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# Impedance Sensing Platform for Detection of the Food Pathogen *Listeria Monocytogenes*

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**Abstract:** A great improvement in food safety and quality controls worldwide has been achieved through the development of biosensing platforms. Foodborne pathogens continue to cause serious outbreaks due to the ingestion of contaminated food. The development of new, sensitive, portable, high-throughput, and automated platforms is a primary objective to allow detection of pathogens and their toxins in foods. *Listeria monocytogenes* is one common foodborne pathogen. Major outbreaks of listeriosis have been caused by a variety of foods, including milk, soft cheeses, meat, fermented sausages, poultry, seafood and vegetable products.

Due to its high sensitivity and easy setup, electrochemical impedance spectroscopy (EIS) has been extensively applied for biosensor fabrication and in particular in the field of microbiology as a mean to detect and quantify foodborne bacteria.

Here we describe a miniaturized, portable EIS platform consisting of a microfluidic device with EIS sensors for the detection of *L. monocytogenes* in milk samples, connected to a portable impedance analyzer for on-field application in clinical and food diagnostics but also for biosecurity purposes. To achieve this goal microelectrodes were functionalized with antibodies specific for *L. monocytogenes*. The binding and detection of *L. monocytogenes* was achieved in the range  $2.2 \times 10^3$  cfu/ml to  $1 \times 10^2$  with a Limit of Detection (LoD) of 5.5 cfu/ml.

**Keywords:** food pathogens; Electrochemical impedance spectroscopy; surface activation;

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## 1. Introduction

Foodborne diseases include several illnesses resulting from ingestion of foods contaminated with microorganisms or chemicals. Food can be contaminated at any stage from production to consumption, because of several factors such as environmental contamination (pollution of water, soil or air) or incorrect conservation and preparation procedures. Although great security awareness and the improvements in food safety and quality control worldwide, foodborne diseases still represent a significant public health problem with significant rate of morbidity and mortality, in both developed and developing countries[1]. According to the WHO studies about 30 hazards (including viruses, bacteria, protozoa, helminths and chemicals) caused 600 million foodborne illnesses globally and 420000 deaths every year[2]. Foodborne diseases represent not only a health problem but has

strong socioeconomic impact since it affects the health care systems, and national economies, tourism and trade. *Listeria monocytogenes* is a gram-positive bacterium and is one of the most common foodborne pathogens affecting millions of people annually. *L. monocytogenes* is a ubiquitous, intracellular pathogen and can cause both invasive or non-invasive listeriosis[3,4]. Outbreaks of listeriosis have been caused by a variety of foods, including milk, soft cheeses, meat, fermented sausages, poultry, seafood and vegetable products[5,6]. This pathogen represents a potential risk to human health since it is able to overcome food preservation and safety barriers thanks to adaptation and survival mechanisms allowing the persistence and growth in different environmental conditions (cold temperatures, low pH and high salt concentration)[7].

FDA regulation for *L. monocytogenes* in ready-to-eat foods impose strict limits and corrective solutions for contaminated food. In particular the limits correspond to 0.04 CFU of *L. monocytogenes* per gram of food that supports the growth of *L. monocytogenes*, while in foods that do not support the growth of the microorganism the limit is extended to 100 CFU/g[8-11].

The high incidence of foodborne pathogen contamination requires the development of new, sensitive, portable, high-throughput, and automated platforms to detect pathogens and their toxins in foods[12]. Due to its high sensitivity and easy setup, electrochemical impedance spectroscopy (EIS) has been widely used for the development of biosensors and in particular for pathogen detection[13]. Electrochemical biosensors, in particular impedance-based systems, present great advantages in terms of ease of miniaturization, lack of reagents, sensitivity, and low cost. Impedance methods have been often used for bacterial identification by monitoring changes in the impedance of the solution induced by the release of ionic metabolites from the living cells (i.e. carbon dioxide and organic acids) as a result of bacterial metabolism and cell growth. The magnitude of these changes in the impedance of the solution and can be correlated to the number of living cells allowing a quantification of bacterial cells. Such an approach has been used by Yang and coworkers who developed an impedimetric detection of viable *Salmonella* spp. through a three-electrodes configuration set-up[14]. The improvements in microfabrication and the trend towards miniaturization allow to translate the traditional electrochemical set-up in compact and portable chips offering new opportunities in the field of bacterial detection thanks to a great improvement in sensitivity. Such chips, being cheap thanks to the mass production, can be used as disposable devices reducing possible cross-contamination errors.

In this respect, Yang et al. in 2004 achieved for a quantitative detection of *Salmonella* spp. in culture and milk samples by using interdigitated microelectrodes (IME) for impedance measurements[15].

In addition, microfabricated systems allow to functionalize the electrode surface with capture molecules specific for one or more pathogens of interests. In this case impedance spectroscopy allows the characterization of solution/electrode interface and can be used to monitor the attachment of molecules[16,17] and cells[18,19]. In this last reference, a gold interdigitated array microelectrode (IDAM) allowed the simultaneous detection of two different food pathogens[19]. Thanks to specific biorecognition reactions, EIS methods can allow the detection of a various pathogen species simultaneously, even in complex matrices, resulting from reduction of the unspecific signals. Moreover, compared with growth-based impedance methods that requires at least 24h, the analysis is much faster, requiring less than 2 h.

Ruan et al. developed an impedimetric biosensor for detection of *E. coli* O157:H7 with a detection limit of  $6 \times 10^3$  cells/mL. The method exploited the immobilization of species-specific antibodies onto

electrode surface in order to capture *E. coli* cells from a solution. A secondary antibody conjugated with alkaline phosphatase was used to amplify the signals[20].

A step forward was done by Yang and colleagues, that avoided the amplification step by development of a label-free immunosensor for detection of the same bacterial strain, *E. coli* O157:H7, using indium tin oxide (ITO) interdigitated electrodes with a size comparable to that of bacterial cells[21]. Bacterial cells attached to the specific antibodies immobilized on the electrodes induced an increase in the electron transfer resistance[13]. A similar approach was used by Radhakrishnan and colleagues: their electrochemical impedance spectroscopy system based on a gold electrode functionalized with an anti-*Listeria* monoclonal antibody was able to detect *L. monocytogenes*. The biochip achieved high sensitivity with a limit of detection (LoD) of 4-5 CFU/mL[12].

The integration of sensors and microfluidics structures led to the development of lab on a chip technology (LOC), opening new scenarios in many fields and in particular in microbiology[18] and food security monitoring[19,22]. LOC platforms may allow to achieve both the isolation and detection of bacterial cells thanks to the integration of biosensors with additional modules and microfluidic components in order to perform all the analytical and pre-analytical procedures on the same chip.

Here we describe a miniaturized, portable EIS biochip consisting of a microfluidic platform with EIS sensors, validated through detection of *L. monocytogenes* in artificially contaminated milk samples.

Microelectrodes were functionalized with antibodies specific for *L. monocytogenes*. The biochip was connected to a portable potentiostat for on-field applications. This platform can be used as point-of-care device for clinical and food diagnostics, as well as for biosecurity purposes.

## 2. Materials and Methods

### 2.1. Microfabrication and functionalization of the biochip

The device consist of an array of gold interdigitated electrodes on glass substrates arranged in four different sensing areas.

The interdigitated electrodes have a line-space period of 10 $\mu$ m and cover a 2mm $\times$ 1.5mm area, they were fabricated by optical lithography (using a Karl Suss MJB3 mask aligner and AZ5214B resist) and thermal evaporation of Cr/Au (3 nm/15 nm). A microfluidic module including channels and microchambers is aligned to microelectrodes in order to handle and confine all the solution and reagents necessary for functionalization of the electrodes.

The microelectrodes were functionalized with antibodies using reagents purchased from Sigma Aldrich at the maximum degree of purity.

The electrodes were incubated overnight with a solution of 11-mercaptoundecanoic acid (MUA) (0.2mM) and 2-mercaptoethanol (2-ME) (1mM) for the deposition of a mixed self-assembling monolayer, then COOH groups of MUA are activated by incubation with a solution of N-hydroxysuccinimide (NHS) and N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) at a ratio of 1:4 in milliQ water for 30 min.

Then a solution of protein A(50  $\mu$ g/ml) in PBS was incubated for 2 h to allow a covalent binding to SAM. Protein A naturally bind the Fc region of antibodies allowing an oriented immobilization of antibodies. Ethanolamine (1M) was used to block reactive groups while BSA (Bovine Serum Albumin) (1mg/ml) to passivate and saturate the unbound surface. As final step each sensing area was functionalized with antibodies specific for *L. monocytogenes*.

### 2.2. EIS measurements on cell chip

A pocketSTAT, a portable potentiostat and galvanostat with integrated impedance analyzer from IVIUM was used for measurements. Impedance spectra were recorded between 0.1 and  $10^6$  Hz using a sinusoidal ac voltage with a 15 mV RMS amplitude and no dc voltage applied ( $V_{DC} = 0$  V). The equivalent circuit consists of the ohmic resistance of the electrolytic solution consisting of PBS added with  $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$  (1:1) 10 mM ( $R_s$ ), a double layer capacitance ( $C_{dl}$ ), an interfacial electron-transfer resistance ( $R_{et}$ ) and a Warburg impedance ( $Z_w$ ) associated to the diffusion of the redox probe. In particular,  $C_{dl}$  and  $R_{et}$  depend on the electrical properties of the solution/electrode interface and can be used to monitor the attachment of molecules and bacterial cells.

### 2.3. Samples preparation

Pathogens growth conditions and measurement of concentration.

*L. monocytogenes* strains used in this study were isolated from food samples, grown in Half Frazer broth followed by a Listeria Enrichment broth (24 LEB) with selective polymixin and quinolone supplements (Oxoid, ThermoFisher, Milano, Italy) and identified by Real Time PCR. Subsequently, an aliquot of exponentially grown bacteria was serially diluted with isotonic solution, and the serial dilutions were plated onto agar plates with solidified Agar Listeria Ottaviani and Agosti (ALOA). The number of CFU/ml was determined by counting the bacteria in plates containing spots well isolated and colonies well spaced from each others. The samples were prepared in two replicates, so that each dilution could be stored frozen for further analyses. For EIS analysis, samples denatured by heating for 10 minutes were used.

*Listeria monocytogenes* suspensions in growth medium and in half-skimmed milk were prepared. Spiked milk samples were made by adding to half-skimmed milk a known concentration of pathogen suspension, in a ratio of 4:1.

### 2.4. Application of EIS biochip to *L. monocytogenes* detection

Pathogens' suspensions were incubated in the device chamber. For dilution of the samples, an uncontaminated growth medium and half-skimmed milk were used. Samples were then transferred into the electrode chamber. In order to calibrate the device for specific detection of the bacteria, different concentrations of *L. monocytogenes* in culture medium and in the milk suspension were incubated above the sensing layer by filling the microfluidic chamber with a volume of 20  $\mu$ l. Using serially diluted samples of *L. monocytogenes*, (i.e.  $2.2 \times 10^1$ ,  $2.2 \times 10^2$ ,  $2.2 \times 10^3$ ), the increase in impedance values was recorded. To obtain the reference EIS value for the negative control, a suspension containing *Salmonella enterica* was used. After 1 h incubation, to allow the interaction between bacteria and antibodies immobilized onto the electrodes, the devices are washed with PBS, and a solution with the redox probes  $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$  (1:1) 10 mM is added. After the redox solution (20  $\mu$ l) is injected in the chamber, EIS measurements are collected (see scheme in figure 1).

## 3. Results

Once the fabrication of the sensing platform was optimized, impedance spectroscopy measurements were performed and plotted as Nyquist curves, in which impedance values were collected in a frequency range from 106 to 0.1 Hz, and the diameter of the semicircle-shaped curves corresponds to the electric transfer resistance at the interface between the electrodes and the solution, in a complex system ( $Z_{re}$  vs  $-Z_{im}$ ). At first, the sensing layer given by immobilized antibodies on the

electrode surface was characterized (functionalization steps, described in Materials and methods). Anti-*L. monocytogenes* antibodies layer resulted in impedance values of around 27 k $\Omega$  (black line, inset in Figure 2). In order to calibrate the device for the detection of the pathogen, different concentrations of *L. monocytogenes* in culture medium suspension were incubated above the sensing layer. After the incubation time and a washing step, redox solution was replaced in the chamber and EIS measurements were collected. As shown in figure 2, a considerable increase in impedance values with respect to the antibody baseline can be associated to the incubation of the stock solution ( $2.2 \times 10^3$  CFU/ml) on the sensing layer (red line). As the concentration of *L. monocytogenes* in suspension decreases, this results in a decrease in impedance values. As can be seen in the diagram, the presence of  $2.2 \times 10^2$  CFU/ml produced EIS values of around 269 k $\Omega$  (blue line).

Using serially diluted samples of *L. monocytogenes*, (i.e.  $2.2 \times 10^3$ ,  $2.2 \times 10^2$ ,  $2.2 \times 10^1$ ), a decrease in impedance values was recorded, from 269 k $\Omega$  to 51 k $\Omega$ . The suspension containing 22 CFU/ml was associated to an increment of impedance values of around 51 k $\Omega$  (pink line, inset).

As a negative control, we incubated a suspension containing  $3 \times 10^3$  CFU/ml of *Salmonella enterica*. Impedance values measured in this case gave values of around 39 k $\Omega$  (green line, inset). Curves related to Ab baseline, lowest concentration of *L. monocytogenes* and negative controls are very close, thus revealing a limit of the detection of the sensing platform realized. The impedance increase was 7 k $\Omega$  in the case of negative control, while the value obtained at the highest concentration tested ( $3 \times 10^3$  CFU/ml) produced values of about 350 k $\Omega$ , showing the high specificity of the optimized sensing system.

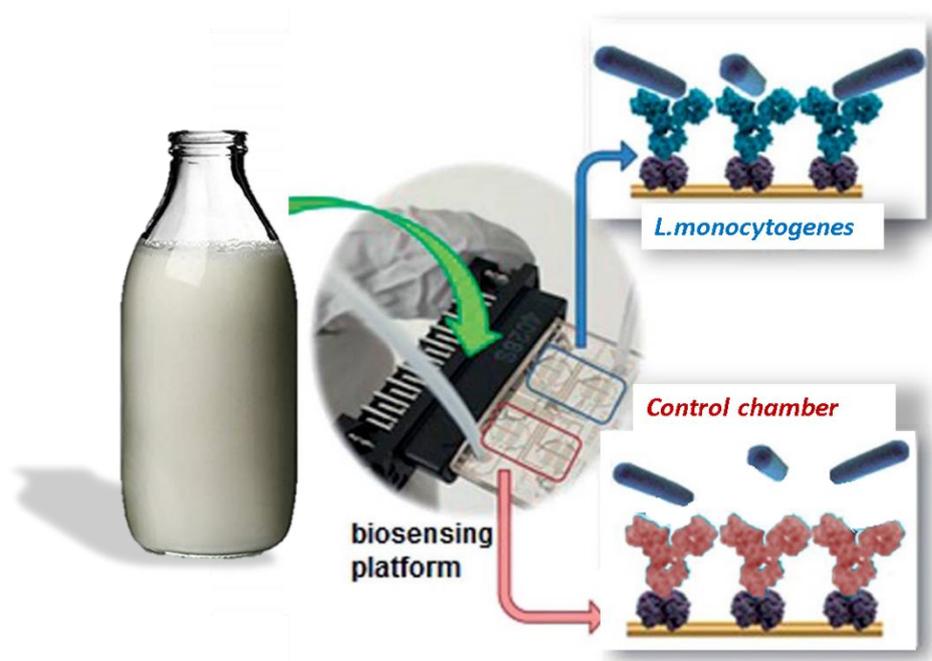
In order to test the possibility to use our optimized device for the detection of *L. monocytogenes* in real samples, we prepared half-skimmed milk spiked samples with different amount of bacteria. As it was not possible to exactly replicate concentrations of *L. monocytogenes* in culture medium, we fixed a milk concentration of 75% and the remaining 25% of the sample was represented by the bacteria suspension, both in the case of *L. monocytogenes* and of negative control (*S. enterica*) (Figure 3).

In this case, we first tested the matrix contribution due to milk (100%) unspecific absorption (red line, inset in figure 3). As a result, a low impedance value (37 k $\Omega$ ) was detected over the Ab baseline fixed at 27 k $\Omega$ , thus confirming once again the specificity of the sensing layer. In this experiment we incubated the first prepared spiked sample (75% spiked milk) containing  $5.5 \times 10^2$  CFU/ml, collecting high impedance values of around 386 k $\Omega$ . At a concentration of  $5.5 \times 10^1$  CFU/ml EIS values resulted in around 196 k $\Omega$ , while in the case of 5.5 CFU in milk we found an increased impedance response (45 k $\Omega$ ) over the baseline related to antibodies and also over the milk matrix contribution, thus revealing a better performance of the assay in spiked milk samples with respect to the same assay carried on culture medium. In order to set up negative control by considering spiked *S. enteric* samples made up of 75% of half-skimmed milk and 25% of culture medium containing  $7.5 \times 10^2$  CFU/ml. In this case the related Nyquist plot had a diameter of 31 k $\Omega$ , thus very close to the antibody baseline (green curve in figure 3).

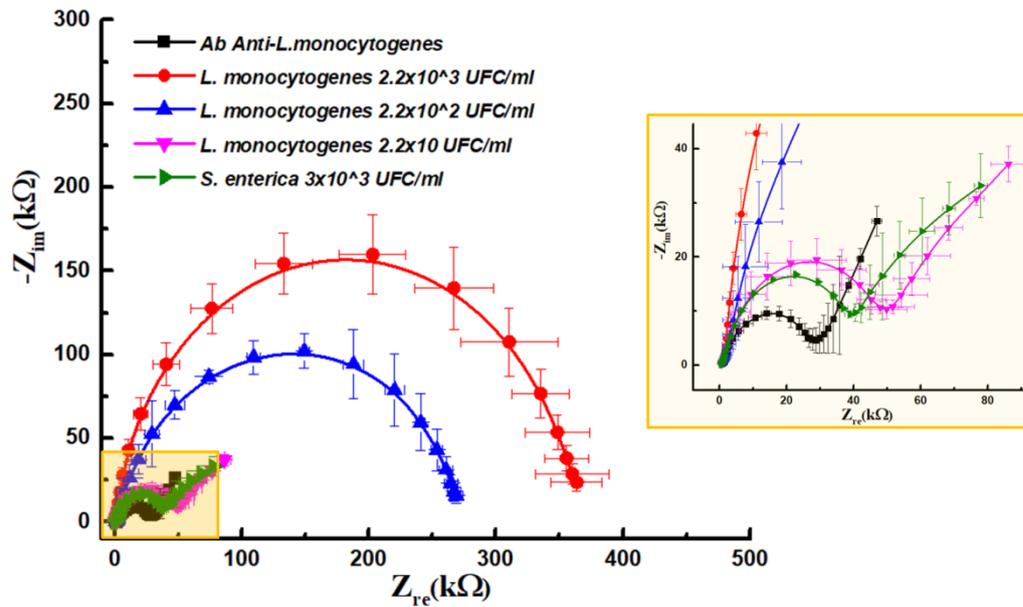
The aim of these experiments was to compare performances of the optimized platform to detect *L. monocytogenes* in culture media and in real samples, in order to verify the possibility to use it as a tool for on-field analysis. To allow this, a direct comparison of impedance values considering concentrations, was not possible, as skimmed milk spiked samples were prepared as 75% of matrix and the remaining 25% of suspended Listeria cells. So, in order to bypass this differences and to

compare the response of the system in presence of a different matrix, we prepared culture medium samples containing the same amount of *L. monocytogenes* cells. As can be seen from figure 4, the two most concentrated suspensions gave a remarkable increase in impedance values over the sensing area. The *L. monocytogenes* suspension containing 5.5 CFU/ml was instead overlapping with the antibody baseline, below or near the value associated with that obtained using *S. enterica* as negative control. These results, combined with the first calibration curves, allowed to set the limit of detection (LoD) for *L. monocytogenes* dissolved in the medium between 22 and 5.5 CFU/ml. Once our platform was calibrated for *L. monocytogenes* detection in medium culture and in milk spiked samples we obtained two calibration curves (Figure 5), using the collected Ret values plotted in function of the different *L. monocytogenes* concentrations.

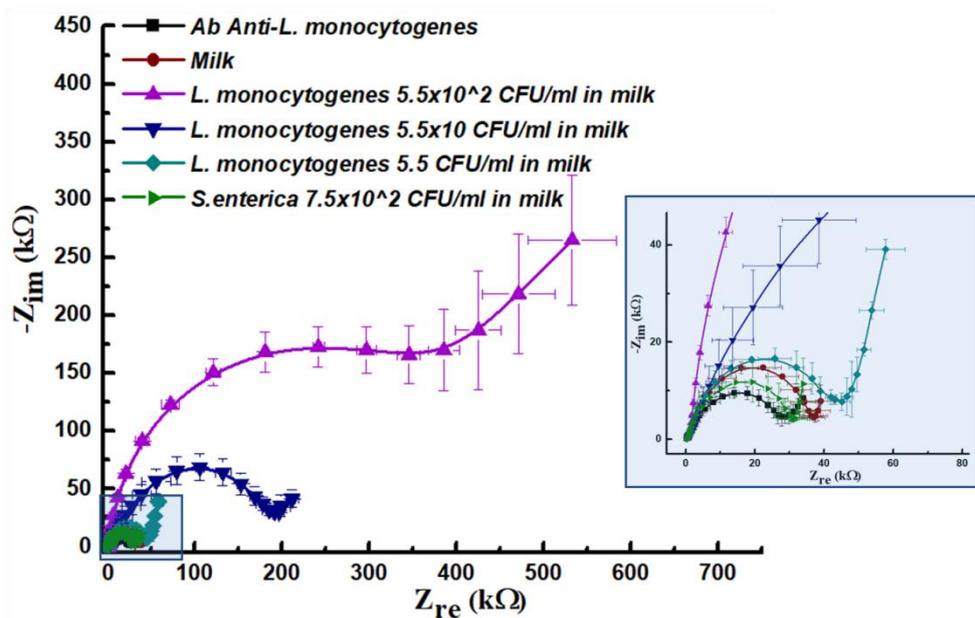
### 3.2. Figures, Tables and Schemes



**Figure 1.** Scheme of performance of EIS biosensing, incubation of antibodies with samples, and specific recognition of antigens by interacting antibodies.



**Figure 2.** Nyquist plots related to the incubation of different concentrations of *L. monocytogenes* on the sensing layer with antibodies against the bacterium. The higher the concentration used, the largest is the diameter of the curve. In the case of unspecific incubation of the sensing layer with *S. enterica* (green line) a slight increase in impedance, compared with antibody layer, was observed.



**Figure 3.** Nyquist spectra collected for milk spiked samples in presence of different concentrations of *L. monocytogenes*. Negative controls included milk matrix and *S. enterica* in milk.

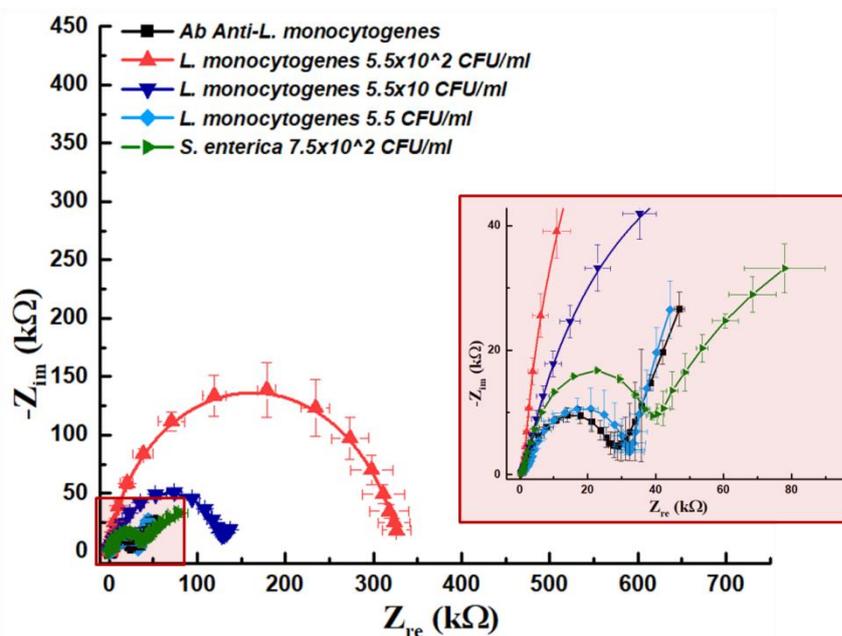


Figure 4. Nyquist Spectra derived from culture medium samples with different concentrations of *L. monocytogenes*

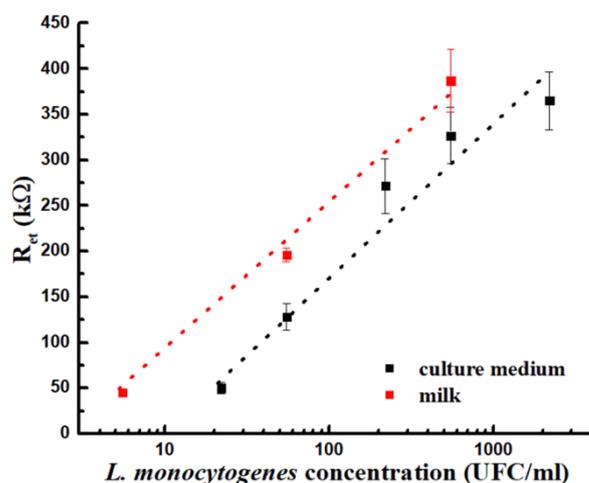


Figure 5. Calibration curves for culture medium (black) and milk (red) spiked samples.

#### 4. Discussion

Food contamination is an eventuality which may happen in critical points of the food supply chain and *Listeria monocytogenes* is one of the most threatening factors of contamination. In order to promptly identify such events, tools for the *on-field* analysis of dairy products with features of low-costs, portability and ease of use are mandatory. In this study, a biosensing platform was realised for the sensitive and specific *on-chip* detection of *L. monocytogenes* both in medium culture (reaching a limit of detection of 22 CFU/ml) and in milk matrix, reaching in this case a lower limit of detection, established as 5.5 CFU/ml, with negligible effects from matrix and unspecific pathogen detection (using *S. enterica* as negative control).

The presence of a matrix introduces a new variable, that in antibody based biosensors consists in aspecific binding of proteins and other solutes to the electrodes. In this study, the good performance of the platform tested with the food matrix could be attributed to intrinsic features of the sample. In

the case of milk, indeed, casein acts as a natural passivation agent[23] and in laboratory protocols of sensing methods it is often added to improve the sensibility of the assay[24]. These characteristics in this optimized assay allowed to match the law's imposed limits of presence for *L. monocytogenes* in foods, fixed at 100 CFU/g of food. However, ready-to-eat (RTE) foods and meats pose serious problems even with a minimum presence of the pathogen. For this reason, US safety agencies adopt different methods than EU and zero tolerance for *L. monocytogenes* in RTE products[10,11].

Other types of substrates used for electrode fabrication have been based on degenerate (highly doped) Si: the doped Si has very weak electrical conductivity, and prevents the formation of a space charge layer during AC interrogation of the sensor interface. Radhakrishnan and Suni presented results demonstrating the possibility to regenerate the Si electrode during experiments during a 30-days storage using KSCN based solution[25,26]. The result showed the possible application and reusability of antibody-coated degenerate Si electrodes for EIS measurements, just performing the calibration on the day of use of electrodes.

Further advancements are envisaged through the application of superior surface modification methods, such as the use of bidentate thiol 16-[3,5-bis(mercaptomethyl)phenoxy]-hexadecanoic acid (BMPHA), the application of proper solutions to stabilize the capture ligands, and the application of AC electrokinetic (ACEK) microfluidics platforms and ACEK microflow mechanisms, such as AC electroosmosis (ACEO) and AC electrothermal (ACET) effects. Furthermore, an increase in the limit of detection may be achieved by combining the biosensor methods with immunoseparation of bacteria from larger volumes[27,28].

Several challenges and obstacles remain to be solved before their use in on-site detection. EIS sensor analysis needs operator skills in data processing. Sample preparation must be simple and performable on site. EIS methods have been often applied successfully to detect pathogens in real samples, such as plant extracts and sera[29,30].

EIS sensing platforms are an appealing device for food industry and health officers: the immediate detection of pathogens[31,32] may allow a quick set up of countermeasures for protection of humans, plants and environment. In a recent study, we used the same anti-*Listeria* antibodies, and obtained reproducible results on the specificity of antibodies[19]. In addition, a protein chip was set up to discriminate five different species of pathogens on the same surface, i.e. *L. monocytogenes*, *S. aureus*, *E. coli*, *Salmonella* spp. and *Campylobacter* spp.[31]. In that setting, the five pathogen-specific antibodies showed no binding to any of the other pathogens, so that specificity was high. Using the arrayed antibodies, Limit of Detection (LOD) of protein chips for *L. monocytogenes* was 2.55 CFU/mL, even in presence of other bacterial species. In that study, the bacteria were from a 24 hour enrichment culture, after immunoseparation from 50 ml or higher volumes using a Pathatrix instrument (Life Technologies). Finally, validation assays remain to be performed, to show the robustness of detection, reproducibility and variability in multi-site installations.

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