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RESEARCH PAPER

Print to detect: a rapid and ultrasensitive phage-based dipstick assay for foodborne pathogens

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Abstract Foodborne pathogens are a burden to the economy and a constant threat to public health. The ability to rapidly detect the presence of foodborne pathogens is a vital component of any strategy towards establishing a safe and secure food supply chain. Bacteriophages (phages) are viruses capable of infecting and replicating within bacteria in a strain-specific manner. The ubiquitous and selective nature of phages makes them ideal for the detection and biocontrol of bacteria. Therefore, the objective of this research was to develop and test a phage-based paper dipstick biosensor for the detection of various foodborne pathogens in food matrices. The first step was to identify the best method for immobilizing phages on paper such that their biological activity (infectivity) was preserved. It was found that piezoelectric inkjet printing resulted in lower loss of phage infectivity when compared with other printing methods (namely gravure and blade coating) and that ColorLok paper was ideally suited to create functional sensors. The phage-based bioactive papers developed with use of piezoelectric inkjet printing actively lysed their target bacteria and retained this antibacterial activity for up to 1 week when stored at room temperature and 80% relative humidity. These bioactive paper strips in combination with quantitative real-time PCR were used for quantitative determination of target bacteria in broth and food matrices. A phage dipstick was used to capture and infect *Escherichia coli* O157:H7, *E. coli* O45:H2, and *Salmonella* Newport in spinach, ground beef and chicken homogenates, respectively, and quantitative real-time PCR was used to detect the progeny phages. A detection limit of 10–50 colony-forming units per millilitre was demonstrated with a total assay time of 8 h, which was the duration of a typical work shift in an industrial setting. This detection method is rapid and cost-effective, and may potentially be applied to a broad range of bacterial foodborne pathogens.

Keywords Bacteriophage · Detection · Bioactive paper · Biosensor

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Introduction

Bacteriophages (or phages) are viruses that specifically infect target bacteria. Infection of bacteria with lytic (or virulent) phages results in their complete lysis and release of numerous progeny phages, which are ready to infect the remaining pathogens in their vicinity [1]. In the preantibiotic era, self-amplification of phages in the presence of the target pathogen was shown to be very effective in preventing the spread of infection [2]. For example, specific phages were used to

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control cholera outbreaks in India from 1933 to 1935 [3]. The average length of the outbreak was 26 days when traditional techniques (vaccination and water disinfection with potassium permanganate) were applied, whereas when phages were introduced into the contaminated well water, the outbreak length was significantly reduced to 48 h. The discovery of penicillin [4] resulted in a decline in interest in phages as antimicrobial agents for both scientific and social reasons. In recent years, however, interest in phages as biological moieties capable of sensing the presence of and killing specific pathogens was reignited because of both concerns with the emergence of antibiotic resistance [5, 6] and the ability of phages to target and kill only specified bacterial species [7], leaving other bacteria in the environment intact. The US Food and Drug Administration has approved the application of phages as food processing aids [8, 9] for control of such important bacterial pathogens as *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157 in foods and on food-contact surfaces [10].

On the other hand, the ability of phages to infect their bacterial hosts in a strain-specific manner provides an excellent opportunity to use them for detection of foodborne bacterial pathogens [7]. Whole phages and their components (such as receptor binding proteins in tail fibres and cell wall binding domains in endolysin) have been used to develop rapid, sensitive and specific bacterial detection assays [11]. Lytic phage infection results in lysis of the target bacterial cells and releases around 150–250 progeny phage particles per infected cell in most cases [12]. This amplification of the phage numbers can be used to enhance the sensitivity of phage-based detection assays. The additional benefit of using lytic phages for detection is their ability to kill the target bacterium during infection. Unlike culture-based bacterial detection techniques, which rely on sample enrichment to provide the required sensitivity, phage-based methods depend on production of multiple progeny phages as an amplification step, which, consequently, results in bacterial destruction rather than bacterial propagation. In this context, biosensors have been developed with the use of phages as specific lytic agents for the target bacteria, and the resulting progeny phages can be detected by culture-based methods, immunoassays or nucleic acid based assays, such PCR [11]. However, to ensure high sensitivity of the assay, the phages originally added should be removed so that only the progeny phages are detected. Various virucidal agents have been used for this purpose [13–15] but with limited success as their application requires more time-consuming and labour-consuming procedures. Use of immobilized phages can help to circumvent this issue as it allows fast and efficient removal of the excess phage particles after infection but before cell lysis.

The development of phage-based materials that are active, stable, easy to store and transport, and user-friendly has been attempted by many researchers [16]. Most of the methods suggested for phage immobilization were developed for biosensing applications [17]. Techniques have been developed for binding phages on gold and silica surfaces, as well as on modified cellulose membranes [18]. All these suggested methods require sophisticated surface treatment to allow the immobilized phage to remain active and hence were labour-intensive, time consuming and costly. For large-scale applications, such as control and detection of bacterial pathogens in the environment, it is imperative that the method of production of the phage-based material is more economically feasible and easily adaptable to industrial conditions, during both manufacturing and application [19, 20].

Paper is made from the most abundant natural polymer on Earth—cellulose [21]. Since its invention in ancient China, paper has been used for many everyday purposes, such as packaging and wrapping, writing and printing, and liquid purification (filtering) as well as for some more sophisticated uses, such as dipsticks (pH indicator paper, urine dipsticks, etc.) and a support for lateral flow devices used in clinical and environmental analyses [22–24].

The modern paper industry produces multiton quantities of various papers with different qualities and properties, and is capable of modifying their surfaces by printing and/or coating, which can be conducted on a large scale and at high speed [25, 26]. Paper is a hydrophilic, porous and chemically quite inert material which carries a slight negative charge at physiological pH [27]. All of these features make paper and other cellulose-based materials ideal as a support for biological entities such as proteins, cells and tissues as they allow maintenance of an aqueous-like environment, which is essential for preserving the native conformation of biological materials required for their activity, whilst at the same time being produced in large quantities at low cost in available industrial facilities [28, 29]. Printing technologies such as thermal inkjet printing and piezoelectric inkjet printing have been used to print biological materials [30–33]. Piezoelectric inkjet printing was used for printing fibroblast cells [34], horseradish peroxidase [30], *E. coli* cells [35], horseradish peroxidase labelled immunoglobulin [36] and DNA microarray [37], and thermal inkjet printing was used for insulin [38], mammalian cells [39] and DNA [40] among other printing endeavours. Gravure printing and blade coating of T4 phage was conducted, and phage survival was observed [41, 42]. In that study, phage susceptibility to dryness was demonstrated, and decreased paper porosity was observed to improve phage infectivity. Other factors such as decreased ink viscosity and increased phage titre were observed to increase phage infectivity. Printing or coating technologies for the immobilization of phages should enable the phages to remain intact and without significant loss of infectivity. The inks and the immobilization technologies used should not have an adverse effect on the phages, and the incorporation of humectants into the inks

may potentially contribute to long-term immobilized phage survival since it was demonstrated that they are susceptible to drying effects [11].

In this work we present a method for production of bioactive paper containing phages that were applied via inkjet printing. The materials were tested for their activity and stability and were further used for detection of *E. coli* O157H7, *E. coli* O45:H4 and *Salmonella* Newport in food matrices.

Materials and methods

Phages, bacteria and culture media

Phage rV5 and its hosts *E. coli* 0157:H7 EC1960264 and *E. coli* 045:H2 EC 19970358 were obtained from the Public Health Agency of Canada (Guelph, ON, Canada). All other phages and bacteria were obtained from the Canadian Research Institute for Food Safety culture collection (Table 1). Bacterial cultures were grown on tryptic soy agar (TSA), tryptic soy broth (TSB) and semisolid TSA (0.5% agar) (Thermo Scientific, Nepean, ON, Canada). Bacterial cultures were stored at -80 °C in 30% glycerol before use. Fresh cultures were revived each month from the frozen stock cultures onto TSA plates and then streaked onto fresh plates each week. Overnight cultures were prepared by addition of a single colony of bacteria to 10 mL of TSB and shaking at 200 rpm for 18 h at 37 °C. Phages were stored and diluted in phage buffer [0.74 g $CaCl_2$, 2.5g $MgSO_4 \cdot H_2O$, 0.05 g gelatin, 1 M tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5 in a final volume of 1 L of Milli-Q water].

Phage propagation and enumeration

A modification of the previously described protocol for phage propagation [43] was used. Phages were added to overnight cultures (incubation time 18 h) of their respective hosts at a multiplicity of infection of 1–10. Phages were allowed to adsorb to the host cells for approximately 15 min. Then 5 mL of molten semisolid TSA (0.5% agar) tempered to approximately 50–55 °C was added per plate. This mixture was added to the surface of the TSA plates to form a double-layer agar plate. The plates were allowed to solidify, and were then incubated upright at 25 °C for 16–24 h. Five to ten plates were used for propagation. Phage buffer was added to plates showing confluent lysis at 4 mL per plate. The top agar and the buffer mixture were scraped off into a sterile tube, and another 1 mL of phage buffer was added to each plate to wash off any remaining semisolid TSA. The resulting suspension was collected in the sterile tube. The tube was placed on ice for at least 15 min before the semisolid agar layer/buffer mixture was centrifuged at 7000*g* for 20 min to precipitate the agar and bacterial cells. The supernatant was removed and filter-sterilized through a 0.45-μm-pore-size syringe filter (Fisher Scientific, Mississauga, ON, Canada). Phage lysates were enumerated by the overlay method described later and then stored at 4 °C.

For large-scale propagation, 1 L of sterile TSB was inoculated with 5 mL fresh overnight culture of host bacterium and incubated for approximately 2 h at 37 °C [optical density at 600 nm of about 0.2, which is approximately 10^8 colony-forming units (CFU) per millilitre). Phage stock lysate was added at 1 mL/L and the mixture was incubated overnight at 25 °C then centrifuged at 7000*g* for 20 min. The supernatant was then filter-sterilized with a Corning 0.45-μm sterile bottle top filter unit (Fisher Scientific, Mississauga, ON, Canada). Phage lysates were enumerated by the spot test technique as previously described [43] and then stored at 4 °C. Briefly, 100 μL of host bacteria was added to 5 mL semisolid TSA and the mixture was then carefully pipetted onto a TSA plate and left for 15 min to solidify. Tenfold dilutions of phage lysate were made, and 10 μL was spotted on the surface of the bacterial lawn. Plates were incubated for 20 h in an upright position at 25 °C. The number of plaques was counted after incubation, and the titre was determined per millilitre of phage suspension.

Table 1 Bacteriophages used in this study and their respective hosts

Bacteriophage	Abbreviation	Family	Bacterial host	Culture collection ID
StyM-AG6	AG6	*Myoviridae*	*Salmonella* Typhimurium, DTI04	C1077
SenS-AG11	AG11	*Siphoviridae*	*Salmonella* Enteritidis	C417
LmoM-AG20	AG20	*Myoviridae*	*Listeria monocytogenes*	C1301
EcoM-AGl0	AG10	*Myoviridae*	*Escherichia coli* O157:H7, ATCC 43888	C899
T4	T4	*Myoviridae*	*E. coli* B	ATCC 11303
MS2	MS2	*Leviviridae*	*E. coli* Famp	
rV5	rV5	*Myoviridae*	*E. coli* O157:H7, ATCC 43888	
AG2A	AG2A	*Podoviridae*	*E. coli* O45:H2	C1328
vB_SnwM CGG4-1	CGG4-1	*Myoviridae*	*Salmonella* Newport	C 389

Real-time PCR detection of phages

Real-time PCR primers were designed to amplify unique sequences in AG11, rV5, AG2A and CGG4-1 phages (130, 96, 133 and 124 bp respectively) and synthesized by Laboratory Services, University of Guelph (Guelph ON, Canada) (Table 2). Each primer (500 nM) was used in a reaction volume (20 μL) containing phage lysate/TSB/food homogenate (6 μL) and Power SYBR Green master mix (10 μL; Applied Biosystems, Life Technologies, Burlington, ON, Canada). The Vii A7 real-time PCR instrument (Life Technologies) was set to fast mode for 40 cycles. The PCR conditions consisted of an initial hold step of 10 min at 95 °C, followed by 40 cycles of a denaturation step for 15 s at 95 °C and a combined annealing and extension step of 60 °C for 1 min, followed by a final melt cycle.

Gravure printing

Gravure printing was performed with an IGT Global Standard Tester 2 printability unit (IGT Testing Systems, Arlington Heights, IL, USA) at Innofibre, Cégep of Trois-Rivières (Trois-Rivières, QC, Canada). Gravure printing experiments were performed on lightweight coated commercial paper provided by Centre International de Couchage (Trois-Rivières, QC, Canada) with a basis weight or grammage of 35 g/m^2 with a clay and calcium carbonate coating according to the protocol used for printing T4 phage [42]. A bioink solution was formulated containing a 1:1 ratio (v/v) of phage lysate and 4% carboxymethylcellulose sodium salt solution in deionized water of medium viscosity (Sigma-Aldrich Canada, Oakville, ON, Canada) to give a viscosity of 50 mPa s. Phages AG11, AG20, MS2 and T4 were used for printing. An IGT gravure disc from IGT Testing Systems numbered 402.151.412 was used. It was engraved with 40 lines per centimetre, had a screen angle of 53° and contained four engravings at cell depths of 65, 70, 75 and 80 μm, corresponding to cell volumes of 14, 17, 20, and 25 mL/m^2 respectively. Printing was conducted at room temperature (20 °C) and at a speed of 0.2 m/s and a printing pressure equivalent to applied pneumatic force of 600 N onto paper (50 mm × 300 mm).

Table 2 Primers used to detect different bacteriophages used in this study

Phage	Amplicon size (bp)	Primer set
AG11	130	F: AAAGCGCATCAGAACATGAA R: GCGCAGGTATTCGAGTTGA
rV5	96	F: GTTCCCTGTTGGAACCATTG R: GCCCACACACAAGAAGAACA
AG2A	133	F: GATGCATATGGGGCTAATCG R: ATCCGTTTCCTATGGCCTTT
CGG4-1	124	F: TGAGTCCGGAGCAATTGACC R: TCTGCCGGGAATAAGCGAAA

F forward, *R* reverse

Blade coating

Blade coating was performed with a CLC-7000 cylindrical laboratory coater (Simutech Group, Toronto, ON, Canada) at Innofibre. This instrument is designed to reproduce the complete coating process on a laboratory scale at speeds (up to 800 m/min) comparable to those of pilot machines. Blade coating was done on a softwood mechanical pulp base paper of 47 g/m^2 basis weight. The bioactive coating solution was formulated with the phages suspended in 5% pork type A gelatin (Fisher Scientific) and 3% carboxymethylcellulose solution (viscosity of 100 mPa s at 45 °C) before coating at a speed of 400 m/min. The bioactive basis weight after coating was 1 g/m^2. CLC-7000 built-in high-intensity 36-kW infrared lamps were used to dry the phage-coated papers for 30 s. The temperature of the phage-coated paper after drying was 66 °C as measured by an OMEGASCOPE OS532 infrared thermometer with laser sight (OMEGA Engineering, Laval, QC, Canada). Phages AG11, AG20, MS2 and T4 were tested with this coating approach.

Piezoelectric inkjet printing

Inkjet printing was used with Whatman no. 1 paper sheets, Whatman no. 1 paper coated with 0.5% polyvinylamine (PVAm), Whatman no. 1 paper coated with 20% w/v poly(oligoethylene glycol methacrylate-*co*-adipic acid dihydrazide-*co*-(dimethylamino)ethyl acrylate) in 1% phosphate-buffered saline and 20% w/v poly(oligoethylene glycol methacrylate-*co*-aldehyde) (hydrogel-coated paper), and Hewlett-Packard Forest Stewardship Council certified multipurpose paper with ColorLok technology (Hewlett-Packard, Mississauga, ON, Canada). Printing was conducted at room temperature (20 °C) with a Dimatix DMP 2800 materials printer (Fujifilm Dimatix, Santa Clara, CA, USA). The bioink was prepared by suspension of the phages (10^9 plaque-forming units (PFU) per millilitre) in 30% glycerol and 2 mM Triton X-100. Phages AG6, AG10, AG11, AG20 and rV5 were used for printing after being stored for 24 h at 4 °C to stabilize the mixture. Approximately 500 drops per centimetre were printed at about 10 pL per drop with the following printing parameters: firing voltage 40V; firing frequency 5 kHz; drop space 20 μm, meniscus vacuum 11.43 cmH_2O (in water column). All jets were set to fire. Nozzle cleaning cycles were performed approximately every 120 s. The bioinks formulated for piezoelectric inkjet printing were printed for approximately 15 min directly into a Petri dish for each of the phages used. The titres of the bioinks before printing and those collected

after printing were compared to determine any possible titre reduction due to shearing effects during the inkjet printing. Furthermore, the bioinks used for piezoelectric inkjet printing were also pipetted onto ColorLok paper circles (2.5-cm diameter) at 100 μL per paper circle as a control to further quantify the potential shearing effects of piezoelectric printing on phage infectivity. This volume provided in the pipetting experiments was approximately four times the amount delivered by piezoelectric inkjet printing. The paper was then left to dry for 0.5 h in a horizontal laminar flow clean bench and then stored at 80% relative humidity. The printing process is summarized in Fig. 1.

Determination of the infectivity of bioactive paper and phage diffusion from paper

The infectivity of the phage-based paper was determined by the overlay technique and/or in liquid medium. The bioactive paper was cut into circles (2.5-cm diameter) and transferred with sterile forceps to the surface of semisolid agar inoculated with the relevant bacterium. The medium was incubated in an upright position overnight at 25 °C for *E. coli* B, *L. monocytogenes* and *Salmonella* and at 37 °C for *E. coli* Famp. Plates were visually examined for zones of growth inhibition around the periphery of the paper containing phages after the incubation period. Positive controls were prepared with paper without phages.

Furthermore, infectivity of the phages incorporated in the paper was determined quantitatively in broth medium. The host bacterium was incubated for 18 h at 37 °C and diluted to achieve a final count of 10^3 CFU/mL in phosphate-buffered saline (Fisher Scientific). A paper disc (2.5-cm diameter) containing the phage was added to 9 mL of the bacterial suspension and incubated for 18 h without shaking. Bacterial suspensions without paper discs and with paper discs without phages were used as controls. The count of the target bacterium in the broth was determined by our plating tenfold serial dilutions in phosphate-buffered saline onto MacConkey agar (BD Diagnostics, Mississauga, ON, Canada), Oxford agar (Oxoid, Nepean, ON, Canada) and xylose lysine deoxycholate agar (BD Diagnostics) or modified brilliant green agar (Oxoid) for enumeration of *E. coli*, *L. monocytogenes* and *Salmonella* respectively. The appropriate medium was inoculated with 100 μL of each dilution in duplicate, and spread across the agar surface with a sterile spreader. Plates were retained in an upright position until the inoculum had been absorbed by the agar, and then they were inverted and incubated for 18 h at 37 °C. After the incubation period, the plates were examined for typical colonies of each host bacterium on the selective plates.

Stability of bioactive phage-based paper

The printed or coated phage-based paper was stored at room temperature (approximately 21–25 °C) in an airtight container with a relative humidity of around 80%. Phage infectivity was determined against the respective host bacteria in broth after storage for up to 1 month for inkjet-printed paper or monthly for up to 2 months for gravure-printed paper and blade-coated paper as described earlier.

Estimation of phage particle number on the inkjet-printed bioactive paper

The number of active phage particles printed on paper was estimated by our investigating the effect of different free

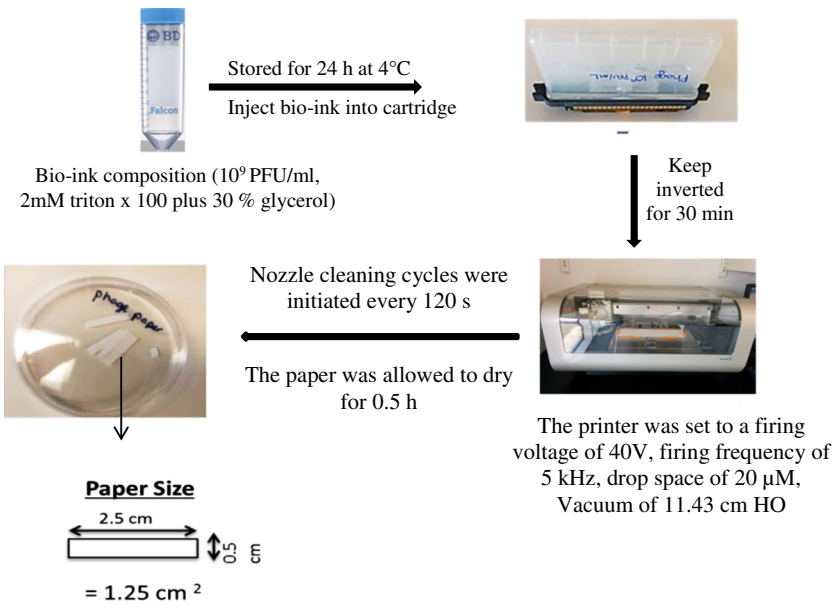

Fig. 1 Flowchart for production of bacteriophage-based bioactive paper for detection of foodborne pathogens. PFU plaque-forming units

phage concentrations on the host bacterium and then comparing the inhibitory effect with that obtained for the phage-based paper. Additionally, phage AG11 was inkjet printed onto ColorLok paper and then the DNA was extracted and quantified by quantitative real-time PCR (qPCR). First, the number of infective phages on the 2.5-cm-diameter phage-based paper disc was estimated by our investigating the effect of six different free phage concentrations for each phage on 10^3 CFU of the respective host bacterium per millilitre. Tenfold dilutions of free phages in phage buffer (103–10^8 PFU/mL) were added to 10^3 CFU of the respective host bacterium per millilitre in TSB, and then the mixture was incubated at 25 °C for 18 h. The surviving bacteria were counted after the plating of tenfold serial dilutions of the broth onto selective media as outlined earlier. The host bacterial counts from the free phage and immobilized phage experiments were compared to estimate the number of infective phage particles present on the paper. Secondly, the amount of AG11 printed onto ColorLok paper was determined by extraction of the phage DNA from the paper and use of qPCR to estimate the phage particle numbers by comparison with samples of known phage titre. Total DNA from the printed paper disc (2.5-cm diameter) was extracted with a plant/fungi DNA isolation kit according to the manufacturer's instructions (Norgen Biotek, Thorold, ON, Canada). Then qPCR was used to detect and estimate the amount of phage DNA present on the paper samples with use of the primer set and PCR conditions described previously.

Detection of *E. coli* O157:H7 in broth using paper strips with phage printed onto ColorLok paper

The phage-printed paper strips (0.5 cm × 2.5 cm) were added to 1.5-mL Eppendorf tubes containing 1 mL of *E. coli* O157:H7 at 1, 10, 50, 100 or 100 CFU/mL in TSB. A phage-printed paper strip was added to 1 mL of sterile TSB as a control. The whole experiment was done in triplicate. The samples were incubated at 37 °C for 20 min. The phage-printed paper strip was then moved to a fresh solution of TSB (1 mL) and incubated for 5 h at 37 °°C in a shaking incubator (100 rpm). The phage-printed paper strips were then removed and the tubes were placed on ice, and 30 μL of chloroform was added. The plaque assay technique and real-time PCR were used to confirm the presence of progeny phage by means of the procedures described earlier.

Detection of foodborne pathogens in food matrices using paper strips with printed phage

The strips of phage-based bioactive paper (inkjet-printed paper strips) were used to detect the target bacterial pathogens (*E. coli* O157:H7, *E. coli* O45:H2 and *Salmonella* Newport) in different food matrices [washed spinach bought locally, extra-lean ground beef (around 5% fat) and chicken respectively] with use of the dipstick approach shown schematically in Fig. 2. Spinach, ground beef and chicken homogenates were prepared by our blending 25 g of each of them in 225 mL of phosphate-buffered saline (pH 7.4) for 1 min at 150 rpm

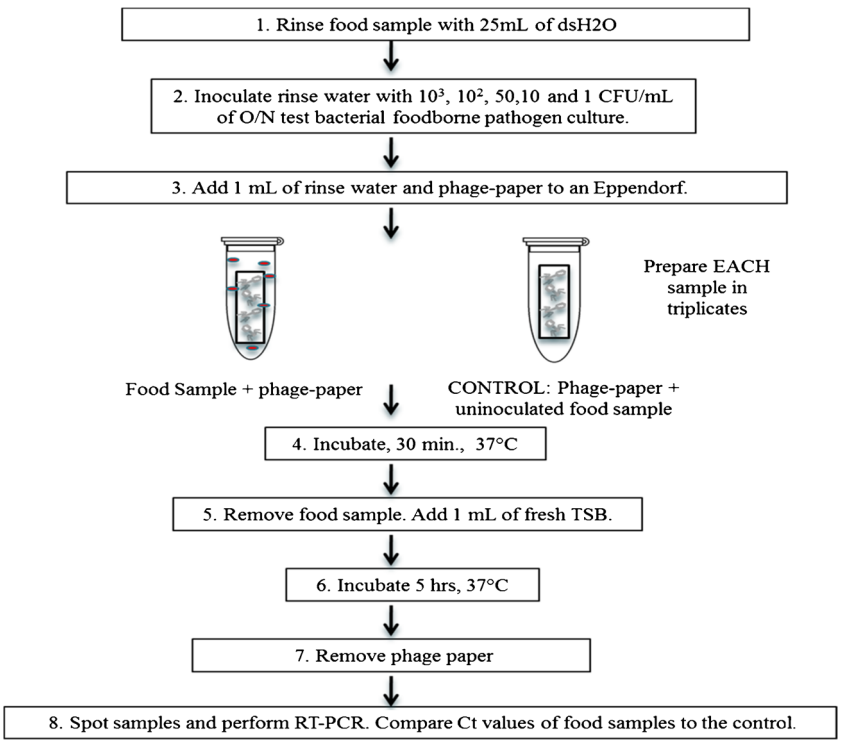

Fig. 2 The phage dipstick approach for detection of foodborne pathogens in a food matrix in combination with quantitative real-time PCR. The total time of the assay is 8 h. CFU colony-forming unit, Ct threshold cycle, dsH2O (distilled water), O/N overnight, RT reverse transcription, TSB tryptic soy broth

(Seward Stomacher® 400 circulator, Cole-Parmer Canada, Montreal QC, Canada). Different dilutions of the target bacteria were prepared in the food homogenate to achieve 10^3, 10^2, 50, 10 and 1 CFU/mL. The strips of phage-printed paper were added to 1.5-mL Eppendorf tubes containing 1mL of food homogenate inoculated with the target bacterium at different dilutions in triplicate. Preparation of dilutions, dispensation in Eppendorf tubes and addition of phage-printed paper strips were done within a few minutes to avoid proliferation of bacterial cells and change in the initial bacterial count. The samples were incubated for 30 min at 37 °C in a shaking incubator to allow the phages to capture their targets, and then the food homogenate was replaced with 1 mL of fresh TSB, and the suspension was further incubated under the same conditions for 5 h. After incubation, the phage strips were removed from each tube, and the suspension in each tube was divided into two equal amounts in separate tubes. One tube was centrifuged at about 1000*g* for 3 min and placed on ice, after which the plaque assay technique was used to count the progeny phages as previously described. The other tube was placed in a water bath for 15 min at 95 °C to release DNA from the progeny phages before detection by real-time PCR. Hence, overlay and real-time PCR techniques were both used to determine the presence of progeny phages in samples. This experiment was performed with immobilized phages stored for 1 day and 1 week after printing to examine the stability of printed phages. Figure 2 summarizes the detection assay steps.

Statistical analysis

The detection experiment was performed as three independents trials, and the averages and standard deviations were determined. The threshold cycle (Ct) values determined by qPCR were averaged for each concentration and compared with the average Ct values for the incubated control paper without phages. An independent-samples *t* test using IBM SPSS statistics, version 23, was applied to the data, and the differences between means were considered significant at $p < 0.05$ for three independent trials.

Results and discussion

Selection of phage immobilization method and paper type and stability of phage-based bioactive paper

Compared with other microorganisms, phages are considered rather stable. Some phage species were found to withstand various adverse environmental conditions, such as high temperature and dryness; however, most phages survive well at temperatures below 60 °C and at pH 5–9 [44]. Desiccation is probably the most damaging process for phages. Multiple studies have shown that exposure to low water activity causes significant irreversible damage to phages [45]. Hence, we selected the printing/coating methods that allow application of an aqueous-based medium (bioink) on the paper, and the bioactive papers with phages applied were stored at 80% relative humidity.

Temperature is another factor affecting survival of phages, with high temperatures having a negative impact on phage infectivity by denaturing viral proteins, including the receptor binding proteins of tail fibres, which initiate the phage–host infection cycle [45]. Hence, the selection of an appropriate coating/printing technique is a crucial step to produce infective phage-based bioactive paper. Drying of wet pulp to a final moisture level between 6% and 7% is a critical step in papermaking, and is usually conducted at temperatures ranging between 110 and 140 °C. Hence, it seems reasonable to apply the temperature-sensitive phages onto a paper after papermaking by means of other techniques, such as blade coating, gravure printing or inkjet printing. Both blade coating and gravure printing provide an opportunity for high-throughput production of phage-based bioactive paper. Phages with different morphology were mixed with carboxymethylcellulose sodium salt and gelatin or carboxymethylcellulose only, and the bioink formed was applied onto the paper by blade coating or gravure printing respectively, and their activity (infectivity) was tested (Table 3). Only one phage, *Salmonella* AG11, was moderately efficient in inhibiting growth of the target bacteria under the conditions required for these coating and printing techniques. On a bacterial lawn, *Salmonella* AG11 phage-printed paper obtained by gravure printing showed a zone of lysis around the paper disc, which indicates that the phage immobilized on the paper retained infectivity. However, the blade-coated phage did not show a clear zone of inhibition (Fig. 3). The fact that a higher AG11 phage titre was initially used (around 1.0×10^{10} PFU/mL) could be the reason for the demonstrated infectivity compared with the other phages (around 1.0×10^9 PFU/mL) that lost their infectivity in the

Table 3 Efficiency of bacteriophage-based bioactive paper produced by Gravure printing or blade coating at inhibiting growth of target bacteria

Bacteriophage	Reduction in bacterial counts in comparison with control (paper with no bacteriophage added), log (CFU/mL)	
	Gravure printing	Blade coating
T4	–0.03 ± 0.17	–0.16 ± 0.03
MS2	0.06 ± 0.07	–0.07 ± 0.11
AG20	0.24 ± 0.02	–0.03 ± 0.04
AG11	1.16 ± 0.17	0.76 ± 0.19

Conditions Whatman no. 1 paper, initial bacterial count 10^3 colony-forming units (*CFU*) per millilitre, incubation time 18 h

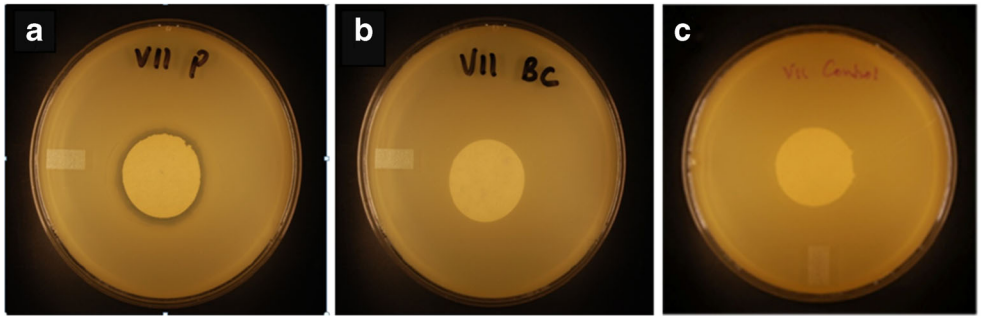

Fig. 3 Infectivity of the gravure-printed or blade-coated AG11-bacteriophage-based paper determined by the overlay method after incubation for 24 h at 25 °C: **a** gravure-printed AG11; **b** blade-coated AG11; **c** paper with no bacteriophage. Gravure bacteriophage-printed paper shows a zone of lysis around the paper disc which reflects the infectivity of bioactive paper

printing/coating process. Moreover, it was reported that *Siphoviridae* phages (such as AG 11) are stabler to drying than *Myoviridae* phages [46]. It is also possible that thermal inactivation of the other phages occurred during the blade coating process since a holding temperature of 45 °C was used before coating and a temperature of 66 °C for 30 s was used to dry the phage coating. Although in gravure printing, exact pinpointing of the cause of phage inactivation during the process is not easy, we suggest that temperature fluctuations of the solution during printing and shear stress might play a significant role in the inactivation of the phage. Since these results showed that both gravure printing and blade coating resulted in substantial loss of phage infectivity, our efforts were redirected towards performing a detailed study on the use of piezoelectric inkjet printing to deposit phages on various types of paper.

Inkjet printing is a common technique for paper surface modification/functionalization but it does not allow the same large-scale productivity as blade coating or gravure printing. Nevertheless, modern printers can reach speeds up to 1000 ft/min, which is sufficient for large-scale production of bioactive papers [47]. Two technologies exist for drop-on-demand inkjet printing: thermal and piezoelectric. In the thermal inkjet process, a very short electric pulse generates an instantaneous temperature rise in the chamber, resulting in rapid vaporization of ink, which creates pressure and forces a droplet of ink onto the paper [48]. In the piezoelectric inkjet process, the applied voltage changes the shape of the piezoelectric material, generating the pressure that forces a droplet of ink from the printer nozzle [49]. Considering that a high temperature of 300 °C for milliseconds is used for ink ejection during thermal inkjet printing [50], piezoelectric inkjet printing done at room temperature (20 °C) was identified as having the greater potential for phage immobilization in this study to avoid the negative impact of high temperature on phage infectivity. Bioinks must exhibit a certain viscosity and surface tension to be compatible with inkjet printing [51]. Glycerol and neutral detergents are the most common viscosity and surface tension modifiers used. The effect of two different concentrations of glycerol (30% and 50%) with 2 mM Triton X on infectivity of non-immobilized phages was determined after 5 h of exposure (Fig. 4). There was no significant difference ($p > 0.05$) between the two glycerol concentrations in the infectivity of phages AG6 and AG11 after 5 h. However, the titres of phages AG10 and AG20 declined by about 2 $\log_{10}$ PFU/mL after 5 h of exposure to glycerol at both concentrations.

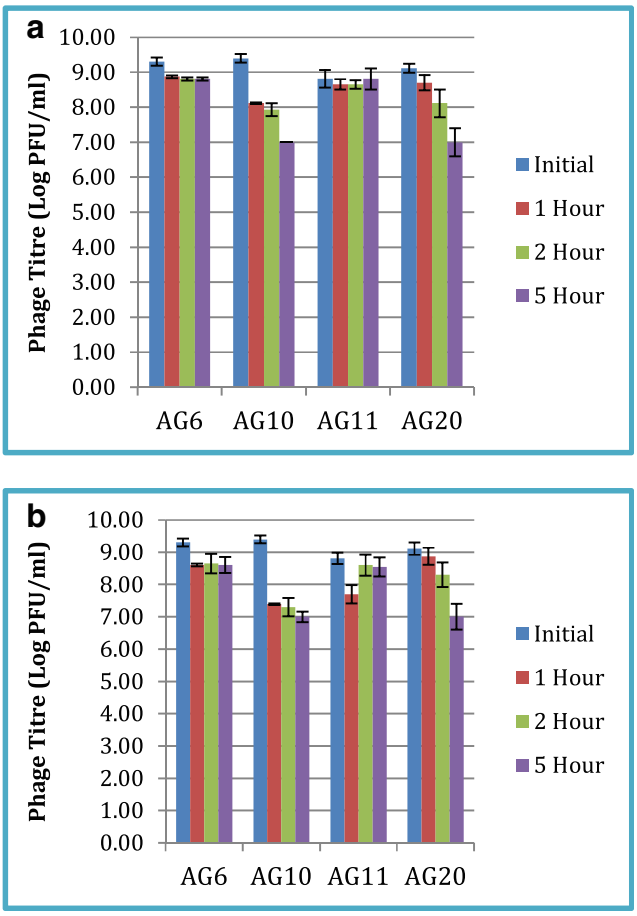

Fig. 4 The effect on bacteriophage titre of bioink containing 30% glycerol (**a**) and 50% glycerol (**b**) with Triton X-100 for piezoelectric inkjet printing. Bacteriophages were resuspended in bioinks, and the titre was determined initially and after 1, 2 and 5 h. PFU plaque-forming units

There was no significant effect from exposure to 30% glycerol for 1 h. Thus, a 30% glycerol concentration was selected for further piezoelectric printing experiments, and the bioink was prepared 30 min before printing.

The major component of any paper is cellulose fibres, but in addition, common printing papers contain up to 30% (w/v) mineral fillers (e.g., calcium carbonate) and certain polymeric additives affecting both the surface properties and the bulk properties of the material, such as wettability, absorption and wet strength [52]. Considering that cellulose carries a slightly negative charge at neutral pH, addition of cationic polymers to paper composites increases their wet strength because of ionic interactions, which is important for further application of bioactive paper in aqueous media [53]. On the other hand, we have shown previously that use of positively charged surfaces for immobilization of phages allows preferential attachment to the surface through their slightly negatively charged heads, leaving the receptor fibres free to capture the target bacterium [18].

Considering these facts, we compared three cationic paper types (hydrogel-coated paper, PVAm-coated paper and commercially available ColorLok paper) with Whatman no. 1 paper (pure cellulose) as materials for phage printing. Printing was done with the piezoelectric inkjet printer, and the bioactive paper samples were compared for their ability to inhibit growth of the target bacterium. The results for *E. coli* phage AG10 are presented in Table 4. The ColorLok bioactive paper produced the highest level of bacterial inhibition, with a more than 2 $\log_{10}$ reduction in count (CFU/mL) when compared with the control (no bioactive paper added). This was followed by the hydrogel-coated paper, with an around 1.5 $\log_{10}$ CFU/mL reduction in the bacterial count. Whatman no. 1 paper and PVAm-coated phage-based bioactive paper did not show a noticeable reduction in the *E. coli* count when compared with the control. Hence, the cationic ColorLok paper was selected for the development of phage-based bioactive paper using the piezoelectric printing technique.

Table 4 Efficiency of bioactive paper produced by piezoelectric inkjet printing of AG10 bacteriophage in inhibiting growth of *Escherichia coli* O157:H7

Type of paper	Average reduction in bacterial counts in comparison with control (paper with no phage added), log (CFU/mL) ± SD
Whatman no. 1 paper	0.24 ± 0.30
PVAm-coated paper	0.1 ± 0.28
Hydrogel-coated paper	1.58 ± 0.63
ColorLok paper	2.28 ± 0.37

Initial bacterial count 10^3 colony-forming units (*CFU*) per millilitre, incubation time 18 h

PVAm polyvinylamine, *SD* standard deviation

The biological activity (infectivity) of the printed *Salmonella* phages (AG6 and AG11), *E. coli* phage (AG10) and *Listeria monocytogenes* phage (AG20) was tested 1 day and 1 month after printing. Paper strips with printed AG 10, AG11 and AG20 phages were able to reduce their bacterial host count by 2.68, 2.11 and 3.89 $\log_{10}$ CFU/mL respectively in comparison with the control (no bioactive paper added) (Table 5). AG11 and AG20 phage-based bioactive paper produced by gravure printing both led to a reduction of the bacterial host count, by 1.16 and 0.24 $\log_{10}$ CFU/mL respectively. The papers produced by blade coating of AG11 and AG20 resulted in 0.76 and 0 $\log_{10}$ CFU/mL reduction of the target bacteria respectively (Table 3). The results obtained with piezoelectric inkjet printing of phages (Table 4) clearly show that this printing method is preferable to create phage-based bioactive paper. Unfortunately, storage of the piezoelectric-printed bioactive paper at room temperature and 80% relative humidity for 1 month resulted in almost complete loss of antibacterial activity. *Salmonella* phage AG6 was not able to survive in the printing process, and the bioactive paper developed was not able to significantly reduce the count of its host in comparison with the control. This supports the notion that optimization of the bioink composition might be required for some phages. Previous results showed that morphologically different phages behave differently when stored in dry conditions [54].

When phage-based ink was applied onto the ColorLok paper by manual pipetting followed by drying and storage at 80% relative humidity and room temperature, infectivity of the phages was mostly retained during 1 month of storage (Table 5). In an attempt to explain these results, we calculated the number of phages deposited by the printing and pipetting approaches. The Dimatix piezoelectric inkjet printer nozzle delivered 10-pL drops and the printer deposited 500 drops per centimetre. Thus, if the initial phage count in the bioink is 1×10^9 PFU/mL, we can calculate that there are around 6×10^5 PFU per square centimetre on the printed paper. When the whole DNA was extracted from the printed paper and used as a template to amplify a unique sequence of the AG11 genome by qPCR, it was estimated that around 10^6 PFU of AG11 per square centimetre were deposited on the paper by the printer, which is comparable to the theoretical calculation. Our calculations (data not shown) show that the amount of bioink pipetted onto the paper did not result in a significantly higher number of phages delivered to the paper per unit area compared with printing. However, the volume of applied bioink was larger in the case of pipetting (100 μL) and, consequently, a larger amount of glycerol was deposited onto the paper surface. This could protect phages from degradation following exposure to desiccation, and might be the cause of the increased stability of bioactive paper produced by pipetting.

Table 5 Efficiency of bacteriophage-based bioactive paper produced by piezoelectric inkjet printing and pipetting in inhibiting growth of target bacteria

Bacteriophage	Reduction in bacterial counts in comparison with control (paper with no bacteriophage added), $\log_{10}$ (CFU/mL) ± SD			
	Printing		Pipetting	
	1-day storage at 80% RH, RT	1-month storage at 80% RH, RT	1-day storage at 80% RH, RT	1-month storage at 80% RH, RT
AG6	0.29 ± 0.23	0.22 ± 0.10	-0.08 ± 0.27	0.15 ± 0.30
AG10	2.68 ± 0.62	–0.08 ± 0.06	2.74 ± 0.69	2.45 ± 0.58
AG11	2.11 ± 0.82	0.13 ± 0.12	3.01 ± 0.96	2.17 ± 0.20
AG20	3.89 ± 0.63	0.05 ± 0.15	4.03 ± 0.33	1.62 ± 2.24

Conditions ColorLok paper, initial bacterial count 10^3 colony-forming units (*CFU*) per millilitre, incubation time 18 h

RH relative humidity, *RT* room temperature

Application of bioactive paper for rapid detection of *E. coli* O157:H7 in broth

Phages can be used for both control of growth of bacterial pathogens and for their quantitative determination [11, 16, 20]. As the bioactive paper demonstrated high infectivity, we decided to use this paper for rapid detection of bacteria. For proof of concept, the rV5 phage was printed onto ColorLok paper with the piezoelectric inkjet printer, and this bioactive paper was used for detection of *E. coli* O157:H7 in TSB as a model system. First, the strip of phage-based bioactive paper (1 cm × 2 cm) was dipped into the sample containing the different concentrations of the target bacterium, *E. coli* O157:H7, ranging from 10 to 1000 CFU/mL and incubated at 37 °C for 30 min. The medium was then removed and replaced with fresh TSB before incubation was continued for another 5 h. As a result of infection, hundreds of new phage particles were released from each infected cell into the medium, and these were subsequently enumerated by a qPCR technique after removal of the bioactive paper strip. The suggested approach provides a two-stage amplification of the target (viz. generation of progeny phages coupled with amplification of the phage nucleic acid sequence by PCR), which results in a higher sensitivity than does use of direct detection of the target bacterium by qPCR. Furthermore, phages infect only live and metabolically active bacteria, so the proposed method is applicable for detection of only live cells, which is important when it is used to assess food and environmental safety [11, 16]. Furthermore, the phage-based detection process results in the death of most of the target bacteria in the sample, which simplifies biological waste disposal, especially when highly pathogenic bacteria are being handled. The use of immobilized phages also allows easy removal of initially introduced phages after the infection process, so the number of phages remaining in the sample is indicative of the number of infected cells present in the initial sample. As a result, the ability to detect low numbers of bacteria in the sample was achieved without the need for enrichment. The whole assay was completed within 8 h. As shown in Table 6, the Ct value obtained from the detection of progeny phages released in tubes containing *E. coli* O157:H7 at 10 CFU/mL was significantly different from that obtained with the control tubes (no bacteria added). Hence, 10 CFU/mL was considered to be the limit of detection for the proposed method.

Detection of bacterial pathogens in foods using phages immobilized on ColorLok paper in combination with qPCR

Phages in nature operate in very complex environments and are capable of efficiently sensing and infecting their target bacterium in the presence of competing microflora and high concentrations of various biologically active compounds. This makes them suitable for in situ monitoring of food and environmental contaminants [55]. Detection of foodborne pathogens in various food commodities using phage-printed ColorLok paper strips in combination with qPCR was

Table 6 Detection of *Escherichia coli* O157:H7 in tryptic soy broth with rV5 bacteriophage-printed paper strips. The progeny bacteriophages were detected by quantitative real-time PCR [threshold cycle (Ct) values]

Bacteria concentration (CFU/mL)	Ct (mean ± SD)
1000	27.35 ± 0.73[a]
100	29.99 ± 0.35[a]
50	30.80 ± 0.37[a]
10	33.24 ± 0.22[a]
0 (control)	33.71 ± 0.09

The data represent the mean ± standard deviation (*SD*) for three replicates.

CFU colony-forming units

[a] Significantly different from the control (no bacteria added), $p < 0.05$

assessed in this study. Spinach, ground beef and chicken homogenates were inoculated with *E. coli* O157:H7, *E. coli* O45:H2 and *Salmonella* Newport respectively at different concentrations. Following addition of the paper strips with immobilized phage, the progeny phages were detected by both the plaque assay and the qPCR technique. Similar detection limits were obtained with both techniques to detect progeny phages, except for *Salmonella* Newport in chicken homogenate, where the plaque assay was more sensitive (10 CFU/mL) than qPCR (50 CFU/mL) (Table 7). However, the total assay time with the plaque assay was around 26 h, while the whole qPCR-based detection assay took around 8 h. The immobilized rV5 phage-based assay was able to detect *E. coli* O157:H7 at 10 CFU/mL in spinach homogenate and the AG2A phage-based assay was able to detect *E. coli* O45:H2 at 10 CFU/mL in ground beef homogenate using both plaque assay and the qPCR technique to detect the progeny phages (Tables 8 and 9). The detection limits achieved are summarized in Table 10. Similar detection limits were obtained when the experiment was repeated with 1-week-old phage-printed paper strips, which were stored at 80% relative humidity and room temperature. The limit of detection of *E. coli* O157:H7 in spinach homogenate and *E. coli* O45:H2 in ground beef homogenate was 10 CFU/mL, while that of *Salmonella* Newport in chicken rinse was 10 CFU/mL with the plaque assay and 50 CFU/mL with qPCR. Phage-based bioactive papers stored for 2 weeks under the same conditions were not able to capture and infect target bacteria, and no progeny phages were detected after the incubation period by the plaque assay or the qPCR technique.

The ability of phages to infect and lyse specific bacteria with the subsequent release of progeny phases provides a useful tool for the detection of pathogens. Several studies have reported the use of phages to detect foodborne pathogens [56–60]. In one such study, free phages were used to detect *Salmonella* and *E. coli* with subsequent detection of progeny phages by ELISA. This resulted in a detection limit of 10^6 CFU/mL [61]. The immobilization of E2 phage onto a magnetoelastic biosensor was applied for capture of the pathogen from artificially contaminated eggshells with a *Salmonella* Typhimurium detection limit of 1.6×10^2 CFU/cm^2 within 30 min [62]. In another study, T4 phage particles were immobilized on a gold surface to ensure uniform and strong binding of the phages. This allowed specific detection of *E.coli* K12 at levels as low as 7×10^2 CFU/mL by surface plasmon resonance [63]. The detection approach presented in this study used phages not only as specific capture agents but also as lytic agents. Furthermore, use of the immobilized phages for detection enhances the efficiency and sensitivity

Table 7 Detection of *Salmonella* Newport in chicken homogenate with CGG4-1 *Salmonella* bacteriophage-printed paper. The progeny bacteriophages were detected by plaque assay [$\log_{10}$ plaque-forming units (PFU)/mL] and quantitative real-time PCR [threshold cycle (Ct) values]

Approximate bacterial count (CFU/mL)	Log AG2A titre ± SD (PFU/mL)	Ct (mean ± SD)
1000	9.48 ± 0.31[a]	17.85± 0.16[a]
100	7.61 ±0.15[a]	21.93 ± 0.89[a]
50	6.77 ± 0.74[a]	25.50 ± 0.76[a]
10	4.75 ± 0.37[a]	26.95 ± 1.13
1	3.41 ± 0.10	27.55 ± 0.19
Control	3.46 ± 0.15	27.45 ± 0.10

The data represent the mean ± standard deviation (*SD*) for three replicates.
CFU colony-forming units
[a] Significantly different from the control (no bacteria added), $p < 0.05$

Table 8 Detection of *Escherichia coli* 0157:H7 in spinach homogenate using rV5 bacteriophage-printed paper strips. The progeny bacteriophages were detected by plaque assay [$\log_{10}$ plaque-forming units (PFU)/mL] and quantitative real-time PCR [threshold cycle (Ct) values]

Approximate bacterial count (CFU/mL)	Log rV5 titre ± SD (PFU/mL)	Ct (mean ± SD)
1000	7.13 ± 0.10[a]	25.58 ± 0.80[a]
100	5.88 ± 0.15[a]	28.36 ± 1.10[a]
50	5.65 ± 0.27[a]	29.27 ± 1.50[a]
10	5.29 ± 0.05[a]	29.70 ± 1.05[a]
1	4.83 ± 0.17	31.39 ± 0.89
Control	4.88 ± 0.42	31.50 ± 1.28

The data represent the mean ± standard deviation (*SD*) for three replicates.
CFU colony-forming units
[a] Significantly different from the control (no bacteria added), $p < 0.05$

Table 9 Detection of *Escherichia coli* O45:H2 in ground beef with AG2A bacteriophage-printed paper strips. The progeny bacteriophages were detected by plaque assay ($\log_{10}$ plaque-forming units (PFU)/mL) and quantitative real-time PCR [threshold cycle (Ct) values]

Approximate bacterial count (CFU/mL)	Log AG2A titre ± SD (PFU/mL)	Ct (mean ± SD)
1000	6.39 ± 0.27[a]	24.06± 0.54[a]
100	6.36 ±0.10[a]	24.91 ± 0.91[a]
50	6.33 ± 0.55[a]	24.56 ± 1.03[a]
10	4.62 ± 0.27[a]	26.77 ± 0.06[a]
1	3.25 ± 0.42	26.68 ± 0.64
Control	3.26 ± 0.24	27.78 ± 0.14

The data represent the mean ± standard deviation (*SD*) for three replicates.
CFU colony-forming units
[a] Significantly different from the control (no bacteria added), $p < 0.05$

Table 10 Summary of the detection limits of bacterial pathogens in different food commodities used in this study with use bacteriophage-based bioactive paper. The progeny bacteriophages were detected by plaque assay (log_{10} plaque-forming units per millilitre) and quantitative real-time PCR (qPCR) (threshold cycle values)

Pathogen	Medium	Total time of progeny bacteriophage detection assay	Detection limit
Escherichia coli 0157:H7	Spinach homogenate	Plaque assay 26 h	10 CFU/mL
		qPCR 8h	10 CFU/mL
E. coli 045:H2	Ground beef	Plaque assay 26 h	10 CFU/mL
		qPCR 8h	10 CFU/mL
Salmonella Newport	Chicken broth	Plaque assay 26 h	10 CFU/mL
		qPCR 8h	50 CFU/mL

CFU colony-forming units

of the detection assay. Firstly, it keeps the phages at concentrations suitable for detection, and the technique allows capture of the target pathogen, which allows its removal from the food matrix. Consequently, it can prevent potential interference between competitor microorganisms and the target pathogen in the food, which may result in false positive results or impede the detection of the targeted pathogen in food. Approaches using immobilized phages can also enhance the efficiency of qPCR for detection in food matrices by removing inhibitors that affect the sensitivity of qPCR [64].

The stability of immobilized phages on paper during storage is an issue, but it can likely be increased by optimization of other factors, such as the relative humidity and temperature of storage after printing on the paper. Other modifications to the paper such as coating with polymers might also help to prolong the stability of the phages, even when they are stored in dry conditions. Our research group has recently shown that the stability of *Salmonella* and *E. coli* O157:H7 phages immobilized in pullulan (a complex and highly branched carbohydrate polymer [65]) was maintained for up to 21 days (unpublished data).

The amount of leached phages from phage-printed ColorLok paper is important for the assay sensitivity. The leached phage counts were determined in the control tubes, and they ranged from 10^3 to 10^4 PFU/mL for all phages used in this study. So optimization of the detection procedure and improvement of the printing technique could further enhance the sensitivity of the assay by decreasing the number of phages released from the paper into the sample. Modification of the paper surface to increase its net positive charge and optimization of bioink composition may help to stabilize the phages incorporated into paper.

Conclusion

In conclusion, a method for producing phage-based bioactive paper using inkjet printing was developed that can efficiently capture and infect target pathogens in broth and food matrices. The phage-based bioactive paper developed was used to develop a specific, rapid and sensitive dipstick detection assay for different foodborne pathogens in food. Further optimization of bioink composition could possibly allow bioactive paper to be produced that demonstrates not only high infectivity but also better stability at room temperature.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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