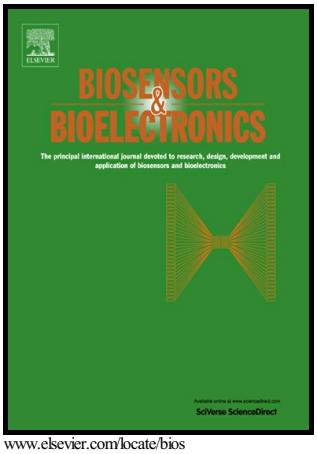

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Micro-nano-bio acoustic system for the detection of foodborne pathogens in real samples

George Papadakis[1*], Pavla Murasova[3], Audrey Hamiot[4], Katerina Tsougeni[5], Georgia Kaprou[2,5], Michael Eck[6], David Rabus[7], Zuzana Bilkova[3], Bruno Dupuy[4], Gerhard Jobst[6], Angeliki Tserepi[5], Evangelos Gogolides[5], Electra Gizeli[1,2*]

[1]Institute of Molecular Biology and Biotechnology-FORTH, 100 N. Plastira Str, Heraklion, 70013, Greece
[2]Department of Biology, Univ. of Crete, Voutes, Heraklion, 70013, Greece
[3]University of Pardubice, 573 Studentska, Pardubice 532 10, Czech Republic
[4]Institut Pasteur, 25-28 Rue du Docteur Roux, Paris Cedex 15, 75724 France
[5]Institute of Nanoscience and Nanotechnology, NCSR-Demokritos, Patriarhou Gregoriou & 27 Neapoleos Str., Aghia Paraskevi 15310, Greece
[6]Jobst Technologies GmbH, Engesserstraße 4b, 79108, Freiburg, Germany
[7]SENSeOR SAS, 18Temis Innovation, 18 rue Alain Savary, 25000 Besançon, France

gpapadak@imbb.forth.gr
gizeli@imbb.forth.gr

[*]**Corresponding authors.**

ABSTRACT

The fast and efficient detection of foodborne pathogens is a societal priority, given the large number of food-poisoning outbreaks, and a scientific and technological challenge, given the need to detect as little as 1 viable cell in 25 gr of food. Here, we present the first approach that achieves the above goal, thanks to the use of a micro/nano-technology and the detection capability of acoustic wave sensors. Starting from 1 *Salmonella* cell in 25 ml of milk, we employ immuno-magnetic beads to capture cells after only 3 h of pre-enrichment and subsequently demonstrate efficient DNA amplification using the Loop Mediated Isothermal Amplification method (LAMP) and acoustic detection in an integrated platform, within an additional ½ h. The demonstrated 4 h sample-to-analysis time comes as a huge improvement to the current need of few days to obtain the same result. In addition, the work presents the first reported Lab-on-Chip platform that comprises an acoustic device as the sensing element, exhibiting impressive analytical features, namely, an acoustic limit of detection of 2 cells/μl or 3 aM of the DNA target and ability to detect in a label-free manner dsDNA amplicons in impure samples. The use of food samples together with the incorporation of the necessary pre-enrichment step and ability for multiple analysis with an internal control, make the proposed methodology highly relevant to real-world applications. Moreover, the work suggests that acoustic wave devices can be used as an attractive alternative to electrochemical sensors in integrated platforms for applications in food safety and the point-of-care diagnostics.

Keywords: Lab-on-a-chip, molecular diagnostics, acoustic biosensor, *Salmonella* detection

1. Introduction

The application of micro-nano/technologies in the life sciences has brought a paradigmatic change in the area of molecular diagnostics and the analysis of genetic biomarkers, especially when combined with a Lab-on-Chip (LOC) platform for target analyte detection.(Jain, 2003; Rosi, 2005) Ultra-sensitive, label-free, fast and integrated systems employing a biosensor as the detection platform have been developed for the analysis of a variety of nucleic acid analytes, including cancer biomarkers such as circulating tumor DNA(Das, 2016), pathogens, such as Salmonella(Cinti, 2017), Escherichia coli and Staphylococcus aureus(Ahmed, 2013; Safavieh, 2014), and infectious diseases, such as influenza virus(Ferguson, 2011), Mycobacterium tuberculosis and Klebsiella pneumonia(Luo, 2014). One of the critical challenges in the development of point-of-care (POC) integrated systems is the sample preparation which is normally required prior to amplification and detection. With the exception of few works(Das, 2015; Ferguson, 2011; Patterson, 2013a), the majority of the reported LOC systems employ synthetic or bench-top pu-

rified genetic material spiked in the working solution, thus, avoiding steps such as cell lysis and nucleic acid extraction. In addition, the vast majority of the LOC reported applications employ electrochemical sensors for analyte detection, normally combined with the hybridization of the amplified target to a surface-bound probe(Hsieh, 2015; Labib, 2016; Patterson, 2013b; Sage, 2014).

Food safety is one of the current societal priorities given the very large number of food-poisoning outbreaks and incidences that occur annually worldwide. Today, culture colony counting is the standard method used for food-pathogen detection. While the big asset of the microbiological method is its high sensitivity and low cost, the main disadvantage is that it suffers from laborious and time consuming protocols, requiring several days (2-5) per pathogen. Alternative molecular diagnostics methods such as PCR and immune-tests have also been developed. These methods take 13-16 h for analysis, are also laborious and require trained personnel for operation. The development of new methodologies advancing the state of the art in foodborne pathogen detection is a challenge for scientists and technologists for two reasons. The first is related to the high complexity of food samples(Mortari, 2014), i.e., a matrix containing high concentrations of nutrients and cells (for example, milk contains 3.5% fat, 3.5% proteins, 5% carbohydrates and 104-105 cells/mL), as opposed to urine or serum. The second concerns the legislation worldwide which requires the "absence of bacteria" in food quantities as large as 25 gr(Cinti, 2017). The latter requirement imposes the necessity for a pre-enrichment step for the microbial growth of viable cells. Moreover, for the development of integrated platforms, the 25 gr (or ml) of sample is a huge amount for miniaturized microfluidic channels, having typical volumes of 10-200 μl. For these reasons, although integrated platforms have been used extensively for applications related to human diseases, they have not been applied to food analysis yet, at least for real life applications starting from 25 gr (ml) of the food sample. Indeed, the pre-enrichment step included in some works, combined with PCR and micro-sensor based DNA detection, was performed in a modular rather than integrated way (Khemthongcharoen, 2015).

In this work, we present for the first time a micro-nano-bio acoustic system that overcomes the existing barriers for the application of microsystems and integrated platforms to food quality control and provides the means for ultra-fast, sensitive and cost-effective analysis, always within the existing legislation framework. The system takes advantage of magnetic immunobeads for capturing bacteria from milk and a LOC platform for the isothermal amplification of target-DNA in a microfluidic chamber followed by detection with a surface acoustic wave (SAW) sensor. Acoustic sensors are an attractive alternative to electrochemical devices, since they also offer label-free detection, have a planar geometry which can be easily integrated with microfluidics, and can be produced in extremely competitive prices since they are used as components in mobile phones(Bo, 2015). In addition, it has been shown that acoustic wave devices are sensitive to the conformation(Mateos-Gil, 2016; Papadakis, 2010, 2012; Tsortos, 2008, 2016) and way of attachment(Milioni, 2017; Wiseman, 2012) of surface-bound molecules; this novel concept allows the design of highly specific genetic assays for DNA-amplicons of a pre-defined geometry(Papadakis, 2013) and/or a distinct way of binding (flat versus floating)(Milioni, 2017). Here, acoustic detection is combined with the Loop Mediated Isothermal Amplification (LAMP) method in a LOC and shown to be able to successfully detect as little as 1 *Salmonella* cell in 25 ml of milk within only 4 hours of total analysis time. An overview of the complete procedure is depicted in Figure 1.

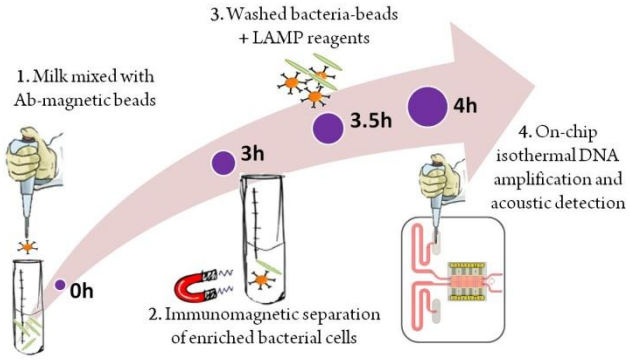

Fig 1. The complete procedure for the detection of food pathogens comprises the following steps: (1) 25 ml of milk are mixed with 1 mg of magnetic particles functionalized with antibodies and incubated at 37°C for 3 hours; (2) and (3) Enriched bacterial cells are captured by an external magnet, washed with buffer and resuspended in 25 μl of a solution containing Triton-X 100 for lysis and the LAMP ingredients; and, (4) The 25 μl is injected in the LOC where isothermal DNA amplification is performed at ~65°C for 30 min followed by rapid acoustic detection (~60 sec) using a SAW sensor. Results can be presented as a "Yes" or "No", or as a real-time graph.

2. Materials and Methods

2.1 Reagents

The sodium salt of N-hydroxysulfosuccinimide (sulfo-NHS), *1*-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 2-(*N*-morpholino)ethanesulfonic acid (MES), Tween 20, were purchased from Sigma Aldrich (St. Louis, MO, USA). Polyclonal antibodies: goat anti-Salmonella sp. Ab were from KPL (Gaithersburg, MD, USA). ProMag™ 1 Series COOH surfactant free magnetic beads (0.8, 1.0 and 3.0 μm) were from Bangs Laboratories, Inc. (Fishers, IN, USA). All others chemicals were reagent grade and obtained from Penta (Chrudim, the Czech Republic). Water used for preparation of buffers was filtered through a TKA Smart2Pure system (Thermo Scientific TKA, Niederelbert, Germany). Whole UHT milk (fat content 3.5%) were bought in a common grocery. PLL(25)-g-PEG(2) was purchased from Nanocs Inc (New York, USA).

2.2 Growth Cell Protocol and Numeration

All experiments have been performed with an attenuated strain of *Salmonella enterica* serovar Thyphimirium. *Salmonella* was grown overnight in Luria-Bertani (LB) medium; cultures were subsequently measured spectrophotometrically (OD_{600}) and adjusted at OD_{600}:1 corresponding to a cell concentration of 1-1,5x10^9 CFU/ml. Serial dilution of the above cells suspension by LB was carried out to reach a concentration of 100 CFU/ml; the latter was used to spike 25 ml of milk with 1-25 CFUs. The spiked milk was incubated under agitation at 37^{o}C and the growth was followed every hour by plating 2x100 of the culture on LB agar plate from 3 to 10 hours. Plates were incubated overnight before numeration. Three independent cultures were numerated for each time growth.

2.3 Preparation of Magnetic Immunosorbent

Magnetic immunosorbent was prepared as described previously (Srbova, 2018). Briefly, goat anti-*Salmonella* sp. antibodies KPL were immobilized on the magnetic particles following a 2 step carbodiimide reaction; 1 mg of particles was washed with 0.1M MES pH 5 and then pre-activated with EDC (7.5 mg in 0.25 mL of 0.1M MES pH 5) in combination with sulfo-NHS (1.25 mg in 0.25 mL 0.1M MES pH 5) in a total volume of 1 mL. After 10 min of incubation the magnetic carriers were washed with 1 mL of 0.1M MES pH 5 and then incubated with 25 μg of the anti-*Salmonella* Ab overnight and at 4°C. Functionalized particles were washed with 0.1M MES pH 5 (x2), 0.1M MES pH 5 with 1M NaCl (x2) and 0.1M MES pH 5 (x5). The capturing efficiency of *S.* Typhimurium was characterized and found to be satisfactory even in the presence of an excess of *E. coli* bacteria (Srbova, 2018).

2.4 Immunomagnetic Separation of Salmonella in Milk

1 mg of immunosorbent was mixed with 25 ml of whole UHT milk spiked with 1- 25 CFU of *Salmonella*. After incubation for 2.5, 3.0 and 3.5 h at 37°C under shaking, the particle-bacteria complexes were placed on a magnetic separator for 20 minutes, followed by washing with 1 ml of PBS buffer containing 0.05% Tween 20 (PBS-T) (x2). For verifying the capture efficiency, magnetically separated particles were resuspended in 100 μL of the same buffer and plated on nutrient agar. After cultivation for 18–24 h at 37° C, bacteria enumeration took place.

2.5 Fabrication of SAW Array

A SAW array (16x16 mm) comprising a 4-acoustic channel design was fabricated on a ST quartz (YXI/36^o) and subsequently fully characterized as described before(Papadakis, 2017a); briefly, each sensor included patterned 150 nm thick aluminium electrodes followed by a split-finger geometry resulting in a 8 μm electrode period and a 32 μm acoustic wave length, i.e., an operating frequency of 155 MHz. A waveguiding layer was formed by spin coating a S1813 photoresist (Shipley, U.S.) guiding layer over the entire array. A follow up photolithographic step was included for opening the electrical pad access after exposure to UV light. Typical insertion losses of the fabricated devices in air were in the 25 dB range.

2.6 Fabrication of the Microfluidic Card and Docking station

The microfluidic cards were created from 75μm thick polyolefin foils structured by a CNC cutter and heat-laminated to a 6-layer stack in the micro-fluidics rapid-layer-manufacture service of Jobst Technologies GmbH, Germany. Delta-shaped ultra-narrow low loss seals to the SAW chip of <100μm seal width were created from MED-6215 silicone of NuSil Technology LLC, USA and directly attached to the cards using a precision milled PMMA mold. The docking station was created by precision CNC machining of polycarbonate parts and PCBs, reactive molding of seals from MED-6215, components soldering for contact to SAW chip and heaters, and final assembly. The docking station also houses a 5x5.5 cm2 printed circuit board (0.8 mm thick) where two resistive microheaters were fabricated to provide (through Joule heating) the temperature necessary for DNA amplification(Moschou, 2014). The microheaters were patterned in the inner (18 μm thick) copper layer of the PCB(Kaprou, 2016). The copper tracks, 200 μm wide, were designed meandering-like to provide an electrical resistance of approximately 40 Ohm. In order to maximize the temperature uniformity across the heating zone, an external copper layer (18 μm thick) of the PCB was used, and during LOC operation was brought into thermal contact with the microfluidic card containing the microchambers for LAMP-based DNA amplification. The designed microheaters were industrially realized at PCB manufacturers, and to provide the desired temperature during operation, they are connected to a temperature controller unit.

2.7 LAMP amplification protocol

Bacterial cells resuspended in PBS buffer were lysed for 10 min with 0.01% Triton-X 100. The *Salmonella* invasion gene invA was targeted by a set of six primers(Chen, 2011), two outer (F3 and B3), two inner (FIP and BIP) and two loop (Loop-F and Loop-B). F3: CGGCCCGATTTTCTCTGG, B3: CGGCAATAGCGTCA-CCTT, FIP: GCGCGGCATCCGCATCAATATGCCCGGTAAACAGATG-AGT, BIP: GCGAACGGCGAAGCGTACTGTCGCACCGTCAA-AGGAAC, Loop-F: GGCCTTCAAATC-GGCATCAAT, Loop-B: GAAAGGGAAAGCCAGCTTTACG. The LAMP reagent mix in a total volume of 25 μl contained 12.5 μl WarmStart 2x Master Mix (New England BioLabs), 1.8 μM FIP and BIP, 0.1 μM F3 and B3, 0.4 μM Loop-F and Loop-B, and 1ul lysed cells in PBS. The minimum required time for successful DNA amplification from ~50 cells was 20 min at 63^{o}C. LAMP products were analyzed using electrophoresis on a 2% agarose gel containing GelRed^{TM} (Biotium) and visualized under UV light.

2.8 Acoustic detection protocol

Surface functionalization was performed by injecting 90 µl of PLL-g-PEG (0.1 mg/mL) in 10 mM Tris pH 7.5 followed by buffer rinsing. The LAMP reactions were subsequently injected and rinsed with buffer. All steps were performed at a flow rate of 20 µl/min. The surface of the sensor could be cleaned several times by rubbing gently with a cotton stick. Real time amplitude and phase changes were monitored using an Agilent Network Analyzer or the electronic measuring system (SAW reader) developed by SENSeOR(Rabus, 2013).

3. Results and Discussion

3.1 Optimizing Pre-Enrichment and Cell Capture with Magnetic-Beads

Our initial aim was to assess the minimum time needed for pre-enrichment in order to comply with the need to grow and detect even one viable cell if present in the sample but, also, have in the end enough bacteria for subsequent LOC amplification and detection. We challenged the traditional foodborne pathogen bacteria pre-enrichment method which includes, first, growth in a non-selective medium followed by a selective enrichment step in order to enhance the number of target cells versus competitor microorganisms. Starting from 1-25 cells in 25 ml of milk, we performed different pre-enrichment times varying from 3 to 10 h at 37 °C without adding any growth medium (selective or non-selective). Around three hours is the minimum time required for cell growth given that cells normally go through an initial lag phase of 2 h before they start growing. From the growth kinetics, (Fig. 2/a) it was observed that around 500 CFUs were reached after 3 h of culturing and approximately twice as many within 4.5 hours. In a following step, magnetic beads functionalized with polyclonal anti-*Salmonella* Typhymurium antibodies were added in milk cultures spiked with *Salmonella* cells. In search of the optimum conditions for bacteria cells capturing, the following parameters were taken into account: size of nano-particles (0.8, 1.0 and 3.0 µm), amount of particles (1.0, 0.5 and 0.2 mg) added in 25 ml of milk, time at which beads were added in the sample (at the beginning or end of the culture period) and cross-reactivity with other cells. Immuno-ProMAG beads (0.88 µm; 1 mg) were selected due to their uniform size, good colloidal stability in food matrices and excellent specificity, even in the presence of an excess of *E. coli* cells(Srbova, 2017). Moreover, it was repeatedly confirmed that bacteria cells were able to grow and divide even when they were captured on the surface of the beads. For this reason, samples containing immunobeads from the start of incubation were cultured for 2.5, 3.0 and 3.5 h and then put into contact with a magnet for 20 min. Re-suspended beads were plated and colonies enumeration showed an average of 85% capturing efficiency for all growth times tested (Fig. 2/b).

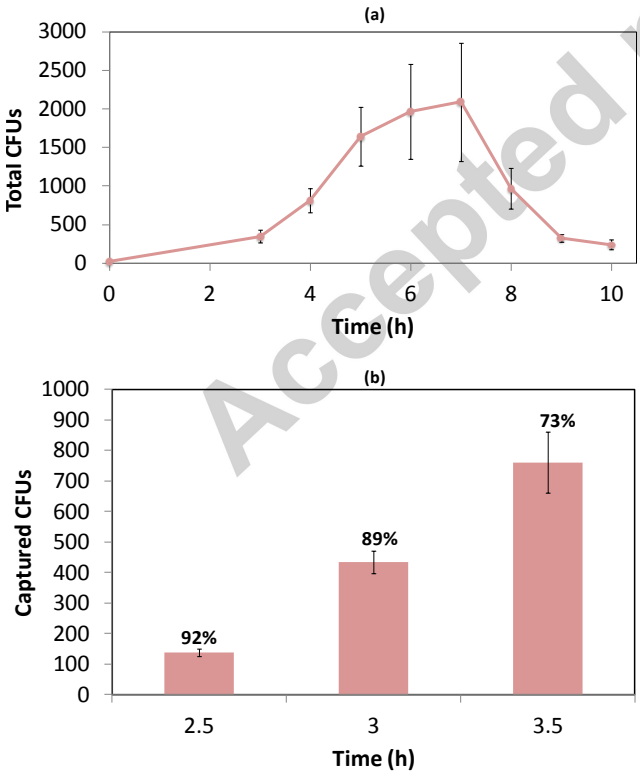

Fig 2. (a) *Salmonella* growth curves obtained directly in 25 ml of milk after incubation at 37°C for up to 10 h; note that after 6 h of incubation *Salmonella* cells stop growing and are lysed. (b) Plating results showing the absolute number of *Salmonella* bacteria extracted with the magnetic immunobeads as a function of the culture time starting from ~1 cell in 25 ml of milk; the capturing efficiency corresponds to N_c / N_o x 100, where N_c is an average total number of cells captured with the immunosorbent (CFU/mL) and N_o is an average total number of cells counted after plat-

ing of the original cell suspension (growing without beads) (CFU/mL). In all cases, immunobeads were added at the beginning of the enrichment step.

3.2 LAMP Amplification Inside Cell Lysates and Acoustic Detection

Based on the previous observations, it was estimated that for a minimum pre-enrichment step of 3h, approximately 400 bacterial cells would be captured for further injection in the LOC system. The ability of the LAMP method to specifically and rapidly amplify the *Salmonella* invasion gene target (*invA*) from whole cell lysates was investigated. Given the fact that thermal lysis was not possible due to the instability of Bst polymerase at elevated temperatures, Triton X-100, a nonionic detergent commonly used during DNA extraction, was employed for chemical lysis. After performing several tests, it was concluded that a final concentration of 0.004% Triton X-100 within the LAMP cocktail was sufficient to lyse up to 10^5 cells within 10 min (SI, Fig. S1). Moreover, acoustic experiments verified that this concentration of the detergent did not affect the surface or signal of the biosensor during the measurement. Cells could also be lysed separately outside the LAMP cocktail with 0.1% - 1% Triton X-100 within 5 min but, in this case, acoustic measurements could not be performed due to the high concentration of the detergent. Following cell lysis within the LAMP cocktail, amplification was performed and found to be efficient and capable of amplifying DNA within 15 min directly from 10 CFUs and without prior DNA extraction (SI, Fig. S2).

The capability of the LAMP for rapid amplification in crude samples was subsequently combined with the detection capability of a 155 MHz acoustic wave device based on a Love wave configuration(Rasmusson, 2001); the latter is a waveguide geometry which, in this case, comprised a thin (1.2 μm) photoresist (S1813) layer deposited on the surface of a shear horizontal SAW device. The sensor surface was pre-functionalized with a PLL-g-PEG copolymer which can be physically adsorbed onto bare gold as well as other polymeric materials (such as photoresist) at physiological pH(Papadakis, 2017b). This co-polymer has also been shown to allow the selective immobilization and simultaneous acoustic detection of DNA molecules in the presence of other proteins or cell debris as is the case with bacteria-cell lysates. We performed acoustic measurements with LAMP amplicons produced off-chip and after chemical lysis of 10^5 *Salmonella* cells in 25 μl of the LAMP cocktail. Acoustic changes monitored through a network analyzer showed that amplified DNA could be successfully detected directly from the complex mixture, without pre- or post-amplification purification steps. It was also observed that changes in the acoustic wave energy, here recorded as amplitude (Fig. 3/a), were more sensitive than phase measurements (Fig. 3/b) and could be used to differentiate positive from negative samples. This difference results from the fact that dsDNA amplicons, found to adsorb loosely and with a flat configuration on the PLL-g-PEG surface (Fig. 3/c), are more dissipative than the non-specific components present in the solution(Papadakis, 2017b). Indeed, while the latter also bind to the surface as indicated by the high mass-sensitive phase signal, these molecules adsorb directly on the polymer surface and in a quite tight manner (Fig. 3/d), resulting in a distinguishable lower energy dissipation signal.

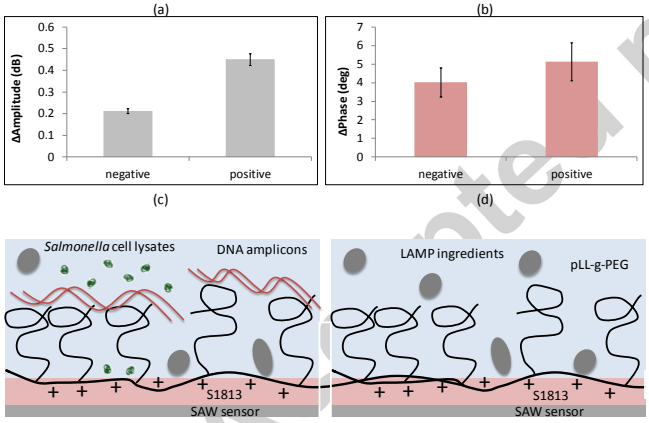

Fig 3. (a) and (b): Amplitude and phase acoustic signals obtained during the detection of dsDNA amplicons produced during LAMP of 10^5 *Salmonella* cells (positive) and samples containing no cells (negative) using an acoustic waveguide device functionalized with PLL(25)-g-PEG(2); (c) schematic representation of the device surface upon addition of a positive sample that contains ds DNA amplicons, cell lysates and LAMP ingredients; and, (d) as before but for a negative sample, i.e., only the LAMP ingredients.

In order to verify that the acoustic detection is possible even after a short (3h) pre-culturing step, we performed the whole process of bacterial growth and capturing, DNA amplification and acoustic detection and managed to detect 5 starting CFUs in 25 ml of milk within 4h in total. When we tested various numbers of cells in the range of 150-1000 CFUs (according to plate enumeration), we found that the change in the amplitude for the positive samples was 0.361±0.029 dB while for the negative samples 0.207±0.039 dB. It is apparent, that implementing a very short pre-enrichment step is feasible due to the high capturing efficiency of magnetic beads in combination with the efficiency of the LAMP method with whole cell lysates and the impressive performance of the acoustic DNA detection platform in complex samples.

3.3 Efficiency of Amplification and Acoustic Detection in the docking station

For the development of a rapid detection assay of foodborne pathogens, the DNA amplification and detection steps were integrated into a LOC system for automatic operation. The ability of a SAW device to operate when the surface is in contact with a 4-channel microfluidic chip has been demonstrated in the past(Mitsakakis, 2008, 2011). In this work, a disposable plastic micro-

fluidic card (Fig. 4/a) was fabricated where two DNA amplification processes could take place inside two micro-chambers; the same card could also lead the liquid from each amplification chamber over the surface of the 4 SAW sensors array (S1-S4). The good quality of the acoustic signal, a prerequisite for the development of a reliable and reproducible LOC system, was ensured after extensive optimization of the geometry of the plastic card/SAW contact area and type of material used for the gasket(Papadakis, 2017a). A docking station for the micro-fluidic cards with integrated resistive microheaters (Fig. 4/b) was created by precision CNC machining. In order to avoid the possibility of contamination of the LOC, the fluidic inlets were introduced on the disposable card so that liquid was loaded directly using a pipette tip. Finally, a portable unit comprising the SAW sensor reader and control units for driving the fluidics (micro-pump) and temperature controller (micro-heaters) was developed(Rabus, 2013) together with a Graphical User Interface (GUI).

The efficiency of LAMP amplification inside the microfluidic chambers was first tested by using cards made of three different materials, i.e., polycarbonate (PC), poly(methyl)methacrylte (PMMA) and polypropylene (PP), and compared to the LAMP efficiency obtained inside a standard microcentrifuge tube in the thermocycler. In all three materials, the amplification was successful and the efficiency was approximately 87±7% of the respective one inside the tube. The difference of 13±7% is probably due to the bigger surface-to-volume ratio of the meander-like microchambers, the lack of uniform heating since the resistive microheaters were located only below the chip and a small degree of evaporation that was evident.

Furthermore, the acoustic response of the biosensor when in contact with the microfluidic card inside the docking station was evaluated with respect to the acoustic detection of LAMP amplicons produced without pre- or post- purification on the PLL-g-PEG functionalized surface. Through the GUI of the system, it was possible to monitor in real-time the injection of the completed amplification reaction over the 4 sensors in a sequential manner. The injection was performed with 20 μl/min flow rate that corresponds to a 60 sec end-point detection step (Fig. 4c). In this way, four measurements could be recorded for each sample which significantly minimized the risk of false results or failed measurements. For all the experiments, amplitude change was preferentially monitored as the most sensitive signal for DNA detection. In addition, for comparison, the amplitude signal changes of positive samples under examination were normalized based on the respective changes of negative samples; i.e., without bacteria in the amplification mixture. The normalized signals shown in Fig. 5, thus, indicate the ratio of positive amplitude value (dB)/average negative reference value (dB).

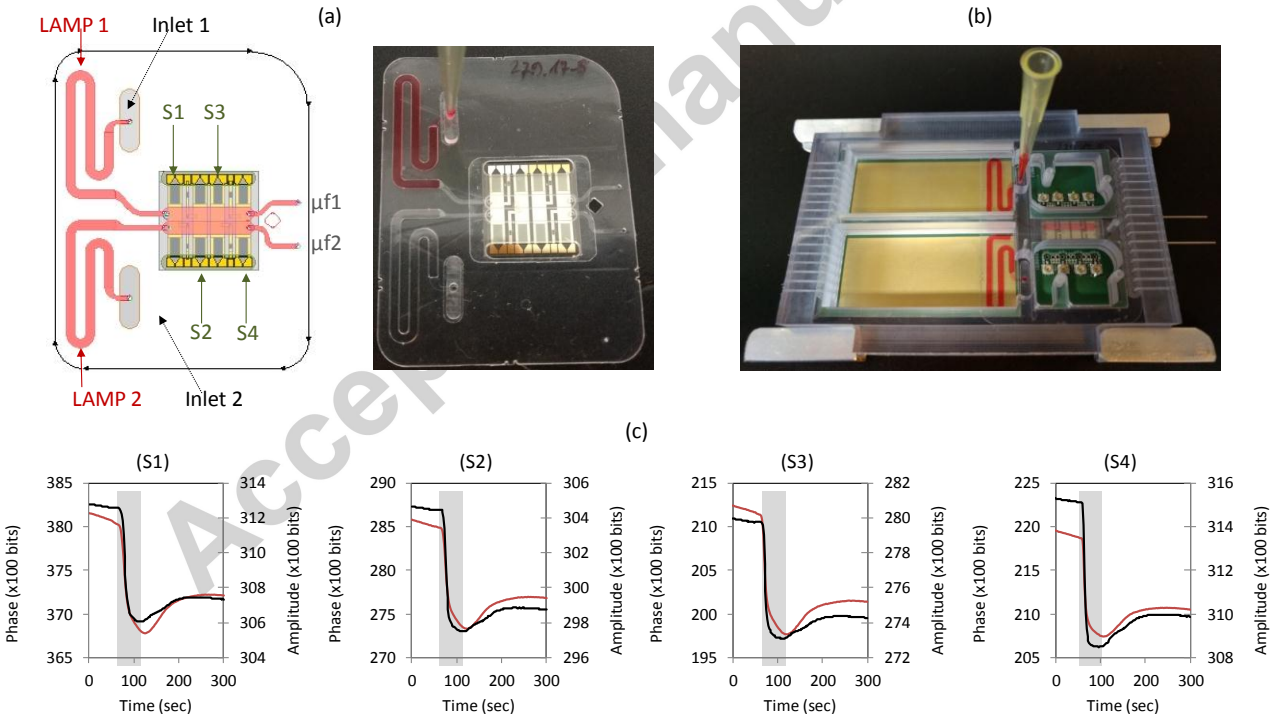

Fig 4. (a) Left: Schematic drawing of the disposable plastic card with two isothermal DNA amplification micro-chambers; each LAMP comprises a 55.5 mm long x 1.5 mm wide and 0.3 mm high microfluidic chamber of 25 μl with one inlet for sample injection and an extended path at the other end that flows over the 4-sensors of the acoustic biochip (μf1 for LAMP 1 and μf2 for LAMP 2). The second LAMP compartment can be used for testing either a second sample or as a control (LAMP reagents). Right: Photograph of the plastic card on top of the SAW biochip; (b) Photograph of the docking station comprising one acoustic biochip and the plastic card with the 2 amplification chambers. The plastic card lies above a PCB, where a microheater is fabricated for on-chip heating. The empty (yellow) space on the left can be used to house another biochip with 2 extra LAMP chambers for the eventual detection of 4 samples per docking station, or three samples and one control. (c) Real-time binding curves of LAMP reactions flowing sequentially over the 4 sensors (S1 to S4) of the acoustic device through μf1 (see Fig. 4/a). The red and black lines correspond to changes in phase and amplitude, respectively. Both phase and amplitude are measured in bits (arbitrary units). The shadowed part corresponds to the time interval during sample flow over the surface, in between buffer flow.

For the evaluation of the acoustic response, LAMP reactions performed in a thermocycler for 15 or 30 min with Bst2.0 warm start (ws) polymerase were compared. A high number of *Salmonella* cells (10^5) was initially spiked in the 25 μl of the reaction. The normalized amplitude change showed that in 30 min the response was higher than in 15 min (Fig. 5/a). By keeping a constant 30 min amplification time we also measured decreasing starting numbers of bacteria in the reaction down to 10^3 CFUs (Fig. 5/b). For 10^4 and 10^5 CFUs the signal was very close, indicating a saturation of the surface by the produced amplicons. For 10^3 CFUs there was a decrease but still significantly higher (at least double) from the baseline (Fig. 5/b). To investigate further the limit of detection of the acoustic sensor in the LOC by using the new portable SAW reader, we performed a time comparison assay with 10^3 CFUs, this time using the Bst2.0(ws) kit. Since 10 additional minutes of amplification (20 vs 30 min) resulted in significantly higher acoustic signal (Fig . 5/c), we subsequently incubated reactions for 30 min containing 50, 10^2 and 10^3 CFUs (Fig. 5/d). Acoustic data showed a decrease in the signal with decreasing number of cells and 50 cells were considered as the limit of detection. Between 10^2 and 10^3 CFUs there was no significant difference indicating, again, that LAMP amplicons reached saturation after 30 min of incubation.

3.4 Integrated Amplification and Acoustic Detection in the LOC

After completing the evaluation of the acoustic response inside the LOC but with off-chip amplification reactions, the integrated LAMP amplification and acoustic detection was performed to estimate the total detection efficiency of the system. For this purpose, 10^3 *Salmonella* whole cells were mixed with the lysis/LAMP cocktail, as described previously.

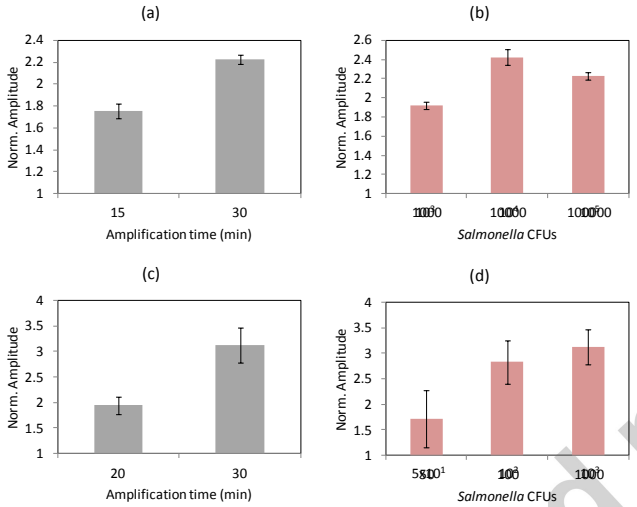

Fig 5. Charts of acoustic response with LAMP reactions performed in 25 μl and for: (a) 15 and 30 min containing10^5 cells, (b) 30 min with 10^3, 10^4 and 10^5 cells. (c) 20 and 30 min with 10^3 cells, (d) 30 min with 50, 10^2 and 10^3 cells. Measurements in the top charts were performed with Bst2.0(ws) polymerase while in the bottom charts with a Bst2.0(ws) LAMP kit.

The mixture was loaded in the system and the procedure was completed within 30 min, including amplification and acoustic detection. The normalized acoustic signal of 19 measurements is depicted in Fig. 6. Based on the presented data, positive reactions in the LOC gave a signal that on average was 2.1±0.6 times above the control baseline. The previously measured signal when LAMP was performed outside the LOC was 3.1±0.3 times higher. This difference, as well as the greater variation (±0.6) in the measurements observed within the LOC can be attributed to the reduced amplification efficiency in the microfluidic chips described previously.

However, even with this lower amplification efficiency, the signal is still twice the one observed with the negative samples and can be used to detect reliably the presence of even 50-100 cells in 30 min according to the results presented in Fig. 5/d.

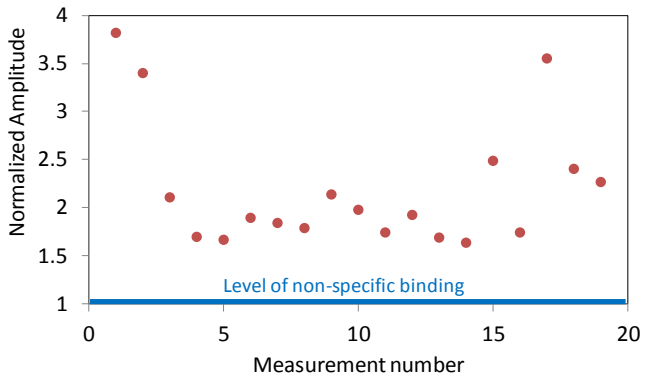

Fig 6. Normalized amplitude changes for the detection of 10^3 whole *Salmonella* cells with the LOC system. Each red point represents one of the 19 acoustic measurements.

Conclusions

The current work demonstrates the first reported LOC platform that employs an acoustic wave device as the detection module. While the combined procedures of amplification and acoustic detection have been demonstrated in applications using PCR or other isothermal DNA amplification methods along with the SAW(Kordas, 2016) or QCM(Grammostianou, 2017; Ittarat, 2013; Jearanaikoon, 2016; Karamollaoglu, 2009; Prakrankamanant, 2013) devices, never before has the integration of the two been achieved in a microfluidic channel and inside a LOC system. Obstacles, regarding the efficient operation of the acoustic chip when clamped inside a LOC as well as the ability/sensitivity of the acoustic signal when in contact with a complex medium, have been the reason for the lack of incorporation of acoustic devices in such platforms. While the solution to the first obstacle requires optimization of the design and material of the fluidics card(Papadakis, 2017a), the latter is more complex and cannot be addressed through the traditional use of acoustic devices as a mass-sensor. The novel biophysical approach applied here that exploits the hydrodynamic nature of acoustic detection(Milioni, 2017; Tsortos, 2015) is apparently a more powerful detection mode for molecular diagnostic. Moreover, the ability to discriminate the sample signature from "noise", allows the direct detection of dsDNA amplicons avoiding the cumbersome steps of denaturation and subsequent hybridization. Through acoustic energy measurement we have achieved an impressive limit of detection of DNA derived from 50 cells in 25 μl of the reaction sample, i.e. a LOD of 2 cells/μl or ~3 aM of DNA target; this was achieved in a crude sample and without the use of any purification step.

Moreover, we demonstrated a micro-nano-bio- acoustic methodology which enables the detection of *Salmonella* cells in milk. The successful combination of a 3 h pre-enrichment step in the presence of immunobeads of high capturing efficiency together with the integrated DNA-amplification and acoustic detection results in a sample-to-answer time of only 4h. This performance breaks not only the barrier of same-day results but also achieves analysis time within the same working shift. Using our methodology and integrated platform, we have achieved the detection of 1 cell in 25 ml of milk. While electrochemical systems have been used in the past for *Salmonella* analysis(Campuzano, 2017; Cinti, 2017; Ferguson, 2009; Hsieh, 2012; Li, 2012; Lubin, 2006; Patterson, 2013b; Yan, 2016) to the best of our knowledge this is the first time that a methodology employing a LOC platform is demonstrated with real food samples and by also including the necessary pre-enrichment step. The combination of the advantages of on-chip DNA amplification with the detection power of surface acoustic wave sensors paves the way to the creation of a new generation of products for rapid and label-free DNA analysis, not only for food safety but also for point-of-care diagnostics.

Supporting Information

Supplementary data associated with this article can be found in the online version

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HIGHLIGHTS

- First ever reported LOC system for foodborne pathogen analysis in a record time of 4 h
- First ever reported LOC system comprising an acoustic device as the sensing element
- Fully integrated DNA amplification and acoustic detection in unpurified samples
- Hybridization-free acoustic detection protocol, sensitive to the way of attachment of double stranded DNA
- Ability to distinguish between live and dead cells
- Holistic approach including the necessary bacteria pre-enrichment step in whole milk (here demonstrated to take only 3 instead of 13-36 hours)