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Detection of Listeria innocua by acoustic aptasensor

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Foodborne pathogens

- Worldwide: 600 milion cases, 420 000 deaths (WHO, 2020)

Prevalent foodborne pathogens contaminating food:

- *Listeria monocytogenes*
- *Escherichia coli*
- *Staphylococcus aureusm*
- *Salmonella enteritica*
- *Bacillus cereus*
- *Campylobacter jejuni*
- *Clostridium perfringens*
- **(Oliver et al., 2005; Scallan et al., 2011; Zhao et al., 2014)**

(https://www.ift.org/news-and-publications/blog/2019/september/top-10-most-common-foodborne-pathogens)

Standardized methods for foodborne pathogen detection

- Highly specific
- Low-cost
- Provide qualitative and quantitative information

- It can take up to 72 hours to confirm negative sample, 7 days to confirm positive

- PCR (Polymerase chain reaction), ELISA (enzyme linked imuno assay), ELFA (Enzyme linked fluorescent assay)
  - Fast detection, examination of large number of samples
  - High reocurring costs restricting their routine use.

(Law et al., 2015)

Biosensors

- Analytical tool
- Consists of a bioreceptor and a physicochemical transducer
- Several types: optical, electrochemical, piezoelectric and calorimetric
- Receptors: antibodies, enzymes, aptamers, bacteriophages, ssDNA/RNA,...

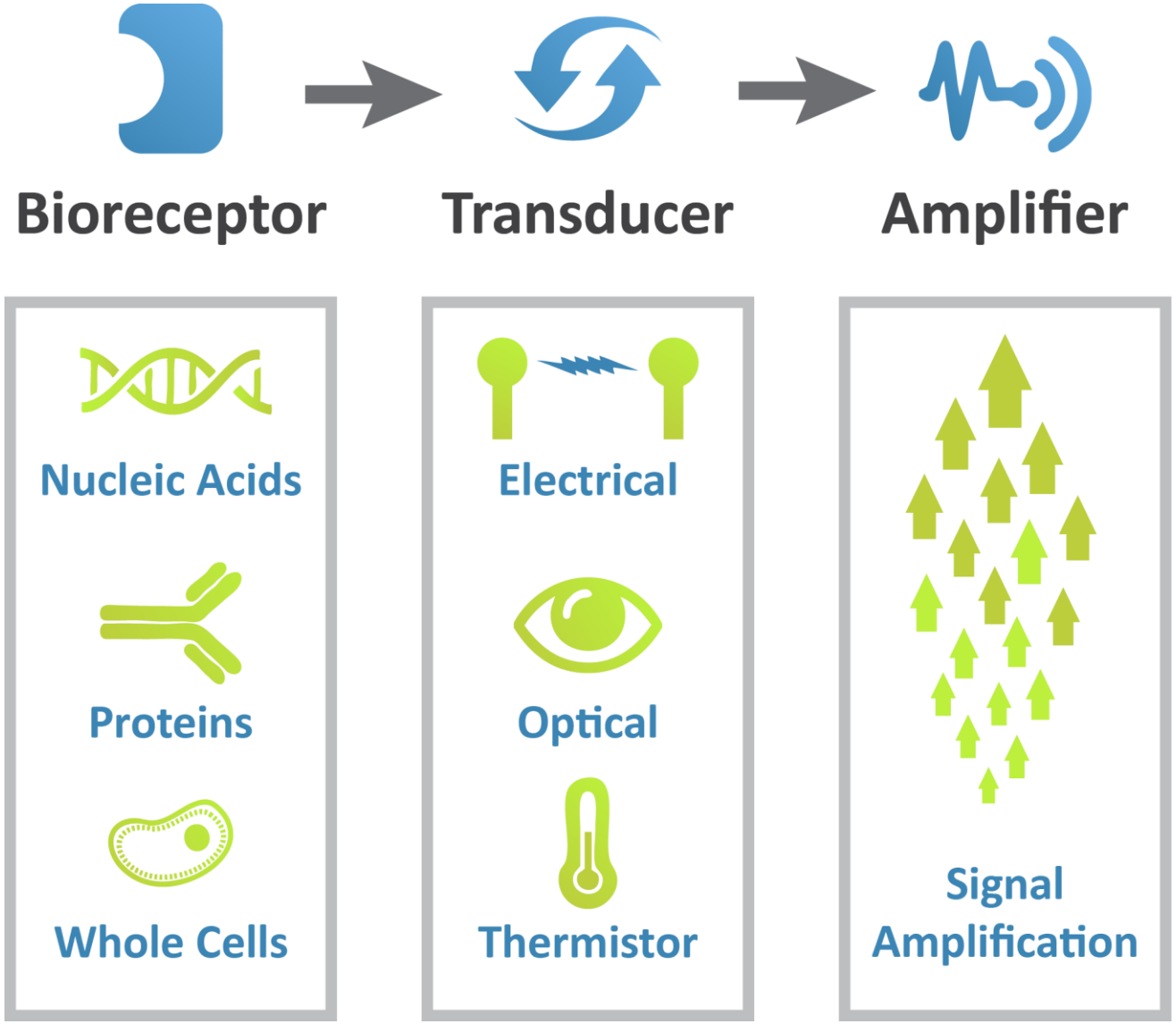


Figure 1: Scheme of biosensor detection. (https://www.innovogene.com/store/pc/viewcontent.asp?idpage=11)

(Salami et al., 2019; Karunakaran et al., 2015; Thévenot et al., 2001; Choi and Chae, 2009)

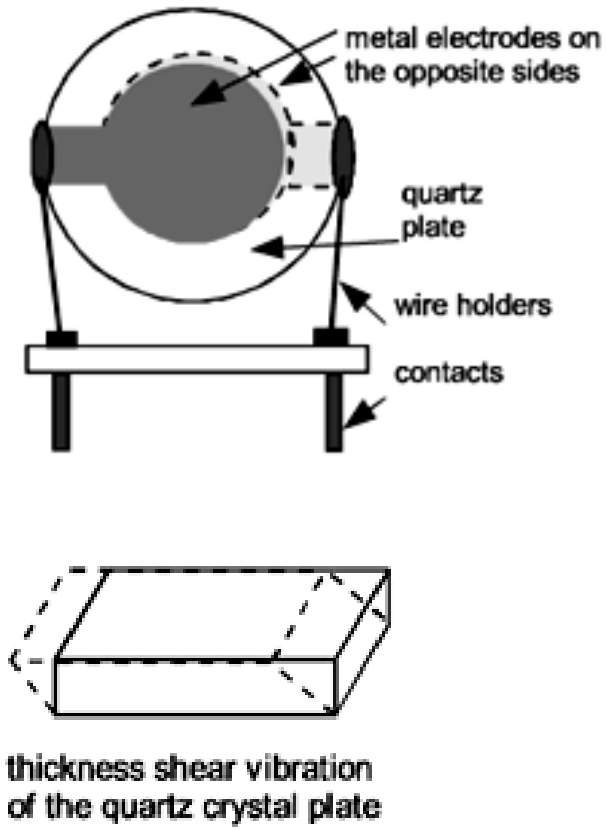
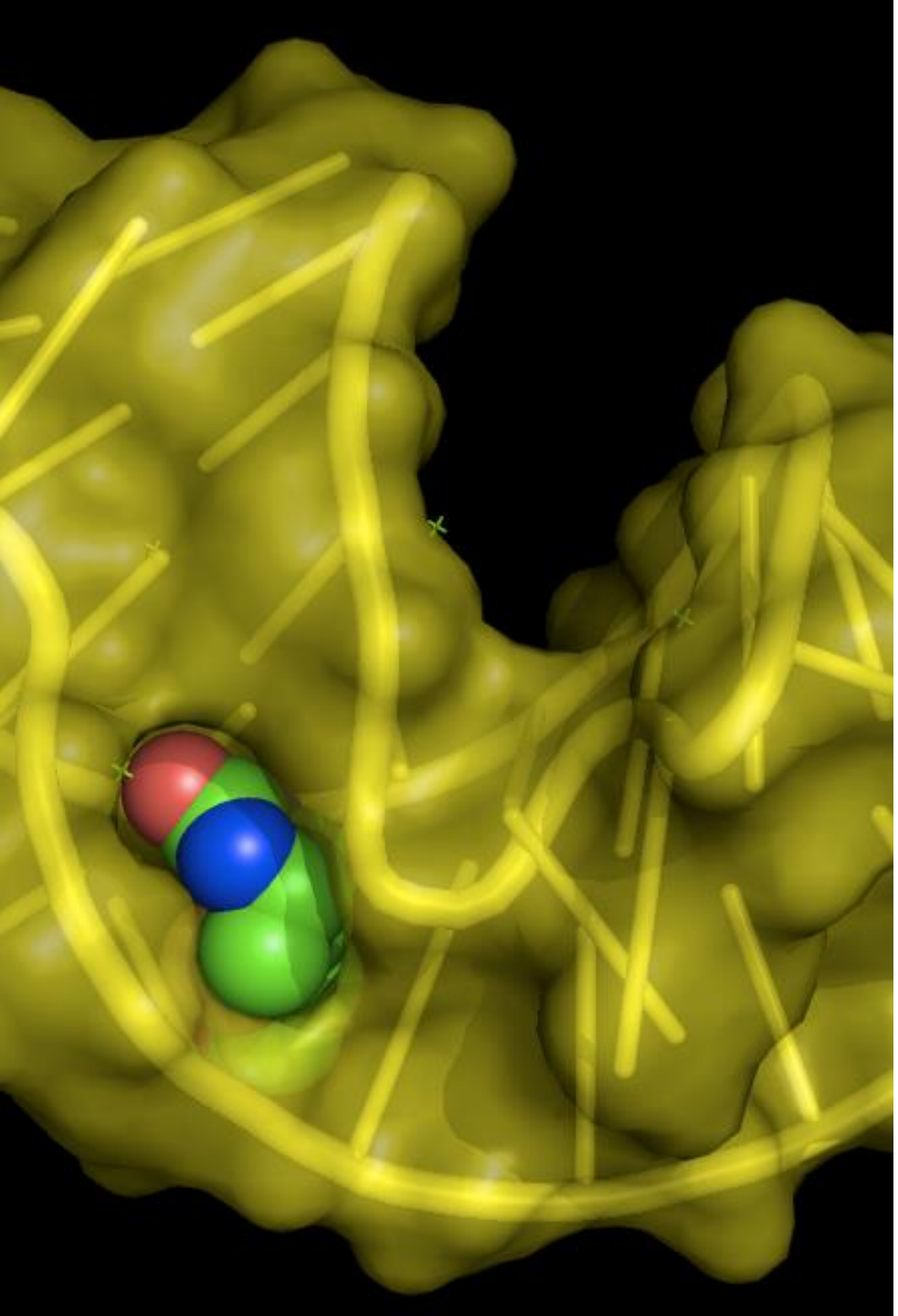


Figure 2: Schematic representation of piezoelectric Quartz Crystal Microbalance and scheme of QCM vibration. (Skládal 2003)

Piezoelectric biosenzors and their application

- Quartz crystal with AT cut is used in the QCM configuration
- Frequency of the piezoelectric crystal is affected by the amount of mass deposited on its surface (Skládal, 2002)
- Detection of bacteria using QCM biosensor:
  - QCM-aptasensor for the detection of *Brucella melitensis* in milk, limit of detection (LOD) = 10^3 CFU/mL (Bayramoglu et al., 2019)
  - QCM aptasensor for detection of *E.coli*, LOD = 34 CFU/mL for t = 40 min. (Yu, 2018)



Aptamers and their application

- Synthetic peptides or single-stranded sections of DNA/RNA oligonucleotides
- Synthesized using SELEX (Systematic Evolution of Ligands by Exponential Enrichment). (Ellington and Szostak, 1990)
- Compose advanced 3D structures, thus creating specific bonding site for target molecule (Yu et al., 2018).
- Advantages compared to antibodies: higher affinity and specificity towards its target molecule, thermostability, low-cost and fast synthezis.
- Possible applications for a wide range of target molecules (Yu et al., 2018).

Aim of work

- Investigating the possibility of using QCM method for the detection of pathogenic bacteria *Listeria innocua* using DNA aptamers as receptors.
- Study of aptamer specificity for the detection of *Listeria innocua.*
- For future experiments we would like to continue in the development of highly sensitive biosensor with the use of nanoparticles modified with aptamers. Our aim is limit of detection 10 CFU/ml.

Hungarian Dairy Research Institute, Mosonmagyaróvár, Maďarsko

MTKI
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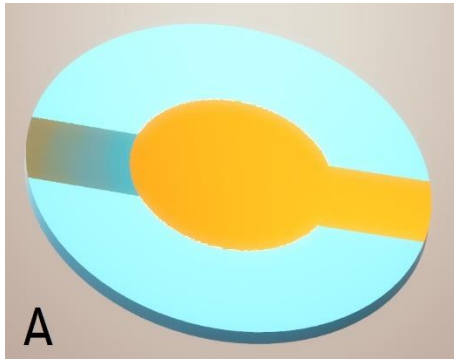
The experiment was performed at the Hungarian Dairy Institute in Mosonmagyaróvár

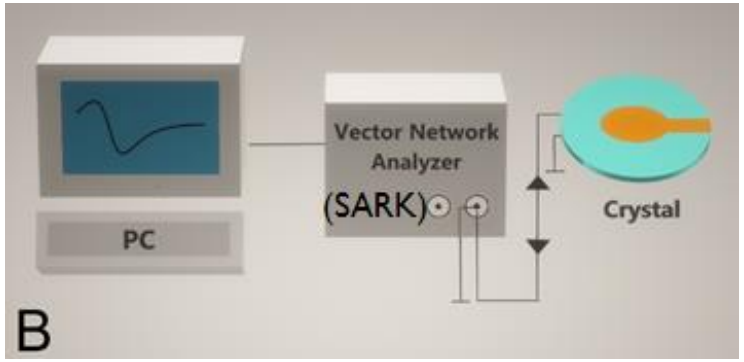
SARK-110

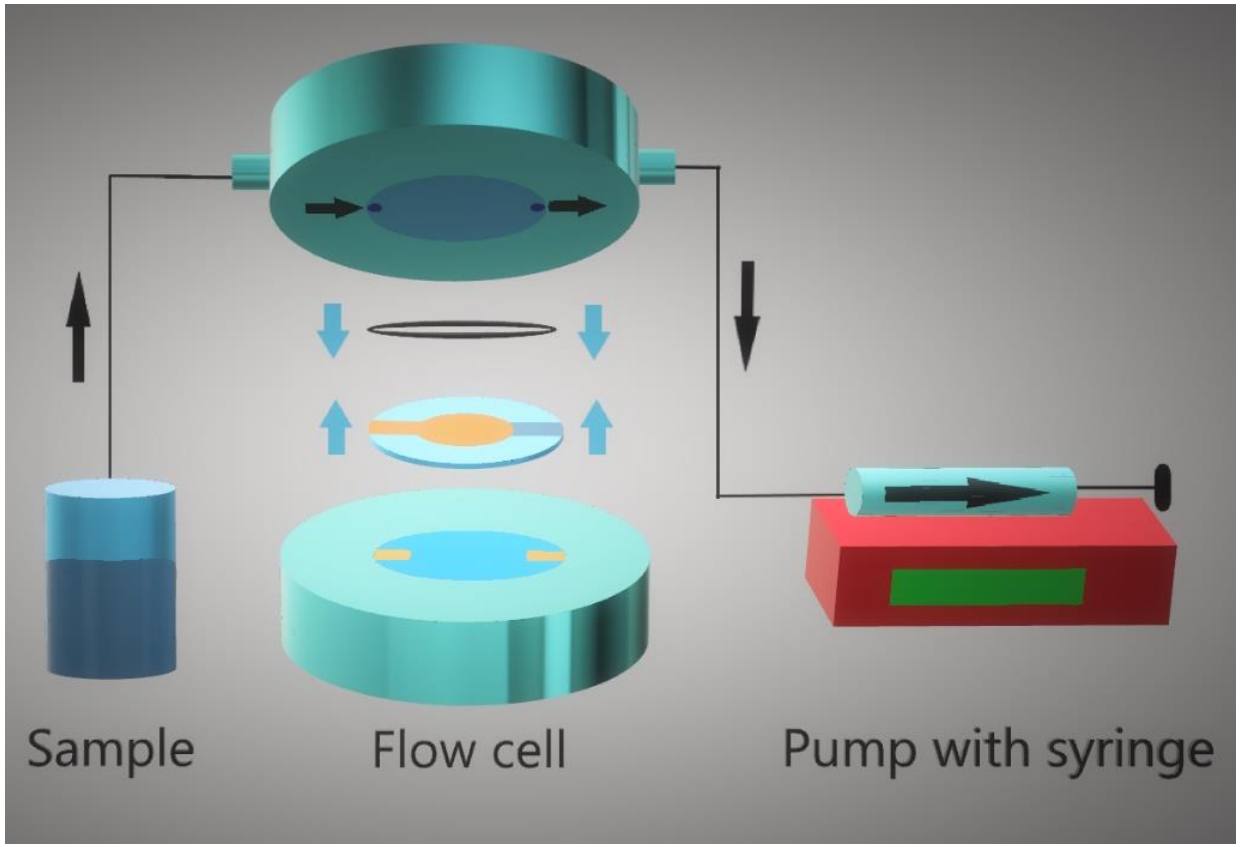
- Vector analyzer

- Multi-purpose
- Portable
- Generates and detects signal

QCM measurements: we measured multi-harmonic frequencies and energy dissipation







Materials and methods - QCM

Figure 3: Scheme of AT- cut crystal with gold electrodes (A) and its implementation into QCM composition (B). The crystal is placed inside the flow cell and the solution is delivered with a pump with a syringe to the surface of the crystal (C).

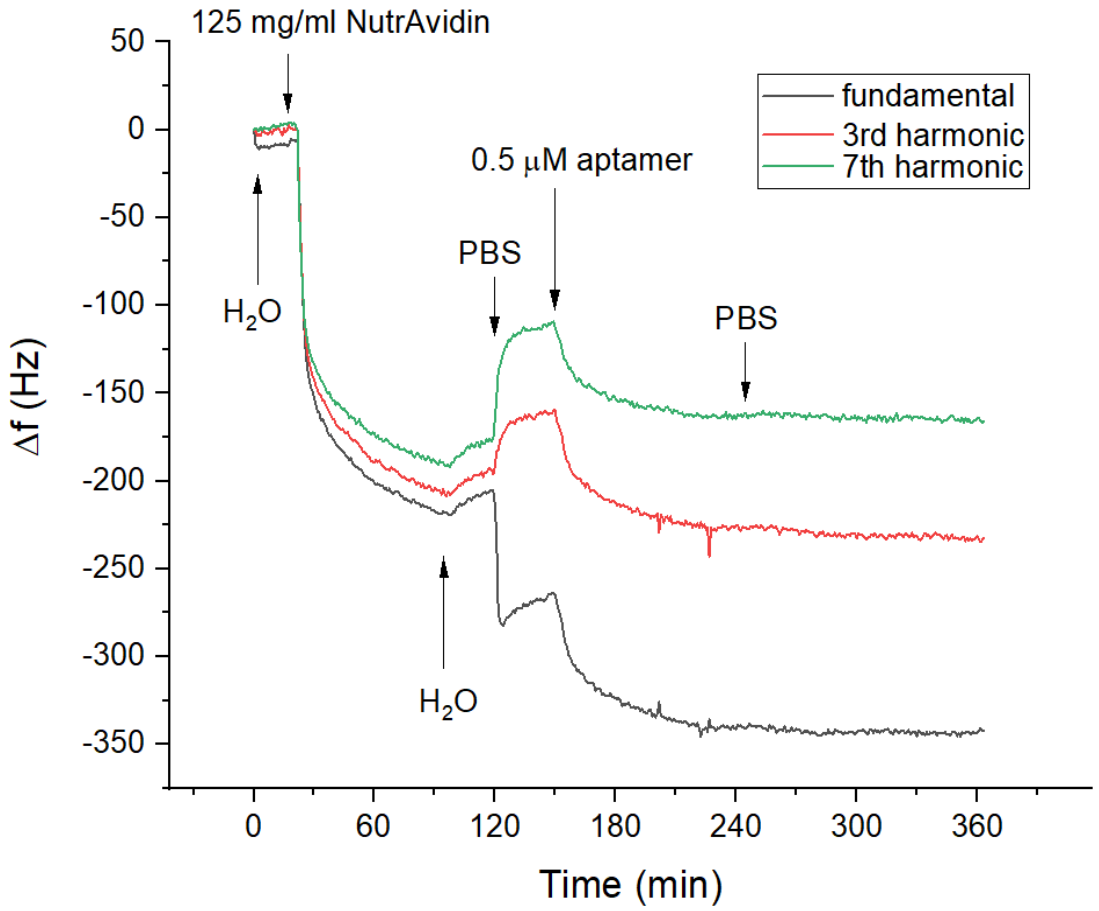
Material and Methods 1

- QCM crystal was purified with basic Piranha solution (mixture of NH_3, H_2O a H_2O_2 in the ratio 1:5:1) for three 25-minute cycles and dried with nitrogen.
- Subsequently, the surface of the crystal was modified with NeutrAvidin and aptamer specific for *Listeria spp*. was immobilized on the surface of the crystal.
- The sample of *Listeria innocua* was prepared followingly: culture of *L. innocua* was innoculated from deep freeze to the surface of bacterial growth promoting agar. After the cultivation, a certain amount was collected and diluted in PBS. Subsequently, the samples were measured by QCM.

Material and Methods 2

- To determine CFU/ml (colony forming units), enumeration plate method on agar was used.
- Following methods wew used to detect foodborne bacteria:
  - Cultivation on agar for the purpose of identification as well as quantification (MSZ EN ISO 11290-1:1996/A1:2005; MSZ EN 11920-2:2012).
  - PCR test (BACGene Listeria spp. KIT, validation EGS 38/02-01/17)
  - Vidas® test (ISO 16140 by AFNOR (BIO 12/33-05/12))

Results: Aptamer layer



Immobilization of layers:
(recalculation by the Sauerbreyer equation)

A) NeutrAvdin:
- 5,627 x 10^{12} molecules/cm^2 bound

B) Aptamer
- 1,079 x 10^{13} molecules/ cm^2 bound

Total number of free biotin binfing sites (per aptamer):
- 1.125x10^{13} molecules/cm^2

Figure 4. 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.

Table 1. Resulting data from the addition of column forming units per milliliter (CFU / ml) at each dilution.

Sample	Dilution	Number of colonies on agar			Average count	Standard deviation	Relative deviation	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%	

BACGene PCR results: Number of tested samples obtained from milk, poultry and semi-finished products: 40 pcs total → 15 pcs positive.

Results: Determination of the CFU/mL value in the samples and testing for the presence of *Listeria spp.* in foods

Results: aptamer Listeria Innocua

Table 2. Change of frequency after the appplication of sample with different concentration.

Concentration x 3.24 CFU/ml	Change in fundamental frequency Δf_s [Hz]
5x 10^3	-2.035
10^4	-4.566
5x 10^4	-8.520
10^5	-13.163

After application of *Listeria innocua*, we observed a decrease in frequency (Fig. 5), which is associated with the uptake of bacteria by the aptamer.

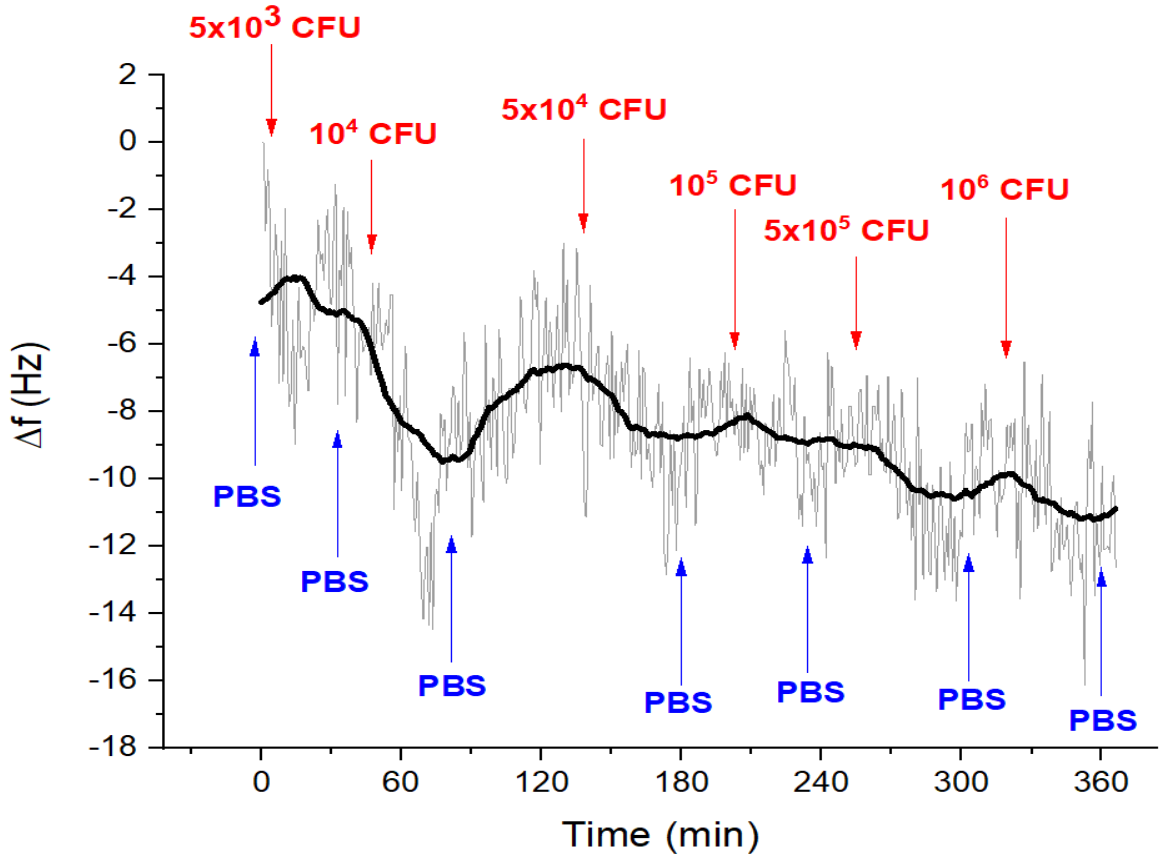


Figure 5: The kinetics of the changes of fundamental frequency of aptasensor following addition of *Listeria innocua*. After each addition the sensor has been washed by PBS to remove weakly adsorbed bacteria.

Results: Calibration curve

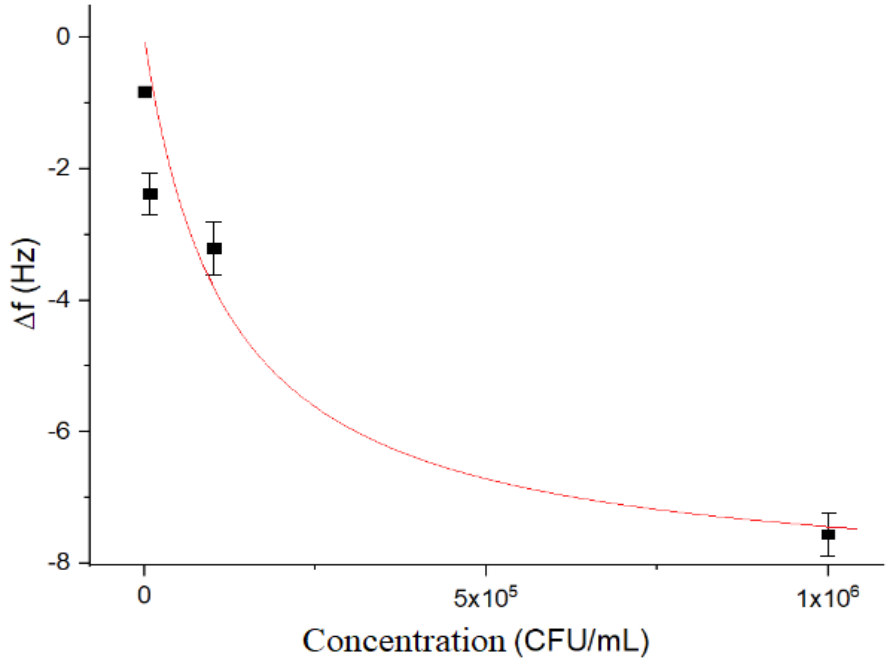


Figure 6: Calibration curve for *Listeria innocua* created by plotting resonance frequency change vs. bacteria concentration and fitted with the Langmuir isotherm using Origin version 7.5. Fitting parameters were Δf_{max}= -8.34 ± 2.09 Hz, K_D = 1.21 ± 1.11 x10^5 CFU/ml, Chi^2 = 2.01.

Table 3: Average frequency changes due to application of a sample of a given concentration resulting from three measurements.

Concentration CFU/ml	Average change of fundamental frequency Δf_s [Hz]	Standard deviation (STDEVA)
10^3	-0.840	0.003
10^4	-2.392	0.311
10^5	-3.198	0.411
10^6	-7.575	0.333

Limit of detection 1,611 x 10e3 ± 0,4 x 10e3 CFU/ml

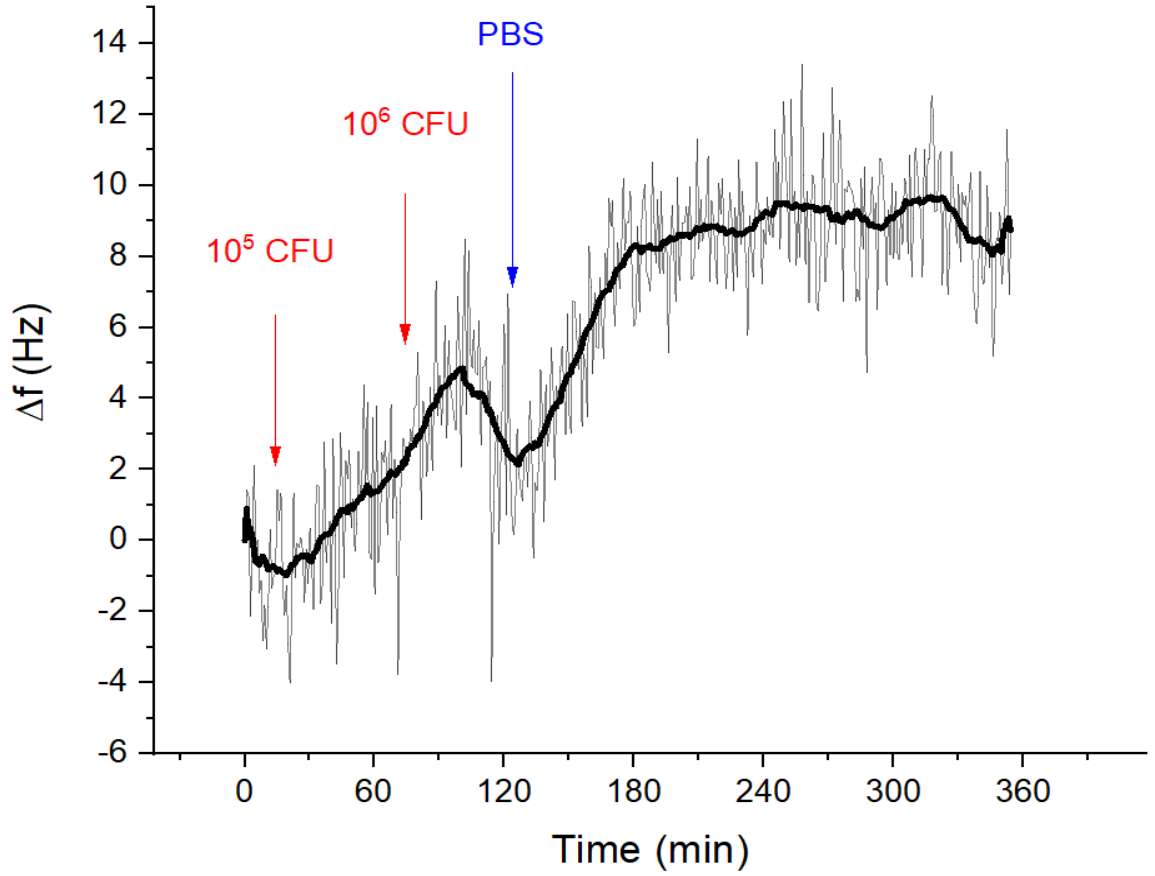


Figure 7: The kinetics of 5th harmonic frequency (values divided by factor n=5) caused by addition of *E. coli*.

Results: Non-specific interactions with E. Coli

- In contrast to Figure 5 after application of bacterial samples, we observe an increase in frequency, i.e. we did not observe uptake of E. coli bacteria by the aptamer.

Conclusion

- We were able to detect Listeria innocua using QCM method.
- **Limit of detection (LOD)** = 1 611 CFU/ml for t = 30 minutes.
- Tested specificity for *Listeria spp.* (absence of signal for bacteria E. coli)
- The method we used proved to be a highly specific, sensitive and rapid form of pathogen detection
- Goals for future experiments: detection of pathogens directly in dairy products.

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