

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 IJABR 2026; SP-10(6): 1409-1416
www.biochemjournal.com
 Received: 19-05-2026
 Accepted: 22-06-2026

Shreya Saha
 ICAR-National Dairy
 Research Institute (NDRI),
 Karnal, Haryana, India

Dr. Pavel Rout
 ICAR-National Dairy
 Research Institute (NDRI),
 Karnal, Haryana, India

Dr. HV Raghu
 ICAR-National Dairy
 Research Institute (NDRI),
 Karnal, Haryana, India

A novel PANI-pectin-based colorimetric assay for rapid total plate count estimation in raw milk

Shreya Saha, Pavel Rout and HV Raghu

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6Sq.8846>

Abstract

Traditional Total Plate Count (TPC) methods for assessing microbial quality of milk are laborious and require 48-72 h to generate results. This study presents a novel, rapid, and cost-effective Polyaniline-Pectin (PANI-Pec) nanoparticle-based colorimetric assay for TPC estimation in raw milk using a simple 2 mL Eppendorf tube format. PANI-Pec nanoparticles (Size: 361 ± 3 nm; zeta potential: -41 ± 3 mV; electrical conductivity: $6.51 \pm 0.02 \times 10^{-4}$ S/cm) were synthesized and incorporated into a 2% agarose matrix within sterile Eppendorf tubes. The sensor detects bacterial growth by measuring pH changes through a visible color transition from blue (emeraldine base form) to green (emeraldine salt form) as bacterial metabolites interact with the PANI-Pec matrix via proton doping. The growth medium composition, consisting of casein enzyme hydrolysate, yeast extract, dextrose, and sodium chloride, was optimized using Response Surface Methodology (RSM), yielding an optimum concentration of 2.75 g/50 mL for enhanced metabolite production and colorimetric sensor response. The sample volume was optimized experimentally, and 250 μ L was selected for subsequent analyses. The assay demonstrated a strong linear correlation ($R^2 = 0.9584$) between bacterial count and detection time, capable of detecting microbial loads as low as 4.0 log CFU/mL within 4.0 ± 0.25 h at 37 ± 2 °C and higher inoculum (8.0 log CFU/mL) within 2.0 ± 0.25 h. Colorimetric sensor assay maintained sensitivity and color intensity for up to two months under room temperature, 4 °C, and -20 °C storage conditions. This Eppendorf tube-based colorimetric sensor assay represents a significant advancement over conventional methods, offering a practical, equipment-free alternative suitable for dairy quality assurance laboratories and raw milk reception docks.

Keywords: Colorimetric sensor; PANI-Pectin nanoparticles; raw milk; microbial quality; rapid detection

Introduction

The rapid detection and continuous monitoring of pathogenic microorganisms have become increasingly important due to the growing incidence of food- and water-borne disease outbreaks worldwide (Valenzuela-Amaro *et al.*, 2023) [35]. Pathogenic and toxin-producing bacteria are responsible for significant public health concerns, causing illness and mortality across populations (Abebe *et al.*, 2020) [1]. Their widespread occurrence in food, drinking water, and environmental matrices necessitates the development of reliable monitoring systems to ensure food safety and quality (Yao *et al.*, 2022) [37].

Microbial quality assessment is particularly important in the dairy industry, where contamination can occur during production, processing, storage, and distribution (Fusco *et al.*, 2020) [12]. Milk provides an ideal environment for the growth of various pathogenic and spoilage-causing microorganisms such as Coliforms, *Bacillus* spp., *Pseudomonas* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* spp. (Haldar *et al.*, 2022) [17]. Conventional microbiological methods, such as the Total Plate Count (TPC) method (IS 5402-1:2021), remain the regulatory standard for evaluating bacterial load. However, these methods are labour-intensive and typically require 48-72 h to produce results, limiting their suitability for modern dairy processing environments where rapid decision-making is essential. In India, the regulatory framework for milk and milk products is primarily governed by the Food Safety and Standards Authority of India (FSSAI), under the Food Safety and Standards Act (FSSA), 2006, which sets mandatory TPC standards for all categories of dairy products (Gandhi *et al.*, 2020) [13].

Corresponding Author:
Dr. Pavel Rout
 ICAR-National Dairy
 Research Institute (NDRI),
 Karnal, Haryana, India

To overcome these limitations, several advanced analytical techniques, including flow cytometry (D-Count), polymerase chain reaction (PCR), and the direct epifluorescent filter technique (DEFT), have been developed (Moldenhauer, 2008) [26]. Although these methods significantly reduce detection time, they require expensive instrumentation, specialized laboratory facilities, and trained personnel. Recent advances in molecular diagnostics, including recombinase polymerase amplification, CRISPR-based assays, and viability-PCR approaches, have further improved detection sensitivity and specificity (Shah *et al.*, 2026) [31]. Nevertheless, these technologies remain largely restricted to centralized laboratories and are often impractical for routine on-site monitoring. Consequently, there is a growing demand for rapid, cost-effective, and user-friendly sensing platforms that can be deployed directly at dairy reception points and processing facilities (Mhapsekar *et al.*, 2025) [25]. Colorimetric sensing has emerged as a promising alternative because of its simplicity, low cost, and ability to provide visual information without the need for sophisticated instrumentation (Kant *et al.*, 2023) [21]. Such sensors can be incorporated into food packaging or used as standalone indicators to monitor microbial contamination and product freshness (Yousefi *et al.*, 2019) [39]. Unlike solution-based colorimetric assays, which may suffer from interference due to sample turbidity, solid-state sensor formats offer greater practicality and ease of interpretation. Conducting polymers, particularly polyaniline (PANI), are attractive materials for colorimetric sensing applications because of their high sensitivity to pH changes and excellent environmental and thermal stability (Wang & Feng, 2022) [36]. PANI undergoes a reversible transition from its insulating emeraldine base (EB) form (blue) to its conductive emeraldine salt (ES) form (green) upon protonation. This unique property enables the direct transduction of bacterial metabolic activity into a visible color response (Thakur *et al.*, 2015) [33]. Previous investigations using PANI-Pectin (PANI-Pec) nanoparticles have demonstrated their suitability for bacterial sensing applications. Anjali *et al.* (2022) [4] reported a PANI-Pec paper strip-based sensor for detecting *Escherichia coli* in milk and milk products, achieving a detection sensitivity of $0.52 \pm 0.17 \log \text{CFU/mL}$ within 10-21 h. More recently, Saha *et al.* (2025) [29] developed a PANI-Pec paper strip sensor for rapid TPC detection in milk and milk products, capable of detecting $4.0 \pm 0.15 \log \text{CFU/mL}$ of microorganisms within $4 \frac{1}{2} \pm \frac{1}{4} \text{ h}$ at $30 \pm 2^\circ \text{C}$. These advances confirm the potential of PANI-Pec systems for dairy quality monitoring; however, further improvements in detection speed, format versatility, and assay simplicity are required. In the present study, a novel PANI-Pec-based colorimetric assay was developed using a 2 mL Eppendorf tube format distinct from paper strip configurations for rapid TPC estimation in raw milk. The sensing mechanism relies on the interaction between bacterial metabolic products and the pH-responsive PANI-Pec matrix embedded in agarose, resulting in a measurable color change correlated with microbial proliferation. By providing a rapid visual indication of bacterial activity within a compact, sterile, and self-contained tube format, the developed assay offers a practical, equipment-free, and cost-effective approach for real-time microbial quality assessment in dairy products.

2. Materials and Methods

2.1. Chemicals and reagents

Aniline (99%, Sigma-Aldrich), ammonium persulfate (Sigma-Aldrich), hydrochloric acid (37%, Sigma-Aldrich),

ethanol (HPLC grade, Sigma-Aldrich), NaOH crystals (HiMedia), pectin (HiMedia), and Milli-Q water (Millipore water purification system) were used. Casein enzymatic hydrolysate, yeast extract, dextrose, and sodium chloride used in the study were obtained from HiMedia (India).

2.2. Synthesis of PANI-pec nanoparticles

PANI-Pec nanoparticles were synthesized using a methodology adapted from Anjali *et al.* (2022) [4], and Thakur *et al.* (2015) [33]. Briefly, aniline was polymerized in the presence of pectin as stabilizer (aniline: pectin weight ratio of 1:2) using ammonium persulfate as the oxidizing initiator and hydrochloric acid as the doping agent. The reaction was carried out at room temperature under constant stirring for 4 h. The resulting PANI-Pec dispersion was precipitated in a 1:1 ethanol-water mixture, filtered, washed, and re-dispersed in Milli-Q water by ultrasonication. The final dispersion was adjusted to pH 7.0 using 1 N NaOH to convert the emeraldine salt (ES) form to the emeraldine base (EB) form, yielding a stable blue-colored nanoparticle dispersion.

2.2. Characterization of PANI-pec nanoparticles

Fourier-transform infrared (FTIR) spectra were recorded on a Shimadzu IR Affinity-1S ($4000\text{--}400 \text{ cm}^{-1}$, 4 cm^{-1} resolution). UV-Vis spectra were obtained using a TECAN Infinite M200PRO plate reader (300-1000 nm). Electrical conductivity was measured with a Eutech PC 2700 multiparameter bench meter. Particle size, polydispersity index (PDI), and zeta potential were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90.

2.3. Fabrication of PANI-pec sensor

An optimized volume of PANI-Pec solution (3 mg/mL) was incorporated into 5 mL of $1\times$ phosphate-buffered saline (PBS) containing 2% (w/v) agarose. The mixture was heated to approximately 70°C with continuous stirring until a homogeneous dispersion was obtained. The optimized volume of the warm PANI-Pec-agarose composite was transferred into sterile 2 mL microcentrifuge (Eppendorf) tubes and allowed to solidify, forming a uniform gel layer. The resulting sensor matrices were sterilized by exposure to UV light for 20 min before further use.

2.4. Optimization of PANI-pec solution, growth medium, and sample volume

2.4.1. Optimization of PANI-pec volume

The volume of PANI-Pec nanoparticle suspension incorporated into the agarose matrix was optimized by evaluating volumes ranging from 100 to 400 μL . Each formulation was tested for its ability to produce a rapid and distinct color transition from blue to green in response to bacterial metabolic activity in the optimized growth medium. The volume exhibiting the fastest response time and the most pronounced visual color change was selected as the optimal concentration for subsequent experiments.

2.4.2. Optimization of growth media

Casein enzyme hydrolysate, yeast extract, dextrose, and sodium chloride were optimized by Response Surface Methodology (Design Expert v10.1). Fifteen medium combinations were evaluated against three criteria: highest optical density (600 nm), lowest pH after incubation at 37°C , and the sensitivity and colour intensity of the resulting PANI-Pec colorimetric assay response.

2.4.3. Optimization of sample volume

Sample volumes ranging from 100-500 μL , spiked reconstitute milk powder to approximately 0.5 log CFU/mL, were evaluated to identify the volume yielding the most rapid colour change.

2.5. Sensitivity of the PANI-pec sensor

Skim milk was spiked with bacterial cell suspensions isolated from raw milk to achieve final counts ranging from 8 to 4 log CFU/mL. The optimized assay protocol was applied, and the time to visible colour change was recorded for each inoculum level.

2.8. Shelf-life study of sensor

Prepared sensors were stored in airtight containers at room temperature ($25 \pm 2^\circ\text{C}$), 4°C , and -20°C . Sensors were evaluated for sensitivity and color intensity at 15-day intervals over a 6-month period using the optimized assay protocol.

2.9. Statistical analysis

Linear regression analysis was performed to establish the correlation between bacterial count (log CFU/mL) and detection time. Pearson correlation coefficient (R^2) was calculated to assess linearity. All experiments were conducted in triplicate, and data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using appropriate software at a significance level of $p < 0.05$.

3. Results

3.1. Synthesis and characterization of PANI-pec nanoparticles

PANI-Pec nanoparticles were successfully synthesized in the emeraldine salt (ES) form, characterized by their green coloration. Following pH adjustment to 7.0 with 1 N NaOH, the nanoparticles were converted to the emeraldine base (EB) form, appearing blue the initial state of the colorimetric sensor. The FTIR spectrum of the synthesized PANI-PEC particles exhibited a broad band at 3445 cm^{-1} , corresponding to O-H and N-H stretching vibrations, indicating interactions between pectin and polyaniline. Peaks at 1734 cm^{-1} and $1638\text{--}1560\text{ cm}^{-1}$ were attributed to C=O stretching of pectin and quinoid/benzenoid ring vibrations of polyaniline, respectively. The characteristic bands at 1304 cm^{-1} , 1143 cm^{-1} , and 1098 cm^{-1} corresponded to C-N stretching of polyaniline and C-O-C/C-O stretching of pectin. These results confirmed the successful formation of the PANI-PEC composite and the presence of intermolecular interactions between the two polymers. The synthesized PANI-Pec nanoparticles exhibited an average hydrodynamic radius of $361 \pm 3\text{ nm}$ (PDI: 0.278 ± 0.002), indicating a narrow size distribution and homogeneous dispersion. The zeta potential was determined to be $-41 \pm 3\text{ mV}$, confirming colloidal stability of the dispersion. Electrical

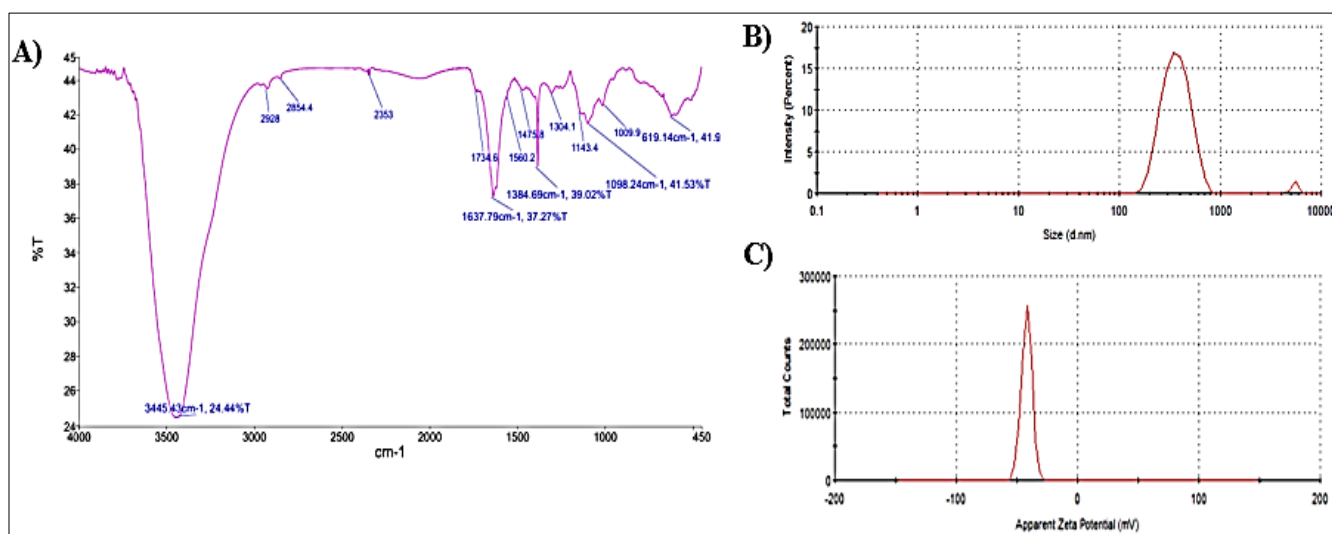


Fig 1: Characterization of polyaniline-pectin particles: (A) FTIR spectroscopy, (B) Size distribution profile, (C) Zeta profile distribution

Conductivity of the PANI-Pec solution at 3 mg/mL was $6.51 \pm 0.02 \times 10^{-4}\text{ S/cm}$, consistent with values reported previously for PANI-Pec nanoparticles (Amarnath *et al.*, 2014) [2]. Pectin served as both a stabilizer and biocompatible coating, facilitating the formation of uniformly sized particles with improved aqueous dispersibility (Fig. 1).

3.2. Principle of the colorimetric sensor

The sensing mechanism of the PANI-Pec Eppendorf tube sensor relies on the pH-responsive behavior of polyaniline. Under neutral or slightly alkaline conditions ($\text{pH} \geq 7$), polyaniline exists predominantly in its emeraldine base (EB) form, exhibiting blue coloration. During bacterial growth, microorganisms metabolize carbon sources (particularly

dextrose) in the optimized growth medium through glycolytic and fermentative pathways, releasing acidic by-products including succinate, acetate, lactate, and formate. These metabolites create an acidic microenvironment within the sensor tube. The resulting protonation of the PANI-Pec matrix triggers the EB-to-ES transition, producing a distinctive and visually detectable color change from blue to Green (Fig. 2). The use of an agarose-embedded semi-solid matrix within sterile Eppendorf tubes offers a significant advantage over liquid-phase monitoring: it minimizes interference from bacterial cell turbidity while maintaining intimate diffusive contact between the sensor matrix and metabolites produced in the overlying liquid phase. This configuration enables reliable and reproducible color change detection across a wide range of bacterial concentrations.

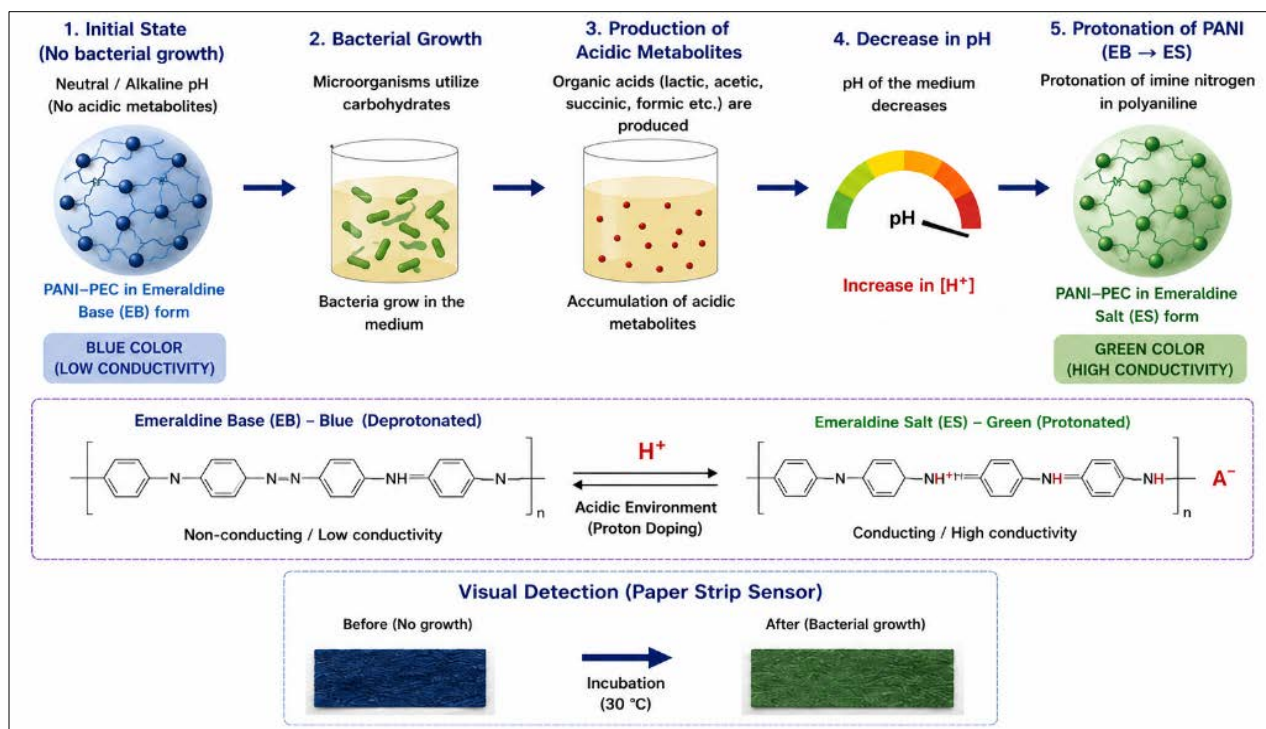


Fig 2: Principle of the colorimetric sensor assay

3.3. Optimization of PANI-pec solution, growth medium, and sample volume

The optimized PANI-Pec nanoparticle volume was found to be 250 μ L, which produced the most intense and visually distinguishable blue-to-green colour transition in response to bacterial metabolite-induced pH changes. This volume provided the highest assay sensitivity and was therefore selected for subsequent studies. RSM-based optimization of the growth medium identified an optimum composition of

2.75 gm/50 mL incorporating casein enzyme hydrolysate, yeast extract, dextrose, and sodium chloride. This medium supported enhanced production of acidic metabolites and ionic compounds during bacterial growth. One-way ANOVA confirmed significant differences among medium combinations ($p < 0.0001$), with R^2 values of 0.9685 (OD at 600 nm) and 0.9452 (pH), indicating a strong correlation between predicted and experimental outcomes.

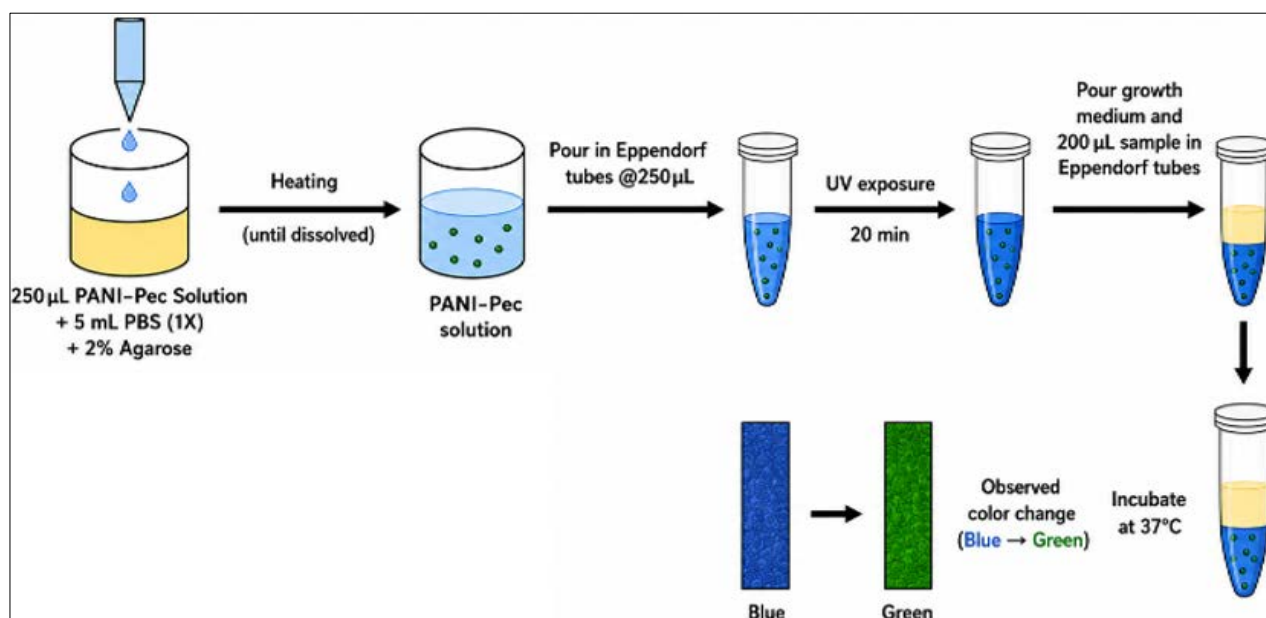


Fig 3: Fabrication of PANI-pec sensor

A sample volume of 250 μ L was found to be optimal for reliable sensor performance in the Eppendorf tube format, yielding the most rapid color change at 37 $^{\circ}$ C with a detection time of 2.0 ± 0.25 h at an initial bacterial concentration of 8.0 log CFU/mL (Fig. 3). This finding parallels the volume optimization reported for PANI-Pec

paper strip sensors, where 500 μ L was optimal for a larger paper strip format (Saha *et al.*, 2025)^[29].

When applied to raw milk samples, the PANI-Pec nanoparticle sensor produced a positive result (Fig. 4), evidenced by a clear blue-to-green color transition within 2.0 ± 0.25 h at 37 $^{\circ}$ C. This color shift was attributed to the

acidic metabolites and ionic by-products released during active bacterial growth inherent in raw milk, which contains a naturally high and diverse microbial load. On the other hand, sterilized milk sample yielded a negative result (Fig. 4), with the sensor retaining its characteristic blue colour throughout the incubation period. The absence of a colour

transition in sterilized samples reflected the significantly reduced bacterial population resulting from thermal processing. These findings demonstrate the ability of the PANI-Pec colorimetric assay to effectively discriminate between microbiologically unsafe raw milk and thermally treated pasteurized milk.

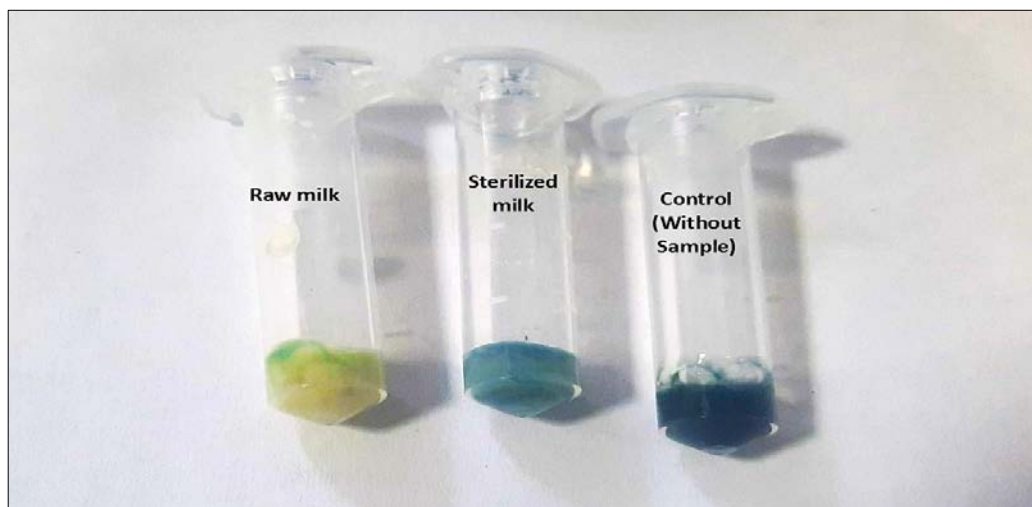


Fig 4: Images of positive result in a raw milk sample and negative result in a pasteurized milk sample

3.4. Sensitivity

The sensitivity of the PANI-Pec Eppendorf tube sensor was evaluated using raw milk spiked with bacterial cell suspensions at concentrations ranging from 8.0 to 4.0 log CFU/mL. A strong linear relationship ($R^2 = 0.9584$) was observed between bacterial count and detection time (Fig. 5), demonstrating the quantitative capability of the assay. At high inoculum (8.0 log CFU/mL), the sensor produced a

visible color change within 2.0 ± 0.25 h, while at low inoculum (4.0 log CFU/mL), detection was achieved within 4.0 ± 0.25 h. This performance compares favorably with existing PANI-Pec paper strip sensors (detection at 4.0 ± 0.15 log CFU/mL within 4.5 ± 0.15 h) and with commercial systems such as the Soleris® NB-100 system (Neogen® Corporation).

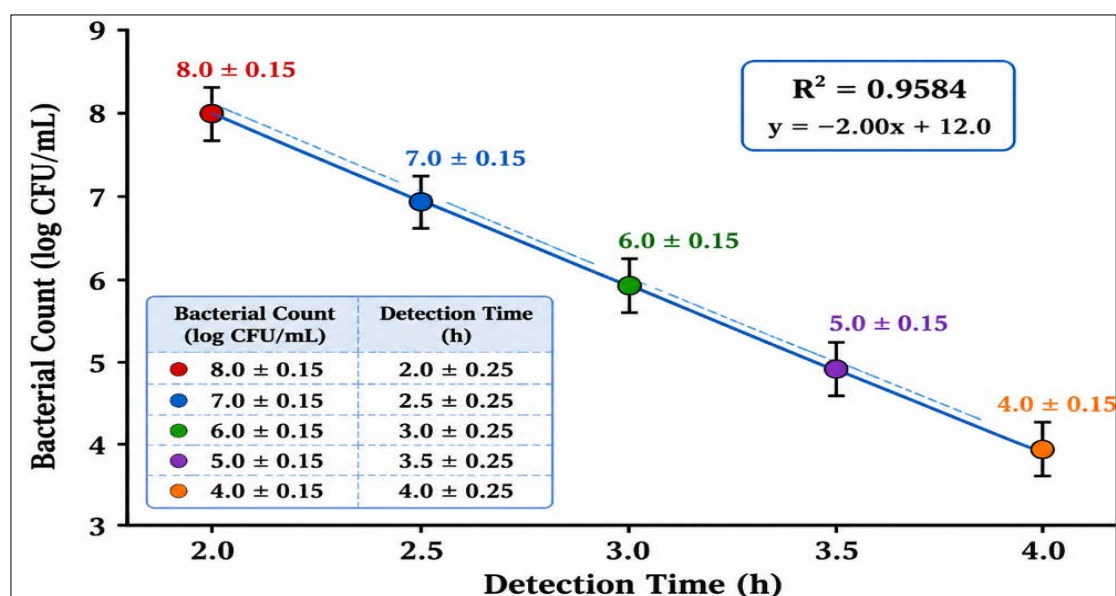


Fig 5: Relationship between bacterial inoculum level and sensor detection time

3.5. Shelf-life study

PANI-Pec Eppendorf tube sensors retained full sensitivity and color intensity for up to two months across all storage conditions tested: room temperature (25 ± 2 °C), 4 °C, and -20 °C. No significant deterioration in sensor performance was observed during this period, confirming the excellent thermal and environmental stability of the PANI-Pec matrix. The absence of biological recognition elements antibodies,

enzymes, or nucleic acids is a key advantage of this system, as it eliminates the risk of biocomponent degradation that typically limits the shelf life of conventional biosensors.

4. Discussion

The present study reports the development of a PANI-Pec (polyaniline-pectin) colorimetric assay for the rapid detection of total plate count (TPC) in raw milk, employing

an Eppendorf tube-based semi-solid agarose matrix as a novel sensing platform. The fundamental sensing mechanism relies on proton doping of polyaniline (PANI) by organic acids generated through bacterial fermentative metabolism, causing a characteristic colour transition from green (emeraldine salt, oxidised conductive form) to yellow (emeraldine base, reduced insulating form) as the local pH decreases (Thakur *et al.*, 2015; Saha *et al.*, 2025)^[29, 33]. This acid-induced chromogenic response has been well established in PANI-based sensor literature (Janata and Josowicz, 2003; Bhadra *et al.*, 2009)^[7, 19]. However, the current work introduces important architectural and medium-optimisation innovations that substantially enhance the practical utility and analytical performance of the PANI-Pec sensor relative to previously described paper strip formats (Thakur *et al.*, 2015; Saha *et al.*, 2025)^[29, 33].

A central innovation in this study is the transition from a PANI-Pec paper strip configuration to an Eppendorf tube-based semi-solid 2% agarose matrix. In liquid-phase biosensing systems, increasing microbial cell density inevitably leads to sample turbidity, which can confound optical measurements and impair visual colour discrimination (Lazcka *et al.*, 2007)^[23]. The semi-solid agarose gel physically confines the PANI-Pec sensor material at the base of the tube, creating a spatial separation between the turbid microbial suspension above and the sensing zone below, while permitting free diffusion of low-molecular-weight metabolites primarily short-chain organic acids such as lactic acid, acetic acid, and formic acid to the sensor surface. This geometry-driven optical isolation is particularly important for raw milk samples, where the intrinsic opacity of the matrix compounds the turbidity problem noted in earlier PANI-Pec work (Thakur *et al.*, 2015)^[33]. Beyond turbidity management, the Eppendorf tube format offers a sealed, contamination-resistant, self-contained reaction vessel that is readily scalable for high-throughput testing, aligning with the logistical demands of modern dairy quality assurance laboratories (Bhunia, 2014)^[8]. The nutrient medium composition was systematically optimised to support broad-spectrum acid-producing microbial growth. The selected formulation casein enzyme hydrolysate (nitrogen source), yeast extract (vitamins, minerals, and co-factors), dextrose (fermentable carbon source), and sodium chloride (osmotic stabiliser) at a combined concentration of 2.75 g/50 mL was designed to support the metabolic requirements of the diverse milk microbiota, including fermentative Gram-positive bacteria (*Lactobacillus*, *Streptococcus*, *Staphylococcus* spp.) and Gram-negative bacteria (coliforms, *Pseudomonas*, *Listeria* spp.) (Boor *et al.*, 2017; Quigley *et al.*, 2013)^[9, 28]. Dextrose serves as the primary fermentable substrate whose catabolism via glycolysis and mixed acid fermentation yields organic acids that protonate the PANI backbone. This formulation mirrors the medium components and concentration ranges reported by Saha *et al.* (2025)^[29] for paper strip-based TPC detection in milk, validating the cross-platform applicability of this medium design. The inclusion of yeast extract and casein enzyme hydrolysate ensures the availability of essential growth factors and assimilable nitrogen that might otherwise limit growth of fastidious organisms at early time points, thereby maximising the kinetic acid output that drives sensor actuation (Atlas, 2010)^[5]. The detection performance of the PANI-Pec Eppendorf tube assay is notable across a range of

initial bacterial concentrations. At an inoculum of 4.0 log CFU/mL representative of the lower regulatory threshold for raw milk microbial quality in many jurisdictions - a definitive colour change was recorded within 4.0 h. This compares favourably with the analogous PANI-Pec paper strip sensor, which required 4.5 ± 0.15 h to achieve a comparable endpoint at the same bacterial load (Saha *et al.*, 2025)^[29], suggesting that the agarose matrix configuration confers a modest kinetic advantage, possibly attributable to the higher local concentration of metabolites at the gel-liquid interface. At elevated bacterial loads of 8.0 log CFU/mL, the assay achieves detection within 2.0 h, approaching real-time monitoring capability. In contrast, conventional standard plate count (SPC) and total viable count (TVC) methods require 48-72 h of incubation before enumeration, a delay that is incompatible with perishable dairy product release decisions (Frank *et al.*, 1992)^[11]. Several other rapid methods, including ATP bioluminescence (Bautista *et al.*, 1992)^[6], flow cytometry (Gunasekera *et al.*, 2000)^[16], and impedance microbiology (Ur and Brown, 1975), reduce analysis time to 3-8 h but require expensive capital equipment and trained operators, limiting their deployment in resource-constrained or field settings. The PANI-Pec assay achieves comparable speed without instrumentation, representing a significant cost-performance advantage.

An important analytical feature of the assay is the linear relationship ($R^2 = 0.9584$) between detection time and initial bacterial count, which enables semi-quantitative estimation of microbial load from a simple visual time-to-positive read-out. This inverse relationship shorter detection time at higher initial concentrations arises from the kinetics of acid accumulation: higher initial cell densities reach the threshold acid concentration required for PANI Protonation more rapidly (Dalgaard and Koutsoumanis, 2001)^[10]. A similar linear detection time versus bacterial load relationship has been documented for impedimetric rapid methods (Gibson *et al.*, 1988)^[14] and for PANI-Pec paper strip sensors (Saha *et al.*, 2025)^[29]. The R^2 value obtained in this study (0.9584) is consistent with values reported for comparable colorimetric and impedimetric rapid methods, demonstrating adequate predictive capability for semi-quantitative milk quality classification (Bhunia, 2014)^[8]. Crucially, this semi-quantitative functionality is achieved without spectrophotometric or fluorometric instrumentation, relying solely on timed visual observation, which is a practical advantage in low-resource dairy testing environments.

The broad-spectrum detection capability of the PANI-Pec assay is a key operational advantage in raw milk quality surveillance, where the microbiota is heterogeneous and dominated by diverse acid-producing species. The assay responds generically to all fermentative acid-producing bacteria encountered in raw milk, including coliforms, lactobacilli, streptococci, staphylococci, pseudomonads, and *Listeria* spp. (Quigley *et al.*, 2013; Boor *et al.*, 2017)^[9, 28]. This is consistent with the principle that TPC methodologies are inherently non-selective, measuring the collective metabolic activity of the viable microbial community rather than targeting individual pathogens (Jay *et al.*, 2005)^[20]. However, a recognised limitation of the present assay shared with PANI-Pec paper strip sensors (Saha *et al.*, 2025)^[29] and related colorimetric acid-response platforms is its inability to detect endospore-forming bacteria such as

Bacillus cereus and *Bacillus licheniformis* within the standard assay window. These organisms characteristically exhibit extended lag phases and reduced acid production rates relative to vegetative fermentative bacteria (Andersson *et al.*, 1995)^[3], and their spores are heat-resistant, rendering them a persistent concern in pasteurised dairy products. Future iterations of the assay could address this gap by extending the incubation window or by incorporating selective pre-enrichment steps for spore-formers (Griffiths, 2010)^[15].

From a practical and economic perspective, the PANI-Pec composite material offers significant advantages over enzyme-based or antibody-based biosensors that are widely investigated for food safety applications. PANI and pectin are both commercially available, chemically stable compounds that can be synthesised and stored without refrigeration or specialised conditions (Stejskal and Gilbert, 2002; Thakur *et al.*, 2015)^[32, 33]. Unlike Enzyme-Linked Immunosorbent Assays (ELISA) or lateral flow immunoassays, the PANI-Pec sensor does not require biological recognition elements that are subject to denaturation, cold-chain dependency, or batch-to-batch variability. The Eppendorf tube format is universally available in microbiology laboratories, requires no custom fabrication, and is compatible with standard biosafety practices. The overall assay cost is low, and the read-out is purely visual, requiring no reader device, power source, or data system. These attributes collectively position the PANI-Pec Eppendorf tube assay as a particularly suitable tool for quality control in small and medium-sized dairy operations, cooperative testing laboratories, and field survey settings in low- and middle-income countries (Lim *et al.*, 2015)^[24].

Contextualising the PANI-Pec assay within the broader landscape of colorimetric microbial detection technologies, several parallels and distinctions are noteworthy. Redox indicator dyes such as resazurin (Sarker *et al.*, 2007)^[30] and tetrazolium salts (Kuhn *et al.*, 2015)^[22] similarly transduce bacterial metabolic activity into a colour signal, but these are typically single-use, soluble dyes that offer no structural confinement and are subject to background interference from coloured food matrices. Carbon nanotube and graphene-based colorimetric sensors offer higher sensitivity but require sophisticated nanomaterial synthesis protocols and are not yet amenable to point-of-use implementation. The PANI-Pec system occupies an advantageous niche: analytically sufficient sensitivity and speed for regulatory-relevant bacterial thresholds, combined with fabrication simplicity and a fully visual read-out. Future work should address the quantitative precision of the time-to-positive relationship through a larger multi-laboratory validation study, assess sensor performance across diverse raw milk compositions (fat content, somatic cell count, antibiotic residue presence), and explore miniaturised or multiplexed configurations that could enable simultaneous TPC and pathogen-specific detection (Neethirajan *et al.*, 2018; Bhunia, 2014)^[8, 27]. Integration of the assay with a simple smartphone-based colour capture application could further standardise the visual end-point determination and facilitate remote data logging (Yetisen *et al.*, 2013)^[38].

5. Conclusion

A novel PANI-Pectin nanoparticle-based colorimetric assay for rapid TPC estimation in raw milk operates on the principle of pH-driven proton doping of PANI-Pec

nanoparticles embedded in an agarose gel matrix, producing a distinct and visually detectable color change from blue to green in response to acidic metabolites generated during bacterial growth. The PANI-Pec Eppendorf tube assay offers several significant advantages over conventional TPC methods and existing rapid technologies: it requires no specialized equipment, is low-cost, provides results within 2-4 h (compared to 48-72 h for plating methods), is compatible with diverse bacterial groups in raw milk, and delivers unambiguous visual results interpretable by the naked eye. From a regulatory and implementation standpoint, the PANI-Pec Eppendorf tube assay is particularly well-suited for deployment at Raw Milk Reception Docks (RMRDs) in India and other developing nations, where access to laboratory infrastructure is limited. The assay offers a compelling alternative to the Methylene Blue Reduction Test (MBRT), which is a conventional indirect rapid screening method but is semi-quantitative, operator-dependent, and unreliable for pasteurized or chilled milk. The PANI-Pec system, in contrast, provides consistent performance across both raw and pasteurized milk and eliminates subjectivity in result interpretation through its unambiguous blue-to-green color transition.

References

1. Abebe E, Gugsu G, Ahmed M. Review on major food-borne zoonotic bacterial pathogens. *J Trop Med*. 2020;2020:4674235.
2. Amarnath CA, Venkatesan N, Doble M, Sawant SN. Water dispersible Ag@polyaniline-pectin as supercapacitor electrode for physiological environment. *J Mater Chem B*. 2014;2(31):5012-5019.
3. Andersson A, Ronner U, Granum PE. What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int J Food Microbiol*. 1995;28(2):145-155. doi:10.1016/0168-1605(95)00053-4
4. Anjali MK, Bharath G, Rashmi HM, Avinash J, Naresh K, Raju PN, *et al.* Polyaniline-pectin nanoparticles immobilized paper based colorimetric sensor for detection of *Escherichia coli* in milk and milk products. *Curr Res Food Sci*. 2022;5:823-834.
5. Atlas RM. Handbook of microbiological media. 4th ed. Boca Raton (FL): CRC Press; 2010.
6. Bautista DA, McIntyre L, Laleye L, Barbut S. The application of ATP bioluminescence for the assessment of milk quality. *J Rapid Methods Autom Microbiol*. 1992;1(1):1-12. doi:10.1111/j.1745-4581.1992.tb00312.x
7. Bhadra S, Khastgir D, Singha NK, Lee JH. Progress in preparation, processing and applications of polyaniline. *Prog Polym Sci*. 2009;34(8):783-810. doi:10.1016/j.progpolymsci.2009.04.003
8. Bhunia AK. One day to one hour: how quickly can foodborne pathogens be detected? *Future Microbiol*. 2014;9(8):935-946. doi:10.2217/fmb.14.61
9. Boor KJ, Wiedmann M, Murphy S, Alcaine S. A 100-year review: microbiology and safety of milk handling. *J Dairy Sci*. 2017;100(12):9933-9951. doi:10.3168/jds.2017-12969
10. Dalgaard P, Koutsoumanis K. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. *J Microbiol Methods*.

- 2001;43(3):183-196. doi:10.1016/S0167-7012(00)00219-0
11. Frank JF, Gillett RAN, Ware GO. Association of *Listeria* spp. contamination in the dairy processing plant environment with the presence of organic matter. *J Food Prot.* 1992;55(6):445-451. doi:10.4315/0362-028X-55.6.445
 12. Fusco V, Chieffi D, Fanelli F, Logrieco AF, Cho GS, Kabisch J, *et al.* Microbial quality and safety of milk and milk products in the 21st century. *Compr Rev Food Sci Food Saf.* 2020;19(4):2013-2049.
 13. Gandhi K, Sharma R, Gautam PB, Mann B. Safety and regulatory aspects of dairy industry. In: Chemical quality assurance of milk and milk products. Singapore: Springer; 2020. p. 273-312.
 14. Gibson AM, Bratchell N, Roberts TA. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. *Int J Food Microbiol.* 1988;6(2):155-178. doi:10.1016/0168-1605(88)90051-7
 15. Griffiths MW. Improving the safety and quality of milk. Vol. 1, Milk production and processing. Cambridge: Woodhead Publishing; 2010.
 16. Gunasekera TS, Attfield PV, Veal DA. A flow cytometry method for rapid detection and enumeration of total bacteria in milk. *Appl Environ Microbiol.* 2000;66(3):1228-1232. doi:10.1128/AEM.66.3.1228-1232.2000
 17. Halder L, Raghu HV, Ray PR. Milk and milk product safety and quality assurance for achieving better public health outcomes. In: Agriculture, Livestock Production and Aquaculture: Advances for Smallholder Farming Systems. Vol. 1. 2022. p. 217-259.
 18. Bureau of Indian Standards. IS 5402-1:2021. Microbiology of the food chain Horizontal method for the enumeration of microorganisms. Part 1: Colony count at 30 °C by the pour plate technique. New Delhi: BIS; 2021.
 19. Janata J, Josowicz M. Conducting polymers in electronic chemical sensors. *Nat Mater.* 2003;2(1):19-24. doi:10.1038/nmat768
 20. Jay JM, Loessner MJ, Golden DA. Modern food microbiology. 7th ed. New York: Springer; 2005.
 21. Kant T, Shrivastava K, Tejawani A, Tandey K, Sharma A, Gupta S. Progress in the design of portable colorimetric chemical sensing devices. *Nanoscale.* 2023;15(47):19016-19038.
 22. Kuhn DA, Vanhecke D, Michen B, Blank F, Gehr P, Petri-Fink A, *et al.* Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages. *Beilstein J Nanotechnol.* 2014;5:1625-1636. doi:10.3762/bjnano.5.174
 23. Lazcka O, Del Campo FJ, Muñoz FX. Pathogen detection: a perspective of traditional methods and biosensors. *Biosens Bioelectron.* 2007;22(7):1205-1217. doi:10.1016/j.bios.2006.06.036
 24. Lim DV, Simpson JM, Kearns EA, Kramer MF. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. *Clin Microbiol Rev.* 2005;18(4):583-607. doi:10.1128/CMR.18.4.583-607.2005
 25. Mhapsekar R, Kilbane D, Davy S, Abraham L, Fenelon M, O'Shea N. A systematic review of the internet of things and artificial intelligence applications in milk quality monitoring and analysis. *Int J Dairy Technol.* 2025;78(3):e70049.
 26. Moldenhauer J. Overview of rapid microbiological methods. In: Principles of bacterial detection: biosensors, recognition receptors and microsystems. New York: Springer; 2008. p. 49-79.
 27. Neethirajan S, Tuteja SK, Huang ST, Kelton D. Recent advancement in biosensors technology for animal and livestock health management. *Biosens Bioelectron.* 2018;98:398-407. doi:10.1016/j.bios.2017.07.015
 28. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, *et al.* The complex microbiota of raw milk. *FEMS Microbiol Rev.* 2013;37(5):664-698. doi:10.1111/1574-6976.12030
 29. Saha S, Singh M, Prajapati M, Mallappa RH, Kumar N, Sharma R, *et al.* Development and evaluation of PANI-PecPaper strip sensor for detection of total plate count in milk and milk products. *Food Control.* 2025;176:111397.
 30. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods.* 2007;42(4):321-324. doi:10.1016/j.ymeth.2007.01.006
 31. Shah K, Khan MI, Guo Y, Adnan M, Wu H. Infectious bacteria as biological warfare agents: mechanisms, epidemiological threats, and defense strategies. *Front Bioeng Biotechnol.* 2026;14:1805294.
 32. Stejskal J, Gilbert RG. Polyaniline. Preparation of a conducting polymer (IUPAC technical report). *Pure Appl Chem.* 2002;74(5):857-867. doi:10.1351/pac200274050857
 33. Thakur B, Amarnath CA, Sawant SN. Pectin coated polyaniline nanoparticles for an amperometric glucose biosensor. *RSC Adv.* 2014;4(77):40917-40923.
 34. Ur E, Brown DFJ. Impedance monitoring of bacterial activity. *J Med Microbiol.* 1975;8(1):19-28. doi:10.1099/00222615-8-1-19
 35. Valenzuela-Amaro HM, Aguayo-Acosta A, Meléndez-Sánchez ER, de la Rosa O, Vázquez-Ortega PG, Oyervides-Muñoz MA, *et al.* Emerging applications of nanobiosensors in pathogen detection in water and food. *Biosensors (Basel).* 2023;13(10):922.
 36. Wang Y, Feng W. Conductive polymers-based sensors. In: Conductive polymers and their composites. Singapore: Springer Nature; 2022. p. 275-347.
 37. Yao Z, Coatsworth P, Shi X, Zhi J, Hu L, Yan R, *et al.* Sensors based for diagnostics, human activity monitoring, food safety and environmental detection. *Sens Diagn.* 2022;1(3):312-342.
 38. Yetisen AK, Akram MS, Lowe CR. Paper-based microfluidic point-of-care diagnostic devices. *Lab Chip.* 2013;13(12):2210-2251. doi:10.1039/C3LC50169H
 39. Yousefi H, Su HM, Imani SM, Alkhaldi K, Filipe CDM, Didar TF. Intelligent food packaging: A review of smart sensing technologies for monitoring food quality. *ACS Sens.* 2019;4(4):808-821.