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Detection of Bacterial Contamination in Food Matrices by Integration of Quorum Sensing in a Paper-Strip Test.

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ABSTRACT: There are an estimated 48 million cases of foodborne illness in the United States every year. In general, these illnesses are the result of unintentional contamination and improper food handling. Because bacterial contamination plays a major role in food spoilage and, hence, in foodborne illnesses, it is important to design easy, portable methods to detect bacteria in food. Quorum sensing (QS) enables bacteria to communicate with one another and by doing so they can modulate their behavior in a cell-density dependent manner. In bacteria, quorum sensing molecules (QSMs) are known to control several factors such as virulence factor production, antibiotic production, biofilm formation, and gene regulation. Herein, we demonstrate the applicability of whole cell biosensing systems for the early identification of food contamination via detection of QSMs. Additionally, we have developed a portable system for detection of bacterial contamination using microdots of immobilized whole cell-based biosensors on paper that boast nanomolar level detection of QSMs in two different food matrices, namely beef and milk. Limits of detection ranged from 1 x 10^{-7} M to 1 x 10^{-9} M with relative standard deviations (RSDs) of 1-16%. This rapid, easy, and portable test could be a useful tool for use in the field and during all stages of food manipulation, i.e., from farms to distribution, storage, sales, and preparation prior to consumption, to ensure that food is free of bacterial contamination.

Introduction

Foodborne illnesses are a major health issue that affect roughly 48 million people and result in 3,000 deaths every year in the United States alone[1, 2]. Most of these diseases are due to pathogens that enter the gastrointestinal tract via consumption of contaminated food. Food spoilage can occur at any stage, from farms and slaughterhouses to distribution at restaurants and shopping centers. This problem is further compounded by the fact that the current market demands that food be stored for long periods of time, which, of course, increases the likelihood of spoilage. The economic and health consequences of food spoilage have fueled efforts to detect contamination as early as possible. The determination of bacterial count, colony forming units (cfu) per gram of food, has been used in a number of applications, ranging from evaluation of food spoilage to assessment of the effectiveness of an agent intended to prevent food spoilage[3, 4]. However, cfu determination cannot be considered accurate because it neglects the bacterial population that cannot grow under the conditions of the cfu assay. Hence, alternative methods to count bacteria have been developed, including epifluorescence[5, 6], an ATP bioluminescence assay[7,8], impedance measurements[9,10,11,12], and spectroscopic methods. Fourier transform infrared (FT-IR) and short-wavelength-near-infrared (SW-NIR) spectroscopy have been used to discriminate among pathogens[13] and to detect overall food spoilage[14], respectively. Furthermore, molecular methods, like polymerase chain reaction[15, 16], and other field amenable tests[17-24] have been developed to detect food spoilage caused by microorganisms. Other assays have been developed to detect biomarkers of bacterial contamination by exploiting specific bacteria's ability to produce toxins, biofilms, volatile organic compounds, and metabolites[25-28]. However, the practicality of these tests is limited due to their requirement of expertise, specialized instrumentation, and detection of only specific bacteria based on its biomarker. Although specificity would be preferable in most circumstances, in this case it makes the design and use of assays difficult, as specific systems need to be engineered for each separate strain of bacteria when food spoilage may occur due to multiple different strains. Thus, the development of an easy, portable method that requires no specialized tools for food spoilage identification based on the detection of a small molecule that serves as a universal biomarker for bacterial contamination would be highly beneficial in the fight against foodborne illnesses.

Bacteria are known to communicate with one another by producing, releasing, and responding to small signaling molecules known as quorum sensing molecules (QSMs)[29]. When these molecules reach a critical threshold concentration corresponding to a given cell density, certain specialized genes are expressed. This type of cell-to-cell communication, termed quorum sensing (QS), enables bacteria to regulate specialized phenotypes, including virulence factor production and biofilm formation, depending on their population size. Among the QSMs employed for bacterial chatter are *N*-acyl homoserine lactones (AHLs) in Gram-negative bacteria, autoinducing peptides (AIPs) in Gram-positive bacteria, and autoinducer-2 (AI-2) in both Gram-negative and Gram-positive bacteria. Since AHLs are found in the quorum sensing systems of nearly every Gram-negative bacteria[30] and AI-2 is the most common known QSM[31], we postulate that they are suitable for use as biomarkers for the presence of bacteria.

Quorum sensing signaling is an important component of bacterial growth, and, therefore, it stands to reason that it would also play a significant role in food spoilage. Previous studies have shown that the QSM population detected in food stored under various conditions differs depending upon the particular condition[32, 33]. Since it is well known that storage conditions impact the rate of bacterial growth, it is reasonable to conclude that those conditions also impact QS signaling[34, 35]. It has also been demonstrated in a number of different foods that elevated levels of QSMs are associated with food spoilage[33, 34, 36]. The impact of QS signaling on food can also be observed by altered QSM concentrations and gene expression in spoiled food[32]. QS is deeply implicated in biofilm formation, enabling bacteria to propagate and establish themselves on contaminated surfaces by generating complex three-dimensional structures where bacteria are included and protected from other bacteria and antibiotics[37]. This may be especially important in food matrices because of the non-uniform nature of food matrices and their bacterial populations. Examples of food-related biofilm-forming bacteria include *Bacillus* spp. from dairy processing plants[38] and *Salmonella* from poultry processing plants[39]. In some foodborne pathogens, biofilm formation has been linked to quorum sensing. Specifically, wild type *Hafnia alvei*, a milk and meat pathogen that employs AHL-based QS signaling for communication, regularly forms biofilms, while a mutant from the same bacterial species *Hafnia alvei* that is unable to synthesize AHLs, cannot form biofilms[40]. It is also known that the expression of food degrading enzymes whose expression is controlled by QS signaling are important in causing food spoilage[32, 41].

In light of the evident important role that QS plays in food contamination and spoilage, it is important to develop methods capable of the rapid, sensitive, and reliable detection of QSMs because methods that can detect QSMs in food samples should allow for the early detection of food spoilage and, thus, the prevention of illnesses caused by foodborne bacterial contamination. Currently, conventional physical-chemical methods, most commonly separation techniques coupled to various detection principles such as mass spectrometry, are used to detect QSMs. However, these techniques require sample preparation, expensive instrumentation, and specialized technical personnel[42]. On the other hand, whole cell biosensing systems are sensitive, with limits of detection in the micromolar to nanomolar ranges[43], rapid, easy to use, cost-effective, and require simple instrumentation with minimal or no sample preparation. By repurposing the regulatory elements of the *las* operon found in *P.* aeruginosa, whole cell biosensors have been developed for the luminescent and colorimetric detection of AHLs[43]. A separate biosensor has been developed for AI-2 detection using a genetically modified strain of *Vibrio harveyi*[44]. Furthermore, these sensors are amenable to multiplexing and high-throughput analysis, as

well as on-site monitoring when incorporated into portable devices as has been illustrated by the development of a paper-based assay designed to detect QSMs engineered using whole cell-based biosensors immobilized on filter paper[45]. To that end, we utilized cell-based biosensing systems to develop a facile, portable analytical method for the quantitative detection of QSMs in food matrices using a rapid, paper-based assay. This manuscript describes the application of whole cell-based biosensors immobilized on filter paper in a field amenable test designed for *in situ* evaluation of bacterial contamination in liquid and solid food.

Experimental

Materials

AHLs, *N*-hexanoyl-DL-homoserine lactone (C-6 HSL), N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C-12 HSL), *N*-dodecanoyl-DL-homoserine lactone (C-12 HSL), ampicillin, and kanamycin were purchased from Sigma (St. Louis, MO). AI-2 was obtained from Omm Scientific (Dallas, TX). Luria Bertani (LB) broth, nutrient broth, and vitamin free casamino acids were purchased from Difco (Sparks, MD). Acetonitrile used in all experiments was of HPLC grade and was from VWR Scientific (Pittsburgh, PA). The 96-well microtiter plates were purchased from Costar (Corning, NY). Lean ground beef (93% lean), fat free milk (skim milk), 2% fat milk, and whole milk were purchased from a local grocery store. Reverse osmosis (RO) filtered water (Milli-Q water purification system, Millipore, Bedford, MA) was utilized in all experiments as needed. Whatman Grade 4 qualitative filter paper was obtained from VWR scientific. The microcentrifuge was purchased from Eppendorf (Westbury, NY). The orbital shaker incubator was from Fisher Scientific (Fair Lawn, NJ). Bioluminescent measurements were performed using the FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC). The Spectronic 21D spectrophotometer used to measure optical density of the bacterial cultures was purchased from Artisan Scientific (Champaign, IL). Wax printing was accomplished using a ColorQube 8570 printer purchased from Xerox Corporation (Norwalk, CT).

Plasmids, bacterial strains, and culture conditions

The plasmids pSB406 and pSB1075 were originally provided by Dr. Paul Williams (University of Nottingham, Nottingham, UK) and were previously transformed into *E. coli* JM109 cells. The plasmid pSD908 was previously constructed in the Daunert laboratory by using PCR to amplify *lasR* and its promoter from pSB1075 and inserting the resulting DNA fragment into the plasmid pSV-β-galactosidase control vector [45]. The transformed AHL-sensing cells were then stored at -80 °C as glycerol stocks. Fresh cell cultures were obtained from the glycerol stocks, grown in LB media (100 µg/mL ampicillin) overnight in an orbital shaker at 37 °C, 250 rpm, refreshed, and allowed to grow until an optical density at 600 nm (OD_{600}) of 0.45-0.50 was reached. *V. harveyi* MM32, *E. coli* AB1157, and *H. alvei* 718 were purchased from American Type Culture Collection (Manassas, VA). Cultures of *V. harveyi* MM32 cells were setup in autoinducer bioassay (AB) media containing 30 µg/mL kanamycin and grown overnight in the orbital shaker at 30 °C, 250 rpm. Overnight cultures were then diluted 1:100 to obtain an OD_{600} of 0.01-0.02. The procedure to prepare AB media has been described elsewhere[46]. *E. coli* AB1157 cells were grown in LB media with no antibiotic at 37 °C, 250 rpm, while *H. alvei* 718 cells were grown in nutrient media with no antibiotic at 30 °C, 250 rpm.

Dose-response curves in water

Commercially purchased *N*-acyl-homoserine lactones were dissolved in acetonitrile to obtain 1 x 10^{-2} M stock solutions, which were serially diluted with RO filtered water to obtain standard solutions at concentrations ranging from 1 x 10^{-4} M to 1 x 10^{-8} M. A 1% solution of acetonitrile in RO filtered water was used as a blank. Upon addition of sample to the bacterial culture, the acetonitrile final concentration was 0.1%, which is not toxic for the sensing cells. A volume of 10 µL of each of these solutions was added in triplicate to a 96-well white polystyrene microtiter plate containing 90 µL/well of cell culture grown to an OD_{600} of 0.45-0.50. C-6 HSL solutions were employed for the whole cell sensor with pSB406, while C-12 HSL solutions were employed for the whole cell sensor with pSB1075. The microtiter plate was then incubated at 37 °C, 175 rpm for two hours. The produced bioluminescence was then measured and the luminescence intensity was expressed as relative light units (RLU). An aqueous stock solution of AI-2 (3.7 × 10^{-3} M) was serially diluted with RO filtered water to prepare AI-2 standard solutions at concentrations ranging from 1 × 10^{-4} M to 1 × 10^{-8} M. RO filtered water was used as a blank. A volume of 10 µL of each of these standard solutions and blank was added in triplicate to a 96-well black polystyrene microtiter plate containing 90 µL/well of *V. harveyi* MM32 cell culture at an OD_{600} nm of 0.1-0.2. The microtiter plate was then incubated in the orbital shaker at 30 °C, 175 rpm for three hours. The produced bioluminescence was then measured and the luminescence intensity was expressed in relative light units (RLU).

Food sample collection and processing

Ground beef was further ground with slow addition of water using a high performance blender to form a uniform suspension. The volume of water was then adjusted to prepare a 10% w/v suspension that was stored as 20 mL aliquots at -80 °C. When needed, beef suspension aliquots were thawed at room temperature and diluted using RO filtered water. All milk samples were stored at -80 °C in 15 mL aliquots. When needed, milk aliquots were thawed at room temperature and diluted using RO filtered water. In order to evaluate the contamination in meat left at room temperature, a store bought package of meat was opened and left on the laboratory bench. Periodically, 1 g samples were taken and processed as described above. These samples were then frozen at -80 °C until use.

Food sample dilution study

To test for potential matrix effects of food on the whole cell sensing systems response, an aliquot of 10% w/v beef suspension was serially diluted with RO filtered water to obtain 1:10, 1:100, and 1:1000 dilutions. At each dilution step, proper care was taken to vortex the suspensions thoroughly to avoid settling of meat particles. Similarly, a fat free milk aliquot was serially diluted with RO filtered water to obtain 1:10, 1:100, and 1:1000 dilutions. To test the beef and skim milk samples with the AHL sensing systems pSB406 and pSB1075, a 10 µL volume of the above beef suspensions and milk solutions was added in triplicate to a microtiter plate followed by addition of 90 µL/well of the sensing bacterial cells grown to an OD_{600} of 0.45-0.50. Similarly, beef and skim milk samples were tested with the *V. harveyi* MM32 whole cell biosensing system by adding a 10 µL volume of the above beef suspensions and milk solutions in triplicate to a microtiter plate followed by addition of 90 µL/well of the sensing bacterial cells at an OD_{600} of 0.1-0.2. The microtiter plates were then incubated for 2 hours at 37 °C (for pSB406 and pSB1075) or 3 hours at 30 °C (for MM32)

Evaluation of food matrix effects in the presence of QSMs

Beef suspensions and skim milk solutions at various dilutions, prepared as described above, were spiked with a fixed concentration of 1×10^{-6} M C-6 HSL, 1×10^{-6} M C-12 HSL, or 1×10^{-5} M AI-2. A 10 µL volume of each of these spiked samples was added in triplicate to a microtiter plate followed by addition of 90 µL/well of the corresponding sensing cell suspension and assays were then performed as in the food dilution studies above

Dose-response curves in sample matrix

Acetonitrile 1×10^{-2} M stock solutions of C-6 HSL and C-12 HSL in acetonitrile were serially diluted with 1:10 beef suspension or undiluted skim milk to obtain AHL solutions of concentrations ranging from 1×10^{-4} to 1×10^{-9} M. A 1% acetonitrile solution in each of the above food sample dilutions was used as a blank. Similarly, a 3.7×10^{-3} M aqueous stock solution of AI-2 was serially diluted with 1:10 beef suspension or undiluted skim milk to obtain AI-2 solutions of concentrations ranging from 1×10^{-4} to 1×10^{-9} M. 1:10 beef suspension and undiluted skim milk served as blanks. A 10 µL volume of each of the obtained solutions and blanks was added in triplicate to a microtiter plate followed by addition of 90 µL/well of cell culture. The assays were then performed as described above. A reference dose-response curve was included in each analytical run.

Food contamination study

To investigate QSM production in food matrices, we contaminated beef and skim milk with AHL producing *H. alvei* 718 and AI-2 producing *E. coli* AB1157. An overnight culture of *E. coli* AB1157 was added into 10% w/v beef suspension to obtain a 1:100 dilution and allowed to grow at 37 °C, 250 rpm. Similarly, an overnight grown culture of *H. alvei* 718 was added into 10% w/v beef suspension to obtain a 1:15 dilution of the original culture and allowed to grow in the incubator shaker at 30 °C, 250 rpm. Procedures similar to those used for contaminating beef suspension were employed to contaminate skim milk. One-milliliter fractions of media from each of the contaminated food samples were collected at each hour for 8 hours, followed by collection of an overnight fraction. The collected fractions were centrifuged at 13,000 rpm for 5 minutes at room temperature and the supernatants were stored at -20 °C until analyzed. To measure the QSMs produced, the supernatant samples were assayed using the respective whole cell biosensing systems as described above.

Preparation of paper strips

Paper strips were prepared as previously reported, with several modifications [45, 47]. Briefly, a wax design was printed onto Whatman #1 filter paper using a Nikon ColorQube 8570 printer. pSD908 harboring cells were grown to an OD_{600} of approximately 0.9. These cells were then harvested via centrifugation, washed with PBS, and resuspended in a drying protectant solution. Three microliters of the resulting mixture was then spotted on the section of the paper strips isolated by wax printing. The strips were then allowed to air dry for ten minutes before being transferred to 50 mL conical centrifuge tubes and freeze dried at -80 °C, 0.004 mbar overnight. Paper strips were then stored at 4° C until use.

Paper-based QSM detection in food matrices

Ground beef and skim milk samples were processed as previously described. QSM standards were prepared by performing a serial dilution of N-(3-oxododecanoyl)-L-homoserine lactone in acetonitrile. The resulting standards were the diluted into beef suspension and

skim milk such that each sample contained 1% acetonitrile. One hundred microliters of spiked food sample was added to 900 µL of LB Miller broth supplemented with ampicillin (100 µg/mL) in a 15 mL polypropylene culture tube and a paper strip was placed inside the tube in a manner that ensured that the immobilized biosensing cells were completely submerged. Paper strips were then incubated at 37 °C for 1.5 hours prior to the application of 10 µL X-gal (50 mg/mL). After addition of the substrate, strips were covered in plastic wrap and allowed to develop for 1.5 hours at room temperature. All assays were performed in triplicate.

Detection of food contamination using paper strips

Overnight cultures of *P. aeruginosa* strain PAO1 were diluted 1:100 into 50 mL of 10% w/v beef suspension or skim milk. The contaminated beef and milk were then incubated at 37 °C, 250 rpm and 1 mL aliquots were taken hourly. These aliquots were immediately centrifuged and the supernatant was decanted and stored at -20 °C until use. Contamination detection using paper strips was performed as previously described, substituting 100 µL of contaminated sample aliquots taken from each time point for spiked samples.

Results and Discussion

The purpose of this work was to develop a portable, paper-based method to detect QSMs as a measure of spoilage and contamination in foods and demonstrate the feasibility of using whole cell biosensing systems towards this purpose. Four different specific biosensing systems were employed in the detection of two different classes of quorum sensing molecules, AI-2 and AHLs (long and short chain), in ground beef and milk. The role of QS in food spoilage has previously been suggested by identification of QSMs in spoiled foods[35, 48]. For example, bacterial presence and AHL molecules were detected in vacuum packed meat samples[35]. In addition, AI-2 has been identified in foods like fish, tomato, carrots, tofu, and milk[48]. The methods that were employed in both of the above studies were time consuming due to a need for processing steps, making their use in an on-site test inconvenient[35]. To the best of our knowledge, this work is the first application of cell-based biosensing systems in the quantitative detection of QSMs in food samples for the early detection of bacterial food contamination.

The *E. coli* whole cell biosensing systems containing plasmids pSB406 and pSB1075 were employed to detect short and long chain AHLs, respectively, and a whole cell biosensing system based on *V. harveyi* MM32 was used to detect AI-2. The plasmids pSB406 and pSB1075 bear recognition and regulatory elements derived from the *Pseudomonas aeruginosa* AHL-dependent RhlR/RhlI and LasR/LasI QS systems[49]. Plasmid pSB406 carries the promoters P_{rhlI} as well as the gene *rhlR*, which encodes for the recognition/regulatory protein RhlR. Similarly, pSB1075 contains the promoter P_{lasI} and *lasR,* the gene coding for the transcriptional activator LasR. Both plasmids contain the *luxCDABE* cassette placed under transcriptional control of the respective promoters. The *luxCDABE* genes encode for bacterial luciferase and the enzymes catalyzing the synthesis of the luciferase substrate (Figure 2). The plasmids lack the *rhlI* and *lasI* genes that code for the AHL synthase enzymes and, thus, exogenous AHLs need to be supplied in order for the sensing cells to produce bioluminescence. When AHLs are present in the environment of the sensing cells, they bind to the recognition/regulatory proteins and trigger the expression of the *luxCDABE* cassette and production of bioluminescence in a manner proportional to the concentration of

AHLs present. The whole cell biosensing systems based on plasmid pSB406 and pSB1075 have been previously characterized and employed to detect AHLs in saliva and stool samples[43]. *V. harveyi* MM32 is a mutant of wild type *V. harveyi* BB120, a marine bioluminescent bacterium that controls light emission through multiple QS systems. Strain MM32 was genetically modified to only emit bioluminescence only in response to AI-2 and to remove its ability to produce AI-2[50]. Consequently, light emission is only triggered by exogenous AI-2. When AI-2 binds its recognition element, the periplasmic binding protein LuxP, the binding event triggers a cascade of phosphorylation and de-phosphorylation processes in a number of proteins that control the expression of *luxCDABE*. Thus far, *V. harveyi* MM32 has been used as a reporter strain in bioassays for a number of applications, including the evaluation of QS regulated functions[51], screening compounds for agonistic and antagonistic activities[52], and to identify bacteria that produce AI-2[53]. Due to the high sensitivity and selectivity demonstrated by the above whole cell biosensing systems, we utilized them as sensitive and rapid tools for the analysis of QSMs in various food matrices because the levels of QSMs should correlate with the extent of bacterial contamination and serve as indicators of food spoilage.

Milk and ground beef were chosen as representative food models to investigate whether whole cell biosensing systems could be used to analyze liquid as well as solid foods. Initially, the analytical parameters of the biosensing systems were determined using prepared standard solutions of QSMs in RO water and the limits of detection for each analyte were found to be 1×10^{-9} M with RSD ranging from 8.8 to 9.9% (Table 1). In addition, dynamic ranges of at least three orders of magnitude were obtained with all biosensing systems. This range spans from 1×10^{-9} M to 1×10^{-6} M for pSB1075 and 1×10^{-9} M to 1×10^{-5} M for pSB406 and MM32.

Next, the matrix effect produced by the food samples on the whole cell biosensing systems response was evaluated. Beef was mixed with RO filtered water, ground, and a 10% w/v suspension was prepared. When beef suspension was analyzed with whole cell biosensing systems, bioluminescence signals lower than those of the controls were observed (Supplementary Figure 1A-C). Such a decrease was probably due to components of the sample matrix diminishing the ability of the system to emit bioluminescence. This matrix effect was eliminated when the beef suspension was diluted 1:10, 1:100, and 1:1000. At these dilutions, bioluminescent signals were similar to those of the controls, confirming that dilution was necessary and that a 1:10 dilution was sufficient to ablate the matrix effect. Similar studies were carried out to investigate the effect of skim milk on QSM detection. When undiluted skim milk was incubated with the whole cell biosensing systems, an emission of bioluminescence signal similar to that of the controls was observed (Supplementary Figure 1D-F). Further, dilutions of skim milk did not alter the bioluminescence signals of the whole cell systems.

Dose-response curves were generated by spiking the beef suspension with a known concentration of a QSM standard solution and using the whole cell biosensors to assay these samples. Analytical characteristics of tests assaying beef samples were similar to those seen when analyzing standards solutions prepared in water (Table 1 & Figure 3A-3C). Therefore, short chain AHLs in the range of 1×10^{-8} M to 1×10^{-6} M, long chain AHLs in the range of 1×10^{-9} M to 1×10^{-7} M, and AI-2 in the range of 1×10^{-8} M to 1×10^{-6} M can be detected in 1:10 beef suspension using whole cell biosensing systems with an RSD of 6.2, 1.2, and 9%, respectively. Similarly, assays performed using spiked milk samples yielded limits of detection and dynamic ranges with moderately higher variance, that were comparable to standard solutions prepared in water with LODs of 1×10^{-7} M for pSB406 and 1×10^{-8} M for pSB1075 and MM32 and RSDs of 16.3, 6.5,

and 7.1% for pSB406, pSB1075, and MM32 respectively (Table 1 and Figure 3D-3F). Dynamic ranges spanned from 1 x 10^{-9} M to 1 x 10^{-7} M for pSB1075 and 1 x 10^{-8} M to 1 x 10^{-6} M for pSB406 and MM32. At least three sets of experiments were performed to verify reproducibility, both with standard solutions and in food matrices. These results prove that biosensing systems could be employed to detect QSMs in food matrices with minimal sample processing.

To further prove that whole cell biosensing systems can be employed in food analysis, these systems were used to detect QSMs in simulated spoiled food samples that were subjected to bacterial contamination. *E. coli* AB1157 and *H. alvei* 718 were chosen as model organisms to artificially contaminate skim milk and beef suspension because they produce AI-2 and AHLs, respectively. To perform spoilage studies, overnight cultures of *E. coli* AB1157 and *H. alvei* 718 were added to beef suspension and skim milk. A 1 mL-volume sample was collected hourly, centrifuged to remove debris, and the supernatant was analyzed for the presence of QSMs using the whole cell biosensing systems. The results obtained with beef suspension contaminated with *H. alvei* 718 validate the ability of the employed sensing system to detect the short chain AHLs produced by *H. alvei* 718 contamination (Figure 4A). Only minor production of long chain AHLs was observed, which is consistent with the fact that *H. alvei* produces and uses short chain AHLs as QSMs[35]. Similarly, when beef was contaminated with AI-2 producing *E. coli* AB1157, the *V. harveyi* MM32 assay was able to detect the AI-2 produced (Figure 4B). The same trends were observed when skim milk was contaminated with *E. coli* AB1157 and *H. alvei* 718. The ability of our sensors to detect contamination in meat left at room temperature without any external contamination was also validated (Figure 4C-4D). After 24 hours, detectable signals were seen for both long chain AHLs and AI-2, demonstrating that it is possible to detect the QSMs of bacteria naturally occurring in and around the meat. Although short chain AHLs were not detectable, this is likely due to the bacterial population found in these particular meat samples. In sum, these data demonstrate that the whole cell biosensing systems can be successfully utilized to detect different QSMs in various contaminated food matrices.

After validation of the efficacy of whole cell biosensing systems for the detection of QSMs in food samples, we decided to adapt our bacterial biosensor into a portable paper-based microdot test. Previously, the plasmid pSD908 was developed for use in a colorimetric assay to detect QSMs. This plasmid places the reporter gene *lacZ* under the control of the *las* regulatory system, which may be used to detect long chain AHLs. The presence of these signaling molecules may be inferred based upon the intensity of the signal produced when β-galactosidase substrates are introduced to the cells following incubation with samples. In this work, cells harboring the plasmid pSD908 were immobilized onto filter paper strips via lyophilyzation, as has been previously described[45]. The analytical performance of the paper strip microdots was then evaluated by incubating the strips with mixtures consisting of 900 µL LB miller broth supplemented with ampicillin (100 µg/mL) and 100 µL of skim milk or 10% w/v beef suspension spiked with N-(3-oxododecanoyl)-L-homoserine lactone. After applying substrate and allowing for color development, a clear response was seen in samples containing as low as 1 x 10^{-9} M of the long-chain AHL, (Figure 5A). Limits of detection were confirmed through analysis via software that correlates the pixels in the image with the levels of the colored product on the microdot (Figure 5C-F). To evaluate the use of these paper strips for the detection of bacterial spoilage in food, the strips were then used to detect contamination in food inoculated with *P. aeruginosa* strain PAO1. Contamination was detectable within 1 hour in both milk and beef samples (Figure 5B). The success of these assays demonstrate

that paper strips sensors that employ whole cell-based biosensors for QSMs are a viable tool for identifying spoilage and bacterial contamination in food.

Conclusion

Incidences of food spoilage and foodborne illnesses have been on the rise and more multistate outbreaks are reported each year. Because numerous bacteria are responsible for these outbreaks, the source of contamination is often difficult to identify. However, a feature common to many bacteria, including foodborne pathogens, is the production of QSMs. Herein, we investigated the feasibility of employing QSMs as markers of bacterial presence in foods. To that end, we have developed an analytical method for the early detection of food contamination and prevention of illnesses caused by food spoilage in food matrices based upon the detection of QSMs. The proposed method takes advantage of whole cell biosensing systems that are sensitive, easy to use, rapid, cost-effective, and amenable to miniaturization, thus exhibiting potential for high throughput and on-site analysis. Each individual assay takes only 3 hours and costs as little as $0.15. The optimized method allowed for detection of QSMs in a sensitive manner with limits of detection reaching nanomolar levels. Additionally, food spoilage studies proved that our whole cell biosensing systems can be successfully employed to detect QSMs in spoiled food, thus suggesting potential usefulness in the early detection of food spoilage and prevention of foodborne illnesses. Furthermore, we have demonstrated that this type of sensor can be used in a portable, paper-based detection system that provides a quick, cheap, and reliable method to detect spoilage without the need for special training or laboratory materials.

ASSOCIATED CONTENT

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Conflicts of Interest

The authors declare no conflicts of interest.

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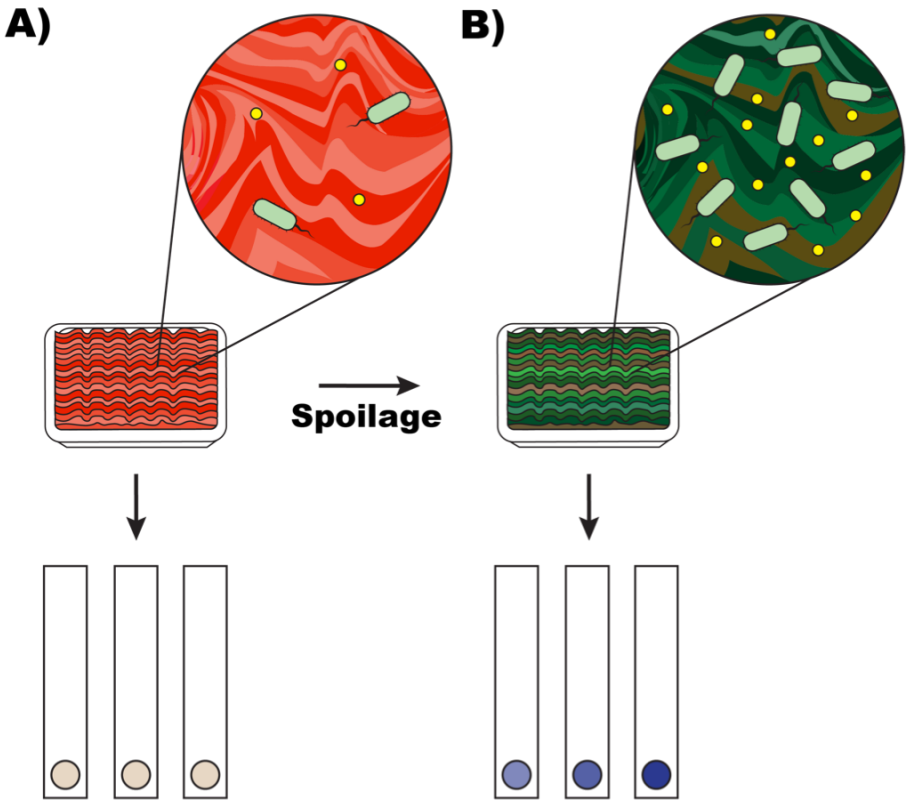

Figure 1 A rapid paper-based test for bacterial pathogens in food. Food may become contaminated with bacterial pathogens at many points between production and the kitchen. The signs of this contamination will likely not be apparent to the consumer in most circumstances. As shown in (A), unspoiled food will have a lower total bacterial population and a lower concentration of QSMs (depicted as yellow circles. However, when spoilage occurs, both the number of bacteria and concentration of QSMs will increase, shown in (B). Because the concentration of QSMs is low in unspoiled food, paper strip tests will turn a shade of blue but, if spoiled food is analyzed, the paper will blue such that the intensity of the color development is proportional to the concentration of QSM.

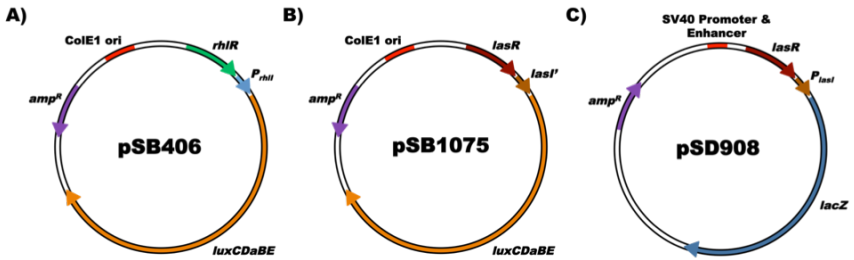

Figure 2. Plasmids pSB406 (A), pSB1075 (B), and pSD908 (C).

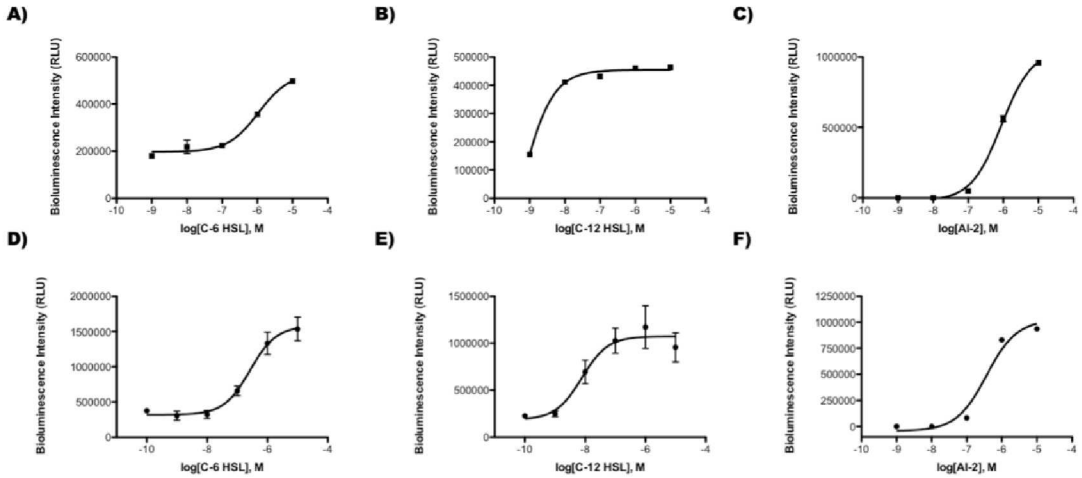

Figure 3 Dose-response curves obtained using (A) beef suspension with whole cell biosensing system containing plasmid pSB406, (B) beef suspension with whole cell biosensing system containing plasmid pSB1075, (C) beef suspension with *V. harveyi* MM32-based whole cell biosensing system, (D) milk with whole cell biosensing system containing plasmid pSB406, (E) milk with whole cell biosensing system containing plasmid pSB1075, and (F) milk with *V. harveyi* MM32-based whole cell biosensing system. Data shown are the average ± one SEM (n=3).

	Limit of Detection (M)			%RSD			Dynamic Range (M)		
Biosensor	Buffer	Beef	Milk	Buffer	Beef	Milk	Buffer	Beef	Milk
pSB406	1×10^{-9}	1×10^{-8}	1×10^{-7}	9.2	6.2	16.3	1×10^{-9} - 1×10^{-5}	1×10^{-8} - 1×10^{-6}	1×10^{-8} - 1×10^{-6}
pSB1075	1×10^{-9}	1×10^{-9}	1×10^{-8}	8.8	1.2	6.5	1×10^{-9} - 1×10^{-6}	1×10^{-9} - 1×10^{-7}	1×10^{-9} - 1×10^{-7}
MM32	1×10^{-9}	1×10^{-8}	1×10^{-8}	9.9	9	7.1	1×10^{-9} - 1×10^{-5}	1×10^{-9} - 1×10^{-7}	1×10^{-8} - 1×10^{-6}

Table 1. Analytical characteristics of QSM detection whole cell-based biosensors.

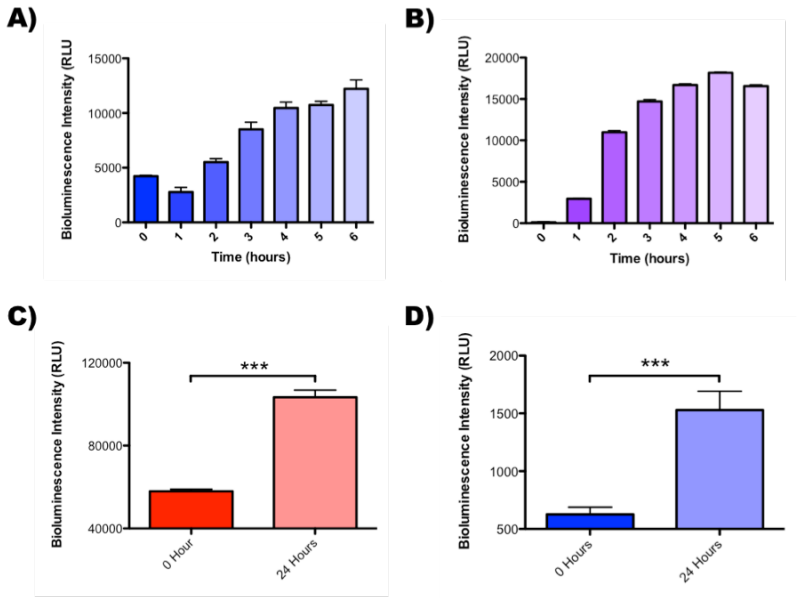

Figure 4. Contamination of food matrix with QSM producing bacteria. (A) Short chain AHL production in beef suspension contaminated with *H. alvei* 718, as monitored with whole cell biosensing system containing plasmid pSB406. Data shown are the average ± one SEM (n=3). (B) AI-2 production in beef suspension contaminated with *E. coli* AB1157, as monitored with *V. harveyi* MM32 whole cell biosensing system. (C) Long chain AHL production in beef left at room temperature for 24 hours, as measured by the pSB1075 system, shown as a percentage of the signal of uncontaminated beef. (D) AI-2 production in beef left at room temperature for 24 hours, as measured by the MM32 system, shown as a percentage of the signal of uncontaminated beef. Data shown are the average ± one SEM (n=3).

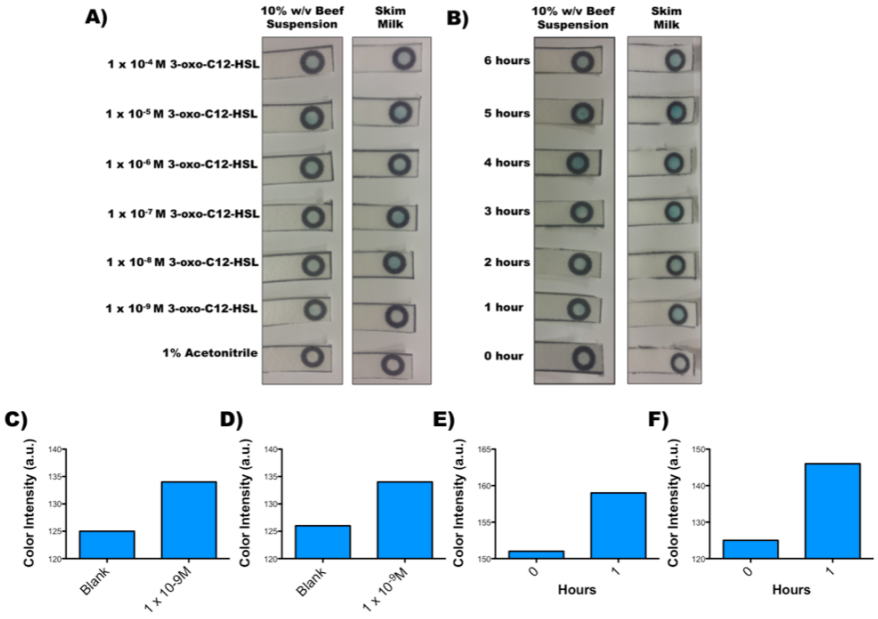

Figure 5. Detection of QSMs and spoilage using paper strips. For the sake of image clarity, contrast and brightness adjustments have been performed. A) When incubated with milk and beef spiked with QSMs, there is a response with as low as 1 x 10-9 M N-(3-oxododecanoyl)-L-homoserine lactone. B) Spoilage in milk and beef samples was detectable in as little as 1 hour following inoculation with *P. aeruginosa*. C) ImageJ analysis comparing color intensity of paper strips incubated with 1% acetonitrile and 1 x 10-9 M 3-oxo-C12-HSL in beef suspension, shown in A. D) ImageJ analysis comparing color intensity of paper strips incubated with 1% acetonitrile and 1 x 10-9 M 3-oxo-C12-HSL in skim milk, shown in A. E) ImageJ analysis comparing color intensity of paper strips incubated with either beef suspension at 0 hours or 1 hour, shown in B. F) ImageJ analysis comparing color intensity of paper strips incubated with either milk at 0 hours or 1 hour, shown in B.