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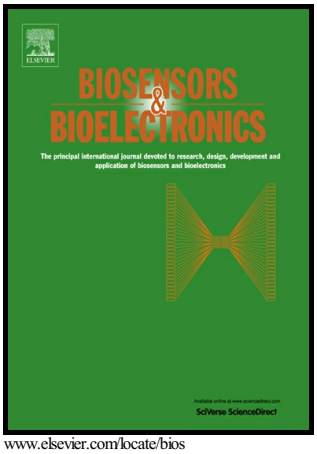

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Rapid calorimetric sensing platform for the detection of *Listeria monocytogenes* foodborne pathogen

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Abstract

L. monocytogenes is a serious cause of human foodborne infections worldwide, which needs spending billions of dollars for inspection of bacterial contamination in food every year. Therefore, there is an urgent need for rapid, in-field and cost effective detection techniques. In this study, rapid, low-cost and simple colorimetric assay was developed using magnetic nanoparticles for the detection of listeria bacteria. The protease from the listeria bacteria was detected using D-amino acid substrate. D-amino acid substrate was linked to the carboxylic acid on the magnetic nanoparticles using EDC/NHS chemistry. The cysteine from the C-terminal of the substrate was used for the self-assembled monolayer formation on the gold sensor surface, which in turn the black magnetic nanobeads will mask the golden color. The color will change from black to golden color upon the cleavage of the specific peptide sequence by the listeria protease. The sensor was tested with serial dilutions of *Listeria* bacteria. It was found that the appearance of the gold surface area is proportional to the bacterial concentrations in CFU/ml. The lowest detection limit of the developed sensor for

listeria was found to be 2.17 X 10^2 colony forming unit/ml (CFU/ml). The specificity of the biosensor was tested against four different foodborne associated bacteria (*E.coli*, *Salmonella*, *Shigella flexnerii* and *Staphylococcus aureus)* against *Listeria* specific substrate. Finally, the sensor was tested with artificially spiked whole milk and ground meat spiked with *listeria*.

Keywords

Listeria monocytogenes, Listeriosis, Colorimetric assay, Self-assembled monolayer, Magnetic nanoparticles, Biosensors.

1. INTRODUCTION

Ingestion of foods contaminated with pathogens such as bacteria, viruses, and parasites leads to the Foodborne diseases (FBD). Foodborne disease pose a significant health problem in the world, with significant rate of morbidity and mortality, in both developed and developing countries . The most common known bacterial foodborne pathogens are *Escherichia coli O157:H7, Salmonella enterica, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni and Bacillus cereus* (Zhao et al. 2014. *Listeria monocytogenes* considered as one of the pathogens causing gastroenteritis, and more likely to cause death unlike other bacteria that cause foodborne disease (Robert et al. 2010; Vazquez-Boland et al. 2001; GarridO' et al. 2008). *L. monocytogenes* has been associated with listeriosis, which has fatality rates up to 30% (Roberts et al. 2010; Auvolat and Gnanou Besse 2016). Listeriosis affect human's health by either an invasive or non-invasive way. The non-invasive gastrointestinal form is characterized by symptoms like fever, muscles ache, nausea and diarrhea. The invasive form symptoms are much more serious, as upon spreading to the central nervous system, it could lead to meningitis, stiff neck, confusion, loss of balance, convulsion and ocular listeriosis (Mead et al. 2006; Shoughy and Tabbar, 2014). Invasive listeriosis is dominant among immunocompromised populations such as pregnant women, elderly people above 65, new born babies and acquired immune deficiency syndrome (AIDS)

patients (Zhao et al. 2014; Centers for Disease prevention, 2013; Swaminathan and Smidt 2007).

In pregnant women disease manifestations include miscarriage, stillbirth, perinatal septicemia and meningitis in new born (Mokta et al. 2010; Bobade et al. 2016).

L. monocytogenes contaminates water, soil, silage, fruits, vegetables and food samples such as dairy product (unpasteurized milk and soft cheese), meat and either raw or cooked sea-food. Animals can also be infected by eating contaminated vegetation or poorly prepared animal feed. In general, *L. monocytogenes* grows best at neutral to slightly alkaline pH, tolerate high stress environment form and wide range of temperature (from -18 to 37°C). Thus contamination of ready to eat food by *L. monocytogenes* increases the risk of foodborne disease (O'Connor et al. 2010; Junttila et al. 1988). Food contamination could occur during food processing, packaging and distribution. In food industry, the time between food packaging, inspection and consumption is very crucial. Therefore, *L. monocytogenes* is considered a serious cause of human foodborne infection worldwide (Roberts et al. 2010; Vazquez-Boland et al. 2001; GarridO, et al. 2008; Liu 2006).

L. monocytogenes detection method is commonly achieved by culture methods, which typically requires few days to confirm the results. Moreover, this method is laborious and require trained-personnel (Jadhav et al. 2012; Alessandria et al. 2010; Kabuki et al. 2004; Frece et al. 2010; Gasanov et al. 2005; Brehm-Stecher and Johnson 2007). Immunoassays based techniques are used, where it involve specific antigen-antibody reaction. The immunoassays suffer from poor sensitivity in comparison with culture based method with detection limit between 10^5 cells/ml -10^4 cells/ml (Gasanov et al. 2005). Other more-sensitive real-time detection kits were developed, which require sample pre-enrichment procedures and very expensive for the routine lab analysis (Chen et al. 2012). Based on the fact that *L. monocytogenes* foodborne disease represents major concern to public health worldwide. Developing a rapid, specific and sensitive *L. monocytogenes* detection method is urgently required.

L. monocytogenes has several virulence factors which contributes to its pathogenicity. During infection, *L. monocytogenes* invade the host cell by lysing the phagocytic vacuoles and proliferate in the cytosol with the help of Listeriolysin O (LLO) and two secreted phospholipases C (PLC): a broad-range phospholipase C (PC-PLC) and a phosphatidylinositol specific PLC (PI-PLC) (Portnoy et al. 1992; Smith et al. 1995). Broad-range phospholipase C is released as an inactive propeptide and is activated by the cleavage of the propeptide by a metalloprotease (MMP) at low pH (Marquis et al. 2000). Therefore,

higher level of MMP would be observed in the host infected with *L. monocytogenes*. This virulence protease could be used as a biomarker for the detection of food contamination. In addition, protease are known to break the peptide bonds in a protein substrate, and some protease are known, to be very specific with regard to the type of peptide bond they are able to cleave (Mitchell 2003; Kasana et al. 2011). Protease enzyme cleave a selective peptide bond at specific site in protein and polypeptide and contribute in the pathogenicity and feasibility of the disease (Kasana 2010). Therefore, proteases has been used as biomarkers for the detection of microbial infection due to the specificity and the stability of the enzyme under diver condition (Suaifan et al. 2013a).

Biosensors are a powerful substitute to conventional analytical techniques, due to their particularly high specification and sensitivity, as well as their small size and cost-effectiveness. Nanoparticles was found to be an excellent tool to be applied for microbe's detection and drug therapy (Suaifan et al. 2013b). Our group has developed a number of colorimetric assay based nanoparticles and peptide sequence for prostate specific antigen (PSA), HIV and periodontitis. The assay employing a monolayer of specific substrate peptide conjugated with magnetic nano-particles which has black color. The color will change upon the protease cleavage of the peptide-magnetic nanobeads conjugate (Suaifan et al. 2013a; Suaifan et al. 2013b **;** Wignarajah et al. 2015**)**.

Overall, simple, cost effective screening assays are needed for the detection of *L. monocytogenes* food contamination from the time it is produced to the time it is purchased. Accordingly, this study aimed towards the development of novel rapid and low-cost colorimetric paper-based detection tool with high sensitivity and specificity. Magnetic nanoparticles conjugated with *L. monocytogenes* protease specific substrate was employed which will be selectively cleaved by the *L. monocytogenes* proteases.

2. Materials and Method

Carboxylic acid terminated magnetic nanoparticles *(*MNPs) with less than 50 nm dispersed as 10 mg/ml in water was purchased form Turbobeads (Zurich, Switzerland). N-Hydroxysussinimide (NHS) and 1-(3-Dimethylaminopropyl)-3-Ethyl-Carbodiimide (EDC) and pH stripes, Potassium phosphate, Sodium Chloride, Bovine Serum Albumin (BSA), Ethylenediaminetetraactic acid (EDTA), Sodium Azide, Tris buffer were purchased from sigma-Aldrich (Dorest, UK). *Listeria monocytogenes* ATCC 19115, *E.coli* ATCC 25922, *salmonella* ATCC13312, *Shigella flexnerii* ATCC 12022 and *Staphylococcus aureus* ATCC 25923 were purchased from LGC Standards (Middlesex, UK).

Peptide sequences with specific cleavage sites for *L monocytogenes* (Gasanov et al. 2005) was synthesized by Pepmic Co., Ltd (Suzhou, China) with a purity of about 90%. Two different peptides substrates with D- and L-amino acids have been synthesized for *L. monocytogenes*. Peptides were provided as lyophilized powder and was then dissolved in Dimethyl sulfoxide (DMSO).

2.1 Bacteria growing

Bacteria were grown in 5 ml of Brain heart infusion (BHI) broth, incubated at 37ºC for 18 hrs. Subsequently, 8 serial dilutions in Phosphate buffer saline (PBS) buffer were prepared to determine the bacterial colony forming unit (CFU) value of the original stocks, using viable count spread dilution method. Subsequently, the bacteria were pelted by centrifuged at 3000Xg for 10 minutes. The supernatant was then filtered with sterile syringe filter 0.22µm purchased from Millipore (Middlesex, UK) to obtain the protease which is directly proportional to each bacterial concentration.

2.2 Conjugation of the MNPs *with L. monocytogenes peptide substrates*

Peptide substrates (1 mg/ml) dissolved in DMSO was mixed with freshly prepared coupling agent EDC (0.57 mg/ ml) and NHS (12 mg/ml) in water and previously washed MNPs. The mixture was gently mixed by end over end at room temperature for 4 hours. After which, uncoupled peptides were removed by washing the beads 3 times with washing buffer. The unreacted carboxylic acids in the beads were blocked by the Tris base in the washing buffer. Finally, the functionalized MNPs were stored at 4° C in storage buffer.

2.3 Preparation of the sensing platform

Self-adhesive sheet was purchased from Whatman (London, U.K) and was then gold plated using sputtering machine at the School of Engineering at Canfield University. The sheet was cut into narrow pieces (1-2 mm width) and then stacked over plastic strip at standardized distance as shown in schematic 1A. A specific amount of the MNPs-peptides solution was placed over the gold sensing platform and allowed to dry at room temperature. Then, a permanent magnet (12.5 X 12.5 X 5 cm), strength (3360 gauss and 573 gauss) was passed over the sensor surface to remove unreacted MNPs-peptide. A change in the gold sensor surface into

black will be observed by the naked eye upon completion of the immobilization step as shown in schematic 1B.

Self-adhesive magnetic sheets were provided from Polarity Magnetic Company was cut into 1mm diameter sphere and was stacked at the back of the strip at a standardized distance of 5 mm from the immobilized sensing surface. This magnet would assist collection of the cleaved MNPs-peptide sequence as shown in Scheme 1C.

2.4 Biosensing of the L. monocytogenes protease

The current colorimetric detection assay is based on the detection of *L. monocytogenes* proteases proteolytic activity using a specific MNPs-peptide-gold sensing probe. Accordingly, *L. monocytogenes protease (bacterial extract containing proteases)* solution (20 µl) was added over the black color sensing probe, which in turn cleave MNPs-peptide complex rendering peptide moiety-gold sensor probe golden color visible to the naked eye. The percentage of the cleaved area was proportional to the protease concentration. Cleaved MNPs-peptide moiety was attracted by the permanent magnetic endorsing a visual colorimetric detection for qualitative evaluation of the tested sample as shown in schematic 1C.

2.5 Food spiking and testing

5 mL of fresh full-fat milk provided from diary company UK and 2g of minced ready to eat meat were mixed with 5 ml sterile water, inoculated with a colony of the *L. monocytogenes* and incubated at 35 C° for 15-18 hr. The samples were centrifuged at 10,000xg and the supernatants containing protease were filtrated with 0.22µm filter (Sigma-Aldrich, UK) and then added to the sensor surface. Negative blank sample containing no bacteria was also prepared in a similar way and tested to check the matrices effect of the milk and meat content on the sensor.

2.6 Quantification measurements

The developed biosensor was intended to be used by visual observation, the colour change (black to golden), as a result of the proteolytic activity of *bacterial* proteases was easily viewed by the naked eye. The appearance of yellow colour was quantified by a simple method using the ImageJ program (a public-domain, Java-based image processing program developed at the National Institute of Health). The images taken from smart mobile phone camera before and after the addition of the bacterial samples were processed using the ImageJ software. The results presented as percentage of the amount of the cleaved magnetic beads (appearance of yellow color) to the total immobilized black magnetic nanobeads (black area).

3. Results and Discussion

Today, *L. monocytogenes* is considered to be one of the most important pathogenic agents causing foodborne diseases. Small undetectable number of microbes can multiply and become life threatening, especially among the immune compromised populations. Ready to eat foods are considered as a vehicle for the transmission of listeria. As ready to eat food goes under different process, they are more susceptible to bacterial contamination especially by listeria. Moreover, listeria has the ability to grow and survive in a wide unfavorable conditions. Thus gives the attentions to assess *listeria monocytogenes* in the food during manufacturing and distribution.

Proteases are enzymes involved in the bacterial growth, multiplication, and pathogenicity. Therefore, the focus of this study is to develop novel portable low-cost colorimetric screening assay for the detection of *Listeria monocytogenes* proteases using nanomagnetic particles.

3.1 Detection using D-peptide sequences

The protease extracted from each *listeria monocytogenes* bacterial concentration was applied to the D-peptide sequence. Figure 1a and 1b show the typical results before and after the addition of various bacterial protease concentrations respectively. It shows that there is clear visible correlation between the proteases concentration and the appearance of the yellow area as a result of the cleavage of the black magnetic nanobeads. Second confirmation spot was formed by the accumulation of the black cleaved magnetic nanoparticles at the paper magnetic area. The gradual increase in the appearance of the golden background, reflect the increase in the concentration of the protease. Figure 2 shows that there is a liner relationship between the amount of cleaved magnetic nanobeads (appearance of the yellow color) and the proteases concentration from various bacterial concentration in CFU/ml.

It is known that the peptide substrate containing D-amino acids are specific and facilitate the prokaryotic cleavages (Kaman et al. 2013). In this study, two peptide sequences using D- and L-amino acids were synthesized to compare the proteolytic activity for *listeria monocytogenes*. Then the selected peptide sequence can be integrated with magnetic nanoparticles for the construction of highly specific peptide sequence which can be cleaved specifically by *listeria monocytogenes proteases*. Serial dilution of the *listeria monocytogenes* was prepared to compare the detection limit as colony forming unit (CFU/ml) for both D and L-peptide sequences peptides. The protease extracted from each bacterial concentration was applied to the peptide sequences containing D-and L-aminoacids. Figure 2 shows the

comparison of the percentage of the cleaved nanobeads area for the D- and L-amino acid sequences as a function of various bacteria concentrations. The results shows that the peptide sequence containing D-amino acid, is highly specific with lower detection concentration 2.17 $X10^2$ CFU/ml within 30 seconds, than the sequence containing L-amino acids which gave higher detection limit 2.1 $X10^3$ CFU/ml in 2 hours. The detection limit of the D-aminoacids probe sensor in this study, without any pre-concentration of the sample is comparable to the other reported methods. For example, Moreno *et al* 2012 was able to detect the *L. monocytogenes* using PCR with D.L $7.4X10^2$ after 7 hour culturing (Välimaa et al. 2015). Fu *et al*. (2013) proposed colorimetric method using gold nanomagnetic particles combined with hyper branching rolling circle amplification (HRCA). The method gave detection limit as low as 100 aM synthetic hly gene targets and about 75 copies of L. monocytogenes (Fu et al. 2013). Law et al. (2015) used DNA microarray assay and Sabat *et al.* (2013) used NGS molecular method for the detection and identification of *L. monocytogenes*. Both methods were highly sensitive and specific but laborious, costly and unsuitable for field applications (Law et al. 2015). Table 1 summarizes the detection limits for the most recent developed assays. Indeed the detection limit from the developed sensor is comparable with the other reported methods but it has alot shorter response time.

3.2 Specificity of the sensor

The sensor was tested for the specificity by using proteases extracted from other commonly associated foodborne bacteria. In this study, proteases extracted from *staphylococcus aureus, Escherichia coli, Shigella and Salmonella* were used to check the cross-reactivity with the Listeria specific substrate. The results in Figure 3 shows the cleavage only occur with the *Listeria* unlike other bacteria where no cleavage was observed. It can be concluded that the listeria peptide probe is highly specific to the *Listeria* bacteria.

3.3 Testing the sensor with food samples

The sensor was tested with the whole milk and the supernatant of the ready to eat meat to check if the food has any proteolytic activity due to Listeria. Figure 4A (before adding the sample) and Figure 4B (B1, meat sample without inoculation) shows that the food supernatant has no effect on the sensor performance. Fresh whole milk and ready to eat meat were spiked with the colony of *listeria monocytogenes* incubated 35^{o}C for 15-18 hour. The concentrations of the bacteria stock after 18 hours for the meat was $13.8X\ 10^7$ CFU/g and the fresh whole milk count was $11.7\ X\ 10^7$CFU/ml. The stock concentration was used to make 8 serial dilutions and

viable count of spread dilution method was used to confirm the correct bacteria number. The negative control sensor (B1 in Figure 4 and Figure 5) shows no any proteolytic activity with the meat and milk ingredient (before spiking). The sensor (B2 in Figure 4 and Figure 5) was tested with the spiked samples directly (without centrifugation) and also the samples were tested after centrifugation (B3 in Figure 4 and Figure 5). It is clear from the amount of the cleaved magnetic nanobeads by the protease on the sensor surface that both give similar detection limit regardless the sample is centrifuged or not. The detection limit of the sensor in the spiked milk was $11.7X10^2$ CFU/mL and in the spiked meat was 13.8X10 CFU/g. The results obtained within 15 minutes and without any pre-enrichment steps.

4. Conclusion

In in this study, the protease produced from *Listeria monocytogenes* was used as biomarker for the detection of pathogenic *Listeria monocytogenes,* using D- amino acid substrate specific to Listeria. The change of the black color to the golden color, was due to the cleaved magnetic nanobeads, which was proportional to the concentration of the *listeria*. The detection limit of the *Listeria* was found to be $2.1 \text{ X } 10^2$ CFU/ml in 30 sec. The specificity of the sensor has been tested using the protease extract from other bacteria associated with foodborne diseases. The developed diagnostic tool is low-cost, rapid, sensitive and simple where does not require instrumentation for the read-out. The reported rapid screening assay can be applied as a point of care detecting the contamination of food by pathogenic bacteria and other sectors such as biomedical, environmental and security.

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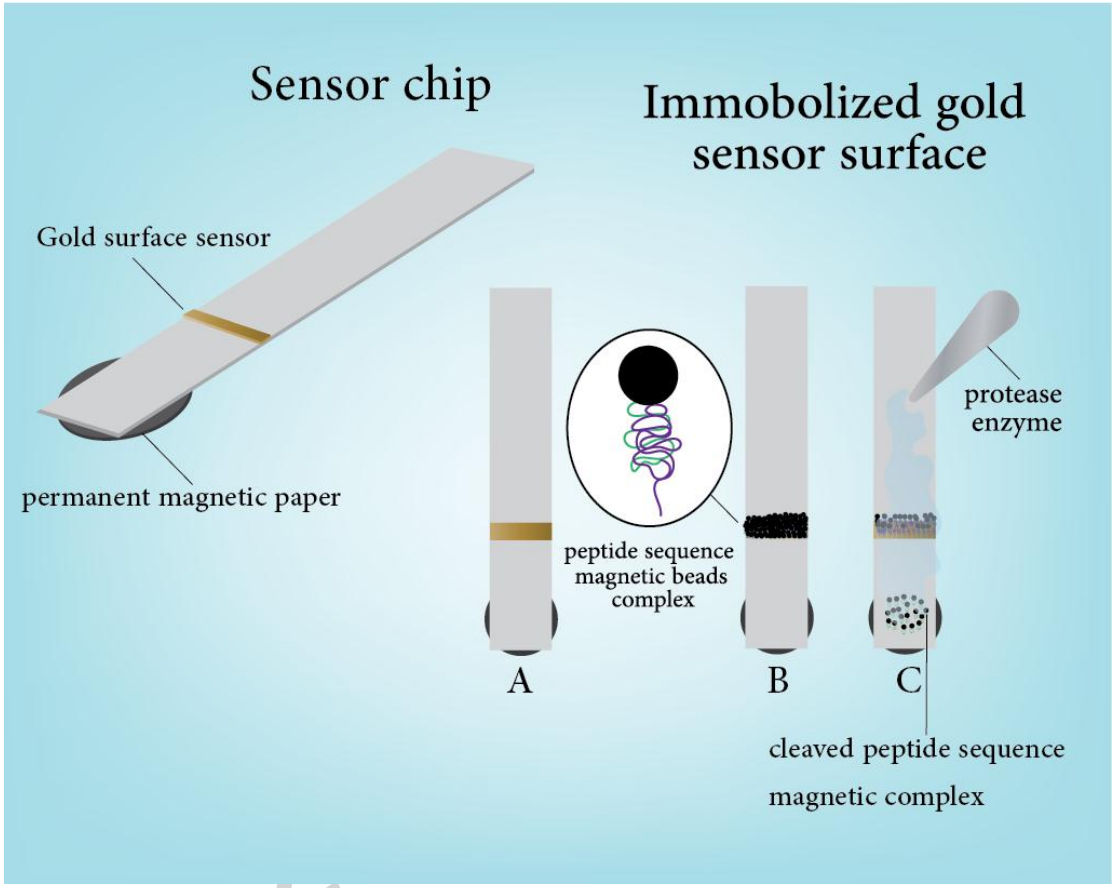

Schematic 1: Schematic image of fabricated sensor assay. The sensor designed specifically for *Listeria monocytogenes* detection. (A). The sensor with gold surface sensor, (B) Immobilized peptide sequence magnetic beads complex over the gold sensor surface masking its gold color, (C) In the presence of protease enzyme of *L. monocytogenes* the proteolytic activity of the enzyme will observed by the dissociation of the magnetic beads complex exposes the gold color back ground.

(a)

(b)

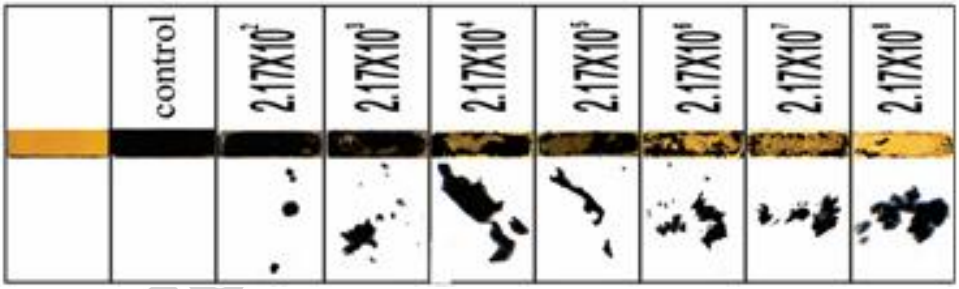

Figure 1: a) Colorimetric *Listeria monocytogenes* protease sensor probe using D–amino acid covalently bound to a magnetic nanobead; a) before adding the sample to the specific listeria monocytogenes protease substrate; b) after adding different concentrations of Listeria monocytogenes protease solutions.

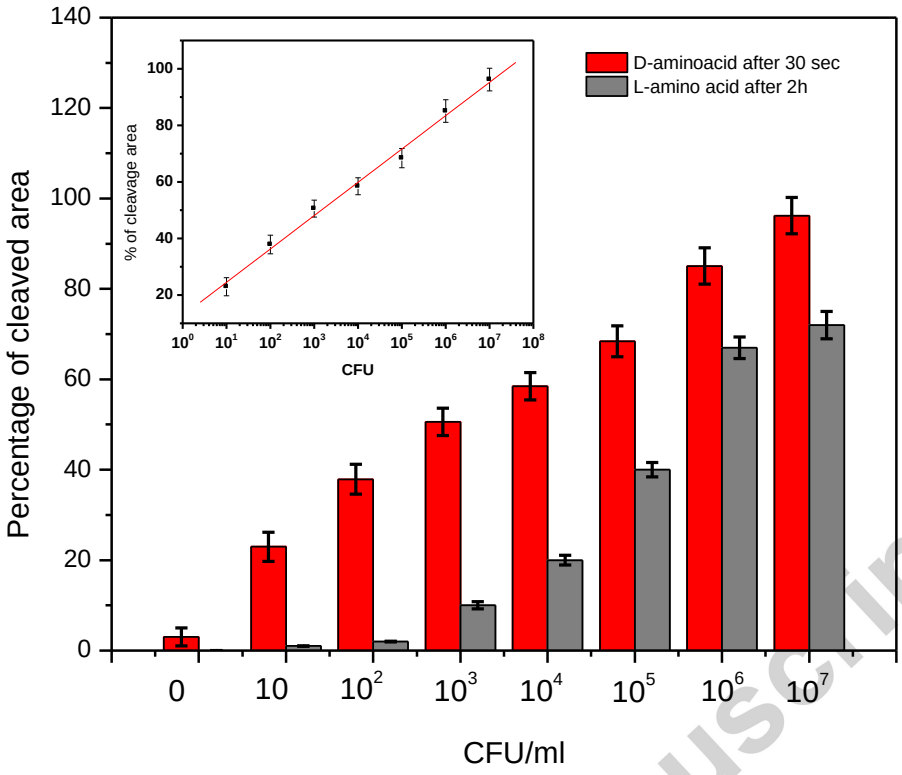

Figure 2: Shows the comparison of using D- and L-aminoacids in the *Lsiteria* peptide sequence probe in sensors with various bacteria concentrations. The Calibration curve is inserted in the figure to show the potential for quantitative analysis.

Table 1

Detection limits of the standard and some alternative, rapid L. monocytogenes (LM) identification methods.

Method Detection	Detection limit	Matrix	Reference
Amperiometric	10^2 CFU/g	Blueberries (A	Valimaa et al. 2015
Impedance spectroscopy	4 CFU/ml	Tomato extract (A	Valimaa et al. 2015
Piezoelectric	$10^2 – 10^3$ CFU/ml	Milk	Valimaa et al 2015
Fibre-optic	10^2 CFU/25 g	Meat	Ohk et al 2010
Fe_3O_4 NPC	5.4 X 10^3 CFU/mL		Zhang et al 2016
ELISA based on VHH clone L5-79 and Monoclonal Ab	1X 10^4 CFU/Ml	Milk	Tu Zhui et al 2016
Platinum interdigitated array Microelectrode	5.39 +_ 0.21 CFU/mL		Sidhu R et al 2016
Metagenomics	<2 X10^2 CFU/ml	soft chees	Wang et al 2016

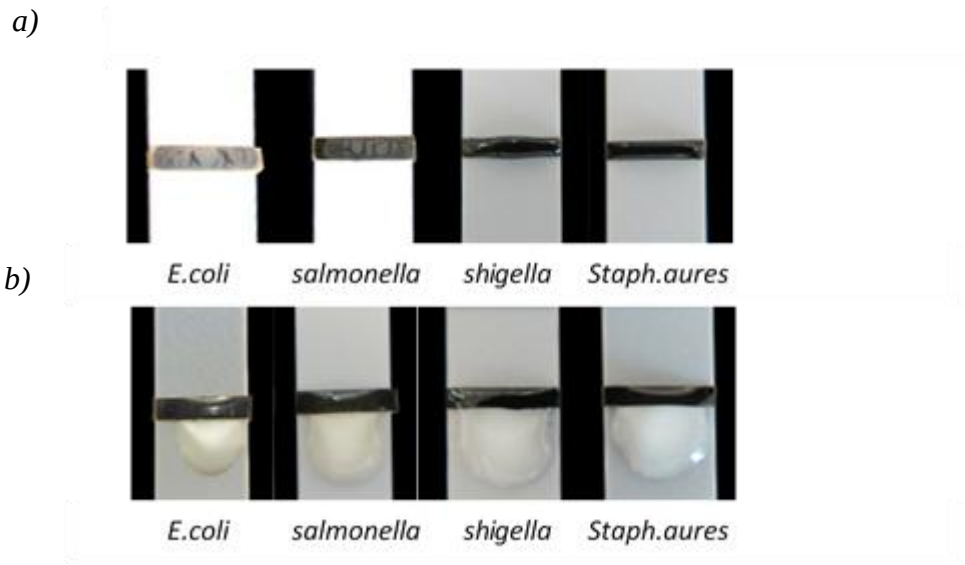

Figure 3: *a) Colorimetric Listeria monocytogenes protease sensor probe with peptide sequence nanomagnetic complex of L. monocytogenes before the application of different bacterial protease .b) sensor probe under the effect of other bacterial protease.*

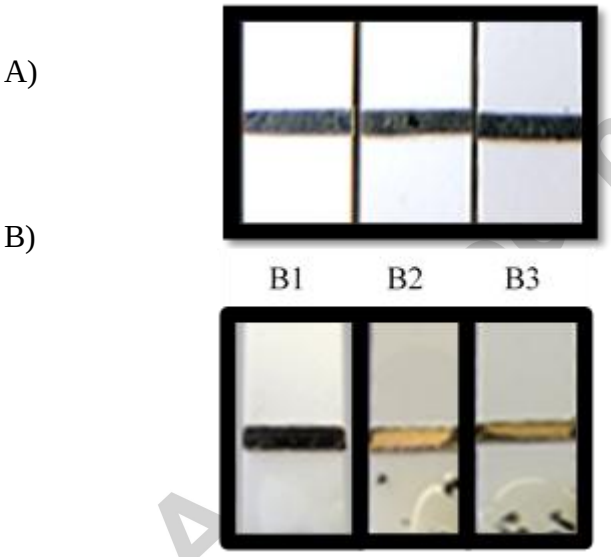

Figure 4: Colorimetric *Listeria monocytogenes* sensor probe with D- amino acid peptide substrate application on spiked meat; A) Sensor chip functionalized with magnetic beads-specific *Listeria monocytogenes* before adding the samples; B) Functionalized biosensor tested with B1) meat supernatant without inoculation. B2) meat supernatant after 18 hours of inoculation. B3) meat supernatant after 18 hours of inoculation centrifuged.

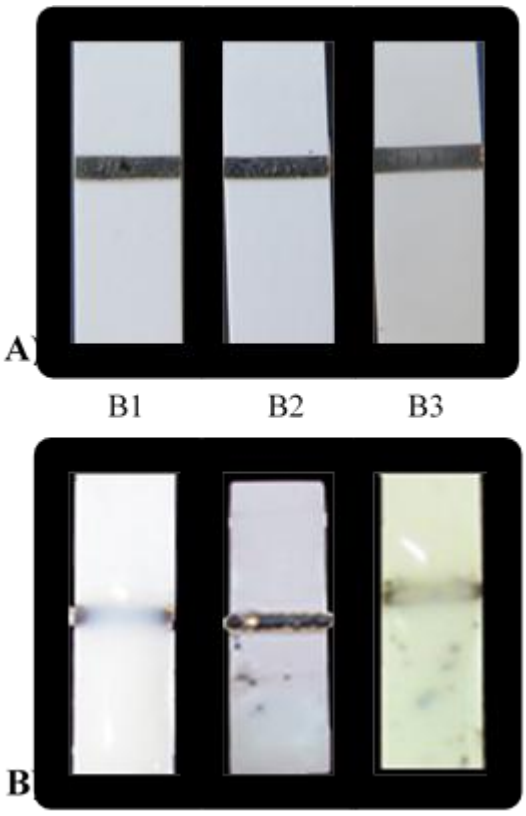

Figure 5: Colorimetric Listeria monocytogenes sensor probe with D- amino acid peptide substrate application on spiked milk. A) Biosensor chip functionalized with magnetic nanobeads-specific Listeria monocytogenes before adding the sample. B) Functionalized biosensor tested with B1) fresh milk without inoculation. B2) Milk after 18 hours of inoculation. B3) Milk supernatant after 18 hours of inoculation.

Highlights

- Novel, ultra-rapid colorimetric biosensor for the detection and identification of Listeria
- The designed colorimetric biosensor detect rapidly the amidolytic activity of *Listeria* protease.
- The detection limits of this colorimetric sensor was 2.17 X 10^2 CFU/ml in less than one minute in milk and meat samples.
- The platform presented herein is considered to be simple, fast and low-cost, thus opening new opportunities for point of care devices for other diseases.