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PhD Thesis

Fast, Affordable and multiplexed foodborne pathogen detection on miniaturized devices

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Doctoral Programme in Innovation in Food Safety and Technology

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TESE DE DOUTORAMENTO

FAST, AFFORDABLE AND MULTIPLEXED FOODBORNE PATHOGEN DETECTION ON MINIATURIZED DEVICES

Sarah Gaspar Ferreira Azinheiro

ESCOLA DE DOUTORAMENTO INTERNACIONAL DA UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

PROGRAMA DE DOUTORAMENTO EN INNOVACIÓN EN SEGURIDADE E TECNOLOXÍA ALIMENTARIAS

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Dª. Marta Prado Rodríguez

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RESUMO

As intoxicacións alimentarias son un problema de saúde pública mundial que afecta non só aos países en desenvolvemento, senón tamén aos países desenvolvidos [1]. A Organización Mundial da Saúde (OMS) destacou que 1 de cada 10 persoas en todo o mundo enfermará debido ao consumo de alimentos contaminados [2]. Diferentes axentes patóxenos son os responsables destas enfermidades de transmisión alimentaria que causan hospitalización e morte, entre eles *Salmonella* spp., *E. coli* produtora de toxina Shiga (STEC) e *L. monocytogenes* son moi problemáticos para a industria alimentaria. Os dous primeiros, presentan o maior número de casos de hospitalización informados polas autoridades europeas, mentres que *L. monocytogenes* segue mostrando unha maior gravidade e taxa de mortalidade de todos os patóxenos de transmisión alimentaria monitorizados [3]. A pesar do esforzo por mellorar os estándares internacionais de seguridade alimentaria, seguen xurdindo novos riscos na cadea de subministración de alimentos [4] e as dificultades para rastrexar as fontes dos brotes aumentan o risco de que haxa máis casos de infección. Existe unha necesidade urxente de métodos máis sensibles e rápidos para detectar microorganismos patóxenos nos produtos alimenticios, para evitar posibles enfermidades e mortes. Os métodos tradicionais para detectar patóxenos transmitidos por alimentos baséanse en cultivos, que son laboriosos, levan moito tempo e requiren persoal de laboratorio capacitado [5]. Estas metodoloxías amplían a análise ata cinco días, sendo non sostibles para produtos de curta vida útil e non encaixando na intensa produción actual. Ademais, a maior demanda de metodoloxías que permitan detectar máis dun patóxeno ao mesmo tempo non se pode alcanzar coas técnicas tradicionais. Nos últimos anos xurdiron novos métodos baseados no ADN ou na análise de proteínas co obxectivo de superar algúns destes inconvenientes [6].

Así, dispositivos como os micro sistemas de análise total (μTAS) representan unha verdadeira vantaxe, aumentando a velocidade de análise e a sensibilidade, diminuíndo ademais o consumo de reactivos, o risco de contaminación cruzada das mostras e abre a posibilidade dunha automatización total [7,8]. Probouse a integración de técnicas de amplificación de ADN en dispositivos miniaturizados, non só para a detección de patóxenos, senón tamén para a identificación de trastornos xenéticos e enfermidades infecciosas [9].

A amplificación enzimática en dispositivos microfluídicos realízase principalmente mediante PCR, o que require un control de temperatura e un aumento rápido da temperatura, aumentando a complexidade da implementación e aumentando o custo do instrumento. Nos últimos anos xurdiron novas técnicas alternativas de amplificación de ADN co obxectivo de ofrecer solucións analíticas a algúns dos inconvenientes do método de referencia para a amplificación in vitro, entre eles as técnicas de amplificación isotérmica de ADN son especialmente interesantes para fins de miniaturización. Debido á sinxeleza do control de temperatura para a amplificación de secuencias, a amplificación isotérmica pódese implementar facilmente en microchips simples sen complicados controles térmicos e/ou fluídos [10].

O obxectivo desta tese foi desenvolver unha metodoloxía mellorada para a detección múltiple de varios patóxenos transmitidos por alimentos baseada na detección de ADN, e a súa integración nun dispositivo miniaturizado. Para acadar este obxectivo abordáronse os diferentes pasos da análise, incluíndo o pretratamento da mostra, a amplificación do ADN e a visualización dos resultados, nos que se avaliaron varios enfoques para a escolla da mellor opción para reducir o tempo de análise, reducir o custo e permitir a detección a simple vista.

Para acadar a sensibilidade requirida da análise para os patóxenos transmitidos por alimentos, é esencial o enriquecemento da mostra. Neste momento, as metodoloxías máis rápidas aínda precisan de 18-48 h de tempo de enriquecemento, sen melloras significativas, sendo este punto o principal pescozo de botella na análise microbiolóxica de alimentos á hora de reducir o tempo de análise. É fundamental mellorar o xeito no que se trata a mostra, e por iso neste proxecto realizouse a optimización do pretratamento da mostra para permitir unha análise máis rápida. Avaliáronse diferentes enfoques para unha recuperación simultánea eficiente de bacterias patóxenas, incluíndo a optimización do medio no enriquecemento estándar, a concentración das bacterias e as estratexias de redución de tempo.

Probouse a influencia de diferentes medios selectivos ou non selectivos, no crecemento das bacterias diana, así como a suplementación con varios compostos. Valorouse o mellor medio para a detección simultánea de *Salmonella* spp., *E. coli* O157 e *L. monocytogenes*. Debido a que *L. monocytogenes* presenta unha menor taxa de crecemento, en comparación coas outras dúas bacterias, a optimización do medio centrouse na mellora da concentración deste patóxeno. O medio xeral, Tryptic Soy Broth (TSB), mostrou ser o medio que permitiu a maior redución da fase de latencia, o que reduciu o tempo de análise coa maior concentración bacteriana obtida. Ademais, probáronse varios suplementos e avaliouse o seu rendemento para mellorar o paso de enriquecemento, incluíndo celobiosa, extracto de levadura, piruvato de sodio, sangue de cabalo hemolisado (LHB), suplemento de crecemento de Campylobacter e suplemento selectivo do Fraser a media concentración. Entre estas, a celobiosa foi a única que permitiu un aumento da concentración final de *L. monocytogenes* despois de 24 h, sen mostrar cambios na concentración das outras dúas dianas. Non obstante, non se viu ningunha mellora na fase de latencia con este composto.

Para aumentar a sensibilidade da metodoloxía, avaliáronse diferentes enfoques para concentrar as bacterias no pretratamento da mostra. A primeira metodoloxía consistiu nunha Separación Inmunomagnética (IMS), onde se avaliaron catro anticorpos comerciais diferentes, específicos de L. *monocytogenes*, comparando a súa pureza e especificidade. O enfoque seleccionado consistiu na funcionalización de nanosferas magnéticas e na súa utilización para analizar mostras de alimentos. O enfoque IMS permitiu un LoD de 9,7 ufc/ 25 g, cando se combina cun enriquecemento selectivo en Half Fraser (HF) durante 24 h e análise de qPCR. A segunda alternativa implicou o uso dun dispositivo microfluídico onde se funcionalizou unha esponxa de polidimetilsiloxano (PDMS) 3D con ligandos específicos para reter as bacterias. Utilizouse un ligando bacteriano inespecífico, a proteína ApoH, para a captura múltiple e un anticorpo anti- *L. monocytogenes* específico (seleccionado anteriormente no enfoque IMS), para unha captura dirixida. Ambos enfoques avaliáronse con cultivos bacterianos puros, e tamén para analizar superficies de aceiro inoxidable contaminado. O ligando inespecífico, a proteína ApoH, permitiu acadar unha eficiencia de captura lixeiramente maior, e a captura múltiple de bacterias dianas, sendo unha vantaxe en comparación cos ligandos específicos, que requirían un anticorpo para cada patóxeno. Non obstante, os ligandos universais non se poden usar en matrices complexas cun alto número de microorganismos de fondo, xa que uniranse aleatoriamente a outros microorganismos. Cando se analizaron as mostras de superficie combinando a esponxa PDMS coa análise de qPCR sen enriquecemento previo, o LoD só está limitado pola propia técnica de amplificación do ADN ou polo proceso de mostraxe. Ambas opcións, IMS e a esponxa funcionalizada mostraron un alto rendemento e permiten unha detección

fiable para diferentes aplicacións. Unha vantaxe da captura das células bacterianas, é a súa separación do resto da solución de enriquecemento, o que permite eliminar posibles compostos inhibidores da reacción de amplificación do ADN. Non obstante, o uso destes ligandos específicos aumenta o custo da análise, xa que os diferentes ligandos, anticorpos ou outros seguen sendo caros.

Co obxectivo de reducir o tempo de análise, probáronse tres alternativas: Ensaio de amplificación de fagos (PAA), lise da matriz e enriquecemento curto. O PAA consiste nunha estratexia indirecta para detectar un microorganismo mediante a detección dun bacteriófago específico que infectará a bacteria diana. Coa replicación máis rápida do fago, obtivemos unha maior concentración do microorganism diana en menos tempo en comparación coa detección directa. Este enfoque aplicouse para a detección de *S.* Enteritidis en mostras de alimentos, permitindo realizar a análise por qPCR en só 10 h coa mesma sensibilidade que as metodoloxías convencionais, acadando unha LoD de 8 ufc/ 25 g. O enfoque de lise da matriz depende dun xeito completamente diferente de tratar a mostra. En lugar de tomar só unha pequena alícuota da mostra enriquecida, como nas análises tradicionais, a mostra enteira é tratada para degradar o máximo posible a matriz e recuperar as bacterias para posterior extracción de ADN. Con certa similitude, o enriquecemento curto consiste nun reducido tempo de incubación en medio de cultivo, e recuperación de todo o líquido da mostra a procesar, para permitir a separación da gran maioría dos microorganismos da mostra do alimento. Ambas metodoloxías foron avaliadas para a recuperación e detección de *L. monoctogenes* en simplex mostrando unha redución do tempo de análise a 5 h e 7 h, con lise da matriz e enriquecemento curto, respectivamente. A lise da matriz presenta un LoD excesivamente alto ($1{,}1 \times 10^5$ ufc/ 25 g) polo que non é apto para a industria alimentaria, sendo descartado como posible opción para a metodoloxía final. O enriquecemento curto mostrou unha sensibilidade moito maior, cunha LoD de 8,6 ufc/ 25 g, debido ao paso de cultivo realizado antes do tratamento da mostra, polo que o uso desta metodoloxía para a detección de *E. coli* O157 en formato simple, e a simultánea detección de *E. coli* O157 e *Salmonella* spp. logrouse con resultados similares (LoD = 3-4 ufc/ 25 g), demostrando a posibilidade de ser utilizado na detección múltiple. Despois de avaliar as diferentes alternativas para o tratamento da mostra, a metodoloxía mostrou os resultados máis prometedores para permitir unha detección múltiple máis rápida e económica de *L. monocytogenes*, *E. coli* O157 e *Salmonella* spp. probouse como o enriquecemento curto.

Como parte da estratexia avaliáronse diferentes alternativas de amplificación e detección de ADN. As técnicas de amplificación isotérmica demostraron varias vantaxes sobre a PCR/qPCR, como ser realizadas a temperatura constante sen necesidade de equipos complexos, como termocicladores. Esta característica foi interesante para o desenvolvemento do proxecto xa que simplifica a análise, reduce o custo e permite unha integración máis sinxela deste paso nun sistema miniaturizado, permitindo ademais un menor consumo de enerxía. Ademais, pódense empregar facilmente diferentes alternativas para a detección a simple vista cando se combinan cunha técnica de amplificación isotérmica. Por este motivo, avaliáronse dúas técnicas de amplificación isotérmica, LAMP e RPA, para detectar os patóxenos diana en formato simple ou múltiple, é obter un resultado visual sinxelo. O primeiro paso foi comprender o rendemento da análise tradicional de qPCR, utilizando un colorante intercalante (SYBR-qPCR) e unha sonda de hidrólise (Probe-qPCR), e comparalos con LAMP e RPA. A súa especificidade, sensibilidade e precisión con diferentes dianas xenéticas avaliáronse con cultivos puros e mostras de alimentos contaminados. O SYBR-qPCR foi un enfoque máis económico xa que non precisa de sondas específicas, non obstante o deseño do ensaio é máis complexo, así como a análise dos resultados cando se require a detección múltiple. Por outra banda, a Probe-qPCR ofreceu unha maior especificidade debido á implementación da sonda. Ambas metodoloxías permitiron realizar detección múltiple con sensibilidades similares. En canto á avaliación da amplificación isotérmica, tanto LAMP como RPA, deron como resultado unha sensibilidade inferior á dos enfoques qPCR, cando se examinou a sensibilidade analítica mediante cultivo puro. Non obstante, cando se

analizaron mostras contaminados combinadas cun enriquecemento en dous pasos, en caldo mTA10 e Full Fraser (FF), conseguiuse un LoD similar (1,4 ufc/ 25 g). Tamén se observou un rendemento lixeiramente superior de RPA, que proporciona resultados máis rápidos en comparación con LAMP. Probáronse tamén diferentes alternativas para a visualización dos resultados dun xeito máis sinxelo para cada técnica de amplificación isotérmica. Para RPA, avaliouse a carga da reacción de amplificación nunha banda de fluxo lateral (LF) e logrouse unha alta sensibilidade e especificidade con este método para a detección de *L. monocytogenes* en mostras de superficie, mostrando un LoD de 18,2 ufc/ cm^2 despois dun enriquecemento de 24 h en o caldo, ONE broth. A desvantaxe do RPA-LF foi o aumento do custo da análise debido á necesidade do uso de cebadores modificados e unha sonda, xunto coas tiras LF. Como alternativa, pódense engadir diferentes compostos á reacción, antes ou despois da amplificación, para conseguir un resultado detectable a simple vista en solución. Neste sentido, avaliouse a adición de SYBR Green, un colorante intercalante de dsDNA, despois da reacción RPA, para permitir a visualización da fluorescencia en mostras positivas cando se expoñan a luz UV.

A detección de *E. coli* O157 en mostras de alimentos foi posible e conseguimos unha LoD_{95} de 19 ufc/ 25 g cando se combina cun pretratamento baseado nun enriquecemento curto. Ademais deste enfoque que presenta un resultado prometedor, o feito de que SYBR Green se una a calquera dsADN presente na mostra, fai que a análise de mostras complexas e a detección múltiplex sexan limitadas, debido á presenza do ruido de fluorescencia de fondo obtido en mostras negativas, o que dificulta para identificar resultados positivos. Para a visualización dos resultados da amplificación LAMP probáronse tres enfoques, incluíndo a turbidez e o cambio de cor mediante o uso de nanopartículas de ouro (AuNPs) ou cunha mastermix colorimétrica comercial. A turbidez na reacción LAMP conséguese pola produción do subproduto insoluble, pirofosfato de magnesio, cando se produce a amplificación. Este enfoque utilizouse para detectar diferentes serovares de *Salmonella* spp., dirixíndose a catro dianas xenéticas, o que provocou diferenzas no rendemento da análise, cando se probaron mostras de alimentos despois un enriquecemento de 24 horas en mTA10. A turbidez pódese controlar en tempo real mediante un turbidímetro ou como detección de punto final mediante a visualización a simple vista dos resultados. Non obstante, a observación a simple vista debe realizarse nun ángulo de luz específico, o que fai que a análise sexa máis subxectiva e complexa. Os AuNPs foron probados para diferentes aplicacións e grazas ás posibilidades de modificar as súas propiedades ópticas en función do estado de agregación, pódese conseguir a detección a simple vista. A funcionalización do AuNP con ácido 11-mercaptoundecanoico (MUA) permite controlar a agregación das nanopartículas cando se orixina o produto de amplificación, provocando diferenzas de cores. Neste sentido, as mostras positivas serán vermellas, mentres que as negativas mostrarán unha cor morada. Este enfoque probouse para a detección de *Salmonella* spp. despois dun enriquecemento de 24 h en BPW. O paso de amplificación realizouse nun dispositivo microfluídico e o produto da amplificación mesturouse co MUA-AUNP, producindo un claro cambio de cor. Outra alternativa para orixinar un cambio de cor na reacción de amplificación é a adición dun colorante sensible ao pH, para detectar a amplificación LAMP. Este enfoque probouse para a detección de *Salmonella* spp. para comprender as posibilidades na metodoloxía final, neste caso o uso dos cebadores Loop, e o tempo de amplificación houbo que optimizar para evitar falsos positivos e obter resultados fiables. Probáronse mostras enriquecidas durante 24 h, presentando un LoD_{95} de 2,1 ufc/ 25 g, ademais avaliouse a combinación cun enriquecemento curto de 6 h mostrando unha total concordancia coa metodoloxía qPCR.

Despois dunha análise en profundidade das diferentes opcións avaliadas para realizar a amplificación do ADN e para permitir un enfoque simplificado para a visualización a simple vista dos resultados, a metodoloxía que permite a detección máis sinxela, rendible e sensible parece ser a LAMP colorimétrica. A desvantaxe do uso da metodoloxía MUA-AuNP relacionouse coa

manipulación do produto de amplificación despois da reacción, que pode provocar contaminacións cruzadas e falsos positivos. Co mastermix colorimétrico de NEB superouse este inconveniente, proporcionando unha solución vantaxosa. Por este motivo, a metodoloxía final deseñouse para combinar a metodoloxía de enriquecemento curto para o crecemento dos tres patóxenos, coa LAMP colorimétrica empregando a mastermix comercial.

Para obter unha detección múltiple de *Salmonella* spp. *E. coli* O157 e *L. monocytogenes*, o enriquecemento curto foi optimizado para permitir o crecemento de todos eles nun único paso de enriquecemento permitindo resultados moi sensibles. Despois de probar medios xerais e selectivos para este fin, o enriquecemento en TSB durante 7 h foi o que deu mellores resultados para a detección de *L. monocytogenes*, xa que este foi o patóxeno máis problemático pola súa menor taxa de crecemento. Avaliouse a metodoloxía completa analizando diferentes tipos de mostras de leite (UHT, Fresco e cru), acadando un LoD aceptable, cunha detección máis sensible de *Salmonella* spp. e *E. coli* O157 cunha LoD_{95} de 1,6 ufc/ 25 mL. Para *L. monocytogenes* foi necesaria unha maior concentración de bacterias para permitir unha detección fiable, obtendo unha LoD_{95} de 79 ufc/ 25 mL. Observouse unha clara influencia da microflora natural para a detección de *L. monocytogenes*, cun aumento asociado da LoD con maiores recontos mesófilos. A caracterización mesófila tamén se realizou mediante a análise de secuenciación MinION onde se identificaron principalmente bacterias acidolácticas.

Despois da avaliación completa do método, probouse a integración do paso de amplificación en dispositivos miniaturizados e comparouse cos resultados anteriores. Comparouse primeiro o rendemento de dous sistemas distintos analizando cultivos puros, para determinar a mellor opción a implementar na metodoloxía final. Un dos dispositivos consistía nun dispositivo integrado que combinaba o control de temperatura e un soporte flexible que permitía o uso de tubos de silicona con diferentes volumes. A outra alternativa consistía nun dispositivo microfluídico con 8 microcanles que se podía colocar nunha incubadora de laboratorio convencional para realizar a reacción. Conseguiuse unha clara diferenciación entre resultados positivos e negativos, cando se integrou a LAMP colorimétrica en ambos os dispositivos, non obstante observouse unha diminución da sensibilidade analítica. O dispositivo de microcanles mostrou os peores resultados, cunha escasa sensibilidade analítica, sendo mesmo imposible detectar *L. monocytogenes* nas concentracións máis altas ensaiadas. Por este motivo, este dispositivo foi descartado para a seguinte análise. Seleccionouse, e avaliouse, o sistema de quentamento integrado para a análise de mostras de leite. A metodoloxía final obtivo un LoD maior en comparación co método termociclador, como se esperaba polos resultados da sensibilidade analítica. Obtívose unha LoD_{95} de 15, 17 e 141 cfu/ 25 mL para *Salmonella* spp. *E. coli* O157 e *L. monocytogenes*, respectivamente. Coas mostras analizadas conseguiuse unha total concordancia coa metodoloxía qPCR.

En conclusión, a metodoloxía desenvolvida neste proxecto permitiu a detección múltiple de *Salmonella* spp. *E. coli* O157 e *L. monocytogenes*, reducindo o tempo de análise a só 9 h fronte a 7 días cando se realiza mediante técnicas tradicionais baseadas en cultivos e proporcionando detección a simple vista. Obtívose unha sensibilidade e especificidade similares cando se comparou co método de referencia, qPCR, sen necesidade dunha instrumentación complexa como un termociclador en tempo real. O uso do enriquecemento curto como pretratamento da mostra permitiu conseguir unha importante redución do tempo de resposta, sen aumentar o custo, o que fai a análise máis accesible para todos. Ademais, a posibilidade de integrar o paso de amplificación nun dispositivo miniaturizado abre a porta ao desenvolvemento de sistemas Point-of-Care (POC), de tamaño reducido e coa posibilidade de automatizar a análise a realizar en configuracións descentralizadas.

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ABSTRACT

Food poisoning is a global public health concern affecting, not only developing, but also developed countries [1]. The World Health Organization (WHO) highlighted that 1 in 10 persons around the world will become ill due to the consumption of contaminated foods [2]. Different pathogens are responsible for these foodborne diseases causing hospitalization and death, among them *Salmonella* spp., Shiga Toxin-producing *E. coli* (STEC) and *L. monocytogenes* are highly problematic for the food industry. The first two, present the highest number of hospitalization cases reported by the European authorities, while *L. monocytogenes* continues to show higher severity and mortality rate from all foodborne pathogens monitored [3]. Despite the effort to improve international food safety standards, new risks in the food supply chain continue to emerge [4], and the difficulties to track outbreak sources, increases the risk for more infection cases. An urgent need exists for more sensitive and faster methods to detect pathogenic microorganism in food products, to avoid potential illness and deaths. Traditional methods to detect foodborne pathogens are culture-based which are laborious, time-consuming and require trained laboratory personnel [5]. These methodologies extend the analysis up to five days, being not sustainable for short shelf-life products and not fitting in the intense production ongoing nowadays. Furthermore, the increased demand for methodologies that can detect more than one pathogen at the same time cannot be reached by the traditional techniques. Novel methods based on DNA, or in protein analysis, have emerged in the last years with the objective to overcome some of these drawbacks [6]. Thus, devices like micro Total Analysis Systems (μTAS) represent a real advantage, increasing the speed of analysis and the sensitivity, furthermore decreasing the reagent consumption, the risk of sample cross-contamination, and opens up the possibility of full automation [7,8]. The integration of DNA amplification techniques in miniaturized devices, not only for the detection of pathogens, but also for the identification of genetic disorders and infectious diseases has been tested [9]. The enzymatic amplification in microfluidic devices is mainly performed by PCR, which requires a temperature control and a rapid temperature ramping, increasing the complexity of the implementation and increasing the instrument cost. Novel alternative DNA amplification techniques have emerged in the last years with the objective of providing analytical solutions to some of the drawbacks of the gold-standard for *in vitro* amplification, among them isothermal DNA amplification techniques are especially interesting for miniaturization purposes. Due to the simplicity of the temperature control for sequence amplification, isothermal amplification can be easily implemented in simple microchips without complicated thermal and/ or fluid control [10].

The objective of this thesis was to develop an improved methodology for the multiplex detection of several foodborne pathogens based in DNA detection, and its integration in a miniaturized device. To achieve this goal, the different steps of the analysis, including the sample pre-treatment, DNA amplification and visualization of the results were addressed, in which several approaches were evaluate in order to choose the best option to reduce the time of analysis, reduce the cost, and allow naked-eye detection.

To achieve the required sensitivity of analysis for foodborne pathogens, the enrichment of the sample is essential. At this moment, the fastest methodologies still need 18-48 h of enrichment time, without significant improvements, this point being the major bottleneck in microbiological food analysis when it comes to reducing the analysis time. It is essential to improve the way on how the

sample is treated, and for this reason, in this project the optimization of the sample pre-treatment was performed to allow a faster analysis. Different approaches were evaluated for an efficient simultaneous recovery of pathogenic bacteria, including medium optimization in standard enrichment, concentration of the bacteria and time reduction strategies.

The influence of different selective or non-selective media, in the growth of the targeted bacteria were tested, as well as the supplementation with several compounds. The best medium was evaluated for the simultaneous detection of *Salmonella* spp., *E. coli* O157 and *L. monocytogenes*. Due to the fact that *L. monocytogenes* presents a lower growth rate, compared to the other two bacteria, the optimization of the medium was focused in the improvement of the concentration of this pathogen. The general medium, Tryptic Soy Broth (TSB), showed to be the medium allowing the highest reduction of the lag phase, which reduced the time of analysis with the highest bacterial concentration obtained. Additionally several supplements were tested and their performance evaluate to improve the enrichment step, including cellobiose, yeast extract, sodium pyruvate, Laked Horse Blood (LHB), Campylobacter Growth Supplement and Half Fraser Selective Supplement. Among these, cellobiose was the only one enabling an increase of the final concentration of *L. monocytogenes* after 24 h, without showing changes in the concentration of the other two targets. However, no improvement in the lag phase was visible with this compound.

To increase the sensitivity of the methodology, different approaches to concentrate de bacteria were evaluated in the sample pre-treatment. The first methodology consisted in an Immunomagnetic Separation (IMS), where four different commercial antibodies, specific for *L. monocytogenes*, were evaluated comparing their purity and specificity. The selected approach consisted on the functionalization of magnetic nanospheres and their use to analyse food samples. The IMS approach allowed a LoD of 9.7 cfu/ 25 g, when combined with a selective enrichment in Half Fraser (HF) for 24 h and qPCR analysis. The second alternative involved the use of a microfluidic device where a 3D Polydimethylsiloxane (PDMS) sponge was functionalized with specific ligands to retain the bacteria. A non-specific bacterial ligand, the ApoH protein, was used for multiplex capture, and a specific anti-*L. monocytogenes* antibody (selected before in the IMS approach), for a targeted capture. Both approaches were evaluated with pure bacterial cultures, and also to analyse spiked stainless steel surfaces. The non-specific ligand ApoH protein allowed to achieve a slightly higher capture efficiency, and multiplex capture of targets, being an advantage compared to the specific ligands, which required an antibody for each pathogen. However, the universal ligands cannot be use in complex matrices with a high background of microorganisms, as it will bind randomly to other microorganism. When the surface samples were analysed combining the PDMS sponge with qPCR analysis without prior enrichment, the LoD is only limited by the DNA amplification technique itself or the sampling process. Both options, IMS and the functionalized sponge showed high performance and allow a reliable detection for different applications. An advantage of the capture of the bacterial cells, is their separation from the rest of the enrichment solution, which allows to eliminate possible inhibitory compounds of the DNA amplification reaction. However, the usage of these specific ligands increases the cost of the analysis, as the different ligand, antibodies or others remain expensive.

With the objective to reduce the time of analysis, three alternatives were tested: Phage Amplification Assay (PAA), Matrix Lysis and Short Enrichment. The PAA consists on an indirect strategy to detect a microorganism by the detection of a specific bacteriophage that will infect the target bacteria. By the faster replication of the phage, we obtained a higher concentration of the target in less time compared to the direct detection. This approach was applied for the detection of *S.* Enteritidis in food samples, allowing to perform the analysis by qPCR in only 10 h with the same sensitivity as the conventional methodologies, reaching a LoD of 8 cfu/ 25 g. The Matrix lysis approach relies in a completely different way to treat the sample. Instead of taking only a small aliquot

of the enriched sample, like the traditional analyses, the whole sample is treated to degrade as much as possible the matrix, and recover the bacteria for downstream DNA extraction. With some similarity, the short enrichment consist in a reduced time of incubation in culture medium, recovery of all the liquid from the sample to be processed, to allow the separation of the vast majority of microorganisms from the food sample. Both methodologies were evaluated for the recovery and detection of *L. monoctogenes* in simplex showing a reduction of the analysis time to 5 h and 7 h, with Matrix lysis and Short enrichment, respectively. The matrix lysis presents an excessively high LoD (1.1×10^5 cfu/ 25 g) making it unsuitable for the food industry, being discarded as possible option for the final methodology. The short enrichment showed a much higher sensitivity, with a LoD of 8.6 cfu/ 25 g, due to the culture step performed before the sample treatment, therefore the use of this methodology for the detection of *E. coli* O157 in simplex, and the simultaneous detection of *E. coli* O157 and *Salmonella* spp. was accomplished with similar results (LoD = 3-4 cfu/ 25 g), demonstrating the possibility to be used in multiplex detection. After evaluating the different alternatives for sample pre-treatment, the methodology showing the most promising results for allowing a faster and cheaper multiplex detection of *L. monocytogenes*, *E. coli* O157, and *Salmonella* spp. was proven to be the short enrichment.

As part of the strategy different alternatives for DNA amplification and detection were evaluated. Isothermal amplification techniques demonstrated several advantages over PCR/ qPCR such as being performed at constant temperature without the need of complex equipment, like thermocyclers. This characteristic was interesting for the development of the project as it simplifies the analysis, reduces the cost and allows an easier integration of this step in a miniaturized system, allowing as well lower energy consumption. Additionally, different alternatives can be easily employed for naked-eye detection when combined with an isothermal amplification technique. For this reason, two isothermal amplification techniques, LAMP and RPA, were evaluated to detect the targeted pathogens in simplex or multiplex, and obtain a simple visual result. The first step was to understand the performance of traditional qPCR analysis, using an intercalating dye (SYBR-qPCR) and a hydrolysis probe (Probe-qPCR), and to compare them against LAMP and RPA. Their specificity, sensitivity and accuracy with different genetic targets were evaluated with pure cultures and spiked food samples. The SYBR-qPCR was a more economic approach as it does not need specific probes, however the assay design is more complex, as well as the analysis of results when the multiplex detection is required. On the other hand, the Probe-qPCR offered higher specificity due to the implementation of the probe. Both methodologies allowed to perform multiplex detection with similar sensitivities. Regarding the evaluation of the isothermal amplification both, LAMP and RPA, resulted in a lower sensitivity than the qPCR approaches, when the analytical sensitivity using pure culture was examined. However when spiked samples were analysed combined with a two-step-enrichment in mTA10 broth and Full Fraser (FF), a similar LoD was achieved (1.4 cfu/ 25 g). It was also noticed a slightly higher performance from RPA, which provide faster results compared to LAMP.

Different alternatives for the visualization of the results in a simpler way were as well tested for each isothermal amplification technique. For RPA the loading of the amplification reaction in a lateral flow strip (LF) was evaluated and a high sensitivity and specificity was accomplished with this method for the detection of *L. monocytogenes* on surface samples, showing a LoD of 18.2 cfu/ cm2 after an enrichment of 24 h in ONE broth. The disadvantage of the RPA-LF was the increase of the analysis cost due to the need of the use of modified primers and a probe, along with the LF strips. As an alternative, different compounds may be added to the reaction, before or after amplification, to achieve a naked-eye detectable result in solution. In this sense, the addition of SYBR Green, a dsDNA intercalating dye, after the RPA reaction was evaluated, to enhance the visualization of fluorescence in positive samples when exposed to UV light. The detection of *E. coli* O157 in food samples was possible and we achieved a LoD_{95} of 19 cfu/ 25g when combined with a pre-treatment based on short

enrichment. Besides this approach presenting promising result, the fact that SYBR Green binds to any dsDNA present in the sample, makes the analysis of complex samples, and multiplex detection limited, due to the presence of the high fluorescence background obtained in negative samples, making it difficult to identify positive results.

For the results visualization of LAMP amplification three approaches were tested, including turbidity and change of colour either with the use of gold nanoparticles (AuNPs) or with a commercial colorimetric mastermix. The turbidity in the LAMP reaction is achieved by the production of the insoluble by-product, magnesium pyrophosphate, when amplification occurs. This approach was used to detect different serovars of *Salmonella* spp., targeting four genetic targets, leading to differences in the analysis performance when food samples were tested after a 24 h enrichment in mTA10. The turbidity can be monitored in real-time using a turbidimeter or as end-point detection by naked-eye visualization of the results. However the naked-eye observation needs to be performed in a specific light angle, which makes the analysis more subjective and complex. The AuNPs have been tested for different applications and thanks to the possibilities to modify their optical properties depending on aggregation state, naked-eye detection can be achieved. The functionalization of the AuNP with 11-mercaptoundecanoic acid (MUA) allows to control the aggregation of the nanoparticles when amplification product is originated, leading to differences in colours. In this sense, the positive samples will be red while negative samples will display a purple colour. This approach was tested for the detection of *Salmonella* spp. after an enrichment of 24 h in BPW. The amplification step was performed in a microfluidic device and the product of amplification mixed with the MUA-AUNP, producing a clear change of colour. Another alternative to originate a colour change in the amplification reaction is the addition of a pH-sensitive dye, to detect the LAMP amplification. This approach was tested for the detection of *Salmonella* spp. to understand the possibilities in the final methodology, in this case the use of the Loop primers, and time of amplification had to be optimized to avoid false positives and to obtain reliable results. Samples enriched for 24 h were tested, presenting a LoD_{95} of 2.1 cfu/ 25 g, additionally the combination with a short enrichment of 6 h was evaluated showing total concordance with the qPCR methodology.

After in-depth analysis of the different options evaluated to perform the DNA amplification and to allow a simplified approach for naked-eye visualization of the results, the methodology allowing the easiest, more cost effective and sensitive detection seems to be the colorimetric LAMP. The disadvantage of the use of MUA-AuNP methodology was related with the manipulation of the amplification product after reaction, which can cause cross-contaminations and false positives. With the colorimetric mastermix from NEB this drawback was overcame, providing an advantageous solution. For this reason, the final methodology was designed to combine the short enrichment methodology for the growth of the three pathogens, with the colorimetric LAMP using the commercial mastermix.

To obtain a multiplex detection of *Salmonella* spp. *E. coli* O157 and *L. monocytogenes*, the short enrichment was optimized to allow the growth of all of them in a single enrichment step enabling a highly sensitive results. After testing general and selective media for this purpose, the enrichment in TSB for 7 h was the one providing better results for the detection of *L. monocytogenes*, as this was the most problematic pathogen due to its lower growth rate. The complete methodology was evaluated analysing different types of milk samples (UHT, Fresh and raw), reaching an acceptable LoD, with a more sensitive detection of *Salmonella* spp. and *E. coli* O157 with a LoD_{95} of 1.6 cfu/ 25mL. For *L. monocytogenes* a higher concentration of bacteria was need to allow for a reliable detection, obtaining a LoD_{95} of 79 cfu/ 25mL. A clear influence of natural microflora was observed for the detection of *L. monocytogenes*, with an associated increase in the LoD with higher mesophilic counts. The mesophilic characterization was also performed by MinION sequencing analysis where mostly lactic acid bacteria were identified.

After full method evaluation, the integration of the amplification step in miniaturized devices was tested and compared against previous results. The performance of two distinct systems was first compared analysing pure cultures, to determine the best option to be implemented in the final methodology. One of the devices consisted on an integrated device combining temperature control and a flexible holder enabling the use of silicon tubing with different volumes. The other alternative involved a microfluidic device with 8 microchannels which could be placed in a conventional laboratory incubator to perform the reaction. A clear differentiation between positive and negative results was achieved when the colorimetric LAMP was integrated in both devices, however a decrease in analytical sensitivity was observed. The microchannel device showed the worst results, with poor analytical sensitivity, being even impossible to detect *L. monocytogenes* in the higher concentrations tested. For this reason this device was discarded for the following analysis. The integrated heating system was selected, and evaluated, for the analysis of milk samples. The final methodology obtained higher LoD compared with the thermocycler approach, as was expected by the results of the analytical sensitivity. A LoD_{95} of 15, 17 and 141 cfu/ 25g was obtained for *Salmonella* spp. *E. coli* O157 and *L. monocytogenes*, respectively. A complete concordance with the qPCR methodology was achieved with the samples tested.

In conclusion, the methodology developed in this project allowed for the multiplex detection of *Salmonella* spp. *E. coli* O157 and *L. monocytogenes*, reducing the time of analysis to only 9 h vs 7 days when performed by traditional culture-based techniques and providing naked-eye detection. A similar sensitivity and specificity was obtained when compared with the gold standard, qPCR, without the need of a complex instrumentation such as a real-time thermocycler. The use of the short enrichment as sample pre-treatment allowed to achieve a significant turnaround time reduction, without increasing the cost, which makes the analysis more accessible to everyone. Furthermore, the possibility to integrate the amplification step in a miniaturize device opens the door for the development of Point-of-Care systems, with reduced size and with the possibility to automatize the analysis to be performed on decentralized settings.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
µmax	Maximum Specific Growth
Ab	Antibody
AC	Relative Accuracy
ALOA	Agar Listeria Ottavani & Agosti
ApoH	Apolipoprotein H
AuNP	Gold Nanoparticles
B3	Backward Outer Primer
BAM	Bacteriological Analytical Manual
BHI	Brain Heart Infusion
BIP	Backward Inner Primer
BLAST	Basic Local Alignment Search Tool
BLEB	Buffered Listeria Enrichment Broth
BPW	Buffered Peptone Water
BSA	Bovine Serum Albumin
CDC	Center for Disease Control and Prevention
CE	Capture Efficiency
CECT	Spanish Type Culture Collection
cfu	Colony Forming Unit
cIAC	Competitive IAC
CNC	Computer-Numerical-Control
Cq	Cycle of Quantification
DAEC	Diffusely Adherent *E. coli*
DAS	Strand Displacement Amplification
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double-Stranded DNA
EAEC	Enteroaggregative *E. coli*
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-Linked Immunosorbent Assay
EPEC	Enteropathogenic *E. coli*

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ETEC	Enterotoxigenic *E. coli*
EU	European Union
F3	Forward Outer Primer
FDA	Food and Drug Administration
FF	Full Fraser
FIP	Forward Inner Primer
FN	False Negative
FP	False Positive
FSIS	Food Safety and Inspection Service
GuSCN	Guanidine Thiocyanate
HACCP	Hazard Analysis for Critical Control Point
HC	Haemorrhagic Colitis
HF	Half Fraser
HRP	Horseradish Peroxidase
HUS	Haemolytic-Uraemic Syndrome
IAC	Internal Amplification Control
IgG	Immunoglobulin G
IMS	Immunomagnetic Separation
ISSO	International Organization for Standardization
k	Cohen's Kappa Index
LAMP	Loop-Mediated Isothermal Amplification
LB primer	Loop Primer B
LB	Luria-Bertani
LEB	Listeria Enrichment Broth
LF	Lateral Flow
LF primer	Loop Primer F
LHB	Laked Horse Blood
LoD	Limit of Detection
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin Broth
MNP	Magnetic Nanospheres
MOPS	3-(N-Morpholino)Propanesulfonic Acid
MPC	Magnetic Particle Concentrator
mTSBn	Modified TSB with Novobiocin
MUA	11-Mercaptoundecanoic Acid
NA	Negative Agreement
NASBA	Nucleic Acid Sequence-Based Amplification
NB	Nutrient Broth
NC-IAC	Non-Competitive IAC
ND	Negative Deviation
NMEC	Neonatal Meningitis-Associated *E. coli*
NPV	Negative Predicted Value
OD	Optical Density

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ODmax	Maximum Optical Density
ON	Overnight
ONE broth	Oxoid Novel Enrichment
PA	Positive Agreement
PAA	Phage Amplification Assay
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Positive Deviation
PDMS	Poly(Dimethylsiloxane)
PMMA	Poly(Methyl Methacrylate)
POC	Point-of-Care
PoD	Probability Of Detection
PPV	Positive Predicted Value
PSR	Polymerase Spiral Reaction
qLAMP	Real-time LAMP
qPCR	Real-time Polymerase Chain Reaction
qRPA	Real-time RPA
RCA	Rolling Circle Amplification
RFU	Relative Fluorescence Units
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
RT	Room Temperature
RTE	Ready to Eat
RT-qPCR	Real-Time Reverse Transcription PCR
RVS	Rappaport-Vassiliadis-Soya Broth
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SE	Relative Sensitivity
SEPEC	Sepsis-Causing *E. coli*
SP	Relative Specificity
SSB	Single-Strand DNA Binding Protein
ssDNA	Single-Stranded DNA
ssRNA	Single-Stranded RNA
STEC	Shiga Toxin-Producing *E. coli*
Stx	Shiga Toxin
TEC	Thermoelectric Cooler
TP	True Positive
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSYEA	Tryptic Soy Yeast Extract Agar
UDG	Uracil-DNA Glycosylase
UHT	Ultra-High Temperature

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UPEC	Uropathogenic *E. coli*
USDA	United States Department of Agriculture
UV	Ultra-Violet
VBNC	Viable But Non-Culturable Cells
WDCM	World Data Centre for Microorganisms
XLD	Xylose Lysine Desoxycholate Agar
YE	Yest Extract
λ	Lag Time

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CHAPTER 1.

INTRODUCTION

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1 INTRODUCTION

1.1 THE IMPORTANCE OF FOOD SAFETY IN FOOD SUPPLY CHAIN

With the growth of the human population worldwide the demand for food has risen, and with this, an intensification of the production, and large-scale food processing and distribution systems. The complexity of the food supply chain leads to higher possibilities for contamination of the food products and additionally, the time for food products to reach the consumer is relatively short, particularly for fresh products, which makes the time to detect possible contaminations very short. Additionally, the increased globalization in the recent years have also affect the food production system, making foodborne disease easily spreading all around the world.

Every year, nearly one in 10 people around the world falls ill after eating contaminated food [2]. Besides foodborne illness are more frequent in developing countries, due to the lack of food safety regulation and lower access to optimal hygienic conditions of the population, developed countries also are affected and suffer with this situation. The Centres for Disease Control and Prevention (CDC) estimates 47.8 million people get sick, 127,839 are hospitalized, and 3,037 die from foodborne diseases every year in the United States [11,12]. In Europe, more than 23 million people fall ill from eating contaminated food every year, resulting in 4,654 deaths and more than 400,000 disability-adjusted life years [13]. The major cause of all these hospitalizations and death are enteric disease, known as intestinal illnesses, caused by different types of microorganisms such bacteria, parasites and even virus. The most common symptoms are related to intestinal disorders, such as diarrhoea, but can have much serious consequences and even lead to long-term effects and death [14,15].

Even if no illness is reported, the recall, due to an identified threat in a food product, can cause important economic lost for the company and originate high food waste. These cases may harm the responsible company not only economically but also tarnish its reputation, by leading to low consumer trust

For all this reasons, important resources have been put in place to control and avoid the contamination of the food value chain with this foodborne pathogens. Monitoring needs to be done from farm to fork in the whole food value chain, as the hazard can be at any point, from the production of the raw material, passing by the processing industry until the transportation and retail of food product. Safety measures and good practices have been implemented in all stages of the supply chain, with the Codex Alimentarius [16] guidelines and the application of Hazard Analysis for Critical Control Point (HACCP). Since the implementation of these standards, more specific regulation were created by different organization for the food industry, in order to avoid and mitigate contamination.

Even with the regulation in place and the strong vigilance existing nowadays, the number of outbreaks related with foodborne pathogens did not shown any decrease in the last years, as reported by European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) **Figure 1.1**. The linkage between cases of illness reported in a specific outbreak is difficult to identify, and the source of contamination can remain undetected, leading to more infections. This highlights the important to identify the contaminated food product before reaching the consumer.

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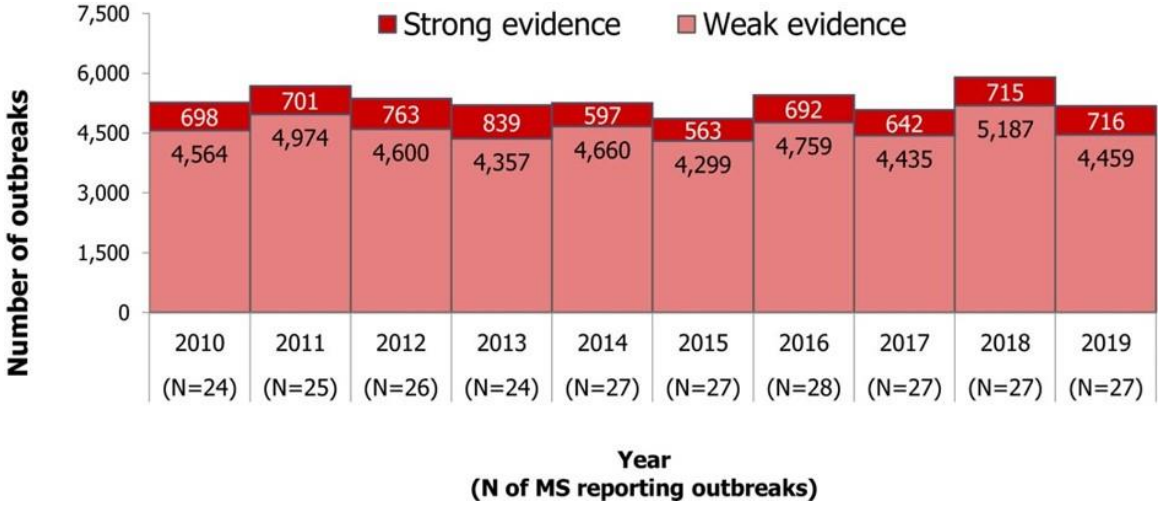

Figure 1.1. Number of foodborne outbreaks, reported by EU member states from 2010 to 2019. Graphical representation from EFSA and ECDC Zoonoses Report [3], indicating the strength of evidence (strong or weak). N represents the number of EU countries reporting outbreaks (image used with permission from European Food Safety Authority).

1.2 PROBLEMATIC PATHOGENS

Different causative agents are already identified by the regulatory authorities, being their incidence monitored in different countries. Between them, bacteria are the most common, reporting high number of outbreak every year, causing zoonotic diseases when this bacteria are from animal origin. The five zoonotic diseases mostly reported by EFSA and ECDC in 2019, are campylobacteriosis, salmonellosis, Shiga Toxin-producing *E. coli* (STEC) infection, yersinosis and listeriosis (**Figure 1.2** and **Table 1.1**).

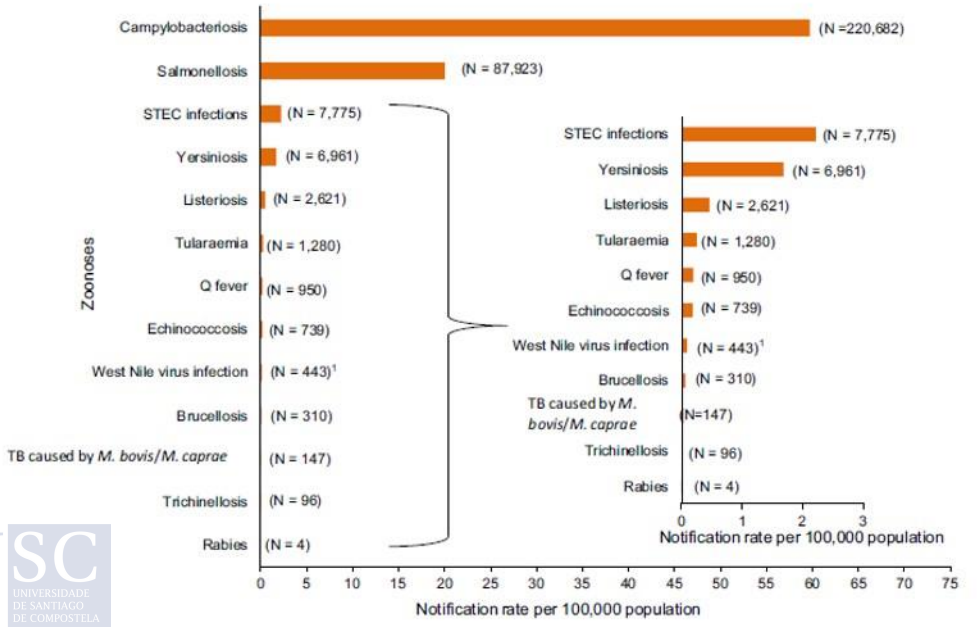

Figure 1.2. Confirmed number of human zoonoses reported in EU in 2019. N indicate the number of total cases. Graphical representation from EFSA and ECDC Zoonoses Report [3] (image used with permission from European Food Safety Authority).

Table 1.1. Top 5 pathogens responsible for the reported cases in Europe

Pathogen	N of cases	N of hospitalization	N of death
Campylobacter spp.	220,682	20,432 (31.8 %)	47 (0.03 %)
Salmonella spp.	87,923	16,628 (42.5 %)	140 (0.22 %)
STEC	7,775	1,100 (37.9 %)	10 (0.21 %)
Yersinia	6,961	648 (33.9 %)	2 (0.05 %)
L. monocytognes	2,621	1,234 (92 %)	300 (17.6 %)

Data reported by EFSA/ ECDC in 2019 [3]

Campylobacter spp., which originate campylobacteriosis disease, present the highest incidence, representing 50% of all the reported cases of zoonosis monitored. Beside the high number of illness caused by this bacteria, the mortality rate have a relatively low value (0.03 %). Equally between the bacteria reporting the highest number of hospitalization cases are *Salmonella* spp. and STEC which present much higher number of death rate (0.22 % and 0.21 %). However, between all pathogens monitored by European authorities, *L.* monocytogenes continue to be the more problematic based in the severity, showing a mortality rate of 17.6 % from reported cases. For this reason, the pathogens with higher incidence and causing the more severe illness are consider to be *Salmonella* spp., STEC and *L. monocytogenes*.

1.3 CHARACTERISTIC OF THE MOST PROBLEMATIC PATHOGENS

1.3.1 *Salmonella* spp.

Salmonelliosis is the second most reported cause of illness by foodborne pathogens in Europe and USA, being caused by *Salmonella* spp.. This pathogen is a Gram-negative bacterium, rod-shaped, facultative anaerobic which belong to the family of *Enterobacteriaceae*. In 1884, the first isolated was *S.* Choleraesuis, during an outbreak of cholera in pigs. This microorganism present a wide range of different serytopes, around 2600 identified, where most of them have the ability to adapt in a different variety of animal hosts, among them also humans. The genus *Salmonella* is classified into two species, *Salmonella enterica* (type species) and *Salmonella bongori*, being further divided in subspecies. Between these subspecies, *S. enterica* subsp. *enterica* are the ones causing 99 % of the *Salmonella* infection in warm-blooded animals and humans [17]. The serovars, Typhi and Paratyphi are human-restricted and responsible for the septic typhoid syndrome (enteric fever), while the others have a broad range of hosts causing gastroenteritis in humans [18]. *Salmonella* Enteritidis and *Salmonella* Typhimurium are the sevorar causing approximately 70 % of the cases of salmonellosis reported each year (**Table 1.2**), but high number of other serovars are associated with this diseases.

Diarrhea, fever and abdominal cramps are the most common clinical manifestations of salmonelliosis, however this illness can evolve to more serious complications, mainly in infant, elderly and immunocompromised patients, causing bacteraemia, which can affect multiple organs, increasing the mortality [19].

Since a first outbreaks reported in Germany in 1888, this pathogen have gained an important role in the development of control measures for poultry meat where its incidence is the highest, but also in the rest of the meat industry. Beside a decrease of 32 % in the number of cases observed in Europe between 2008 and 2012 [20], EFSA report of 2019 show that this type of food matrix, particularly

broilers and turkey fresh meat, present the higher number of positive samples [3]. This bacteria have been also found in different Ready to eat (RTE) product and also infant formula. Recently in the beginning of this year a multi-country outbreak of monophasic S. Typhimurium infections were linked to chocolate products by a company in Belgian [21], leading to hospitalization in 41% of the cases, being most of them children.

Table 1.2. Serovars of Salmonella spp. responsible for confirmed cases of salmonellosis in EU in 2019

Serovar	Cases	EU Member states	%
Enteritidis	39,865	27	50.3
Typhimurium	9,404	27	11.9
Monophasic Typhimurium 1.4.[5].12:i:-	6,491	18	8.2
Infantis	1,924	26	2.4
Newport	870	24	1.1
Derby	721	23	0.9
Stanley	560	19	0.7
Kentucky	545	24	0.7
Napoli	508	18	0.6
Agona	503	20	0.6
Virchow	477	21	0.6
Coeln	455	18	0.6
Bovismorbificans	454	19	0.6
Java	440	14	0.6
Mikawasima	415	15	0.5
Chester	350	17	0.4
Bareilly	321	17	0.4
Saintpaul	302	20	0.4
Branderup	300	18	0.4
Hadar	298	17	0.4
Other	14,097	–	17.8

Data reported by EFSA/ ECDC in 2019 [3]

1.3.2 Shiga Toxin-producing *E. coli* (STEC)

Escherichia coli is well known as a natural host of the gut microbiota, not only of humans but also other warm-blooded mammal animals. For this reason, this bacteria is normally used as an indicator of environmental faecal contamination [22]. If its prevalence in healthy intestinal persons was discovered long time ago, the capacity to cause disease in this same natural habitat was not so clear and evidence arrived slowly. Only in the 1987, this microorganism was recognized as a primary pathogen, when several outbreaks have emerged causing infantile diarrhea, associated with a strain of *E. coli* [23].

E. coli is a member of the *Enterobacteriaceae* family, and characterized by being a bacillus Gram-negative bacteria, and facultative anaerobic. The different *E. coli* stains are serotyped based in their lipopolysaccharide (O) and flagellar (H) surface antigen profile. Some of this serotypes showed to be pathogenic, causing different symptoms depending on the pathotype and the system of infection, being classified in enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC), and other non-gastrointestinal pathogenic *E. coli* including uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC) [24–26].

The STEC are a group of *E. coli* which produce a toxin called shiga toxin (Stx), due to its structural and functional similarity to the toxin produced by *Shigella dysenteriae* [27]. This toxin was previously called "verotoxin" by its cytotoxic activity on Vero cells, and for this reason the strains of *E. coli* bearing this gene were referenced as verotoxin-producing *E. coli* (VTEC) [28]. Among STEC there is a subset which corresponds to enterohemorrhagic *E. coli* (EHEC), and are associated with severe clinical symptoms, which can pass from a minor diarrhoea to haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS), the main cause of acute renal failure in children [29]. Although a wide range of serotypes have been implicated in human STEC infections, as presented in **Table 1.3**, the majority of the cases, and outbreaks of HC and HUS, are caused by a limited number of serotypes including O157:H7, O26:H11, O103:H2, O111:H8, O145:H28 and their non-motile derivatives [30]. *E. coli* O157:H7 differentiates from the other serotypes from its increase probability to cause more severe symptoms [31], leading to more hospitalization and in some cases death.

E. coli O157:H7 is the most commonly reported STEC serotype, member of the EHEC. The first outbreak associated to this pathogen was reported in 1982, and it was traced to hamburgers in a restaurant chain in USA. Since then new outbreaks have been reported every year all over the world [28,29,32,33] and specific methods were created to identify this pathogen in food products, as ISO 16654:2001 [34] and BAM Chapter 4A (k) [35]. This specific strain presents distinct growth and metabolic characteristics compared to other *E. coli* strains, for instance the restriction in the temperature of growth at 45.5°C, the inability to ferment sorbitol in 24 h and additionally the absence of β-glucuronidase production that could facilitate its differentiation from the other strains by culture based methods [36,37].

The most common source of human infection with STEC, especially O157:H7, are ruminants with a higher incidence in bovine meat [38]. However other associated product, as raw milk and milk product, also reported cases with a lower incidence [39,40]. EFSA and ECDC reported that in 2016 12.7 % of a herd of 2496 cattle, tested positive for STEC [41], and in 2019 this value increased to 17 % in a total of 1493 cattle sampling units [3], evidencing these animals as a reservoir of this pathogen. The transmission of this pathogen to surrounding environment has been reported, extending the source of contamination to water and soils, spreading to other animals, but also to crops by the use of manure as fertilizer and contaminated water for irrigation [42]. For this reason several outbreaks were reported associated with fresh vegetables [43,44] including spices and herbs, as well as fruits. Additionally, RTE foods were found to be contaminated with STEC [45,46], what arose more concern due to the absence of treatment aimed to reduce or eliminate the possible presence of this pathogen.

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Table 1.3. Most Frequent serogroups of human STEC infections in EU in 2019

Serogroup	Cases	EU Member states	%
O157	1,195	22	26.6
O26	722	16	16.0
Non-typable*	572	11	12.7
O146	220	11	4.9
O103	213	13	4.7
O91	181	12	4.0
O145	162	11	3.6
O128	113	12	2.5
O80	80	9	1.8
O111	63	12	1.4
O63	62	8	1.4
O113	60	10	1.3
O117	52	6	1.2
O76	48	9	1.1
O27	44	6	1.0
O55	36	10	0.8
O8	36	7	0.8
O78	30	8	0.7
O121	29	8	0.6
O182	28	7	0.6
Others	554	–	12.3

Data reported by EFSA/ ECDC in 2019 [3]
* Non-typable, when the identification of the O-serogroup by the laboratory was not successful, depending on the serological or molecular techniques for typing.

1.3.3 L. monocytogenes

Among the most commonly reported foodborne pathogens, *L. monocytogenes* is the bacterium causing the most severe disease, and presenting the highest mortality rate in humans. In healthy individuals this pathogen normally expresses light symptoms, such as gastroenteritis, however in some cases the bacteria can cross the intestinal barrier and invade the rest of the body, being accumulated in the liver and spleen [47]. Septicaemia, meningitis and meningoencephalitis are the complications of listeriosis. This disease can lead to spontaneous abortion, still birth, or foetal infection in pregnant women.

Between 2008 and 2019, the number of confirmed cases reported of listeriosis in EU has increased 90 % (**Figure 1.3**). *L. monocytogenes* rises high concern in the food supply chain, and this increase in confirmed cases can be due to the higher detection capability of the food industry.

However only a slight decrease in the mortality rate was observed, with 17.6 % of fatality cases from reported cases in 2019.

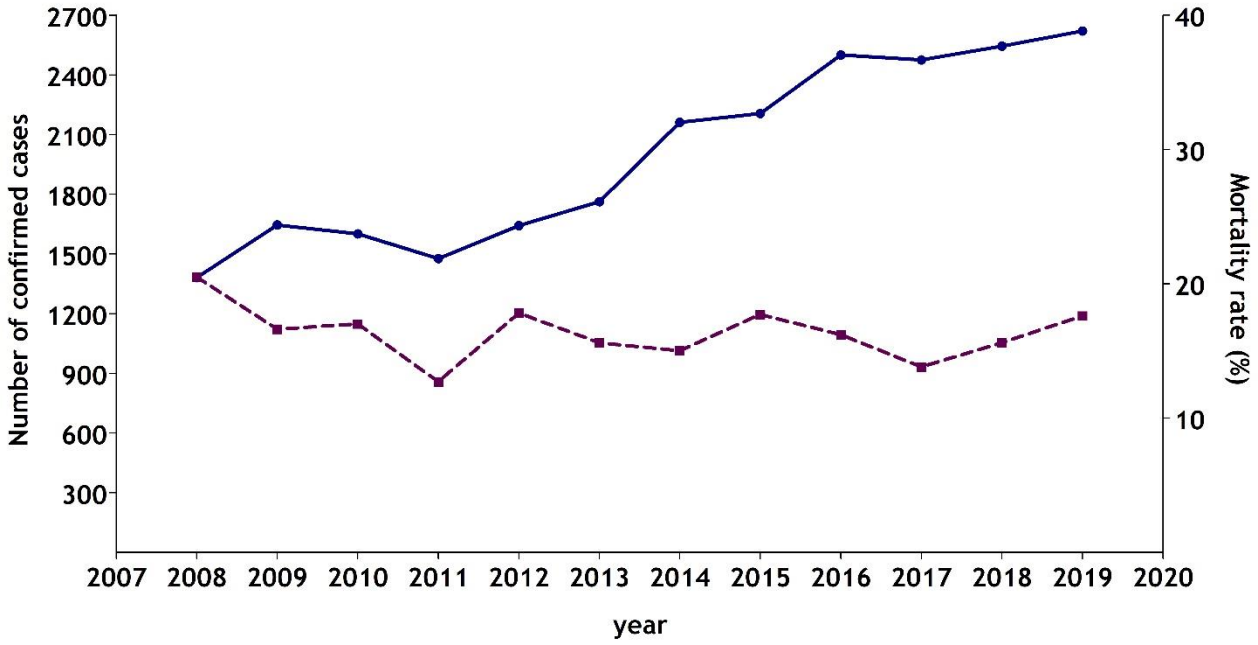

Figure 1.3. Evolution of listeriosis cases and related case fatality rates in EU between 2008 and 2019. The solid blue line correspond to the number of confirmed cases reported and the dashed purple line indicate the corresponding to the mortality rate in percentage.

This pathogen is a facultative anaerobic Gram-positive bacterium, small rod shaped-bacteria and motile under specific conditions, as regulated by temperature [48]. The genus *Listeria* has 20 species, but only two, *L. monocytogenes* and *L. ivanovii* are considered pathogenic. The species *L. monocytogenes* is divided in four evolutionary linages and 13 serotypes, being 98 % of the listeriosis cases associated with lineage I, serotypes 1/2b and 4b, and lineage II, serotype1/2a [49].

Unlike *Salmonella* spp. or STEC, *L. monocytogenes* is a ubiquitous organism, widely distributed in the environment, which increases the contamination routes. With its ability to survive in harsh environments, such as refrigerated temperatures [50], high salt concentrations [51], and low pH [52], *L. monocytogenes* can persist in the food supply chain, even with regular sanitation, which may lead to cross-contamination of food. RTE products are for this reason a direct risk for the consumer, as no treatment that could eliminate or reduce the bacterial concentration are required before consumer consumption . In 1980 an outbreak of listeriosis was identified in Canada, but was not considered relevant, since this disease was associated with sheep and cattle infection, and human cases were considered rare by then [53]. However more outbreaks occurred associated to pasteurized milk and milk products, starting to bring awareness in the food industry, and regulation authorities, triggering exponential research on this pathogen [54]. Nowadays, the association of listeriosis with the contamination of RTE product is clear, not only in milk related product, but also all sorts of food products, including fruit, cheese, meat and fish [3].

1.4 REGULATION IN THE FOOD INDUSTRY

For the protection of public health, the legislation stipulates maximum levels of defined pathogens. In USA, two regulatory authorities, Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) are responsible for the establishment of this legislations [55], while in Europe the European Comission (EC) state this criteria in the Regulation 2073/2005 [56] and its different amendment.

This microbiological criteria vary according to the pathogen itself, and the category of the food. Different regulatory authorities can also state distinct limits, showing variations between countries.

Regarding *L. monocytogenes* a zero tolerance in USA is considered for any RTE food [54], while in the European Union the regulation is not so strict, due to the fact that this pathogen is ubiquitous in the environment, and a concentration of <100 cfu/ g is not enough to cause illness [57]. For this reason, up to 100 cfu/ g are allowed in Europe in foods that do not allow its growth during self life. However, the absence of *L. monocytogenes* is sustained in RTE food which support its growth during self-life, and also in infant products and for special medical purposes [56,58]. Implying the need for the food producers to ensure the failure of the pathogen to grow in the specific food product.

Similar regulations are provided by both USA and European regulations, regarding the presence of *Salmonella* in food products. In the Regulation 2073/2005 strict criteria have been specified for minced meat, meat preparations and meat products intended to be eaten raw or cooked, where the absence in 25 g or in 10 g, respectively, need to be complied. The processing of these types of meat product provides an opportunity for the pathogens present on the carcase surface or in the close environment to be spread into the product. Although the foodstuff will be cooked, the bacteria may not be destroyed in the centre of the product, leading to unsafe consumption [59].

Being eggs an important source of contamination, the same criteria of absence of *Salmonella*, need to be followed also in egg products and RTE product containing raw egg, excluding products where the manufacturing process or the composition of the product will help to minimise the *Salmonella* risk. Some other RTE food, as raw milk products, milk and whey powder, cooked shellfish, sprouted seeds, fruits and vegetables are also included in this criteria due to the possibility of cross-contamination.

Even though STEC were identified as foodborne pathogens back in 1982, it was only recently when specific legislation was put into place for certain types of foods. After the occurrence of several outbreaks linked to vegetables, an amendment to the EU No. 2073/2005 (No. 1441/2007, [60]) was put into place and specified the absence in 25 g of sprouts. However, in Europe, meat from ruminants is out of the regulation due to the lack of data available with STEC-contaminated food [61]. On the contrary, in the regulation from the USA, this type of product was already included [62], and also encourages importers companies to comply with the same criteria.

The failure to meet these criteria, implies the recall of the product from the market in order to avoid, or at least reduce, the risk for the consumer's health. For this reason detection methodologies for the target pathogens need to be reliable, accurate and as fast as possible.

1.5 DETECTION METHODOLOGY

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1.5.1 Gold standards culture based analysis

The use of culture media is the classical methodology for the detection of foodborne pathogens. Their sensitivity has shown to be high, allowing reliable results and high cost-effective performance, as the resources needed for this approach are relatively affordable. The media used can be of three different categories: general, selective and differential [63]. The General medium are mostly

employed to perform a pre-enrichment were no selective compounds are added to inhibit the non-targeted microorganism, but allowing the growth of the target microorganism. This can also be used for the recovery of stressed bacteria in some type of samples.

Selective media contain inhibitory agents, such as antibiotics, which constrain the growth of the other non-target bacteria. The concentration of these additives must be carefully evaluated, as interference in the growth of the target microorganism may occur.

Differential media make easier the identification of the bacteria of interest as they will develop distinct characteristics in comparison to other microorganisms which may also grow, for instance, the presence of chromogenic compounds will produce a specific colour change. The ISO methodologies for the detection of foodborne pathogen have been focus in this type of approach for recovering, growth, isolation and identification of a specific microorganism.

The EN ISO 6579-1 specify a horizontal methodology for the detection of *Salmonella* spp. in the food supply chain, not only in products intended for human consumption, but also in animal feeding and environmental samples. The recent update in 2017 also included testing in milk and milk product, as well as the testing in animal faeces, dust and boot socks with the objective of preventing cross-contamination and detect the contamination source [64], the procedure is depicted in **Figure 1.4**. As it may be observed, it follows the standard approach in the sense of a first pre-enrichment in a general medium, BPW, followed by a selective enrichment in two different media, RVS and MKTTn. After the enrichment both broths are plated on two selective and differential media, which can be XLD and a second medium which is open for the laboratories to choose, and finally, if typical colonies are observed they are purified on a general agar medium, typically Nutrient Agar (NA), for subsequent biochemical identification, and serological analysis if needed.. The full methodology takes a total of 6 days, being extremely laborious, lengthy and time-consuming.

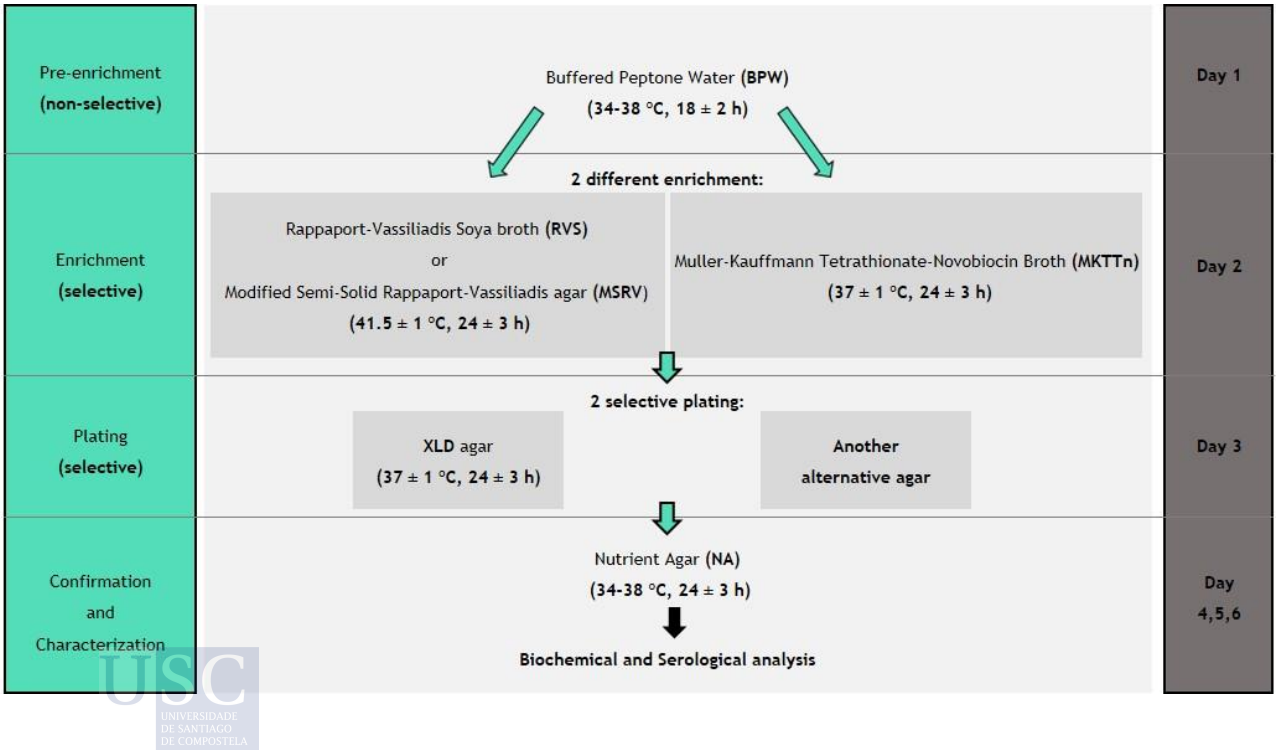

Figure 1.4. Scheme of horizontal method for the detection and serotyping of *Salmonella* (ISO 6579-1:2017)

For the detection of *E. coli* O157 a slightly different approach is followed in order to differentiate this specific serogroup from all other *E. coli*. Attending to the ISO 16654:2001 (**Figure 1.5**), after a selective enrichment an immunomagnetic separation (IMS) is performed taking advantage of specific antibodies to recover this pathogen. The captured bacteria are then plated on two different solid media, and the subsequent steps are as those for *Salmonella* spp., re-isolation of typical colonies, followed by biochemical and serological analysis. This approach delivers the final results in 5 days, and by the use of the IMS the process is less laborious than the protocol for the detection of *Salmonella* spp.

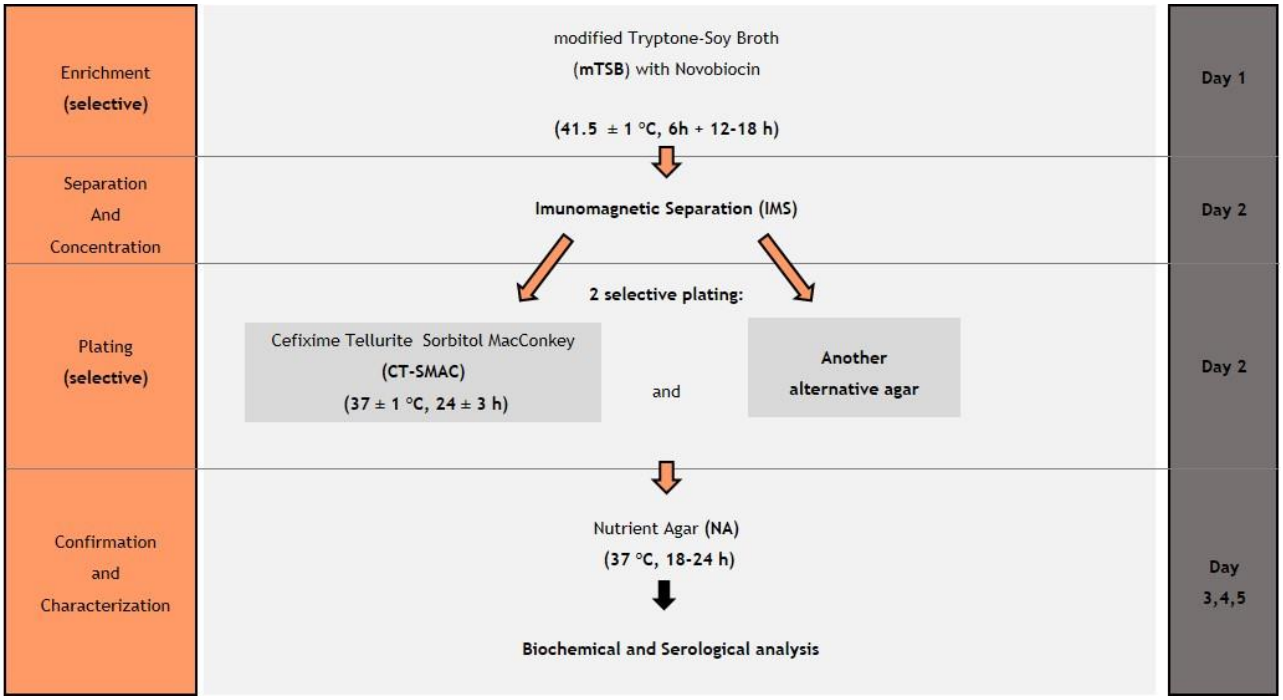

Figure 1.5.Scheme of horizontal method for the detection of *E. coli* O157 (ISO/TS 16654:2001)

The detection of *L. monocytogenes* needs more time than the protocols previously presented, taking the full analysis 7 days, following the procedure described in the ISO 11290-1:2017 (**Figure 1.6**). The slower growth of this bacterium influences the time of analysis, and the need for the use of selective medium also delays its growth. One semi-selective enrichment, in HF, followed by a selective enrichment, in FF, are needed (24 h each), and then platting on two solid media, ALOA and second selected by the laboratory, for up to 48 h.

The culture-based approaches make the analysis for the detection of foodborne pathogens very laborious and lengthy and the time of analysis is also not compatible with the intense demands of current food production system existing nowadays. These fact highlight that the food industry is in need of better methodologies.

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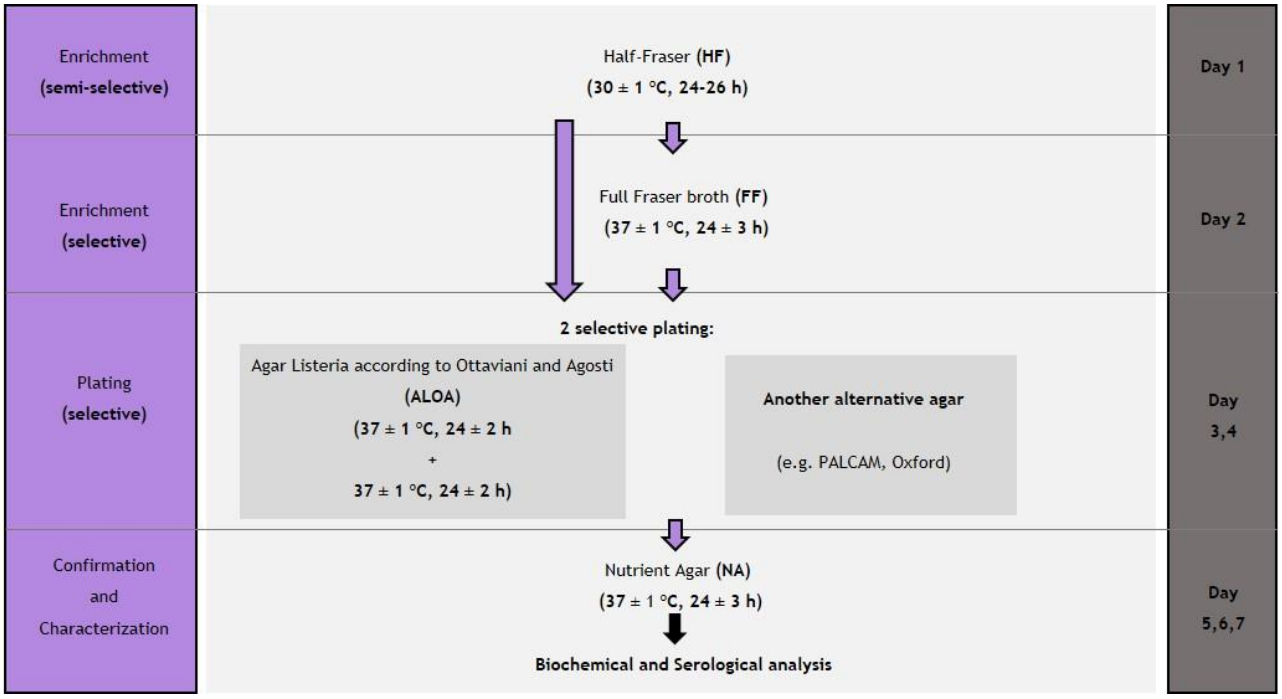

Figure 1.6. Scheme of the horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. (ISO 11290-1:2017)

1.5.2 Commercial methods available for improved analysis

To cover the needs of the food industry, new products are being introduced into the market to reduce hands-on time, increase sensitivity or to allow the detection of several pathogens in multiplex. To assist the culture-based methodologies, new type of solid media have been developed, as Petrifilm (3M), Dryplate (MICROKIT, Spain) or SimPlate (IDEXX Laboratories, USA) (**Figure 1.7 A, B, C**), with the advantage of being ready–to-use, and removing agar preparation. Regarding the colony counting for microbial concentration determination, can also be improved by other technologies, like Spiral Plates counter (**Figure 1.7 D**) which allows automatization of the process. Bacterial quantification by Most Probable Number can also be automatized as exemplified by the TEMPO system (BioMérieux, France) (**Figure 1.7 E**) avoiding the usual tedious preparation. All of them have the aim to provide a faster and simpler analysis, however the culture-based methodologies continue to require a long period of time to reach the results.

Different molecular alternatives have emerged, being immunological methodologies well established in the market, with different lateral flow products (**Figure 1.7 F**) which are based on colloidal gold immunoassay strips, and are sold by different companies (Biocontrol, Merck, Neogen among others) for different pathogens [65]. The major drawback of this approach falls on its sensitivity, being a good methodology for preliminary analysis, but always with the need to be complemented with a more sensitive detection. Automated system for immunological detection have also been developed and commercialized, as VIDAS® from BioMérieux (France) (**Figure 1.7 G**).

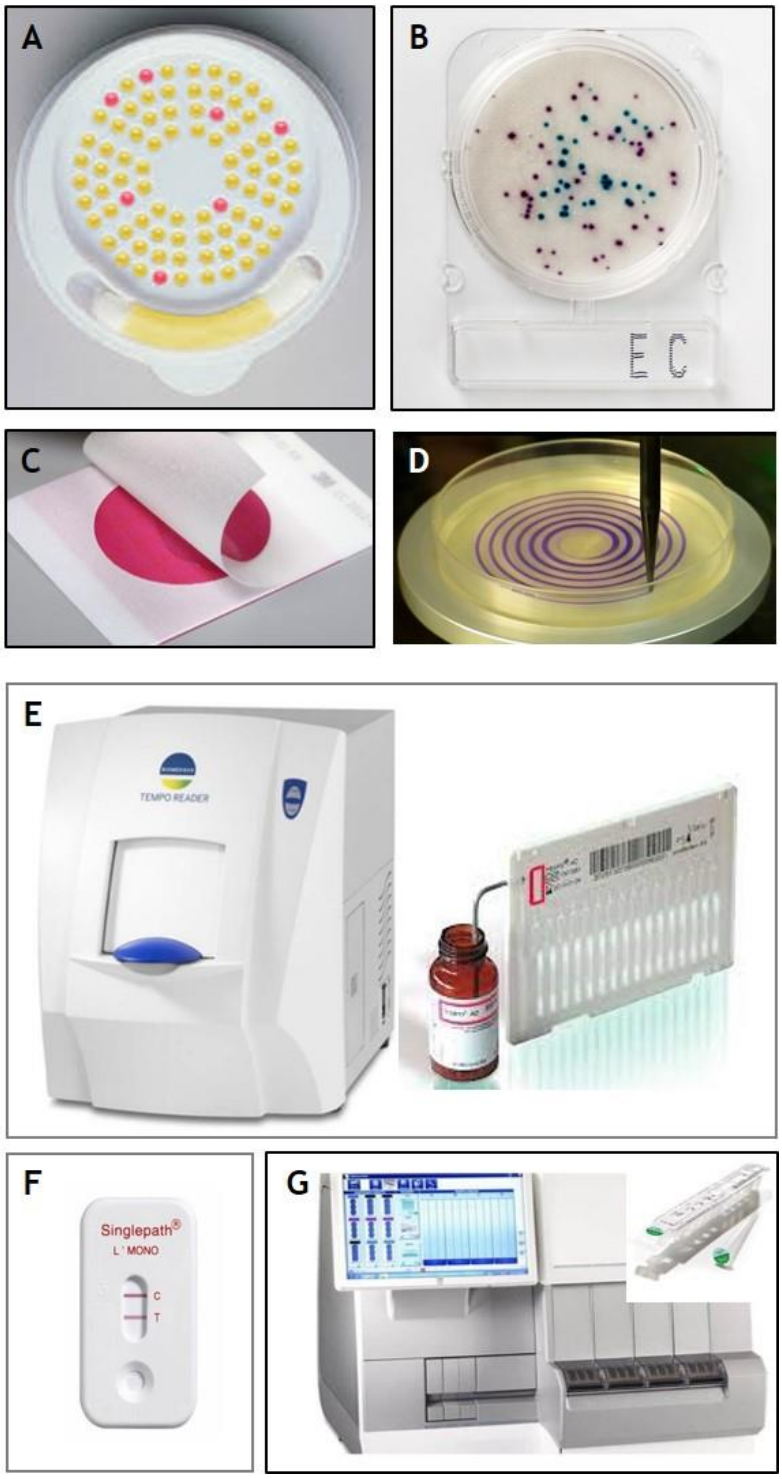

Figure 1.7. Examples of product in the market developed to improve the food analysis and detection of pathogens. (A) SimPlate, Easy-to-count wells with change of colour for bacteria quantification; (B) Dryplate, ready to use plates with dehydrated media; (C) Petrifilm, ready selective culture system in a disk for fast enumeration; (D) Spiral Plater, automatic and standardized plating of a sample generating decimal dilution on a single plate; (E) TEMPO, fully automated enumeration system, using the most probable number method; (F) Lateral Flow , immunochromatographic test, based on gold-labelled antibodies. (G) VIDAS, automated benchtop immunoanalyzer, based on the Enzyme Linked Fluorescent Assay (ELFA) technology.

1.5.3 Alternative sample pre-treatment strategies

Most of the efforts for reducing the time of analysis without affecting the reliability and sensitivity of the traditional methodologies in food analysis have been focused in molecular techniques. However, sample pre-treatment continues to be the bottleneck of food analysis, as the enrichment continues to be a crucial step to increase the concentration of the targeted bacteria to obtain a sensitive detection, particularly when the target pathogen tends to be present in low concentration. Additionally, the current legislation requires in some cases to ensure the absence of the pathogens in the food product and to fulfil this requirement the detection methodology needs to be able to detect 1 cfu/ 25 g of samples. The turnaround time of the molecular approaches is not limited by the technology itself but by the need to always perform a pre-enrichment step of at least 16h to 24h. Extensive selection of media have been formulated with this aim, and alternatives are constantly appearing in the market to allow faster growth or reduce the natural microflora present in the sample, with the addition of proper selective agents. However, alternatives to significantly reduce the time needed for the sample pre-treatment are not available in the market.

Different approaches have been more recently studied to overcome this fact with alternatives to concentrate the bacteria, reduce or substitute the enrichment time. Improvements in the ISO protocols were already implemented, with the inclusion of the Immunomagnetic Separation (IMS) for the detection of *E. coli* O157 [34] as previously described, to capture the specific bacteria cells, and separate them from the remaining microorganism present, to allow a specific detection with downstream culture-based methodology. IMS consist in the binding of a specific antibody to a magnetic particle to allow its recovery by magnetic force, which has been studied to be use for the detection of several pathogen to shorten the enrichment or even replace it completely thanks to the possibility of concentrating the microorganisms of interest [66–68]. Similar approaches involving the use of specific ligands, as antibodies in a solid phase have emerge with the same objective [69,70], and having the advatage to concentrate the bacteria, separating them from the food matrix and enrichment solution, allowing the removal of inhibiting compounds of the DNA amplification reaction or other molecular approaches [71].

Other alternatives for an indirect detection of the pathogen, as Bacteriophage amplification assays (PAA), have been reported to decrease the analysis time [72]. With the addition of a specific phage which will infect only living cells, the method takes advantage of its faster replication compared to the bacteria. Besides reducing the time of analysis, this methodology also allow to detect only viable pathogen cells and has been combined with qPCR for the detection of plant and human pathogens [73,74].

The matrix lysis approach developed by Rossmanith et al., [75], and further optimized later [76] relies in a completely different strategy, where the enrichment step is totally removed. The method consists on the degradation and solubilisation of the whole sample in order to obtain a sufficiently small pellet capable to be fully processed in the DNA extraction step for later DNA amplification analysis. However the authors were only capable to perform the analysis in 6.25 to 12.5 g or mL of sample, as the bigger quantity of starting sample, the bigger the pellet recovered will be and this makes it harder to process. This novel approach showed to be able to detect different foodborne pathogens, such as *S. aureus*, *Salmonella* spp. and *L. monocytogenes* in several food matrixes ([77–79]).

With the same purpose to decrease the time spent in the sample pre-treatment, a different procedure, known as short enrichment, has been developed combining a reduced incubation in an enrichment medium, with the degradation of the food debris recovered from the liquid portion, and the recovery of the bacteria by centrifugal steps. Fachmann et al. use this methodology allowing the detection of *Salmonella* spp. in meat samples in a short enrichment of 3 h [80].

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Overall different strategy could be optimized to reduce the time of sample pre-treatment and allow a sensitive detection of pathogens in food commodities. Some of them, presented in **Figure 1.8** were evaluate in this project in order to understand their advantage and limitations.

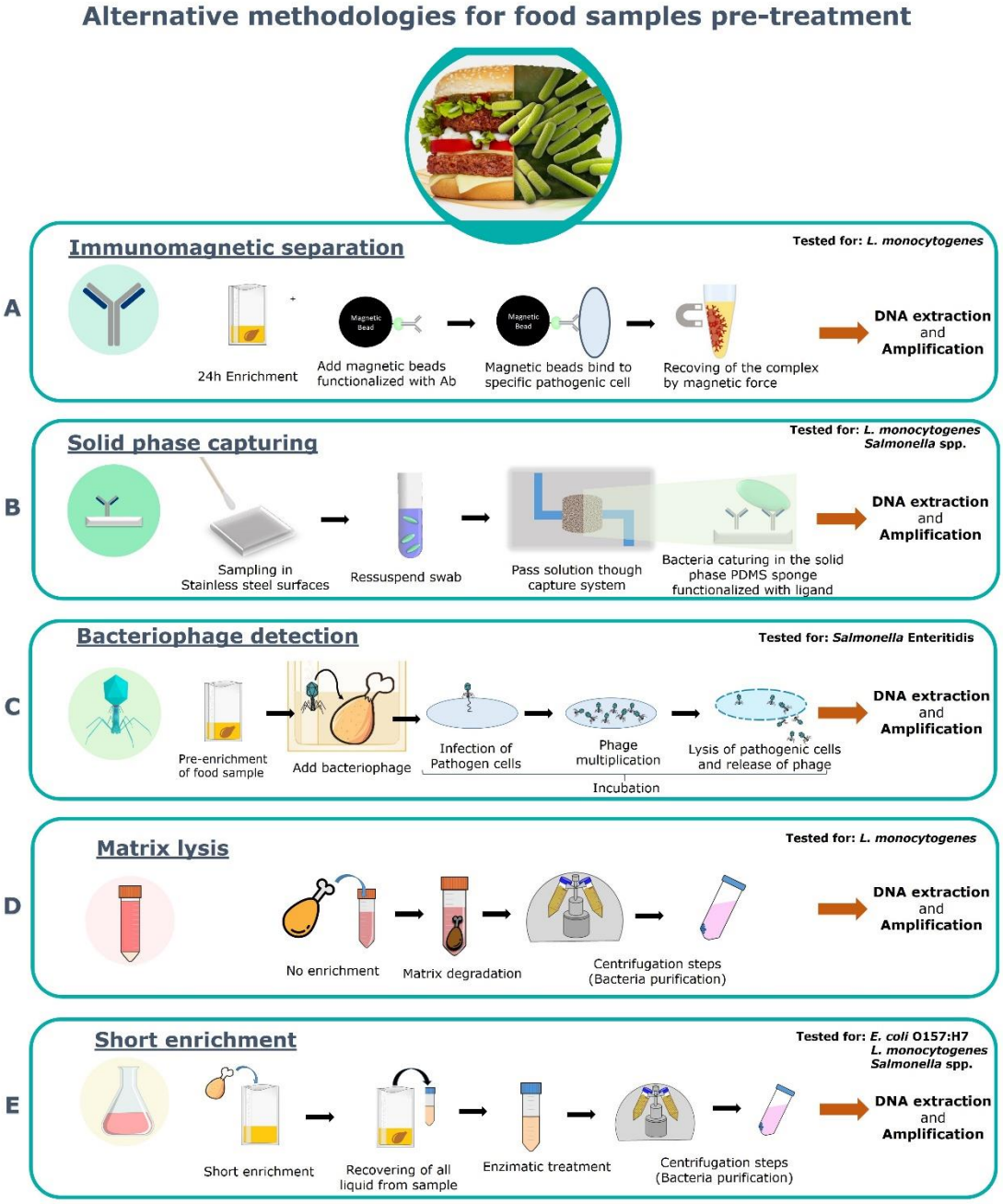

Figure 1.8. **Graphical representation of the alternative methodologies to improve the sample pre-treatment tested in this project.** (A) For the Immunomagnetic separation (IMS) the magnetic beads were functionalized with a specific antibody for the detection of *L. monocytogenes*. (B) The solid phase capturing approach was tested using a PDMS sponge inserted in a microfluidic device: for the specific detection of *L. monocytognes* when the sponge was functionalized with a specific antibody; or for multiplex detection of *L. monocytogenes* and *Salmonella* spp. by a non-targeted detection using a universal ligand, ApoH protein. (C) The indirect detection using bacteriophage was also tested for the specific detection of *S.* Enteritidis. (D) The matrix lysis approach was first developed by Rossmanith et al., [75] for smaller samples size and in this work the detection of L. monocytogenes in 25 g of samples was evaluated. (E) Finally the short enrichment reported by Fachmann et al [80] approach was tested for the detection of *L. monocytogenes* and *E. coli* O157 in simplex and a multiplex detection of *E. coli* O157 and *Salmonella* spp..

1.5.4 Polymerase Chain Reaction (PCR) and real-time PCR (qPCR)

Trying to overcome the problems found in the traditional culture-based methodologies, molecular approaches, such as those based on nucleic acids have been studied. Amplification methods due to their ability to increase the concentration of DNA are especially important for the detection of microorganisms in very low numbers. PCR is the gold standard amplification technique. The technique consist on the denaturisation of the double-stranded DNA (dsDNA) at high temperature (95 ºC), then the temperature is decreased to enable the annealing of the primers (56 ºC) and finally the temperature is increased again (72 ºC) to proceed with the extension of the primers using as template the complementary sequence. This last two step can be combined in one single step at an intermediate temperature (60-65 ºC). For the conventional PCR, also called end-point PCR, the results need to be visualized after reaction in an agarose gel after electrophoretic separation. The PCR reaction can also be performed in real-time by the monitoring of a fluorescent dye, not requiring additional manipulations of the amplification product. In real-time PCR (qPCR), the fluorescence obtained is plotted against the quantification cycle (Cq), when the florescence amount is higher than the background. Thanks to this, an inverse correlation can be established between the Cq and the concentration of DNA loaded, serving as a relative quantification of the target.

The fluorescence signal can be generated by two different chemistries. The first approach consists on the binding of a DNA intercalating dye to the dsDNA molecules generated during amplification, being SYBR Green the most commonly used for this purpose. One of the inconvenients of this method is that these dyes will bind to any dsDNA present in the reaction, and if non-specific amplification are originated that can lead to false positive results. However this effect can be avoided by the analysis of melting curves, which allow to differentiate between the different amplification products. The melting analysis consists in the assessment of the dissociation characteristics of the amplicon fragment generated, being the value of melting temperature (Tm) obtained when 50% of the DNA molecules are single-stranded (ssDNA). The melting temperature is influenced not only by the length of the DNA fragment, but also by the guanine-cytosine (GC) content, which will allow to discriminate between the specific and non-specific amplicons presents in the reaction [81].

Another way to obtain the fluorescent signal tracked in the real-time approach is the use of dual-labelled probes, also called hydrolysis or TaqMan™ probes, which are complementary to the amplicon, increasing the specificity of the reaction. This probe have a fluorophore attached to the 5' and a quencher to the 3' which absorbs the fluorescent signal when the probe is intact. However, over the amplification the probe is hybridised to its target site in the annealing step, and in the extension step, the polymerase will cleave the probe due its 5'-> 3' exonuclease activity, separating the fluorophore from quencher and allowing the detection of the signal.

qPCR are now routinely used for the detection of pathogens in different type of samples in the clinical field. Also in the food industry the ISO regulation already integrated this technology in different standards, being the first one, ISO 13136:2012 for the detections of STEC and determination of O157, O111, O26, O103 and O145 serogroups, and more recently the ISO 15216:2019 for the detection of hepatitis A virus and norovirus by RT-qPCR. Different guidelines have been set in order to develop a PCR/qPCR methodology and evaluate its performance and validate its implementation in the food industry [82–84].

An important reason for the use of DNA-based techniques in the detection of pathogens is the decrease in the analysis time. By the ISO 13136:2012 the STEC identification can be obtained in only 3 days (**Figure 1.9**), comparing with the 5 days with the previous protocol.

Additionally to the protocols already implemented in the ISO regulation, different PCR kits have been validated according with ISO 16140, in order to be used for the detection of other foodborne

pathogens, as BAX® System (Hygiena), BACGene (Eurofins), SureTect™ (Thermo Scientific) among others.

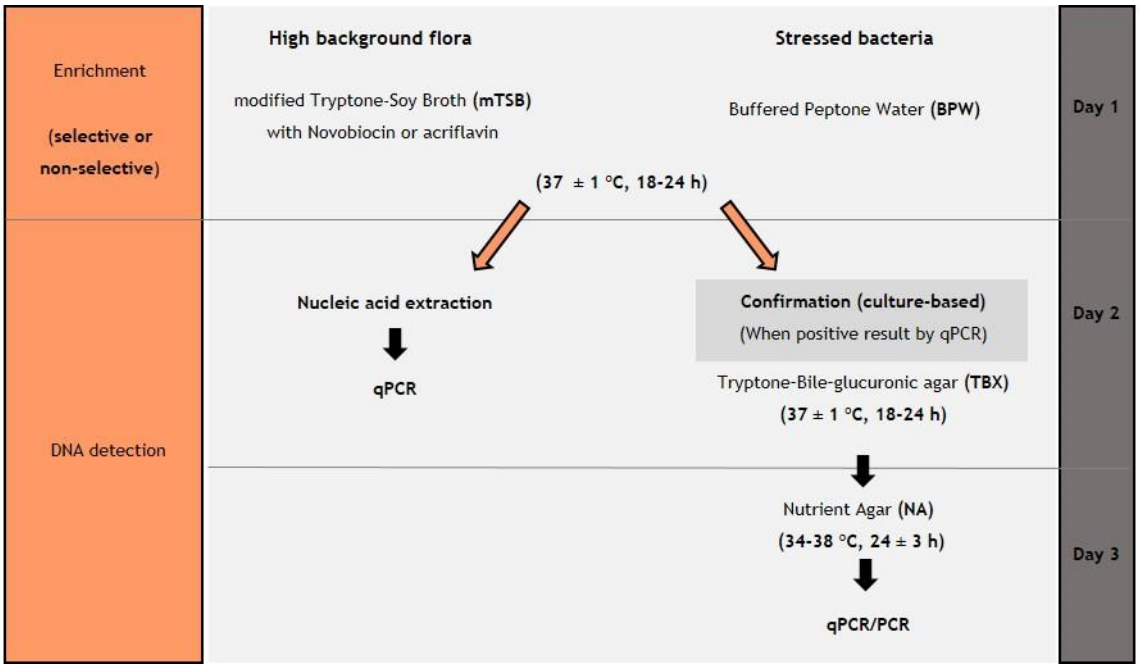

Figure 1.9. Scheme of the horizontal method for the detection of Shiga Toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups (ISO/TS 13136:2012). In this methodology the sample pre-treatment was also improved, specifying distinct enrichment depending on the type of samples analysed, being mTSB supplemented with Novobiocin or acriflavin where high background microflora is normally present, and BPW, a general medium, when stressed bacteria could be present.

1.5.5 Alternative isothermal amplification

As PCR needs complex equipment to perform fast, and accurate changes of temperature, the development of isothermal amplification techniques have arisen interest. These allowed to reduce the cost of the analysis and made easier their integration in portable platforms, as well as to reduce energy consumption.

Many different techniques, performing isothermal amplification, have been described in the literature, being the first one Nucleic acid sequence-based amplification (NASBA) developed in 1991, which allowed to amplify single-stranded RNA (ssRNA). Since its development, other options have emerged not only for RNA but also for DNA amplification with diverse enzymatic mechanisms such as Loop-mediated isothermal amplification (LAMP), Recombinase polymerase amplification (RPA), Rolling circle amplification (RCA), Strand displacement amplification (SDA), Polymerase spiral reaction (PSR) among others [85]. In this project, RPA and LAMP will be the alternative amplification techniques tested due to their advantage and possibilities for naked-eye visualization of the results.

1.5.5.1 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is the most studied technique for isothermal DNA amplification [86]. It was originally described in 2000 [87] attracting attention due to its potential to substitute the traditional PCR analysis, by the rapidity and accuracy of the reaction

achieved in constant temperature (60-65 ºC), with results between 30 min to 1 h . The amplification is performed thanks to a DNA polymerase with strand-displacement activity and 4 to 6 primers (outer, inner and loop primers). To begin with, the formation of loops at the end of the specific sequence need to occur to allow the exponential cycling amplification and elongation. In this sense, first the 2 inner primers, FIP and BIP, which are composed by two different region recognition (F2c-F1 and B1-B2c), hybridize with the complementary target in the F2c/B2c region, leaving the F1/B1 part of the primer free. The sequence is then extended by the polymerase. This product is displaced by the syntheses of a second strand, initiated by the outer primers (F3/B3). The ends of the first product are now free do form a loop with the hybridization of the F1/B1 region, called dumbbell structure. In this format the sequence contain multiple site to initiate the syntheses in the open loop. The amplification proceed from these multiple sites, where the products grows and forms long concatamers. The loops primers (LF/LB) are optional and accelerate the reaction, by providing additional starting points for the polymerase, **Figure 1.10** illustrate the LAMP reaction. Different ways to visualize the results can be achieved by this technique, as presented in section 1.5.5.3.

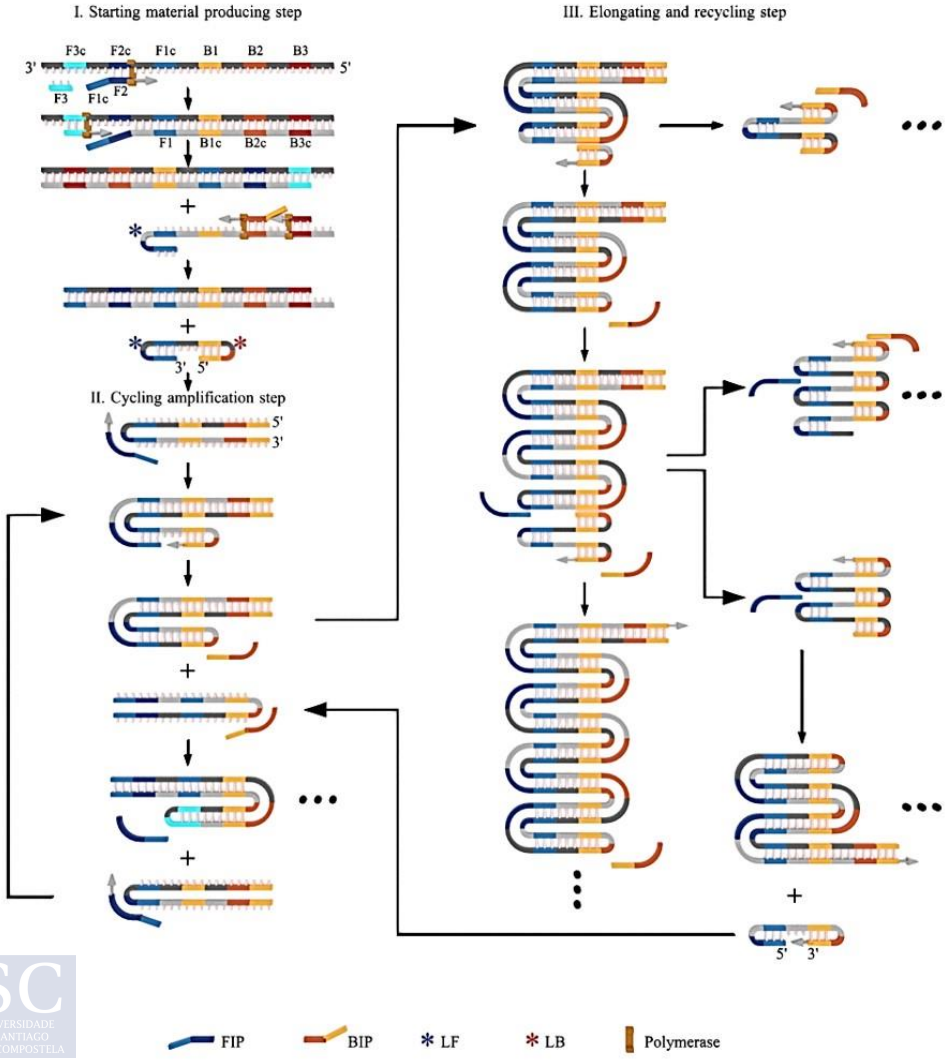

Figure 1.10. Loop-mediated isothermal amplification (LAMP) reaction (image used with licence from JOHN WILEY AND SONS [88]).

1.5.5.2 Recombinase polymerase amplification (RPA)

Recombinase polymerase amplification (RPA), first described in 2006 by Piepenburg et al. [89], has attracted the attention of many researchers due to its specificity, cost-effective approach and for allowing further simplification of assay development and optimization. This technique only needs two primers (PCR primers are fully compatible), the results can be obtained in 10-20 min, and it works a low operation temperature (37–42 °C).

As the isothermal amplification does not denatures the double stranded DNA by temperature increase, RPA relies on 3 key proteins for this step, a Recombinase (T4 UvsX protein), a Recombinase loading factor (T4 UvsY protein) and a Single-strand DNA binding protein (SSB) (T4 gp32). To achieve the hybridization of the primers to their target sequence, the Recombinase binds to the primers supported by the Recombinase loading factor, forming the nucleoprotein filament. This complex will scan the double-stranded DNA sequence for the target region and when it founds the homologous sequence, the invasion occurs, creating a D-loop. To avoid rebinding of the double stand, the SSB stabilize the single stranded DNA unbounded and the recombinase is then released leaving the primers available for the DNA polymerase to synthetize the complementary sequence [90]. The scheme of the RPA reaction is presented in **Figure 1.11**.

Different alternatives to the basic reaction have been created depending on the detection method desired, as presented below.

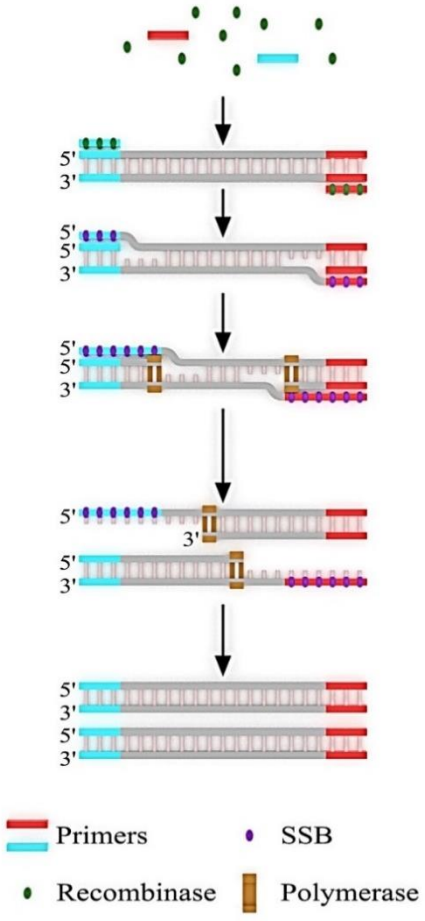

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Figure 1.11. Recombinase polymerase amplification (RPA) reaction. ((image used with licence from JOHN WILEY AND SONS [88]))

1.5.5.3 Results visualization methodologies available for LAMP and RPA

Several kits sold by different companies are in the market to perform these DNA amplification techniques for different applications in health but also for the food industry, and the results observation have also suffer growing improvement

The amplicons generated by these techniques can all be visualized by gel electrophoreses, being this approach the first used for PCR analysis. Electrophoresis remains a good way to confirm the presence of the specific amplicon, but also serves to verify the presence of non-specific amplicons. However this technique is time-consuming and laborious. For this reason different alternatives have been developed to allow a real-time, or a naked-eye, end-point detection for isothermal amplification. The real-time detection by LAMP can be achieved by the addition of a florescent intercalating dye, while for RPA a probe based approach must to be employed for this purpose.

Even though the real-time approach opens other possibilities, such as the quantification of the target or the discrimination between specific or non-specific amplicons, it needs specific and expensive equipment to visualize the results. For this reason, naked-eye detection simplifies the analysis, giving the possibility to be performed by unspecialized personnel, and making the analysis inexpensive.

Different alternatives are available to be used with each one of these isothermal amplification techniques. The amplification by LAMP can be observed by the monitoring of a white precipitate originated by the presence of insoluble $Mg_2P_2O_7$, a by-product of the reaction, being possible to follow the results in real-time using a photometer as Loopamp™ (Eiken Chemical, Japan) or as end-point analysis, identifying the presence of turbidity. This has been the standard, most common approach used for LAMP product detection, but others have emerged, such as the addition of dyes before or after the incubation to allow a colorimetric reaction [91–93].

For the RPA technique, also different strategies have been described to allow a visualization of theresult, without the need of a thermocycler. TwistDX, the developer and supplier of the RPA reagents, besides the basic kit, intended for gel electrophoresis detection, and the probe based real-time mastermix, also offers a lateral flow option [89]. Other studies have been conducted to develop more alternatives, like fluorescence [94,95] or change of colour [96,97].

1.6 DEVICES FOR DNA AMPLIFICATION

1.6.1 Equipment in the market

Automatic systems have also been developed implementing isothermal techniques. The Molecular Detection System from 3M use the isothermal DNA amplification, LAMP, combined with bioluminescence detection for the identification of several foodborne pathogens. Also Optigene, integrated the same technique in the automatize Genie® device, which rely on the detection of fluorescence. On the other hand, AXXIN developed different systems with multichannel fluorescence detection that can be used for both RPA and LAMP reaction, as a temperature range of 37˚C to 65˚C is possible. The preparation of the reaction can also be performed in a workstation, like the one from Hamilton® foodInspect™ NIMBUS®. However this platforms represent a high investment as this equipment are complex and required to be set in a laboratory with highly experienced personal. Furthermore, these workstation go against the need for on-site analysis, without any possibility to be portable.

1.6.2 Devices under study

Lab-on-chip devices, where all steps of the analysis are integrated in a miniaturized platform, are possible, due to the rapid advances in micro- and nano-frabrication. Besides the portability, which allows for an *in-situ* analysis, the low volume of sample and reagents, low energy consumption and rapid response makes these technologies ideal for point-of-care testing. Isothermal amplification has the advantage of being easy to integrate in miniaturized devices, without the need for instrumentation with high power consumption due to the requirements of fast ramps of temperature changes such as in PCR.

Different studies have reported the development of microdevices for the detection of foodborne pathogens. Lee et al. described a paper-infused LAMP reaction incorporate in plastic portable microdevice to detect *E. coli* O157:H7, *Salmonella* spp.,and *S. aureus* in milk [98]. Also RPA was reported to be suitable for integration in miniaturized systems as exemplified in a study using a centrifugal microfluidic platform for *Salmonella* enterica, *E. coli* O157:H7, and *Vibrio parahaemolyticus* [99]. This two examples clearly demonstrate the possibility for ease of multiplexing in miniaturized devices. However, none of these systems have successfully reached the market, as further improvements in their development are required.

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CHAPTER 2.

OBJECTIVES

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2 OBJECTIVES

2.1 MAIN OBJECTIVES

The main objective of the present work was the development of a new analytical method to enable the detection and identification of three of the most problematic foodborne pathogenic bacteria, *Salmonella* spp., *E. coli* O157 and *L. monocytogenes* in food samples reducing the time of analysis and evaluating the possibility of its integration in miniaturized devices.

2.1.1 Specific Objectives

To fulfil this main objective, the specific objectives were

- To develop, optimize and test and improved sample pre-treatment strategy for optimal recovery of the three foodborne pathogens of interest in multiplex, allowing a reduction in the time spent in this critical part of the analysis.

- To develop, optimize and compare highly specific isothermal amplification methods, LAMP and RPA, to obtain a sensitive analysis detection and allowing a naked-eye detection of the three pathogens of interest.

- To develop an optimized methodology based in the results obtained with the previous approaches tested and to integrate the DNA amplification step in a miniaturized device.

- To evaluate the novel protocol and device against traditional detection methods.

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2.2 THESIS STRUCTURE

The thesis was divided in seven chapters.

- Chapter 1 correspond to a general introduction, where the importance of the detection of foodborne pathogen was state and the different methodology use in the food industry for this purpose were presented. Beside traditional techniques (culture-based), a brief explanation of DNA amplification by PCR/qPCR and isothermal amplification (RPA and LAMP) was included, and as well as the different product and devices available in the market;

- Chapter 2 state the objective of the project;

- Chapter 3 describe the Methodology employed to perform the project;

Results and Discussion was separated in three chapters (sample pre-treatment approaches, DNA amplification/ detection methods and Final methodology):

  - Chapter 4: results of the evaluation of different approaches to improve the Sample pre-treatment;

  - Chapter 5: the assessment of results testing several alternatives for the DNA amplification, in order to obtained a isothermal amplification allowing a naked-eye detection;

  - Chapter 6: the optimization and validation of the final methodology, and the integration on miniaturized device, testing two different systems;

- Chapter 7: summarize the final conclusions of the work presented and future work is proposed to complement the results obtain and improve the methodology for the detection of foodborne pathogens in food samples.

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CHAPTER 3.

METHODOLOGY

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3 METHODOLOGY

3.1 INTRODUCTION

In order to develop a new methodology for a faster, but reliable detection, the different steps of the analysis were optimized as defined in the objective of the project, involving the improvement of sample pre-treatment, DNA amplification and optical detection.

To improve the sample pre-treatment different approaches were evaluated to concentrate the bacteria or reduce the time of analysis. First, the analysis of a standard 24 h enrichment was tested with several selective and non-selective media to enhance the growth and competiveness between the targeted microorganisms. The concentration of bacteria to increase the sensitivity of the methodology was evaluated by Immunomagnetic separation (IMS) and secondly using a functionalized solid phase to capture the bacteria. Finally, protocols for the reduction of the enrichment time were developed and tested, including an indirect detection using bacteriophages and other two approaches involving the degradation of the matrix and a shorter enrichment.

The second step, consisted on the evaluation of different DNA amplification techniques with the aim to obtain a methodology which would allow naked-eye detection and could be easily integrated in a miniaturized device. We focused in the study of two main isothermal amplification techniques, RPA and LAMP, which were first compared against qPCR implementing two different detection chemistries (SYBR-qPCR and Probe-qPCR). Two naked-eye detection strategies were evaluated for RPA (RPA-LF and RPA-SYBR), and three other for LAMP (Turbidity, MUA-AuNP and colorimetric mastermix).

All these approaches were used for the analysis of different samples and targeting different pathogens, namely *L. monocytogenes*, *Salmonella* spp. or *E. coli* O157, in order to compare their performance.

After the final methodology was selected, the ability to detect these three microorganisms was evaluated, as well as the integration of the DNA amplification part in a miniaturized device. This chapter describes the protocols and reagents used to perform this complete analysis and optimization to reach the final methodology.

3.2 REFERENCE BACTERIA STRAINS USED

To perform all experiments developed in this project, reference strains acquired from the Spanish Type Culture Collection (CECT), presented in **Table 3.1**, were used, including *Listeria monocytogenes* serovar 4b (WDCM 00021), *Escherichia coli* serotype O157:H7 (WDCM 00014) and *Salmonella enterica* serovar Typhimurium (WDCM 00031). The code provided for these strains correspond to the reference from the World Data Centre for Microorganisms (WDCM). A non-toxicogenic strain of *E. coli* O157:H7 was selected for safety reasons. A wild strain of *Salmonella enterica* serovar Enteritidis (S1400) from poultry, was an exception, belonging to the private collection of the University of Bristol.

Table 3.1. List of reference strains use in the approaches tested

Bacteria species	WDCM reference	Acquired from
L. monocytogenes	WDCM 00021	CECT
E. coli O157	WDCM 00014	CECT
Salmonella enterica serovar Typhimurium	WDCM 00031	CECT
Salmonella enterica serovar Enteritidis	-*	S1400 (UB)

WDCM: World Data Centre for Microorganisms; CECT: Spanish Type Culture Collection; UB University of Bristol
* Wild strain obtained from UB private collection. Do not have a WDCM reference

All the spiking experiments were performed with fresh cultures, which were prepared by inoculating a single colony of the corresponding bacterium in 4 mL of a general media: Buffered Peptone Water (BPW, Biokar diagnostics S.A., France) or Nutrient Broth (NB, Biokar Diagnostics S.A., France). The culture was incubated overnight (ON) at 37 °C. Concentration of bacteria used in each assay, was calculated by preparing ten-fold serial dilutions of the initial culture in the same general media and two dilution were plated in duplicated on Tryptic Soy Agar (TSA) (Biokar Diagnostics S.A., France) for *E. coli* O157:H7 and *Salmonella* spp., and on Tryptic Soy Yeast Extract Agar (TSYEA, Biokar Diagnostics S.A., France) for *L. monocytogenes*. The plates were incubate ON, at 37 °C, and resulting colonies counted. The following formula was used to obtained the starting bacterial concentration, as specified in the FDA/BAM standard method [100] :

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2)] \times (d)} \qquad \text{Eq. 1}$$

N: plate counts in a sample

C: The total number of colonies on the plates

N_1: The number of colonies on the plates of the first proper dilution degree;

N_2: The number of colonies on the plates of the number of colonies on the plates of the second proper dilution degree;

D: Dilution Factor (the first dilution degree).

3.3 DNA EXTRACTION

3.3.1 Pure culture

To obtain the DNA extract from pure culture to be used in the following experiments a simple thermal lysis was performed. Briefly, 1 mL of a ON cultures obtained as described in section 3.2, was first centrifuged at 16,000 × g for 5 min to concentrate the bacteria, the supernatant was removed, the pellet resuspended in 1 mL of TE 1X (10 mM Tris-HCl, 1mM EDTA (Sigma–Aldrich, USA, pH 7.5) and centrifuged again in the same conditions. Again, the supernatant was discarded, the pellet was resuspended in 300 µL of TE 1X. This new bacterial suspension was incubated for 15 min at 99 °C, with constant agitation (1400 rpm), to lyse the cells in a Thermomixer comfort (Eppendorf AG, Germany).

Finally, the thermally lysed bacteria were centrifuged at 16,000 × g for 5 min at 4 °C, to separate the DNA (supernatant) from the other cell debris (pellet). The supernatant was transferred to a clean tube, and stored at −20 °C until needed. This methodology was performed to obtained DNA standard from the targeted pathogens, but also from other strains to evaluate the inclusivity and exclusivity of the amplification techniques.

3.3.2 Complex food matrixes

For the DNA extraction from food matrixes, two different approaches were performed depending on the type of bacteria to be detected, unless otherwise specified. In this sense, the extraction of DNA from *E. coli* O157 and *Salmonella* spp., which are Gram-negative bacteria, was performed by thermal lysis. For *L. monocytogenes*, this step needed the addition of an enzymatic mixture in order to cause disruption of the cell wall, due to the strong peptidoglycan barrier, characteristic of Gram-positive bacteria. Before starting with the DNA extraction process, the samples were submitted to a washing step. Briefly, 1 mL of the pre-enriched sample was centrifuged at 380 × g, for 2 min to pellet any large food particles, and the supernatant was transferred to a new tube, which was centrifuged at 16,000 × g for 5 min. The supernatant was discarded, and the pellet was resuspended in PBS. The suspension was centrifuged again under the same conditions. The supernatant was discarded again, and the pellet was processed for thermal lysis or enzymatic lysis as described in section 3.3.2.1 and 3.3.2.2.

3.3.2.1 Thermal lysis

The thermal lysis consisted in the resuspension of the pellet in 300 µL of 6 % Chelex®100 (w/v. Bio-Rad Laboratories, Inc., USA) and incubated at 56 °C for 15 min. The addition of 25 µL of Proteinase K (10 mg/ mL, Macherey-Nagel, Germany) was also included in this first incubation step for the RPA-LF approaches (section 3.5.3.1.3) when surface samples were analysed. The lysis is then performed by heating the samples at 99 °C for 10 min. Both incubation steps were performed with constant agitation in a Thermomixer comfort (Eppendorf AG, Germany). Finally, the samples were centrifuged at 16000 × g at 4 °C for 5 min and the supernatants were transferred to new, clean, and sterile tubes.

3.3.2.2 Enzymatic lysis

The DNA extraction from food samples spiked with *L. monocytogenes* was performed based on the Lysis-GuSCN method described by Kawasaki et al., [101] and modified by Garrido et al., [102]. The pellet obtained from section 3.3.2 was treated with 200 µL of an enzymatic solution containing 1 mg/ mL of achromopeptidase (Sigma–Aldrich, USA) and 20 mg/ mL of lysozyme (Sigma–Aldrich, USA) in TE 2X with 1.2% of Triton X-100 (Sigma–Aldrich, USA). The samples were incubated for 30-60 min at 37 °C, with constant agitation in a Thermomixer comfort (1400 rpm). After the incubation, 300 µL of a solution containing 4 M of Guanidine isothiocyanate (Sigma–Aldrich, USA) and 1 % of Tween 20 (Sigma–Aldrich, USA) were added, and 400 µL of this solution were transfer to 400 µL of 100 % isopropanol (Sigma–Aldrich, USA), and centrifuged for 10 min at 16,000 × g. The pellet was rinsed with 1 mL of 75 % isopropanol, resuspended in 160 µL of sterile Milli-Q water, and incubated at 70 °C, for 3 min. The DNA was separated from any remaining debris by a 5 min centrifugation at 16,000 × g and 4 °C, and supernatant use for DNA analysis.

3.4 PROTOCOLS FOR IMPROVED SAMPLE PRE-TREATMENT

3.4.1 Comparison of different media for standard 24 h enrichment

3.4.1.1 Growth kinetic model and statistical analysis

According with the regulation, different selective media have to be used for the detection of specific pathogens, as described in Chapter 1, section 1.5.1. However none of them allows the recovery of different types of pathogens at the same time, making necessary a specific medium for the recovery and isolation of each targeted bacteria. The aim of this project was to develop a full methodology for the multiplex detection of three different pathogens, for this reason, the first task was the optimization of the enrichment medium, in order to improve the growth of all three target microorganisms, particularly *L. monocytogenes*, as it presents a lower growth rate compared with *Salmonella* spp. and *E. coli* O157. Additionally, the competition for nutrients between organisms can also delay the growth of this pathogen, thus the major concern was to improve its growth rate, allowing higher competitiveness. For this reason several media were tested and compared to determine which one was the best for the recovery of *L. monocytogenes*. Attending to this, to perform an analysis of food samples with the aim of detecting *L. monocytogenes*, different selective media are specified in EN ISO 11290-1:2017 [103], USDA-FSIS [104] and FDA/BAM chapter 10 [105]. Half Fraser (HF) (ISO method), Buffered Listeria Enrichment Broth (BLEB) (BAM Media M52), Listeria Enrichment Broth (LEB) (USDA-FSIS method) and Oxoid Novel Enrichment (ONE broth) a commercial selective medium, were first evaluated as an option to allow the growth of *L. monocytogenes*, inhibiting the growth of the other two pathogens.

Then three non-selective media with different modifications on the formulation, as specified in **Table 3.2**, were tested to enhance the growth of this bacterium, trying to reduce the lag phase and increase the final concentration of bacteria obtained after 24h. Three different version of mTA10 media, were tested. First the basic formulation containing 10 g/ L Tryptose (Pronadisa, Spain), 5 g/ L Beef extract (Scharlau Chemie S.A., Spain), 5 g/ L Yeast extract (Difco, BD & Co., USA), 5 g/ L NaCl, 3.4 g/ L KH_2PO_4 and 19.3 g/ L Na_2HPO_4, then replacing the KH_2PO_4 and Na_2HPO_4 by 8.5 g/L of 3-(N-morpholino)propanesulfonic acid (MOPS) and 13.7 g/ L MOPS sodium salt [106,107], and also testing the addition of 0.5 g/ L of glucose in mTA10-MOPS. D-(+)-Cellobiose showed promising results to enhance the growth of *L. monocytogenes* in previous studies [108,109], and for this reason the effect of different concentration (0.25, 5, 20 g/ L) of this compound in mTA10-MOPS was tested.

Additionally, two general media, Tryptic Soy Broth (TSB) (Biokar diagnostics S.A., France) and Brain Heart Infusion (BHI) (Biokar diagnostics S.A., France), were evaluated in their original formulation, and supplemented with yeast extract (6 g/ L) and sodium pyruvate (1 g/ L). All the chemical for media formulation and supplements, including the glucose and cellobiose were acquired from Sigma-Aldrich (USA).

To evaluate the different media, the growth of *L. monocytogenes* was monitored over 24 h. In 96 well-plates, 200 µL of each medium were inoculated with 2 µL of fresh bacterial culture prepared as described above in section 3.2, with a final concentration of 10^2–10^3 cfu. The absorbance at 600 nm was measured every 30 min during 24 h in a Microplate Reader (Synergy, BioteK H 1, USA), with constant agitation.

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Table 3.2. Non-selective media formulation and variations tested

Based medium	Composition	Modification 1	Modification 2
mTA10	10 g/ L Tryptose, 5 g/ L Beef extract 5 g/ L Yeast extract, 5 g/ L NaCl, 3.4 g/ L KH_2PO_4 19.3 g/ L Na_2HPO_4	- - - - 8.5 g/L MOPS 13.7 g/ L MOPS sodium salt 0.5 g/ L of glucose*	- - - - 8.5 g/L MOPS 13.7 g/ L MOPS sodium salt 0.25, 5, 20 g/ L Cellobiose
TSB	17 g/ L Tryptose 3 g/ L Papaic digest of soybean meal 2.5 g/ L Glucose 2.5 g/ L K_2HPO_4 5 g/ L NaCl	- - - - - 6 g/ L Yeast extract	- - - - - 6 g/ L Yeast extract 1 g/ L Sodium pyruvate
BHI	17.5 g/ L Pork brain heart infusion 10 g/ L Pancreatic digest of gelatin 5 g/ L NaCl 2.5 g/ L Na_2HPO_4 2 g/ L Glucose	- - - - - 6 g/ L Yeast extract	- - - - - 6 g/ L Yeast extract 1 g/ L Sodium pyruvate

mTA10 is a non-commercial medium, TSB and BHI commercial ones;
* mTA10 MOPS medium was tested with and without the addition of glucose.

After obtaining the data, microbial growth kinetics were modelled using a logistic equation, reparametrized according to Zwietering et al. [110] to explicitly show those parameters with a biological meaning:

$$OD(t) = \frac{OD''max''}{1 + e^{\left(\frac{4\,\mu''max''}{OD''max''}(\lambda - t) + 2\right)}} \qquad \text{Eq. 2}$$

where OD(t) represents the optical density, measured at 600 nm, at time "t", ODmax represents the maximum optical density, μmax is the maximum specific growth rate in h^{-1}, and λ is the lag time in h. Data fitting, assessment of the model parameters significance (Student t-test; $\alpha = 0.05$), and consistency of the mathematical model (Fisher's F test; $p < 0.05$) were performed with Mathematica 9 (Wolfram Research, Inc., UK).

After the parameters were adjusted with the model, the results were statistically analyzed with GraphPad Prism 5.0 in order to determine if significant differences were observed ($p < 0.05$) in the maximum absorbance, maximum rate of growth or lag phase of *L. monocytogenes* in the different medium tested, to this end Mann–Whitney U test was selected.

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3.4.1.2 Pure culture mix in different media

Competition among the different pathogens may occur and affect their growth, and consequently influence the sensitivity of the methodology. This aspect highlights the importance of determining the capacity of the target pathogens to grow simultaneously, and for this reason mixed cultures were performed and evaluated. Mixed cultures were prepared in 50 mL of mTA10-MOPS supplemented with the different compounds, and were inoculate with $10–10^2$ cfu of each target species and incubated at 37 °C, 24 h. After the incubation, ten-fold serial dilutions of the culture were plated on the following selective media: COMPASS Listeria (Biokar diagnostics S.A., Allonne, France), CHROMagar™ *Salmonella* Plus (CHROMagar, Paris, France) and CHROMagar™ *E. coli* O157 for the quantification of *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157, respectively. All plates were incubate at 37 °C, ON.

3.4.2 Concentration of bacteria

3.4.2.1 Immunomagnetic separation (IMS)

Even with the optimization of the enrichment conditions to improve the growth of targeted pathogens, the capacity to concentrate the bacteria present in a sample, represents a real advantage. Antibodies have been extensively used in the detection step of the analysis, where the binding of the target generate a reaction or a signal leading to the result, as happens in an ELISA assay. However, antibodies can also be used to concentrate the target pathogen when combined with DNA-based detection. This approach not only allows a more sensitive detection, but also allows to wash out several compounds capable of inhibiting the amplification reaction. The immunomagnetic separation (IMS) emerge from the developments in micro- and nanoscale technologies, with the functionalization of magnetic beads with specific antibodies. This allows the separation of the target bacteria by magnetic force, from the rest of the sample. To evaluate the IMS as an alternative to the usual 48 h of enrichment, for a reliable detection of *L. monocytogenes*, different commercial antibodies were evaluated, as well as the optimal protocol for the functionalization of the magnetic beads.

3.4.2.1.1 Antibody evaluation

After the study of the different antibodies available in the market, claiming to be specific for *L. monocytogenes*, four alternatives were chosen and evaluated attending to their purity and specificity. The ones providing the best results were further evaluated attending to their capture efficiency (CE) in the functionalized magnetic nanospheres (MNP). In **Table 3.3**, information about the two polyclonal and two monoclonal antibodies selected is detailed, as well as the secondary antibodies used for indirect ELISA.

In order to determine the purity of the commercial antibodies, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reduced conditions, using a protocol based on the method of Laemmli et al., [111]. Three dilutions of each antibody were prepared in order to obtain a final concentrations of 100, 10 and 1 µg/ mL. Before proceeding with the electrophoresis, the dilutions were reduced in an equal volume of Laemmli sample buffer (Bio-Rad Laboratories, Inc., USA) supplemented with 5% (V/V) of 2-mercaptoethanol and incubated at 95 °C

during 5 min. Electrophoresis was performed using 4–15% Mini-PROTEAN TGXTM Pre-cast gel (Bio-Rad Laboratories, Inc., USA), where 5 µL of ruler and 20 µL of reduced samples were loaded and run first 5 min, at 50 V, then the voltage was increased to 100V, and ran approximately 1 h. The detection of the protein in the polyacrylamide gel was achieved by Silver Stain PlusTM Kit (Bio-Rad Laboratories, Inc., USA), and the gels visualized by Gel DocTM EZ Imager, using 4.1 Image LABTM Software (Bio-Rad Laboratories, Inc., USA).

Table 3.3. Commercial antibodies evaluated

Name	Company	Clone name	Class	Isotype	Host
MA1-20271	ThermoFisher	LZF7	Monoclonal	IgG2a	Mouse
PA1-7230	ThermoFisher	-	Polyclonal	IgG	Rabbit
MAB8953	Abnova	3a15	Monoclonal	IgG2b	Mouse
MD-05-0329	RayBiotech	-	Polyclonal	Not defined	Goat
Goat Anti-Mouse IgG-HRP*	SantaCruz Biotechnology	-	Polyclonal	IgG	Goat
Goat Anti-Rabbit Ig, Human ads-HRP*	SouthernBiotech	-	Polyclonal	IgG	Goat
Chicken Anti-Goat IgG, HRP*	Novus Biologicals	-	Polyclonal	IgG	Chicken

*Secondary antibodies used for the indirect ELISA.

To evaluate the specificity of each commercial antibody, an indirect ELISA was performed, against the reference *L. monocytogenes*, and S. Typhimurium mentioned above (section 3.2.), as well as *L. innocua* WDCM 00017. In a 96 well Nunc MaxiSorpTM plate (Thermo Fisher Scientific, USA), 200 µL of a fresh bacterial culture, with a concentration of approximately 10^8 cfu/ mL, was added and incubated ON at 50 °C to allow the evaporation of the medium and the full coating of the plate with the bacteria. The following day, 100 µL of the antibody to be tested, diluted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to a final concentration of 10 µg/ mL, was incubated 1 h at room temperature (RT), with agitation. The plate was then washed three times with 200 µL of PBS to remove unattached antibodies, and a blocking step with 5% BSA in PBS, for 1 h, at RT with agitation was performed to fill empty spots where the secondary antibody could bind. To finalize the assay, after a new washing step, the deposition of 100 µL/ well of the respective secondary antibody, diluted 1: 500 in PBS, was performed and incubate again 1h at RT, with agitation. Finally the plate was washed one last time and the measurement of the fluorescence or chemiluminescence was done, using Synergy H1 Multi-Mode Microplate Reader (BioTek, USA).

For fluorescent detection, 100 µL of PBS was added to the wells and the reading protocol was fixed according with the Absorption/ Emission spectra of the antibody. The chemiluminescent detection, was performed using western blotting detection kit from GE Healthcare Life Sciences, where 100 µL of two reagents mixture (1:1) were added to the wells and the luminescence resulting from the reaction was read.

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3.4.2.1.2 *Magnetic beads functionalization and IMS protocol*

To use the commercial antibody with the best specificity results in the IMS approach, AbraMag Magnetic Nanospheres (MNPs, average size of 500 nm) coated with protein A, were purchased from Abraxis Inc. (Warminster, USA). To this end, first the MNPs were washed twice with 1 mL of 0.1 M sodium phosphate buffer with Tween20 (PBT, 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 0.05% Tween20, pH 7.4), being recovered with a magnetic particle concentrator (MPC) (Invitrogen, USA)) for 2 min. This step allowed for the recovery of only MNPs which retain magnetic properties. An antibody concentration of 60 µg/ mL was added to magnetic beads, in a final volume 10 times higher, to allow the distribution of the antibody through the particles. The solution was incubated for 1h at RT in a Mini Tube Rotator (Fisher Scientific) set at 10 rpm. Finally, the MNPs were washed again twice as described above, to remove the unbound antibody.

After the functionalization was completed, the MNP were ready to be used for the concentration of the targeted bacteria. Twenty microliters of MNPs were added to the solution to be treated (pure culture or sample), and incubated at room temperature for 15 min under constant mixing in a Mini Tube Rotator at 10 rpm. After incubation, the MNPs were separated with the MPC for 3 min, the buffer was removed and the MNPs with the bacteria cells attached were used for DNA extraction as described in section 3.3.2.2, with enzymatic lysis of 1h, and the qPCR was performed as detailed below in **Table 3.8** of section 3.5.2.2.

3.4.2.1.3 Capture efficiency

With the objective to understand the ability of the functionalized MNPs to retain the target bacteria, the capture efficiency (CE) was evaluated, based on the protocol described by Varshney et al.[112]. In this sense, a pure culture of *L. monocytogenes* was ten-fold serially diluted and 1 mL of each dilution was used for DNA extraction as described in section 3.3.1. In parallel, these dilutions were plated in TSYEA in order to plot cfu/ mL vs Cq values for the construction of a standard curve. After the standard curve was constructed, a diluted pure culture, with a theoretical concentration of 10^4 cfu/ mL, was treated in triplicate, following the IMS protocol as detailed in section 3.4.2.1.2, which correspond to the cells bound to the MNPs (Cb). Another three aliquots of the same dilution were not treated with IMS, representing the total number of cells in the sample (C0). DNA was extracted from all aliquots and the bacterial concentration was calculated by qPCR as the protocol described above in **Table 3.8** of section 3.5.2.2.

The CE was calculated with the following formula:

$$CE\ MNP\ (\%) = \left(\frac{Cb}{C0}\right) x\ 100 \qquad \text{Eq. 3}$$

This approach was used for the optimization of the IMS protocol. In addition to the protocol detailed for food samples in section 3.4.2.1.2, the inclusion of three additional washing steps were also tested as specified by the supplier protocol.

3.4.2.1.4 Analysis with complex food matrixes

Different types of food samples were tested with the IMS methodology including meat (chicken breast), dairy (hard and fresh cheese), and fish (anchovies) with different spiking levels. To proceed with their analysis, 25 g of corresponding food sample were weighted, mixed with 225 mL of HF broth and homogenized for 2 min at 230 rpm in a Stomacher 400 Circulator (Seward Limited, UK). The matrix was incubated at 30 °C for 24 h. One mL of 24 h enriched HF broth was taken to proceed with the IMS as described in section 3.4.2.1.2

For confirmation purposes, after enrichment a loop-full was streaked on COMPASS Listeria agar (Biokar Diagnostics S.A., France), the plates were incubated up to 48 h at 37 ºC and examined for typical colonies.

3.4.2.2 Miniaturized micro-device for bacteria concentration

A similar approach to the IMS is the concentration of the bacteria with a solid phase functionalized with a specific ligand. To this end, a methodology where a 3D Polydimethylsiloxane (PDMS) sponge integrated in a microfluidic device was developed to allow the specific or non-specific detection. This methodology was tested for the simplex detection of *L. monocytogenes* when a specific antibody is used, and to recover both Gram-positive and Gram-negative bacteria for a multiplex detection, when a universal ligand is employed.

3.4.2.2.1 Fabrication of PDMS sponge

To get a PDMS sponge with a defined pore size, NaCl (Sigma-Aldrich, USA) was grinded in order to obtained particles around 80–100 μm and then squeezed into an empty syringe working as a mold. A mixture of PDMS (Sylgard 184) with curing agent in ration of 10: 1 was then loaded into the syringe and using a vacuum force, the syringe was degassed to allow the PDMS to infiltrate into the salt template through capillarity, and to fill the air gaps present. The salt templates with the absorbed mixture were then incubated at 65 °C for 4 h and the cured PDMS was washed in a water bath, under continuous steering, and periodically renewed to completely dissolve the salt and form the microporous PDMS sponges. A specially designed microfluidic device was previously fabricated to incorporate the PDMS sponge with the fluidic connections as presented in section 3.7.1.

3.4.2.2.2 PDMS functionalization with ligand

After the fabrication of the sponge, the modification of the PDMS surface was performed to allow the binding of the ligand. To do so, the PDMS surface was first cleaned with isopropanol, dried with a nitrogen stream, and then treated with oxygen plasma for 2 min under vacuum using a plasma cleaner (Harrick, Germany). Right after surface hydroxylation, the samples were immersed in a freshly prepared 5% v/v solution of (3-Aminopropyl) triethoxysilane in ethanol, and incubated for 150 min at RT. The samples were then washed gently with ethanol, cured at 80 °C for 1 h, and sonicated in ethanol for 10 s to remove the physically unbound (3- Aminopropyl) triethoxysilane molecules. After a second rinsing with ethanol, a drying step was performed with nitrogen stream, and the obtained epoxide functionalized PDMS structures were then stored in a sealed container at RT until use. After surface

modification, the sponges were functionalized with either 5 μg/ mL of ApoH protein (ApoH-Technologies, France), for a non-specific bacteria targeting or with 10 μg/ mL of anti- *L. monocytogenes* antibody (Ab) (MAB8953, Abnova, Taiwan), previously evaluated and used in the IMS approach described above, for specific *L. monocytogenes* targeting. Two hundred microliters of the solution were added to a tube with the sponge, vortexed vigorously and incubated ON at 4 °C to allow the binding of the protein or antibody to the sponge. The sponge was then washed three times with PBS, and stored at 4 °C until use.

3.4.2.2.3 Bacteria concentration using pure cultures

To evaluate the capacity of the device with the PDMS sponge to concentrate the bacteria, the capture efficiency was calculated. For this, a pure bacterial solution of *L. monocytogenes* and *Salmonella* spp. separated and in co-culture, were flown through the sponge, with a controlled flow rate of 10 μL/ min. A washing step with 800 μL of PBS at the same flow rate was performed to recover all bacterial cells unbound to the sponge. The outlet solution was collected and used for culture plate counting to confirm the capture efficiency in the sponge. To determine the concentration of the non-captured bacteria, serial dilutions were made in PBS and plated on COMPASS and Xylose Lysine Desoxycholate Agar (XLD, Biokar diagnostics S.A., France) for the isolation of both pathogens separately. The capture efficiency was calculated using the following equation:

$$\text{CE sponge device (\%)} = \frac{\text{Nt} - \text{Ne}}{\text{Nt}} \text{ x } 100 \qquad \text{Eq. 4}$$

Where Nt is the number of bacterial cells in the sample introduced in the device and Ne is the number of uncaptured bacterial cells, recovered from the device outlet.

Concentrations between 10^3-10^5 cfu of each microorganism, in pure or in mix cultures, were passed through the device containing the sponge functionalized with ApoH protein and the anti-Listeria antibody, not only to determine the capture efficiency but also to define the limit of detection by qPCR using this approach. For the Limit of Detection (LoD) evaluation, after the sample solution passed through the device, DNA extraction from the sponge was performed by enzymatic lysis as described in section 3.3.2.2 with incubation of 30 min for downstream qPCR analysis, as described in section 3.5.2.2 in **Table 3.8**.

3.4.2.2.4 Evaluation of spiked surface sample

To ensure the reliability of the results, the detection of *L. monocytogenes* and *Salmonella* spp. were tested spiking stainless steel surfaces in order to test the applicability of the developed methodology in the evaluation of cleaning procedures in the food industry. To contaminate the surfaces, an ON culture was diluted and 10^5 cfu of a bacterial mixture was spread on the surface and it was allowed to dry at RT. The bacteria were recovered with a cotton swab pre-moisturised in PBS with 0.01% of Tween 80, and re-suspended in 2 mL of PBS by vortexing. One mL of the solution was passed through the device as specified in section 3.4.2.2.3, and the sponge treated for downstream DNA extraction and qPCR. The capture efficiency was also determine for these surface samples.

3.4.3 Protocols for time reduction

3.4.3.1 Phage Amplification Assay (PAA)

Another approach tested to improve the sample pre-treatment, is the phage amplification assay (PAA), as an indirect detection strategy. This approach was used for the detection of *S.* Enteritidis using the *Salmonella* phage vB_SenS_PVP-SE2 (GenBank accession no. MF431252.1), previously named ϕ38, isolated by Sillankorva et al., [113].

3.4.3.1.1 PAA optimization

For the determination of the phage stock concentration, ten-fold serial dilutions of the phage prepared in SM buffer (100mM NaCl, 50mM Tris, 8000mM $MgSO_4 \cdot 7H_2O$, pH 7.5) were performed, and a mixture containing 5 mL of molten semi-solid Luria-Bertani (LB) (7.5 g/ L agar), 100 µL of an ON culture of *S.* Enteritidis and 100 µL of the corresponding phage dilution were poured on solid LB. These plates were incubated at 37 °C, ON.

This methodology is based in the detection of the phage DNA, being limited by the ability of the amplification technique to detect it. For that reason, the first step was to establish the concentration to be use to infect the *S.* Enteritidis in the samples, which originate a positive result by qPCR. Ten-fold dilutions of the pure virus in BPW were directly analysed by qPCR, as describe in **Table 3.8** of section 3.5.2.2. The phage stock (dilution 0) was diluted 1:2 and this was used as the highest concentration.

The minimum time of enrichment to reach detectable levels of phage was also determined in spiked samples. For this, after a pre-incubation to grow *Salmonella* cells and allow a higher quantity of cells to be infected by the virus, the time to let the phage multiply inside the cells was analysed. To performed this evaluation, samples were treated as describe in section 3.4.3.1.2 and aliquots of 1 mL taken after different times of the second enrichment, 3h and 6h, and compare with samples without this incubation (T0).

3.4.3.1.2 Evaluation with complex food matrixes

Raw chicken breast samples were contaminated with four different contamination level: <10, 10-10^2, 10^2-10^3, >10^3 cfu/ 25g. The analysis was performed as following: 25 g were weighed and 225 mL of 37 °C pre-warmed BPW were added, the matrix was homogenized for 30 s in a Stomacher 400 Circulator (Seward Limited, UK); then 1 mL of the appropriate dilution of S. Enteritidis, prepared as mentioned above, was added and homogenized again for 30 sec. These samples were first incubated for 3 h at 37 °C with agitation (120 rpm). After this initial incubation step, 10^3-10^4 pfu/ mL vB_SenS_PVP-SE2 phages were added (concentration selected after evaluation of pure phage LoD) and the matrix was re-incubated to perform the second enrichment.

Confirmation of the presence of *S.* Enteritidis in spiked food samples after pre-enrichment, was performed by streaking the pre-enriched samples on XLD. The plates were incubated at 37 °C overnight. A different thermal lysis DNA extraction protocol was performed, were no washing step was made, and 1mL of sample was directly heated to lyse the bacteriophage as describe in section 3.3.1. Once finished, the samples were centrifuged at 4000 × g for 10 min and 4 °C.

To verify that the proposed methodology only detected viable bacteria, 6 additional samples were spiked with dead *S.* Enteritidis. Non-viable bacteria were obtained by an autoclave step at 121 °C for 30 min to completely inactivate the bacteria. Once the treatment was completed, 10^3 10^5 and 10^7 cfu/ mL of dead bacteria were added to the corresponding food sample, and processed as described above. The bacteria were plated before autoclaved to determine the concentration of dead cells.

3.4.3.2 Matrix lysis

In a molecular analysis, the standard procedure is to do an enrichment and take a small aliquot, normally 1 mL to be tested. In the Matrix lysis methodology the whole food sample was treated to degrade the tissue and concentrate the bacteria in a small pellet. The protocol was based on the one described by Rossmanith et al. [75] with some modifications in order to process 25 g of sample instead of the 6.25-12.5 g described by the authors, and in this study, this approach was developed for the recovery of *L. monocytogenes* from the sample. Thereby, 25 g of sample were added to a stomacher bag with filter, and homogenize with 40 mL of sucrose buffer (0.25 M sucrose, 1 mM EDTA, 0.05 M Tris, pH 7.6), for 30 s in a Stomacher 400 Circulator [76]. The liquid part was recovered and transferred to a clean 50 mL tube. Additional 10 mL of the sucrose buffer were added to the bag, homogenized for an additional 10 s, and the extra liquid was added to the same 50 mL tube, to reach 40-45 mL. The tubes were centrifuged at 8960 × g for 10 min, the supernatant was decanted and the pellet, containing bacteria and food debris, were resuspended in PBS to which 5 mL of the protease buffer (1/100 dilution in PBS of Alcalase and Neutrase, Novozymes, USA) were added. The samples were incubated horizontally at 37 ºC for 30 min with constant agitation (200 rpm), then the tubes were centrifuged again under the same conditions and once more the supernatant was decanted and the new pellet was resuspended in the lysis buffer as specified by different authors [76,114,115] containing 8 M urea, 1 M $MgCl_2$, 50 mM Tricine and 0.35 % of a surfactant mixture with a hydrophilic/ lipophilic balance equivalent to that of Lutensol AO-07 [76]. The mixture was incubated once more at 37 ºC for 30 min with constant agitation, and was followed by a new centrifugation and the supernatant was discarded. The pellet was resuspended in washing buffer containing PBS with 0.35 % of a surfactant mixture as above mentioned, incubated 37 ºC for 30 min with constant agitation, and centrifuged again. Finally, the pellet was recovered in 1.5 mL of PBS, transferred to a clean tube and centrifuged at 11000 × g for 5 min. The resulting pellet was used for DNA extraction as described above in section 3.3.2.2, reducing the incubation with the enzymatic solution to 30 min and qPCR process as mentioned in **Table 3.8** of section 3.5.2.2.

3.4.3.3 Short enrichment

Another approach to reduce the time of analysis, is based on the performance of a shorter enrichment and recovery of the total liquid from the enrichment for downstream treatment to remove the remaining food debris and obtained the bacteria cells pellet. This will allow to reach a lower LoD, thanks to this step of bacteria growth, when compared with the matrix lysis. The methodology was tested for *L. monocytogenes* and *E. coli* O157 in simplex, and for *E. coli* O157 and *Salmonella* spp. in multiplex. The modifications between the protocols used for each one is present in **Table 3.4**, as the enrichment conditions, type of samples analyzed, centrifugation speed and time, and type of DNA extraction applied.

Table 3.4. Short enrichment conditions

Pathogen tested	Type of samples	Enrichment media	Centrifugation conditions	DNA extraction used
E. coli O157	Ground beef Leafy greens	mTSBn (3h)	4700 × g, 5 min	Chelex®100
E. coli O157 *Salmonella* spp.	Ground beef Chicken breast	BPW + 0.4 % Tween 80 (3h)	8960 × g, 10 min	
L. monocytogenes	Smoked salmon	TSB (5h)		Lysis-GuSCN method

mTSBn correspond to the commercial modified TSB supplemented with 20 mg/ L of novobiocin.

3.4.3.3.1 Protocol optimization and sample treatment

Starting with *L. monocytogenes* detection, two aspect of the protocol were optimized, the volume of broth used to dilute the sample and proceed with the enrichment (25 mL vs 50 mL), and whether or not to perform the incubation under constant shacking (200 rpm). To perform this evaluation, the samples were spiked with 10^2-10^3 cfu/ mL, an aliquot was taken at T0, and a second one after 4 h of incubation (T4) under each condition. This aliquots were ten-fold serially diluted, plated on TSYEA and incubated overnight at 37 ºC, to determine the bacterial increase.

After this condition sets, the samples were analysed with the following established protocol. Twenty five grams were weighted in a stomacher bag with filter (< 250 μm) and the corresponding bacterial concentration was added, along with 25 mL of enrichment medium mentioned in **Table 3.4** pre-warmed at 37 °C. The matrix was homogenized for 30 s in a Stomacher 400 Circulator (Seward Limited, UK) or hand-massaged in the case of the multiplex detection, due to the type of sample analysed. The samples were incubated at 37 °C with constant agitation (200 rpm) for the corresponding time. After incubation, the whole liquid was recovered and transferred to a conical 50 mL tube. The tube was centrifuged at 8960 x g for 10 min and the supernatant was discarded, the pellet resuspended in 45 mL of protease buffer, as described above in section 3.4.3.2, and was incubated horizontally at 37 °C for 10 min at 200 rpm. After digestion, the samples were centrifuged again in the same conditions. Once more the supernatant was discarded, and the pellet was resuspended in washing buffer, as described in section 3.4.3.2 followed by a new centrifugation step. Finally, the new pellet was resuspended in 1.5 mL of washing buffer, transferred to a clean tube and centrifuged for 5 min at 11000 × g, and the pellet used for corresponding DNA extraction depending on the pathogen targeted, and downstream qPCR analysis as detailed in **Table 3.8** of section 3.5.2.2.

3.5 EVALUATION OF THE DNA AMPLIFICATION ALTERNATIVES

3.5.1 Primers design

For all amplification approaches specific primers had to be designed in conserved regions, in order to allow a specific and reliable detection of the pathogens. To ensure that the primers are targeting a common region within the gene of interest a consensus sequence was generated after the alignment of the target sequences with CLC Sequence Viewer (C L C Bio-Qiagen 2016). This consensus sequence was used as a template for the primer and probe design. For qPCR and RPA approaches the free online

software Primer3 [116] was used, and Primer Explorer V4 for LAMP primers (https://primerexplorer.jp/e/index.html). Different parameters were evaluated to confirm the primer performance, as the GC content, melting temperature (Tm), the probability to form secondary structures and primer dimers. Different genetic targets were tested and the choice of the target gene was made based in previously published studies describing their specificity to discriminate among strains. All primers were evaluated after being designed in terms of specificity by BLAST analysis.

In order to develop the amplification techniques for the detection of *L. monocytogenes*, three different genetic targets were tested, *actA*, *hlyA*, *plcA*, which belongs to a same gene cluster, named Listeria Pathogenicity Island 1 (LIPI-1)[117]. The *actA* is a virulence-associated gene, coding for a protein involved in the actin filament assembly and shows a high discriminatory power between *L. monocytogenes* strains and subtyping [29], furthermore, it has already been used in different qPCR approaches [118,119]. The *hlyA* gene has also been applied in a variety of qPCR [75,120–122], the gene encodes for the hemolysin listeriolysin, a major virulence factors involved in host-pathogen interactions [123]. *plcA*, coding for the phosphatidylinositol-specific phospholipase C, is also a virulence factor, being less reported for the detection of *L. monocytogenes* by qPCR [124]. This last one was only used for the LAMP reaction.

To detect *E. coli* O157, the gene *rfbE*, which encodes for the "O" antigen, was targeted. This gene has been highly used for the development of PCR assays to detect this pathogen, and demonstrated its good performance [125–127].

For the detection of *Salmonella* spp. three different genes, *fimA*, *ttr* and *invA*, were evaluated and used in different methodologies. The *fimA* is identified as one of the major fimbrial subunit genes of *Salmonella* spp., wherein several qPCR targeted it because of its discriminative capacity with other species [128–130]. The gene operon implicated in tetrathionate respiration (*ttrRSBCA*) with the same purpose was tested for the detection of this pathogen by qPCR, and also proved its specificity in previous studies [131–133]. Another genetic target, which encoded the invasion protein gene (*invA*), was used in LAMP reactions for real-time and naked-eye approaches development, and as *fimA* and *ttr*, this gene was targeted also by different authors [134,135].

In a different approach, primers for the detection of specific serovars of *Salmonella* species were designed. *safA* gene was targeted for the specific identification of *S.* Enteritidis. This gene encodes the major subunit of *S. enterica* atypical fimbriae, involved as a virulence factor in the host-restricted colonization of the porcine ileum, [136] and STM4497, coding for a putative cytoplasmic protein, for *S.* Typhimurium [137].

For the qPCR approach two different Internal Amplification Controls, one competitive (cIAC) and another non-competitive (NC-IAC), were developed to detect reaction inhibition. Both use a similar sequence for the amplification only varying in the 3' and 5' end with the addition of the respective primers. DNA sequence of the IAC was designed using http://usersbirc.au.dk/biopv/php/fabox/random_sequence_generator.php to generate a random DNA fragment and this sequence was subsequently used as template for primer and probe design as previously described. The cIAC was developed to be amplified with the same pair of *hly* primers by qPCR, having the simultaneous amplification of both targets (hly and cIAC). For this reason the cIAC can only be used in the detection of *L. monocytogenes*. The NC-IAC has specific primers to amplify the IAC fragment, and offer a wide range of possibilities, and the capacity to be used for the detection of other targeted pathogens.

The details about the sequence of the these primers and probes, used for the different methodologies tested, qPCR, RPA and LAMP is provided in **Table 3.5**, **Table 3.6**, **Table 3.7**, and their specificity was

verified in silico with BLAST® (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE =BlastHome).

All primers and probes, as well as the IAC DNA fragment, were purchased from Integrated DNA Technologies Inc. (IDT, Belgium), except the RPA probes (hly-exo-P, and hly-RPA-LF), which were ordered from Eurogentec (Eurogentec, Belgium).

Table 3.5. Primer used in qPCR reaction

Target microorganism	Target gene	Sequence 5'-3'	Concentration used
L. monocytogenes	*hly* F	GCAACAAACTGAAGCAAAGGAT	200 nM
	hly R	CGATTGGCGTCTTAGGACTTGC	
	hly P	FAM-CATGGCACC-ZEN-ACCAGCATCTCCG-IABkFQ	150 nM
	actA F	TTAAGACTTGCTTTGCCAGAGAC	200 nM
	actA R	GGTGGTGGAAATTCGAATGAGC	
	actA P	CY5-AATGCTCCT -TAO- GCTACATCGGAACCGA-IAbRQSp	150nM
Salmonella spp.	*ttr* F	GGCTAATTTAACCCGTCGTCAG	200 nM
	ttr R	GTTTCGCCACATCACGGTAGC	
	ttr P	NED-AAGTCGGTCTCGCCGTCGGTG-MGBNFQ	150 nM
	fimA F	CACTAAATCCGCCGATCAAACG	100 nM
	fimA R	TTCAGGACGATGGAGAAAGGC	
E. coli O157	*rfbE* F	TCAACAGTCTTGTACAAGTCCAC	200 nM
	rfbE R	ACTGGCCTTGTTTCGATGAG	
	rfbE P	FAM-AC TAG GAC C-ZEN-G CAG AGG AAA GAG AGG AA-IABkFQ	150 nM
NC-IAC	NC-IAC F	TTAAGACTTGCTTTGCCAGAGAC	100 nM
	NC-IAC R	GGTGGTGGAAATTCGAATGAGC	
	IAC P	YY-AGT GGC GGT -ZEN- GAC ACT GTT GAC CT- IABkFQ	
Salmonella spp. (PAA)	*RBP* F	CCGAACAACAGTCTCACCGA	100 nM
	RBP R	CTACAATTTTACCGGCG GCG	
	RBP P	FAM- AACAACAAG-ZEN-GCGCGCCCGTACGA-3IABkFQ	150 nM

NC-IAC correspond to the Non-Competitive Internal Amplification Control which allow the identification of amplification inhibition, to avoid false-negative results;
The probe for the IAC can be used for both competitive (cIAC) and non-competitive (NC-IAC);
FAM, CY5 NED, YY (Yakima Yellow), MGBNFQ (Minor Groove Binder nonfluorescent quencher), IABkFQ (Iowa Black®FQ), IAbRQSp (Iowa Black RQ quencher), ZEN (secondary, internal quencher) are trademarks from IDT.

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Table 3.6. Primer used in RPA reaction for *L. monocytogenes* detection

Target microorganism	Target gene	Sequence 5'-3'
qRPA	hly-RPA-F	TTACACTTATATTAGTTAGTCTACCAATTGCG
	hly-RPA-R	TCCAATCCTTGTATATACTTATCGATTTCATC
	hly-PCR-F	GCAACAAACTGAAGCAAAGGAT
	hly PCR-R	CGATTGGCGTCTTAGGACTTGC
	hly-exo-probe	TCTGCATTCAATAAAGAAAATTCAATTTCATCZATGGCACCACCAGCATC
RPA-LF	hly-RPA-F	TTACACTTATATTAGTTAGTCTACCAATTGCG
	hly-RPA-R	Biotin-TCCAATCCTTGTATATACTTATCGATTTCATC
	hly-PCR-F	GCAACAAACTGAAGCAAAGGAT
	hly-PCR-R	Biotin-CGATTGGCGTCTTAGGACTTGC
	hly- LF-probe	FAM-TCTGCATTCAATAAAGAAAATTCAATTTCATC- THF-ATGGCACCACCAGCATC-SpC3

hly-P3F and *hly*-P3R are the same primer sequence used in qPCR methodology. For the RPA-LF assay, the reverse was modified with a biotin on 5'.*"Z" indicates the position of the THF.

Table 3.7. Primer used in LAMP reaction

Target microorganism	Target gene	Primer	Sequence 5'-3'	Concentration used
L. monocytogenes	*plcA*	*FIP*	GCAGCGCTCTCTATACCAGGTACAttttAATGTCCATG TTATGTCTCCGTTA	1000
		BIP	AGGTTTGTTGTGTCAGGTAGAGCGttttCGCTTAATA ACTGGAATAAGCCAA	
		F3	TGTGTTTGAGCTAGTGGTTTGG	200
		B3	CCCATTAGGCGGAAAAGCATAT	
		LB	CATCCATTGTTTTGTAGTTACAGAG	500
Salmonella spp.	*InvA*	HK-FIP	GACGACTGGTA CTGATCGATAGTTTTTCAACGTTTCCTGCGG	700nM
		HK-BIP	CCGG TGAAATTATCGCCACACAAAACCCACCGCCAGG	
		HK-F3	GG CGATATTGGTGTTTATGGGG	100nM
		HK-B3	AACGATAAACTGGACC ACGG	
		HK-LF	GACGAAAGAGCGTGGTAATTAAC	50nM
		HK-LB	GGGCAATTCGTTATTGGCGATAG	

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Continuation Table 1.7				
S. Typhimurium	STM4497	STM-FIP	ACC TGC AGC TCA TTC TGA GCA G-TCA AAA ACA ACG GCT CCG G	400
		STM-BIP	GAA AAG GAC CAC AAG TTC GCG C-TCA GTG AGC ATG TCG ACG AT	
		STM-F3	AGC CGC ATT AGC GAA GAG	100
		STM-B3	GCG GTC AAA TAA CCC ACG T	
		STM-LF	TCA AAA ATC CAG AAC CCA ATC TCA	200
	typh	Typh FIP	TGC TGC TGT GCT TAT TAC TTT GTA AGT ATT TGT TCA CTT TTT ACC CCT	1600
		Typh BIP	GAT GCG CAG TGC CTA TTA AAC CTT AAG GCA ACG TAT CCT CTC	
		Typh F3	CAT CGT TGC GCA ATA GCT	400
		Typh B3	GTT TTT CAA CAC CAT TTT TCA AC	
S. Enteritidis	*safA*	SEN-FIP	AGC CCA CAG TGA GTA TCG TG-CGC TGC TGG TAG TGC ATG G	600
		SEN-BIP	CAG AGG TCA TGG CGC GCA AAT-GGC ATT GGT ATC AAA GGT GA	
		SEN-F3	GTT GCT AAC ACG ACA CTG GAC	100
		SEN-B3	GTG GGA TAT TCT GAG CCC CTA T	
		SEN-LB	GTG GAA TGG GAG GAG CTG GT	300
		SEN-F3	GTT GCT AAC ACG ACA CTG GAC	
	Sdf I	Sdf-FIP	CAT GCT CGC TGC ACA AAA G C-GAG AGG CGG TTT GAT GTG	800
		Sdf-BIP	CTG GAA AGC CTC TTT ATA TAG CTC A-TGA TAT ACT CCC TGA ATC TGA GA	
		Sdf-F3	GGG AGG AGC TTT AGC CAA	200
		Sdf-B3	ATG GTG AGC AGA CAA CAG	
		Sdf-LF	GCC TAA AAA ATC AGT GAC GAA CCA A	400
		Sdf-LB	CTG ACC TCT AAG CCG GTC AAT G	
E. coli O157	*rfbE*	*rfbE*-FIB	TGCCAATATTGCCTATGTACAGCTAttttGACAAAACA CTTTATGACCGTTG	-
		rfbE-BIP	GGATGACAAATATCTGCGCTGCTATttttTCAGCAATT TCACGTTTTCGT	
		rfbE-F3	GGTGGAATGGTTGTCACGAA	
		rfbE-F3	GTGGACTTGTACAAGACTGTTGAT	
		rfbE-LB	AGGATTAGCCCAGTTAGAACAAGC	

The final methodology was performed using the *plcA*,*invA* and *rfbE* set of primers with the concentration mentioned in section 3.6.

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3.5.2 Real-time amplification

The analysis were performed in a StepOne Plus™ Real- Time PCR system with StepOne™ Software v2.2.2, or in a QuantStudio 5 Real-Time PCR System with the QuantStudio™ Design and Analysis Software v1.4.3. Both were obtained from the same supplier (Applied Biosystems™, USA)

3.5.2.1 SYBR-qPCR for multiplex detection

Different intercalating dyes, such as SYBR Green, have been extensively used to monitor the amplification curve, but also to allow the visualization of melting curves of the amplicons generated. In this way, the identification, and differentiation, between different targets in a same sample is possible.

In order to evaluate this detection methodology, a SYBR Green multiplex qPCR methodology was developed for the simultaneous identification of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157 in food samples, by melting curve analysis. Three sets of primers were designed as described in M&M and optimized to allow the detection of the three targets. The genetic targets chosen were *actA*, *fimA* and *rfbE* for *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157, respectively. The primers were designed to have different melting temperatures in order to discriminate between the pathogens based on their Tm. To do so, the melting curve of the fragments obtained by the sets of primers designed were predicted and analysed using uMELT online software (https://www.dna.utah. edu/umelt/umelt.html), to ensure the distinction between the melting peak generate. The theoretical peak pattern was correlated with the one experimentally determined over the analysis of the samples. NC-IAC was also introduced in the reaction to ensure a reliable result and avoid false negative results due to reaction inhibition.

After optimization, the qPCR reaction was performed in final volume of 25 µL with 3 µL of template. The reaction was carried with a primer concentrations of 900 nM for *actA* and 100 nM for *fimA rfbE* and NC-IAC, 1 µL of NC-IAC DNA (926 copies/ µL), and 15 µL of PowerUp. SYBR® Green Master Mix (Applied Biosystems™, USA). The thermal profile consisted on a first step of UDG treatment at 50 °C for 2 min (avoid carryover contamination), followed by a hot-start activation of the polymerase at 95 °C during for 2 min, and 40 cycles of 95 °C for 15 s and 63 °C for 1 min. The melting curve stage was performed by heating at 95 °C for 15 s, cooling to 70 °C for 1 min, and increasing back to 95 °C with continuous increments of 0.015 °C/ s. Once reached the final temperature it was kept for 15 s.

Infant milk formula was selected as reference food matrix for the evaluation of the developed methodology. Twenty-five mL of milk sample were inoculated with different contamination levels of each target microorganism, and diluted 1:10 with 225 mL of mTA10-MOPS (as describe in section 3.4.1.1). The matrix was homogenized in a Stomacher 400 Circulator, at 230 rpm, for 30 s. The pre-enrichment was performed at 35 °C for 24 h and after incubation 1 mL was used to perform the DNA extraction as described in section 3.3.2.2, by enzymatic lysis with an incubation of 30 min.

3.5.2.2 qPCR with hydrolysis probe (Probe-qPCR)

The major advantage of the hydrolysis probes approach is that they increase the specificity of the assay, and do not require post-PCR analysis such as melt curve. The Probe-qPCR also simplify the detection of several targets at the same time in qPCR reaction, due to the possibility of multiple combinations between different sets of primers, without the designed complexity demonstrate by the SYBR-qPCR.

This approach was evaluated for the detection of *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 in simplex, but the primers designed could be combined in case of the need for multiplex detection. The primers and probes sequences and concentrations used are presented in **Table 3.5** All pre-treatment approaches were evaluated using this amplification technique as described in **Table 3.8**.

Table 3.8. qPCR conditions for each pre-treatment approach evaluation

Methodology	Bacteria	Target	MMix	Thermal profile	Reaction	
					Template	Vf
IMS	*L. monocytogenes*	*hlyA*	Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA)	50 °C, 2 min 95 °C, 10 min 40 cycles of 95 °C, 15 sec 63 °C, 60 sec	2µL	20uL
PAA	*Salmonella* spp.	*ttr*				
Matrix Lysis	*L. monocytogenes*	*hlyA*	TaqMan®Fast Advanced Master Mix supplier (Applied Biosystems™, Foster City, CA, USA)	50 °C, 2 min 95 °C, 2 min 40 cycles of 95 °C, 1 sec 63 °C, 20 sec	5µL	
Short enrichment	*L. monocytogenes*	*hlyA*				
	E. coli O157	*rfbE*			3µL	
	Salmonella spp.	*ttr*				
	E. coli O157	*rfbE*				
Sponge device	*L. monocytogenes*	*actA*				
	Salmonella spp.	*ttr*				

The thermal profile was based on the manufacturer standard protocol adapting the annealing/extension step to the optimized temperature.

3.5.2.2.1 L. monocytogenes

For *L. monocytogenes* detection by Probe-qPCR two different primers were tested. *actA*, previously used in the SYBR-qPCR approach and *hlyA* another genetic target.

Regarding *hly*, the reaction using these primers and probes were combined with the amplification of the cIAC, as mentioned in the section 3.5.1 section. The optimization of this assay was mainly focused in the determination of the appropriate concentration of cIAC DNA to be added, in order to avoid interference with the amplification of *hly*, thus a range of concentrations between 20000 to 1000 copies per reaction were tested.

Once the optimal concentration of cIAC was determined and no interference with the amplification of *hly* was confirmed, the evaluation of the qPCR reaction was performed as detailed below.

The reactions were performed in 20 µL including 10 µL of Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., USA), 200 nM and 150 nM of *hly* primers and probe respectively, 2000 copies of cIAC DNA and 100 nM of its probe. Two µL of template were added per reaction. The thermal profile selected consisted in 2 min at 50 °C for Uracil- DNA Glycosylase (UDG) treatment, followed by 10 min at 95 °C hot-start polymerase activation, and 40 cycles of denaturation at 95 °C for 15 s and annealing- extension at 63 °C for 30 s.

Another genetic target was also tested for the detection of *L. monocytogenes*, *actA*. The cIAC could not be used in combination with this set of primer, but the developed NC-IAC also used for the multiplex SYBR-qPCR approach can be incorporate in the reaction using the probe designed for the cIAC previously mentioned, which was suitable to work in both approaches.

The evaluation of *actA* target was made in a multiplex reaction, including also the amplification of this NC-IAC. The reaction was performed in a final volume of 20 µL and 3 µL of sample with 10 µL of TaqMan™ Fast Advanced Master Mix (ThermoFisher, USA) and 1 µL of NC-IAC DNA (926 copies/µL). The thermal profile used was the recommended by the manufacturer for the fast format, with an optimized annealing/extension temperature. A step for UDG treatment at 50 °C for 2 min was first performed, followed by a hot-start activation of the polymerase at 95 °C during for 2 min, and 40 cycles of 95 °C for 1 s and 63 °C for 20 s.

3.5.2.2.2 E. coli O157 and Salmonella spp.

For the detection of *E. coli* O157, *rfbE* was chosen as targeted gene due to previous studies showing its specificity. The evaluation of *rfbE* as target for qPCR reaction was performed as described for *actA*, also integrating the NC-IAC in order to avoid false negative results.

In order to specifically detect *Salmonella* spp. the genetic target chosen was *ttr*, as numerous study have shown its use for the detection of this pathogen. The evaluation of the set of primers targeting this gene was also performed with the inclusion of the NC-IAC.

3.5.2.3 qRPA

RPA can also be monitored using a real-time PCR equipment by adding fluorescent probes specially designed for this purpose.

To test this amplification methodology, the primers developed for Probe-qPCR targeting *hly* for the detection of *L. monocytogenes*, were used and combined with a specific probe for qRPA reaction. This probe, mentioned in **Table 3.6** was designed following TwistDx recommendations, being the sequence length of 50 bp and was located between the primers mentioned.

The qRPA reactions were run in a final volume of 25 µL, containing 420 nM primers (hly-P3F and hly-P3R) and 120 nM probe *hly*-exo-P, the rest of the reagents (rehydration buffer and magnesium acetate) were used as recommended by the manufacturer of TwistAmp®exo kit (TwistDx, UK). The samples were incubated at 39 °C during 40 min when using the qPCR primers. The fluorescence was measured every 30 s.

3.5.2.4 qLAMP

As previously mentioned for RPA reaction, also LAMP can be monitored in real-time by the addition of an intercalating dye in the reaction. This approach was also tested for the detection of *L. monocytogenes*, targeting *plcA* gene, with primers specifically designed for LAMP assays.

All qLAMP assays were performed in a final volume of 25 µL with 15 µL of Isothermal Master Mix (OptiGene Ltd., UK), 1.2 M Betaine (Sigma-Aldrich, USA), 3 µL of DNA template, with the primers and concentrations specified in **Table 3.7**. The reactions were performed at 65 °C for 50 min with a melt

curve step consisting on heating up to 98 °C for 15 s, cooling down to 80 °C for 1 min and then raising the temperature again up to 95 °C for 15 s acquiring fluorescence every 0.3 °C.

3.5.2.5 Comparison between real-time approaches

To understand the differences in the performance of the four approaches, with a real-time analysis, including SYBR Green and hydrolysis probe qPCR, and the two isothermal amplification techniques, qRPA, and qLAMP, food samples were analysed and results compared for the detection of *L. monocytogenes*, targeting *hlyA* and *plcA*, respectively. The qRPA was performed using the qPCR primers and the Exo probe previously described in section 3.5.2.3, and SYBR-qPCR with 200 nM of F3/B3 primers designed for the qLAMP approach.

For this propose, different types of matrixes were tested performing a two step-enrichment, first an incubation in a non-selective medium, mTA10 broth, followed by a selective enrichment in Full Fraser (FF, Biokar Diagnostics S.A., France), as specified in ISO 11290-1 [138] . For this propose 25 g of sample were homogenized by hand with 225 mL of mTA10 and incubated for 24 h at 35 °C. Then 100 µL of the matrix were transferred to 10 mL of FF and incubated at 35 °C, for another 24 h. Once the enrichment was completed, the samples were plated on COMPASS Listeria, and 1 mL was taken for DNA extraction, as described in section 3.3.2.2. by enzymatic lysis for 1 h.

3.5.3 RPA combined with naked-eye detection

3.5.3.1 RPA combined with lateral flow (RPA-LF) and evaluation of its applicability

LF has been applied for a variety of detection methods, and the combination with RPA reaction can allow a simple and decentralized analysis. For this reason, this approach was tested to be used in the detection of our targets.

Two different sets of primers for the detection of *L. monocytogenes* were tested to evaluate which one provided the best results in terms of sensitivity and specificity by the RPA assay, in combination with a novel LF probe. Both sets targeted the *hlyA* gene, one designed for qPCR analysis previously mentioned, and the other specifically developed for RPA following the recommendation of TwistDx (TwistDX, UK), being these longer than the other primers. The reverse primers were modified in this study, labelling the 5’ end with biotin, to allow the lateral flow detection. The LF probe was also designed following TwistDX guidelines, with FAM on the 5’ end, an internal tetrahydrofuran residue (THF) and a C3 spacer (SpC3) on the 3’ end. The detailed sequence of each primer and probe used with their specific modifications are presented in **Table 3.6**.

3.5.3.1.1 RPA reaction and LF detection

The RPA reactions were performed in a Veriti Thermal Cycler (Applied Biosystems™, USA) using the primers designed for qPCR (hly-P3F/hly-P3R) in a concentration of 420 nM and 120 nM of the LF probe (hly-LF-P), in a final volume of 25 µL with 2 µL of the template using the TwistAmp® nfo kit (TwistDX, UK). After optimization, the amplification was performed at 39 °C for 40 min. Once the amplification reaction was completed, the LF was done using Milenia® HybriDetect universal test strip (dipstick). To do so, a 1/ 50 dilution in HybriDetect Assay Buffer (Milenia Biotec GmbH, Germany)

or PBS was done, and 10 µL were loaded directly on the sample application area of the LF strips (Milenia Biotec GmbH, Germany). The strips were introduced in a clean 1.5 mL tube containing 100 µL of the same buffer used for the dilution, and incubated for 5 min until the control band was clearly visible.

3.5.3.1.2 Evaluation of applicability for surface contamination analysis

In order to test the RPA reaction with the primers presenting the best results for surface analysis, stainless steel coupons of 100 cm^2 (10 cm × 10 cm) were used to mimic food processing plants, and the detection of *L. monocytogenes* in surface was evaluated.

Before use, the surfaces were sterilized (autoclaved at 121 °C for 15 min) and cleaned (cleaned with 10% bleach and 70% ethanol, air dried and finally exposed to UV light for 15 min). After the sterilization and cleaning process was completed, 100 µL of the corresponding bacterial dilution, was added to the surface in droplets and uniformly spread on the coupons until dry. The inoculated surfaces were incubated 30 min at room temperature before sampling. For the recovery of the bacteria, two different tools were tested, a Rayon Sterilin™ Plain Swab (Thermo Fisher Scientific Inc., USA) and 3M™ Sponge-Sticks (3M, USA). The surface was scratched 10 times in each direction (horizontal, vertical, and diagonal) with the swab or sponge pre-moistened in PBS with 0.01% Tween 80. The swab/ sponge was introduced in 3 mL of a ONE broth selective medium for *L. monocytogenes*, homogenized manually and incubate at 30 °C.

3.5.3.1.3 Enrichment optimization and samples analysis

The time of enrichment was also evaluated to understand the effect on the Limit of Detection (LoD). A 2 mL aliquot was taken right after sampling (without enrichment), after 8 h and 14 h. For each time point three samples were analysed, spiked from 10^4 cfu/ cm^2 to 10^2 cfu/ cm^2. The results obtained were compared to those of a 24 h enrichment.

After the enrichment step, 2 mL of the sample was taken and a washing step performed as described in section 3.3.2. The DNA extraction was performed with the NucleoSpin® Tissue kit (Macherey-Nagel, GmbH & Co KG, Germany), using the manufacturer support protocol established for bacteria with some modifications. Briefly, after the washing with PBS the enzymatic lysis was performed as described in section 3.3.2.2 for 30 min, and with the addition of 25 µL of Proteinase K. Then the protocol was followed as described by the manufacturer, continuing from step 3 with the addition of 200 µL of Buffer B3. The elution was modified, by being performed in two times, adding 50 µL in each time. The DNA extracts were stored at 4 °C until use. Additionally, 100 µL of enrichment medium were transfer to 10 mL of FF broth and we observed if the medium turned dark after 24–48 h at 37 °C, and streaked on COMPASS plates to confirm the presence of *L. monocytogenes*. Plates were incubated under the same conditions detailed above.

3.5.3.2 RPA with colorimetric SYBR GREEN detection (RPA-SYBR)

Another way to perform naked-eye detection by RPA, is the addition of a fluorescence intercalating dye, in this case not for the real-time analysis but for end-point detection of positive amplification. With the addition of this dye at the end of the reaction the binding to the DNA can produce a change of colour under white light, or emit fluorescence when exposed to UV light. The detection of

E. coli O157 in food samples was evaluated by this methodology combined with the short enrichment approach described in section 3.4.3.3

3.5.3.2.1 Evaluation with complex food matrixes

To evaluate the RPA-SYBR approach, 13 samples of ground beef were spiked with concentrations of *E. coli* O157 between 1.10×10^2 to 2.5 cfu/ mL. The samples were then process following the short enrichment methodology for the detection of this pathogen in simplex, as detailed in section 3.4.3.3. In brief, 25 mL of mTSBn, pre-warmed at 37 °C, were added to 25g of sample and incubated at 37 °C with constant agitation (200 rpm) for 3 h. Sample pre-treatment and DNA extraction from the pellet were performed as described in section 3.3.2.1, being the extraction performed with Chelex®100 resin.

The RPA reaction was performed in a final volume of 25 µL with 2 µL of template DNA, using TwistAmp® Basic kit, following supplier's recommendations for the reaction preparation. For this analysis qPCR primers mentioned before targeting *rfbE* gene were used at a concentration of 480 nM to specifically detect *E. coli* O157. The RPA amplification was accomplished the same Veriti™ Thermal Cycler (Applied Biosystems™), as mentioned before, at 39 °C, and after 4 min the reaction was vortex and the amplification resumed until 15 min of total amplification time.

To perform the naked-eye detection, a solution of SYBR™ Green 10000X (Invitrogen, USA) was diluted in DMSO to obtain a working solution of 400X from which 2 µL were added to the RPA reaction and mixed. The tubes were expose to UV light and also loaded in a 2% agarose gel to visualize the results. The results were compared to qPCR analysis, as describe in section 3.5.2.2.

3.5.3.2.2 Agarose gel electrophoresis

To confirm the amplification of the expected fragment, an electrophoresis was performed in a 2% agarose gel prepared with Sodium Borate buffer as previously described by Brody and Kern, 2004 [139], and stained with 4 µL of Midori Green (Nippon Genetics Europe GmbH, Germany). The gel was loaded with 1µL of 6X DNA loading dye (Thermo Fisher Scientific, Inc., USA) mixed with 5 µL of amplification product. The electrophoresis was run for 30 min at 300 V, and the results were observed in a GelDocTM EZ Imager (Bio-Rad Laboratories, Inc., USA).

3.5.4 LAMP reaction coupled with naked-eye detection

Different variations of LAMP have been developed with the objective of allowing to visualize the result of the amplification reaction, by turbidity or by colour change, showing promising results. Three approaches were tested for the detection of *Salmonella*, the first one using a commercial turbidity mastermix, a second one with the functionalization of AuNP to enhance a change of colour, and the last one using a colorimetric commercial mastermix.

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3.5.4.1 Turbidity

The turbidity approach was tested in this study for the specific detection and differentiation of two different serovars of *Salmonella* spp., namely *S.* Enteritidis and *S.* Typhimurium. Two genetic targets

were evaluated for each serovar. The targets chosen were *safA* and STM4497 based on the primers designed by Garrido-Maestu et al. (2017) [140] with the addition of newly designed loop primers, while *Sdf I* and *typh* were designed by Yang et al. (2010) [141] and Pavan Kumar et al. (2014) [142], respectively (**Table 3.7**). The selection of the primers for *safA* and STM4497, was based on the fact that they exhibited excellent performance (accurate, sensitive, and specific detection) in food samples including chicken and turkey, obtaining a κ index of 0.98 and 0.97 respectively [140]. On the other hand, the other two sets of primers reported to have high specificity when tested 14 bacteria for *Sdf I* primers [141] and against a large panel including 56 strains for *typh* primers [142].

3.5.4.1.1 Evaluation with complex food matrixes

To evaluate all these sets of primers, different types of foods were analysed covering those of high risk for *Salmonella* contamination, including raw chicken, turkey, as well as raw and cooked egg products. Twenty-five grams of each food sample were weighed and 225 mL of mTA10 broth and enriched at 37 °C for 18 to 24 h. After incubation, the DNA was extracted using Chelex®100 protocol, as described in section 3.3.2.1 and the LAMP reaction was performed in a Loopamp Realtime Turbidimeter LA-500 (Eiken Chemical Co., Ltd., Japan) in order to monitor the turbidity increase in real-time. The reactions were prepared in a final volume of 25 µL, with 1 M Betaine (Sigma–Aldrich, USA), 12.5 µL of Isothermal Master Mix (OptiGene Ltd., UK) and the corresponding amount of primers (see **Table 3.7**), and 3 µL of template DNA. The amplification was accomplished at 65 °C for 1 h. Even though the amplification was performed in 1 h, only those food samples reporting positive within the first 30 min were considered as such for *safA*, *Sdf I*, and STM4497, while for *typh*, due to the lack of loop primers, up to 40 min were considered acceptable.

3.5.4.2 Naked-eye detection approach by combination of MUA and AuNP

In order to obtain a change of colour to visualize the LAMP results, gold nanoparticles (AuNP) were functionalized with 11-mercaptoundecanoic acid (MUA) to confer negative charge to the surface of the particles. This characteristic will allow to control the aggregation between nanoparticles in absence of target sequence, as Mg^{2+} remains present in the reaction and will serve as a bridge between AuNP bringing them close together, however when amplification occurs the resulting production of $P_2O_7^{4-}$ will capture the Mg^{2+} and AuNP stay free and in suspension [143].

AuNP were previously produce by another group within INL as described by Garrido-Maestu et al. [144], By this protocol, the addition of 5 mL of 1% solution of trisodium citrate (Sigma-Aldrich, USA) to a boiling solution of gold chloride (Sigma-Aldrich, USA) was performed with a strong magnetic stirring during 5 min, where the solution turned an intense red colour

The functionalization with MUA was performed following the protocol described by Wong et al. (2014) [143]. Briefly, 20 nM AuNPs and 2 mM MUA (freshly prepared in DMSO) were mixed, and incubated for 24 h at room temperature with constant agitation, at 1400 rpm.

To evaluate this methodology, spiked samples including chicken breast, turkey and egg product (fresh egg and omelette) were analysed after an enrichment step in BPW following the protocol also use for the LAMP turbidity approach detailed in section 3.5.4.1.1. After the DNA extract was obtained, the LAMP amplification was performed targeting *invA* gene of *Salmonella* spp. with the concentration described in **Table 3.7**. The reaction was prepared in a final volume of 25 µL with 3 µL of DNA template

2.5 µL of 10X Isothermal Amplification Buffer (New England BioLabs, Inc., USA), 1 M betaine (Sigma-Aldrich, USA), 0.35 mM dNTP mix (Thermo Fisher Scientific, Inc., USA), and 8 U Bst 2.0 WarmStart DNA Polymerase (New England BioLabs, Inc., USA). Twenty µL of this mixture was then loaded in a microfluidic device in PDMS (detailed in section 3.7.2.2) and properly sealed to proceed with the amplification in a conventional laboratory incubator (Memmert GmbH, Germany) with the temperature set at 65 °C for 1 h.

After amplification, in a final volume of 15 µL, 4 µL of the LAMP product were diluted with 8.5 µL sterile milli-Q water and mixed with the functionalized AuNP for a final concentration of 6 nM, and results generated were observed by naked-eye. The red colour was interpreted as a positive result while purple was identified as negative. The UV-vis spectrum was also measured using a NanoDrop 2000c.

3.5.4.3 Commercial colorimetric mastermix

Several mastermixes enabling a naked-eye detection of LAMP results are available in the market. One of them, from New England Biolabs (NEB) allows a very clear colour change, from pink to yellow. This mastermix was tested for the detection of *Salmonella* spp. targeting *invA* gene using the primers previously described for the AuNP-MUA approach (**Table 3.7**) and the primer concentration recommended by the mastermix manufacturer (NEB, Massachusetts USA,) (1600 nM FIP/ BIP, 200 nM F3/ B3, 400 nM LF/ LB).

The addition of Loop primers was first tested, comparing the performance of LF and LB separately in order to improve the reaction in terms of efficiency and sensitivity, and also to reduce the amplification time. A concentration of 400 nM was tested for both primers, as recommended by the mastermix manufacturer (NEB, USA,). For this experiments, different samples both spiked and non-spiked with *Salmonella*, were analysed.

The LAMP reaction for all experiments consisted in a final volume of 25 µL with 3 µL of DNA template, 15 µL of WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA), 40 mM of guanidine hydrochloride (Sigma–Aldrich, USA) and the recommended concentration of primers being 1600 nM of FIP and BIP, 200 nM of F3 and B3 and 400 nM of LF or LB depending on the assay. The amplification reaction was performed for 30 min at 65 °C in a thermocycler and results were directly visualized and photographed with a cell phone camera.

Different milk samples (UHT, Fresh and raw) were tested to evaluate this approach. First a set of samples with a 24 h enrichment in mTA10 MOPS were analysed simultaneously with multiplex SYBR-qPCR as described in section 3.5.2.1. Another set of samples were analyzed after a 6 h short enrichment in TSB. These last samples were also confirmed by Probe-qPCR. The DNA of the two sets of samples was extracted by the enzymatic lysis as described in section 3.3.2.2.

3.6 OPTIMIZED SELECTED METHODOLOGY

After the evaluation of the different approaches, for both pre-treatments of the samples and DNA amplification techniques, a final methodology was chosen and optimized.

Short enrichment was chosen to reduce the time of sample pre-treatment, and different media to perform the enrichment were tested to allow the growth of the three target bacteria in order to have a positive result as fast as possible. The short enrichment, as described in section 3.4.3.3, was performed in TSB for 7 h and the DNA extraction as detailed in section 3.3.2.2.

The DNA amplification was performed by colorimetric LAMP to enable naked-eye detection, and allow for a more economic analysis. *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 were detected targeting the *plcA*, *invA* and *rfbE* genes, respectively, and the primers used are described in **Table 3.7**, wherein the loop primer used for *Salmonella* detection was the LF. The reaction was performed in a final volume of 25 µL, 15 µL of mastermix and supplemented with GuHCl as mentioned in section 3.5.4.3, and the primers concentration added was the recommended by the mastermix manufacturer (NEB), 1600 nM FIB/ BIP, 200nM F3/ B3 and 400 nM of the loop primers, with the exception of *plcA* LF which was used in a higher concentration, 600 nM. For the LAMP targeting *Salmonella* spp. and *E. coli* O157, a mixture of the primers 10X concentrated was prepared in order to be use in the different experiments, and adding 3 µL of DNA template. However for *L. monocytogenes* the volume of the template had to be increased for higher sensitivity, to 6 µL, and the primer mix was concentrated 20X in order to fit all reagent in the reaction. The amplification was performed at 65 °C, for 30 min for the two Gram-negative bacteria, and 1 h to detect *L. monocytogenes*. The newly developed methodology was tested performing the DNA amplification in a thermocycler, but also integrated in the two alternative, miniaturized amplification devices described below, in sections section 3.7.2 and section 3.7.3

The methodology was evaluated analysing different milk samples (UHT, fresh and raw) spiked with different combinations of *L. monocytogenes*, *S.* typhimurium and *E. coli* O157 with several contamination levels ranging from 6 x 10^2 to 1 cfu/ 25 g of sample. The results were confirmed by Probe-qPCR, and also plating in selective media, COMPASS *Listeria*, CHROMagar™ *Salmonella* Plus and CHROMagar™ *E. coli* O157.

3.7 MINIATURIZED DEVICES

The aim of this project was to develop an improved methodology for the detection of pathogens in food samples, and its integration in a miniaturized device to allow an automated and portable analysis. For this last part, three different devices previously designed and fabricated at INL were tested. One of the devices was evaluated to improve the sample pre-treatment, allowing the concentration of bacteria as mentioned in section 3.4.2.2 and the two other were tested for the integration of the isothermal amplification approaches.

3.7.1 Microfluidic device for capture and concentration of bacteria

To improve the sample pre-treatment, a microfluidic device was developed with an embedded functionalized PDMS sponge to capture and concentrate the bacteria [145]. This device was composed by two parts made of PMMA with the same dimensions (25 mm × 25 mm × 10 mm) and assembled with 4 screws at each corner. A chamber in each part of the device was created to allocate the sponge, with an internal diameter of 7 mm with 5 mm depth in the top and 1 mm depth in the bottom part. An O-ring was also introduced in order to properly seal the chamber. The schematic representation and real device are presented in **Figure 3.1**. The device was developed with 4 inlet/outlet, but only 2 were used in this study to allow the entrance of the sample and recovery of the liquid after passing by the device as exemplified in **Figure 3.1 D**. The device also included an electrode for electrochemical analysis, not needed for the concentration of the bacteria.

The device was designed using a 3D CAD software (SolidWorks, Dassault Systèmes Corp., Waltham, MA) and then transferred to Art-Cam software to generate the G-Code for high speed milling.

A computer numerical control (CNC) machine (FlexiCAM, Germany) performed the milling process using 1 mm and 3 mm End-mill tips (DIXI, Switzerland).

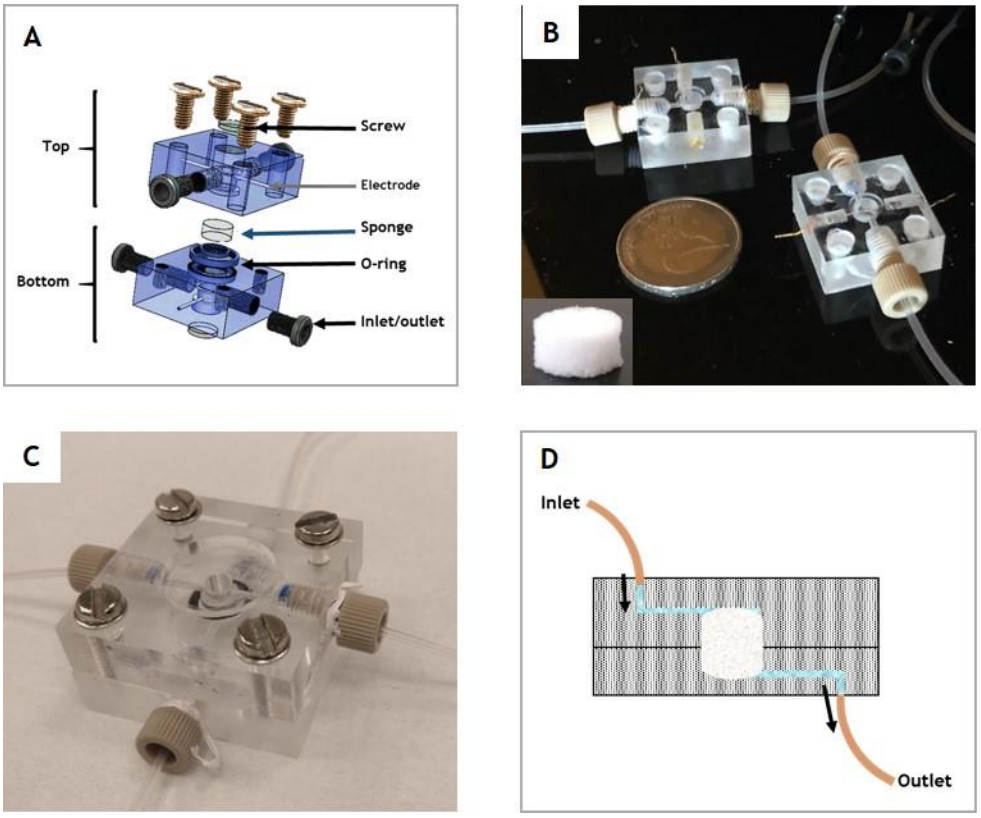

Figure 3.1. Microfluidic device with PDMS sponge for bacteria concentration. (A) Design in SolidWorks, displaying the different components. (B) The two parts of the device separated and image of the PDMS sponge. (C) Device assembled and ready to use. (D) Schematic representation of the flow passing through the device with the sponge.

3.7.2 PDMS channels prototype

3.7.2.1 Device design and fabrication

A microfluidic device composed by 8 capillarity-driven microchannels was designed to perform the amplification reaction at constant temperature (**Figure 3.2**). The final dimensions of the device were 4 mm (thickness) × 76 mm (length) × 26 mm (width), and each channel presented a geometry of 40 mm (length) × 800 µm (depth) × 600 µm (width) allowing the loading of a maximum reaction volume of 20 µL, and presenting one inlet and one outlet.

For the fabrication of this device, a mold was designed by AutoCAD software and fabricated in poly(methyl methacrylate) (PMMA) using a computer-numerical-control (CNC) miller (FlexiCAM Viper 606). After the mold completed, the PDMS replica was produced, mixing the base with the curing agent, from the Sylgard 184 silicone elastomer kit, in a proportion of 10:1 and centrifuge 6 min at 3000

x g to remove the air bubbles. The PDMS was then poured onto the PMMA mold and placed under vacuum for 20 min, to ensure that the last bubbles were eliminated. Finally the replica was cured in an incubator at 65 °C for 1 h. After cooling, the replica was removed from the mold and the bonding to a glass slide was performed using oxygen plasma to seal the microfluidic channels.

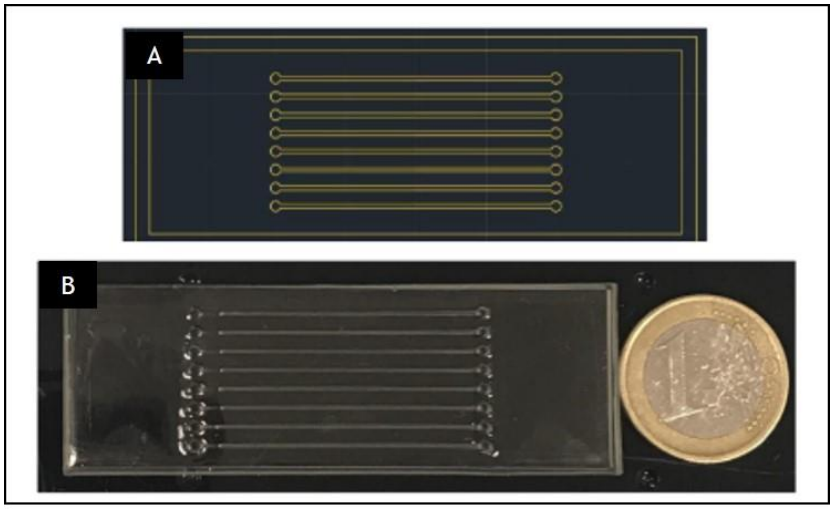

Figure 3.2. Microfluidic PDMS device with 8 capillarity-driven microchannels to performed DNA amplification (PDMS channels prototype). (A) Design in AutoCad and (B) PDMS replica after fabrication

3.7.2.2 DNA amplification experiment

After the bonding was performed, the device was ready to be use. The amplification reaction was first prepared in 0.2 mL PCR tubes in a final volume of 25 µL, and 20 µL were loaded into the channels. Finally, the inlet and outlet were covered with another glass slide with the same dimensions and held together with a clamp to avoid evaporation during incubation. The amplification was then performed in conventional laboratory incubator as described in section 3.5.4.2.

3.7.3 Milled channels prototype

The second device used to perform the DNA amplification included an integrated heating system where silicone tubing loaded with the reaction could be placed, and therefore not requiring additional instrumentation.

3.7.3.1 Prototype design, fabrication and temperature control

Figure 3.3 illustrates the prototype including different units: heating elements, temperature control, fan for cooling, electrical wires, and a display. A Peltier module allowed to maintain a constant temperature and the combination with a standard passive aluminium heat sinks created a uniform temperature distribution. A platform and holder with milled channels to maintain the tubes close to the

aluminium part, and decrease variations of temperature during the incubation, was fabricated in PMMA using a laser cutter Widlaser LS1390 Plus (Widinovations, Portugal). This holder present different possibilities to accommodate tubes with different lengths. The volume capacity of the device is flexible, between 10 to 30 µL, depending on the length of the tube selected.

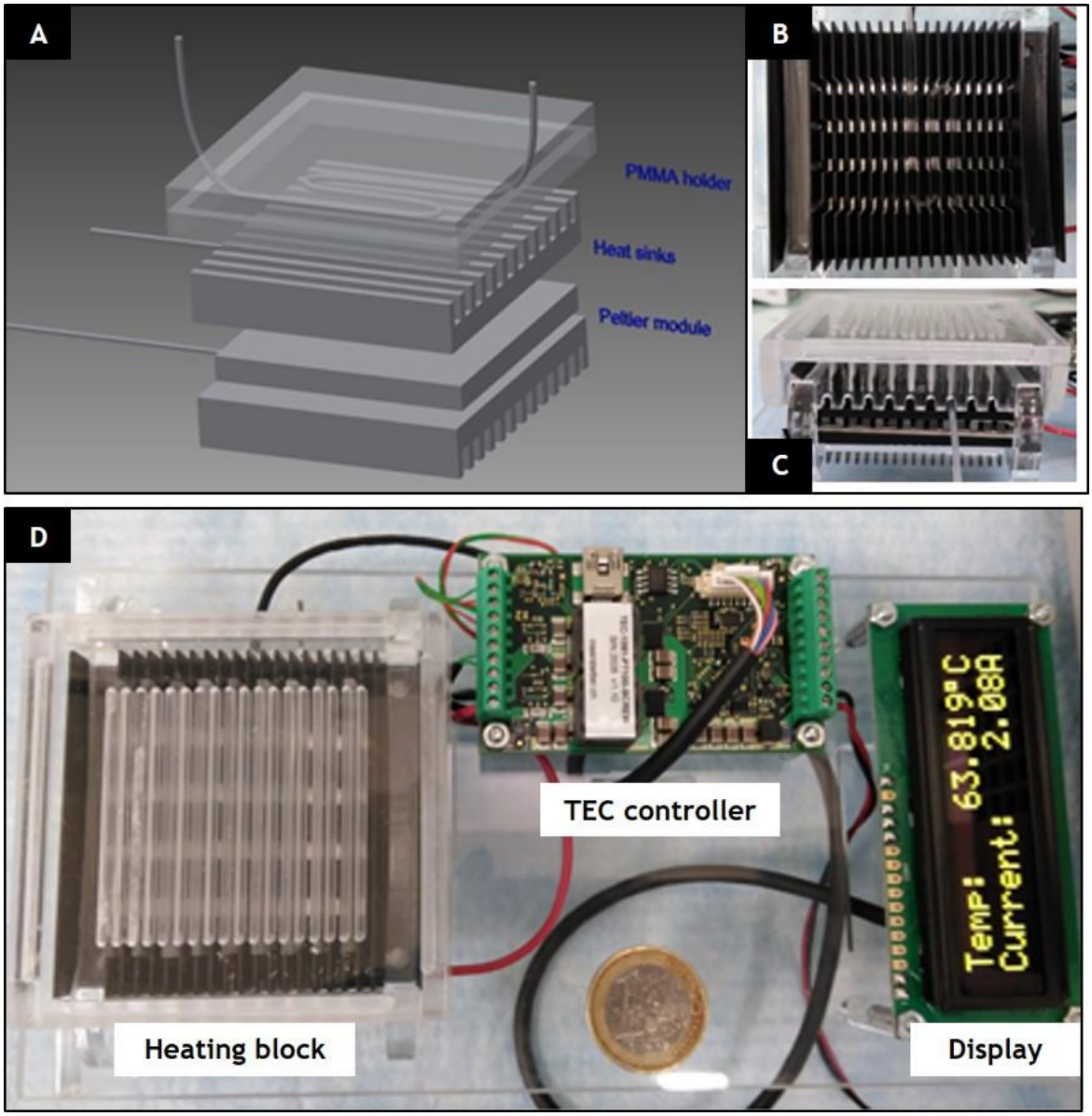

Figure 3.3. Miniaturized device using silicon tubing in integrated heating system for DNA amplification (Milled channels prototype). (A) Schematic representation of the Peltier module with standard passive aluminum heat sinks, and PMMA platform and holder. (B) Tubing chamber. (C) PMMA platform and holder. (D) Completing device including the heating block where the amplification reactions are placed, the TEC controller and the display.

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To control the temperature a thermoelectric cooler (TEC) controller, TEC-1091 (Meerstetter Engineering GmbH, Switzerland), was used for high precision and stability and the monitoring of the temperature was achieved by a precision platinum sensor, connected to the high-performance closed-loop Peltier controller. A DPY-1113 TEC status display (Meerstetter Engineering GmbH, Switzerland), was connected to the device to allow the visualization of the temperature and current parameters.

The miniaturized device did not need to be connected to a computer to work, being the parameters set before experiment with the TEC Service Software (Meerstetter Engineering GmbH, Switzerland), and only when some modification was required, the connection was established again by a standard USB connection port to a PC running Windows.

3.7.3.2 DNA amplification experiment

After the setup of the equipment was completed, the device was pre-heated until the desired temperature was reached. The amplification reaction was prepared as for the previous device, first in 0.2 mL PCR tubes and then the whole volume was transferred to a standard silicone tubing with inner and outer diameters of 1.02 mm and 2.16 mm, respectively. The tubing with the reactions were then introduced in the device, and the holder placed over them. The incubation was performed during the desired time depending on the targeted pathogen and, when finished, the tubes were removed in order to be observed and photographed.

3.8 DNA AMPLIFICATION EVALUATION

To evaluate the performance of the different amplification approaches the efficiency and dynamic range of the assay, as well as the inclusivity/ exclusivity was determined, in order to understand which could be the best option to integrate a micro-device for the detection of the three target microorganisms. This parameters were tested for the different sets of primers designed for each target.

For the evaluation of the dynamic range and analytical sensitivity, two different approaches were followed, the first allowed to determine the lowest DNA concentration needed to have a positive result by the amplification technique. To accomplish this, ten-fold serial dilutions of the corresponding DNA extract were made, and used as the template in the amplification reaction. The extracts were obtained from the target microorganism after thermal lysis as described in section 3.3.1.

For some of the amplification approaches, the lowest concentration of bacteria capable to give a positive result was also determined. For this second one, ten-fold serial dilutions of an ON pure culture were done and the cell lysates were prepared from each one of the dilutions.

Through these results, the efficiency of the reaction was also evaluated for the real-time techniques performed in a real-time qPCR equipment, and also for the LAMP with turbidity detected by the Loopamp Realtime Turbidimeter LA-500. This parameter was calculated using the following equation [146]:

$$E = 10^{1/slope} - 1 \qquad \text{Eq. 5}$$

where “E” is the efficiency, and the slope was calculated by linear regression.

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Values between 0.9 and 1.1, -3.1 and -3.6, and >0.99 of the efficiency, slope and coefficient of correlation (R^2), respectively, are related with a good performance of the reaction.

The inclusivity and exclusivity of the amplification reactions were evaluated, in order to confirm empirically the specificity observed by the BLAST analysis when the primers were designed. For this purpose, an ON culture was performed as mentioned in section 3.2 from each strain to be tested, and the DNA extraction achieved by thermal lysis as described in section 3.3.1. This DNA extracts were then used as template in the amplification reactions.

3.9 EVALUATION OF THE DEVELOPED METHODOLOGIES

The methodologies tested to reduce the enrichment time and to optimize the DNA amplification step were evaluated for the detection of one, or more, foodborne pathogens in complex food matrixes. This evaluation was accomplished by the determination of several parameters. First the Limit of Detection (LoD) was established and then the analysis of the fitness for purpose performed as presented in **Table 3.9**.

Table 3.9. Methodologies employed for the valuation of the developed protocols

Type of approach	Methodology	Bacteria	LoD	Fitness for purpose
Sample pre-treatment	IMS	*L. monocytogenes*	90% positives	ISO
	PDMS Sponge device	*L. monocytogenes* *Salmonella* spp.	*Analytical sensitivity**	
	PAA	*S. Enteritidis*	90%	ISO
	Matrix lysis	*L. monocytogenes*	PoDLoDu	ISO
	Short enrichment	*L. monocytogenes*	PoDLoD	ISO
		E. coli O157	PoDLoD	ISO
		E. coli O157 *Salmonella* spp.	90%	ISO
DNA amplification	SYBR-qPCR	*L. monocytogenes* *E. coli* O157 *Salmonella* spp.	PoDLoD	ISO
	Real-time comparison	*L. monocytogenes*	90%	ISO
	RPA-LF	*L. monocytogenes*	PoDLoD	*Nordval*
	RPA-SYBR	*E. coli* O157	PoDLoD	ISO
	LAMP turbidity	*S. Enteritidis* *S.* Tiphimurium	*Analytical sensitivity**	*ISO*
	LAMP MUA-AuNP	*Salmonella* spp.	90%	ISO
	LAMP colorimetric	*Salmonella* spp.	PoDLoD	*Nordval*

The LoD was obtained by two different approaches: when at leat 90% of the samples were positives; and by the PoDLoD analysis;
The fitness for purpose was evaluate by two different methodologies: ISO refers to ISO 16140-2003criteria [147]; and NordVal refers to NordVal International Protocol [148];
* The fitness for purpose of the methodology was only evaluate with pure cultures, not with food samples.

3.9.1 Evaluation with complex food matrixes

All approaches, for samples pre-treatment and DNA amplification were evaluated analysing different types of matrixes, and targeting different pathogens. A summary of the analysis performed is presented in **Table 3.10** and **Table 3.11**. The conditions for the new methodology are detailed in **Table 3.12**. All contamination of the samples were confirmed by a culture-based methodology, plating the enrichment on a selective medium in order to isolate the target pathogen, and allow the growth of typical colonies and identification of the bacteria present. With this analysis the LoD was determined and the parameters for the fitness for purpose calculated.

Table 3.10. Samples analysis for the evaluation of sample pre-treatment approaches

Approach	Pathogen tested	Type of samples	Enrichment conditions	DNA extraction	DNA amplification	Confirmation
IMS	*L. monocytogenes*	Chicken breast Hard and fresh cheese Fish	HF (24 h)	Enzymatic lysis	Probe-qPCR	Plating in selective media
Sponge device	*L. monocytogenes* *Salmonella* spp.	Surfaces	-			
Matrix lysis	*L. monocytogenes*	Smoked salmon	-			
Short enrichment	*L. monocytogenes*	Smoked salmon	TSB (5 h)			
	E. coli O157	Ground beef Leafy greens	mTSBn (3 h)	Chelex®100		
	E. coli O157 *Salmonella* spp.	Ground beef Chicken breast	BPW + 0.4 % Tween 80 (3 h)			mTSBn + Chr O157 RVS + XLD or TBX
PAA	*S. Enteritidis*	Chicken breast	BPW (3+6 h)			Plating in selective media

For the confirmation of the short enrichment for the multiplex detection of *E. coli* O157 *and Salmonella* spp a selective second enrichment was performes in mTSBn and RVS, respectively, before plating in a selective agar.
The other confirmations were performed plating directly in the respective selective medium. COMPASS or PALCOM for *L. monocytogenes*, XLD or CHROMagar™ Salmonella Plus for *Salmonella* spp. and Tryptone Bile X-Glucuronide Agar (TBX) or CHROMagar™ *E. coli* O157 for *E. coli* O157

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Table 3.11. Samples analysis for the evaluation of DNA amplification approaches

Approach	Pathogen tested	Type of samples	Enrichment conditions	Sample Pre-treatment	DNA extraction	Confirmation
SYBR-qPCR	*L. monocytogenes* *E. coli* O157 *Salmonella* spp.	UHT milk	mTA10 (35 °C, 24 h)	-	Enzymatic lysis	Plating in selective media
Real-time comparison	*L. monocytogenes*	Smoked salmon	mTA10 (35 °C, 24 h) + FF (35 °C, 24 h)			
RPA-LF	*L. monocytogenes*	Surfaces	ONE broth (30 °C, 24 h)			FF (24 h- 48 h) + COMPASS
RPA-SYBR	*E. coli* O157	Ground beef	mTSBn (3 h)	Sort enrichment	Chelex®100	Plating in selective media
LAMP turbidity	*S. Enteritidis* S. Tiphimurium	Egg product Chicken Turkey	mTA10 (37 °C, 18 h-24 h)	-		
LAMP MUA-AuNP	*Salmonella* spp.		BPW + (37 °C, 18 h-24 h)	-		
LAMP colorimetric	*Salmonella* spp.	UHT milk	mTA10 (35 °C, 24 h) TSB (6 h)	Sort enrichment	Enzymatic lysis	

For the confirmation of the RPA-LF a second enrichment in Full Fraser (FF) was performed and only then plated in a selective medium (COMPASS). The other confirmations were performed plating directly in the respective selective medium. COMPASS or PALCOM for *L. monocytogenes*, XLD or CHROMagar™ Salmonella Plus for *Salmonella* spp. and CHROMagar™ *E. coli* O157

Table 3.12. Optimized condition for the selected methodology

Pathogen detected	Type of samples	Samples pre-treatment			DNA amplification		Confirmation	
		Approach	Enrichment conditions	DNA extraction	Approach	Genetic targets	qPCR	Culture
L. monocytogenes *E. coli* O157 *Salmonella* spp.	Milk (UHT, Fresh, Raw)	Short enrichment	TBS (7 h)	Enzymatic lysis	LAMP colorimetric	*plcA*, rfbE, invA	Probe-qPCR	Plating in selective media

Confirmations were performed plating directly the enrichment in the respective selective medium. COMPASS for *L. monocytogenes*, CHROMagar™ Salmonella Plus. and CHROMagar™ *E. coli* O157

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3.9.2 LoD determination

To determine the minimum concentration of targeted bacteria providing a positive result in 25 g of food sample, two different approaches were followed.

In the first, the LoD was identified as the lowest, spiked bacterial concentration which could be reliably detected. To determine this parameter 10 samples were inoculated with less than 10 cfu of the targeted bacteria, and after analysis (enrichment, IMS, DNA extraction and qPCR) at least 9 had to be positive (90%).

The other approach to determine the LoD was performed analysing several samples inoculated with different contamination levels until obtained negative results. The LoD_{50} and LoD_{95} were calculated using PoDLoD calculation program, version 9, which also allowed to predict the Probability of Detection (PoD). This approach provided more robust results.

Extra, non-inoculated samples were also analysed to assure absence of the pathogen in the original matrix.

3.9.3 Fitness for purpose

To assess if the different methodologies developed were suitable to be used in the food industry and allowed reliable results, with high sensitivity, different parameters were calculated following the criteria of ISO 16140-2003 [147] and NordVal International Protocol [148].

The samples analysed were classified as positive and negative agreement (PA/ NA), and Positive and Negative deviation (PD/ ND) comparing the result obtained after analysis, with the expected results or a reference methodology. ND are the number of samples expected positive with a negative result, and PD, are the number of samples expected negative with a positive result.

In the NordVal regulation, three other concepts are identified when a confirmation to the reference method is applied. ND are False Negative (FN) when the reference method is confirmed positive. PD becomes True Positive (TP) when the alternative method is confirmed positive, and False Positive (FP) when the confirmation was negative

Using these data, the relative sensitivity, specificity, accuracy (SE/ SP/ AC) and the Cohen's kappa, or κ, were calculated according to ISO and Nordval criteria, and the positive and negative predictive values (PPV/ NPV) following previous studies [149,150].

SE was defined as the percentage of positive samples giving a correct positive result.

$$SE = \frac{PA}{(PA + ND)} \times 100 \qquad \text{(ISO)} \qquad \text{Eq. 6}$$

$$SE = \frac{PA + TP}{(PA + FN)} \times 100 \qquad \text{(NordVal)} \qquad \text{Eq. 7}$$

SP was defined as the percentage of negative samples giving a correct negative result.

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$$SP = \frac{NA}{(PD + NA)} \times 100 \qquad \text{(ISO)} \qquad \text{Eq. 8}$$

$$SP = \frac{NA}{(FP + NA)} \times 100 \qquad \text{(NordVal)} \qquad \text{Eq. 9}$$

AC is defined as the degree of correspondence between the response obtained by the expected result and the method on identical samples. P_0

$$AC = \frac{(PA + NA)}{N} \times 100 \qquad \text{(ISO)} \qquad \text{Eq. 10}$$

$$p_0 = AC = \frac{(PA + NA + TP)}{N} \times 100 \qquad \text{(NordVal)} \qquad \text{Eq. 11}$$

N= total number of samples analysed.

PPV and NPV are measures of the performance of the method by giving the probability of a sample being really positive or negative when the method shows a positive or negative result.

$$PPV = \frac{PA}{(PA + PD)} \times 100 \qquad \text{Eq. 12}$$

$$NPV = \frac{NA}{(NA + ND)} \times 100 \qquad \text{Eq. 13}$$

Finally, the index kappa (κ) of concordance shows the degree of concordance between the method and the expected result. The following equation refers to the calculation of the κ by ISO standard:

$$\kappa = 2 \text{ x} \frac{(\text{PA x NA}) - (\text{ND x PD})}{(\text{PA} + \text{PD}) \text{ x } (\text{PD} + \text{NA}) + (\text{PA} + \text{ND}) \text{ x } (\text{ND} + \text{NA})} \qquad \text{Eq. 14}$$

To calculate the κ following the NordVal regulation another parameter need to be calculated, the expected frequency of agreement, the expected accuracy, or repeatability by chance (p_e). The κ was then obtained using the p_e and the previously calculated accuracy (p_0)

$$p_e = \frac{(\text{PA x FN}) \times (\text{TP} + \text{NA} + \text{FP}) + (\text{PA} + \text{TP}) \times (\text{FN} + \text{NA} + \text{FP})}{N^2} \qquad \text{Eq. 15}$$

$$\kappa = \frac{p_0 - p_e}{1 - p_e} \qquad \text{Eq. 16}$$

When the kappa value is between 0.81 and 1.00 the results are interpreted as “nearly complete concordance”, showing a very good performance of the methodology [148]. For the new methodologies to fit the purpose a result between this values is required.

3.10 MESOPHILIC BACTERIA ANALYSIS IN FOOD SAMPLES

To evaluate the microbial composition of the raw milk samples used to evaluate the selected methodology and understand their influence in the detection of the targeted pathogens, the concentration of the natural microbiota was assessed, following ISO 4833-1:2013 method, and characterized by Next Generation DNA Sequencing (NGS)

3.10.1 Enumeration of microorganisms

To know the concentration of mesophilic microorganism present in the raw milk samples, the enumeration was performed following ISO 4833-1:2013 standard. For this, 10 mL of sample were mixer with 90 mL of BPW in triplicates and homogenized in a Stomacher for 30 sec. To assess the total counts, dilutions were then performed, plated in PCA medium and incubated at 30 °C for 72 h.

3.10.2 Long-read next generation DNA sequencing

To identify the microorganisms presented in the raw milk sample a long-read next generation DNA sequencing was performed in 3 non-spiked samples obtained after performing a 7 h short enrichment methodology and downstream DNA extraction with enzymatic lysis protocol, describe in section 3.3.2.2. The miniaturized sequencing MinION device (Oxford Nanopore Technologies, UK) with the Flongle flow cells (FLO-FLG001) were used to perform the microbial characterization of the samples. The libraries were prepared following Rapid Barcoding Kit (SQK-RBK004) standard protocol, including purification with Agencourt AMPure XP beads (Beckman Coulter Inc., USA). Summarizing, 3.75 µL of the DNA extract was mixed with 1.25 µL the corresponding barcode and incubate at 30 °C for 1 min and 80 °C for another 1 min. Then, the reactions were pooled and purification was performed, where equal volume of AMPure beads were added and incubated in a Mini Tube Rotator for 5min. The beads were washed twice with 200 µL of 70% ethanol assisted by a MPC, and DNA resupended in 5 µL of elution buffer (10 mM Tris-HCl, 50 mM NaCl, pH 7.5-8.0), incubated 2 min, and after beads recovered by magnetic force, the 5 µL were transferred to another tube. To the purified DNA, 0.5 µL of RAP was added and incubated 5 min, followed by the addition of 13.5 µL of Sequencing Buffer (SQB) and 11 µL of Loading Beads (LB). Before loading the sequencing reaction, the flow cell was primed with the mix of Flush Buffer (FB) and Flush Tether (FLT). The sequencing was performed for 4 h and base calling done in real-time. The resulting sequences were analysed with EPI2ME™ software (https://epi2me.nanoporetech.com) using “What’s In My Pot” (WIMP) workflow.

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CHAPTER 4.

RESULTS –

SAMPLE PRE-TREATMENT ALTERNATIVES

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4 RESULTS - SAMPLE PRE-TREATMENT ALTERNATIVES

4.1 INTRODUCTION

The detection of pathogenic bacteria has been the subject of an important number of studies in the last decades with the objective of improving sensitivity, accuracy and specificity. Most of the advances in this area have been focused on the specific detection of microorganism, in particular by DNA-based methods, however, little progress has been made on improving the enrichment and culture based steps as discussed in the Introduction section.

Most of the advances in the area of enrichment and culture-based techniques, are related with the development of different specific media to allow the recovery of the pathogen of interest, inhibiting the growth of competitive organisms which can interfere with the detection of the target. The validated methodologies for food analysis, still need a long time of enrichment to allow a reliable detection of the microorganisms of interest. This is considered to be the major bottleneck for faster foodborne pathogen detection.

In this chapter, different variations and improvement of the traditional enrichment were evaluated with the aim of developing a faster protocol of analysis, when combine with specific DNA-amplification based detection. The developed pre-treatment methodologies were analyse and compare by qPCR analysis.

This chapter includes the results obtained for this different samples pre-treatment approaches developed and tested with three objective:

- **Improvement of the standard enrichment,** where different media and the addition of supplements were evaluate for a 24 h incubation, to improve the growth of *L. monocytogenes* in simplex and for the three pathogens in co-culture.

- **Concentration of the bacteria**, evaluating the performance of IMS methodology and the use of a microfluidic device containing a functionalized PDMS sponge, as a solid-phase capturing.

- **Time reduction,** where an indirect detection by PAA and the pre-treatment of the sample by matrix lysis and the short enrichment protocol were evaluated

4.2 IMPROVEMENT OF THE STANDARD ENRICHMENT STEP

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As the focus of this project was to develop a multiplex detection for three important pathogens, *L. monocytogenes*, *Salmonella* spp, and *E.coli* O157, with different growth requirement, the enrichment step needed to be improved to allow the best growth of the targets, reducing the time of analysis. *L.*

monocytogenes is observed as the most challenging from the bacteria targeted, with a slower growth rate and for this reason the attention was focus in the improvement of the enrichment step for this pathogen, evaluated in simplex and in co-culture.

4.2.1 *L. monocytogenes* growth in simplex enrichment

A first evaluation of the effect of different media in the growth of *L. monocytogenes* was accomplished to understand which could be the best option to include as an alternative enrichment medium in the final methodology to be developed. For this purpose, the growth of the pathogen was followed in a microplate reader during 24 h, measuring the absorbance at 600 nm each 30 min, and kinetic curves were obtained plotting the OD versus the time of measurement. The results are presented in **Figure 4.1**. After the data obtained and modelled by the respective equation, the main parameters were extracted and are summarized in **Table 4.1**. The experimental data showed an excellent correlation, with adjusted correlation coefficients (adjusted R^2) higher than 0.99.

Table 4.1. Evaluation of the *L. monocytogenes* kinetic growth in different medium formulations.

		OD600 max	μmax	λ
30 ºC	HF	2.334 ± 0.036	0.900 ± 0.061	16.3 ± 0.3
	LEB	-	-	-
	BLEB	-	-	-
	ONE	2.708 ± 0.030	0.636 ± 0.012	14.3 ± 0.2
30 ºC	mTA10	0.288 ± 0.004	0.074 ± 0.004	14.2 ± 0.2
	mTA10 MOPS	0.261 ± 0.013	0.078 ± 0.003	14.0 ± 0.2
	mTA10 MOPS+Glu	0.328 ± 0.010	0.088± 0.001	14.8 ± 0.1
35 ºC	mTA10	0.242 ± 0.003	0.089 ± 0.017	12.4 ± 0.1
	mTA10 MOPS	0.202 ± 0.006	0.095 ± 0.003	13.1 ± 0.2
	mTA10 MOPS+Glu	0.292 ± 0.002	0.085 ± 0.004	13.4 ± 0.1
37 ºC	mTA10 MOPS	0.468 ±0.033	0.115 ± 0.033	11.0 ± 1.0
	0.25g/ L	0.693 ± 0.046	0.243 ± 0.021	12.0 ± 0.8
	5g/ L	0.382 ± 0.033	0.135 ± 0.008	11.3 ± 0.1
	20g/ L	0.990 ± 0.003	0.120 ± 0.009	11.7± 0.2
37 ºC	TSB	0.944 ± 0.025	0.215 ± 0.006	9.9 ± 0.2
	TSB+YE	1.085 ± 0.040	0.290 ± 0.020	12.4 ± 0.2
	TSB+YE+SP	1.082 ± 0.020	0.266 ± 0.034	11.7 ± 0.6
	BHI	1.006 ± 0.028	0.171 ± 0.006	11.2 ± 0.2
	BHI+YE	1.040 ± 0.016	0.189 ± 0.012	11.4 ± 0.3
	BHI+YE+SP	1.100 ± 0.018	0.193 ± 0.007	11.3 ± 0.3

OD600 max correspond to the maximum optical density; μmax represent the maximum specific growth rate; λ the lag time in hours.
Results were given by the model.

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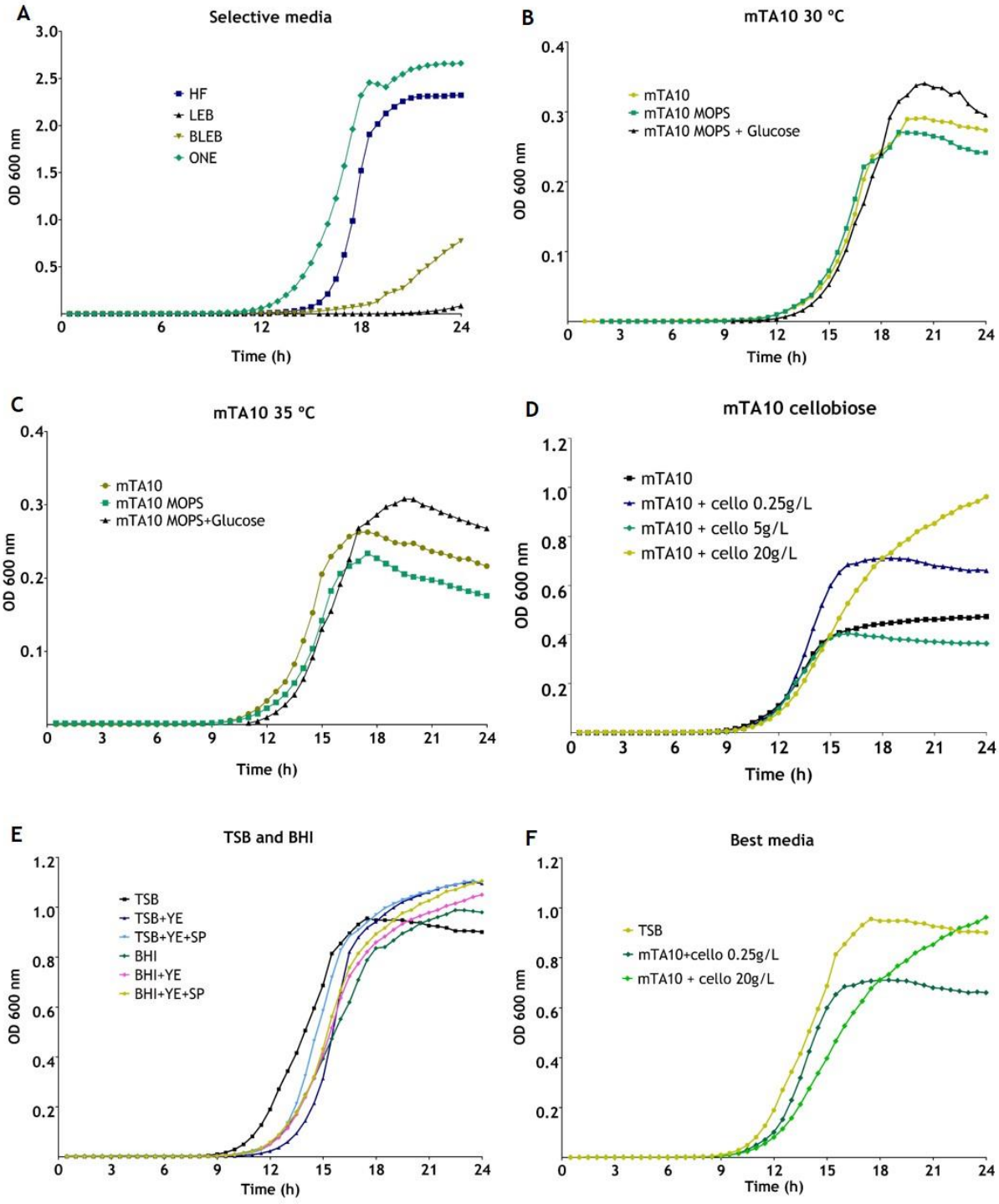


Figure 4.1. Evaluation of the growth of *L. monocytogenes* within 24 h in different selective and general media. (A) Selective media (HF, LEB, BLEB, ONE) at 30 ˚C. (B-D) Variation of mTA10 medium. MOPS buffering and 5 g/L glucose addition at 30 ˚C (B) and 35 ˚C (C) and supplementation with cellobiose (0.25, 5, 20 g/ L) at 37 ˚C (D). (E) TSB and BHI, with the addition of yeast extract (6 g/ L) and sodium pyruvate (1 g/ L) were also evaluated at 37 ˚C. (F) Comparison of the best media evaluated.

The standard protocols, from both ISO and FDA regulation authorities, specify several selective media for the enrichment of samples with the objective to identifying *L. monocytogenes*. These media allows optimal conditions for the specific bacteria to grow and, most importantly, inhibits the development of interfering microorganisms that may delay the growth of the target microorganism. With this in mind, several selective media, normally used in the reference protocols, were selected and tested, between them HF, LEB and BLEB, including also a commercial selective medium, ONE broth, with the same objective (**Figure 4.1 A**). LEB and BLEB showed an important delay in growth, and the model was not capable to calculate the parameters. However, the BLEB medium seemed to allow a lower lag phase than LEB, as can be observed in **Figure 4.1 A**. Comparing the two other media, ONE broth showed a shorter lag phage ($\lambda = 14.3 \pm 0.2$) and also showed a higher maximum optical density (OD600 max = 2.708 ± 0.03) compared to HF ($\lambda = 16.3 \pm 0.3$; OD600 max = 2.334 ± 0.036). The selective media were tested at an incubation temperature of 30 °C, as recommended by suppliers and the regulation. ONE broth showed better performance not only to achieve higher *L. monocytogenes* concentration, but also to inhibit the growth of naturally present microorganisms as already reported [151]. The use of selective media has its advantage when complex samples are analysed, however may not always be the best choice, not even when the aim is to target a single microorganism. In samples where stressed or injured bacteria may be present, the selective media can interfere with the recovering process of such cells [152], being a pre-enrichment in non-selective medium an advantage in these situations. Additionally, in some cases, some competitive microorganisms can present a faster growth in selective media than *L. monocytogenes*, as reported for *L. innocua* [153,154]. Finally, if a multiplex enrichment is the aim of the methodology, selective medium will not be able to allow the best growth of the three targets and for this reason is not compatible with all approaches.

The TA10, commercial name of the original No. 17 broth, was already improved twice, first by Omiccioli et al., [155] removing the dextrose, and later then adapted by Garrido et al., [156] by changing the amount of buffering salts used, increasing the final pH of the broth. However, when used in high concentration, phosphate salts were reported to have a toxic effect in bacteria [157]. Previous studies have described the use of MOPS to recover stressed *L. monocytogenes* cells and can be use in higher concentration to stabilized pH during enrichment, the substitution of the KH_2PO_4 and Na_2HPO_4 by MOPS was evaluated [157,158]. No statistically significant differences ($p > 0.05$) in lag phase, maximum OD600, growth rate between the use of the two different buffers were observed (**Figure 4.1 B and C)**.

The addition of 0.5 g/ L of glucose was evaluated in the new broth containing MOPS and an increase in the maximum concentration of bacteria was achieved obtaining a value of 0.328 ± 0.010 and 0.292 ± 0.002 when growth performed at 30 ºC and 35 ºC respectively. However, regarding the other parameters, μmax and λ no statistical differences were observed when glucose was added.

Three different temperatures were also analysed for the different formulation, and the results showed that even though a 1h reduction was observed between the enrichment at 35 °C compared to 30 °C, no statistical difference were observed in all parameters evaluated (**Table 4.1**). Additional the mTA10 MOPS was tested with the incubation performed a 37 °C and the OD 600 presented significantly higher value (0.468 ± 0.033) when compared with 30 °C (0.288 ± 0.004) and 35°C (0.242 ± 0.003).

With this results in mind, modifications to this medium were tested with the higher temperature (37 °C). Different concentration of cellobiose (0.25, 5, 20 g/ L) were tested in mTA10 with MOPS (**Figure 4.1 D**) and the data obtained from the growth studies, are in agreement with the results previously reported, showing a significantly higher growth rate when this carbohydrate is added to the medium [108,109], even when lower concentrations (0.25 g/ L) of cellobiose were used. The addition of 20 g/ L showed the same growth rate and lag phase as the control without cellobiose, however during the

stationary phase the bacteria concentration did not stabilized as observed in the other kinetics and a slower growth continued, allowing to have the highest concentration after 24h, comparing with all other concentrations of cellobiose tested. The results can possibly be explained, by the capacity of *L. monocytogenes* to use cellobiose as an alternative carbon source [159,160], but continue to have other preferential compounds, more efficiently metabolized as glucose-6-phosphate [159,161], being cellobiose the choice when the other sources are exhausted. By this, the growth of the bacteria can be extended by the metabolization of cellobiose in a later stage.

Two additional general media, TSB and BHI, were analysed by the same methodology in their original formulation and supplemented with yeast extract (YE) and sodium pyruvate (SP). The results are represented in **Figure 4.1 E**. The shortest lag phase was obtained with native TSB (9.9 ± 0.2). The supplementation of the medium with yeast extract (YE) and/ or sodium pyruvate (SP) obtained results similar to those of mTA10. No significant effect was observed on the other parameters evaluated. Regarding BHI, all variations obtained a similar result in all parameters, between 1-1.1, 0.17-0.19 and 11.2-11.4 for the OD600 max, μmax, and λ, respectively.

For the detection of *L. monocytogenes* in a methodology where the enrichment is performed in 24 h, the best option may pass by the use of mTA10 broth supplemented with 0.25 g/ L or 20 g/ L of cellobiose, as both are able to improve the growth of this pathogens and may allow to recover stressed cells (**Figure 4.1 F**). This medium can also gave some advantage regarding the competition existing with other target and interfering bacteria presented in the sample. On the other hand, since the objective was to reduce the enrichment time to have a same day detection, the medium needed to reduce the lag phase, as the bacteria will never reach the stationary phase, being the final concentration of bacteria after 24 h not relevant for this approach. For this end, TSB is the medium which gave the best results and have the ability to achieve higher *L. monocytogenes* concentration in less time **Figure 4.1 F**.

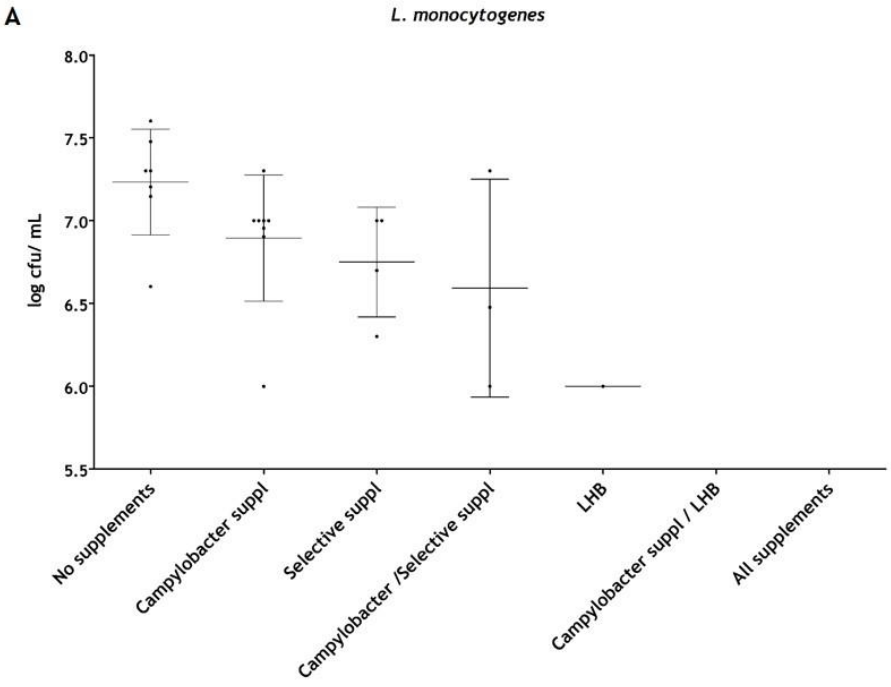
4.2.2 Growth of the three targets in co-culture

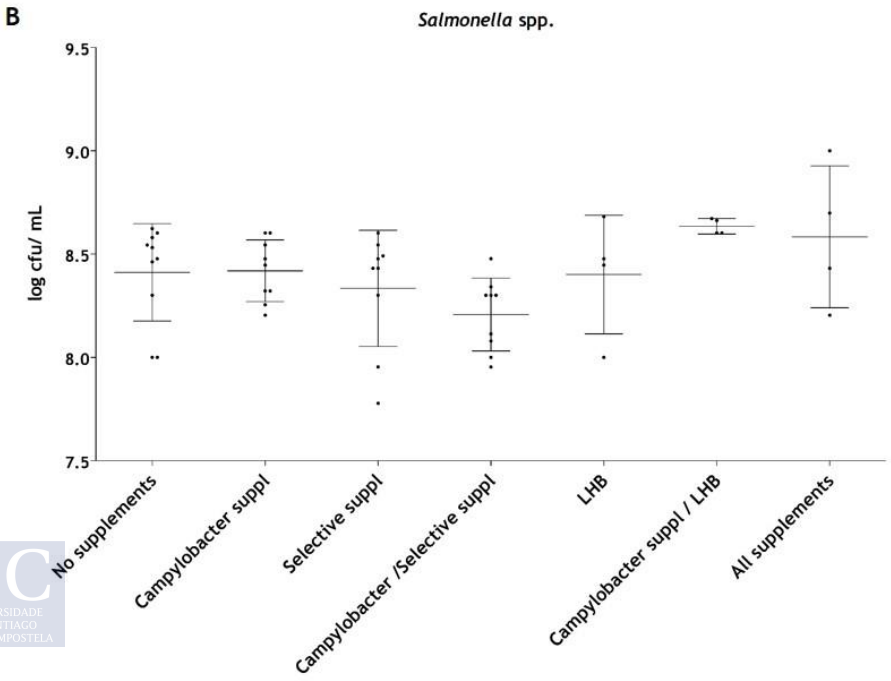
After studying the growth of *L. monocytogenes* in simplex to identify which medium could provide the best conditions to enhance the final concentration obtained, different variations of mTA10 MOPS were tested for the growth in co-culture of *L. monocytogenes, Salmonella* spp. and *E. coli* O157.

Laked Horse Blood (LHB), *Campylobacter* Growth Supplement and Half Fraser Selective Supplement were chosen to evaluate their effect alone and combined in the growth of the three pathogens (**Figure 4.2**). The results presented in **Figure 4.2 B** show that LHB and *Campylobacter* Supplement combined improve the growth of *Salmonella* spp., but not in a statistically significant manner, the plate counts were higher with a small standard deviation. No major effects, promoting or inhibiting, were observed on the plate counts of *E. coli* O157 **Figure 4.2 C**. However, none of them were able to improve the growth of *L. monocytogenes*, actually had a detrimental effect even though it was not statistically significant. **Figure 4.2 A** shows lower concentration of this bacterium after 24 h of incubation in mTA10-MOPS supplemented with the different compounds in co-culture. LHB seems to originate the lower concentrations, and its combination with any of the other supplements increase this effect, being the plate counts for these lower than 6 log cfu/ mL.

Having in consideration the results obtained by the kinetic analysis, mTA10 modified with MOPS buffer and 0.25 g/ L cellobiose was similarly evaluated to enhance the growth of *L. monocytogenes* in co-culture for a multiplex enrichment in 24 h, the results are presented in **Figure 4.2 D**. A statistically significant difference was observed in the growth of *L. monocytogenes*, increasing 2.3 log cfu/ mL the

final concentration obtained. The results are in concordance with the kinetic assay, confirming the improvement in the growth of *L. monocytogenes* when cellobiose was added to the medium. The final concentration of *Salmonella* and *E. coli* O157 did not show any difference with the implementation of cellobiose, which benefit the detection of *L. monocytogenes*, improving its competiveness.





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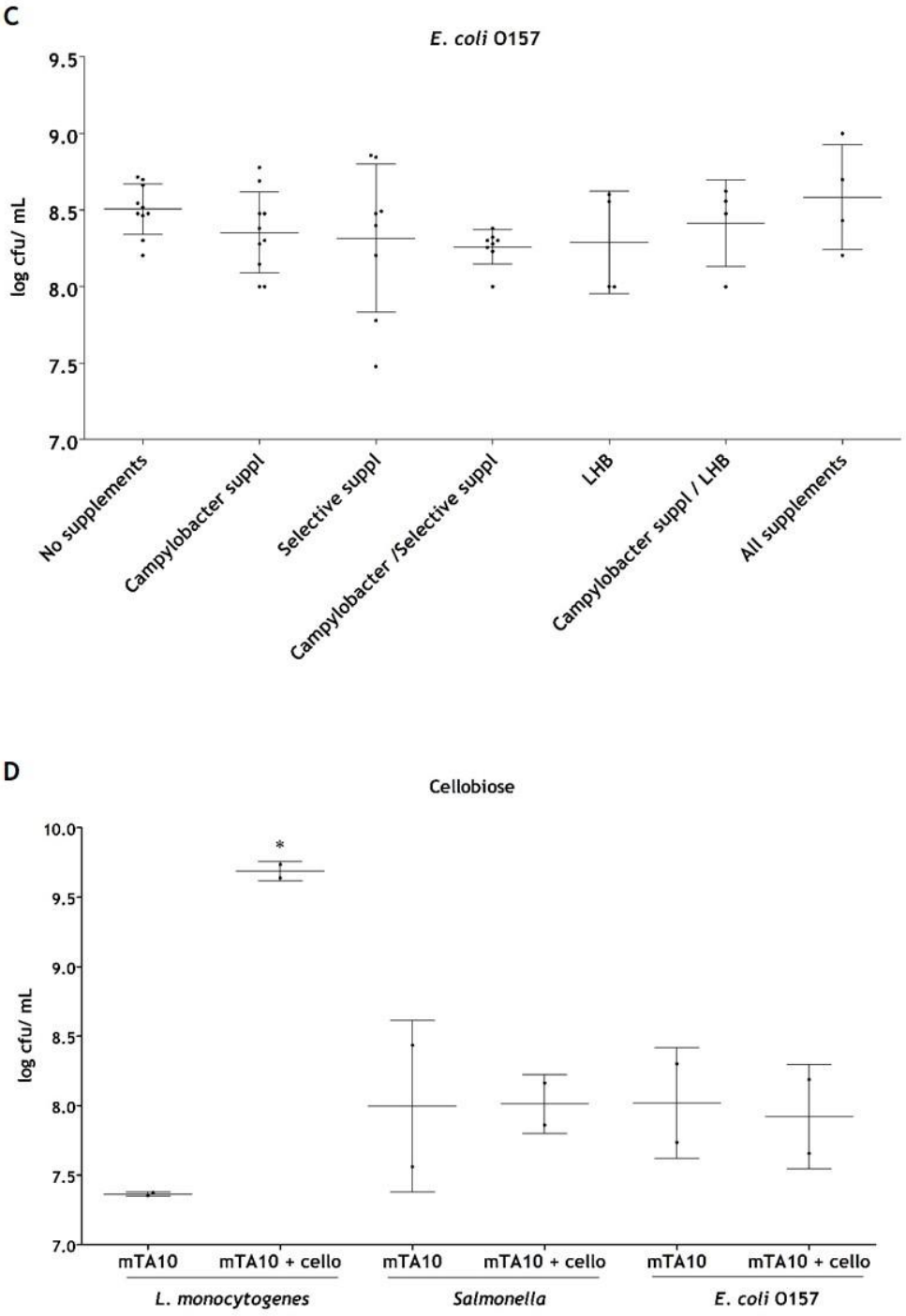

Figure 4.2. Optimization of mTA10 MOPS medium. (A-C) Plating results of mix cultures of *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157 after 24 h of incubation in mTA10 MOPS with the different supplements: 4 mL/L Campylobacter Growth Supplement (Campylobacter suppl.) (Oxoid, UK), 225 µL/225 mL Half Fraser Supplement (Selective suppl.) (Biokar Diagnostics S.A., France), 50 mL/L Laked Horse Blood (LHB) (Oxoid, UK), and as well the combinations of these compounds simultaneously. (D) Co-culture results of the supplementation with 0.25 g/ L of cellobiose for the three pathogens.

4.3 CONCENTRATION OF BACTERIA

4.3.1 Immunomagnetic separation (IMS)

The first step performed to develop the IMS methodology was the evaluation of different commercial antibodies in terms of purity and specificity in order to choose the best option to functionalize the magnetic beads to concentrate *L. monocytogenes*.

4.3.1.1 Commercial antibodies evaluation

To screen the purity of the antibodies SDS-PAGE was performed and the resulting gel is presented in **Figure 4.3**. The results shows that all antibodies presented two similar main bands, one about 20–25 kDa and another one at 55 kDa, corresponding to the light and heavy chains of the Abs. No additional bands are present in the two monoclonal antibodies (MA1-20271 and MAB8953), however the two polyclonal antibodies contain other contaminant proteins showing a band with a molecular weight around 66 kDa, which can correspond to BSA. Another protein is present in the goat pAb (MD-05-0329) with higher molecular weight between 70–100 kDa, which could also represent some intact antibody, due to incomplete reduction by Laemmli buffer.

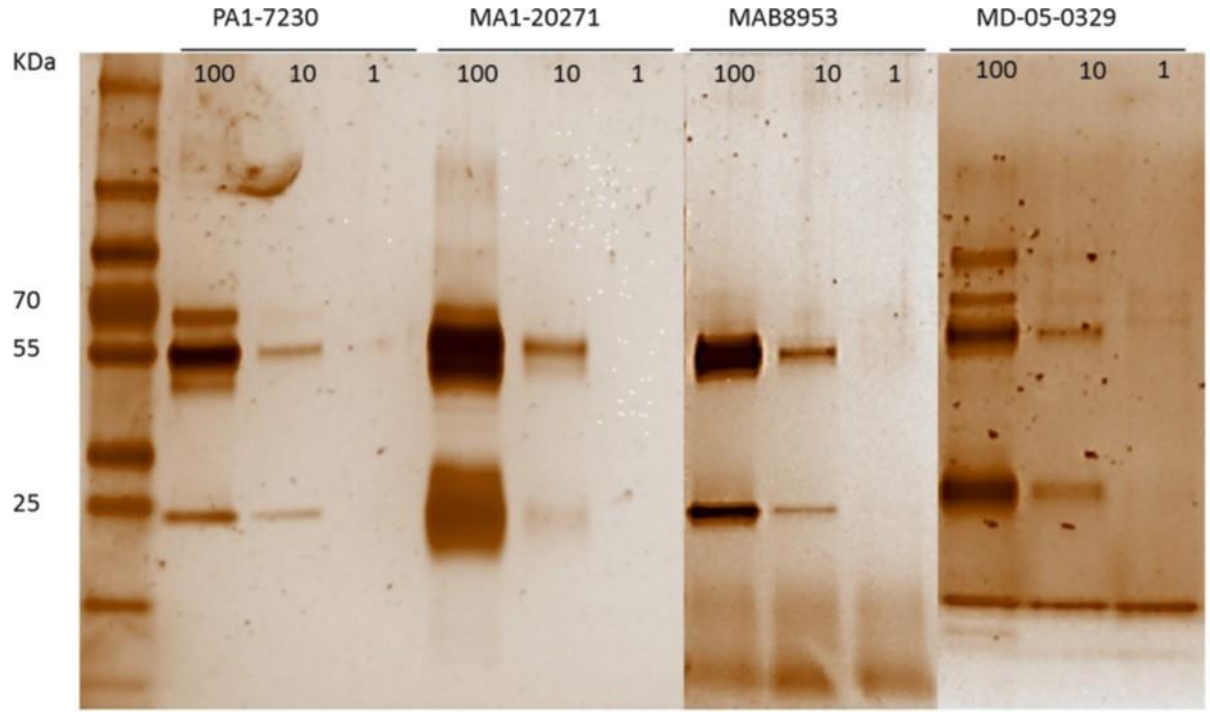

Figure 4.3. SDS-PAGE results obtained for the evaluation of the purity of the evaluated Ab. Three concentrations of each Ab were loaded: 100, 10 and 1 µg/ mL

To evaluate the specificity of the antibodies to bind to *L. monocytogenes*, an indirect ELISA was performed with other microorganisms, including *S.* Typhimurium and *L. innocua*. As shown in **Figure 4.4**, cross-reactivity against the non-specific targets was exhibited by both polyclonal antibodies. The monoclonal antibody MA1-20271, in addition to cross-reactivity resulted in a very low signal for *L. monocytogenes*. The only antibody which presented acceptable results in terms of specificity and signal intensity was Ab MAB8953, with a high signal for the target pathogen and only a residual cross-reactivity for the other. If cross-reactivity with other bacteria is present the recovering of the target can be compromised, as they will compete for the antibody binding, reducing the capture efficiency, and consequently the limit of detection of the methods being tested [162].

As expected, all the antibodies tested were relatively pure, but exhibited great differences in terms of specificity. The mAb MAB8953 showed the highest specific for *L. monocytogenes*, and thus selected for nanoparticle functionalization.

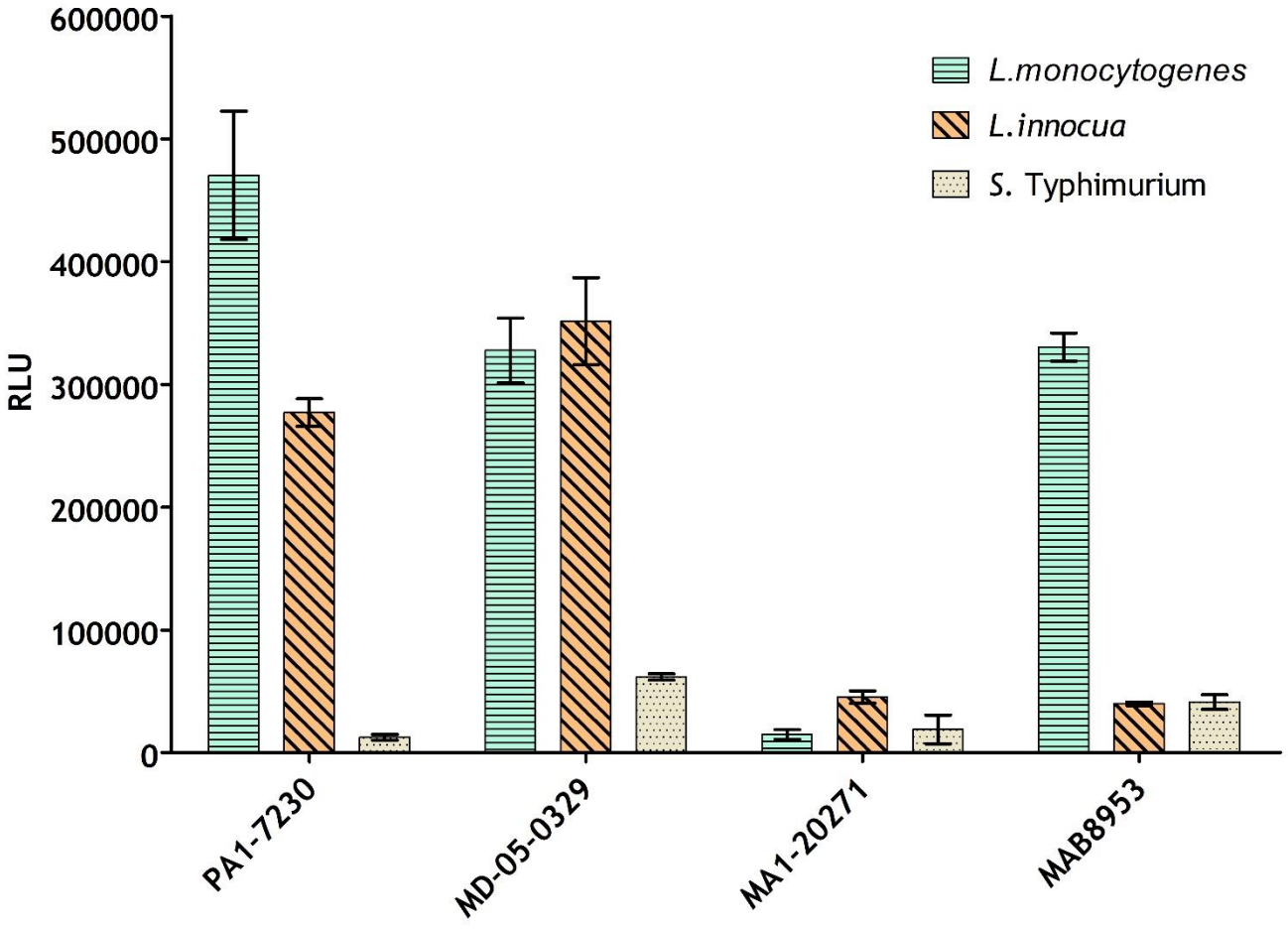

Figure 4.4. Specificity of the antibodies tested for IMS. PA1-7230 and MD-05-0329 are polyclonal antibodies, while MA1-20271 and MAB8953 are monoclonal. Signal intensity is presented in Relative Luminescence Units (RLU)

4.3.1.2 Capture efficiency

The optimization of the IMS protocol was made evaluating the capture efficiency (CE) of different alternatives to the standard protocol recommender by the MNPs supplier. The quantification results obtained by qPCR are presented in **Figure 4.5**, expressed as log cfu/ mL, and the CE was calculated based on these results. Performing Three bead washing steps, prior to further analysis, provided lower CE values than only one washing step, The direct analysis of the MNPs provides a CE of 95 % (Cb = 3.8; C0 = 4.0), while extensive washing, as indicated in the MNPs protocol, reduces this value to 75 % (Cb = 3.6; C0 = 4.8), which will compromise the sensitivity of the methodology. For this reason, no extensive MNPs washing was included in the final protocol.

The influence of the washing step in the capture of the target bacteria was also reported by other authors [163,164], showing a decrease of sensitivity when too many washing steps were performed. The use of IMS reported in other studies also obtained similarly high CE values [165].

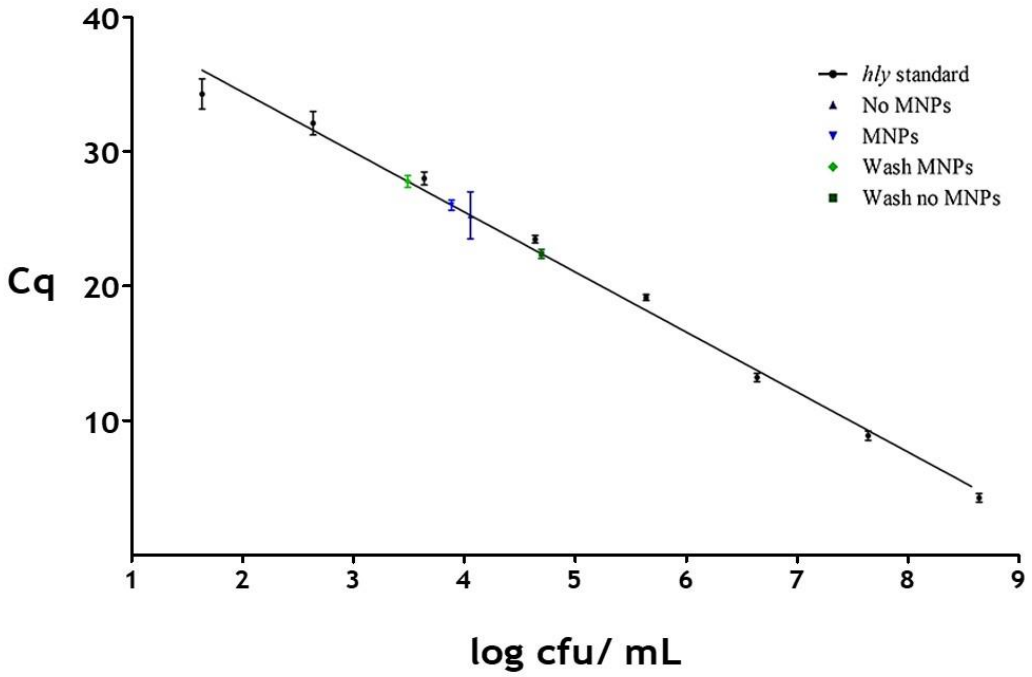

Figure 4.5. Quantification performed by qPCR for the determination of the CE. In green are represented the results for the standard IMS protocol, including three washing steps with PBS (dark green for the direct quantification and light green after the MNPs treatment). The blue points indicate the optimized IMS, with direct separation from sample enrichment, without washing (dark blue for the direct quantification and light blue after the MNPs treatment)

4.3.1.3 Evaluation with food matrixes

The optimized protocol was tested and evaluated in spiked samples from different types of food product and the results obtained by qPCR analysis are presented in **Table 4.2**. From this total of 42 samples, 10 samples of fresh cheese samples, were spiked with a lower bacterial concentration (9.7 cfu/ 25 g) where the LoD was established, as describe in Chapter 3, section 3.9.2. Additionally it was possible

to detect the presence of *L. monocytogenes* in samples with a lower concentration ranging between 4.6 to 7.1 cfu/ 25 g, showing the ability of the methodology to reach higher sensitivity. All the remaining samples inoculated with higher concentration obtained 100 % positive results and the remaining non-inoculated samples were negative, presenting a k index of 1.0.

Table 4.2. Samples analysed by IMS

Type of food	N	Inoculation level (cfu/ 25 g)	IMS- qPCR	Plate confirmation
Milk	2	-	-	-
	1	< 10	+	+
	1	10-10^2	+	+
	1	10^2 - 10^3	+	+
Hard cheese	1	-	-	-
	4	< 10	+	+
	1	10-10^2	+	+
	2	10^2 - 10^3	+	+
	1	> 10^3	+	+
Anchovy	3	-	-	-
	1	< 10	+	+
	1	10-10^2	+	+
Chicken	4*	10-10^2	+	+
	2*	< 10	+	+
	2	< 10	+	+
	2	10-10^2	+	+
	2	10^2 - 10^3	+	+
Fresh cheese (LoD)	10	< 10	+	+
	1	-	-	-

*** These samples were not originally inoculated, but were naturally contaminated with *L. monocytogenes***

Over this study, it was observed that two batches of chicken samples, which included 6 non-inoculated samples, presented a positive result. Further analysis of the enriched samples following the ISO 11290 method confirmed that these samples were naturally contaminated with *L. monocytogenes*. These samples were also analysed according to the 11290-2:2017, in triplicate to determine the concentration of *L. monocytogenes*, which was determined to be lower than 10 cfu/ 25 g for one batch, and between 10 and 100 cfu/ 25 g for the other. With total concordance between the results obtained by IMS-qPCR methodology and the culture-based confirmation, the performance parameter resulted on a value of 100 % and a kappa index of 1.00, which demonstrate the reliability of the developed methodology. Comparable results were obtained in other studies using IMS combined with a DNA amplification methods, not only PCR/ qPCR based methods but also isothermal approaches [135,163,164].

The developed approach optimized for the detection of *L. monocytogenes* demonstrated a real advantage when implemented in the analysis by simplifying the sample pre-treatment before the DNA extraction. Several steps to remove the food particles and chemicals, as filtrations, centrifugation and washing steps are replaced by the magnetic separation, reducing not only the time but also the sample manipulation [166,167]. With the analysis of different food samples, the methodology demonstrated to be suitable for a wide range of complex matrixes, avoiding inhibition of the qPCR reaction.

Different aspects can influence the capacity to capture the bacteria by the magnetic bead. The performance of the IMS can be improved when MNPs are used instead of the microspheres [168,169] due to the higher surface-to-volume ratio of the first ones, and possibility of higher antibody binding, improving the CE and faster binding kinetics.

The possibility to functionalize the nanospheres with any antibody allows to control and improve the specificity of the analysis for different targets. This approach has been developed not only for *L. monocytogenes* but also for the detection of other foodborne pathogens, [66,165,168,170], showing to be a real advantage.

4.3.2 PDMS sponge

4.3.2.1 Non-targeted bacterial capturing with ApoH protein

The capacity of the sponge to retain the bacterial cells was evaluated testing two different bacteria, *L. monocytogenes* as a Gram-positive, and *Salmonella* spp. as Gram-negative. A control was performed to confirm that the bacteria capture was due to the interaction with ligand and not due to a non-specific capture in the pores of the sponge. Without any functionalization of the sponge a capture efficiency of 19.7 ± 2.4 was observed, when passing a bacterial concentration of 10^4 cfu/ mL through the device. This result show some retention in the pores of the sponge, but the use of a ligand allowed to significantly improve the bacterial capture capacity of the device. The results indicated that the sponges functionalized with ApoH had a capture efficiency between 88.6 ± 10.4 and 92.3 ± 0.1 for *L. monocytogenes* and 78.6 ± 10.3 to 90.5 ± 3.0 for *Salmonella* spp., see **Table 4.3** and **Figure 4.6 A**, depending on the concentration of bacteria tested. This results are comparable with previous studies using concentration methodologies for pathogen detection. Fakruddin et al. report the use a solid phase in microplate to capture different bacteria from food samples, obtaining a capture efficiency of 80% until a log 6 cfu/ mL with an incubation of 6 h [171]. With higher concentrations, this value decreases rapidly, probably due to having reached the capture limit of the system. The same is observed by Li et al. (2019), using IMS for the concentration of *E. coli* who obtained a capture efficiency of 81% for a bacteria concentration of 2×10^2 CFU/mL [172]

A mixture with the two pathogens was also tested and results showed a capture efficiency of 70.9 ± 2.9 and 72.6 ± 12.9 for *S.* Typhimurium and *L. monocytogenes*, respectively, when tested in the range of 10^5 cfu, presenting in the later case a higher deviation (**Table 4.3** and **Figure 4.6 B**). However an increase in this value (98.7 ± 1.8 and 97.2 ± 2.5) with lower deviation was noticed when using a lower bacterial concentration (range of 10^3 cfu). This fact may be due to the competition existing between the bacterial cells to bind to the ApoH protein when higher concentration is used, as when the capture efficiency for one pathogen increased, a decrease was visible for the other one. The deviation existing in the capture efficiency between replicates can also be explained by the fact that not all sponges have the same size, as the cutting was made manually and the porosity can also vary between sponges. This will influence

the concentration of immobilized protein or antibody into the sponge and also the capacity of the flow to penetrate.

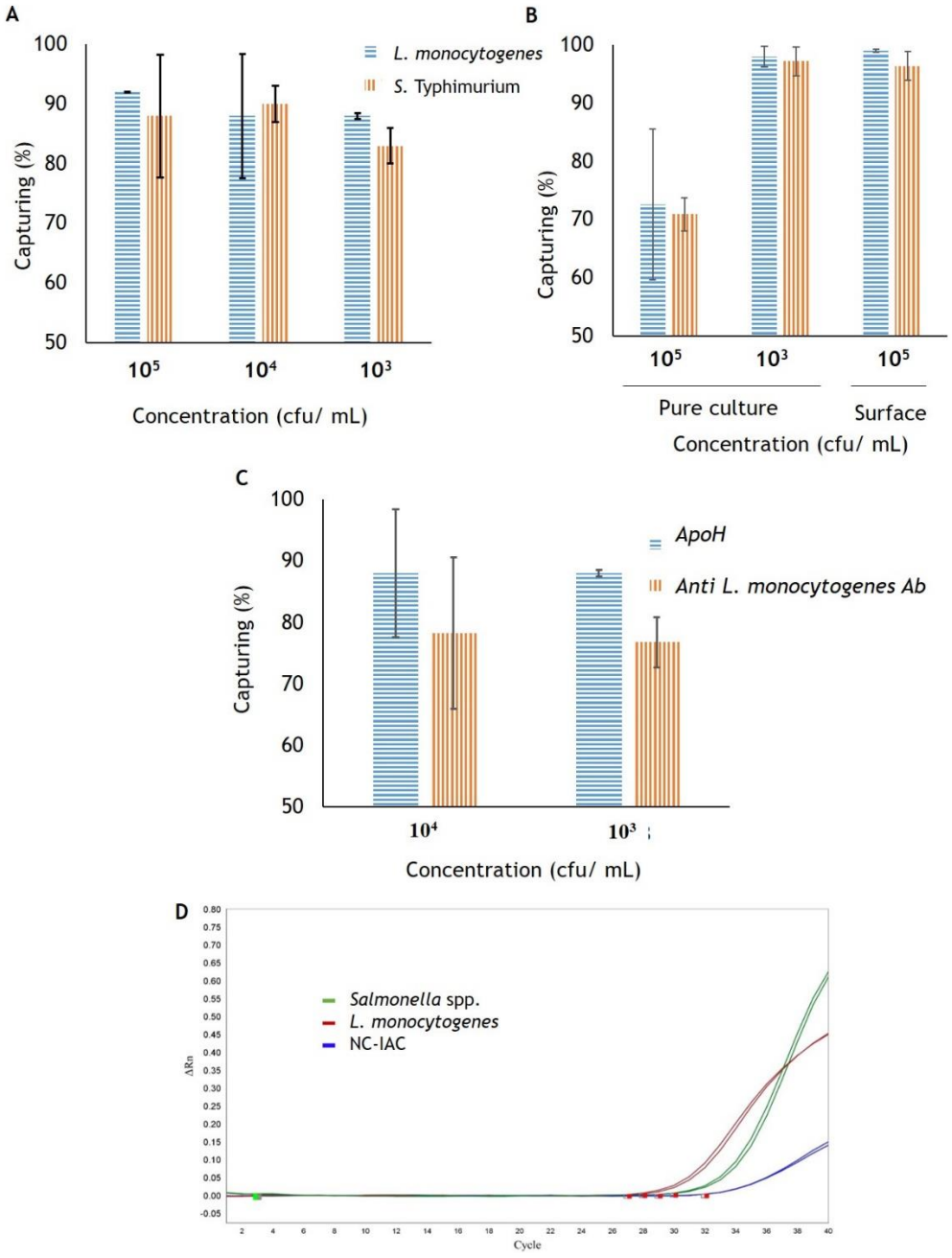

Figure 4.6. Graphical representation of the capture efficiency for *L. monocytogenes* and *Salmonella* spp., using the PDMS sponge functionalized into the device. (A) Simplex and (B) Multiplex detection of pure culture or passing a surface sample, using ApoH protein functionalization. (C) Comparison of capture efficiency using PDMS sponge functionalized with ApoH protein and Anti- *L. monocytogenes* specific antibody. (D) qPCR amplification plots detecting *L. monocytogenes* () and *Salmonella* spp. in a concentration of 10^5 cfu/ mL. NC-IAC was included in the reaction to detect inhibition and avoid false negative results due to reaction inhibition.

Table 4.3. Capture efficiency of the PDMS sponge functionalized with ApoH protein

Bacteria concentration	Pure culture			Mixed culture		Surface
	10^5	10^4	10^3	10^5	10^3	10^5
L. monocytogenes	92.3 ± 0.1	88.6 ± 10.4	88.8 ± 0.5	72.6 ± 12.9	98.7 ± 1.8	99.7 ± 0.3
S. Typhimurium	78.6 ± 10.3	90.5 ± 3.0	83.8 ± 3.0	70.9 ± 2.9	97.2 ± 2.5	96.4 ± 2.5

Capture efficiency is expressed in % and the bacteria concentration in the range of cfu/ mL.

4.3.2.2 Comparison between ApoH and Ab functionalization

ApoH has the ability to bind to both, Gram-positive and Gram-negative, thus being non-specific. To test if the results provided by the use of this protein instead of a specific target is as efficient for bacterial capture, a specific anti-*L. monocytogenes* antibody was used to compare the ability to capture this pathogen. Two *L. monocytogenes* concentrations, 10^4 and 10^3 cfu/ mL, were tested showing capture efficiencies of 78.3% ± 12.3 and 76.8% ± 4.1, respectively. Higher capture efficiency was observed using the ApoH protein, being 88.6% ± 10.4 and 88.8% ± 0.5, respectively (**Table 4.4**). The comparison between these two different functionalization is represented in **Figure 4.6 C**, showing an advantage in using the non-targeted approach. However, even if ApoH had a higher capture efficiency, it must be taken in consideration the fact of this approach being a non-specific capture, meaning that is not suitable in samples with high bacterial background, as other bacteria will compete with *L. monocytogenes* or other targeted pathogen. This effect can be avoided with the use of a specific capture.

Table 4.4. Comparison capture efficiency with ApoH and antibody

	L. monocytogenes	
	10^4	10^3
Ab	78.3 ± 12.3	76.8 ± 4.1
ApoH	88.6 ± 10.4	88.8 ± 0.5

Capture efficiency is expressed in % and the bacteria concentration in the range of cfu/ mL

4.3.2.3 qPCR analysis

The detection by qPCR was performed to evaluate if the sponge-based concentration method was compatible with this type of DNA-based detection, and additionally to allow the determination of the LoD of the full method. The amplification results are presented in **Table 4.5** and **Figure 4.6 D**. Bacterial concentrations below the range of 10^3 cfu/ mL were not detectable by qPCR. This results agree with previous reports, being a limitation of the qPCR [173]. For *Salmonella* spp., levels of 10^3 cfu/ mL were achieved, however for *L. monocytogenes*, the lowest concentration which showed amplification in all replicates were 10^4 cfu/ mL, separately and in mixture. *L. monocytogenes* is more difficult to lyse and

for this reason a lower concentration of DNA is recovered from the sample and resulting in lower LoD. A possibility to overcome the limitation of the qPCR reaction and improve the sensitivity, is the addition of an enrichment step to the methodology in order to reach a detectable concentration of bacteria in the sample.

Table 4.5. qPCR results obtained after PDMS sponge concentration and DNA extraction from the sponge.

	ApoH						Ab	
	Separately			In mixture		Surface	Separately	
	10^5	10^4	10^3	10^5	10^3	10^5	10^4	10^3
L. monocytogenes	+	+	-*	+	-	+	+	-
S. Typhimurium	+	+	+	+	+	+		

Result consider positive when both technical replicate amplified;
* 1 in 4 samples showed amplification;
Bacteria concentration in the range of cfu/ mL.

4.3.2.4 Evaluation with spiked surface samples

Surface samples were inoculated to test the applicability of the methodology to capture and detect different pathogens at the same time in a real case scenario in the food industry. A mixture of both bacteria was spread over the surface, then recovered by sponge sampling method and passed through the device. All bacteria passed through the device were retained by the sponge as the capture efficiency showed to be 99.7 ± 0.3 and 96.4 ± 2.5 for *L. monocytogenes* and *S.* Typhimurium, respectively (**Table 4.3** and **Figure 4.6 B**). Previous steps, performed before the introduction of the sample into the device, can result in loss of bacteria. Namely, the sampling process from the surface and the elution of bacteria retained in the sponge to the PBS solution is not 100% efficient, leading to an incomplete recovery of the bacteria, and lost regarding the initial bacteria concentration spiked on the surface. This results in a lower bacterial concentration passed and retained in the sponge, and increase of the LoD when analysing surface samples. Besides this fact, the detection *L. monocytogenes* and *Salmonella* spp. was successfully accomplished by qPCR for both targets using 10^5 cfu of bacterial cells.

This methodology represents a real advantage giving the possibility of full integration in a lab-on-chip system, as the DNA extraction and amplification step can be incorporated in a miniaturized device [174,175].

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4.4 TIME REDUCTION

4.4.1 PAA

4.4.1.1 Protocol optimization

In order to define the concentration of bacteriophages to be use to infect the *Salmonella* cells present in the sample, the minimum concentration that could be detected by qPCR was determined, showing to be possible to detect the pure bacteriophages from 6.6×10^{11} pfu/mL down to 1.3×10^{3} pfu/ mL (**Figure 4.7**). As the limit of detection was 10^{3} pfu/ mL it was decided to add a final concentration between 10^{3} - 10^{4} pfu/ mL to each sample. Even if the qPCR limit of detection showed to be 100 times higher than other studies [176], this difference did not affect the final performance of the methodology developed, when samples were analysed.

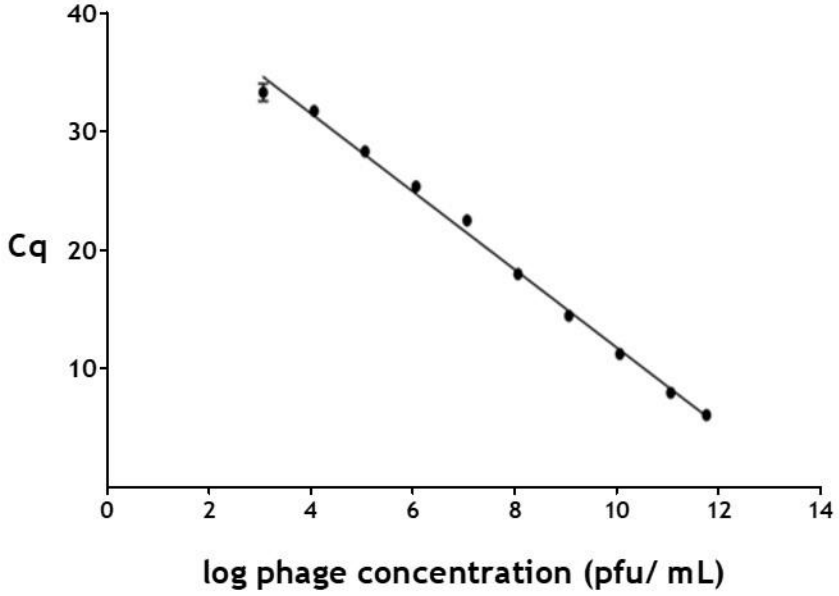

Figure 4.7. Detection of Bacteriophage DNA with Probe-qPCR. Efficiency of the amplification obtained after ten-fold serially diluting the pure Bacteriophage vB_SenS_PVP-SE2.

To process the samples before qPCR analysis, a thermal lysis was added to release the bacteriophage DNA inside the *Salmonella* cells, in case of incomplete lytic cycle, and also from the bacteriophage capsids. This type of DNA extraction allow higher sensitivity of the methodology in a simple and practical way, taking only 10 min to be performed.

For the methodology to have the best performance, the time of enrichment after the addition of the bacteriophage was optimized, performing the DNA extraction in different time points, right after the addition of the bacteriophages, 3 and 6 h after (T0, T3 andT6). The percentage of positive samples obtained for each enrichment time was plotted and the results are presented in **Figure 4.8**. As expected

in T0 no amplification was observed, as the bacteriophage did not had time to replicate. This test also allowed to confirm that the positive results only were associated with the increase of viral DNA, resulting from the infection of viable *Salmonella* cells by the bacteriophage. At the same time, T0 served as a baseline to compare T3 and T6. A decrease in the cycle of quantification (Cq) value was observed when the time of enrichment and the bacterial concentration in the samples was also increased, due to the increase of the bacteriophage DNA. The results show the possibility to detect higher bacterial concentration, 10^2 cfu/ 25 g, with only 3 h of enrichment, but only a concentration of 10^4 cfu/ 25 g allow to detect 100 % of the samples. To reach lower detection levels, a 6 h incubation is needed, allowing to detect < 10 cfu/ 25 g. The bacteriophage selected underwent a lytic infectious cycle, with infection resulting in rapid lysis and dead of bacteria within a very short time [113]. This process can occur as long as the bacterial host is present in sufficient number to support the replication. To have enough bacteriophage replicated before exhaustion of the bacterial cells, a pre-enrichment step of 3 h was included in the protocol. However this step could be optimized and reduced to shorten the analysis time below 10 h, maintaining the sensitivity.

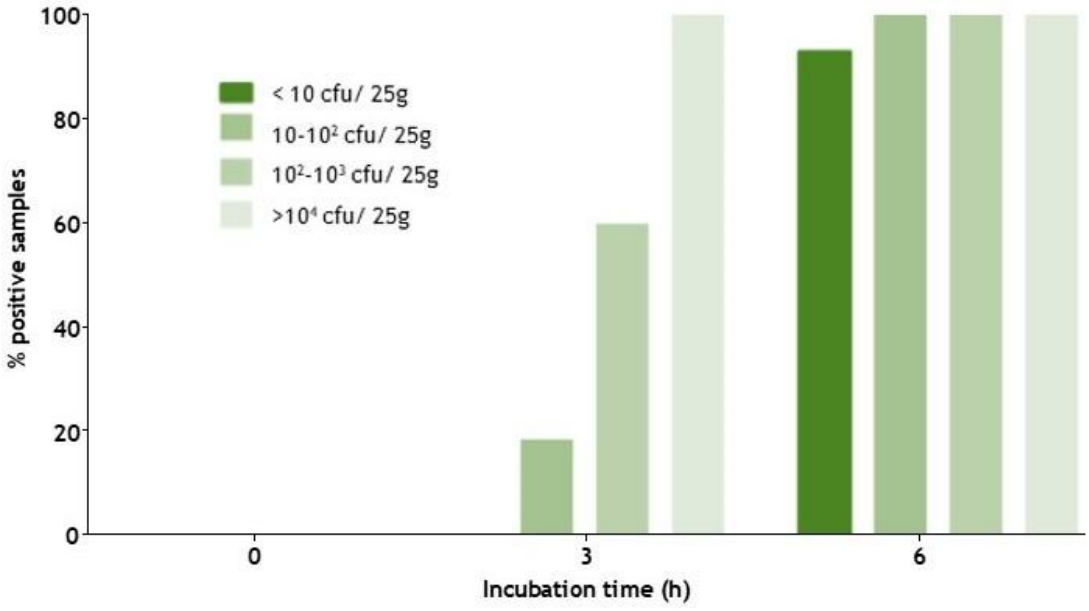

Figure 4.8. Optimization of the incubation time for Bacteriophage multiplication. Percentage of positive samples at different co-incubation times (3 h pre-enrichment + the indicating time after bacteriophage addition).

4.4.1.2 Evaluation with food matrixes

A total of 41 samples, summarized in **Table 4.6**, were analysed, following the optimized protocol, to evaluate the methodology and establish the LoD. This approach specifically detects viable bacteria, as the replication of the bacteriophage is only possible in viable cells. To confirm this statement, 6 samples were spiked with different contamination levels of non-viable *S.* Enteritidis, between 10^3 and 10^7 cfu/ 25 g, and the analysis was performed as described previously. After qPCR analysis, even in the higher

concentration no positive result was observed, confirming the detection of only viable *Salmonella* cells. With the analysis of samples spiked with viable bacteria, only one ND was observed in samples with a concentration close to the LoD (9 cfu/ 25 g), resulting in a SE, SP and AC of 96.6 %, 100 % and 97.6 % respectively. The PPV and NPV obtained values of 100 % and 92.3 % respectively, representing results in high degree of concordance with the expected result, obtaining a κ of 0.94 (**Table 4.7**).

Table 4.6. Samples analysed by PAA methodology

Spiking	N	Inoculation level (cfu/ 25g)	PAA- qPCR
Live Bacteria	10*	8	+
	3	5/10/0	+/+/-
	5	5.2/9.8/9.4/8.6**×10	+
	5	$5.2/9.8/9.4/8.6\times10^{2}$	+
	2	9.8×10^{3}	+
	2	9.8×10^{4}	+
	2	9.8×10^{5}	+
	6	-	-
Non-viable Bacteria	2	8.0×10^{7}	-
	2	8.0×10^{5}	-
	2	8.0×10^{3}	-

*** Samples used to determine the LoD;**
**** Two samples were spiked with this concentration.**

Table 4.7. Evaluation of PAA methodology

N	PA	PD	NA	ND	SE	SP	AC	PPV	NPV	κ	LoD (cfu/ 25 g)
41	28	0	12	1	96.6	100	97.6	100	92.3	0.94	8

ND spiked with 9 cfu/ 25g;
Chicken breast samples were used for the evaluation.

A LoD of 8 cfu/ 25 g was obtained, resulting from the analysing of 10 samples spiked with a concentration <10 cfu/ 25 g, but lower concentration, down to 5 cfu/ 25 g could also be detected.

In conclusion, the developed method enables the detection of *S.* Enteritidis cells with high sensitivity in a total analysis time of 10 h, and has the capacity to specifically detect viable *S.* Enteritidis. This represents a significant time reduction with respect to the standard culture-based, and other molecular biology-based methods [142,167,177], which need between 20 to 48 h to have the results.

Due to the high confidence of the results obtained, this method can be suitable for the implementation on routine laboratories. Further optimization can be done to allow a faster analysis, as the selection of bacteriophage with shorter latent periods, or for a multiplex detection a phage cocktail can be developed [178].

4.4.2 Matrix lysis

The matrix lysis approach was developed by Rossmanith et al., [75], and optimized later [76]. Due to quantity of food debris recovered by this protocol, the authors were only capable to perform a reliable analysis until 12.5 g or mL of sample. The European regulation advise the sampling of 25 g from the foodstuff [179], and for this reason, to be able to process a higher sample size, the original protocol was modified. Instead of taking all the sample, after homogenisation with the sucrose buffer, the mixture was recovered through the filter of a stomacher bag with a pore size of <250 µm, working as a barrier for most of the larger food debris, reducing the pellet obtained. This step was introduced as it was observed that the protease treatment, and lysis buffer, were not enough to reduce the pellet recovered to a size suitable for direct DNA extraction.

The modified methodology tested for 25 g of sample provided a LoD_{95} of 1.1 x 10^5 cfu/ 25g, and for the 16 samples above this concentration, the analysis performed correctly, with no deviation observed and provided a κ of 1.0 (**Table 4.8**). From 14 samples spiked below the LoD, it was observed that 7 of them gave a positive result even at a concentration of 8 x 10^3 cfu/ 25 g. However, the original protocol obtained a lower LoD of 7.3 cfu/ mL for the analysis in milk samples [75], but also had similar values in other types of liquid samples (7.8 cfu/ mL) when analysed 12.5 mL. It was observed a slight increase when 6.25 g of solid foodstuff was process (15.4 cfu/ g) [76], showing the influence of the type of matrix analysed. The increase of the samples from 6.25 g to 25 g could be the reason of the difference in the LoD, as the proportion of matrix recovered compared with the bacteria is much higher and can interfere with the qPCR reaction.

Other studies have shown the application of this approach for different purposes, such as the recovery of Mycobacteria from animal tissues [180], detection of *Staphylococcus aureus* in milk [78] and cheese [181], among others.

This methodology still needs further optimization to reach a lower LoD, down to 100 cfu/ g, to be apply in the food industry for the detection of *L. monocytogenes*, in foods which do not support its growth.

Table 4.8. Evaluation of Matrix lysis approach

N	PA	PD	NA	ND	SE	SP	AC	PPV	NPV	κ	LOD_{95} (cfu/ 25 g)
16	13	0	3	0	100	100	100	100	100	1.00	1.1 x 10^5

Salmon samples were used to evaluate the methodology.

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4.4.3 Short enrichment

4.4.3.1 Protocol optimization

After the results obtained by the matrix lysis methodology, showing not being adequate for the detection of *L. monocytogenes* in food samples due to the high LoD value obtained, an alternative approach was tested based on the protocol developed by Fachmann et al. [80] for the detection of *Salmonella* in fresh meat. In their study a short enrichment associated with a matrix degradation was performed. To apply this approach for the detection of *L. monocytogenes* different aspects of the protocol were optimized. Fist the volume of enrichment medium to be added to the sample was tested, and no significant difference was observed between the use of 25 mL and 50 mL of TSB to growth *L. monocytogenes* in simplex, with a variation of 1.08 ± 0.12 and 1.07 ± 0.26 log cfu/ g, respectively, however 25 mL showed a smaller deviation between replicates (**Figure 4.9**). The final protocol was for this reason performed with 25 mL, which also make the analysis less expensive and the smaller volume simplify the following step. The need to use agitation during the enrichment was evaluated in the same way, resulting in an statistically significant increase of bacteria growth when constant shaking at 200 rpm is used (variation of 1.40 ± 0.02 log cfu/ g), compared with static incubation (variation of 1.02 ± 0.16 log cfu/ g) (**Figure 4.9**). Even if this optimization was performed with an enrichment time of 4 h, the results obtained by qPCR when the samples were analysed in these conditions, were not consistent, not allowing to achieve a proper LoD. The enrichment required a longer incubation time to provide reliable results, therefore the following sample analysis was accomplish with the optimized protocol, where 25 mL of TSB were added to the sample and incubated with constant shacking at 37 ºC for 5 h.

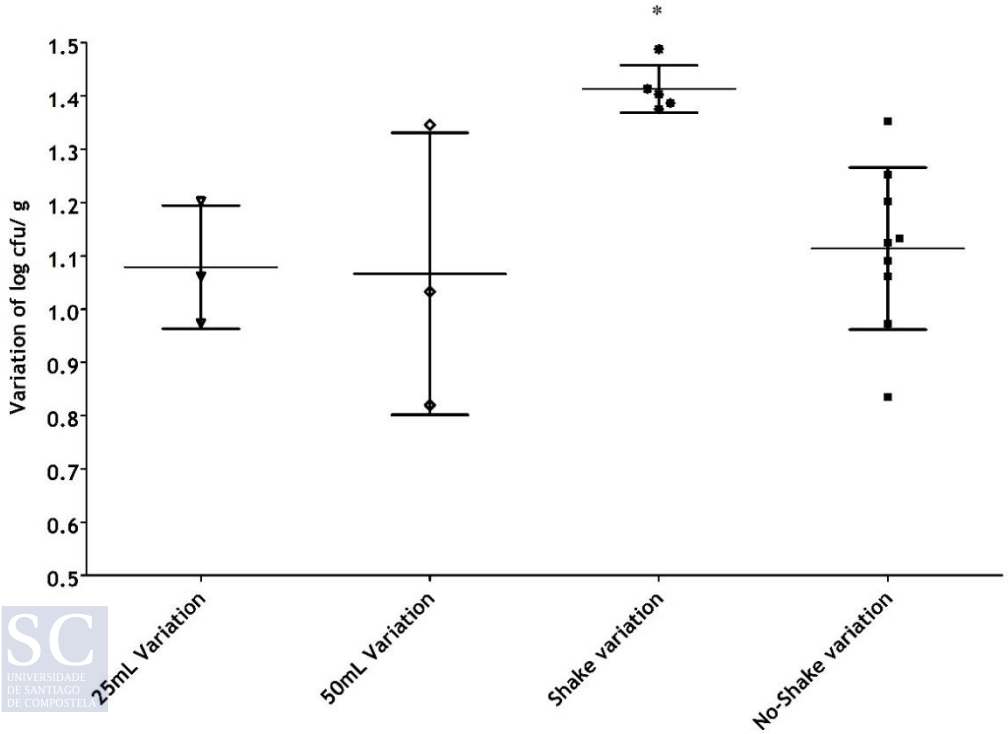

Figure 4.9. Evaluation of different conditions for short enrichment optimization. Bacterial growth variation between T0 and T4 (4 h incubation). * indicates statistically significant differences

4.4.3.2 Evaluation with food matrixes

The methodology was tested for the detection of *L. monocytogenes* and a similar approach was also tested for *E. coli* O157 and the multiplex detection of *E. coli* O157 and *Salmonella* spp. with the enrichment condition specified in **Table 3.4** of Chapter 3, section 3.4.3.3. The results obtained for the analysis of different samples is presented in **Table 4.9**, including the parameters to evaluate the fitness for purpose, but also the LoD achieved for each one of them. For all pathogens, it was possible to detect a concentration of targeted bacteria lower than 10 cfu/ 25 g, being 8.6 cfu/ 25 g for *L. monocytogenes* in simplex and between 3 - 4 cfu/ 25 g for the *E. coli* O157 in simplex and multiplex detection of *E. coli* O157 and *Salmonella* spp.. Comparing with the previous study in which the method was based, *Salmonella* and *E. coli* O157 obtained a lower value, as the authors observed a LoD_{50} of 8.8 cfu/ 25 g. However *L. monocytogenes* showed higher values, which could be related with the target itself as *L. monocytogenes* has a slower growth rate and the DNA extraction of this pathogen present more difficulties than for the other targets. Another cause for this higher value could the type of matrix analysed. The sample type could make the recovery of the bacteria difficult, due to several factors. The size of the debris generated and the fat content could block the filter of the stomacher bag not allowing the passage of all bacterial cells, as may also interfere with the washing steps and protease treatment [80].

Table 4.9. Short enrichment evaluation and comparison

Bacteria	Food sample	N	PA	PD	NA	ND	SE	SP	AC	PPV	NPV	κ	LoD_{95} (cfu/ 25 g)
L. monocytogenes	Salmon	17	12	0	5	0	100	100	100	100	100	1.00	8.6
E. coli O157	Ground beef	19	14	0	4	1	93	100	95	100	80	0.85	3.9
	Leafy green	20	12	0	8	0	100	100	100	100	100	1.00	3.3
	Combined	39	26	0	12	1	96	100	97	100	92	0.94	3.6
Salmonella spp. *E. coli* O157 (Multiplex)*	Ground beef and Chicken breast	39	30	0	8	1	97	100	97	100	89	0.92	3.4
		39	30	0	8	1	97	100	97	100	89	0.92	3.7
		78	60	0	16	2	97	100	97	100	89	0.92	3.6

The "combined" results indicate the values obtained for the all methodology considering both type of samples (ground beef and leafy green) tested;
* Results presented are for the detection of *Salmonell*a spp. *E. coli*, and combined results in that order.

On the other hand, some specific components may not be washed in the pre-treatment and inhibit the qPCR reaction [182], leading to a False negative result, and compromising the reliability of the method. The leafy green samples demonstrate this effect, as the use of a specific plant kit (NucleoSpin® Plant, Macherey-Nagel, Germany) to purify these samples after the DNA extraction was required, and the DNA extract obtained needed to be additionally diluted ½ to obtain positive results. Several studies already reported the inhibition of qPCR by plant component [183,184], and the need to adequate the protocol to overcome this effect. Another aspect that can interfere with the detection of the targeted pathogen is the presence of interfering microorganism in high levels in certain types of samples [151]

With a κ index of the method for the detection of different pathogens, with values ranging between 0.85 and 1.00, the short enrichment methodology show a real advantage to be used in the food industry, allowing a same day detection with an enrichment of 3 or 5 hour depending on the pathogen to be

detected. An important fact to be mentioned is the relevance of proper defrost of the samples before the enrichment step and to add the medium pre-warmed at 37 ºC, to allow the appropriate growth of the bacteria.

A ND was observed in the simplex *E. coli* O157 detection. It was related with a sample where the ON defrosting was not properly performed. This could lead to decrease the temperature of the starting enrichment, delaying the growth of the bacteria that was already spiked in a low concentration (5.1 cfu/ 25 g).

When the methodology was evaluated for the multiplex detection of *E. coli* O157 and *Salmonella* spp, the recovery and detection of stressed bacteria were also tested. Before spiking the samples, both bacteria went through a heat treatment at 60 ºC for 10 min with constant agitation (1000 rpm) and diluted in PBS to contaminate 8 samples at a final concentration lower than 10 cfu/ 25 g. Half of these samples were stored at 4-8º C during 24 h and the other half during 48 h and then the protocol for the short enrichment was applied. Regarding *Salmonella*, the detection of 3 of 4 spiked samples was possible, regardless of the treatment. The same results were observed for *E. coli* O157 when the samples were refrigerated for 24 h. However when this period is extended to 48 h only 2 of the 4 samples gave a positive result.

The influence of non-viable pathogens, was also determined, in order to understand if the methodology could allow to specifically detect viable bacteria. For this evaluation, the approach for the multiplex detection was applied in 8 samples where different concentration, ranging from10^3 cfu/ 25 g to 10^6 cfu/ 25 g of non-viable bacteria were spiked from both targets. The LoD was calculated, and showed that it was necessary at least 3.9 x 10^5 cfu/ 25 g and 2.9 x 10^5 cfu/ 25 g of dead *Salmonella* spp. and *E. coli* O157 respectively, to originate false positives associated with the presence of DNA from the non-viable pathogen cells.

This methodology showed promising results, allowing to perform a same-day detection, and with further optimization could be applied to different food commodities. The type of matrix will be the principal factor that influence the performance of the method, and variation in the protocol may be needed for the methodology to fit in a wide range of foodstuffs. The pore size of the stomacher bags, the homogenisation of the sample with the enrichment medium (manual or automatic), and the protease used for the degradation of food debris, are some of the conditions to be improved for better recovering of the bacteria and a cleaner DNA extract [80].

4.5 COMPARISON OF SAMPLE PRE-TREATMENT APPROACHES

After the analysis of the four different approaches to improve the sample pre-treatment (**Table 4.10**), all of them presented some advantages when compared with the traditional methodologies based on culture techniques. Even if the IMS did not present a significant difference in terms of time of the analysis comparing with different qPCR approach developed with the same objective [121,167,185], the concentration of the target bacteria allowed to separate them from the rest of the sample and enrichment media, removing most inhibitors present in this mixture. With the analysis of different type of foodstuff, the presented IMS, demonstrated to be suitable for the analysis of a wide variety of matrix, as milk, cheese, anchovies and chicken.

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Table 4.10. Comparison of the different approaches

Methodology	Analysis time	LoD (cfu/ 25g)	Cost	Advantage	Disadvantage
IMS	26 h (*L. monocytogenes*)	9.7	+8€	Elimination of inhibitors	Still need long enrichment
PDMS Sponge	> 5h [a] (*L. monocytogenes Salmonella* spp.)	- [b]	*	Elimination of inhibitors Automatize analysis	Not suitable for food with large debris
PAA	10h (*S.* Enteritidis)	8	-**	Viable bacteria differentiation	Need to growth bacteria before
Matrix lysis	< 5 h (*L. monocytogenes*)	1.1 x 10^5	+ 4€	More bacteria recovered	Inhibitors remaining
Short enrichment	5h (*Salmonella* and *E. coli* O157) 7h (*L. monocytogenes*)	3-4 8.6	+ 2€		

The cost column refers to the approximation in the cost increase per sample, performing the respective pre-treatment methodology, without enrichment or DNA extraction steps. Only including reagents and specific compounds required;
[a] The analysis time do not include enrichment step
[b] The LoD was not determined in complex samples. For surface samples without enrichment a bacteria concentration of 10^5 was detected.
* The cost will depend in the fabrication price of the device to allocate the sponge;
** No additional cost, only the purchase of the bacteriophage at the beginning that can be replicate posteriorly.

In a very similar way, the use of PDMS sponge to concentrate the bacteria allowed to wash the sample recovered, from PCR inhibitors. In this case, the approach was evaluate for detection of pathogens on surface samples, which many times contain cleaning agents, known to interfere with PCR reaction, leading to false negative results [186]. The multiplex detection by IMS was already reported [170,187] but required the use of a specific antibodies for each target pathogen, making the analysis much more expensive. Universal ligands as ApoH protein have the advantage to bind to any bacteria, virus or fungi allowing an easier and cheaper multiplex recovery, however its use is incompatible with most complex matrix with a high concentration of background microorganisms, being suitable only for samples subjected to sterilization process as pasteurization or more intense heat treatment [188], which have a very low initial natural microorganism content.

These two methodologies are the unique from the approaches tested, which allowed the purification from inhibitors compounds. Replacing the washing step normally performed before DNA extraction by the concentration, we also simplified the process and reduced the sample manipulation, improving the sensitivity of the analysis. The automatization of the concentration process was demonstrated by the integration onto a micro-device, which simplified and reduced the hands-on time for the sample pre-treatment.

The use of specific bacteriophages to improve the sensitivity of the analysis present some advantages, such as the specific detection of viable pathogens in 10 h. The need for the differentiation between viable and non-viable bacteria is still controversial in the food industry. Most of the companies will want to know if the bacteria are still alive to determine the safeness of their product. However if the DNA of the pathogen was found in the product, it means that the pathogen was present at some point, and maybe the sample taken does not have any viable pathogens, but it does not assure that the rest of the production line is safe [189].

Some studies have combined the use IMS to recover the target bacteria from the enrichment medium, and allow removal of inhibitors, with the bacteriophages infection, to increase the signal [190,191], enhancing a more sensitive and reliable detection. Both approaches, PAA and IMS, are the most complex

to be developed. The PAA requires to understand the dynamics between the bacteriophage and its host, and for this both elements need to be well characterized. With the need to find a specific antibody and bacteriophage for each target to be detected and make sure to do not have interference among them. Also the use of these extra components in the protocol makes the analysis more expensive.

In terms of time of analysis, the methodologies which present a significant improvement were the matrix lysis and the short enrichment, allowing a same-day detection. This could be a real advantage in the food processing chain taking into account the intense production existing nowadays and the fast delivery of the products. However the matrix lysis was discarded, as the results of the LoD obtained were too high to suite the limit required by the legislation. Regarding the short enrichment approach, a LoD < 10 cfu/ 25g was achieved for all targeted pathogens, showing the possibility of application in the industry, The only limitation of this approach could be the interference of the matrix in qPCR reaction, due to the presence of high levels of inhibitors, which required extra measures in certain types of samples. The combination of the short enrichment with the IMS could be an option to overcome this limitation, however the cost of the analysis will consequently increase, as purchase of specific antibody could be expensive.

For these reasons, the short enrichment was chosen as the most adequate option for the development of a faster, and economic methodology to detect in multiplex *L. monocytogenes*, *E. coli* O157, and *Salmonella* spp.

The evaluation of a wide range of media were tested, having in attention the reduction of the lag phase, growth rate and maximum concentration of *L. monocytogenes* achieved, as this was the pathogen with more restrained growth, when compared with *E. coli* O157 and *Salmonella* spp.. Even if mTA10 supplemented with cellobiose showed promising results to be used in a 24 h enrichment, the most important factor for the medium to be integrated in the short enrichment protocol, was to begin the exponential phase as soon as possible, and TSB provided a significantly shorter lag phase, around 10 hours, demonstrating to be the most suitable from all media tested for this type of approach.

4.6 CONCLUSIONS

After intense analysis of different approaches to improve the specificity, sensitivity, and time of the analysis, all of them demonstrate several advantages regarding the existing, and implemented, methodologies used in the food industry.

Standard enrichment

- By the analysis of different media variation, as expected general media allow to improve the growth of *L. monocytogenes* when compared with the selective ones, being TSB the one which was able to have higher reduction in the lag phase
- The cellobiose was the only supplementation allowing a significantly improvement in the final concentration of *L. monocytogenes* obtained, without having any effect in growth of the *Salmonella* spp. and *E. coli* O157. This improves the competiveness of this Gram-positive bacteria.

Concentration of the bacteria

- The IMS allow to concentrate the bacteria from the enrichment medium, achieving a sensitive detection with the advantage of the elimination of possible inhibitors.

- Regarding the functionalized PDMS sponge, with the use of the non-specific ApoH ligand, higher capture efficiency was achieved comparing with the use of specific antibody.

Time reduction

- The PAA approach using bacteriophage allowed to reduce the time of the analysis to a total of 10 h for the detection of *S.* Enteritidis.
- The analysis by matrix lysis can be faster, but due to the very high limit of detection obtained, this methodology was not consider suitable to be applied in regular testing.
- The shot enrichment approach allows a fast same day detection, and achieved a very low limit of detection for the three pathogens targeted.

The presented study had as objective the development of a fast, affordable and multiplexed foodborne pathogen detection, and with this in mind, the short enrichment was chosen as appropriate approach for the detection of *L. monocytogenes*, *E. coli* O157 and *Salmonella* spp. The reason for this choice, is the significant time reduction, as the experiments performed for the detection of *L. monocytogenes* in simplex demonstrated a decrease from 7 days by the ISO method [103,192], or 26 h for alternative qPCR methods [122,193] to just 6-7 h. Another aspect to have in consideration is the simplicity of the protocol, without the need a specific compounds, as antibodies used for the IMS approach and bacteriophages for the PAA, which allow a low-cost but sensitive detection.

4.7 PUBLICATION OF THE RESULTS

The results presented in this chapter were published in the following scientific articles:

Azinheiro, S., Carvalho, J., Fuciños, P., Pastrana, L., Prado, M., & Garrido-Maestu, A. (2022). Short pre-enrichment and modified matrix lysis. A comparative study towards same-day detection of Listeria monocytogenes. LWT, 154, 112900. https://doi.org/10.1016/J.LWT.2021.112900

Azinheiro, S., Kant, K., Shahbazi, M. A., Garrido-Maestu, A., Prado, M., & Dieguez, L. (2020). A smart microfluidic platform for rapid multiplexed detection of foodborne pathogens. Food Control, 114, 107242. https://doi.org/10.1016/j.foodcont.2020.107242

Garrido-Maestu, A., **Azinheiro, S**., Carvalho, J., Espiña, B., & Prado, M. (2020). Evaluation and implementation of commercial antibodies for improved nanoparticle-based immunomagnetic separation and real-time PCR for faster detection of Listeria monocytogenes. Journal of Food Science and Technology, 1–9. https://doi.org/10.1007/s13197-020-04450-1

Garrido-Maestu, A., **Azinheiro, S.**, Roumani, F., Carvalho, J., & Prado, M. (2020). Application of Short Pre-enrichment, and Double Chemistry Real-Time PCR, Combining Fluorescent Probes and an Intercalating Dye, for Same-Day Detection and Confirmation of Salmonella spp. and Escherichia coli O157 in Ground Beef and Chicken Samples. Frontiers in Microbiology, 11. https://doi.org/10.3389/fmicb.2020.591041

Garrido-Maestu, A., Fuciños, P., **Azinheiro, S.**, Carvalho, C., Carvalho, J., & Prado, M. (2019). Specific detection of viable Salmonella Enteritidis by phage amplification combined with qPCR (PAA-qPCR) in spiked chicken meat samples. Food Control, 99, 79–83.

https://doi.org/10.1016/J.FOODCONT.2018.12.038

Garrido-Maestu, A., **Azinheiro, S.**, Carvalho, J., Fuciños, P., & Prado, M. (2019). Optimized sample treatment, combined with real-time PCR, for same-day detection of E. coli O157 in ground beef and leafy greens. Food Control, 106790. https://doi.org/10.1016/j.foodcont.2019.106790

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CHAPTER 5.

RESULTS -

DNA AMPLIFICATION APPROACHES

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5 RESULTS - DNA AMPLIFICATION APPROACHES

5.1 INTRODUCTION

The DNA-based detection of pathogens allows high sensitivity and specificity and enables the identification of microorganisms which are difficult of culture or viable but non-culturable cells (VBNC) and contribute to reduce the time of analysis after sample pre-treatment. For this reason qPCR has been integrated in some standard protocols for foodborne pathogen detection. However in order to simplify the amplification analysis, different isothermal amplification techniques have been studied to substitute the need of real-time thermocycler and allow an easier analysis of results.

Two isothermal amplification techniques, LAMP and RPA have been studied in this project, together with several naked-eye based detection methods and their performance has been compared against qPCR analysis.

In this chapter the results obtained for the different approaches of DNA amplification combined with several results visualization possibilities were presented including:

- **Real-time amplification by two qPCR alternatives,** using intercalating dye (SYBR-qPCR) and hydrolysis probe (Probe-qPCR)

- **Real-time isothermal amplifications (qLAMP and qRPA),** where their performance where compared against qPCR methodologies.

- **Naked-eye RPA approaches,** where the combination with a Lateral Flow strip (RPA-LF) and the addition of SYBR Green (RPA-SYBR) after RPA reaction for a colour change/fluorescence detection was accomplished

- **Naked-eye LAMP approaches,** with the detection of turbidity and colour change by two different strategies, with the addition of gold nanoparticle (MUA-AuNP) and employing a commercial mastermix with a pH sensitive dye.

5.2 REAL-TIME AMPLIFICATION

In order to evaluate alternatives for DNA amplification, several real-time approaches were tested and compared. Two qPCR techniques, including the use of intercalating dye and hydrolysis probe, were first tested for the detection of the three foodborne pathogens targeted, being them *Salmonella* spp., *E. coli*

O157 and *L. monocytogenes*. This was followed by the comparison with the two isothermal amplification techniques LAMP and RPA.

5.2.1 SYBR-qPCR for multiplex detection

The first method developed included the use of an intercalating dye to detect in multiplex *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157. The melting peaks obtained were evaluated, as well as the performance of the qPCR inquiring the inclusivity/ exclusivity and efficiency of the reaction, and at last, the analysis of spiked milk samples was performed.

5.2.1.1 Melting curves analysis

The difficulty in the development of this SYBR green qPCR approach was the design of the primers in order to have the amplification of the targeted fragments with enough differentiation between melting peaks to identify each pathogen.

After *in silico* analysis with uMELT software, the predicted values determined were 77.5, 79.5, 84 and 82.5 ºC and the sample obtained an average melting peak of 77.45 ± 0.13, 79.47 ± 0.11, 83.20 ± 0.13, 82.67 ± 0.16 ºC for the identification of *E. coli* O157, *L. monocytogenes*, *Salmonella* spp. and NC-IAC, respectively. Only the peak of *Salmonella* presented a lower Tm compared to the predicted one, being closer to the peak of NC-IAC. Despite this proximity, it can clearly distinguish both targets. The predicted and the experimental melting temperatures obtained with pure culture and within the sample analysis melting peaks are represented in **Figure 5.1**.

It is worth to mention that over the evaluation of the methodology, it was observed that the reference strain *L. monocytogenes* (WDCM 00021), generated two peaks, one more predominant with the expected melting temperature (Tm) (79.47 ± 0.11 °C), and a smaller and wider one (76.30 ± 0.5 °C). None of the other strains presented this second peak. The amplification products of the reference strains were analyzed by gel electrophoresis and presented in **Figure 5.2**, showing only one single fragment when this strain of *L. monocytogenes* was tested. This second peak appears close to the one of *E. coli* O157 (77.45 ± 0.13 °C), but still clearly differentiated, as it can be observed in **Figure 5.1 C.**

The adequacy of the proposed genes for the detection of these pathogens, in simplex, was well demonstrated in previous studies, and the primers designed in this study confirm those results, as all the strain tested were correctly identified.

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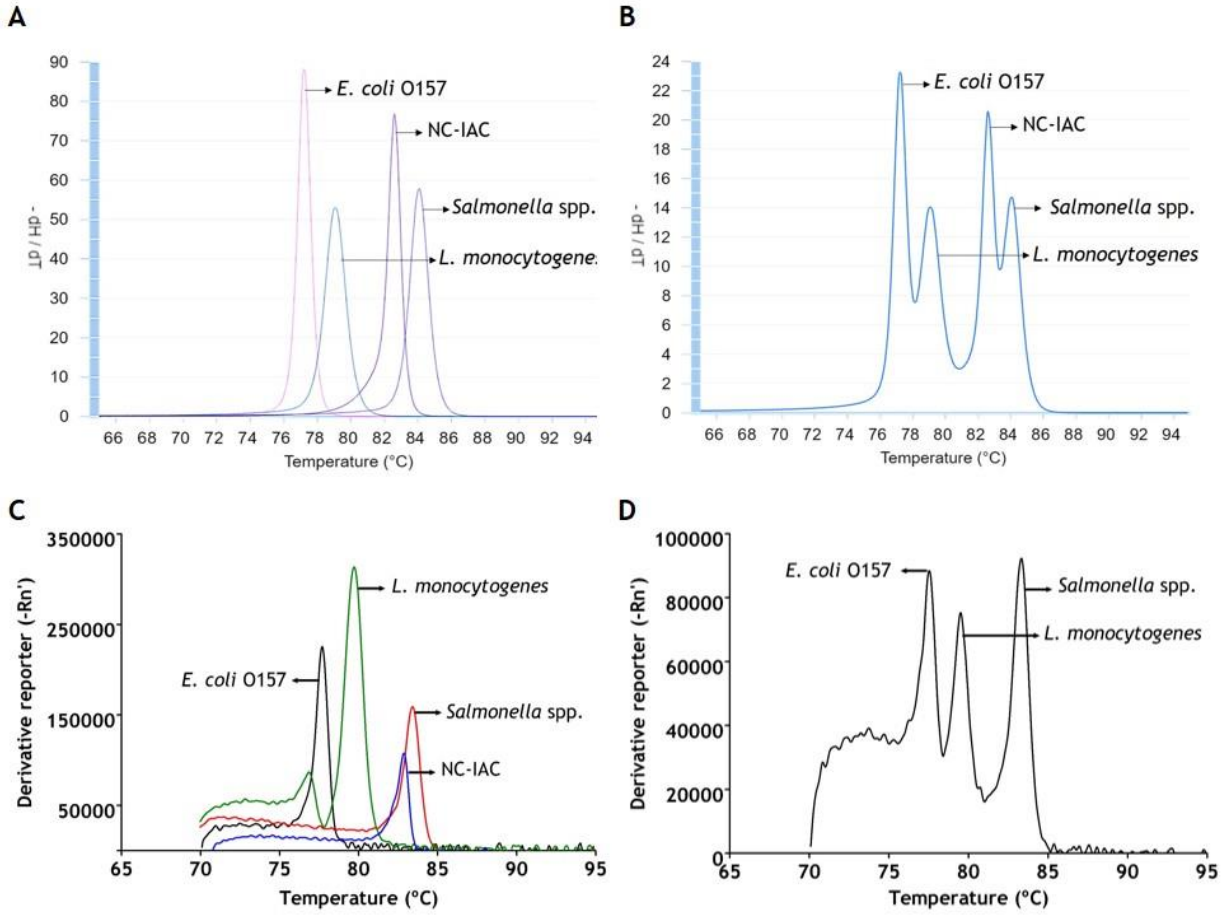

Figure 5.1. Comparison of expected and obtained melting curves with the SYBR-qPCR approach. Predicted melting curves generated by uMELT online software in simplex (A) and multiplex (B) reaction. Experimental melting curves obtained by pure culture in simplex (C) and by sample analysis in multiplex (D).

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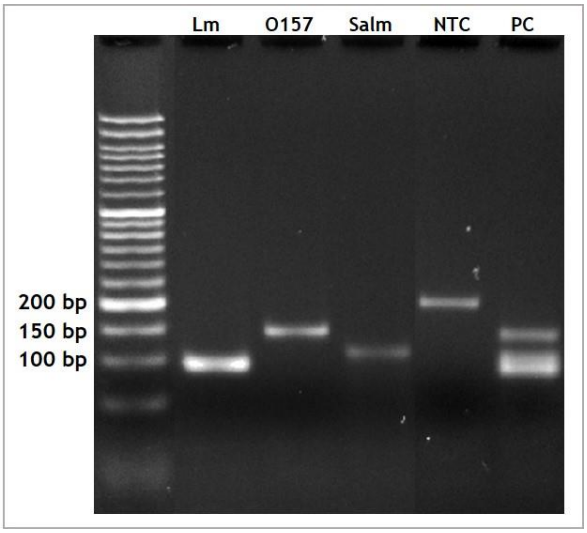

Figure 5.2. **Agarose gel presenting the amplicons originated by multiplex SYBR-qPCR.** The amplification using as template a DNA extract from a pure culture of *L. monocytogenes* (Lm), *E. coli* O157 (O157), S. Typhimurium (Salm). A negative control with water as template (NTC) showed the amplification of the NC-IAC and a positive control (PC) when a mixture of DNA from three pathogens is loaded. The theoretical amplicon size was 89, 142, 105 and 200 bp for *hlyA*, *rfbE*, *fimA* and NC-IAC, respectively, being consistent with the results obtained.

5.2.1.2 Evaluation of the SYBR-qPCR reaction

To evaluate the performance of the optimized qPCR method with *actA, fimA, rfbE* and NC-IAC primers, the inclusivity/exclusivity and efficiency were evaluated.

A total of 45 pure cultures from different strains were tested to evaluate the inclusivity/exclusivity of the qPCR and the results are presented in **Table 5.1**. The inclusivity of the multiplex qPCR was evaluated with, 13 *Salmonella* spp., 18 *L. monocytogenes* and 1 *E. coli* O157, presenting all the strains the expected melting peak. Regarding the exclusivity, 13 other bacteria were evaluated, including two other *Listeria* species and two *E. coli* strains. As expected, all non-target microorganism amplified with a Cq values of 35.36 ± 0.81 with a melting peak of 82.78 ± 0.09, specific for NC-IAC amplification.

The amplification efficiency of the qPCR was also evaluated, in simplex and multiplex. The lowest DNA concentration detected for *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 was 1.4, 1.6, 1.9 pg/ µL respectively, see **Figure 5.3 A, B and C**. Regarding the multiplex detection of the three targets simultaneously, the qPCR reaction showed a LoD 10 times higher of 11 pg/ µL when a mixture of the three DNA extract from each bacteria was loaded (**Figure 5.3 D**). This result could be due to the fact that the peaks for the other targets are more predominant than the one for *E. coli* O157, causing some interference in its detection when a lower range of DNA concentration is present **Figure 5.4 D**. However, it is still possible to detect and identify correctly *L. monocytogenes* and *Salmonella* spp. even when present at a 10 times lower DNA concentration. The amplification efficiency was calculated after plotting the standard curves, and the determined values were 98.2 %, 93.2 %, 92.6 % for *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 respectively; and 91.4 % for the multiplex format. These results are shown in **Figure 5.3** and the values are between the previously reported acceptable limits (90–110 %) [35].

Table 5.1. List of bacteria strain used to evaluate the inclusivity/exclusivity of the primers and SYBR-qPCR

Bacteria species	Source	N	*fimA*	*actA*	*rfbE*	IAC
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00031)	15	+	-	-	-*
L. monocytogenes	WDCM 00021, Mollusk, chestnut, chicken	16	-	+	-	-*
L. ivanovii	WDCM 00018	1	-	-	-	+
L. innocua	WDCM 00017, CECT 5376, 4030, 1325, 1141, 2110	6	-	-	-	+
S. aureus	WDCM 00034, 00033	2	-	-	-	+
Staphylococcus coagulase +	Proficiency test	1	-	-	-	+
C. coli	University of Minho	1	-	-	-	+
E. coli	WDCM 00013, 00012	2	-	-	-	+
E. coli O157	WDCM 00014	1	-	-	+	-

N: number of strains; IAC amplification allow to prove that any inhibition is present when the absence of amplification is observed in the rest of the targets;
* The absence of IAC amplification is due to the amplification of at least one of the targeted bacteria.

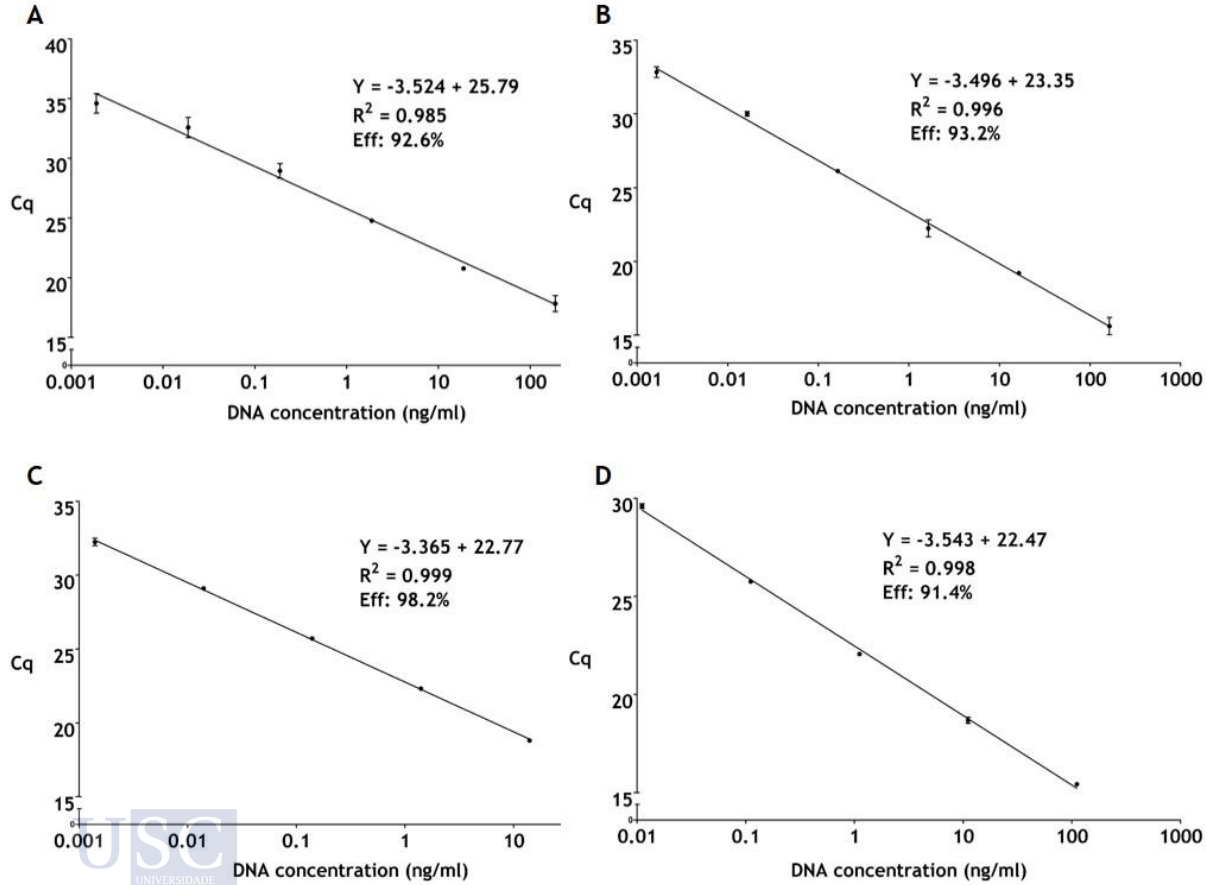

Figure 5.3. SYBR-qPCR reaction evaluation. The efficiency, dynamic range and coefficient of determination was obtained in simplex for *E. coli* O157 (A), *Salmonella* spp. (B) and *L. monocytogenes* (**C**) and multiplex (**D**). Standard for each situation were obtained by three replicates of ten-fold serial dilutions of a DNA extract from each pathogen, and a mixture of the three extracts for multiplex experiment.

The influence of the DNA concentration in the melting peak is presented in **Figure 5.4**, for simplex and multiplex detection as reported above, showing the decrease of the peak intensity with the decrease of DNA concentration in the qPCR reaction.

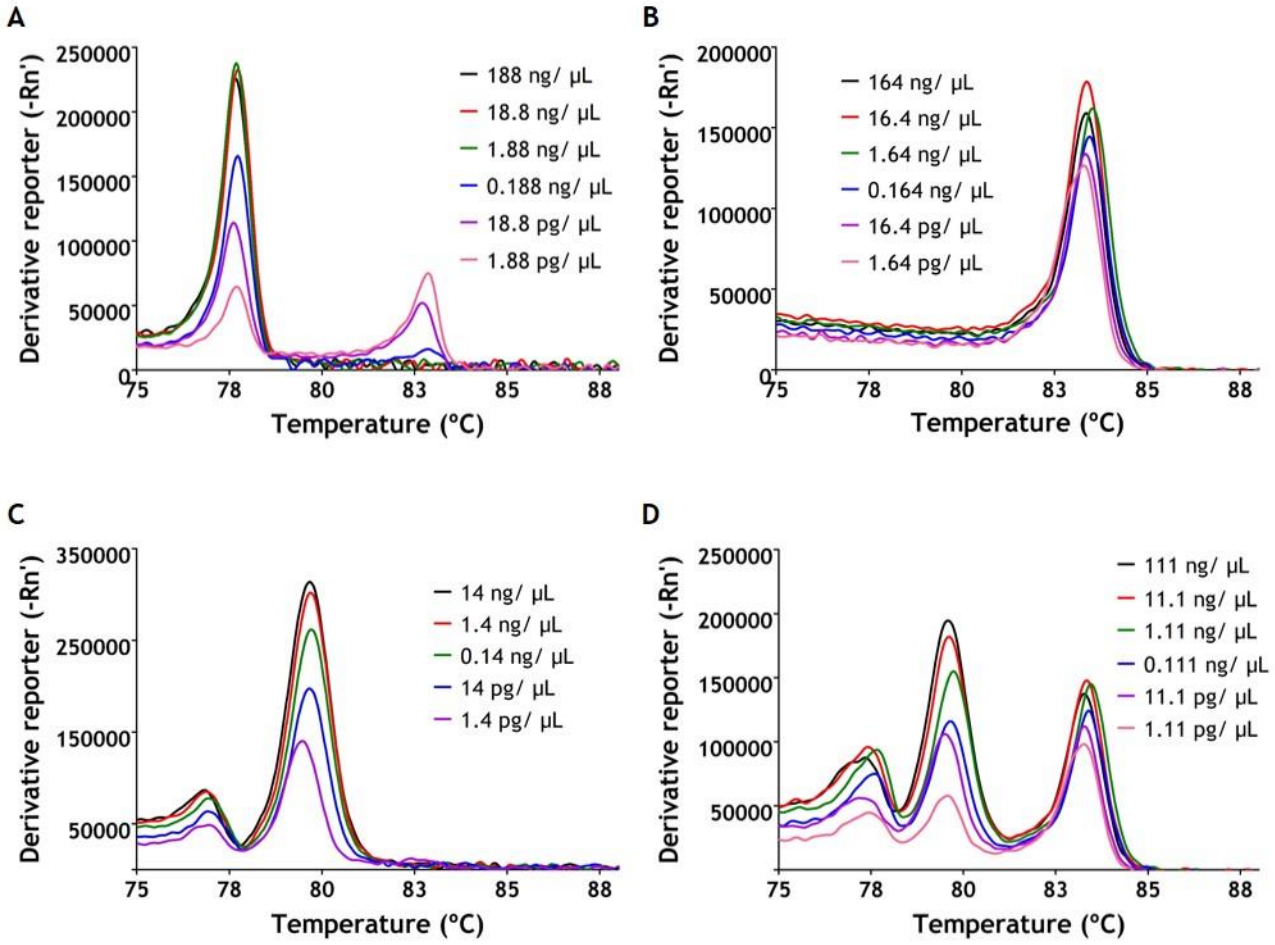

Figure 5.4. Melting analysis from SYBR-qPCR efficiency. In simplex for *E. coli* O157 (A), *Salmonella* spp. (B) and *L. monocytogenes* (**C**) and multiplex (**D**) reaction.

5.2.1.3 Evaluation with food matrixes

The methodology was evaluated in a panel of 44 samples spiked at different contamination levels. The results are summarized in **Table 5.2** for the four targets. All negative samples for one or more pathogens were correctly identified by qPCR, and in the total absence of the targeted pathogen, a Cq higher than 34 was observed, originate by the amplification of the NC-IAC, presenting only its respective melting peak. All positive samples were detected with the correct pathogen identification and none PD were observed, allowing a relative specificity, sensitivity and accuracy, of 100 % and a Cohen's κ of 1.

Table 5.2. Spiked samples to evaluate SYBR-qPCR approach

Type of sample	Contamination level (cfu/ 25 g of sample)*			N	SYBR-qPCR			
	L. monocytogenes	*S.* Typhimurium	*E. coli* O157		*actA*	*fimA*	*rfbE*	IAC
Infant Milk (LoD)	16.6	7.4	5.5	6	+	+	+(2ND)	-
	10.2	5.5	5	6	+	+	+	-
	5.1	2.8	2.5	6	+	+	+	-
	2.2	1.1	0.8	6	+ (2ND)	+ (3ND)	+	-
	2.3	0.9	0.145	6	+	+	+(3ND)	-
Infant Milk	-	-	-	4	-	-	-	+
	9	6	4	2	+	+	+	-
	9.1	13	8	1	+	+	+	-
	9	6		1	+	+	-	-
		6	4	1	-	+	+	-
	9		4	1	+	-	+	-
	91	1.3×10^3		1	+	+	-	-
	9		8.0×10^2	1	+	-	+	-
		1.3×10^4	8.0×10^3	1	-	+	+	-
	9.1×10^3	1.3×10^4	8.0×10^3	1	+	+	+	-

N: number of samples; ND: Negative deviation;
* The contamination level correspond to concentration of bacteria inoculate before enrichment and was obtained by results of the plating in TSA for *S.* Typhimurium and *E. coli* O157 and TSYEA for *L. monocytogenes*; The ND obtained were all below the LoD, and for this reason were not consider as so for the methodology evaluation.

The determination of LoD was done using PoDLoD analysis. A total 30 samples were tested with five different levels of contamination. The LoD_{50} was calculated to be 0.1, 0.5 and 0.6 cfu/ 25 g and the LoD_{95} 0.6, 2.1 and 2.6 cfu/ 25 g for *E. coli* O157, *Salmonella* spp. and *L. monocytogenes*, respectively. The LoD of the multiplex detection was also evaluated, being the LoD_{50} 0.4 and the LoD_{95} 1.7 cfu/ 25 g.

In the current study, the primer concentration for *actA* had to be increased, with respect to the other targets in order to improve the amplitude of the peak, and so the LoD. This could be due to the lower concentration of *L. monocytogenes* after the enrichment, or to the preferential binding of SYBR Green to specific DNA fragments, previously it has been reported a preference of SYBR Green for G+C rich sequences [194,195]. The G-C content of the fragment generated in this studies were 40.1, 54.3 and 47.2% for *rfbE*, *fimA* and *actA* respectively, what would agree with the fact that *fimA* had a bigger peak with a lower primer concentration.

The detection of the different microorganisms, spiked at different concentration levels, in the same sample, was successfully accomplished. Simultaneous detection has advantages in terms of cost savings and shorter time to result. Overall the methodology proved to be reliable and sensitive, as 100% of the results obtained were in concordance with the expected results for all evaluated parameters, and had comparable results to other studies using qPCR with probes for multiplex detection [196–198].

5.2.2 Probe-qPCR

qPCR with hydrolysis probes has been extensively used for a broad range of applications. This approach has the advantage of being easily multiplexed, without the difficulties of the primer design presented in the intercalating dye qPCR. Different sets of primers were evaluated using this technique, targeting specific genetic targets of the three pathogens. For *L. monocytogenes* the comparison between *actA* primers, used in the SYBR-qPCR approach, and *hly* primers combined with their respective probe, was performed. Regarding *Salmonella* spp. and *E. coli* O157, *ttr* and *rfbE genes* were chosen. The efficiency and inclusivity/ exclusivity of this primers/probes were analysed in simplex or multiplex with an IAC.

5.2.2.1 L. monocytogenes

When targeting *hly*, a cIAC was implemented. This approach has the advantage of using the same primers to amplify both targets, the internal amplification control sequence and the specific target sequence. For this reason the first step was to optimize the concentration of the IAC to avoid interference in the amplification of the main target.

It was observed that the addition of 100 copies/ µL (2000 copies per reaction) of cIAC DNA, provided optimal results, as lower concentrations presented higher replicate deviations as well as a final Cq value excessively high (>33 cycles), these results are depicted in **Figure 5.5 A**.

After the conditions of the reaction were optimized, it was confirmed that the IAC DNA did not interfere with the amplification of *hly*, as no preferential amplification over the samples was seen. The same was observed for the interaction between the different probes and targets, as no amplification was originated when the *hly* probe was used with IAC DNA, or when the IAC probe was added with DNA from *L. monocytogenes*. These results confirmed the appropriate design of the reaction and implementation of the IAC.

The efficiency of the qPCR reaction was evaluated only implementing *hly*, and compared with the results obtained in multiplex when both targets, *hly* and IAC, were co-amplified. An efficiency of 92 % and 90 % in simplex and multiplex (co-amplification of IAC) format was obtained, respectively. In both situations, the R^2 of the equation was higher than 0.99 and covered 6 consecutive dilutions from 120 ng/ µL to 1.2 pg/ µL, as shown in **Figure 5.5 B and C**. This experiment also allowed to determine the lowest concentration of DNA providing positive amplification of *hly*. Both formats needed a minimum concentration of 1.2 pg/ µL from *L. monocytogenes* total DNA to have a positive result. It was observed that when high concentrations of pathogen DNA was amplified, presenting a low Cq value for *hly*, the amplification of IAC could be delayed or even completely absent. However as the IAC was integrated in the reaction as control to identify false negatives results when no amplification is detected for *hly*, therefore the absence of IAC amplification when *hly* amplify was not considered problematic.

For the detection of *L. monocytogenes* targeting *actA* gene, a NC-IAC was tested as a different approach. The reaction efficiency for this combination is represented in **Figure 5.5 D**, showing a value of 93.4 %, slightly higher than the results with the *hly* multiplex method detailed above, but once more between the acceptable values. The lowest concentration of bacterial DNA detected was 0.2 pg/ µL, 6 times lower than in the case of the *hly* reaction.

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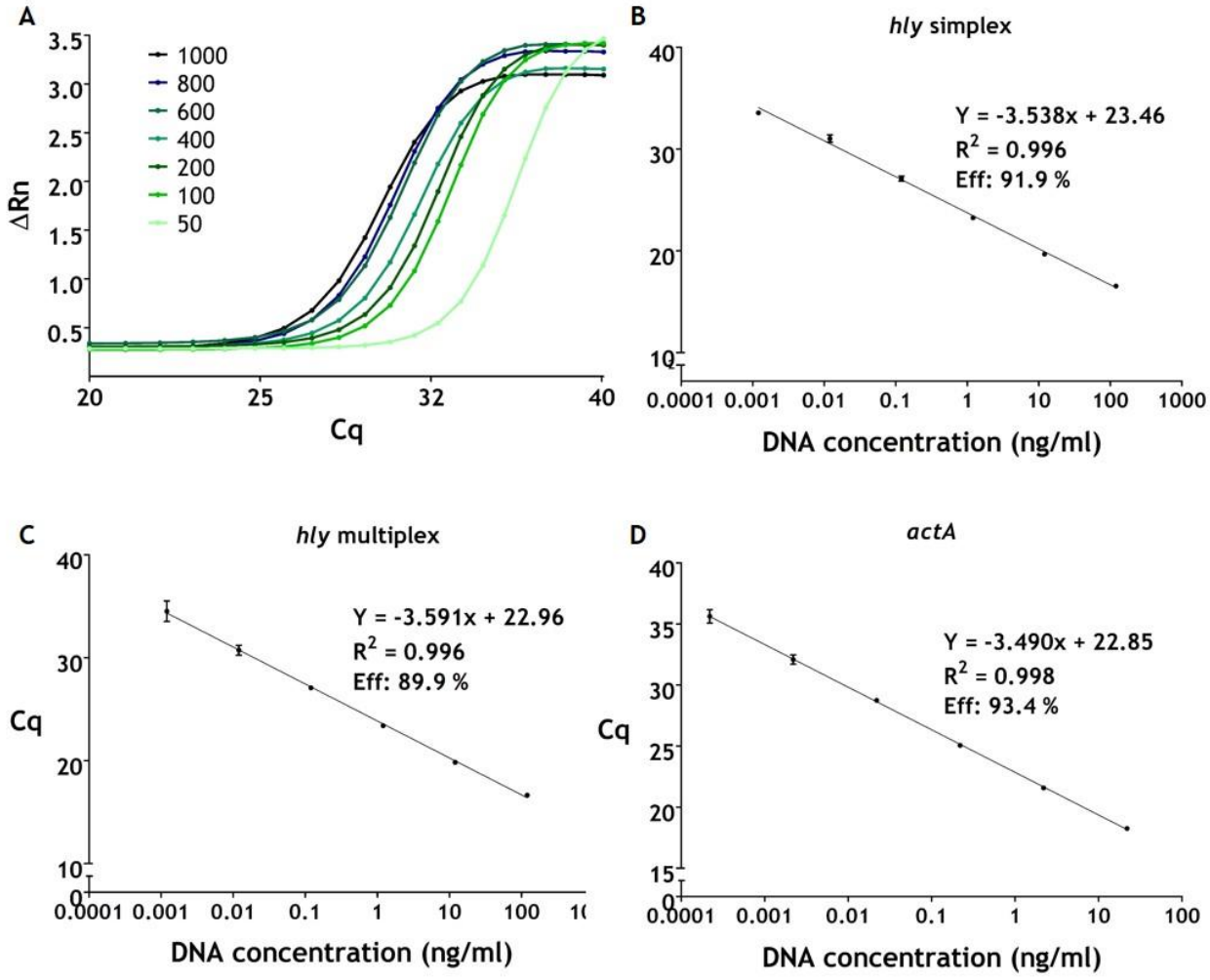

Figure 5.5. Probe-qPCR optimization and evaluation for the detection of *L. monocytogenes* using different genetic targets, *hly* and *actA*. (A) Concentration optimization of competitive IAC to be used with *hly* primers. The specified quantities are expressed in copies/ mL. (B) and (C) Efficiency of *hly* detection in simplex and multiplex format with simultaneous detection of both targets (*hly* with 100 copies/µL of cIAC DNA), respectively. (D) qPCR efficiency targeting *actA* and NC-IAC simultaneously. Efficiency curves were obtained by three replicates of ten-fold serial dilutions of a pure DNA extract.

Beyond the efficiency and dynamic range, the inclusivity/ exclusivity of both primer sets were evaluated, testing the methods with the different bacterial stains presented in **Table 5.3**. The optimized qPCR protocol using *actA* and *hly* combined with their respective IACs, provided positive results exclusively with the 16 strains of *L. monocytogenes*. The other 8 *Listeria* spp., and extra 22 bacterial stains, were all negative, confirming the optimal specificity of this genetic targets.

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Table 5.3. Inclusivity and exclusivity evaluation of Probe-qPCR

Bacterium	Source	N	*hly*	*actA*	*rfbE*	*ttr*
E. coli O157:H7	WDCM 00014, clinical isolated (AMC)	2			+	
E. coli O157:H7	In silico PCR	5			+	
E. coli	WDCM 00013, 11 Mollusk, Cow stool (AMC 275), Intestinal biopsy (LSP-389-99), Sea water (AMC 176)	15	-*	-*	-	-*
E. coli	*In silico* PCR	60			-	
L. monocytogenes	WDCM00021, Mollusk, chestnut, chicken	16	+	+	-	-**
L. seeligeri	CECT 917	1	-		-	-
L. ivanovii	WDCM00018	1	-	-	-	-
L. innocua	WDCM00017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	-	-	-	-
C. coli	UM	1	-	-	-	-
C. freundii	CECT 401					-
E. faecalis	WDCM 00009					-
S. sonnei	CECT 413					-
S. aureus	WDCM 00034, 00033					-
Proteus spp.	Mollusk (AMC 178)	1			-	
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00031)	15	-	-	-	+

Evaluation of the inclusivity and exclusivity of the qPCR reaction using *rfbE* primers in simplex. All *E. coli* O157 strains were correctly identified and all non-target bacteria were not detected in the reaction;
N: number of strains;
* only tested against WDCM 00013;
** refers to results obtained with the strains from mollusk source.

Both primer sets performed well, showing high specificity and sensitivity for the detection of *L. monocytogenes*, with lowest concentration of DNA detected by *actA*. In both cases no interference with the IAC was observed when a multiplex reaction was performed. The gene *actA* has been extensively used to detect *L. monocytogenes* by qPCR [193,199,200], as well as *hly* [115,120,121,201]. The competitive format of the IAC is recommended when only one target is detected in order to minimize the chances of undesired interactions among several primers [202], however in a multiplex reaction targeting different pathogens a NC-IAC is more convenient to test the overall reaction inhibition, without the competition for the same primers [203]. The two sets of primers, and respective probes, were both used to evaluate several of the pre-treatment approaches as mentioned in Chapter I, proving their performance to detect *L. monocytogenes*.

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5.2.2.2 *E. coli* O157

For the detection of *E. coli* O157, *rfbE* was the genetic target chosen. As performed for *actA* and *hlyA*, these primers were evaluated in terms of efficiency and specificity.

Both simplex and multiplex, including a NC-IAC, approaches were evaluate for the detection of ten-fold serial dilution of of *E. coli* O157 DNA (**Figure 5.6 A and B**) and with dilution of the bacterial culture followed by DNA extraction (**Figure 5.6 C and D**). The analysis of the dilutions of the pure *E. coli* O157 DNA, showed no impact when the NC-IAC was included in the qPCR assay, as there were no major differences between the results obtained from the simplex and multiplex assays, 99.4 and 100.7 % respectively. Concentrations of DNA ranging from 252 ng/ µL to 0.0252 pg/ µL produced a reproducible amplification, being 0.0252 pg/ µL the lowest DNA concentration which could be detected. Similar efficiency was obtained when DNA extracted from the diluted pure cultures of the pathogen were tested as template. In simplex and multiplex, the efficiency calculated was 101.3 and 103.8 % respectively. And as for the other approach 8 orders of magnitude, from 1.7×10^8 to 17 cfu/ mL were covered by the dynamic range and the lowest bacterial concentration to give a reliable amplification was 17 cfu/ mL.

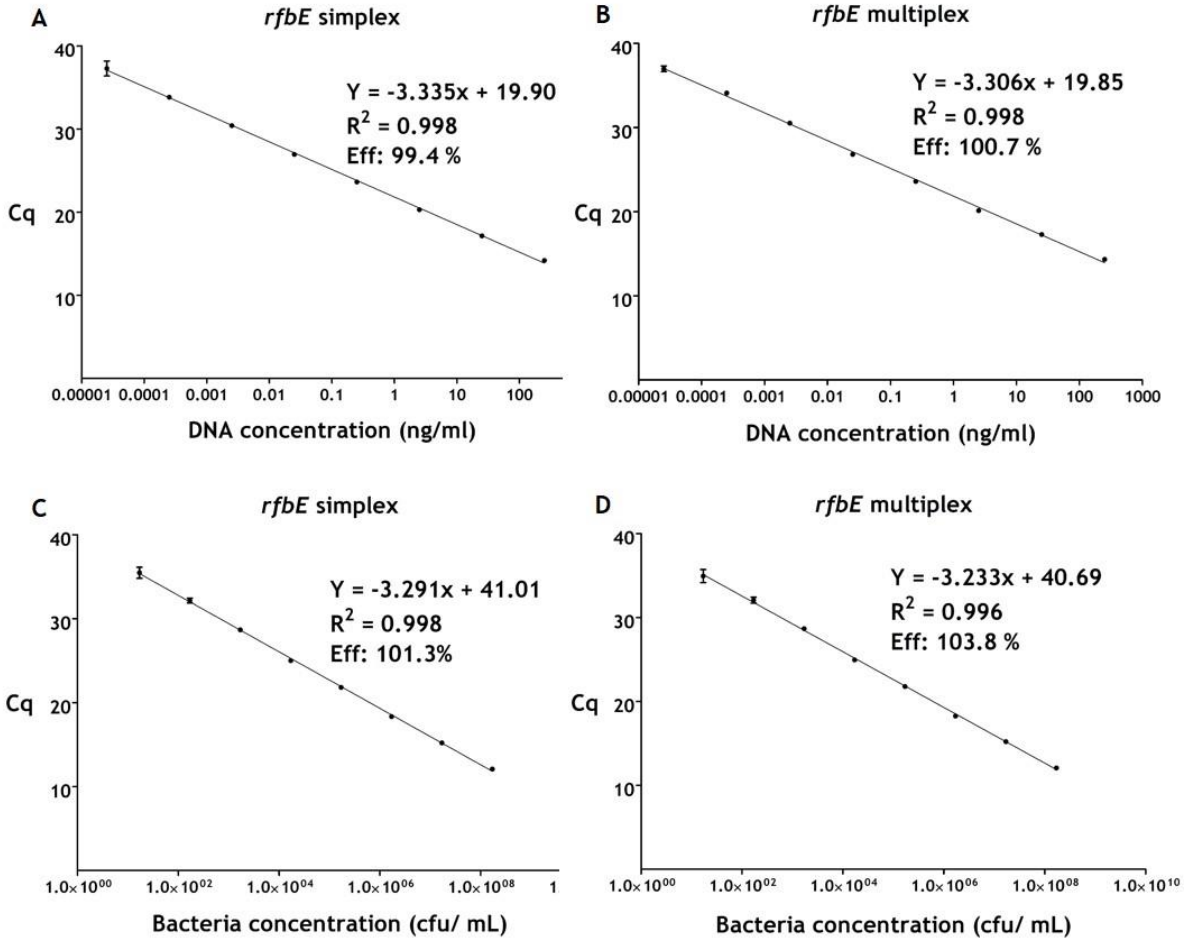

Figure 5.6. Evaluation of the Probe-qPCR reaction targeting *rfbE* in simplex or multiplex with simultaneous amplification of NC-IAC. (A) and (B) represent the ten-fold dilutions of pure bacterial DNA, and (C) and (D) ten-fold dilutions of bacterial culture followed by DNA extraction from each of the dilutions.

The specificity of the set of primers was not only tested *in vitro*, performing the qPCR reaction using pure DNA extracts of 2 *E. coli* O157 and 15 non- O157 strains, but also *in silico*, where an online software was used (http://insilico.ehu.es/PCR/) to test 5 *E. coli* O157 and 60 non- O157. Additionally, the cross-

reactivity against a panel of 41 non-target microorganisms was also evaluated , which included 15 *Salmonella* spp., 16 *L. monocytogenes*, 8 *Listeria* spp., 1 *Proteus* spp. and 1 *Campylobacter coli*. All targeted strains were well identified and no amplification was originated by the non-targeted microorganisms, confirming the specificity of the reaction, as presented in **Table 5.3**

The multiplex qPCR protocol using this primers and the NC-IAC, was used for the detection of *E. coli* O157 to evaluate the short enrichment approach, described in Chapter 4, section 4.4.3, allowing a LOD_{95} 3.6 cfu/25 g and a κ of 0.94 [204].

5.2.2.3 *Salmonella* spp.

For the detection of *Salmonella* spp. by Probe-qPCR, *ttr* was selected as genetic target, and the efficiency and specificity of the reaction were evaluated for the simplex and multiplex detection when the NC-IAC was also implemented. A dynamic range of five ten-fold serial dilutions was obtained, being possible to detect *Salmonella* DNA between 193 ng/ mL and 0.0193 ng/ mL (**Figure 5.7**). This shows lower detection levels that the ones obtained for *hly*, *actA* and *rfbE* which ranged from 0.2 to 0.02 pg/ mL. Additionally a lower efficiency value was observed with this set of primers, obtaining a value of 84% and 89 %, for the simplex and multiplex approach, respectively.

In order to test the specificity of this set of primers and probe, 20 non-*Salmonella* strains, covering 10 different species, including other bacteria frequently found in food samples, such as *E. coli* or *S. aureus*, were analysed (**Table 5.3**). All the 15 Salmonella strains were correctly identified, while the non-targets obtained negative results. The multiplexing of these primers and probe with the ones targeting *actA* and NC-IAC were used for the detection of the respective pathogens using the PDMS sponge for bacterial concentration described in Chapter 4, section 4.3.2. High sensitivity and specificity with a LoD of 10^3 cfu/ mL was achieved, similar to other qPCR approach using hydrolysis probes [205], while previous similar studies reported a higher LoD (10^4 cfu/ mL) [206].

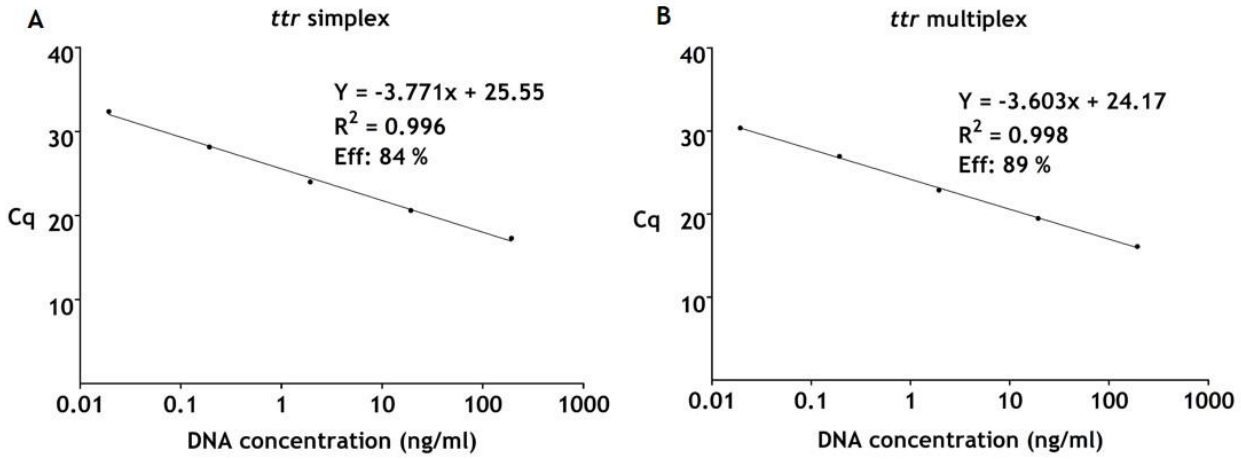

Figure 5.7. Efficiency and dynamic range of the Probe-qPCR targeting *ttr* gene. (A) Simplex detection (B) Multiplex detection, implementing NC-IAC. Efficiency curve obtained by three replicates of ten-fold dilutions of pure bacterial DNA.

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5.2.3 qLAMP, qRPA and comparison between real-time techniques

A comparison between qPCR, qRPA and qLAMP approach was performed for the detection of *L. monocytogenes*. Primers were designed for the LAMP assay targeting *plcA* gene, and the F3/ B3 were used to perform the qPCR assays using intercalating SYBR-qPCR, while the primers *hly* designed for qPCR were used for the Probe-qPCR methodology, and combined with the Exo probe for RPA amplification. All approaches were evaluated assessing the dynamic range, detection probability, inclusivity/ exclusivity and also the detection of the pathogen in spiked samples.

5.2.3.1 Evaluation of the different amplification reactions

Concentrations between 120 ng/ µL and 0.000012 ng/ µL in 9 replicates were tested by each approach to evaluate the dynamic range. The results are presented in **Figure 5.8 A**. In both isothermal amplification techniques, qLAMP and qRPA, a concentration down to 0.12 ng/ µL was detected with a 100 % probability. However, the traditional qPCR methodology achieved a lower minimum concentration of DNA with the same 100 % of detection probability, being 0.012 ng/ µL and 0.0012 ng/ µL for the SYBR-qPCR and Probe-qPCR respectively (**Figure 5.8 B**).

While previous studies presented comparable results, showing a decrease in sensitivity using isothermal approaches [207,208], others reported similar or even higher detection sensitivity in LAMP and RPA over standard qPCR methodologies [134,209].

On the other hand, the fastest methodology showed to be qRPA with positive amplification after only 4 min with the highest concentration tested, while qLAMP required 15 min in order to enable a positive result with the same concentration. Comparatively, the qPCR methodologies needed more time than qRPA to achieve similar results.

The specificity of qPCR and qRPA using the same primers, as well as the SYBR-qPCR with F3/ B3 and qLAMP was compared and results are detailed in **Table 5.4**. The qLAMP primers F3/ B3 were first used to confirm their specificity *in silico* by qPCR. All *Listeria* spp. from the data bank were tested, and included 43 strains (1 *L. innocua*, 1 *L. ivanovii*,1 *L. seeligeri*,1 *L. welshimeri* and 39 *L. monocytogenes*), and only *L. monocytogenes* showed amplification. The *in vitro* tests were equally performed with all primers, confirming 100 % specificity when tested against 16 *L. monocytogenes* strains, and 30 non-target strains, including 6 *L. innocua*, 1 *L. seeligeri* and 1 *L. ivanovii*, without any cross-reactivity.

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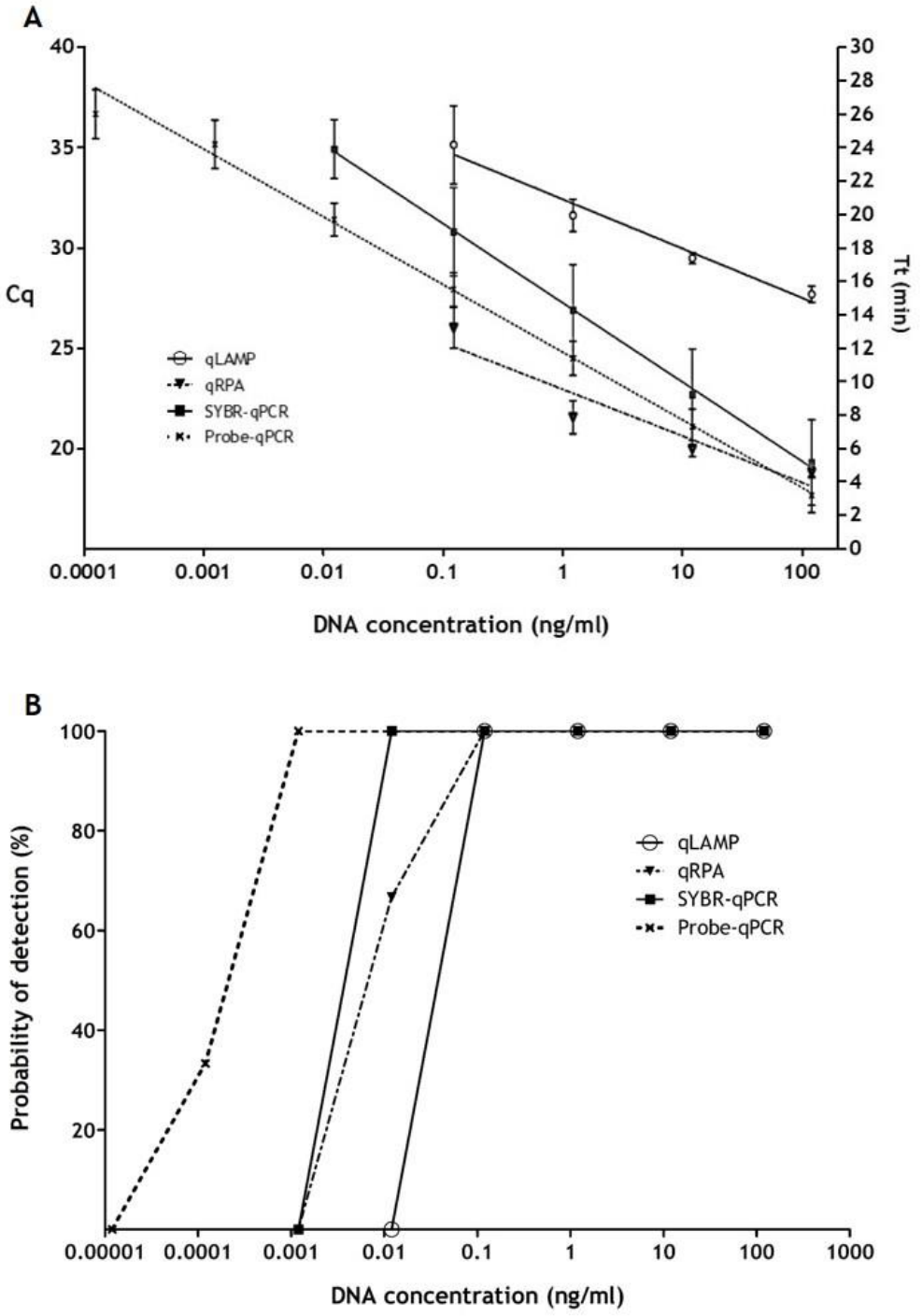

Figure 5.8. Comparison between the four approaches tested, SYBR-qPCR, Probe qPCR, qLAMP, qRPA. (A) Dynamic range. (B) Probability of detection.

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Table 5.4. Strain list and specificity results for the real-time DNA amplifications

Table 5.4. Strain list and specificity results for the real-time DNA amplifications

Bacterium	Source	N	Probe-qPCR	qRPA	SYBR-qPCR	qLAMP
L. monocytogenes	WDCM 00021, Mollusk, chestnut, chicken	16	+	+	+	+
L. monocytogenes	In silico PCR	39			+	
Listeria spp.	In silico PCR (*L. innocua, L. ivanovii, L. seeligeri, L. welshimeri*)	4			-	
L. seeligeri	CECT 917	1	-		-	-
L. ivanovii	WDCM00018	1	-	-	-	-
L. innocua	WDCM 00017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	-	-	-	-
C. coli	UM	1	-	-	-	-
C. freundii	CECT 401		-	-	-	-
E. faecalis	WDCM 00009		-	-	-	-
S. sonnei	CECT 413		-	-	-	-
S. aureus	WDCM 00034, 00033		-	-	-	-
E. coli	WDCM 00013	1	-	-	-	-
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00031)	15	-	-	-	-

Pobe-qPCR and qRPA were performed using the same *hly* primers designed for PCR with the respective probes, SYBR-qPCR was performed with the F3/B3 primers of the LAMP reaction targeting *plcA*;
N: number of strains.

5.2.3.2 Evaluation with complex food matrixes

Fifty six samples, including different types of matrixes, from fish to poultry meat were spiked with a range of *L. monocytogenes* concentrations between 1 and 10^7 cfu/ 25 g, further details are provided in **Table 5.5**. The results were compared between all methodologies and confirmed by plating the secondary enrichment on COMPASS. The LoD was determined in smoked salmon samples inoculated with 1.4 cfu/ 25 g. All the methodologies obtained a positive amplification in 9 of the 10 samples, establishing this value as the lowest detectable concentration. Two blanks of this matrix were used as a negative control to ensure the absence of the pathogen in the original samples, which were negative by all molecular alternatives and after plating confirmation.

A total of 55 food samples were analysed and correctly classified by the approaches tested, with the exception of 1 PD by qLAMP in a non-spiked sample, which was negative after diluting 1/10 the DNA extract. This result may suggest the presence of an interfering compound in the sample, influencing the qLAMP reaction. Additionally, a ND obtained during the determination of the LoD, was consider in the published version of this work. However, as this sample was also determined to be negative by the plating methodology, the deviation was not considered here. This negative result was probably due to the low concentration of bacteria (1.4 cfu/ 25 g) present in this sample.

Table 5.5. Spiked samples compare the performance of the real-time methodologies

Type of sample	Contamination level (cfu/ 25 g of sample)	N	SYBR-qPCR	Probe-qPCR	qRPA	qLAMP	Plates*
Tuna in oil	-	1	-	-	-	-	-
Hamburger	-	1	-	-	-	+ (PD)	-
Smoked Salmon	-	4	-	-	-	-	-
	1.4	10	+ (1-)[a]	+ (1-)[a]	+ (1-)[a]	+ (1-)[a]	+ (1-)[a]
	1.8 x 10	2	+	+	+	+	+
	1.8 x 10^2	2	+	+	+	+	+
	1.8 x 10^3	2	+	+	+	+	+
	1.8 x 10^4	2	+	+	+	+	+
	3.6 x 10^5	2	+	+	+	+	+
	3.6 x 10^6	2	+	+	+	+	+
	3.6 x 10^7	1	+	+	+	+	+
Sardine in oil	-	3	-	-	-	-	-
	3.1 x 10	5	+	+	+	+	+
	6.8 x 10	2	+	+	+	+	+
	6.8 x 10^2	2	+	+	+	+	+
	6.8 x 10^3	2	+	+	+	+	+
Mussel in brine	-	1	-	-	-	-	-
	3.1 x 10	2	+	+	+	+	+
Tuna	-	1	-	-	-	-	-
	3.1 x 10	3	+	+	+	+	+
RTE turkey	-	2	-	-	-	-	-
	3.6 x 10^7	1	+	+	+	+	+
Chicken	3.1 x 10	1	+	+	+	+	+
Turkey	2.4 x 10	1	+	+	+	+	+
	2.4 x 10^2	1	+	+	+	+	+

* The plating results were obtained after plating on COMPASS, the secondary enrichment performed in Fraser Broth;
** The PD was eliminated after 1/10 dilution of the crude DNA extract;
[a] Samples used to determine the LoD, where 1 in 10 samples gave a negative result;
RTE refers to ready-to-eat turkey presented as bite-size cubes.

With these results, qRPA and both qPCR approaches obtained a κ of 1, and a lower but still acceptable value of 0.95 was observed for qLAMP technique, see **Table 5.6**. This values correspond to "very good concordance" [149,210], and were comparable to those reported in other similar studies using LAMP, RPA and qPCR for the detection of *L. monocytogenes* and other pathogens [185,211–213]. When

performed in real-time, using an intercalating dye, the LAMP reaction allow the analysis of the melting curves, which can increase the specificity of the assay.

The monitoring of the isothermal approaches was performed using a qPCR thermocycler. However, due to the fact that the software is not specific for these techniques, this could lead to errors in the determination of the *Tt*, resulting in over or infra-estimation of these values. For this reason, a mathematical modelling of the qLAMP/RPA kinetics is required.

Both isothermal approaches presented excellent performance, and even though the amplification sensitivity was lower than that of qPCR, at the end the same sensitivity was observed when spiked samples were tested, confirming their fit to be use in the food industry, allowing the detection of very low concentrations of bacteria when combined with an enrichment.

Table 5.6. Evaluation of the results obtained by real-time amplification approaches

Table 5.6. Evaluation of the results obtained by real-time amplification approaches											
Approach	**N**	**PA**	**PD**	**NA**	**ND**	**SE (%)**	**SP (%)**	**AC (%)**	**PPV (%)**	**NPV (%)**	***k***
qLAMP	55	40	1	13	0	100	93	96	98	100	0.95
qRPA	55	41	0	13	0	100	100	100	100	100	1
SYBR-qPCR	55	41	0	13	0	100	100	100	100	100	1
Probe-qPCR	55	41	0	13	0	100	100	100	100	100	1
Different types of samples were evaluated from meat fish and RTE products.											

5.3 RPA COMBINED WITH NAKED-EYE DETECTION

Due to the fact that RPA showed good performance in the experiments achieved in real-time, it was consider a suitable option to be included in the final methodology and two alternative naked-eye detection, RPA-LF and RPA-SYBR, were evaluated.

5.3.1 RPA- Lateral flow (RPA-LF)

The product of the RPA reaction can be loaded in a lateral flow strip in order to visualize it. The primers needed to be modified as describe in Chapter 3, section 3.5.3.1 and a specific probe needed to be designed. This approach was evaluated for the detection of *L. monocytogenes*, comparing the performance of two sets of primers, to include the more reliable one in a methodology to analyse food-contact surfaces.

5.3.1.1 Evaluation of the RPA-LF reaction and Primers comparison

To determine the best primer set to be used with the RPA in combination with LF detection, first the lowest concentration of a *L. monocytogenes* DNA, yielding a positive result was determined; and second, the concentration of *L. monocytogenes* cells needed to have a positive result by RPA-LF was also determined. This was performed by preparing ten-fold dilutions from an ON culture and preparing cell lysates from each dilution. Both sets of primers obtained comparable results, as depicted in **Figure**

5.9 A and B. It was possible to reliably detect down to 1 pg/ µL. The use of qPCR primers seemed to generate slightly less intense bands. When the evaluation was focused on bacterial cultures, it was observed that a concentration of 8×10^4 cfu/ mL was needed with both sets of primers for reproducible detection, as shown in **Figure 5.9 C and D**.

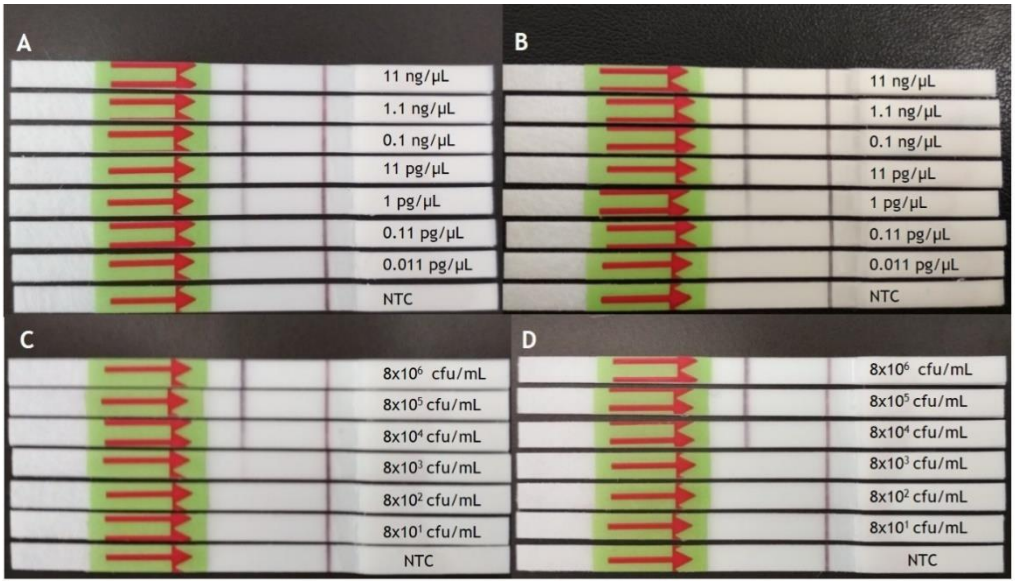

Figure 5.9. Results of the analytical sensitivity for *L. monocytogenes* detection for RPA-LF reaction. (A) and **(B)** show the lowest concentration of DNA possible to detect with qPCR and RPA primers, respectively. **(C)** and **(D)** present the results of the lowest bacteria concentration detected by RPA-LF using qPCR and RPA primers, respectively. NTC correspond to the "no template control

Regarding the inclusivity and exclusivity, the DNA from a total of 42 cell lysates from the strains detailed in **Table 5.7**, were tested to evaluate the RPA-LF assay. For the inclusivity, a total of 11 *L. monocytogenes* strains were analysed, while for the exclusivity 7 other *Listeria* spp., and 24 different bacterial strains were screened. All *L. monocytogenes* strains were correctly identified by RPA-LF. Faint bands, as exemplified in **Figure 5.10**, were observed in 4 strains for each set of primers. These results allowed to establish a threshold in the intensity of the band to consider a positive result.

It was previously reported that extended incubation times, while performing the LF, could lead to the appearance of faint band [214], in the same way previous studies have indicated that applying a higher dilution factor could solve this issue [215]. It was also recently reported that faint bands may be visible in LF assays linked to the structure and size of the probes, which may not be related with cross-reactivity [216], this seems like a feasible explanation of the results observed as the lack of cross-reactivity has been described previously using the same probe but in Exo RPA [217,218]. As commented above, this was not considered an issue as these results served to determine a band intensity threshold, and so to avoid any possible sample misidentification.

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Table 5.7. Strain list and specificity results for RPA-LF with both sets of primers

Bacterium	Source	N	PCR primers	RPA primers
L. monocytogenes	WDCM 00021, Mollusk, chestnut, chicken	11	+	+
L. seeligeri	CECT 917	1	-	
L. ivanovii	WDCM00018	1	-	-
L. innocua	WDCM 00017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	-* 1141	-
C. coli	UM	1	-*	-*
E. faecalis	WDCM 00009	1	-	-
S. aureus	WDCM 00034, 00033	2	-	-* WDCM 00033
Staphylococcus coagulase +	Proficiency test	1	-	-
E. coli	WDCM 00013, 00012, 00014	3	-	-
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00031)	15	-* 84, 261	-* 198, 238

The inclusivity and exclusivity were tested for both sets of primers for the RPA amplification and detection performed by Lateral Flow (LF) using TwistAmp® nfo and Milenia HybriDetect 1 strips;
Positive results were observed when two bands appeared in the strips, the control band and the result band;
* Strain where a faint band was observed in the test line; N: number of strains.

Figure 5.10. Faint band with non-target DNA. Lateral flow strips showing the difference in the intensity of the test band with a specific (*L. monocytogenes*) and non-specific (*C. coli*) detection obtained with RPA amplification, using PCR primers

5.3.1.2 Sample pre-treatment optimization and method evaluation for surface contamination analysis

To evaluate this approach in a real context for surface contamination analysis, spiked stainless steel were sampled and an enrichment performed to allow a reliable detection by RPA-LF. To reach the lowest limit of detection, the full methodology was optimized, to improve the recovery of the bacterial cells from the surface, and the enrichment step. Two different tools, swab and sponge were compared to determine the optimal procedure to recover *L. monocytogenes*. Both approaches were used in the same way, regarding the way to moisten the device, the same sampling surface, the number of times and the direction in which the devices were passed, and after enrichment, the limit of detection by RPA-LF was evaluated. The swabs reached a LoD_{50} of 391 cfu/ cm^2, while for the sponge it was calculated to be 4.2 cfu/ cm^2, showing that the sponge allowed to enhance the recovery of the bacterial cells from the surface by 2 logs, compared to the swab. The results obtained are in agreement with previous studies [219]. The higher contact area of the sponge, and the robustness of the stick, allowed for a stronger rubbing of the

surface and the recovery of more bacteria than the swab, which was smaller and more fragile. Swabs have been reported to be more appropriate to be used in hard-to-reach small areas and sponges are normally preferable in bigger areas [219].

The LoD achieved in the analysis of samples differ from those presented for the dynamic range, as this data was generated after sample enrichment, allowing the bacteria growth.

The time of enrichment was also evaluated and the LoD at different times was determined. The LoD_{50} values obtained were 117, 21, 27 and 4 cfu/ cm^2 without enrichment, and after 8 h, 14 h and 24 h of enrichment in ONE broth respectively. In **Figure 5.11**, the difference between these results is shown, highlighting the reduction of the LoD by 1.4 log when a 24 h enrichment was performed, compared with the value obtained without enrichment. A reduction of 0.76 ± 0.07 log is observed with enrichment times of 8 h and 14 h, providing these two a similar LoD. Other samples presented in **Table 5.8** were analysed to determine the LoD and evaluate the methodology. The LoD_{50} and LoD_{95} of the RPA-LF methodology with and without enrichment were compared and summarized in **Table 5.9**, presenting a LoD_{95} of 509.3 and 18.2 cfu/ cm^2 respectively.

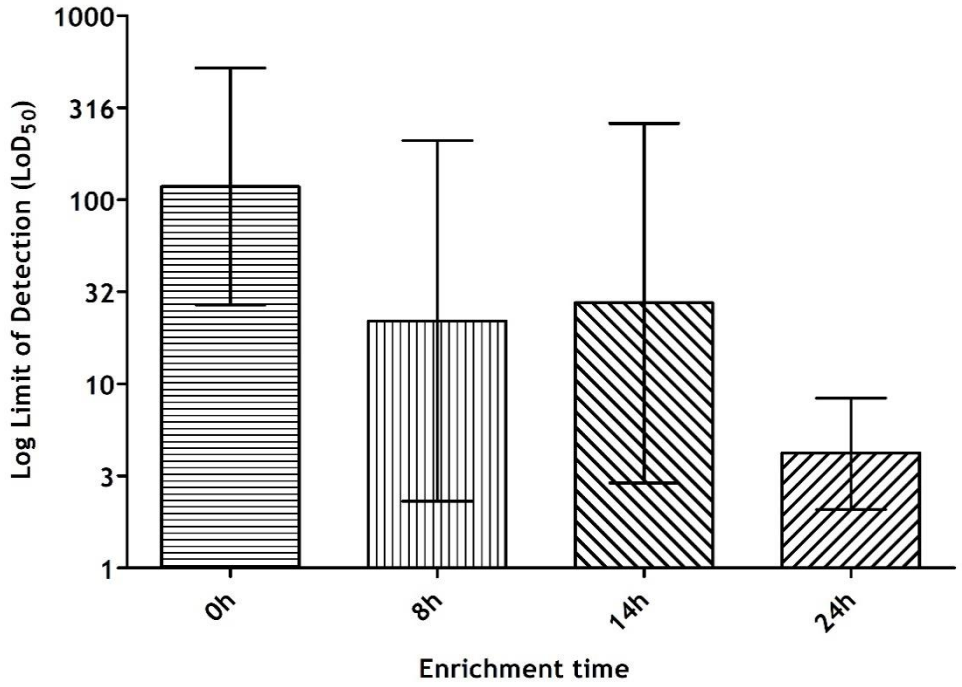

Figure 5.11. Optimization of enrichment time for RPA-LF methodology. Comparison of the LoD_{50}, with their upper and lower limits, obtained with different enrichment times (0, 8 h, 14 h, 24 h) in ONE broth. LoD_{50} calculated with PoDLoD software and represented in cfu/ cm^2.

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Table 5.8. Surface samples analysis by RPA-LF, qPCR and culture-based approaches

Contamination level (CFU/ cm^2)	N	RPA-LF	qPCR	COMPASS	Full Fraser 24 h	Full Fraser 48 h
8×10^5	2	+	+	+	+	+
8×10^4	2	+	+	+	+	+
8×10^3	2	+	+	+	+	+
8×10^2	2	+	+	+	+	+
8×10^1	1	+	+	+	+	+
7×10^1	1	+	+	+	+	+
5×10^1	3	+	+	+	+	+
2×10^1	2	+	+	+	+	+
	1	+	-	-	-	-
9	2	+	+	+	+	+
	2	-	-	-	-	-
8	2	+	-	+/-*	+	+
	2	+	+	+	+	+
7	1	-	-	-	-	-
4	2	+	+	+	+	+
	3	-	-	-	-	-
1	1	-	-	-	-	-
1	1	-	-	-	-	-

* Out of the 2 samples, 1 was positive and the other negative. Contamination level refers to the concentration of bacteria inoculated in the surface by cm^2 before enrichment in ONE broth. The samples were analysed by RPA-LF and qPCR and the results were compared with the reference culture-based methodology;
"+" in "COMPASS" refers to the presence of typical colonies observed after enrichment in ONE broth (molecular method), and "+" in "Fraser" observed when media turned dark and typical colonies were observed after plating on COMPASS;
N corresponds to the number of samples analysed with the respective bacterial concentration.

Table 5.9. Limit of Detection obtained for *L. monocytogenes* with RPA-LF

Methodology	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
RPA-LF without enrichment	117.9	26.9	516.0	509.3	116.3	2230.0
RPA-LF with enrichment	4.2	2.1	8.5	18.2	9.1	36.5
qPCR	8.3	4.1	16.8	36.1	17.9	72.5

* cfu/ cm^2;
qPCR methodology was accomplish with a 24 h enrichment in ONE broth to be compared with RPA-LF.

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In RTE products, where absence of *L. monocytogenes* is required, it is essential to ensure that the food processing plants are clean to avoid cross-contamination. For this reason the methodology combining a sampling step with the sponge, and a 24 h enrichment in ONE broth was deemed the most suitable, in order to reach a lower LoD. The length of this step is comparable to the time reported in other studies dealing with the detection of *L. monocytogenes* when applying isothermal [217,220] or other molecular techniques [166,221,222].

The results obtained with RPA-LF were compared against the other approaches (qPCR and the culture-based as the reference). The results are summarized in **Table 5.10**. Overall, similar results were obtained with the novel RPA-LF and qPCR, and both provided values higher than 90 % for most parameters evaluated. Only two deviations were observed, one for each methodology. Regarding qPCR one FN result was obtained, and was associated to a sample very close to the calculated LoD_{50}, while for the RPA-LF one FP was observed, related to a positive sample which was negative by both qPCR and the culture-based method.

Table 5.10. Evaluation of the results obtained by RPA-LF and comparison with qPCR

Approach	N	PA	NA	FN	TP	FP	AC (%)	SE (%)	SP (%)	p_0	p_e	κ
qPCR	32	21	10	1	0	0	97	95	100.0	0.97	0.44	0.94
RPA-LF	32	23	8	0	0	1	97	100	89	0.97	0.40	0.95

Evaluation performed analyzing surface samples.

It was observed that the limit of detection was not restricted by the detection methodology (RPA-LF, qPCR, or culture-based) but by the sampling process, as this step might limit the number of bacteria recovered from the surface. Several studies have reported bacterial loses in the sampling step, influenced by the tool employed for the sampling procedure, as well as the type of surface tested [219,223].

In this study, after inoculation of the surfaces, the bacteria were air dried and recovered 30 min later in an attempt to more closely mimic what happens in the food industry. This process can hinder the recovery of the bacteria, as different studies reported the ability of this pathogen to attach quickly, in less than 20 min, to different food contact materials [224], showing a reduction of 2 log cfu in stainless steel surfaces after 30 min [225]. Dry surfaces also increase de adhesion of the bacteria that is also the reason behind the European guidelines recommendation to use of moistened wiping devices for the recovery. The degree of pressure applied on the swabbing device also influences the recovery of bacteria, which can affect the repeatability of the method, producing different results in samples contaminated with the same bacterial concentrations [219]. These facts may explain why, even though a sample inoculated with 20 cfu/ cm^2, which is above the LoD_{95} of the RPA-LF (18 cfu/ cm^2), provided a negative result. Also, it is worth to mention that even though both samples were analysed using the same DNA extract, this was not considered as a ND by qPCR because the calculated LoD_{95} for this particular technique was higher than the inoculation level (36 cfu/ cm^2).

Nevertheless, the high values obtained in the performance parameters along with the positive comparison versus previously published studies, confirms the good performance of the novel method. Furthermore, the values obtained for SE and κ fulfil the requirements of the NordVal regulation for alternative methods, as the values obtained were higher than 95 % and 0.80 respectively, thus demonstrating the reliability of the novel assay [148].

5.3.2 RPA with SYBR Green (RPA-SYBR)

Trying to simplify the process for a naked-eye detection, SYBR Green was added to the RPA reaction after amplification, with the objective of having a change of colour at naked-eye, or end-point fluorescence discrimination when exposed to UV light.

Initially, when the amplification was performed during 30 min, a high background was observed in RPA reactions using non-target strains as template, being impossible to differentiate them from the positive samples. It was decided to reduce the amplification time from 30 min to 15 min to decrease the background obtained in the negative samples. **Figure 5.12 A** shows the presence of non-specific bands in the agarose gel, which are avoided when the amplification time is reduced. The correspondent fluorescence observed when SYBR Green was added, was also reduced in the negative reactions, confirming that the background was due to non-specific amplification occurring during RPA reaction.

Different concentration of SYBR Green were tested to improve the differentiation between negatives and positives, and results are presented in **Figure 5.12 B**. The increase in the volume added until 2 µL with 400X concentrated dye, gave better results, and the fluorescence of the reaction could be clearly observed when exposed to a UV light using a hand-held lamp, as well as when using the Gel DocTM EZ Imager. When the volume of fluorescent dye was increased to 4 µL, a general intensification of fluorescence emitted was observed, which also caused higher background in the negative reactions.

Thirteen ground beef samples, where the short enrichment methodology was applied for the detection of *E. coli* O157, were analysed by this alternative end point naked-eye analysis. The change in colour was reported in previous studies when SYBR Green was used for a similar approach [226]. However, the experiment performed during the optimization and analysis of this samples did not provided a similar results, even if most of the samples presented a slight difference, the change of colour was not intense enough to have a reliable differentiation between positives and negatives. This approach was, for this reason, excluded as a possible way to observe the results and the detection of *E. coli* O157 was performed by exposure to UV light.

After the sample analysis, a LoD_{95} and LoD_{50} of 19 and 4 cfu/ 25 g respectively was obtained. All the positive samples were correctly identified with the exception of 1 FN associated to a sample with low contamination level (2.5 cfu/ 25 g) that gave a positive results by qPCR. The samples tested and results obtained are presented in **Table 5.11**, and the result after the addition of SYBR Green and fluorescence obtained shown in **Figure 5.12 C**.

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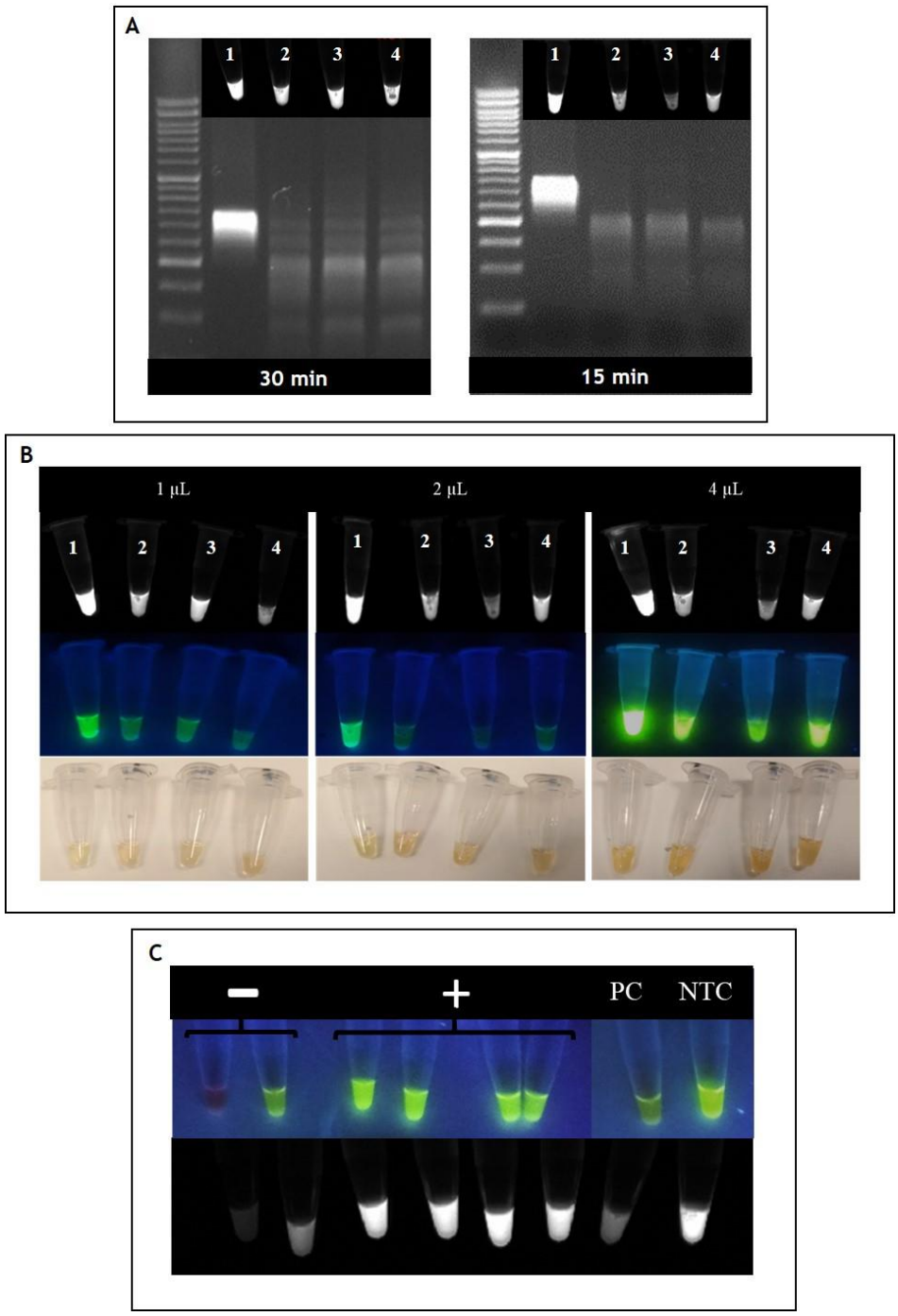

Figure 5.12. Optimization of the RPA amplification combined with SYBR GREEN naked-eye detection. (A) Effect of the RPA amplification time, comparing 30 min and 15 min of amplification. (B) Results obtained with different volume (1 µL, 2 µL, 4 µL) of 400X SYBR Green I solution after RPA amplification for 15min. In both Figures (A and B), different strains were tested: 1- *E. coli* O157, 2- *L. monocytogenes*, 3- S. Typhimurium, 4- NTC (C) Results obtained with some of the ground beef sample, including negatives (-) and positive (+) samples. PC is the positive control performed with a pure DNA extract of *E. coli* O157, while NTC represent the no template control, using water as template in the RPA reaction

Table 5.11. Spiked samples to evaluate the naked-eye RPA- SYBR approach

Type of sample	Contamination level (cfu/ 25 g of sample)	N	RPA- SYBR	Probe-qPCR
Ground beef	-	2	-	-
	1	1	+	+
	2.5	1	-	+
	1.0 x 10	1	+	+
	1.1 x 10	3	+	+
	7.3 x 10	1	+	+
	9.6 x 10	1	+	+
	1.1 x 10^2	3	+	+

RPA-SYBR and Probe-qPCR was performed both using the same *rfbE* primers; Probe-PCR also used the corresponding probe.

When evaluating the different parameters, a κ index of 0.81 was determined (**Table 5.12**), presenting a nearly complete concordance with the few samples tested. To properly evaluate this methodology a higher number of samples must be analysed. A similar approach was reported by Yu et al., [227] but adding the fluorescent dye to a LAMP reaction, demonstrating the possibility of implementation in other isothermal amplification approaches.

Table 5.12. Evaluation of the results obtained by RPA- SYBR

N	PA	PD	NA	FN	AC (%)	SE (%)	SP (%)	*k*
13	9	0	3	1	90	100	92	0.81

Ground beef samples treated with short enrichment methodology were used for the evaluation.

However this methodology exhibits some limitations due to some background observed in negative samples. This effect can be induced by the non-specific amplifications or primer dimers originated during the RPA reaction, as the reduction of the amplification time showed a decrease in the non-specific bands obtained in the agarose gel, but also in the fluorescence intensity observed after the addition of SYBR Green (**Figure 5.12 A**). Additionally, the initial DNA concentration can increase this background and interfere with the differentiation between positive and negative samples. This could be a problem in samples with higher DNA concentration due to the type of sample, or the presence of interfering microorganisms.

By these results the RPA-SYBR methodology may not be compatible with the non-specific enrichment to be use in the final methodology of this project. As the objective is to grow three pathogens in a same medium, the DNA content obtained from this enrichment will be higher, increasing the background effect.

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5.4 LAMP COMBINED WITH NAKED-EYE DETECTION

LAMP also showed promising results when performed in real-time for the analysis of several samples to detect *L. monocytogenes*. For this reason different naked-eye detection approaches were evaluated combined with LAMP to determine the possible applications. Turbidity and two different colorimetric approaches were tested for the detection of *Salmonella* spp.

5.4.1 Turbidity

The first naked-eye detection strategy developed with LAMP reaction was turbidity due to LAMP's ability to originate magnesium pyrophosphate an insoluble by-product. This approach was evaluated to compare different genetic targets for *S*. Enteritidis and *S*. Typhimurium, and the increase in turbidity was monitored using Loopamp Realtime Turbidimeter LA-500.

5.4.1.1 LAMP reaction performance

The lowest DNA concentration detectable for each target was evaluated through the dynamic range.

For *S*. Enteritidis detection, *safA* allowed the detection of a concentration down to 0.00144 ng/ µL, while *Sdf I* only enable the detection of 0.144 ng/ µL, as presented in **Figure 5.13 A**. Regarding the genetic targets for *S*. Typhimurium, a DNA concentration of 0.00438 ng/ µL was achieved with STM4497, however a much higher value of 4.38 ng/µL was obtained using *typh*, as seen **Figure 5.13 B**.

A panel of 34 strains, detailed in **Table 5.13** were tested in order to evaluate the specificity of the assay for each set of primers. Both *safA* and *Sdf I* got a positive result with a *Tt* value below 30 min for both *S*. Enteritidis strains tested while all the other non-target strains were negative. However, late amplification occurred with a *Tt* value higher than 35 min with certain strains when *safA* was used, while this was not observed targeting *Sdf I*. Regarding the identification of *S*. Typhimurium, all three target strains were detected with STM4497, without any interference due to non-target species. On the contrary, a total of four strains were misidentified targeting *typh* gene (one *S*. Typhimurium was not detected, and three non-target obtained positive results). It is worth considering that the specificity problems were only associated with *Salmonella* strains, as all the 19 non-*Salmonella* isolates were correctly identified as negative.

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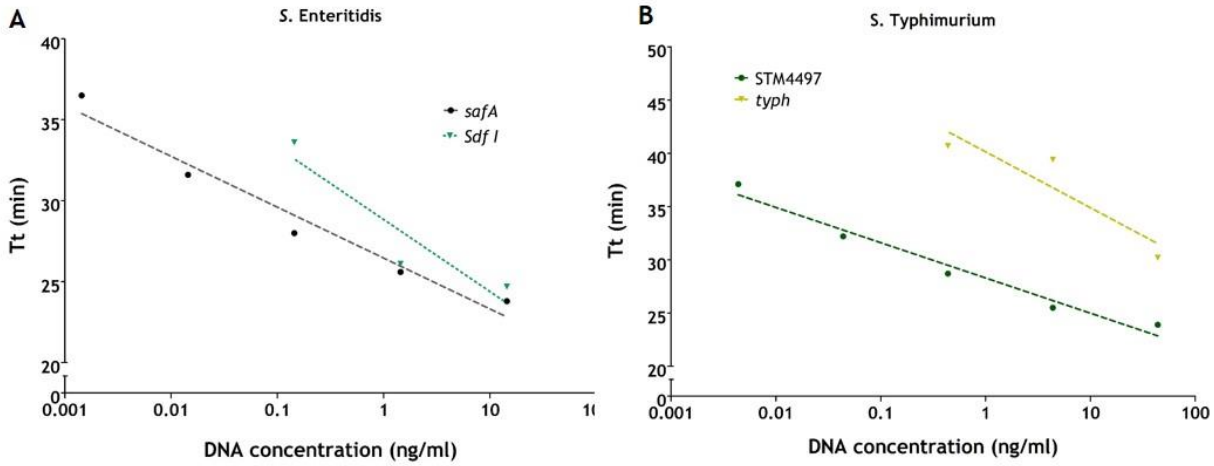

Figure 5.13. Determination of the dynamic range of the different targets evaluated by the LAMP turbidity approach. (A) *S.* Enteritidis genes *safA* and *Sdf I*. (B) *S.* Typhimurium genes STM4497 and *typh*. The Time to threshold (*Tt*) plotted against the DNA concentration of strain. Ten-fold serial dilution were obtained from S1400 for S. Enteritidis or WDCM 00031 for *S.* Typhimurium.

Table 5.13. Strain list selected for the evaluation of the specificity of the LAMP turbidity

Bacterium	Source	N	*Sdf* I	*safA*	STM4497	*typh*
L. monocytogenes	WDCM 00021, Mollusk, chestnut, chicken	16	-	-	-	-
L. seeligeri	CECT 917	1	-		-	-
L. ivanovii	WDCM00018	1	-	-	-	-
L. innocua	WDCM 00017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	-	-	-	-
C. coli	UM	1	-	-	-	-
S. Typhimurium	WDCM 00031	1	-	-	+	+
S. Typhimurium	P.T. AMC 96	1	-	-	+	-*
S. Typhimurium	Mollusk AMC 238	1	-	-	+	+
S. Enteritidis	UB (S1400)	1	+	+	-	-
S. Enteritidis	P.T. AMC 82	1	+	+	-	-
S. Veneziana	Mollusk AMC 200	1	-	-	-	-
S. Wentworth	P.T. AMC 84	1	-	-	-	+*
S. Oranienburg	Mollusk AMC 28	1	-	-	-	+*
S. Anatum	P.T. AMC 60	1	-	-	-	-
S. Liverpool	P.T. AMC 198	1	-	-	-	-
Salmonella spp.	Mollusk AMC 253	1	-	-	-	+*
Salmonella spp.	Mollusk AMC 90, 255	2	-	-	-	+*
Salmonella spp.	Unknown AMC 260, 261	2	-	-	-	-

N: number of strains;
* Strain misidentified

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5.4.1.2 Evaluation with spiked complex food matrixes

The different primers were also evaluated in spiked food matrixes, including chicken, turkey, eggs, and egg products. A total of 87 samples were spiked at different concentrations, and with different combination of each strain. The results obtained are summarized in **Table 5.14**.

Table 5.14. **Spiked samples summary for the evaluation of the LAMP turbidity approach**

Sample	N	Bacteria spiking *		Genetic targets				Observations**
		SE	*ST*	*Sdf I*	*safA*	STM4497	*typh*	
Egg	5	-	-	-	-	-	+*	4 PD
	3	<10	<10	+	+	+	+*	1 ND
	1	-	$10-10^2$	-		+	+	
	2	-	10^2-10^3				+*	1 ND
	1	-	10^8	-	-	+	+	
	1	10^2-10^3	-	+	+	-	+*	1 PD
	1	10^8	-	+	+	-	+*	1 PD
	1	$10-10^2$	10^3-10^4	+	+	+	+	
	1	10^2-10^3	10^2-10^3	+	+	+	+	
	1	10^3-10^4	10^3-10^4	+	+	+	+	
	1	10^3-10^4	$10-10^2$	+	+	+	+	
	1	10^4-10^5	10^4-10^5	+	+	-*	+	1 ND
	1	10^8	10^8	+	+	+	+	
Omelette	3	-	-	-	-	-	+*	1 PD
	1	$10-10^2$	-	+	+	-	-	
	1	10^2-10^3	-	+	+	-	+*	1 PD
Chicken	9	-	-	-	-	-	-	
	3	-	<10	-	-	+	+	
	13	<10	-	+	+	-	-	
	2	<10	<10	+	+	+	+*	1 ND
	1	<10	10^8	-*	-*	+	+	1 ND
	1	10^8	<10	+	+	-*	-*	1 ND
Turkey	5	-	-	-	-	-	-	
	11	-	<10	-	-	+	+*	3 ND
	1	-	$10-10^2$	-	+*	+	+	1 PD
	1	-	10^2-10^3	-	-	+	+	
	10	<10	-	+*	+	-	-	1 ND
	2	$10-10^2$	-	+	+	-	-	
	1	10^2-10^3	-	+	+	-	-	
	1	$10-10^2$	<10	+	+	+	+	
	1	<10	$10-10^2$	+	+	+	-*	1 ND

N: number of samples; SE: *S.* Enteritidis; ST: *S.* Typhimurium;
* Range of concentration spiked from each bacteria in cfu/ 25 g;
** Deviations obtained per simple type and inoculation range.

Both *S.* Enteritidis targets gave similar positive results with the correct identification of all positive samples, except for 2 ND. The same was observed with *S.* Typhimurium when targeting the STM4497 gene, while with the *typh* gene target, 16 of the 87 samples were misidentified. These results correlate to those obtained with DNA isolated from pure bacterial cultures, where STM4497 proved more sensitive than *typh*. With *Sdf I* both ND were associated with a sample with <10 cfu/2 5 g of *S.* Enteritidis.

Based on the results obtained from the spiked food samples, it was determined that both genetic targets for *S.* Enteritidis provided good results, with minor differences among them. This was not the case for *S.* Typhimurium, as major differences were observed when targeting STM4497 and *typh*. This last one showed the worst performance of all the genes evaluated in terms of sensitivity and specificity, resulting in a κ value of 0.62, which should be interpreted as "substantial agreement," while for the rest, values higher than 0.9 were obtained, interpreted as "almost complete concordance"[148] (**Table 5.15**).

Table 5.15. Evaluation of the different genetic target used for LAMP turbidity approach

Gene	N	PA	PD	NA	ND	SE	SP	AC	PPV	NPV	κ	AL
safA	87	44	1	41	1	97.8	97.6	97.7	97.8	97.6	0.95	0-2
Sdf I	87	45	0	40	2	95.7	100.0	97.7	100.0	95.2	0.95	0-2
STM4497	87	45	0	40	2	95.7	100.0	97.7	100.0	95.2	0.95	2-2
typh	87	27	8	44	8	77.1	84.6	81.6	77.1	84.6	0.62	0-16

N: number of samples; PA: Positive Agreement; PD: Positive Deviation; NA: Negative Agreement; ND: Negative Deviation; SE: relative sensitivity; SP: relative specificity; AC: relative accuracy; PPV: positive predictive value; NPV: negative predictive value; κ: index kappa of concordance;
Evaluation performed by the analysis of chicken breast, turkey and egg product with an enrichment in BPW for 24h.

When the results obtained were compared with those previously published for each target, some differences were observed. For *safA* and STM4497 it was possible to reach a lower LoD, with DNA from pure cultures, than previously reported by our the Food Quality and Safety group at INL [140], but minor differences were observed with spiked samples. These discrepancies may be associated with small changes in the method, such as the inclusion of loop primers, which were not used in the original study, as well as the different mastermix used and the increased amount of template added to the reaction.

The specificity results obtained with *Sdf I* matched those previously published by Yang et al., but the LoD was higher in the current study [141]. Greater differences were observed for *typh*, with respect to the study of Pavan Kumar et al., who reported excellent specificity [142]. However, in the present study, these primers were not able to correctly discriminate all the strains tested. This is in agreement with the fact that BLAST testing of these primers reported the same results for *S.* Typhimurium as for other serovars. Regarding the LoD, once more, in the present study, the results were worse than those reported in the original paper, as we could only detect 4.3 ng/ µL, while it was indicated that 0.002 ng/ µL could be reached. As mentioned previously, the discrepancies found among this and the original studies may be related with slight differences in the methodology followed, i.e., small differences in the amplification temperature, end-point results with respect to real-time turbidity tracking, application of gel electrophoresis, among others. Additionally, the reasons behind the overall differences in performance obtained by these four genetic targets may be of diverse origin, from the quality of the selected sequences, the primer design process, to specific assay optimization. This highlights that caution needs to be taken when directly implementing previously published studies in routine laboratory testing.

Regarding the performance of the turbidity detection, reliable results can be obtained using the turbidimeter, or other similar device that could read optical density, despite the increase in cost of the analysis by the use of a more expensive equipment. Unfortunately, when it comes to the naked-eye detection, the turbidity did not allow a straightforward observation, and the tubes needed to be placed in the right angle against the light to allow for the differentiation of most positives and negatives. Other authors have also reported this drawback, and worked with the addition of another compounds to promote a change of colour [92,228,229]. This issue made the analysis more difficult and so this approach cannot be used by any person, as a trained eye is required.

5.4.2 Naked-eye detection approach by combination of MUA and AuNPs

The optical properties of gold nanoparticles (AuNP) can be modified by changing their size, shape, surface chemistry, or aggregation state. This single proprieties can allow a change of colour and in this way provide a naked-eye detection. The functionalization with MUA was already reported by Wong et al., to allow a change of colour from red to purple by the aggregation/ disaggregation of the AuNP, based on the presence/ absence of Mg^{2+} [143]. In this study the functionalized AuNPs were added before the LAMP reaction, which caused particle aggregation. After DNA amplification, in order to see colour differences, ultrasounds had to be applied to re-disperse the particles in positive samples. This simple treatment, may be problematic when thinking of *in situ* analyses. For this reason, the addition of the AuNPs after DNA amplification, in a microfluidic device, was developed and evaluated.

As mentioned, the properties of the AuNP can be affected by its size and shape, thus after synthesis, the AuNP were characterized by TEM to confirm the correct morphology, and spheres with an average size of 13.3 ± 1.2 nm were observed (**Figure 5.14 A**). Additionally, UV-vis spectrum was measured before and after functionalization with MUA, showing the same peak at ≈520 nm, as presented in **Figure 5.14 B**.

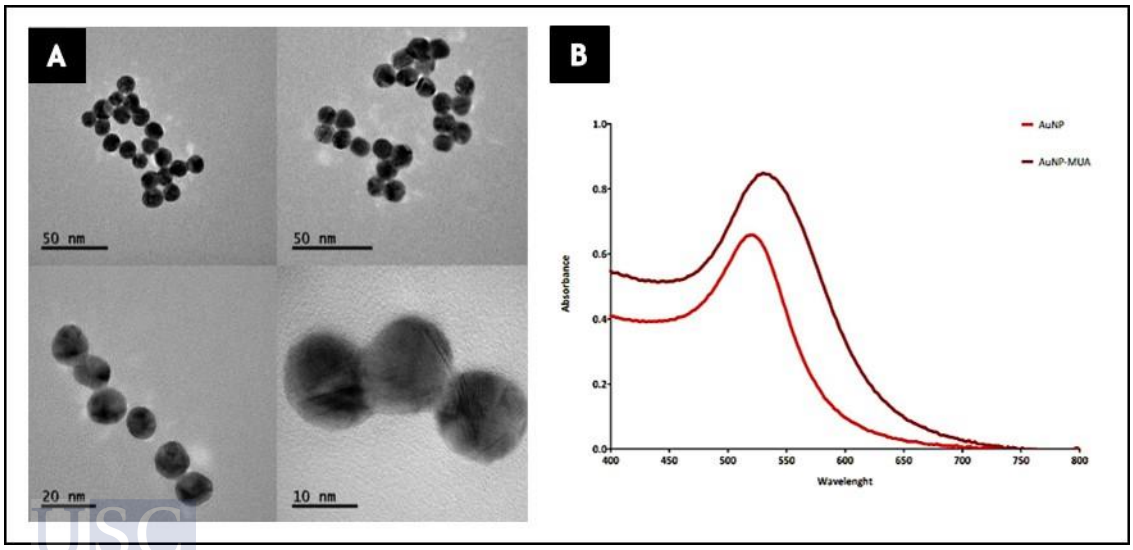

Figure 5.14. AuNP characterization for colorimetric LAMP approach. (A) AuNP TEM images at different magnifications. (B) UV-Vis of AuNPs before and after MUA functionalization.

The LAMP reaction was performed in the microfluidic device with 8 capillarity-driven microchannels and successfully accomplished as observed in the agarose gel of **Figure 5.15 A**, showing the characteristic banding pattern of this amplification technique. This confirmed the possibility of integrating the LAMP reaction in a microfluidic system.

After the MUA-AuNP addition, a clear difference of colour was obtained between positive (red) and negative (purple). These differences were also visible by UV-Vis (**Figure 5.15 B and C**).

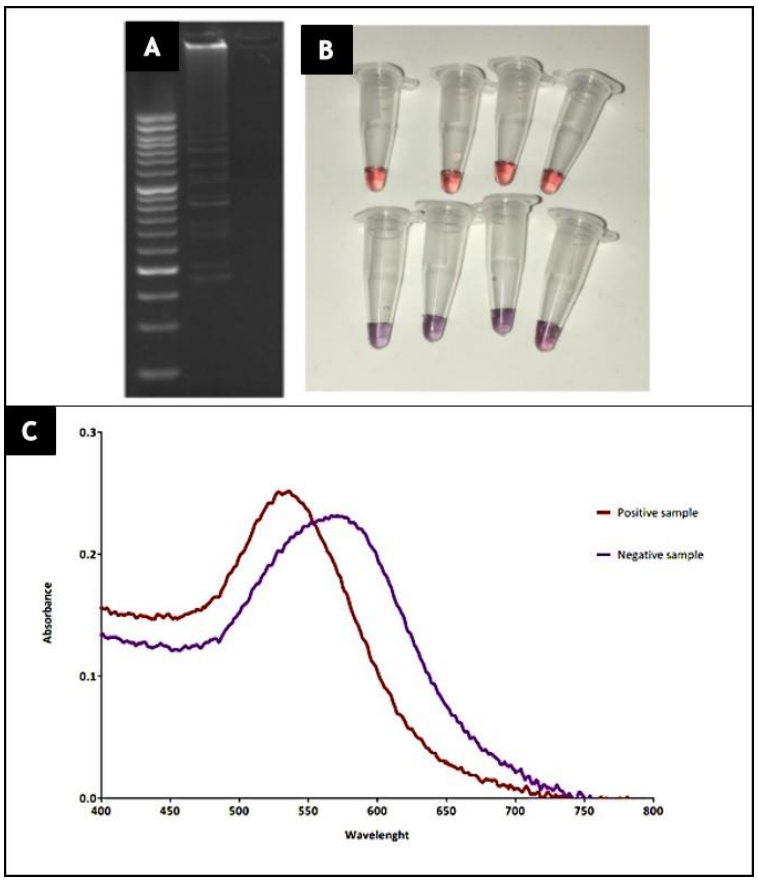

Figure 5.15. Results of microfluidic-LAMP amplification and colorimetric MUA-AuNPs detection. (A) Gel electrophoresis of a positive and negative sample after LAMP DNA amplification in the microfluidic device. (A) Results of positive (red) and negative (purple) samples for *Salmonella* spp. after LAMP DNA amplification in the microfluidic chip, and the addition of MUA-AuNPs. (C) UV-Vis spectra obtained for positive and negative samples after the addition of MUA-AuNPs.

Poultry meat and egg samples, also used to evaluate the LAMP with turbidity naked-eye approach, were analysed also for this alternative with AuNP to determine the LoD and calculate the parameters for the fitness for purpose. After the analysis of 39 samples was done, a perfect performance was obtained with a κ index of 1 (**Table 5.16**), and a LoD of 10 cfu/ 25g. These results confirmed the reliable detection of *Salmonella* spp., and the applicability of the method in the food industry. These results are similar to those obtained with other conventional LAMP methods [230,231], or even with other DNA amplification techniques [136,198].

Table 5.16. Evaluation of the results obtained by colorimetric LAMP using MUA-AuNP

N	PA	PD	NA	ND	SE (%)	SP (%)	AC (%)	PPV (%)	NPV (%)	κ
38	24	0	14	0	100	100	100	100	100	1.00

N: number of samples; PA: Positive Agreement; PD: Positive Deviation; NA: Negative Agreement; ND: Negative Deviation; SE: relative sensitivity; SP: relative specificity; AC: relative accuracy; PPV: positive predictive value; NPV: negative predictive value; κ: index kappa of concordance;
Evaluation performed analysing chicken breast, turkey and egg product with an enrichment in BPW for 24h

The results obtained by this methodology were compared with amplification performed in tubes instead of the microfluidic device, and one negative sample misidentified on tubes was clearly identified as negative with the device, demonstrating a higher specificity when the microfluidic approach was used.

This LAMP MUA-AuNP allowed a naked-eye detection by the simple addition of the AuNP after amplification and the integration of the amplification part in a microfluidic device was successfully accomplished. One of the disadvantages of this approach was the need to open the tubes or removing the reaction from the microfluidic system in order to add the AuNP. This process can lead to cross-contaminations, which can originate false positives, as also reported by other studies [232,233]. However, a fitness-for-purpose redesign of the microfluidic device could overcome this drawback, by enabling an integrated mixture with the AuNPs after isothermal amplification.

5.4.3 Colorimetric mastermix

An alternative to MUA-AuNP, also allowing a colour change was the use of a mastermix already combined with pH-sensitive dyes to detect changes in pH resulting from proton accumulation due to dNTP incorporation. This approach was evaluated for the detection of *Salmonella* spp. using *invA* primers.

To enhance the reaction, the incorporation of loops primers was tested, comparing LB and LF results with negative samples, in order to understand if the addition of this primers in the reaction lead to false positive results. In fact, after 30 min of amplification the use of LB already shows the change in colour from pink to yellow, while for LF none of the negatives presented this change, remaining pink **(Figure 5.16 A)**. Only after 40 min this false positives appears and lead to not reliable results. With this in mind the LF primers were used to analyze more samples, including positive samples and further evaluate the best amplification time to obtain consistent results. The results are presented in **Figure 5.16 B**, confirming that in 40 min false positives start to appear, and in order to avoid this effect, the reaction needed to be stopped in 30 min. The amplification of the positive samples is achieved within this time and the differentiation between positives and negative was still visible.

A total of 22 samples were used to evaluate this methodology, 18 analysed after a 24 h enrichment and 4 following the short enrichment approach. These samples were also spiked with different contamination levels of the three pathogens to be targeted in the final approach, but only *Salmonella* spp. was detected by colorimetric LAMP. The samples with their corresponding spiking level, and the results obtained by colorimetric LAMP and qPCR are presented in **Table 5.17**, and the colour change after amplification can be observed in **Figure 5.17**. All samples presented results consistent with the ones obtained by qPCR. Three of them contaminated with *Salmonella*, obtained a negative result. These samples were spiked with a concentration of bacteria below the LoD and were the only ones that gave a negative result also by qPCR. Some of the negative samples tested were also contaminated with the two

other pathogens, and the absence of amplification (reaction remained pink) confirmed that the presence of *L. monocytogenes* and *E. coli* did not interfere with the amplification of *Salmonella* and also did not generate any false positives. This approach obtained a κ of 1.0 showing total concordance with reference qPCR approach, and also the same LoD_{95} of 2.1 cfu/ 25 g. Like the other colorimetric LAMP study, this one presented similar results to other amplification methodologies.

It was also noticed that, when the samples were allowed to cool down, a more intense colour differentiation was observed, turning the more orange negative samples into the expected intense pink. However the reaction needed to be well optimized in order to avoid false positives, as it was noticed that the precise time of reaction was critical to have reliable results. If the amplification was extended intermediate colour (orange) could be obtained making difficult the interpretations of the results.

The use of this mastermix to improve naked-eye detection has been applied for a broad range of different applications [234–237], producing a clear differentiation between positive and negative results.

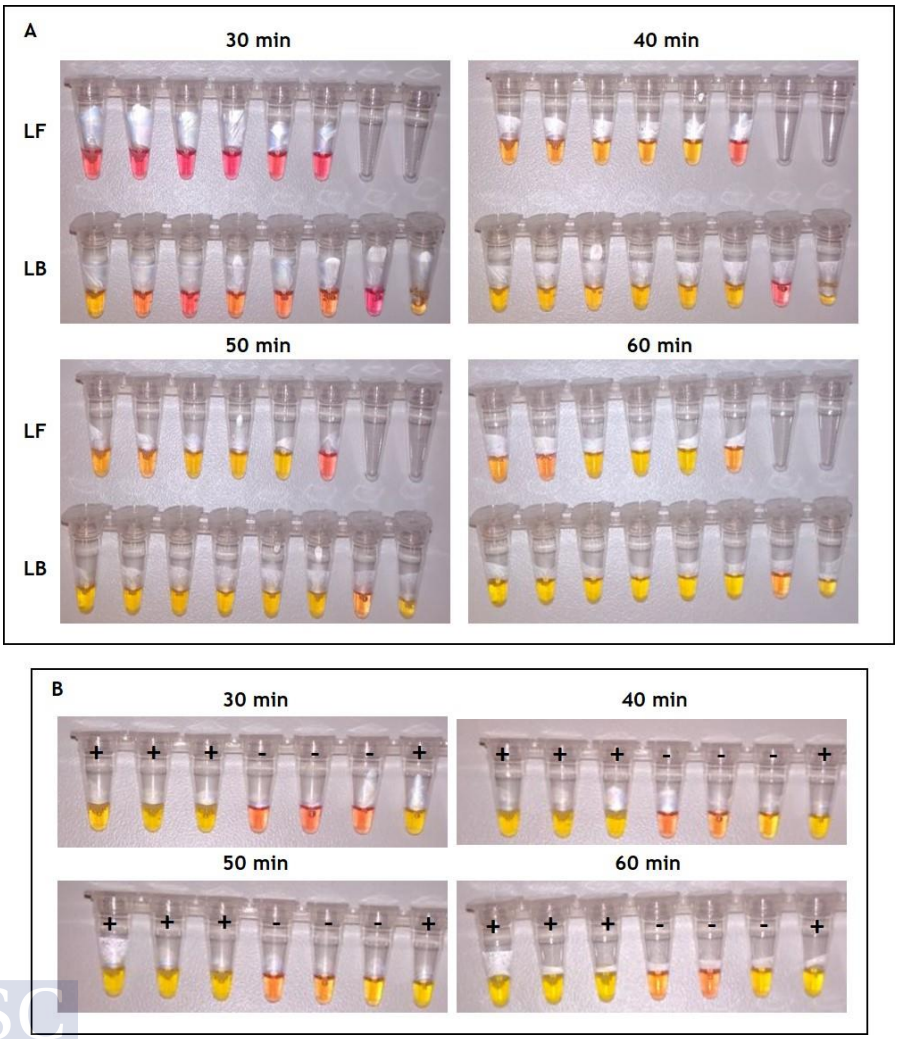

Figure 5.16. **Optimization of the colorimetric LAMP for the detection of *Salmonella* spp.** (A) Comparison between the use of LB or LF loop primers when negative samples were tested. (B) Optimization of the time of amplification using LF primer, with both negative (-) and positive samples (+).

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Table 5.17. **Spiked samples to evaluate the colorimetric LAMP with commercial mastermix**

Type of sample	Contamination level (cfu/ 25 g of sample)*			N	*Salmonella* detection	
	L. monocytogenes	*S.* Typhimurium	*E. coli* O157		ColorLAMP	qPCR
24 h enrichment (mTA10-MOPS)	5.1	2.8	2.5	3	+	+
	2.3	0.9	0.145	3	+	+
	2.2	1.1	0.8	6	+ (3); - (3)	+ (3); - (3)
	-	-	-	2	-	-
	9	6	4	1	+	+
	9		4	1	+	-
	91	1.3×10^3		1	+	+
	9		8.0×10^2	1	+	-
Short enrichment (TBS, 6 h)	10	15	6	1	+	+
	100	150		1	+	+
	100		60	1	-	-
	-	-	-	1	-	-

N: number of samples;
* The contamination level correspond to concentration of bacteria inoculate before enrichment.

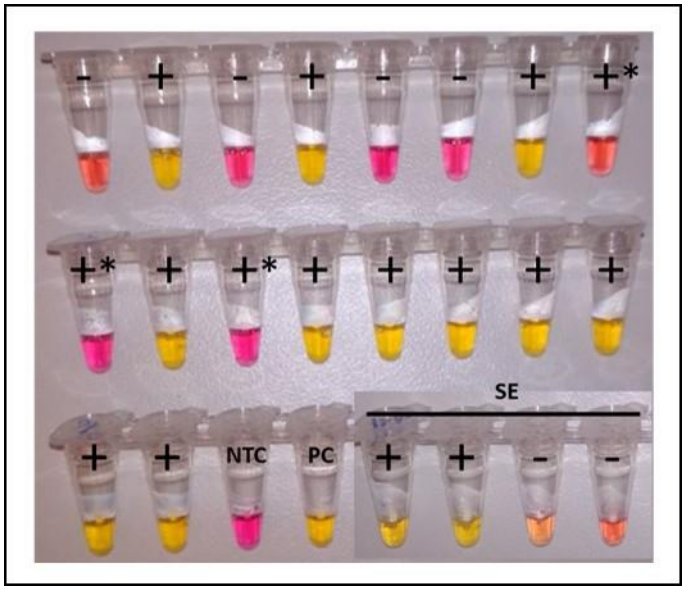

Figure 5.17. Sample analysis for the evaluation of LAMP colorimetric methodology using commercial mastermix to detect *Salmonella* spp. Samples analysis performed with a 24h enrichment in mTA10, with the exception of the ones identified with "SE" where the short enrichment methodology was accomplished in TSB for 6h. The "+" and "-" indicate if the sample was contaminated or not, respectively, with S. Typhimurium. * identify the samples contaminated which gave a negative result. The same samples were also negatives by Probe-qPCR.

5.5 COMPARISON OF THE DNA AMPLIFICATION AND DETECTION APPROACHES

The increased acceptance of molecular methods for the detection of different bacterial pathogens in foods has led to the appearance of a great number of approaches. Several of them based in DNA detection, allow a real-time monitoring and others an endpoint detection, for different types of pathogens of concern for the food industry. Both approaches were evaluated in this project and different methods, allowing a naked-eye detection, presented reliable results with excellent sensitivity and specificity, higher than 90 %.

For the real-time detection, qPCR is the most extensively applied technique for the detection of foodborne pathogens, with some protocols validated and implemented in the food industry. Most multiplex qPCR assays developed for the identification of different foodborne pathogens use hydrolysis probes [238,239] or high resolution melting analysis [240], which need appropriate reagents and a more complex downstream analysis, increasing the price in the first case or the time of the analysis in the second. The use of a common fluorescent intercalating dye (SYBR Green) avoids the cost increase due to use of expensive probes. However, some loss in specificity may be noticed, as the hydrolysis probe allow a second degree of specificity due to their hybridization, in addition to the primers. The design of these assays in multiplex is also more complex to develop and the results more difficult to analyze. On the other hand the use of hydrolysis probes simplify the combination of reactions for the detection of different targets, and thus ease of multiplexing.

Alternatively to PCR/ qPCR, isothermal amplification techniques have emerged allowing to solve some of the disadvantages associated with PCR/ qPCR, namely reducing the cost, by the removal of complex equipment, such as the thermocycler, and facilitating the miniaturization of amplification devices. Two isothermal amplification approaches were tested and evaluated, first in real-time and later on for naked-eye detection. This approaches were later compared with qPCR in terms of performance. Regarding the real-time evaluation, qPCR showed a higher sensitivity for the detection of DNA, reaching a lower concentration when compared with qLAMP and qRPA analysis, however this discrepancy did not influence their performance when spiked food samples were analysed after enrichment, achieving a similar LoD. In general, both isothermal approaches provided excellent results, being slightly better the method that implemented RPA, as the DNA amplification was faster, the primer/ probe design was simpler, as less primers are needed avoiding the possibility of interaction between them. Additionally, a lower number of discrepancies with the expected results, were observed with RPA.

With this in mind, several naked-eye detection approaches with both, LAMP and RPA, techniques were analysed in order to understand which could be the best combination for the goal of detecting the three different pathogens in foodstuffs. All of them obtained accurate results when applied for the detection in food products, so their advantages and drawbacks needed to be consider to combine this detection with a pre-treatment based on a short enrichment.

The combination of RPA amplification with LF was tested showing to be a less time consuming approach when compared with the culture-based methodologies, it simplifies the assessment of positive/ negative samples removing any complicated analysis for the result interpretation, However, the development of this kind of methodology needed the design of specific probes, and also the modification of the primers, as well as the purchase of the lateral flow strips, what represented an increase in cost to perform the analysis. On the other hand, the addition of SYBR Green dye for the detection of fluorescence, made the analysis less costly as only needed a UV light source to see the result. This approach showed promising results, however also presented limitations in complex samples, due to the fact that the SYBR Green will bind to any DNA present in the sample. This could be problematic for the

final methodology, knowing that the growth of three pathogens in the same matrix can produce high DNA concentration, and consequently increase the background observed in the negative reaction, being impossible to differentiate from positive results.

Regarding the LAMP methodology three different naked-eye detection strategies were evaluated. Turbidity was the first one, and depending on the set of primers used the result varied but in general a consistent analysis could be performed. In this case the turbidity could be monitored with a turbidimeter as well as being observed at the end by naked-eye, however the observation needs to be performed in a specific angle under white light to differentiate between negatives and positives, making the result visualization more complex than expected. The change of colour produced by the addition of MUA-AuNP, and the use of the colorimetric mastermix allowed to perform the clearest visualization discrimination. The LAMP amplification product contains multiple inverted repeated sequences, which makes cross-contaminations very easy when tubes are opened, and different authors reported the development of close tubes reactions to prevent false positives [232,233]. For this reason the opening of the tube after reaction, to add the MUA-AuNP can lead to this cross-contamination. The use of DNA-functionalized AuNP has been described by several authors [241,242], to increase the specificity of the assay, however the use of these probes increased the cost of the analysis. Additionally, as reported by Wong et al., [143] the addition of the MUA-AuNP before the reaction started required a sonication step, due to the aggregation particles when in contact with LAMP buffer. This sonication step represented an important limitation with the objective of integrating the analysis in a miniaturized device.

In order to overcome this drawback, and avoid false positives results, the use of the colorimetric mastermix demonstrated to be the best option for the naked-eye detection. This approach is simple to performe, did not required additional instrumentation, and the first trial combined with a short enrichment proved to allow the detection of *Salmonella* spp. Furthermore, the integration in a miniaturized device can be easily done with the colorimetric mastermix.

5.6 CONCLUSIONS

Real-time comparison

- Real-time qPCR is extremely sensitive and allows multiplexing for the detection of several pathogens at the same time. However, it needs expensive equipment to be performed, and trained personal to analyse the results.
- Isothermal amplification techniques can be performed in a simple heating equipment, and allow a broad range of naked-eye detection strategies to visualize the results.
- For real-time detection, LAMP and RPA, were less sensitive than the qPCR when evaluating the ability to detect the DNA of the targeted pathogens. However, when samples were analysed, this lower sensitivity did not affect the performance of the methodology, reaching the same limit of detection.

RPA naked-eye detection

- RPA-LF obtained high sensitivity and specificity with a clear result with the visualization of a band on the strips. However, the need for modified primers, probes and lateral flow strips makes the analysis more costly.
- RPA-SYBR on the other hand, is simpler to perform, but have some limitations due to the background signal in negative samples.

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LAMP naked-eye detection

- The use of turbidity provided clear results when using a proper equipment to monitor the reaction, but the visualization of the results by naked-eye showed to be quite subjective. Additionally the study of the different genetic targets, demonstrate the need to be careful when implementing published studies.
- Two colorimetric LAMP assays were evaluated, one based on AuNP and the second on a pH-sensitive commercial colorimetric mastermix. Both allowed for perfect differentiation of negative and positive results. The disadvantage of the first one was the need to open the reaction tube after amplification to add the AuNP, what could lead to cross-contamination.
- At the end the colorimetric mastermix was chosen to perform the LAMP amplification in the final methodology for the detection of *L. monocytogenes, Salmonella* spp. and *E. coli* O157. This approach allowed a clear result observation without the need for downstream processing.

5.7 PUBLICATION OF THE RESULTS

The results presented in this chapter were published in the following scientific articles:

Azinheiro, S., Roumani, F., Rodríguez-Lorenzo, L., Carvalho, J., Prado, M., & Garrido-Maestu, A. (2022). Combination of Recombinase Polymerase Amplification with SYBR Green I for naked-eye, same-day detection of Escherichia coli O157:H7 in ground meat. Food Control, 132, 108494. https://doi.org/10.1016/J.FOODCONT.2021.108494

Azinheiro, S., Carvalho, J., Prado, M., & Garrido-Maestu, A. (2020a). Application of recombinase polymerase amplification with lateral flow for a naked-eye detection of listeria monocytogenes on food processing surfaces. Foods, 9(9). https://doi.org/10.3390/foods9091249

Azinheiro, S., Carvalho, J., Prado, M., & Garrido-Maestu, A. (2020b). Multiplex Detection of Salmonella spp., E. coli O157 and L. monocytogenes by qPCR Melt Curve Analysis in Spiked Infant Formula. Microorganisms, 8(9), 1359. https://doi.org/10.3390/microorganisms8091359

Garrido-Maestu, A., **Azinheiro, S.**, Fuciños, P., Carvalho, J., & Prado, M. (2020). Comparative study of multiplex real-time recombinase polymerase amplification and ISO 11290-1 methods for the detection of Listeria monocytogenes in dairy products. Food Microbiology, 92, 103570. https://doi.org/10.1016/j.fm.2020.103570

Garrido-Maestu, A., **Azinheiro, S.**, Carvalho, J., & Prado, M. (2018). Rapid and sensitive detection of viable Listeria monocytogenes in food products by a filtration-based protocol and qPCR. Food Microbiology, 73, 254–263. https://doi.org/10.1016/j.fm.2018.02.004

Azinheiro, S., Carvalho, J., Prado, M., & Garrido-Maestu, A. (2018). Evaluation of Different Genetic Targets for Salmonella enterica Serovar Enteriditis and Typhimurium, Using Loop-Mediated Isothermal AMPlification for Detection in Food Samples. Frontiers in Susteinable Food Systems, 2(February), 1–8. https://doi.org/10.3389/fsufs.2018.00005

Garrido-Maestu, A., **Azinheiro, S.**, Carvalho, J., Abalde-Cela, S., Carbó-Argibay, E., Diéguez, L., Prado, M. (2017). Combination of Microfluidic Loop-Mediated Isothermal Amplification with Gold

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Nanoparticles for Rapid Detection of Salmonella spp. in Food Samples. Frontiers in Microbiology, 8, 2159. https://doi.org/10.3389/fmicb.2017.02159

Garrido-Maestu, A., **Azinheiro, S.**, Carvalho, J., Fuciños, P., & Prado, M. (2017). Development and evaluation of Loop-mediated isothermal amplification, and Recombinase Polymerase Amplification methodologies, for the detection of Listeria monocytogenes in ready-to-eat food samples. Food Control, 86. https://doi.org/10.1016/j.foodcont.2017.11.006

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CHAPTER 6.

RESULTS – SELECTED METHODOLOGY AND INTEGRATION ON MINIATURIZED DEVICE

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6 RESULTS – SELECTED METHODOLOGY AND INTEGRATION ON MINIATURIZED DEVICE

6.1 INTRODUCTION

After careful evaluation of the developed approaches to enable a faster and yet reliable detection of bacterial pathogens in food products, those developments providing the best results were selected. The full method combines a short enrichment, isothermal amplification by LAMP, and naked-eye detection by colour change, using the pH sensitive mastermix. The integration of the DNA amplification in a miniaturized device was also part of the objective of this project. In this regard, two different systems were tested and evaluated. The first one consisted on silicon tubing integrated in a miniaturized device with temperature control, while the second consisted on microfluidic channels made in PDMS, which were heated in a conventional laboratory incubator.

In this chapter, the results obtained for the development the selected methodology are presented, including:

- **Optimization of the selected methodology** for multiplex short enrichment and colorimetric LAMP reaction for the different targets;
- **Evaluation with food samples**, in order to understand the advantages and limitation of the full methodology;
- **Integration of the colorimetric LAMP reaction** on two **miniaturized devices** prototypes, and selection of the one with better performance;

6.2 SELECTED METHODOLOGY

6.2.1 Optimization

The method was first optimized to be able to detect the three target pathogens, *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 by combining the simultaneous short enrichment of the three microorganisms and allowing the downstream amplification of each target separately.

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6.2.1.1 Multiplex short enrichment for the three targets

The short enrichment approach was selected from the sample pre-treatment methodologies tested, due to the significant reduction of time obtained, compared with traditional enrichment. This methodology was proven to be able to recovered *L. monocytogenes* in simplex and as well for the multiplex recovery of *E.coli* O157 and *Salmonella* spp.. The optimization of the short enrichment is crucial for the successful detection of the three pathogens, especially *L. monocytogenes*, which represent a challenge due to its slower growth rate, compared to the other two bacteria. Different alternatives, including the combination of a general enrichment with a selective step, were tested to try to improve the detection of this last target. **Figure 6.1**, present the different protocols tested to improve the recovery of *L. monocytogenes*, and consequently its detection. The results of the qPCR analysis targeting *hly* are presented in **Figure 6.2** and **Table 6.1**, showing the amplification curves and the Cq value obtained.

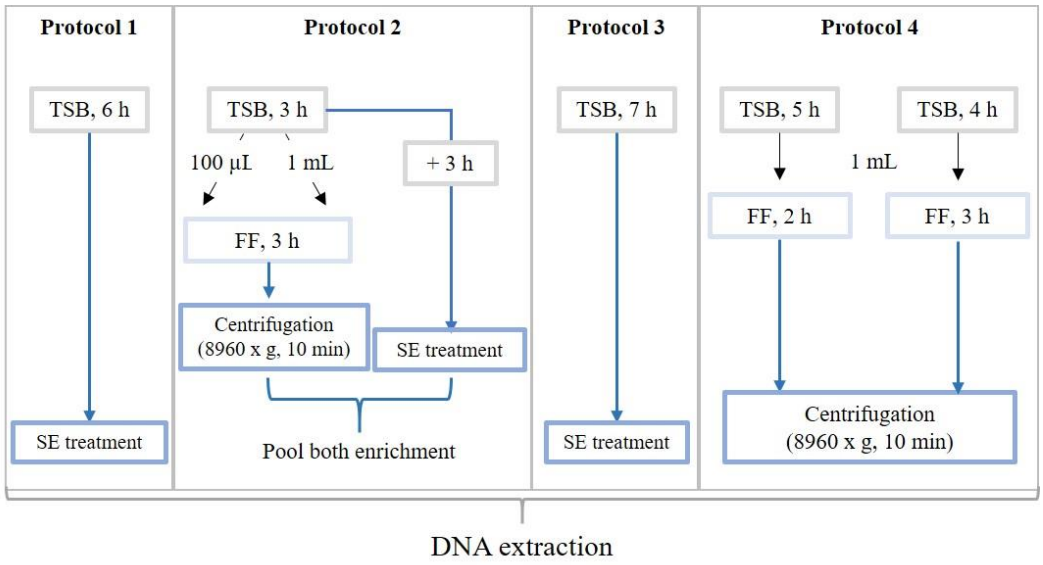

Figure 6.1. Optimization of the short enrichment (SE) for the final methodology, testing different alternatives. All enrichment were performed at 37 ºC with constant agitation at 200 rpm

Considering the results obtained for the short enrichment of *L. monocytogenes*, where an incubation in TSB was performed in 5 h (Chapter 4, section 4.4.3), a first test increasing the time to 6 h was evaluated with different combinations of the targeted pathogens (Protocol 1). By colorimetric LAMP the detection of the Gram-negative bacteria, *E. coli* O157 and *S.* Typhimurium was possible even in lower concentrations, 6 and 16 cfu/ 25g, however no change of colour was visible for *L. monocytogenes* by colorimetric LAMP using the commercial mastermix. After performing the enrichment in TSB for 6 h, no amplification was detected either by qPCR for the low contamination levels, and a very high Cq of 39.98 was observed for the highest concentration, which explained the lack of detection by the colorimetric LAMP. However, when the samples were spiked with *L. monocytogenes* along with only one of the other targeted pathogens, lower Cq value was obtained, 35.11 and 37.39 when in combination with S. Typhimurium and *E. coli* O157 respectively (**Figure 6.2 A**). This decrease in the Cq, showed that the lower competition lead to lower interference for the detection of *L. monocytogenes*.

In order to attempt to improve competiveness and final concentration of *L. monocytogenes*, the TSB pre-enrichment was combined with a second enrichment in Full Fraser (FF). For this, after a 3 h incubation in TSB, 100 µL or 1 mL of the pre-enrichment were transferred to 10 mL of FF and both, TSB and FF were further incubated with agitation for another 3 h. The 6 h TSB was treated for the recovery of the bacteria cells with the short enrichment protocol and the 3 h FF was centrifuged in order to recover the bacterial pellet. Both resulting enrichment solutions of enrichment were then pooled together to proceed with the DNA extraction (Protocol 2). This approach allowed to decrease the Cq obtained for both volumes, being the transfer of 1 mL to FF better to increase the final concentration of *L. monocytogenes*, allowing to obtain a Cq of 28.11 (**Figure 6.2 B**). However, when the samples were analysed by colorimetric LAMP, it was still not possible to observe any colour change.

For this reason, the total incubation time was increased to 7 h, Protocol 3, only in TSB and also testing the combination with 5 or 4 h incubation in TSB along with 2 or 3 h in FF, respectively, after adding 1 mL from TSB, as described in Protocol 4 of **Figure 6.1**. The resulting enrichment from just the FF medium was analysed in order to increase the concentration of *L. monocytogenes* without the interference of *Salmonella* spp. and *E. coli* O157 and allow a more sensitive detection of this pathogen (**Figure 6.2 C**). The qPCR results for the 2 h and 3 h enrichment are similar, obtaining a Cq of 34.00 and 33.55, respectively, for the medium spiking, and with samples contaminated with low concentration, no detection or a Cq of 39.43 was observed. None of these experiments were positive by colorimetric LAMP. The enrichment of 7 h in TSB reported lower Cq values, of 28.03, for the detection of *L. monocytogenes* by qPCR analysis, similar to the mixture of TSB and FF for 6 h. However, unlike the mixture, this protocol was the only one to give a clear change of colour by LAMP and improved the detection of *L. monocytogenes* after spiking the sample with 93 cfu/ 25 mL. On the other hand, none of the protocols tested allowed to detect this pathogen in the lower spiking level (≤ 10 cfu/ 25 mL) when the other two pathogens are also present.

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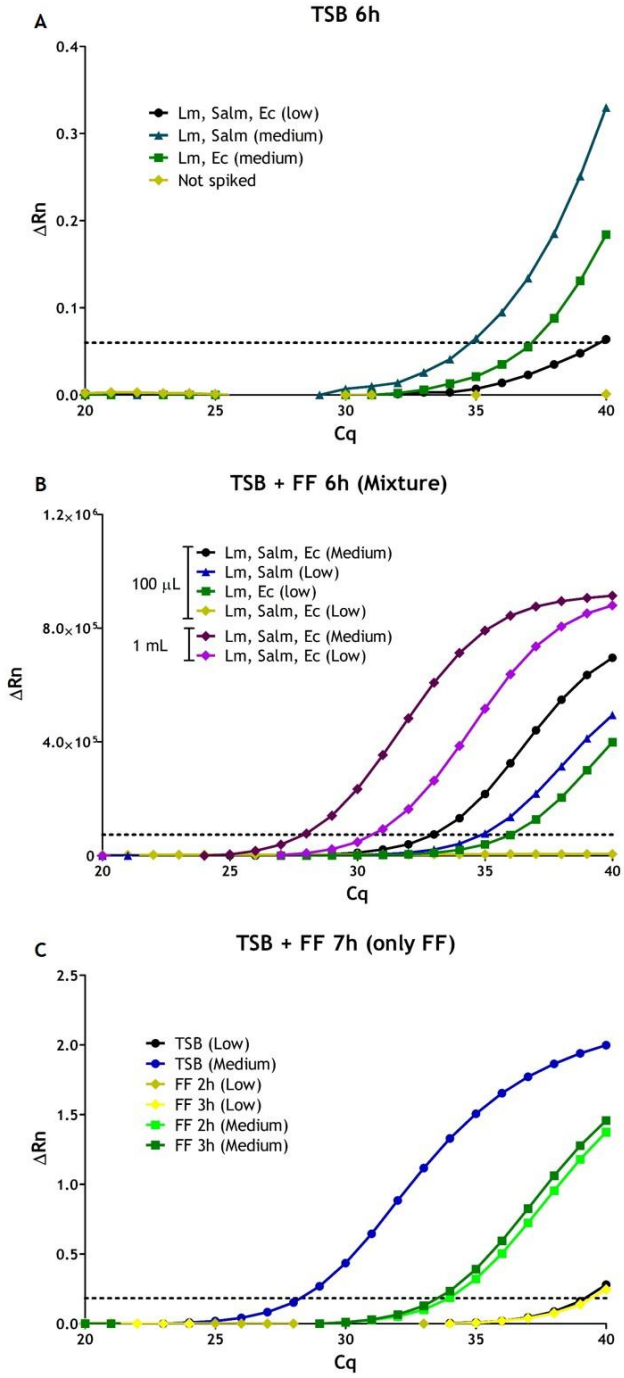

Figure 6.2. **Amplification curves showing the optimization of the enrichment in UHT milk samples, analysing qPCR results for *L. monocytogenes* detection.** The three targeted pathogens, *L. monocytogenes* (Lm), *Salmonella* spp. (Salm) and *E. coli* O157 were spiked in different concentrations: "Low" for 1-20 cfu/ 25 mL, and "Medium" for 60-160 cfu/ 25 mL. (A) Enrichment in TSB for 6 h (Protocol 1); (B) Pre-enrichment in TSB for 3 h and transference of 100 µL, or 1 mL to FF for additional incubation of 3h. At the end the two enrichment were pooled before DNA extraction (Protocol 2); (C) Comparison among enrichment in TSB 7 h (Protocol 3) and a pre-enrichment of 4 h or 5 h in TSB and transfer of 1 mL to FF for additional 3 h or 2 h in FF, respectively. The FF curves correspond to DNA extracts coming only from the secondary enrichment in FF (Protocol 4).

Table 6.1. Cq values obtained using different enrichment media

	Type of enrichment	Bacteria concentration			qPCR (Cq)	ColorLAMP
		L. monocytogenes	*S.* Typhimurium	*E. coli* O157		
Protocol 1	TSB 6h	10	16	6	-	-
		100	155		35.11	-
		100		60	37.39	-
		-	-	-	-	-
		81	116	76	39.98	-
Protocol 2	TSB 3h + 100µL FF 3h (mixture*)	85	118	68	32.97	-
		9	12		34.89	-
		9		7	35.98	-
		9	12	7	-	-
	TSB 3h + 1mL FF 3h (mixture*)	102	102	37	28.11	-
		10	10	4	30.84	-
Protocol 3	TSB 7h	8	7	6	39.34	-
		81	66	62	28.03	+
Protocol 4	TSB 5h + FF 2h (only FF**)	8	7	6	-	-
		81	66	62	33.95	-
	TSB 4h + FF 3h (only FF**)	8	7	6	39.43	-
		81	66	62	33.55	-

Bacteria concentration expressed in cfu/ 25 mL of UHT milk sample;
* The samples analyzed represent the TSB and FF treatment polled;
** The samples analyzed represent only the treatment of the FF enrichment;
The protocols presenting the best results by qPCR, showing the lowest Cq value are highlighted in green.

In the samples where the FF was used to perform part of the enrichment, an initial change of colour was observed in the LAMP reaction before performing the DNA amplification, as presented in **Figure 6.3 A**. This could suggest an interference of this medium with the amplification reaction, explaining why the mixture of TSB and FF incubated for a total of 6 h, which obtained a similar Cq to that of TSB 7 h, did not develop any colour change, in this regard. The colorimetric reaction implements phenol red, a pH-sensitive dye, to generate the colour change [243], making the solution more sensitive to different compounds present in the DNA extract. The manufacturer of the mastermix also reports the interference of some solutions, recommending the elution of the DNA in water, as other buffers may interfere with the pH when added in higher volume than 10 %, what emphasizes the possible interference.

Diluting ½ the DNA extract before loading in the LAMP reaction seemed to decrease this effect, which was completely solved when a kit for DNA purification was used to purify the DNA extract (**Figure 6.3 A**). However the positive samples still did not change their colour after amplification (**Figure 6.3 B**).

For this reason the final methodology will include an enrichment in TSB for 7 h, as the experiments in this conditions seemed to allow a better sensitivity with consistent results (**Figure 6.3 C**).

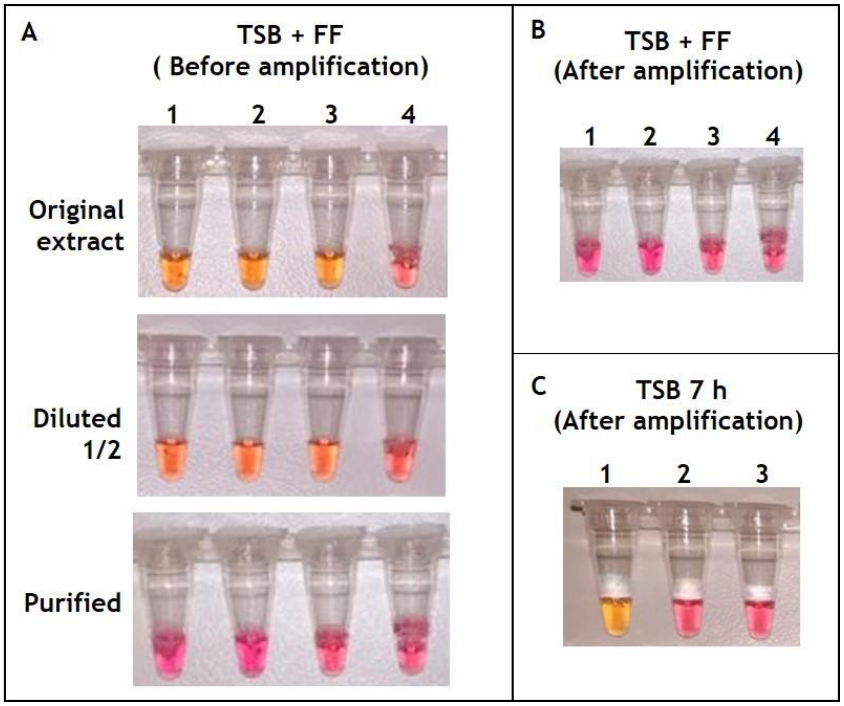

Figure 6.3. Full Fraser broth influence in the colorimetric LAMP reaction. The figure represents the detection of *L. monocytogenes*, in different samples. (A) LAMP reaction before incubation with samples obtained with Protocol 2, originating a DNA extract with the enrichment results of TSB and FF combined. A visible change of colour from pink to orange is seen when the DNA extract is added into the reaction and a decrease of this effect is observed after a dilution ½ and treatment with NucleoSpin™ gDNA Clean-up Kit (Purified). (B) LAMP reaction of the same samples after 1 h incubation at 65 ºC, using purified samples. No change of colour is visible, indicating lack of amplification. 1- Medium concentration, 2- Low concentration, 3- Negative sample, 4- Negative template control (water). (C) LAMP reaction testing samples obtained with an enrichment in TSB for 7 h (Protocol 3). 1- Medium concentration, 2- Low concentration, 3- Negative sample. The medium concentration represents samples spiked with 81, 66 and 62 cfu/ 25 mL and the low 8, 7 and 6 cfu/ 25 mL of *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157, respectively

6.2.1.2 Colorimetric LAMP for *L. monocytogenes* and *E. coli* O157 detection

The best conditions to perform the colorimetric LAMP targeting *invA*, *plcA* and *rfbE* were selected for the detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157, respectively. The reaction for the detection of *Salmonella* spp. was already optimized as previously described in Chapter 5, section 5.4.3.

The optimal amplification time was also evaluated to perform the detection of *E. coli* O157 and *L. monocytogenes*. For the first pathogen, a similar result than the one obtained for *invA* was obtained. In 40 min, non-specific amplification was observed, limiting the amplification to 30 min (**Figure 6.4 A**). However for *L. monocytogenes* a longer incubation time was needed, at least 1 h, to obtain the change of colour. Additionally, this longer amplification had to be combined with higher template volume (6 µL) and higher Loop primer concentration (600 nM) to increase the sensitivity of this reaction.

As also performed for the LAMP reaction targeting *invA* gene, previously described in Chapter 5, section 5.4.3, the specificity and applicability of *E. coli* O157 and *L. monocytogenes* detection with the optimized reaction were evaluated with different milk samples analysed after a 24 h enrichment, but also with the short enrichment in TSB for 7 h, as detailed in **Table 6.2**. All samples were correctly identified, developing the expected change of colour (**Figure 6.4 B**). To confirm the results, positive and negative reactions of the different targets were analysed by gel electrophoresis (**Figure 6.5**). All positive samples presented the typical LAMP pattern, while the negatives did not show any bands. For the reaction targeting *Salmonella* spp. a false positive result was obtained when the amplification time was extended up to 40 min. This sample turned to yellow and bands were present in the gel, however with different pattern from the positive sample. The results obtained with the *invA* primers, emphasize the need to stop the reaction in 30 min for the reliable detection of *Salmonella* spp. Regarding the detection of *E. coli* O157, one negative sample, also with an extended amplification time of 1 h was performed, and besides false negatives where observed in this conditions during the optimization, this sample did not present a positive result by colorimetric LAMP, remaining with the initial pink colour, and in the gel no amplification was observed. The results presented also highlighted that the extension of the amplification time do not always produce false positive.

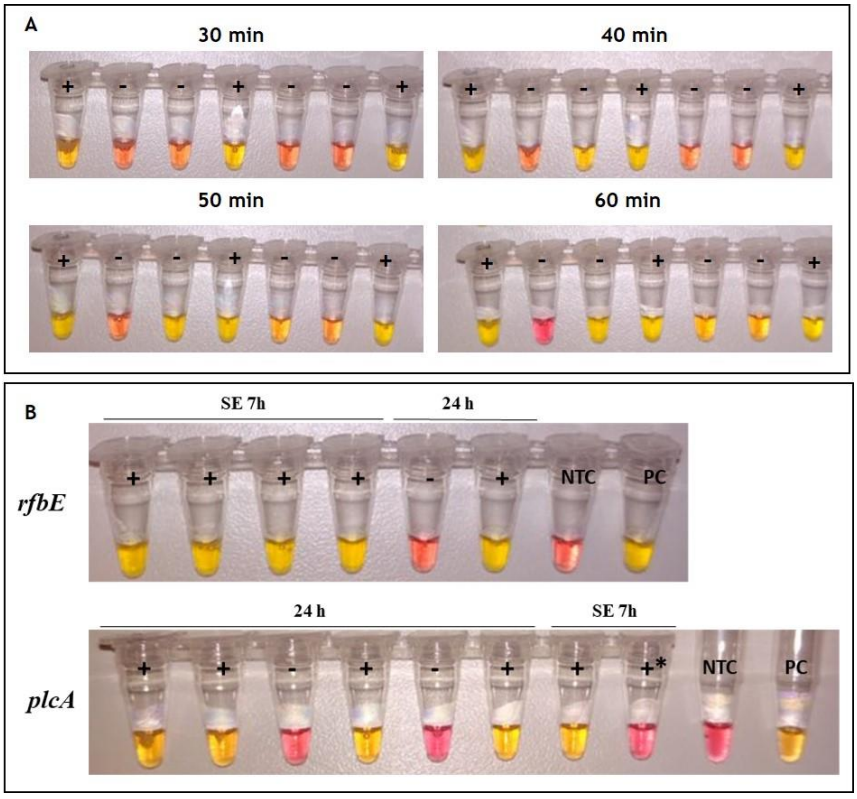

Figure 6.4. LAMP reaction optimization. (A) Optimization of the time of amplification targeting *rfbE* with both negative (-) and positive samples (+). (B) Test of different samples from a 24 h enrichment and a 7 h short enrichment (SE 7 h) after a 30 min amplification for *rfbE* target and 1 h for *plcA*. The positive results are yellow and the negatives pink. NTC: Non-Template Control (water); PC: Positive control (DNA from pure bacterial culture); * Sample spiked with lower contamination level (8 cfu/ 25mL), not detected by colorimetric LAMP

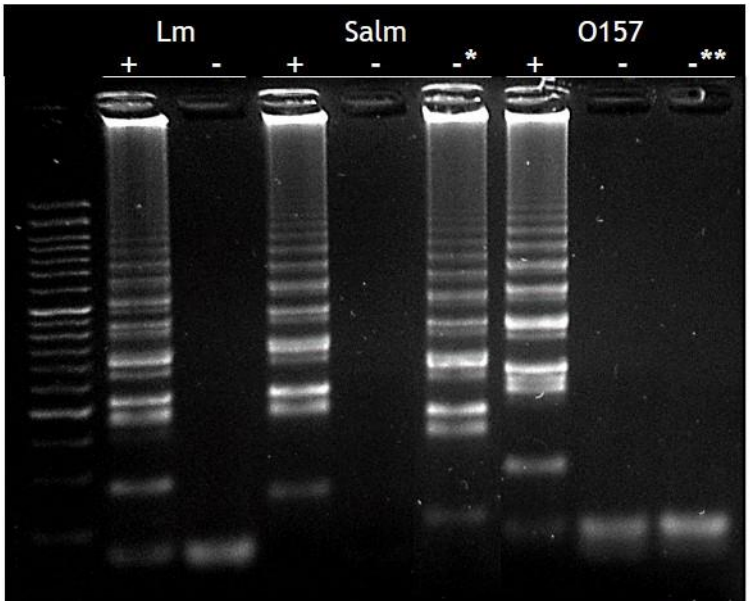

Figure 6.5. Amplification product from the colorimetric LAMP reaction for de detection of *L. monocytogenes* (Lm), *Salmonella* spp. (Salm) and *E. coli* O157 (O157). "+" and "-" represent positive and negative samples obtained by the optimized short enrichment analysis (TSB 7h). * negative sample which changed colour for *invA* when the amplification was extended up to 40 min is performed. This gel of this sample shows that amplification product is present, however with a different pattern; ** negative sample with no change of colour for *rfbE* when an extended amplification of 1 h is performed. No amplification is visible in the gel for this negative sample.

Table 6.2. **Samples tested for the colorimetric LAMP optimization**

Sample pre-treatment	Bacteria concentration			ColorLAMP*	
	L. monocytogenes	*E. coli* O157	*Salmonella* spp.	*plcA*	*rfbE*
24 h enrichment	9		6	+	-
	9	4	6	+	+
	-	4	6	-	
	9	4	-	+	
	-	-	-	-	
	9	4	6	+	
7 h Short enrichment	8	6	7	-	+
	81	62	66	+	+
	9	2	9		+
	93	18	88		+

Bacteria concentration expressed in cfu/ 25mL of UHT milk sample;
* Visualization of the change of colour after colorimetric LAMP reaction after 1 h for *plcA* and 30 min for *rfbE*.

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6.2.2 Evaluation of the colorimetric LAMP reaction

After the reaction of the colorimetric LAMP optimized, the lowest concentration of DNA from the targeted pathogen was evaluated, as well as the analytical sensitivity for the bacteria concentration.

For the first approach ten-fold serial dilutions of a DNA obtained from a pure culture of the corresponding target was used to perform the LAMP assay (**Figure 6.6 A**). For *E. coli* O157, a dynamic range of 5 dilutions was achieved, allowing the detection of 14.8 pg/ µL. Similar results were obtained for the reaction targeting *invA* gene of *Salmonella* spp., where it was possible to detect down to 19.3 pg/ µL. The dynamic range for *L. monocytogenes* was smaller, 4 dilutions, due to the lower concentration of the initial extract, however it was possible to detect a concentration of DNA in the same range of the two other targets, being the lowest concentration 22 pg/ µL. The same concentrations were also tested by qPCR, obtaining similar results than those of the colorimetric LAMP, as it can be observed in **Figure 6.7**.

To evaluate the lowest concentration of bacteria needed to originate a positive result by colorimetric LAMP, ten-fold dilutions of the bacteria were used, and their DNA was obtained by thermal lysis of each dilution (**Figure 6.6 B**). For the two Gram-negative targets the analytical sensitivity was on the same logarithmic range, 3.3 x 10^5 cfu/ mL and 1.3 x 10^5 cfu/ mL for *E. coli* O157 and *Salmonella* spp. respectively. Regarding *L. monocytogenes* a higher value was obtained, 6.9 x 10^6 cfu/ mL. This difference, can be explained by the fact that the DNA from the bacteria were all obtained by a simple thermal lysis. The lytic efficiency on Gram-positive bacteria, like *L. monocytogenes*, by this methodology is reduced due to the presence of a thick peptidoglycan layer. It is for this reason that the DNA extraction in the full methodology was performed implementing an enzymatic solution combining lysozyme and achromopeptidase. Several studies have shown the potential of these enzymes to improve the lysis of both Gram-negative and positive microorganisms. Lysozyme is extensively used to lyse bacteria [244,245], however some bacteria have developed resistance mechanisms for lysozyme, which is the case of *L. monocytogenes*, highly resistant to this compound [246,247]. For this reason achromopeptidase was also added to the lysis buffer [248].

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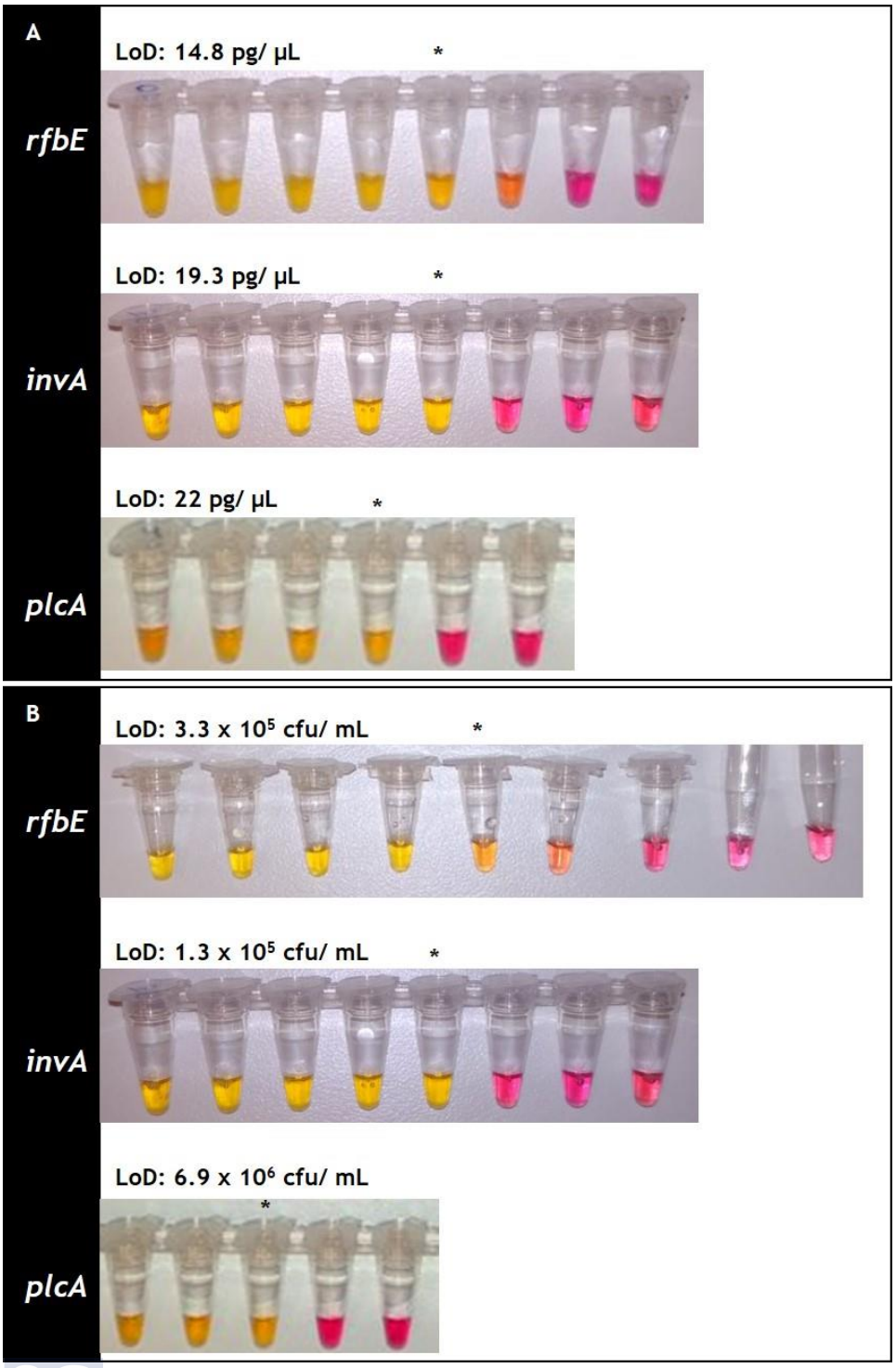

Figure 6.6. Dynamic range of the colorimetric LAMP for the different targets. (A) Determination of the lowest DNA concentration detectable, using a ten-fold serial dilutions of DNA extracts obtained from pure cultures of each pathogen. (B) Determination of the lowest concentration of bacteria detectable, using ten-fold serial dilutions of a pure bacterial cultures. * represent the last dilution to be considered positive.

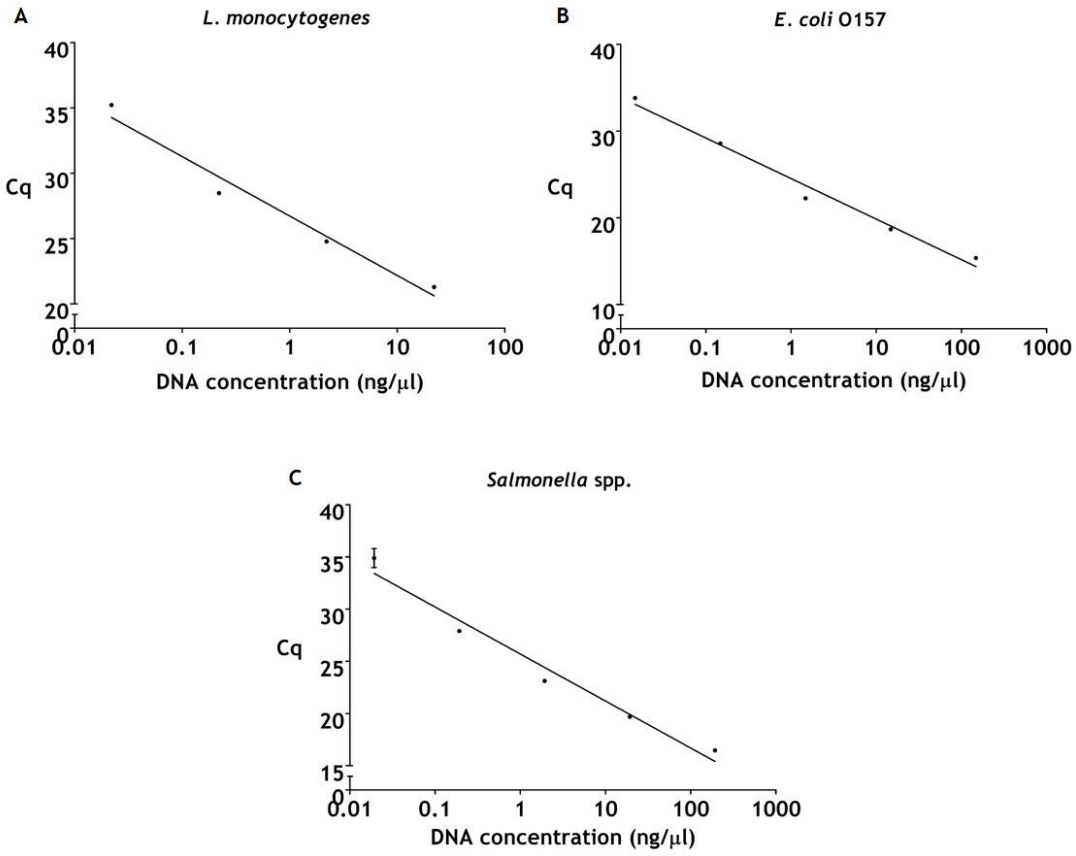

Figure 6.7. Lowest detectable DNA concentration obtained by multiplex qPCR for the different targets *hly* (A), *rfbE* (B) and *invA* (C). Ten-fold serial dilutions of the DNA extracted from pure cultures of each pathogen were tested in duplicate.

When these bacterial dilutions where analysed by qPCR (**Figure 6.8**) it was possible to detect 3.3 x 10^4 cfu/ mL and 1.3 x 10^4 cfu/ mL for *E. coli* O157 and *Salmonella* spp, respectively. For *L. monocytogenes*, the reaction was able to detect 2 log more comparing with the colorimetric LAMP, until 6.9 x 10^4 cfu/ mL, with higher deviation (2.7) in the Cq of the last dilution.

It was previously reported in this project that LAMP presented a lower analytical sensitivity than qPCR (Chapter 5, section 5.2.3), and the decrease of performance when this isothermal amplification is used was also describe by other authors [249–251].

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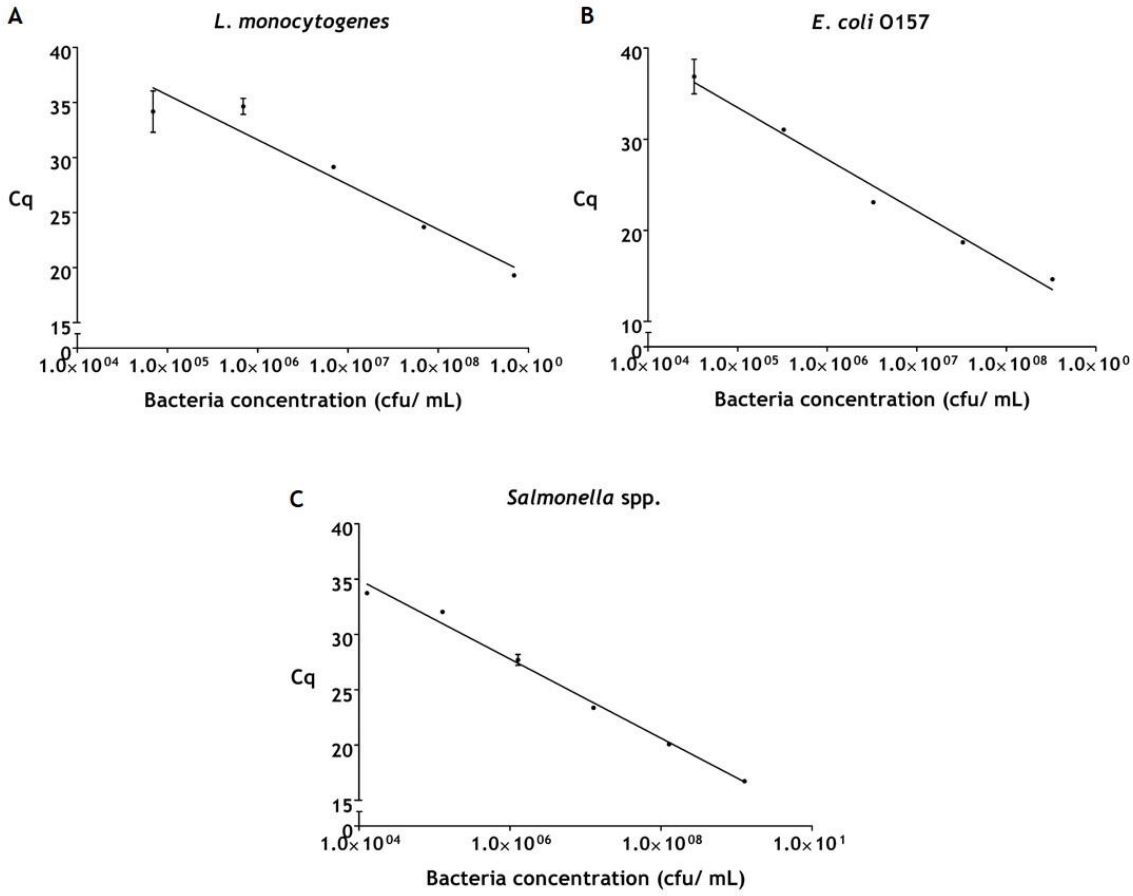

Figure 6.8. Lowest bacterial concentration detectable by multiplex qPCR for the different targets *hly* (A), *rfbE* (B) and *invA* (C). DNA extracted from ten-fold serial dilutions of a pure cultures.

A total of 52 different strains were tested by colorimetric LAMP with the three sets of primers to evaluate the specificity. This panel of bacteria included: 15 *Salmonella* spp., 17 *L. monocytogenes* and 2 *E. coli* O157:H7 and 18 non-specific strains, as non-O157 *E. coli*, other *Listeria* spp. (*L. ivanovii* and *L. innocua*) *S. aureus*, *Y. enterocolitica* and *C. coli*. All the strains were correctly identified by colorimetric LAMP, as summarized in **Table 6.3**.

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Table 6.3. Specificity of colorimetric LAMP for the different targets

Bacterium	Source	N	*plcA*	*rfbE*	*invA*
L. monocytogenes	WDCM 00021, Mollusk, chestnut, chicken	17	+	-	-
L. seeligeri	CECT 917	1	-	-	-
L. innocua	WDCM 00017, CECT 5376, 4030; CUP 1141, 1325, 2110	6	-	-	-
C. coli	UM	1	-	-	-
S. aureus	WDCM 00033, WDCM 00034	2	-	-	-
Y. enterocolitica	WDCM 00038	1	-	-	-
E. coli	WDCM 00013, AMC 73, 75,81, 171, 178, 278	7	-	-	-
E. coli O157:H7	WDCM 00014, AMC 76	2	-	+	-
Salmonella spp.	(AMC 28, 60, 82, 84, 90, 96, 198. 200, 238, 253, 255, 260, 261, UB, WDCM 00031)	15	-	-	+

N: number of strains; WDCM: World Data Centre for Microorganisms reference; CUP: Catholic University of Porto; UM: University of Minho; UB: University of Bristol;
The *plcA* was selected for the detection of *L. monocytogenes*, the *rfbE* for *E. coli* O157 and the *invA* for *Salmonella* spp.

6.2.3 Evaluation of the full methodology

For the evaluation of the optimized methodology, a total of 109 milk samples, representing three different types of milk, 51 UHT, 32 Fresh and 26 raw milk, were spiked with different bacterial concentrations and analysed. The results obtained are presented in **Table 6.4**

In line with the observations previously described, a lower LoD was achieved for *E. coli* O157 and *Salmonella* spp. obtaining both a LoD_{95} of 1.6 cfu/ 25 mL, for the combined analysis of the different types of samples (**Table 6.5 and Table 6.6**). No significant differences in the probability of detection were observed between UHT, fresh and raw. However, for *L. monocytogenes* a higher LoD was obtained, with a difference of 1 log compared to that of the Gram-negative bacteria, being the LoD_{95} 79 cfu/ 25 mL (**Table 6.7**). These results were in concordance with those obtained when performing the enrichment optimization. In addition to this, a matrix effect was observed in the detection of this particular pathogen, as the LoD_{95} from UHT, fresh, and then raw milk, was calculated to be 52, 82 and 130 cfu/ 25mL, respectively.

For most cases, the absence of *L. monocytogenes* in 25 g is required by the European regulation, but in some type of foods, not supporting the growth of this pathogens during shelf-life, up to 100 cfu/ g (250 cfu/ 25ng) are allowed. This regulation was established after epidemiological studies performed in different countries, showing that a concentration of *L. monocytogenes* not exceeding 100 cfu/ g when the food is consumed, represented a low risk for illness [252,253].

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Table 6.4. Milk samples analyzed and results obtained by colorimetric LAMP and confirmation by qPCR and plating

Sample	Contamination level				Results								
	Lm	ST	Ec O157	N	*L. monocytogenes*			*Salmonella* spp.			*E. coli* O157		
Milk					LAMP	qPCR	Plate	LAMP	qPCR	Plate	LAMP	qPCR	Plate
UHT	89	124	30	6	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	38	48	24	6	+	+	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	23	49	16	6	+	+	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	34	6	5	6	+(2) -(4)	+(2) -(4)	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	11	18	3	6	-	-	+*	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	7	17	4	6	-	-	+(3)*-(3)	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	39	4	2	6	+	+	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	37	1	1	6	+	+	+	+(3) -(3)	+(2) -(4)	+(2) -(4)	+	+	+
	30	1	1	6	+	+	+	+(3) -(3)	+(5) -(1)	+(5) -(1)	+(5) -(1)	+(5) -(1)	+
	67	-	4	1	+ [a]	+ [a]	+ [a]	- [a]	- [a]	- [a]	+ [a]	+ [a]	+ [a]
	-	-	-	2	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]
Fresh	81	88	12	3	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	61	66	9	3	+	+	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	40	57	11	6	+(5) -(1)	+(5) -(1)	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	20	28	13	6	+(5) -(1)	+(5) -(1)	+	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]	+ [a]
	36	2	1	5	+(2) -(3)	+(4) -(1)	+	+ [a]	+ [a]	+ [a]	+(4) -(1) [a]	+(4) -(1) [a]	+(4) -(1) [a]
	20	1	1	6	+(3) -(3)	+	+	+(3) -(3)	+(4) -(2)	+(4) -(2)	+	+	+
	-	-	-	3	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]
Raw	-	636	195	1	- [a]	- [a]		+ [a]	+ [a]		+ [a]	+ [a]	
	407	636	-	1	+ [a]	+ [a]		+ [a]	+ [a]		- [a]	- [a]	
	407	-	195	1	+ [a]	+ [a]		- [a]	- [a]		+ [a]	+ [a]	
	122	191	59	3	+	+		+ [a]	+ [a]		+ [a]	+ [a]	
	96	1	2	5	+	+		+(4) -(1)	+(4) -(1)		+ [a]	+(4) -(1) [a]	
	61	1	1	6	+(3) -(3)	+		+	+		+(3) -(3)	-	
	36	3	1	6	+(4) -(2)	+		+ [a]	+ [a]		+(3) -(3)	-	
	-	-	-	3	- [a]	- [a]		- [a]	- [a]		- [a]	- [a]	

The contamination level is expressed in cfu/ 25 g of sample. Lm, ST and Ec O157 stand for *L. monocytogenes*, *S.* Typhimurium, *E. coli* O157 respectively;
N: indicates the number of samples;
* only observed between 1-4 colonies;
[a] (in green) samples considered for the evaluation of the fitness for purpose, which were above the LoD_{95}.

Table 6.5. Limit of Detection (LoD) calculated for *E. coli* O157

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	0.2	0.1	0.5	1.1	0.5	2.3
Fresh	0.3	0.1	0.6	1.1	0.5	2.5
Raw	0.6	0.3	1.2	2.6	1.3	5.0
Combined	0.4	0.3	0.6	1.6	1.1	2.4

* cfu/ 25 mL.

Table 6.6. Limit of Detection (LoD) calculated for *Salmonella* spp

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	0.5	0.2	1.2	2.1	0.9	5.0
Fresh	0.5	0.2	1.2	2.1	0.9	5.2
Raw	0.2	0.1	0.6	1.1	0.5	2.4
Combined	0.4	0.2	0.6	1.6	1.0	2.7

* cfu/ 25 mL.

Table 6.7. Limit of Detection (LoD) calculated for *L. monocytogenes*

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
UHT	12.1	7.5	19.5	52.3	32.4	84.2
Fresh	18.9	11.5	31.0	81.5	49.6	133.8
Raw	30.1	16.7	54.2	130.0	72.2	234.1
Combined	18.3	13.6	24.6	79.0	58.7	106.4

* cfu/ 25 mL.

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For the evaluation, samples above the LoD_{95} were consider and results are presented in **Table 6.8** for the different pathogens. Both *L. monocytogenes* and *Salmonella* obtained a relative accuracy, sensitivity and specificity of 100 %, leading to a κ index of 1. Regarding *E. coli* O157, one false positive was observed in a sample with a concentration of 2 cfu/ 25 mL, giving a positive result by LAMP while the qPCR was negative. Due to this deviation, the κ index was lower (94 %) than for the other pathogens and the values for the relative accuracy, sensitivity and specificity were 99, 100, and 90 % respectively.

In raw milk samples the confirmation in selective plates was not possible due to a higher concentration of background flora, which make the identification and isolation of typical colonies impossible (**Figure 6.9**), and for this reason the results were only compared against qPCR analysis.

Table 6.8. Results of the fitness for purpose for each pathogen detected

Target	N	PA	NA	FN	TP	FP	AC	SE	SP	p0	pe	*k*
L. monocytogenes	21	12	9	0	0	0	100	100	100	1.00	0.49	1.00
Salmonella spp.	87	77	10	0	0	0	100	100	100	1.00	0.20	1.00
E. coli O157	87	74	9	0	0	1	99	100	90	0.95	0.20	0.94

N: number of samples, PA: positive agreement, NA: negative agreement, FN: false negative, TP: true positive, FP: false positive, AC: relative accuracy (%), SE: relative sensitivity (%), SP: relative specificity (%), p0: proportion of agreement, pe: expected frequency of agreement, *k*: Cohen's kappa. Samples above the LoD_{95} were consider for these analysis

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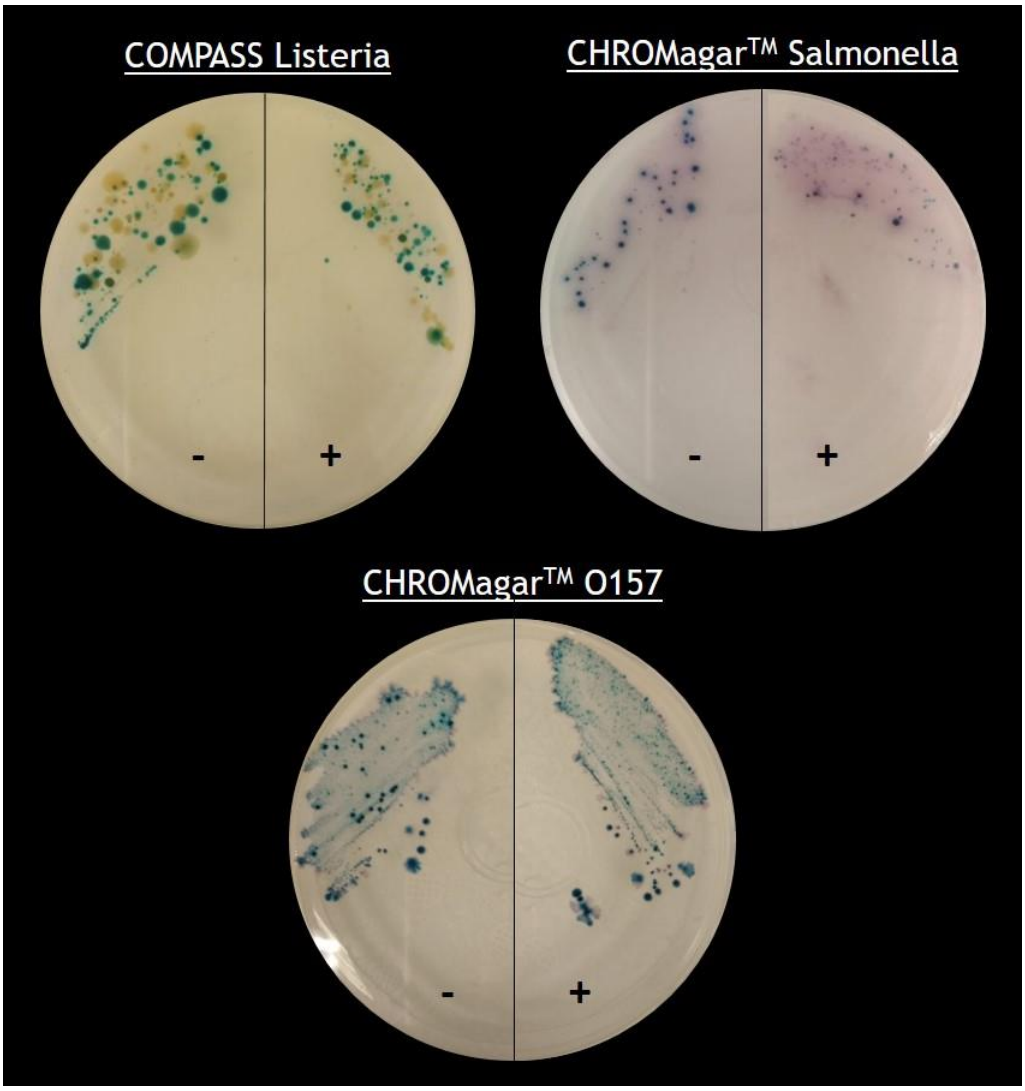

Figure 6.9. Confirmation methodologies for raw milk samples in selective chromogenic Agar. The differentiation between negative (-) and positive (+) is hampered by the natural microflora existing in the sample.

6.2.4 Analysis of mesophilic bacteria

When analysing more complex samples, TSB, as non-selective medium will allow the growth of other natural bacteria present. Al-Zeyara et al. [151] observed that the number of *L. monocytogenes* recovered after enrichment in TSB was inversely related to the concentration of initial aerobic bacteria present, when different type of samples were analysed. The inhibition of the growth of *L. monocytogenes* is influenced not only by the microbial numbers but also by the composition of the microflora. Lactic acid bacteria showed to have a critical role in this inhibition followed by the *Enterobacteriaceae*.

For this reason the mesophilic bacteria concentration and the identification of the species present in each type of sample was evaluated, to understand their influence in the growth of *L. monocytogenes*.

In order to know the concentration of natural contaminant bacteria of the samples tested, the ISO 4833-1:2013 method was followed, showing no presence of colonies on the plates for UHT milk, (< 10 cfu/ mL). For the fresh milk, one of the triplicates showed a colony in the first dilution (10 cfu/ mL), however the rest of replicates remained without colonies (< 10 cfu/ mL). Lastly, in raw milk an average counts of 8.7 x 10^5 cfu/ mL was observed, explaining the higher limit of detection obtained with this type of samples.

Additionally, in order to understand the composition of the microbial community existing in the raw milk samples, a long-read next generation DNA sequencing was performed, for the DNA extracted of a non-spiked sample obtained by the 7 h short enrichment methodology. The analysis was achieved using the MinION device from Oxford Nanopore Technologies and the data analysis was performed with the software EPI2ME, with the workflow "What's in my Pot" (WIMP). This workflow allows to perform whole genome data analysis [254]. The results obtained by this analysis are presented in **Figure 6.10**.

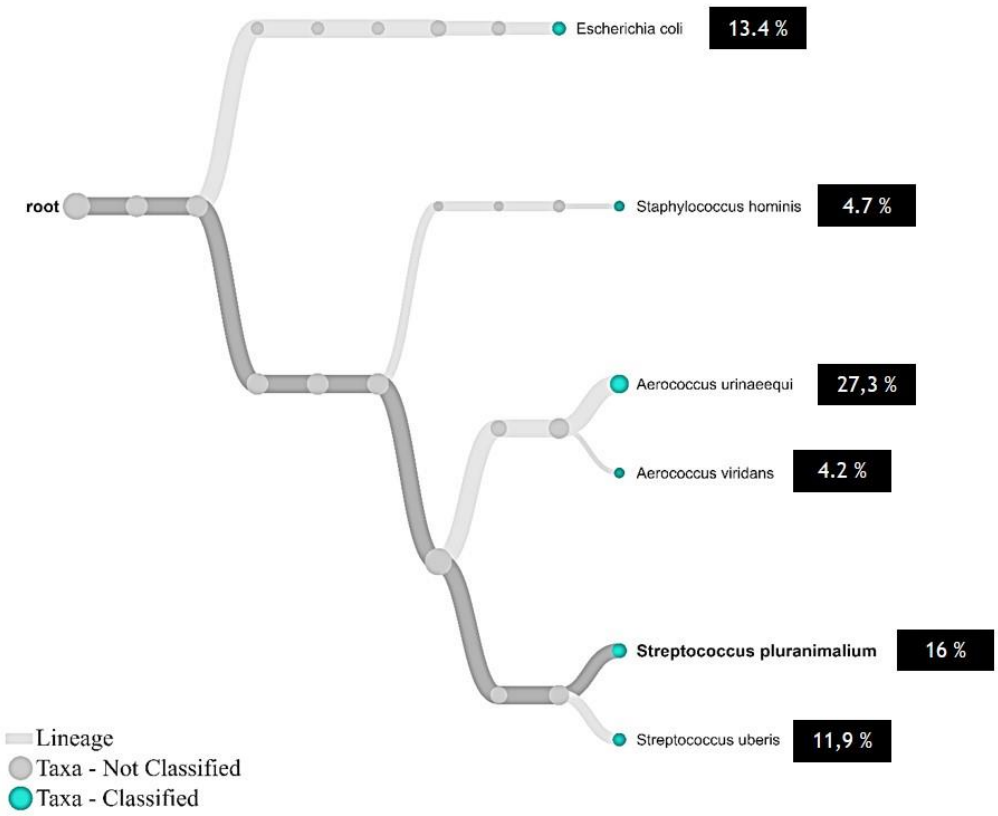

Figure 6.10. MinION sequencing results obtained for a non-spiked sample to identify the mesophilic microorganisms of the raw milk. A threshold of 3% was established, and the percentage of reads obtained for each species were calculated based on the total number of reads classified.

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A total of 519 reads were obtained and analysed, being 337 classified. The highest percentage of reads classified belonged to *Aerococcus urinaeequi* (27.3 %), followed by *Streptococcus pluranimalium* (16 %), *Escherichia coli* (13.4 %), *Streptococcus uberis* (11.9), *Staphylococcus hominis* (4.7%) and *Aerococcus viridans* (4.2%). *Aerococcus* has been reported to be the major bovine faecal enterococci [255,256] explaining its presence in the raw milk. This also applies for *E. coli*. *S. pluranimalium*, which was already related with several diseases in cows, including bovine reproductive diseases [257], valvular endocarditis and septicaemia [258]. Additionally, *S. uberis* is one of the most prevalent pathogens causing mastitis in cows [259], as well as *S. hominis* [260], which explains their presence in raw milk samples.

Lactic acid bacteria (LAB) have been previously reported to inhibit the growth of *L. monocytogenes* [151] which seems in line to what has been observed in this study, since the majority of microorganisms found in raw milk samples were identified as *Aerococcus* and *Streptococcus*, which belong to LAB category. The delay in the growth obtained for *L. monocytogenes* is consistent with this results and explain the increase in the LoD.

6.3 INTEGRATION ON THE AMPLIFICATION PROTOCOLS ON MINIATURIZED DEVICES

After the new methodology optimized and evaluated, two different miniaturized devices were tested to integrate the DNA amplification step. The first device included heating elements and temperature control, as well as a display, enabling to maintain a constant temperature and to monitor the temperature in real-time as described in Chapter 3 section 3.7.3. This device included a flexible holder allowing the use of tubes with different lengths depending on the volumes of reaction. The amplification reactions were performed in the silicon tubing placed in milled channels of the flexible holder. The second approach was a system with microfluidic channels and the amplification reaction was performed in a regular incubator. Both were evaluated for the three target pathogens, comparing both devices in terms of the dynamic range for the detection of the target DNA, and also the determination of the lowest concentration of bacteria needed to have a positive result.

6.3.1 Milled channels prototype

The milled channel prototype enabled to perform the amplification reactions in disposable silicon tubing, being the change of colour clearly visible. **Figure 6.11** shows the results obtained with this approach.

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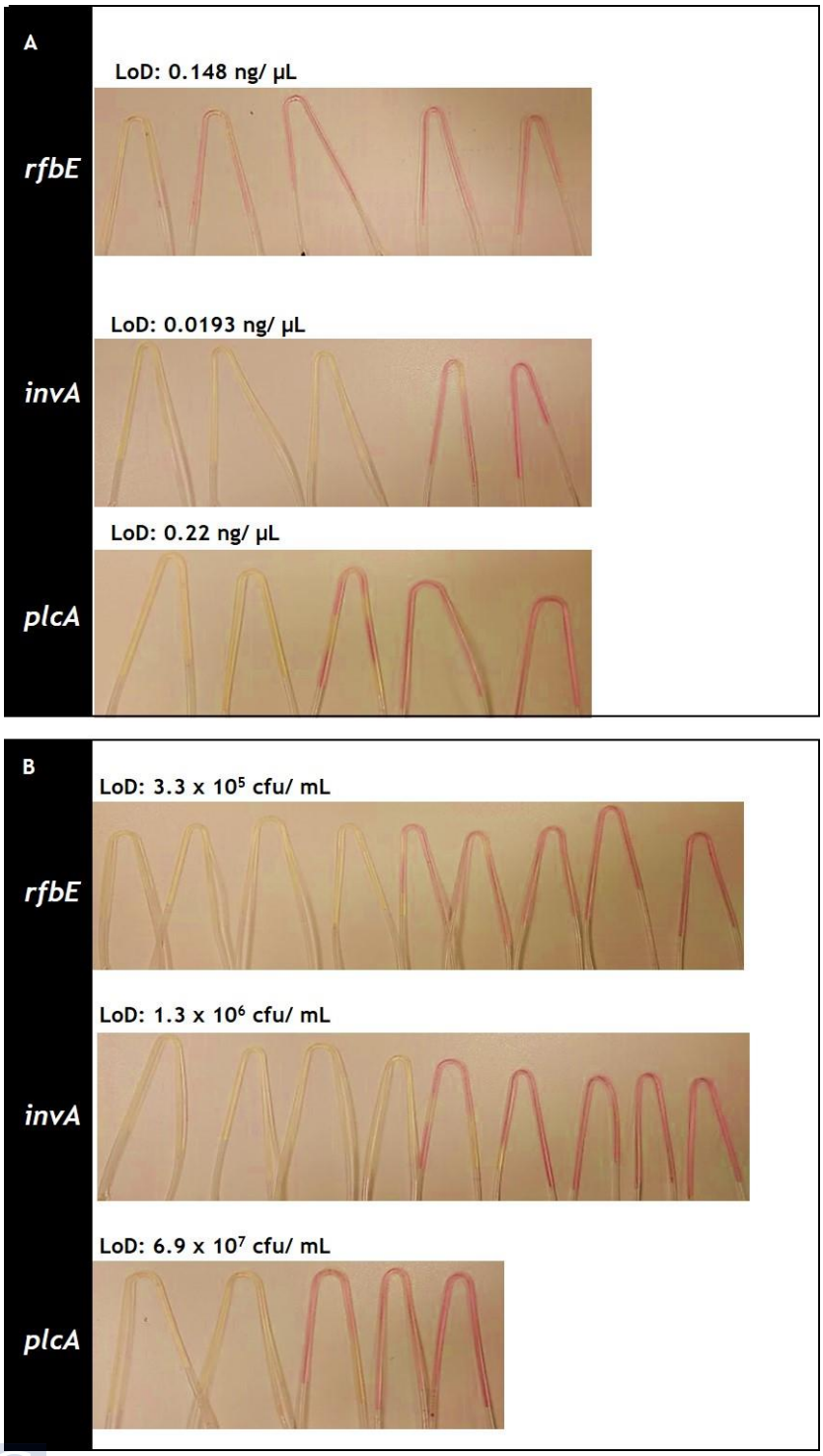

Figure 6.11. Determination of the dynamic range of the colorimetric LAMP reaction integrated in the silicon tubing system. (A) Determination of the lowest DNA concentration detectable, using a ten-fold serially diluted DNA from pure cultures of each pathogen. (B) Determination of the lowest concentration of bacteria detectable, using ten-fold serial dilutions of pure cultures.

When targeting *Salmonella* spp., the same analytical sensitivity for DNA was achieved with the tubing system and with the thermocycler (0.0193 ng/ µL). However, for *E. coli* O157 and *L. monocytogenes*, a decrease of 1 log was observed, being able to only detect and exhibit colour change with a concentration of 0.148 ng/ µL and 0.22 ng/ µL, respectively. The gel electrophoresis in **Figure 6.12 A** performed for *plcA* reaction shows a specific patter until the 0.22 ng/ µL, and a different pattern was visible in the following dilution, which did not produce any visible change of colour.

For the bacterial concentration, different results were observed. For *E. coli* O157 the same concentration was reached with both systems, being the lowest 3.3×10^5 cfu/ mL. Regarding *Salmonella* spp. 1 log additional is needed to be able to have a positive result when the reaction was performed in the miniaturized device, being only able to detect 1.3×10^6 cfu/ mL. One additional log was requires in the analysis of *L. monocytogenes* with an analytical sensitivity of 6.9×10^7 cfu/ mL. Besides the absence of colour change for *L. monocytogenes* in concentration of 6.9×10^6 cfu/ mL, in the gel, a ladder pattern was visible (**Figure 6.12 A**), suggesting that the colorimetric reaction is less sensitive. For *Salmonella*, the amplification product was visible in the gel down to the last dilution detected by colorimetric LAMP and the following dilution presented a different pattern with less intensity (**Figure 6.12 B**).

It was also observed that in the silicon tubing, a two colour pattern, with sections turning yellow and others remaining pink, was visible in some of the dilutions below the last dilution positive, instead of having an intermedium colour as happened when the reaction was performed in tubes (thermocycler). This makes an easier analysis, and most importantly, a better differentiation between positive and negative.

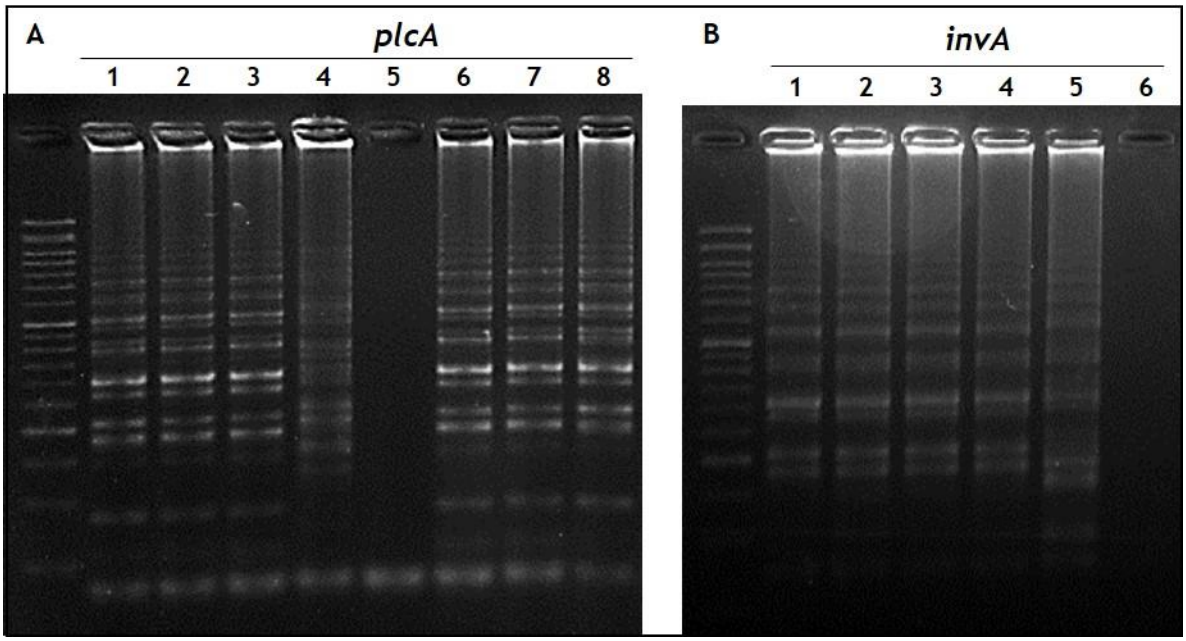

Figure 6.12. Amplification product of the colorimetric LAMP targeting *plcA* (A) and *invA* (B) after performing the reaction in silicon tubing on the milled channels prototype. (A) 1-4 Ten-fold serial dilutions of DNA extracts, being 1 the highest concentration, 22 ng/ µL; 5- Non Template Control (NTC); 6-8 Ten-fold serial dilutions of a pure bacterial culture, being 6 the highest concentration with 6.9×10^8 cfu/ mL. (B) 1-5 Ten-fold serial dilutions of a pure bacterial culture, being 1 the highest concentration of 1.3×10^9: 6-NTC

6.3.2 PDMS channels prototype

A different approach, using a PDMS based prototype with a parallel channel geometry, was also tested to integrate the DNA amplification step. However when the reaction was performed in this device lower sensitivity was observed, compared to the results obtained performing the reaction in the thermocycler, as well as with the previous miniaturized system tested.

Regarding the lowest concentration of DNA detectable, an analytical sensitivity of 1.48 ng/ µL and 1.93 ng/ µL was reached for *E. coli* O157 and *Salmonella* spp., respectively. For *L. monocytogenes* none of the concentration tested originated a change of colour (**Figure 6.13 A**). However, after DNA electrophoresis, the band pattern was visible in these reactions (**Figure 6.14 A**), suggesting an increased difficulty in the change of colour even if amplification is visible in the agarose gel.

When analysing pure bacterial cultures, for both, *E. coli* O157 and *Salmonella* spp., it was only possible to detect 3.3×10^6 cfu/ mL and 1.3×10^7 cfu/ mL of the respective bacteria, when the reaction is performed in the PDMS channels prototype (**Figure 6.13 B**), showing an increase of 1 log compared to the results obtained with the other prototype. This is consistent with the results previously commented analysing pure DNA, where a decreased sensitivity was noticed. For *L. monocytogenes*, a colour change was visible in the first dilution, but never reached the characteristic intense yellow colour of the positive results, as in this case the reactions turned orange. The electrophoresis of this reaction showed the typical banding pattern, which indicates that the amplification happened, however some interference in the colour change must be occurring, as previously observed (**Figure 6.14 A**). For *Salmonella*, one more dilution showed the specific pattern of bands in the agarose gel, although with less intensity that the previous dilutions which changed colour (**Figure 6.13 B**)

To understand if the loss of sensitivity was due to the device itself, or due to the different heating conditions, PCR tubes with the reactions were also placed in the incubator and the amplification was performed at the same time than the PDMS channels prototype. For *E. coli* O157, comparable results were observed. However, for *L. monocytogenes* and *Salmonella* spp., a higher analytical sensitivity was obtained, which may indicate a more efficient reaction in this format. However the presence of more positive results may also be due to the production of false positives, caused by the instability of the temperature in the conventional laboratory incubation, which leads to unprecise amplification results.

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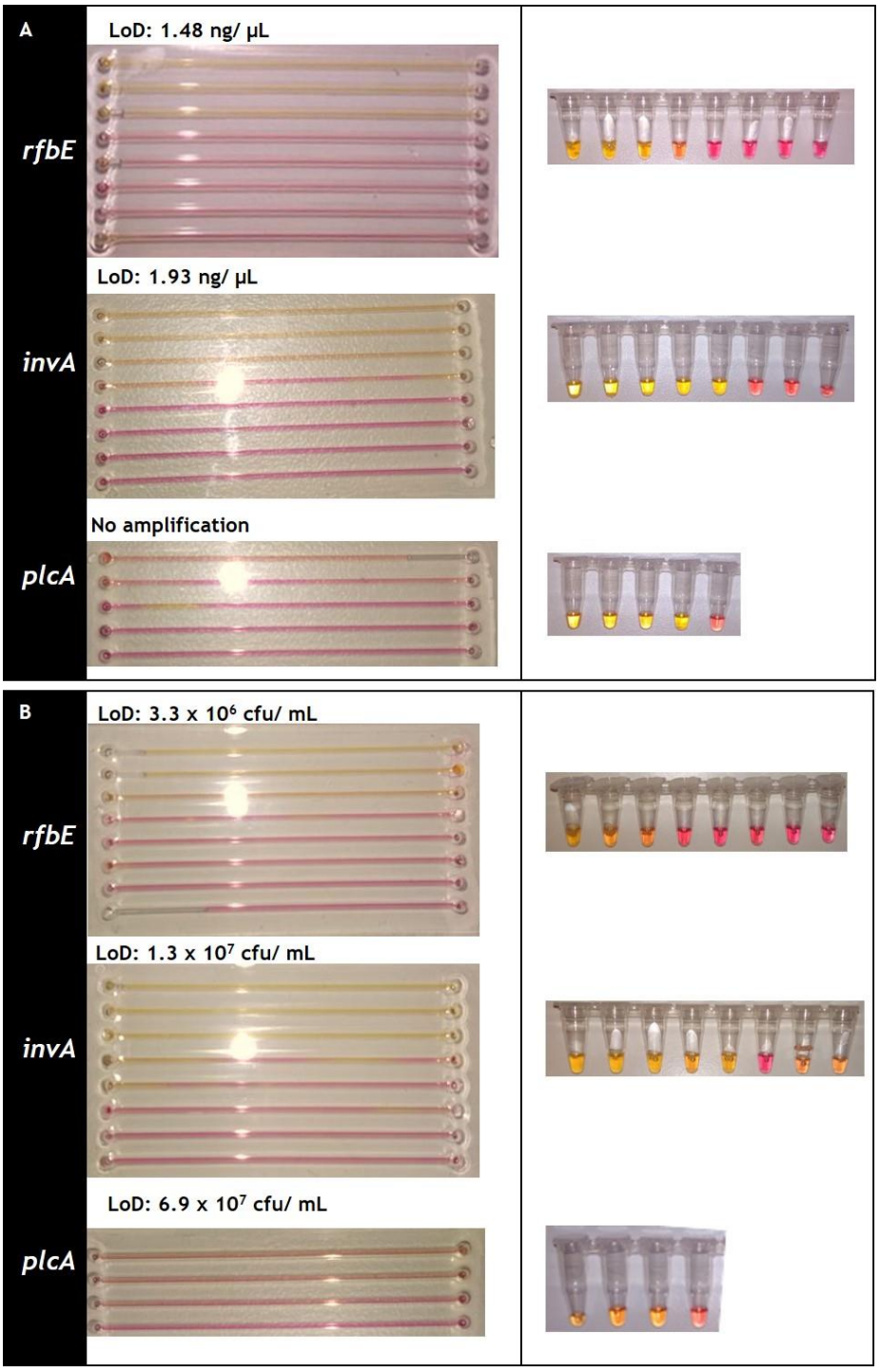

Figure 6.13. Determination of the dynamic range of the colorimetric LAMP reaction integrated in a microfluidic channels system. The device was heated in a conventional laboratory incubator, and the reaction was also performed in 0.2 mL PCR tubes for comparison. (A) Determination of the lowest DNA concentration detectable, using a ten-fold serially diluted DNA extracts obtained from a pure cultures of each pathogen. (B) Determination of the lowest concentration of bacteria detectable, using ten-fold serial dilutions of pure cultures.

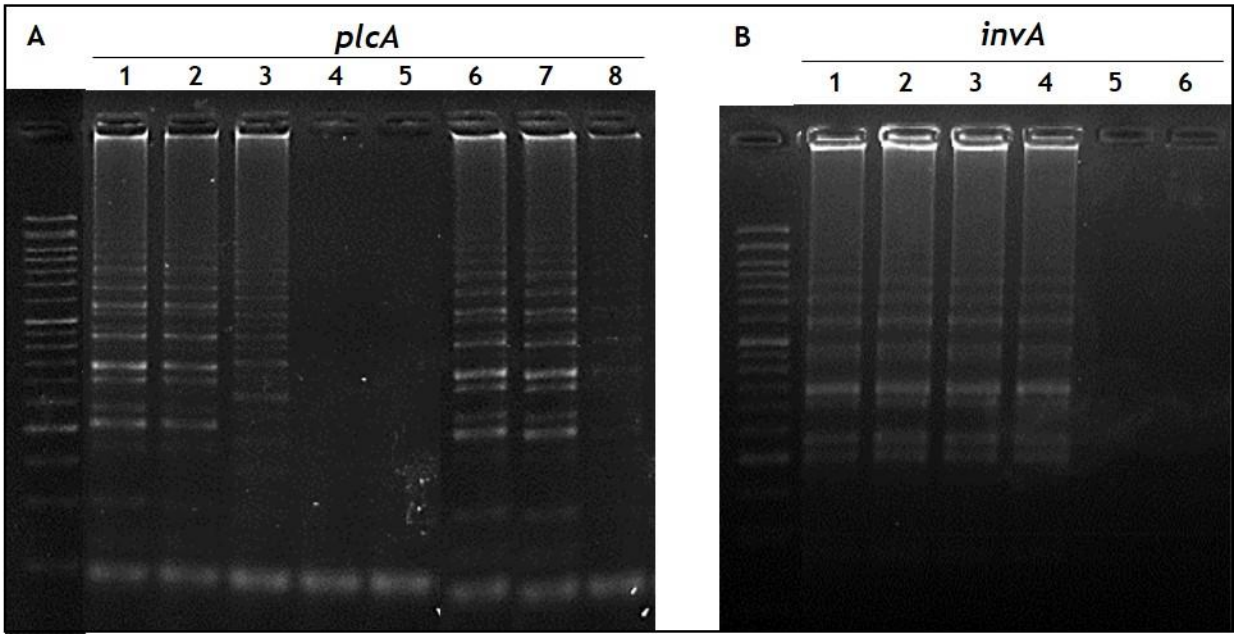

Figure 6.14 Amplification product of colorimetric LAMP targeting *plcA* (A) and *invA* (B) performing the reaction in microfluidic channels. (A) 1-4 Ten-fold serial dilutions of DNA extract, being 1 the highest concentration,22 ng/ µL; 5- Non Template Control (NTC) 6-8 Ten-fold serial dilutions of a pure bacterial culture, being 6 the highest concentration with 6.9 x 10^8 cfu/ mL. (B) 1-5 Ten-fold serial dilutions of a pure bacterial culture, being 1 the higher concentration of 1.3 x 10^9: 6-NTC

6.3.3 Comparison between thermocycler and miniaturized devices

Table 6.9 compares the performance of the different amplification methodologies in terms of sensitivity. For all targeted pathogens the same sensitivity was obtained between qPCR and colorimetric LAMP performed in the thermocycler, when dilutions of pure DNA were tested. The same was observed when the milled channels prototype was used for the detection of *Salmonella* spp. However, for the other two targets, a 1 log decrease of sensitivity, was observed. Regarding the detection of bacterial dilutions, the decrease in sensitivity was more pronounced, as for *L. monocytogenes* 2 log more are needed to have a positive result by the LAMP on thermocycler approach compare to qPCR, and both *E. coli* O157 and *Salmonella* spp. needed 1 log additional. The integration in the milled channels prototype also decrease the sensitivity to 1 log extra for *L. monocytogenes* and *Salmonella* spp., while for *E. coli* O157 the same sensitivity was obtained comparing with performing the amplification on the thermocycler. On the other end, the PDMS channels prototype had a very poor performance compared to all the other methodologies, with the worst sensitivity for the three pathogens.

After the results were evaluated, it was decided to exclude this last miniaturized device, the PDMS channels prototype, from the subsequent sample analyses, and the milled channels system was chosen to couple with the colorimetric LAMP reaction.

Table 6.9. Comparison between qPCR and colorimetric LAMP performed by different methodologies

Bacteria target	Methodology	LoD	
		DNA (ng/ µL)	Bacteria (cfu/ mL)
L. monocytogenes	qPCR	0.022	6.91×10^4
	LAMP in Thermocycler	0.022	6.91×10^6
	LAMP in Milled channels prototype	0.22	6.91×10^7
	LAMP in PDMS channels prototype	-*	-*
E. coli O157	qPCR	0.0148	3.27×10^4
	LAMP in Thermocycler	0.0148	3.27×10^5
	LAMP in Milled channels prototype	0.148	3.27×10^5
	LAMP in PDMS channels prototype	1.48	3.27×10^6
Salmonella spp.	qPCR	0.0193	1.28×10^4
	LAMP in Thermocycler	0.0193	1.28×10^5
	LAMP in Milled channels prototype	0.0193	1.28×10^6
	LAMP in PDMS channels prototype	-*	1.28×10^7

* Not possible to determine the value, as the initial DNA extract (not diluted) gave a negative result.

6.3.4 Evaluation of the method integration on the milled channels prototype

In order to evaluate the colorimetric LAMP integrated in the milled channels prototype, the samples were analysed in parallel with the thermocycler and the LoD determined. A variety of UHT, fresh and raw milk samples, were tested. The results of the PoDLoD analysis are presented in **Table 6.10**. For the three targeted pathogens, an increase in the LoD was observed, which was expected considering the previous findings with pure bacterial cultures. The LoD_{95} obtained for *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 were 141, 15 and 17 cfu/ 25 mL, and LoD_{50} of 33, 3 and 4 cfu/ 25 mL, respectively. All positive samples above the LoD_{95} were successfully identified and the negative samples remained without any change of colour. With this results a κ index of 1 was reached.

The integration on the milled channels prototype decreased the initial sensitivity of the colorimetric LAMP reaction developed. Different factors can influence the performance of the LAMP in miniaturized devices, including the heat transfer, and the size of the channels. The heat transfer is normally more efficient in these devices as the small size of the silicon tubes and microchannels provides a higher surface area of contact with the reaction [261]. However the same characteristic hinders the diffusion of the different components inside the LAMP reaction [262], which is facilitated in PCR tubes due to convective motions of the liquid. This may explain the decrease in the performance of the reaction in both miniaturized systems.

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Table 6.10. Limit of Detection (LoD) obtained on the miniaturized device

Sample type	LoD_{50}*			LoD_{95}*		
	LoD	Lower conf. Limit	Upper conf. Limit	LoD	Lower conf. Limit	Upper conf. Limit
L. monocytogenes	32.5	17.7	59.7	140.6	76.6	258.1
Salmonella spp.	3.4	1.6	7.2	14.7	7.0	31.2
E. coli O157	3.9	2.1	7.0	16.7	9.1	30.4

* cfu/ 25 mL;
LoD determined using UHT, fresh and raw milk samples.

6.4 CONCLUSIONS

The full development of a new methodology (**Figure 6.15**) was accomplished for a multiplex detection of the most problematic foodborne pathogen, *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157. The newly methodology reduce to only 9 h the time of analysis, representing a significant improvement compared with culture-based traditional method, which takes between 5 to 7 days depending on the pathogen to be detected. The protocol involved a multiplex short enrichment of 7 h, followed by a sample treatment to recover the bacteria and remove as much as possible the food debris. The DNA was then extracted by an enzymatic lysis, and after purification, used to perform isothermal amplification by colorimetric LAMP, targeting in a simplex approach *plcA*, *invA* and *rfbE* genes. On this way, a naked-eye analysis was obtained, which simplify the results interpretation.

Thermocycler approach:

- The methodology reached an acceptable LoD for all pathogens and an almost complete concordance with the reference methodologies was achieved, showing a very good performance.
- The detection of *Salmonella* and *E. coli* O157 showed to be more sensitive, with a LoD_{95} of 1.6 cfu/ 25mL
- *L. monocytogenes*, obtained higher LoD, as it needs to be present in a higher concentration to be detected, still being possible to use this methodology for specific foodstuffs.
- The concentration of the natural microbiota present in foods affects the detection of *L. monocytogenes*, observing a decrease in the LoD with high mesophilic counts.

Miniaturized devices:

- A decrease in sensitivity was observed when the colorimetric LAMP reaction was integrated in both miniaturized devices tested. The PDMS channels prototype was the one giving worse results, being not suitable with the purpose of the project.
- The milled channels prototype, due to its reduced size and good performance in terms of DNA amplification, opens the door for the development of a Point-of-Care system, as it allows to perform the amplification on-site, without the need of complex equipment.
- The miniaturize device is a low cost equipment presenting a low energy consumption due to the simplified thermal control when using isothermal amplification techniques.

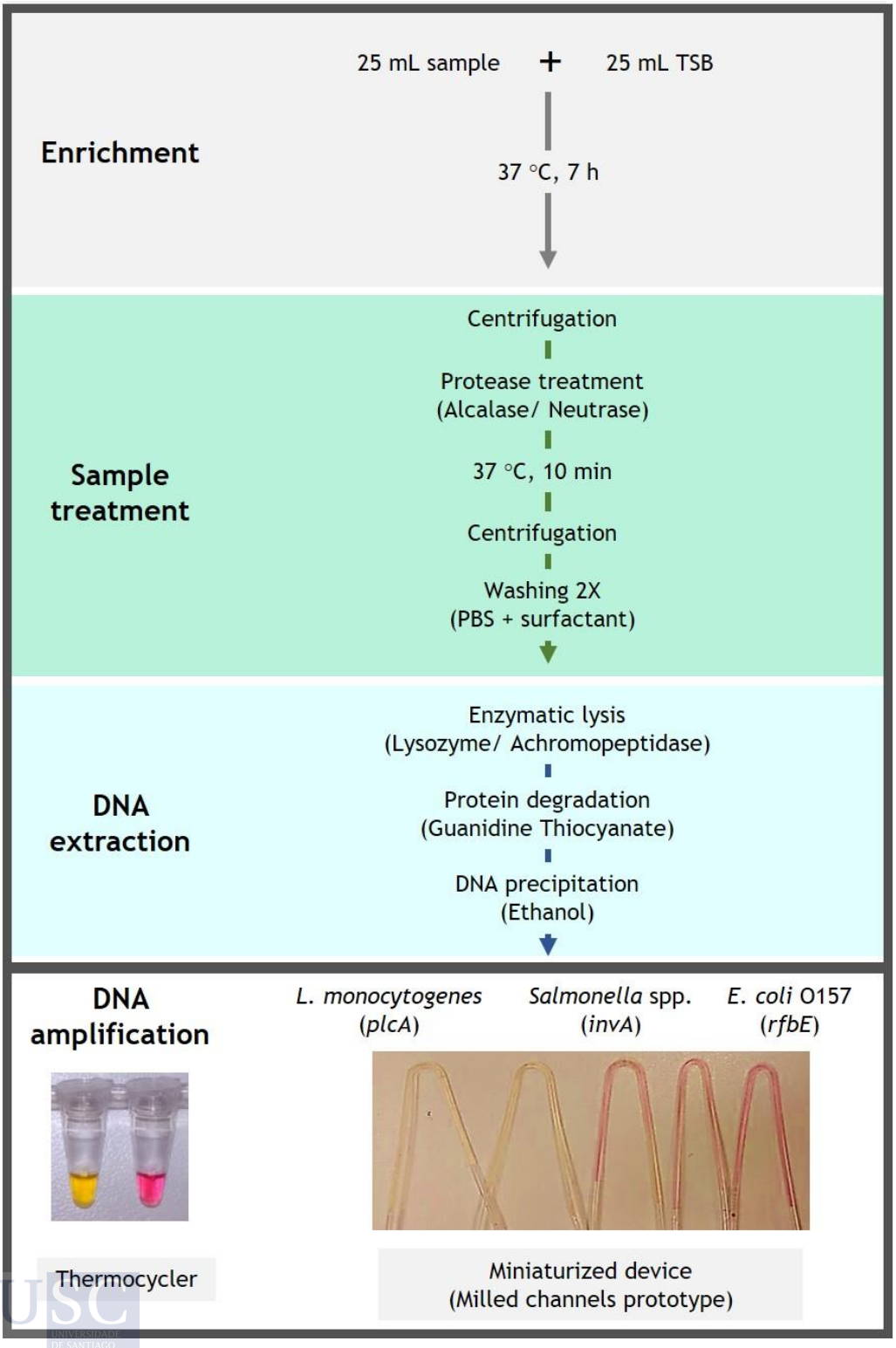

Figure 6.15. Workflow of the newly developed methodology for the detection of *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157 in 9 h.

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CHAPTER 7.

FINAL CONCLUSIONS AND FUTURE WORK

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7 FINAL CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS

7.1.1 Major conclusion

With the work developed in this thesis it was possible to obtain a faster, multiplexed and reliable method for the detection of *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157. The turnaround time is decreased to 9 h, comparing to 7 days with standard methods used nowadays by the food industry and laboratories of control, and besides the difficulties faced in the detection of *L. monocytogenes*, the methodology showed the potential to be applied in the food industry for selected complex food matrixes. The integration of the amplification step in a miniaturize device shows the possibility of automatization of the analysis.

7.1.2 Specific conclusions

- A protocol based on short enrichment methodology has been developed and optimized for the detection of *L. monocytogenes*, *E. coli* O157 and *Salmonella* spp. allowing a multiplex 7h enrichment, which shows a significant reduction on time compared to traditional sample pre-treatment, from 2 days for *Salmonella* spp. and *L. monocytogenes*, and at least 18 h for *E. coli* O157. Additionally, this approach do not increase the cost and allow multiplex analysis.

- Isothermal DNA amplification methods for the specific detection of *L. monocytogenes*, *E. coli* O157 and *Salmonella* spp., namely RPA and LAMP based methods, were developed and evaluated demonstrating a comparable performance with qPCR for the detection of these foodborne pathogens in different type of complex food matrixes and allowing alternative naked eye detection of positive amplification, without the need of complex laboratory equipment.

- The combination of LAMP based methods with colorimetric master mix has demonstrated to provide a clear and unequivocal naked-eye detection of positive amplification.

- The developed protocol consists on a multiplex 7 hours short enrichment in TSB, combined with colorimetric LAMP reaction to detect each targeted pathogen separately providing an ultrafast detection when compared with standard methods, being the total analysis time of 8.5 hours for *E.coli* O157 and *Salmonella* spp., and 9h for *L. monocytogenes* versus 5, 6 and 7 days with standard methods, respectively.

- Two different miniaturized devices were successfully tested, including a PDMS microfluidic channel prototype and a milled channels prototype with embedded silicon tubing system coupled with integrated temperature control, being the second prototype the best in terms of performance.

- A commercial miniaturized nanopore based DNA sequencer was used to characterize the mesophilic microbiota present in food matrixes (raw milk) to evaluate the effect observed in the growth of *L monocytogenes.* The majority of microorganisms present were identified as Lactic acid bacteria, already reported for interfere with the growth of *L. monocytogenes*.

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7.2 FUTURE WORK

To ensure that the new methodology have the possibility to be applied in the food industry, It would be interesting to further test the methods with a larger number of food matrixes, like cheese or other dairy, or meat and fish based RTE products.

The integration of the DNA amplification step in miniaturized devices brings several advantages, such as the possibility for automatization, low energy consumption, and enabling a cost effective alternative to the use of the thermocyclers as in PCR-based methods. The devices tested allowed to perform the reaction, however a decrease in sensitivity was observed, showing some limitations in the analysis and highlighting the need for additional improvements.

Future works need to be developed in order to optimize the colorimetric LAMP reaction in the miniaturized devices so that a higher sensitivity can be obtained. Also the design of the miniaturized device may be modified testing different approaches, such as the use of reservoirs instead of long channels which may help the mixing of reagents during amplification.

Lyophilisation of some of the LAMP reagents inside the miniaturized device can be performed and will surely contribute to the ease of use of the devices, as well as facilitate its integration with DNA extraction and purification module to obtain a Lab-on-chip system. This full automation of the analysis, allows to remove the need of specialized personal and avoid cross-contaminations between samples, as the manipulation steps are reduced.

The developed method allowed a complete analysis of 8 h and 30 min for the detection of *Salmonella* spp. and *E. coli* O157 and 9 h for *L. monocytogenes*, being the hands-on time of 8 h for all targets. Additionally, the result visualization can also be automatized, which gives the possibility to obtain the results remotely, allowing data connectivity and analysis. Different alternatives have already been studied [263], like the use of digital cameras, or sensors to differentiate between positive and negative results and communicate the analysis outcomes. On the other hand, the use of these technologies will increase the price of the analysis.

The development and optimization of a methodology will always have to be oriented to the need of the final users and will depend on the goal established, which can pass by the increase sensitivity, faster analysis, and automatization or cheaper and more accessible protocols. Usually if a more rapid methodology for the detection of pathogens is performed, the sensitivity, or the cost, will be affected. A balance between these improvements have to be reached.

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LIST OF FIGURES

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LIST OF EQUATIONS

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APPENDIX

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APPENDIX

APPENDIX I – PUBLICATIONS

PUBLICATION 1

Azinheiro, S. [1,2], Roumani, F. [1,2], Rodríguez-Lorenzo, L. [1], Carvalho, J. [1,2], Prado, M. [1], & Garrido-Maestu, A. [1] (2022). Combination of Recombinase Polymerase Amplification with SYBR Green I for naked-eye, same-day detection of *Escherichia coli* O157:H7 in ground meat. *Food Control*, *132*, 108494.

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PUBLICATION 2

Azinheiro, S. [1,2], Carvalho, J. [1,2], Fuciños, P. [1], Pastrana, L. [1], Prado, M. [1], & Garrido-Maestu, A. [1] (2022). Short pre-enrichment and modified matrix lysis. A comparative study towards same-day detection of *Listeria monocytogenes*. *LWT*, *154*, 112900.

[1] International Iberian Nanotechnology Laboratory, Braga, Portugal
[2] Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences, University of Santiago de Compostela, Santiago de Compostela, Spain

DOI:	https://doi.org/10.1016/J.LWT.2021.112900
Journal:	LWT - Food Science and Technology
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Impact Factor (2020):	FOOD SCIENCE & TECHNOLOGY – SCIE 4.952 Q1 (29/143)
Contribution of Azinheiro S. (CRediT taxonomy):	Investigation, Writing, Review and Editing
Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 3

Azinheiro, S. [1,2], Carvalho, J. [1,2], Prado, M. [1], & Garrido-Maestu, A. [1] (2020). Application of recombinase polymerase amplification with lateral flow for a naked-eye detection of Listeria monocytogenes on food processing surfaces. Foods, 9(9).

[1] Food Quality and Safety Research Group, International Iberian Nanotechnology Laboratory,Av. Mestre JoséVeiga s/n, 4715-330 Braga, Portugal;
[2] Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences,University of Santiago de Compostela, Campus de Lugo, 27002 Lugo, Spain

DOI:	https://doi.org/10.3390/foods9091249
Journal:	Foods
Year:	2021
Impact Factor (2020):	FOOD SCIENCE & TECHNOLOGY – SCIE 4.350 Q2 (37/143)
Contribution of Azinheiro S. (CRediT taxonomy):	Performed the analysis and wrote the manuscript.
Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 4

Azinheiro, S. [1,2], Carvalho, J. [1,2], Prado, M. [1], & Garrido-Maestu, A. [1] (2020). Multiplex Detection of *Salmonella* spp., *E. coli* O157 and *L. monocytogenes* by qPCR Melt Curve Analysis in Spiked Infant Formula. *Microorganisms*, *8*(9), 1359.

[1] Food Quality and Safety Research Group, International Iberian Nanotechnology Laboratory, Av. Mestre JoséVeiga s/n, 4715-330 Braga, Portugal
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DOI:	https://doi.org/10.3390/microorganisms8091359
Journal:	Microorganisms
Year:	2020
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Contribution of Azinheiro S. (CRediT taxonomy):	Performed the experiments
Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 5

Azinheiro, S. [1], Kant, K. [1], Shahbazi, M. A. [2], Garrido-Maestu, A. [1], Prado, M. [1], & Dieguez, L. [1] (2020). A smart microfluidic platform for rapid multiplexed detection of foodborne pathogens. Food Control, 114, 107242.

[1] Department of Life Sciences, International Iberian Nanotechnology Laboratory (INL), Braga, Portugal
[2] Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Finland

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Journal:	Food Control
Year:	2020
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Contribution of Azinheiro S. (CRediT taxonomy):	Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Data curation, Writing - original draft
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PUBLICATION 6

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Carvalho, J. [1], Espiña, B. [1], & Prado, M. [1] (2020). Evaluation and implementation of commercial antibodies for improved nanoparticle-based immunomagnetic separation and real-time PCR for faster detection of *Listeria monocytogenes*. *Journal of Food Science and Technology*, 1–9.

[1] Department of Life Sciences, Nano4Food – Food Quality and Safety Research Group, International Iberian Nanotechnology Laboratory, Av. Mestre Jose´ Veiga s/n, 4715-330 Braga, Portugal

DOI:	https://doi.org/10.1007/s13197-020-04450-1
Journal:	Journal of Food Science and Technology
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PUBLICATION 7

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Fuciños, P. [1], Carvalho, J. [1], & Prado, M. [1] (2020). Comparative study of multiplex real-time recombinase polymerase amplification and ISO 11290-1 methods for the detection of *Listeria monocytogenes* in dairy products. *Food Microbiology*, *92*, 103570.

[1] International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330, Braga, Portugal

DOI:	https://doi.org/10.1016/j.fm.2020.103570
Journal:	Food Microbiology
Year:	2020
Impact Factor (2020):	MICROBIOLOGY - SCIE 5.516 Q1 (29/136)
Contribution of Azinheiro S. (CRediT taxonomy):	Investigation, Writing, Review and Editing
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PUBLICATION 8

Garrido-Maestu, A. [1], **Azinheiro, S.** [1,2], Roumani, F. [1,2], Carvalho, J. [1,2], & Prado, M. [1] (2020). Application of Short Pre-enrichment, and Double Chemistry Real-Time PCR, Combining Fluorescent Probes and an Intercalating Dye, for Same-Day Detection and Confirmation of *Salmonella* spp. and *Escherichia coli* O157 in Ground Beef and Chicken Samples. *Frontiers in Microbiology*, *11*.

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DOI:	https://doi.org/10.3389/fmicb.2020.591041
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Contribution of Azinheiro S. (CRediT taxonomy):	Conducted the experimental part
Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 9

Garrido-Maestu, A. [1], Fuciños, P. [2], **Azinheiro, S.** [1], Carvalho, C. [3,4], Carvalho, J. [1], & Prado, M. [1] (2019). Specific detection of viable *Salmonella* Enteritidis by phage amplification combined with qPCR (PAA-qPCR) in spiked chicken meat samples. *Food Control*, *99*, 79–83.

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[3] Department of Nanoelectronics Engineering, Nanodevices Research Group, International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330, Braga,
Portugal
[4] Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal

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Journal:	Food Control
Year:	2019
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Contribution of Azinheiro S. (CRediT taxonomy):	Performed the experiments, review, editing
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PUBLICATION 10

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Carvalho, J. [1], Fuciños, P. [2], & Prado, M. [1] (2019). Optimized sample treatment, combined with real-time PCR, for same-day detection of *E. coli* O157 in ground beef and leafy greens. *Food Control*, 106790.

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[2] Department of Life Sciences, Nano4Food Unit, Food Processing Research Group, Av. Mestre José Veiga s/n, 4715-330, Braga, Portugal

DOI:	https://doi.org/10.1016/j.foodcont.2019.106790
Journal:	Food Control
Year:	2019
Impact Factor (2020):	FOOD SCIENCE & TECHNOLOGY - SCIE 5.548 Q1 (19/143)
Contribution of Azinheiro S. (CRediT taxonomy):	Performed the experiments, Writing - review & editing
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PUBLICATION 11

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Carvalho, J. [1], & Prado, M. [1] (2018). Rapid and sensitive detection of viable *Listeria monocytogenes* in food products by a filtration-based protocol and qPCR. *Food Microbiology*, *73*, 254–263.

[1] International Iberian Nanotechnology Laboratory, Av. Mestre Jos_e Veiga s/n, 4715-330, Braga, Portugal

DOI:	https://doi.org/10.1016/j.fm.2018.02.004
Journal:	Food Microbiology
Year:	2018
Impact Factor (2020):	MICROBIOLOGY - SCIE 5.516 Q1 (29/136)
Contribution of Azinheiro S. (CRediT taxonomy):	Performed the experiments, Writing - review & editing
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PUBLICATION 12

Azinheiro, S. [1], Carvalho, J. [1], Prado, M. [1], & Garrido-Maestu, A. [1] (2018). Evaluation of Different Genetic Targets for *Salmonella enterica* Serovar Enteriditis and Typhimurium, Using Loop-Mediated Isothermal AMPlification for Detection in Food Samples. *Frontiers in Sustainable Food Systems*, *2*(February), 1–8.

[1] Department of Life Sciences, Nano4Food – Food Quality and Safety Research Group, International Iberian Nanotechnology Laboratory, Braga, Portugal

DOI:	https://doi.org/10.3389/fsufs.2018.00005
Journal:	Frontiers in Sustainable Food Systems
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Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 13

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Carvalho, J. [1], Abalde-Cela, S. [1], Carbó-Argibay, E. [1], Diéguez, L. [1], Prado, M. [1] (2017). Combination of Microfluidic Loop-Mediated Isothermal Amplification with Gold Nanoparticles for Rapid Detection of *Salmonella* spp. in Food Samples. *Frontiers in Microbiology*, *8*, 2159.

[1] International Iberian Nanotechnology Laboratory, Braga, Portugal

DOI:	https://doi.org/10.3389/fmicb.2017.02159
Journal:	Frontiers in Microbiology
Year:	2017
Impact Factor (2020):	MICROBIOLOGY - SCIE 5.640 Q1 (28/136)
Contribution of Azinheiro S. (CRediT taxonomy):	Performed the DNA amplification experiments and AuNPs evaluation
Journal Authorization for publication in the PhD thesis:	Open Access

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PUBLICATION 14

Garrido-Maestu, A. [1], **Azinheiro, S.** [1], Carvalho, J. [1], Fuciños, P. [1], & Prado, M. [1] (2017). Development and evaluation of Loop-mediated isothermal amplification, and Recombinase Polymerase Amplification methodologies, for the detection of *Listeria monocytogenes* in ready-to-eat food samples. *Food Control*, *86*.

[1] Department of Life Sciences, International Iberian Nanotechnology Laboratory, Av. Mestre Jose´ Veiga s/n, 4715-330 Braga, Portugal

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Journal:	Food Control
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Food poisoning is a global public health concern where several pathogens are
responsible for high number of hospitalization and death. The objective of this thesis
was to develop an improved methodology for the multiplex detection of Salmonella
spp., Shiga Toxin-producing E. coli (STEC) and L. monocytogenes in food, based in
DNA detection, and its integration in a miniaturized device. The different steps of the
analysis, including the sample pre-treatment, DNA amplification and visualization of the
results were addressed in order to reduce the time of analysis, reduce the cost, and
allow naked-eye detection. The methodology developed allowed for the multiplex
detection of the three targets, reducing the time of analysis to