocytogenes*.

L. grayi is not detected by the first set of assays because *L. grayi* is a species that genetically differs significantly from the other species of the *Listeria* genus (e.g. 56% amino acid homology for *iap* gene between *L. monocytogenes* and *L. grayi*) (Schmid *et al.* 2005). This was confirmed by the multiple alignment of the amplicons of *iap*-50-deg and *prs*-2-deg assays (data not shown). It is also important to note that the newly characterized species of *L. marthii* and *L. rocourtiae* strains have not been tested *in situ* since they were not available at the time of the experiments.

These qualitative SYBR®Green qPCR assays to detect *Listeria* spp. and discriminate *L. monocytogenes* were developed to be run simultaneously using a uniform PCR programme. Furthermore, in order to avoid any false negative detection due to sequence variation between strains, two SYBR®Green qPCR assays have been developed for each level of specificity: *Listeria* genus and *L. monocytogenes*.

The SYBR®Green chemistry has been chosen for these qualitative detection assays since it is important to detect all strains. Indeed, with the TaqMan® technology, two primers and one probe should be designed instead of only two primers for the SYBR®Green technology. A higher homology of the amplicon sequence would then be required with TaqMan® technology. For instance, in the 37 nucleotides length between the two primers of *iap*-50-deg assay, at least seven mismatches were observed among the different *Listeria* species (data not shown). Moreover, the SYBR®Green chemistry is less expensive than TaqMan® and allows a post-amplification verification of specificity of the amplicon (Postollec *et al.* 2011). All of these advantages make SYBR®Green technology the best choice for a screening system.

Most published PCR and qPCR assays to detect foodborne pathogens are not homogeneously validated (O'Grady *et al.* 2008; Oravcova *et al.* 2006; Rossmanith *et al.* 2006). ISO 22119 (Anonymous 2011c) gives the general definitions and requirements about the use of qPCR in food microbiology but does not give performance criteria and their acceptance limits to validate a qPCR assay. To date, the evaluation of the application of qPCR assay in food microbiology is performed according to ISO 16140 (Anonymous 2003), e.g. Rossmanith *et al.* (2006). This ISO 16140 norm gives the guidelines and acceptance criteria to compare an alternative method with the ISO reference method but does not allow the validation of the developed qPCR assays itself. In the present study, we propose a guideline to validate qPCR assays applied to food microbiology. All of the developed assays were evaluated for a set of performance criteria, specific to the qPCR applied to food microbiology. These performance criteria are not listed in an available guideline, so they were extracted from two available guidelines (Anonymous 2011b, 2008a). The first guideline is giving performance criteria to evaluate a PCR assay in food-microbiology (Anonymous 2011b), while the second (Anonymous 2008a) gives a list of performance criteria and their acceptance limits specific to the qPCR to evaluate a qPCR assay applied to genetically modified organisms (GMO) detection.

Table 13 : Reproducibility test of the four SYBR®Green *Listeria* spp. and *L. monocytogenes* detection assays.

Sample	Copy number	<i>L. mono</i> 12b (ATCC 51780)		<i>L. mono</i> 4b (BCRL:10/47)		<i>L. mono</i> 3b (Würzburg)		<i>L. mono</i> 4a (ATCC 8144)		<i>L. mono</i> 1/2c (BCRL:10/49)		<i>L. mono</i> 3a (BCRL:9/09)		<i>L. mono</i> 4c (EU-RL:09LEB41)		Uncertainty (99%)
		20	50	100	200	500	1000	2000	5000	10000	20000	50000	100000	200000	500000	
<i>iap</i> -50-deg	Cq	29.58	28.16	27.45	26.80	28.46	29.57	29.49	31.59	144						
	Average Lab 1	29.15	27.37	27.65	26.67	27.29	28.45	28.52	32.03							
	Average Lab 2	103	2.03	0.50	0.35	2.98	2.72	2.35	0.96							
	RSD _R (%)	77.00	77.00	76.50	76.75	76.05	76.50	77.00	76.50	121						
	Tm	76.35	76.35	75.50	76.05	76.05	76.50	76.50	76.05							
<i>prfA</i> -2-deg	Cq	28.38	26.62	26.04	25.44	27.31	28.36	28.45	30.03	0.95						
	Average Lab 1	27.91	26.48	25.42	25.72	26.46	28.23	27.93	30.56							
	Average Lab 2	119	0.35	170	0.78	2.24	0.35	131	124							
	RSD _R (%)	71.75	71.75	71.50	72.00	71.75	71.50	71.75	71.50	0.46						
	Tm	71.60	71.60	71.45	71.45	71.60	71.60	71.45	71.45							
<i>hlyA</i> -177	Cq	30.28	28.33	27.51	29.32	28.83	30.05	29.57	31.88	130						
	Average Lab 1	29.16	27.63	27.60	28.53	28.29	29.05	29.74	31.61							
	Average Lab 2	2.66	177	0.23	192	1.33	2.41	0.40	0.61							
	RSD _R (%)	74.00	74.00	74.00	74.00	73.50	74.00	74.00	74.00	0.88						
	Tm	73.75	73.75	73.40	73.40	73.20	73.75	73.40	73.40							
<i>hlyA</i> -146-deg-ironic	Cq	29.90	28.14	27.65	27.20	28.95	30.37	30.53	32.65	3.72						
	Average Lab 1	27.65	26.03	26.23	25.70	26.75	28.46	27.96	31.28							
	Average Lab 2	5.53	5.50	3.74	4.02	5.58	4.61	6.19	3.02							
	RSD _R (%)	75.00	75.00	74.75	75.00	73.50	73.75	74.00	74.75	142						
	Tm	74.50	74.35	74.05	74.05	73.05	73.20	73.05	73.75							
	RSD _R (%)	0.47	0.62	0.67	0.90	0.43	0.53	0.91	0.95							

Indeed, in the GMO field, qPCR is the golden standard for GMO detection in food and feed (Marmiroli *et al.* 2008). The evaluation of these performance criteria, according to these two guidelines, showed that the four developed SYBR®Green qPCR assays, *iap*-50-deg, *prs*-2-deg (for *Listeria* spp. (except *L. grayi*) detection) and *hlyA*-177, *hlyA*-146-deg-tronc (for *L. monocytogenes* detection), are highly accurate for their targets with 98.08 and 100% accuracy, respectively. These assays are also very sensitive, with LOD between two and five copies per reaction (LOD should be between one to ten copies (Anonymous 2011b)). They are also efficient, with PCR efficiency between 97 and 107%. They are repeatable, with RSD_r values far below the requested 25%, and they are reproducible, with RSD_R values also far below the requested 35%.

Besides the lack of performance criteria for qPCR in food microbiology, the qPCR assays to detect foodborne pathogens described so far are designed using different methodologies (classical PCR, real-time PCR using the TaqMan® or SYBR®Green chemistry) as well as different PCR programmes and protocols. This makes the simultaneous use of all of these methods impossible. However, such a simultaneous detection may turn out to be extremely useful when a more global approach is necessary or when a rapid identification of the foodborne pathogens is requested in a bio-emergency or outbreaks of unknown origin.

Table 14 : Matrix of targets amplification with the four SYBR®Green qPCR assays.

Genus	Species	<i>iap</i> -50-deg	<i>prs</i> -2-deg	<i>hlyA</i> -177	<i>hlyA</i> -146-deg-tronc
<i>Listeria</i>	<i>monocytogenes</i>	+	+	+	+
<i>Listeria</i>	<i>ivanovii</i>	+	+	-	-
<i>Listeria</i>	<i>seeligeri</i>	+	+	-	-
<i>Listeria</i>	<i>welshimeri</i>	+	+	-	-
<i>Listeria</i>	<i>innocua</i>	+	+	-	-
<i>Listeria</i>	<i>grayi</i>	-	-	-	-

The four SYBR®Green assays were developed to be run using the same PCR programme. They can be assembled on a single plate in order to save time and reduce plate-to-plate variation. The combination of these four assays, based on two levels of detection, results in a high-quality screening system and a remarkable food surveillance tool. This qPCR system will give an answer on the presence/absence of *Listeria* spp. in the sample and will at the same time indicate if the detected *Listeria* is *L. monocytogenes* which is the strain mandatory in EU regulation 2073/2005

(Anonymous 2005). The detection of other *Listeria* species may be useful to uncover other origins of contamination by the *Listeria* genus as few cases of listeriosis have been attributed to *L. ivanovii* (Cummins *et al.* 1994; Guillet *et al.* 2010; Lessing *et al.* 1994), *L. innocua* (Perrin *et al.* 2003) and *L. seeligeri* (Rocourt *et al.* 1986). Moreover, the post-amplification dissociation curve, a tool inherent to SYBR®Green chemistry, will give information about the specificity of the amplicon, further reducing false positive conclusions as well as giving information about the species or the serotypes amplified (Table 9). A similar qPCR detection system that detects both *L. monocytogenes* and the other *Listeria* species simultaneously is already available (Pall GeneDisc® *Listeria* DUO) (http://www.pall.be/pdfs/Biopharmaceuticals/nexidia_Listeria_id_genesystems_aoac_2009-v2.pdf). However, contrary to the CoSYPS Path Food system, this commercial kit is not modular. Indeed the CoSYPS Path Food system could be easily adapted to target other important foodborne pathogens such as *Salmonella* spp., *Campylobacter* spp. or verotoxin-producing *Escherichia coli* using the same screening platform. The detection assays will be added to or removed from the screening system in function of the bacteria sought in the sample. A future study will focus on the validation of the present qPCR system compared with the ISO reference method (microbiological methods) (Anonymous 1996a) on food samples according to ISO 16140 (Anonymous 2003).

Table 15 : Matrix of targets amplification with the four SYBR®Green qPCR assays: experimental verification

Sample Name	Detector	<i>iap</i> -50-deg		<i>prs</i> -2-deg		<i>hlyA</i> -177		<i>hlyA</i> -146-deg-tronc	
<i>L. monocytogenes</i> 4b	Cq	23.45	22.99	21.66	21.57	22.95	22.75	22.48	22.58
	Tm	76.9	76.9	71.7	71.7	74.1	74.1	74.7	74.7
	Conclusion	<i>Listeria</i> present	spp	<i>Listeria</i> present	spp	<i>Listeria monocytogenes</i> present		<i>Listeria monocytogenes</i> present	
	Average Cq	22.55							
	St dev Cq	0.65							
<i>L. ivanovii</i>	Cq	23.69	23.29	22.23	22.11	Und	Und	34.67	38.67
	Tm	75.9	76.3	71.7	71.7	72.6	74.7	74.7	71.7
	Conclusion	<i>Listeria</i> present	spp	<i>Listeria</i> present	spp	Below LOD		Below LOD	
	Average Cq	22.83				na			
	St dev Cq	0.78							
<i>L. seeligeri</i>	Cq	26.73	26.09	25.02	25.48	34.72	37.91	Und	Und
	Tm	75.9	75.9	71.7	71.7	74.1	74.1	73	72.1
	Conclusion	<i>Listeria</i> present	spp	<i>Listeria</i> present	spp	Below LOD		Below LOD	
	Average Cq	25.83				na			
	St dev Cq	0.74							
<i>L. grayi</i>	Cq	Und	35.02	34.17	Und	Und	35.86	Und	Und
	Tm	70	74.4	71.5	71.7	74.4	74.4	70	70
	Conclusion	Below LOD		Below LOD		Below LOD		Below LOD	

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Part II: Results, Chapter III: Development of the CoSYS Path Food detection system

Section 1: Development and validation of *Listeria* qPCR assays

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Section 2: Development and validation of *Salmonella* qPCR assays

The second phase of the CoSYPS Path Food detection system development was the development of SYBR®Green qPCR assays for the detection of all *Salmonella* spp. and the discrimination of the *Salmonella enterica* species and *Salmonella enterica* subsp. *enterica* which regroups most of the serovars of the *Salmonella* genus. To be run on a single plate with the qPCR previously developed from *Listeria* spp. detection and discrimination of *L. monocytogenes*, they were developed to be run with the same PCR program. The developed assays were all assessed using the validation process developed in the previous section. Moreover, the CoSYPS detection system developed in this section was compared with available commercial qPCR detection assays.

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METHODS AND PROTOCOLS

SYBR®Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels

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Abstract

In this work, a three-level *Salmonella* detection system based on a combination of seven SYBR®Green qPCR was developed. This detection system discriminates *Salmonella* at the genus, species and subspecies levels using a single 96-well plate. The SYBR®Green qPCR assays target the *invA*, *rpoD*, *iroB* and *safC* genes, as well as the STM0296 locus, putatively coding for a cytoplasmic protein. This study includes the design of primer pairs, *in silico* and *in situ* selectivity, sensitivity, repeatability and reproducibility evaluations of the seven SYBR®Green qPCR assays. Each detection level displayed a selectivity of 100%. This combinatory SYBR®Green qPCR system was also compared with three commercially available *Salmonella* qPCR detection kits. This comparison highlighted the importance of using a multi-genes detection system to be able to detect every target strain, even those with deletion or mutation of important genes.

Keywords

Real-Time PCR, SYBR®Green, Foodborne pathogens, Detection, *Salmonella*

I. Introduction

The food safety is an important concern worldwide. Foodborne diseases cause not only an enormous economic burden due to sick leaves, treatments, hospitalisations and mortality (Scharff 2012) but also economical losses of farmers and industry. The recent outbreak of the O104:H4 serotype of *Escherichia coli* in Germany was a dramatic illustration of all the consequences of a severe outbreak (European Commission 2011). Currently, the official methods for the detection of pathogenic bacteria in food samples are norms from the International Organization for Standardization (ISO). These norms are culture-based methods. They are reliable but focus on individual pathogenic species, are time consuming and labour intensive (e.g. at least 5 days for *Salmonella* detection (ISO 6579 norm 2002). Therefore, developing fast and simple foodborne pathogens detection tools, able to simultaneously detect several pathogens, remains a not only necessity but also a real challenge.

Real-time PCR (qPCR) assays have gained confidence as faster and reliable alternatives (Postollec *et al.* 2011), and several qPCR assays have already been

developed (e.g. Beutin *et al.* 2009; Botteldoorn *et al.* 2003; Fukushima *et al.* 2009; Garrido *et al.* 2012b; Kim and Cho 2010; Löfström *et al.* 2010; Singh *et al.* 2012). However, their use all together into the same detection system is hampered by several drawbacks: they are using different technologies (e.g. TaqMan® and SYBR®Green), they are using different protocols and they were developed for different purposes and validated following different ways. Moreover, current detection assays are mostly based on the detection of a single virulence gene (e.g. *invA* for *Salmonella* or *hlyA* for *Listeria* detection). Yet, it has now been documented that *Salmonella* can lose an entire Pathogenicity island while remaining infectious (Hu *et al.* 2008). Single-gene target detection systems could, in this case, lead to a false negative signal.

In Europe, *Salmonella* is the first cause of notification of foodborne contamination due to microorganisms into the Rapid Alert System for Food and Feed (European Commission 2012) and the first detected causative agent in foodborne outbreaks reported, representing ca. 30% of the total number of reported outbreaks in Europe in 2011 (European Food Safety Authority, European Centre for Disease Prevention and Control 2013). Salmonellosis is caused by the consumption of contaminated food (Gomez-Aldapa *et al.* 2012) (e.g. meat, raw eggs, cheese, fruit or vegetables) and, to a lesser extent, person-to-person transmission (Medus *et al.* 2006), waterborne transmission and environmental or animal exposure. The reservoirs are mainly poultry but also involve cattle, swine, sheep, birds, rats and reptiles (Bertrand *et al.* 2008; Pui *et al.* 2011). In developing countries, some *Salmonella* serovars can provoke typhoid and paratyphoid fevers. These serovars are not transmitted by food but mainly by drinking water spoiled by urine and faeces of infected individuals.

The *Salmonella* genus includes two species: *Salmonella enterica* and *Salmonella bongori*, both pathogenic for humans (Guibourdenche *et al.* 2010). The *S. enterica* species is itself divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Baumler 1997). The subspecies *enterica* is the most frequently found in food samples and is therefore the most relevant for human illnesses (Bertrand 2010). *S. bongori* and the other subspecies of *S. enterica* are rarely found in salmonellosis cases since they are associated with cold-blood invertebrates (Bertrand *et al.* 2008).

This paper reports on the development and validation of a *Salmonella* spp. detection system based on three detection levels. The first level detects all *Salmonella* spp.,

the second level detects all *S. enterica* and the third level detects all the *Salmonella enterica enterica*. Each level is composed of two to three SYBR®Green qPCR assays. The seven qPCR assays forming this detection system were developed to be run simultaneously on a single 96-well plate. Finally, this Combinatory SYBR®Green qPCR System (CoSYPS) is compared with commercial single-gene target *Salmonella* detection kits.

II. Material and methods

A. Bacterial strains

The bacterial strains used in this study are listed in Table 16. A panel of 111 bacterial strains (72 *Salmonella* strains and 39 other genera), two mould strains, two yeast strains and two viruses have been tested. These strains were obtained from National Reference Centres and Laboratories.

B. Bacterial growth conditions, DNA extraction and DNA quantification

Overnight cultures of each bacterial strain were grown in brain-heart infusion (BHI) broth or Bolton broth (for *Campylobacter*) at the adequate temperature and oxygen condition. The total DNA from bacterial strains was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (gDNA) from yeast was extracted using the Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH). Fungal gDNA was extracted with ZR Fungal/Bacterial gDNA extraction (Zymo Research). Viral RNA was extracted using the RNeasy mini kit (Qiagen), and cDNA was obtained by reverse transcription using the Transcriptor high fidelity cDNA synthesis kit (Roche). The cDNA amplificability was checked with a specific PCR amplification. All kits were used according to the manufacturer's recommendations. The DNA quality was controlled on agarose gel, and the DNA concentration was measured using a Nanodrop® 2000 device according to the manufacturer's recommendations.

Table 16 : Specificity assessment of the seven SYBR®Green qPCR methods: “*invA* - 5”, “*rpoD*-20”, “*iroB*-192”, “*iroB*-12”, “*saI*C-10”, “*saI*C-13” and “STM0296”.

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	<i>invA</i> - 5	<i>rpoD</i> - 20	<i>iroB</i> - 192	<i>iroB</i> - 12	<i>saI</i> C - 10	<i>saI</i> C - 13	STM0296
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Paratyphi A	C:2 (A)	-	1	10.03665	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Agona	C:4 (B)	-	1	NH.II.18.12	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Brandenburg	C:4 (B)	-	1	H.V.62.54	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Derby	C:4 (B)	-	1	H.II.34.34	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Heidelberg	C:4 (B)	-	1	NH.III.67.26	+	+	+	+	+	-	-
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Paratyphi B	C:4 (B)	-	1	H.VII.64.65	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Thyphimurium	C:4 (B)	-	1	H.II.13.13	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	var. Copenhagen	C:4 (B)	-	1	H.II.32.32	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Thyphimurium	C:4 (B)	-	1	NH.III.66.30	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Infantis	C:7 (C1)	-	1	H.III.52.53	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Ohio	C:7 (C1)	-	1	H.I.21.21	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Paratyphi C	C:7 (C1)	-	1	H.I.21.21	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Virchow	C:7 (C1)	-	1	H.V.42.56	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Montevideo	C:7 (C1)	-	1	H.V.33.50	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Kentucky	C:8 (C2-C3)	-	1	H.I.70.26	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Muenchen	C:8 (C2-C3)	-	1	H.I.79.35	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Newport	C:8 (C2-C3)	-	1	H.V.70.42	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Enteritidis	C:9 (C1)	-	1	H.VI.6.32	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Typhi	C:9 (C1)	-	1	H.III.28.24	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Baldon	C:9.46 (D2)	-	1	H.III.30.26	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Anatum	C:3.10 (E1)	-	1	H.II.76.36	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Give	C:3.10 (E1)	-	1	H.V.52.50	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	London	C:3.10 (E1)	-	1	H.II.60.20	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Gnesta	C:13.19 (E4)	-	1	H.II.47.7	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Kouka	C:13.19 (E4)	-	1	H.II.41.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Krefeld	C:13.19 (E4)	-	1	NH.I.50.10	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Liverpool	C:13.19 (E4)	-	1	H.II.52.12	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Sentenberg	C:13.19 (E4)	-	1	H.II.50.10	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Sentenberg	C:13.19 (E4)	-	1	H.II.48.8	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Vilvoorde	C:13.19 (E4)	-	1	H.VI.10.55	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Marseille	C:11 (F)	-	1	H.III.55.3	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Tours	C:11 (F)	-	1	H.VI.76.12	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Poona	C:13 (G)	-	1	NH.II.32.2	+	+	+	+	+	+	+

Table 16 (continued)

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	invA -5	rpoD -20	iroB -92	iroB -12	saIC -10	saIC -13	STM 0295
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Florida	O:6,14 (H)	-	1	H IV 28.8	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Gaminara	O:16 (I)	-	1	H III 74.12	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Camel	O:17 (J)	-	1	H III 79.3	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Cerro	O:18 (K)	-	1	H IV 16.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Minnesota	O:21 (L)	-	1	H IV 20.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Cotnam	O:28 (M)	-	1	H VII 63.23	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Pomona	O:28 (M)	-	1	H VII 24.27	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Urbana	O:30 (N)	-	1	H IV 52.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Adelaide	O:35 (O)	-	1	H IV 35.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Monschau	O:35 (O)	-	1	NH II 6.2	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Inverness	O:38 (P)	-	1	H VII 23.12	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Perth	O:38 (P)	-	1	H VI 24.6	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Anto	O:39 (Q)	-	1	NH III 58.2	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Champaign	O:39 (Q)	-	1	H IV 62.2	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Hoffi	O:39 (Q)	-	1	H IV 63.3	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Kokomlele	O:39 (Q)	-	1	H II 47.8	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Namur	O:39 (Q)	-	1	H VII 35.5	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Wardsworth	O:39 (Q)	-	1	H IV 36.1	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Johannesburg	O:40 (R)	-	1	H VII 29.6	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Waycross	O:41(S)	-	1	H VI 30.7	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Fredericksburg	O:42 (T)	-	1	H VII 65.5	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Berkeley	O:43 (U)	-	1	H IV 73.4	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Lauria	O:44 (V)	-	1	H IV 55.3	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Tomow	O:45 (W)	-	1	NH III 15.2	+	+	+	+	+	+	+
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Djakarta	O:48 (Y)	-	1	H IV 45.1	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIa)	<i>arizonae</i> (IIa)	51:24223 :-	O:51	-	1	H VI 61.2	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	47:IV235	O:47 (X)	-	1	NH III 6.6	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	53:20235	O:53	-	1	H VII 42.1	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	57:--	O:57	-	1	NH IV 4.1	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	59:Z52Z53	O:59	-	1	H V 27.1	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	60:rz	O:60	-	1	H V 70.2	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	61v15.7	O:61	-	1	H IV 77.1	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>arizonae</i> (IIb)	<i>arizonae</i> (IIb)	65(k)35	O:65	-	1	H V 13.3	+	+	+	+	+	+	-

Table 16 (continued)

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	invA	-5	rpoD	-20	iroB	-102	iroB	-12	sa/C	-10	sa/C	-3	STN0296
<i>Salmonella</i>	<i>enterica</i>	<i>noventae</i> (IV)	44Z423-	O:44 (V)	-	1	H IV 40.1	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>noventae</i> (IV)	50Z4232	O:50 (Z)	-	1	H V 9.5	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>noventae</i> (IV)	50Z423-	O:50 (Z)	-	1	H V 7.3	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>indica</i> (VI)	16,14,25:a:enx	O:6,14 (H)	-	1	1240K	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>salamae</i> (II)	48 ± 15	O:48 (Y)	-	1	H VII 48.10	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>enterica</i>	<i>salamae</i> (II)	58cz5	O:58	-	1	H VII 72.10	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Salmonella</i>	<i>bongori</i>	NA	66z41-	O:66	-	2	122472	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>Pseudomonas</i>	<i>aeruginosa</i>	NA	NA	NA	-	3	LM G 6395	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Vibrio</i>	<i>parahaemolyticus</i>	NA	NA	NA	-	3	TIAC 610	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aeromonas</i>	<i>hydrophila</i>	NA	NA	NA	-	4	M/2862	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Citrobacter</i>	<i>freundii</i>	NA	NA	NA	-	3	TIAC 554	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Citrobacter</i>	<i>diversus</i>	NA	NA	NA	-	4	M/649	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterobacter</i>	<i>cloacae</i>	NA	NA	NA	-	3	TIAC 445	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterobacter</i>	<i>gergoviiae</i>	NA	NA	NA	-	4	5502	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterobacter</i>	<i>aerogenes</i>	NA	NA	NA	-	4	M/3785	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia</i>	<i>coli</i>	NA	NA	NA	-	3	ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia</i>	<i>coli</i>	NA	NA	NA	-	5	EH 630	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia</i>	<i>fergusonii</i>	NA	NA	NA	-	5	95/394	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia</i>	<i>hermannii</i>	NA	NA	NA	-	5	Div 2663	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia</i>	<i>vulneris</i>	NA	NA	NA	-	5	Seq048	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Hafnia</i>	<i>alvei</i>	NA	NA	NA	-	4	786	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Klebsiella</i>	<i>oxytoca</i>	NA	NA	NA	-	3	TIAC 695	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Klebsiella</i>	<i>pneumoniae</i>	NA	NA	NA	-	3	TIAC 446	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Morganella</i>	<i>morganii</i>	NA	NA	NA	-	4	M/100	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Proteus</i>	<i>mirabilis</i>	NA	NA	NA	-	3	TIAC 726	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Proteus</i>	<i>vulgaris</i>	NA	NA	NA	-	4	M/654	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Providencia</i>	<i>reitteri</i>	NA	NA	NA	-	4	M/831	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Serratia</i>	<i>fonticola</i>	NA	NA	NA	-	4	3429	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Serratia</i>	<i>marcescens</i>	NA	NA	NA	-	4	705	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Serratia</i>	<i>odorifera</i>	NA	NA	NA	-	4	3436	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Shigella</i>	<i>boydii</i>	NA	NA	16	-	1	10-02874	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Shigella</i>	<i>dysenteriae</i>	NA	NA	5	-	1	10-01857	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Shigella</i>	<i>flexneri</i>	NA	NA	2a	-	1	10-03891	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Shigella</i>	<i>sonnei</i>	NA	NA	NA	-	1	10-03865	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 16 (continued)

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	invA -5	rpoD -20	iroB -192	iroB -12	satC -10	satC -13	STI 0295
<i>Yersinia</i>	<i>enterocolitica</i>	NA	NA	NA	-	3	LM G 45558	-	-	-	-	-	-	-
<i>Campylobacter</i>	<i>coli</i>	NA	NA	NA	-	3	TIAC 539	-	-	-	-	-	-	-
<i>Campylobacter</i>	<i>jejuni</i>	NA	NA	NA	-	3	ATCC 33291	-	-	-	-	-	-	-
<i>Campylobacter</i>	<i>lari</i>	NA	NA	NA	-	3	TIAC 544	-	-	-	-	-	-	-
<i>Clostridium</i>	<i>perfringens</i>	NA	NA	NA	+	3	ATCC	-	-	-	-	-	-	-
<i>Bacillus</i>	<i>cerus</i>	NA	NA	NA	+	3	ATCC 4579	-	-	-	-	-	-	-
<i>Brachotrix</i>	<i>thermosphaera</i>	NA	NA	NA	+	3	TIAC 400	-	-	-	-	-	-	-
<i>Listeria</i>	<i>innocua</i>	NA	NA	NA	+	6	CIP 8011	-	-	-	-	-	-	-
<i>Listeria</i>	<i>ivanovii</i>	NA	NA	NA	+	6	CIP 7842	-	-	-	-	-	-	-
<i>Listeria</i>	<i>monocytogenes</i>	NA	NA	12a	+	6	ATCC 51772	-	-	-	-	-	-	-
<i>Staphylococcus</i>	<i>aureus</i>	NA	NA	NA	+	3	ATCC 25923	-	-	-	-	-	-	-
<i>Lactobacillus</i>	<i>acidophilus</i>	NA	NA	NA	+	3	TIAC 642	-	-	-	-	-	-	-
<i>Aspergillus</i>	<i>fumigatus</i>	NA	NA	NA	NA	7	ECCM /IHE M 13436	-	-	-	-	-	-	-
<i>Clado sporium</i>	<i>sphaerospermum</i>	NA	NA	NA	NA	7	ECCM /IHE	-	-	-	-	-	-	-
<i>Saccharomyces</i>	<i>cerevisiae</i>	NA	NA	NA	NA	7	M 24474 ECCM /IHE	-	-	-	-	-	-	-
<i>Candida</i>	<i>parapsilosis</i>	NA	NA	NA	NA	7	M 3961 ECCM /IHE	-	-	-	-	-	-	-
<i>Hepatitis A Virus</i>		NA	NA	NA	NA	3	M 6478	-	-	-	-	-	-	-
<i>Norovirus GI</i>		NA	NA	NA	NA	3	27 (VZ)	-	-	-	-	-	-	-
No template control					NA	NA	2593	-	-	-	-	-	-	-
					NA	NA	NA	-	-	-	-	-	-	-

+ There is an amplification and a Tm value similar for all corresponding strains; - No amplification; 1: Scientific Institute of Public Health, Salmonella and Shigella National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; 2: French National Reference Center Salmonella, Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris Cedex 16, France; 3: Scientific Institute of Public Health, Food pathogens laboratory, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; 4: Scientific Institute of Public Health, Quality of food laboratory, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; 5: Department Microbiology and Infection Control, VTEC National Reference Centre, Free University of Brussels, Laarbeeklaan 101-1090 Brussel; 6: Scientific Institute of Public Health, Listeria National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; 7: Mycology & Aerobiology, Scientific Institute of Public Health, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; NA, not applicable

C. Development and *in silico* assessment of primer pairs

A uniform primer design approach was applied in the development of all primer pairs. The first step consisted of identifying genes of interest, either genus or species specific, by means of a bibliographic study (Chen *et al.* 1997; Galan *et al.* 1992; Gonzalez-Escalona *et al.* 2009; Parkhill *et al.* 2001; Porwollik *et al.* 2002; Soyer *et al.* 2009). The second step included the collection of DNA sequences relevant for the chosen targets retrieved from the NCBI public database (<http://www.ncbi.nlm.nih.gov/sites/entrez>). The primer pairs were designed, preferentially within conserved regions, using the "Primer 3" program (<http://frodo.wi.mit.edu/primer3/>) (Rozen and Skaletsky 2000) with the "product size range" specification set at "60 to 120 bp" and "primer size" optimal set at "22 bases". An *in silico* test of the primer pairs was then performed. It consisted in a bioinformatic analysis carried out with the "wprimersearch" software available on wEMBOSS Open Source Software package (<https://wemboss.uio.no/wEMBOSS/>) (Rice *et al.* 2000; Sarachu and Colet 2005). This software mimics the PCR amplification of the tested primers on a database of bacterial genomes sequences from NCBI of 217 bacteria, representing 103 species belonging to 61 genera. When mismatches between primer and one of its targets were observed, degenerate nucleotides were introduced into the primer sequence. However, primer pairs with no degenerate nucleotides were always preferred. Only primer pairs that gave the expected *in silico* amplification were retained for the following steps.

D. Qualitative SYBR®Green qPCR assay and optimal primer concentration

All qPCR assay reactions were performed according to the protocol described in Barbau-Piednoir *et al.* (2013). For the interpretation of a SYBR®Green qPCR assay, two criteria were taken into consideration: the quantification cycle (Cq) value and the melting temperature of the amplicon (Tm). The Cq value represents the fractional cycle at which the PCR amplification reaches the threshold level for the reaction (Bustin 2000). Since it is a screening assay, a qualitative respond is required. To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level, with a single peak upon melting analysis giving a unique Tm value. A signal was considered as negative when no Cq value was obtained. The optimal concentration of the selected primer pairs was determined by testing different concentrations of each primer, between 250 and 1,000 nM.

Table 17: Primer pairs and amplicon size for each SYBR®Green qPCR assay.

Targeted genus	Targeted species	Targeted subspecies	Targeted sequence	qPCR assay name	Primer name	Primer Sequence (5' → 3')	Primer concentration in nM	Product Size (bp)	Tm amplicon	References
<i>Salmonella</i>	all	all	Sigma factor (<i>rpoD</i>)	<i>rpoD</i> -20	<i>rpoD</i> -20-F <i>rpoD</i> -20-R	ACA TGGGTATTCAGGTAA TGGAA TTTTCA CR GTGCTGGTGGTATTTTCA	1000	75	77-78°C	Adapted from Botfeldboom <i>et al</i> , 2006 This study
			Invasine gene (<i>invA</i>)	<i>invA</i> -5	<i>invA</i> -5-deg-F <i>invA</i> -5-R	GATY TGAAR GCGGGTATTATG ATAAACCTTCATCGCACCGTCA	250	107	80-82°C	
	<i>enterica</i>	all	C-glycosyltransferase (<i>iroB</i>)	<i>iroB</i> -92	<i>iroB</i> -92-F <i>iroB</i> -92-R	CCAAAGATCTGGCGTGATAG TATTGCATGGAGATAACCGCT	250	86	78°C (<i>S. enterica salamae</i>) 79°C (<i>S. enterica enterica</i> and <i>S. enterica arizonae</i>) 80°C (<i>S. enterica douerinae</i> and <i>S. enterica diarizonae</i>)	Adapted from Murphy <i>et al</i> , 2007
				<i>iroB</i> -12	<i>iroB</i> -12-deg-F <i>iroB</i> -12-R	GGR ACAAAAATGGGGM ACTTCT AAGGGAGGR TAGACGATGA	500	105	80.5-83°C	This study
	<i>enterica</i>		Outer membrane usher protein (<i>safC</i>)	<i>safC</i> -10	<i>safC</i> -10-F <i>safC</i> -10-R	CACTAAAAAGTCGTCTGACTCTG CGGTAAATGGCACACATCAAAAT	250	72	75-76°C	This study
				<i>safC</i> -13	<i>safC</i> -13-F <i>safC</i> -13-R	ATGATGACAGCTACGGACTGAGT CTGTTTGTATCCAGTTCAATGAC	250	76	77-78°C	This study
			STM0296 (Acc n°AL0253)	STM0296	STM0296-F STM0296-R	AMGCCCA TCCCTTCAATACAG GTGATYACGCCGCA GCA TACCC	500	56	76-78°C	This study

Degenerated nucleotides: R=A or G, Y=C or T, M=A or C

The concentrations giving the lowest Cq value without formation of a high level of primer dimer were selected. When using a positive sample at a concentration around the limit of detection (LOD), the primer dimer dissociation peak should not be higher than the positive sample dissociation peak. The primer pairs and their concentrations used in this study are presented in Table 17.

E. Selectivity test and accuracy calculation

Primer pairs selected from the *in silico* evaluation were tested *in situ*. In brief, the selectivity of the SYBR®Green qPCR assay is tested according to the ISO 22118 norm (2011) on target and non-target strains. The selectivity of an assay is assessed by inclusivity (target strains giving a positive signal) and exclusivity (non-target strains not giving a positive signal). The selectivity test is fully described in Barbau-Piednoir *et al.* (2013). From the selectivity test, the accuracy can be calculated. The accuracy represents the closeness of agreement between a test result and the accepted reference value (ISO 3534–1 1993). Its formula is found in ISO 16140 norm (2003).

F. Amplicon cloning and sequencing

In order to check the correctness of the amplicon obtained with each developed primer pair, the amplicons obtained by the amplification of *S. enterica enterica* Enteritidis (H.V.6.32) were cloned, sequenced and verified using the same approach as recently described by Barbau-Piednoir *et al.* (2013). Sequencing reactions were run on an ABI 3130xl at the Platform Biotechnology and Molecular Biology at the Scientific Institute of Public Health.

G. Full genome sequencing

The total DNA from *S. enterica enterica* serovar Namur was extracted with Blood & Cell Culture DNA Midi kit (Qiagen). The quality and quantity of the DNA were controlled with the 260/280-nm ratio and by spectrophotometer measurement, respectively. This DNA was sent to Baseclear for sequencing using the Illumina technology.

H. Dynamic range and calculation of the PCR efficiency

The dynamic range of a qPCR assay is the range of concentrations where it performs in a linear manner. It was assessed by the analysis in duplicate of a serial dilution in a carrier DNA (4 ng/μL Calf Thymus DNA (CTD) (Invitrogen) of pure strain DNA (1,000 to 0.01 theoretical genomic copies) of *S. enterica enterica* Enteritidis

(H.V.6.32) and *S. enterica enterica* Typhimurium (H.II.32.32). The carrier DNA avoids the improper dilution due to low concentration of gDNA. This analysis allows the assessment of the coefficient of determination (R^2) and the PCR efficiency (E) for each SYBR®Green qPCR assays. The coefficient of determination (R^2) is an indicator of the correlation of data regarding the linear regression curve. The R^2 of the dynamic range curve should be above 0.98 (European Network of GMO Laboratories 2008). The PCR efficiency (E) should be between 89.6 and 110.2% and can be calculated according to the formula reported by Rutledge and Cote (2003).

I. Sensitivity test

Primer pairs passing the selectivity test were subsequently examined for their sensitivity. Using serial dilution, the LOD of each SYBR®Green qPCR assays was evaluated. The LOD is defined as the lowest concentration of an analyte giving a positive result with a probability of 95% (European Network of GMO Laboratories 2008). The strains used were *S. enterica enterica* Enteritidis (H.V.6.32) and *S. enterica enterica* Typhimurium (H.II.32.32). The calculation of the target genomic copy numbers for each dilution point was performed according to Barbau-Piednoir *et al.* (2013), considering the gDNA size of *S. enterica enterica* Enteritidis and Typhimurium as 4,685,848 and 4,857,432 bp (Acc. Num.: AM933172 and AE006468, respectively). To determine the LOD, a range of copy number between 10 and 0.1 theoretical copies was tested (i.e. 10, 5, 2, 1, 0.5, 0.2 and 0.1). Each dilution was tested in six replicates per plate, for both strains. Moreover, the analysis was performed three independent times, under repeatable conditions, resulting in 36 repeats for each dilution point. It has to be noticed that the dilution points beyond the theoretical single copy were carried out to assess the dilution series correctness. Indeed, as it is statistically impossible to get amplification in all replicates with the dilution points beyond one theoretical copy, none of these dilution points should give 100% of positive signals.

J. Repeatability calculation

To evaluate the repeatability of the assay, independent tests were performed with the same protocol, with the same samples, by the same operator using the same apparatus within a short interval of time (ISO 3534–1 norm 1993). The repeatability limit (r) and the relative standard deviation of repeatability (RSD_r) were calculated according to ISO 16140 norm (2003). RSD_r should be $\leq 25\%$ for all the dilution range above the LOD (European Network of GMO Laboratories 2008). The RSD_r and r

values of the Cq values were calculated at each dilution point while the RSD_r and r values of the T_m values were calculated with all the T_m values coupled with amplification (Cq <40).

K. Reproducibility study and calculation

To evaluate the reproducibility of the assays (ISO 3534–1 norm 1993), independent tests were performed with the same protocol, using the same samples, in two different laboratories, by two different operators using different apparatus (Bio-Rad iQ5 and ABI 7300). The nine tested samples were all gDNA from *S. enterica enterica* strains at different concentrations between 20 and 100 genomic copies per reaction. Each sample was analyzed in duplicate by each operator.

Two reproducibility measures can be calculated from these results: the relative standard deviation of reproducibility (RSD_R) and the expanded uncertainty (U). RSD_R represents the absolute value of the coefficient of variation. It is expressed in percent and is obtained by multiplying the reproducibility standard deviation by 100 and dividing this product by the reproducibility median (ISO 16140 2003). The RSD_R should be ≤35% for all the tested samples (European Network of GMO Laboratories 2008). The RSD_R of the Cq values are calculated for the nine tested samples. The RSD_R of the T_m values are calculated with all the T_m values coupled with an amplification (Cq <40).

The uncertainty is “the parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand”. The uncertainty can be expressed by the expanded uncertainty (U) which is the quantity defining “an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand”. The expanded uncertainty (U) is obtained by multiplying the combined standard uncertainty by a coverage factor (Joint Committee for Guides in Metrology 2008).

L. CoSYPS *Salmonella*

The seven qPCR SYBR®Green assays have been run, on the same plate with the same PCR program (Barbau-Piednoir *et al.* 2013) using the appropriate concentration of each primer (Table 17) on 10⁴ copies of five gDNA extraction from pure culture of *S. enterica enterica* Enteritidis (H.VI.6.32), *Salmonella enterica*

salamae (H.VII.72.10), *Salmonella enterica arizonae* (H.VI.61.2), *Salmonella enterica diarizonae* (NH.IV.4.1) and *S. bongori* (1224/72).

M. Comparison of CoSYPS *Salmonella* with other commercial kits

The CoSYPS *Salmonella*, composed of the seven SYBR®Green qPCR assays as detection/identification system, was compared with three *Salmonella* detection kits: iQ-Check™ *Salmonella* II from Bio-Rad, foodproof® *Salmonella* Detection Kit from Bioteccon and GeneDisc® *Salmonella* spp. from Pall® Life Sciences (according to manufacturer's protocol). Eleven gDNA *Salmonella* samples from pure culture at 10⁴ copies per assay have been tested in duplicate with the three detection systems.

III. Results

A. *In silico* selection of the primer pairs and optimisation of primer concentration

As a first step, specific genes of *Salmonella* genus, *S. enterica* species and *S. enterica enterica* subspecies were identified. For the specific detection of the *Salmonella* genus, *invA* and *rpoD* were retained. The *rpoD* gene encodes the RNA polymerase sigma 70 factor (Bai *et al.* 2012), while *invA* plays an important role during invasion of the intestinal cells (Galan *et al.* 1992). *invA* is located in the *Salmonella* Pathogenicity Island I (SPI-I) (Baumler *et al.* 1997) shown to be present in all phylogenetic lineages of the *Salmonella* genus (Li *et al.* 1995). For the specific detection of the *S. enterica*, the chosen gene was *iroB*, which belongs to the Fur regulon that mediates gene regulation in response to iron availability (Ernst *et al.* 1978). This gene is not only present in *S. enterica* but also in all its subspecies (Porwollik *et al.* 2002). Finally, for the specific detection of *S. enterica* subsp. *enterica*, the *safC* and *ratB* genes were selected (Porwollik *et al.* 2002). *SafC* belongs to the *safABCD* operon (Folkesson *et al.* 2002) located in the *Salmonella* Pathogenicity Island 6 (SPI-6) (Parkhill *et al.* 2001). This operon encodes the *Salmonella* atypical fimbriae (Saf) pilus (Salih *et al.* 2008). *ratB* encodes an outer membrane protein essential in the colonization of the caecum by *Salmonella*.

During *in silico* evaluation of these primer pairs, some nucleotides were degenerated when necessary. Twenty primer pairs were first tested *in situ* with the "light" selectivity test (data not shown). From these, seven primer pairs were retained: *invA*-5 and *rpoD*-20 for the *Salmonella* spp. specific detection, *iroB*-192, *iroB*-2 and *iroB*-12 for the *S. enterica* specific detection and *safC*-10 and *safC*-13 for the *S. enterica*

enterica detection. The optimal concentration of the seven primer was also evaluated (Table 17). These assays were then evaluated *in vivo* for their selectivity.

B. *In situ* optimisation of primer pair and targets

During *in situ* selectivity test, the primer pairs *invA*-5 and *iroB*-12 were found to less efficiently (latter Cq value) amplify *S. bongori* and *Salmonella enterica houtenae*, respectively, while strains *S. enterica enterica* Senftenberg, Namur and Krefeld were not amplified by the *safC* assays.

C. Optimisation of *invA* -5 and *iroB* -12 primer pairs

The locus containing the primer pair *invA*-5 in *S. bongori* and *iroB*-12 in *S. enterica houtenae* was sequenced (data not shown) and aligned with the published sequences in order to identify mismatches. The new degenerated primers *invA*-5-deg-F and *iroB*-12-deg-F are displayed in Table 17. The new assays performed with these degenerated primers amplified *S. bongori* and *S. enterica houtenae*, respectively, as efficiently (giving a comparable Cq value at the same concentration) as the other positive strains.

D. Optimisation of *S. enterica enterica* target

SafC could not be found in the genomes of both *S. enterica enterica* Senftenberg (CAGQ000000000.1 and AFCU000000000.1) and Namur (AWGG000000000) (see below). However, a short region (56 bp) into the STM0296 gene of *S. enterica enterica* Typhimurium (AE006468), upstream of the *saf* operon, was found to be highly conserved in *S. enterica* subsp. *enterica* including Senftenberg and Namur. Therefore, a new primer pair, named STM0296, was designed to amplify this 56-bp sequence (Table 17).

E. Determination of SYBR®Green qPCR assay selectivity

The primer pairs *invA*-5 and *rpoD*-20 amplified 100% (72/72) of the *Salmonella* spp. tested strains, none (0/45) of the non-target strains nor the no template control (NTC) (Table 16). The *iroB*-192 and *iroB*-12 assays for *S. enterica* detection gave a specific amplification with 100% (71/71) of *S. enterica* tested strains and 0% (0/46) of the non-target strains and NTC (Table 16). Thus, these four assays were considered 100% accurate for their target (*S. enterica*) giving 0% of false positive and 0% of false negative.

The four *safC* assays amplified 94.6% (52/55) of the tested serovars and 0% (0/61) of the non-target strains and NTC (Table 16). Primer pairs *safC*-10 and *safC*-13 were selected among four candidates since they amplified different loci of *safC* and gave the smallest amplicon size with two distinct *T_m* values (Table 17). These two assays give 0% of false positive and 5.45% (3/55) of false negative (serovars Senftenberg, Namur and Krefeld). The accuracy of these two assays is 97.4% for detection of *S. enterica enterica*.

The STM0296 assay amplifies 90.9% (50/55) of the tested serovars and 0% (0/61) of non-target strains and NTC (Table 16). This assay gives 0% of false positive and 9.1% (5/55) of false negative (serovars Heidelberg, Virchow, Perth, Hofit and Djakarta). The accuracy of this assay is 95.7% for detection of *S. enterica enterica*. However, 100% (55/55) of the tested serovars were amplified by at least one *S. enterica enterica* assay. Then, the combination of *safC* and STM0296 assays forming the level detection system was 100% accurate for the specific detection of *S. enterica enterica*.

These seven assays tested on *S. enterica enterica* Enteritidis showed a unique band at the expected size upon agarose gel analysis (data not shown). The sequence of each amplicon was determined and shown to correspond to the sequence of *S. enterica enterica* Enteritidis (data not shown). In addition, the seven detection assays gave a unique melting peak with a specific melting temperature for each assay (Table 17).

F. *SafC* presence in *S. enterica enterica* Namur, Krefeld and Senftenberg

As indicated above, the genome of *S. enterica enterica* Namur was fully sequenced (Appendix C). The presence of the *Salmonella* atypical fimbrial (*saf*) operon was neither been found in the Senftenberg strain whole genome shotgun contigs (CAGQ00000000.1 and AFCU00000000.1), nor in the Namur strain sequence (AWGG00000000). The *S. enterica enterica* Senftenberg, Namur and Krefeld strains were also PCR-tested using primers flanking, or located inside, the *saf* operon. None of them was positive confirming the absence of this operon in these three strains (data not shown). The lack of this *saf* operon is in accordance with the phylogenetic study of den Bakker *et al.* (2011) demonstrating the diversification of virulence factors content, mainly fimbrial operons, between two clades of *S. enterica enterica*.

Table 18 : Standard curve, amplification efficiency, coefficient of correlation, LOD, repeatability and reproducibility of the seven *Salmonella* assays.

	Accepted range	rhoD -20	lnvA -5	iroB -32	iroB -32	iroB -32	saC -12	saC -10	saC -13	STM0296
Standard curve equation	-3.2slope<-3.6	-3.2399 (logX) +34.657	-3.3546 (logX) +35.473	-3.3386 (logX) +34.290	-3.3477 (logX) +35.651	-3.3477 (logX) +35.651	-3.3765 (logX) +34.951	-3.3765 (logX) +34.951	-3.2543 (logX) +35.771	-3.3976 (logX) +33.795
Amplification efficiency (E)	89.6 - 110.2%	104%	99%	100%	99%	99%	98%	98%	103%	106%
Coefficient of correlation (R ²)	>0.98	0.997	0.982	0.985	0.986	0.986	0.988	0.988	0.999	0.998
LOD	na	2-5 copies	2-5 copies	2-5 copies	2-5 copies	2-5 copies	5-10 copies	5-10 copies	2-5 copies	1-2 copies
<i>Salmonella</i> <i>enterica</i> <i>enteritidis</i>	≤ 25%	2.2	5.1	3.2	2	2	3.7	3.1	3.1	2.9
Cq RSDr (%)	na	2	4.7	2.9	19	19	3.5	2.9	2.9	2.8
Tm-value±SD (°C)	na	77.5±0.2	80.3±0.4	78.7±0.3	81.1±0.3	81.1±0.3	75.0±0.4	75.0±0.4	77.6±0.2	76.1±0.22
Tm RSDr (%)	≤ 25%	0.2	0.4	0.4	0.3	0.3	0.5	0.2	0.2	0.3
Tm r	na	0.5	1	0.9	0.8	0.8	1	0.5	0.5	0.6
Cq RSDr (%)	≤ 25%	2.2	3.5	2	2.9	2.5	2.5	2.2	2.2	1.7
Cq r	na	2	3.1	1.9	2.7	2.7	2.2	2	2	1.5
Tm-value±SD (°C)	na	77.2±0.3	80.6±0.4	78.6±0.3	81.2±0.3	81.2±0.3	75.0±0.3	75.0±0.3	76.9±0.3	77.7±0.29
Tm RSDr (%)	≤ 25%	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4
Tm r	na	0.7	1	0.7	0.8	0.8	0.9	0.9	0.9	0.8
RSD _r (%) (min - max)	≤ 35%	164 - 479	0.73 - 4.2	1.35 - 3.11	2.57 - 5.05	2.57 - 5.05	3.62 - 7.34	3.11 - 8.25	3.11 - 8.25	0 - 195
Uncertainty at 99%	na	0.36 - 0.96	0.13 - 0.66	0.27 - 0.63	0.35 - 0.78	0.35 - 0.78	0 - 0.84	0.05 - 1.01	0.05 - 1.01	0 - 0.37
Cq	na	2.41	2.68	18	3.27	3.27	4.38	4.78	4.78	0.92
Tm	na	132	0.94	1.17	124	124	1.14	1.14	1.14	0.39

Standard curves, E and R² were obtained from two replicates for each concentration (from 1,000 to 1 copies) from two different strains; LOD is based on 36 repetitions with the two genomic targets as DNA template; repeatability is based on 18 repetitions with the genomic target as DNA template; X: copy number; LOD: limit of detection, na: not applicable.

G. Determination of SYBR®Green qPCR assay dynamic range and PCR efficiency

The seven developed assays performed in a linear manner between 1 and 1,000 copies as their R^2 values were between 0.982 and 0.999, above the required 0.98 (ENGL 2008) (Table 18). From the dynamic range analyses, the PCR efficiency (E) of each assay was calculated. The seven assays displayed PCR efficiencies ranging between 98 and 106%, falling into the accepted limits of 89.6–110.2% (ENGL 2008) (Table 18).

H. Determination of SYBR®Green qPCR assay sensitivity and repeatability

The LOD of the seven assays fell between two and ten copies (Table S1: Appendix B) complying with the requirement “between 1 and 10 CFU” (ISO 22118). The r values at the LOD of the Cq values ranged between 1.9 and 4.7 Cq and those of the Tm values ranged between 0.5 and 1°C (Table 18). The RSDr values at LOD of the Ct values of the seven assays were between 1.7 and 5.1% while those of the Tm values ranged between 0.2 and 0.5% (Table 18). All these RSDr values were below ≤25% as requested by the ENGL guidelines (ENGL 2008).

I. Determination of SYBR®Green qPCR assay reproducibility

For all the developed SYBR®Green qPCR assays, the RSDr values were below ≤35% as requested by the ENGL guideline (ENGL 2008): between 0 and 1.01% for the Tm values and between 0.00 and 7.34% for the Ct values (Table S2 Appendix B). The expanded uncertainty at 99% of confidence was also calculated from the reproducibility data from the eight samples. U was ranging between 0.39 and 1.47°C for the Tm values and between 0.92 and 4.78 for the Cq values (Table S2 Appendix B).

J. CoSYPS *Salmonella*

The seven SYBR®Green qPCR assays were run together on the same plate. *S. enterica enterica* Enteritidis was amplified by the seven assays, whereas *S. enterica arizonae*, *S. enterica salamae* and *S. enterica diarizonae* subspecies were amplified by the four assays amplifying all the *S. enterica* subspecies. The *S. bongori* species was only amplified by the two assays that are amplifying all the species of the *Salmonella* genus (Table 19).

Table 19 : Matrix of amplified targets with the different SYBR®Green qPCR assays.

Genus	Species	Sub-species	Serovars	<i>rpoD</i> -20	<i>invA</i> -5	<i>iroB</i> -192	<i>iroB</i> -12	<i>safC</i> -10	<i>safC</i> -13	STM0296
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i> (I)	Sentftenberg, Namur and Krefeld	+	+	+	+	-	-	+
		<i>enterica</i> (I)	Djakarta, Heidelberg, Hofit, Perth, Virchow	+	+	+	+	+	+	-
		<i>enterica</i> (I)	Others	+	+	+	+	+	+	+
		<i>salamae</i> (II)	na	+	+	+	+	-	-	-
		<i>arizonae</i> (IIIa)	na	+	+	+	+	-	-	-
		<i>diarizonae</i> (IIIb)	na	+	+	+	+	-	-	-
		<i>houstenae</i> (IV)	na	+	+	+	+	-	-	-
		<i>indica</i> (VI)	na	+	+	+	+	-	-	-
	<i>bongori</i> (V)	na	na	+	+	-	-	-	-	-

na: not applicable

K. Comparison with other *Salmonella* qPCR detection kits

The CoSYPS Path Food detection/identification system based on seven qPCR assays allowed the detection of the eleven tested samples. The iQ-Check™ *Salmonella* II did not detect *S. enterica enterica* Senftenberg, isolated from a Chinese outbreaks that do not contain the SPI-1 (Hu *et al.* 2008) and the GeneDisc® *Salmonella* spp. did not detect *S. bongori*. The foodproof® of Bioteccon detects all tested strains (Table 20).

Table 20 : Comparison CoSYPS *Salmonella* with other commercial qPCR *Salmonella* detection kits

	CoSYPS <i>Salmonella</i>	FoodProof® <i>Salmonella</i>	GeneDisc® <i>Salmonella</i> spp.	iQ-Check <i>Salmonella</i> II
Control positif kit	+	+	+	+
No template Control	-	-	-	-
<i>S. bongori</i>	+	+	-	+
<i>S. enterica indica</i>	+	+	+	+
<i>S. enterica houtenae</i>	+	+	+	+
<i>S. enterica arizonae</i>	+	+	+	+
<i>S. enterica diarizonae</i>	+	+	+	+
<i>S. enterica salamae</i>	+	+	+	+
<i>S. enterica enterica</i> Typhimurium	+	+	+	+
<i>S. enterica enterica</i> Senftenberg	+	+	+	+
<i>S. enterica enterica</i> Senftenberg (-SPI-1)	+	+	+	-
<i>S. enterica enterica</i> Enteritidis	+	+	+	+
<i>S. enterica enterica</i> Namur	+	+	+	+

IV. Discussion

In order to significantly reduce the incidence of foodborne outbreaks, food chain surveillance is necessary (Official journal of the European Union 2004). Although standard protocols based on microbiology to detect foodborne pathogens are available (ISO 11290–1 norm 1996; ISO 16654 norm 2001; ISO 6579 norm 2002; ISO 10272 norm 2006), there are time consuming, labour intensive and target only single pathogenic bacteria. On the contrary, real-time PCR is much more rapid to detect foodborne pathogens (Postollec *et al.* 2011), and several assays have already been developed and published (e.g. Amagliani *et al.* 2010; Chen *et al.* 2000; Fukushima *et al.* 2009; Garrido *et al.* 2012a; Hein *et al.* 2006; Lund *et al.* 2004; McCabe *et al.* 2011; Perelle *et al.* 2004; Sharma 2002; Sharma and Dean-Nystrom 2003). Unfortunately, these assays are not harmonized: they are using different chemistry (SYBR®green or TaqMan®), different protocols and different validation procedures, hampering their use as coherent detection system. Moreover, these assays target a single DNA sequence, often virulence or associated virulence genes, potentially leading to false negative signal when the target is mutated or absent. Such drawbacks could be avoided in this study. For instance, the primers for *invA* and *iroB* were successfully degenerated to better amplify *S. bongori* and *S. enterica houtenae*, respectively, illustrating the variability of target genes. Also, comparison of different *Salmonella* qPCR detection kits demonstrated that single target kits were unable to detect *S. bongori* or an atypical *S. enterica enterica* Senftenberg lacking the entire Pathogenicity Island I (Hu *et al.* 2008) (Table 20). Therefore, strategy targeting several genes is highly advisable for the appropriate and efficient detection of pathogenic bacteria.

In this study, seven simplex qualitative detection SYBR® Green qPCR assays, targeting all *Salmonella* spp., one of the most important foodborne pathogen (European Food Safety Authority, European Centre for Disease Prevention and Control 2013), were successfully developed and in-house validated. Considering that all *Salmonella* spp. are pathogenic for human, these assays were developed targeting five distinct loci (*invA*, *rpoD*, *iroB*, *safC* and STM0296) for a full coverage of all the *Salmonella* species, subspecies and serovars. These assays were developed and validated, to be run simultaneously with the same PCR program, according a harmonized standard procedure described in Barbau-Piednoir *et al.* (2013).

Figure 12: Alignment of *invA* amplicon from different *Salmonella* species and subspecies.

M90846.1-S.-ent.-ent.-Typhimurium	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
DQ644626-S.-ent.-houteanae	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
FJ496648.1-S.-ent.-salamae	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
CP001113.1-S.-ent.-ent.-Newport	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
AE014613-S.-ent.-ent.-Typhi	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
AM933172S.-ent.-ent.-Enteritidis	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
CP001127.1-S.-ent.-ent.-Schwarzengrund	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGTGT
CP000880.1-S.-ent.-arizonae	GATTTGAAGGCCGGTATTATTGATGCGGATGCCGCGCGAACGGCGAAGCGT
FR877557.1-S.-bongori	GATCTGAAAGCCGGTATTATTGATGCGGATGCCGCGCGTGAACGGCGAAGCGT
	*** **
M90846.1-S.-ent.-ent.-Typhimurium	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
DQ644626-S.-ent.-houteanae	ACTGGAAGGGAAGCCAGCTTTATGGTTCCTTGACGGTGCGATGAAGTTTAT
FJ496648.1-S.-ent.-salamae	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
CP001113.1-S.-ent.-ent.-Newport	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
AE014613-S.-ent.-ent.-Typhi	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
AM933172S.-ent.-ent.-Enteritidis	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
CP001127.1-S.-ent.-ent.-Schwarzengrund	ACTGGAAGGGAAGCCAGCTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
CP000880.1-S.-ent.-arizonae	ACTGGAAGGGAAGTCACTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
FR877557.1-S.-bongori	ACTGGAAGGAGAAAGTCACTTTACGGTTCCTTGACGGTGCGATGAAGTTTAT
	***** **

framed: *invA*-5 primers

The seven assays allowed a three-level detection system for the discrimination between the different species and subspecies of *Salmonella* (Table 19). Two assays allow the detection of the *Salmonella* genus bacteria based on *invA* and *rpoD* detection. Two assays permit the specific identification of the species *S. enterica* based on *iroB* detection. A third level is composed of two assays based on *saftC* and an additional one targeting the STM0296 sequence. The last assay was developed to detect *S. enterica enterica* serovars presenting a deletion of *saftC*. This deletion seems to be relatively frequent: present in three serovars (Senftenberg, Krefeld and Namur) among the 55 tested. This type of operon deletion was also reported in den Bakker *et al.* (2011) and hypothetically explained by a selective pressure. The three assays (*saftC*-10, *saftC*-13 and STM0296) specifically and exhaustively detected *S. enterica* subsp. *enterica* (Table 19), amplifying all the serovars tested in this study (Table 16).

The SYBR®Green chemistry was preferred to the commonly used TaqMan® chemistry since SYBR®Green assays present the advantage of allowing the use of only two primers in the conserved region surrounding the more polymorphic sequence. As shown in Figure 12, the design of a TaqMan® probe between *invA*-5 primers would have been impossible as polymorphisms are observed between all the *Salmonella* species and subspecies. In addition, SYBR®Green assays are cheaper and permit post-amplification verification of the amplicon specificity by melting temperature analysis (Postollec *et al.* 2011).

The seven SYBR®Green qPCR assays for *Salmonella* detection developed and validated in this paper could be combined with the four assays for *Listeria* detection

previously described (Barbau-Piednoir *et al.* 2013) and run all together on a single plate. Since this approach is modular, detection assays can be added to detect a wider range of foodborne pathogens or emerging pathogens. The only requirement is to develop an assay able to be run with the same conditions, allowing its use in high throughput on the same 96-well plate.

Combined with a decision support system (Van den Bulcke *et al.* 2008; 2009), such detection method represents a very useful approach in managing the experimental analysis of samples for a high-quality screening system and a remarkable food surveillance tool which can be modulated in response to the laboratory needs. It will also considerably reduce the time and the cost of a sample analysis. Such simultaneous detection may be extremely useful when a global screening and rapid identification of the foodborne pathogens is requested, as in the case of a bio-emergency or outbreaks of unknown origin.

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Part II: Results, Chapter III: Development of the CoSYS Path Food detection system

Section 2: Development and validation of *Salmonella* qPCR assays

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Section 3: Development of the Decision Support System

I. Introduction

In order to facilitate the data analysis and to provide a harmonised interpretation of the results from the eleven SYBR®Green qPCR assays developed for *Listeria* spp. (Chap III, section 1) and *Salmonella* spp. (Chap III, section 2), a support tool has been developed. This support tool is an Excel file composed of 5 sheets. The first three sheets ("Plate set-up", "Primer pairs" and "Screening Mix-PCR program") provide a technical support whereas the two last ("Results analysis" and "Conclusion screening") bring a decision support system (DSS) to obtain an automatic and harmonised conclusion of the qPCR results. The combination of these 11 qPCR assays and the related decision support system (DSS) is named CoSYPS Path Food for Combinatory SYBR®Green qPCR Screening system applied to the detection of pathogens in food samples. This system is constructed on the same principle that the CoSYPS previously developed for the detection of Genetically Modified Organisms (GMO) (3;4). The CoSYPS Path Food system intends to be used in routine analysis, for laboratories working under accreditation. A positive result obtain with this detection system is a presumptive positive result. It will have to be confirmed by microbiological culture on selective medium (1;2).

II. Description of the support tool

A. Technical support

1) Plate set-up of the CoSYPS Path Food analysis

In the "Plate set-up" sheet, different set-ups, including all controls, are proposed regarding the number of samples to test. The use of pre-defined plate set-ups saves time during analysis preparation and manipulation.

2) Primer pairs

The Excel sheet "Primer pairs" gives all information about primer name, sequence, primer concentration, product size, origin of the primer pairs and expected T_m-value

for each SYBR®Green qPCR assay (Table 9 and Table 17). This information sheet allows the operator to rapidly check all required information about the used primers.

3) PCR mix and PCR program

The Excel sheet "Screening Mix-PCR program" provided a PCR mix automatic calculation tool. The operator includes the number of reactions to perform and Excel automatically calculates the amount of each component to prepare the PCR mix. The automatic calculation is made for qPCR assay according to its optimal concentration of each primer (Table 9 and Table 17). This automatic calculation reduces the time of preparation of the CoSYPS Path Food analysis and the risk of calculation mistakes.

B. Decision support system

The decision support system (DSS) has been developed on the basis of the DSS developed by Van den Bulcke *et al.* in 2010 (4) for the CoSYPS GMO applied to detect genetically modified organisms into food samples (3).

The CoSYPS Path Food system is composed of levels of detection which are genus, species and subspecies levels. Each level of detection is composed of two to three genetic markers (qPCR assays). The DSS automatically interprets the CoSYPS Path Food results. In first, each SYBR®Green qPCR assay controls are evaluated. Then, the presence/absence (below LOD) of each genetic marker into the tested sample is identified. Finally, the conclusion of the presence or absence (below LOD) into the tested sample of the different level of detection is performed. The different steps included in the DSS are described below.

1) Decision about each SYBR®Green qPCR analysis

The output values of a SYBR®Green qPCR analysis are the quantification cycle (Cq-value) and the melting temperature of the amplicon (Tm-value). The Cq and Tm-values obtained in each analysed well are introduced into the "Results analysis" sheet. The DSS automatically compares the obtained Cq-values and Tm-values with the Cq-value of the limit of detection (LOD) of each assay and the Tm-value interval. The values of both references criteria were identified during the validation of the different qPCR assays (Chap. III, section 1 and 2). The accepted Tm-interval is performed with the minimal and maximal Tm-values obtained with the positive strains during the inclusivity test.

The DSS concludes that a SYBR®Green qPCR signal is positive if i) the Cq-value is inferior or equal to the [Cq-value at the LOD] +1Cq (to cover the measurement uncertainty) and ii) the Tm-value is comprised in the interval of the minimum Tm-value -0.5°C and the maximum Tm-value + 0.5°C.

The DSS concludes that a SYBR®Green qPCR signal is below LOD if the Cq-value is superior to the [Cq-value at the LOD] + 1Cq or if the Tm-value is outside the interval [min Tm-value-0.5°C and max Tm-value+0.5°C].

a) Conclusions of control analysis

Three different controls are included into the CoSYPS Path Food analysis in order to verify the efficiency of the PCR reaction (positive control: PC), the non-contamination of the PCR mix (no template control: NTC) and the quality of the extracted DNA (extraction and inhibition control: EIC). The DSS attribute a "TRUE" or "FALSE" conclusion for each control. To get a "TRUE" conclusion, PC and EIC should give a positive signal (defined previously) whereas the NTC should give a below LOD signal.

Afterwards, the combination of the three controls per qPCR assay is automatically analysed. A general conclusion about the three controls gives information about the reliability of the tested sample results obtained with the respective qPCR assay. This general conclusion about all controls is set as "Valid" if every control is "TRUE". If one or more controls are concluded as "FALSE", the general conclusion about all controls is set as "Not Valid" and the Excel sheet cell becomes red. If a PC or NTC is concluded as "FALSE" for a qPCR assay, all the results obtained within this qPCR assay are unreliable, and the analysis of all samples should be repeated with this qPCR assay. Indeed, a "FALSE" PC or NTC means that the PCR reaction mix was problematic. If an EIC is concluded as "FALSE", this suggests the presence of PCR inhibitors in the gDNA extracted sample. If the tested sample gives a "present" conclusion, the "FALSE" signal obtained with the EIC control is informative but not problematic. On the contrary, if the tested sample gives "no amplification" or "suspected presence below the LOD", the EIC and the sample have to be diluted (e.g. 2 and 10 times) and are retested. A "no amplification" or "suspected presence below the LOD" conclusion into the tested sample could be accepted only if the EIC gives a "TRUE" conclusion.

Decision Support System of the CoSYPS Path Food analysis (p2)						
Matrix						
<i>Salmonella</i> spp.	5	1	1	1	1	5
<i>Salmonella enterica</i>	5	7	1	1	1	35
<i>Salmonella enterica enterica</i>	5	7	11	1	1	385
<i>Listeria</i> spp.	1	1	1	13	1	13
<i>Listeria monocytogenes</i>	1	1	1	13	17	221
Sample results analysis						
<i>Salmonella</i> spp.	invA-5-deg			5	presence (1) or absence (0) of the marker into the tested sample	Result for each marker: presence (prime number) absence (1)
	rpoD-20-deg			5		5
<i>Salmonella enterica</i>	iroB-alt-192			7		7
	iroB-12-deg2			7		7
	safC-10			11		11
<i>Salmonella enterica enterica</i>	safC-13			11		11
	STM0296			11		11
<i>Listeria</i> spp. (except <i>L. grayi</i>)	iap-50-deg			13		1
	prs-2-deg			13		1
<i>Listeria monocytogenes</i>	hlyA-177			17		1
	hlyA-146-deg-tronc			17		1
					GPPs: G��del prime product sample	1630475

Figure 13 (part 1): Conclusion sheet of CoSYPS Path Food DSS Excel file.
 Example of sample results analysis into the DSS for a *S. enterica enterica* positive sample. For the details of the decision see text: part B.2.

Salmonella spp.							Division GPPs by GPpt
Salmonella enterica							326095
Salmonella enterica enterica							46585
Salmonella enterica enterica							4235
Listeria spp.							125421.1538
Listeria monocytogenes							7377.714932
Result							
Salmonella spp.							Conclusion
Salmonella enterica							Present
Salmonella enterica enterica							Present
Salmonella enterica enterica							Present
Listeria spp. (except L. grayi)							Below LOD
Listeria monocytogenes							Below LOD

Figure 13 (part 2): Conclusion sheet of CoSYPS Path Food DSS Excel file.
Example of sample results analysis into the DSS for a *S. enterica enterica* positive sample. For the details of the decision see text: part B.2.

b) Conclusion of the tested sample

Three different conclusions could be given concerning the presence of each genetic marker targeted by its specific qPCR method into the tested sample analyses: i) Present, ii) Suspected presence above LOD and iii) No amplification.

To be concluded as “Present”, the signals obtained with the two repeats of the tested sample should give a positive signal (described previously). To be concluded as “Suspected presence above the LOD”, the signals obtained with the two repeats of the tested sample should give a below LOD signal (described previously) but with the T_m -value of one or both repeats falling into the accepted T_m -interval. To be concluded as “No amplification”, the signals obtained with the two repeats of the tested sample should give a below LOD signal (described previously) with both T_m -value out of the accepted T_m -interval.

2) Decision about the presence of the targets in the tested samples

The decision about the presence of *Listeria* spp. or *Salmonella* spp. into the tested sample, and the discrimination among the species and subspecies, is built on i) two to three qPCR assays (genetic marker) per level of detection (genus, species and subspecies) and ii) a mathematical model based on prime numbers developed by Van Den Bulcke *et al.* in 2010 (4). A prime number is a natural number that is dividable only by 1 and it-self. Into the CoSYPS Path Food DSS, a unique prime number is assigned to each level of detection, in other words, each qPCR assay of a level of detection has the same assigned prime number (Figure 13, part 1: Matrix part). The conclusions “Present”, “Suspected presence above LOD” or “No amplification” of each marker into the tested sample are transformed to a number. If the marker is concluded as “Present” into the tested sample, the specific prime number is given to this marker. If the marker is concluded as “No amplification” or “suspected presence below the LOD” into the tested sample, the number 1 (neutral element in a multiplication) is given to this marker (Figure 13, part 1: Sample results analysis part). By multiplying all the assigned number for each marker, the algorithm calculates the Gödel prime product of the sample (GPPs). For each targeted strain, a Gödel prime product of the target (GPpt) is calculated by multiplying the prime number of the each level of detection expected as present into this target. Then, the GPPs is divided by the GPpt of each targeted strains. If the quotient is an integer number, the corresponding strain is concluded as potentially present into the tested sample (Figure 13, part 2). As explain previously, each marker of detection level has

the same assigned prime number. Thus, only one positive marker is required to get a positive signal for the respective level of detection.

It has to be noticed that the DSS can be used even if not all of the qPCR assays are run. If the markers are not tested they are considered as negative into the DSS.

The strain detected as potentially present into the sample, by the CoSYPS Path Food system, have to be confirmed by microbiological culture on selective medium (1;2).

III. Practical cases

The DSS have been tested by analysing gDNA extracts from 5 *Listeria* spp. and 5 *Salmonella* spp. strains. The qPCR were performed as described in sections 1 and 2 with a template of 10^4 bacterial chromosome copies per assay. The SYBR®Green qPCR assays set for *Listeria* spp. detection and discrimination (section 1) was analysed on *Listeria* samples and the SYBR®Green qPCR assays set for *Salmonella* spp. detection and discrimination (section 2) was analysed on *Salmonella* samples. All the tested strains were detected and identified by the CoSYPS Path Food system using the DSS (Table 21).

IV. Discussion and conclusion

The developed support tool for the CoSYPS Path Food system in a routine laboratory is an Excel tool designed to facilitate and to accelerate the work of the operator: it reduces the time required to prepare the analysis and reduces the risk of mistakes during calculation and manipulation. Moreover the DSS allows a harmonised analysis and interpretation of the results which is essential for an accredited use of the CoSYPS Path Food system.

To our knowledge, none of the other available foodborne detection kits, except the GeneDisc system (Pall®), provide such a complete support system. Indeed, the CoSYPS Path Food system contains the qPCR detection system associated with a technical support tool for PCR mix calculation and automatic conclusions about the controls, the tested markers and the detected strains. Moreover, the CoSYPS Path Food system has the advantage to be modular which is not the case of the GeneDisc system.

A web application of this support tool has already been developed and is in use with the CoSYPS GMO. A comparable web application could be developed for the

CoSYPS Path Food. This web application of the support tool would permit easier improvements and management of the support system tool than in Excel.

To conclude, the support system developed to help the operator to perform and automatically interpret the results of the CoSYPS Path Food system is an efficient and complete tool, very useful for analysis of samples under accreditation.

Table 21: Decision support system practical test on 5 *Listeria* spp. and 5 *Salmonella* spp. gDNA extracts.

Genre	Species	Subspecies	serovar/ serotype	CRL Reference	Output of the DSS
<i>Listeria</i>	<i>ivanovii</i>	-	-	CIP 7842	Potential presence of <i>Listeria</i> spp. (except <i>L. grayi</i>)
<i>Listeria</i>	<i>seeligeri</i>	-	-	ATCC 35967	Potential presence of <i>Listeria</i> spp. (except <i>L. grayi</i>)
<i>Listeria</i>	<i>monocytogenes</i>	-	1/2a	10/13	Potential presence of <i>L. monocytogenes</i>
<i>Listeria</i>	<i>monocytogenes</i>	-	1/2b	ATCC 51780	Potential presence of <i>L. monocytogenes</i>
<i>Listeria</i>	<i>monocytogenes</i>	-	4b	ATCC 51777	Potential presence of <i>L. monocytogenes</i>
<i>Salmonella</i>	<i>bongori</i>	-	Brookfield, 66:z41:-	1224/72	Potential presence of <i>Salmonella</i> spp.
<i>Salmonella</i>	<i>enterica</i>	<i>arizonae</i>	51:24z23:-	H.VI.61.2	Potential presence of <i>S. enterica</i>
<i>Salmonella</i>	<i>enterica</i>	<i>salamae</i>	58:c:z6	H, VII, 72, 10	Potential presence of <i>S. enterica</i>
<i>Salmonella</i>	<i>enterica</i>	<i>diarizonae</i>	57:-:-	NH, IV, 4, 1	Potential presence of <i>S. enterica</i>
<i>Salmonella</i>	<i>enterica</i>	<i>enterica</i>	Enteritidis	H, VI, 6, 32	Potential presence of <i>S. enterica enterica</i>

* As explained in section 1, *L. grayi* is not detected by the *Listeria* spp. qPCR assay.

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Part II: Results, Chapter III: Development of the CoSYSP system

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Chapter IV: Discrimination of dead bacteria using molecular methods

In the Chapter III, the SYBR®Green qPCR assays for the detection of *Listeria* spp. and *Salmonella* spp. have been developed and validated. Moreover, a decision support tool has been developed to ease the interpretation of the results from these assays. The detection system it-self is thus available. In the present chapter, a preliminary point of the detection step is addressed. Indeed, an important drawback of the qPCR-based detection of bacteria is the possible detection of both viable and dead cells. In this chapter the viability qPCR (v-PCR) using propidium monoazide (PMA) has been evaluated.

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Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis

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Abstract

The propidium monoazide (PMA) coupled with PCR (viability PCR) is used in foodborne pathogen detection in order to detect only viable bacteria. Originally presented to fully remove the signal of dead bacteria, the limits of the viability PCR rapidly came out in the literature. In this study, the use of PMA in a viability-qPCR (v-qPCR) was assessed on viable and dead cells of *Salmonella enterica* subsp. *enterica* serovar Enteritidis. The PMA treatment protocol was modified (dark incubation duration, concentration of PMA) to evaluate if a complete negative signal of dead *Salmonella* was possible. However, none of these modifications was found to improve the removal of the remaining qPCR signal observed in the presence of dead bacteria. The present research also underlines that PMA may unexpectedly decrease the qPCR signal observed on living *S. Enteritidis* at low concentration. Finally, the use of *S. Enteritidis* cells killed by processes altering, or not, the cell-wall/membrane gives us a clue to answering the question about the non-total extinction of the signal of dead cells sample in the v-qPCR assay. Indeed, the data strongly indicate that the remaining qPCR signal observed in non-culturable cells does not only depend on the cell-wall/membrane integrity of the bacteria. According to these results, the authors suggest that for a rapid and reliable foodborne bacteria detection system, an enrichment followed by a qPCR analysis should be preferred to a v-qPCR.

Keywords

Propidium monoazide, Real-Time PCR, SYBR®Green, detection, *Salmonella*

I. Introduction

Foodborne pathogens are an important concern as illustrated for Europe in 2012, where 91,034 human cases have been reported, resulting in 61 deaths (EFSA and ECDC 2014). To be able to prevent the occurrence of such outbreaks, foodstuffs are monitored according to the Regulation EC 2073/2005 in which microbiological criteria are given for each food category (Commission of EU 2005). In this regulation, the reference methods used to search for the presence of foodborne pathogens are mostly culture-based (e.g. ISO 2006a; ISO 2006b; ISO 1996a; ISO 1996b; ISO 2001; ISO 2002). These conventional methods are efficient and detect only viable bacteria but are time-consuming and labour-intensive. Hence, they are not suited in case of outbreaks where a rapid answer is necessary.

Molecular methods are progressively recognised as valuable alternatives since they are fast, sensitive and specific. However, polymerase chain reaction (PCR) or real-time PCR (qPCR) amplify DNA from both dead and viable bacteria as DNA remains stable after the death of bacteria (Li 2013b; Masters 1994; Wolffs 2005). Two techniques can potentially be used to detect only viable bacteria. The first one is based on the detection of mRNA by the use of reverse-transcriptase (RT) (q)PCR (Gonzalez-Escalona 2009; McIngvale 2002; Yaron 2002). However, this detection technique requires expression of the targeted gene(s), which can vary under conditions of stress. Furthermore, RNA is very sensitive to degradation in complex matrices such as food. Overall the use of RT-(q)PCR technology is more adapted for gene expression studies than as a detection system for foodborne pathogens (for a recent review, see (Postollec 2011)). The second available technique relates to the viability PCR (v-PCR) or viability qPCR (v-qPCR) that are based on DNA detection of cells with intact cell/wall membranes. In v-(q)PCR, before (q)PCR amplification, a viability discrimination step is performed. In this step two known molecules can be used: ethidium monoazide (EMA) and propidium monoazide (PMA), derivatives from ethidium bromide and propidium iodide, respectively. EMA and PMA intercalate DNA every 4-5 nucleotides (Waring 1965). They are positively charged molecules thus they are excluded by intact, negatively charged, bacterial cell-walls but can enter bacteria with damaged cell-wall/membranes (Nocker 2006b). EMA was firstly reported to be useful to quantify viable bacteria by v-PCR (Nocker 2006a; Rudi 2005) but can penetrate and be toxic for viable bacteria (Nocker 2006b; Pan 2007). Afterwards, PMA was used as a non-toxic alternative (Nocker 2006b; Pan 2007) able of covalent cross-linkage with DNA under light exposure (Coffman 1982). The precise mode of action of PMA remains unclear: the DNA charge changes (Nocker 2006a) and/or the DNA is cleaved (Soejima 2007), leading to reduced ability to extract or amplify DNA, respectively. Since PMA is not able to penetrate bacteria with intact cell-wall/membranes, only the DNA from bacteria with compromised cell-wall/membranes is bound and is thus not amplified by (q)PCR (Nocker 2006b; Shapiro 2003).

The definition of "bacterial viability" is still a subject of controversy. The most usual one is: when a sample is plated out on an appropriate solid medium, dead bacteria are unable to produce colony forming unit (CFU) whereas a viable bacteria is able to form CFU (Trevors 2012). In this definition, the cell wall/membrane integrity of the different states is not taken into account. Another approach, based on culturability,

metabolic activity and membrane integrity has been described (Nocker 2009). In this model, four different states are postulated. The “living” bacteria are defined as culturable, metabolically active and with an intact cell-wall/membrane. The “viable but non-culturable” (VBNC) bacteria are defined as metabolically active, with an intact cell-wall/membrane but non-culturable. The “ghost” bacteria have an intact cell-wall/membrane but are metabolically inactive and non-culturable. Finally the “membrane compromised” cells have a compromised cell-wall/membrane, are non-culturable and have no metabolic activity.

The use of PMA was recently coupled with PCR (Banihashemi 2012; Cawthorn 2008; Yang 2012) or qPCR (Dinu 2013; Elizaquivel 2012a; Elizaquivel 2012b; Josefsen 2010; Kim 2012; Liang 2011; Mamlouk 2012; Singh 2013; Soejima 2012; van Frankenhuyzen 2011) in order to detect only viable bacteria. PMA was originally reported to fully remove the signal of dead bacteria in v-PCR (Nocker 2006b) and v-qPCR analysis (Josefsen 2010), but later several drawbacks of the technique were reported. Indeed, PMA treatment does not always lead to complete removal of the qPCR signal of dead bacteria (see review in (Fittipaldi 2010)). In particular, recent studies have showed that PMA treatment does not fully remove the signal from dead bacteria if i) the amplicon size of the qPCR assay is short (Li 2013a; Luo 2010; Martin 2013; Schnetzinger 2013), ii) the target bacteria is at high concentration (Elizaquivel 2012c; Li 2013a; Pacholewicz 2013; Zhu 2012), iii) the concentration of Mg^{2+} in the PCR reaction is not adapted (Nocker 2006b), or iv) the fat content of food sample is high (Yang 2011), and may also vary according to the “killing” treatment (Kobayashi 2010; Liang 2012; Nocker 2007; Yang 2011).

In this study, optimisation of the PMA protocol (by variation of dark incubation and duration and PMA concentration) was first assessed to achieve full extinction of the dead bacteria qPCR signal. Afterwards, to evaluate the dynamic range of the v-qPCR assay, different amounts of living and isopropanol-killed *Salmonella* Enteritidis were tested. Finally, for a better understanding of the mode of action of PMA, cells of *S. Enteritidis* killed by different processes affecting, or not, the cell-wall/membrane integrity were simultaneously analysed with v-qPCR, culture-based and microscopic observation. The general applicability of v-qPCR for a *S. Enteritidis* detection system is also discussed.

II. Material and methods

A. Bacteria strain and growth conditions

S. enterica enterica Enteritidis (H,VI,6,32 from Belgian *Salmonella* NRC) was used as model bacteria for Gram-negative foodborne pathogens. One single typical colony was inoculated in 10 ml of sterile Brain Heart Infusion (BHI) broth, vortexed and incubated at 37°C for about 16 h. Ten microliters of this overnight (ON) culture were inoculated into a new 10 ml sterile BHI broth and incubated without shaking for 3.5 to 4 h at 37°C to get a culture at an exponential growth phase (OD 600 nm between 0.3 and 0.6).

B. Enumeration of *S. Enteritidis*

The initial concentration of *S. Enteritidis* was determined by performing a tenfold serial dilution, 100 µl of the dilutions -4 to -7 were plated on Nutrient Agar (NA) and incubated at 37°C overnight. Plates with less than 300 CFU were retained for enumeration and calculation of the initial bacterial counts expressed as colony forming units/ml (CFU/ml).

C. Death control

The killing process used on viable *S. Enteritidis* was evaluated by enumerating the growing cells by streaking 100 µl of the treated sample on NA plate followed by 24 h incubation at 37°C.

D. PMA treatment

One hundred microliters of tested sample were transferred in a 1.5 ml Eppendorf tube. In a dark room (PMA is sensitive to light), 75 µM or 150 µM of PMA 20 mM (Biotium: BTIU40019) were added to each tube. Tubes were incubated in a dark room under rotating agitation for 5 or 60 min. At the dark incubation step, PMA penetrates the permeable cell-wall/membranes and get access to the DNA of cell-wall/membrane compromised bacteria. Tubes were transferred to the PhAST blue lamp (GenIUL) to undergo the LED-light exposure for 15 min. The light treatment covalently binds PMA to DNA. Free PMA was removed by harvesting the bacteria 10 min at 6,000 x g. The pellet was re-suspended in 100 µl of DNase/RNase free water (Acros, Geel, Belgium).

E. Processes used to kill the *S. Enteritidis* cultures

The killing processes were chosen for their possible occurrence in food product (heat and freezing) or for the membrane status of the cell after treatment (isopropanol and kanamycin). All killing processes were applied to a culture at the exponential growth phase (between 10^6 - 10^8 CFU/ml).

- Isopropanol 70% was added to aliquots of *S. Enteritidis* culture and the bacterial suspension was incubated for 10 min. Isopropanol was removed by harvesting the *S. Enteritidis* 10 min at 6,000 x g prior to re-suspension in the initial volume of BHI.
- Heat: 400 or 500 µl of *S. Enteritidis* culture were heated for 15 min at 99°C in a thermoblock (dry bath).
- Kanamycin: 5 ml of *S. Enteritidis* culture (sensitive to kanamycin) were supplemented with kanamycin at 1 mg/ml and incubated for 16-18 h at 37°C.
- Freezing: 500 µl of *S. Enteritidis* culture were immersed into liquid nitrogen for 15 min and transferred to -20°C for 16-18 h. Note that freezing is a stress process that leads to a portion of dead *S. Enteritidis*.

F. DNA extraction

DNA extraction was performed by heat-lysis for 15 min at 99°C in a thermoblock. Cell debris were removed by centrifugation 10 min at 6,000 xg. The supernatant (containing the DNA) was collected in a new Eppendorf tube.

G. Real-Time PCR assays

The qPCR assays were performed on an iCycler iQ™5 Real-Time PCR Detection System (BioRad) with iCycler iQ™ PCR plates, 96 wells (BioRad) closed with the PCR Sealers Microseal B films (BioRad). The reaction was performed in a final volume of 25 µl containing 5 µl of template, 1X SYBR®Green PCR Mastermix (Diagenode), and 250 nM of each primer (*safC*-10 *Salmonella* spp. detection qPCR assay (72 bp) (Barbau-Piednoir 2013)). The following thermal program was applied: a single cycle of DNA polymerase activation for 10 min at 95°C followed by 40 amplification cycles of 15 sec at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step). Subsequently, melting temperature analysis of the

amplification products was performed by gradually increasing the temperature from 60°C to 95°C over 20 min ($\pm 0.6^\circ\text{C}/20$ sec). Positive control using gDNA of *S. Enteritidis* at 10^4 copies and negative control using DNase and RNase free water (Acros, Geel, Belgium) were included in each qPCR reaction.

H. Data analysis

1) SYBR®Green qPCR assay

For the interpretation of SYBR®Green qPCR assays, two criteria were taken into consideration: the quantification cycle (Cq) value, and the melting temperature of the amplicon (Tm). The Cq-value represents the cycle at which the PCR amplification reaches the threshold level of the reaction (Bustin 2000). To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level associated with the specific Tm-value of the amplicon.

2) PMA effect

To evaluate the effect of PMA treatment on a sample, the ΔCq was calculated. The ΔCq of a sample is the difference between the Cq-value obtained with PMA treated sample and the Cq-value obtained with not treated sample: $\Delta\text{Cq} = (\text{Cq}_{\text{sample w PMA}} - \text{Cq}_{\text{sample w/o PMA}})$.

3) Statistical analysis

The data were analysed with Splus 8.0 for Linux. A linear mixed model was set up with conditions as fixed factors and date of analysis and aliquot number as nested random factors. Predefined contrasts between conditions were calculated and their corresponding P-values were corrected for simultaneous hypothesis testing according to the Sidak correction. Differences with P values < 0.05 were considered statistically different.

I. Live/Dead® BacLight™ Bacterial Viability kit analysis

In order to discriminate by microscopy bacteria with compromised cell-wall/membranes (dead) from those with non-compromised cell-wall/membranes (viable) the LIVE/DEAD® BacLight™ Bacterial Viability kit (L7007 from Molecular probes) was used according to the manufacturer's recommendations. This kit provides a two-colour fluorescence assay: Syto®9 green-fluorescent nucleic acid stain (480/500 nm) and propidium iodine (PI) red-fluorescent nucleic acid stain

(490/635 nm). Syto®9 stains all bacteria in a population whereas PI penetrates only cell-wall/membrane compromised bacteria and causes a reduction in SYTO®9 stain fluorescence when both are present. This kit displays an appropriate mixture of SYTO®9 and PI stains leading to green fluorescence with intact cell-wall/membranes (live) bacteria and red-fluorescence for bacteria with damaged cell-wall/membrane (dead).

A 100 µl aliquot of each tested sample was transferred into a sterile 1.5ml Eppendorf and the pellet was harvested by centrifugation 10 min at 6,000 x g. The pellet was re-suspended in 100 µl of physiologic water and stained with 0.3 µl of (v/v) mixture of components A and B (L7007 LIVE/DEAD® BacLight™ Bacterial Viability kit). Samples were mixed thoroughly and incubated at room temperature for 15 min in the dark. Hundred microliter of agarose 2 % in physiological water were added to each sample (final concentration 1 % agarose). Seventy-five microliters were trapped between a slide and a 18 mm square cover slip. Confocal fluorescence microscopy (CFM) images were taken using an LSM710 Microscope (Zeiss) with a 40x oil immersion objective and 1x zoom. Fluorescent images of SYTO®9 and PI stained bacteria were acquired at 488 and 543 nm, respectively. The count of dead (red) and live (green) bacteria was performed using the Imaris Software.

III. Results and Discussion

A. Optimisation of the PMA protocol

To obtain full extinction of the dead bacteria qPCR signal using v-qPCR, optimization of the PMA protocol was sought by varying the dark incubation duration and PMA concentration.

1) Effect of increased dark incubation time on qPCR signal reduction

To investigate whether duration of the PMA incubation in the dark has an effect on the extinction of the dead *S. Enteritidis* qPCR signal, 5 and 60 min dark incubation times were compared. Two concentrations of PMA, 0 and 75 µM, were used in both conditions, with *S. Enteritidis* suspensions at concentration of ca. 8.7 log CFU/ml (4.7×10^8 and 6.4×10^8 CFU/ml for the two independent analyses). Samples were analysed in triplicate in each independent analysis ($n = 6$) and qPCR was performed in duplicate on each sample. The qPCR positive and negative controls gave the expected positive and negative results, respectively.

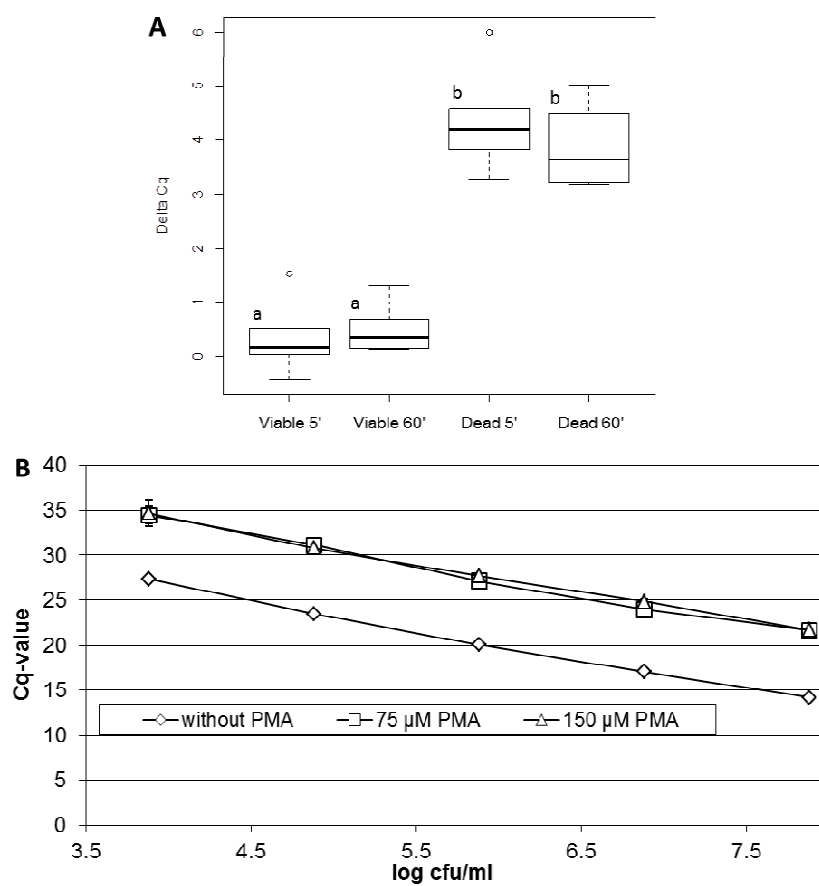


Figure 14: Effect of dark incubation time and PMA concentration on viable and dead cultures of *S. Enteritidis*.

A. Boxplot of different PMA incubation periods (5 and 60 min) on viable ($n = 6$) and dead ($n = 6$) bacteria at 8.7 log CFU/ml. Delta Cq: qPCR signal reduction between samples treated and not treated with PMA 75 μ M, different letter denote values significantly different at $P \leq 0.05$.

B. Effect of three PMA concentrations (0, 75 and 150 μ M) on five different suspensions of dead cells: 7.9, 6.9, 5.9, 4.9 and 3.9 CFU/ml ($n = 1$).

For the dead samples, one of the most common killing processes, isopropanol 70 %, was used to eliminate the bacteria. Death was checked on plate and around 37 CFU/ml (first repetition) and 0 CFU/ml (second repetition) of *S. Enteritidis* were recovered after isopropanol treatment.

As shown in Figure 14A, no effect of PMA treatment was observed on living *S. Enteritidis* for both dark incubation times since their average signal reductions (ΔCq) were not significantly different. Also, as expected, PMA treatment induced a significant reduction of the qPCR signal ($P = 0.0001$) on isopropanol-killed *S. Enteritidis*. However, no significant difference was observed between 5 and 60 min of dark incubation ($P = 0.85$). The average Cq reduction was ca. 4 for both incubation periods. Therefore, increasing the incubation time from 5 to 60 min had no significant effect on the ΔCq of dead *S. Enteritidis* treated by PMA and thus cannot be used to improve the protocol.

2) Effect of PMA concentration on qPCR signal reduction

To investigate whether PMA concentration has an effect on the reduction of the qPCR signal associated with dead *S. Enteritidis*, 0, 75 and 150 μM PMA were compared (5 min dark incubation was used for all PMA concentrations). A dynamic range of *S. Enteritidis* culture of tenfold dilution from 7.9 to 3.9 log CFU/ml (7.6×10^7 CFU/ml to 7.6×10^3 CFU/ml) were used. Killing was performed by isopropanol 70 %. The efficiency of the killing procedure was verified for each *S. Enteritidis* dilution by the absence of growth on nutrient agar. The Cq -value of the dead samples not treated with PMA (0 μM), treated with 75 and 150 μM were compared (Figure 14B). The qPCR positive and negative controls gave the expected positive and negative results, respectively.

As expected, a higher Cq -value was observed with the PMA treatments ($\Delta Cq = \text{ca. } 7$) than with no PMA treatment (0 μM). Yet, for all the tested dilutions, the Cq -values obtained with 75 and 150 μM of PMA were not significantly different. Interestingly, these observations indicate that the increase in PMA concentration does not further reduce the qPCR signal of dead *S. Enteritidis* samples.

B. PMA effect on different *S. Enteritidis* concentrations

To assess whether the amount of bacteria in a sample may have an impact on the reduction of the v-qPCR signal of dead bacteria, four *S. Enteritidis* concentrations were used: 3.7, 4.8, 6.8 and 8.8 log CFU/ml.

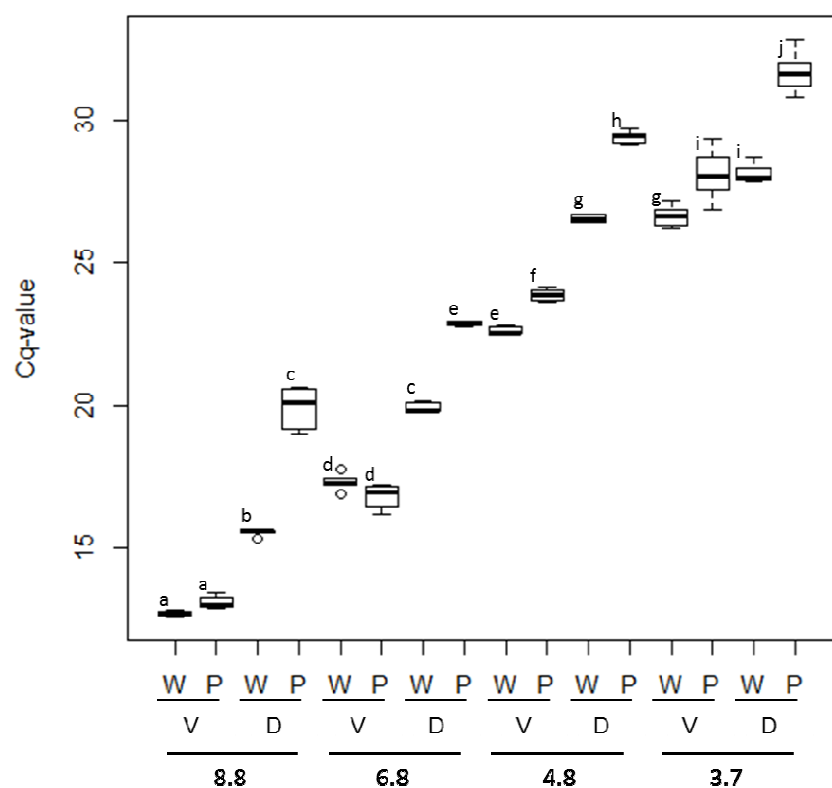


Figure 15: Effect of PMA on the Cq-values of different concentrations of viable and dead *S. Enteritidis* (n = 3).

W: without PMA, P: with PMA 75μM, V: Viable, D: Dead. The numbers 8.8, 6.8, 4.8 and 3.7 indicate the cell concentration in log CFU/ml. Different letters denote values significantly different at $P \leq 0.05$.

Each sample was tested in triplicate and qPCR was performed in duplicate on each replicate. The qPCR positive and negative controls gave the expected positive and negative results, respectively. Living and 70 % isopropanol-killed *S. Enteritidis* were tested. The efficiency of the killing procedure was verified by the absence of colony on plate for all tested concentrations of *S. Enteritidis*. The Cq-values obtained with each type of sample were recorded (Figure 15).

This experiment gave three distinct results. Firstly, the Cq-values obtained with living and dead samples not treated with PMA present an unexpected significant shift between 1 and 4 Cq for the four tested concentrations of cells. This illustrates that the death itself already induces a reduction of the qPCR signal which is probably due to the loss of DNA occurring at the harvesting cell step, from dead cells with highly damaged membrane/cell wall. Secondly, for dead *S. Enteritidis*, as expected, the Cq-values observed with PMA treated samples were significantly higher than with not PMA treated samples for all the studied concentrations (Figure 15: 2: 8.8D, 6.8D, 4.8D and 3.7D). The shift observed was significantly higher for 8.8 log CFU/ml with a ΔCq ca. 4.5 whereas it was the same for the 3 other concentrations with a ΔCq between 2.7 and 3.9. These results confirm the effect of PMA on dead sample but also demonstrate again that PMA is not able to completely remove the signal of dead cells at all studied concentrations. Thirdly, as previously shown, on living samples (8.8A and 6.8A) at high concentration (8.8 and 6.8 log CFU/ml), PMA treatment has no effect on the signal reduction since no significant differences were observed between Cq-values obtained on living cells (Figure 15: 8.8A and 6.8A) treated or not with PMA.

On the contrary, at lower concentrations (3.7 and 4.8 log CFU/ml), PMA has a significant effect on viable cells. Indeed, the Cq-values obtained with living samples treated with PMA are significantly higher, with a shift between 1 and 2.6 Cq, than the not treated living samples (Figure 15: 4.8A and 3.7A). This reduction of the signal of living cells at low concentration may lead to underestimation of viable bacteria and, in the worst case scenario, may lead to false negative results with very low amount of viable bacteria. This reduction of viable cells signal was previously observed on *Legionella* spp. (Yanez 2011) and on *Escherichia coli* (Liu 2014).

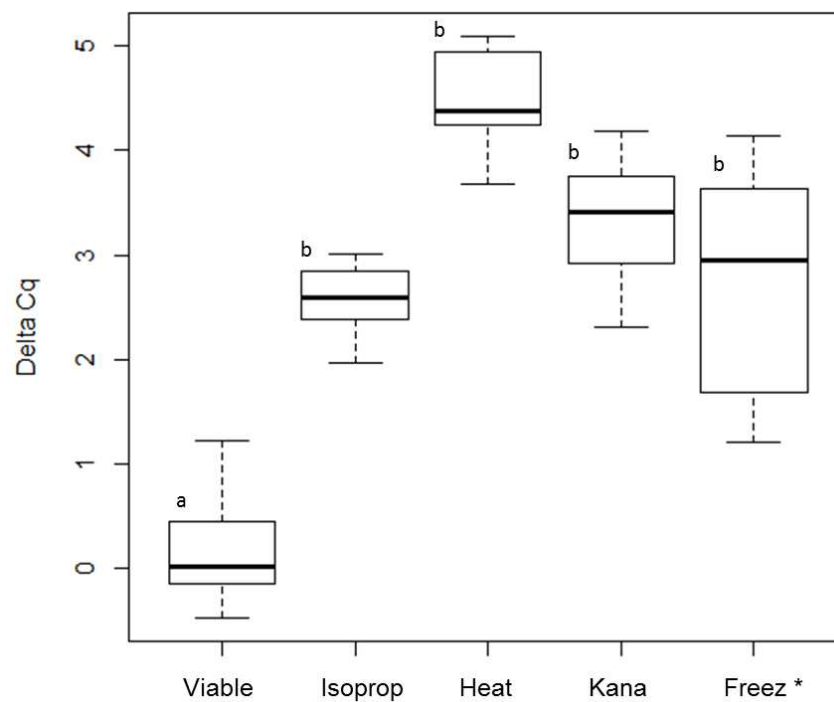


Figure 16: Boxplot of the effect of PMA 75 µM on qPCR signal reduction of *S. Enteritidis* cells treated with isopropanol, heat, kanamycin or a freezing treatment (n = 9).

*: not 100% dead; different letter denote values significantly different at $P \leq 0.05$

C. Effect of different killing processes to the v-qPCR response

To better understand why PMA treatment does not completely remove the qPCR signal of dead *S. Enteritidis* (between 7.9 and 11 log CFU/ml), four distinct killing or stress processes (heat, isopropanol, antibiotic or freezing) were analysed using three different viability evaluation protocols: i) samples were plated on nutrient agar (conventional microbiological technique), ii) samples were analysed with v-qPCR (molecular technique) with 75 μ M PMA and 60 min dark incubation, and iii) samples were dyed with the Live/Dead® BacLight™ Bacterial Viability kit and observed microscopically (microscopic technique). The four distinct killing or stress processes were chosen to act differently on the cell-wall/membrane integrity. Alcohols (such as isopropanol) dehydrate bacteria, disrupt cell-wall/membranes and cause coagulation of proteins. Heat denatures the proteins of the cell-wall and membrane, the phospholipids become more fluids, leading to disruption of the integrity of the cell-wall and membrane. Freezing forms ice crystals inside the bacteria that can rupture the cell-wall/membrane. Isopropanol, heat and freeze-killed samples should induce a high percentage of dead bacteria with disrupted cell-wall/membranes. Kanamycin has a different mode of action. By interacting with the 30S subunit of ribosomes, it induces mistranslation and indirectly inhibits protein synthesis (Misumi 1980). Kanamycin-treated samples should therefore contain a high rate of dead bacteria with intact cell-wall/membrane (ghost cells: Nocker 2009).

Compared to the control experiment using viable bacteria, the v-qPCR, displayed significant reduction of the signal (between 2.5 to 4.5 Cq) on bacteria samples (n = 9) killed by the four different processes (Figure 16). Unexpectedly however, no significant difference in the Cq signal reduction was recorded between the different killing processes (Figure 16). The qPCR positive and negative controls gave the expected positive and negative results respectively.

To further investigate these data in relation to cell-wall/membrane integrity, v-qPCR results were compared with plate counts and Live/Dead kit microscopic observations (Table 22). Plate count assesses the “culturability” of the bacteria whereas Live/Dead kit discriminates bacteria on the basis of their cell-wall/membrane integrity. The first interesting observation was that even if no culturable bacteria were observed, bacteria with intact cell-wall/membrane remained visible (e.g. isopropanol, kanamycin and heat treatments).

Treatment	Microscopic observation					Plate count		v-PCR	
	Alive cells (green)	Dead cells (red)	Total count cells	Percentage of cells with intact membrane	Percentage of membrane-compromised cells	CFU/ml	Percentage of "culturable" cells	Percentage of non "culturable" cells	$\Delta Cq\text{-PMA}$
No (living cells)	101	15	116	87	13	$8.1 \cdot 10^7$	Theoretically 100%	Theoretically 0%	0.9 ± 0.4
Isopropanol	38	252	290	13	87	< 3	0	100	2.3 ± 0.2
Kanamycin	290	74	364	80	20	< 3	0	100	3.1 ± 0.7
Heat	19	355	374	5	95	< 3	0	100	4.5 ± 0.3
Freezing	17	904	921	2	98	$4.05 \cdot 10^3$ to $1.35 \cdot 10^{10*}$	0.5 to 1.7*	98.3 to 99.5*	2.5 ± 0.6

Table 22: Comparison of different assays used to evaluate the percentage of dead *S. Enteritidis* in a sample.

* none countable (bacterial lawn): approximated from other freezing experiments

Heat and isopropanol treated samples included bacteria presenting an intact cell-wall/membrane (5 and 13 %) whereas no culturable bacteria were observed on plate (Table 22). For freezing, 2 % of the cells had an intact membrane and between 0.5 and 1.7 % were culturable. These intact cell-wall/membrane cells among non-culturable cells may explain the remaining qPCR signal of dead cells, as previously reported (Kralik 2010; Lovdal 2011; Pan 2007). Contrary to heat and isopropanol treatments and as expected, for kanamycin treated samples, a high percentage of bacteria with intact cell-wall/membranes (80%) was observed whereas none of the bacteria in the kanamycin treated sample were culturable. According to the PMA mode of action, this must have a drastic effect on the v-qPCR signal reduction: in such samples, no or very low extinction of the signal (ΔC_q) should have been observed. However, this was not the case since the kanamycin treated samples ΔC_q were not significantly different from the other killing processes (Table 22 and Figure 16). These observations demonstrated that the v-qPCR results do not strictly correlate with the physical status of the bacterial cell-wall/membrane.

IV. Conclusion

As the rapid detection of foodborne pathogens remains a challenge, the improvement of the currently available and internationally accepted methods is needed. The ISO/TS 13136 for the detection of Shiga toxin-producing *E. coli* (ISO 2012) was the first reference methods using qPCR instead of culture-based method as a screening system. This opened the way for internationally accepted qPCR detection systems in foodborne pathogens detection. However, there is still room to speed up the detection method by replacing the enrichment step which takes around 18 h. The use of PMA treatment instead of enrichment before qPCR analysis, called v-qPCR, was proposed as a potential alternative.

The aim of this study was first to optimise the v-qPCR protocol to obtain full extinction of the dead bacteria signal and secondly to study the results of v-qPCR on *S. Enteritidis* cells when different killing and stress conditions are applied.

The present study confirms previous observations where v-qPCR was shown as inefficient to completely remove qPCR signal of dead *Mycobacterium avium*, *Listeria innocua* and *Listeria monocytogenes* (Kralik 2010; Lovdal 2011; Pan 2007). Indeed, in this study, from 3.7 to 8.8 log CFU/ml of dead *Salmonella enterica*, full extinction of the v-qPCR signal was never reached, only a reduction of the signal between 2 to 7 C_q was observed in the different experiments reported in this study. Neither longer

dark incubation nor higher concentration of PMA resulted in a significant improvement of the signal reduction. Other studies describe possibilities of improvements of the v-qPCR by further disrupting of the cell-wall/membranes of dead *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* before PMA treatment (Nkuipou-Kenfack 2013; Wang 2014a; Wang 2014b; Yang 2011; Yang 2014). In these papers, even if the reduction of the signal is better, a signal is still observed with dead bacteria. This may lead to overestimation of the presence of viable bacteria in a sample and a false positive signal with dead bacteria. This is not a major issue for detection purposes since detected bacteria should always be confirmed by isolation of the bacteria.

Our research also underlines that the PMA may reduce the signal of viable bacteria at low concentration and confirms other recent studies (Liu 2014; Yanez 2011). This represents a risk of false negative results if the reduced signal falls below the LOD of the qPCR assay. These false negatives are not acceptable in a foodborne pathogen detection system where zero tolerance is the rule, as is the case for *S. Enteritidis* (Commission of EU 2005; Commission of EU 2013).

The last experiment performed on *S. Enteritidis* killed by processes altering or not the cell-wall/membrane gave some clues to the question about the non-total extinction of dead samples signal in v-qPCR assay. Indeed, a portion of the non-culturable bacteria (expected to be dead) have an intact membrane/cell-wall. Since PMA is able to penetrate only bacteria with compromised cell-walls/membranes, in v-qPCR assay, dead bacteria with intact membrane/cell-wall should give a signal similar to that of viable bacteria. However, the kanamycin-treated samples raised incoherence about the mode of action of the v-qPCR. Indeed, these types of inactivated samples displayed a high percentage of non-culturable bacteria with intact cell-wall/membrane although they unexpectedly gave one of the best ΔCq in v-qPCR. These results strikingly illustrate that the v-qPCR results are not only based on the bacterial cell-wall/membrane integrity.

To conclude, according to the results of this study and of a recent review (Fittipaldi 2012), for a qPCR foodborne pathogen detection system, it is not recommended to replace the enrichment step by a PMA treatment, in its current form. At this stage of current knowledge on foodborne pathogenic bacteria detection systems, the use of enrichment before qPCR analysis followed by confirmation of positive results with culture-based methods continues to remain as the most appropriate approach.

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Chapter V: Validation of the entire system on swab samples

The previous work led to a complete CoSYPS Path Food workflow consisting of enrichment, qPCR detection and discrimination system, isolation and confirmation of the detected strains. In the present chapter, this workflow will be compared with the reference culture methods and validated for its use on beef carcass swab samples according to the ISO 16140:2003.

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Fast and discriminative CoSYPS detection system of viable *Salmonella* spp. and *Listeria* spp. in carcass swab samples

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Abstract

In this study, the complete CoSYPS Path Food workflow was validated on beef carcass swabs. This workflow includes all steps from swab sample enrichment, SYBR®Green qPCR detection of *Salmonella* spp. and *Listeria* spp. to isolation and confirmation of the detected strain. To perform the validation, the results of the complete CoSYPS Path Food workflow were compared, according to the ISO 16140:2003, with the ISO reference methods ISO 11290-1:1996 and ISO 6579:2002 for detection, isolation and confirmation of *Listeria monocytogenes* and *Salmonella* spp., respectively. The results showed that the relative level of detection and the limit of detection of the complete CoSYPS Path Food workflow and ISO reference methods are in a range of 2 to 16 CFU/swab for both bacteria. The relative specificity, relative sensitivity and relative accuracy identified during this validation were all 100 % since the results obtained with the complete CoSYPS Path Food workflow and the ISO reference methods were identical (Cohen's kappa index = 1.00). In addition, it was shown that the complete CoSYPS Path Food workflow is able to provide detection results in half the time (two days) needed for the ISO reference methods (four to five days). These results demonstrate that the performance of the complete CoSYPS Path Food workflow is not only comparable to the ISO reference methods but also provides a faster response for verification of beef carcasses before commercial distribution.

Keywords

ISO 16140:2003, *Listeria*, *Salmonella*, swabs, CoSYPS Path Food

I. Introduction

Worldwide there are annually 1.3 billion cases of human gastro-enteritis due to *Salmonella* spp. (Bhunia 2008b). In European Union (EU), *Salmonella* is the first notification cause of microbial food-borne contamination (Commission of EU 2012), and the main reported causative agent in food-borne outbreaks causing ca. 90,000 confirmed human cases in 2012 (EFSA and ECDC 2014). The reservoirs are mainly poultry, but also cattle, swine and sheep (Pui 2011). Human salmonellosis is mainly caused by contaminated food consumption (EFSA and ECDC 2014).

L. monocytogenes has a low annual incidence worldwide. About 1,500 cases per year are recorded in EU and 2,500 per year in US (Bhunja 2008a; EFSA and ECDC 2014). However, because of its high mortality rate (between 20 and 30 %), listeriosis ranks among the most frequent human death causes due to food-borne illnesses in USA and in EU (Barton Behravesh 2011; EFSA and ECDC 2014). *Listeria* spp. principal reservoirs are soil, forage, water and the domestic animal intestinal tract (cattle, sheep, goats, etc.) (Bhunja 2008a). The main transmission route to humans is contaminated food consumption (EFSA and ECDC 2014).

As such food-borne pathogens reservoirs are mainly farm animals, foodstuffs from animals are controlled according to regulation 854/2004 (Commission of EU 2004). Meat is one of the most important food-borne pathogens vehicles, giving the highest number of non-compliant samples with the EU *Salmonella* spp. criteria (Commission of EU 2005). Meat contamination is usually a surface contamination occurring during slaughter process by faecal contamination during evisceration (FSA 2002).

Regulation 2073/2005 (EFSA and ECDC 2014) gives the official analytical reference methods to detect food-borne pathogens and the microbiological acceptance limits for each food category. For *Salmonella* spp. and *L. monocytogenes* detection, the reference methods are culture-based (ISO 1996; ISO 2002). Although efficient, these methods are time-consuming and labour-intensive, i.e. each target bacterium has its own protocol and up to 7 days are required to confirm *L. monocytogenes* or *Salmonella* spp. presence. This hampers a rapid answer in case of outbreaks where a swift (re-)action is required.

Molecular methods are increasingly accepted as good alternatives since they are fast, sensitive and specific. At present, several real-time PCR (qPCR) assays have been developed for detection of *Salmonella* spp. (e.g. Hein 2006; Josefsen 2007; Liming 2004; Malorny 2004; Malorny 2007; Pasquali 2013; Perelle 2004; Seo 2004; Wang 2010) and *L. monocytogenes* (e.g. Berrada 2006; Hough 2002; Nogva 2000; O'Grady 2008; O'Grady 2009; Oravcova 2007; Rossmannith 2006; Rudi 2005) in food products. These systems provide single-genus and even single-species detection system for *Salmonella* spp. and *L. monocytogenes*, respectively. Moreover, they target a single gene with a single assay, which is the case in most of qPCR detection systems described in the literature. This could lead to false negative results in case of targeted gene mutation or deletion (Barbau-Piednoir 2013b; Hu 2008). To mitigate these two inconveniences, approaches targeting two genes for *Salmonella* spp.

detection (Gonzalez-Escalona 2012) or targeting several bacteria at the same time (Garrido 2012a; Garrido 2012b; Ma 2014; Köppel 2013; Singh 2012) have been developed. Recently, this strategy was further improved with the combinatory SYBR®Green qPCR screening (CoSYPS) system, able to detect in a single step both *Salmonella* spp. and *Listeria* spp., and to give information about species and subspecies detected (Barbau-Piednoir 2013a; 2013b). This system contains several target genes per bacterium to create a multi-levels detection system. All SYBR®Green qPCR assays of this CoSYPS system have been validated (Barbau-Piednoir 2013a; 2013b) and can be used together as a single-plate detection system. This detection system is also part of the complete CoSYPS Path Food workflow which includes all steps from swab sample enrichment, DNA extraction, *Salmonella* spp. and *Listeria* spp. qPCR detection, isolation and confirmation of the detected strains.

In order to fully validate the complete CoSYPS Path Food workflow that includes qPCR detection, isolation and confirmation, as an alternative method to the reference ISO methods, a final evaluation is required. The ISO 16140:2003 (ISO 2003) describes the process to validate an alternative method by comparing it with a reference method. The complete validation is composed of two steps: i) a comparison study of the alternative method *versus* the reference method carried out in the organizing laboratory, and ii) an inter-laboratory study performed with both methods in parallel. The first part allows alternative method to be used in the laboratory under accreditation. The second part is mainly performed for commercialisation purposes.

To assess potential meat contamination by food-borne pathogens, carcasses monitoring at slaughterhouse is an important tool. Sampling carcasses can be performed by destructive (excision or drilling) or by non-destructive methods (swabbing). The latter presents several advantages: i) it is non-destructive and causes no damage to the carcasses (no commercial impact), ii) it allows to sample a large surface, i.e. from 400 cm²/carcass (Commission of EU 2005) to 1400 cm²/carcass and 1600 cm²/carcass in particular regulations (Estonia Belgium, Sweden and Finland) (EFSA and ECDC 2014). This large surface sampling is an important advantage for detection of low contamination levels (Lindblad 2007).

In this paper, the *Salmonella* spp. and *Listeria* spp. detection in carcass swab samples by the complete CoSYPS Path Food workflow was validated by comparing

its results to those of reference methods (ISO6579:2002 and ISO11290-1:1996 (ISO 1996; ISO 2002)). The methods limits of detection were also determined. Different validation criteria such as relative detection level, relative sensitivity, relative specificity, relative accuracy, Cohen kappa index and practicability of both detection methods were investigated. Finally the advantages of using the complete CoSYPS Path Food workflow are discussed.

II. Materials and methods

A. Bacterial strains and spiked preparation

S. enterica enterica Enteritidis (H,V1,6,32 from Belgian *Salmonella* NRC) and *L. monocytogenes* serotype 1/2a (ATCC 51772) were used to artificially contaminate the swab samples. A single colony was inoculated in 10 ml of Brain Heart Infusion (BHI) broth and cultured at 37°C without shaking for 16-18 h. This culture was diluted in sterile BHI broth to get an OD_{600nm} at 1 (around 5.10⁸ CFU/ml). This dilution called D0 was used as starter in a 10-fold serial dilution until D-9 in buffered peptone water (BPW). To perform the enumeration of D-6 to D-9, 100 µl of these dilutions were plated in triplicate on nutrient agar plates. These four dilutions were used to spike the swab samples.

B. Spiked carcass swab samples

To create artificial beef carcass swab samples containing the same resident microflora as the genuine beef carcass swab samples, 25 g of minced meat (100 % beef) (free of *Salmonella* spp. and *Listeria* spp.) from a retail shop were grinded in 225 ml of BPW medium in a filter stomacher bag. A BPW-hydrated sponge was introduced into a new filter stomacher bag and soaked with 10 ml of the minced meat juice. To spike these swabs, 100 µl of D-6 to D-9 were added onto the sponge swab. The spiked swabs were stored for 18 ± 2 h at fridge temperature to mimic the storage and transport steps that are undergone by real swab samples between sampling and analysis (ISO 2004).

C. Methods for *Listeria* spp. and *Salmonella* spp. detection, isolation and confirmation

The workflow of the procedure followed by all the detection methods is shown in detail in Figure 17.

1) Common pre-enrichment step

Spiked swab samples were pre-enriched in 90 ml BPW at 37°C without shaking. As requested by ISO16140:2003, the same sample was used for the analysis with the ISO reference methods as well as with the complete CoSYPS Path Food workflow.

2) ISO reference methods

The reference method for *L. monocytogenes* detection was the ISO 11290-1:1996 amended by ISO11290-1/A1:2005 (ISO 1996; ISO 2005). A variation from this protocol was performed. BPW was used instead of Fraser-demi for the pre-enrichment to be able to perform *Salmonella* and *Listeria* detection at the same time, on the same sample. The reference method used for *Salmonella* spp. detection was ISO 6579:2002.

a) Enrichment step

After 24 h incubation, for *Listeria* detection, pre-enrichment broths were used to inoculate the selective Fraser enrichment broth. For *Salmonella*, the enrichment media, i.e. Rappaport-Vassiliadis Soja (RVS) broth and Müller-Kauffmann tetrathionate-novobiocine (MKtn) broth, were inoculated with the 24 h pre-enrichment broth.

b) Isolation

The isolation was performed by plating out the selective enrichment broth on selective solid media. For *Listeria* spp., the isolation was performed on Agar *Listeria* according to Ottaviani and Agosti (ALOA) and Rapid L'Mono (RLM) agar, while for *Salmonella* spp., the isolation media were Xylose-Lysine-Deoxycholate (XLD) agar and ChromID™ *Salmonella* (SMID) agar.

c) Confirmation

For *Listeria* spp., no confirmation was performed. Indeed, the presence of typical colonies on selective plates was enough to get a positive result as they were spiked samples.

For *Salmonella* spp., one typical colony on each of the selective plates, i.e. two typical colonies were selected. These colonies were biochemically confirmed. If the selected colony was negative, four other typical colonies were tested.

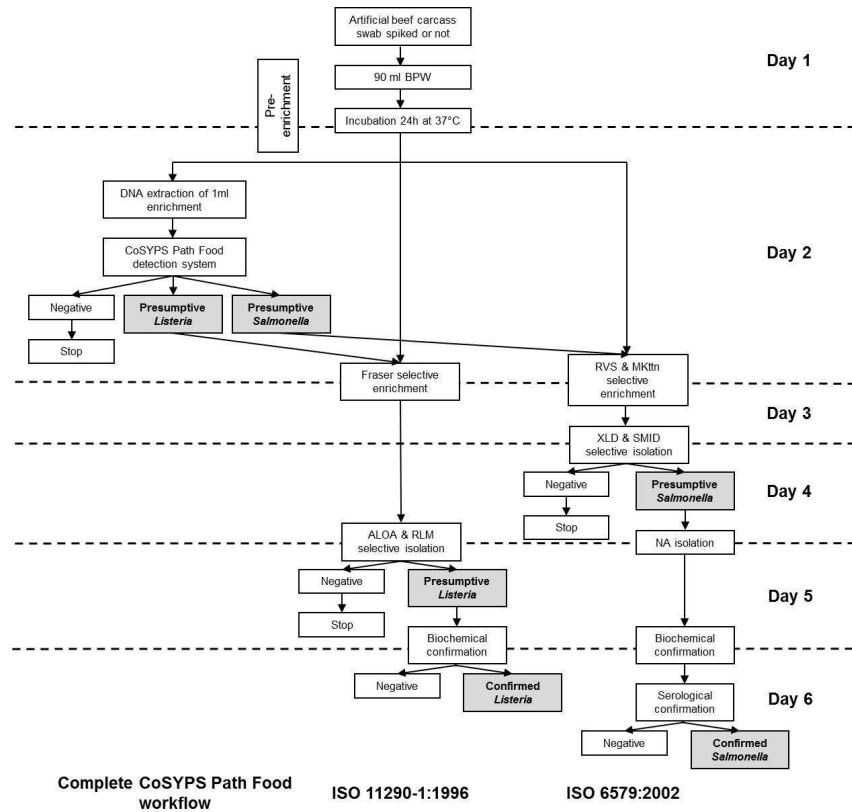


Figure 17: Flow chart illustrating the different steps of the entire CoSYPS Path Food workflow, ISO 11290-1:1996 and ISO 6579:2002 on beef carcass swab samples. Abbreviations are as follows: BPW: Buffer peptone water, RVS: Rappaport-Vassiliadis Soja, MKtn: Müller-Kauffmann tetrathionate-novobiocine, XLD: Xylose-Lysine-Deoxycholate, SMID: ChromID™ *Salmonella*, NA: Nutrient Agar, ALOA: Agar *Listeria* according to Ottaviani and Agosti and RLM: Rapid L'Mono.

In this validation, the expected feature on Kligler iron agar was considered as a positive result since the sample was *Salmonella*-spiked. Neither Rapid ID32E nor serological confirmation were performed.

3) The complete CoSYPS Path Food workflow

a) DNA extraction

After 24 h of pre-enrichment, one ml of the pre-enrichment broth was transferred into a 1.5 ml micro-centrifuge tube, centrifuged for 10 min at 6,000 x g at room temperature and the supernatant was discarded. The pellet was extracted with the Nucleospin food kit (Macherey-Nagel®) according to the manufacturer's recommendations.

b) CoSYPS analysis

The *Salmonella* and *Listeria* spp. detection were performed using the CoSYPS Path Food detection system. This system is composed of respectively 7 and 4 SYBR®Green qPCR assays, for the detection of *Salmonella* spp. and *Listeria* spp., respectively, in a tested sample (Barbau-Piednoir 2013a; 2013b). These 11 qPCR assays can be run separately or together on the same plate (as 11 singleplex). All qPCR assays were performed on an iCycler iQ™5 Real-Time PCR Detection System (Bio-rad) with iCycler iQ™ PCR plates, 96 wells (Bio-rad) closed with the PCR Sealers Microseal B films (Bio-rad). All qPCR assay reactions were performed according to the same protocol: the reactions were performed in a final volume of 25 µl containing 5 µl of the diluted DNA extract (1/2 for *Listeria* qPCR assays and 1/1000 for *Salmonella* qPCR assays), 1X SYBR®Green PCR Mastermix (DMSG-2X-A300, Diagenode), and the appropriate concentration of each primer (Barbau-Piednoir 2013a; 2013b).

Primers were purchased from Eurogentec (Belgium). The following thermal program was applied: a single cycle of DNA polymerase activation for 10 min at 95°C followed by 40 amplification cycles of 15 s at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step). Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60°C to 95°C over 20 min ($\pm 0.6^\circ\text{C}/20\text{s}$). The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit was set manually at the beginning of the exponential amplification phase.

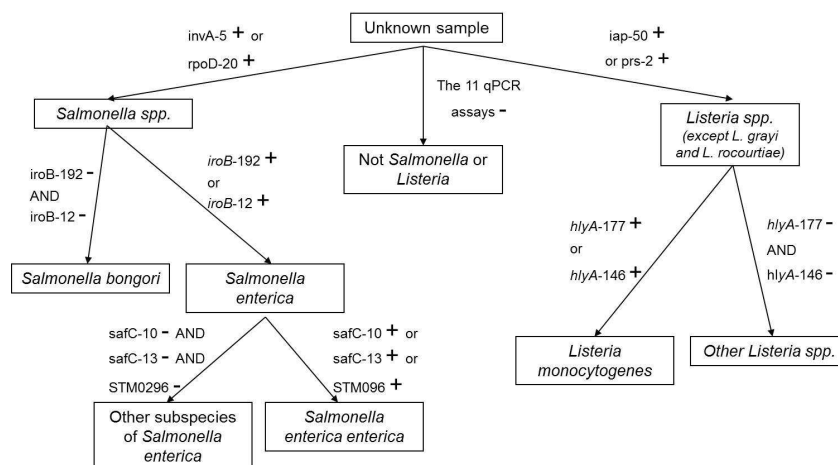


Figure 18: Decision tree of the complete CoSYPS Path Food workflow.

The CoSYPS Path Food detection system is multi-species (*Listeria* and *Salmonella*) and multi-levels (Genus, species and subspecies) SYBR®Green qPCR system. Each level of detection is performed by two qPCR assays: *iap*-50 and *prs*-2 for *Listeria* spp (except *L. grayi*); *hlyA*-177 and *hlyA*-146 from *L. monocytogenes*; *invA*-5 and *rpoD*-20 for *Salmonella* spp.; *iroB*-192 and *iroB*-12 for *Salmonella enterica*; and *safC*-10, *safC*-13 and *STM* for *Salmonella enterica enterica*. Regarding the positive or negative signal observed with each assay, the presumptively present strain is identified. More details in (Barbau-Piednoir *et al.*, 2013a, b). Abbreviations for the genes are as follows: *iap*: invasion-associated protein, *prs*: phosphoribosylpyrophosphate synthetase, *hlyA*: Listeriolysin O, *invA*: invasine gene, *rpoD*: RNA-polymerase sigma factor, *iroB*: C-glycosyltransferase, *safC*: *Salmonella* atypical fimbriae outer membrane usher protein and *STM0296*: putative cytoplasmic protein AAL19253.

“No Template” Controls (NTC) using DNase and RNase free water were included in each reaction to assess primer dimer formation or non-specific amplification. For the interpretation of a SYBR®Green qPCR assay, two criteria were taken into consideration: the quantification cycle (C_q) value, and the melting temperature of the amplicon (T_m). The C_q-value represents the fractional cycle at which the PCR amplification reaches the threshold level for the reaction (Bustin 2000). Since it is a screening assay, only a qualitative response is required. To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level, with a single peak upon melting analysis giving a unique T_m-value. A signal was considered as negative when no C_q-value was obtained. The combination of positive assays generates the list of bacteria possibly present into the sample (presumptive positive) according to the decision tree presented in Figure 18.

c) Selective enrichment, isolation and confirmation

The selective enrichment, isolation and the confirmation were performed only if a presumptive positive result was obtained. All these steps were performed as previously described in the section on the ISO reference methods.

D. Validation of the complete CoSYPS Path Food workflow

The complete CoSYPS Path Food workflow was validated for the enrichment, detection, isolation and confirmation of the presence of *Listeria* spp. and *Salmonella* spp. in beef carcass swab samples. The samples were analysed in parallel by the reference ISO methods and the complete CoSYPS Path Food workflow as depicted in Figure 17. The results obtained with each method were compared using the criteria described in the ISO 16140:2003, the NordVal guidelines and the AFNOR technical board listed 13 practicability criteria (AFNOR 2013; ISO 2003; NordVal 2009)

According to ISO 16140:2003 at least three levels of contamination should be tested. In this study, four levels were analysed (D-6 to D-9). They were spiked on four different swabs. Four independent analyses of these four swabs were performed for each tested bacteria. As the ISO 16140:2003 requires at least 6 repetitions, the dilution identified as relative detection level with each individual detection method was re-analysed with 6 swabs.

The ISO 16140:2003 also requires the use of 20 samples to validate a system on a food category, with 50% positive and 50% negative samples. The same samples should be analysed by both the alternative and the reference methods. In this study, 20 samples were prepared presenting different spike concentrations and samples were inoculated with one or both targets (*Salmonella* spp. and *Listeria* spp.) of the complete CoSYPS Path Food workflow: i) five samples containing none of the targets were used as negative samples; ii) six samples contained only one of the targets at the LOD, 10 times or 100 times the LOD; iii) one sample contained both targets at the LOD; iv) four samples contained one target at the LOD and the other target 10 or 100 times above the LOD; v) two samples contained one target 10 times above the LOD and the other target 100 times above the LOD; vi) one sample contained both targets 10 times above the LOD and vii) one sample contained both targets 100 times above the LOD.

E. Validation criteria

1) Limit of detection and relative detection level

The limit of detection of each method is defined as the lowest number of microorganisms per assay that is positive in 95% of occasions (ISO 2011). The relative detection level is the smallest number of culturable microorganisms that can be detected in the sample in 50% of the occasions by the alternative and reference methods.

2) Relative sensitivity, relative specificity and relative accuracy

The relative specificity (SP) is the ability of the alternative method to not detect the analyte when it is not detected by the reference method (ISO 2003). The relative sensitivity (SE) is the ability of the alternative method to detect the analyte when it is detected by the reference method (ISO 2003). The relative accuracy (AC) is the degree of correspondence between the response obtained by the reference method and the one obtained by the alternative method on identical samples (ISO 2003). They were determined by comparing results obtained by analysing 20 samples of spiked artificial swabs with the ISO methods (reference methods) and with the complete CoSYPS Path Food workflow (alternative method).

$$SE = \frac{PA \times 100}{PA + ND}$$

$$SP = \frac{NA \times 100}{NA + PD}$$

$$AC = \frac{(PA + NA) \times 100}{PA + NA + PD + ND}$$

PA is the positive agreement: number of samples that are positive by both reference and alternative methods. NA is the negative agreement: number of samples that are negative by both reference and alternative methods. PD is the positive deviation: number of samples that are negative with the reference method and positive with the alternative method. ND is the negative deviation: number of samples that are positive with the reference method and negative with the alternative results.

3) Cohen kappa index

The Cohen kappa index, expressing the degree of acceptance between two methods was calculated according to the following formula (Cohen 1960):

$$k = \frac{p_o - p_c}{1 - p_c}$$

where

$$p_o = \frac{PA + NA}{PA + NA + PD + ND}$$

$$p_c = \frac{[(PA + ND) \times (NA + PD)] + [(PA + PD) \times (NA + ND)]}{(PA + NA + PD + ND)^2}$$

Cohen kappa values are categorised as follow: ≤ 0.20 poor agreement, between 0.21 and 0.40 fair agreement, between 0.41 and 0.60 moderate agreement, between 0.61 and 0.80 good agreement, and ≥ 0.81 very good agreement (Landis 1977; NordVal 2009).

4) Practicability

AFNOR technical board listed 13 practicability criteria (AFNOR 2013). Some of these criteria, judged as relevant by the authors, were evaluated in the present study: the training of the operator, the lab equipment and the time required to get results.

Method	Listeria detection				Salmonella detection			
	Spike: CFU/swab (dilution)	Positive	Negative	Percentage of positive	Spike: CFU/swab (dilution)	Positive	Negative	Percentage of positive
ISO	77-150 (D-6)	4/4	0/4	100	30-60 (D-6)	4/4	0/4	100
CoSYPS		4/4	0/4	100		4/4	0/4	100
ISO	4-16 (D-7)	10/10	0/10	100	2-11 (D-7)	10/10	0/10	100
CoSYPS		10/10	0/10	100		10/10	0/10	100
ISO	0-2 (D-8)	2/4	2/4	50	0-1 (D-8)	1/4	3/4	25
CoSYPS		1/4	3/4	25		1/4	3/4	25
ISO	0-1 (D-9)	1/4	3/4	25	0 (D-9)	1/4*	3/4	25
CoSYPS		1/4	3/4	25		1/4*	3/4	25
ISO	0 (C-)	0/10	10/10	0	0 (C-)	0	10	0
CoSYPS		0/10	10/10	0		0	10	0

Table 23: Comparative results of swab spiked with a 10-fold serial dilution of *S. Enteritidis* or *L. monocytogenes* tested in parallel with the ISO reference methods or the CoSYPS Path Food workflow.

ISO Methods are ISO 11290-1 and ISO 6579 for *Listeria* and *Salmonella*, respectively.

CoSYPS: complete CoSYPS Path Food workflow

* this positive is not a false positive, it is due to the bias in the spike enumeration, i.e. the spike is evaluated by enumeration of 3 repetitions of the spike (a enumeration of the spike itself is not possible).

III. Results and Discussion

A. Limit of detection and the relative level of detection determination

Beef carcass swab samples spiked with different *L. monocytogenes* or *S. enterica enterica* concentrations were analysed in parallel with the ISO reference methods and the complete CoSYPS Path Food workflow. The swabs spiked with the dilutions D-6 and D-7 were positive in the four independent repeats with the complete CoSYPS Path Food workflow as well as with the ISO reference methods. The D-8 gave 50% (2/4 repeats) of positive with ISO 11290-1:1996 and 25% of positive with the ISO 6579:2002 and the complete CoSYPS Path Food workflow. The D-9 gives 25% of positives with all the tested methods. Considering these results, the limit of detection (LOD) of both conventional and CoSYPS methods as well as the relative detection level (RDL) are at dilution -7 (D-7), i.e. between 4 and 16 CFU/swab for *L. monocytogenes* detection and between 2 and 11 CFU/swab for *S. enterica enterica* detection. To confirm these LOD and RDL, six additional swabs spiked with D-7 were analysed. These six additional repeats gave all positive results, confirming both criteria (Table 23). The study demonstrated that the complete CoSYPS Path Food workflow is as efficient as the reference ISO methods to detect low concentration of targets.

B. Relative specificity, relative specificity and relative accuracy and Cohen's kappa index determination

Twenty beef carcass swab samples spiked with different *L. monocytogenes* and/or *S. enterica enterica* concentrations were analysed in parallel with the ISO reference methods and the complete CoSYPS Path Food workflow. Each of the swabs spiked with the different concentrations of bacteria gave the expected positive signal (12/12) with both approaches, whereas the non-spiked swabs gave all a negative signal (8/8) (Table 24). This demonstrates that the complete CoSYPS Path Food workflow was able to detect both target bacteria in a tested sample, even when one target was present at very low concentration.

This is particularly relevant for samples containing low *Listeria* spp. numbers. Indeed, *Listeria* spp. grow slower than *Enterobacteriaceae* and its growth could be inhibited by the beef carcass co-resident microflora, and by *Salmonella* spp. in case of double contamination. Moreover, the pre-enrichment in BPW instead of Fraser-demi is less optimal for *Listeria* spp. growth.

	<i>Listeria</i> detection comparison				<i>Salmonella</i> detection comparison			
	Spiked CFU <i>Listeria</i> /swab	CoSYPS <i>Listeria</i>	ISO 11290	Comparison	Spiked CFU <i>Salmonella</i> /swab	CoSYPS <i>Salmonella</i>	ISO 6579	Comparison
Sample 1	0	-	-	=	0	-	-	=
Sample 2	10-19	+	+	=	8-10	+	+	=
Sample 3	121-131	+	+	=	56-74	+	+	=
Sample 4	1105-1555	+	+	=	760-786	+	+	=
Sample 5	1105-1555	+	+	=	0	-	-	=
Sample 6	121-131	+	+	=	8-10	+	+	=
Sample 7	10-19	+	+	=	56-74	+	+	=
Sample 8	0	-	-	=	760-786	+	+	=
Sample 9	121-131	+	+	=	0	-	-	=
Sample 10	1105-1555	+	+	=	8-10	+	+	=
Sample 11	0	- *	-	=	56-74	+	+	=
Sample 12	10-19	+	+	=	760-786	+	+	=
Sample 13	10-19	+	+	=	0	-	-	=
Sample 14	0	-	-	=	8-10	+	+	=
Sample 15	1105-1555	+	+	=	56-74	+	+	=
Sample 16	121-131	+	+	=	760-786	+	+	=
Sample 17	0	-	-	=	0	-	-	=
Sample 18	0	-	-	=	0	-	-	=
Sample 19	0	-	-	=	0	-	-	=
Sample 20	0	-	-	=	0	-	-	=
PA		12				12		
NA		8				8		
ND		0				0		
PD		0				0		
SE (%)		100				100		
SP (%)		100				100		
AC (%)		100				100		
Cohen's k index		100				100		
Cohen's k conclusion		very good agreement				very good agreement		

Table 24: Comparative results of the culture-based reference methods with the CoSYPS *Salmonella* and *Listeria* on the detection in spiked artificial swabs of beef meat.

* : this sample had to be reextracted since in first extract a false positive was observed; +: positive result; -: negative result; PA: positive agreement; NA: negative agreement; ND: negative deviation; PD: positive deviation; SE: relative sensitivity; SP: relative specificity; AC: relative accuracy

From these results, the positive and negative agreements (PA and NA), the positive and negative deviation (PD and ND) were assessed. The PA is 12, the NA is 8 and the ND and PD are 0 for both targets. These values allow the calculation of a 100% relative sensitivity (SE), 100% relative specificity (SP) and 100% relative accuracy (AC) and a Cohen's kappa index of 1.00 of the results obtained with the complete CoSYPS Path Food workflow compared with the results obtained with the ISO methods (Table 24). These values demonstrate the complete CoSYPS Path Food workflow as efficient as the reference methods in detecting *Salmonella* spp. and *L. monocytogenes* in beef carcass swab samples

C. Practicability

To perform the ISO reference methods, as well as the complete CoSYPS Path Food workflow, classical microbiological equipments are required (e.g. L2 laboratory facility with conventional equipment for food microbiology). In addition, the complete CoSYPS Path Food workflow required qPCR well-trained personnel operating a properly maintained qPCR apparatus.

The ISO 11290-1:1996 and ISO 6579:2002 comprise a pre-enrichment step, a selective enrichment step and isolation on selective plates (Figure 17). These different steps need four and five days of works for *Salmonella* spp. and *L. monocytogenes* detection, respectively, since each culture step requires an 18 to 24 h incubation period (48 h for Fraser selective enrichment). If no typical colonies are observed on selective plates, the sample is concluded as containing no pathogen (*Salmonella* spp. or *L. monocytogenes*) and the analysis is stopped. If typical colonies are observed on the selective plates, it is a presumptive positive result, and further biochemical confirmations are performed, which takes an additional day (Figure 17). The complete CoSYPS Path Food workflow comprises a pre-enrichment step, followed by a DNA extraction and a CoSYPS detection system (qPCR analysis). These steps can be completed within two days (including an overnight enrichment) as DNA extraction and CoSYPS analysis are easily performed within a single day. Indeed, DNA extraction required at most 3 h and the CoSYPS analysis needs around 4 h (preparation, running and interpretation included). If the CoSYPS analysis result is negative, the sample is concluded as containing neither *Salmonella* spp. nor *Listeria* spp. and the analysis is stopped. If the CoSYPS analysis result is positive, it is a presumptive positive. Thus, the time of analysis of the CoSYPS Path Food workflow to get a negative or presumptive positive result is divided by two (from

four-five days to two days) compared to the ISO detection methods. This reduced time is an important advantage, especially in case of outbreaks and for self-control of short-life products. Moreover, for a food business operator a presumptive positive is enough to take action.

To be confirmed, a presumptive positive sample must continue the complete workflow with the selective enrichment, isolation on selective plate and confirmation of the isolated strain which require four additional days (Figure 17). Thus, a confirmed positive result requires the same number of days, i.e, 6 days for *Salmonella* spp. and *Listeria* spp. analysis.

IV. Conclusion

This validation study confirms that the complete CoSYPS Path Food workflow is as efficient as the reference methods in detecting *Salmonella* spp. and *L. monocytogenes* in beef carcass swab samples. Thus, it is a valuable alternative to ISO reference methods for beef carcasses control before commercial distribution. Moreover, the complete CoSYPS Path Food workflow presents several advantages. Firstly, as a multi-genera system, this workflow is able to detect the presence of both pathogens in a single plate and from a single sample. Secondly, as a multi-levels system, it has the advantage over other previously developed qPCR-based detection systems to provide information about detected strain species and/or subspecies. Thirdly, it gives presumptive positive or negative results in two days whereas four and five days are required for ISO 6579:2002 and ISO 11290-1:1996, respectively. Finally, it presents an additional advantage of great flexibility over other available qPCR-based detection systems. The CoSYPS Path Food qPCR detection step is indeed adaptable to the sample requirements: i) the tested targets list can be adapted to the analysis purpose and ii) new foodborne pathogens can be added into the qPCR detection system as soon as new qPCR assays targeting these newly targeted pathogens are developed to be run with the same PCR conditions as the already included qPCR assays (Barbau-Piednoir 2013a; 2013b). The selective enrichment, isolation and confirmation steps would have to be specific of each added foodborne pathogen, using the protocols provided into the respective ISO reference methods (when available). Thus, this workflow could be upgraded with additional foodborne pathogen targets with a limited amount of work.

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Part III: General discussion, conclusions and perspectives

Chapter VI: General discussion

World Health Organisation estimates that diarrhoeal diseases, of which a considerable proportion is foodborne, kill around 2.2 million people each year in the world (218). In Europe in 2011, 27,000 deaths were due to diarrhoeal diseases (219). As foodborne diseases are not limited to diarrhoeal symptoms, the total death number due to foodborne diseases is likely to be higher than the above figures. To be able to decrease the incidence of foodborne diseases, and to protect the consumer, the control of food products is the most important tool. Foodborne diseases can be caused by chemical or harmful substances but they are mainly caused by foodborne pathogens such as bacteria, viruses and protozoa amongst which bacteria are the most important causative agents.

Conventional and standard methods to detect foodborne bacteria in food products are culture-based techniques that are time consuming and labour intensive. In the first part of this work (Chapter III), a faster alternative method using qPCR-based detection assays namely CoSYPS Path Food detection system was developed. First (Chapter III, section 1), a two-level detection system for detection of all *Listeria* spp. and the discrimination of the most important species of this genus, i.e. *Listeria monocytogenes*, was developed. These two levels are composed of two assays per level to reduce the probability of false negative resulting from mutation or deletion of the target sequence(s). These four SYBR®Green qPCR assays were also validated following a new procedure for qPCR assays applied to food microbiology. These assays were developed to be run on a single-plate, using the same PCR program.

The second part of this work (Chapter III, section 2) was the development and the validation of a three levels detection and discrimination system for *Salmonella*. For the same reasons as presented above, each level is composed of at least two SYBR®Green qPCR assays. The first level (two assays) detects all *Salmonella* spp., the second level (two assays) discriminates the *Salmonella enterica* species and the third level (three assays) discriminates the *Salmonella enterica* subsp. *enterica*. These eleven assays were also developed to be run on a single-plate with the previously developed *Listeria* qPCR assays (same PCR program). Moreover, the *Salmonella* CoSYPS detection system was compared with available commercial qPCR detection assays.

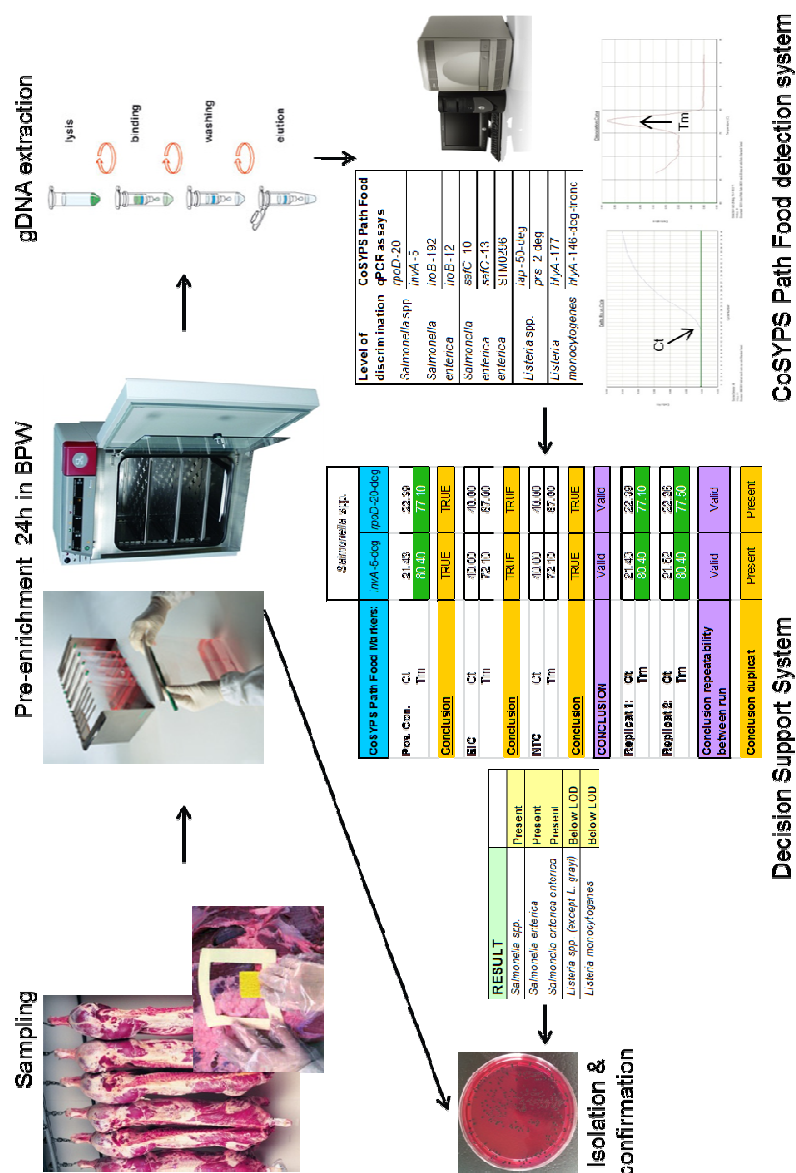


Figure 19: Schematic representation of the complete CoSYPS Path Food workflow.

The complete CoSYPS Path Food workflow is a qPCR-based detection system including all steps from sample enrichment to isolation and confirmation of the detected strain. The swabbing is performed at the slaughterhouse and the swabs are sent to the laboratory where they are enriched for 24h in buffered peptone water (BPW). One ml of enrichment is used to perform a DNA extraction using the Nucleospin Food kit (Macherey-Nagel®). The DNA extracts are analyzed by the CoSYPS Path Food detection system composed of four *Listeria* spp. and seven *Salmonella* spp. detection and discrimination SYBR®Green qPCR assays. The results of the CoSYPS Path Food detection system are introduced into the decision support system to obtain the list of possibly present *Salmonella* spp. and *Listeria* spp. To confirm the presence of the detected strains, isolation on selective plates and confirmation are performed according to the respective ISO methods for *Salmonella* and *Listeria*, i.e ISO 6579:2002 and 11290-1:1996 (38;68).

This comparison demonstrates that single-target detection system can lead to false negative signal with atypical strains. This highlights the importance of using a multi-targets detection system to be able to detect every target strain, even those with deletion or mutation of important genes. In the third part of this work (Chapter III, section 3), a support tool was developed to facilitate the run and data analysis of the *Salmonella* and *Listeria* CoSYPS detection system. This support tool provides a technical support (i.e. primer list, PCR mixes automatic calculation, optimum plate setup) and a decision support system (DSS) to obtain an automatic and harmonised conclusion from the qPCR results. The combination of the eleven qPCR assays (four for *Listeria* and seven for *Salmonella*) and the related DSS is named “CoSYPS Path Food” for Combinatory SYBR®Green qPCR Screening system for pathogens detection in food samples. This CoSYPS Path Food intends to be used in routine analysis, for laboratories working under accreditation.

In Chapter IV, a preliminary step to the detection is addressed. Indeed, an important drawback of the qPCR-based detection of bacteria is the possible detection of both viable and dead cells. Propidium monoazide (PMA) coupled with qPCR (viability-qPCR: v-qPCR) was assessed on viable and dead cells of *S. enterica* subsp. *enterica* serovar Enteritidis. This study demonstrated that none of the modifications of the PMA treatment protocol totally removed the qPCR signal of dead bacteria. This study also underlined that PMA may unexpectedly decrease the qPCR signal observed on viable *S. Enteritidis* at low concentration. Finally, the study gave a clue to answer the question about the non-total extinction of the signal of dead samples in v-qPCR assay. Indeed, the data strongly indicated that the remaining qPCR signal observed in non-culturable cells did not only depend on the cell-wall/membrane integrity of the bacteria. According to these results, an enrichment followed by a qPCR analysis should be preferred to a v-qPCR for a rapid and reliable foodborne bacteria detection system.

The previous parts of this project led to the design of a complete qPCR-based detection system which includes all the steps, from food sample enrichment to isolation and confirmation of the detected strain, and is entitled “complete CoSYPS Path Food workflow”. The complete workflow is detailed in Figure 19. In the following part (Chapter V), this workflow was validated for detection, isolation and confirmation of *Salmonella* spp. and *Listeria* spp. on beef carcass swab samples. The ISO 16140:2003-validation showed that the relative level of detection and the limit of detection of the complete CoSYPS Path Food workflow and ISO reference methods

were the same for both bacteria. The relative specificity, relative sensitivity and relative accuracy identified during this validation were all 100 % since the results obtained with the complete CoSYPS Path Food workflow and the ISO reference methods were identical (Cohen's kappa index = 1.00). In addition, it was shown that the complete CoSYPS Path Food workflow is able to provide detection results in half the time (two days) compared to the ISO reference methods (four to five days). These results demonstrate that the performance of the complete CoSYPS Path Food workflow is not only comparable to the ISO reference methods but also provides a faster response for verification of beef carcasses before commercial distribution.

During the development and validation of this complete CoSYPS Path Food workflow, several questions arose and are discussed below.

I. The enrichment: an unavoidable step ?

To protect the consumers from foodborne illnesses, the EU commission imposes food microbiological criteria (154), e.g. viable *Salmonella* absent in 25g of meat or on a swab (Table 6 and Table 7). Therefore, to be able to state that a food sample is conform to the legislation, the detection methods should be able to detect one CFU (viable cell) in a tested food portion. The viability of the bacteria is usually determined by their ability to multiply (220).

The enrichment step, which allows multiplication of the bacteria, is thus a critical step to ensure detection of viable bacteria. It is performed by using a non-selective or a selective enrichment broth allowing the growth of the target bacteria.

A. The dream of an universal enrichment broth

The complete CoSYPS Path Food workflow, developed in this work, is a multi-genera detection system since it allows the detection of *Salmonella* spp. and *Listeria* spp. simultaneously into a single sample. To be able to detect both genera simultaneously, an appropriate enrichment broth allowing the growth of both targets was used (Chapter V). Such enrichment media are available on the market (e.g. from BD, accumedica®, FLUKA or Himedia®) but are limited to the simultaneous optimal growth of *Listeria* spp. and *Salmonella* spp. To extend this workflow to other foodborne bacteria, a more "universal" enrichment broth should be used, such as the one developed by Köppel and coll. (214) for *Salmonella* spp., *L. monocytogenes* and Shiga-toxin producing *E. coli* (STEC). Yet, it was also shown that *Campylobacter* spp., a micro-aerophilic bacterium, could not grow in the same enrichment broth. This

illustrates the potential bottleneck to use a single sample for a multi-genera detection system.

B. Possible alternatives to enrichment

In a food sample, the target bacteria can be present at very low number or can be dead (non-infectious). An enrichment step is thus essential since it allows the viable cells to multiply, which results in an increased viable to dead bacteria ratio. The major drawback of an enrichment step is the time it requires. A possible way to reduce this detection time is the replacement of this enrichment by a shorter step. Several alternative approaches have been recently developed: filtration (221;222), buoyant density gradient centrifugation (BDC) (223-227), immunomagnetic separation (IMS) (215;228) and propidium monoazide (PMA) (229).

PMA is specifically used before PCR or qPCR-based detection method. Indeed, it is known that DNA of dead bacteria remains detectable into a sample for several days (110). On spiked samples, the occurrence of this false-positive signal due to dead cells varies according to the type of food matrix (110) and the type of death (109). However, to evaluate the importance of these false positive signals on real-life sample, it would be interesting to perform a survey on its occurrence when using qPCR in real foodstuffs.

Filtration, BDC and IMS can be used before PCR or qPCR but also before the culture-based isolation step. They intend to separate the target bacteria from the food product particles, the PCR inhibitors and the background microflora. They also concentrate the target bacteria to facilitate their detection and isolation. Filtration can be directly performed on highly aqueous sample such as water, milk or juice, but with more solid food matrices, a preliminary filtration with large size-pore filter, to remove large debris, that could clog the filter, is required (222). BDC is claimed to be able to separate dead from viable cells (223). BDC could be used to replace enrichment since it is faster and concentrates the viable cells, but it is too complex to be done for a routine analysis.

IMS have been shown to have a high detection limit, but a low specificity for their target (215;230), which can result in false-negative or false-positive results. Moreover, IMS as filtration are able to concentrate bacteria but do not discriminate viable from dead cells (221;231). On contrary, PMA potentially discriminates viable from dead cells but does not concentrate the target bacteria, and this concentration is

crucial when absence in the tested portion is requested by the EU regulation (154). To overcome this drawback, protocols combining filtration and PMA (232) or IMS and PMA (231;233) have been developed. However, as presented in Chapter IV, PMA presents two other important weaknesses. The first one is that PMA is not totally removing the signal of dead cells and can even reduce the signal of viable cells at low concentration. So even combined with IMS-PMA or filtration-PMA will not efficiently replace the enrichment step.

In the present work, some potential improvements of the PMA protocol (higher PMA concentration, longer incubation) have been tested but none led to the total extinction of the qPCR signal from dead cells. Other improvements such as the use of sarkosyl or sodium deoxycholate before PMA treatment were also evaluated in the literature (144;234-237) but even though the signal from dead cells was further reduced, it was never totally removed. The second additional weakness is that the dead cells signal reduction due to PMA varies according to the different killing processes applied to the cells (144-147). For example, PMA is inefficient on cells killed by U.V. as the cell wall/membrane is not altered by U.V. treatment (147).

All these observations on the limitations of alternative methods to the enrichment are in the favour of keeping this enrichment step. Indeed, the latter remains the simpler, safer and most effective method to perform the multiplication of viable cells and the discrimination between dead and viable bacteria. Li and Mustapha (238) demonstrated that a short enrichment, i.e. 8 h, is not enough to sufficiently increase the viable to dead cells ratio when a high concentration of dead cells is present into the sample. In the complete CoSYPS Path Food workflow, a 24 h enrichment step is performed, which resulted in a 100% detection of the target bacteria in artificially contaminated beef carcass swab samples.

II. How to obtain a reliable and informative qPCR-based detection system ?

In the recent years, many commercial and non-commercial qPCR-based detection systems have been developed for the detection of *L. monocytogenes* (60;117;203-208) and *Salmonella* spp. (191-202) in food samples. However, they present two major disadvantages: i) they only target a single genus at a time and ii) they target a single gene with a single assay. To circumvent these inconveniences, approaches targeting two genes for *Salmonella* spp. detection (210) or targeting several bacteria

at the same time (211-215) were developed. The CoSYPS Path Food detection system, tackled both obstacles at the same time as a multi-genera, multi-levels and multi-targets detection system.

The detection of *Salmonella* spp. and *Listeria* spp. by the CoSYPS Path Food detection system is reliable as i) it uses a multi-targets detection system and ii) each assay was validated for several criteria. Moreover the CoSYPS Path Food detection system provides additional information about the species and the subspecies detected.

A. Reliable ?

1) Multi-target

The multi-target detection is performed by at least two qPCR assays per level of detection. This multi-targets detection system is of particular interest if the strain present into the sample misses or is mutated for the target gene(s). Indeed, it has previously been shown that some strains can still be pathogenic whereas they miss specific sections of their genome, such as pathogenicity islands (PAIs) (209). As it was demonstrated in the Chapter III (section II), such strains would give false negative results with a single-target detection system but are detected by the multi-targets CoSYPS Path Food detection system (Table 20, p109).

2) Validation of each assay

Each qPCR assay used into the CoSYPS Path Food detection system have been validated for selectivity, dynamic range, PCR efficiency, sensitivity, repeatability and reproducibility (Chapter III, sections 1 and 2). This extensive validation of the qPCR assays guaranties that they will correctly work on a wide range of strains, on a high range of template concentration, with different operators and even in different laboratories using different qPCR platforms.

B. Informative ?

The discrimination of *Salmonella* spp. and *Listeria* spp. by the CoSYPS Path Food detection system is informative as it is performed using different levels (genus, species and subspecies). This provides additional information on the detected strains. An excellent illustration of the benefit of this multi-levels system was encountered in November 2012. An atypical *Salmonella* strain was sent to the Belgian National Reference Center (NRC) for *Salmonella*. This strain was isolated

from broilers (*Gallus gallus*) fresh meat by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) at processing plant during official control. This strain presented no agglutination with the specific sera of *Salmonella* spp. and non-typical biochemical characteristics (Lactose⁺, H₂S⁻). This led the NRC to conclude this strain as a non-*Salmonella* strain. However, this strain gave a positive signal with a commercial qPCR *Salmonella* detection kit. To clarify the situation and to confirm this result, the CoSYPS Path Food detection system was performed on the strain. The results using the eleven assays of the *Salmonella* qPCR detection method concluded this strain as *Salmonella* spp. positive [*rpoD*+; *invA*+], *Salmonella enterica* positive [both *iroB* assays +], *Salmonella enterica* subsp. *enterica* positive [*safC*-; STML0296+]. This pattern, i.e. all markers positive except *safC*, is a pattern encountered only with the serovars Senftenberg, Namur and Krefeld during the validation of the method (Table 16, p 94-95). The strain was further characterized by the *Rijksinstituut voor Volksgezondheid en Milieu* (RIVM), the Netherlands, which confirmed this strain as *S. enterica* subsp. *enterica* serovar Senftenberg. Such lactose positive, H₂S-negative *S. Senftenberg* and *S. Regent* strains were also reported from imported poultry in UK in 2012, and were defined as "not reliably identified on standard isolation media" (239).

This example shows that the CoSYPS Path Food detection system is also a tool able to detect atypical strains.

III. How to correctly control the CoSYPS workflow ?

As in every qPCR-based detection system, necessary controls need to be included. Because the qPCR is preceded by a DNA extraction, an extraction control should be included. For the qPCR-based foodborne detection system, two ISO norms detail the controls to include (176;181). These qPCR controls verify the efficiency of the PCR reaction (positive control), the non-contamination of the PCR mix (negative control) and the quality of the extracted DNA (extraction and inhibition control). The inhibition control is crucial in a foodborne qPCR detection system because of the presence of potential inhibitory substances (94). In 2004, Hoorfar *et al.* proposed the internal amplification control (IAC) to be mandatory in PCR diagnostics (240) and gave advice to design such IAC (93). By definition an IAC is performed in the same well as the detection assay, as a multiplex assay. In the case of the complete CoSYPS Path Food workflow, the use of an IAC is inappropriate since SYBR®Green technology is not adapted to multiplexing as SYBR®Green dye is not specific to one target but

dyes all amplified DNA. An external amplification control, i.e. performed on the same sample but in another well, is a better choice.

In the CoSYPS Path Food system, the inhibition control is coupled with the extraction control. This Extraction and Inhibition Control (EIC) evaluates both the efficiency of the extraction and the presence of PCR inhibitor into the tested sample. This EIC is the tested sample spiked with the target micro-organism. The EIC is extracted in parallel of the tested sample and the qPCR analysis is performed on the same plate, with the same PCR mix than the tested sample and with each of the tested qPCR assays. All EIC controls should give a positive signal to demonstrate that the extraction and the amplification were performed correctly. If the EIC gives the expected signal, no inhibition is foreseen in the sample, on the contrary, if the EIC is inhibited (lower signal than expected or no signal), the reaction with the tested sample is most probably inhibited as well. The only way to reduce inhibition is to dilute the DNA extract. It must be kept in mind that such dilution of the extract will dilute inhibitors as well as the detection target gene, increasing the risk to fall below the LOD of the method.

The choice of an appropriate kit to extract DNA from enrichment broth of food matrices is important in reducing the presence of inhibitors into the extracted DNA. Most commercial kits include a DNA extraction kit. For the complete CoSYPS Path Food workflow, the Nucleospin Food kit (Macherey-Nagel®) was selected as it is specialized for food extraction and is efficient in removing usual PCR inhibitors encountered in food matrices such as blood and fat.

IV. What are the advantages of the complete CoSYPS Path Food workflow ?

A. The qPCR-based detection system is fast

As extensively described in Chapter V, the complete CoSYPS Path Food workflow considerably reduces the time of analysis compared to the respective ISO methods for the detection of *Listeria* spp. and *Salmonella* spp. (38;68). Indeed, this workflow gives a negative or presumptive positive result within only two days (Figure 20). This is of primary importance in case of foodborne outbreak since the faster the source of the outbreak is identified (pathogen and food matrix involved), the faster and better the corrective actions can be taken to control the further spread of the outbreak. It is also interesting for the control of short shelf-life products.

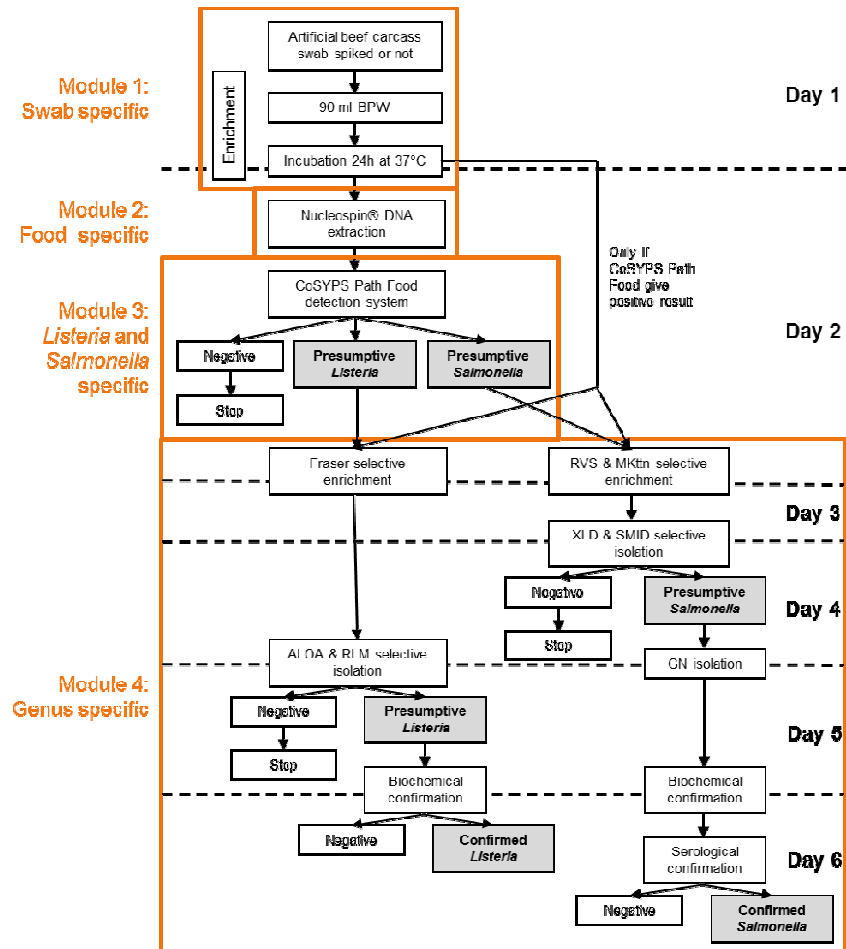


Figure 20: Complete CoSYPS Path Food workflow truncated into four different independent modules. Each of these modules can be changed for different application of the system.

Moreover, such a fast and simple foodborne qPCR detection system will promote industrial auto-controls, which will contribute to safer foodstuffs on the market.

B. It is also selective, sensitive, repeatable and reproducible

As described in Chapter III, sections 1 and 2, the SYBR®Green qPCR assays forming the CoSYPS Path Food detection system have been validated according to a newly design process based on guidelines of food microbiology PCR validation (ISO 22118 (180)) and guidelines of European Network GMO Laboratories for qPCR validation (186). This newly design validation process evaluates the selectivity, the sensitivity, the repeatability and the reproducibility of the design qPCR assays and evaluates if they are acceptable in comparison with validation criteria from the two previous guidelines. This newly developed food microbiology qPCR assays validation process is an important advance in food microbiology qPCR assay validation and gives an important advantage to the developed CoSYPS Path Food detection system.

C. The decision support system provides reproducible interpretation

The CoSYPS Path Food system Excel support tool, developed in the course of this work (Chap III, section 3), facilitates and accelerates the work of the operator, allows a fast, automatic and reproducible analysis and interpretation of the results of the CoSYPS Path Food system. Indeed, the decision support system providing an automatic analysis and interpretation of the qPCR results will give the same conclusion regardless the operator performing the analysis. This last point is an important advantage for analysis of samples under accreditation.

D. The complete CoSYPS Path Food workflow is modular

The complete CoSYPS Path Food workflow was developed to be used on swab samples. However, each step (module) can be used independently (Figure 20). For example, as illustrated previously, the CoSYPS Path Food detection system can be used directly on isolated strains, and can be particularly useful for the correct identification of strains with atypical biochemical patterns. It will indeed provide information about the genetic markers present into the strain and it will allow to conclude whether the strain is the expected target or not. This kind of discrimination analysis does not require the enrichment and the Nucleospin® food kit extraction as they are two modules of the complete CoSYPS Path Food workflow which are specific of food analysis. In the case of pure strain, its DNA can be extracted by a

simple boiling protocol (99) and used directly within the CoSYPS Path Food qPCR detection system.

Moreover, the CoSYPS Path Food qPCR detection step is adaptable to the sample requirements. In other words, the list of tested assays can be adapted to the purpose of analysis. For instance, if only *Salmonella* spp. needs to be tested, the seven assays for the *Salmonella* detection and discrimination system can be run alone. If general information is required about the presence of *Listeria* spp. and *Salmonella* spp., only genus-specific qPCR assays can be run.

The third aspect of the modularity of the complete CoSYPS Path Food system is that additional genera could be added (module 3, Figure 20). The only condition is that the new qPCR assays targeting these other pathogens should be developed to be run with the same PCR conditions than the already included qPCR assays (241;242). Moreover, the selective enrichment, isolation and confirmation steps (module 4, Figure 20) would have to be specific of each added foodborne pathogen, using the protocols provided by the respective ISO reference methods (when available).

E. The complete CoSYPS Path Food system is adapted to the labs needs

Finally, the complete CoSYPS Path Food workflow is a workflow easily implementable in the food microbiology laboratories where qPCR technology is already used for further strain characterization. Therefore, such laboratories already have qualified analysts for qPCR technology, which is absolutely not the case for LAMP, Luminex or NGS. Moreover, the SYBR®Green chemistry used into the CoSYPS Path Food detection system allows this detection system to be run on every qPCR apparatus and is relatively affordable compared to other qPCR technologies (SYBR®Green is around 40% less expensive than the TaqMan® chemistry). Thus this alternative method would be a good choice for all laboratories controlling food products such as National Reference Laboratories, hygiene-control or quality-control laboratories.

Chapter VII: Conclusions and perspectives

The detection of foodborne pathogens is crucial for public health since it ensures safe food products on the market. The traditional culture-based methods for detection and identification of foodborne pathogens are sensitive and reliable, but their major drawback is the time required to obtain results. In this project, a complete CoSYPS Path Food workflow allowing qPCR detection, culture-based identification and biochemical confirmation of *Listeria* and *Salmonella* spp. in beef carcass swab samples was developed and validated. This complete workflow is faster than the traditional methods and was shown to be a reliable and appropriate alternative to the ISO reference methods for control of beef carcasses before commercial distribution.

In this study, the complete CoSYPS Path Food workflow was validated on artificially contaminated swab samples. Every sample was analysed in parallel with the complete CoSYPS Path Food workflow and the ISO reference methods and gave the same results with both methods. Moreover, the complete CoSYPS Path Food workflow presents the advantage to give results for both targets within a single analysis whereas two different culture-based methods are required for the two targets. Although this validation is sufficient to use the complete CoSYPS Path food on “real-life samples”, for its full implementation in a laboratory, real-swab samples should be tested in parallel with the ISO reference methods. This would confirm its reliability and consequently, the current ISO methods could be totally replaced by the complete CoSYPS Path Food workflow.

To further validate the complete CoSYPS Path Food workflow, an inter-laboratory study should also be performed. This inter-laboratory study would demonstrate the transferability of the system between laboratories. It should be conducted according to the annex H of ISO 16140:2003 (188). Several inter-laboratory trials of qPCR-based detection systems have been reported (243-245). This inter-laboratory trial will be performed the same way than the validation was performed in this study (Chap V) but instead of involving only one laboratory, it should involve at least 10 collaborative laboratories (188). For commercialisation, the inter-laboratory study need to be carried out by recognised organisms such as AFNOR certification, NordVal or MicroVal.

In this study, the complete CoSYPS Path Food workflow was validated on beef swab samples. To extend its use to all food matrices, five food categories (e.g. meat products, dairy products) need to be validated with at least three different types of product (e.g. raw, heat processed, frozen) per category. Each type of products should include 20 samples and each food category at least 60 samples with ca. 50% of positive and 50% of negative samples (188). The different food categories and types of products that can be used into a validation protocol are details into the Annex B of ISO 16140: 2003 (188). This validation of the CoSYPS Path food on five food categories, to be able to use it on all food matrices, would thus include at least 300 samples.

Another perspective would be the miniaturization of foodborne pathogens detection systems (246-248). This would allow, for example, to include them into the package of the food product or to sell consumer ready-to-use foodborne pathogens tests. These types of devices are called “lab-on-a-chip biosensors” and some are already available. They consist of chemical variation measurement sensors, immuno-, or PCR-assays that are miniaturized on a chip. They can be potentially used in farms, packaging/processing facilities, delivery/distribution systems and at consumer level (247). However, several drawbacks remain to be solved, i.e., the pre-treatment of samples, the proper storage of reagents, the full integration into a battery powered system and the sensitivity (247).

The complete CoSYPS Path food workflow developed and validated in this study is a qPCR-based technology. qPCR is a powerful detection tool as it is used in many fields such as GMO detection (249), human, animal or plant viruses detection (250-252), fungus, parasite or cancer detection (253). However, recent molecular technologies such as loop-mediated isothermal amplification (LAMP) (254), Luminex and Next-Generation Sequencing (NGS) are now available and could be envisaged as potential alternatives.

In the LAMP technology, the monitoring of the DNA amplification can be done by visual inspection (turbidity or fluorescence). It is a very simple and rapid detection tool that can be performed on site. It does not require a thermal cycler, a heating block is enough. LAMP analysis can be performed, for the quickest, within 10 to 15 min (255). Some LAMP assays have been designed for foodborne pathogens detection in food samples (e.g. (256)). However, LAMP technology requires four to six specific primers spanning eight distinct sequences of a target gene (254;255).

This makes it a really specific detection system but considering the difficulty of designing two primers amplifying all species of a genus, the use of four to six primers is considerably increasing the difficulty of developing a genus specific assay. Since LAMP is to be performed on site, no enrichment is achieved and the problematic of dead bacteria detection and low level presence remains a major drawback for this technology.

The Luminex is a relatively new technology. It is a multiplex detection system. Different devices (Luminex® corp.) are available with different multiplexing capacity, from 50-plex (MagPix®) up to 500-plex (FlexMap® 3D). Luminex assays can target DNA, proteins, or enzymes. The major drawback of this technology is the very extensive work required to develop such high multiplex assays. Therefore, it is not appropriate in case of outbreak where a fast development is required. Immunological and nucleic acid-based Luminex assays have already been developed for the genetic characterization of strains (97-100) or for pathogens detection in human stool samples for clinical purposes (101-105). No Luminex assay have been designed so far to be used as a screening system on food sample and further studies are required to evaluate if Luminex could replace qPCR screening, especially for the sensitivity.

The NGS is used to get information on all the sequences present in a sample. No specific amplification is required. Using NGS on food sample, all DNA present into the sample is sequenced, e.g. beef, chicken, vegetables, bacteria or fungus. After the sequencing, an extensive processing of data by bioinformaticians is needed to identify all sequenced genes and organisms they originate from. Even if the price of NGS has seriously decreased over the last years, this technology remains very expensive and the food microbiology laboratories are not yet equipped or qualified for this kind of analysis, thus, this technology cannot be used yet in a routine detection system. Moreover, no NGS detection system for foodborne pathogens has been already developed. However, NGS could be a very useful technology in case of outbreak since it gives a “global view” of what is present in the sample without the need of screening for specific genes. It was effectively the case in 2011 during the O104:H4 *E. coli* German outbreak (107). Moreover, a previous step to NGS consisting in targeting especially specific sequences in the samples, such as the Target Locus Amplification (TLA) technology (257), could decrease the amount of data generated and ease the detection of foodborne pathogens. However, such detection systems are not yet developed; they are still at the theoretical status.

Thus, the qPCR-based detection, used into the complete CoSYPS Path Food workflow, developed in this study, is so far the faster and more appropriate alternative to culture-based methods to perform a quick and reliable screening of food samples. Moreover, in this workflow, the drawbacks of the available qPCR-based detection systems were taken into consideration and solved by the multi-levels and multi-targets detection system. Furthermore, the complete CoSYPS Path Food workflow is a global tool since all steps from food sample enrichment to the confirmation of the detected strain were developed. The replacement of the enrichment and discrimination of living and dead cells, in order to further reduce the time required to get results, was assessed. This study demonstrates that the alternative methods, although faster, display too important drawbacks to efficiently replace the enrichment step. The last part of this work was the successful validation of the complete CoSYPS Path Food workflow on beef carcass swab samples. This allows the developed workflow to be used as alternative method to traditional culture-based methods for fast screening of beef carcass swab samples for *Salmonella* spp. and *Listeria* spp. detection, isolation and confirmation.

To conclude, the complete CoSYPS Path Food workflow considerably reduces the time to get presumptive positive and negative results compared to traditional culture-based methods. This is especially convenient in case of the identification of outbreak causative agent or for short shelf-life products release. Indeed, with the complete CoSYPS Path Food workflow, the detection result is obtained after two days whereas four to five days are needed with conventional methods. Three days are then required in case of presumptive positive results to isolate and confirm the strain, if needed. Moreover, due to its multi-targets strategy, it is a highly reliable system. Indeed, a lower number of false negative are expected with atypical strains than when using a single target qPCR detection systems. The multi-levels system gives additional information about the detected strain (species, subspecies). This qPCR system, associated with the developed decision support system is easily implementable in routine analysis under accreditation as i) assays have been validated and published (241;242), ii) the decision support system allows easy and automated interpretation of the multiple qPCR signals (217) iii) this laboratory are already equipped and trained to use qPCR technology. In addition, the CoSYPS Path Food detection system is flexible as new targets can easily be developed and added into the system in a relatively short period of time. Moreover, for routine analysis, the tested targets list could be adapted to each sample regarding the request. This

complete CoSYPS Path Food workflow is therefore a good alternative to conventional culture-based methods for all laboratories controlling food products such as National Reference Laboratories, hygiene-control or quality-control laboratories of food business operators. Moreover, the complete CoSYPS Path Food workflow is fast and relatively simple, its use will promote industrial auto-controls that will contribute to a better microbiological control of the food chain, and as a consequence, will decrease the risks of foodborne bacteria for the public health.

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Appendix A: Scientific communications

Oral presentations

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2013, **SYBR@Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels**, 18th Conference on Food Microbiology, 12-13 September 2013, Brussels.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2012, **Development and validation of *Listeria* spp. and *Listeria monocytogenes* qPCR screening assays to be used in a combinatory system to detect foodborne pathogens**, 17th Conference on Food Microbiology, 20-21 September 2012, Brussels.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2012, **Development and validation of *Listeria* spp. and *Listeria monocytogenes* qPCR screening assays to be used in a combinatory system to detect foodborne pathogens**, Food Micro 2012, 3-6 September 2012, Istanbul.

Posters

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2014, **Validation of the complete CoSYPS Path Food workflow for qPCR detection, identification, isolation and confirmation of food-borne pathogenic bacteria present in carcass samples**, Rapid Methods Europe (RME) 2014, 31 March-02 April 2014, Leiden.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2013, **SYBR@Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels**, 18th Conference on Food Microbiology, 12-13 September 2013, Brussels.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2012, **Development and validation of *Listeria* spp. and *Listeria monocytogenes* qPCR screening assays to be used in a combinatory system to detect foodborne pathogens**, 17th Conference on Food Microbiology, 20-21 September 2012, Brussels

Barbau-Piednoir E., ., Mahillon J., Roosens N., Botteldoorn N., 2011, **Development of qPCR screening assays to be used in a combinatory system to detect foodborne pathogens**, Scientific Meeting of the Young researchers of the “Réseau des Instituts Pasteur”, 10 November 2011, Paris.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2011, **Development of qPCR screening methods to be used in a combinatory system to detect food-borne pathogens**, 16th Conference on Food Microbiology, 22-23 September 2011, Brussels.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2011, **A combinatory qPCR screening system to detect food-borne pathogens**, Sfam Summer conference, 5-7 July 2011, Dublin.

Barbau-Piednoir E., Mahillon J., Roosens N., Botteldoorn N., 2010, **A combinatory qPCR screening system to detect food-borne pathogens**, 15th Conference on Food Microbiology, 16-17 September 2010, Ghent.

Appendix B : supplementary data

Supplementary data of Chapter III, Section 2:

Table S1: Limit of detection determination for all *Salmonella* SYBR®Green qPCR methods.

		Mean Ct value $\pm$ SD (% positive)									
		<i>Salmonella</i> spp. methods			<i>Salmonella enterica</i> methods			<i>Salmonella enterica</i> criteria			
Theoretical copy number/ assay		<i>rpoD</i> -20	<i>mva</i> -5	<i>mva</i> -92	<i>mva</i> -2	<i>saftC</i> -10	<i>saftC</i> -13	<i>saftC</i> -13	<i>saftC</i> -13	STM 0296	
Serial dilution points	10	32,54 $\pm$ 0,59 (100)	32,34 $\pm$ 1,18 (100)	31,90 $\pm$ 0,59 (100)	32,95 $\pm$ 0,87 (100)	32,42 $\pm$ 1,30 (100)	31,51 $\pm$ 0,75 (100)	31,51 $\pm$ 0,75 (100)	30,76 $\pm$ 1,10 (100)		
	5	33,50 $\pm$ 0,72 (97,2)	33,08 $\pm$ 1,15 (100)	32,96 $\pm$ 0,87 (100)	33,72 $\pm$ 0,89 (100)	33,00 $\pm$ 0,93 (94,4)	32,71 $\pm$ 0,88 (97,2)	32,71 $\pm$ 0,88 (97,2)	31,66 $\pm$ 1,01 (100)		
	2	35,00 $\pm$ 1,14 (88,9)	33,64 $\pm$ 0,84 (88,9)	33,85 $\pm$ 0,81 (77,8)	34,81 $\pm$ 1,02 (72,2)	33,95 $\pm$ 1,01 (83,3)	33,62 $\pm$ 0,74 (83,3)	33,62 $\pm$ 0,74 (83,3)	32,98 $\pm$ 1,35 (100)		
	1	35,56 $\pm$ 0,88 (86,7)	34,60 $\pm$ 1,16 (63,9)	34,64 $\pm$ 1,14 (63,9)	34,85 $\pm$ 1,05 (38,9)	34,57 $\pm$ 0,85 (69,4)	34,04 $\pm$ 0,72 (50)	34,04 $\pm$ 0,72 (50)	33,68 $\pm$ 1,27 (91,7)		
	0.5	35,95 $\pm$ 0,85 (44,4)	35,11 $\pm$ 0,96 (41,7)	35,01 $\pm$ 0,83 (63,9)	35,50 $\pm$ 0,57 (36,1)	34,97 $\pm$ 0,92 (22,2)	33,94 $\pm$ 0,69 (25)	33,94 $\pm$ 0,69 (25)	34,25 $\pm$ 0,87 (63,9)		
Negative controls	0.2	36,48 $\pm$ 0,93 (22,2)	35,07 $\pm$ 0,90 (3,9)	34,65 $\pm$ 1,15 (25)	35,53 $\pm$ 0,56 (0,4)	34,87 $\pm$ 0,44 (8,33)	35,29 $\pm$ 2,07 (0,4)	35,29 $\pm$ 2,07 (0,4)	34,71 $\pm$ 0,62 (41,7)		
	0.1	36,57 $\pm$ 1,17 (6,7)	35,31 $\pm$ 0,90 (11,1)	34,71 $\pm$ 0,89 (25)	36,10 $\pm$ 0,69 (11,1)	34,76 $\pm$ 0,86 (25)	35,58 $\pm$ 0,26 (5,56)	35,58 $\pm$ 0,26 (5,56)	34,95 $\pm$ 0,65 (25)		
	Dilution buffer control	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD		
	Water control	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD		
		Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD		

Results based on 36 repetitions with genomic targets as DNA template; Average, standard deviation and percentage of positive signal of Ct and Tm-values obtained at each dilution point; LOD : Limit of detection

Table S2: continued

Sample	Copy number	Agona	Derbi	Sentent.	Sentent.	Sentent.	Frederiksberg	Sentent.	London	Marseille	Muenchen	Sentent.	Paratyp C	Sentent.	Poona	Typhi	Sentent.	Method	Reproducibility	Uncertainty
iroB -f2	Cq	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07
		Average Lab 1	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07	29.07
		Average Lab 2	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36	30.36
		RSD _R (%)	3.29	2.57	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43
	Tm	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4
		Average Lab 1	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4	27.4
		Average Lab 2	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00
		RSD _R (%)	0.43	0.43	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78
sarC -f0	Cq	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93
		Average Lab 1	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93	27.93
		Average Lab 2	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40	29.40
		RSD _R (%)	3.62	4.00	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38
	Tm	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
		Average Lab 1	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
		Average Lab 2	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50	75.50
		RSD _R (%)	0.00	0.47	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
sarC -f3	Cq	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37
		Average Lab 1	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37	27.37
		Average Lab 2	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53	29.53
		RSD _R (%)	5.37	5.12	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11	3.11
	Tm	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65
		Average Lab 1	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65	4.65
		Average Lab 2	77.25	77.25	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40	77.40
		RSD _R (%)	0.68	0.32	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82
STM 0296	Cq	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17
		Average Lab 1	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17	28.17
		Average Lab 2	28.21	28.21	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66	28.66
		RSD _R (%)	0.10	0.195	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95
	Tm	50	29	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
		0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
		Average Lab 1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
		Average Lab 2	77.45	77.45	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25	75.25
		RSD _R (%)	0.14	0.05	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14

Appendix C : *Salmonella* Namur full genome sequencing

During the development of the qPCR assays for the detection and discrimination of *Salmonella* spp. (Chapt III; section 2), the serovar Namur have been identified as lacking the *saftC* gene identified as *S. enterica enterica* marker. To check this lack and further investigate it, the full genome sequencing of the serovar Namur was performed.

This work has been published in Genome Announcement⁸ and the sequence is publicly available in NCBI database under accession number [AWGG000000000](#).

⁸ Barbau-Piednoir E, Bertrand S, Roosens NH, De Keersmaecker SCJ. 2014. **Genome sequence of the *Salmonella enterica* subsp. *enterica* serovar Namur strain 05-2929, lacking the *Salmonella* atypical fimbrial operon.** Genome Announc. 2(2):e00299-14. doi: [10.1128/genomeA.00299-14](#)

Genome Announc. (2014) 2(2):e00299-14. doi:10.1128/genomeA.00299-14.



Genome sequence of the *Salmonella enterica* subsp. *enterica* serovar Namur strain 05-2929, lacking the *Salmonella* atypical fimbrial operon

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Abstract

This paper announces the genome sequence and annotation of *Salmonella enterica* subsp. *enterica* serovar Namur strain 05- 2929. *S. Namur* is a new serovar (39:z4,z23:-) that was isolated from a patient with salmonellosis in 2005 in Namur, Belgium, and has been identified as lacking the *Salmonella* atypical fimbrial (*saf*) operon.

Salmonella is one of the major causes of food-borne zoonoses. Between 90,000 and 135,000 cases per year of human salmonellosis were reported in Europe in the period 2008 to 2012 (1). The genus *Salmonella* includes 2 species, *i.e.*, *S. enterica* and *S. bongori*, which are both pathogenic for humans (2). *S. enterica* is further divided into 6 subspecies and 2,587 serovars, which are often named according to the place of first isolation. The serovars are unequally distributed, since 1,547 belong to the subspecies *S. enterica* subsp. *enterica* (subsp. I) (2), which is most often found in human and food samples (1). In 2005, a *Salmonella* strain (05-2929) was isolated at the Centre Hospitalier Régional de Namur, Belgium, from a stool sample of a patient with salmonellosis. The serotyping of this strain was performed at the Belgian National Reference Center for *Salmonella* and *Shigella*. It was characterized as a *Salmonella enterica* subsp. *enterica* (subsp. I) serovar, presenting a new antigenic formula, *i.e.*, 39:z4,z23:-. This was confirmed by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute, Paris, France. This new serovar was added to the White-Kauffmann-Le Minor scheme in 2010 as the *S. Namur* serovar (2).

During the selectivity test of the validation of SYBR®green real-time PCR (qPCR) assays for detection in food samples of *Salmonella* spp. based on genus-, species-, and subspecies-specific marker genes, genomic DNA isolated from this *S. Namur* serovar was identified as not amplified by two qPCR assays targeting *safC* (3). *safC* belongs to the *Salmonella* atypical fimbrial (*saf*) operon (4) and was reported to be specific for *S. enterica* subsp. *enterica* (5).

To further characterize this *S. Namur* 05-2929 strain, wholegenome sequencing was performed on an Illumina HiSeq 2000 run using a paired-end and a 4-kbp insert mate-pair library (performed by BaseClear B.V., Leiden, the Netherlands). Sequencing yielded 4,901,007 paired-end and 11,099,754 mate-pair reads (50

cycles, 439-fold coverage), which were assembled *de novo* using the “*de novo* assembly” option of the CLC Genomics Workbench version 5.1 (CLC bio). The assembly generated 21 scaffolds consisting of 95 contigs, with a maximum scaffold size of 3,131,320 bp and a minimum size of 323 bp. The total sequence length is 4,842,244 bp and has a G_C content of 51.96%.

Genome annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline (2013) and predicted 4,616 genes, including 4,501 protein-coding genes. This annotation confirmed the deletion of the entire *saI* operon in this *S. Namur* strain. Strains of other subspecies I serovars lacking *saI* have been previously reported (6). Moreover, based on the presence and absence of clade-specific genes and previously identified single nucleotide polymorphisms (7), this *S. Namur* strain can be further classified as an *S. enterica* subsp. *enterica* clade A strain.

It would be interesting to further investigate these deletion and diversification events using the newly available *S. Namur* sequence and to extend this analysis to other strains of the *S. Namur* serovar.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. [AWGG00000000](#). The version described in this paper is version AWGG01000000.

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