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Functionalized screen-printed electrodes for the thermal detection of *Escherichia coli* in dairy products

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ABSTRACT

Accurate and fast on-site detection of harmful microorganisms in food products is a key preventive step to avoid food-borne illness and product recall. In this study, screen-printed electrodes (SPEs) were functionalized *via* a facile strategy with surface imprinted polymers (SIPs). The SIP-coated SPEs were used in combination with the heat transfer method (HTM) for the real-time detection of *Escherichia coli*. The sensor was tested in buffer, with a reproducible and sensitive response that attained a limit of detection of 180 CFU/mL. Furthermore, selectivity was assessed by analyzing the sensor's response to *C. sakazakii, K. pneumoniae* and *S. aureus* as analogue strains. Finally, the device was successfully used for the detection of *E. coli* in spiked milk as proof-of-application, requiring no additional sample preparation. These results suggest the proposed thermal biosensor possesses the potential of becoming a tool for routine, on-site monitoring of *E. coli* in food safety applications.

1. Introduction

According to the World Health Organization, microorganisms are the most common cause of foodborne disease worldwide. Diarrhoeal diseases, which are responsible for 70 % of the total burden, are the cause of the majority of deaths associated with foodborne diseases (World Health Organization (WHO), 2015). *Escherichia coli*, a key cause of foodborne diarrhea, is a widespread bacterium found in the human intestinal tract. The presence of this microorganism is employed as hygiene environmental indicator (Ssemanda et al., 2017). The reliable detection of *E. coli* and other pathogens that can be transmitted through the food chain can lead to adequate interventions that prevent disease, but also economic loss related to the recall of contaminated products.

The advantages and disadvantages of traditional and molecular techniques for the detection *E. coli* and other microorganisms of interest for the agro-food sector have been thoroughly reviewed (Ge & Meng, 2009; Valderrama et al., 2016). Plate counting, which is still the gold standard methodology in most cases, requires long laboratory incubation times that can take several laboratory hours and transport to a lab facility, often external, which stretches the entire procedure over a few days (Hameed et al., 2018). Polymerase-chain reaction (PCR) is a promising alternative that is much faster and possess sensitivity but also has some obstacles that impede its wide-spread implementation since it can be costly and requires specialized equipment and skilled operators (Umesha & Manukumar, 2018). In view of this, detection technologies that overcome these drawbacks are of research interest in order to develop fast, cost-effective and accurate detection tools that can be used for on-site, routine screening of food products for microbial contamination.

Biosensors have been explored for their application in diverse fields, including food monitoring. The multiple combinations available of receptor layers and transducing technologies have enabled their use for the detection of chemical (pesticides, adulterants, etc.) as well as biological contaminants (Xie et al., 2022). Sensing platforms developed for *Escherichia coli* consist commonly on biological receptors such as antibodies and aptamers in combination with optical, electrochemical and micro gravimetric readouts (Huang et al., 2022a). Two of the main challenges that biosensors face in food monitoring are the sensitivities required and the ability of perform in complex samples. In this context, biological receptors present limitations due to their poor stability in different conditions (pH, temperatures, etc.) (Luong et al., 2008). Synthetic alternatives for biological receptors represent a suitable solution

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to overcome these flaws. Molecularly imprinted polymers have proven to be suitable for the direct or indirect detection of chemical and biological analytes in combination with various transducers including impedance, calorimetry, quartz microbalance, etc. (Arreguin-Campos, Jiménez-Monroy, et al., 2021).

The heat-transfer method (HTM) is a translating technology that monitors the thermal transport along the receptor-liquid sample interface during the recognition event (van Grinsven et al., 2012). Some of the major advantages of HTM include the requirement of little instrumentation, making it low cost, as well as relatively simple data interpretation in comparison with other platforms based on e.g. electrochemical or piezoelectric transducers. This readout was introduced for the detection of *E. coli* employing surface imprinted polymers a few years ago. (van Grinsven et al., 2016). However, application in food safety was only considered as outlook due to the sensitivity limitations of the sensor. Although research efforts have focused on the optimization of this aspect of the technology (Cornelis et al., 2019), attention should be also drawn to the fact that in order to reach applicability, the readout and its elements should remain simple to facilitate its use in diverse fields. That being said, the introduction of specialized and complex preparation protocols for imprinted polymers for the sensitive recognition of the target might now compromise batch-to-batch reproducibility of the receptors and hinder their eventual up-scalability.

Attaining sensitivity and selectivity of the sensor in combination with simple imprinting protocols that yield reproducible results for bacteria sensing in food samples are the key elements for bringing this new detection technology forward to application. Recently, our group has reported a novel and facile sedimentation technique for the preparation of synthetic receptors that overcame some of the challenges of bacteria imprinting (Arreguin-Campos et al., 2022). The current study is a further step in the translation process from "lab-to-application". Screen-printed electrodes (SPEs) have become increasingly popular for biosensor applications in recent years due to their low-cost and scalable production process that enables for the mass production of highly reproducible sensor chips. SPEs have been explored in combination with electrochemical and optical transducing platforms for the detection of proteins and small molecules (Li, Li, Fossey, & Long, 2010; Song et al., 2018; Song, Li, Liang, Qiang, & Xu, 2014). Nonetheless, these substrates have not been used as imprint-transducer interface for the thermal sensing of bacteria despite of their excellent thermal conductivity (Suresh et al., 2021). Hereby, we present the combination of SPEs together with a simple surface-imprinting process for their functionalization that represents an easily scalable strategy for the production of synthetic receptors, which is crucial in order to close the gap for the translation of this technology into application settings. The material proposed for imprinting is Polydimethylsiloxane (PDMS), a commercially available resin that is widely employed in soft lithographic applications. Its use reduces batch to batch variability in receptor preparation since no synthesis is required, and offers the ability to bind and sort bacteria when acting as recognition element (Ren & Zare, 2012). These characteristics of imprinted PDMS are combined in this study with the well-known attributes of SPE such as portability, low cost and easy handling (Torre et al., 2020). The materials and techniques hereby presented lead to the preparation of low-cost disposable functional interfaces that align with the already mentioned advantageous characteristics of the thermal sensor. The use of functionalized SPEs could therefore bring us one step closer to commercial application of the biomimetic thermal detection of bacteria. To further illustrate this commercial potential, the developed disposable sensor was used for *E. coli* detection in dairy products. The presence of this microorganism as well as its pathogenic strains is a concern in the dairy industry since cattle act as reservoir for them, increasing the chances of contaminating stock material for the production of multiple type of products and especially those that are consumed raw without pasteurization (cheese, yogurt, etc.).

2. Materials and methods

2.1. Chemicals and reagents

*Escherichia coli (*ATCC 8739), *Cronobacter sakazakii* (ATCC 29544), *Klebsiella pneumoniae* (ATCC 4352) and *Staphylococcus aureus* (ATCC 6538) strains were purchased from DSM-Z (Braunschweig, Germany). Polydimethylsiloxane Sylgard 184 elastomer kit from Mavom N.V. (Schelle, Belgium). Lysogeny (LB) culturing broth, phosphate buffer saline (PBS), anhydrous tetrahydrofuran (THF), safranin and sodium dodecyl sulfate (SDS) with a minimum purity of 99.9 % were used as received from Merck (Diegem, Belgium).

2.2. Bacteria cultures and preparation of bacteria suspensions

Liquid growth mediums were prepared following the standard protocols. A single colony of bacteria were inoculated in 20 mL of the corresponding broths and kept overnight in a shaking incubator at 37 °C. Following, 0.5 mL of the cultures were diluted in 4.5 mL of fresh broth and incubated for further growth for 2 h. OD600 was measured in order to determine the final concentrations of bacteria. The cultures were then centrifuged at 3000 rpm for 5 min and the pellets were re-suspended in PBS. After washing the bacteria an additional time with PBS, dilutions were performed with sterile PBS in order to obtain the desired concentrations.

2.3. Fabrication of screen-printed electrodes

Briefly, the SPEs were fabricated in-house using a stencil design to achieve a 3.1 mm diameter working electrode with a 32 mm connection length using graphite ink (Product Ink: C2000802P2; Gwent Electronic Materials Lts., Pontypool, United Kingdom) and were printed using a DEK 248 screen printer machine (DEK, Weymouth, United Kingdom) onto a polyester flexible film (250 μm thickness; Autostat, Milan, Italy). The graphite layer was printed and then cured in a fan oven at 60 °C for 30 min. Next, a silver/silver chloride reference electrode was introduced by screen-printing Ag|AgCl paste (Product Code: D2070423D5; Gwent Electronic Materials ltd., Pontypool, United Kingdom) onto the polyester substrates and cured in a fan oven for 30 min at 60 °C. Finally, a dielectric paste (Product Code: D2070423D5; Gwent Electronic Materials ltd., Pontypool, United Kingdom) was then printed onto the polyester substrate to cover the connections and cured for an additional 30 min at 60 °C before use.

2.4. Functionalization of the SPEs via interfacial polymer imprinting

PDMS was prepared following the manufacturer's protocol, including the suggested base to curing agent ratio of 10:1 (w/w). Furthermore, the viscous mixture was diluted with tetrahydrofuran (10 % (w/w) in order to obtain a stock solution for imprint preparation.

Interfacial imprinting (Arreguin-Campos et al., 2022) was adapted for the screen-printed electrodes as follows. Screen-printed substrates were spin-coated for 60 s at 5000 rpm with 50 μL of PDMS stock solution. Subsequently, the PDMS-covered substrates were placed in an oven at 65 °C for 10 min in order to achieve a partial curing of the resin. Past this time, the films were taken out of the oven and placed on a flat surface at room temperature. Subsequently, a droplet of 150 μL of *Escherichia coli* template suspension ($1E^8$ CFU/mL) was applied onto the surface of the PDMS-coated substrates and left for sedimentation for 20 min. Without removing the template solution, the substrates were then placed again in the oven at 65 °C (3 h) for further curing of the PDMS and evaporation of the liquid, leaving only the bacteria and salt residues on the surface of the polymer. After imprinting, the substrates were rinsed with distilled water to remove residual salts from the evaporated buffer, SDS 3 % to remove the template and finally distilled water again to rinse the surfactants remanent.

2.5. Optical characterization of polymer imprint's surfaces

In order to characterize the imprint's surface, PDMS interfacial imprinting was performed on glass slides and bright-field microscopy was employed for assessing the synthetic receptors (LEICA DM 750 optical microscope). Safranin as staining solution was used with the purpose of enabling the visualization of the bacteria. The average surface coverage of *E. coli* imprints on the layers was calculated using ImageJ 1.44O (National Institute of Health, Bethesda, MA) employing three films from independent batch samples and three different locations on each imprint.

Scanning electron microscopy was carried out at DSM, Geleen, Netherlands. The prepared imprinted polymers on screen-printed electrodes as well as on glass slides were used in order to confirm the presence of the cavities on the surface.

3. Sensing setup

The thermal setup has been described thoroughly in previous publications (Lowdon et al., 2019; van Grinsven et al., 2017). In short, the sensor setup consists of a copper chip holder, which serves as a heat provider. The temperature underneath the sample, T_1, is stringently controlled by means of a thermocouple, a proportional-integral-derivative (PID) controller (settings 10-8-0) and a power heater. The temperature is transported through the functionalized layer into the flow cell that allows for administering test liquids. The temperature inside this flow cell is monitored by a second thermocouple. Before each experiment, the flow cell is filled with PBS or non-spiked milk and the chamber is allowed to stabilize. Subsequently, 2 mL of the desired bacteria suspension is introduced at a controlled flow rate of 2 mL/min. Dose-response curves for HTM were constructed from the raw temperature data as it has been reported previously for chemo-sensing employing the HTM (Caldara et al., 2021; Diliën et al., 2017).

3.1. Real sample measurements

As application case, supermarket-bought milk was employed for thermal measurements. The pasteurized milk was used as obtained without further sample preparation and it was spiked with *E. coli* and serial-diluted to obtain the required concentrations.

4. Results and discussion

4.1. Functionalization of screen-printed substrates

SPEs were covered with PDMS and imprinted with *E. coli* via the interfacial sedimentation of the bacteria on the polymer. This protocol consists of three steps. First, the substrate is spin-coated with PDMS and partially cured, obtaining a semi-rigid film. This step is followed by the direct addition of the template in buffer in order to sediment the cells and allow their free assembly on the PDMS. Finally, the polymer is fully cured in the presence of the template with the aim of fixing the shape of the bacteria as well as some chemical functionalities of their membrane, creating complementary cavities on the PDMS. The substrates were assessed with scanning electron microscopy before and after the imprinting process. In Fig. 1A, the structure of the bare SPEs can be observed. The surface is rough due to the ink, which contains micrometric carbon flakes. After the deposition of PDMS on the surface of the SPEs (Fig. 1B), it can be noticed that the roughness of the surface is smoothed due to the creation of a polymeric layer on the substrate that coats the carbon. Finally, in picture 1C it can be noticed that the surface exhibits pattern that is derived from the sedimentation of the bacteria on the PDMS. This pattern is formed by heterogeneously distributed and agglomerated small cavities (1–3 μm) that result from the curing of the polymer in the presence of *E. coli* cells. Additional optical characterization of the films on smooth glass slides was performed in order to depict the cavity formation derived from the imprinting procedure. Brightfield and Scanning Electron Microscopy images can be found on Supplementary information (Fig. S1). The surface coverage of the template bacteria on the polymer's surface was calculated as 22.67 ± 1.3 %, which represents an improvement on the obtained density of cavities obtained with other imprinting protocols, such as microcontact (van Grinsven et al., 2016).

4.2. Bacteria detection: heat-transfer measurements

The functionalized SPE chips were then employed for the quantification of the target in PBS solutions. For this purpose, the imprints were cut into 1x1 cm² squares and placed into a polycarbonate flow cell (Fig. 2A), which is directly connected to the HTM. Temperature control 1 for the copper block was set at 37 °C for all the measurements. In an initial step, the chamber was filled only with buffer and stabilized for 20 min in order to create a baseline for each run. Subsequently, increasing
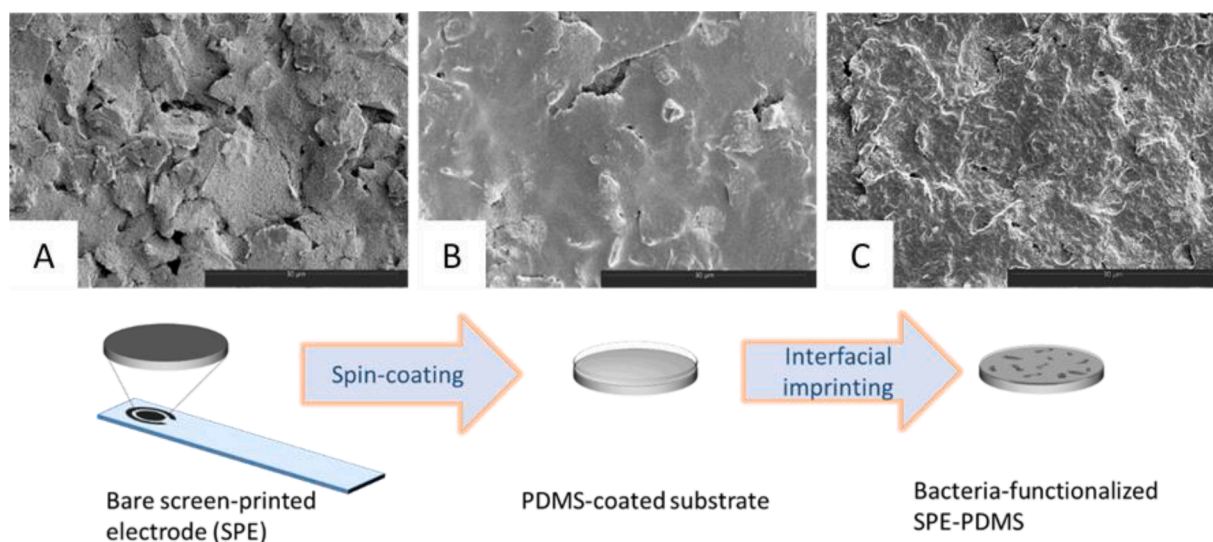

Fig. 1. Schematic representation of the bacteria-functionalization of screen printed substrates with scanning electrode microscopy images for: A) Bare substrates, B) Polymer-coated substrates and C) Bacteria-functionalized substrated. Scale bars are 30 μm.
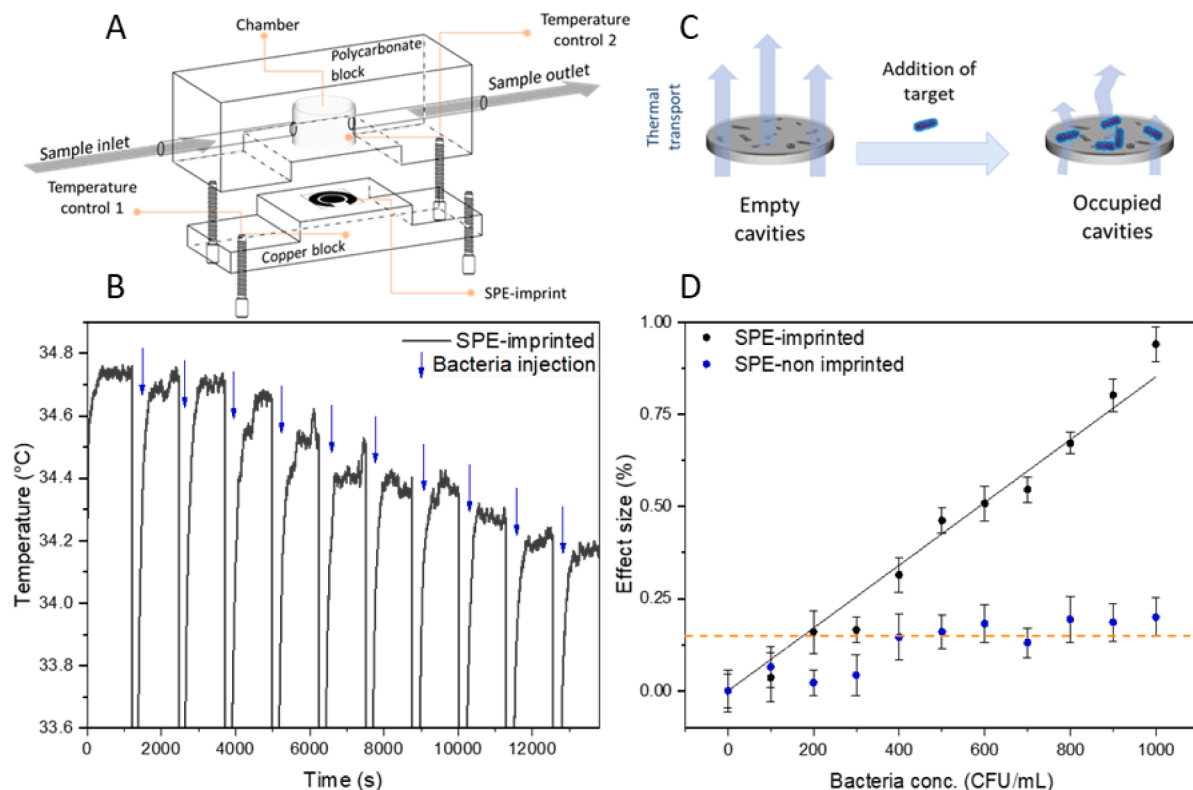
Fig. 2. A) Schematic representation of the flow cell employed for the thermal measurements. B) Representation of the thermal recognition of E. coli through the cell-functionalized substrates. C) Real-time temperature signal of the sensor (arrows indicate the injections of 100 CFU/mL). D) Dose-response curve of the sensor, the dashed line indicates the limit of detection, defined as three times the average the error in the datasets. Error bars are calculated making use of the noise in the signal of the sensor.

concentrations of *E. coli* (from 0 to 1000 CFU/mL) in PBS were injected into the system in a controlled manner with an automated pump built on the HTM. A thermocouple placed above the imprint monitors the temperature (temperature control 2) during the sample additions and stabilizations. The raw data for temperature 2 of a representative run is presented on Fig. 2B, where it can be observed that the signal decreases over time with each bacteria injection into the flow cell. This can be explained by the increment of the thermal resistance upon the occupation of the functional cavities in the imprinted polymer-SPEs when rebinding occurs (Fig. 2C) (Eersels et al., 2015).

The real-time temperature data was employed to construct a dose–response curve for the sensor. Once stabilization of the signal is achieved, mean temperature values for each incubation step were determined from the raw data, and the effect sizes resulting from the exposition of the SPE to the bacteria suspensions were calculated by dividing the observed temperature decrease (Y) for each concentration (t = c) by the average baselines (t = 0) employing the following formula:

$$Effect size (\%) = \frac{\Delta Y_t(t=c)}{Y_t(t=0)} x 100$$

The resulting dataset was fitted with OriginPro to a linear (y = ax + b) equation and this analysis was performed to three independent SPE measurements in order to obtain the dose–response curve, obtaining an R-square of 0.99. The same procedure was performed for SPEs that were not imprinted with bacteria, and used as blank for the calculations. From these results (Fig. 2D), it can be observed that the sensor's response when testing imprinted SPEs follows a linear regression along all the bacteria concentrations analyzed, which contrasts for the blank, for which a non-linear signal that falls within the noise of the method is observed. The limit-of-detection (LoD) was determined as the lowest concentration at which the effect size is higher than three times the averaged error for all the data sets (orange line, 3 σ method), with a

value of 180 ± 60 CFU/mL.

4.3. Selectivity of the receptor layer against enterobacteriaceae C. sakazakii

An advantage of functionalizing SPEs with imprinted polymers is that these synthetic receptors possess the ability to bind selectively to the desired target, which can be exploited for the construction of specific sensing devices. For this study, the enterobacteriaceae *C. sakazakii and K. pneumoniae* as well as *S. aureus* were employed for testing the selectivity of the SPE-HTM sensor. These three bacteria are relevant food contaminants encountered in the the dairy industry with the potential of causing foodborne disease related to poor hygienic practices (Martin, Trmcic, Hsieh, Boor, & Wiedmann, 2016). Moreover, the last two are a major cause of mastitis in dairy cows (Davis & Price, 2016; Fetsch & Johler, n.d.). The *E. coli* imprints were exposed to subsequent concentrations of the non-template strains and thermal measurements were taken in real-time (representative thermal curves can be found in Supplementary information Fig. S3). The average of multiple experiments is presented on Fig. 3. It can be noticed that the device's response towards *E. coli* is around three times higher when compared to *C. sakazakii* and *S. aureus*, and around twofold regarding *K. pneumoniae*. The quantitative difference of the signals would indicate that the layers are able to distinguish between the bacteria tested. Moreover, the signals for *C. sakazakii and S. aureus* fall within the noise of the method for the majority of the concentrations tested, leaving only 900 and 1000 CFU/mL with a response that exceeds the error, indicating that only high levels of non-template species would lead to a non-specific signal (at which point the sample is not suitable for consumption). In the specific case of *K. pneumoniae*, the signal becomes significant at concentrations above 500 CFU/mL. Since the recognition mechanism for imprinted polymers derives from the complementarity in shape and chemical
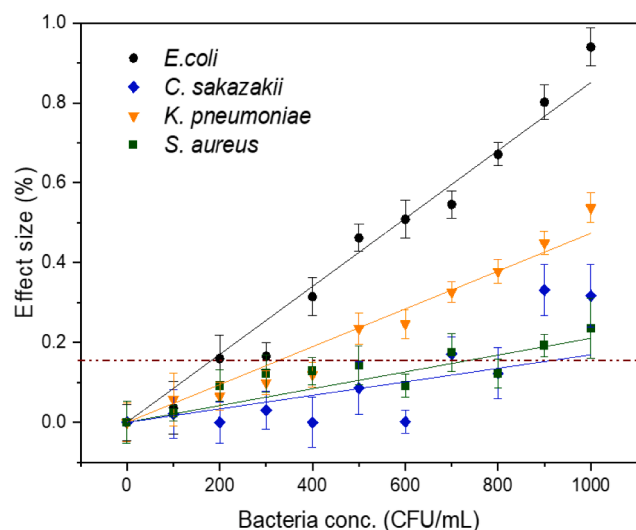
Fig. 3. Effect sizes obtained for the assessment of the selectivity of the sensor. Substrates imprinted with E. coli were exposed to C. sakazakii. The dashed line indicates three times the average of the error on the datasets.

functionalities present in the cavities of these synthetic receptors (Yongabi et al., 2018), this would indicate that the sensor's sensitivity will depend on the layer's capability to distinguish the bacteria based on these two main features. Results would be supported by the differences in gram group and morphology for *E.coli* and *S.aureus* as well as in membrane composition for *C. sakazakii* and *K. pneumoniae*. The results in this study indicate that it is difficult for the current sensor to distinguish between *E. coli* infected samples and samples that are contaminated with large amounts of other pathogenic bacterial strains. However, the sensor presented in this work is proposed as a rapid prescreening tool that can be used in combination with current gold standard practices, and is able to identify food samples with a high bacterial load. Although it is possible to achieve a higher selectivity by chemically modifying the PDMS layers (Wolf, Salieb-Beugelaar, & Hunziker, 2018), the goal in the current investigation is to make a device that is easy to mass produce and provides end-users with a low-cost and fast tool for on-site hygiene indication.

4.4. Application case: detection of target in milk and comparison against relevant reported biosensors

To further illustrate the direct application of the thermal biosensor as a hygiene indicator tool in food samples, it was tested in untreated, spiked milk samples. The samples were directly spiked with different concentrations of *Escherichia coli* without the need of further sample preparation. The thermal measurements in real-time were conducted following the same conditions as for buffer, maintaining T1 at 37 °C and controlled injections into the flow cell. The raw data of a representative temperature run (T2) is exhibited in Fig. 4A, where the concentration-dependent trend is observed over time. The results from multiple and independent measurements were compiled in a dose–response curve following the same protocol followed for PBS. These are presented in Fig. 4B, where these results are compared to the response of the sensor in buffer. It can be noticed that the overall response of the sensor in milk is very similar to the performance observed in PBS. This is also evident when calculating the LoD calculated milk, which is 130 ± 60 CFU/mL and therefore falls within the reproducibility range of the sensor in PBS. These results suggest that the technology can be applied for rapid, low-cost screening of milk-based products.

In order to provide further context for the results of this study, we present the comparison of the sensor developed herein against recently reported biosensors for the detection of *E. coli* based on antibodies and aptamers, which are the most common biological receptors employed (Huang et al., 2022b). The review has been further narrowed down to devices that have been tested in milk samples in order to present a fair comparison regarding the inherent characteristics of milk that could interfere with the recognition such as the presence of biomolecules (proteins, fats, etc.) as well as the physical characteristic of the sample such as viscosity.

From Table 1, it can be highlighted that the biomimetic SPE-HTM platform is competitive with natural receptor-based ones. Biosensors employing electrochemical readouts are well known for their high sensitivity, which can be noticed in the slightly better limits of detection these devices attain. On the other hand, the thermal sensor displays a better performance in terms of limit of detection against most optical devices reported in this comparison. Whereas mot studies focus on analyzing up to high concentrations of bacteria for their linear range, this specific investigation has selected the lower range since at such high *E. coli* counts the foodstuff would be unacceptable for the application. It can be also noticed from the summarized table that sample pretreatment (typically centrifugation or dilution) is required in most of
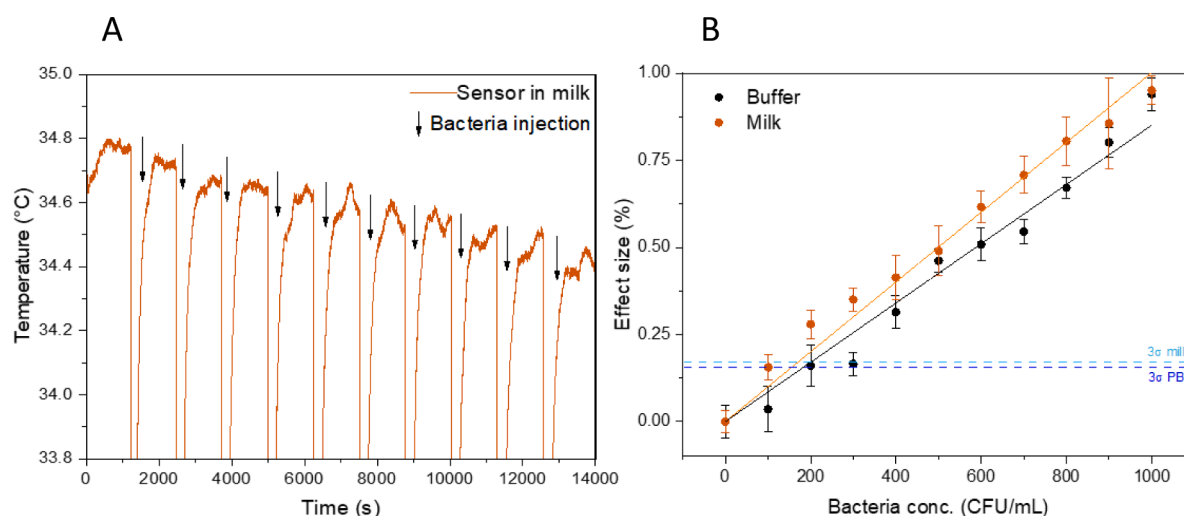

Fig. 4. A) Real-time temperature signal of the sensor in milk (arrows indicate the injections of 100 CFU/mL). B) Dose-response curves of the sensor in both media. The dashed line indicates the limit of detection, defined as three times the average the error in the datasets. Error bars are calculated making use of the noise in the signal of the sensor.

Table 1

Comparison of recently developed biosensors for Escherichia coli in milk.

Recognition element	Translating technology	LoD	Linear range	Pre-treatment to milk	Ref.
Antibody	Electrochemical	1 cells/mL	10^1-10^7 cells/mL	Dilution	(Dhull et al., 2019)
	Electrochemical	12 CFU/mL	10^1-10^5 CFU/mL	Dilution	(Yao et al., 2018)
	Electrochemical	79 CFU/mL	10^2-10^7 CFU/mL	None	(F. Huang et al., 2018)
	Electrochemical	2840 CFU/mL	8.9×10^3-8.9×10^9 CFU/mL	Dilution	(Mo et al., 2019)
Aptamer	Naked eye	250 CFU/mL	500-5×10^7 CFU/mL	None	(Li et al., 2020)
	Optical	3 CFU/mL	5–10^6 CFU/mL	None	(Jin et al., 2017)
	Electrochemical	1 CFU/mL	10^1-10^6 CFU/mL	Centrifugation	(Kaur et al., 2017)
	Optical	835 CFU/mL	10^2-10^6 CFU/mL	Dilution	(Ren et al., 2021)
	Optical	500 CFU/mL	10^1-10^6 CFU/mL	Centrifugation	(Song et al., 2022)
Imprinted polymer	Thermal	2000 CFU/mL	Not reported	None	(Arreguin-Campos, et al., 2021)
	Thermal	170 CFU/mL	10^2-10^3 CFU/mL	None	This study

the cases with the purpose of removing some matrix components that might influence the device's signal. In this regard, the proposed methodology is advantageous for application in the food industry as it offers the possibility of detecting the target without the need of additional sample modification.

5. Conclusions

In summary, this work introduces a novel, thermal biosensor based on imprinted-polymer functionalized screen-printed electrodes for the detection of *Escherichia coli*. The biomimetic sensor demonstrated reproducible and sensitive results in buffer. Moreover, the device exhibits excellent selectivity against *C. sakazakii* and *S. aureus* with no significant response of the sensor observed below concentrations of 1000 CFU/mL and 500 CFU/mL against *K. pneumoniae*, and even at high concentrations of this non-targeted bacterium, the sensor displays a higher effect size for *E. coli*. The combination of a simplified imprint preparation together with screen-printed substrates offers versatile functional interfaces that are not limited to a thermal transducer. The imprints could be used with any commercial or self-made flow cell compatible with screen-printed electrodes, enabling their usage with other readouts such as electrochemical.

Finally, the imprinted SPE-HTM platform was used for the detection of *E. coli* in milk samples, exhibiting results in alignment with the ones obtained for buffer and without the need of sample preparation. The detection can be performed real-time and the technology allows the label-free quantification of the microorganism with experimental procedures that require little to no instrumentation. Further research should be aimed at testing the sensor in increasingly complex food products, ranging from increasingly viscous dairy products to even (extracted) solid food products that might influence the selectivity or sensitivity of the device.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134653.

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