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Développement d'un test d'immunodétection rapide d'*E.coli* O157:H7 pour les outils et surfaces de travail des industries alimentaire.

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« Apprendre sans réfléchir est vain. Réfléchir sans apprendre est dangereux. »

Confucius

AVANT-PROPOS

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RÉSUMÉ

Les produits issus des industries alimentaires sont souvent la principale cause des toxi-infections alimentaires dues aux contaminations croisées aussi bien dans les foyers familiaux que dans la restauration collective. De ce fait, il est important de s'assurer de l'innocuité de l'aliment et plus spécifiquement de l'environnement de travail, principalement les surfaces de travail et des outils utilisés lors de la transformation, dans lesquels le pathogène est souvent présent en faible quantité et accompagné d'autres germes. *E. coli* O157:H7 représente le sérotype du pathogène le plus incriminé dans les éclosions et ceci par sa production de vérotoxines causant des diarrhées sanglantes et pouvant aller jusqu'au syndrome urémique. Dans cette optique, ce projet de maîtrise consiste à développer un test permettant la détection rapide et spécifique de ce pathogène à partir des échantillons des surfaces et des outils de travail dans les industries alimentaires. Les objectifs consistent ainsi en premier lieu à développer un support de détection permettant une capture optimale du pathogène durant la phase de l'enrichissement. Le deuxième objectif est de développer un test immunoenzymatique pour une détection spécifique d'*E.coli* O157:H7. Troisièmement, le mémoire consiste à optimiser la formulation du milieu d'enrichissement afin de favoriser la détection de ce pathogène par rapport aux autres germes.

Les résultats des travaux sur le support de détection ont permis non seulement de déterminer les composants affectant l'immobilisation des anticorps (pour le test de détection en ELISA) mais aussi la modélisation du signal de ces derniers en fonction des composants du support. Les résultats des travaux du milieu d'enrichissement ont permis d'optimiser un milieu assurant une

meilleure croissance d'*E.coli* O157:H7 et une augmentation de la production de Shiga toxine 2. D'autres part, un test d'immunodétection, ELISA indirect, a été mis au point en utilisant un anticorps polyclonal dirigé vers la sous unité B de la Shiga toxine 2 et le support développé pour la capture de la toxine au cours de la phase d'enrichissement.

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ABSTRACT

Products from the food industry are often the main cause of food poisoning due to cross-contamination in both family homes and in catering services. Therefore, it is important to ensure the safety of the food and more specifically of the work environment, mainly the work surfaces and tools used during processing, in which the pathogen is often present in the food in low amount and accompanied by other microorganisms.

E. coli O157:H7 represents the serotype of the most incriminated pathogen in outbreaks and this by its production of the verotoxins causing bloody diarrhea and up to the uremic syndrome. Thus, this master's project consists in developing a test allowing the rapid and specific detection of this pathogen from samples of surfaces and tools used in the food industry. The objectives are primarily to develop a detection medium for optimal capture of the pathogen during the enrichment phase. The second objective is to develop an immunoenzymatic test for a specific detection of *E. coli* O157: H7. Third is to optimize the formulation of the enrichment medium to promote the detection of this pathogen compared to other microorganisms.

The results of the studies on the detection medium showed that it is possible not only to determine the components affecting the immobilization of the antibodies (for the ELISA detection test) but also the modeling of the signal of the immobilization according to the components of the support. The result of this study optimized an enrichment medium ensuring a better growth of *E. coli* O157:H7 with an increased production of Shiga toxin 2.

. On the other hand, an immunodetection test, Indirect ELISA, was developed using a polyclonal antibody directed to the B subunit of Shiga toxin 2 and the support developed for toxin capture during the enrichment phase.

Amina Baraketi, étudiante

Monique Lacroix, directrice de recherche

LISTE DES ABRÉVIATIONS

ACIA: Agence Canadienne d'Inspection des Aliments

AFNOR: Association Française de Normalisation

ANOVA: Analyse de la variance

AOAC: Association of Analytical Communities

BBD: Box-Behnken design

BSA: Bovine serum albumin

CFU: Unité Formatrice de Colonies

CHI : Chitosane

CNC: Nanocrystals de cellulose

DNA: deoxyribonucleic Acid

EHEC: *Escherichia coli entérohémorragiques*

ELISA: enzyme-linked immunosorbent assay

FFD: factorial fractional design

g : gramme

GA: glutaraldehyde

GLY: Glycerol

GMP: Good manufacturing practices

HACCP: Hazard analysis critical control point

HMC: Heppe Medical Chitosan

HRP : horseradish peroxidase

HUS : hemolytic uremic syndrome

IS: immobilization signal

kDa: kilodalton

LAMP: loop-mediated isothermal amplification

LOD: limit of detection

mAb: Monoclonal antibodies

MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

ml: millilitre

MPN: probable number method

mRNA: messenger ribonucleic acid

NASBA: nucleic acid sequence-based-amplification

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline-Tween

PCR: The polymerase chain reaction

RNA: ribonucleic acid

rRNA: Ribosomal ribonucleic acid

RTE: ready to eat

SPR: surface plasmon resonance

STEC: Shiga-like toxin producing *E. coli*

Stx : Shiga-Like toxin

TMB: 3,3′,5,5′-Tetramethylbenzidine

TPP: Tripolyphosphate

TS: tensile strength

TM: tensile modulus

v/v: Volume/Volume

VBNC: viable but nonculturable cells

w/v: Weight/Volume

µg: microgramme

CHAPITRE 1 : INTRODUCTION GÉNÉRALE

Chaque année, l'Agence Canadienne d'Inspection des Aliments (ACIA) réalise en moyenne 3000 enquêtes, à la suite d'une contamination d'aliments présents sur le marché. Une partie importante est due à une potentielle contamination par un agent microbiologique dont 13000 cas de toxi-infections engendrés par *E. coli* O157:H7. *Escherichia Coli*, est habituellement une bactérie commensale qui fait partie de la flore intestinale de l'homme. Néanmoins certaines souches sont pathogènes plus particulièrement les *E. coli* O157:H7.

E. coli O157:H7 fait partie de la famille des *Escherichia coli* Enterohémoragiques (EHEC) causant des troubles de santé allant des diarrhées hémorragiques jusqu'au Syndrome hémolytique et urémique (SHU). Ces toxi-infections alimentaires ont un important degré de sévérité principalement chez les personnes âgées, les enfants ainsi que les personnes dont le système immunitaire est affaibli.

Escherichia coli Enterohémoragiques (EHEC) fait aussi partie du groupe des Shiga-Like toxine producing *E. coli* (STEC). Les STEC sont connues par la production de deux toxines nommées Shiga-Like toxines en raison de leur similarité avec la Shiga toxine produite par *Shigella dysenteriae.* Ces toxines sont composées d'une sous-unité A et de cinq sous-unités B identiques. Formant un anneau, les sous-unités B, fixent la toxine sur un récepteur membranaire spécifique (Gb3) afin d'assurer l'internalisation de la toxine dans la cellule. Quant à la sous unité A, une fois scindée en deux parties, elle va désactiver la sous-unité 60S du ribosome ce qui va conduire à l'arrêt des synthèses protéiques et ainsi la mort cellulaire. Les Shiga-Like toxines sont les

principaux facteurs de virulence des *Escherichia coli* Enterohémoragiques. Alors que la Stx1 présente une homologie de 99% en termes de séquence d'acides aminés avec la toxine de *Shigella dysenteriae*, Stx2 présente presque 60% d'homologie avec la séquence de Stx1.

L'industrie de la viande est la principale source de contamination par ce pathogène causant ainsi plusieurs rappels de produits déjà présents sur le marché. En effet, *E. coli* O157:H7 provient de l'appareil digestif des animaux et contamine une large gamme d'aliments aussi bien d'origine animale, tel le lait cru et ses dérivés et la viande, que végétale tel que les fruits et légumes crus, qu'ils soient utilisés dans les foyers familiaux pour la consommation directe ou encore qu'ils subissent des procédés de transformations dans les industries. De ce fait, il est important de s'assurer de l'innocuité de l'aliment et de l'environnement de travail, principalement les surfaces de travail et les outils utilisés lors de la transformation, dans lesquels le pathogène est souvent présent en faible quantité et accompagné d'autres germes. Cela souligne l'importance de test simple et rapide pour les surfaces et les outils de travail. Il existe de nombreuses méthodes bactériologiques, pour l'isolement et l'identification de ces pathogènes à partir des échantillons humains et environnementaux mais aussi d'autres méthodes immunologiques plus sensibles qui ont été développées et validées. Toutefois, le temps d'incubation assez long, le matériel lourd et couteux, l'exigence d'un personnel qualifié, le nombre important de manipulation et l'utilisation de plusieurs réactifs sont les principaux freins de celles-ci. Malgré l'existence de ces différentes techniques, des rappels à la suite de la contamination des produits alimentaires par le pathogène *Ecoli* O157:H7 ont encore lieu. À ce sujet, ce projet de maîtrise vise à développer un test simple, rapide et compétitif comparativement aux autres méthodes qui permettra de confirmer la présence ou l'absence du pathogène *E. coli* O157:H7 et/ou ses toxines sur les surfaces et outils de travail,

permettant ainsi une prise de décision rapide quant à la validation de la libération d'un lot de produits de l'industrie.

Le but de ce projet est de développer une trousse commerciale d'immunodétection rapide d'*Escherichia coli* O157:H7 sur les surfaces de travail et d'outils dans les industries alimentaires, faisant appel à un film intelligent à base de chitosane/nanocellulose pour la capture du pathogène pendant la phase d'enrichissement. Les objectifs du projet sont ainsi: 1) Développer un support de détection par l'optimisation d'une méthode de réticulation et de renforcement du chitosane. 2) Développer une méthode immunoenzymatique pour la détection du pathogène *E. coli* O157:H7, immobilisé sur le support de détection. 3) Optimiser un milieu d'enrichissement permettant une détection rapide du pathogène.

CHAPITRE 2:

Foodborne pathogens detection: Persevering worldwide challenge

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Amina Baraketi a rédigé le chapitre de livre.

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Abstract:

According to Center for Disease Control and Prevention (CDC), 48 million people get sick, 128,000 are hospitalized and 3,000 dies due to foodborne diseases each year in the United States. Cross contamination from the raw materials, during the process or on working surface has to be rapidly detected. Good manufacturing practices (GMP) and hazard analysis critical control point (HACCP) can help to reduce the incidence of contamination. However, the development of sensitive and rapid methods of detection are still an important need. Standard culture-based methods require the consumption of large amounts of media, are time-consuming and interferences can occur when samplings are done in complex food matrices. The polymerase chain reaction (PCR)-based methods are new technologies. These methods show high level of specificity and sensitivity because they can detect nucleic acid sequences of target bacteria. However, they require an expensive instrumentation and trained scientific technicians. This review highlights several innovative strategies on the development of novel technologies that is simple, sensitive, specific, time consuming in order to detect quickly foodborne pathogens for application in 38 food industries.

Keywords: Foodborne pathogens, rapid technologies, food industries, food safety

1. Introduction

Large-scale foodborne outbreaks are still an ever-present threat to public health, particularly for very young and elderly people as well as pregnant women, and people susceptible to a weakened immune system (Scallan *et al.*, 2011). The global incidence of foodborne disease is difficult to estimate, but it has been reported that every year, foodborne pathogens cause millions of infections and intoxications as well as thousands of deaths. Moreover, outbreaks generate billions of dollars in worth of damage, public health problems, and agricultural product losses (Yeni *et al.*, 2016).

The etiology was determined in the United States in the period from 1993–1997 and reported outbreaks showing that bacteria caused 75% of outbreaks and 86% of cases (Olsen *et al.*, 2000). Furthermore, among the 31 pathogens identified as causing foodborne illnesses, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, and *Escherichia coli* O157:H7 have been incriminated for the large majority of illnesses, hospitalizations and deaths (Zhao *et al.*, 2014). On the other hand, *Salmonella* spp, *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus* are on the top of the list for the largest number of outbreaks, cases, and deaths (Oussalah *et al.*, 2007) (Wu *et al.*, 2016).

The frequent occurrence of foodborne diseases in previous years is mainly based on five factors, inter-related and difficult to control to a large degree involving environmental conditions, health system including infrastructure, social situation, behaviour and lifestyles, health and demographic situation and food supply system (Motarjemi & Käferstein, 1999). Although pathogen detection is a growing concern for three main application areas including water, environment quality control (Leonard *et al.*, 2003) (O’Kennedy *et al.*, 2005) and clinical diagnosis, food industry still remains the major area

concerned with 38% of the relative number of works appeared in the literature about the detection of pathogenic bacteria (Lazcka *et al.*, 2007).

In industrialized countries, the public health authorities set up strict measures and regulations for food control systems such as Hazard Analysis Critical Control Point system (HACCP) and Good Manufacturing Practice (GMP) in order to overpower the spread of these diseases at the level of the food processing and the food supply chain. HACCP is a method of food safety assurance based on the application of good hygiene practices. The HACCP system identifies any additional or more specific control measures necessary in food operations, places an additional emphasis on those points of good hygienic practices, foresees corrective measures if monitoring results indicate a loss of control, and finally provides more training and responsibility to operators (Motarjemi & Käferstein, 1999). Thus, the detection of foodborne pathogenic bacteria is an important key to the prevention and the control of some hazardous points in food processing or supply systems. Traditional detection methods may take up to a week to yield a confirmed result, challenging many researchers to gear their efforts towards the development of rapid methods for obtaining analytical results in the shortest time. The present chapter attempts to compare the different methods of pathogens detection currently used in the food industry as measures of prevention from foodborne diseases. Certainly, it is essential to be well informed about the different methods of pathogens detection but it is as much interesting to find out the possible sources of contamination.

2. Sources of contamination

Foodborne diseases are induced by the consumption of foods or water contaminated by pathogens (Dwivedi & Jaykus, 2011). **Figure 2.1** shows most of the pathways leading to the presence of foodborne pathogens in daily food products for nowadays consumers. These food products include

fresh produce such as fruits, vegetables, herbs, seeds and nuts, milk and dairy products, meat products as well as poultry and eggs. From the pre-harvest phase, most of these products go through either a local distribution directly from the farmer to the consumer, or a wider distribution to the industry. In industrialized countries, consumers get these raw materials for home use through the supermarkets. In all cases, food is an excellent source of energy and nutrition, not only for human and animals but also for the proliferation of microorganisms.

The contamination by the fresh produce has been well discussed by (Yeni *et al.*, 2016). Food manufacturing mostly relies on fresh produce, as raw materials that offer to consumers a wide range of benefits such as nutrients, vitamins and fibers. From farm to fork, the contamination of fresh produce by pathogens may occur at any stage during the transformation process from the preharvest to the postharvest phase. In the field, contamination can occur through some elements of nature (water, soil, seeds, insects, dust, etc.) whereas the central part of contamination during the postharvest phase is related to handlers and equipment during processing, transportation and preparation (Gorny, 2006). The risk for this kind of products is that they are usually consumed in raw state or not heat-treated, avoiding the elimination of pathogens before consumption (Ribot *et al.*, 2008). *Salmonella* spp, pathogenic *E. coli*, *L. monocytogenes*, *S. aureus*, *Shigella spp*, *Yersinia spp*, *Clostridium spp* are the main pathogens contaminating fresh produce.

On another side, as described by (Marriott & Gravani, 2006), healthy cattle may, hideaway in their liver, kidneys, lymph nodes, and spleen human pathogenic microorganisms. Beginning from slaughtering, the first step in meat processing, carcasses is exposed to microorganisms in animal intestinal tracts and consequently could contaminate other cut surfaces and carcasses. Thus, carcass contact surfaces, water, air and staff during processing and distribution channels are potential

contamination sources of meat and meat products. Concerning poultry products, critical steps that may lead to contamination are defeathering and evisceration with higher probability in the case of contaminated hands and toll workers. The pathogens that threaten these products are *Salmonella* and *Campylobacter. L. monocytogenes* is the most incriminated pathogen in the contamination of dairy products, which are vulnerable to the risks from udders of cows and milk equipment.

It is obvious that the high volume of food production may lead to a greater likelihood of a cross-contamination as previously described and consequently a high spread the disease. This finding was also supported by (Angulo *et al.*, 2006) mentioning that in industrialized countries, the amounts of outside food consumption including international travels as well as the increasing demand for minimally processed ready-to-eat (RTE) products are increasing the risk of foodborne diseases. In a large case-control, 20% of infections with *E. coli* O157:H7 was associated to eating at a table-service restaurant, 35% of infections with *S. enteritidis* with egg consumption in a restaurant and 35% were attributed to eating chicken prepared out of home.

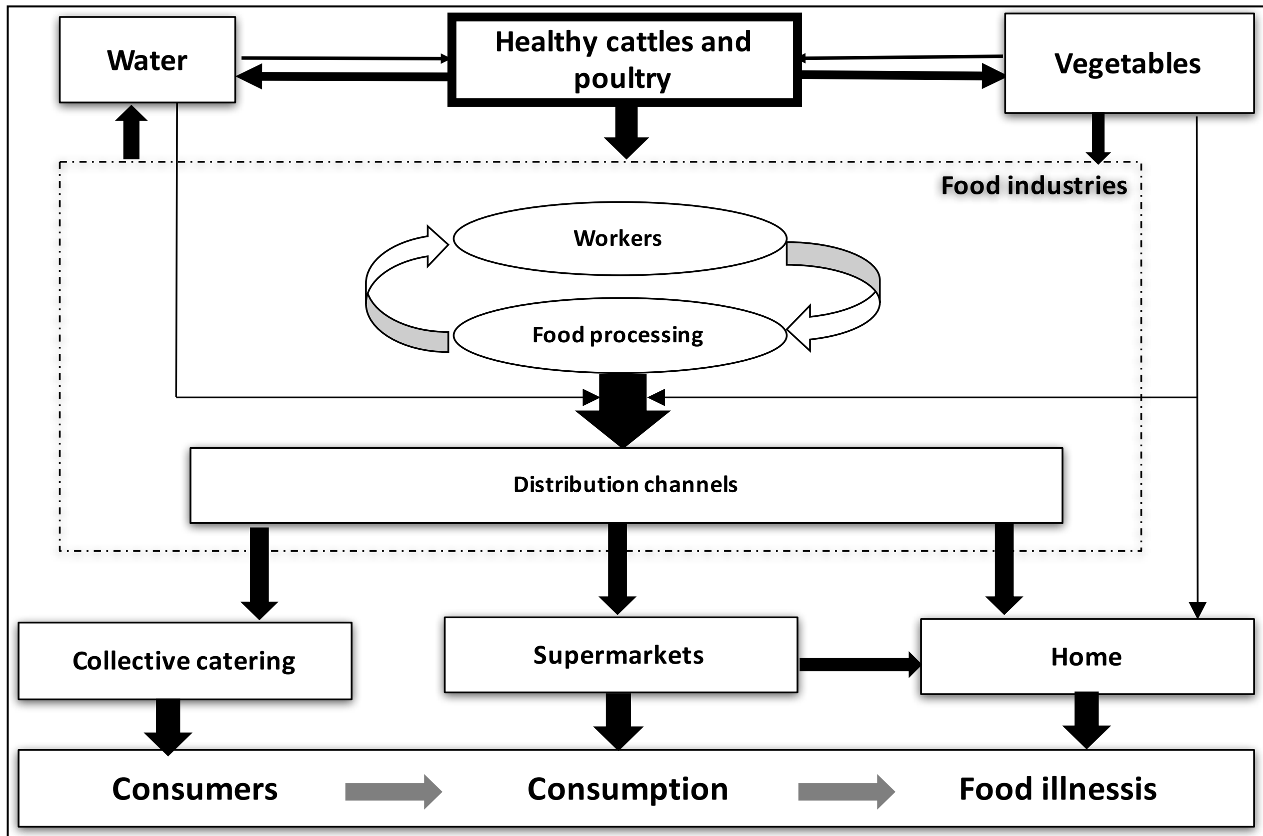

Figure 2.1: Potential flow of food contamination (adapted from (Andral *et al.*, 2003)

Although fresh produce, red meat, poultry and milk are the raw materials for the food industry and restaurants, supermarket RTE food products themselves are the raw materials for consumers' homemade meals (Goldburn, 2009).

To avoid cross-contamination from raw materials, it is essential to wash hands, tools and prepare surfaces before and after processing. Also, food products that are already prepared/cooked have to be refrigerated at 4ºC. However, hot foods should be kept at above 60ºC. Besides, it is recommended to split large volumes of food into small portions for rapid cooling in the refrigerator as well as heating canned foods fully before tasting.

The large number of interconnected factors increases the risks of cross-contaminations. To control the spread of these pathogens, there is first a need for monitoring the contamination of raw materials from suspected sources to the end of the supply chain by applying hygiene and sanitation practices and also the advent of new rapid technologies of detection.

3. Conventional methods

According to (Doyle & Buchanan, 2012), conventional microbiological methods are usually performed for the isolation and enumeration of pathogens in food samples. Nowadays, these standard culture methods are still considered as the "gold standard" as they are sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of microorganisms present in food samples.

On the other side, conventional methods are time-consuming considering all basic pre-enrichment, enrichment and plating steps needed. They mainly rely on specific media to enumerate and isolate viable bacterial cells in food. The pre-enrichment of the food samples, in a non-selective or selective broth culture, can be used to increase the number of injured but viable bacteria that can be a potential threat to human health, to a detectable level (Zhao & Doyle, 2001). Pre-enrichment recovers a larger proportion of bacteria from food matrices and is usually followed by sub lethal stressors such as heating, cooling, acids or osmotic shocks (Harrigan, 1998). In addition to that, the occurrence of toxin production in food requires that the cell pathogen concentration reaches a specific level as much as 5 log CFU/g of *Staphylococcus aureus and Bacillus cereus,* 3 log CFU/g of *Clostridium botulinum* (CFU referring to colony-forming unit). Thus, all existing detection technologies have to be preceded by an enrichment step (Wang & Salazar, 2016).

Enrichment steps (selective enrichment, selective plating) may require an additional period of 8–24 h before the enumeration or the detection can be completed and mostly they will be followed by biochemical screening and serological confirmation (Gracias & McKillip, 2004). A variety of chromogenic and fluorogenic culture media are available for selective isolation and differentiation of food-associated spoilage bacteria by incorporation of enzyme substrates. As no single microbiological test, among these standard culture methods provides a confirmed identification of any unknown microorganism, there is a need for several additional series of analysis (Ivnitski *et al.*, 1999).

Conventional methods can be laborious too as they usually require the preparation of culture media and colony counting with the most probable number method (MPN) (Mandal *et al.*, 2011). The duration of these methods depends on the ability of the microorganisms to grow in pre-enrichment, selective enrichment and selective plating media. This process is often slow and takes 48 to 72 hours for preliminary identification and more than a week for the confirmation of the pathogen species (Zhao *et al.*, 2014).

Qualitative culture methods are only used to determine the absence or presence of microorganisms in food samples. However, the quantitative ones are preferred for enumeration. The limit of detection (LOD) or sensitivity, the minimum amount of detectable cells, is defined by the presence of microorganisms in 25 g of food examined for qualitative methods and a concentration of <10-100 MPN of bacteria per gram or >10-100 viable counts for quantitative methods (López-Campos *et al.*, 2012) considering that the LOD for plating methods is 1 CFU/g.

Regarding to the high spread of foodborne pathogens illness, the inspection regulations are very strict with the requirements for process control. The LOD for food pathogens is restrained at 1 cell per unit

of food sample (Gill, 2017). Depending on the target pathogen and the food sample, the analytical unit may be considered from 25 to 325g.

These methods are recognized for their low cost and ease of use that are relatively interesting compared with alternative methods (Gracias & McKillip, 2004). Despite these traditional methods are still used due to their high selectivity (Lazcka *et al.*, 2007), they are laborious, time-consuming, and may be limited by their low sensitivity (Lee *et al.*, 2014) compared to other rapid methods. In addition, there is a probability that false negative results may occur due to viable but nonculturable (VBNC) cells.

The challenge of pathogen detection in food matrix, as reported by (Mandal *et al.*, 2011) and (Doyle & Buchanan, 2012), resides in the presence of pathogens in low numbers and uniformly distributed in a food heterogenic matrix with the presence of non-pathogenic microorganisms that may interfere with the identification step. Food matrices can be found in different physical states (powder, liquid, gel or semi-solid) and contain a wide range of ingredients that may interfere with the detection.

4. Alternative methods for the detection of foodborne pathogens

To overcome the limitations of conventional methods, various rapid methods have been developed and are commercially available to meet the needs of the food industry. Considering that commercialized rapid detection methods should be validated from a recognized organization such as the Association Française de Normalisation (AFNOR) in the European Union or the Association of Analytical (AOAC International) in the United States, most of kits of detection are validated according to their sensitivity and specificity (Beumer & Hazeleger, 2009). Ideally for industrial applications, rapid methods should be characterized by their specificity, high sensitivity and fast performance. Nowadays, current rapid methods are able to detect pathogens in raw and processed foods in low numbers to avoid the risk of

infection, are more time-efficient, labor-saving and prevent human errors (Mandal *et al.*, 2011). Currently, the range of detection time for available rapid methods is estimated from a few minutes to a few hours. Nevertheless, the sensitivity and specificity still have to be improved for testing foods samples without the needs to be pre-enriched before analysis (Feng, 1997). Indeed, the enrichment step is considered as the main limitation in most of the methods but remains essential for the revival of stressed or injured cells, the differentiation of viable from nonculturable cells and the dilution of inhibitors present in the food sample (Feng, 2001).

Rapid detection methods can be categorized into biosensors, immunological methods and nucleic acid-based methods (Figure 2.2). Simple polymerase chain reaction (PCR), multiplex PCR, real-time PCR, nucleic acid sequence-based-amplification (NASBA), loop-mediated isothermal amplification (LAMP) and oligonucleotide DNA microarray are classified as nucleic-based methods. Biosensors-based methods include optical, electrochemical and mass-based biosensors. Finally, enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay are recognized as immunological-based methods (Law et al., 2015). Several publications have already detailed the principle of each of these methods (Gopinath et *al*., 2014; Law et *al*., 2015; Valderrama et *al*., 2016; Zhao et *al*., 2014). However, the aim of this work is to focus on the advantages and limitations of these methods for application in food industry. The development of new immunology-based methods and PCR are well documented conventional techniques for the detection of pathogens (Alahi & Mukhopadhyay, 2017).

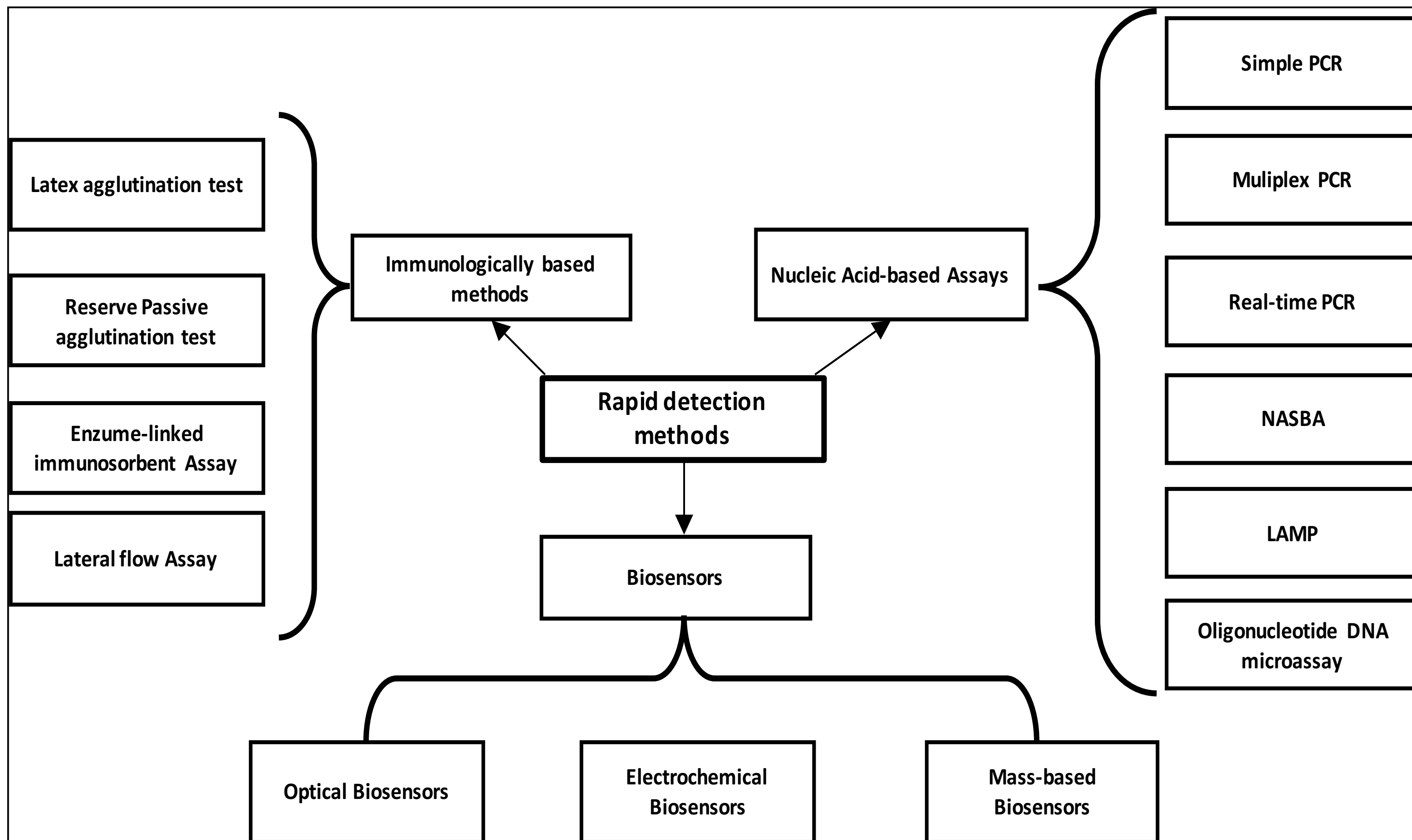

Figure 2.2: Mapping of rapid detection technologies for foodborne pathogens (Valderrama *et al.*, 2016)

4.1. Nucleic Acid-based methods

Nucleic acid-based methods prevent ambiguous or wrongly interpreted results. They operate by detecting specific deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences in the target pathogen and hybridizing the target nucleic acid sequence to a synthetic oligonucleotide, which is complementary to the target sequence (Zhao *et al.*, 2014). Invented 20 years ago, simple PCR (Velusamy *et al.*, 2010) is widely used for the detection of *L. monocytogenes* (Manzano *et al.*, 1997), *E. coli* O157:H7 (Lindqvist, 1997), *S. aureus* (Johnson *et al.*, 1991)*, Campylobacter jejuni* (Wang *et al.*, 1999)*, Salmonella spp* (Cheah *et al.*, 2008) and *Shigella spp* (Lindqvist, 1999). The presence of sufficient numbers of target molecules, the purity of the target template, the complexity of food matrices containing potentially inhibitory compounds may affect the reliability of PCR amplification (Lampel *et al.*, 2000).

Through the years, PCR techniques have undergone significant improvements for faster detection with the development of real-time PCR for monitoring PCR amplification products, in addition to the methods of simultaneous detection such as multiplex PCR and oligonucleotide DNA microarray that can detect up to five or more pathogens simultaneously (Chen *et al.*, 2012) such as *Salmonella enteritidis, S. aureus, Shigella flexneri, L. monocytogenes*, and *E. coli* O157:H7 (Kim *et al.*, 2007).

Presently, as shown in **Table 2.1,** there is an important selection of commercially available kits based on nucleic acid methods for the detection of foodborne pathogens. However, although these techniques are automated for reliable results and characterized with high sensitivity and specificity, they induce some disadvantages such as difficulties to differentiate viable from nonculturable cells and the design of the primers. In some case, they require trained staff in order to minimize the occurrence of cross-

contamination. According to (Leone *et al.*, 1998), the isothermal amplification method for nucleic acids, NASBA, an amplification system for RNA analytes (e.g., viral genomic RNA, mRNA or rRNA) could be extended from viral diagnostics to the gene expression and cell viability. Despite, the low cost of these methods and the non-requirement of thermal cycling system, post-NASBA product detection is still considered labor-intensive.

Otherwise, the LAMP method, can provide a large amount, usually 10^3 higher to simple PCR, of DNA with rapidity under isothermal conditions (Zhao *et al.*, 2014), lower detection limits compared to conventional PCR (Xu *et al.*, 2012) (Zhao *et al.*, 2010) and higher specificity due to the use of four primers targeting six specific regions (Hara-Kudo *et al.*, 2005).

Table 2.1: Commercially nucleic acid-based methods available for the detection of foodborne pathogens

Pathogen	Method	Commercially available kits	Sensitivity	Catalog number	Sample matrix	Company
Staphylococcus	PCR	BAX® System Real-Time PCR Assay	10^4 CFU/mL, after enrichment	D12762689	Powdered infant formula, ground beef, soy protein isolate	HYGIENA
Salmonella spp.	PCR	BAX® System Standard PCR Assays for *Salmonella*	10^4 CFU/mL, after enrichment	D11000133 - D14368501	Poultry, dairy, fruits, vegetables, bakery products, pet food--and environmentals	HYGIENA
Salmonella spp.	Real-Time PCR	BAX® System Real-Time PCR Assay for *Salmonella*	10^4 CFU/mL, after enrichment	D14306040	Meat, poultry, dairy, fruits, vegetables, bakery products, pet food--and environmentals	HYGIENA
E. coli O157:H7	multiplex PCR	BAX® System PCR Assay for *E. coli* O157:H7 MP	10^4 CFU/mL, after enrichment	D12404903	Raw ground beef, beef trim, produce	HYGIENA
Salmonella	DNA hybridization test	GeneQuence® for *Salmonella*	1–5 CFU/25 g	6700 -	food and environmental samples	NEOGEN
stx and eae genes - STEC Screening	Real-time PCR assay	BAX® System Real-Time PCR STEC Assay	10^4 CFU/mL, after enrichment	D14642964	Raw ground beef, beef trim, produce	HYGIENA
E. coli O26, O111, O121 -	Real-time PCR assay		10^4 CFU/mL, after enrichment	D14642970	Raw ground beef, beef trim, produce	HYGIENA
E. coli O45, O103, O145	Real-time PCR assay		10^4 CFU/mL, after enrichment	D14642987	Raw ground beef, beef trim, produce	HYGIENA
E. coli O157:H7	Real-time PCR assay	BAX® System Real-Time PCR Assay for *E. coli* O157:H7	10^4 CFU/mL, after enrichment	D14203648	Raw ground beef, beef trim, produce	HYGIENA
Listeria spp	PCR	BAX® System *Listeria* spp	10^5 CFU/mL, after	D11000147	Food and environmentals	HYGIENA

			enrichment			
Listeria spp (except L. grayii)	PCR	BAX® System PCR Assay for Genus *Listeria* 24E	10^4 CFU/mL, after enrichment	D13608135	Dairy, meat, fish, vegetables, environmentals	HYGIENA
Listeria species	Real-time PCR assay	BAX® System Real-Time PCR Assay for Genus *Listeria*	10^4 CFU/mL, after enrichment	D15131113	Dairy, ready-to-eat meat, seafood, vegetables, environmentals	HYGIENA
Listeria monocytogenes	PCR	BAX® System PCR Assay for *L. monocytogenes*	10^5 CFU/mL, after enrichment	D11000157	Variety of food types	HYGIENA
Listeria monocytogenes	PCR	BAX® System PCR Assay for *L. monocytogenes* 24E	10^4 CFU/mL, after enrichment	D13608125	Dairy, meat, fish, vegetables, environmentals	HYGIENA
Listeria monocytogenes	Real-time PCR assay	BAX® System Real-Time PCR Assay for *L. monocytogenes*	10^4 CFU/mL, after enrichment	D15134303	Dairy, ready-to-eat meat, seafood, vegetables, environmentals	HYGIENA
Listeria spp.	DNA hybridization test	GeneQuence® for *Listeria*	1–5 CFU/25 g	6708	Food and environmental samples	NEOGEN
Listeria monocytogenes	DNA hybridization test	GeneQuence® for *L. monocytogenes*	1–5 CFU/26 g	6709	Food and environmental samples	NEOGEN

(modified from (Valderrama *et al.*, 2016).

4.2. Immunology-based methods

The most successful and popular technology in the field of the detection of bacterial cells, spores, viruses and toxins is represented by immunological methods. This technology is faster, more robust and has the ability to detect contaminating organisms as well as their biotoxins. However, they are less specific and less sensitive than nucleic acid-based detection (Iqbal *et al.*, 2000). Compared to traditional counting methods, antibody-based methods generate less assay time but present a lack of ability to detect microorganisms in “real-time” mode if the quantity of pathogens is not high enough to provide real-time information. As reported by (Meng & Doyle, 2002), problems that may emerge are the low sensitivity of the assays, low affinity of the antibody to the pathogen or other analytes being measured, and potential interference from contaminants.

Among other immunological methods, both ELISA and lateral flow immunoassay are mainly used for the detection of foodborne pathogens. ELISA is specific and labor saving as it allows the detection of bacterial toxins and can handle large number of samples. However, this technology presents several disadvantages such as the need for trained staff and the possibility of false negative results due to the cross-reactivity with closely related antigens. As immunoassays rely on the specific binding of an antibody to an antigen, the response of the test depends on the amount of the antigen in the sample and the availability of the binding sites. Thus, the low sensitivity of this technology, in the field of the detection of foodborne pathogens, requires a pre-enrichment step to reach a detectable level of antigen in the sample as well as a labelling of antigens and antibodies (Park *et al.*, 2014; Zhang, 2013). On the other hand, lateral flow assay is low cost, reliable, easy-to-operate, sensitive, specific and allows the detection of bacterial toxins but still requires labelling of antigens and antibodies (Zhao *et al.*, 2014). Commercialized kits of these two techniques are summarized in **Table 2.2**. Towards the progress of

rapid methods, new antibody-based methods have been coupled with other methods for pathogen detection, such as immunomagnetic separation on magnetic beads coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) for detection of staphylococcal enterotoxin B (Schlosser *et al.*, 2007) and combination of immunomagnetic separation with flow cytometry for the detection of *L. monocytogenes* (Hibi *et al.*, 2006).

Table 2.2: Commercially immunologically based methods available for the detection of foodborne pathogens

Pathogen	Method	Commercially available kits	Sensitivity	Catalog number	Sample matrix	Company
Shiga Toxin-producing *E. coli* (STEC) including *E.coli* O157:H7 and Verotoxin	Lateral flow Assay	Food check *E.coli* O157 test kit, Carcass Sponge Kit, Assay Cassettes	1 CFU/375 g of ground beef	FCEC-001, FCEC-005, FCEC-006	Raw ground beef, beef trims and carcass	Foodchek Systems Inc
		RapidChekO *E. coli* O157 (including H7) Test Kit	1 CFU/25 g of food.	7000157, 7000158, 7000161, 7000165	Boneless beef trim and ground beef	Romer Labs
		Transia Card *E.coli* O157	-	-	Raw ground beef Raw beef product	Raisio Diagnostics
		Reveal® for *E. coli* O157:H7	1 CFU/25 g; 1 CFU/375 g	9714		NEOGEN
	Enzyme-Linked Immuno Sorbent Assay	3MTM TecraTM *E. coli* O157 VIA	1-5 CFU/25 g sample	ECOVIA48 ECOVIA96	NR	3M Canada
		Assurance® EIA EHEC	-	4000 01	Meat, dairy, poultry, fruit, nuts, and more	BioControl
Listeria	Enzyme-Linked Immuno Sorbent Assay	3MTM TecraTM *Listeria* VIA	1-5 CFU/25 g sample or 1-5 CFU/swab	LISVIA48	NR	3M Canada
		Assurance *Listeria* EIA	-	67000-96	Environmental surfaces and food samples.	BioControl
	Lateral flow Assay	Reveal®2.0 for *Listeria*	1 CFU/analytical unit	9707	Food and environmental samples	NEOGEN

Salmonella spp	Enzyme-Linked Immuno Sorbent Assay	3MTM TecraTM *Salmonella* Visual Immunoassay (VIA)	1-5 CFU/25 g sample	SALVIA48	All Foods	3M Canada
		3MTM TecraTM *Salmonella* ULTIMA VIA	1-5 CFU/25 g sample	SALULT96	All Foods	3M Canada
		MaxSignal® *Salmonella* Test Strip Kit	1x10^5 CFU - 1x10^6 CFU/mL	BO_1063-01	Food and Feed Products	Bioo Scientific
	Lateral flow Assay	RapidChek® *Salmonella*	-	7000183-7000167	Raw ground beef (25 g, 375 g), raw ground chicken, chicken carcass rinsates, liquid eggs, sliced cooked turkey, environmental samples and peanut butter.	SDIX
		RapidChek® SELECT™ *Salmonella*	-	7000190 - 7000195 - 7000198		SDIX
		RapidChek® SELECT™ *Salmonella enteritidis*	-	7000220 - 7000222	Food samples	SDIX
		TRANSIA™ PLATE *Salmonella* gold	-	SA0180	All foods	BioControl
		Reveal® 2.0	1 CFU/analytical unit 10^6 CFU/mL post enrichment	9706	Chicken carcass rinse, raw ground turkey, raw ground beef, hot dogs, raw shrimp, ready-to-eat meal products, dry pet food, ice cream, fresh spinach, cantaloupe, peanut butter, swabs from stainless steel surfaces, and sprout irrigation water	NEOGEN

Staphylococcus aureus	Enzyme-Linked Immuno Sorbent Assay	3MTM TecraTM *S. aureus* VIA (3M)	1-5 CFU/25 g sample	STAVIA96	Food samples	3M Canada
		3MTM TecraTM Staph Enterotoxin VIA (3M)	1 ng/mL of sample extract	SETVIA48	Food samples	3M Canada
	Lateral flow Assay	TRANSIA® PlATe Staphylococcal Enterotoxins	0.25 ng S. enterotoxins/g sample	ST0796	Milk and dairy products	BioControl
		TRANSIA™ PLATE Staphylococcal Enterotoxins Plus	0.25 ng S. enterotoxins/g sample	ST0777	Milk and dairy products	BioControl
		TRANSIA™ PLATE Staphylococcal Enterotoxins ID	20-60 pg/mL of each serological group (A-E)	ST0712	Milk and dairy products, Meat, poultry and eggs, Seafood and other foods, Feed products	BioControl
		TRANSIA®IAc Staphylococcal Enterotoxins	0.1ng S.enterotoxins/g sample	ST0705	Milk and dairy products	BioControl
		TRANSIA® TUBe Staphylococcal Enterotoxins	0.5 ng S. enterotoxins/g	ST724B	Milk and dairy products	BioControl

NR: not reported

(modified from(Valderrama *et al.*, 2016).

4.3. Biosensors

Nowadays, the use of biosensors is increasing in the field of food pathogen detection using nucleic acid- and immunology-based methods considered as conventional ones. In recent years, there has been much research activity in the area of biosensors development for detecting pathogenic microorganisms. Comparing to standard methods, biosensors are more favorable for checking the safety foods, throughout the production process, due to their real time response (Bahadır & Sezgintürk, 2015). Biosensors are a powerful analysis tools covering a wide range of applications particularly food quality monitoring, disease detection, toxins of defenses interest, environmental monitoring, soil quality monitoring, drug discovery and prosthetic devices (Bhalla *et al.*, 2016).

As defined by (Velusamy *et al.*, 2010), biosensor devices are constituted with two main parts: the bioreceptor (biological material recognizing the analyte) and the transducer (converting the bio-recognition energy into optical or electrical signals). A bioreceptor can be a microorganism, cell, enzyme, antibody, nucleic acid, aptamers or biomimic. However, the transduction may be optical, electrochemical, thermometric, piezoelectric, magnetic and micromechanical or combinations of the above techniques.

The classification of the several types of biosensors is based on their bioreceptors or transducers, as described by (Velusamy *et al.*, 2010). Electrochemical, mass-based and optical biosensors are the mainly used biosensors for the detection of foodborne pathogens (Zhang, 2013), especially surface plasmon resonance (SPR) biosensors due to their high sensitivity (Velusamy *et al.*, 2010).

Few commercial biosensors for the detection of foodborne pathogens are nowadays available. **Table 2.3** presents the rare commercially available devices of biosensors for food analysis (da Costa Silva *et*

al., 2013). Unlike nucleic-acid-based methods and immunological methods, biosensors are easy-to-operate and they do not require any pre-enrichment step (Singh *et al.*, 2013).

Optical biosensors are very suitable for the detection of pathogens substances in food as they detect analytes with no need of special sample treatment even in complex matrices, in addition to the lower interference and the low loss of signal. As described by (Narsaiah *et al.*, 2012), optical biosensors are based on the measurement of the change in amplitude, phase, frequency or polarization of light. Also, optical devices are more specific and more sensitive than the other biosensors, with a compact design minimally invasive. However, the enhancement of stability of immobilized biocomponents is still a challenge. The main inconvenient of these biosensors is that their commercialization is slower than other rapid methods due to several factors such as their high cost in quality assurance, stability and sensitivity issues, and instrumentation design (Velasco-Garcia & Mottram, 2003).

Electrochemical biosensors, the second type of biosensors, can handle large numbers of samples and are label-free detection devices but they have low sensitivity, and analysis may be interfered with by food matrices in addition to the many washing steps required, all not suitable for analyzing samples containing low amount of microorganisms. Finally, mass-based biosensors are cost-effective, easy-to-operate, label-free and real-time detection devices but with low specificity and low sensitivity with a long incubation time of bacteria and many required washing/drying steps, in addition to the regeneration of the crystal surface which may be problematic (Ivnitski *et al.*, 1999).

Table 2.3: Commercially available biosensors devices for the detection of foodborne pathogens.

Pathogen	**Method**	**Commercially available kits**	**Sensitivity**	**Sample matrix**	**Company**	**references**
Escherichia coli 0157:H7	*Optical immunosensor based on selective antibody expressed by human cell line*	CANARY™ system	*500 CFU/g*	Lettuce	Massachusetts Institute of Technology	(Anonyme, 2004)
Escherichia coli O157:H7 and *Salmonella*	Eectrochemical immunosensor based on the assembly of three nanoparticle	Michigan State Electrochemical Biosensor:	10^1 to 10^6 CFU/ml	Fresh produce and meat products	Michigan State University	(Wang *et al.*, 2015)
Detection of *Salmonella* and *Campylobacter*	Interferometric Biosensor	Georgia Tech Interferometric Biosensor	5,000 cfu/ml for Salmonella 500 CFU/ml for Campylobacter	Poultry products	Georgia Research Tech Institute	(Anonyme, 2004)
Staphylococcal enterotoxin B and Botuminum toxin A	Fluorescencent immunoassay biosensor	Naval Research Laboratory array biosensor	From 20 to 500 ng/ml for Botuminum toxin A From 0.1 to 0.5 ng/ml for Staphylococcal enterotoxin B	Tomatoes, sweet corn, beans and mushrooms	Naval Research Laboratory	(Sapsford *et al.*, 2005)
Escherichia coli 0157, *Salmonella, Listeria* and *Campylobacter*	Electro-immunoassay biosensor	Detex Pathogen Detection System	NR	Chicken breast	Molecular Circuitry Inc.	(Anonyme, 2000)

CANARY™ *: (Cellular Analysis and Notification of Antigen Risks and Yields)*

(modified from (Ivnitski *et al.*, 1999)

5. Conclusion

The first step to ensure food safety resides in prevention by raising industry and consumer awareness. Few primary daily actions can prevent food diseases. Despite the fact that conventional methods are often regarded as the "Gold standard" for their specificity and reliability, in addition to their low cost and simplicity, they remain time-consuming and laborious. Over the years, many rapid methods for the detection and identification of foodborne pathogens have been developed to overcome the limitations of their conventional counterparts. Several different types of nucleic-based methods, immunology-based methods and biosensors have been developed and discussed in a large number of publications. Each one offers advantages depending on the target pathogen and the food sample. But also, several disadvantages and downfalls have to be solved for practical applications in the food industry.

Comparatively to conventional microbiological methods, rapid commercially available technologies that are sensitive enough to detect pathogens, are expected to be more time-efficient, labor saving and able to reduce human errors significantly. Although they are expensive and require a trained technical staff, they are not yet practical for daily industrial uses.

Nowadays, novel detection methods are released regularly but their acceptance by the industry depends not only on speed but also on initial investment, operating cost, technical support, and usability. Indeed, advanced researches have converged to rise to the challenge of developing new simple, sensitive, specific, time-saving technologies for foodborne pathogens detection that could be practical in the food industry.

CHAPITRE 3:

Development of a support based on chitosan and cellulose nanocrystals Optimization of the monoclonal anti-Shiga toxin 2 antibody immobilization method

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Contribution des auteurs

Amina Baraketi a réalisé les manipulations et la rédaction de l'article.
Sabato D'Auria, Co-Directeur de recherche, a participé à la correction de l'article.
Carole Fraschini a fourni la cellulose nanocrystals et a participé à la correction de l'article.
Stéphane Salmieri a participé au bon déroulement des expériences et à la correction de l'article.
Majid Jamshidian a participé aux analyses des résultats préliminaires par le logiciel statistique.
Marie Christine Etty a participé aux résultats préliminaires de cette étude.
Affef Sahraoui a participé à la préparation des membranes.

Monique Lacroix: Directrice de recherche, responsable scientifique et coordinatrice du projet de recherche, a participé à la planification des expériences et à la correction de l'article.
L'article sera soumis au Journal Carbohydrate Polymer.

ABSTRACT

This work describes the development of a new support system and the optimization of its formulation for the immobilization of antibodies in order to carry out immunoassay detecting of *E. coli* O157:H7. The aim of this study was to evaluate the effect of cellulose nanocrystals (CNC), chitosan (CH) and glycerol (GLY) on the immobilization of antibodies. Further, a factorial fractional design (FFD) was applied to screen the effects of compounds by considering major interactions and significant factors introduced in a Box-Behnken design (BBD) to optimize their concentrations. The study demonstrated that the use of 0.6% (w/w) cellulose nanocrystals improved significantly the immobilization of antibodies. The innovation of this work was the new formulation of an immobilization support for future pathogen detection.

Keywords:

Cellulose nanocrystals, chitosan, antibody immobilization, fractional factorial design, Box-Behnken design.

1. INTRODUCTION

Escherichia coli O157:H7 is one of the top five pathogenic Shiga toxin-producing *E. coli* (STEC) involved in foodborne outbreaks worldwide. The foodborne pathogen causes bloody diarrhea, abdominal cramps and can also lead to hemolytic uremic syndrome (HUS), renal failure, and occasionally to death (Vogt *et al.*, 2005).

The detection of foodborne pathogens in raw food materials and sample surfaces in the food industry still rely on traditional culturing techniques that require usually up to 3 days (Yun (Zhang *et al.*, 2017). Sandwich ELISA is one of the most commonly effective immunological methods used for the detection of foodborne pathogens (Zhao *et al.*, 2014). A primary very selective antibody is usually immobilized onto the walls of the micro-plate wells for the capture of the antigen (Zhang *et al*, 2013). Despite the development of an automated ready-to-use format, a liquid enriched sample is still needed to get results of detection, (Glynn *et al.*, 2006). Considering that these systems are limited by the low volume required, there is an urgent need for the development of a new adaptable immobilization for a wide range of sample volumes to overcome this limitation.

Cellulose nanocrystals (CNC) represent the amorphous crystalline region of cellulose, the most abundant biopolymer in the world (Lu *et al.*, 2010). The production of CNC, considered renewable, low energy and non-toxic for human health and the environment. (Mariano *et al.*, 2014), has increased significantly in several industrial applications such as cosmetic products as well as food products and packaging (Endes *et al.*, 2016). Mostly produced in North America, CNC also presents a high commercial potential for biomedical products applications such as enhancers for covalent attachment of active molecules, compatibilizers for interactions between materials and human tissues and co-stabilizers of polymeric excipients. In addition to its nano-reinforcement, biological and mechanical properties, CNC is characterized with a large surface chemical reactivity allowing the adsorption of proteins due

to the negative sulfate esters (Lin *et al.*, 2014). Furthermore, the reactive hydroxyl side groups allow hydrogen bonding interactions of CNC with other matrices (George *et al.*, 2015) along with as higher mechanical properties and a lower density making this stiff nanoparticle considered as one of the most interesting reinforcing polymer filler (Moon *et al.*, 2011).

Chitosan is considered nowadays as one of the most abundant polysaccharide. This biopolymer has a linear structure showing α-(1→4)-linked 2-amino-2-deoxy-D-β-glucopyranose, which is obtained from *N*-deacetylation of chitin and is characterized by reactive amino groups. As a seafood waste product, chitin and chitosan have shown interesting chemical and biological properties in several industrial applications specifically in water engineering, pulp and paper, textile, food processing, agriculture (Dutta *et al.*, 2004), pharmaceuticals, bio-medicals, cosmetics, dermatological field and enzyme immobilization (Hamed *et al.*, 2016). However, compared to synthetic polymers, chitosan films exhibit poor mechanical and barrier properties confirming that the incorporation of nanofillers and plasticizers is essential to improve films properties for efficient applications (Azeredo *et al.*, 2010). Moreover, previous studies showed that the molecular weight and deacetylation degree of chitosan, as well as glycerol (Fundo *et al.*, 2011) and chitosan concentrations (Park *et al.*, 2002) affect the physicochemical properties of chitosan-based films.

Chitosan, has been widely used for several applications including cells, enzymes and particularly antibodies immobilization (Ricardi *et al.*, 2018). Due to the hydroxyl and amino groups resulting from the deacetylation of the chitin, chitosan is an excellent biopolymer for adsorption and covalent linkage. Activated by the glutaraldehyde, chitosan can be used as an immobilization support in various forms: gels, membranes and particularly as nanocomposite beads due to their important contact surface. For beads application, alginate was cited as necessary for its binding properties to calcium. Indeed, it has been reported that alginate-chitosan based beads showed promising results for their application as proteins

immobilization support. Although the immobilization of proteins on chitosan-based beads has been very successful, their stability is affected by ions that can induce conformational changes of the proteins (Albarghouthi *et al.*, 2000, Singh *et al.*, 2011).

Plasticizers are usually used not only to improve water vapor barriers properties but also to overcome film brittleness and to avoid film cracking (Suyatma *et al.*, 2005). Despite the existence of several other plasticizers, such as fatty acids and sorbitol, glycerol was selected as a suitable plasticizer, for this study, due to its good plasticization efficiency, large availability, and low exudation (Epure *et al.*, 2011). Furthermore, among the main non-volatile plasticizers, glycerol, sorbitol, propylene glycol or polyethylene glycol (Kolhe *et al.*, 2003), the choice of glycerol as a membrane stabilizer is based on its humectant properties, low volatility and high hydrophilicity providing a good polar affinity with chitosan and CNC. Glycerol is known to improve the flexibility of films by reducing intermolecular forces and increasing the mobility of the polymer chains, but it can also increase membrane dehydration by the same mechanism (Srinivasa *et al.*, 2007). Furthermore, recent studies have shown that imidazolium ionic liquid could be used as a better performing plasticizer of chitosan-based films by generating lower water vapor permeability than in the presence of glycerol. However, the inconvenience is that this plasticizer reacts with chitosan amino groups and may consequently decrease the immobilization process onto the chitosan surface (Boesel, 2015).

The aim of the study was to develop and optimize a novel chitosan/CNC-based nanocomposite support for antibody immobilization as a first part of the Sandwich ELISA method for the detection of pathogenic bacteria onto industrial food surfaces. In this work, the effect of film composition *i.e.* chitosan parameters, Glycerol and CNC concentrations, as well as their interactions, were evaluated in order to determine an optimal film formulation enhancing the immobilization of antibodies, and therefore improving the detection of *Escherichia coli* O157:H7 into food surfaces, with a high specificity.

2. MATERIALS AND METHODS

2.1 Materials

High-purity chitosan (CHI) for pharmaceutical applications was purchased from Heppe Medical Chitosan GmbH, (HMC^+, Halle, Germany). Nanocellulose crystals (CNC) were provided by FPInnovations (Pointe-Claire, QC, Canada). Monoclonal antibodies (mAb) were produced by Genscript USA Inc. (Piscataway, NJ, USA). Horseradish peroxidase (HRP) conjugated anti-mouse secondary antibodies were purchased from Jackson Immuno Research (PA, USA). Glycerol (GLY) and sulfuric acid were from Laboratoire Mat (Beauport, QC, Canada). Tripolyphosphate (TPP) and glutaraldehyde (GA) solutions were from Sigma-Aldrich (Mississauga, ON, Canada).

2.2 Methodology

2.2.1. Preparation of the membrane as a support for antibody immobilization

CNC was suspended in distilled water for 1 h at room temperature under magnetic stirring. After addition of acetic acid 1% (v/v), sonication (1 kJ/g of CNC), CHI and GLY were added. The suspension was stirred for 4 h at room temperature under vigorous stirring. Once the mixture was completely homogenized, the suspension was cast in a polystyrene 24-well micro-plate and air dried at room temperature for 5 days until the membrane became completely dried. After complete drying, 1 mL of 10% tripolyphosphate (TPP) solution was added for 10 min under stirring and then rinsed with distilled water to suppress TPP residues. Then, 300 µL of 0.2% (v/v) of glutaraldehyde (GA) were added and the micro-plate was incubated for 2 h at room temperature then rinsed with distilled water to eliminate GA residues. After 3 washings with phosphate buffered saline-Tween® (PBS-T), 1% (w/v) bovine serum albumin (BSA) in PSB was added to each well to block non-specific binding sites and

the micro-plate was stored at 4 °C overnight. The obtained membrane was designated as CCG membrane.

2.2.2. Measurement of the detection signal of immobilized antibody

Immobilized antibodies were detected using polyclonal anti-mouse as a secondary antibody coupled to HRP. BSA was used as a protein blocking buffer to bind to free open spaces on the surface of the membrane and to block them in order to reduce non-specific binding in the step of the detection of immobilized antibodies. Then, the wells were washed once with PBS-T for 10 min. Thereafter, the secondary antibody was diluted (1/10000) in PBS-T with 0.25% BSA and was added and incubated for 30 min at 37 °C under shaking. After 3 washes with PBS-T, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added as substrate for the horseradish peroxidase (HRP). For all immobilization experiments, the reaction time of horseradish peroxidase (HRP)-TMB (enzyme-substrate) allowed was 1 min. Then, the reaction was stopped by the addition of 2M H_2SO_4. Results were determined by transferring 300 µL of each well solution to a 96-well micro-plate and absorbance was read at 450 nm using a Biotek micro-plate reader (BioTek Instruments Inc., Winooski, VT, USA) and Gen 5 2.07 software.

2.2.3. Effect of the nanocomposite components and their concentrations on antibody immobilization

2.2.3.1. Effect of the chitosan molecular weight, deacetylation degree and concentration on antibody immobilization

As a first step, membranes were prepared as described previously with addition in the formulation solution, 2% (w/v) CHI and 0.6% (w/v) GLY, using 3 several CHI differing by their molecular weight and deacetylation degree: HMC CHI 85/1000 (% deacetylation degree/viscosity mPa.s), HMC CHI 85/2500 and HMC CHI 95/2500, thereby suggesting proportional reactive amino groups with the deacetylation degree and proportional viscosity of film suspension with the molecular weight of CHI. Monoclonal antibodies were used at a

concentration of 20 µg/ml. Then, 1, 2 and 4% (w/v) of 95/2500 HMC CHI were compared in the film formulation in presence of 0.6% (w/w) GLY.

2.2.3.2. Effect of the volume of chitosan solution on antibody immobilization

The volume of CHI solution cast in each well has an influence on the membrane thicknesses. The membranes were prepared as described previously with addition in the formulation solution of 2% (w/v) CHI and 0.6% (w/v) GLY, and volumes of 0.5, 1 and 2 mL were cast per well.

2.2.3.3. Effect of the concentration of cellulose nanocrystals on antibody immobilization

The membranes were prepared as described previously with 0.2, 0.4, 0.6, 0.8 and 1% (w/v) of CNC. Negative control was prepared without CNC. All these membrane solutions contain 0.6% (w/v) GLY and 2% (w/v) 95/2500 CHI. Monoclonal antibodies concentrations tested were 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL and 32 µg/mL. After 2 h incubation at room temperature, the supernatant of each well of the first immobilization was transferred in another 24-well micro-plate with the same membrane formulation as used for the first immobilization. The same immobilization steps were applied for the first and each corresponding second plate. The interest of this step was to estimate the signal intensity obtained from the quantity of non-immobilized antibody for each concentration and to compare it to the corresponding asset of the first signal.

2.2.3.4. Effect of the concentration of glycerol on antibody immobilization

The membranes were prepared as described previously with the presence of 2% (w/v) 95/2500 HMC CHI reinforced with 0.6% (w/v) CNC using 0.2, 0.6 and 1% GLY in the formulation solution. For this experiment, monoclonal antibodies were used at a concentration of 20 µg/ml.

2.2.4. Fractional factorial design

Five (5) factors with 2 variation levels were incorporated into a 2-level fractional factorial design (FFD) by Design Expert Software 7.0.0, to evaluate the effect of each membrane parameter on the antibody immobilization after analysis of variance (ANOVA). Level of significance was considered at p-value ≤ 0.05. Studied factors, summarized in **Table 3.1**, included: concentration of CHI (*A*), concentration of CNC (*B*), concentration of GLY (*C*), thickness (*D*), and concentration of antibody *(E)*. As the 3rd resolution FFD neglects interactions between 3 or more factors, the test was constituted of 29 runs including 3 repetitions and 5 central points compared to 96 runs required for a full factorial design.

Table 3.1: Summary of 2-Level Factorial Fractional design (FFD with reduced 2FI) .

Factor	Name	Units	Low Actual	High Actual
A	Chitosan concentration	(% w/w)	1.00	2.00
B	Nanocellulose concentration	(% w/w)	0.000	0.60
C	Glycerol concentration	(% w/w)	0.20	0.60
D	Film thikness (weight solution/well)	(g)	0.50	1.00
E	Antibody concentration	(µg/ml)	10.00	40.00

2.2.5. Optimization of the immobilization signal level

Parameters having significant effect on the signal of immobilization identified in the previous section and summarized in **Table 3.2**, were set as parameters for additional optimization. Box-Behnken design (BBD) was applied with the 3 factors previously selected, for the optimization of signal, using Design-Expert Software 7.0.0. Thirteen (13) formulations of CNC- CHI-GLY (CCG) membranes including 3 center points were analyzed. The aim of

using Box-Behnken design (BBD) lies in its rotability and the less formulation suggested for the experiment especially for sensitive and costly studies. Indeed, this design has been applied for the optimization of spectro analytical method, chromatographic methods, capillary electrophoresis, sorption processes, enzymatic procedures, water disinfection processes and micro-plate assay for detection. Box-Behnken design (BBD) is more efficient than three-level full factorial designs and central composite designs. It avoids experiments performed at their lowest or highest levels that may generate undesirable results (Ferreira *et al.*, 2007).

Table 3.2: Summary of Box-Behnken Design (BBD).

Factor	Name	Units	Low Actual	High Actual
A	Chitosan concentration	(% w/w)	1.00	2.00
B	Nanocellulose concentration	(% w/w)	0.000	0.60
C	Antibodies concentration	(% w/w)	10.00	40.00

3. RESULTS AND DISCUSSION

3.1 Screening of membrane components affecting antibody immobilization

The first step of the screening was the selection of the type of CHI suitable for this work. The characteristics of immobilization with several CHI used in membrane formulation without CNC are presented in **Fig. 3.1** and show that the best signal was obtained for the CHI 95/2500, having both the highest deacetylation degree (95%) and molecular weight (2500 mPa.s corresponding to 250-600 kDa). Lower decreasing signals were obtained with the other CHI, respectively 85/2500 and 85/1000. Therefore, both highest molecular weight and highest deacetylation degree allowed to significantly enhance ($P \leq 0.05$) the signal by 83% (from 85/1000 to 85/2500) and 58% (from 85/2500 to 95/2500) respectively. It can be hypothesized that both CHI-based films (85/2500 and 95/2500) were strengthened by 0.6% CNC and

induced the same level of detection. Other studies reported that increasing the molecular weight of CHI improved the mechanical properties of CHI-based films by increasing the tensile strength (TS) and tensile modulus (TM) (Chen *et al.*, 1996, Park *et al.*, 2002). In addition, Leceta *et al.* (2015) reported that CHI with a high molecular weight showed an improved stability of derived films during storage. Consequently, in order to maximize the immobilization signal and thereby enhance the detection signal level, the CHI 95/2500 was selected for the next steps of this study.

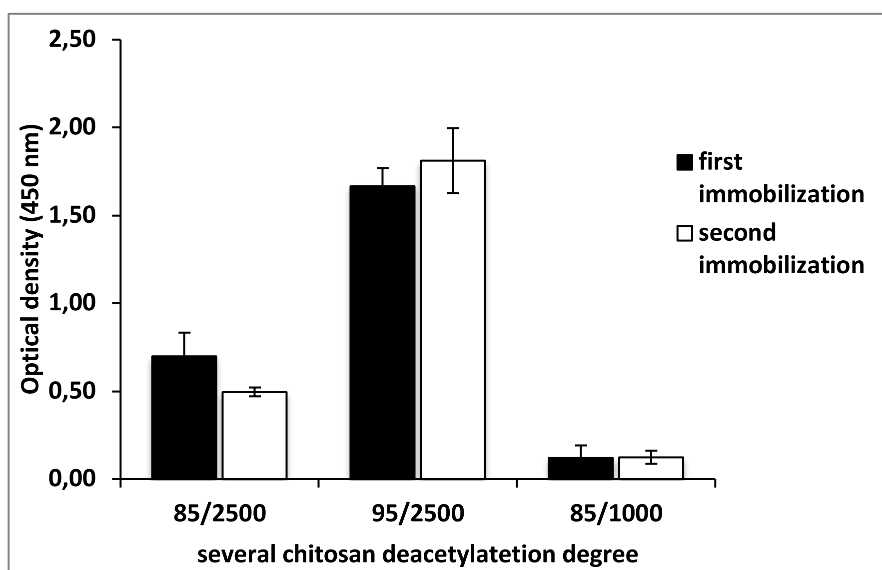

Figure 3.1: Effect of the deacetylation degree and molecular weight of CHI on the immobilization signal of 20 µg/ml Anti-Shiga toxin 2B monoclonal antibodies.

The second step consisted in the determination of the optimal concentration of the selected CHI. **Fig, 3.2** shows that increasing the concentration of CHI from 1 to 2% (w/v) in film-forming suspension increased the antibody immobilization signal by 70%. In this context, increasing the CHI concentration can improve the mechanical properties of films, as reported by other studies (Ren *et al.*, 2017).

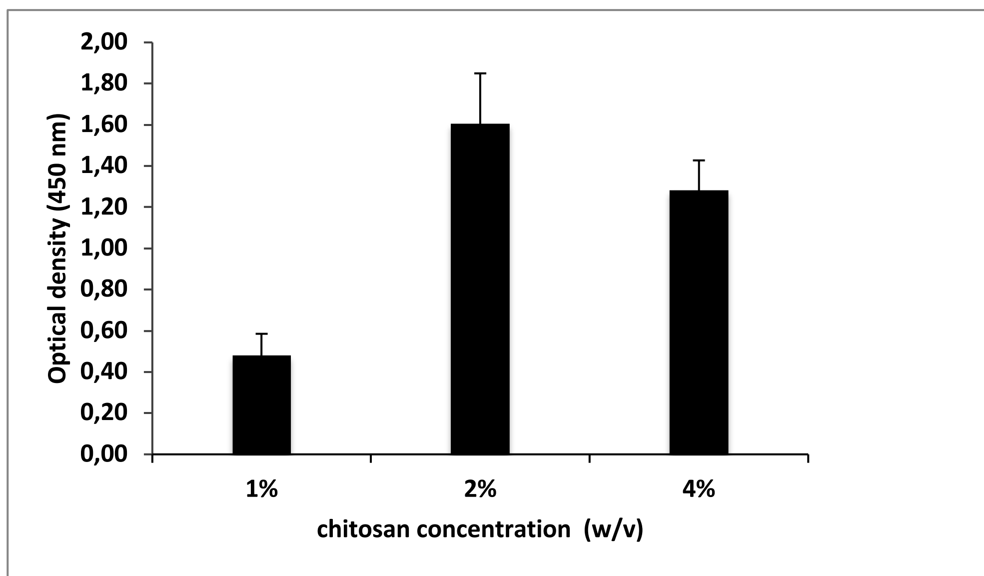

Figure 3.2: Effect of the concentration of CHI on the immobilization signal of 20 µg/ml Anti-Shiga toxin 2B monoclonal antibodies.

Results of varying the GLY concentration (**Fig. 3.3**) showed that the incorporation of 0.6% GLY increased the immobilization signal by 29% compared to that obtained with the control (membrane without GLY). Beyond 0.6% GLY, no significant improvement was observed ($P > 0.05$).

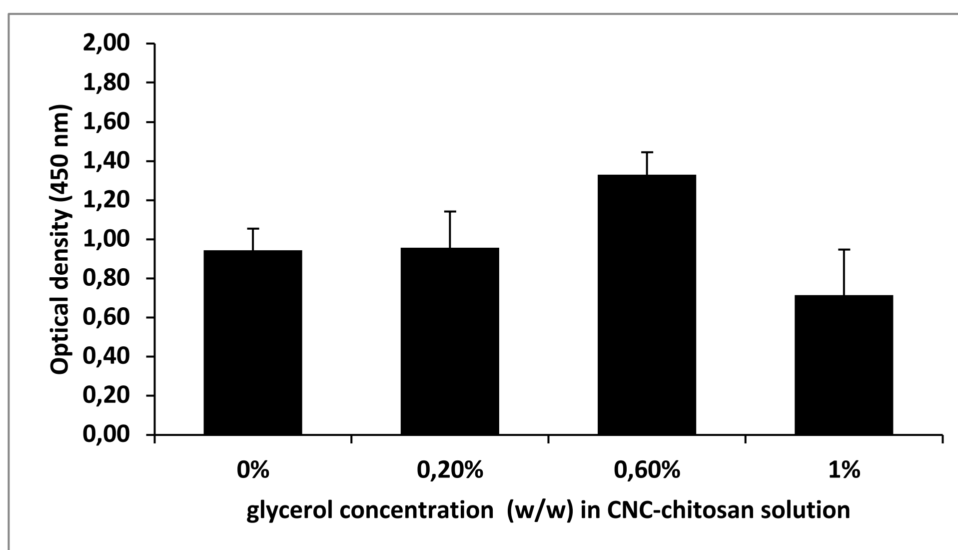

Figure 3.3: Effect of the concentration of GLY on the immobilization signal of 20 µg/ml Anti-Shiga toxin 2B monoclonal antibodies.

Varying the CNC concentration (**Fig. 3.4**) showed that for the same concentration of monoclonal antibodies, the signal increased significantly by increasing the CNC concentration ($P \leq 0.05$). Furthermore, for the same concentration of CNC, the signal also increased significantly with the concentration of monoclonal antibodies ($P \leq 0.05$). The maximum immobilization level was observed at the maximum tested concentration of 32 μg/mL of antibodies with a film containing 0.6% CNC (w/v). Beyond this concentration, a little reduction of signal was measured with 0.8% ($P > 0.05$) and 1% CNC ($P \leq 0.05$). Thus, it is suggested that CNC improved significantly the signal of antibody immobilization while reinforcing the mechanical properties of the CCG membrane. The lower signal with CNC concentrations > 0.6% can be explained by side-effects caused by CNC agglomeration. Khan *et al.*, (2012) mentioned that strong interactions (mainly hydrogen bonding) between CNC and CHI matrix generated a significant ($P \leq 0.05$) increase of TS and TM up to 24 and 87% respectively compared to the control (CHI-based films without CNC). This effect was clearly shown with an addition of only 5% of CNC (w/w, dry material). Also, Huq *et al.* (2012) confirmed the effect of CNC addition in alginate-based films by reporting that the addition of CNC up to 5% (w/w) to alginate based-films promoted a significant increase of TS and TM up to 37 and 75% respectively compared to native films. Moreover, both studies confirmed that the presence of CNC decreased the water vapor permeability of the films.

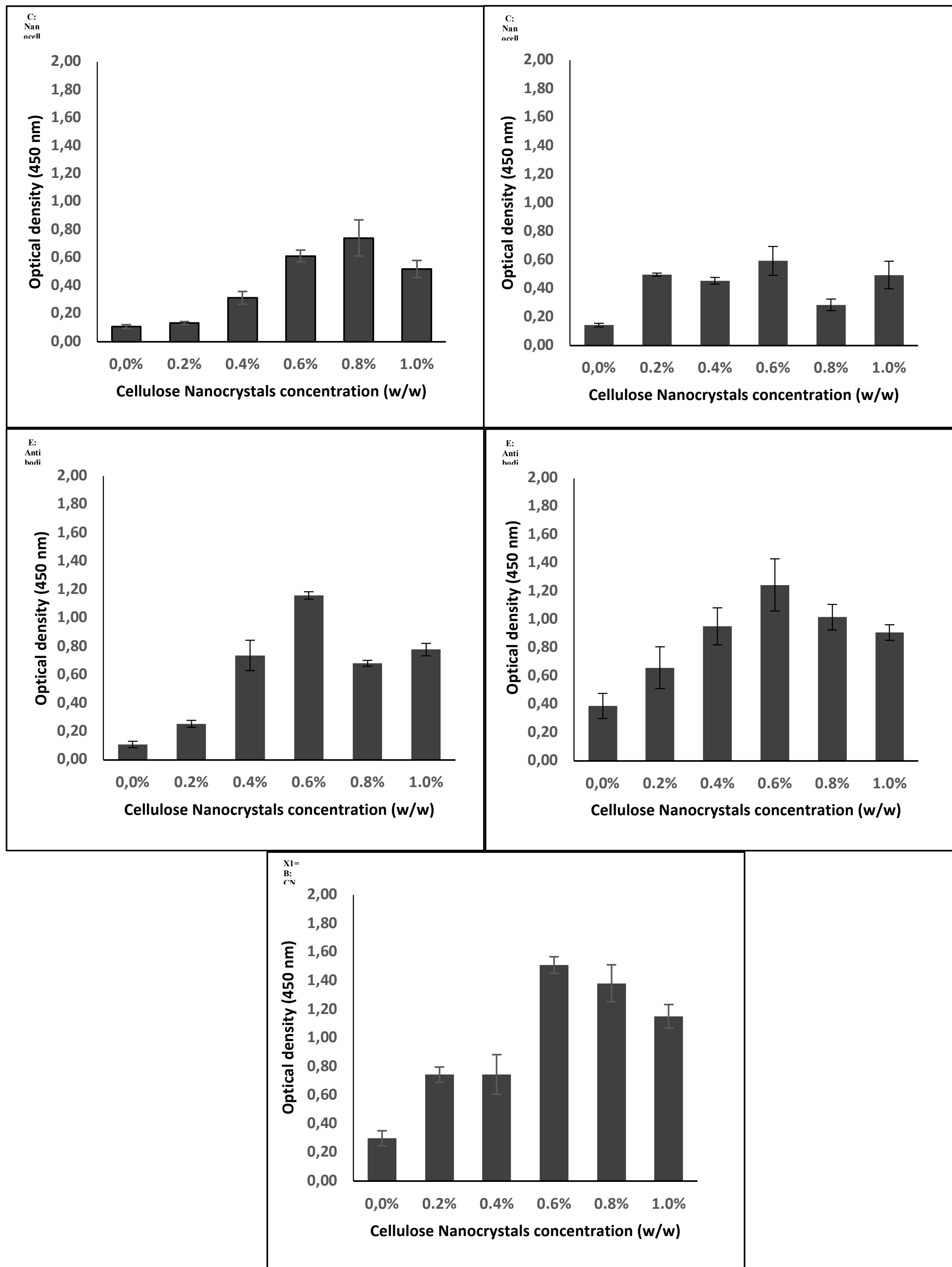

Figure 3.4: Effect of the concentration of CNC on the immobilization signal of Anti-Shiga toxin 2B monoclonal antibodies: a) 2 μg/mL, b) 4μg/mL, c) 8 μg/mL, d) 16 μg/mL, e) 32 μg/mL.

The increase of signal immobilization could be explained, also, by the improvement of the availability of the chitosan amino groups on the CCG membrane surface. In this context, in addition to the reinforcement properties in polymer matrices, it has been reported that the incorporation of the CNC to chitosan films improves their microstructure with better homogenization on the film surface (Khan *et al.*, 2012). Combining the high specific surface area of the CNC to functional amino groups of the chitosan, increases the immobilization on the nanocomposite surface (Mohammed, 2017). This hypothesis is based on the influence of both CNC concentrations and CHI molecular weight suggesting that their interaction allowed a higher availability of reactive amino groups from CHI film by increasing the contact surface and consequently the signal of immobilization.

During the experiments, it was noted that the appearance of the CCG membrane between throughout the immobilization reaction, suggesting that the step of insolubilization with TPP, GA and incubation time had an effect on the membrane structure. CNC, acting as a nanofiller, improved considerably the mechanical properties of the CCG membrane, which also resulted in an enhancement of antibody immobilization but also a higher resistance of the membrane to the insolubilization treatments facilitating a possible dehydration of the membrane. In agreement with the results showed in **Fig. 3.5**, an increase of film thickness related to volumes from 0.5 to 1 mL volumes in the micro-plate well resulted in exalting the immobilization signal level by 35%. However, for a volume of 2 mL (higher thickness), the signal decreased by 65% compared to the optimal signal obtained with 1 mL (optimal thickness).

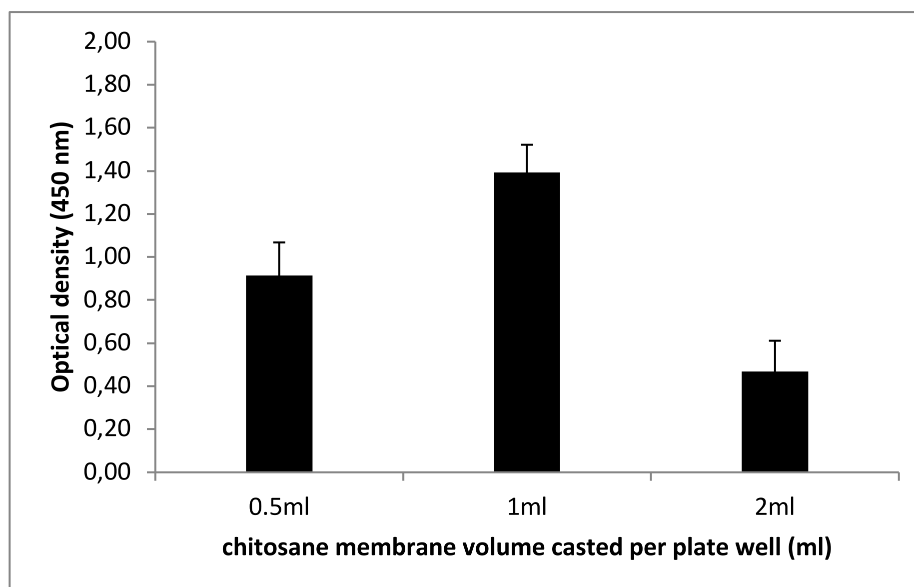

Figure 3.5: Effect of the thickness of the CCG membrane on the immobilization signal of 20 µg/ml Anti-Shiga toxin 2B monoclonal antibodies.

At this step of the study, results showed that the highest immobilization signal was obtained with 32 µg/mL of antibodies, using a CCG membrane composed of 2% (w/v, in film suspension) CHI 95/2500, 0.6% CNC and 0.6% GLY in film formulation under solution. Hence, this composition was selected for the subsequent steps of investigation. However, preliminary results with this optimal composition also indicated that the detection level increased with higher concentrations of antibody (> 32 µg/mL), hence requiring the determination of the maximum detection signal. From this, the amount of non-immobilized antibodies had to be determined. Results in **Fig. 3.6** show that the immobilization signal increased significantly ($P \leq 0.05$) with the concentration of antibody. But it also rises the signal of non-immobilized antibody for the same concentration. In order to verify this artifact, the signals obtained for the concentrations of 40, 60, 80 and 100 µg/ml were compared to the concentration showing the least loss of antibody at the first immobilization (20 µg/ml) representing the signal difference between the first and second immobilization at these concentrations (**Fig. 3.2**). This comparison shows that the concentration of 20 µg/ml of

antibody provided the lowest loss of antibody from 11% to 51% and therefore can be considered as leading to the highest yield of antibody immobilization.

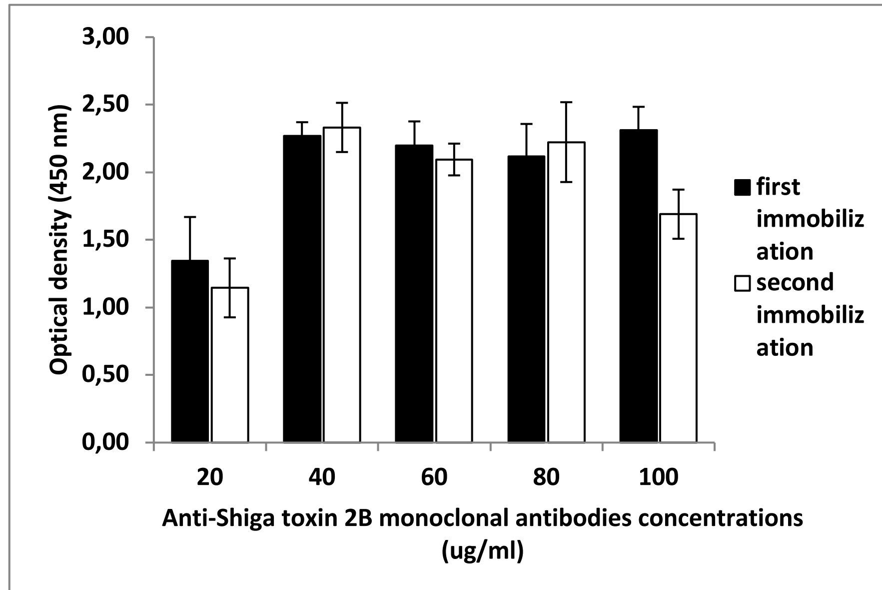

Figure 3.6: Effect of the concentration of Anti-Shiga toxin 2B monoclonal antibodies on the immobilization signal from the CCG membrane.

3.2 Optimization with statistical tools

The experiments were conducted in a randomized order and the analysis of variance (ANOVA) was obtained through 2^{5-3} for the FFD, as presented in **Table 3.3**. The mathematical model, in terms of coded factors, which gives the immobilization signal *(IS)* as function of significant effects, is represented by equation **(1)**:

IS = -0.067056 + 0.15325 *A + 0.72620 * B + 0.19708 * C - 0.030500 * D + 0.035006 * Antibodies - 0.88125 * CNC * Glycerol -6.95370E-003 * CNC * Antibodies (1)

The model F-value of 200.3 implies that the model is robust. Values of "Prob > F" less than 0.05 indicate significant model terms. In this case A, B, E, BC, BE are significant model terms. For simultaneous variations of all factors, thickness had no direct effect which can be

explained by the fact that the variation of the nanofiller concentration itself increases the thickness of the film enough to ensure good immobilization.

Table 3.3: Fractional factorial design (FFD) experiment with ANOVA results.

Source	Sum of Squares	DF	Mean square	F value	P-value
Model	6.18	7	0.88	200.29	< 0.0001
A- Chitosan concentration	0.14	1	0.14	31.99	< 0.0001
B- Nanocellulose concentration	0.086	1	0.086	19.59	0.0003
C- Glycerol	4.347E-003	1	4.347E-003	0.99	0.3324
D- Thickness	1.395E-003	1	1.395E-003	0.32	0.5798
E- Antibodies concentration	5.85	1	5.85	1328.59	< 0.0001
BC	0.067	1	0.067	15.23	0.0009
BE	0.024	1	0.024	5.34	0.0317
Curvature	0.47	1	0.47	5.34	< 0.0001
Pure Error	0.088	20	4.405E-003	107.28	
Cor Total	6.74	28			

The "Curvature F-value" of 107.3 implies that there is a significant curvature (as measured by a difference between the average of the center points and the average of the factorial points)

in the design space. The "Pred R-Squared" equal to 0.97 is in reasonable agreement with the "Adj R-Squared" of 0.98. "Adeq Precision" measures the signal to noise ratio. This ratio of 32.96 indicates an adequate signal. As a ratio higher than 4 is desirable, this model can be used to navigate the design space (Maran *et al.*, 2013). A significant "Curvature F-value" indicates that a Response Surface Methodology is necessary for optimization.

It is interesting to note that two interesting interactions were found: CNC-GLY and CNC-antibody. **Fig. 3.7** illustrates the CNC-GLY interactions and shows that for 0.6% (w/v) GLY, the signal was not affected by the variation of CNC concentration.

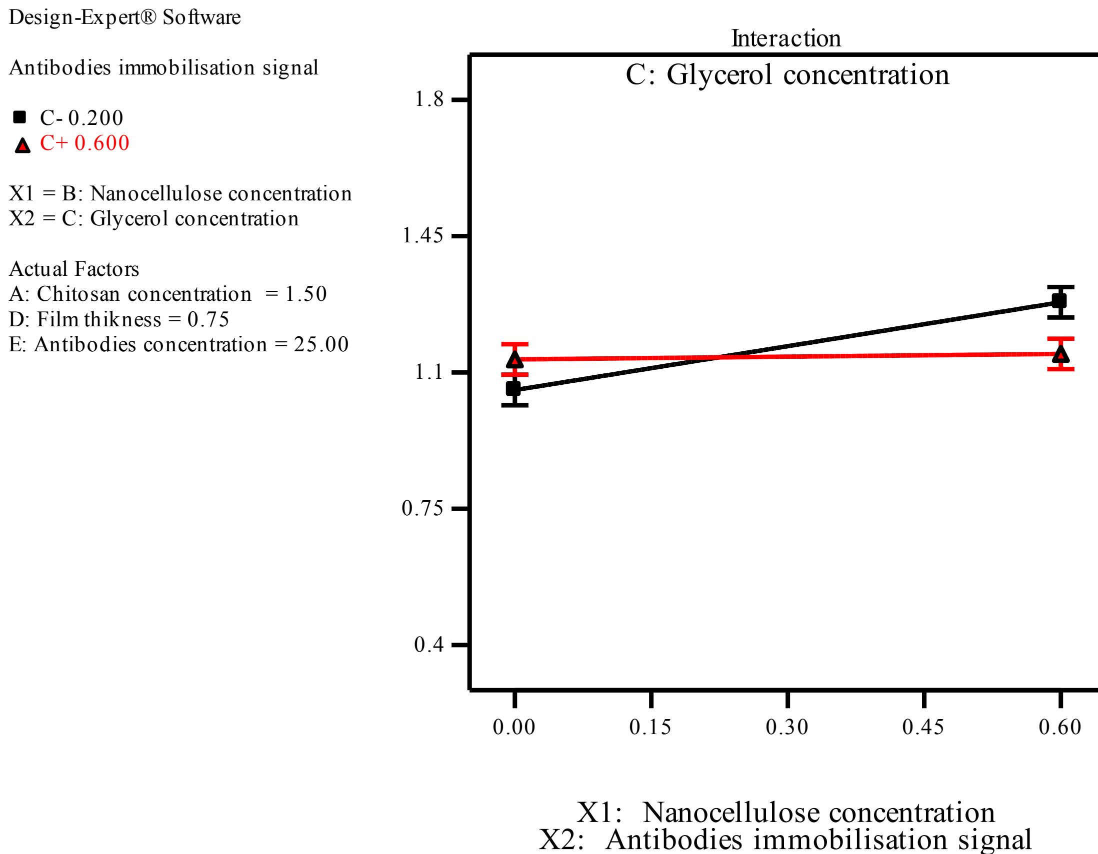

Figure 3.7: Interactions of the effects between the factors for the concentration of GLY and the concentration of the CNC.

However, the signal increased with CNC concentrations at 0.2% GLY. Usually, CNC- GLY interactions tend to decrease the opacity of the membrane (Azeredo *et al.*, 2017). In this case, the interaction can be explained by the fact that lower GLY leaves more biopolymer interspace for CNC in CHI-GLY phase, which improves antibody immobilization.

Fig. (3.8), (3.9), (3.10) et (3.11) illustrates the CNC-antibody interaction and shows that an increase of CNC concentration enhanced antibody immobilization, for different concentration of CHI and GLY, hence confirming the results obtained in previous experiments.

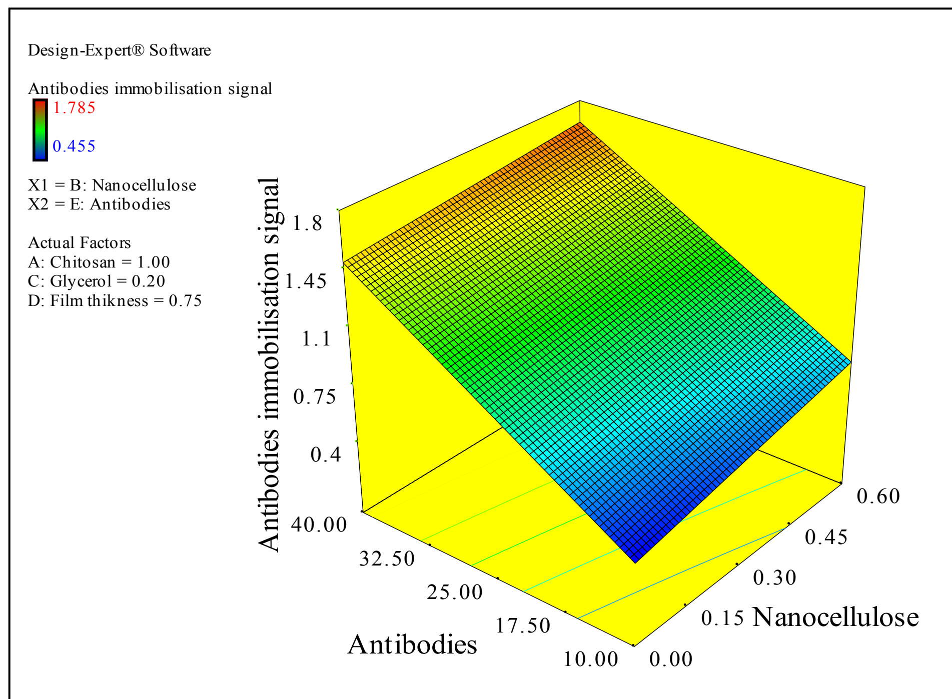

Figure 3.8: 3D Response surface of antibody immobilization signal as a function of CNC and antibody concentrations with 1% chitosan and 0.2% glycerol.

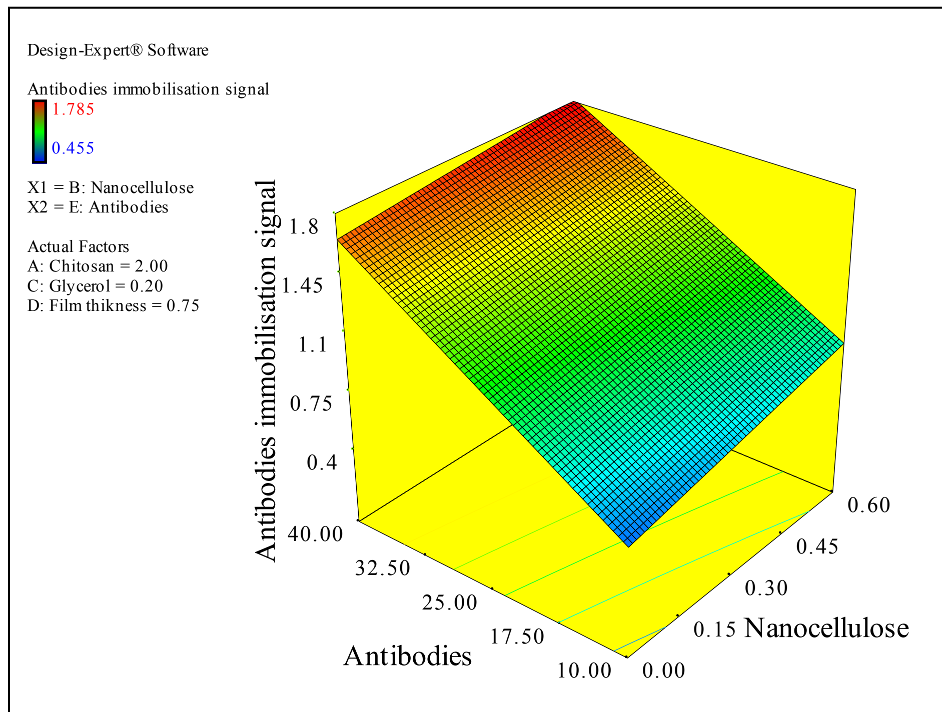

Figure 3.9: 3D Response surface of antibody immobilization signal as a function of CNC and antibody concentrations with 2% chitosan and 0.2% glycerol.

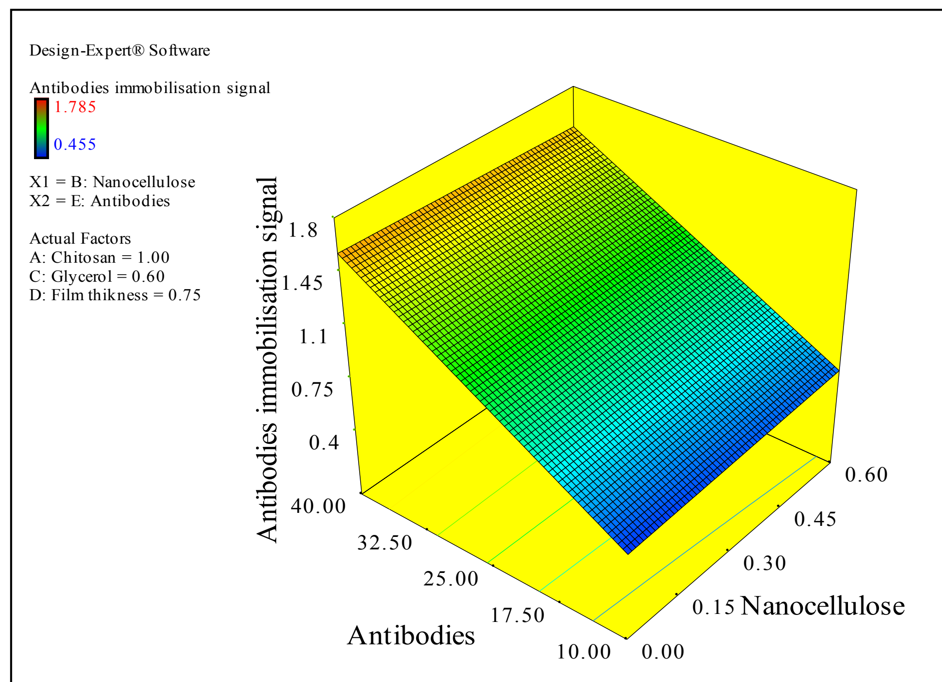

Figure 3.10: 3D Response surface of antibody immobilization signal as a function of CNC and antibody concentrations with 1% chitosan and 0.6% glycerol.

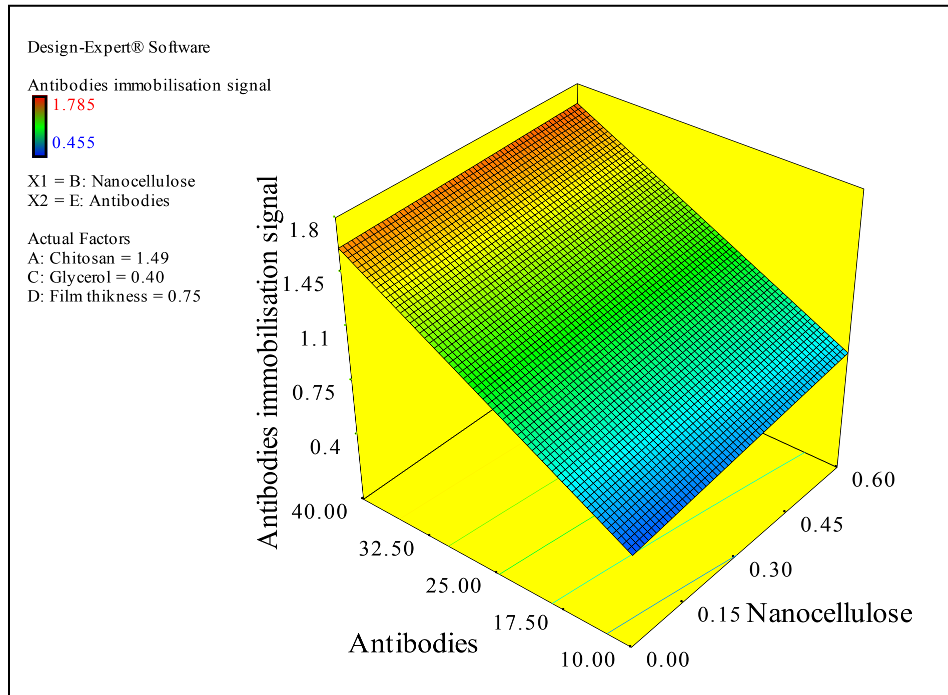

Figure 3.11: 3D Response surface of antibody immobilization signal as a function of CNC and antibody concentrations with 1.49% chitosan and 0.4% glycerol.

For the 3 main significant parameters, based on FFD results and studied in Box–Behnken Design (BBD), 17 experiments were performed. The significance probability was evaluated by ANOVA considering that the studied parameter *p*-values ≤ 0.05 affected the immobilization signal model. The ANOVA obtained through BBD is presented in **Table 3.4**. The immobilization signal (IS) as a function of significant effects is represented by equation **(2)**:

$$\mathbf{IS = +\,0.92442 - 0.91787 * A + 8.40139E\text{-}003 * E + 0.3560 * A^2} \qquad \textbf{(2)}$$

The Model F-value of 28.9 implies that the model is significant. Values of "Prob > F" lower than 0.05 indicate significant model terms. In this case A, C, A^2 are significant model terms. Values > 0.1 indicate the model terms are not significant. If there are many insignificant model terms, then the model reduction may improve the

model, and consequently CNC was removed and included in the intercept. The "Lack of Fit F-value" of 1.3 implies the Lack of Fit is not relatively significant to the pure error. Despite 42.6% chance that a "Lack of Fit F-value" could occur due to noise, non-significant lack of fit is good for the model to fit. The "Pred R-Squared" of 0.77 is in reasonable agreement with the "Adj R-Squared" of 0.84. "Adeq Precision" measures the signal to noise ratio and the model ratio of 17.6 indicates an adequate signal. This model can be used to navigate the design space considering that a ratio > 4 is desirable.

By comparing FFD and BBD analyses, the incorporation of CNC, with the studied factors levels, in the CCG membrane, had a significant effect ($P \leq 0.05$) in improving the immobilization signal. However, for mathematical modeling, CNC was removed as a factor and then coincidental with the intercept.

Table 3.4: Box-Behnken Design (BBD) experiment with ANOVA results.

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	0.21	3	0.069	28.87	< 0.0001
A- Chitosan concentration	0.045	1	0.045	19.03	0.0008
E- Antibodies concentration	0.13	1	0.13	53.45	< 0.0001
A^2	0.034	1	.034	14.12	0.0024
Residual	0.031	13	2.377E-003		
Lack of Fit	0.023	9	2.563E-003	1.31	0.4259
Pure Error	.837E-003	4	1.959E-003		
Cor Total	0.24	16			

CONCLUSION

According to the results obtained in this study, the incorporation of CNC in CHI membranes modifying mechanical properties and increasing the contact surface, showed an effect on the signal of antibody immobilization. The study showed that CNC concentrations and the type of CHI (based on molecular weight and deacetylation degree) increased the signal level. In particular, increasing CNC concentrations up to 0.6% (w/v) improved significantly ($P \leq 0.05$) the immobilization signal. CHI highest molecular weight (250-600 kDa) also improved the signal presumably by improving the mechanical properties of the CCG membrane. The presence of GLY showed no significant signal improvement ($P > 0.05$) with increasing GLY concentrations beyond 0.6% (w/v).

FFD study confirmed the effect of each compound on the signal. However, the BBD analysis provided a correlation between the amount of antibody to be immobilized and the concentration of CHI. The variation interval fitting with the model and suggested for prediction is so limited suggesting that the optimal formulation was set to 2% (w/v) CHI, 0.6% CNC and 0.2% GLY for an immobilization of 13 µg/mL of antibody. This formulation will be used in the following studies for development of a detection support.

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CHAPITRE 4:

Novel spider web trap approach for the detection of *Escherichia coli* O157:H7 on food surfaces

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Contribution des auteurs

Amina Baraketi a réalisé les manipulations et la rédaction de l'article.

Sabato D'Auria, Co-Directeur de recherche, a participé à la correction de l'article.

Carole Fraschini a fourni la cellulose nanocrystals et a participé à la correction de l'article.

Stéphane Salmieri a participé au bon déroulement des expériences et à la correction de l'article.

Joshua Menissier a participé à la répétition des manipulations.

Monique Lacroix: Directrice de recherche, responsable scientifique et coordinatrice du projet de recherche, a participé à la planification des expériences et à la correction de l'article.

L'article sera soumis au Journal of Sensors and Actuators B: Chemical.

ABSTRACT

The aim of this study was to develop a novel approach allowing simultaneous enrichment and specific and fast detection of *Escherichia coli* O157:H7. The study demonstrated that the performance of a newly developed indirect ELISA method using an optimized support was able to address not only the speed but also the simplicity of the detection of this foodborne pathogen. Therefore, combining the step of the capture of the pathogen and enrichment steps for the microbial growth led to a high detection signal at a low inoculation level without cross-reaction with several *Pseudomonas* and *Salmonella* strains, known as very common food pathogens in raw meat products. The detection was performed by several incubation periods and different levels of inoculations. Among the composition of the tested supports and samples, the signal of detection in samples incubated with the chitosan-based support reinforced with cellulose nanocrystals (CNC) and directly from *E.coli* O157:H7 bacterial culture was much higher as compared to CNC-free support with cell-free supernatant samples. The adjustment of this innovative spider web trap approach (SWTA) could minimize the risks of cross-contaminations and consequently food product recalls by facilitating significantly the detection of foodborne pathogens in samples collected from tools and work surfaces in the food processing industry.

Keywords: *E.coli* O157:H7, enrichment, cellulose nanocrystals, capture support, food safety, rapid detection.

1. INTRODUCTION

Nowadays, food safety is a major public health concern attracting increasing attention to minimize foodborne illnesses. Due to the high cross-contamination risk in food industries, the consumption of minimally processed and ready-to-eat (RTE) products is the primary source of the occurrence and the spread of foodborne diseases (Srey *et al.*, 2013). According to the recent census of The Centers for Disease Control and Prevention (CDC) (2011), foodborne illnesses caused 3,000 deaths, 128,000 hospitalizations and 48 million infections every year in the United States. Among these cases, there are 20 deaths, 2100 hospitalizations and 63,000 infections due to food contamination by *E. coli* O157:H7, one of the top five food pathogens (Xu *et al.*, 2017a). In Canada, *E. coli* O157 serotype, count for up to 500 cases annually, according to a Public Health Agency of Canada report (2015) in addition to 20 unreported cases in the community for every one reported case (Thomas *et al.*, 2013).

Escherichia coli O157:H7 is the most incriminated Shiga toxin-producing *E. coli* (STEC) serotype, in worldwide food outbreaks due to the very low of the infective dose, estimated between 50 and 100 cells, and its ability to produce the Shiga toxins (Zhang *et al.*, 2017). Shiga toxins are composed of two subunits A and B. The subunit A has a catalytic function, cleaving adenosine residue from 28S rRNA. The subunit B mediates targeting and binding Gb3 receptors (Skinner *et al.*, 2013). The infection is associated with abdominal cramps, bloody stools, little or no fever, asymptomatic shedding and non-bloody diarrhoea (Karmali *et al.*, 1983).

Thw meat industry still remains the major source of human infection by foodborne *E. coli* O157:H7. During the slaughter process, cross-contamination can occur through contact with personnel, equipment’s and carcasses directly in contact with each other (Elder *et al.*, 2000). In addition, ground beef and derived products such as hamburger patties are well-identified as

efficient transmission vehicles of the *E. coli* O157 infections and have been associated with a high number of outbreaks (Cassin *et al.*, 1998, Ferens *et al.*, 2011, Morton *et al.*, 2017, Tuttle *et al.*, 1999).

The increase occurrence of foodborne outbreaks obliges regulatory agencies and industries to control the transmission routes and vehicles of foodborne pathogens in order to ensure food safety by reducing/eliminating the risks for consumer infection. Traditional culture-based *E. coli* O157:H7 detection methods are inexpensive and simple but involve pre-enrichment, selective enrichment, biochemical screening and serological confirmation making such testing impractical for routine, large-scale food industry use (Ratnam *et al.*, 1988). Over the years, alternatives rapid methods of detection have been developed to overcome conventional methods limitations, although they are expensive and require trained technical staff (Law *et al.*, 2015). Indeed, nucleic acid-based methods are highly sensitive, highly specific and labor-saving but may be affected by PCR inhibitors, require DNA purification and have implied difficulties to distinguish between viable and non-viable cells (Mandal *et al.*, 2011, Zhang, 2013). On the other hand, biosensor-based methods are cost effective, easy-to-operate, label-free and provide real-time detection but they could have low specificity and be unsuitable for analyzing samples with low amounts of microorganisms while requiring long incubation time for the bacteria (Ivnitski *et al.*, 1999). Furthermore, immunological-based methods may have low sensitivity, could generate false negative results as well as requiring a pre-enrichment step to produce enough the cell surface antigens (Zhao *et al.*, 2014). Indeed, the reduction and/or elimination of culture enrichment essential in the quest for truly real-time detection methods (Dwivedi *et al.*, 2011).

The recent trends in the immunoassay development are based on biopolymers as support for immobilization. Several studies have described the application of chitosan, one of the most abundant biopolymers in nature, as support of immunoassay for the immobilization of antibodies

as well as support for immobilization of proteins. Chitosan is characterized by the functional amino groups that can allow strong covalent linkage with glutaraldehyde activation. Several researches have implemented Chitosan film-based sensing format for the detection of pathogenic *E. coli*. Similarly, Dogan *et al* (2016) and Kozitsina *et al* (2016) developed a new detection method by using fluorescent or magnetic nanoparticles for the immbolilization of the antibodies on chitosan surface (Dogan *et al.*, 2016, Kozitsina *et al.*, 2016). Though these methods are sensitive, selective and fast, they require complementary fluorescence and UV-vis spectroscopy for the detection (Sadat Ebrahimi *et al.*, 2015).

Chitosan detection supports have also been used with nanocomposite for electrochemical detection of *E. coli* O157:H7, such as graphene oxide/chitosan nanocomposite (Xu *et al.*, 2017b) and graphene oxide–nickel ferrite–chitosan nanocomposite (Tiwari *et al.*, 2015). Despite the important yield and the high sensitivity of these methods, they are still expensive for industrial application in addition to the need of special training for their use.

Although the inspections and strict monitoring regulatory requirements, novel simple, rapid and specific approach recognizing small numbers of pathogens still need to overcome actual methods limitations for *E. coli* O157:H7 detection. In this study, in order to reduce duration and cost of the test for pathogen detection, a new approach, intended for sample surfaces, raised the challenge to ensure a quick simple method, specific, sensitive and inexpensive by performing simultaneous enrichment and detection of *E. coli* O157:H7 using an innovating biodegradable nanocomposite. The membrane used in this work is based on previous work for the development and optimization of a low cost multi-compatible support for immobilization.

The aim of this study is to develop a new method allowing the simultaneous enrichment and capture for the detection of *E. coli* O157:H7 in order to apply it on a user-friendly swab device for surface sampling and detection in the food industry. This approach may reduce the duration of

the test and improve the detection of the pathogen by coupling pre-enrichment and enrichment steps by increasing the amount of toxins captured on the support. In this case, the efficiency of this approach highly depends on the specificity of the immunoassay, the strength of the support, the preparation of the sample and the enrichment medium.

2 MATERIALS AND METHODS

2.1 Chemicals

High-purity HMC$^+$, 95/2500, pharmaceutical chitosan (CHI), was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). Cellulose nanocrystals (CNC) were provided by FPInnovations (Pointe-Claire, QC, Canada). Laboratory grade Glycerol (GLY), 90% glacial acetic acid and pure sulfuric acid were from Laboratoire Mat (Beauport, QC, Canada). Polyclonal antibodies Anti-Shiga Toxin 2 of purified rabbit IgG (Stx2) were purchased from Cedarlane (Burlington, ON, Canada). Horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Step Ultra TMB-ELISA substrate solution was purchased from Thermo Fisher Scientific (Burlington, ON, Canada), gelatin from BioRad (Mississauga, ON, Canada)**,** and Bovine Serum Albumin (BSA) from Santa Cruz Biotechnology Inc. (Mississauga, ON, Canada). Trypto Soy Broth (TSB), Brain Heart Infusion (BHI) and modified *E. coli* medium (mEcoli) were purchased from Alpha Biosciences (Baltimore, MD, USA). Tween® 20, Universal Pre-enrichment Broth, (UPEB), tripolyphosphate (TPP), glutaraldehyde (GA) solution, powder mitomycin C (MC) and powder norepinephrine (NE) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

2.2 Preparation of the membrane

CNC was suspended in distilled water for 1 h at room temperature under magnetic stirring. After addition of acetic acid 1% (v/v) and sonication treatment (1 kJ/g of CNC), CHI and GLY were added under vigorous stirring at room temperature, for 4 h. After complete homogenization, the suspension was cast in a polystyrene 24-well microplate and air-dried at room temperature for 5 days until the membrane became completely dried.

2.3 Preparation of bacterial cultures

A total of 8 laboratory strains were used in this study. Strain EDL 933 *(E.coli* O157:H7), a non-pathogenic *E.coli* strain, three *Pseudomonas* strains (*Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*) and three *Salmonella* strains (*Salmonella enterica* serovar Typhi, *Salmonella* typhimurium, *Salmonella enterica* serovar *Hadar*) were revived from cryovials stored at -80 °C and inoculated onto Tryptic Soy Agar plates for 24 h at 37 °C. Cultures were prepared from isolated colonies of each strain and grown in 10 mL Tryptic Soy Broth at previous conditions. Plate counting method with serial dilutions was performed for the enumeration of bacterial cultures.

2.4 Detection of *E. coli* O157:H7 by SWTA

Once completely dry, the membrane fixed in each well was treated for chemical reaction with amino groups. A volume of 1mL of 1% TPP solution was added for 10 min under shaking and rinsed with distilled water to suppress TPP residues. Secondly, 300 µL of a 2% (v/v) GA solution were added in each well, under rotating stirring (150 rpm) for 1h at room temperature and rinsed with distilled water to eliminate unreacted GA residues. A volume of 2 mL from each culture, was added into each well and sterile TSB was considered as negative control. Plates were incubated for 24 h under agitation of 150 rpm and after 5 washings of 10 min with phosphate buffered saline-Tween® (PBS-T), 1% (w/v) of gelatin solubilized in PBS was added to each well to block non-specific binding sites, under shaking at room temperature (20 ˚C ± 2 °C).

Rabbit IgG, Anti-Shiga Toxin 2 was used as detection antibody with a concentration of 2 µg/mL. After incubation for 1 h at 37 °C and five washing steps of 10 min with PBS-T, polyclonal anti-rabbit was used as secondary antibody coupled to HRP and incubated for 30 min at 37 °C under shaking. After 3 washes with PBS-T, TMB was added as substrate for the HRP. The reaction was

stopped by the addition of H_2SO_4 (2M). Results were determined by transferring 300 µL of each well solution to a 96-well microplate and absorbance was read at 450 nm using a Biotek microplate reader and Gen 5 2.07 software. Detection test was performed with approximately 10^3 cells/mL from each strain culture, followed by 24 h incubation under 150 rpm shaking at 37 °C resulting in approximately 10^9 cells/mL in a stationary phase culture. The SWTA detection method was performed not only on the 24 h resulting (10^9 cells/mL) bacterial culture of *E. coli* O157:H7 but also on the unfiltered supernatant and cell-free supernatant using the membrane with and without CNC.

2.5 Optimization of enrichment medium

The objective of this step was to screen out the potential medium showing the fastest growth of pathogens in order to improve the detection of Shiga-Like Toxin 2 (STX2). The Bioscreen C automated microbiology Analysis System was used to perform continuous 24 h kinetic growth curve of *E. coli* O157:H7. TSB, BHI UPEB and mEcoli were tested as enrichment media. Mitomycin C from *Streptomyces caespitosus* and norepinephrine were added in the medium to reach a final concentration of 50 ng/mL and 50 µmol/mL respectively. The production of STX2 was evaluated with western blot. Cell-free supernatant of EDL933 was obtained from the filtration of 24 h cultures and then, concentrated using a 30 kDa Amicon Ultra centrifugal filter unit. The proteins were separated using 12% polyacrylamide gel for SDS-Page. After electrophoresis, transfer and blocking steps, Anti-Shiga Toxin 2 antibodies and HRP-labelled secondary polyclonal anti-rabbit were added for the detection. The protocol of detection was performed to evaluate the effect of these media on the detection level of the toxin.

2.6 Adaptation to swab model

The CCG solution was prepared as described previously. For the swab model, the suspension was cast in a glass tube and air-dried at room temperature until the membrane became completely dry.

Then, 1% TPP followed by 2% (v/v) of GA were added in each tube as previously described. Each 9 mL of medium was inoculated with one fresh colony and incubated at 37°C under 150 rpm shaking added in each tube in triplicate. To determine the minimum enrichment duration necessary to obtain a signal of detection, the culture growth was monitored to each hour. After 5 washings with 5 s vortexing with PBS-T, 1% (w/v) of gelatin in PSB was added for 2 h under shaking at room temperature to block the non-specific binding sites. Rabbit IgG, Anti-Shiga Toxin 2 and polyclonal anti-rabbit were used for the detection of immobilized proteins with the same previously established concentrations. The revelation was performed with the TMB and the reaction was stopped by the addition of 2 M H_2SO_4. Results were determined by transferring 300 µL of each well solution to a 96-well microplate and absorbance was read at 450 nm using a Biotek microplate reader.

2.7 Statistical analysis

Each experiment was done in triplicate (n=3). Analysis of variance (ANOVA), Duncan's multiple range tests for equal variances and C de Dunnett's C test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, USA). Differences between means were considered significant when the confidence interval was lower than 5 % ($P \leq 0.05$).

2. RESULTS AND DISCUSSION

3.1 Detection of *E. coli* O157:H7 using the SWTA approach

The method developed is this work was strongly based on the support system whose function was to ensure the capture of toxins present in a sample and to resist to all treatments during the immunoassay steps. Previous works for the development of a support, based on CHI and CNC for antibody immobilization, suggested that CNC improved significantly the signal of immobilization by the reinforcement of the mechanical properties. In addition to that, an optimal formulation was done showing that 2% (w/v), chitosan (95% deacetylation degree), 0.6% CNC and 0.2% GLY could ensure an optimal immobilization of antibodies. As shown in **Figure 1**, the support reinforced with 0.6% CNC improved the detection signal of the whole *E. coli* O157:H7 culture by 25% compared to that of the whole culture using the support without CNC, as well as for the unfiltered and cell-free supernatants.

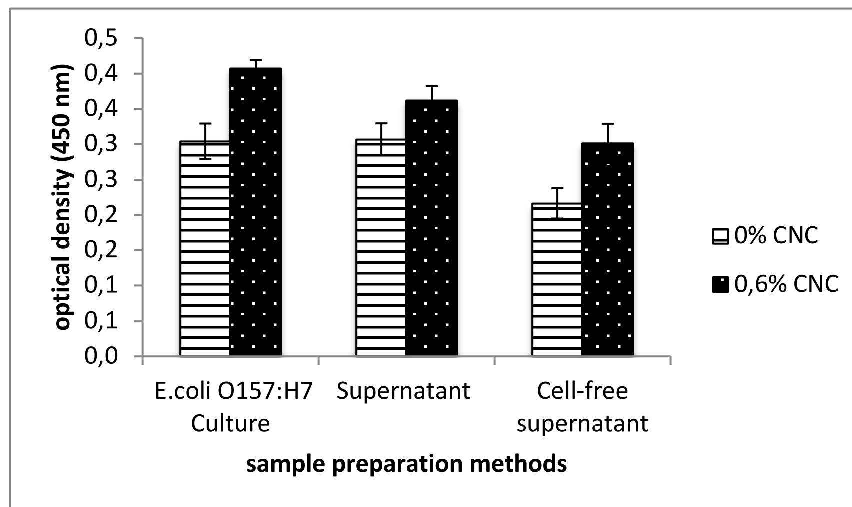

Figure 4.1: Detection signal of *E.coli* O157:H7 for a whole 24 h-culture, unfiltered supernatant and cell-free supernatant in mEcoli broth.

(*) level of signification ($P \leq 0.05$)

These finds confirm that CNC improved the capture of the protein and consequently the detection signal of the pathogen. **Figure 4.1** also shows that the whole bacterial culture allowed a higher signal than both unfiltered and cell-free supernatant, which highlights the fact that the developed SWTA did not require any sample preparation compared to traditional methods. *Salmonella* spp, *Pseudomonas* spp and non-pathogenic *E.coli* are very common contaminants in the meat industry (Bakhtiary *et al.*, 2016). Thus, the first step in this work relied on validating the specificity of this method against *E. coli* O157:H7 by evaluating also against these bacteria. **Table 4.1** presents the results of SWTA analyses for these strains of cultures using the CNC-reinforced CHI capture support, and the results showed that no cross-reaction was significantly observed with the other strains. Indeed, the polyclonal antibody used was produced using a recombinant Stx2a obtained by changing glutamic acid in the active site into glutamine. Indeed, the rabbit Anti-Shiga Toxin 2 binds specifically to the Shiga toxin 2 without reacting with Stx1(He *et al.*, 2013).

Table 4.1: Optical density obtained by the SWTA using polyclonal anti-Stx2 antibodies and samples from 24-h culture of different strains in TSB culture medium. (a, b, c, d and e) are significantly different ($P \leq 0.05$)

Organisms	Optical density (450 nm)
***E. coli* 8739 (non-pathogène)**	0,036±0,010 [b]
***E. coli* O157:H7 EDL 933**	0,436±0,032 [e]
***Pseudomonas aeruginosa* ATCC 15442**	0,003±0,025 [ab]

***Pseudomonas fluorescens* CRDA V49**	-0,020±0,012 [a]
***Pseudomonas putida* CRDA V376**	-0,001±0,029 [a]
***Salmonella enterica serovar Hadar* (ATCC® 51956™)**	0,008±0,007 [ab]
***Salmonella enterica serovar Typhi.* (ATCC® 19430**	0,133±0,015 [d]
***Salmonella Typhimurium* SL 1344**	0,098±0,06 [c]

3.2 Optimization of the enrichment medium

The pre-enrichment and enrichment steps increase the amount as well as the selectivity of pathogen cells enough to reach a detectable level with high specificity and revive physiologically stressed or injured cells. In addition, culture enrichment broths are necessary to dilute the inhibitory substances that may be present in the sample and could interfere with the analysis (Hahm *et al.*, 2015). Nevertheless, enrichment steps still remain time-consuming for the detection of food pathogens, which usually occur with low amounts in large sample volume (Leonard *et al.*, 2003). Despite the availability of several enrichment broths, the detection of *E. coli* O157:H7 remains challenging and consequently underlines the need for improving a new medium allowing a rapid detection of *E. coli* O157:H7.

TSB and mEcoli are commonly used as supports for the detection of *E. coli* O157:H7 in several food matrices (Vimont *et al.*, 2006). UPEB is known for its ability to revive injured foodborne pathogens (Zhao *et al.*, 2001). Finally, BHI was also reported to increase rapidly the growth of *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Salam *et al.*, 2010).

To select the broth allowing the maximum growth, these four enrichment broths were compared in 24 h growth kinetic. The overall growth profiles of EDL933 cultures in TSB, BHI, UPEB and mEcoli are shown in **Figure 4.2**. The results show a higher number of bacteria in mEcoli culture than in BHI and TSB while the lower bacterial number was observed in UPEB. This can be explained by the fact that the incorporation of the bile salts to mEcoli delayed the Jameson effect and improved STEC growth (Vimont *et al.*, 2007). Jameson effect has been defined as a phenomenon of growth inhibition of other microbial species, that could be present in a culture, to the benefit of another species by the production of specific inhibitors for other competitors (Mellefont *et al.*, 2008). Indeed, the addition of bile salts in mEcoli broth increases the growth of the Shiga toxins producing *E. coli* (STEC) and the production of the toxins, which leads to the acidification of the culture medium and consequently the inhibition of growth of other bacteria.

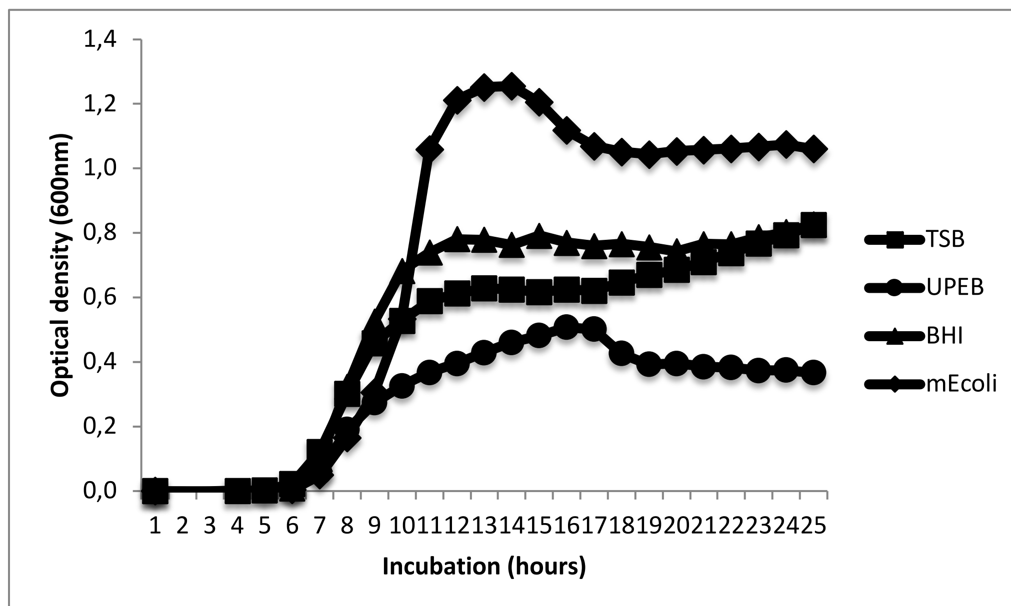

Figure 4.2: Growth curve of *E. coli* O157H7 in TSB, UPEB, BHI and mEcoli.

Besides, other studies confirmed that mEcoli broth enhanced significantly STEC growth (Stromberg *et al.*, 2015). Thus, mEcoli appears to be the most suitable medium for the growth of this pathogen. This broth could be improved by the addition of substances that could increase the production of the Shiga toxin 2 such as antibiotics and/or hormones. **Figure 4.3** shows the western blot result for the production of the STX2 comparing TSB as conventional medium and mEcoli, the optimal medium for the growth of EDL933. The amount of STX2 produced for the TSB (**A**) is as low as undetectable by western blot suggesting that no production occurred, as opposed to the result obtained for mEcoli broth (**B**) where the band clearly appeared (Figure 3). Indeed, the addition of the mitomycin C for TSB (**C**) and mEcoli broth (**D**) increased the STX signal. However, the addition of the norepinephrine to TSB (**E**) and mEcoli broth (**F**) did not show any difference with the negative control (**A** and **B** respectively). In counterpart, as shown by (**G**) and (**H**), combining the mitomycin C and the norepinephrine to mEcoli broth showed a band with high intensity while their addition to TSB gave similar results (**E**) as the addition of mitomycin C only (**B**). Hence, these findings are explained by the fact that the mitomycin C induced the production of the STX2, while the norepinephrine had an effect on detecting the amount produced specifically by mEcoli broth.

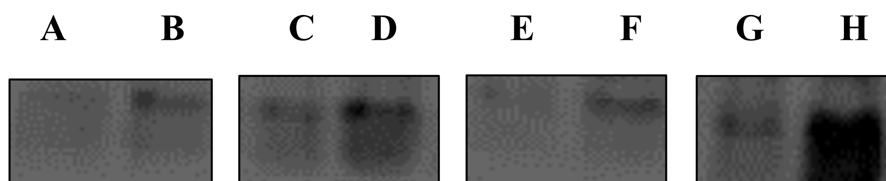

Figure 4.3: Western blot analysis on the effect of the medium on the production of Shiga Like toxin 2 produced by *E. coli* O157:H7. **A**: culture in TSB, **B**: culture in mEcoli, **C**: culture in TSB supplemented with mitomycin C, **D**: culture in mEcoli supplemented with mitomycin C, **E**: culture in TSB supplemented with norepinephrine, **F**: culture in mEcoli supplemented with

norepinephrine, **G**: culture in TSB supplemented with mitomycin C and norepinephrine, **H**: culture in mEcoli supplemented with mitomycin C and norepinephrine.

Furthermore, a strong association between mitomycin C and the production of Shiga Like toxin 2 was reported (Kimmitt *et al.*, 2000, Wagner *et al.*, 2001). Indeed, mitomycin C could damage the DNA and so activate the SOS response which may induce an increase of STX2 production (Yee *et al.*, 1993). On the other hand, it has been reported that norepinephrine, a catecholamine stress hormone, stimulates the growth of enterohemorrhagic *E. coli* (EHEC) and increases the production of the Shiga toxin (Yang *et al.*, 2014). As described in Lyte *et al.* (1996), norepinephrine does not enhance the growth as a nutritional source but could increase the production of the toxin by up to 100 folds. In summary, the chemical signaling by norepinephrine is highly dependent on the sensor kinase receptors QseC sensor kinase that may activate QseF sensor which regulates the production of the Shiga Like (Lustri *et al.*, 2017).

Finally, the new formulation of the enrichment broth was tested with this novel approach and the results presented in **Figure 4.4** show that mEcoli supplemented with 50 ng/mL mitomycin C and 50 µmol/mL norepinephrine ensured an optimal detection signal compared to the signal of non-supplemented mEcoli broth and to TSB.

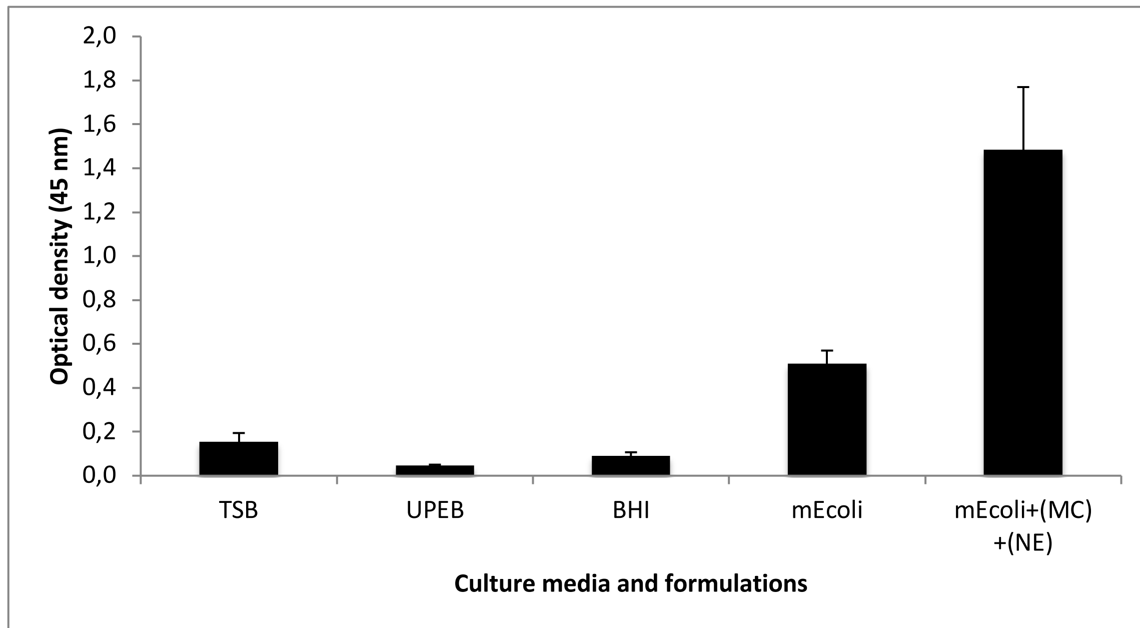

Figure 4.4: Detection signal of *E. coli* O157:H7 after 24 h of incubation at 37 °C under 150 rpm agitation in function of different culture media.

(*) level of signification ($P \leq 0.05$)

Therefore, the application of the SWTA on glass tubes showed promising results. **Figure 4.5,** starting from one fresh plate colony, shows that this approach allows the detection of *E. coli* O157:H7 after only 4 h of enrichment without any requirement of sample preparation. In addition, no other step of capture is needed, and the support chemically resists to all steps of analysis.

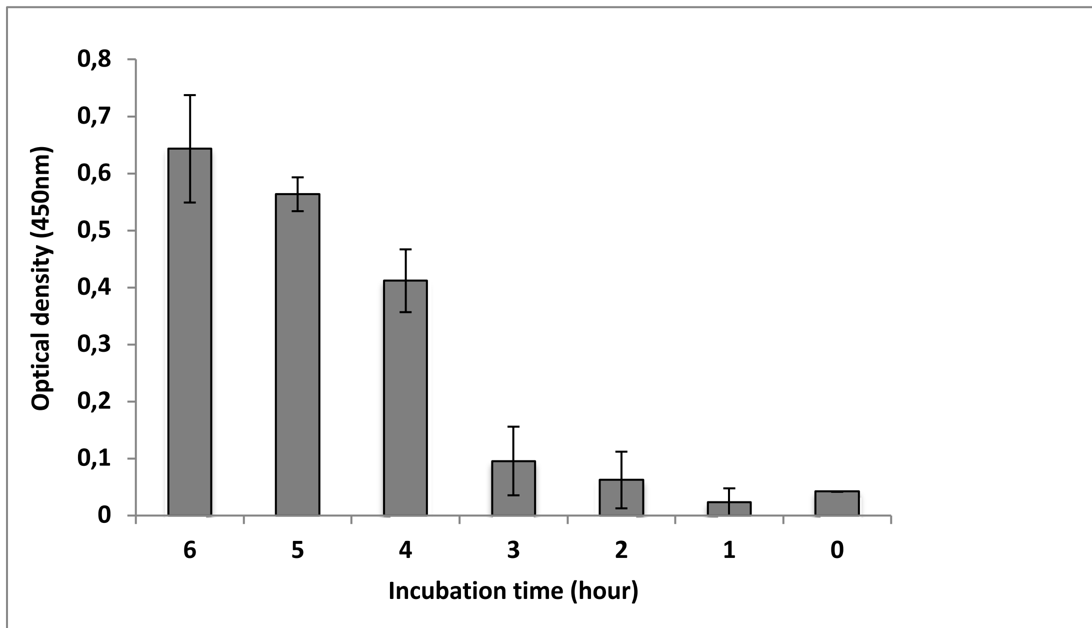

Figure 4.5: Detection signal of *E. coli* O157:H7 after 12 and 24 h of incubation at 37 °C under 150 rpm agitation in mEcoli additionned with mitomycin C and nnorepinephrine. (a, b and c) are significantly different ($P \leq 0.05$).

3. CONCLUSION

The spider web trap approach for detection of *E. coli* O157:H7 allowed the detection of 10^3 cells/mL after only 4 h of enrichment compared to 12 hours with conventional methods. The enrichment medium optimized in this study was improved specifically for the production of the Shiga toxin 2 and consequently enhanced the detection signal.

The adaptation of this approach to Swab test model would lead to a promising feasible commercial detection kit of pathogen *E. coli* for surfaces and work tools sampling in the food industry. Hence, the developed target enrichment strategy with a visually detectable signal would be a viable option for routine monitoring of pathogens in large volume samples requiring enrichment before detection.

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CHAPITRE 5 : CONCLUSION GÉNÉRALE

En conclusion, les travaux réalisés pour le développement du kit de détection, les travaux réalisés pour le développement du kit de détection du pathogène *E. coli* O157:H7 ont permis d'optimiser le milieu d'enrichissement permettant une meilleure production de la Shiga-Like Toxine 2 et assurant ainsi une détection au bout de 4 heures seulement d'enrichissement comparativement à 12 heures en temps normal. Deuxièmement, le test immunoenzymatique de détection de ce pathogène a été optimisé par l'utilisation d'un simple anticorps polyclonal spécifique à la Shiga-Like toxine 2 et a été adapté sur la membrane CCG développée et optimisée au cours des travaux précédents. La spécificité de ce test a également été confirmée.

Pour résumer, au cours de ces dernières années, une nouvelle méthode de détection rapide et spécifique pour *E. coli* O 157 :H7 a été développée. Elle est basée sur la méthode d'ELISA indirecte, l'utilisation d'un support à base de biopolymère et un révélateur de détection fluorescent. Toutefois, la trousse finale développée est destinée pour l'instant à la détection d'*E. coli* O157 :H7 se trouvant en faible quantité sur les surfaces et des outils de travail des industries alimentaires. Il s'agit d'une trousse contenant un écouvillon muni d'une membrane, développée et optimisée au cours de ce projet qui va permettre la capture de la Shiga-Like Toxine 2 simultanément au cours de l'enrichissement, les réactifs nécessaires pour l'activation de la membrane ainsi que l'étape de la détection et la révélation visuelle ainsi que le milieu d'enrichissement dont la formule a été optimisée lors de ces travaux.

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