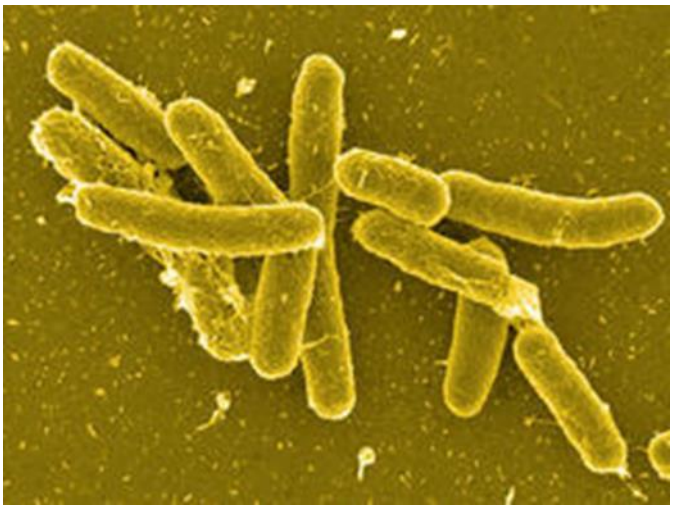
University of Crete,
Biology Department
MSc Program:
Protein Biotechnology

MASTER THESIS
Chapter 1

LAMP for food pathogens acoustic detection using liposomes.



Author: Kontogianni Georgia-Ioanna, AM 862

Supervisor: Prof. Gizeli Electra

Heraklion, October 2019

Contents

1. Abstract 5
2. Περίληψη 6
3. Acknowledgements 7
4. Introduction 8
   4.1 *Salmonella* 8
   4.2 Loop-mediated isothermal amplification (LAMP) 8
   4.3 Acoustic biosensors 9
      4.4.1 Quartz Crystal Microbalance (QCM) 9
      4.4.2 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) 10
   4.4 Liposomes 11
   4.5 Aim of the study 11
5. Materials and Methods 13
   5.1 Materials 13
   5.2 Methods 14
      5.2.1 Loop-mediated Isothermal amplification (LAMP) 14
      5.2.2 Gel Electrophoresis 15
      5.2.3 Liposomes preparation 15
      5.2.4 QCM-D 15
6. Results 16
   6.1 Loop-mediated Isothermal amplification (LAMP) 16
      6.1.1 *invA* gene 16
      6.1.2 *bcfD* gene 17
   6.2 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) 17
      6.2.1 invA gene 18
      6.2.2 bcfD gene 20
7. Conclusion and discussion 25
8. References 27

Figure contents

Figure 1: LAMP amplification scheme. (1) Initiation at F end of target sequence via FIP and displacement of nascent strand by synthesis initiating at F3. (2,3) Synthesis and displacement at B end of target resulting in (4), the seed structure for exponential LAMP amplification (Tanner and Evans 2014). 9

Figure 2: Description of the main components in QCM-D. a: Typical QCM-D sensor with gold electrodes. b: Quartz crystal with alternating current applied across electrodes. c: Short circuiting the alternating current. d: The oscillatory decay as the quartz disk comes to rest. The frequency of the oscillating crystal, shown in b, is related to the total oscillating mass adsorbed on the surface, while the energy dissipation, shown in c, is related to the viscoelastic properties of the oscillating mass. Thus, changes in adsorbed mass of, for example, a rigid protein provide a change in frequency, but for viscoelastic masses such as biomacromolecules, there is a change both in frequency and dissipation (Dixon 2008). 10

Figure 3: Scheme of a liposome formed by phospholipids in an aqueous solution. 11

Figure 4: *Detection of labeled LAMP amplicons on the (a) surface of neutravidin-coated well. (b) The biotinylated LAMP amplicons bind to the neutravidin-coated gold biosensor surface. (c) The cholesterol loop-specific oligonucleotide probes with complementary sequences to the single stranded loops of the Salmoella LAMP amplicons bind to the corresponding areas on the DNA. (d) POPC liposomes injected for the identification of the probes binding. (Image not in scale).* 12

Figure 5: A representative gel image after 40 min of electrophoresis. In lane (I), is the well containing the ladder, with 12 fragments starting at 100 bp and going up to 3000 bp, in lane (II) is the negative control showing a band corresponding to the primers of the invA gene (from 17 to 40bp) and in lane (III) the LAMP product of the invA gene with the primer band after 20 min post reaction. 16

Figure 6: A representative gel image after 40 min of electrophoresis. In lane (I) is the well containing the ladder containing 12 fragments starting at 100 bp and going up to 3000 bp, in lane (II) is the negative control showing a band corresponding to the primers of the bcfD gene (primers from 18 to 42 bp) after 10 min post reaction with the loop primers , in lane (III) is the LAMP product after 10 min post reaction with the loop primers for the bcfD gene, in lane (IV) is the negative control corresponding to the primers of the bcfD gene (primers from 19 to 42 bp) after 35 min post reaction without the loop primers and in lane (V) is the LAMP product after 35 min post reaction with the loop primers for the bcfD gene. 17

Figure 7: Negative control (left) and LAMP product (right) after 15 min post reaction with the cholesterol loop primers. 22

Graph contents

Graph 1: (A) The sensorgram of the negative reaction for the invA gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the invA gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm). 18

Graph 2: (A) ΔF, (B) ΔD and (C) Acoustic ratio of the invA gene LAMP amplicons (after 20 min LAMP post reaction) injection. 19
Graph 3: (A) ΔF, (B) ΔD and (C) Acoustic ratio for the liposomes (100 nm diameter) binding on the cholesterol of the hybridized probe for the invA gene (after 20 min LAMP post reaction of the gene). 19
Graph 4: (A) The sensorgram of the negative reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm). 20
Graph 5: (A) The sensorgram of the negative reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm). 21
Graph 6: The sensorgram of the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA with cholesterol, c) Liposomes (100nm). 22
Graph 7: (A) ΔF, (B) ΔD and (C) Acoustic ratio of the bcfD gene LAMP amplicons binding in different times and conditions. 23
Graph 8: (A) ΔF, (B) ΔD and (C) Acoustic ratio for the liposome (100 nm diameter) binding on the cholesterol of the hybridized probe. 24

Table contents

Table 1: Components and their amounts for the invA gene. 14
Table 2: Components and their amounts for the bcfD gene. On the right table with loop primers and on the left table without loop primers. 14
Table 3: Average values of ΔF, ΔD and acoustic ratio ΔD/ΔF with the standard deviation of each value. 24

1. Abstract

Outbreaks linked to *Salmonella*-contaminated products produce the need to develop simple, rapid and accurate detection methods, even if *Salmonella* is present in low amounts. In this study, we examined a novel strategy for the rapid detection and quantification of viable S*almonella* by coupling a simple acoustic detection of biotinylated amplicons on neutravidin modified surface of a QCM-D sensor with loop-mediated isothermal amplification (LAMP).

We first designed and optimized a LAMP assay targeting *invA* gene of *Salmonella* and secondly *bcfD* gene with biotinylated FIP primers. For low amounts of nucleic acids to be detected, 100nm POPC liposomes were used as a label in order to amplify the acoustic detection; this need for liposome binding in the LAMP products is the reason why we used loop forward and loop backward primers modified with cholesterol. 500 cells were used for LAMP amplification in the initial experiments (aiming to lower this threshold) and the reaction time had a range from 15 to 45 minutes, while control reactions took place to avoid the false negative and false positive results.

In the case of *invA* gene the cholesterol probes were injected in the biosensor chamber after the injection of the biotinylated DNA and no signal was observed, so their presence was identified upon injection of POPC liposomes. For *bcfD* gene, two different approaches were used; in the first the injection of cholesterol probes in the biosensor chamber took place after the injection of the biotinylated DNA and in the second case, the cholesterol probes were used directly during the amplification (LAMP), so that the final product before the injection in the biosensor chamber contains biotin and cholesterol. In the first case no signal was observed after the addition of the POPC liposomes on the biosensor chamber regardless of the use or not of loop primers in the LAMP reaction. Nevertheless, in the case of the insertion of the cholesterol primers in the LAMP reaction, by-products were observed in the negative control reaction and similar signal shifts as in the positive reactions were recorded in QCM-D, leading to the conclusion that we cannot rely on those results.

2. Περίληψη

Οι εκδηλώσεις που συνδέονται με προϊόντα μολυσμένα με Σαλμονέλα προκαλούν την ανάγκη ανάπτυξης απλών, γρήγορων και ακριβών μεθόδων ανίχνευσης, ακόμη και αν η παρουσία Σαλμονέλας είναι σε χαμηλά επίπεδα. Σε αυτή τη μελέτη, εξετάσαμε μια νέα στρατηγική για την ταχεία ανίχνευση και ποσοτικοποίηση βιώσιμης Σαλμονέλας με σύζευξη απλής ακουστικής ανίχνευσης βιοτινυλιωμένων μορίων DNA σε επιφάνεια τροποποιημένη με νιουτραβιδίνη ενός αισθητήρα QCM-D μετά από ισοθερμική ενίσχυση με μεσολάβηση βρόχου (LAMP).

Σχεδιάσαμε και βελτιστοποιήσαμε αρχικά μια LAMP δοκιμή που στοχεύει το γονίδιο *invA* της Σαλμονέλας και δεύτερον το γονίδιο *bcfD* με βιοτινυλιωμένους FIP εναρκτήρες. Για χαμηλές ποσότητες νουκλεϊνικών οξέων που θα ανιχνευθούν, χρησιμοποιήθηκαν λιποσώματα POPC με διάμετρο 100nm ως σήμανση για να ενισχυθεί η ακουστική ανίχνευση; αυτή η ανάγκη για δέσμευση λιποσώματος στα προϊόντα LAMP είναι ο λόγος για τον οποίο χρησιμοποιήσαμε βρόχο προς τα εμπρός και βρόχο προς τα πίσω εκκινητές τροποποιημένους με χοληστερόλη. Χρησιμοποιήθηκαν 500 κύτταρα για την ενίσχυση του σήματος με τη χρήση της LAMP στα αρχικά πειράματα (με στόχο τη μείωση αυτού του ορίου) και ο χρόνος αντίδρασης είχε εύρος από 15 έως 45 λεπτά, ενώ πραγματοποιήθηκαν αντιδράσεις ελέγχου για να αποφευχθούν τα ψευδώς αρνητικά και τα ψευδώς θετικά αποτελέσματα.

Στην περίπτωση του γονιδίου *invA* οι ανιχνευτές χοληστερόλης εισήχθησαν στον θάλαμο βιοαισθητήρα μετά την έγχυση του βιοτινυλιωμένου DNA και δεν παρατηρήθηκε σήμα, οπότε η παρουσία τους ταυτοποιήθηκε κατά την εισαγωγή των λιποσωμάτων POPC. Για το γονίδιο *bcfD*, χρησιμοποιήθηκαν δύο διαφορετικές προσεγγίσεις. Στην πρώτη, η έγχυση ανιχνευτών χοληστερόλης στον θάλαμο βιοαισθητήρα έλαβε χώρα μετά την έγχυση του βιοτινυλιωμένου DNA και στη δεύτερη περίπτωση οι ανιχνευτές χοληστερόλης χρησιμοποιήθηκαν απευθείας κατά τη διάρκεια της ενίσχυσης του σήματος (LAMP), έτσι ώστε το τελικό προϊόν πριν από την εισαγωγή στο θάλαμο του βιοαισθητήρα να περιέχει βιοτίνη και χοληστερόλη. Στην πρώτη περίπτωση δεν παρατηρήθηκε κανένα σήμα μετά την προσθήκη των λιποσωμάτων POPC στο βιοαισθητήρα ανεξάρτητα από τη χρήση ή όχι των εκκινητών βρόχου στην αντίδραση LAMP. Παρ 'όλα αυτά, στην περίπτωση της προσθήκης των εκκινητών χοληστερόλης στην αντίδραση LAMP, παρατηρήθηκαν παραπροϊόντα στην αρνητική αντίδραση ελέγχου και παρόμοια μετατόπιση σήματος όπως στις θετικές αντιδράσεις καταγράφηκαν στον QCM-D, οδηγώντας στο συμπέρασμα ότι δεν μπορούμε βασίζονται σε αυτά τα αποτελέσματα.

3. Acknowledgements

I would like to express my sincere appreciation to my supervisor Prof. Electra Gizeli from Biosensors lab and my co-supervisor, for the second part of my thesis, David Tomas Fornes from Nestle for their constant guidance encouragement and trust, without which this work would not have been possible. Also Dr. Dimitra Milioni and Dr. George Papadakis, from Biosensors lab, for their support and precious help during my thesis, who were always there for all the troubleshooting problems and the planning of my work with great patience and understanding and finally Mrs. Maria Megariti for all the new techniques that she taught me and the knowledge she shared with me.

I would also like to thank Prof. Irene Athanassakis and Prof. Dimitris Tzamarias who accepted to be the committee members of my master thesis.

Finally, I would like to thank my colleagues from Biosensors lab and Nestle, my family and my friends, who with patience and courage offered me the necessary mental support for completing my master thesis with calmness and consistency.

4. Introduction

The increasing demands for pathogen testing call for methods that can screen for major pathogens, such as *Salmonella*, with rapidity, reliability, and robustness. Despite being sensitive and reliable, current *Salmonella* testing relies primarily on culture-based methods, which are time-consuming and labor intensive. Nucleic acid amplification tests (NAATs) including PCR and real-time quantitative PCR (qPCR) have gained some popularity in this front (Maciorowski, Pillai et al. 2005, Balachandran, Friberg et al. 2012, Lofstrom and Hoorfar 2012) however, they require sophisticated thermal cyclers and are susceptible to many assay inhibitors present in food (Maciorowski, Pillai et al. 2005).

4.1 *Salmonella*

Salmonella is a gram-negative bacterium of the family Enterobacteriaceae and is a leading cause of food-borne illness worldwide. *Salmonella* symptoms vary depending on the type of *Salmonella* that has caused the infection. Most *Salmonella* infections lead to problems with digestion known as gastroenteritis, though some strains of the bacteria can cause typhoid fever.

Not all Salmonella bacteria are the same. In fact there are more than 2,500 types of Salmonella. Each type is identified and labeled as a different serotype. Some of these serotypes will only infect one particular animal, or only exist in one specific place. Of these, less than 100 are responsible for the majority of human infections. Knowing the serotype of a given organism it is important for scientists who want to observe and control the spread of outbreaks. More details about *Salmonella* will be given in Chapter 2.

4.2 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP), is a NAAT that has recently emerged as a promising alternative to PCR for pathogen detection in food testing and clinical diagnostics (Mori and Notomi 2009).

LAMP is a method for the amplification of nucleic acids under isothermal conditions (60-65 °C) with high specificity and sensitivity, using a set of four specially designed primers and a *Bst* DNA polymerase to produce a target-specific stem-loop DNA structure during initial assay steps. The addition of one to two loop primers accelerates the LAMP reaction by their

hybridization to stem-loop DNAs and the facilitation of strand displacement and amplification (Figure 1).

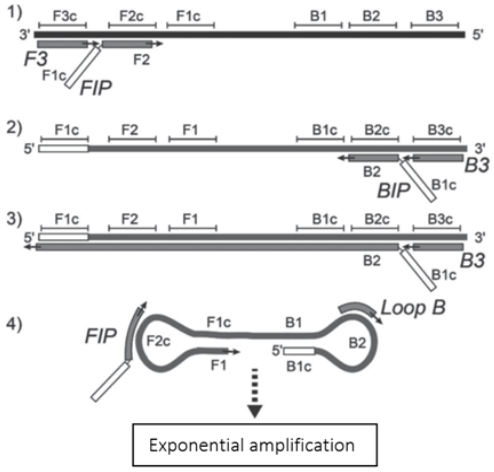


Figure 1: *LAMP amplification scheme. (1) Initiation at F end of target sequence via FIP and displacement of nascent strand by synthesis initiating at F3. (2,3) Synthesis and displacement at B end of target resulting in (4), the seed structure for exponential LAMP amplification (Tanner and Evans 2014).*

Because the procedure takes place under isothermal conditions, LAMP can be performed in much simpler instruments such as a heater or a water bath. Without the need of a denatured DNA template normally required for PCR and due to the auto-cycling strand displacement nature of *Bst* DNA polymerase with high strand displacement activity, LAMP is a powerful tool for nucleic acid amplification and it has already been widely used in pathogen detection (Zhuang, Gong et al. 2014). LAMP results can be interpreted with naked eye techniques, gel electrophoresis, real-time monitoring etc. Acoustic wave devices are an attractive alternative due to their simplicity in operation, label free nature and high sensitivity (nanogram of the analyte).

4.3 Acoustic biosensors

Acoustic wave devices operate by coupling the measurand (analyte) as a modulation in the properties of the acoustic wave that can then be correlated to the mass deposited on the surface, the viscoelasticity of the analyte and/or other properties.

4.4.1 Quartz Crystal Microbalance (QCM)

Quartz Crystal Microbalance (QCM) is an extremely sensitive mass balance that measures nanogram to microgram level changes in mass per unit area. The heart of the technology is a quartz disc; a piezoelectric material that oscillates at a defined frequency by applying an appropriate voltage usually via metal electrodes. The frequency of oscillation can be affected by the addition or removal of small amounts of mass onto the electrode surface. This change

in frequency can be monitored in real time to obtain useful information about molecular interactions or reactions taking place at the electrode surface, such as film growth, oxidation, corrosion/decay, etc (Dixon 2008).

4.4.2 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

The introduction of QCM-D has enabled monitoring of the dissipation, factor which gives information on energy loss due to the mass deposited on the surface. QCM-D is a bulk acoustic wave (BAW) sensor comprising a quartz crystal between two gold circular electrodes (Figure 2a). The piezoelectric nature of the quartz imposes the deformation of the crystal upon application of an alternating current (AC) voltage between the electrodes (Figure 2b), as said previously, and causes the crystal to oscillate at its acoustic resonance frequency, creating a wave that propagates through the biosensor chamber. When the AC voltage is turned off (Figure 2c), the oscillation decays exponentially (Figure 2d) and with addition of mass the decay is quicker. The propagating wave is recorded and the changes on the frequency (F) and energy dissipation (D) are measured (Fogel, Limson et al. 2016). The frequency change (ΔF) is related to the mass (m) of the adsorbed entities, the dissipation change (ΔD) is related to the viscoelasticity of the adsorbed analyte and the ratio ΔD/ΔF is related to the energy loss per surface coupled unit mass (Tsortos, Papadakis et al. 2016). The surface of the chip can be coated with different chemical compounds depending on the analyte to be bound.

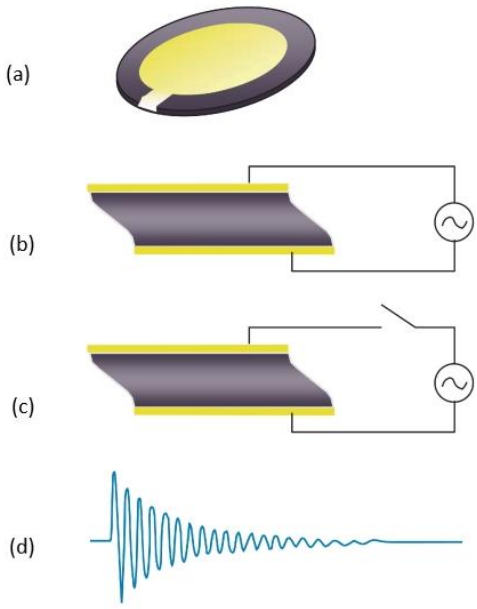


Figure 2: *Description of the main components in QCM-D. a: Typical QCM-D sensor with gold electrodes. b: Quartz crystal with alternating current applied across electrodes. c: Short circuiting the alternating current. d: The oscillatory decay as the quartz disk comes to rest. The frequency of the oscillating crystal, shown in b, is related to*

the total oscillating mass adsorbed on the surface, while the energy dissipation, shown in c, is related to the viscoelastic properties of the oscillating mass. Thus, changes in adsorbed mass of, for example, a rigid protein provide a change in frequency, but for viscoelastic masses such as biomacromolecules, there is a change both in frequency and dissipation (Dixon 2008).

4.4 Liposomes

A liposome, as can be seen in figure 3, is a spherical vesicle having at least one lipid bilayer. The latter can be produced from natural non-toxic phospholipids -especially phosphatidylcholine, but may also include other lipids, such as cholesterol. Liposomes can be prepared by different methods. They may vary in their dimensions, composition and charge. The major types of liposomes are the multilamellar vesicles (MLV, with several lamellar phase lipid bilayers with diameters 200nm-3μm), and the unilamellar vesicles divided into 3 categories depending on their size: a) the small unilamellar liposome vesicles (SUV), with one lipid bilayer and diameters 20nm-100nm, b) the large unilamellar vesicles (LUV) with diameters 100nm-400nm and c) the giant unilamellar vesicles (GUV) with diameters up to 1μm and larger (Torchilin 2006, Akbarzadeh, Rezaei-Sadabady et al. 2013). Chemical modification of liposomes with certain surface ligands or modified lipids (e.g. fluorescent) may be desired for facilitating their attachment to unhealthy tissue with high specificity or for the stabilization of those vesicles for increasing their shelf life *in vivo* (Torchilin 2006).

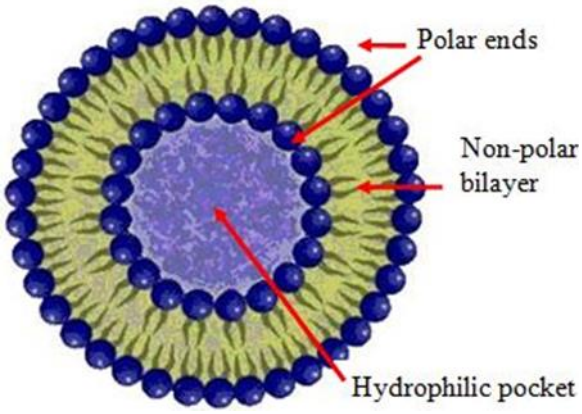


Figure 3: *Scheme of a liposome formed by phospholipids in an aqueous solution.*

4.5 Aim of the study

This study aimed to evaluate the detection of *Salmonella* using QCM-D. The *Salmonella* DNA was amplified using LAMP, targeting *invA* (Chen, Wang et al. 2011) or *bcfD* (Zhuang, Gong et al. 2014) genes. The ultimate goal of this project is the parallel detection of more than one bacteria in the same sample. This is the reason why cholesterol-modified single stranded DNAs complementary to single stranded areas of *Salmonella* LAMP products were injected; any liposome binding recorded would reveal *Salmonella* presence in the initial sample.

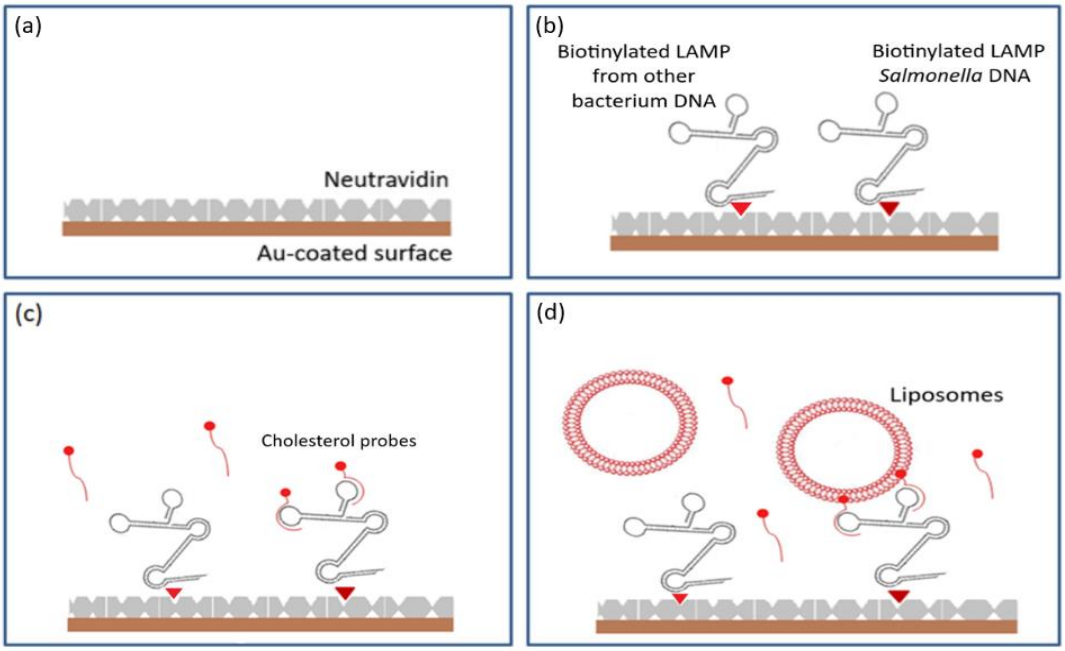


Figure 4: *Detection of labeled LAMP amplicons on the (a) surface of neutravidin-coated well. (b) The biotinylated LAMP amplicons bind to the neutravidin-coated gold biosensor surface. (c) The cholesterol loop-specific oligonucleotide probes with complementary sequences to the single stranded loops of the Salmoella LAMP amplicons bind to the corresponding areas on the DNA. (d) POPC liposomes injected for the identification of the probes binding. (Image not in scale).*

5. Materials and Methods

5.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and the phosphate buffered saline (PBS) tablets (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) from Sigma Aldrich, Germany.

The mini-extruder and the filter supports were obtained from Avanti Polar Lipids, 100nm pore-sized polycarbonate membranes from Avestin (Ottawa, Canada) and the LAMP kit (containing *Bst* 2.0 WarmStart Polymerase, 10X isothermal Amplification Buffer and dNTPs) from New England BioLabs. The MWD100 ladder was obtained from Nippon Genetics Europe, Germany.

The set of primers for the *invA* and the *bcfD* genes respectively were the followings: (F3: 5'-CGGCCCGATTTTCTCTGG-3', B3: 5'-CGGCAATAGCGTCACCTT-3', FIP: 5'-biotin-GCGCGGCATCCGCATCAATA –TGCCCGGTAAACAGATGAGT-3', BIP: 5'-GCGAACGGCGAAGCGTACTG –TCGCACCGTCAAAGGAAC-3', LF: 5'-GGCCTTCAAATCGGCATCAAT-3', LB: 5'-GAAAGGGAAAGCCAGCTTTACG-3', chol-LF: 5'-chol-GGCCTTCAAATCGGCATCAAT-3', chol-LB: 5'-chol-GAAAGGGAAAGCCAGCTTTACG-3'), (F3: 5'-CCGGACAAACGATTCTGGTA-3', B3: 5'-CCGACATCGGCATTATCCG-3', FIP: 5'-biotin-TGCACTTTACCGGTACGCTGAA-TACAGCGGCAATTTCAACCA-3', BIP: 5'-CGGTCTGGATTCGCAGGTCAAA –GCGATAGCCTGGGGAAC-3', LF: 5'-TACCCCCTCCGGCTTTTG-3', chol-LF: 5'-chol-TACCCCCTCCGGCTTTTG-3' , LB: 5'-ACAATGCGTCTTATCGCTACG-3', chol-LB: 5'-chol-ACAATGCGTCTTATCGCTACG-3') and they were purchased from Metabion international AG. The QCM-D crystals were obtained from AW Sensors, Spain and the Neutravidin used for this study from Thermo Fisher Scientific, USA.

5.2 Methods

5.2.1 Loop-mediated Isothermal amplification (LAMP)

All the components are put into the reaction tube, in the order that is shown below, in Table 1 for the first case and the *invA* gene and in Table 2 for the *bcfD* gene:

COMPONENTS	*25µl RXN*	*FINAL CONCENTRATION*
2X LAMP mix	12.5µl	1X
b-FIP/BIP (45µM)	1µl	1.8 µM
F3/B3 (10µM)	1µl	0.4 µM
LF/LB (2.5µM)	1µl	0.1 µM
DNA sample	1µl	500 cells
ddH_2O	5.5µl	
Total Reaction Volume	25µl	

Table 1: *Components and their amounts for the invA gene.*

COMPONENTS	*25µl RXN*	*FINAL CONCENTRATION*	*OR*	*25µl RXN*	*FINAL CONCENTRATION*
2X LAMP mix	12.5µl	1X		12.5µl	1X
b-FIP/BIP (40µM)	1µl	1.6 µM		1µl	1.6 µM
F3/B3 (20µM)	1µl	0.8 µM		1µl	0.8 µM
LF/LB (5µM)	1µl	0.2 µM			
DNA sample	1µl	500 cells		1µl	500 cells
ddH_2O	5.5µl			7.5µl	
Total Reaction Volume	25µl			25µl	

Table 2: *Components and their amounts for the bcfD gene. On the right table with loop primers and on the left table without loop primers.*

In the first case the mixture was incubated for 20 minutes at 63°C.

In the second case the mixture was incubated at 64°C with various reaction times. 15-25 minutes when the loop primers were used, and 35-45 minutes without the loop primers.

In each case the final volume for each reaction was 25µl. The template DNA was extracted from *Salmonella Typhimurium* strains. The final concentration of bacterial culture was 10^5

cells/µl. The amplification and the detection of a negative control were also important to rule-out the false-positive or false-negative results in the amplification assay.

5.2.2 Gel Electrophoresis

The purity of the LAMP product was evaluated by Agarose gel electrophoresis. Electrophoresis uses an electric field to move the negatively charged DNA through the matrix. The DNA molecules were therefore separated according to their MW. The concentration of the gel affects the resolution of DNA separation. A big Gel-Cast is prepared with 100ml 1x TBE-Buffer containing 2 g of Agarose (2%). After boiling, 10 µl of gel Red are added and the mixture is spread in the prepared cast. A mixture of 1 µl loading dye and 5 µl of LAMP product is added in each well. The negative and positive products on the gel can be seen after 40 min under UV light.

5.2.3 Liposomes preparation

2 mg of POPC lipids previously diluted in Chloroform:Methanol (4:1) were placed in a glass flask. The solvent was evaporated under a thin nitrogen gas stream (for ~30 min), allowing a thin lipid film to be formed. Afterwards, 1ml of PBS was added dropwise and the flask was vortexed forming multilamellar vesicles (MLVs) for ~1 hour.

Liposome extrusion

Extrusion converts MLVs to LUVs. The suspension of MLVs is extruded 21 times through uniform cylindrical pores of a track-etched polycarbonate membrane (100nm, Whatman) yielding smaller vesicles with 100nm diameter. At the beginning of this procedure, a small volume of PBS buffer is used to wash the extruder and ensure a high liposome recovery.

5.2.4 QCM-D

Prior to mounting, the QCM-D crystals were rinsed with ethanol and water and then cleaned in a UV-ozone cleaner (Ossila, UK) for 20 min. QCM-D measurements were performed at several harmonics (3, 5, 7, 9 and 11) using a QCM-D E4 system (Q-Sense AB, Sweden). The frequency and dissipation shifts were plotted using the OriginPro 8 software. The presented frequency shifts were obtained at the 7th harmonic (35MHz).

The experiment was carried out as follows: Neutravidin (0.2 µg/mL, 200µl in PBS) solution was injected followed by rinsing with PBS buffer. Biotinylated LAMP products were injected into the chamber and bound to the neutravidin-coated gold biosensor surface and rinsed. The injection of single stranded DNA probes (LF-chol: 100pmol, LB-chol: 100pmol) with complementary sequences to the single stranded areas of the LAMP DNAs already

immobilized on the surface and functionalized with cholesterol on their 5' side followed. The probes signal being very low, the system was left to equilibrate in PBS and then POPC liposomes (0.1mg/ml in 1ml PBS) were injected for the identification of the probes binding.

6. Results

6.1 Loop-mediated Isothermal amplification (LAMP)

6.1.1 *invA* gene

The positive sample in LAMP method displayed a specific characteristic ladder-like pattern (Figure 5). The amplification of *Salmonella* DNA by LAMP was accelerated with the addition of loop forward and backward primers. After 20 min the LAMP product was visible in the gel, while no pattern was observed for the negative control reaction, apart from the primers used.

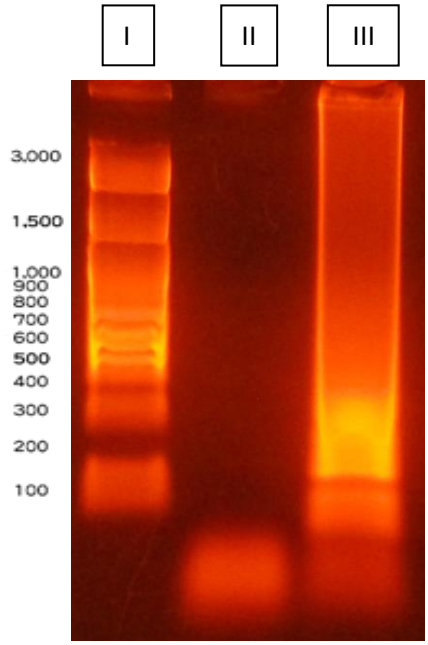


Figure 5: *A representative gel image after 40 min of electrophoresis. In lane (I), is the well containing the ladder, with 12 fragments starting at 100 bp and going up to 3000 bp, in lane (II) is the negative control showing a band corresponding to the primers of the invA gene (from 17 to 40bp) and in lane (III) the LAMP product of the invA gene with the primer band after 20 min post reaction.*

6.1.2 *bcfD* gene

The extracted DNA from *Salmonella Typhimurium* for this gene became positive 10 min post reaction when the loop primers were added and 35 min post reaction in the absence of loop primers (Figure 6). So the reaction time was reduced from 35 to 10 min with the addition of loop primers. Again, no pattern was observed for the negative control reactions.

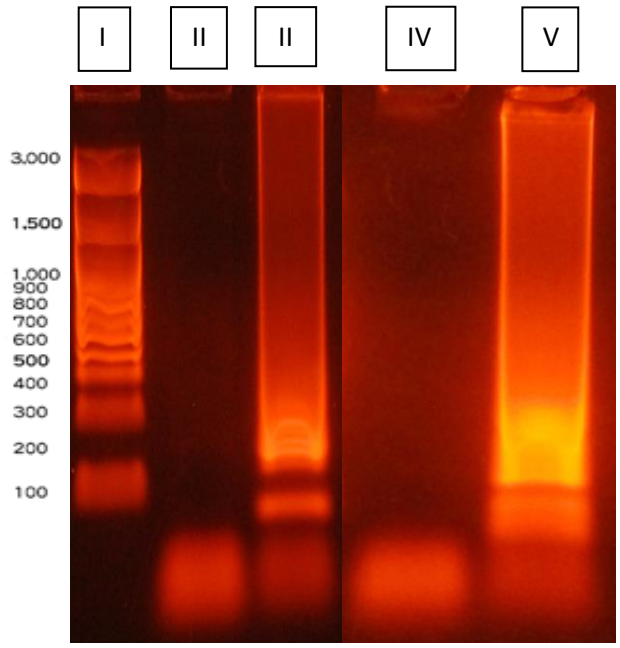


Figure 6: *A representative gel image after 40 min of electrophoresis. In lane (I) is the well containing the ladder containing 12 fragments starting at 100 bp and going up to 3000 bp, in lane (II) is the negative control showing a band corresponding to the primers of the bcfD gene (primers from 18 to 42 bp) after 10 min post reaction with the loop primers , in lane (III) is the LAMP product after 10 min post reaction with the loop primers for the bcfD gene, in lane (IV) is the negative control corresponding to the primers of the bcfD gene (primers from 19 to 42 bp) after 35 min post reaction without the loop primers and in lane (V) is the LAMP product after 35 min post reaction with the loop primers for the bcfD gene.*

6.2 Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

For the QCM-D studies, LAMP amplicons from different reaction times were used and the frequency and dissipation changes were recorded.

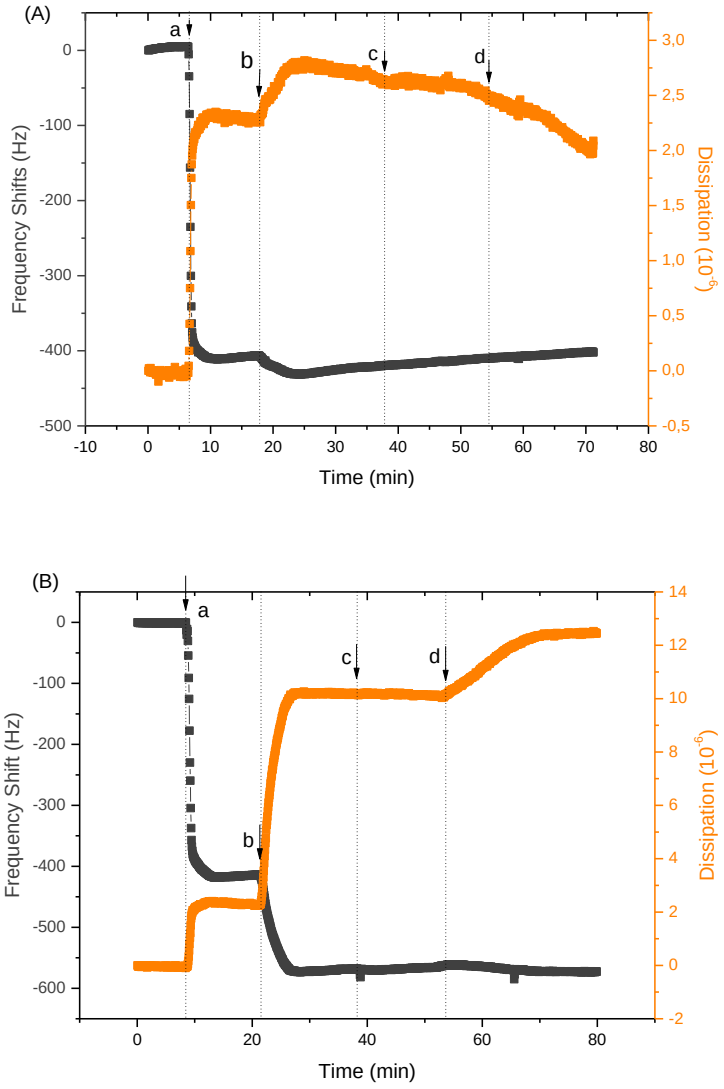
The immobilization of neutravidin caused 480 ± 50 Hz decrease in frequency in 10 min and 3.2 ± 0.6 increase in energy dissipation, which confirmed the successful adsorption of a neutravidin layer. The F and D shifts provoked by the binding of the LAMP DNA products depended on the reaction time used in LAMP and will be presented below.

The injection of the complementary cholesterol-modified probes did not cause any measurable shift of the signal, because on the biosensor surface we already have a high

amount of previously amplified DNAs, and their presence could only be identified with the addition of POPC liposomes. The latter have the ability to bind to the cholesterol of the probes and, thus, cause a decrease in frequency and an increase in the dissipation.

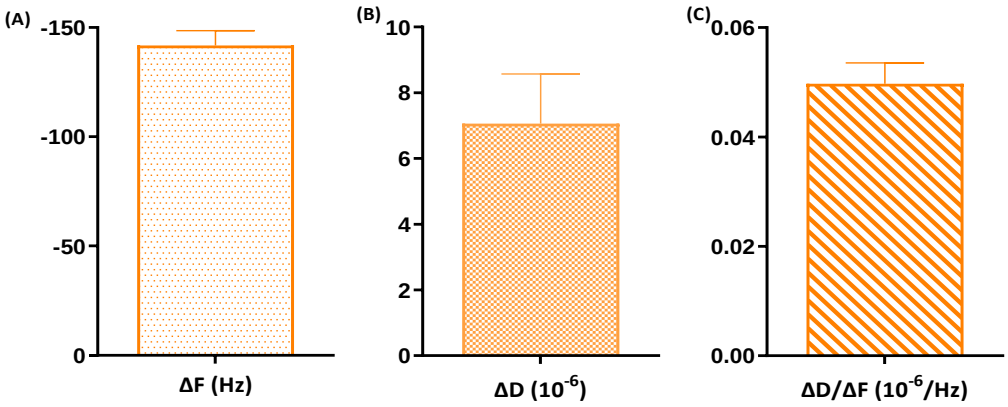
6.2.1 invA gene

In the experiments carried out using the first set of primers (*invA* gene), the liposomes were bound successfully, showing a successful binding of the probes on the DNA. A typical experiment sensogram is shown in graph 1.



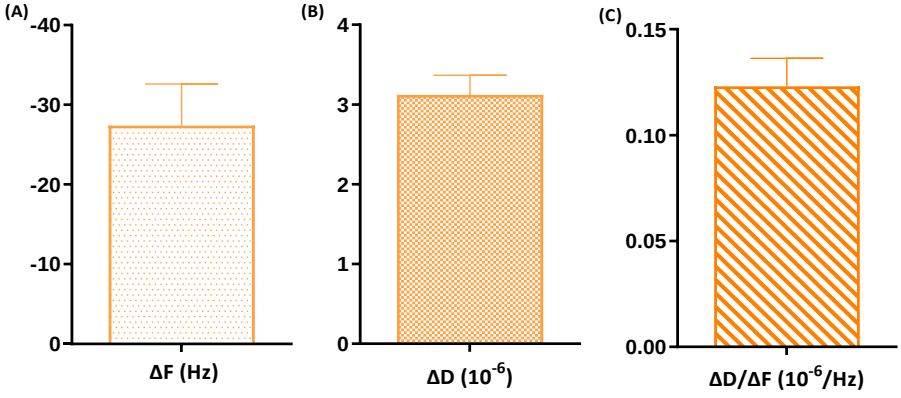
Graph 1: *(A) The sensorgram of the negative reaction for the invA gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the invA gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm).*

As previously mentioned, for the *invA* gene all the LAMP reactions were performed at 63°C for 20 min. The different F and D signals obtained from the LAMP product injection (step b in graph 1) are presented in Graph 2. The ΔD/ΔF ratio is also shown in the same graph. The average values are (-141.755 ± 6.788) Hz for ΔF, (7.060 ± 0.356) 10^{-6} for ΔD and (0.050 ± 0.001) 10^{-6}/Hz for ΔD/ΔF. The experiments have been repeated at least 6 times.



Graph 2: *(A) ΔF, (B) ΔD and (C) Acoustic ratio of the invA gene LAMP amplicons (after 20 min LAMP post reaction) injection.*

The respective values for the liposome injection (step d in Graph 1) are shown in Graph 3. These values correspond to the liposome binding on the cholesterol of the hybridized probe proving the binding of the probe on the LAMP product. The average values are (-27.391 ± 11.682) Hz for ΔF, (3.121 ± 0.547) 10^{-6} for ΔD and (0.123 ± 0.03) 10^{-6}/Hz for ΔD/ΔF.

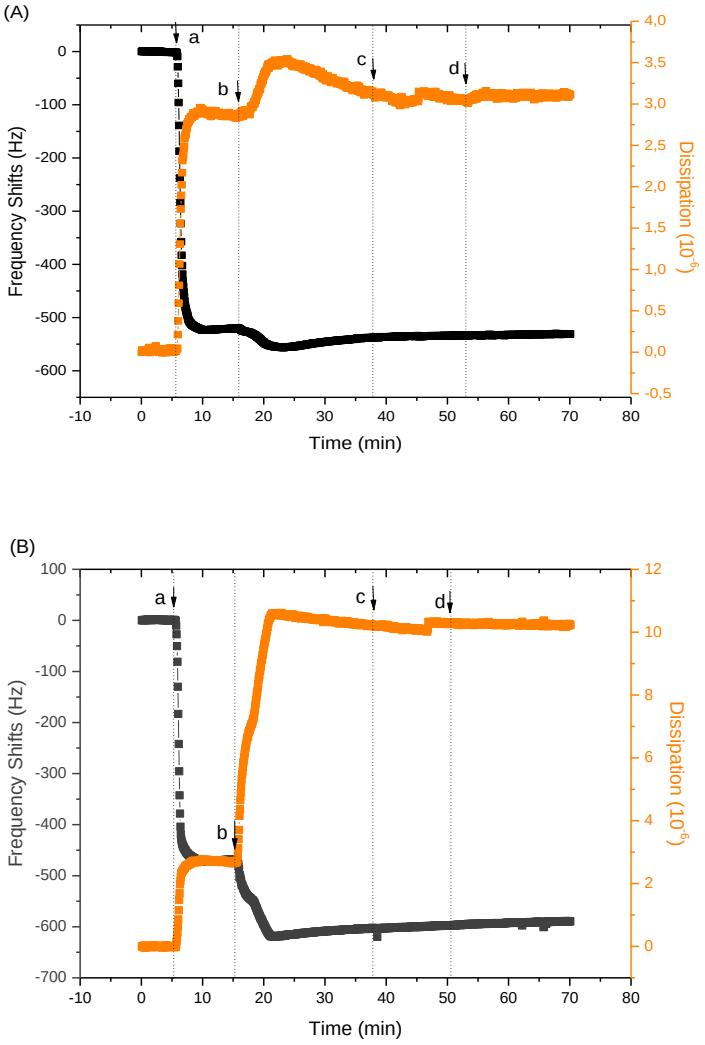


Graph 3: *(A) ΔF, (B) ΔD and (C) Acoustic ratio for the liposomes (100 nm diameter) binding on the cholesterol of the hybridized probe for the invA gene (after 20 min LAMP post reaction of the gene).*

6.2.2 bcfD gene

6.2.2.1 LAMP reaction with loop primers

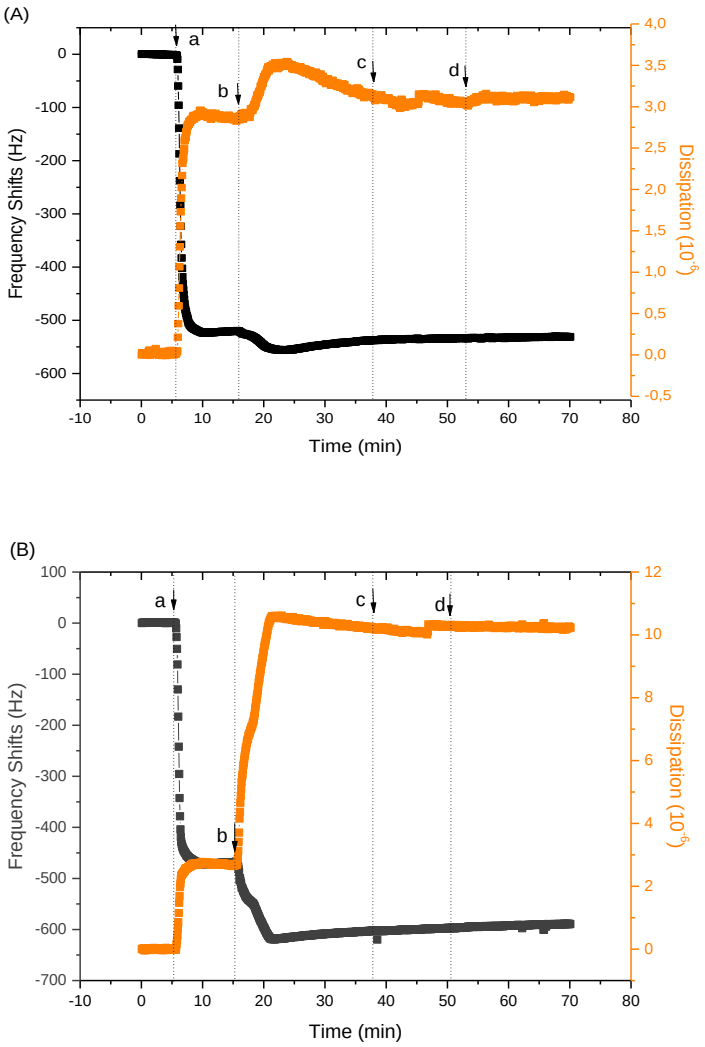
For the second set of primers (*bcfD* gene), no signal for the probes nor for the binding of the liposomes was measured (Graph 4). This is the reason why various LAMP protocols have been tried. Wide range of presence or absence of loop primers, with or without cholesterol loop primers.



Graph 4: *(A) The sensorgram of the negative reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm).*

6.2.2.2 LAMP reaction without loop primers

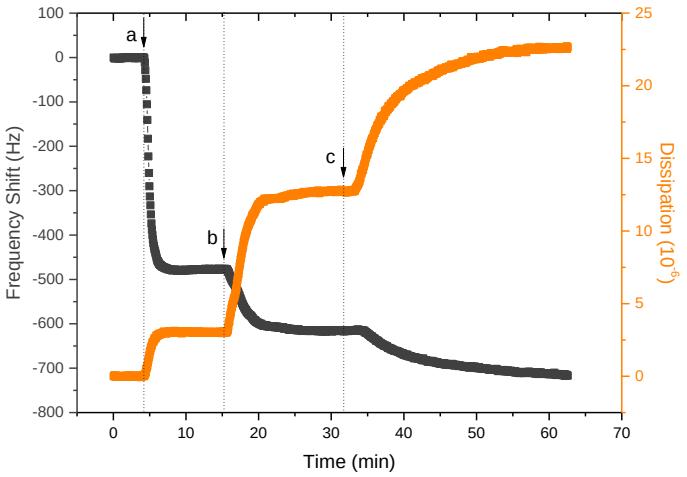
Because we did not observe and changes in the F and D shifts after the injection of the POPC liposomes in the previous case, we tried to perform a LAMP reaction without loop primers, because we taught that the single stranded areas of the DNA are saturated and there is no space for the hybridization of the cholesterol probes on the QCM-D surface. But again no signal was observed after the addition of POPC liposomes (Graph 5).



Graph 5: *(A) The sensorgram of the negative reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) negative product, c) Probes with cholesterol and d) Liposomes (100nm). (B) The sensorgram of the positive reaction for the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA, c) Probes with cholesterol and d) Liposomes (100nm).*

6.2.2.3 LAMP reaction with cholesterol loop primers

The only case for which liposome binding was observed was when the used loop primers used in the reaction were functionalized with cholesterol molecules on their 5' ends (LB-chol and LF-chol) instead of LF-LB (Graph 6).



Graph 6: *The sensorgram of the bcfD gene detection. The times of injection are indicated by small arrows for: a) Neutavidin, b) biotinylated LAMP DNA with cholesterol, c) Liposomes (100nm).*

Nevertheless, in this case by-products were observed on the negative control reaction due to the injection of the cholesterol in the LAMP reaction mix (Figure 7) and when we tried to inject the negative control in the biosensor chamber we took the same signal as in the case of the positive control (Data not shown).

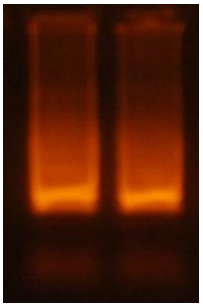
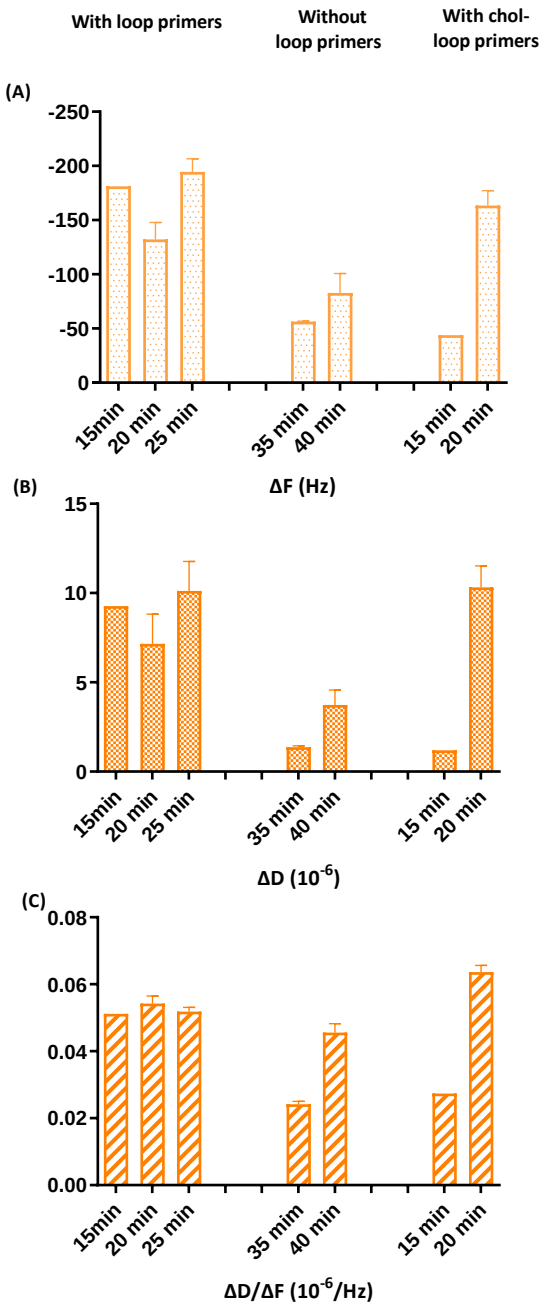


Figure 7: *Negative control (left) and LAMP product (right) after 15 min post reaction with the cholesterol loop primers.*

6.2.2.4 Comparison of different experimental conditions

The different F and D signals obtained in some preliminary experiments for the LAMP product injection (step b in Graphs 4,5 & 6) for different conditions have been traced and are presented in Graph 6. The ΔD/ΔF variation is also shown in the same graph.



Graph 7: (A) ΔF, (B) ΔD and (C) Acoustic ratio of the bcfD gene LAMP amplicons binding in different times and conditions.

ΔF (Hz) *ΔD (10^{-6})* *ΔD/ΔF (10^{-6}/ Hz)*

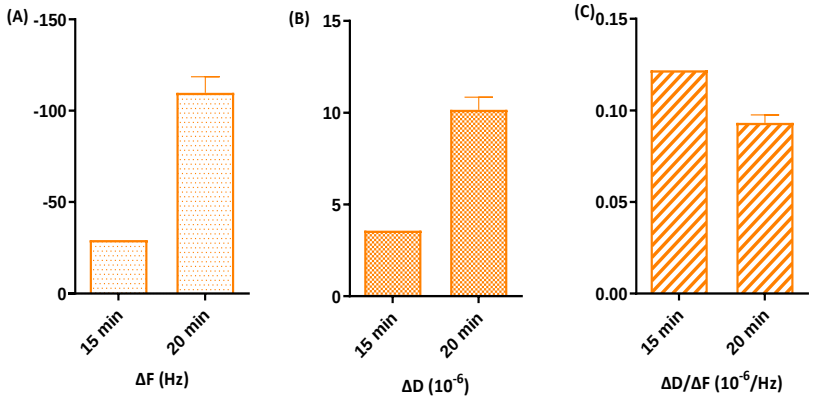
	Time	Mean Value	SD	Mean Value	SD	Mean Value	SD
With loop primers	15 min	-181,140	0,000	9,264	0,000	0,051	0,000
	20 min	-132,209	31,225	7,154	1,669	0,054	0,005
	25 min	-194,578	23,835	10,121	1,651	0,052	0,003
Without loop primers	35 min	-56,377	0,683	1,363	0,083	0,024	0,001
	40 min	-82,839	25,437	3,725	0,847	0,046	0,004
With cholesterol loop primers	15 min	-43,825	0,000	1,200	0,000	0,027	0,000
	20 min	-163,357	27,832	10,316	1,209	0,064	0,004

The average values for the ΔF, ΔD and ΔD/ΔF of the DNA amplicons for the different conditions of the LAMP reaction are shown in Table 3:

***Table 3**: Average values of ΔF, ΔD and acoustic ratio ΔD/ΔF with the standard deviation of each value.*

In the case of loop primers we can observe that the reaction time does not affect the signal much and the ratio ΔD/ΔF is almost stable, but in the two other cases (without the loop primers and with cholesterol loop primers) we can see differences in the signal and in the ΔD/ΔF ratio. In lower LAMP reaction times there is lower signal, and this can happen due to the different conformations of the products on the surface.

As mentioned before no binding of the liposomes on the cholesterol probes was observed in this case (*bcfD* gene), unless the loop primers were functionalized with cholesterol. Graph 8 shows the different F and D signals for the liposome binding on the cholesterol of the DNA amplicons (step c in Graph 6). The average values for the 15 min LAMP reaction are (-29.318) Hz for ΔF, (3.572) 10^{-6} for ΔD and (0.122) (10^{-6}/Hz) for ΔD/ΔF. For the 20 min LAMP reaction the respective values are (-109.784 ± 17.645) Hz for ΔF, (10.159 ± 1.381) 10^{-6} for ΔD and (0.093 ± 0.009) 10^{-6}/Hz for ΔD/ΔF.



***Graph 8:** (A) ΔF, (B) ΔD and (C) Acoustic ratio for the liposome (100 nm diameter) binding on the cholesterol of the hybridized probe.*

As we can observe in Graph 8, the F and D signals and also the ΔD/ΔF ratio vary. The F and D for the 15 min LAMP reaction are much lower than the same shifts for the 20 min LAMP reaction. These measurements result in a higher ΔD/ΔF ratio for the 15 min reaction due to the differences between the previously bound LAMP products. We are not able yet to explain these differences, but we can suggest that the liposomes adopt a different configuration on the surface affected by the prior binding of the DNA.

7. Conclusion and discussion

In the present study, we managed to immobilize DNA molecules previously amplified by LAMP on the biosensor surface. The *Salmonella invA* and *bcfD* LAMP assay was rapid, specific and due to the parallel use of the QCM-D we were able to see differences in the two previously amplified genes and between the different reaction times that were used for the amplification method. We managed to amplify and detect 500 cells per reaction and it seems that this limit can be much lower. Different amplification conditions were used and the F and D signals for each one of the amplified products were measured.

The signal of the biotinylated DNA after 20 min LAMP reaction for *invA* gene was (-141.755 ± 6.788) Hz for ΔF, (7.060 ± 0.356) 10^{-6} for ΔD and (0.050 ± 0.0009) 10^{-6}/Hz for ΔD/ΔF and for the liposomes was (-27.391 ± 11.682) Hz for ΔF, (3.121 ± 0.547) 10^{-6} for ΔD and (0.123 ± 0.03) 10^{-6}/Hz for ΔD/ΔF. For *bcfD* gene, two different approaches were used, in the first, the injection of the cholesterol probes in the biosensor chamber took place after the injection of the biotinylated DNA and in the second, the cholesterol probes were used directly during the amplification (LAMP), so that the final product before the injection in the biosensor chamber had biotin and cholesterol. In the first case on the biosensor chamber regardless of the use or not of loop primers in the LAMP reaction no signal was observed after the addition of the POPC liposomes. The signal from the biotinylated DNA for 15 min of LAMP with loop primers was (-181.140) Hz for ΔF, (9.264) 10^{-6} for ΔD and (0.051) 10^{-6}/ Hz for ΔD/ΔF, for 20 min (-132.209 ± 31,225) Hz for ΔF, (7.154 ± 1.669) 10^{-6} ΔD and (0.054) 10^{-6}/ Hz for ΔD/ΔF and for 25 min (-194.578 ± 23.835) Hz for ΔF, (10.121 ± 1.651) 10^{-6} for ΔD and (0.052 ± 0.003) 10^{-6}/ Hz for ΔD/ΔF. The signal from the biotinylated DNA for 35 min of LAMP without loop primers was (-56.378 ± 0.683) ΔF, (1.363 ± 0.083) 10^{-6} for ΔD and (0.024 ± 0.001) 10^{-6}/ Hz for ΔD/ΔF and for 40 min (-82.840 ± 25.437) Hz, (3.725 ± 0.847) 10^{-6} for ΔD and (0.046 ± 0.004) 10^{-6}/ Hz for ΔD/ΔF. In the second case, when LAMP reaction was held with cholesterol loop primers, after the injection of POPC on the biosensor surface signal changes were recoded. The shift from the biotinylated DNA with the cholesterol loop primers for 15 min LAMP reaction was (-4.825)

Hz for ΔF, (1.200) 10^{-6} for ΔD and (0.027) 10^{-6}/ Hz for ΔD/ΔF and for 20 min of LAMP reaction was (-163,357 ± 27.832) Hz for ΔF, (10.316 ± 1,209) 10^{-6} for ΔD and (0.063 ± 0.004) 10^{-6}/ Hz for ΔD/ΔF. In the case of loop primers we can observe that the reaction time does not affect the signal much and the ratio ΔD/ΔF is almost stable, but in the two other cases (without the loop primers and with cholesterol loop primers) we can see differences in the signal and in the ΔD/ΔF ratio. In lower LAMP reaction times there is lower signal, and this can happens due to the different conformations of the products on the surface. The average values for the immobilization of the POPC liposomes for the 15 min LAMP reaction were (-29.318) Hz for ΔF, (3.572) 10^{-6} for ΔD and (0.122) 10^{-6}/Hz for ΔD/ΔF. For the 20 min LAMP reaction the respective values were (-109.784 ± 17.645) Hz for ΔF, (10.159 ± 1.381) 10^{-6} for ΔD and (0.093 ± 0.009) 10^{-6}/Hz for ΔD/ΔF. From those results it is clear that for *invA* and *bcfD* genes for 20 min LAMP reaction the F and D shifts and the ratio ΔD/ΔF have similar values, and for the latter no liposomes binding was observed.

Although we have tried many different conditions, more studies need to be done in order to reduce any experimental errors and to find out why no signal is observed for the binding of the liposomes after the injection of cholesterol probes in the case of *bcfD* gene. This could be due to the way the product binds on the biosensor surface and in order to further investigate this issue, we can try to relocate the biotin tag from the FIP primer to LB or LF and thus change its configuration on the biosensor surface.

The combination of LAMP and QCM-D seem to be able to perform diagnostics in a parallel, multiple and integrated format. During this study we were able to detect the target DNAs using liposomes as tags for specific targets. Such a set-up may help us in the future for the parallel detection of two or maybe more bacteria in the same sample with the aid of multiplexed LAMP.

8. References

Akbarzadeh, A., R. Rezaei-Sadabady, S. Davaran, S. W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi and K. Nejati-Koshki (2013). "Liposome: classification, preparation, and applications." Nanoscale Res Lett **8**(1): 102.

Balachandran, P., M. Friberg, V. Vanlandingham, K. Kozak, A. Manolis, M. Brevnov, E. Crowley, P. Bird, D. Goins, M. R. Furtado, O. V. Petrauskene, R. S. Tebbs and D. Charbonneau (2012). "Rapid detection of Salmonella in pet food: design and evaluation of integrated methods based on real-time PCR detection." J Food Prot **75**(2): 347-352.

Chen, S., F. Wang, J. C. Beaulieu, R. E. Stein and B. Ge (2011). "Rapid detection of viable salmonellae in produce by coupling propidium monoazide with loop-mediated isothermal amplification." Appl Environ Microbiol **77**(12): 4008-4016.

Dixon, M. C. (2008). "Quartz crystal microbalance with dissipation monitoring: enabling real-time characterization of biological materials and their interactions." J Biomol Tech **19**(3): 151-158.

Fogel, R., J. Limson and A. A. Seshia (2016). "Acoustic biosensors." Essays Biochem **60**(1): 101-110.

Lofstrom, C. and J. Hoorfar (2012). "Validation of an open-formula, diagnostic real-time PCR method for 20-h detection of Salmonella in animal feeds." Vet Microbiol **158**(3-4): 431-435.

Maciorowski, K. G., S. D. Pillai, F. T. Jones and S. C. Ricke (2005). "Polymerase chain reaction detection of foodborne Salmonella spp. in animal feeds." Crit Rev Microbiol **31**(1): 45-53.

Mori, Y. and T. Notomi (2009). "Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases." J Infect Chemother **15**(2): 62-69.

Tanner, N. A. and T. C. Evans, Jr. (2014). "Loop-mediated isothermal amplification for detection of nucleic acids." Curr Protoc Mol Biol **105**: Unit 15 14.

Torchilin, V. P. (2006). "Multifunctional nanocarriers." Adv Drug Deliv Rev **58**(14): 1532-1555.

Tsortos, A., G. Papadakis and E. Gizeli (2016). "On the Hydrodynamic Nature of DNA Acoustic Sensing." Anal Chem **88**(12): 6472-6478.

Zhuang, L., J. Gong, Q. Li, C. Zhu, Y. Yu, X. Dou, X. Liu, B. Xu and C. Wang (2014). "Detection of Salmonella spp. by a loop-mediated isothermal amplification (LAMP) method targeting bcfD gene." Lett Appl Microbiol **59**(6): 658-664.

University of Crete,
Biology Department

Institute of Food & Analytical Sciences,
Food Safety Research Department, R&D Nestlé, Lausanne, Switzerland

MSc Program:
Protein Biotechnology

MASTER THESIS
Chapter 2

MICROST: Microbiological analysis improvement by optimisation of sample treatment. Methods for Salmonella spp.

Author: Kontogianni Georgia-Ioanna, AM 862

University professor: Prof. Gizeli Electra
Nestlé Supervisor: David Tomas Fornes

Heraklion, October 2019

Contents

1 Abstract 33

2 Περίληψη 34

3 Introduction 35

3.1 BACKROUND 35

3.2 AIM OF THE STUDY 38

4 Methodology and Trials 39

4.1 BACTERIAL STRAINS 39

4.2 REFERENCE MATERIAL PREPARATION 39

4.3 STRESS MODEL SELECTION 39

4.3.1 Heat stress 39

4.3.2 Dry stress 40

4.3.3 Growth curves 40

4.4 RAW MATERIALS 40

4.5 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) 41

4.6 NEUTRALIZER TRIALS 42

4.6.1 Procedure 43

5 Results 46

5.1 STRESS MODEL SELECTION (HEAT STRESS) 46

5.2 MINIMUM INHIBITORY CONCENTRATION (MIC) 47

5.3 NEUTRALIZERS TOXICITY AGAINST *S.TYPHIMURIUM* AND *S.ENTERITIDIS* 47

5.4 NEUTRALIZER EFFICACY 48

5.4.1 Neutralizers with inhibitory compounds 49

5.4.2 Neutralizers with raw materials 53

6 Discussion 58

7 Conclusions 62

8 Recommendations 64

9 References 65

10 Appendices 68

10.1 APPENDIX 1: *SALMONELLA* SPP. STRAINS INCLUDED IN THIS STUDY 68

10.2 APPENDIX 2: MEDIA,REAGENTS AND EQUIPMENT 68

10.3 APPENDIX 3: COMPOSITION OF DEY-ENGLEY 69

10.4 APPENDIX 4: TABLE WITH THE GROWTH CURVES DATA. 69

10.5 APPENDIX 5: ENUMERATION RESULTS FOR HEAT STRESS. 70
10.6 APPENDIX 6: TESTED CONCENTRATIONS THAT WAS USED FOR THE DETERMINATION OF MIC FOR *S.TYPHIMURIUM* AND FOR *S.ENTERITIDIS*. 70
10.7 APPENDIX 7: TOXICITY OF NEUTRALIZING BROTHS ((CFU/ML)/(CFU/ML)). 72
10.8 APPENDIX 8: TABLE WITH RESULTS FROM RAW MATERIALS AND NEUTRALIZERS. 73

Figure contents

Figure 1: Salmonella outbreaks per year in USA. 35
Figure 2: Locations and mechanisms in the bacterial cell thought to be sites of action for the components of the spices and herbal species: degradation of the cell wall; damage to cytoplasmic membrane; damage to membrane proteins; leakage of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Sara Burt et al., 2004). 36
Figure 3: Raw materials with the included inhibitory compounds: 1) Carvacrol in oregano, 2) Eugenol in cloves, 3) Cinnamaldehyde in cinnamon, 4) Caffeine and Chlorogenic acid in coffee and 5) Vanillin in vanilla. 41
Figure 4: Growth curves for Salmonella Typhimurium: (a) before thermal treatment and (b) after thermal treatment. 46

Graph contents

Graph 1: Results from enumeration on selective and non selective media S.Typhimurium and S.Enteritidis, before and after heat treatment. 47
Graph 2: Toxicity of neutralizing broths in comparison with the viability control. 48
Graph 3: The recovery of the cells (log(cfu/ml)) from 1 mg/ml carvacrol against the five neutralizers (n=3). 49
Graph 4: The recovery of the cells (log(cfu/ml)) from 2 mg/ml eugenol against the five neutralizers (n=3). 50
Graph 5: The recovery of the cells (log(cfu/ml)) from 1 mg/ml cinnamaldehyde against the five neutralizers (n=3). 50
Graph 6: The recovery of the cells (log(cfu/ml)) from 5 mg/ml caffeine against the five neutralizers (n=3). 51
Graph 7: The recovery of the cells (log(cfu/ml)) from 40 mg/ml chlorogenic acid against the five neutralizers (n=3). 51
Graph 8: The recovery of the cells (log(cfu/ml)) from 10 mg/ml vanillin against neutralizer 3 (n=3). 52
Graph 9: The recovery of the cells (log(cfu/ml)) from 1.5 mg/ml thymol against the five neutralizers (n=3). 52
Graph 10: The recovery of the cells from oregano against four neutralizers and under different conditions and concentrations (n=3). 54
Graph 11: The recovery of the cells from cloves against four neutralizers and under different conditions and concentrations (n=3). 55

Graph 12: The recovery of the cells from cinnamon against four neutralizers and under different conditions and concentrations (n=3). 55
Graph 13: The recovery of the cells from coffee against four neutralizers and under different conditions and concentrations (n=3). 56
Graph 14: The recovery of the cells from vanilla against four neutralizers and under different conditions and concentrations (n=3). 57

Table contents

Table 1: Inhibitory compounds and concentrations. 41
Table 2: Neutralizers recipes that were used against inhibitory matrices. 42
Table 3: Inhibitory compounds concentrations with the CFUs that were tested against 5 neutralizers. 43
Table 4: Spices concentrations with the CFUs that were tested (with or without an extra stress for the already heat treated cells) against neutralizers (the results of those experiments are in: 3.4 Neutralizer efficacy). 45
Table 5: MIC for all the tested inhibitors. The range of the tested concentrations is in Appendix 6. 47
Table 6: Used names for each one of the tested samples. 48
Table 7: Inhibitor concentrations in the raw materials that were used for this study. 53
Table 8: Previously reported efficacy of some of chemical ingredients of the studied neutralizers. 58
Table 9: Comparison of the composion of N1 and N3. 59
Table 10: Comparison of the composition of N2 and N4. 60
Table 11: Tested concentrations of the inhibitors as pure compounds and as a content in the raw materials. 61
Table 12: The range of different concentrations that was used for the determination of MIC for carvacrol against growth for S.Typhimurium and for S.Enteritidis. 70
Table 13: The range of different concentrations that was used for the determination of MIC for eugenol against growth for S.Typhimurium and for S.Enteritidis. 70
Table 14: The range of different concentrations that was used for the determination of MIC for cinnamaldehyde against growth for S.Typhimurium and for S.Enteritidis. 71
Table 15: The range of different concentrations that was used for the determination of MIC for caffeine against growth for S.Typhimurium and for S.Enteritidis. 71
Table 16: The range of different concentrations that was used for the determination of MIC for chlorogenic acid against growth for S.Typhimurium and for S.Enteritidis. 71
Table 17: The range of different concentrations that was used for the determination of MIC for vanillin against growth for S.Typhimurium and for S.Enteritidis. 72
Table 18: The range of different concentrations that was used for the determination of MIC for thymol against growth for S.Typhimurium and for S.Enteritidis. 72
Table 19: Table with results from oregano and the neutralizers. 73
Table 20: Table with results from cloves and neutralizers. 73
Table 21: Table with results from cinnamon and neutralizers. 74
Table 22: Table with results from coffee and neutralizers. 74

Table 23: Table with results from vanilla and neutralizers. 75

1 Abstract

A neutralizers evaluation study was made for five plant occurring antimicrobial phenolic compounds (inhibitors) against two *Salmonella* strains: *Salmonella Typhimurium* and *Salmonella Enteritidis.* The five inhibitors, isolated as pure chemical compounds, were tested alone against the two heat treated strains in order to identify the minimum inhibitory concentration of those and then with the addition of five previously tested neutralizer recipes and twelve homemade. The heat treatment applied to the cells to check the recovery of the latter after the addition of an extra stress, except from the one because of the inhibitory matrices. The first screening of the five neutralizers was held against the pure chemical compounds and the second against raw materials in which it is contained each inhibitor. From the experiments we saw that *Salmonella Enteritidis* is more sensitive in the application of the thermal treatment and the majority of the experiments was performed with this strain. Even if after the first screening of the neutralizers against the pure chemical compounds we saw almost full recovery of the cells, on the second screening there was almost zero recovery in most of the trials. Those results indicate that the absence of recovery may be because the concentration of the neutralizers ingredients is not high enough to overcome the inhibitory effects since in all the toxicity and viability controls for the neutralizers there is full recovery.

2 Περίληψη

Διεξήχθη μελέτη αξιολόγησης απενεργοποίησης για πέντε φυτικές αντιμικροβιακές φαινολικές ενώσεις (αναστολείς) έναντι δύο στελεχών της Σαλμονέλας: Σαλμονέλα Typhimurium και Σαλμονέλα Enteritidis. Οι πέντε αναστολείς, που απομονώθηκαν ως καθαρές χημικές ενώσεις, δοκιμάστηκαν μόνες τους έναντι των δύο θερμικά επεξεργασμένων στελεχών προκειμένου να αναγνωριστεί η ελάχιστη ανασταλτική συγκέντρωση αυτών και στη συνέχεια με την προσθήκη πέντε προηγουμένως δοκιμασμένων και δώδεκα μη δοκιμασμένων συνταγών εξουδετέρωσης. Η θερμική επεξεργασία εφαρμόστηκε στα κύτταρα για να ελέγχει η ανάκτηση αυτών μετά την προσθήκη ενός πρόσθετου στρες, εκτός από το ένα λόγω της χρήσης των ανασταλτικών ουσιών. Η πρώτη διαλογή των πέντε απενεργοποιητών πραγματοποιήθηκε έναντι των καθαρών χημικών ενώσεων και η δεύτερη έναντι των πρώτων υλών στις οποίες περιέχεται κάθε αναστολέας. Από τα πειράματα είδαμε ότι η Σαλμονέλα Enteritidis είναι πιο ευαίσθητη στην εφαρμογή της θερμικής επεξεργασίας και η πλειονότητα των πειραμάτων πραγματοποιήθηκε με αυτό το στέλεχος. Ακόμη και αν μετά την πρώτη διαλογή των απενεργοποιητών έναντι των καθαρών χημικών ενώσεων είδαμε σχεδόν πλήρη ανάκτηση των κυττάρων, στη δεύτερη εξέταση υπήρχε σχεδόν μηδενική ανάκτηση στις περισσότερες από τις δοκιμές. Τα αποτελέσματα αυτά δείχνουν ότι η απουσία ανάκτησης μπορεί να οφείλεται στο ότι η συγκέντρωση των συστατικών εξουδετέρωσης δεν είναι αρκετά υψηλή για να ξεπεραστούν τα ανασταλτικά αποτελέσματα, καθώς σε όλους τους ελέγχους τοξικότητας και βιωσιμότητας για τους απενεργοποιητές υπάρχει πλήρης ανάκτηση.

3 Introduction

3.1 BACKROUND

Salmonella represent the most common and primary cause of food poisoning in many countries for at least over 100 years and can cause serious and sometimes fatal infections in young children, frail or elderly people, and others with weakened immune systems. Healthy persons infected with *Salmonella* spp. often experience fever, diarrhea (which may be bloody), nausea, vomiting, and abdominal pain. Despite well-established instructions and measures for preventing salmonellosis (*Salmonella* food poisoning), the incidence of human salmonellosis have significantly increased over the last years (Figure 1) (National Outbreak Reporting System (NORS)).

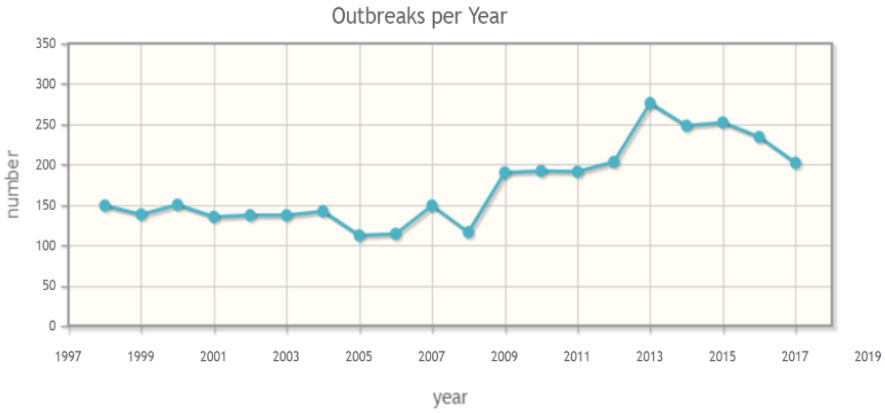


Figure 1: *Salmonella outbreaks per year in USA.*

Salmonella enterica is the representative pathogen causing salmonellosis in humans and in animals and is sub-classified into more than 2500 serovars. From those, *Salmonella Enteritidis* and *Salmonella Typhimurium* are the most important agents of foodborne salmonellosis in humans (Popoff MY *et al.,* 2003) in the United States and in European countries. *Salmonella* is not considered to be fatal to healthy people or as a bioweapon agent.

Despite the non-fatal effect of *Salmonella* on humans and animals, efforts have been made to develop and improve detection of food borne pathogens (e.g. *Salmonella*) in food industries for the improvement of food safety and public health because it may cause devastating foodborne illness. Thus, industries try to keep all the processes under control, perform and improve the methods that those already have for the foodborne bacteria.

Spices and herbal plant species, except from their already established antioxidant activity, have been recognized to possess a broad spectrum of active constituents that exhibit antimicrobial activity, most of which are phenols or their oxygen-substituted derivatives (Radulović NS *et al.*, 2013). They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases (Tepe B *et al.,* 2004).

Although the antimicrobial properties of spices and herbal plant species and their components have been reviewed in the past (Shelef *et al.,* 1983), the mechanism of action has not been studied in great detail (Lambert *et al.,* 2001). Considering the large number of different groups of chemical compounds present in those compounds, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (*Skandamis et al., 2001)*. The locations or mechanisms in the bacterial cell thought to be sites of action for the components of the spices are indicated in Figure 2.

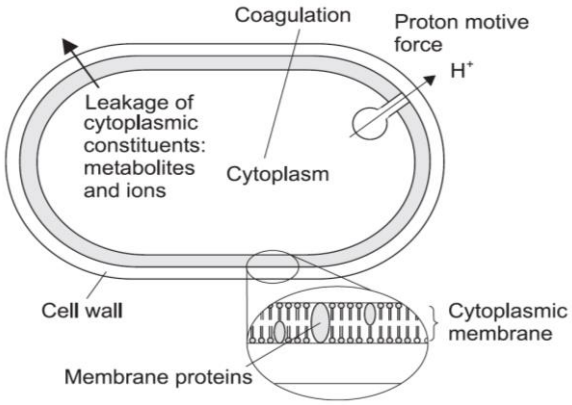


Figure 2: *Locations and mechanisms in the bacterial cell thought to be sites of action for the components of the spices and herbal species: degradation of the cell wall; damage to cytoplasmic membrane; damage to membrane proteins; leakage of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Sara Burt et al., 2004).*

Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted. An important characteristic of those components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Sikkema *et al.,* 1994). Leakage of ions and other cell contents can then occur (Ultee *et al.,* 2002).

Generally, those components possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol (Farag *et al.,* 1989; Cosentino *et al.,*

1999; Lambert *et al.,* 2001). It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Davidson, 1997).

Components also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al.,* 1989). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid–protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Sikkema *et al.,* 1995). Some others could act on the enzymes involved in the energy regulation or synthesis of structural components (Conner *et al.,* 1984).

For years it has been recognized that organisms which have been in contact with those active inhibitory matrices (e.g. plant origin phenolic compounds) and fail to grow *in vitro* and thus persumed not present, may in fact, still be alive (Qing Liu *et al.*,2017), so there is a need for a combination of chemical molecules that will inactivate the each time inhibitory matrice (e.g. phenolic compound from raw materials in our case), so if *Salmonella* is present, we will be able to detect it.

Common methods for deactivation of the action of the "inhibitors" include dilution or chemical neutralization. Chemical agents commonly known as neutralizers or inactivators are often used for (i) the bactericidal evaluation of antimicrobial agents, antiseptics and disinfectants, (ii) the evaluation of preservative efficacy in any pharmaceuticals and cosmetic products and (iii) the microbial limit testing of products containing antimicrobial agents (MacKinnon, I.H. et al., 1974). The choice of an inactivator (e.g. neutralizer for inhibitory compounds) should be strictly limited by certain criteria. It must, by definition, neutralize the inhibitor it is used against. It should not give rise to any inhibiting effect, so if *Salmonella* is present, we are able to avoid the false negatives. Its action (e.g. neutralizer) should also be fairly rapid; slow neutralization allows continued bactericidal effect long after any timed period has ended.

Complete neutralization of inhibitors is important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survivors with time and inhibition of microbial

growth by low levels of residual biocide would lead to exaggerated measures of microbicidal activity (Cremieux, A. *et al.,* 1983). A convenient method for this neutralization is through the use of recovery diluents designed to neutralize commonly used antimicrobials. A number of reagents are used in this regard (Russell, A. D. *et al.,* 1981).

3.2 AIM OF THE STUDY

Taking into consideration of what was mention above, this research project aimed to improve the *Salmonella* detection in raw materials (species) with inhibitory ingredients (e.g. carvacrol, eugenol, cinnamaldehyde, caffeine, chlorogenic acid and vanillin). Dedicated sample preparation protocols tried to be developed to replace the high dilution factors currently applied to overcome the inhibitory compounds and avoid false negative results, allowing also to reduce the high analytical cost of those procedures.

4 Methodology and Trials

All the used media and equipments are in Appendix 2.

4.1 BACTERIAL STRAINS

In this study, different *Salmonella* spp. were initially tested to determine the stress. However, reference strains from ISO 6579-1:2017 were used in the main experiments: *S. Tympimurium* WDCM00031 and *S. Entetitidis* WDCM00030, detailed information is present in Appendix 1.

Beads from frozen cultures at -80°C obtained from Nestlé Research Center strains collection were added to Brain Heart Infusion (BHI) and grown overnight at 37°C. A loop from the BHI was streaked on Tryptone Salt Agar (TSA) medium and after incubation, colonies were kept at 4°C for 1 month, as stock/working cultures.

4.2 REFERENCE MATERIAL PREPARATION

The enumeration procedure for *Salmonella* spp. Is the following: One colony of each strain were inoculated into one 10 ml Buffered Peptone Water (BPW) tube separately. The tubes including a negative control (BPW) were incubated 37 °C, until stationary phase (e.g. 18h).

From the cells/sample suspension, 1:10 dilutions were done to achieve lower dilutions with a countable range of colonies (i.e. 15-150 cfu) (e.g. 1ml in 9ml diluent liquid), from one appropriate dilution. A total of 1 ml was plated in triplicates on XLD and 100 ul was plated in triplicates on TSA and (Xylose Lysine Deoxycholate Agar) XLD media and incubated at 37°C for 24h.

4.3 STRESS MODEL SELECTION

In this research, it was intended to mimic realistic conditions for *Salmonella* spp that often are present in food processing lines which includes stress (e.g. dry, chemical, cold, heat). Therefore, in this research one of the given stress instructions were tested.

4.3.1 Heat stress

After the selection of the appropriate dilution, heat stress applied to all the replicates of the selected dilution. The dilutions where perfomed as many times were needed, depended on the number of replicates of each sample for each experiment. Heat stress at 50°C for 15 min of culture was done in a thermoblock. Thermal treatment was performed in 1.5 ml Eppendorf

tubes containing the desirable concentration of cells. When treatment time was completed, all tubes were put in ice for 10 minutes before starting the enumeration and before adding the stressed culture to each sample. After stress, a dilution with a countable number of colonies stress was selected. This dilution was plated according ISO 4833-2 and were incubated for 24 ± 3h (i.e. spread 0,1 ml from each tube on two TSA plates and incubate at 37 °C ± 1 °C for 24 h).

4.3.2 Dry stress

After the application of the heat stress and the spiking to the raw materials (2.5 Raw Materials) in some cases there was applied an extra stress. After the spiking, raw materials were left drying at room temperature for 1 or 24 hours. After this extra stress the selected neutralizer was added in each of the samples and the protocol was proceeded as described below.

4.3.3 Growth curves

Lag phase was studied to be able to see differencies between heat treated and non heated cells. This study had been done only in *S.Typhimurium*. For that purpose one colony of *S.Typhimurium* was inoculated into one 10 ml BPW tube. The tube including a negative control (BPW) were incubated 37 °C, until stationary phase (e.g. 18h). From the cells/sample suspension, 1:10 dilutions were done to achieve lower dilutions with a countable range of colonies (i.e. 15-150 cfu) (e.g. 1ml in 9ml diluent liquid), from one appropriate dilution.
Each growth curve for the heat treated prepared four times and for the non heated cells prepared three times. For each one of the replicates 500 ul of the appropriate dilution were transferred in 49.5 ml of BPW and emumeration has been made for 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours with incubation at 37°C in between.

4.4 RAW MATERIALS

For the validation of *Salmonella* detection, 3 spices, 1 coffee product and 1 natural product (raw materials with the inhibitors that will be tested below) were tested in different amounts, with different dilution factors and with a range of concentration of the spiked cells and with the application of an extra drying stress for 1 or 24h (Table 4) if needed. Raw materials contain inhibitory compounds (that will be tested isolated, as pure chemical compoumds) that inhibit the growth of *Salmonella* during pre-enrichment (Figure 3), so there is a need for the development of preparation protocols in order to overcome the inhibitory compounds effect and avoid false negative results.

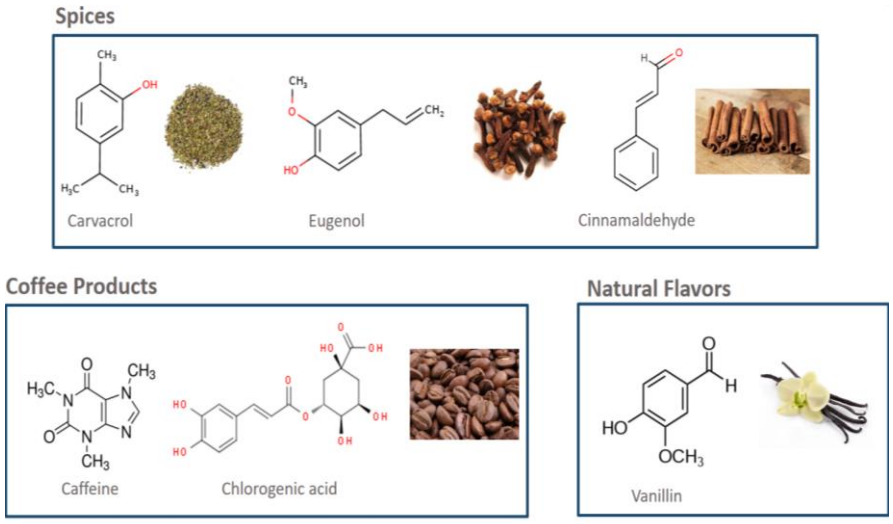


Figure 3: *Raw materials with the included inhibitory compounds: 1) Carvacrol in oregano, 2) Eugenol in cloves, 3)Cinnamaldehyde in cinnamon, 4) Caffeine and Chlorogenic acid in coffee and 5) Vanillin in vanilla.*

4.5 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of different plant occurring Inhibitory compounds was measured by broth dilution method using BPW by using a range of different concentrations (Table 1). The previously thermal treated cultures of *Salmonella* were spiked in the inhibitory matrices. The tubes were incubated at 37 ◦C for 24 h and all the MIC tubes (100ul of culture from each tube) were then used for spreading on XLD plates for colony counting. The plates were incubated at 37 ◦C for 24 h and the colonies were calculated. The concentration at which no growth was observed was determined MIC. All the determinations were performed in triplicates.

		Concentration (mg/ml)									
Inhibitor	Code	I	II	III	IV	V	VI	VII	VIII	IX	X
Carvacrol	A	1	0.5	0.25	0.1	0.01	0.005	0.0025			
Eugenol	B	2	0.2	0.1	0.05	0.01					
Cinnamaldehyde	C	1	0.5	0.25	0.1	0.01	0.005	0.0025			
Caffeine	D	5	0.5	0.1	0.05	0.025	0.01	0.005	0.0025		
Chlorogenic acid	E	40	30	20	15	10					
Vanillin	F	1	2	3	4	5	6	7	8	9	10
Thymol	G	0.15	0.3	0.45	0.6	0.75	0.9	1.05	1.2	1.35	1.5

Table 1: *Inhibitory compounds and concentrations.*

4.6 NEUTRALIZER TRIALS

All neutralizers were prepared as cited, with the final concentrations as listed in Table 2. Neutralizer evaluation procedure in this study involves three categories:

- The ***neutralizer efficacy*** which can be determined by evaluating the growth in the neutralizing broth in the presence of the inhibitory matrices compared with the reference (growth in standard BPW).
- The ability of the neutralizer alone to allow the survival and growth of the target bacteria. The ***neutralizer toxicity*** can be determined by comparing survivors in the neutralizing broth alone without the inhibitor with the control growth in standard BPW.
- The ***inhibitory properties*** of the inhibitory matrices (BPW with the inhibitor or raw material containing the latter) to check the inhibition of *Salmonella.*

	N1 (NR1)	N2 (UG1)	N3 (UG2)	N3.2	N4 (ISO 21149 D1)	N5 (NR2)	N6	N7
Ingredient	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***
Charcoal	5					10	5	
D/E	39							
Tween 80		24	60	30	30	30	60	30
L-histidine		0,24			1		0,24	
Sodium bisulphite			0,4	0,4			0,4	
Sodium thiosulfate 5 H2O			7,84	7,84			7,84	
Sodium Thioglycollate			5	2			5	
L-cysteine			1,5	0,5			1,5	
Magnesium chloride						1		
Whey protein						20		
Sodium pyruvate						7		
BPW (powder)	25,5	25,5	25,5	25,5	25,5	25,5	25,5	25,5
		PH 7.00+Filtration	PH 7.00+Filtration	PH 7.00+Filtration	PH 7.00+Filtration		PH 7.00+Filtration	
Lecithin		7,5	2	2	3		2	

	N8	N9	N10	N11	N12	N13	N14	N15	N16
Ingredient	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***	***g-ml/L (BPW Merck)***
Charcoal									5
D/E									
Tween 80	40	50							
L-histidine									
Sodium bisulphite									
Sodium thiosulfate 5 H2O									
Sodium Thioglycollate			2	3	4				
L-cysteine						0,1	0,5	1	
Magnesium chloride									
Whey protein									
Sodium pyruvate									
BPW (powder)	25,5	25,5	25,5	25,5	25,5	25,5	25,5	25,5	25,5
Lecithin									

Table 2: *Neutralizers recipes that were used against inhibitory matrices.*

D/E on the table 2 is Dey-Engley, a neutralizing broth that its composition is described on Appendix 3.

This procedure performed twice. Firstly, the efficacy of the neutralizer was tested against the inhibitors in concentrations higher that the MIC. In those experiments a first evaluation of the neutralizer toxicity against the target bacteria was held. In the second part of the experiments the ''good'' candidates from the first trials, were tested against the raw materials, which

contain the inhibitory compounds in unknown concentrations. The concentration of those inhibitory compounds will be evaluated externally.

4.6.1 Procedure

This procedure provides two treatment strains for comparison, as described previously, *S. Tympimurium* and *S. Entetitidis.*

4.6.1.1 Neutralizers with inhibitory compounds

A specific amount of each inhibitor (Table 3) was added in two tubes containing:

- 10 ml of the neutralizing broth and
- 10 ml of BPW (negative control) and these suspensions were incubated for 15 minutes at 37°C.

A third, containing 10 ml of the neutralizer (toxicity control) and a fourth, containing 10 ml of BPW (viability control) were prepared also. Each one of these solutions was inoculated with ~10^2 CFU of the target heat stressed organism (~10 CFU/ml final concentration). 1 ml of the viability control was plated on three XLD plates (333,3 ul each), 0.1 ml was plated in triplicates on XLD and TSA plates to ensure the concentration of the cells in each sample. Those tubes were incubated at 37°C for 24 hours.

Inhibitors	Concentration(mg/ml)	Strain	N1	N2	N3	N4	N5
Carvacrol	1	S498					
Eugenol	2	S498					
Cinnamaldehyde	1	S498					
Caffeine	5	S498	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml
Chlorogenic acid	40	S498					
Thymol	1.5	S498					
Vanilin	10	S498					

Inhibitors	Concentration(mg/ml)	Strain	N1	N2	N3	N4	N5
Carvacrol	1	S497					
Eugenol	2	S497					
Cinnamaldehyde	1	S497					
Caffeine	5	S497	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml	10^2 CFU in 10ml
Chlorogenic acid	40	S497					
Thymol	1.5	S497					
Vanilin	10	S497					

Table 3: *Inhibitory compounds concentrations with the CFUs that were tested against 5 neutralizers.*

Recovery of the studied organisms was performed the next day by plating 0.1 ml from all the samples and from different dilutions on XLD plates and incubating at 37°C for 24 hours. All

the plates were examined for the recovery of CFUs for the selection of the candidates for the next part of the study.

4.6.1.2 Neutralizers with raw materials

A specific amount of each raw material was added to a tube containing each time a different amount of the neutralizing broth with the inhibitor and to a tube containing BPW (negative control). A third, containing only the neutralizer (toxicity control) and a fourth, containing BPW (viability control) were prepared also. Each one of these solutions was inoculated with 1 - 100 CFU of the challenge organism (Table 4). 1 ml of the viability control was plated on three XLD plates (333,3 ul each), 0.1 ml was plated in triplicates on XLD and TSA plates to ensure the concentration of the cells in each sample. Those tubes were incubated at 37°C for 24 hours.

Recovery of the studied organisms was performed the next day by plating 0.1 ml from all the samples and from different dilutions on XLD plates and incubating at 37°C for 24 hours. All the plates were examined for the recovery of CFUs.

			N3		N4		
Raw Materials	**Concentration**	**Strain**	**Dry stress 24h**	**Dry stress 1h**	**Dry stress 24h**	**Dry stress 1h**	**No dry stress**
Oregano (Carvacrol)	1 g/10ml	S498					
Cloves (Eugenol)	1 g/10ml	S498					
Cinnamon (Cinnamaldehyde)	1 g/10ml	S498	*10 CFU in 10ml*	*10^2 CFU in 10ml*	*10 CFU in 10ml*	*10^2 CFU in 10ml*	*10^2 CFU in 10ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498					
Vanilla (Vanillin)	1 g/10ml	S498					

			N3	N4
Raw Materials	**Concentration**	**Strain**	**No dry stress**	**No dry stress**
Oregano (Carvacrol)	1 g/20ml	S498		
Cloves (Eugenol)	1 g/20ml	S498		
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	*$2*10^2$ CFU in 20ml*	*$2*10^2$ CFU in 20ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/20ml	S498		
Vanilla (Vanillin)	1 g/20ml	S498		

			N3	N4	N16
Raw Materials	**Concentration**	**Strain**	**No dry stress**	**No dry stress**	**No dry stress**
Oregano (Carvacrol)	10 g/100ml	S498	*1 CFU in 100ml*	*1 CFU in 100ml*	*1 CFU in 100ml*
Cloves (Eugenol)	10 g/200ml	S498	*1 CFU in 200ml*	*1 CFU in 200ml*	*1 CFU in 200ml*
Cinnamon (Cinnamaldehyde)	10 g/100ml	S498	*1 CFU in 100ml*	*1 CFU in 100ml*	*1 CFU in 100ml*
Coffee (Chlorogenic acid + Caffeine)	10 g/100ml	S498	*1 CFU in 100ml*	*1 CFU in 100ml*	*1 CFU in 100ml*
Vanilla (Vanillin)	10 g/100ml	S498	*1 CFU in 100ml*	*1 CFU in 100ml*	*1 CFU in 100ml*

			N6
Raw Materials	**Concentration**	**Strain**	**No dry stress**
Oregano (Carvacrol)	1 g/10ml	S498	*10^2 CFU in 10ml*
Cloves (Eugenol)	1 g/20ml	S498	*$2*10^2$ CFU in 20ml*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	*$2*10^2$ CFU in 20ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	*10^2 CFU in 10ml*
Vanilla (Vanillin)	1 g/10ml	S498	*10^2 CFU in 10ml*

			N7	N8	N9	N10	N11
Raw Materials	**Concentration**	**Strain**	**No dry stress**	**No dry stress**	**No dry stress**	**No dry stress**	**No dry stress**
Oregano (Carvacrol)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*
Cloves (Eugenol)	1 g/50ml	S498	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*
Vanilla (Vanillin)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*

			N12	N13	N14	N15	N3.2
Raw Materials	**Concentration**	**Strain**	**No dry stress**	**No dry stress**	**No dry stress**	**No dry stress**	**No dry stress**
Oregano (Carvacrol)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*
Cloves (Eugenol)	1 g/50ml	S498	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*	*1 CFU in 50ml*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*	*1 CFU in 20ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*
Vanilla (Vanillin)	1 g/10ml	S498	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*	*1 CFU in 10ml*

			N3	N4
Raw Materials	**Concentration**	**Strain**	**No dry stress**	**No dry stress**
Oregano (Carvacrol)	1 g/20ml	S497		
Cloves (Eugenol)	1 g/20ml	S497		
Cinnamon (Cinnamaldehyde)	1 g/20ml	S497	*$2*10^2$ CFU in 20ml*	*$2*10^2$ CFU in 20ml*
Coffee (Chlorogenic acid + Caffeine)	1 g/20ml	S497		
Vanilla (Vanillin)	1 g/20ml	S497		

***Table 4**: Spices concentrations with the CFUs that were tested (with or without an extra stress for the already heat treated cells) against neutralizers (the results of those experiments are in: 3.4 Neutralizer efficacy).*

5 Results

5.1 Stress model selection (Heat stress)

To generate realistic food processing scenarios where *Salmonella* spp. are often under stress conditions growth curves of *S.Typhimurium* for before (Figure 4a) and after the thermal treatment (Figure 4b) performed, to see the differencies between the growth rates. It was interesting to observe that actively growing cells of *S.Typhimurium* without thermal treatment (as described in 2. Methodology and Trials) are leaving the lag phase after 1.578±0.299 hours but with thermal treatment after 3.42 ± 0.195 hours. So with thermal treatment there is an increased lag phase time (Appendix 4). Additional work has been made for the validation of the growth under thermal stress for *S.Enteritidis* (data not shown) from other colleagues. Those data shown that *S.Enteritidis* is more sensitive than *S.Typhimurium* with a more extended lag phase after the thermal treatment in comparison with *S.Typhimurium*.

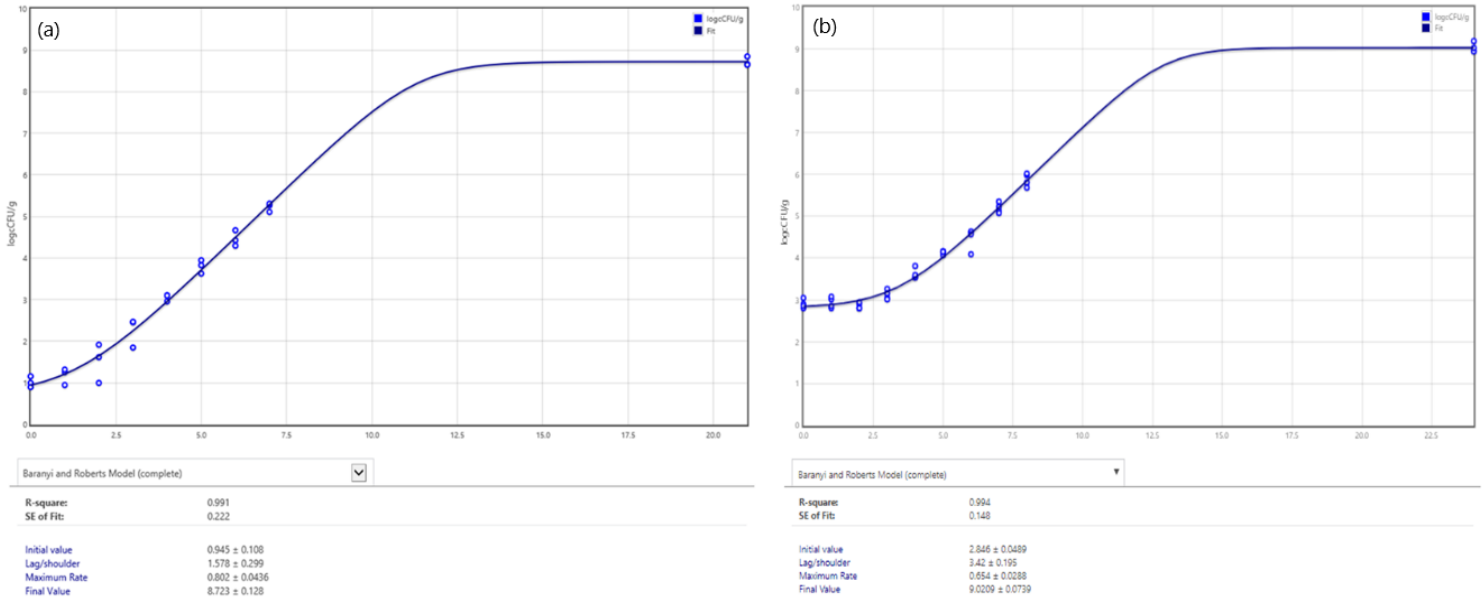
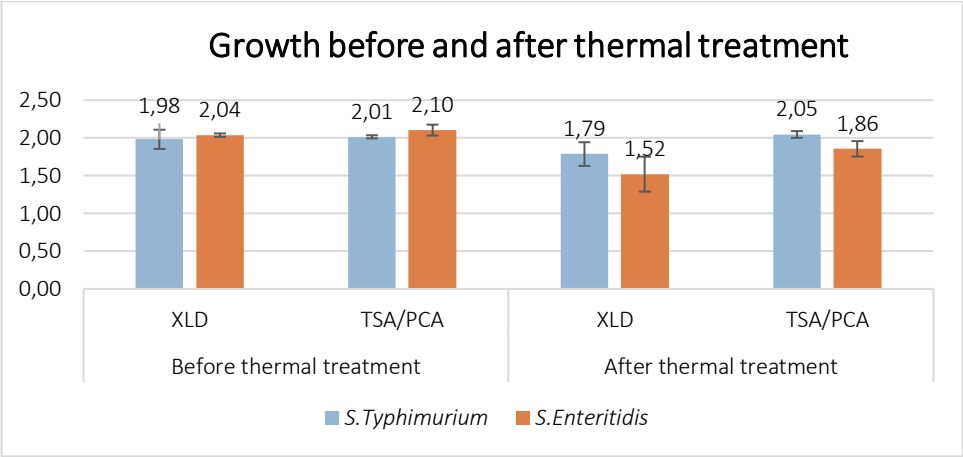


Figure 4: *Growth curves for Salmonella Typhimurium: (a) before thermal treatment and (b) after thermal treatment.*

Additionaly a comparison between the two strains has been made, to see if there are any differences related to the heat treatment. For that purpose plating before and after the heat treatment has been performed on selective and non selective media for both strains (Graph 1, Appendix 5). From this figure it is clear that even if the initial suspension of the strains is the same, after the thermal treatment *S.Enteritidis* is more affected than *S.Typhimurium.* So

the first one is more sensitive than the latter. For that reason the majority of the neutralizer trials has been performed with *S.Enteritidis:* to check the worst case scenario.



Graph 1: *Results from enumeration on selective and non selective media S.Typhimurium and S.Enteritidis, before and after heat treatment.*

5.2 Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of seven inhibitory matrices was evaluated *in vitro* in this study. The concentration at which no growth was observed was determined as MIC. A range of different concentrations was tested against *S.Typhimurium* and *S.Enteritidis* (Appendix 6, Tables 12-18). The MIC was shown in the table below:

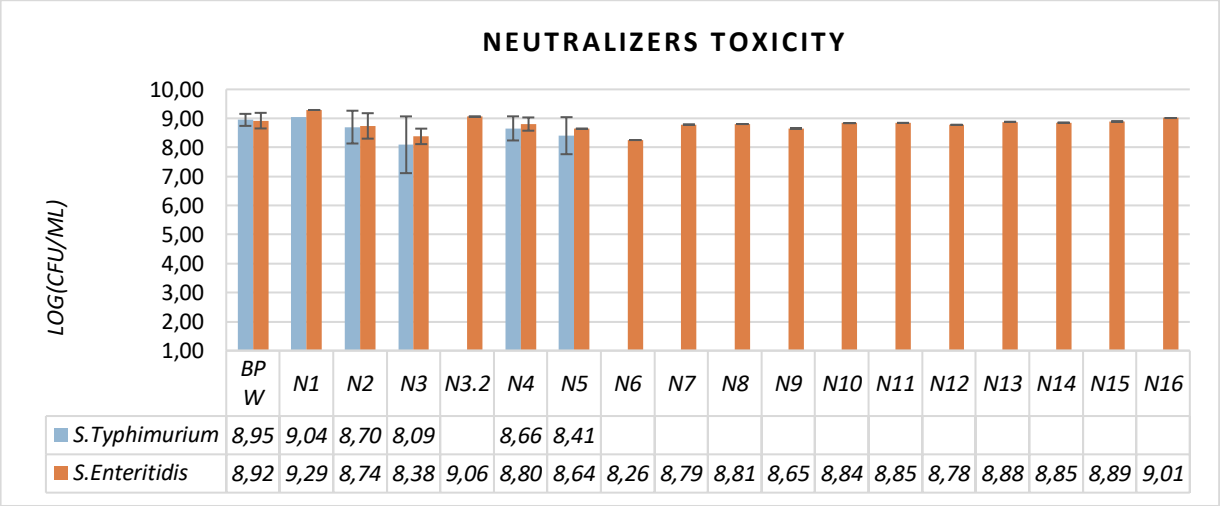
	MIC (mg/ml)	
Inhibitor	***S.Typhimurium***	***S.Enteritidis***
Carvacrol	0.1-0.01	0.1-0.01
Eugenol	2-0.2	2-0.2
Cinnamaldehyde	0.1-0.01	0.1-0.01
Caffeine	5-0.5	5-0.5
Chlorogenic acid	30-20	30-20
Vanillin	2-1	3-2
Thymol	1.2-1.05	0.9-0.75

Table 5*: MIC for all the tested inhibitors. The range of the tested concentrations is in Appendix 6.*

5.3 Neutralizers toxicity against *S.Typhimurium* and *S.Enteritidis*

Evaluation of the neutralizer toxicity was performed by the comparison between the viability population (strain in BPW) and the neutralizer exposed population (strain in BPW with the

neutralizer)(Graph 2). Neutralizer toxicity ratios were determined for all neutralizer - target organism combinations. These results are shown in Appendix 7. None of the examined neutralizers showed toxicity against the *S.Typhimurium* or *S.Enteritidis* and in some cases there is better growth with the neutralizing broth in comparison with the viability control, so the neutralizers had no antibacterial activity, except from one case. After the change of the supplier for one of the ingredients (sodium thioglycolate from Alfa Aesar to Sigma Aldrich) for the N3 we noticed no recovery results in the toxicity control.



	BPW	N1	N2	N3	N3.2	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16
S.Typhimurium	8,95	9,04	8,70	8,09		8,66	8,41											
S.Enteritidis	8,92	9,29	8,74	8,38	9,06	8,80	8,64	8,26	8,79	8,81	8,65	8,84	8,85	8,78	8,88	8,85	8,89	9,01

Graph 2: *Toxicity of neutralizing broths in comparison with the viability control.*

5.4 Neutralizer Efficacy

Determination of neutralizer efficacy requires evaluation of the neutralizing broth's ability to neutralize the inhibitor at a specified dilution. We evaluated neutralizer efficacy by the comparison between the toxicity and viability control, the inhibitor exposed population (strain with the inhibitor in BPW) and the neutralizer plus inhibitor population (strain with the inhibitor and the neutralizer in BPW). Acceptable neutralization is defined when the viability and toxicity control population have comparable values with the neutralizer plus inhibitor population and the growth in the inhibitor is negative.

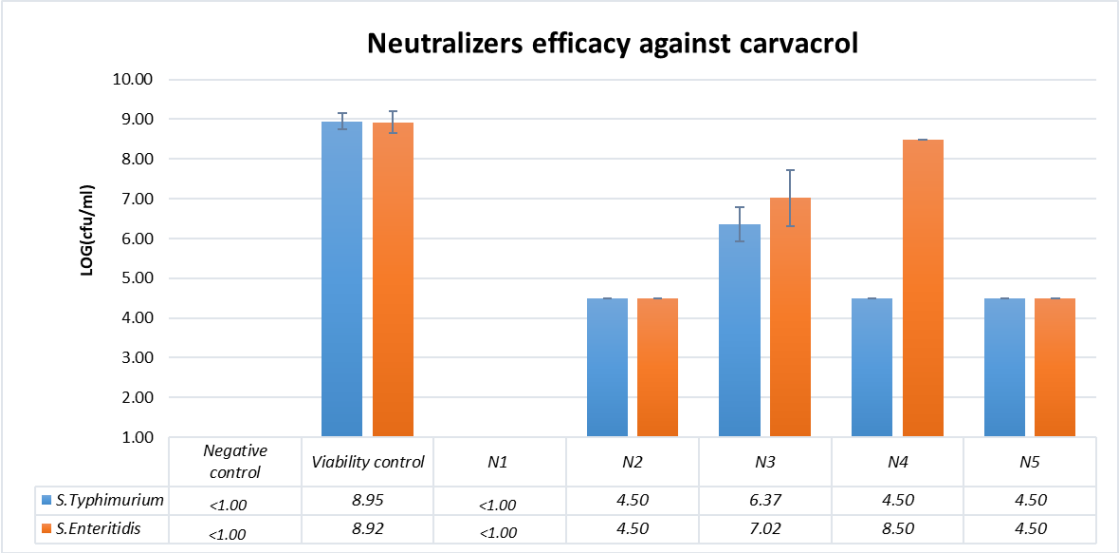
Viability control	Toxicity control	Negative control	Efficacy control
BPW + strain	BPW+Neutralizer + Strain	BPW+Inhibitor + strain	BPW+Neutralizer + Inhibitor + strain

Table 6: *Used names for each one of the tested samples.*

5.4.1 Neutralizers with inhibitory compounds

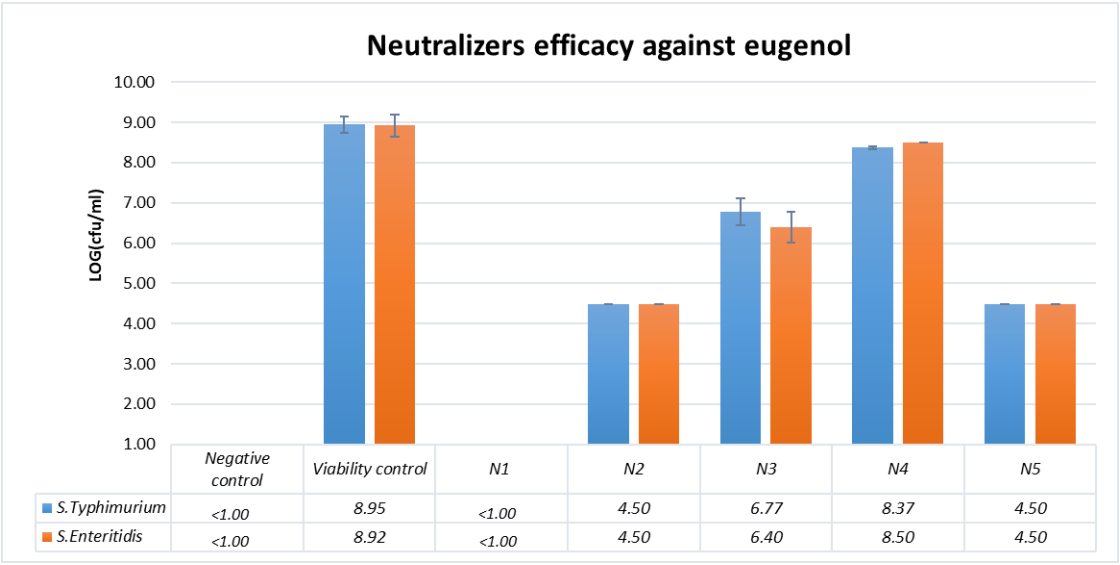
After the determination of the MIC there was a first screening of the effectivity of the five basic neutralizers that were used in this study. For these trials inhibitory concentrations higher than the MIC were tested against the neutralizers. For all the experiments there were two controls as reference: the negative and the recovery of the cells in the viability. In the first case we are not able to see growth because of the inhibitory effects, and in the second the average number of recovery in BPW for *S.Typhimurium* is 8.95 log and for *S.Enteritidis* 8.92 log (Figure 5).

For carvacrol, the MIC as said previously (Table 5) is between 0.1-0.01 mg/ml and for the neutralizers trials 1 mg/ml tested (~10x higher than MIC). From the results (Graph 3), there is clear that with N1 there is no growth (same results as the reference), the best candidate for *S.Tympimurium* is N3 with 6.37 growth and for *S.Enteritidis* is N4 with 8.50 log growth.



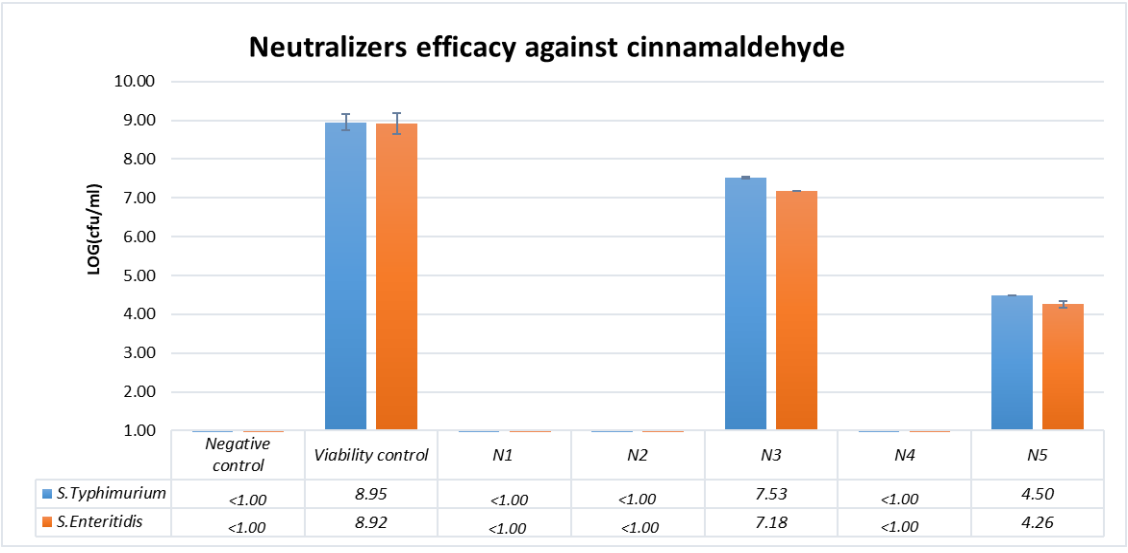
Graph 3: *The recovery of the cells (log(cfu/ml)) from 1 mg/ml carvacrol against the five neutralizers (n=3).*

For eugenol, the MIC as said previously (Table 5) is between 2-0.2 mg/ml and for the neutralizers trials 2 mg/ml tested (~MIC). From the results (Graph 4), there is clear that with N1 there is no growth (same results as the reference) and the best candidate for both strains is N4 with 8.37 growth for *S.Typhimurium* and 8.50 for *S. Enteritidis*.



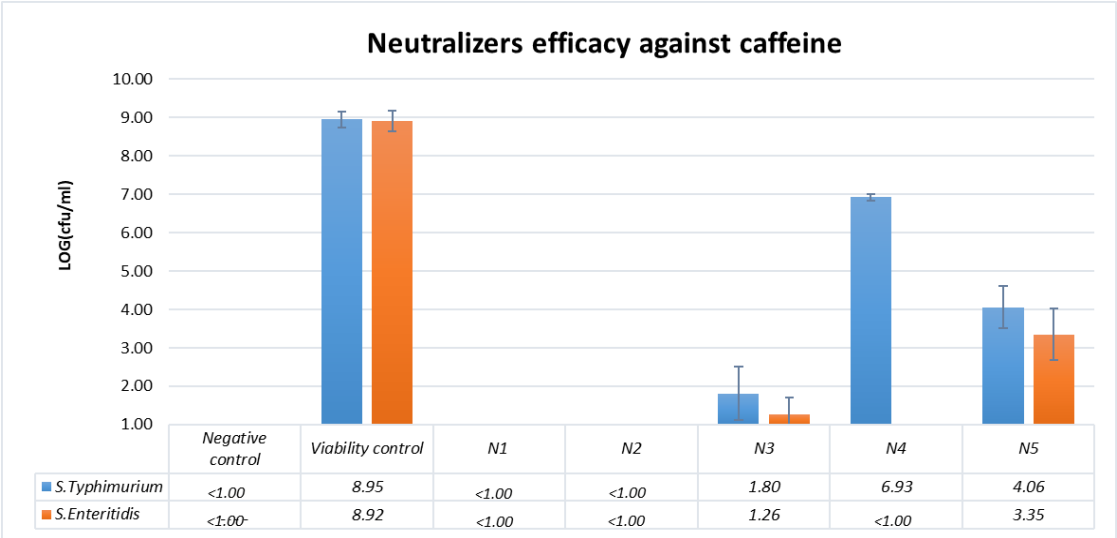
Graph 4: *The recovery of the cells (log(cfu/ml)) from 2 mg/ml eugenol against the five neutralizers (n=3).*

For cinnamaldehyde, the MIC as said previously (Table 5) is between 0,1-0.01 mg/ml and for the neutralizers trials 1 mg/ml tested (~10x higher than the MIC). From the results (Graph 5), there is clear that with N1, N2 and N4 there is no growth (same results as the reference) and the best candidate for both strains is N3 with 7.53 growth for *S.Typhimurium* and 7.18 for *S. Enteritidis*.



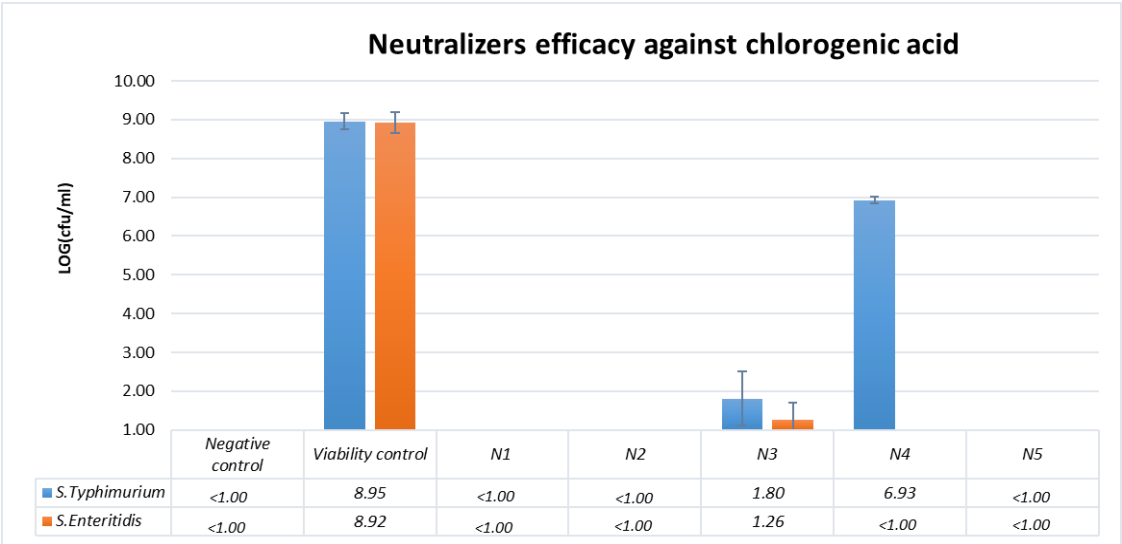
Graph 5: *The recovery of the cells (log(cfu/ml)) from 1 mg/ml cinnamaldehyde against the five neutralizers (n=3).*

For caffeine, the MIC as said previously (Table 5) is between 5-0.5 mg/ml and for the neutralizers trials 5 mg/ml tested (~MIC). For N3 and N4 instead of testing caffeine, in the final volume of each neutralizer 1 gr of coffee was added (5% caffeine). From the results (Graph 6), there is clear that with N1, N2 and N4 (only for *S.Enteritidis*) there is no growth (same results as the reference) and the best candidate for *S.Typhimurium* is N4 with 6.93 growth and N5 for *S. Enteritidis* with 3.35 growth.



Graph 6: *The recovery of the cells (log(cfu/ml)) from 5 mg/ml caffeine against the five neutralizers (n=3).*

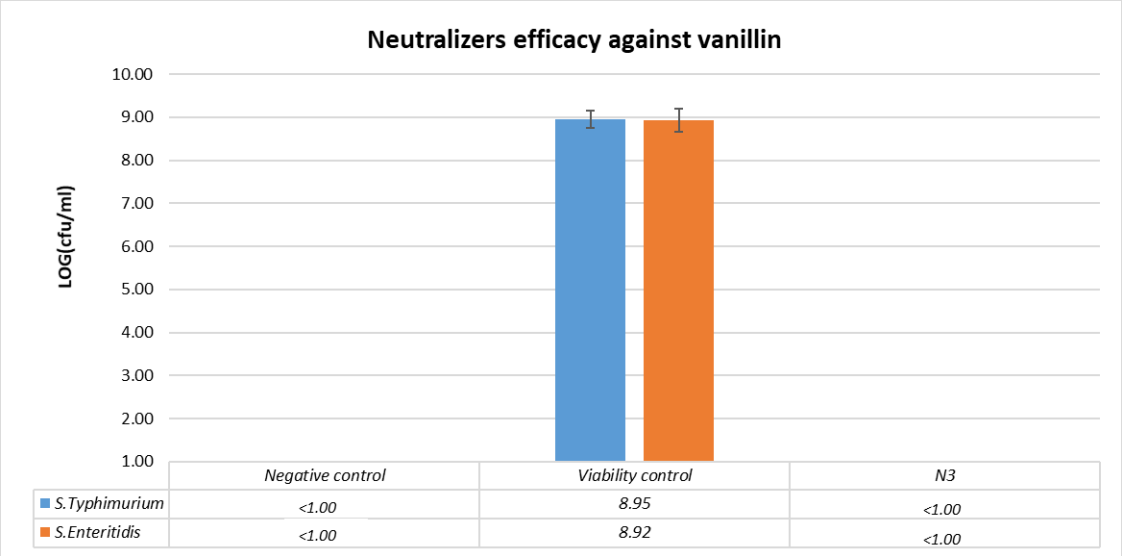
For chlorogenic acid, the MIC as said previously (Table 5) is between 30-20 mg/ml and for the neutralizers trials 40 mg/ml tested (~2x higher than the MIC). For N3 and N4 instead of testing chlorogenic acid, in the final volume of each neutralizer 1 gr of coffee was added (10% chlorogenic acid). From the results (Graph 7), there is clear that with N1, N2, N4 (only for *S.Enteritidis*) and N5 there is no growth (same results as the reference) and the best candidate for *S.Typhimurium* is N4 with 6.93 growth and N3 for *S. Enteritidis* with only 1.80 growth.



Graph 7: *The recovery of the cells (log(cfu/ml)) from 40 mg/ml chlorogenic acid against the five neutralizers (n=3).*

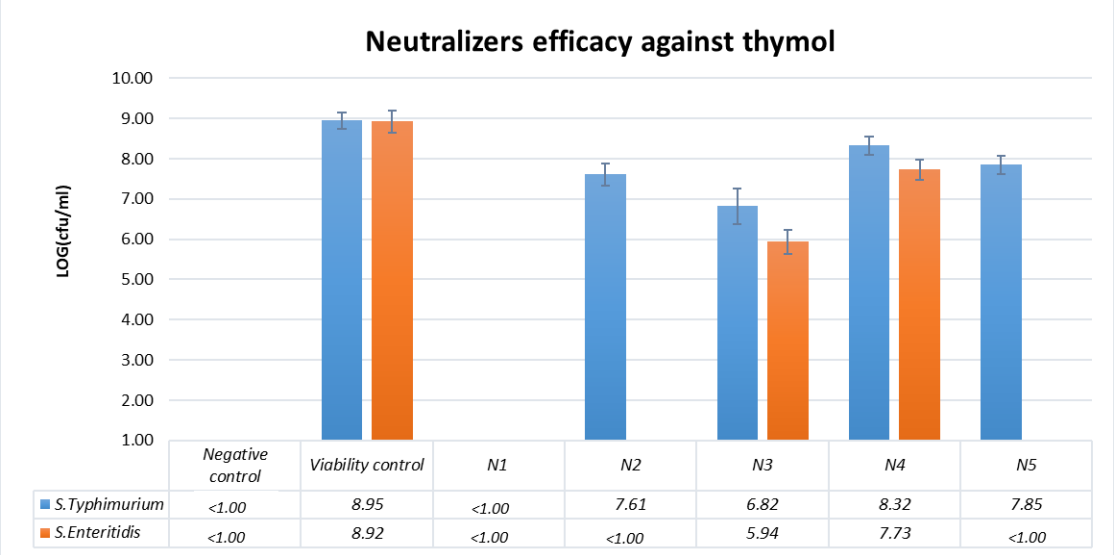
For vanillin, the MIC as said previously (Table 5) is between 2-1 mg/ml for *S.Typhimurium* and 3-2mg/ml for *S.Enteritidis* and for the neutralizers trials 10 mg/ml tested (~5x higher than the

MIC). For vanillin only N3 tested. From the results (Graph 8), there is clear that there is no growth (same results as the reference).



Graph 8: *The recovery of the cells (log(cfu/ml)) from 10 mg/ml vanillin against neutralizer 3 (n=3).*

For thymol, the MIC as said previously (Table 5) is between 1.2-1.05 mg/ml for *S.Typhimurium* and 0.9-0.75 mg/ml for *S.Enteritidis* and for the neutralizers trials 1.5 mg/ml tested (~MIC). From the results (Graph 9), there is clear that with N1, N2 (only for *S.Enteritidis*) and N5 (only for *S.Enteritidis*) there is no growth (same results as the reference) and the best candidate for both strains is N4 with 8.32 growth for *S.Typhimurium* and 7.73 growth for *S. Enteritidis*.



Graph 9: *The recovery of the cells (log(cfu/ml)) from 1.5 mg/ml thymol against the five neutralizers (n=3).*

5.4.2 Neutralizers with raw materials

After that first screening of the effectivity of the five basic neutralizers that were used in this study against the pure chemical inhibitors, the decision to proceed with only two of them has been made for the trials in spices. Those two are N3 and N4 because they seem to be the more effective for the most of the inhibitors. Different recipes (Table 2) of neutralizers were also tested on the second part of the neutralizer trials.

The efficacy of the neutralizers was tested against five different species, which contain the compounds that was previously studied alone (e.g. carvacrol, eugenol, cinnamaldehyde, caffeine, chlorogenic acid and vanillin). The concentration of each inhibitor in those spices is unknown but according to the literature the reference values are in Table 7.

Raw materials	*Active ingredient (maximal concentration in finished products)*
Cinnamon	Cinnamaldehyde (4%)
Cloves	Eugenol (20%)
Coffee	Caffeine (5%), Chlorogenic acids (10%)
Oregano	Carvacrol (2%)
Vanilla	Vainillin (4-hidroxi-3-metoxibenzaldehíde) (2%)

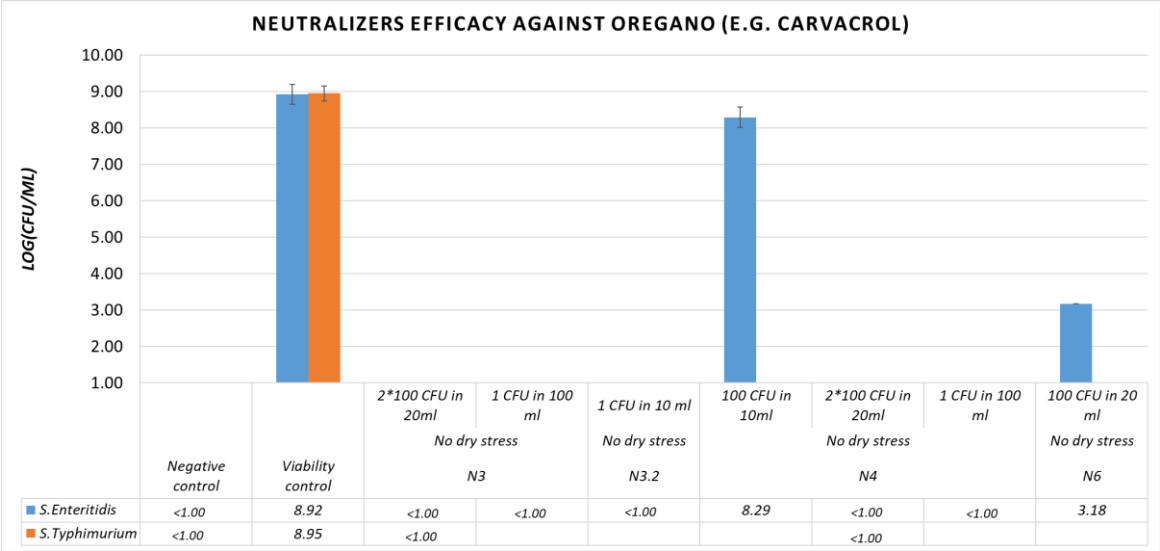
Table 7: *Inhibitor concentrations in the raw materials that were used for this study.*

In the next graphs some of the results are highlighted. Details about the amount of each raw material, the spiked colonies, the extra stress that was applied and the results from all the used neutralizers can be seen in Appendix 8. The detection of *S.Typhimurium* and *S.Enteritidis* in all those experiments was possible in the toxicity controls and the positive controls for all the trials and the different dilutions factors (Graph 2).

The extra drying test was tested with N3 and N4 for 24 hours with 1CFU/ml with all the five species and for 1 hour with 10 CFU/ml and applied during the spiking procedure. It was not effective for any of the spices-neutralizer combination and it was also negative for the viability control (data not shown). So the cells may be dead and we are not able to see any recovery effects (data not shown in graphs but in Appendixes 7, 8, 9, 10 and 11) .

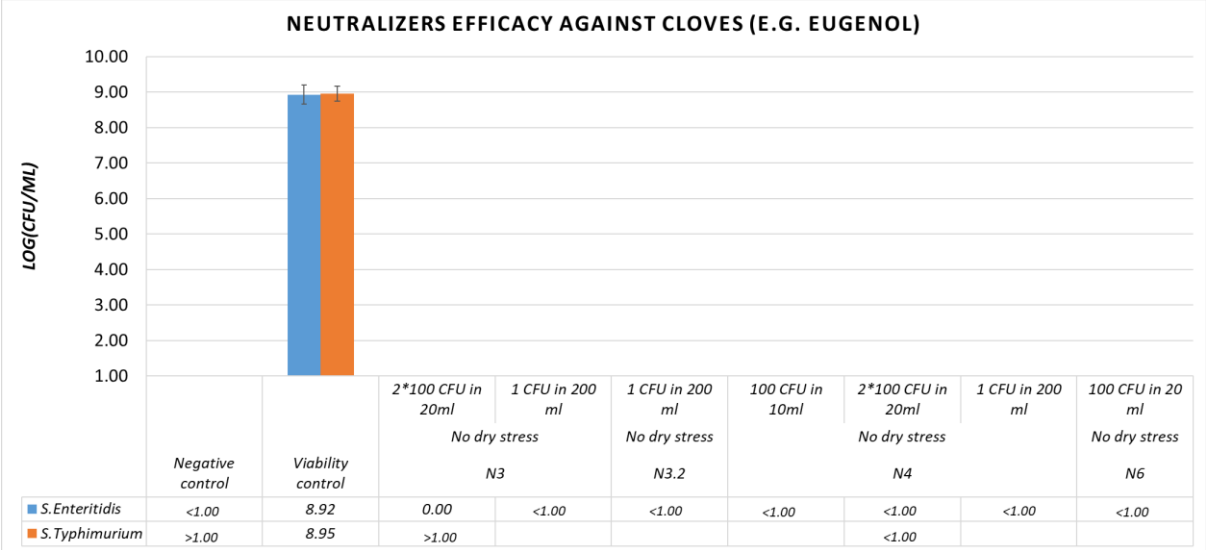
For oregano (e.g. carvacrol) the only result with recovery comparable to the viability control (8.92 log growth for *S.Enteritidis*, Figure 5) was in 1/10 dilution with ~10 cfu/ml final concentration for N4. In all the other cases (Graph 10, Appendix 8, Table 19) and because none of the samples are sterile there were backround flora from contamination, so either the

backround flora does not allow the target microorganism to grow or all the other trials are not effective for the concentration of carvacrol in oregano. Either way futher investigation is needed with more replicates for the already tested combinations to check if there is reproducibility on the results.



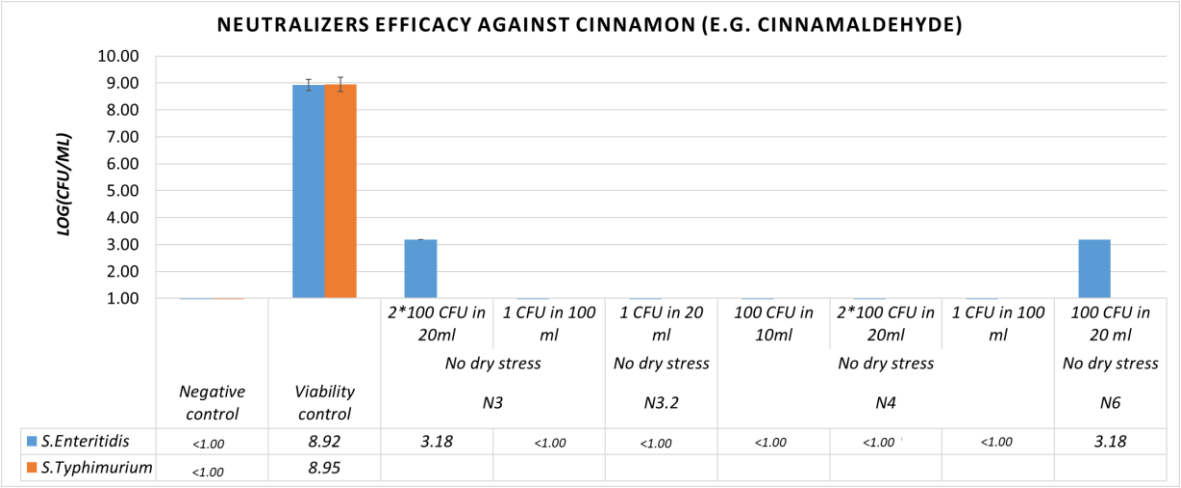
Graph 10: *The recovery of the cells from oregano against four neutralizers and under different conditions and concentrations (n=3).*

For cloves (e.g. eugenol) there are no recovery results (Graph 11, Appendix 8, Table 20) in none of the trials, showing that the recovery of the stressed organisms is difficult in dilutions until 1:20 for cloves, althought in all the toxicity and viability controls the growth was between 8-9 log.



Graph 11: *The recovery of the cells from cloves against four neutralizers and under different conditions and concentrations (n=3).*

For cinnamon (e.g. cinnamaldehyde) there is recovery of S.*Typhimurium* with 10 cfu/ml, without drying stress for N3 only. For *S.Enteritidis* without drying stress for N3 (10 cfu/ml) and N6 (5 cfu/ml). The recovery for those is 3.18 log and in comparison with the viability controls (8.95 log for *S.Typhimurium* and 8.92 log for *S.Enteritidis*) and the toxicity controls (8.26 log for *S.Enteritidis*) is too low. So even if there is growth, still the neutralizers are not effective for dilutions until 1:20 (Graph 12, Appendix 8, Table 21).

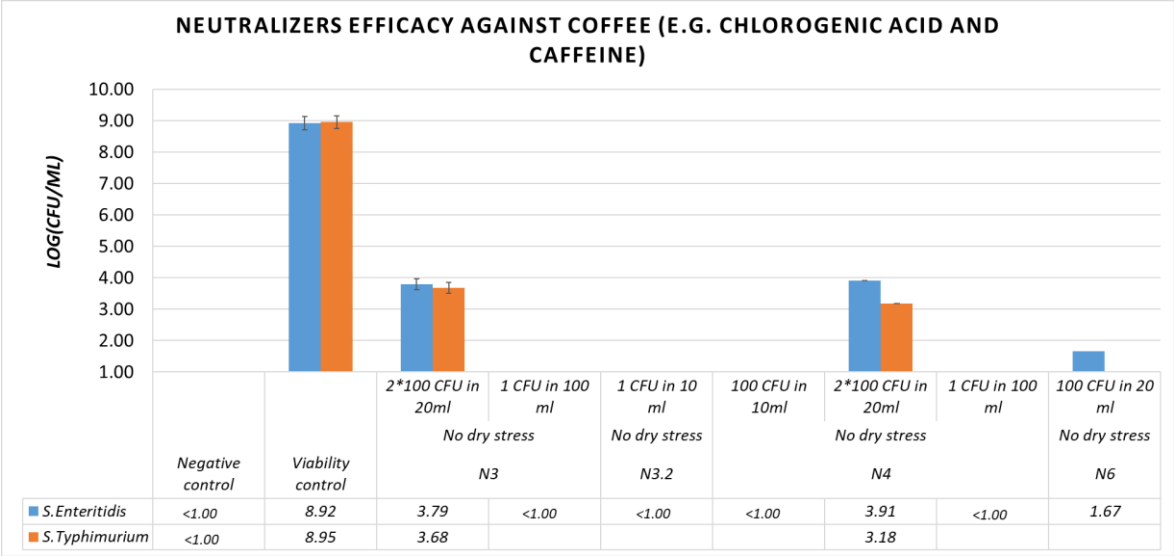


Graph 12: *The recovery of the cells from cinnamon against four neutralizers and under different conditions and concentrations (n=3).*

For coffee (e.g. caffeine and chlorogenic acid) there is recovery of S.*Typhimurium* without drying stress with 10 cfu/ml for N3 and N4. For N3 the growth is 3.79 log, for N4 is 3.18 log and from the comparison of those values with the toxicity (8.09 and 8.66 respectively) and

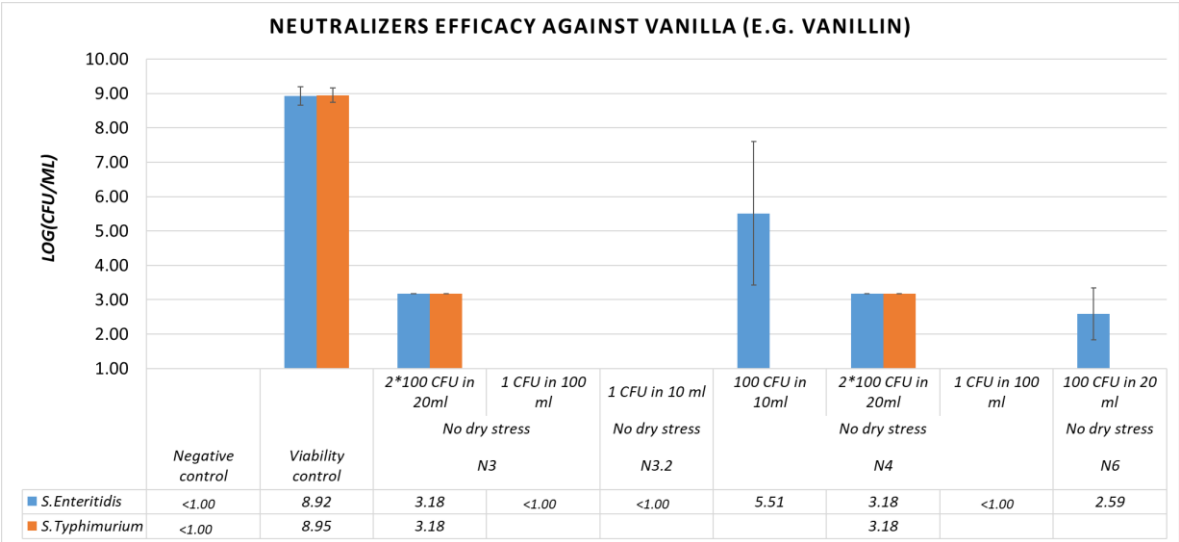
viability controls (8.95), the conclusion that those two neutralizers are not effective for coffee results.

For *S.Enteritidis* there is growth without drying stress for N3 (10 cfu/ml), N4 (10 cfu/ml) and N6 (5 cfu/ml). The recovery for those is 3.79, 2.94 and 2.00 log respectively. The viability controls (8.92 log) and the toxicity controls (8.38 for N3, 8.80 for N4 and 8.80 fo N6) have almost 5 logs higher recovery, so even if there is growth, still the neutralizers are not effective for those dilutions (Graph 13, Appendix 8, Table 22).



Graph 13: *The recovery of the cells from coffee against four neutralizers and under different conditions and concentrations (n=3).*

For vanilla (e.g, vanillin) and S.*Typhimurium* the three from the four trials gave positive results. For N3 and N4 with 10 cfu/ml. for both of them the average growth was 3.18 log, a very low value in comparison with the controls (8.95 log for the viability control, 8.09 and 8.66 log for the toxicity controls respectively. For *S.Enteritidis* positive results gave N3 with 10 cfu/ml and 1/20 dilution for vanilla (3.18 log), N4 with 10 cfu/ml and 1/10 dilution , N4 with 10 cfu/ml and 1/20 dilution and N6 with 5 cfu/ml and 1/20 dilution (Graph 14, Appendix 8, Table 23).



Graph 14: *The recovery of the cells from vanilla against four neutralizers and under different conditions and concentrations (n=3).*

6 Discussion

The effective neutralization of a molecule that inhibits the growth of a microorganism is critically important for the food safety regulations. In this study we studied several recommended neutralizing broths cited in the literature. The basic five neutralizers that tested are cited on Table 2.

In the table below there are some previously reported results for the effectivity of some of the neutralizer contents. All the others that are not reported on the table below are not studied alone but only as an ingredient and its not known yet against which inhibitory matrices are effective. Some of the already reported components seem to have antibacterial activity also such as lecithin (MacKinnon I. H., *et al.* 1974), magnesium chloride (Oyarzúa *et al.* 2014) and sodium thioglycollate (Hibbert H. R. *et al.* 1970).

Ingredient	*Inhibitory matrice*	*Reference*
Charcoal	Adsorbing toxic materials, soaking them up like a sponge	Robert W. Derlet, *et al.* 1986
Tween 80	Phenolic compounds, hexachlorophene, Quaternary Ammonium Compounds (QACs), Iodine	MacKinnon I. H., *et al.* 1974 Brown, M. R. W., *et al.* 1964
Sodium bisulphite	Glutaraldehyde, Formaldehyde	Russell A. D. *et al.* 1976, Cox C. B. *et al.* 1973 MacKinnon I. H., *et al,* 1974
Sodium thiosulfate 5 H2O	Mercurials, Halogens, Glutaraldehyde	MacKinnon I. H., *et al.* 1974 Cox C. B. *et al.* 1973
Sodium Thioglycollate	Mercurials	MacKinnon I. H., *et al.* 1974 Hibbert H. R. *et al.* 1970
Sodium pyruvate	Hydrogen peroxide	
Lecithin	Quaternary Ammonium Compounds (QACs), Parabens, Biguanides, Phenolic compounds	MacKinnon I. H., *et al.* 1974 Russell A. D., *et al.* 1979
Dey Engley	Quaternary Ammonium Compounds (QACs), Phenolic compounds	Engley and Dey *et al.* 1995

Table 8: *Previously reported efficacy of some of chemical ingredients of the studied neutralizers.*

For the first screening of the neutralizers efficacy we tested pure chemical inhibitors with the five basic neutralizers. N1 shown none recovery against all of the inhibitor – strain combinations. On the other hand N3 was one of the most effective against both stains and all

the seven inhibitors. If we compare the ingredients of N1 and N3 (Table 9) we can see that those two have many common components, the only difference is the concentration of those.

	Neutralizer	
	N1	**N3**
Ingedient	***g-ml/L***	***g-ml/L***
Charcoal	5	
casein enzymatic hydrolysate	5	
Yeast extract	2.5	
Dextrose	10	
Sodium thiosulfate	6	7.84
Sodium thioglycollate	1	5
Sodium bisulphite	2.5	0.4
Lecithin	7	2
Tween 80	5	60
L-cysteine		1.5

Table 9: *Comparison of the composion of N1 and N3.*

For N1 there is charcoal, casein enzymatic hydrolysate, yeast extract and dextrose extra. From those casein enzymic hydrolysate provides essential nutrients (carbon and nitrogen source), dextrose is an energy source (fermentable carbohydrate source) and yeast extract is also a rich source of vitamin B-complex (Engley and Dey *et al.* 1970), so those are not helping in neutralizing procedure but in the enhancement of the microorganism growth. According to previous research D/E is a good neutralizing broth (Engley and Dey *et al.* 1995) but in combination with charcoal in this study gave zero effectivity.

On the other hand, N3 has the same ingredients in higher concentrations and extra 1.5 g/l L-cysteine, and regarding the fact that this neutralizer is effective against most of the inhibitory matrices we can conclude that for the fail of recovery for N1 is responsible:

- Charcoal: Low concentration or no effective (because of bad quality) which is working synergically and affects the effectivity of D/E also.
- The concentration of the neutralizer ingredients is not high enough to overcome the inhibitory properties of the inhibitors upon the cells.

N5 contains also charcoal (10g/l) and Tween 80 (30ml/l) but in higher concentrations. This neutralizer shown some partially effectivity against some of the inhibitor – strain

combination. If we take into consideration that for both of them we are using the same quality of charcoal we can conclude that responsible for the non effectivity of N1 is either the combination of charcoal with the D/E (and the low concentration of the D/E ingredients) or the low amount of charcoal.

If now we compare the composition of N2 and N4 we can see that those two have the same components but in different concentrations (Table 10).

	Neutralizer	
	N2	**N4**
Ingedient	***g-ml/L***	***g-ml/L***
Lecithin	7.5	3
Tween 80	24	30
L-histidine	0.24	1

Table 10: *Comparison of the composition of N2 and N4.*

The N4 was the best candidate of all the neutralizer and N2 was partly effective with low growth neutralizer. N2 has higher lecithin concentration and N4 Tween 80 and L-histidine. The incomplete effectivity of N2 can be due to:

- The previously reported toxicity upon the cells from lecithin. The already stressed and exposed to the inhibitor cells can be further damaged from the high concentration of lecithin.
- The lower concentration of L-histidine.

The difference of the Tween 80 concentration between the 2 neutralizers is not that big, but it may be a reason for the difference in the effectivity between the those two.

For the second screening of the neutralizers we tested the two best candidates (N3 and N4) against oregano, cloves, cinnamaldehyde, vanilla and coffee. N4 was effective for oregano and vanilla in 1/10 dilution and 10^2 CFU spiked. All the other examined cases for both of the neutralizers were shown zero recovery.

To examine further the action of the neutralizers we tested different ingredients of the neutralizers alone and in different concentrations in BPW:

- Tween 80: 30 ml/l, 40 ml/l and 50 ml/l
- Sodium thioglycollate: 2 g/l, 3 g/l and 4 gr/l

- L-cysteine: 0.1 g/l, 0.5 g/l and 1 g/l
- Charcoal: 5 g/l

To examine the zero recovery of the strains in the tested neutralizers, we have to take a look of the concentrations of the inhibitors in the raw materials in comparison with the pure inhibitors

	Concentration (mg/ml)	
Inhibitor	**Pure inhibitor**	**Raw material (range)**
Carvacrol	1	1, 2
Eugenol	2	4, 10, 20
Cinnamaldehyde	1	2, 4
Caffeine	5	2.5, 5
Chlorogenic acid	40	5, 10
Vanillin	10	1, 2

Table 11: *Tested concentrations of the inhibitors as pure compounds and as a content in the raw materials.*

As we can see in most of the cases the concentration of the inhibitors in the raw materilas in much higher than the tested of the pure compound, in addition in the raw materials there are also other chemical molecules that can work synergically with the inhibitors and can decrease the efficacy of the neutralizers, so the concentration of the neutralizer ingredients is not high enough to overcome the inhibitory properties.

7 Conclusions

The ultimate goal of this study was to overcome the inhibitory effects of same spice ingredients for avoiding the false negative results with the use of neutralizers, which have been shown that can overcome the inhibition of the target microorganism, so if in that case, *Salmonella* is present we will be able to detect it and see the growth. For that purpose several experiments were held:

- Because of the need to mimic realistic conditions, thermal treatment applied in each experiment to the target strains.
- The MIC of different spices ingredients was determined, as pure chemical compounds.
- Growth curves were prepared so we were able to see if there is a difference between the heat and non heat treated cells.
- Different neutralizer recipes were tested in the first place against the pure inhibitory matrices and secondly against the raw materials that include those compounds.

The comparison of the heat treated cells with the non heated shown that for the stressed cells there is a longer lag phase that the non stressed. Also in selective media we shown slower growth rates but not significally important. From the comparison also of the plating before and after the thermal treatment, we noticed that *S.Enteritidis* is more sensitive to the apllying stress that *S.Typhimurium.*

After the determination of the Minimum Inhibitory Concentration and some first trials with the inhibitor (in concentration higher than the MIC) we decided to proceed with neutralizers 3 and 4 in further analysis with the spices. Even if those two neutralizers were the most effective, without toxicity against the *Salmonella* strains, in the experiments with the spices there was almost none recovery of the injured cells.

Neutralizer results require the use of an appropriate neutralizer, but in most cases there is lack of a 'universal' one. For that purpose other homemade neutralizers have been tested and shown the same result: No toxicity but also no effectivity. The extra drying stress that was tested in experiments shown zero recovery to the viability controls also, so after the extra stress most probably the cells are dead either with to small recovery and we are not able to detect them.

The absence of recovery may be because the concentration of the neutralizers ingredients is not high enough to overcome the inhibitory effects since in all the toxicity and viability controls (except from those with the extra drying stress) there is full recovery between 8-9 logs.

8 Recommendations

From the study, some recommedations for future similar studies are given :

- Flow cytometry method may be applied to evaluate alterations in bacterial cell membrane permeability due to the presence of the inhibitory matrices in the spices and in combination with neutralizers to monitor bacterial growth and metabolism. This will help to understand the time of action of the neutralizers and decrase the incubation times.

- The identification of the concentration of the inhibitory matrices in the used spices will help for the neutralizers optimization.

- More experiments for oregano with the ISO 6579-1-2017 have to be performed to see if the backround flora in the contaminated plates is because of other *Salmonella* strains or not and all the experiments have to be repeated to be able to see if *Salmonella* is present in the samples even if it is in too low concentrations.

- Evaluate charcoal reference from another supplier successfully tested previously.

9 References

B. P. Dey and F. B. Engley. Comparison of Dey and Engley (D/E) Neutralizing Medium to Letheen Medium and Standard Methods Medium for recovery of Staphylococcus aureus from sanitized surfaces. Jr. Journal of Industrial Microbiology, (1995) 14, 21-25 9 1995 Society for Industrial Microbiology.

Brown, M. R. W. and R. M. E. Richards, "The effects of Tween 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation," *J. Pharm. Pharmacol.,* 16, 51-55 (1964).

Conner, D.E., Beuchat, L.R., 1984. Effects of essential oils from plants on growth of food spoilage yeasts. Journal of Food Science 49, 429– 434.

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., Palmas, F., 1999. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. Letters in Applied Microbiology 29, 130– 135.

Cox, C. B., J. C. Feeley, and M. Pittman, "Sterility testing: Detection of fungi and yeasts in the presence of preservatives," J. Biol. Stand., 1, 11-19 (1973).

Cremieux, A. and J. Fleurette, 1983. Methods of testing disinfectants. In: Disinfection, Sterilization and Preservation, 3 Ed., S.S. Block, (ed.), Philadelphia: Lea and Febiger, Philadelphia.

Davidson, P.M., 1997. Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM, Washington, pp. 520–556.

Engley and Dey, 1970. Chem. Spec. Manuf. Assoc. Proc., Mid-Year Meet., p. 100.

Farag, R.S., Daw, Z.Y., Hewedi, F.M., El-Baroty, G.S.A., 1989. Antimicrobial activity of some Egyptian spice essential oils. Journal of Food Protection 52 (9), 665– 667.

Hibbert, H. R. and R. Spencer, "An investigation of the inhibitory properties of sodium thioglycollate in media for the recovery of bacterial spores," J. Hyg. (Camb.), 68, 131-135 (1970).

ISO 16140-2:2016 - *Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.*
International Organization for Standardization, Geneva, Switzerland.

ISO 6579-1:2017 - *Microbiology of the food chain -- Horizontal method for the detection, enumeration and serotyping of Salmonella -- Part 1: Detection of Salmonella spp.*
International Organization for Standardization, Geneva, Switzerland.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H., Weis, N., 1989. Antibacterial and antifungal properties of essential oil components. Journal of Essential Oil Research 1, 119– 128.

Lambert, R.J.W., Skandamis, P.N., Coote, P., Nychas, G.-J.E., 2001. “A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol”. Journal of Applied Microbiology 91, 453–462.

MacKinnon, I. H., “The use of inactivators in the evaluation of disinfectants,” J. Hyg. (Camb.), 73, 189-95 (1974).

National Outbreak Reporting System (NORS): https://wwwn.cdc.gov/norsdashboard

Oyarzúa Alarcón P, Sossa K, Contreras D, Urrutia H, Nocker A. Antimicrobial properties of magnesium chloride at low pH in the presence of anionic bases. Magnes Res 2014; 27(2): 57-68 doi:10.1684/mrh.2014.0362

Popoff MY, Bockemühl J, Gheesling LL.: Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res Microbiol.* 2003 Apr;154(3):173-4.

Qing Liu, Xiao Meng, Ya Li, Cai-Ning Zhao, Guo-Yi Tang, and Hua-Bin Li (2017): Antibacterial and Antifungal Activities of Spices. Int J Mol Sci. 2017 Jun; 18(6): 1283.

Radulović NS, Blagojević PD, Stojanović-Radić ZZ, Stojanović NM (2013): Antimicrobial plant metabolites: structural diversity and mechanism of action. Curr Med Chem. 2013;20(7):932-52.

Robert W. Derlet, MD, and Timothy E. Albertson, MD, PhD, Sacramento, California: Activated Charcoal-Past, Present and Future. (Derlet RW, Albertson TE: Activated charcoal-Past, present and future. West J Med 1986 Oct;
145:493-496)

Russell, A. D. and D. Hopwood, “The biological uses and importance of glutaraldehyde,” In Progress in Medicinal Chemistry, v13. G. P. Ellis and G. B. West (eds). Amsterdam, North Holland Publ. Amsterdam, 1976, pp.271-301.

Russell, A.D., 1981. Modification of the bacterial cell envelope and enhancement of antibiotic susceptibility. In The control of antibiotic-resistant bacteria. C.H. Stuart-Harris and D.M. Harris, editors. London, England: Academic Press, Ltd., pp: 119-16.

Russell, A. D., I. Ahonkhai, and D. T. Rogers, “Microbiological applications of the inactivation of antibiotics and other antimicrobial agents.” J. Appl. Bact., 46, 207-245 (1979).

Sara Burt, “Essential oils: their antibacterial properties and potential applications in foods—a review”, International Journal of Food Microbiology 94 (2004) 223– 253

Shelef, L.A., 1983. “Antimicrobial effects of spices”, Journal of Food Safety 6, 29– 44.

Sikkema, J., De Bont, J.A.M., Poolman, B., 1994. “Interactions of cyclic hydrocarbons with biological membranes”. Journal of Biological Chemistry 269 (11), 8022–8028.

Sikkema, J., De Bont, J.A.M., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59 (2), 201– 222.

Skandamis, P.N., Nychas, G.-J.E., 2001. “Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres”, Journal of Applied Microbiology 91, 1011 –1022.

Tepe B, Daferera D, Sokmen M, Polissiou M, Sokmen A: In vitro antimicrobial and antioxidant activities of the essential oils and various extracts of *Thymus eigii* M. Zohary et P.H. Davis. *J Agric Food Chem* 2004, 52:1132-1137.

Ultee, A., Bennink, M.H.J., Moezelaar, R., 2002. “The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*”. Applied and Environmental Microbiology 68 (4), 1561–1568. PhD thesis, ISBN 90- 5808-219-9.

10 Appendices

10.1 APPENDIX 1: *SALMONELLA* SPP. STRAINS INCLUDED IN THIS STUDY

Strains	Microorganisms code	Nestlé code
S.Typhimurium	WDCM00031	S497
S.Enteritidis	WDCM00030	S498

10.2 APPENDIX 2: MEDIA, REAGENTS AND EQUIPMENT

Media	Catalog Reference	Brand & Origin
Buffered Peptone Water (BPW)	1.07228.5000	Merck®, Darmstadt, Germany
Brain Heart Infusion (BHI)	CM1135	Oxoid, Hampshire, UK
Tryptone Salt Agar (TSA)	CM0131	Oxoid, Hampshire, UK
Xylose Lysine Deoxycholate Agar (XLD)	43563	Biomérieux®, Genève,Switzerland
Carvacrol	W224511	Sigma-Aldrich, USA
Eugenol	E-51791-100g	Sigma-Aldrich, USA
Cinnamaldehyde	W228613	Sigma-Aldrich, USA
Caffeine	C0750-5G	Sigma-Aldrich, USA
Chlorogenic acid	C3878-5G J60457	Sigma-Aldrich, USA Alfa Aesar, Kandel, Germany
Vanillin	W310727-100G-K	Sigma-Aldrich, USA
Thymol	T0501-100G	Sigma-Aldrich, USA
Oregano		Worleé, Ratingen, Germany
Cloves		Euroma, Zwolle, The Netherlands
Cinnamon		
Coffee		Nestlé, Oinofita, Greece
Vanilla		
Charcoal	87126.230	VWR®, Radnor, USA Nature's Way Kohle Hevert, Hevert-Arzneimittel GmbH & Co. kG
Dey-Engley	D3435	
Tween 80	P1754-500ML	Sigma-Aldrich, USA
L-histidine	VWRC24581.134_P	VWR®, Radnor, USA
Sodium bisulphite	243973-100G	Sigma-Aldrich, USA
Sodium thiosulfate 5 H2O	1.06516.0500 1.06516.0500	Fluka, USA Merck®, Darmstadt, Germany
Sodium Thioglycollate	T0632-100G 90404-500G	Alfa Aesar, Kandel, Germany Sigma-Aldrich, USA
L-cysteine	30129-100g	Sigma-Aldrich, USA
Magnesium chloride	1.05833.0250	Merck®, Darmstadt, Germany
Whey protein		Sponser
Sodium pyruvate	SLBH3762V	Sigma-Aldrich, USA
Lecithin	36486.30	Alfa Aesar, Kandel, Germany
Sodium Chloride	K44165104	Merck®, Darmstadt, Germany
Tryptone	LP0042	Oxoid®, Hampshire, UK

Equipments	Brand & Origin
Eppendorf Thermomixer	Eppendorf®, Hamburg, Germany
Laboratory Weighing	Seven multi, Mettler Toledo (Schweiz) GmbH
Spiral plate	IUL, S.A., Barcelona, Spain

PH meter	Seven multi, Mettler Toledo (Schweiz) GmbH

10.3 APPENDIX 3: COMPOSITION OF DEY-ENGLEY.

D/E ingredients	g/L
casein enzymatic hydrolysate	5
Yeast extract	2.5
Dextrose	10
Sodium thiosulfate	6
Sodium thioglycollate	1
Sodium bisulphite	2.5
Lecithin	7
Tween 80	5
pH 7.6	

10.4 APPENDIX 4: TABLE WITH THE GROWTH CURVES DATA.

- Non Heated cells

plating on TSA			
	S.Typhimurium (a)	*S.Typhimurium* (b)	*S.Typhimurium* (c)
Hours	log (CFU/ml)	log (CFU/ml)	log (CFU/ml)
0	1.00	0.900	1.160
1	0.950	1.260	1.320
2	1.00	1.620	1.920
3	1.850	2.460	2.470
4	2.960	3.110	3.100
5	3.630	3.830	3.950
6	4.300	4.430	4.670
7	5.110	5.270	5.310
24	8.670	8.650	8.850

- Heat treated cells

plating on TSA				
	S.Typhimurium (a)	*S.Typhimurium* (b)	*S.Typhimurium* (c)	*S.Typhimurium* (d)
Hours	log (CFU/ml)	log (CFU/ml)	log (CFU/ml)	log (CFU/ml)
0	3.04922	2.90309	2.80618	2.85733
1	2.80618	3.01703	3.07918	2.85733
2	2.80618	2.90309	2.80618	2.94448
3	3.04922	3.01703	3.26482	3.15836
4	3.53656	3.54654	3.59329	3.8118
5	4.07143	4.14324	4.16345	4.15346
6	4.09259	4.61909	4.63548	4.56971
7	5.1356	5.0763	5.34948	5.22454
8	5.68124	5.80618	5.98227	6.01703

24	8.94448	9.18184	8.94	9.01703

10.5 APPENDIX 5: ENUMERATION RESULTS FOR HEAT STRESS.

Experiment	Plating	Strain	Replicates	Log (cfu/ml)		Injury	Stress time	Temperature
				TSA	XLD			
Heat treatment		S497	8	2.05	1.79	-0.26	15 min	50°C
Heat treatment		S498	8	1.86	1.52	-0.34	15 min	50°C

10.6 APPENDIX 6: TESTED CONCENTRATIONS THAT WAS USED FOR THE DETERMINATION OF MIC FOR *S. TYPHIMURIUM* AND FOR *S. ENTERITIDIS*.

Carvacrol					
S.Typhimurium					
Concentration (mg/ml)					
	1	0.5	0.25	**0.1**	**0.01**
A B C	No growth				Growth
S.Enteritidis					
Concentration (mg/ml)					
	0.25	**0.1**	**0.01**	0.005	0.0025
A B C	No growth		Growth		

Table 12: The range of different concentrations that was used for the determination of MIC for carvacrol against growth for S.Typhimurium and for S.Enteritidis.

Eugenol					
S.Typhimurium					
Concentration (mg/ml)					
	2	**0.2**	0.1	0.05	0.01
A B C	No growth	Growth			
S.Enteritidis					
Concentration (mg/ml)					
	2	**0.2**	0.1	0.05	0.01
A B C	No growth	Growth			

Table 13: The range of different concentrations that was used for the determination of MIC for eugenol against growth for S.Typhimurium and for S.Enteritidis.

Cinnamaldehyde					
S.Typhimurium					
Concentration (mg/ml)					
	1	0.5	0.25	**0.1**	**0.01**
A B C	No growth				Growth
S.Enteritidis					
Concentration (mg/ml)					
	0.25	**0.1**	**0.01**	0.005	0.0025
A B C	No growth		Growth		

Table 14: *The range of different concentrations that was used for the determination of MIC for cinnamaldehyde against growth for S.Typhimurium and for S.Enteritidis.*

Caffeine					
S.Typhimurium					
Concentration (mg/ml)					
	5	**0.5**	0.1	0.05	0.01
A B C	No growth	Growth			
S.Enteritidis					
Concentration (mg/ml)					
	0.05	0.025	0.01	0.005	0.003
A B C	Growth				

Table 15: *The range of different concentrations that was used for the determination of MIC for caffeine against growth for S.Typhimurium and for S.Enteritidis.*

Chlorogenic acid					
S.Typhimurium					
Concentration (mg/ml)					
	40	**30**	**20**	15	10
A B C	No growth		Growth		
S.Enteritidis					
Concentration (mg/ml)					
	40	**30**	**20**	15	10
A B C	No growth		Growth		

Table 16: *The range of different concentrations that was used for the determination of MIC for chlorogenic acid against growth for S.Typhimurium and for S.Enteritidis.*

Vanillin										
S.Typhimurium										
Concentration (mg/ml)										
	10	9	8	7	6	5	4	3	**2**	**1**
A B C	No growth									Growth
S.Enteritidis										
Concentration (mg/ml)										
	10	9	8	7	6	5	4	**3**	**2**	1
A B C	No growth								Growth	

Table 17: The range of different concentrations that was used for the determination of MIC for vanillin against growth for S.Typhimurium and for S.Enteritidis.

Thymol										
S.Typhimurium										
Concentration (mg/ml)										
	1.5	1.35	1.2	**1.05**	**0.9**	0.75	0.6	0.45	0.3	0.15
A B C	No growth					Growth				
S.Enteritidis										
Concentration (mg/ml)										
	1.5	1.35	1.2	1.05	0.9	**0.75**	**0.6**	0.45	0.3	0.15
A B C	No growth						Growth			

Table 18: The range of different concentrations that was used for the determination of MIC for thymol against growth for S.Typhimurium and for S.Enteritidis.

10.7 APPENDIX 7: TOXICITY OF NEUTRALIZING BROTHS ((CFU/ML)/(CFU/ML)).

Strain	N1	N2	N3	N3.2	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16
S.Typhimurium	1,01	0,97	0,9	-	0,97	0,94	-	-	-	-	-	-	-	-	-	-	-
S.Enteritidis	1,04	0,98	0,94	1,02	0,99	0,97	0,93	0,98	0,99	0,97	0,99	0,99	0,98	1	0,99	1	1,01

10.8 APPENDIX 8: TABLE WITH RESULTS FROM RAW MATERIALS AND NEUTRALIZERS

Raw Materials	Concentration	Strain		Negative controls		N3		N4		
						Dry stress 24h	Dry stress 1h	Dry stress 24h	Dry stress 1h	No dry stress
				10 CFU spiked	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10^2 CFU spiked*
Oregano (Carvacrol)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	3.41E+08
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	9.50E+07
			C	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	2.31E+08

Raw Materials	Concentration	Strain		Negative control	N3	N4
					No dry stress	No dry stress
				$2*10^2$ CFU spiked	*$2*10^2$ CFU spiked*	*$2*10^2$ CFU spiked*
Oregano (Carvacrol)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4	N16
					No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Oregano (Carvacrol)	10 g/100ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N6
					No dry stress
				10^2 CFU spiked	*10^2 CFU spiked*
Oregano (Carvacrol)	1 g/20ml	S498	A	<1.00E+01	>1.5E+03
			B	<1.00E+01	>1.5E+03
			C	<1.00E+01	>1.5E+03

Raw Materials	Concentration	Strain		Negative control	N3.2	N7	N8	N9	N10
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Oregano (Carvacrol)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N11	N12	N13	N14	N15
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Oregano (Carvacrol)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Table 19: *Table with results from oregano and the neutralizers.*

Raw Materials	Concentration	Strain		Negative controls		N3		N4		
						Dry stress 24h	Dry stress 1h	Dry stress 24h	Dry stress 1h	No dry stress
				10 CFU spiked	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10^2 CFU spiked*
Cloves (Eugenol)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4
					No dry stress	No dry stress
				$2*10^2$ CFU spiked	*$2*10^2$ CFU spiked*	*$2*10^2$ CFU spiked*
Cloves (Eugenol)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4	N16
					No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cloves (Eugenol)	10 g/200ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N6
					No dry stress
				10^2 CFU spiked	*10^2 CFU spiked*
Cloves (Eugenol)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3.2	N7	N8	N9	N10
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cloves (Eugenol)	1 g/50ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N11	N12	N13	N14	N15
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cloves (Eugenol)	1 g/50ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Table 20: *Table with results from cloves and neutralizers.*

Raw Materials	Concentration	Strain		Negative controls		N3		N4		
						Dry stress 24h	Dry stress 1h	Dry stress 24h	Dry stress 1h	No dry stress
				10 CFU spiked	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10^2 CFU spiked*
Cinnamon (Cinnamaldehyde)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4
					No dry stress	No dry stress
				$2*10^2$ CFU spiked	*$2*10^2$ CFU spiked*	*$2*10^2$ CFU spiked*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	A	<1.00E+01	>1.5E+03	<1.00E+01
			B	<1.00E+01	>1.5E+03	<1.00E+01
			C	<1.00E+01	>1.5E+03	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4	N16
					No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cinnamon (Cinnamaldehyde)	10 g/100ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N6
					No dry stress
				10^2 CFU spiked	*10^2 CFU spiked*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	A	<1.00E+01	>1.5E+03
			B	<1.00E+01	>1.5E+03
			C	<1.00E+01	>1.5E+03

Raw Materials	Concentration	Strain		Negative control	N3.2	N7	N8	N9	N10
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N11	N12	N13	N14	N15
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Cinnamon (Cinnamaldehyde)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	5.50E+04

Table 21: *Table with results from cinnamon and neutralizers.*

Raw Materials	Concentration	Strain		Negative controls		N3		N4		
						Dry stress 24h	Dry stress 1h	Dry stress 24h	Dry stress 1h	No dry stress
				10 CFU spiked	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10^2 CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			C	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N3	N4
					No dry stress	No dry stress
				$2*10^2$ CFU spiked	*$2*10^2$ CFU spiked*	*$2*10^2$ CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	1 g/20ml	S498	A	<1.00E+01	3.96E+03	<1.00E+01
			B	<1.00E+01	4.40E+03	>1.5E+03
			C	<1.00E+01	1.36E+04	4.40E+04

Raw Materials	Concentration	Strain		Negative control	N3	N4	N16
					No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	10 g/100ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N6
					No dry stress
				10^2 CFU spiked	*10^2 CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	1 g/20ml	S498	A	<1.00E+01	<1.00E+01
			B	<1.00E+01	1.00E+02
			C	<1.00E+01	1.00E+03

Raw Materials	Concentration	Strain		Negative control	N3.2	N7	N8	N9	N10
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N11	N12	N13	N14	N15
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Coffee (Chlorogenic acid + Caffeine)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	6.40E+04

Table 22: *Table with results from coffee and neutralizers.*

Raw Materials	Concentration	Strain		Negative controls		N3		N4		
						Dry stress 24h	Dry stress 1h	Dry stress 24h	Dry stress 1h	No dry stress
				10 CFU spiked	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10 CFU spiked*	*10^2 CFU spiked*	*10^2 CFU spiked*
Vanilla (Vanillin)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	2.80E+04
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	8.20E+07
			C	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	1.50E+04

Raw Materials	Concentration	Strain		Negative control	N3	N4
					No dry stress	No dry stress
				$2*10^2$ CFU spiked	*$2*10^2$ CFU spiked*	*$2*10^2$ CFU spiked*
Vanilla (Vanillin)	1 g/20ml	S498	A	<1.00E+01	>1.5E+03	>1.5E+03
			B	<1.00E+01	>1.5E+03	>1.5E+03
			C	<1.00E+01	>1.5E+03	>1.5E+03

Raw Materials	Concentration	Strain		Negative control	N3	N4	N16
					No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Vanilla (Vanillin)	10 g/100ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N6
					No dry stress
				10^2 CFU spiked	*10^2 CFU spiked*
Vanilla (Vanillin)	1 g/20ml	S498	A	<1.00E+01	2.72E+03
			B	<1.00E+01	1.00E+02
			C	<1.00E+01	2.20E+02

Raw Materials	Concentration	Strain		Negative control	N3.2	N7	N8	N9	N10
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Vanilla (Vanillin)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01

Raw Materials	Concentration	Strain		Negative control	N11	N12	N13	N14	N15
					No dry stress	No dry stress	No dry stress	No dry stress	No dry stress
				1 CFU spiked	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*	*1 CFU spiked*
Vanilla (Vanillin)	1 g/10ml	S498	A	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01
			B	<1.00E+01	<1.00E+01	<1.00E+01	<1.00E+01	3.00E+06	7.00E+07

Table 23: *Table with results from vanilla and neutralizers.*