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Detection of *Listeria innocua* by Acoustic Aptasensor

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Received: date; Accepted: date; Published: date

Abstract: Early detection of foodborne pathogens is significant for ensuring food safety. Nowadays, the detection of pathogens found in food can take up to 72 h and it might take a week to confirm a positive sample. While standardized methods give test results in a shorter period, the reoccurring costs for each measurement are high. Therefore, it is necessary to develop technology that will be low-cost, fast, simple and accurate enough. Biosensors in combination with nucleic acid aptamers offer such possibilities. This work is focused on the development and testing of a biosensor based on DNA aptamers for detection of pathogenic bacteria *Listeria innocua* using the method of multi-harmonic quartz crystal microbalances (QCM). The aptasensor was prepared on the surface of a piezo crystal, whose frequency was affected by deposited mass. An aptamer specific to the genus *Listeria* spp. was used for the detection of this pathogen, which includes 16 subspecies, out of which 3 are excluded as their antigen structure differs from other species (*L. murrayi*, *L. grayi*, *L. ivanovii*). We found that addition of the pathogens at the surface of QCM transducer modified by aptamers resulted in the decrease of the resonant frequency in concentration depending manner. We also confirmed the specificity of the aptamer used for *Listeria innocua*, as neglected response of the sensor took place for *E. coli* for which *Listeria* spp. has some partial antigens identical and thus can cause cross-reactions in serological tests. The developed aptasensor showed promising sensitivity and specificity for real-time detection of *Listeria innocua*, with a detection time of 30 min. The achieved limit of detection was approximately 1.6×10^3 CFU/mL.

Keywords: *Listeria innocua*; biosensor; QCM; multi-harmonic analysis; aptamer

1. Introduction

1.1. Bacteria and Detection Methods

Early pathogen detection in food products is an important aspect for prevention of diseases that originate from consumption of contaminated food and meals. Commonly used commercial methods are time-consuming and lack in effectiveness [1]. Except for typical methods, that use cultivation of living cells capable to form colonies, methods like real-time PCR (polymerase chain reaction), ELISA (Enzyme-Linked Immunosorbent Assay) or ELFA (Enzyme-Linked Fluorescent Assay) are being used. Their main disadvantage is a high cost for repeatable measurements and in case of the PCR, information is being produced also from dead cells. Therefore, there is demand on development of the assay, that would be cheap, fast, easy to control and precise. Biosensors combined with nucleic acid aptamers can offer such a possibility.

Acoustic biosensors are one of the most used sensors, as they provide real-time monitoring of affinity interactions at surfaces. QCM (quartz crystal microbalance) in combination with nucleic acid aptamers as receptors is an effective option for quantification of cell count as it has high specificity

and low limit of detection (LOD). This work reports the development of QCM aptasensor for the detection of foodborne pathogen—*Listeria innocua*.

Pathogens causing contamination of food product are bacteria, viruses, moulds, and parasites [2]. Common sources of pathogens in food that cause diseases are *Listeria monocytogenes*, *Escherichia coli O157:H7*, *Staphylococcus aureus*, *Salmonella enterica*, *Bacillus cereus, Vibrio* spp., *Campylobacter jejuni, Clostridium perfringens,* and Shiga-toxin-producing *Escherichia coli (STEC)* [2–4].

The majority of the diseases that originated from bacterial pathogens are caused by consumption of insufficiently heated or minimally processed meat (sausages, salami, meat conserves), milk products (spreads prepared from unpasteurized milk, ice-cream, butter, etc.) or fruit (apple cider, strawberries, cantaloupe, etc.). The biggest reservoir of pathogens is still poultry and other raw meat [5]. Poultry is problematic as pathogens are present not only on the surface of the meat but also inside the muscle tissue [6]. The serious problem represents also insufficiently heated seafood because the minimal infective dose of its pathogen is relatively low (10–1000 CFU/mL) [5]. Among them the most dangerous genus are *Vibrio*, *Listeria*, *Yersinia*, *Salmonella*, *Shigella*, *Clostridium*, *Campylobacter and Hepatitis A.*

1.2. Standard Methods of Bacteria Detection

Standard methods of bacteria detection are based on specific media that has the capability to isolate viable bacteria cells from food products. These methods are highly specific and can offer qualitative and quantitative information about microorganisms in food products [7]. Classic methods of bacteria detection include following basic steps: enrichment, selective enrichment, inoculation on selective agar, biochemical analysis, serologic confirmation [8]. Such a complex analysis is necessary for confirmation of correct pathogen identification [9]. Also, the preparation of cultivation media, agar inoculation and colony counting make this method rather time-consuming. Because these methods are based on the capability of microorganisms to reproduce themselves into visible colonies, this golden standard for pathogen identification can take up to several days [5].

Currently, the pathogen detection in the industrial food laboratories is based on the identification of genetic material (DNA, RNA) of microorganisms using PCR. The immunological methods such as ELISA and ELFA are also used as a standard (ISO norms). These methods have advantages of shorter detection time and the possibility to inspect a large number of samples. Despite high sensitivity (up to 10 CFU/mL), the main problem of these assays is relatively high cost as well as the necessity of qualified staff [10].

1.3. Biosensors and Their Application

Present consumer requirements demand effective detection methods that are of similar precision, but faster and cheaper than conventional methods. Biosensors offer such capabilities. Biosensor consists of bioreceptor—element sensitive on signal of biological origin, which relates to physico-chemical transducer. Transducer converts biological and chemical signal into electric, optic, or gravimetric signal and offers information about quantity/semiquantity of analyte [11,12]. Depending on the type of transducer optical, electrochemical, piezoelectric, and calorimetric biosensors can be distinguished [13]. To this day, various types of receptors were used in biosensor development, such as antibodies, enzymes, nucleic acid and peptide-based aptamers, bacteriophage, ssDNA/RNA probes etc. [14].

Basic element of piezoelectric biosensors is transducer—piezocrystal, that under mechanic strain generates electric potential [15]. In an inverted piezoelectric effect, the high-frequency voltage applied to the piezocrystal resulted in generation of deformation and acoustics waves. Several publications confirmed the effectiveness of QCM biosensor for detection of bacteria. Bayramoglu et al. [16] developed nanoparticle-mediated detection by QCM-aptasensor for *Brucella melitensis* in milk with LOD of 10^3 CFU/mL. In PhD. thesis of Yu QCM aptasensor for *E. coli* with LOD of 34 CFU/mL and with the detection time of 40 min was reported [17]. Such biosensor included application of RCA method (rolling cycle amplification) for production of multivalent, aptamer system for bacteria

detection. Wang et al. [18] developed QCM aptasensor for detection of *Salmonella typhimurium* with LOD of 10^3 CFU/mL and time of detection t = 60 min.

1.4. Aptamers and Their Application for Bacteria Detection

Aptamers are synthetic peptides or single-strand sequences of DNA/RNA oligonucleotides. They are produced by SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) [19,20]. In solution, the aptamers fold into complex 3D structures forming a binding site for their target [21]. Compared to commonly used antibodies, aptamer offer similar and even higher affinity and specificity to the target. They also offer more advantages, such as thermostability, absence of immunogenicity, low-cost and rapid production. They can be produced for wide spectrum of target such as small molecules, proteins, cells, bacteria, viruses, or tissues [21]. To this day, aptamers were used in variety of research as biorecognition element for detection of various types of bacteria [21,22], toxins [23,24], cancer cells [25], proteins [26] and various other targets [27].

However, only a few articles reported QCM biosensor for detection of *Listeria spp*., but none of them used aptamer for detection of this pathogen. Vaughan et al. [28] detected *Listeria monocytogenes* using antibodies immobilized on QCM surface with LOD of 10^7 CFU/mL. The main problem of this method was that antibodies were partially inhibited. The reason is that the antibodies are immobilized at the surface through amino-groups including those in a binding site of antigen-binding fragment (Fab). Therefore, a certain portion of binding site is blocked and cannot interact with the target. Sharma and Mutharasan [29] reported sensors based on piezoelectric cantilever, using antibodies for *Listeria monocytogenes*. They achieved LOD of 10^2 CFU/mL in milk. However, such sensitivity was possible only after tertiary binding of antibodies to amplify detected signal. Such detection setup was rather complicated and time-consuming. Similar approach was reported by Yu [21], for detection of *E. coli* O157:H7 with LOD 1.46×10^3 CFU/mL and time of response of 50 min. In this work, we are reporting aptasensor for detection of *Listeria innocua* using multi-harmonic QCM.

2. Materials and Methods

2.1. Chemicals

For the cultivation of bacteria and QCM measurements, following chemicals were used: PBS (Phosphate Saline Buffer, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl diluted in distilled water with final pH 7.4), deionized water MiliQ, ethanol, TSA-YE (Triptone Soyb Agar with yeast extract), TSB-YE (Tripton Soy Broth with yeast extract), NaCl (2M), NH_3, H_2O_2, Neutravidin (all chemicals were from Sigma Aldrich, Germany), DNA aptamer of the composition 5′-biotin-TAC TAT CGC GGA GAC AGC GCG GGA GGC ACC GGG GA-3′ was from Eurogentec (Belgium) [30]. Aptamer was modified by biotin at its 5′ end.

2.2. Standard Methods of Foodborne Pathogens Detection

Commercial detection methods follow European ISO standards. *Listeria* spp. strains were detected by the following methods: cultivation on agar for identification as well as quantification (MSZ EN ISO 11290-1:1996/A1:2005; MSZ EN 11920-2:2012), PCR examination (BACGene *Listeria* spp. KIT, validation EGS 38/02-01/17), Vidas® examination (ISO 16140 by AFNOR (BIO 12/33-05/12)).

The procedure for the identification method of agar culture was as follows. (1) Initial enrichment was performed in a selective enrichment solution with a reduced concentration of active ingredients (half-Fraser broth). The initial suspension was incubated at 30 °C for 24–26 h. (2) The secondary inoculation of the sample from the previous step was performed in a non-diluted enrichment solution (Fraser broth). The incubation time was 24 h at 37 °C. The next step was streaking sample on agar plate and identification. Samples (1) and (2) were cultured on two different selective solid media: (a) *Listeria* agar according to Ottaviani and Agosti (ALOA, ISO. 11290-1) [31]; (b) any other selective medium complementary to Ottaviani and Agosti Agar *Listeria*. This was followed by incubation of

the agar at 37 °C for 24–48 h. After 24 h, the agars were examined. In case of the visible presence of *Listeria* spp. the incubation was stopped.

The following steps were performed to quantify the number of colony-forming units (CFU) per ml. The sample was dissolved in half-Fraser broth for 45 min (initial suspension). 0.1 mL of the initial suspension and 0.1 mL of further decimal dilutions were used for inoculation. Samples were inoculated onto a 140 mm Petri dish containing Agar *Listeria* according to Ottaviani and Agosti. The agar was incubated at 37 °C for 24 h. After incubation, Petri dishes were examined for the presence of *Listeria spp*. and colonies were counted [31].

VIDAS® *Listeria* is an enzyme-linked immunoassay for the detection of *Listeria* antigens contained in the samples, it is an Enzyme Linked Fluorescent Assay (ELFA) method. Sample preparation was as follows: approximately 25 mg of sample (raw meat/other) and 225 mL of half-Fraser broth (1:9 ratio) were added to special mixing bags. This is followed by incubation for 20–26 h at 30 °C. After incubation, 1 mL of suspension was dissolved in 10 mL of Fraser broth and incubated for 20–26 h at 30 °C. After incubation, the sample was heated according to the VIDAS® manual and then the VIDAS® test was performed, giving a positive/negative result.

BACGene is a testing tool for quantitative real-time PCR. Real-time PCR is a highly specific and sensitive DNA amplification and detection method that uses the sequences of nucleotides found in the bacterial genome. The procedure for sample preparation was as follows: The initial suspension was prepared according to the CFU quantification procedure. Further sample enrichment was performed in preheated (37± °C) Actero™ *Listeria* Enrichment Media. Subsequently, the sample was lysed using proteinase K, lysozyme, and lysis buffer L. Lysis was performed at 37 °C for 20 min, 95 °C for 10 min, and 4 °C for 5 min. After centrifugation, samples were analysed according to BACGene procedures.

2.3. Preparation of Bacteria Samples (Listeria innocua)

The genus *Listeria* represents short gram-positive rods 0.5–2 μm long with 1–4 flagella. They are aerobic or facultative anaerobic bacteria. According to somatic and flagellar antigens, *Listeri*a spp. has up to 16 serovars due to its complex structure. Two types—*Listeria murrayi* and *L. grayi* have the same antigen ensemble, making them completely different from other types. Apart from *L. ivanovii*, there is no correlation between antigen structure and affiliation to a particular *Listeria* species. Some partial body antigens of *Listeria* are also common with other bacteria such as *staphylococci*, *enterococci* and *E. coli*, which may cause cross-reactions in serological tests. The genus *Listeria* is resistant to changes in the external environment and multiplies even at high salt concentrations (10% NaCl), which allows their long-term survival outside the host organism [32].

Deep-frozen (−80 °C) *Listeria innocua* samples were inoculated with inoculum rod on a Petri dish plate with TSA-YE solution. This was followed by incubation for 24 h at approximately 29 °C. After growth of visible colonies, a sample was harvested and dispersed in TSB-YE solution (a), followed by incubation for 12–18 h. For further measurements, the bacterial solution was centrifuged and then diluted in PBS (b) to the desired concentration for the experiment. The same procedure was applied for preparation *Escherichia coli*, which served as a control to determine aptamer specificity.

The CFU/mL (colony forming units) was calculated using the formula (1) [33], for which we needed information on the number of live bacteria capable of forming colonies at different dilutions on special agar. The procedure was as follows: solution (a) was diluted by decimal dilution from 10^8 up to 10. Individual dilutions were applied (0.1 mL) each to 3 different TSA-YE agars (Agar A; B; C). This was followed by incubation for 24 h, after which the colonies were counted. The results are shown in Table 1.

$$\frac{CFU}{ml} = \frac{(Averrage\ number\ of\ colonies)}{(Dilution)*(amount\ of\ inoculated\ sample[ml])} \quad (1)$$

2.4. Preparation of the Aptasensor

In our experiments, a piezocrystal with fundamental frequency of 8 MHz (Total Frequency Control, UK) was used. Both sides of crystal had thin gold layers serving as electrodes. Working surface of the crystal has a circular shape of an area 0.2 cm^2. Prior aptamer immobilization the crystal was carefully cleaned with basic Piranha solution (29% NH_3, 30% H_2O an H_2O_2 with volumetric 1:5:1 ratio respectively) for 25 min. After this treatment, the crystal was washed three times with deionized water and stored in ethanol. After drying in a flow of nitrogen the crystal was placed in an acryl flow cell connected to the syringe pump (Genie Plus, USA). Then 125 μM of neutravidin (NA) dissolved in deionised water could flow at the crystal surface with flow rate of 50 μl/min during approx. 30 min. Neutravidin is deglycolised avidin that contains SH groups that allow chemisorption at the gold surface. This process was controlled by measurements of the resonant frequency of the crystal. As soon as the frequency stabilized, the crystal was washed by deionised water to remove weakly adsorbed neutravidin molecules. The washing by PBS was then followed. Finally, the biotinylated aptamers dissolved in PBS in a concentration of 0.5 μM could flow with the same rate during approx. 30 min. Due to high affinity of biotin to the neutravidin a self-assembly aptamer monolayer was formed [34–36]. The sensing surface was ready for the study of interaction with bacteria.

2.5. Detection of bacteria by QCM method

Thanks to simple configuration and sensitivity, the QCM method is one of the most widely used acoustic methods. The principle of the method is to measure the change in mass depending on the change in frequency of the crystal resonator. The resonator is a parallel quartz crystal disk with electrodes sputtered on both sides. To ensure a minimum dependence of the crystal oscillation on the temperature, the quartz crystal is cut with so-called. AT section at an angle of 35°15′ to the *Z*-axis [37].

In crystal modifications, for example, by adsorption of a layer on the crystal surface, the resonant frequency change is described by the Sauerbrey equation (valid only in a vacuum) [38]:

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}} \quad (2)$$

in which f_0 is the resonance frequency, Δm is the change in the mass (adsorbed layer on the surface of the crystal), A is effective crystal area, $\mu_q = 2.947 \times 10^{11}$ g.cm^{-1}.s^{-2} is the shear modulus of elasticity and $\rho_q = 2.648$ g.cm^{-3} is the crystal density [38].

In a water environment, the frequency can be affected also by viscous forces, therefore the Sauerbrey equation must be modified

$$\Delta \mathrm{f} = -2f_0^{\frac{3}{2}}\sqrt{\frac{\eta_l \rho_l}{\pi \mu_q \rho_q}} \quad (3)$$

where η_l represents the dynamic viscosity of the liquid and ρ_l the density of the liquid [39].

The acoustic waves in QCM transducer are generated by applying a high-frequency voltage to the electrodes sputtered at both sides of the crystal [40]. Depending on the thickness of the crystal, the fundamental resonant frequency is defined, mostly in the range of 5–30 MHz [40].

The QCM experiments were performed using the computer-controlled Sark 110 vector analyser (Seeed, China). The device allowed measurement of fundamental and higher harmonic frequencies. The sensitivity of measurement frequency changes increased linearly with the harmonic number. All measurements were performed in a flow mode (see Section 2.4). The bacterial suspension of the concentration determined by the standard method (see Sections 2.2 and 2.3) has been allowed to flow with a flow rate of 50 μl/mL at the surface of the crystal modified by DNA aptamers. The changes of fundamental and higher harmonic frequencies were continuously monitored. All experiments were performed at ambient temperature around 20 °C. For apparatus set up see Dizon et al. [41]

3. Results and Discussion

3.1. Determination of the Concentration of Bacteria by the Standard Method

Before starting the QCM experiments, we determined the concentrations of *Listeria innocua* (*L innocua*) and *E. coli* by standard methods described in Sections 2.2 and 2.3. Final CFU was determined by equation (1) and are presented in Table 1. From 40 samples collected from various food products (milk, poultry, swabs), that was tested firstly by method absence/presence, we found out that 15 samples contained *Listeria* spp. Presence in these samples was further confirmed by BACGGene method, that again confirmed *Listeria* spp. in these 15 samples. The sample tested has been diluted by PBS in desired concentrations for sensor analysis.

Table 1. Data from counting of colony-forming units per milliliter for specific dilutions.

Sample	Dilution	Number of Colonies on Agar			Average Number [pcs]	SD	RD	Final CFU/mL
		A	B	C				
1. L. innocua	10^{-5}	272	294	297	288	13.7	5%	2.91×10^8
	10^{-6}	33	32	32	32	0.6	2%	
2. E. coli	10^{-5}	382	358	368	369	12.1	3%	3.71×10^8
	10^{-6}	44	46	26	39	11.0	28%	
3. L. innocua	10^{-5}	290	346	311	316	28.3	9%	3.24×10^8
	10^{-6}	34	58	30	41	15.1	37%	

3.2. QCM Measurement with Listeria Innocua

3.2.1. Formation of Aptamer Layer

In the first series of measurement, we monitored adsorption kinetics of neutravidin on gold surface and those of biotinylated aptamers to neutravidin layer. Clean crystal mounted in an acryl flow cell was first washed with deionized water during several minutes in a flow mode until the resonance frequency stabilized. The flow of neutravidin dissolved in deionized water with a concentration of 125 μM resulted in sharp changes in the fundamental frequency as well as harmonic frequencies (Figure 1). Washing the surface by deionized water resulted in a much smaller increase of the frequency, which correspond to the removal of the weakly adsorbed neutravidin molecules from the surfaces. The resulting frequency change due to neutravidin adsorption determined from 5 experiments were Δf_s = −203 ± 18.3 Hz. In our previous work [42], we showed that formation of neutravidin layers is accompanied only by small changes in motional resistance, R_m, which is evidence of negligible effect of the surface viscosity into the resonant frequency changes. Thus, the neutravidin layer can be considered as a rather rigid structure allowing to apply Sauerbrey equation for determination of the surface density of neutravidin molecules (see below).

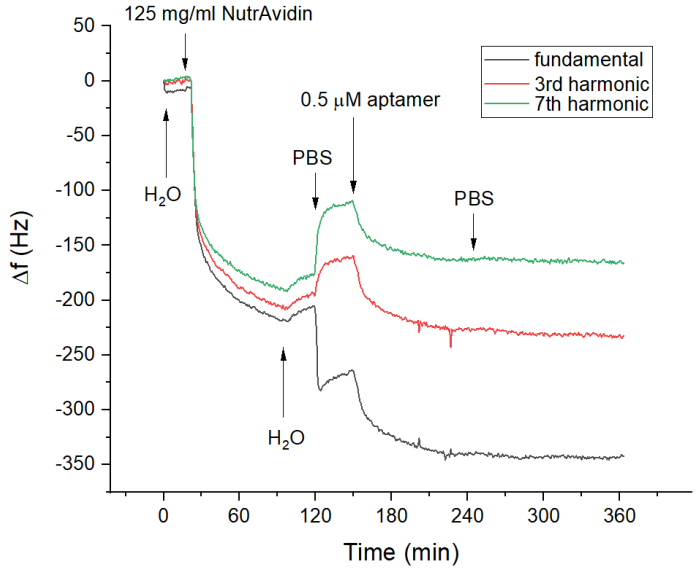

Figure 1. Kinetics of the changes of fundamental frequency, 3rd and 7th higher harmonic frequencies (values divided by their harmonic order number n = 3, 7) vs. time following addition of neutravidin and DNA aptamers. The moment of addition of neutravidin and aptamers as well as washing the surface by water and PBS are shown by arrows.

After formation of neutravidin layer the surface has been washed by PBS. This is since aptamers were dissolved in PBS and the changes in ionic composition can affect the surface properties of the layer and as a result also the resonant frequency. Certainly, this effect was observed in Figure 1. These changes can be attributed to influence of ionic strength on surface viscosity [43]. Addition of 0.5 μM DNA aptamer dissolved in PBS resulted in frequency decrease of Δf_s = −82.0 ± 2.1 Hz. Afterwards, PBS wash caused only a slight increase in frequency, suggesting that stable aptamer layer was formed on the surface of neutravidin layer. Based on resonance frequency changes after application of neutravidin and aptamers, it is possible to determine surface mass density using Sauerbrey equation as 5.6 × 10^{12} molecules of neutravidin and 1.08 × 10^{13} molecules of aptamer per cm^2. Because neutravidin has 4 binding sites for biotin and two of them becomes unavailable due to the immobilization on crystal surface, total number of available neutravidin binding sites is 1.12 × 10^{13} per cm^2. That means that number of immobilized aptamer molecules is almost identical with the number of the free binding sites. This result suggests that aptamers are bound on almost all available binding sites of neutravidin. However, this is only rough estimation since adsorption of aptamers is accompanied also by increase of motional resistance. Thus, the Sauerbrey equation is not strongly valid in this case.

3.2.2. Detection of *Listeria innocua*

The kinetics of frequency changes following the addition of *Listeria innocua* at the surface of QCM aptasensor is presented in Figure 2. Addition of bacteria in the range of 5 × 10^3–10^6 CFU/mL resulted in the frequency decrease. The final changes of the frequency corresponding to the bacteria concentrations of 5 × 10^3–10^6 CFU/mL are presented in Table 2.

However, because the measurement took several hours after cultures extraction, we can assume that the final concentration of bacteria can be higher, as there are continual cell division and growth.

Table 2. Average changes in frequency caused by application of sample with given concentration of Listeria innocua for three independent measurements.

Listeria innocua, CFU/mL	Frequency Changes, Δf_s, [Hz]
10^3	−0.840 ± 0.003
10^4	−2.392 ± 0.311
10^5	−3.198 ± 0.411
10^6	−7.575 ± 0.333

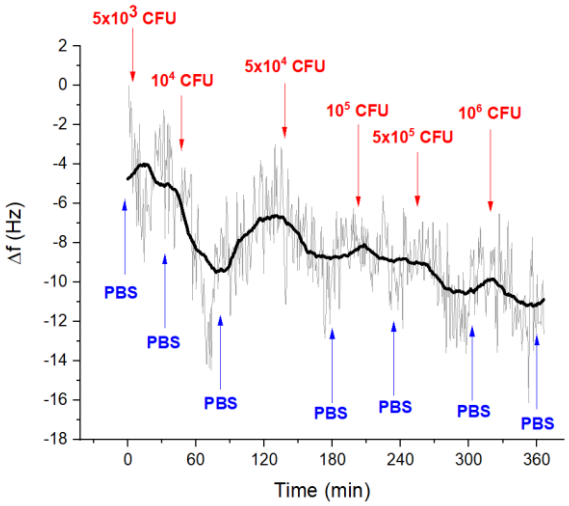

Figure 2. The kinetics of the changes of fundamental frequency of aptasensor following addition of *Listeria innocua*. After each addition the sensor has been washed by PBS to remove weakly adsorbed bacteria.

Based on kinetics experiments and obtained changes in the frequency we constructed the plot of frequency changes vs. concentration of bacteria. The results are presented in Figure 3. The frequency dependence has been fitted by Langmuir isotherm $\Delta f = \Delta f_{max} * c/(K_D + c)$, where K_D is dissociation constant and Δf_{max} is maximal frequency change.

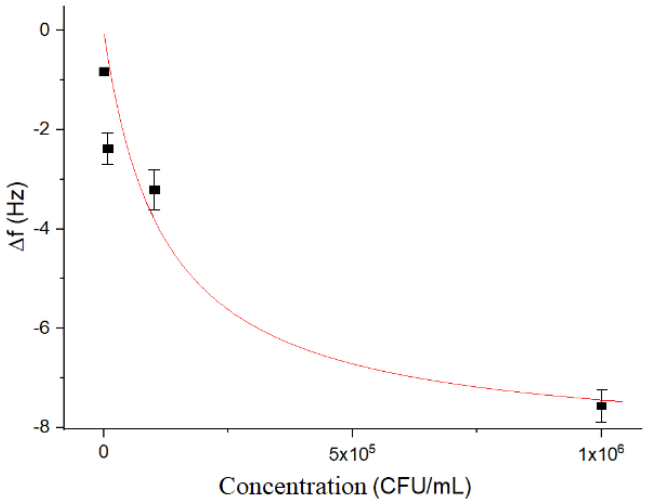

Figure 3. Calibration curve for *Listeria innocua* created by plotting resonance frequency change vs. bacteria concentration and fitted with the Langmuir isotherm using Origin version 7.5. Fitting parameters were Δf_{max}= −8.34 ± 2.09 Hz, K_D = 1.21 ± 1.11 × 10^5 CFU/mL, Chi^2 = 2.01.

In thesis by Tatarko [44] the detection of *Lactobacillus acidophilus* by QCM method has been reported. However, the frequency changes in comparable concentrations of bacteria were 6.2-times larger. This could be since *Lactobacillus* can be 3.1- to 28.8- times larger in comparison with *Listeria*. *Lactobacillus* size is 0.5–1.2 × 1.0–11.0 μm, while *Listeria* size is 0.4–0.5 × 0.5–2.0 μm.

From linear part of the calibration curve at small concentration of bacteria we determined limit of detection (LOD) according to formula: LOD = 3 SD/k = 1.6 × 10^3 CFU/mL, where SD is standard deviation of the lowest detectable concentration and k is the slope of the curve. The obtained LOD value shows sufficient ability of aptasensor to detect *Listeria innocua* concentration that is even lower than the minimal infective dose of 10^6 CFU/mL [45].

3.2.3. Aptamer Specificity Testing with *E. coli*

To confirm the specificity of the aptasensor we used *E. coli* as a control. These bacteria have identical partial antigens with 13 subspecies of *Listeria* spp. (including *Listeria innocua* and *Listeria monocytogenes*) and is causing cross-reaction in serological tests. Formation of the aptamer layer was identical with those presented above. In measurements, we tested two *E. coli* samples on aptasensor with relatively high concentration: 10^5 CFU/mL and 10^6 CFU/mL. Figure 4 shows the kinetics of the changes of the 5th harmonic frequency, as its changes were the most prominent compared to all other higher harmonic frequencies. Application of any concentration of *E. coli* caused only increase in the frequency. In comparison, *Listeria innocua* caused significant frequency decrease already at 5 × 10^3 CFU/mL.

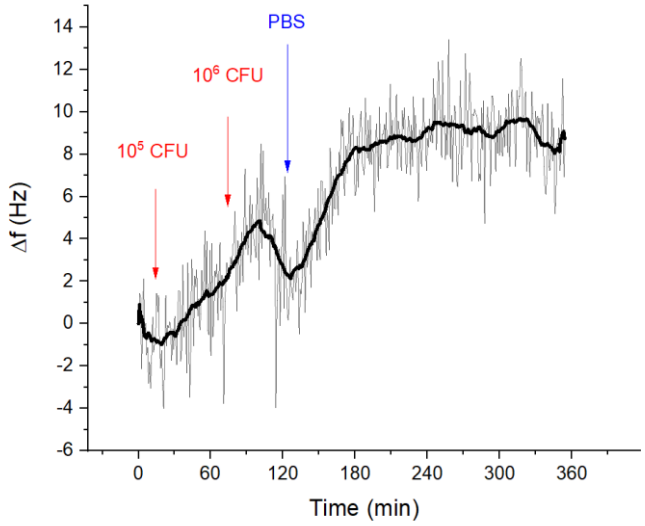

Figure 4. The kinetics of 5th harmonic frequency (values divided by factor n = 5) caused by addition of *E. coli*.

4. Conclusions

In this study, we developed acoustic aptasensor for detection of bacterial pathogen *Listeria innocua*. Using DNA aptamers specific to *Listeria* spp. we reached LOD of 1.6 × 10^3 CFU/mL. No interferences were registered at application of control *E. coli* sample with identical partial antigens to *Listeria* spp. Thus, we confirmed the aptasensor specificity. The obtained results suggest that aptamer based QCM sensor is specific and sufficiently rapid for pathogen detection with relatively low LOD. More detailed research is, however, needed to improve the detection limit. Further specificity test is also necessary to approve the effectiveness of the proposed assay including measurements in real milk samples.

Author Contributions: conceptualization, T.H., V.O. and M.T.; methodology, M.T., J.S., M.H. and Z.K..; software, M.T.; validation M.T. and V.O.; formal analysis, M.T., M.H. and Z.K.; investigation, M.T. and V.O.; resources, T.H. and Z.K.; data curation, M.T.; writing—original draft preparation, V.O.; writing—review and editing, T.H., J.S. and A.H.; visualization, M.T.; supervision, T.H. and Z.K.; project administration, T.H. and A.H.; funding acquisition, T.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the European Union, project Horizon 2020, Marie Skłodowska-Curie, contract number 690898 and was also supported by the Science Grant Agency VEGA, project number: 1/0419/20.

Acknowledgments: Measurements for this work were performed at Hungarian Dairy Research Institute Ltd., -HDRI Ltd., Mosonmagyaróvár, Hungary. Samples and materials for handling the samples were granted with support from MTKI during Horizon 2020 project cooperation.

Conflicts of Interest: The authors declare no conflict of interest.

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