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Article

Salmonella Detection in Food Using a HEK-hTLR5 Reporter Cell-based Sensor

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Abstract: The development of a rapid, sensitive, specific method for detecting foodborne pathogens is paramount in supplying safe food to enhance public health safety. Despite the significant improvement in pathogen detection methods, key issues are still associated with rapid methods, such as distinguishing living cells from dead, pathogenic potential or health risk of the analyte at the time of consumption, detection limit, and sample-to-result. The mammalian cell-based assays analyze pathogens' interaction with the host cells and are responsive to only live pathogens or active toxins. In this study, a human embryonic kidney (HEK293) cell line expressing Toll-Like Receptor 5 (TLR-5) and chromogenic reporter system (HEK dual hTLR5) was used for the detection of viable *Salmonella* in a 96-well tissue culture plate. This cell line responds to low concentrations of TLR5 agonist flagellin. Stimulation of TLR5 ligand activates nuclear factor- κ B (NF- κ B) - linked alkaline phosphatase (AP-1) signaling cascade inducing the production of secreted embryonic alkaline phosphatase (SEAP). With the addition of a *p*-nitrophenyl phosphate as a substrate, a colored end product representing a positive signal is quantified. The assay's specificity was validated with the top 20 *Salmonella enterica* serovars and 19 non-*Salmonella* spp. The performance of the assay was also validated with spiked food samples. The total detection time (sample-to-result), including shortened pre-enrichment (4 h) and selective enrichment (4 h) steps with artificially inoculated outbreak-implicated food samples (chicken, peanut kernel, peanut butter, black pepper, mayonnaise, and peach) was 15 h when inoculated at 1–100 CFU/25 g sample. These results show the potential of HEK-Dual™ hTLR5 cell-based functional biosensors for rapid screening of *Salmonella*.

Keywords: *Salmonella*; cell-based sensor; HEK-dual hTLR 5; detection; flagella; immunomagnetic separation; food safety

1. Introduction

Salmonella is a member of the Enterobacteriaceae family and is a Gram-negative, non-spore-forming motile bacillus with peritrichous flagella [1]. Salmonellae are widespread in the environment and found in various foods and ingredients, posing serious problems to the food industry [2–6]. Non-typhoidal *Salmonella* (NTS) infection is the second most common zoonotic disease after campylobacteriosis in the European Union member states, which reported over 91,000 cases in 2018. According to the Centers for Disease Control and Prevention (CDC), *Salmonella* causes about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year [7,8]. *Salmonella enterica* outbreaks have been associated with a variety of foods, including spices, nuts, flours, bakery products, fresh produce, and poultry, due to their robust physiological adaptability in harsh conditions [9–11].

Noteworthy outbreaks are reported for *Salmonella enterica* serovar Oranienburg (*S. Oranienburg*) involving black pepper [12] and onion [13,14]; *S. Montevideo* in black and red peppers [15]; *S. Mbandaka*, *S. Stanley* and *S. Newport* in peanut and peanut butter [16,17]; *S. Agona*, *S. Anatum*, *S. Braenderup*, *S. Dessau*, *S. Hartford*, *S. Meleagridis*, *S. Muenchen*, *S. Rodepoort*, *S. Tennessee*, and *S. Tornow* in shelled peanuts [18]; *S. Enteritidis* in fresh peaches [19], *S. Heidelberg*, *S. Infantis*, and *S. Tennessee* in chicken [20–23] and *S. Typhimurium* definitive type 49 in eggs and mayonnaise prepared with eggs [24], and *S. Indiana* associated with egg mayonnaise sandwiches [24,25]. Moreover, in Brazil, potato salad made with homemade mayonnaise was associated with *S. Enteritidis* [26], and buffet dishes containing mayonnaise were associated with *S. Enteritidis* outbreak in Germany [27].

Numerous rapid methods, including miniaturized biochemical tests, physicochemical methods that measure bacterial metabolites, nucleic acid-based tests, bacteriophage-based sensors, and antibody-based methods, have been developed [28–30]. Conventional methods for detecting *Salmonella* include pre-enrichment, selective enrichment, selective and differential plating, biochemical phenotyping of suspect colonies, and serotyping [28]. These procedures are time-consuming, laborious, and may take 5 to 7 days [31–35]. For consumer protection, risk-based rapid methods with high specificity and sensitivity are necessary to provide confirmed results in a day, and the quality of these results should at least be as reliable as the reference methods [36].

There is an increasing interest in the applications of biosensors that incorporate mammalian cells to detect foodborne pathogens to differentiate viable from non-living cells or toxins, which is a critical determinant for the food industry since nonviable pathogens are not considered a threat [35,37–39]. Cell-based biosensors (CBBs) have been applied to several detection systems and continue to serve as a reliable method to probe for the presence of pathogens in clinical, environmental, or food samples [40]. Mammalian CBBs exploit host-pathogen interactions, including pathogen adhesion, activation of host cell signaling events, cell-cycle arrest, apoptosis, and/or cytotoxicity [35,39,41]. The ability to detect host-pathogen interaction makes CBB a functional test, thus setting it apart from conventional methods. Most CBBs measure the optical properties of cellular metabolites or intracellular enzymes released after the pathogens' interaction with mammalian cells. Cytotoxicity assays also have been developed independently to detect *Salmonella* spp., using the Caco-2 cell line [42], RAW264.7 [43], and J774 macrophage cell lines [44]. However, due to the complex nature of food matrices, their specificity or sensitivity cannot be guaranteed only based on cytotoxicity analysis. Alternatively, an analyte-specific amplified cell signaling cascade approach could be adopted to improve the specificity and sensitivity of CBB.

In this study, our goal was to use an engineered human embryonic kidney (HEK293) cell line expressing Toll-Like Receptor 5 (TLR5) and chromogenic reporter system (HEK dual hTLR5) as the sensor platform. This cell line responds to low concentrations of TLR5 agonist flagellin [45,46]. Stimulation of TLR5 ligand activates canonical nuclear factor- κ B (NF- κ B) - linked alkaline phosphatase (AP-1) signaling cascade inducing the production of SEAP (secreted embryonic alkaline phosphatase) (Figure 1a). With the addition of p-nitrophenyl phosphate as a substrate, a colored end product representing a positive signal can be detected by the naked eye or quantified spectrophotometrically. Not all bacterial flagellin, but *Salmonella* flagellin is strongly recognized by TLR5 (Andersen-Nissen et al. 2005). In addition, employing an immunomagnetic separation (IMS) system such as anti-*Salmonella* Dynabeads (Invitrogen) can also improve assay specificity and sensitivity by capturing and concentrating target pathogens from complex food matrices before exposure to HEK dual hTLR5 cells [47–50].

Overall, the data showed that the HEK dual hTLR5 cell-based sensor could detect viable ~50-100 *S. Enteritidis* cells both in spiked food samples (black pepper, chicken, mayonnaise, peanut kernel, peanut butter, and peach) in 15 h (including enrichment steps) with high accuracy and specificity. Our results suggest the potential application of HEK-Dual™ hTLR5 cell-based functional biosensors for rapid detection of *Salmonella*.

2. Results

2.1. Optimization of HEK Dual hTLR5 Sensor for Detection of *Salmonella*

HEK-Blue-hTLR5 cells (InvivoGen, CA, USA) were constructed with the human TLR5 gene and an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene (Figure 1a). The SEAP gene is placed under the control of the IFN β minimal promoter fused to five NF- κ B and AP-1-binding sites. Stimulation with a TLR5 ligand activates NF- κ B and AP-1, which induce the production of SEAP. The amount of SEAP production can be determined qualitatively with the naked eye or quantified with the HEK-Blue Detection system (Quanti Blue substrate) by measuring absorbance at 620-655 nm (Figure 1b). Initial experiments were conducted to determine if HEK-Blue-hTLR5 cells are responsive to *Salmonella*. At multiplicity of infection (MOI) 10, HEK cells exposed to *Salmonella enterica* ser Enteritidis (PT21) for 6 h showed a significantly higher response (~6-fold increase; $p < 0.0001$) than *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* (Figure 1c). These data indicate that HEK-Blue-hTLR5 can be used to detect *Salmonella* in a cell-based sensor.

Bacterial motility assay results on agar plates at different concentrations (0.3%, “swim plates”; 0.5%, 0.7%, and 1%, “swarm plates”) [51,52] confirmed *S. Enteritidis* to be highly motile and the most evident colony diameter differences were observed at 0.3% agar (swim plates) than the other bacteria tested (Figure S1). Significantly higher flagellar expression in *Salmonella* compared to other bacterial genera tested may contribute to the observed high signal with the HEK dual hTLR5 sensor than the other pathogens (Figure 1c).

Next, we determined the optimal *Salmonella* cells to HEK cell ratio (multiplicity of infection, MOI) needed to show a positive response after 4 or 6 h of incubation. At 4 h (Figure 1d), MOI 1 and 10 showed 6.7 and 8.7-fold higher signals than the uninfected controls, while at 6 h (Figure 1d), MOI 0.1, 1, and 10 showed 14.2, 15.3, and 10.3-fold higher signal, respectively. *E. coli* O157-493-89, a non-motile strain, was used as a negative control. These data indicate that an MOI of 1 exhibits a positive signal after 4 h of exposure, while an MOI of 0.1 is sufficient after 6 h of exposure. However, TLR-5 response to MOI 0.1 compared to MOI 1 at 6 h did not significantly differ. Therefore, we used MOI 1 (4 h and 6 h) for further experiments.

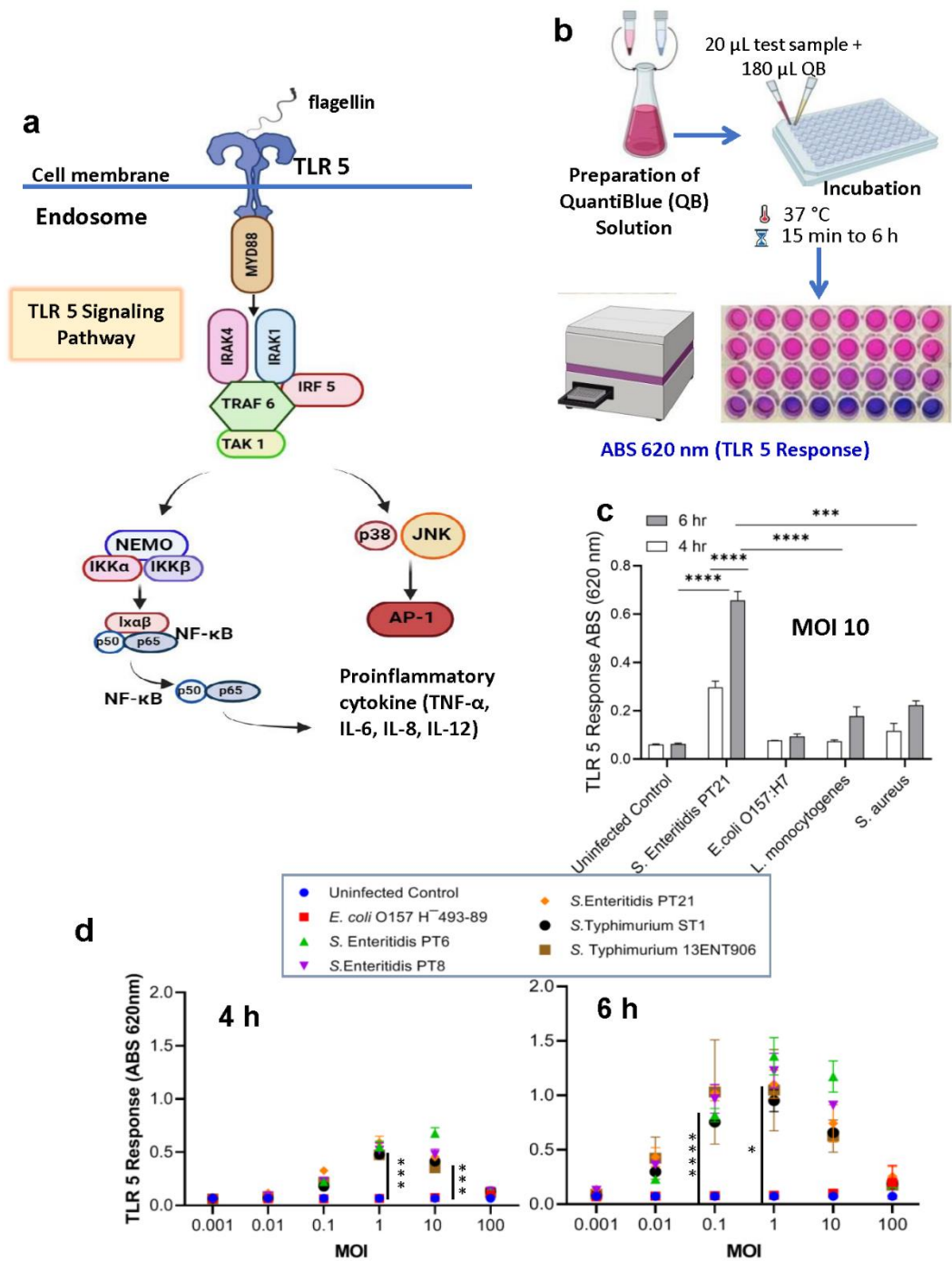


Figure 1. Cell-based assay for detection of *Salmonella* using HEK dual TLR5 sensor. (a-b) Schematic Representation: This panel illustrates the flow diagram of the cell-based assay. The process begins with preparing the Quantitblue solution to track the TLR5 response. HEK cells engineered to express TLR5 response are then exposed to varying concentrations of *Salmonella* bacteria, leading to an observable color change. The schematic details the steps in the assay, including cell culture, interaction with the bacteria, and the TLR5 response mechanism triggered by the bacterial flagella. Image created using Biorender.com. (c) This panel represents the analysis of the HEK dual TLR5 sensor's response to various foodborne bacterial pathogens tested at MOI of 1 for h and 6 h. (d) These panels present the analysis of the signal response from the HEK dual TLR5 sensor cells at different multiplicities of infection (MOI), examining the ratio of HEK cells to bacteria. The MOI ratios analyzed include various levels to determine the sensitivity and dynamic range of the assay. Left panel shows the signal response after 4 hours of incubation, while right panel depicts the response after 6 h. Data in c-d represent the average results from three independent experiments presented as mean $\pm$ SEM.

2.2. Specificity of HEK dual TLR5 sensor for detection of *Salmonella* serovars

The specificity of the HEK dual hTLR5 sensor for *S. enterica* was determined by testing the top 20 *S. enterica* serovars and non-*Salmonella* bacteria [53]. An MOI 1 and the bacterial exposure time of 4 and 6 h was used, and the cell supernatants were tested for AP activity. All 20 tested *Salmonella* strains showed positive signals and values were ~8 and 16-fold higher than the negative controls at 4 h and 6 h, respectively (Figure 2a). *E. coli* O157-493-89, a non-motile strain, was used as a negative control and baseline to ensure that observed TLR5 responses were motility-dependent (Figure 2a).

Compared to *Salmonella* Enteritidis (PT21), most non-*Salmonella* bacteria, including *Hafnia alvei*, *Serratia marcescens*, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and few *E. coli* O157:H7, O26 and O103 strains showed a 2~3-fold decrease in TLR-5 response. In contrast, 2 of 3 *Citrobacter freundii* and *E. coli* O157:H7 strains showed higher responses (Figure 2b). The cytotoxic effect of the test strains on HEK dual hTLR5 cells was analyzed by monitoring the release of lactate dehydrogenase (LDH; Figure 2c). In general, cytotoxicity values were below 20% and significantly lower than the positive control (1% Triton X-100 or *L. monocytogenes*), suggesting that the flagella-mediated signal response obtained was largely independent of cellular damage. These results indicate that the HEK dual TLR5 sensor can be used to detect *Salmonella* serovars due to TLR-5 recognizable flagellin expression [54]. Any non-*Salmonella* bacteria that produce a TLR-5 response, could be eliminated during selective enrichment and/or IMS separation.

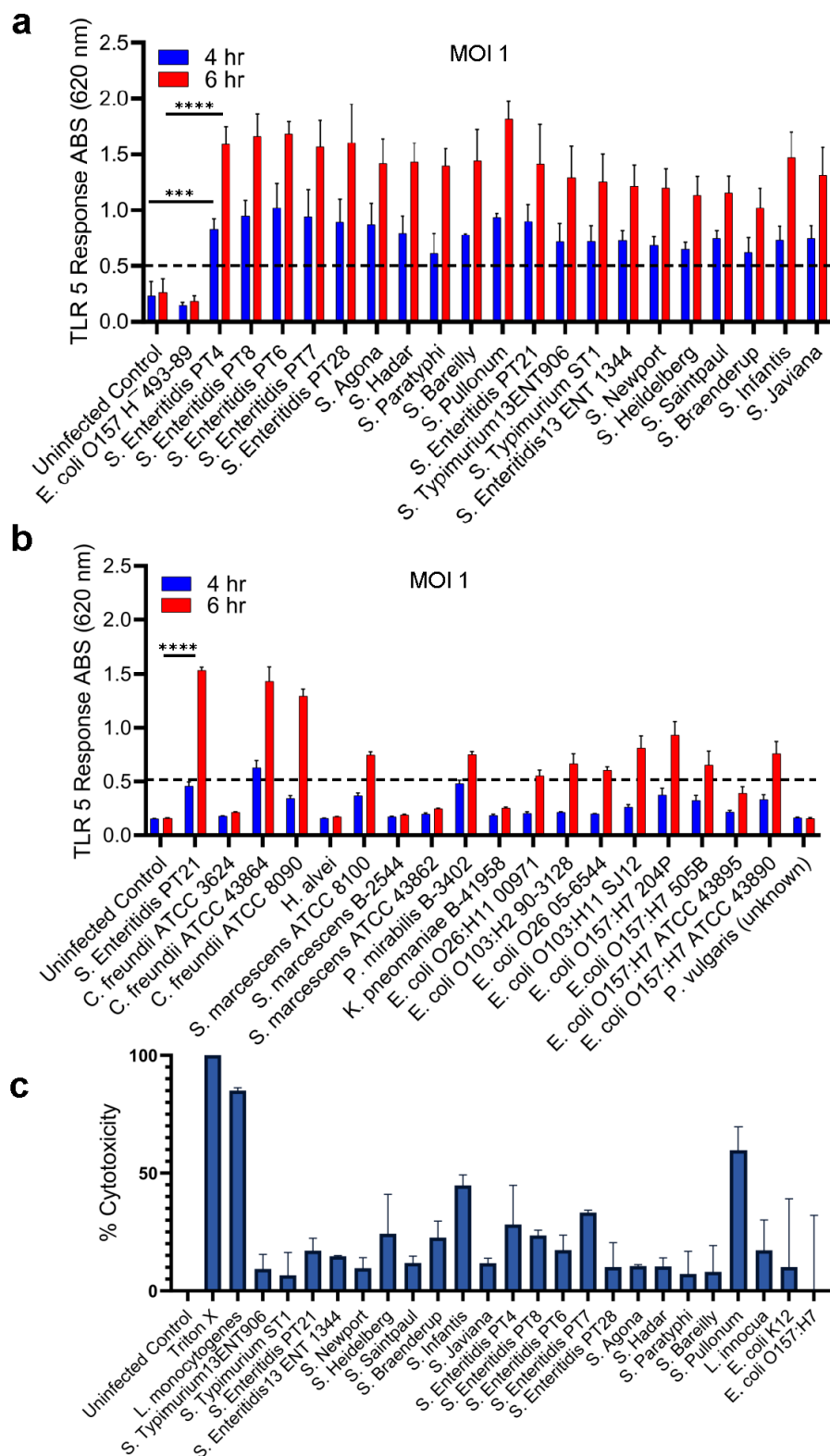


Figure 2. Specificity analysis of the HEK dual TLR5 sensor's response to various *Salmonella* serovars. (a) Specificity analysis of the HEK dual TLR5 sensor's response to various *Salmonella* serovars. The assay was conducted at a multiplicity of infection (MOI) of 1, with 4 and 6 hours exposure times. The data illustrate the sensor's ability to specifically detect different *Salmonella* serovars. *E. coli* O157:H4, a non-motile strain, was used as a negative control. (b) Specificity analysis of the HEK dual TLR5 sensor's response against non-*Salmonella* bacteria: This panel shows the analysis of the HEK dual TLR5 sensor's response to a range of non-*Salmonella* bacteria. Like panel (a), the assay was performed at an MOI of 1, with signal responses measured after 4 and 6 hours of

exposure. *Salmonella* PT21 was used as a positive control. (c) Cytotoxicity was determined by using an LDH release assay. The assay was conducted at a multiplicity of infection (MOI) of 1, with 6-hour exposure times. A 1% Triton-X100 was used as a positive control (100% cytotoxicity). Data in a-c represent the average results from three independent experiments presented as mean $\pm$ SEM.

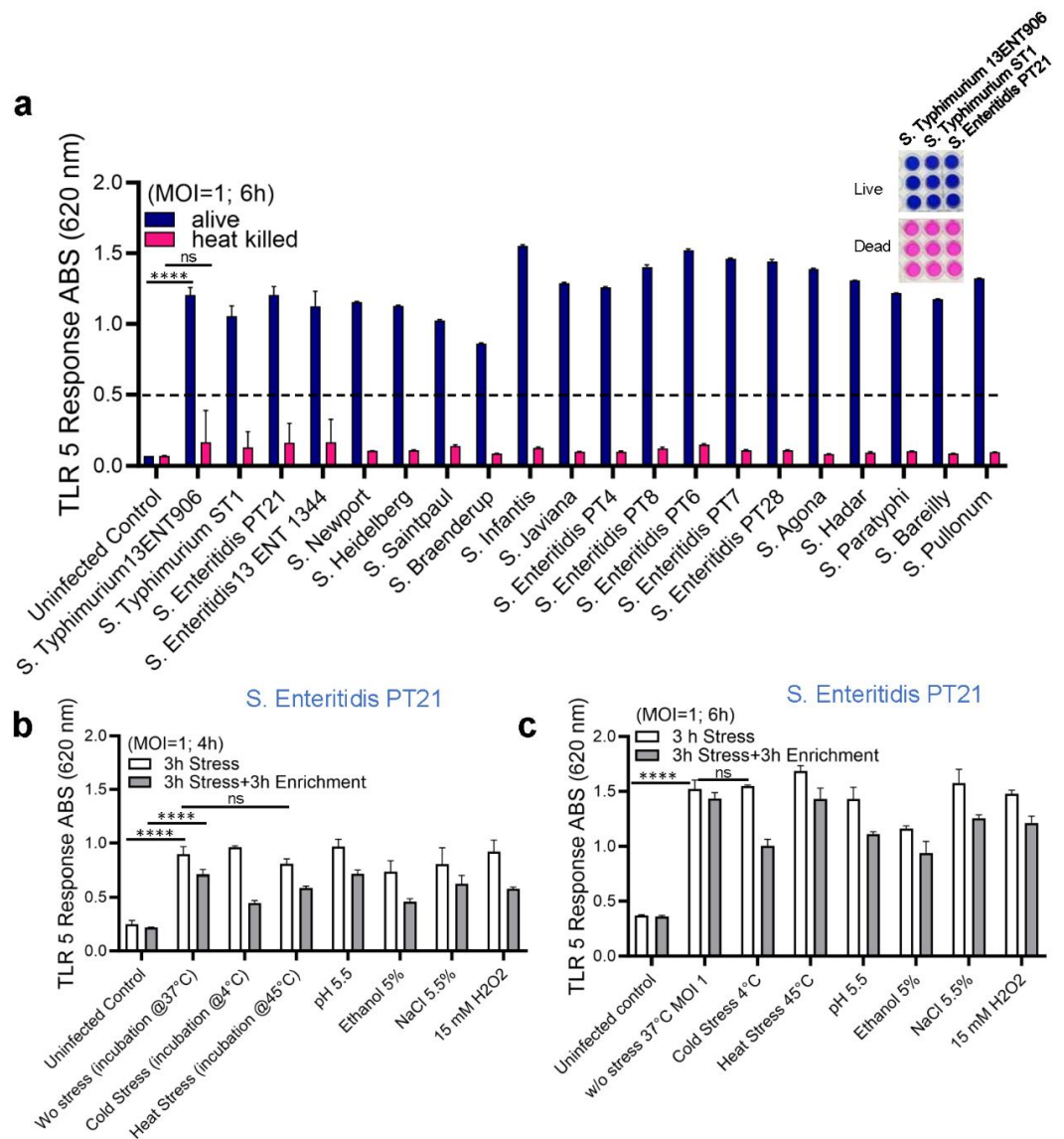


Figure 3. Analysis of the HEK Dual TLR5 Sensor's Response in the presence of stressors. (a) Response to Live and Dead Bacteria: This panel illustrates the HEK dual TLR5 sensor's response to both live and dead bacteria after 6 hours of exposure at a multiplicity of infection (MOI) of 1. Panels on the top right depict representative color changes of the HEK dual TLR5 sensors between live (purple) and dead (pink) tested samples. (b, c) Effects of Stressors on TLR5 Response: These panels investigate how different stressors affect the TLR5 response of *Salmonella* PT21. Panel (b) shows the TLR5 response after 4 hours of exposure, while panel (c) presents the response after 6 h of exposure, both tested at an MOI of 1. Data in a-c represent the average results from three independent experiments presented as mean $\pm$ SEM.

2.3. Detection of Live and Stress-Exposed *Salmonella* Using HEK dual TLR5 Sensor

We next tested if the HEK dual hTLR5 sensor could differentiate live from dead cells since processed food products may carry dead cells. To determine the sensor's response to live and dead cells, overnight-grown fresh cultures of different *Salmonella* serovars were prepared, and MOI was adjusted to 1. *Salmonellae* were treated with heat (80°C for 10 min) and plated on TSA to ensure cells

were inactivated (non-viable). AP levels were monitored after 6 h exposure of HEK dual hTLR5 sensor to *Salmonella*. Only the live cells of *S. enterica* serovars showed positive signals (~10-fold increase), while heat-inactivated dead cells did not yield a signal (**Figure 3a**)

Next, we tested the effects of various stressors on detecting *S. Enteritidis* PT21 strain that was subjected to multiple stressors, including cold (4°C), heat (45°C), acidity (pH 5.5), ethanol (5%, v/v), NaCl (5.5%), and oxidative stress (H₂O₂, 15 mM) for 3 h followed by 3 h resuscitation in TSBYE [37,55]. Bacterial cell suspensions were plated to assess cell viability and cell counts (**Table 1**) to ensure the viability of *Salmonella* following stress induction and to confirm the desirable MOI before exposure to the HEK dual hTLR5 sensor. All stressors showed positive results in the tested *Salmonella* PT21 strain at both 4 and 6 h, respectively (**Figs. 3b and 3c**); however, there was a slight but negligible reduction in signal response (**Figs. 3b & 3c**). These results suggest that the HEK dual hTLR5 sensor can detect viable or stress-exposed *Salmonella* cells.

Table 1. Effect of 3 h environmental stress on *Salmonella* Enteritidis PT21 viability and growth.

	Log ₁₀ (CFU/mL) ± SEM (N=3)						
	No stress	Cold Stress	Heat Stress	Low pH	Ethanol	NaCL	H ₂ O ₂
3 h stress	9.86±0.19	9.38±0.21	9.35±0.11	9.67±0.14	9.60±0.2	9.24±0.28	9.33±0.21
					2		
3 h	10.49±0.2	10.44±0.2	10.01±0.2	10.21±0.2	9.69±0.2	10.33±0.1	10.65±0.2
stress+3 h	2	4	1	4	9	9	4
enrichment							

2.4. Detection of *Salmonella* from Spiked Food Samples Using HEK dual TLR5 Sensor

The performance of the HEK dual hTLR5 sensor was validated using artificially spiked food samples. Initially, the limit of detection (LOD) for the sensor was determined from spiked chicken and peanut butter samples. Background flora on tested chicken thigh meat samples was 1.92 CFU/cm² but tested negative for *Salmonella*. Chicken thigh meat samples were artificially contaminated with different levels of *S. Enteritidis* PT21 cells (7.2 × 10⁵ CFU/cm², 7.2 × 10⁴ CFU/cm², 7.2 × 10³ CFU/cm², 7.2 × 10² CFU/cm², 7.2 × 10¹ CFU/cm²) and incubated overnight in the cold room (4°C). Skin surfaces were swabbed using Dacron swabs, transferred to tubes containing 1 ml of PBS (pH 7.2), serially diluted, and 100 µl samples added to HEK dual hTLR5 cells growing in a 96-well cell culture plate and incubated for 4- 6 h. Bacterial counts in the inoculums were enumerated to be 4.3 ×10⁶, 3.6 × 10⁵, 2.7 × 10⁴, 3.2 × 10³, and 1.0 × 10³ CFU/mL, respectively. Relative to uncontaminated meat, *S. Enteritidis* cells from spiked samples at all inoculation levels showed positive signals at 4 h and 6 h. The values were 2.5-6-fold higher (*p* < 0.05) than the uninoculated sample (Figure 4a). Visual examination of color changes in the wells (6 h) corroborates with absorbance readings (Figure 4a, bottom panel). These data indicate that the TLR5 sensor could detect as few as 100 *Salmonella* cells from the chicken samples.

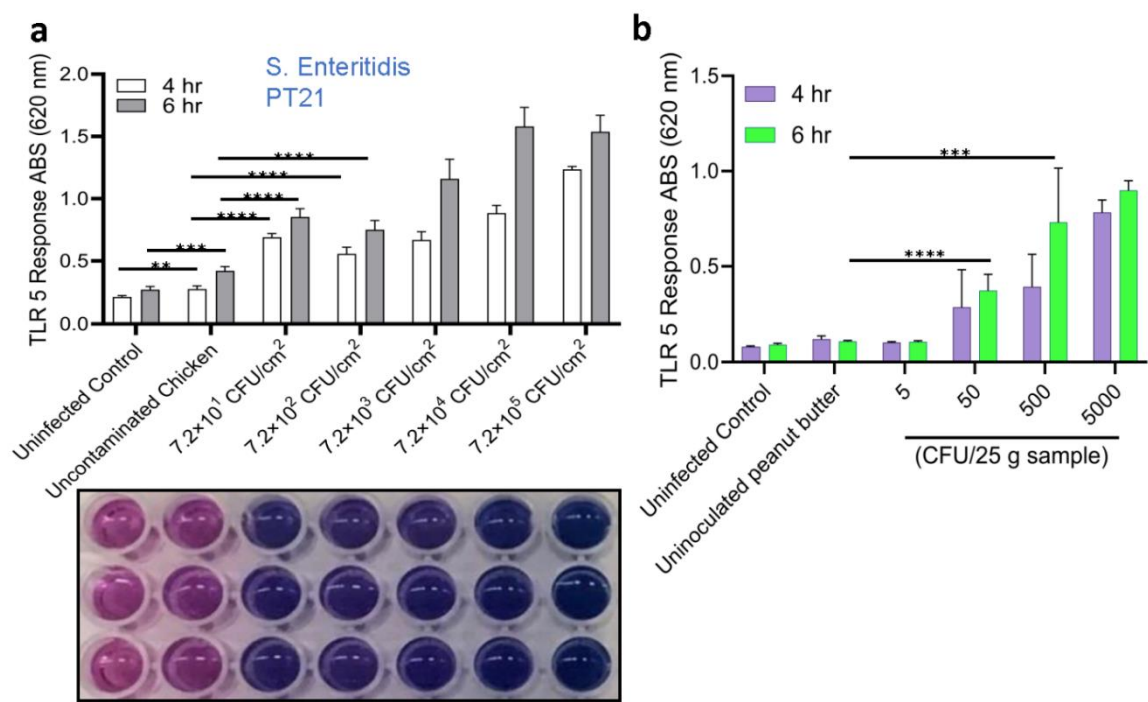


Figure 4. Limit of detection analysis of HEK dual TLR5 sensors to detect *Salmonella* from spiked chicken skin and peanut butter samples. (a) TLR 5 sensors response after 4 and 6 h incubation of spiked chicken samples. Plate image representative showing color development at 6 h (below). (b) TLR 5 sensors response after 4 and 6 h incubation of spiked peanut butter samples. Data in a-b represent the average results from three independent experiments presented as mean ± SEM.

The LOD of the sensor was also determined using peanut butter. No background flora was found in tested peanut butter samples, which were also negative for *Salmonella* (Table 2). Peanut butter (25 g each portion) was spiked with *S. Enteritidis* PT21 to obtain an initial inoculum of about 5, 50, 500, and 5000 CFU /25 g, and enrichment steps followed a shortened USDA-FSIS procedure as described before and confirmed via plate CFU counts (Table 2) (Singh et al. 2014; USDA-FSIS). The initial inoculum containing $50 - 5 \times 10^3$ CFU/25 g showed positive signals, and the incremental values were proportional to the initial inoculum levels. Furthermore, these values significantly differed from the signals obtained from uninoculated or peanut butter inoculated with 5 CFU/25g (Figure 4b). These data indicate that the sensor can detect about 50 *Salmonella* cells (CFU/25g) (as an initial inoculum) without interference from peanut butter samples.

Table 2. Recovery of *Salmonella* Enteritidis PT21 from contaminated peanut butter at each step of sample enrichment.

Inoculation Level (CFU/25g sample)	Log ₁₀ (CFU/ml) ± SEM (N=3)			
	Pre-Enriched		Enriched	
	TSA	XLD	TSA	XLD
0	No growth	No growth	No growth	No growth
~5	No growth	No growth	2.86±0.21	3.09±0.18
~50	2.68±0.17	2.51±0.11	4.06±0.14	4.18±0.19
~500	3.94±0.21	3.85±0.19	6.32±0.22	6.39±0.24
~5000	4.35±0.13	4.44±0.18	6.60±0.23	6.42±0.14

TSA, Tryptic soy agar; XLD, Xylose lysine deoxycholate.

2.5. Validation of Sensor with Spiked Food Samples

Next, we tested the HEK dual hTLR5 sensor with additional spiked food samples (100 CFU/25g; chicken thigh meats, black pepper, peanut kernel, peanut butter, mayonnaise, peach) where sample preparation and enrichment steps followed a shortened USDA-FSIS procedure [53,56] (Table 3). One hundred microliters of enriched samples were tested for their response to HEK dual hTLR5 cells. Each experiment was repeated five times for all food samples on different days.

All *S. Enteritidis*-inoculated samples, irrespective of food types, produced ~5-10-fold higher signals ($p < 0.001$) than the uninoculated food samples (Figure 5a). Interestingly, the signal from inoculated mayonnaise was lowest among all the food samples tested. Furthermore, Microscopic observation of HEK dual hTLR5 cells after exposure to various food samples also did not affect the cell monolayer integrity or cellular morphology (Figure 5b). Parallely, the same set of samples was tested with plate counting (on XLD) methods and PCR for verification. Compared to the TLR5 sensor, PCR could only detect *Salmonella* in peanut kernel, while other spiked food samples post-enrichment tested negative (Table 3). Negative PCR results from inoculated food samples are attributed to the presence of PCR inhibitors. These data suggest that the TLR5 cell sensor can detect as low as 100 CFU/25g of pre-enriched food samples with complex food matrix.

Table 3. Recovery of *Salmonella* Enteritidis PT21 from spiked (~100 CFU/25 g, except peach (~100 CFU/100 g) food samples at each step of sample enrichment and concentration.

Log ₁₀ (CFU/ml) ± SEM (N=3)								
Food Sample	After Selective Enrichment		Without IMS		IMS		Counts (CFU/100 μL)	PCR (<i>invA</i> 796 bp)
	TSA	XLD	TSA	XLD	TSA	XLD	XLD	
Black pepper (U)	0	0	0	0	0	0	0	-
Black pepper (I)	3.91±0.18	4.04±0.11	3.87±0.14	4.05±0.21	3.98±0.08	4.07±0.14	3.07±0.11	-
Chicken (U)	0	0	0	0	0	0	0	-
Chicken (I)	3.85±0.15	3.92±0.08	3.94±0.19	4.10±0.21	3.91±0.15	4.10±0.11	3.1±0.09	-
Mayonnaise (U)	0	0	0	0	0	0	0	-
Mayonnaise (I)	3.94±0.22	4.02±0.14	3.72±0.18	3.94±0.12	3.72±0.21	3.92±0.18	2.92±0.13	-
Peanut kernel(U)	0	0	0	0	0	0	0	-
Peanut kernel (I)	3.92±0.26	4.09±0.19	4.26±0.22	4.29±0.18	4.07±0.14	4.13±0.19	3.13±0.18	+
Peanut butter (U)	0	0	0	0	0	0	0	-
Peanut butter (I)	5.53±0.24	5.18±0.18	5.36±0.25	5.46±0.28	5.39±0.22	5.47±0.24	4.47±0.16	-
Peach (U)	0	0	0	0	0	0	0	-
Peach (I)	3.71±0.14	3.88±0.16	3.72±0.12	4.08±0.19	3.91±0.16	4.33±0.14	3.33±0.11	-

U, uninoculated; I, inoculated; IMS, Immunomagnetic separation.

Next, we employed immunomagnetic separation (IMS) to concentrate *Salmonella* from enriched food samples before testing with the HEK dual hTLR5 sensor to determine if the signal could be augmented [47]. Before conducting food analysis using the IMS method, we investigated the immunomagnetic bead (IMB) based capture rate of freshly grown diluted (8.99 to 1.99 log CFU/mL bacteria) cultures of *S. Enteritidis* by plating. The capture rate was calculated to be >90% for all concentrations, regardless of the bacterial load (Table S1). The application of IMS significantly improved the signal except for the inoculated black pepper (Figure 5a). The sample-to-result time was estimated to be 15 h.

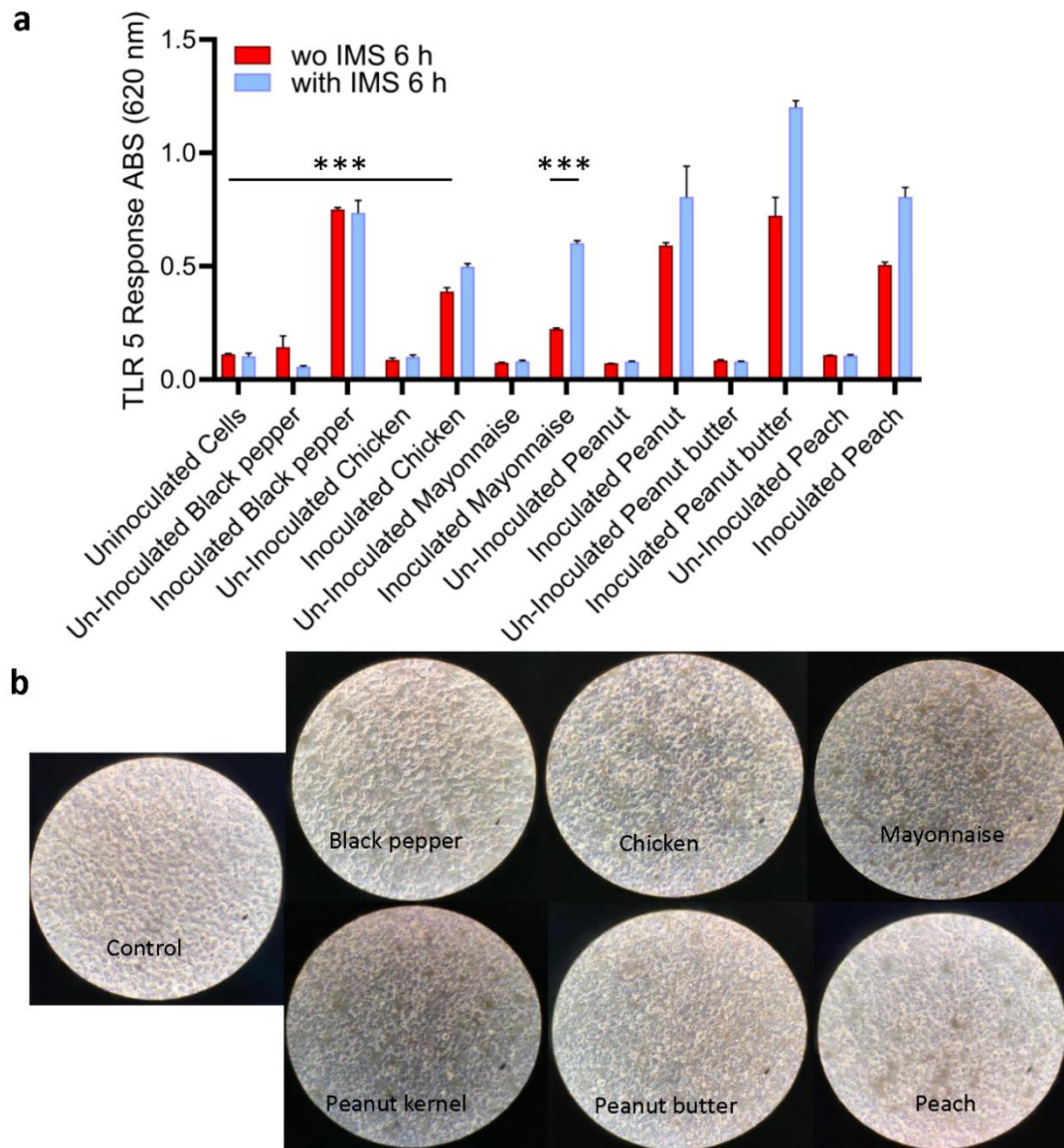


Figure 5. Detection of *Salmonella* using HEKdualTLR 5 sensor from spiked food samples. (a) TLR 5 sensors response after 6 h incubation of spiked food samples. Spiked food samples were subjected to 4-h pre-enrichment and 4-h selective enrichment (Initial contamination level was ~100 CFU /25 g food sample). *Salmonella* was captured and concentrated using immunomagnetic beads (IMB) in tests with IMS. Data represent the average results from three independent experiments presented as mean $\pm$ SEM. (b) Representative microscopic images show an intact cell monolayer treated with bacterial suspension concentrated from all food samples.

3. Discussion

The risk-based rapid and sensitive detection of *Salmonella* species is highly desirable to protect consumers from food-associated illnesses and promote food safety and biosecurity. Although various detection platforms have been developed in recent years, the traditional culture-based detection methods require 4–7 days to yield results [37,56–58], whereas rapid methods still take at least 24–48 h [28,38,59–61]. This delay poses a significant inconvenience to the food industry, especially for products with short shelf lives. Additionally, the requirement to store products until microbiological safety is confirmed increases storage costs. Therefore, products often enter the supply chain before test results are available, which is costly and results in hundreds of recalls each year and millions of pounds of food wasted, which is unsustainable to meet the future food demand [62,63]. This situation creates a high demand for quick, accurate, easy-to-use pathogen detection tools to reduce recalls,

minimize food waste and financial losses, and prevent foodborne illnesses. Mammalian cell-based assays are highly promising for functional screening because they can detect viable pathogens in real-time [39,64]. These assays monitor host-pathogen interactions [40,41], ensuring that non-pathogenic, non-hazardous, dead, or non-toxic agents do not produce false results.

This study uses a human embryonic kidney (HEK293) cell line expressing Toll-Like Receptor 5 (TLR-5) and a chromogenic reporter system to detect viable *Salmonella* in a 96-well tissue culture plate. Flagellin is a bacterial protein that polymerizes into the flagellar filament and allows bacteria to be motile. During the bacterial invasion of the host cell, flagellin is recognized by TLR5 and activates innate immune signal transduction cascades [54,65,66]. TLR5 is an innate immune receptor located on the cell surface and consists of an extracellular leucine-rich repeat (LRR), transmembrane, and intracellular domains [67]. TLR5 uses the extracellular domain to recognize flagellin as a pathogen-associated molecular pattern (PAMP). It activates the MyD88-dependent signaling pathway and NF- κ B-mediated production of proinflammatory cytokines, and this response can be measured using the chromogenic detection system (Figure 1) [45].

To test our hypothesis that the HEK-TLR-5 sensor can detect viable *Salmonella* selectively, we first determined the motility of different types of common foodborne bacterial pathogens (*Salmonella* Enteritidis PT21, *E. coli* O157:H7, and *Listeria monocytogenes*). The motility test (plating results performed on agar surface) and the signals obtained from the HEK dual hTLR5 sensor correlated as *Salmonella* was found to be the most motile bacteria with the highest swimming ability, and the sensor with a flagella recognition feature showed the highest response when tested with *Salmonella* Enteritidis PT21.

Furthermore, flagellar expression is critical for obtaining positive signals. We verified relative flagellar expression in test strains by measuring their motility on agar plates containing variable amounts of agar (0.3 – 1.0%; w/v) [52]. Swimming motility is the movement in liquid or low-viscosity conditions (up to 0.3% agar concentration). Unlike swarming motility, swimming motility requires a functional flagellum, but neither quorum sensing systems nor biosurfactants [51,52]. Plates with up to 0.3% agar concentration are referred to as 'swim plates' because highly motile flagellum-dependent bacteria can swim through the porous medium, leading to a large area of colonization [51]. Plates with agar concentration higher than 0.3% are known as 'swarm plates' because the reduced pore size prevents swimming through the agar, and extensive colonization of the plate occurs only through bacterial migration across the surface.

Specificity testing of the HEK-hTLR5 Reporter Cell-Based Sensor showed a high response (6-fold increase; Figure 2b) with all the top 20 *Salmonella* serovars tested. Testing with the non-*Salmonella* organisms suggested minimal signals with several common foodborne bacterial pathogens such as Enterohemorrhagic *Escherichia coli* (EHEC) strains, *H. alvei*, *S. marcescens*, *K. pneumoniae*, and *Proteus vulgaris*, suggesting their flagellin molecules may be poorly recognized by TLR5 [54]. However, 2 of 3 *Citrobacter* strains showed cross-reactivity, which may be attributed to high TLR-5 recognizable flagellin expression. More importantly, when tested with food samples, the sensor was specific as it showed no response to natural microflora or several uninoculated food samples. Black pepper, chicken, mayonnaise, peanut kernels, peanut butter, and peaches were chosen because these products are commonly linked with *Salmonella* outbreaks, and some are consumed without heat treatment. In addition, they have high protein, fat, and carbohydrate contents, which may cause interferences. Our data demonstrate that the tested uninoculated food samples had minimal or no interference with sensor response, but *Salmonella* inoculated samples showed a strong response. Surprisingly, PCR assay with inoculated food samples gave negative PCR results (except peanut kernel) (Table 3) and the negative PCR results are attributed to the presence of PCR inhibitors in these samples [68].

The sensitivity of the cell-based sensor in pure culture suggested that an MOI of 0.1 in the 10^5 seeded HEK-hTLR5 reporter cells on a 96-well plate elicited a positive signal (Figure 1d). These data indicate that the LOD in pure culture is $\sim 10^5$ CFU/mL (10^4 CFU/well in 100 μ L). Our results are comparable with the LOD of the recently developed Next-Generation Sequencing Assay for direct detection of *Salmonella* [69]. Further testing of LOD in spiked food samples suggested the LOD is as low as 50-100 CFU/25g of food samples, which has the potential to outperform other molecular

detection methods such as ELISA or PCR [61]. Our results are comparable with previous research showing an IMS-PCR procedure designed to isolate and detect *S. enterica* in vegetable salad, egg, and pork meat with a detection sensitivity of 10² CFU/mL [70]. However, one significant advantage of the HEK-hTLR5 cell-based sensor is its ability to selectively detect viable *Salmonella* and not respond to dead cells (Figure 3a). Additionally, although the sensitivity of the sensor may be high, accurate results may not be obtained due to the low level of pathogen survival in stressed food conditions. The HEK-hTLR5 reporter cell-based sensor was further validated and tested positive for the detection of *Salmonella* in stressed conditions such as cold (4°C), heat (45°C), acid (pH 5.5), ethanol (5%), NaCl (5.5%), and oxidative stress (15 mmol H₂O₂). A brief sample enrichment step allowed the resuscitation of stressed or injured cells before detection.

Our approach of combining enrichment steps with IMS improved the TLR-5 sensor sensitivity in certain food products, such as mayonnaise and yielded comparable results with other studies that used IMS tandem fluorescent probes based on quantum dots-antibody (QDs-Ab) for *Salmonella* testing [71]. However, the sensitivity of this assay was lower than our cell-based sensor (500 CFU/mL). Similarly, a protein chip immunosensor has been exploited to detect *Listeria* spp. by lowering the detection limit to 2.5 CFU/mL with the pre-enrichment culture of IMS [72]. While IMS can be evaluated as a potential pretreatment tool for the rapid isolation and enrichment of many foodborne pathogens in food, the bacterial surface antigens from dead cells could still bind the capture antibody. Therefore, our TLR-5 cell-based sensor combined with the IMS method is significantly more advantageous when viable pathogens are sought. Thus, developing cell-based assays is critical for functional screening and eliminating false-negative results from dead, non-hazardous, or non-pathogenic bacteria.

In conclusion, our two-step method (enrichment and TLR-5 sensor), with a significantly lower detection time of 15 h from sample to result, was found to be highly specific for the detection of live salmonellae in complex food matrixes in the presence of stressors with a sensitivity of 50 CFU/25 gram of food. Therefore, the TLR-5 sensor could be used as a risk-based screening tool for viable *Salmonella*.

4. Materials and Methods

4.1. Bacterial Cultures and Motility Testing

Bacterial strains (Table 4) were stored as 10% glycerol stocks at -80°C [53]. To recover from frozen stock, each strain was streaked onto tryptic soy agar (TSA) (Thermo Fisher Scientific, Rochester, NY, USA) plate and incubated at 37°C for 18 h to obtain pure colonies. A single colony of each strain was inoculated and propagated in tryptic soy broth (TSB) with 0.5% yeast (Thermo Fisher Scientific, Rochester, NY, USA) at 37°C for 18 h with shaking at 120 rpm.

For bacterial motility (swimming vs swarming) assay, a previous method [52] was followed. Briefly, bacteria were spot inoculated onto TSA plate containing either 0.3% w/v (for swimming motility) or 1.0% w/v (swarming motility) agar, incubated at 37°C for 18-24 h and recorded colony diameter.

Table 4. List of bacterial strains used in the study from our culture collection.

<i>Salmonella enterica</i> serovars	Non- <i>Salmonella</i> cultures
Enteritidis PT4	<i>Escherichia coli</i> O157 H ⁻ 493-89 (flagella Negative control)
Enteritidis PT8	<i>E. coli</i> O26:H11 00971
Enteritidis PT6	<i>E. coli</i> O103:H2 90-3128
Enteritidis PT7	<i>E. coli</i> O26 05-6544
Enteritidis PT28	<i>E. coli</i> O103:H11 SJ12
Enteritidis PT21	<i>E. coli</i> O157:H7 204P
Enteritidis 13ENT1344	<i>E. coli</i> O157:H7 505B

Typhimurium 13ENT906	<i>E. coli</i> O157:H7 ATCC 43895
Typhimurium ST1	<i>E. coli</i> O157:H7 ATCC 43890
Agona 12ENT1356	<i>Citrobacter freundii</i> ATCC 3624
Hadar 13ENT979	<i>Citrobacter freundii</i> ATCC43864
Paratyphi 11J85	<i>Citrobacter freundii</i> ATCC8090
Bareilly 12ENT1164	<i>Hafnia alvei</i>
Pullorum DUP-PVUII 1006	<i>Proteus vulgaris</i> DUP-10086
Newport 13ENT1060	<i>Proteus mirabilis</i> B-3402
Heidelberg 18ENT1418	<i>Serratia marcescens</i> ATCC 8100
Saintpaul 13ENT1045	<i>S. marcescens</i> B-2544
Bradenderup 12ENT1138	<i>S. marcescens</i> ATCC 43862
Infantis 13ENT866	<i>Klebsiella pneumoniae</i> B-41958
Javiana 13ENT86F	<i>Listeria monocytogenes</i> 104033S

4.2. Cell Culture

HEK-Dual hTLR 5 (NF-KB-IL8) cells were purchased from InVivoGen (San Diego, CA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM) containing heat-inactivated 10% fetal bovine serum, antibiotics (Penicillin-Streptomycin, Normocin, Hygromycin B Gold and Zeocin). Cells were incubated at 37°C with 5% CO₂ in a humidified incubator. Cells were seeded in tissue culture flasks, and medium was refreshed twice a week until cells reached the desired confluency. HEK 293 hTLR 5 cell monolayer was maintained and harvested using trypsin, and a hemocytometer with Trypan blue staining determined viable cell concentrations. A cell concentration of $\sim 5 \times 10^5$ /ml were added to each well of a 96-well cell culture plate and incubated overnight at 37°C under 5% CO₂ in a humidified incubator for 24 h. Then, HEK293 cells were treated with bacteria to obtain the desired multiplicity of infection (MOI) value.

4.3. Cell-Based Detection of Bacterial Culture

HEK cell monolayers were prepared and maintained as described above in a 96-well plate. Overnight bacterial cultures were obtained and diluted to the desired concentration using PBS, which was then transferred to DMEM media. Heat-killed bacteria suspensions were prepared with heat treatment at 80°C for 10 min. Lethality of all bacteria spp. was confirmed by plating on tryptic soy agar (TSA) (Thermo Fisher Scientific, Rochester, NY, USA) plates. A 100 μ L aliquot of culture suspension in DMEM was added to each well. After 4 and 6 h of incubation at 37°C, a 20 μ L aliquot of the supernatant was collected from each well and transferred into another empty 96-well plate and mixed with 180 μ L of the Quanti Blue Solution (InVivoGen, San Diego, CA, USA) used as a detection reagent to quantify Secreted Embryonic Alkaline Phosphatase (SEAP) activity. After one hour of incubation at room temperature in the dark, absorbance at 620 nm was measured using a microplate spectrophotometer (BioTech Epoch). SEAP is a truncated form of placental AP and is widely used as a reporter gene. SEAP is secreted into cell culture supernatant and offers many advantages over intracellular reporters. It allows the determination of reporter activity without disturbing the cells, does not require the preparation of cell lysates, and can be used for kinetic studies.

4.4. Specificity/Selectivity of Cell-Based Sensor

HEK Dual hTLR 5 (NF/IL8) reporter cells were generated from HEK-Dual Null cells by stable transfection of the human TLR5 (hTLR5) gene (InVivoGen, San Diego, CA). They respond to low concentrations of TLR5 agonist flagellin. To test the specificity and selectivity of this cell line, a non-motile *E. coli* O157-493-89 and several non-Salmonella cultures were used (Table 5).

4.5. Limit of Detection of Cell-Based Sensor

HEK Dual hTLR 5 (NF/IL8) reporter cell line was used to detect bacterial flagellin. NTS salmonellae express flagellin and are motile. To determine the limit of detection of this sensor, we prepared *Salmonella* cultures with different dilution levels. The MOI was adjusted to 100, 10, 1, 0.1, 0.01, and 0.001 (HEK cell count was seeded to $\sim 10^5$ bacteria/well). After treating HEK Cells with specified bacteria levels for 4 h and 6 h, the SEAP level was assayed for each well by measuring the absorbance values at 620 nm. The lowest concentration of bacteria, which caused a significant TLR 5 response change, was considered the limit of detection for the sensor. Depending on the experimental design, in most cases, an absorbance (620 nm) cut-off value was set to 0.5.

4.6. *Salmonella* Enteritidis Analysis in Spiked Food Samples

Twenty-five grams of each food sample (except peach, which was sampled at 100 ± 5 g) was artificially contaminated with 500 μ L of *S. Enteritidis* PT21 suspension and placed in sterile sampling bags in a biosafety cabinet. The contamination level was set to approximately 1×10^2 CFU/g. All samples were mixed with 225 ml (peach - 150 mL) of buffered peptone water and incubated at 37°C for 4 h. Then 1 ml of pre-enriched sample was transferred to 10 mL of RV Broth for selective enrichment and incubated at 42°C for 4 h. Finally, 1 mL of selective enriched sample mixed with 20 μ L of Anti-*Salmonella* Dynabeads (Dynabeads™ anti-*Salmonella*. Catalog number: 71002; ThermoFisher) for immunomagnetic separation (IMS). Plating was performed after each step to determine the microbial load. After bead separation, bacteria were suspended in DMEM for cell-based analysis. A 100 μ L of sample/well were transferred to the HEK cell monolayer in 96-well plates. TLR 5 response analyses were performed following 4 and 6 h of bacteria-cell interaction.

4.7. Detection of Stressed Cells

S. Enteritidis PT21 cells were freshly prepared in TSB and then exposed to cold (4°C), heat (45°C), acid (pH 5.5), ethanol (5%), NaCl (5.5%), and oxidative stress (15 mmol H₂O₂) for 3 h [55]. Bacterial cells were washed with PBS, resuspended in DMEM media, added to the HEK cell monolayer, and assayed as above.

4.8. Cytotoxicity Assays

To determine HEK cells' viability during pathogen exposure, supernatants from the wells were assayed for lactate dehydrogenase release (Cayman). Two controls were included for calculating percent cytotoxicity (LDH release). The low control consisted of supernatant from untreated cells with no exposure to bacteria. The high control was from cells treated with 0.1% Triton X-100 for one minute. For plate-based bacterial motility assay, a previous method [52] was followed.

4.9. Polymerase Chain Reaction (PCR) confirmation

Black pepper, Chicken thighs, mayonnaise, peanut kernel, peanut butter, and peach samples were inoculated with approximately 10^2 CFU/25 g of *S. Enteritidis* PT21 and were placed in 37°C for 4 h in pre-enrichment media (Buffered Peptone Water). After 4 h of pre-enrichment, samples were enriched selectively in Rappaport-Vassiliadis (RV) broth at 42°C for 4 h. Then, samples were subjected to testing using the TLR cell-based sensor platform. Parallely, the same set of samples was tested with plate-counting methods. PCR was performed to verify the accuracy of both detection methods. DNA was extracted from the enriched samples using boiling methods.

A PCR reaction was used to amplify the gene targeting the *Salmonella* Invasion protein A (*invA*) with an amplicon size of 796 bp [37]. The forward primer sequence used was 5'-CGGTGGTTTAAAGCGTACTCTT3', and the reverse primer sequence used was 5'-CGAATATGCTCCACAAGGTTA3'. The reaction mixture (25 μ L) contained 1 μ g of DNA template, 0.2 μ M of each primer, 2.5 mM MgCl₂, 200 μ M of dNTP, 1 $\times$ GoTaq Flexi buffer and 1 U of GoTaq Flexi DNA polymerase (Promega). The reaction took place in the Profelx PCR system with an initial denaturation at 94°C for 3 min, 35 amplification cycles consisting of 1 min of denaturation at 94°C, 1.5

min of annealing at 50, and 1.5 min of elongation at 72°C. DNA amplicons (*invA* 796 bp) were detected in agarose gel (1.5%, wt/vol) containing 1 µg of ethidium bromide/mL [37].

4.10. Statistical Analysis

All data were analyzed through GraphPad Prism software. An unpaired t-test was used when comparing two datasets. ANOVA with Tukey's multiple comparison test was used to compare more than two datasets. At least 3 independent experiments were performed for all data presented with mean ± standard error of the mean (SEM).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Analysis of Bacterial Motility on Agar Plates; Table S1: Performance of Anti-Salmonella Dynabeads.

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