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Summary

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Foodborne diseases represent a serious public health issue. For example in the USA it is estimated that the total economic impact is $50 to $80 billion annually in health care costs, lost productivity, and diminished quality of life (Byrd-Bredbenner et al. 2013). For this reason, food safety authorities around the world have realized the need for a strict regulatory framework, including an exhaustive food testing regime.

In the European Union (EU) the Comission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs has been established for food pathogens including *Listeria monocytogenes*. According to the regulation the manufactures and other food business operators are responsible for the production and delivery of safe food. The follow up will be carried out by self-monitoring methods. Conventional methods are often sensitive, but extremely time-consuming. Depending on the target microorganism, it may take from several days to over two weeks to obtain a fully confirmed positive test result (Velusamy et al. 2010). In present food business this timescale is too long. Because of that Fast Microbe Analysis (FMA) solution was developed in this project.

The target of microbiological part of the study was to shorten the lag phase time in *L. monocytogenes* enrichment procedure and determine the selectivity of growth media combined with IMS. It was clearly seen that it is really difficult to make remarkable improvements in shortening the lag phase time. The selectivity of growth media combined with immunomagnetic separation concluded that, the developed method is applicable in *Listeria* spp. detection, but not specific for *L. monocytogenes* detection.

By combining surface enhanced Raman spectroscopic (SERS) detection with the sample concentration the detection limit of 10^4 CFU/ml was obtained. SERS was based on the hybrid nanoparticle and corrugated substrate configuration, while immunomagnetic bead separation and hydrophobic surfaces were utilized to concentrate samples.

Business research in FMA project included indetification of market opportunities for developed FMA solution, identification of the food safety business ecosystem and the related possible ecosystem business model for the developed solution. Business opportunities for FMA solution in other industries were also analyzed.

Keywords: *Listeria monocytogenes*, nanotechnology, SERS, fast analysis, business ecosystem

Contents

1. Introduction

1.1. Need for microbial analysis

Foodborne diseases represent a serious public health issue. For this reason, food safety authorities around the world have realized the need for a strict regulatory framework, including an exhaustive food testing regime. Food safety has become an important, global issue as a result of environmental pollution, increased consumption of processed food and long transportation distances. The incidence of epidemics related to food pathogens has increased significantly due to the greatly accelerated range and speed of distribution that has resulted from the increasingly global trade network for food products. The WHO estimates that annually more than two billion illnesses and the deaths of more than two million children are caused by unsafe food. To guarantee safe food, sensitive, specific and rapid detection methods are needed to minimize the health risk factors in food production chain.

Foodborne diseases cause enormous economic cost for society and trade. For example in the USA it is estimated that the total economic impact is $50 to $80 billion annually in health care costs, lost productivity, and diminished quality of life (Byrd-Bredbenner et al. 2013). For food trade food pathogen contamination in foods causes direct and indirect financial losses due to sample reinspection, analysis and review of records, which can result in product expiration and product recalls (Norhana et al. 2010).

Different kind of microbes, including zoonotic bacteria, can cause foodborne diseases. Zoonotic bacteria are naturally transmissible directly or indirectly between animals and humans. In humans, they cause infections and diseases called zoonosis whose severity varies from mild to fatal symptoms (EFSA 2013a).

Listeria monocytogenes is a zoonotic bacterium causing listeriosis which is a severe threat to human health. The mortality rate of listeriosis can be high, approximately even 20–30% (Todd & Notermans 2011). In Finland the fatality rate of listeriosis in the case associated with butter was about 40% (Lyytikäinen et al. 2000). In the EU, the fatality rate of listeriosis was 12.7% in 2011, but an increasing trend in incidence of listeriosis can be seen since 2008 (EFSA 2013a). In developing countries, listeriosis is one of the most important causes of death among foodborne diseases (Jemmi & Stephan 2006). A large variety of raw and processed foods contaminated during and/or after processing can be a source of *L. monocytogenes*.

In the European Union (EU) the Comission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs has been established for food pathogens including *L. monocytogenes*. According to the regulation the manufactures and other food business operators are responsible for the production and delivery of safe food. The follow up will be carried out by self-monitoring methods.

Currently, there exist several quantitative (enumeration) or qualitative (detection, testing of presence or absence of pathogens) techniques for the detection of foodborne bacteria such as conventional, immunological and molecular methods.

The conventional methods are based on culturing the microorganisms on (selective) plating media followed by morphological, biochemical, physiological, and/or serological conformation tests. Pre-enrichment and selective enrichment steps are carried out prior to the plating. Classical reference/standard methods are typical conventional methods. They are sensitive, but extremely time-consuming. Depending on the target microorganism, it may take from several days to over two weeks to obtain a fully confirmed positive test result (Velusamy et al. 2010).

Immunological methods can be regarded as rapid methods. The technique is based on antibody-antigen interactions. Enrichment is needed before detection. The sensitivity and specificity of immunological-based methods are determined by the binding strength of an antibody to its antigen. Advantages include rapidity and they are less sensitive to food interference. Addition to detect contam-

inating organisms they are able to detect their toxins as well (Jasson et al. 2010, Velusamy et al. 2010).

In molecular methods, nucleic acids are amplified by polymerase chain reaction (PCR). Advantages of molecular methods over conventional methods include rapidity, sensitivity and selectivity. However, components in complex food matrixes may reduce or even block amplification reactions resulting in the underestimation or producing of false negative results. Enrichment is needed prior to detection. (Rodríguez-Lázaro et al. 2010).

The major challenges in microbial analysis are: the slow microbial analysis due to the length of pre-treatment, the relatively high detection levels and the rather high price of analysis due to time consuming laboratory analysis. Raman spectroscopy is a promising new methodology for bacteria detection, with many advantages including identification of the specific species of the bacteria, rapid detection, multiple simultaneous analyses and being label free.

1.2. Objectives

The current available analytical methods own a detection levels for bacterial concentration around 10^8 CFU/ml (Colony Forming Unit/ml). However, regulatory agencies demand to detect a single bacteria cell in 25g of food sample, which means that 10^4–10^5 CFU/ml levels should be detectable. Such low concentrations require a time consuming pre-enrichment step or novel analytical methods to tackle this challenge.

The FMA project concentrated on development of efficient sample pre-treatment method combined with novel analytical technique. The main technical tasks of the research were:

- Shorten the time from sampling to analysis by efficient concentration and separation methods
- Lower the detection limit of microbes by using improved measurement technology
- Lower the costs of the substrate production to meet the requirements of a consumer produc by developing a low-cost, high-volume, large area production process for nano-structured SERS sensors

The solution for the problem related to the detection limits was Surface Enhanced Raman Scattering (SERS) combined with effective sample pre-treatment methods. SERS is a combination of conventional Raman measurement with the substrate surface that will intensify the Raman scattering by a factor of 106. In SERS the measured molecules, or in this case food contaminants, are placed on a rough or nano-structured metal surface. The SERS method can be used to identify and quantify molecules, viruses and bacteria in very low quantities. The sensitivity of SERS detection is a result of interaction between metal substrates or colloids and the incident light.

The objectives for business research in FMA project were related to identifying the new value adding food safety related service possibilities along the food industry supply chain. Therefore, the business analysis took an ecosystem-level perspective and to study how food safety influences different parts of the food value chain, thus shedding light on how new business model can take into account the value of the entire food safety business ecosystem. Also other possible application industries for developed FMA solution were scrutinized. The objectives were:

- Analysis of the existing food safety market and identification new value adding opportunities
- Identification of the FMA solution business ecosystem
- Identification of the ecosystem business model
- Analysis of market opportunities in other industries

2. Background survey

2.1. Foodborne outbreaks

Different kind of microbes, such as bacteria, viruses and parasites can cause foodborne outbreaks. Zoonotic bacteria are naturally transmissible directly or indirectly between animals and humans. In humans, they cause infections and diseases called zoonosis whose severity varies from mild to fatal symptoms (EFSA 2013a).

In 2010, there were nearly 1.5 million deaths globally caused by diarrhoeal diseases. *Vibrio cholerae*, *salmonella, shigella*, *Escherichia coli* and *campylobacter* caused about 500 000 deaths (Lozano ym. 2012). In the EU, it was reported about 350 000 confirmed human zoonoses cases in 5,648 foodborne outbreaks in 2011 (EFSA 2013a). Campylobacteriosis was the most commonly reported zoonosis with 220,209 confirmed human cases, followed by salmonellosis with 95,548 confirmed human cases, verotoxigenic *E. coli* (VTEC) infections with 9,485 confirmed human cases and yersiniosis with 7,017 human cases. Listeriosis (caused by *L. monocytogenes*) was quite rare reported zoonosis with 1,476 confirmed human cases. However, it is the most severe zoonosis in the EU, since the fatality rate was high 12.7%, while the fatality rate of campylobacteriosis, salmonellosis and VTEC- infections were 0.04% 0.12% and 0.75%, respectively.

Several foodstuffs can be a vehicle for foodborne outbreaks (Figure 1.). In 2011, eggs and egg products were responsible for the majority (21.4 %) of the strong evidence outbreaks.

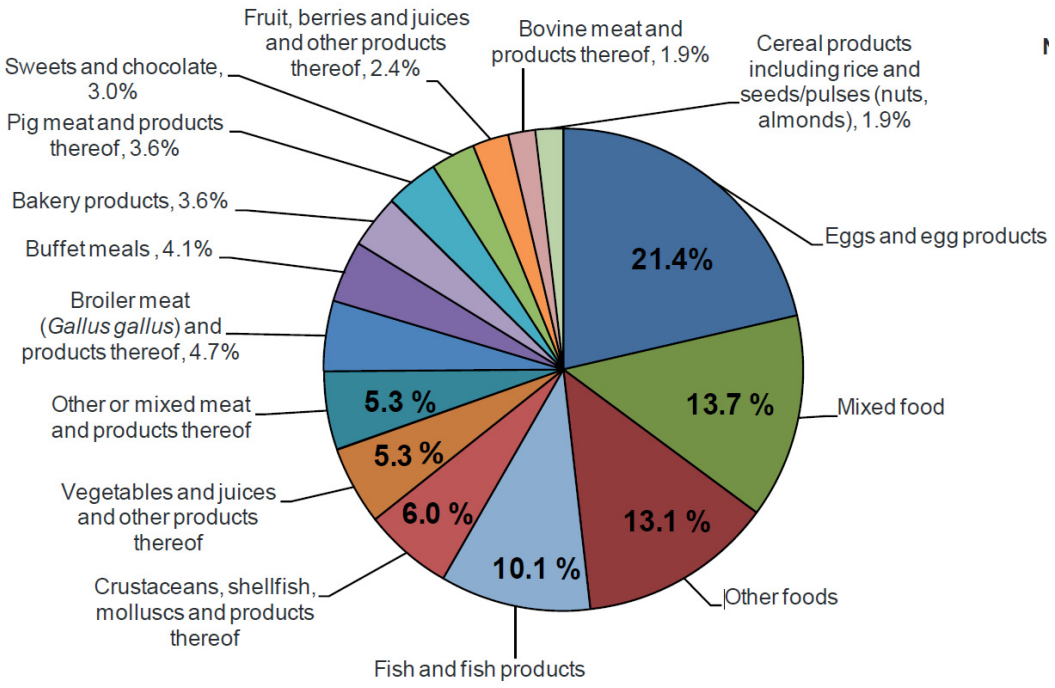


Figure 1. Food vehicles in the strong evidence foodborne outbreaks in the EU in 2011. Data from 701 outbreaks. Other foods include: canned food products, cheese, dairy products (other than cheeses), drinks, herbs and spices, milk, tap water and other foods. (adapted from EFSA 2013a).

Listeria monocytogenes

The genus *Listeria* comprises fifteen species. Two of them are pathogenic to humans and in particular *L. monocytogenes* represents a significant public health threat (Weller et al. 2015). The bacterium is ubiquitous: it has been isolated from soil, vegetation, sewage, water, animal feed, and in the faeces of healthy animals and humans (McLauchlin et al. 2004).

Typical physiological characteristics of *L. monocytogenes* include ability to grow over a temperature range from -0.4 °C to 45 °C (Junttila et al. 1988, Walker et al. 1990), over a pH range from 4.0 to 9.6 (Farber & Peterkin 1991), and both with or without oxygen. It is also able to survive at low water activity (aw) level (Farber et al. 1992), and tolerate high salt concentrations 25.5 % NaCl (Shahamat et al. 1980). Additionally, this bacterium has a capacity for adhering to a variety of food contact surfaces (Silva et al. 2008), and forming persistent strains that can live in food processing facilities even for years and may contaminate foods during processing (Orsi et al. 2008).

A large variety of raw and processed foods contaminated during and/or after processing can be a source of *L. monocytogenes.* The big threat of this pathogen is associated with ready-to-eat (RTE) foods. They are refrigerated products, packaged in vacuum or modified atmosphere having a long shelf life. They are generally consumed with little or no cooking. Foods of animal origin, such as fishery products, heat-treated meat products, and cheese, are associated with *L. monocytogenes* contamination (EFSA 2013b), but also foods from non-animal origin, like coleslaw and cantaloupe, have been vehicles for foodborne *L. monocytogenes* infections (EFSA 2013c).

Listeriosis

Listeriosis is a zoonose caused by *L. monocytogenes*. Especially pregnant women, infants, the elderly, and immunocompromised individuals have an increased risk to get this infection. Among them, listeriosis may cause spontaneous abortion or stillbirth, septicemia, pneumonia or meningitis and serious infections of the nervous system. The mortality rate of listeriosis can be high, approximately 20–30% (Todd & Notermans 2011). In the EU, the fatality rate of listeriosis was 12.7% in 2011 (EFSA 2013a). In developing countries, listeriosis is one of the most important causes of death among foodborne diseases (Jemmi & Stephan 2006). The first well-documented outbreak of foodborne listeriosis was reported in Canada in the 1990's (Schlech et al. 1983), and since then, several foodborne listeriosis outbreaks have been reported mainly from industrialized countries, including from Finland.

In the EU, an increasing trend in the amount of human listeriosis cases can be seen since 2008 (EFSA 2013a). The increased immunocompromised population due to the widespread use of immunosuppressive medications, changed consumer lifestyles such that more RTE and takeaway foods are consumed are considered to be the reasons for this trend. Additionally, changes in food production and technology enable to produce foods with longer shelf-lives. In these products, Listeria risk is relatively high, because the bacteria have time to multiply, and the food is consumed without a listericidal process, such as heating (Allerberger & Wagner 2010).

According to epidemiological studies listeriosis are mainly caused by consumption of contaminated food. The minimal infectious dose is arbitrarily defined to be 10^5 CFUs per gram or millilitre of foodstuff (Allerberger & Wagner 2010). For a healthy human being it is unlikely to get listeriosis when consuming foods containing low levels (<10^2 CFU/g) of *L. monocytogenes* (Chen et al. 2003).

Listeriosis causes enormous economic cost for society and trade. The illness accounts for about 1600 cases with 250 deaths in the USA annually (Scallan et al. 2011). The total economic impact is nearly US$ 2,040,000,000. This consists of health care costs, lost productivity, and diminished quality of life (Byrd-Bredbenner et al. 2013). *L. monocytogenes* contamination in foods causes direct and indirect financial losses for trade due to sample reinspection, analysis and review of records, which can result in product expiration and product recalls (Norhana et al. 2010).

2.2. Legislation addressing *L. monocytogenes* contamination in food

Legislation addressing *L. monocytogenes* contamination in food differs among regions. For example in the USA, in RTE seafood products *L. monocytogenes* must be absent in 25 g of food sample (Jami et al. 2014). In the EU, foods not exceeding the limit of 100 CFU/g are considered safe for healthy people (EC 2005). The microbial criteria for *L. monocytogenes* in RTE food are defined as follows:

- RTE foods intended for infants and RTE foods for special medical purposes: absence in 25 g
- RTE foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes: 100 CFU/g, absence in 25 g (If the food processor cannot demonstrate that this limit is not exceeded during the shelf life, *L. monocytogenes* must be absent.)
- RTE foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes: 100 CFU/g

2.3. Analytical microbial methods for foodborne pathogens

Diverse microbiological quantitative (enumeration) or qualitative (detection, testing of presence or absence of pathogens) analytical methods have been used for many decades for the detection of foodborne bacteria. Based on technology used, the analytical methods can be divided into molecular (nucleic acid-based), immunological and conventional methods.

The conventional methods are based on culturing the microorganisms on (selective) plating media followed by biochemical identification tests. Pre-enrichment and selective enrichment steps are carried out prior to the plating. They are sensitive, reliable in efficiency, and usually inexpensive. Yet, they are extremely time-consuming, often taking several days to get results. Additionally, they are labour intensive (Velusamy et al. 2010).

Classical reference/standard methods are typical conventional methods. Those detection limit (DL) is approximately 1–5 CFU/test portion (Jasson et al. 2010). They comprise a two-step enrichment procedure: a pre-enrichment and a selective enrichment steps. In pre-enrichment step, the sample is suspended in a non- or half selective medium to resuscitate sub-lethally injured cells, and to promote microbial growth. The incubation time (from few hours to overnight) and temperature is dependent on the target microorganisms. In the second enrichment step a selective medium is used to suppress the background flora and to enable the target pathogen to multiply to a detectable level (Dwiwedi & Jaykus 2011, Brehm-Stecher et al. 2009). After enrichment steps the target pathogen is isolated on a selective differential agar medium. The presumptive colonies are confirmed by morphological, biochemical, physiological, and/or serological tests. Depending on the target microorganism, it may take from several days to over two weeks to obtain a fully confirmed positive test result (Velusamy et al. 2010).

In immunological methods, the technique is based on antibody-antigen interactions. Enrichment is needed before detection. The sensitivity and specificity of immunological-based methods are determined by the binding strength of an antibody to its antigen, and may not always be high enough. Sensitivity is lower compared to nucleic acid-based methods. Advantages include that the tests can be automated and are fast, reproducible, and less sensitive to food interference (Jasson et al. 2010, Velusamy et al. 2010). Enzyme-linked immunosorbent assay (ELISA) is widely used immunological methods in food diagnostics.

In molecular methods, nucleic acids are amplified by polymerase chain reaction (PCR). Advantages of molecular methods over conventional methods include rapidity, sensitivity and selectivity. However, components in complex food matrixes may inhibit or even block amplification reactions resulting in the underestimation or producing of false negative results. Enrichment is needed prior to detection. (Rodríguez-Lázaro et al. 2010).

The Comission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs has been established for pathogenic micro-organisms, and their toxins or metabolites in various food commodities. According to the regulation the manufactures and other food business operators are responsible for the production and delivery of safe food. The follow up will be carried out by self-monitoring methods.

The acceptable testing methods are defined in the regulation. Those methods include reference methods, alternative methods and proprietary methods. The reference methods are official ones and standardized by international standardization bodies such as the European Committee for Standardization (Comité Européen de Normalisation (CEN)) or International Organization for Standardization (ISO). The use of alternative or proprietary analytical methods are allowed if they have been shown to provide equivalent results compared to reference methods and they are validated according to internationally accepted protocols by international validation organizations e.g. NordVal (Nordic Committee on Food Analysis), MicroVal (European Validation and Certification Organization) (MicroVal) and AFNOR (French Standardization Organization). (Evira 2009).

Validated methods

The French Standardization Organization AFNOR validated during 2003–2011 altogether 108 microbiological methods food analytical use. These methods were divided technology basis to culture media, immuno-enzymatic methods, immunological tests, molecular hybridization methods and molecular (PCR) methods. Almost 50% of tested methods belonged to culture media, e.g. chromogenic agars. Immuno-enzymatic and immunological methods covered about 30% from the validated methods. Molecular methods have come more common last years, covering about 30% of all the validated methods.

More information about validated methods is available in AFNOR's web page: http://www.afnor-validation.com/afnor-validation-validated-methods/validated-methods.html

Microbial identification by Raman spectroscopy and/or Surfaced enhanced Raman spectroscopy (SERS)

According to literature survey, commercial Raman spectroscopy based microbiological methods are very rare. In the webpages of rapid micro methods (http://rapidmicromethods.com/files/matrix.php) two such methods are presented. Battelle has developed Raman spectroscopy (product name REBS) and rap.ID (product name Bio Particle Explorer BPE) Viable Staining and Imaging LED Raman Spectroscopy methods for identification and enumeration microorganisms. *L. monocytogenes* was not mentioned separately. Detection time is very rapid, only few minutes, but apparently pretreatment is needed, because detection is taking place from cells from colony or liquid medium. Sensitivity is one cell. The workflow for REBS is the following: After sample material is retained on a supported film, the area is examined for microscopic particles using Raman spectroscopy. A spectral signature is provided for each particle, and the spectral signatures are statistically correlated to a library of known microbes. The workflow for Bio Particle Explorer BPE is the following: After the sample material is collected on metal foil and viability staining is performed, automated image analysis using dark field illumination detects viable particle quantity, shape, and size for particles ranging from 0.5 µm and larger. Subsequently, Raman spectroscopy is performed on each viable particle. A spectral signature is provided, and the spectral signatures are statistically correlated to a library of known microbes. Both methods are non-destructive and samples can be used for further analysis.

2.4. Pre-analytical sample preparation: enrichment, separation and concentration

As far as is known, there exist none technology for detection of foodborne pathogens directly from food samples. Therefore, pre-analytical sample preparation meaning enrichment, separation and concentration is necessary part of food microbiology test procedures. It aims to recover intact, viable target bacterial cells for the detection. Sample pre-handling has remarkable effect to the test result.

Before separation and concentration steps the preparation of a sample suspension is required, i.e. the sample have to be suspended in a large volume of liquid, typically diluent or growth medium. The purpose of suspension is to “release” the target pathogen cells from the food sample. Ideally, the suspension is homogeneous containing as little food debris as possible.

Commonly food samples are suspended by stomacher-type paddle blenders or pulsifier-type blenders. In stomacher-type blender, two paddles crush the sample and drive liquid from one side of the bag to the other. In the pulsifier blender type, an oval metal ring surrounding the bag applies a high frequency beating action to it. When combined with shock waves and intense stirring, microbes are transferred into suspension. The benefit of pulsifier-type blender is the smaller amount of food debris than in stomachered suspensions. However, in this study only stomacher type blender was available. (Fung et al. 1998, Wu et al. 2003).

Pre-analytical sample preparation should result in separation and concentration of target cells (sub-lethally injured cells and cells in dormancy state as well) from the food matrices and from food associated background microflora into a detectable level of the chosen detection technology, removal of inhibitory substances (e.g. fat that can interfere with antibody binding, and complex carbohydrates that can inhibit nucleic acid amplification), reduction of sample volume and produce a homogeneous sample. (Dwiwedi & Jaykus 2011, Brehm-Stecher et al. 2009).

Pre-analytical sample preparation in food analytics is challenging due to the complexity of food matrices. Foods consist of many different compounds, such as proteins, fats and oils, sugars and complex polysaccharides in a complex three-dimensional structure. Certain foodstuffs contain high concentration of non-pathogenic microorganisms. Additionally, the concentration of target pathogen cells is very low and they are not evenly distributed in food matrix (Brehm-Stecher et al. 2009).

Generally, there are two pre-analytical sample preparation approaches: non-specific and target-specific ones. Non-specific approaches depend on physical and/or chemical principles. Centrifugation and filtration methods and adsorptive processes including metal hydroxides and ion exchange resins belong to this category (Dwiwedi & Jaykus 2011). Although physical methods like centrifugation and filtration are effective to separate pathogens from food, they normally need to be followed by more refined methods (Brehm-Stecher et al. 2009).

Target-specific pre-analytical sample preparation approaches based on bioaffinity, in which ligands such as antibodies, bacteriophages, nucleic acid aptamers, and lectins recognize and bind to specific cell surface receptor(s), and they pose high selectivity and binding affinity compared to the non-specific approaches. By means with bioaffinity ligands live cells can be captured (Dwiwedi & Jaykus 2011).

Every pre-analytical sample preparation methods have advantages and disadvantages. Hence, combination of non-specific and target-specific approaches is often used to meet the best possible result in each case.

3. Development of pre-analytical sample preparation protocols

3.1. Bacterial strain selection

In this study bacterial strain selection was comprised of two parts: bacterial strain selection for development pre-analytical sample preparation for *L. monocytogenes* and bacterial strain selection for SERS measurements.

Bacterial strain selection for development pre-analytical sample preparation for *L. monocytogenes*

L. monocytogenes is divided into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) of which serotypes 1/2a, 1/2b, 1/2c and 4b cause most cases (95%) of human listeriosis (Cossart 2011, Pontello et al. 2012). From uncommon serotypes, the serotype 3a has caused serious human listeriosis cases in Finland (Lyytikäinen et al. 2000) and recently in Italy (Pontello et al. 2012). Because the new method should be able to detect *L. monocytogenes* despite of the serotype, several different serotypes were selected for testing.

The test methods are normally categorized by sensitivity and specificity. Sensitivity of test method tells how low level of target microbes can be detected. Specificity describes the ability of the test method to detect only the target micro-organism. In order to clarify these properties of the developed method, inclusivity and exclusivity tests were performed. According to the Food Safety and Inspection Service (FSIS 2010) definition "inclusivity measures the ability of a test to detect a wide variety of strains representing the target pathogen. Exclusivity measures the ability of a test to resist interference by cross-reactivity with non-target organisms likely to be found in the tested food."

For inclusivity tests twenty six of *L. monocytogenes* strains belonging to five serotypes (1/2a, 1/2b, 1/2c, 3a and 4b) were selected. For exclusivity test six strains of other species of *Listeria* genus (*Listeria innocua, Listeria ivanovii subs. ivanovii, Listeria seeligeri, Listeria welshimeri, Listeria grayi*) and nine of non-*Listeria* strains belonging to the families *Aerococcaceae, Bacillaceae, Enterococcaceae* and *Staphylococcaceae* were selected. The bacterial strains were obtained from the culture collection of the Finnish Food Safety Authority (Evira), and from the American Type Culture Collection (ATCC). In this report, the strains are intentionally introduced at the specie or at the family levels with certain exceptions.

Bacterial strain selection for SERS measurements

Because there was not a possibility to use pathogenic strains in VTT Oulu, a non-pathogenic *L. innocua* was selected as a model in the SERS measurements due to the facts that it is closely related strain belonging to the same Listeria genus than *L. monocytogenes* (Cossart 2011), and the morphologic structure of *L. innocua* is similar *to L. monocytogenes* and their Raman/SERS-spectra are quite similar (Mendonça et al. 2012).

3.2. Sample pretreatment

As far as is known, there exists none technology for detection of foodborne pathogens directly from food samples. Therefore, enrichment, separation and concentration steps are the necessary prior to subsequent detection. Before the aforementioned steps the sample has to be suspended in liquid, typically diluent or growth medium. The purpose of suspension is to "release" the target pathogen cells from the food sample. Ideally, the suspension is homogeneous containing as little food debris as possible. In this study, a stomacher-type paddle blender was used. Food sample was placed to the blender in a sterile filter bag with growth media. The liquid phase was wrung out of the solids and

filtered. Possible microbes were placed in liquid, which could easily been separated with this technique.

3.3. Development of enrichment procedure

The target enrichment development procedure was to find a media that speed up the growth of *L. monocytogenes* in such a way that the lag phase becomes shorter. The lag phase of the microbial growth cycle is the time period needed for microorganisms to adapt to a new environment before cell begins to multiply. Additionally, an enrichment media was aimed to be applicable in IMS protocols and in SERS-measurements. In this study various non-selective and selective growth media were tested as such or modified and compared to the enrichment medium used in the standard methods.

Growth inducible activity screening for *L. monocytogenes* was performed using an automated incubator and a turbidity reader (Bioscreen C, Oy Growth Curves Ab Ltd, Finland) which is widely used in various applications. It is applicable in microbiological screening tests because it enables the simultaneous testing of 200 samples and monitoring of bacterial growth in real time during the test (Välimaa et al. 2007).

Screening tests: non-selective growth media

Inducible activity of modified non-selective broths for the growth of *L. monocytogenes* was carried out in screening tests. The selected broths are rich media containing no suppressing agents, such as different salts and antibiotics. In the screening tests, Tryptone Soy Broth, (TSB) (Lab M) and Brain Heart Infusion Broth (BHIB) (LAB M) were supplemented with different buffers (V L3, V L6, V M L3, V M L6 V, L L3, L L6, M L L3, M L L6). Bacterial growth was monitored at 37 °C for 24 hours. The turbidity of each well was measured every 15 min. Shorter lag time was not achieved in these screening tests (Figure 2).

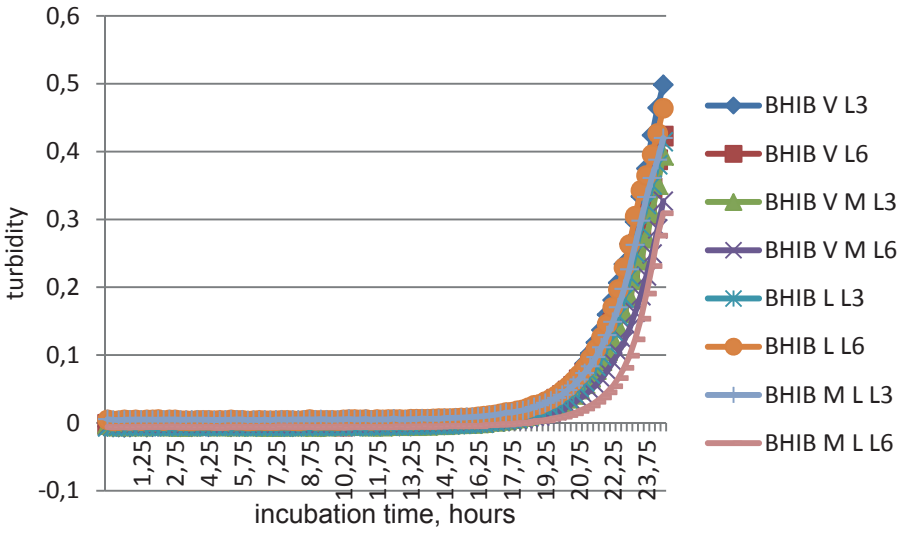


Figure 2. The screening of inducible activity for the growth of *L. monocytogenes* at 37 °C for 24 hours using the BHIB broth supplemented with different buffers

Screening tests: selective growth media

Inducible activity tests for the growth of *L. monocytogenes* were continued with a selective broth. It contains suppressing agents, such as different salts and antibiotics, which role is suppressing the

background flora while increasing the target pathogen concentration. The literature survey revealed that there are various media for enrichment of *L. monocytogenes* and *Listeria* spp. from food matrices. LPT (Listeria Phage Technology) broth (bioMerieux) was selected, because it is used typically in immunoassays, particularly in the enzyme linked fluorescent assay (ELFA) for enrichment in a day (http://www.biomerieux-industry.com/food/vidas-detection-listeria-spp).

The screening tests were performed using LPT broth supplemented with differents buffers. Bacterial growth was monitored at 37 °C for 24 hours. The turbidity of each well was measured every 15 min. In the screening test the results didn't show the shortened lag time using the LPT supplemented with the selected buffers compared to the original LPT (Figure 3).

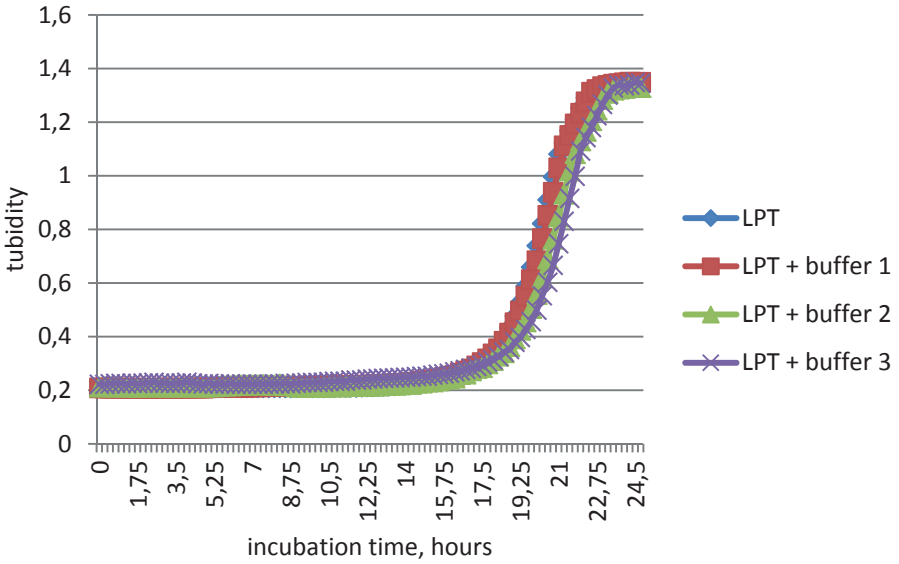


Figure 3. The screening tests of *L. monocytogenes* at 37 °C using the LPT broth supplemented with different buffers

Enrichment procedure for IMS

Immunomagnetic separation (IMS) was selected as preanalytical separation and concentration method. It is based on nanobiotechnology and the combination of immunoassay with SERS technology offered a novel way for lower detection levels.

Because the selected magnetic nanoparticles were covered by antibodies, the broths intented to immunoassays were selected as enrichment broths. Two promising options, LPT broth and LEE broth, were found. According to manufactures' recommendations, Half Fraser broth was used as a reference broth.

LPT broth (bioMerieux) was selected, because it is used in immunoassays for enrichment in a day. The studies demonstrated that LPT broth is applicable in the IMS process (data not shown). Regardless of LPT growth media being a good broth for enrichment of *L. monocytogenes* and *Listeria* spp., it possesses some drawbacks. In routine use it will also be quite costly. For that reason a novel LEE Broth enrichment medium was selected for further studies.

The novel LEE Broth (Listeria Express Enrichment Broth) is a selective enrichment broth for the detection of *Listeria*. According to the manufacturer, the broth enhances the expression of target antigens for most commercially available immunological test kits/methods while suppressing the growth of potential non-target organisms.

Comparison of different enrichment broths for detecting *L. monocytogenes*

The novel enrichment broth (LEE) was compared to the Half Fraser broth (1/2 F) for detecting *L. monocytogenes.* The tests were performed using a *L. monocytogenes* (serotype 1/2a, Evira) strain incubated at 30°C and 37°C for 18 and 24 hours. The results (Figure 4.) indicated that the broths tested are comparable. However, it seems that the novel broth stimulates growth better than Half Fraser broth when incubating at 30°C for 18 hours thus making it a better choice over Half Fraser broth for the rapid microbiological assays.

During food processing (heating, freezing, freeze-drying, drying) or due to various preservative agents (salts, acids, antimicrobial substances), pathogens may be sub-lethally injured and/or entered a dormancy state. Therefore, tests under food processing conditions are required to ensure recovery of these injured cells during enrichment procedures.

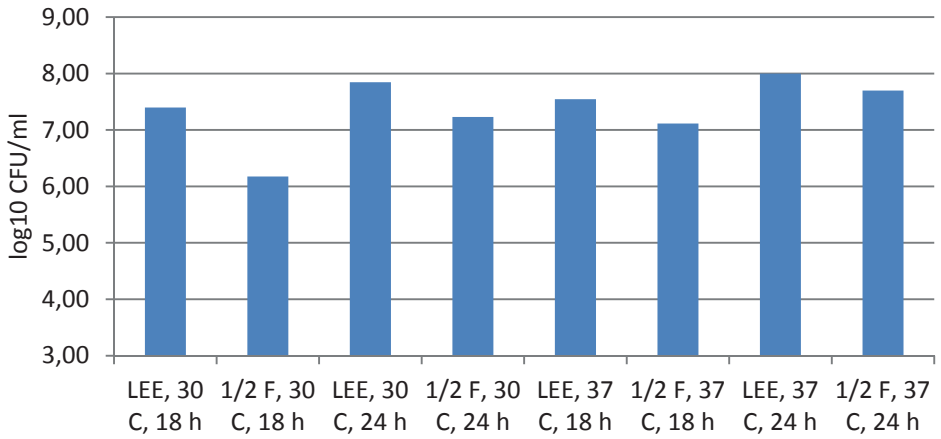


Figure 4. Growth performance of *L. monocytogenes* (serotype 1/2a, Evira) in the novel enrichment broth (LEE) and Half Fraser broth (1/2 F)

Comparison of different enrichment broths for detecting *Listeria species*

If a food sample contains multiple species of *Listeria* genus, *L. monocytogenes* may be overgrown by them, particularly by *L. innocua*, which may lead to false negative results (Gnanou Besse et al. 2010). The novel broth is a selective enrichment broth for the detection of *Listeria*, meaning that beside *L. monocytogenes* it is able to induce the growth of other species of *Listeria* genus as well. In order to clarify the indusible effect of the media on the growth of other Listeria species, the tests using *L. innocua* ATCC 33090 were performed with incubating at 30 °C for 24 hours. According to the results (Figure 5.), the concentrations of the bacteria tested were higher when using the novel broth in incubating at 30°C for 24 hours. Compared to the results of the tests of *L. monocytogenes,* it seems that *L. innocua* ATCC 33090 grows stronger than the tested *L. monocytogenes* strain. It may indicate that in enrichment *L. monocytogenes* may be overgrown by *L. innocua* resulting in false negative results.

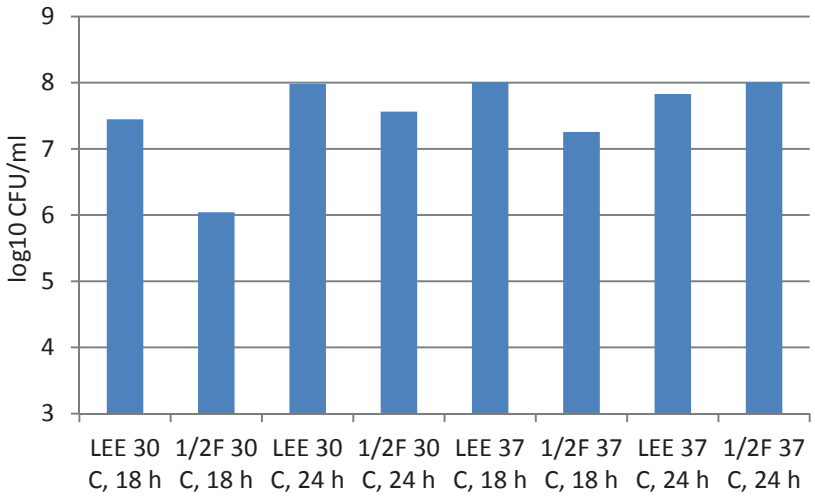


Figure 5. Growth performance of *L. innocua* ATCC 33090 in the novel enrichment broth (LEE) and Half Fraser broth (1/2 F)

Exclusivity tests

The manufacturer notifies that the novel broth suppresses the growth of potential non-target organisms adequately. To test the statement, exclusivity tests were performed using nine of non-*Listeria* strains belonging to the families *Aerococcaceae, Bacillaceae, Enterococcaceae* and *Staphylococcaceae*. Detection was carried out by plating on TSA (Tryptone Soy Agar, Labema). All the tested strain belonging to the families *Aerococcaceae, Enterococcaceae* and *Staphylococcaceae* grew in the novel broth incubated at 30°C for 24 hours (Table 1.). Instead, 50 % of the tested strain belonging to the *Bacillaceae* family grew and 50 % did not grow at the same conditions. These indicate cross-reactivity between *Listeria* and non-*Listeria* species which may lead to false positive results. In any case, serological and biochemical tests are needed to confirm the final test results.

Table 1. Exclusivity tests of the novel enrichment broth using non- *Listeria* strains belonging to the families *Aerococcaceae, Bacillaceae, Enterococcaceae* and *Staphylococcaceae*.

Family	Number of the tested strains	Growth /no growth on TSA
Aerococcaceae	1	Growth
Bacillaceae	4	Growth/no growth
Enterococcaceae	2	Growth
Staphylococcaceae	2	Growth
totally	9	Growth

3.4. Immunomagnetic separation (IMS)

Immunomagnetic separation (IMS) was selected as preanalytical separation and concentration method. IMS -technology is a target-specific pre-analytical sample preparation approach, and it is based on nanobiotechnology. In the IMS, magnetic nanoparticles (MN), typically size of 1–100 nm, are made of compounds of magnetic elements such as iron, nickel and cobalt and can be manipulated using magnetic fields. The large surface-to-volume ratios of MNs allow high capture efficiency. They can be coupled to a biorecognition element specific to the target organism such as antibodies.

In separation and concentration process MNs are mixed with enriched sample. During the incubation the target organism is attached to a biorecognition element on the surface of the MNs. A powerful magnetic field is then used to concentrate and separate the MNs from the matrix. After washing steps MNs coupled with the target organism can be detected typically by culture, PCR and ELISA – methods (Figure 6). Main advantage of IMS methods is rapidity. (Gilmartin & O'Kennedy 2012). Recently, Mendonça et al. (2012) developed highly specific fiber optic immunosensor coupled with IMS for detection of *L. monocytogenes* and *L. ivanovii.* The detection limit of 3 × 10^2 CFU/mL was achieved.

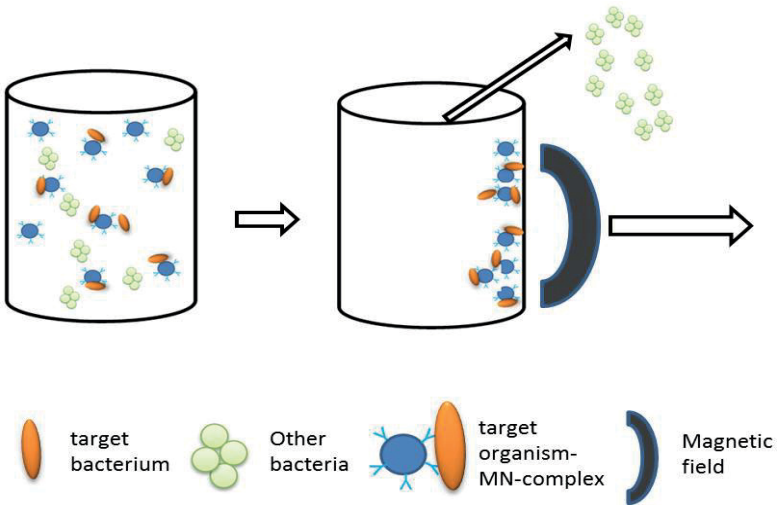


Figure 6. Schematic illustration of immunomagnetic separation.

In this study IMS was performed using Dynabeads® anti-Listeria (Life Technologies Invitrogen), and a Dynal Magnetic Particle Concentrator DynaMag™-2 (Invitrogen Dynal) for both developments with L. monocytogenes and L. innocua.

The workflow was briefly following: The bacterial strains were cultivated in LEE broth. Concentration was analyzed spectrophotometrically and confirmed by plate counting. The cell density was adjusted to the test concentrations. IMS was performed as follows: 1 ml volumes of bacterial culture was added to each of the microcentrifuge tubes containing Dynabeads® anti-Listeria followed by incubation at room temperature for 10 min with continuous mixing. The beads were concentrated by magnetic field (MPC-M) onto the side of the tube, supernatants were carefully aspirated and the samples were washed with the washing buffer. After that the beads were concentrated by magnetic field and the supernatant removed. Finally, the bead–bacteria complexes were resuspended into washing buffer for the subsequent detection by solid culture media Tryptone Soy Agar (TSA) (Labema) or by the SERS technique.

Novel selective growth medium combined to IMS

To clarify the performance of the novel enrichment LEE broth and IMS together, inclusivity and exclusivity tests were carried out.

The new method should be able to detect *L. monocytogenes* despite of the serotype. Inclusivity tests were performed using twenty six of *L. monocytogenes* strains belonging to five serotypes (1/2a, 1/2b, 1/2c, 3a and 4b), and all the tested strains gave positive results in the LEE-IMS tests (Table 1.). Since serotypes 1/2a, 1/2b, 1/2c and 4b cause most cases (95%) of human listeriosis (Cossart 2011, Pontello et al. 2012), this may indicate 100% sensitivity for *L. monocytogenes* detection.

Table 2. Inclusivity tests of the novel enrichment broth combined to IMS using of *L. monocytogenes* strains belonging to five serotypes (1/2a, 1/2b, 1/2c, 3a and 4b).

L. monocytogenes serotype	Number of the tested strains	IMS-result positive/negative
1/2a	8	positive
1/2b	4	positive
1/2c	6	positive
3a	2	positive
4b	6	positive
totally	26	all positive

Exclusivity tests were performed using six strains of *Listeria* genus *L. innocua, L. ivanovii* subs. *ivanovii, L. seeligeri, L. welshimeri* and *L. grayi* (*Listeria* spp. exclusivity tests) and nine of non-Listeria strains belonging to the families *Aerococcaceae, Bacillaceae, Enterococcaceae* and *Staphylococcaceae* (non-*Listeria* exclusivity tests).

In *Listeria* spp. exclusivity tests all the tested strains gave positive results in the LEE-IMS tests. This means that combination of LEE Broth and IMS is not capable in distinguishing *L. monocytogenes* from the other strains of *Listeria* genus. Therefore, in order to confirm the final results, serological and biochemical tests are needed. Accordingly, the developed LEE-IMS method is not specific for *L. monocytogenes* detection.

The results obtained from non-*Listeria* exclusivity tests were controversial: the tested strains belonging to the *Bacillaceae* family gave both positive and negative results depending on the strain used, whereas the tested strains belonging to the families *Aerococcaceae, Enterococcaceae* and *Staphylococcaceae* gave positive results in the LEE-IMS tests. These indicate cross-reactivity between *Listeria* and non-*Listeria* species which may result in false positive results. In any case, serological and biochemical tests are needed to confirm the final test results. To conclude, the developed LEE-IMS method seems to be applicable in *Listeria* spp. detection.

3.5. Reference tests

According to the Comission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, the use of alternative or proprietary analytical methods are allowed if they have been shown to provide equivalent results compared to reference methods. In order to get a first comparison, the reference tests were carried out for the method developed in this project.

Vacuum packed smoked rainbow trout, obtained from a local retail market, was homogenized (BagMixer). The amount needed was divided into portions of 25 g aseptically into sterile filter stomacher bags, and frozen for further analysis. Prior to inoculation the absence of *L. monocytogenes* in the fish sample was confirmed by the official immuno-enzymatic reference method at the reference laboratory.

Defrost samples were inoculated with *L. monocytogenes* strains comprising of three different serotypes (1 /2a, 1 /2c, 4b). Two inoculum levels were used: a lower inoculum level of 7–15 cfu/25 g and a higher inoculum level of 70–150 cfu/25 g. Uninoculated samples were used as negative controls. All samples were carried out as a triplicate. To simulate the natural contamination conditions and to stress the bacteria, the samples were kept under refrigeration for 24 h before testing.

The measurements were carried out at the same time by Luke and the reference laboratory (Oulun kaupungin elintarvike- ja ympäristölaboratorio). The reference laboratory carried out the tests using official reference methods *L. monocytogenes*/25 g Vidas LMO2 (immuno-enzymatic method), *L. monocytogenes*/25 g ISO 11290-1:1996/amd.1:2004 (culture method) and ISO 11290-

1:1996/ amd.1:2004 biochemical and serological confirmations. For comparison, Luke used the new developed Lee Broth-IMS separation and concentration method (FMA).

The results obtained from the tests with the new FMA method compared to the immuno-enzymatic and culture based reference methods were similar (Table 3.) All uninoculated samples were detected as negative and all inoculated (with three different strains) samples with a lower inoculum level and with a higher inoculum were detected as positive by all methods. These tests indicate that novel Fast Microbial Analysis (FMA) method performs comparable results compared to the official reference methods.

Table 3. FMA method compared to the official reference methods.

	Tests in Luke	Tests in the reference laboratory	
Test methods	New FMA method	Immuno-enzymatic method	Culture method
Uninoculated fish samples (negative control)	Negative	Negative	Negative
Inoculated fish samples	Positive	Positive	Positive

4. Development of microbial analysis with SERS

The development of microbial analysis with SERS covered the fabrication of sensor substrates and evaluation of their performance. As small molecules are less complex over the microbial analytes in terms of the formation of Raman spectra and sample handling, small molecules were used to make assessment of the sensor substrate performance, to study surface interactions and evaluate the influence of setting in the. This part of the work is described in Paragraph 4.1 while the usage of sensor surface in microbial analysis is discussed in 4.2.

4.1. SERS substrate development

Polymer materials are particularly attractive in optical sensing because of their ability to be processed rapidly and cost-effectively with high yields. Polymers attain a large number of good optical properties, including high optical transmittance, versatile processability at relatively low temperatures, and the potential for low-cost mass-production. UV lithography has been widely used in the fabrication of conventional optical devices. The resolution obtained with this technique is limited by the effects of wave diffraction and scattering. Compared with conventional techniques, UV-imprint lithography is easy to perform, requires low-cost equipment, and can provide high-resolution nanoscale features down to sub-100 nm (Chou et al. 1995). UV-imprint lithography is performed by pressing a mould onto a UV-sensitive precursor resin (UV-curable acrylate or hybyrid organic-inorganic Ormocor polymer) coating on a substrate, and by curing under UV light; a replica of the mould is formed. This process is illustrated in Figure 7. The process takes place at room temperature and does not require high pressure during the imprinting process. As SERS is based on the plasmon oscillation occurring in metals, the structure is subsequently coated with a thin layer of metal with a thickness of about 100-300 nm. Most widely used metals are silver and gold. In this study, gold was used as plasmon active material as it does not show degradation due to oxidation allowing more stable sensor operation.

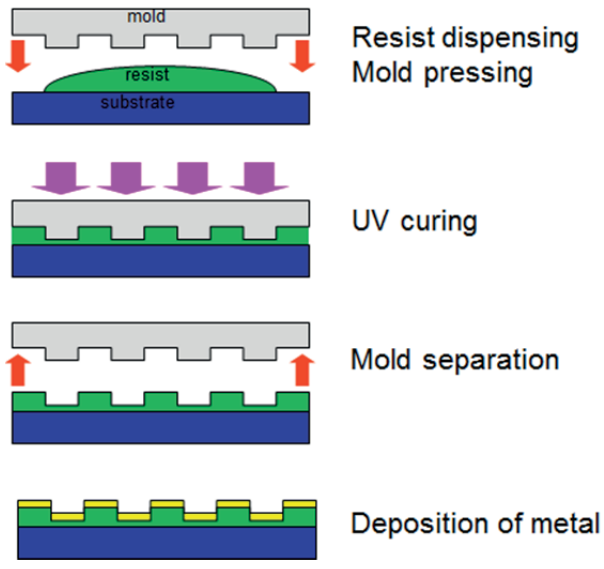


Figure 7. Illustration of UV-imprint method to fabricate SERS-sensor structures.

According to the previous study with a benzyl mercaptan ($C_6H_5CH_2SH$) test molecule on top of the UV-imprint produced SERS structure without fluidics integration, the used SERS surface can provide up to 10^7 a enhancement factor with good reproducibility (5%) (Oo et al. 2013). Therefore, in this work, the studies were initiated, prior the investigation of microbial analytes, by investigating the SERS-sensor operation with small molecules.

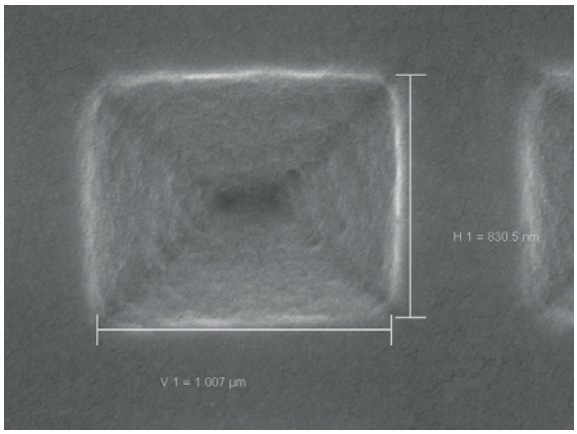
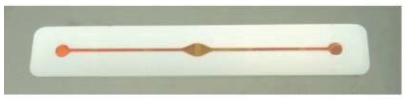


Figure 8. Close-up picture of the pyramid-shaped well of the UV-imprint fabricated SERS surface.

The optical capabilities of the sensor were studied with Rhodamine 6G (R6G) model analyte. The purpose was to confirm the signal enhancement and to study the dynamic signal behaviour as analyte molecules accumulate on the sensor surface. A SEM image of the UV-imprint patterned SERS surface and a close-up picture of one pyramid-shaped well are shown in Figure 8.

Sensor configuration in small molecule studies

The picture of the SERS-sensor configuration used to study small molecule adsorption and dynamic signal behaviour can be seen in Figure 9. The microfluidic circuits were cut from double-sided adhesive. The detection chamber of the fluidic circuit had an oval shape for optimal liquid filling with a steady fluid front, and the chamber sample volume was 2 µl. The chamber dimensions were 220 µm height and 2.67 mm maximal width. The channels leading into the chamber were 400 µm wide. To minimise the effect of the chamber lid on the Raman signal, the microfluidic circuit was lidded with a polyolefin diagnostic adhesive (3M 9795R), which declares high optical clarity and minimal autofluorescence.



1. Polyolefin adhesive lid

2. Cut-out adhesive microfluidic layer

3. Polymer SERS platform with evaporated gold coating

Figure 9. Optofluidic SERS chip with an oval detection chamber: Lid layer polyolefin adhesive patterned with a cutting plotter, middle layer 3M adhesive patterned with the cutting plotter, and bottom layer patterned SERS substrate and metal on surface.

Basic theory analyte transport

To understand the effect of flow dynamics on the detected optical signal, the phenomena behind the transport of the sample molecules from the bulk flow into the detection surface must be considered. With optical detection surfaces, such as in SPR and planar SERS, the flow in a microchannel has a strong influence on the recorded signal. The bulk flow in the microfluidic channels is often produced by pump-inflicted pressure. The pressure-driven flow carries the sample molecules into the detection area, where the induced signal can be observed. In SERS, the sample molecules arriving on top of the detection area need to be in the near vicinity of the plasmonic surface to be detected. Typically, the induced signal originates from the sample molecules adsorbed onto the detection surface. The fluid has the highest velocity in the middle of the channel, and the velocity reduces as the observation point moves nearer to the walls. Typically the fluid velocity vanishes completely at a distance of one molecule layer from the wall. This is called the non-slip condition, in which the molecules adjacent to the channel wall do not move with the flow due to the friction between the wall and the molecules. Since the flow velocity diminishes near the walls, the transport of the sample molecules inside this region by convection is negligible. Figure 10. depicts a situation where the flow velocity of the fluid is at maximum in the middle of the channel, and the convective flow vanishes near the walls.

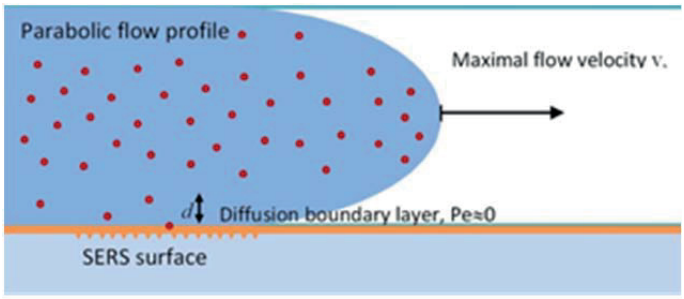


Figure 10. A schematic of the relation between convective and diffusive flow in a microchannel used to study dynamic Raman signal generation with small molecules.

The zone near the wall, where diffusion is the dominant transport mechanism, is called the diffusion boundary layer. There are several studies on the effect of the diffusion boundary layer on the detection mechanism of the biosensor analysed using empirical and computational methods. The phenomena affecting the results include the binding reaction of the analytes to the sensor surface (association and dissociation rate constants) and the relation of convection and diffusion in the diffusion boundary layer. The optical signal response depends on the flow dynamics through the limitations of mass transport of molecules and kinetic binding reactions. In mass transport limited flow, the transport of analytes to the sensor surface is so slow that the signal rise times are growing by the lack of analyte molecules in the vicinity of the surface. This phenomenon includes the effects of insufficient molecule transport to the diffusion boundary layer by convective flow and the effect of the diffusion boundary layer. While insufficient molecule transport can cause analyte depletion near the sensor surface, the effect of the diffusion boundary layer in non-slip conditions makes the signal rise times longer due to slow molecule diffusion. As the bulk flow velocity increases, the effect of transport limitation decreases. This is due to the disappearance of the depletion effect caused by the slow convective flow. By using high enough flow rates, the concentration of the analyte at the surface can be the same as that in the bulk, and the measured signal reflects binding kinetics. However, with too high flow rates, the signal response can encounter a new limitation due to the reaction kinetics of the analyte binding. This kinetic limitation occurs when the binding rates are slow and bulk flow velocity is high. The analytes are transported over the detection zone so rapidly that very few of them have enough time to bind to the surface. When maximal surface coverage of analytes is de-

sired, the used flow rate is often a compromise between the efficient transport of analyte molecules to the surface and the suitable flow velocity for adsorption.

Measurement set-up

Rhodamine 6G (dye content ~95%, SigmaAldrich) solution diluted in di-ionised water was used as a model analyte to analyse the functioning of the polymer-based SERS chip. The optical properties of the chip were studied by filling the chip with the R6G samples and DI H2O serving as a reference medium for the R6G in water solutions. Water is the preferred medium to be used in Raman spectroscopy as a basis for the sample solutions and as a reference, because it does not produce Raman peaks itself. In flow trials, the chip was filled with under-pressure suction produced by a syringe pump (Nexus 3000). The sample was injected into a 2 ml Eppendorf tube, from where it was transferred through the chip and Dolomite flow meter into a syringe. A schematic of the flow system is presented in Figure 11.

Figure 11. The set-up for the flow studies with sample vial, chip holder, chip, Raman microscope, flow meter, and syringe pump.

The surface-enhanced Raman spectra were recorded using a BaySpec Nomadic Raman microscope with a 785 nm excitation wavelength. The power of the laser was set at 40 mW and a 20X magnifying objective was used in the experiments. Integration times were varied between 15 s and 30 s depending on the used R6G concentration. The BaySpec camera was used to focus the system by adjusting a sharp edge between the patterned SERS area and the smooth gold area through the polyolefin lid of the chamber before each Raman spectrum acquisition. In a continuous flow study, the fluid flow velocity was varied from 25 µl/min to 1000 µl/min. To separate the effect of the convective flow of molecules and the mass transport of molecules on the detection surface, we measured the flow of the bulk liquid using fluorescence microscopy and the arrival of the molecules to the detection surface with SERS. To our knowledge, this is a novel method for analysing the dynamic behaviour of an optofluidic chip. 0.5 mM R6G was used as the model analyte. R6G fluoresces around the 570 nm wavelength. Water was first flowed by a syringe pump induced under pressure into the detection chamber before filling the system with dilute 0.5 mM R6G in DI H_2O. The actual flow velocities were observed during the trials with a Dolomite Mitos flow sensor. The flow was recorded as avi-files using a Zeiss fluorescence microscope camera time lapse mode with a 10 ms exposure time and 1 s interval. The same flow trial was executed for SERS detection under the BaySpec Nomadic Raman microscope and the surface-enhanced Raman spectra were recorded with a 15-second integration time and 1 s interval.

Measurements, results, and discussion

With a novel polymer-based SERS sensor, we have to first validate the function of the sensor. To see if the recorded signal is surface enhanced, we began the validation by comparing the SERS signals of the R6G sample on top of the patterned SERS structure and the smooth gold coating. The used integration time for the SERS signal recording was 15 seconds. The chip was filled with DI water to gain the reference Raman spectrum caused by the polyolefin lid. Water was replaced by a 1 mM R6G

sample and the Raman spectra were detected with 2*3 point image mapping on top of the patterned SERS detection area and the smooth gold area without patterning. The Raman spectra with subtracted background spectrum can be seen in Figure 12. a and b with a linear and logarithmic y-axis scale. Since the distinctive peaks for R6G are found in the Raman shift area of 1100 1/cm to 1800 1/cm, this range has been used in the spectrum analysis. The results showed high R6G peaks for the patterned SERS area in comparison to the smooth gold. The peak height difference is more than 30 folds. The result can be compared to the result of (Liu et al 2005) with low intensity R6G peaks for smooth a Ag/PDMS structure. As Liu states, the metal coating alone can enhance the Raman signal, although with less intensity. The results suggest that the detected signal could be SERS originated.

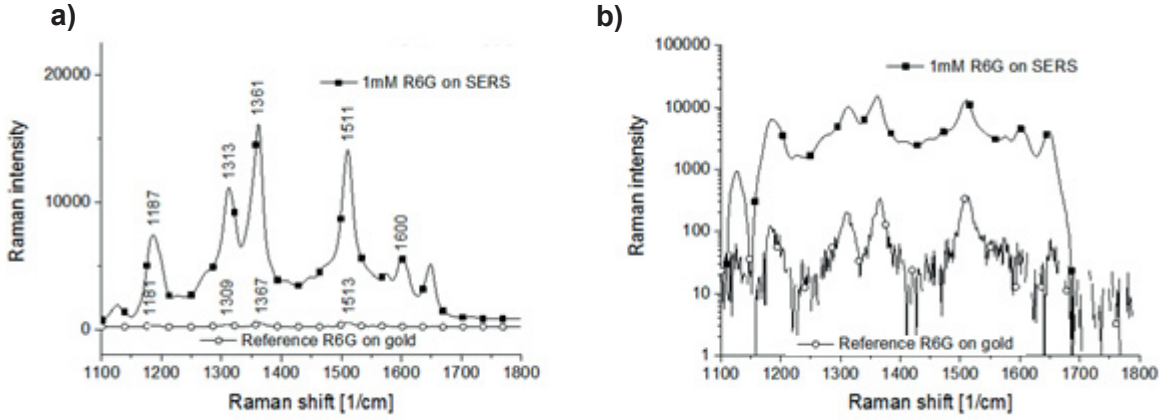


Figure 12. a) The Raman spectra for a 1mM R6G solution on top of the SERS patterned area and the smooth gold area; b) The difference in the intensity can be estimated on a logarithmic scale.

To confirm the prior analysis of the SERS, and to see the effect of the optical focus on the detected signals, we conducted a trial in which we changed the focus depth of the detection. The focus was misaligned by lowering the chip to see if the R6G signal remains constant as the signal is collected from the bulk sample above the SERS surface. If the signal is generated by the non-enhanced Raman from bulk R6G in DI water solutions, the signal intensity should remain constant without varying along the change of focus depth. As we can see from the results in Figure 13., the signal intensity drops as the chip is lowered (focus level raised from the SERS surface), and thus we can, together with the observations shown in Figure 12., confirm that we are detecting surface-enhanced Raman instead of conventional Raman.

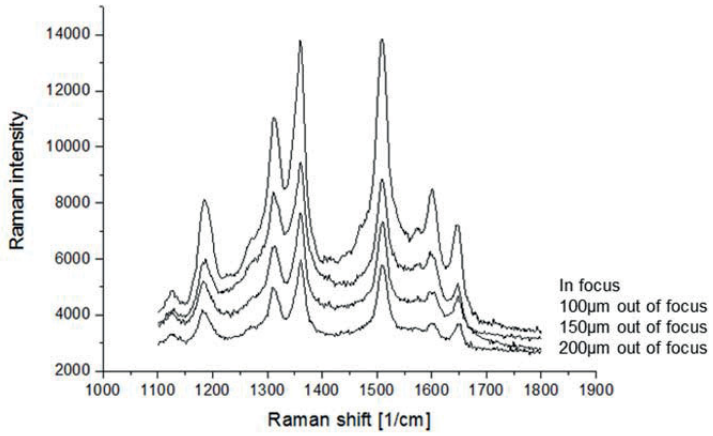


Figure 13. Misalignment of the focus of the Raman microscope from SERS surface.

To investigate the effect of the polyolefin lid on the SERS response, we measured the R6G spectra with 10 µM and 100 µM concentrations. The used integration time for the SERS signal recording was 30 seconds. The chip was filled with DI water to gain the reference polyolefin spectrum. Water was replaced by R6G samples and the Raman spectra were detected. Figure 14.a shows the Raman spectra of the R6G samples and the polyolefin reference, and Figure 14.b shows the 10 µM and 100 µM R6G spectra with subtracted polyolefin reference. The results show that although the polyolefin lid induces low Raman peaks, it has a minor effect on the R6G spectra. The effect of the lid can be further minimised by subtracting the reference spectrum from the R6G spectra.

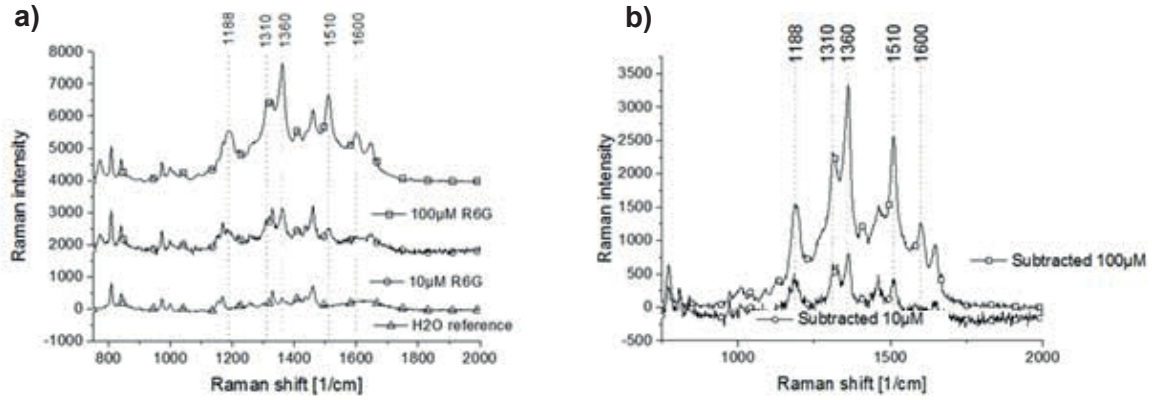


Figure 14. a) The Raman spectra for 10 µM and 100 µM R6G solutions are compared to the polyolefin reference (785 nm laser, 40 mW power, 20 X objective and 30 s integration time); b) The reference spectrum has been reduced from the 10 µM and 100 µM R6G spectra.

The effect of flow dynamics on the optical SERS signal was studied with a continuous flow with 0.5 mM R6G solution in DI H_2O. A similar study has been conducted previously by Hüttner et al. with a glass slide-based optofluidic SERS chip using R6G molecules in ethanol with preceding and following pure ethanol cycles (Hüttner et al. 2012). In our experiment, we focused more on the dynamics of the optical signal response to the used flow velocity than on the relation of sample concentration to the signal intensity.

In the study, the fluid flow velocity was varied from 25 µl/min to 1000 µl/min. The rise of the R6G signal was measured with a Raman microscope and a fluorescence microscope, as described in the Methods, to obtain the effect of the molecule diffusion and the partial mass transport limitation, and the effect of the convective flow. An image of the R6G Raman signal growth with a 50 µl/min flow velocity can be seen in Figure 5. The baseline tilt of the Raman spectrum was removed from the results for the analysed Raman shift area: 1100 1/cm to 1800 1/cm. Peak intensity for the main R6G peaks (1188 1/cm, 1310 1/cm, 1360 1/cm, 1510 1/cm and 1600 1/cm) was counted and averaged from 5 pixels. The change in the peak intensity as a function of time was calculated. Results of the measured signals were normalised and the average signal of 5 repeated measurements was calculated.

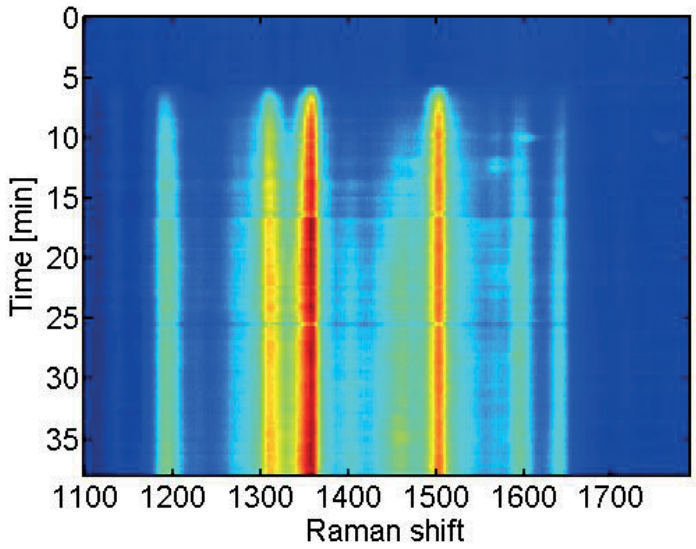


Figure 15. Image of the signal growth during the flow trial of 0.5 mM R6G with 25 µl/min flow velocity.

We recorded the signal rise without the dissociation phase, because the R6G molecules did not detach from the surface by washing with the H_2O flow. The binding strength of the R6G molecules to the gold surface was too strong, and the signal did not return to zero intensity. A cleaning step was carried out by oxygen plasma etching (5 min 300 W) between the flow runs. Each flow velocity was recorded 5 times and each chip was reused 3 times. The detected average fluorescence and SERS signals for the measured flow velocities are depicted in Figure 6a and b as a function of time.

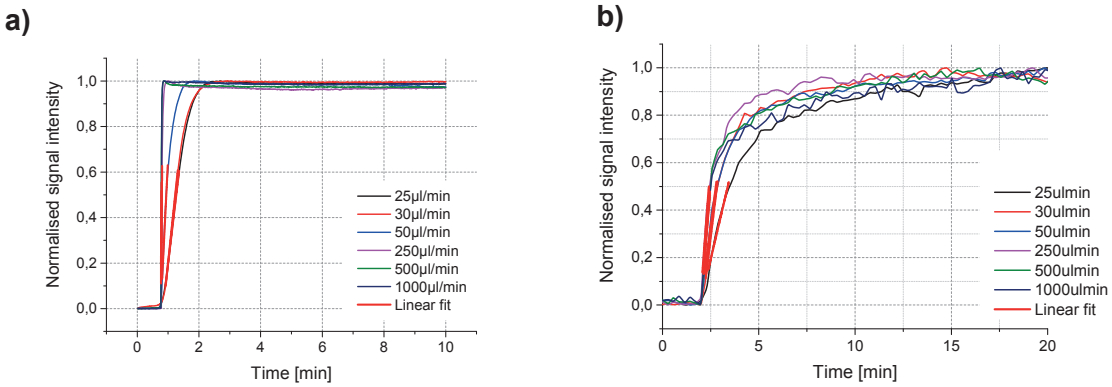


Figure 16. a) Fluorescence signals as a function of time; b) SERS signals as a function of time. Linear function has been fitted for the rising edge of signals.

To analyse the results, linear functions were fitted on the rising edge of the fluorescence and SERS signals. In Figure 6a, the fitted functions are depicted for the fluorescence, and in Figure 6b for the SERS signals. Linear functions are fitted for the range of 10% to 60% of the maximum intensity. The slope values attained are used to calculate the rise time of the signals for the aforementioned range. Figure 17 presents the comparative results of the SERS signal rise times and the fluorescence signal rise times. When comparing the results, it can be seen that the detected SERS signal rise is slower than the fluorescence signal rise of the R6G with all velocities in the study. The median of the lag time between the arrival of R6G molecules in the detection chamber by convective flow and the arrival and binding of R6G molecules on the SERS surface is 40.7 seconds.

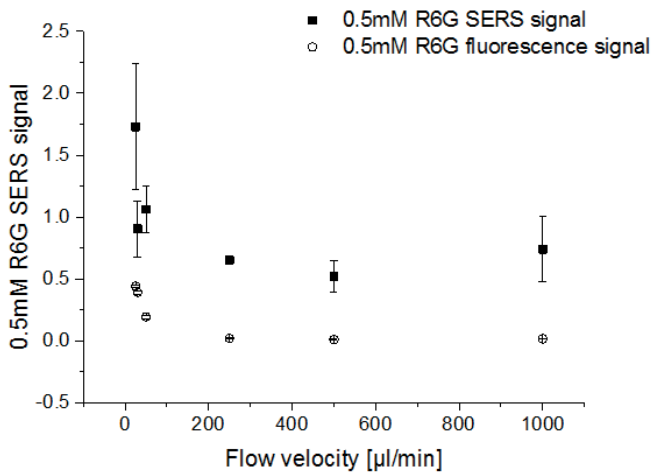


Figure 17. Signal rise times for the 0.5 mM R6G SERS signal and the fluorescence signal.

From the results, we can see that the lag time is larger for slower flow velocities. This could be due to insufficient molecule transport to the diffusion boundary layer. The lag time settles for the higher velocities and the dynamics of the diffusion and surface binding turn constant. In the future, these results will help us to plan studies with bioanalyte samples and active ligands on the surface, through the knowledge of the influence of an increasing mass transport limitation with flow velocities of 50 µl/min and less.

4.2. Analysis with microbial samples

The objective of this study was to develop a simplified method for label-free detection of *Listeria* with high sensitivity that is possible to perform on a disposable SERS platforms based on the results obtained with the small molecule detection. The overall concept is illustrated in Figure 18, where the SERS surface is integrated with very thin polydimethylsiloxane (PDMS) wells for controlled sample appliance. This SERS platform is suitable for low cost large volume production and is practical for one-time use, which diminishes contamination issues of the detection process. The patterned surface was coated by a gold layer and gold colloids, instead of the more SERS active silver, were used for extra enhancement. The method uses immuno-magnetic separation (IMS) beads as bacteria cell concentrators and the only washing steps occur during the pre-enrichment phase. SERS enhancement of different types of gold nanoparticles with Listeria was studied and the colloids with the best enhancement effect were used in combination of R2R nanostructured gold SERS substrates.

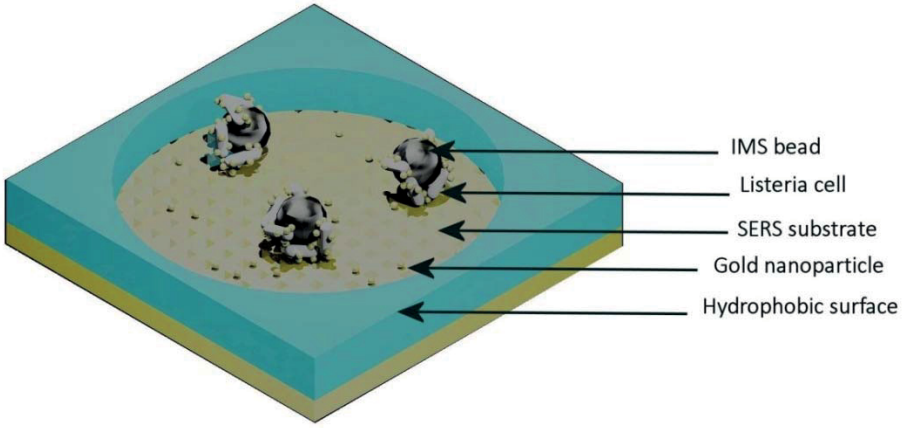


Figure 18. A picture of PDMS well on top of a patterned SERS substrate with gold surface. The immuno-magnetic particle bound *L. innocua* and AuNPs are concentrated inside the PDMS well in a more repeatable way than a free droplet on top of the substrate would.

Sample wells were created into 1 mm thick PDMS sheets (Wacker, Elastosil) by biopsy punches of a diameter of 1–2 mm. These PDMS wells have been bonded onto the polymer SERS substrates by physical adsorption. The hydrophobicity of the wells forces the sample to retreat inside the PDMS well and have contact with the gold layered patterned SERS surface.

Sample treatment for *L. innocua* with IMS bead separation

The bacteria samples were grown in LEE broth cultivation media (Labema, Lab M Limited, pH 7.2 ± 0.2) at 35 °C for 20 h without shaking. Spectrophotometry (Dynamica HALO DB-20S) was used for concentration analysis and samples were diluted into concentration series (10^3 CFU/ml – 10^9 CFU/ml). IMS was performed using Dynabeads® anti-Listeria (Life Technologies) and a Dynal Magnetic Particle Concentrator DynaMag™-2 (Invitrogen Dynal). 1 ml volumes of bacterial culture was mixed with a 20 µl volume of Dynabeads® anti-Listeria (Dynal) followed by incubation at room temperature for 10 min with continuous mixing. Magnetic field was used for bead concentration onto the side of the tube for three min, supernatants were removed and samples were washed with the washing buffer (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4 with 0.05 % Tween 20). In the end the IMS bound bacteria were resuspended into 100 ml of washing buffer for the SERS detection. A reference concentration analysis was done with 50 µl volumes of bead–bacteria complexes streaked onto differential selective ALOA chromogenic agar (Labema) and incubated at 35 ± 0.5 °C for 24 – 48 h.

SERS spectral acquisition of *L. innocua* and post-processing of data

L. innocua was detected with an in-house built Raman device integrated into an Olympus microscope with a 785 nm continues wave (cw) laser. The Surface-enhanced spectrums were excited with patterned SERS substrate, combination of patterned SERS substrates and AuNPs and with AuNPs on their own. Samples were placed into PDMS wells integrated on top of SERS-active substrates or silicon wafers. AuNPs were added into wells sequentially after bacteria samples. The minimum laser power irradiation used was 10 mW with 40x magnification to excite the samples. A maximum of 40 mW was used in combination with low magnification (20x). The signal collection time was 5 seconds with no averaging. The acquired data was baseline corrected with a simple linear algorithm in Matlab (release 2015a, Mathworks Inc., USA) after opening the data with the PLS toolbox, version 2.0 (Eigenvector Research Inc., Manson, WA, USA). Further data handling and figure plotting was executed with Origin Pro (version 9.4, OriginLab corp., USA).

Methods for the detection of *L. innocua*

Typically *Listeria* spp. has been identified by the SERS method from concentrations of 10^7 CFU/ml – 10^{10} CFU/ml. With lower concentrations the intensity of the detected Raman peaks diminishes and many of the peaks disappear from the spectrum. Identification of bacteria from incomplete spectra is difficult and the bacteria can be identified as other bacterial species. To avoid such misidentification the bacteria can be captured by specific proteins while SERS is used for the detection. Previously bacteria has been captured to the surface of SERS substrate with an antibody layer (Grow et al. 2003), but this method weakened the SERS signal. This could have been due to the increased separation distance between the surface and the bacteria, which makes the method not optimal for bacteria detection on SERS substrates. Another possibility for capturing bacteria is the use of immunomagnetic separation beads. Inb previous studies the IMS beads have been removed before detection or used with a sandwich assay and SERS labels. We simplified the detection by having IMS beads present during the SERS detection in a label free manner.

In this method the samples were placed into the hydrophobic PDMS well on top of the SERS substrate. The well prevented the liquid from spreading uncontrollably on the SERS surface as it would be it a droplet on surface. A more stable signal was achieved with the IMS beads present during detection due to more constant settling of the heavy IMS beads on to the sensor surface. A close-up TEM picture of an IMS bead and a SEM picture of IMS beads on top of the patterned SERS substrate

can be seen in Figure 19 a) and b). To further enhance the SERS signal gold nanoparticles were added around the bacteria bound IMS beads.

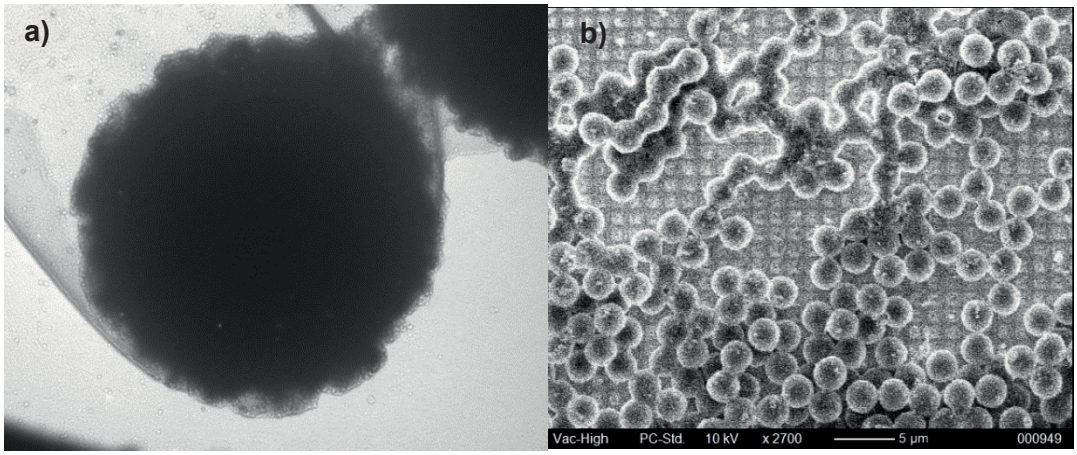


Figure 19. a) TEM picture of an IMS bead b) SEM-image of IMS beads on top of SERS substrate.

Gold nanoparticle characterization

To find an optimal Au nanoparticle (AuNPs) for bacteria detection with SERS, 3 candidates were chosen: ultrapure small AuNPs fabricated by femtosecond laser fragmentation, synthesized medium size AuNPs and synthesized larger AuNPs. Small AuNPs could be more biocompatible than synthesised particles as they lack the traces of non-reacted starting reagents, by-products, ions and surfactants, and have an additional advantage of lower background signal. The medium and large particles were analysed to see the effect of nanoparticle size to the detection of microbes. The size and morphology of the fabricated AuNPs were retrieved by transmission electron microscopy (TEM) from a 10µl droplet of aqueous nanostructure suspension deposited onto a carbon-coated copper grid. Figure 20 shows the TEM images, the corresponding size distributions of the AuNP and the UV-VIS spectra of the AuNP. For the ultra-pure AuNPs the maximum size was found to be around 50 nm. The medium sized AuNPs showed a maximum of 60 nm. The large AuNPs had a maximum of 85 nm. From the UV-VIS spectra in Figure 20 g) it can be seen how the maximum absorption peak of AuNPs shifts closer to 600nm wavelength as the maximum size of the particles grows from 50nm to 85nm.

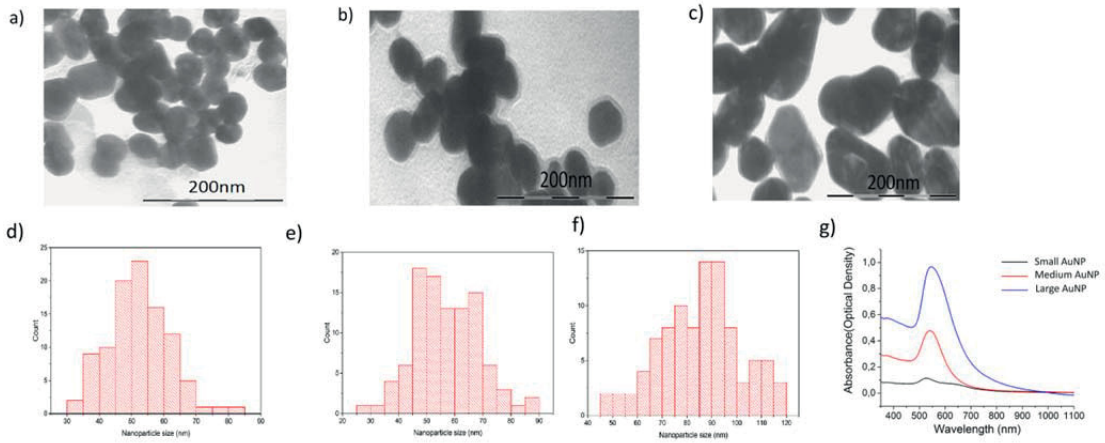


Figure 20. a-c) Transmission electron microscopy images of the small, medium and large AuNPs. d-f) The corresponding size distribution histograms calculated from TEM images of the AuNPs. g) UV-VIS spectrum for the differently sized AuNPs.

The SERS effectiveness of the different sized AuNPs was studied by pipetting 5 µl of bacteria sample and 2 µl of concentrated NP solution into PDMS wells positioned on top of the patterned SERS substrate. According to the bar plot of the intensity of 737 cm-1 peak presented in Figure 21, the median size and the large size particles gave similar intensities for a bacteria concentration of 5 × 10^5 CFU/ml. However, the large nanoparticles were chosen for further studies because the maximun of their UV-VIS spectra was closest to 785nm. Thus larger nanoparticles seem to enhance the signal more than small round ones for microbe detection. Fabrication by physical ablation could not benefit the detection compared to the synthesised. The advantage of the size and the shape of the particles were more important.

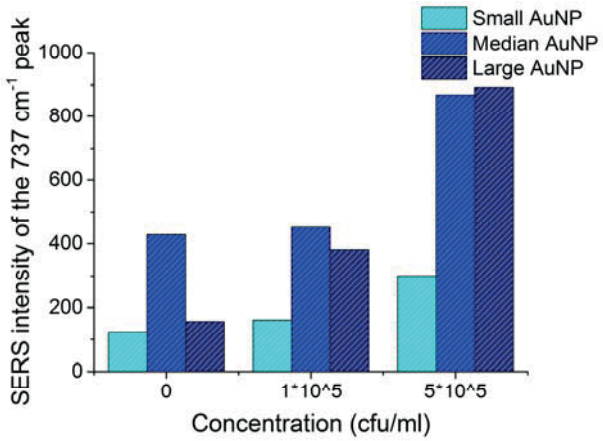


Figure 21. A bar plot of the SERS intensity for the dominant *L. innocua* ATCC 33090 peak at 737 cm^{-1} for different concentrations with the AuNPs inside a PDMS well on top of patterned SERS surface.

The development of the detection process

The SERS signal of IMS bead bound bacteria, possibly due to accumulation of more bacterial cells inside the excitation laser spot, was 20 times stronger than the signal recorded without the beads. The Figure 22 shows the results for the comparison of studies with and without IMS beads. The detection of *L. innocua* with the IMS beads was further studied on top of the patterned SERS substrate without AuNPs, as well as on top of silicon wafer with AuNPs and on top of patterned SERS substrate with AuNPs to see if there was an advantage in combining the SERS substrate with the AuNPs for bacteria detection.

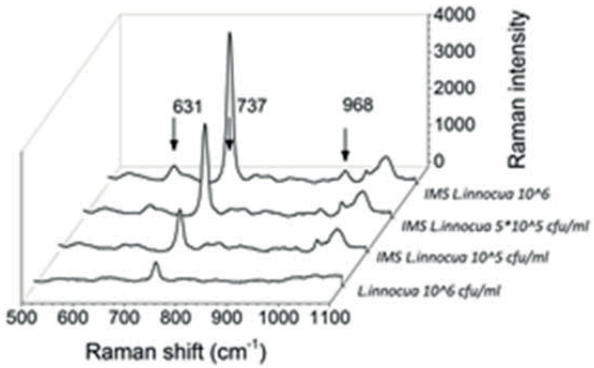


Figure 22. The effect of IMS concentration to the *L. innocua* ATCC 33090 SERS intensity with the AuNPs inside a PDMS well on top of patterned SERS surface.

Figure 23 represents the intensity differences between the measurements and it can be seen that the best intensities for the main dominant peak of 737 cm^{-1} were reached with the combination of the SERS substrate and AuNPs.

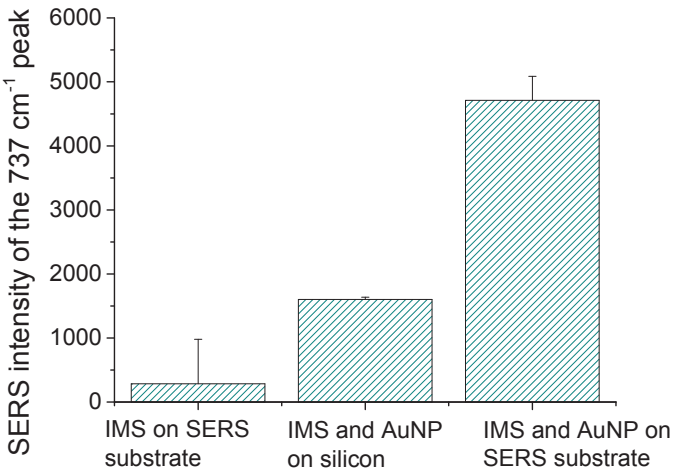


Figure 23. A bar plot of the SERS intensity for the dominant *L. innocua* ATCC 33090 peak 737 cm^{-1} for IMS bound 1×10^7 CFU/ml *L. innocua* ATCC 33090 inside a PDMS well on top of patterned SERS surface without AuNP, on top of a silicon wafer with large AuNP and on top of a patterned SERS surface with large AuNP.

The detected SERS lines

The captured *L. innocua* ATCC 33090 was detected inside a PDMS well in a liquid state with a 40 mw laser power and a 20x magnification. Figure 24 a) shows how the Raman peaks change with different bacteria concentration. As the bacterial amount diminishes, some lines stay constant showing the lines created by the traces of cultivation media and buffer liquids. From the spectra in Figure 24 a) it can be seen that 9 Raman bands were initiated by the bacterial cells. Figure 24 b) shows the Raman bands created by the LEE broth. Most of the background bands seem to originate from traces of the LEE broth.

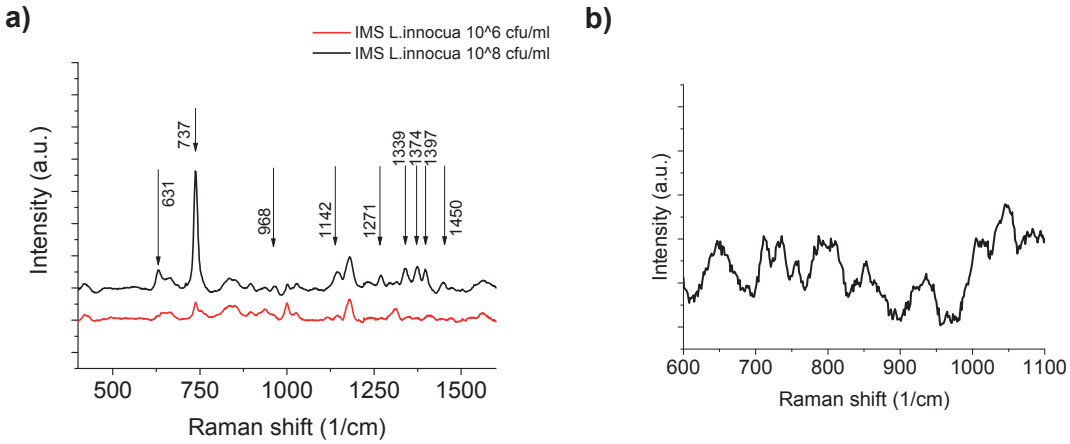


Figure 24. a) SERS spectra of *L. innocua* ATCC 33090 with large AuNPs inside a PDMS well on top of SERS substrate with IMS beads. b) Raman spectrum of the culturing media, i.e. LEE broth.

The nine Raman lines detected for *L. innocua* ATCC 33090 are listed in Table 4 with tentative assignments found in literature references. The dominant peak at 737 cm^{-1} could be coming from a glycosidic ring, adenine or CH2 rocking. Since the presence of adenine in the surface of the bacterial cell is unlikely and the outer wall structure of gram-positive bacteria such as *Listeria* spp. does consist of a thick peptidoglycan structure rich in N-acetyl D-glucoseamine (NAG), the origin of the peak is more likely caused by a glycosidic ring mode of NAG than adenine. The three close aligned lines in the range of 1300 – 1400 cm^{-1} have not been detected with *Listeria spp.* in previous studies. The line 1339 cm^{-1} has been detected ans assigned as a shifted line 1331 cm^{-1} which would come from CH_2 deformation. However, there are closer assignments to the detected 1339 cm^{-1} listed in *E. coli* studies as originating from amide III or as adenosine monophosphate and guanosine monophosphate coming from aromatic amino acids tyrosine and tryptophan. The second line 1374 cm^{-1} has been assigned to DNA. The last line of the group 1397 cm^{-1} is most likely due to the symmetric deformation of CH_3 group which has also been detected for the case of *E. coli*.

Table 4. Raman bands detected for *L. innocua* ATCC 33090.

Detected lines	Raman shift	Tentative assignments	Reference
631	**627/620**	Phenylalanine (skeletal)	Luo, Lin 2008, Maquelin et al. 2002
737	**732**	glycosidic ring mode of D-glucoseamine (NAG), adenine or CH_2 rocking	Luo, Lin 2008, Cui et al. 2015
968	**955**	N-C stretching	Vohnik et al. 1998
1142	**1134/1130**	C-N and C-C stretch (carbohydrates)	Fan, Hu 2011, Chen et al 2015
1271	**1230-1295**	Amide III	Liu, Chen 2007, Lu, Al-Qadiri 2011, Maquelin et al. 2002
1339	**1334/1339/1338**	Deformation CH/Amide III/ signature of adenosine monophosphate and guanosine monophosphate, aromatic amino acids tyrosine and tryptophan	Maquelin et al. 2000 Vohnik et al. 1998 Harz, Rösch, Popp 2008
1374	**1371**	DNA	Harz, Rösch, Popp 2008 Virhe. Viitteen lähdettä ei löytynyt.
1397	**1392/1398**	Symmetric deformation of CH_3 groups	Fan, Hu 2011 Al-Qadiri, Lin 2011
1450	**1453**	CH_2 deformation (lipids)	Fan, Hu 2011, Cui et al. 2015

When comparing the Raman bands detected for *L. innocua* with previous research, it is clear that the SERS spectrum in different studies varies. Liu et al. among others has stated that this could be due to the differences in the measurement conditions such as the cultivation broth and temperature, excitation wavelength of the laser or the SERS enhancer. To see the effect of excitation wavelength or the metal enhancer we recorded the SERS spectra of the same *L. innocua* ATCC 33090 sample with changed SERS conditions. In the first condition the combination of AuNPs on top of the patterned SERS substrate with 785 nm cw excitation were used, while in the second condition a SERS spectrum was detected from the same sample on top of a glass slide with AgNPs and pulsed laser excitation at a different wavelength of 532 nm. The results were consistent. Results are also similar to the third condition published by Luo et al. with silver colloid enhancer with 785 nm excitation wavelength and another research group of Kairyte et al. using silver colloid enhancer with 1064 nm. Clearly there is no connection between the variations in spectra and the enhancer used (silver/gold). Additionally the excitation wavelength does not seem to affect the detected spectrum.

To develope the detection process further laser power of the Raman device was lowered to 10 mW which enabled the use of larger objective magnification with the microscope without burning of the dried sample during detection. The media was disturbing the SERS signal if an excessive amount

of traces had dried on top of the SERS substrate. This is why the sample density was lowered to have less of an effect. Figure 25 shows the mean intensity changes of the dominant peak of 737 cm-1 as a function of *L. innocua* concentration with 40x magnification and 10 mW laser power for several concentration series.

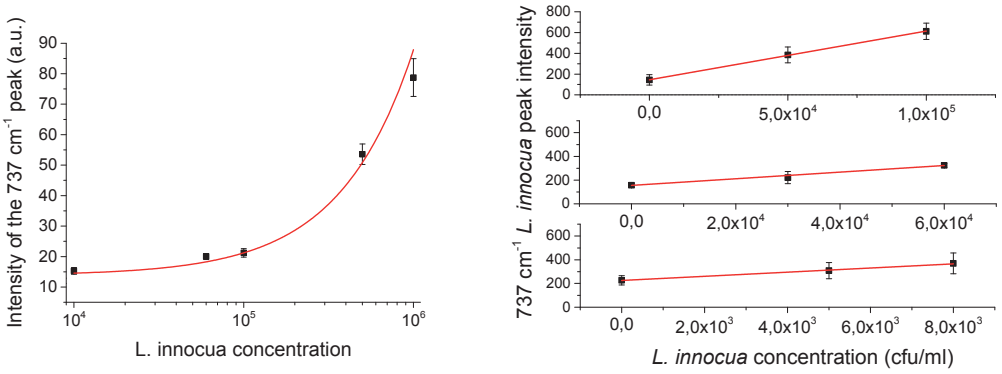


Figure 25. a) An exponential fit for the normalised concentration series in logarithmic scale for the entire series for IMS bound *L. innocua* ATCC 33090. b) Comparison of the 737 cm^{-1} peak intensity for different concentration series for dried IMS bound *L. innocua* ATCC 33090 samples with AuNPs in a PDMS well on top of SERS substrate.

The intensity of the dominant 737 cm^{-1} peak is displayed in Figure 25 a) as a function of logarithmic *L. innocua* ATCC 33090 concentration that follows an exponential curve. For the concentration range below 10^5 CFU/ml the relation was found linear. Since the blank 0 CFU/ml sample exhibit a signal at 737 cm^{-1}, the lowest limit of detection was considered through the deviation of the background signal generated by the sample matrix. According to the international union of pure and applied chemistry, IUPAC, the limit of detection can be defined as the smallest concentration detected with reasonable certainty, and derived from

$$LOD = ks_{bi}S, \qquad \text{(Eq. 1)}$$

where s_{bi} is the standard deviation of the blank measures, k=3 is a numerical factor of confidence level approved by IUPAC and S is the slope of the calibration curve. S is defined as

$$S=\Delta c/\Delta I\ , \qquad \text{(Eq. 2)}$$

where Δc is the change in concentration and ΔI is the change in Raman intensity. By using equation 1 and determining S from linear fit, the LOD was calculated to be 1.4×10^4 CFU/ml. The concentration series shown in Figures 25 a) and b) confirm the LOD, since the deviations of the concentrations below 10^4 CFU/ml coincide with the deviations of the mean blank samples. This means that samples with lower concentrations cannot reliably be detected.

As a summary, we demonstrated in this study the use of disposable SERS platforms and AuNPs with integrated sample wells for fast and simple detection of *L. innocua*. The factors leading the LOD of about 10^4 CFU/ml is listed in Figure 26.

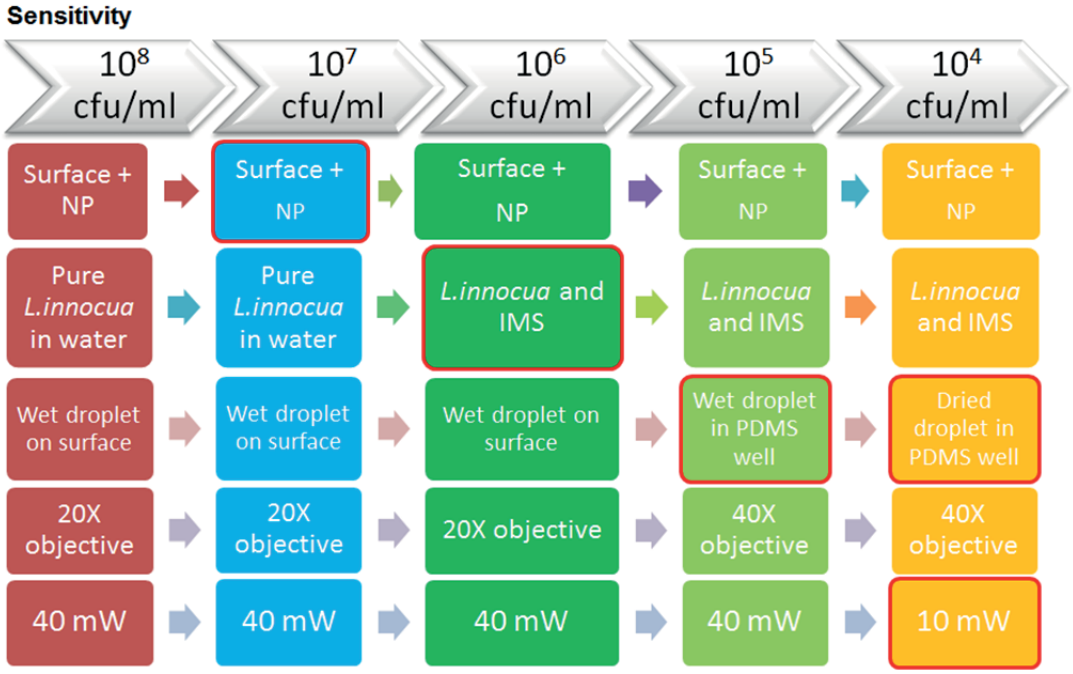


Figure 26. Summary of the factors improving the detection limit of *L. innocua* ATCC 33090 from 10^8 to 10^4 CFU/ml range.

As the size of the microbes is fairly large compared the plasmonic field inducing the Raman signal enhancement, the significant improvement was obtained by combining the surface and nanoparticle effects resulting in higher signal that either factor produced separately. Furthermore, we showed how the capture and deposit of the IMS bound bacteria cells onto the SERS substrate benefits the detection by concentrating bacteria volume. Further concentration was obtained by utilizing hydrophobic PDMS wells when the aquous samples dried locally collecting bacteria into local spots. The instrumental factor was optimal, when 40X objective was used producing smallest available beam waist and also collecting isotropic Raman scattering efficiently. High optical field localization resulted easily into sample burning effect, which could be avoided by adjusting the power close the threshold level that the sample could tolerate.

5. Value chain and service business analysis

5.1. From Food Value Chain to Food Safety Ecosystem

A thorough survey of food value chain studies reveals that food production is characterized by value creation being spread across a range of actors, from primary production via various levels of processing to catering and retailing (Grunert et al., 2008; Minten et al., 2013; Gómez et al., 2011). Food value chains (FVCs) as a definition is created within food industry. Minten et al. (2013) defined FVCs as comprising of all activities necessary to bring farm products to consumers, including agricultural production, processing, storage, marketing, distribution, and consumption.

The changing requirements of consumers and the ever-increasing awareness on food safety issues have led to a restructuring of food supply chains (Minten et al. 2013). Garrett et al. (2003) argue that a complicating factor in determining the economics of food safety systems is the diversity in business models currently used. In an attempt to reduce liability, food producers are promoting strict safety expectations through purchasing specifications and third party audit requirements for their suppliers before any standards have been identified and tested for effectiveness. These requirements continue to drive the supply side of the food industry to seek proper safety programs because the industry needs to reduce food safety associated risks.

Therefore, the FMA project took an ecosystem-level perspective and studied how food safety influences different parts of the food value chain, thus shedding light on how new business model can take into account the value of the entire food safety business ecosystem, creating shared value among ecosystem actors. In general, there are three drivers that guide the development of FMA business model and new food safety service design:

Need for a new business model in food industry

Referring to Grunert et al. (2005), consumer demands (such as on food safety) and the changing nature of technology in food industry lead to a new distribution of innovation across the actors in a food chain, resulting in new types of innovation. It thus arises the question to find or develop the optimum kind of business model and associated strategy for various actors in the field.

While there has been considerable research on supply chain management (Grunert et al, 2005), we identified that the studies seeking to innovate food industry from the business model side are rather limited. Hence, there is a need to carry out the quest for new business models for food industry, especially with the involvement of a range of players in the development of new food technology and innovation (Grunert et al, 2005).

Digitalization of food related business

It is well-known that information and communication technologies (ICT) has deeply impacted the human society (Ganascia 2015). The evolution of ICT has strong dependency on the hyper-connectivity in which it is rooted (Ganascia, 2015). Hagström (2012) suggests that the combination of hyper-connectivity, big data and analytics could empower citizens, governments, businesses, and consumers in three key ways: the ability to know, the ability to dialogue and the ability to innovate.

A recent study by The Economist (2015) suggests that hyper-connectivity is high on the corporate agenda. The common understanding out of the survey of global survey of 561 executives, confirmed that failure to adapt to the digital transformation is the biggest risk the organizations could face in the digital age. However, the extent to which organizations have adapted is limited. While experts warn of the need for the fundamental change to meet the challenges of hyper-connectivity and digitalization, only a fraction of the participating organizations expect to restructure “radically” as it intensifies in the future. Thus, this implies a deficit in leadership, and the hyper-connected economy calls for new ways of organizing and managing (The Economist 2015). Particularly, in the food technology arena, Lu and Wang (2016) suggest an emerging trend of using Internet of Things

(IoT) applications and cloud computing to monitor food quality in a cost-effective way, as the business environment has been moving toward the world of ubiquitous computing (Lee and Kim 2007).

Need for ecosystem-level thinking

Business model in general can be defined as a vehicle that is built to exploit a business opportunity (Zott and Amit 2010). A business model connects the firm and its external business environment, customers, competitors, and society (Teece 2010).

Looking at business model conceptualizations in general, many of the modern frameworks are created at the company level, which serves cases like traditional firm-centric business model well. However, it is less suited for analyzing the interdependent nature of the growth and success of companies that are evolving in the same business ecosystem (Westerlund et al. 2014; Weiller and Neely, 2013). Also, the role of key players and their interactions in the business model level have also been neglected (Wirtz et al. 2015). This results in companies (including food safety sector)' difficulties to capture the unprecedented ecosystem complexity and to develop adequate business models (Turber et al. 2015).

5.2. The need in Food safety testing

Prior to the development of new business model, the FMA project conducted a survey of existing offers in food safety testing industry, aiming to establish an in-depth understanding of the competitive landscape and how the customers' or end users' needs are met.

Based on Bettencourt and Ulwick (2008)'s argument, the innovation journey for many firms is rather identical to a hopeful wandering through customer interviews. Such unsystematic inquiry may occasionally find interesting information, but it rarely uncovers the best ideas or a comprehensive set of opportunities for growth. Therefore, the FMA project developed and employed the opportunity mapping tool to systematically study the opportunity arising from the poorly satisfied customer needs. The opportunity study went through a process of de-constructing the need and value in food safety testing from beginning to end in the business ecosystem. With such process in place, the project gains a complete view of all the points at which a food testing customer/end user might demand from food safety testing solutions and services.

With the practice of opportunity mapping, the project identified that reliability, sensitivity and adaptability to specific testing are the main customer needs and the driving factors for the research and development activities in the food safety industry, which matches the reality. As Mehrotra (2004) suggested, the food industry is consumer driven. There are many opportunities that will drive this paradigm shift. For instance, consumer awareness and concern for food safety is growing throughout Europe. While European consumers have long been concerned with the presence of chemicals and various additives in their food, a series of food safety scandals over the past decades has fundamentally shaken the confidence of some consumers regarding the safety and integrity of some food products and undermined their confidence in national and community systems of regulation and safety enforcement (JaVee and Masakure 2005).

On the other hand, ease of use, rapid testing result and cost reduction are becoming the key concerns of the customers and the users of food test kits, especially in the coming years. There is a clear indicator that the main need of customers (such as reliability, sensitivity and specificity) have become the industry norm, there is high degree of saturation and competition in these areas. In contrast, the demand in usability and rapid testing results, coupled with need for low cost testing methods are expected to drive the market movement. On top of that, there is an emerging trend that the testing industry in general will witness a major change in the coming five years, which is driven by the integration of sample pretreatment and the adoption of Internet of Things (IoT) and sensor technology are likely to change the industry landscape, thus creating opportunity for new type of innovations to meet customers' needs, as shown in FIgure 27.

The findings resonate with on-going trend in food industry, as Grunert (2005) suggest food processing industry realized that competing on price alone is not necessarily the most attractive business strategy. New competitors have been entering the competitive arena, and old competitors have been catching up on their competencies in efficient production and quality control. As a result, many sectors in food business today compete not only on efficiency and quality control, but on adding value.

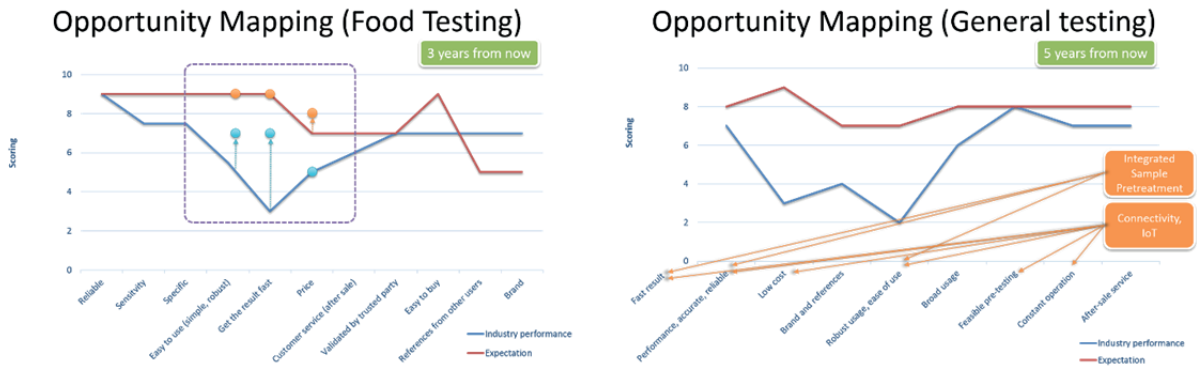


Figure 27. The emerging opportunity in food safety testing and general testing industry

5.3. Dis-bundling and creation of ecosystem business model

Hagel and Singer (1999) suggest that when looking under the surface of most companies, there can be three kinds of business functional areas, customer relationship, product innovation, and infrastructure business. Although organizationally intertwined, these businesses are actually very different. Bundling them into a single corporation inevitably forces management to compromise the performance of each area in ways that do not maximize the performance of the company.

Dis-bundling of the traditional business model

As Hagel and Singer (1999) suggested, when the three business functions are bundled into a single corporation, their divergent economic and cultural imperatives inevitably conflict. It is also in the case of FMA that scope, speed, and scale cannot be optimized simultaneously. Trade-offs have to be made. Thus, an integrated corporate business model as many of the existing incumbent food safety companies is not the optimal solution. The project proposed a new process of dis-bundling the three indispensable business functional areas as shown in Figure .

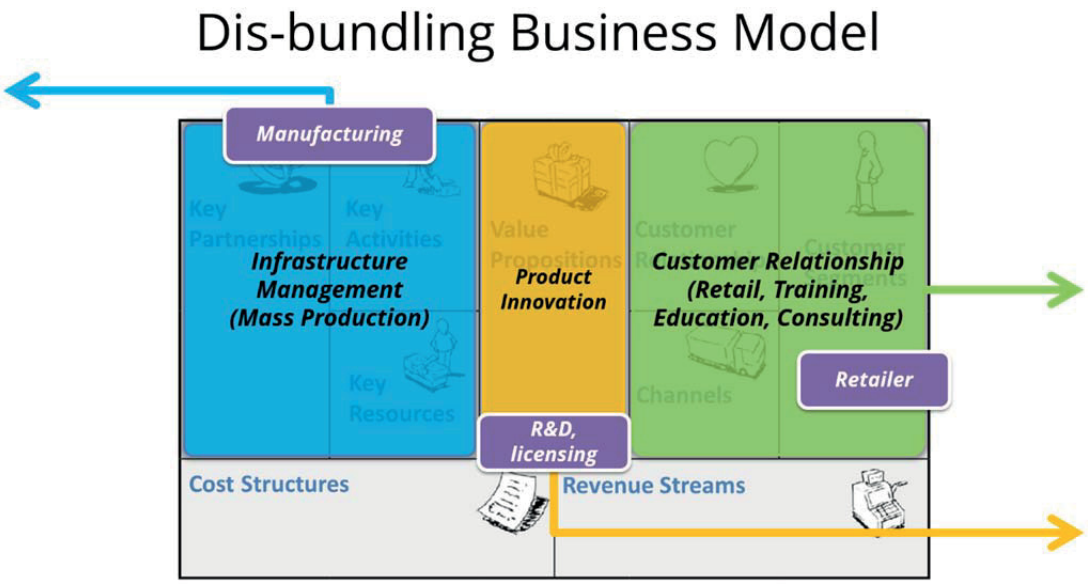


Figure 28. Dis-bundling of traditional food safety business model.

Creation of the ecosystemic business model

"The business ecosystem concept helps to think about how to respond to the dynamic and changing business environment, and how to move towards dynamic and adaptive business ecosystems" (Mazhelis et al. 2013). An ecosystemic business model includes the ideas of open innovation, expanding the boundaries of a company to create and capture value by collaborating with others (Chesbrough and Vanhaverbeke 2014), working as an open innovation platform (Xu et al. 2016).

Through the dis-bundling process, the FMA project de-constructed Hagel and Singer (1999)'s three business functions that could cause organizational tension and in-efficiency and re-allocate them within the food safety ecosystem to align and harmonize the different needs of the ecosystem actors. This results in the development and proposition of the digitalized ecosystemic business model as presented in Figure 29.

This model re-distributes the value creation and capture function into the food safety ecosystem which is consisted of six key actor groups: FMA food safety platform provider, physical testing device producers, research and development community, various testing service providers, logistics providers for the delivery of physical FMA chips and devices, and the end customer groups. Overall, it is a re-organization of the food safety actor groups from a traditional, centralized value chain type of thinking to an ecosystemic type of platform thinking. Under the new setting, the tensions of customer relationship, innovation, and infrastructure management sides of the traditional business are solved. For instance, the physical device producers can focus on improving economic of scale and reducing product unit cost to enable the adoption of wider customer groups from laboratory, to business customers and eventually to the end consumers in the future. The R&D community and service providers can concentrate on the development of new generations of food testing technology while incorporating the specific needs of the end user groups. The FMA platform provider will integrate the distributed and un-organized product offerings, resources and capabilities in the ecosystem into one platform. On the customer layer/side of the platform, the FMA platform providers can become the digital service interface for the customers by providing transaction service, marketing communication, customer education and engagement. One the business layer/side, the platform provider will connect complementary testing services to the platform. Finally, on the technical layer, the platform provider will build the data communication and storage infrastructure and develop

common technical standards to enable service providers create compatible food safety services and solutions that comply with the food safety regulations.

Overall, the goal of digitalized ecosystemic business model is to maximize the shared value at ecosystem-level, while taking into consideration the upcoming digital transformation in sensing and testing industry by incorporating IoT and digital capabilities into the new business model.

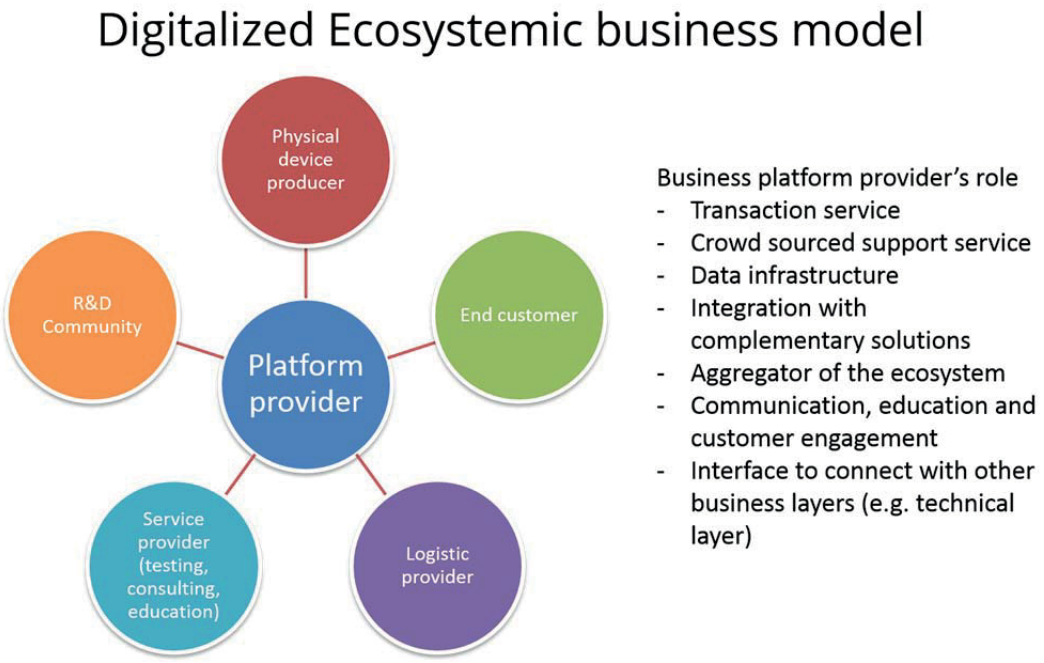


Figure 29. Digitalized ecosystemic business model for FMA.

6. Conclusions and outlook

The target of microbiological part of the study was to shorten the lag phase time in *L. monocytogenes* enrichment procedure and determine the selectivity of growth media combined with IMS. The enrichment efficiency was studied by screening tests where several growth media were modified with different supplements. It was clearly seen that it is really difficult to make remarkable improvements in shortening the lag phase time. Only small differences were detected in these tests. The selectivity of growth media combined with immunomagnetic separation was studied both inclusivity and exclusivity tests. The novel medium accelerates the growth of different *Listeria* strains and was not observed to suppress the growth of non- *Listeria* microbes adequately. Because of that biochemical methods will be needed to confirm the analytical results. To conclude, the developed FMA method seems to be applicable in *Listeria* spp. detection, but not specific for *L. monocytogenes* detection.

In the analytical part of the study the use of different types of AuNPs in addition to a patterned SERS substrate for listeria detection were analyzed. The benefit of usage of immuno-magnetic separation beads as an accumulation assistant of the bacteria for enhanced signal intensity was also demonstrated. The use of novel hydrophobic PDMS wells for sample preparation on chip enabled controlled sample appliance and reduced mean absolute deviation of SERS signals. The limit of detection in this methodology was determined to be in the range of $\sim 10^4$ CFU /ml. The established sensor platform is suitable for low-cost high-volume production.

The business analysis highlighted the importance of ecosystem mindset in new business model development. The business ecosystem concept helps to think about how to respond to changing business environment, and how to move towards flexible business ecosystems and ecosystemic business models, which include the ideas of open innovation and value co-creation. Overall, there is a need of a re-organization of the food safety actor groups from a traditional, centralized value chain type of thinking to an ecosystemic type of platform thinking. Under the new setting, the tensions of customer relationship, innovation, and infrastructure management sides of the traditional business are solved.

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