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Development of a point-of-care technology for bacterial identification in milk

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ABSTRACT

Rapid and sensitive methods for the detection of pathogenic microorganisms are required to monitor contamination in milk and assure public health. A colorimetric antibody-based stack pad system (biosensor) was developed to detect microbiological content of milk based on nitrocellulose membranes. This modification of the lateral flow immunoassay uses a multiple-membrane biosensor containing (from top to bottom): a sample pad, a conjugation pad (with anti-*Escherichia coli* antibodies conjugated to horseradish peroxidase), six *Escherichia coli* strain DH5α bacteria-blocking layers and an absorption pad with dry substrate. Different milk samples were tested, with and without DH5α bacteria. This multistep diagnostic assay was up to 1000-fold more sensitive than the standard laboratory ELISA. This is the first step in the development of a simple and portable device for milk-safety monitoring during all processing stages, with the aim of ensuring fresh and healthy milk for consumption.

1. Introduction

There are more than 6 billion consumers of milk and milk products worldwide, most of them in developing countries [1]. In addition to its excellent nutritional factors, milk is a good growth medium for many bacterial pathogens (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* spp.). The microbiological content of milk and its derivatives affects quality, shelf life, and safety, and thus human health; therefore, constant monitoring of bacterial contamination is important [2,3]. Milk products may become contaminated with pathogens when they come into contact with infected sources in the environment (e.g., colonization of pathogens in the dairy plant environment) [4].

There are several methods available for the detection and enumeration of microorganisms in processed milk; however, many of these are modifications of traditional culture techniques, with test times that have been reduced from several days to approximately ~24 h [2,3,5–7]. Other current analytical techniques (i.e., culture-independent methods), such as real-time polymerase chain reaction (PCR) or immunoassays, often require prior enrichment steps to meet their limit of detection; moreover, they are relatively expensive and complicated, and require a laboratory with specialized equipment [2,8,9]. All of these conventional procedures require 1–7 days on average for complete testing of a milk product; however, in most cases, the industry cannot hold the product for such long periods before it goes to market. Thus, the dairy industry is looking for alternative rapid, low-cost, and simple methods that have been verified with approved standard methodologies [10,11].

With today's continual rise in the use of point-of-care tests (POCT), prioritizing POC applications might provide relevant information in a short time and at minimal cost without the need for a clinical diagnostic laboratory [10,12]. POCT-based sensors (i.e., biosensors) combine a biochemical molecule with a physical signal that can be translated into an indication of the safety or quality of a sample. Such sensitive, selective, rapid, cost-effective, and portable approaches may provide an alternative to existing conventional techniques [10,11]. A wide range and growing number of portable POCT-based sensors that provide rapid 'on-site' results are now available. The lateral flow-based immunoassay is the best-known commercially available application [13]. This assay combines different formats, molecule-biorecognition labels, detection systems and applications [14,15], and has been used for bacterial detection [12,16–19]. The main problems with lateral flow immunoassay approach are its low sensitivity to target microorganisms (10^5 to 10^6 cfu/mL), reduced specificity, and concerns about accuracy, over-reliance on the tests and limited benefits [12,13].

Described here is the construction of a lateral-flow membrane-based POCT device for pathogen detection in milk. Similar to a previous study, these membranes are stacked, and the sample migrates from the

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upper to lower layers. The membrane order is similar to previous setups, i.e., sample, conjugation, blocking and absorption pads [12]. After addition, a sample diffuses from the upper membrane to the next conjugation layer, where it conjugates with the anti-analyte antibodies conjugated to horseradish peroxidase (HRP) that are lying in wait. The resultant complex then migrates to the following (blocking) layer. This nitrocellulose membrane layer is treated with an immobilized analyte (in this case, *Escherichia coli* strain DH5α), which selectively binds to, and stops the migration of free/unbound antibodies while allowing the antibody–analyte complex to pass through. This selectivity is due to the antibodies that are conjugated with the analyte (bacteria) in the sample, which cannot interact with the cells that are immobilized on the membrane and will be free to continue flowing to the bottom layer. In this latter, the color of the absorption pad layer changes, indicating positive detection. The color is produced by the enzymatic reaction of the HRP conjugated to the antibodies. The output intensity may be further correlated to *E. coli* concentration. This approach enables the development of a simple, low-cost, rapid (within min of sample application) and portable tool for measurement of the pathogenic bacteria in milk, for which an antibody is available.

2. Materials and methods

2.1. Reagents and membranes

Amersham Protran 0.45 μM nitrocellulose membranes (cat. no. 10600044), skim milk powder (cat. no. 70166) and 3,3′,5,5′-tetramethylbenzidine (TMB) (cat. no. T0440) were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) tablets (cat. no. P4417), lysogeny broth (LB) (L3022) Lennox Lc ×4 Broth (10 g/L Tryptone; 5 g/L Yeast Extract; 5 g/L NaCl) and Tween-20 (cat. no. P7949) were purchased from Sigma Aldrich (St. Louis, MO, USA). PBS (0.05% v/v)–Tween solution (PBST) was prepared by adding 0.5 mL Tween-20 solution to 1 L PBS (0.203 g/L NaH_2PO_4, 1.149 g/L Na_2HPO_4, 8.5 g/L NaCl, pH 7.2). Skim milk powder solution (5% w/v) was prepared by adding5 g skim milk powder to 100 mL PBST. Milli-Q ultrafiltered H_2O (18.2 MΩ x cm at 25 °C, (Milli-Q, Millipore Co., Bedford, MA, USA) was used in the preparation of all solutions. The pads include: sample (cat. no. GFB-R4), absorbent (cat. no. AP-080), conjugate-release (cat. no. PT-R5) pads, and 5 μM nitrocellulose membranes (cat. no. CNXX9607XXXX103), were purchased from Advanced Microdevices Pvt. Ltd. (Ambala, India).

2.2. Antibodies

Rabbit monoclonal antibodies against *E. coli* (IgG) with (VIR-1004, ViroStat) or without (VIR-1001, ViroStat) conjugated HRP were purchased from ENCO (Petach Tikvah, Israel).

2.3. Bacterial growth

Five bacterial strains were used in this study: two *E. coli* (strains DH5α and K-12) and three *Salmonella typhimurium* (strains R306, SA2386, and TA10104) all obtained from Robert Marks (Ben-Gurion University, Beer-Sheva, Israel). For each experiment, bacteria were cultured in 10 mL fresh prepared LB [20] and incubated overnight at 37 °C in a rotary thermoshaker (Gerhardt, Konigswinter, Germany) at 120 rpm. Then cultures were diluted with fresh LB and regrown at 26 °C, without shaking, to the early log phase of 10^7 cell/mL (optical density at 600 nm (OD_{600}) = 0.2) as determined by an ultrospec 2100 Pro spectrophotometer (Amersham, Cambridge, UK). For each bacterial strain, the 25 mL culture was pelleted (centrifuged at 10,800 × *g* for 5 min at 24 °C; MiniSpin plus, Eppendorf, Hamburg, Germany) and washed with double-distilled water followed by further homogenization by gentle pipetting. This step was repeated three times. The bacteria were then diluted (1:2000) to the final test concentrations ($5*10^3$ cells/mL).

2.4. Device construction

2.4.1. Assay reagent-immobilization procedures

Substrate membranes were made using 6-mm diameter circles cut with a scissor from absorption pads, wet with 30 μL TMB solution and drying in the dark for 1 h at 37 °C. Conjugation membranes (6 mm diameter) were prepared by adding 80 μL anti-*E. coli* (DH5α)–HRP antibodies (diluted with PBST) and drying for 1 h at 37 °C. Blocking pads were made by exposing the nitrocellulose membrane to DH5α for 1 h, prepared as described in section 2.3. Then these membranes were washed three times with PBST and incubated at 24 °C with blocking solution (5% skim milk and 0.05% Tween 20 in PBS) for 1 h. Finally, the nitrocellulose membranes were washed three times and dried at 30 °C.

2.4.2. Assembly of the stacked immunoassay system

The system was assembled by stacking all prepared membranes, one on top of the other, in the order (from top to bottom): sample, conjugation, blocking and absorbent (substrate) pads (Fig. 1). To direct flow toward the center of the pads, provide space and time for the immunoreactions and allow flow with directional integrity, each active layer was separated by a sample membrane and the entire setup was placed in a homemade plastic holder.

2.4.3. Measuring procedure

The milk sample (150 μL) was applied to the top of the sample pad in the plastic holder. The milk was observed to seep through the sample

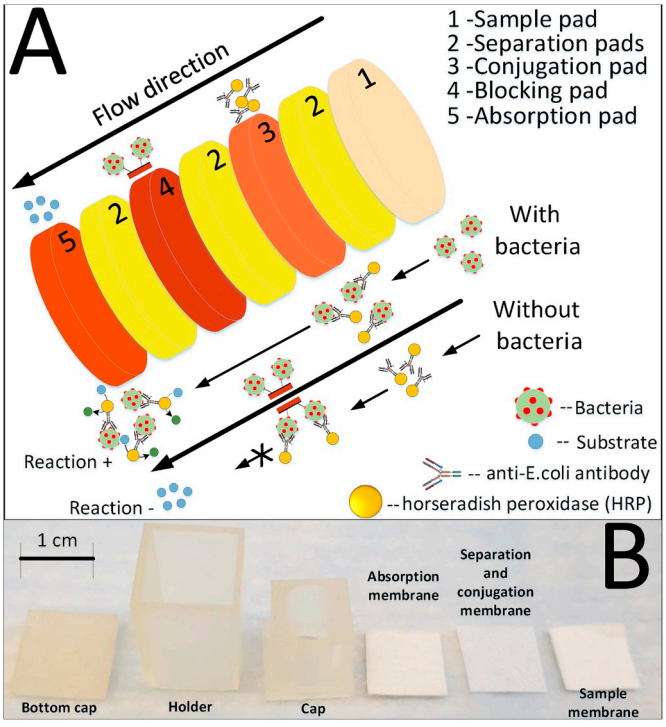

Fig. 1. A. Schematic representation of the stack pad immunoassay and the measurement process. (A) Setup built from a series of layers (1 – sample pad, 2 – separation pad, 3 – conjugation pad, 4 – blocking pad, 5 – absorption pad). Sample is added to the top layer and migrates through the pads to the bottom layer. Antibodies in the conjugation pad interact with any target bacteria in the sample, and only a few, if any, will be available to capture the target cells immobilized in the blocking layer. Thus, the target-positive cluster will flow to the bottom layer of the setup, and a signal will be generated. In samples without bacteria, unbound antibodies from the conjugation pad, dissolved by the sample medium, will flow to the blocking layer and interact with the immobilized cells; as they do not continue on to the bottom layer, no signal is generated. B. Picture of all components used in the StackPad biosensor.

pad and migrate, by either capillary or gravitational force, from the top to the bottom layer. Target analytes first diffused through the layer containing the HRP-labeled antibacterial antibodies (conjugation pad), and then moved through the blocking layer to the absorption pad containing the dried substrate for reaction with the HRP conjugated to the marker antibody. In the absorption layer, milk sample with conjugated HRP rehydrated the substrate and initiated color changes via enzymatic reactions.

2.4.4. Instrumentation

Assays color changes were captured with a cellphone camera (Galaxy S5 SM-G900F, Samsung, Gumi, Korea) placed 30 cm above the absorption layer. The setup was photographed 5 min after addition of the sample. The optimization procedures were done using the more sensitive chemiluminescence reaction as this enabled better quantitation. Substrate solution (luminol:H_2O_2 (1:1, v/v), cat. no.1705040, BioRad, Rehovot, Israel) was layered on the tested pad (i.e., lower layer) and the light response was captured with a Charged Coupled Device (CCD) camera (IVIS Lumina Series III Imaging System, Caliper Life Sciences Inc., Hopkinton, MA, USA) located 30 cm above the membranes. Serial pictures (device photodetector with exposure time of 10–60 s) were taken and analyzed using Living Image® software (IVIS Imaging System, Caliper Life Sciences Inc., Hopkinton, MA, USA).

2.5. Optimization steps

2.5.1. Optimizing the blocking efficiency of the immunoassay

The blocking efficiency in the target-capture layer was optimized by testing several setup configurations)1 to 6 antibody-modified (1:5000 dilution rate) blocking pads). To prevent undirected flow, each blocking membrane was separated with a cotton membrane. In the control setup, unmodified nitrocellulose membranes (0.45 and 5 μm) were used. Each membrane was blocked with the 5% skim milk–PBST blocking solution. Each stack pad configuration was exposed to 150 μL of clear or milk spiked with DH5α bacteria (10^6 cell/mL). The color changes were photographed and analyzed as described in section 2.4.4.

2.5.2. Effect of solution composition on the enzymatic reaction

The effect of the milk on the enzymatic reaction was tested by using two dilutions of the anti-*E. coli*–HRP antibody (1:5000 and 1:10,000) in the different solutions (Pure Milk (i.e., 100%), 10% (v/v) milk diluted with PBST). After initial measurement with a Luminoskan Ascent Luminometer (Thermo Fisher Scientific, San Jose, CA, USA), 100 μL of analyte was added to each well of a 96-well microtiter plate (MaxiSorp, Nunc, Roskilde, Denmark) containing a 100 μL mixture of oxidizing (H_2O_2) and luminol-enhancing reagents (1:1, v/v). Data were collected and the mean and standard deviation of triplicates were calculated for each point, with signals reported in relative light units (RLU) determined by the software with the instrument.

2.5.3. Effect of solution composition on antibody diffusion through the system

Effect of the different milk dilutions on the efficiency of antibody migration was tested through the fully assembled system. The membranes were ordered as follows (top to bottom): sample, conjugation, blocking, separation, blocking, separation, blocking, separation, blocking, separation, blocking, separation, blocking, separation and substrate (absorption pad).

At this stage, only blocking and conjugation layers were modified with immobilized DH5α bacteria (10^6 cfu/mL) and deposited anti-*E. coli*–HRP (1:5000 dilution rate) antibodies, respectively. After setup assembly, 150 μL of the test solutions (0, 10, 50% (v/v) (i.e., diluted with PBST) and pure (i.e., 100%) milk) with anti-E. coli–HRP antibodies (diluted 1:5000) were layered onto the sample pad. After 5 min incubation, all layers were disassembled, placed on a flat surface, introduced into 30 μL substrate solution (luminol:H_2O_2) and photographed with a CCD camera (as described in section 2.4.4).

2.6. ELISA as a benchmark for stack pad sensitivity

The sensitivity of the stack pad immunoassay to the target DH5α bacterial cells was compared with that of ELISA. Each approach was exposed to different concentrations of DH5α (0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu/mL) in milk samples. The highest concentration of 10^6 cfu/mL in PBS was used as the positive control. All setup preparation and operational conditions were as described in sections 2.1–2.5. The ELISA protocol was as follows: 100 μL anti-*E. coli* antibody (diluted 1:2000) solution was added to each well of a 96-well microtiter plate which was then sealed to avoid evaporation, and antibodies were allowed to coat the wells overnight at 4 °C. After incubation, the coating buffer was decanted and the plate rinsed with PBST. Skim milk–PBST solution (150 μL/well of a 5% solution) was added to reduce the overall background and increase the sensitivity of the assay. The plate was then incubated for 1 h at 37 °C and the wells washed three times with PBST. Aliquots (100 μL) of different bacterial concentrations (0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 cell/mL) were added to each well in triplicate and incubated for 1 h at 37 °C. Additional empty wells and wells that were not coated with bacteria were used to check the level of background signal. The wells were washed three times with PBST and 100 μL/well of anti-*E. coli*–HRP antibodies (1:5000 dilution rate) was added. The plate was incubated for 1 h at 37 °C, and washed three times with PBST; 100 μL of the substrate TMB was added to each well and the plate was incubated at 24 °C for 15 min in the dark, at which point 50 μL/well of freshly prepared 2 N H_2SO_4 was added to stop the chemical reaction. Absorbance at 450 nm was determined with a Synergy™ Neo2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Mean and standard deviation of the triplicates were calculated for each point and the signal reported as OD_{450}.

2.7. Sensitivity of the stack pad immunoassay

The immunoassay's sensitivity to the target DH5α cells was compared to the ELISA results. Both approaches were exposed to different concentrations of DH5α (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu/mL) and pure water. All setup preparation and operational conditions were as described in sections 2.1–2.5. The setup composition was as explained in section 2.4.2, using 6 blocking layers and a 1:5000 dilution of anti-*E. coli*–HRP. The various bacterial solutions (150 μL) were added to each setup, and the color changes were photographed and analyzed as described in section 2.4.4.

2.8. Specificity of the stack pad bioassay

Specificity of the stack pads was tested with 5 different bacterial strains: DH5α and K-12, and *S. typhimurium* R306, SA2386, and TA10104. Each strain was diluted with 3% (w/v) homogenized pasteurized milk (Tnuva, Rehovot, Israel) milk to 10^6 cfu/mL prior to exposure to the sensor. The different bacterial solutions (150 μL) were added to each setup, and the color changes were photographed and analyzed as described in section 2.4.4.

2.9. Effect of milk type on setup efficiency

Experiments were done with 4 samples of commercially processed milk from various sources: three different samples of (pasteurized and homogenized) fresh 3% (w/v) fat content milk (from Tara (Noam, Israel), Tnuva, and Yotveta (Kibbutz Yotveta, Israel)), and one sample of 3% (w/v) fat content UHT (ultra-high temperature-processed) milk from Tnuva. The stack pads were exposed to the 4 different commercial milk types spiked with 10^3 cfu/mL DH5α cells. The stack pad sensitivity

to the target DH5α bacteria was compared with that of the ELISA, which was run as described in section 2.6, except that the 96-well plate was incubated with 4 different commercial milk types positively spiked with 10^3 cfu/mL DH5α cells.

2.10. Reproducibility of the stack pad system

For each development stage, a minimum of 30 separate and different setups were exposed to the examined parameters. Reproducibility of the results was estimated by the standard deviation values as shown in Fig. 5.

2.11. Image analysis

Membrane color intensity, recorded in JPEG files, was analyzed with ImageJ software (US National Institutes of Health, https://imagej.nih.gov/ij/). RGB-colored pad images were transformed to a 32-bit format and inverted (converted to pixels along the black and white axis). Then, an average of the pixel intensity was analyzed for each tested pad.

2.12. Statistics

Paired t-tests were used with Excel software (Microsoft, Redmond, WA, USA) to compare between the different *E. coli* DH5α concentrations in milk and negative controls. In this study, a P-values of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Optimization steps and first experimental run

A POCT application for monitoring microbial contaminants in milk was developed. This colorimetric antibody-based stack pad system makes use of the same membranes used in the lateral flow immunoassay, stacked vertically (Fig. 1). In this setup, a hydrophobic layer (i.e., nitrocellulose membrane modified with immobilized 'target' *E. coli* bacterial strain DH5α) acts as the capture layer (blocking pad). The first stage in the optimization procedure was to determine the optimal number of blocking membranes (Fig. 2) that would ensure that HRP-conjugated antibodies that are not bound to bacteria are filtered out of the seeping sample liquid as it migrates to the lower layers. Each system included the anti-*E. coli*–HRP antibodies (in the conjugation pad), the substrate (in the adsorption membrane) and an incremental number (1–6) of nitrocellulose layers (0.45 or 5 μm pore size) carrying immobilized cells. The main hypothesis was that the concentration of antibodies must be lower than that of the available binding epitopes on the bacteria fixed to the blocking membranes. Thus, increasing the number of blocking layers in the system should produce higher capture efficiency, which can be evaluated by color intensity (produced by antibodies that were not blocked) on the bottom layer. Fig. 2 shows the correlation between the number of blocking layers and the device's blocking efficiency. Setups using 5-μm pore size membranes as blocking layers showed similarly high enzymatic activity responses in the configuration with 1–4 active layers. This high enzymatic activity indicated that the setup, in these configurations, was unable to stop antibody diffusion through the system. Setups with 5 and 6 layers showed much lower enzymatic activity—the color intensity in the system with six layers reached background levels and was at least 2-fold lower than in the setups with fewer active blocking layers. This lower response, similar to background values, showed the efficiency of the proposed system at stopping uncontrolled migration of antibodies to the substrate layer that could produce false-positive results.

The effect of pore cutoff size on blocking efficiency was also tested, with 0.45 vs. 5 μm nitrocellulose membranes. The 5 μm membrane showed a dose-dependent response, with an increase in the number of

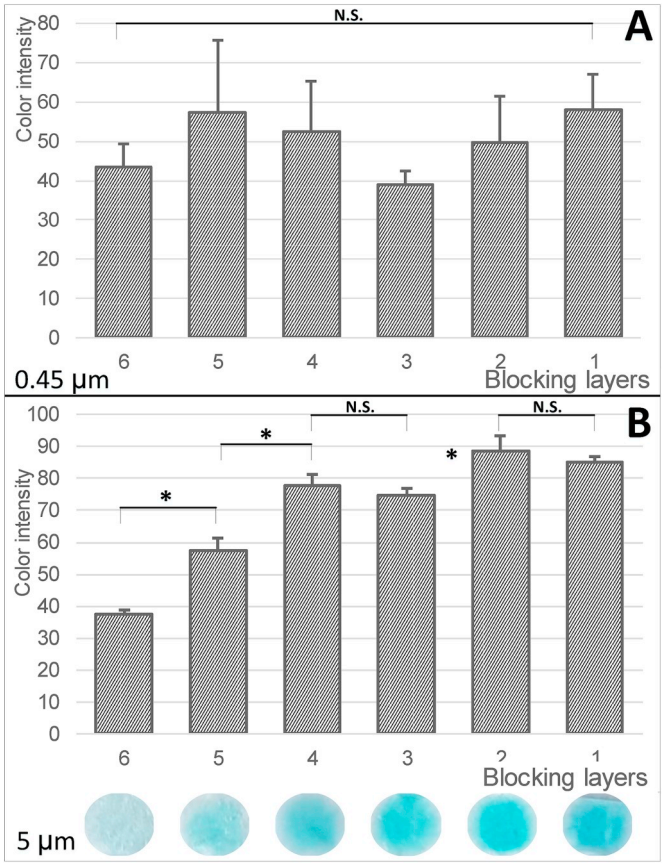

Fig. 2. Effect of membrane pore size ((A) 0.45 μm, (B) 5 μm) and number of blocking layers on the setup's blocking efficiency. N.S.- not significant. *P $<$ 0.001 by pair *t*-test between two near setups with different blocking layers number.

blocking layers enhancing blocking efficiency (Fig. 2B). Responses of the setup with 0.45 μm membranes were not consistent for all tested configurations, but gave values close to background level for all tested conditions (Fig. 2A). *E. coli* are 1–3 μm long and ~0.25 μm in diameter [21], and previous studies have shown these cells' ability to diffuse through 0.45 μm nitrocellulose membranes [12,22]. Moreover, several studies have reported diffusion of bacteria through membranes with a nominal pore size smaller than that of the bacteria [23,24]. The main difference in this study from previous experiments is that the bacteria were in milk and not water. The complex milk matrix components may produce fouling of the 0.45 μm membrane, thereby reducing pore size and preventing bacterial migration through the setup. The similar responses for the 0.45 μm membrane-based setup with all conditions, and their proximity to background levels (Fig. 2A) support this assumption. With the 5 μm membranes, despite the reducing effects, the pore size is still large enough to allow bacterial diffusion through the system. This may be observed as the dose-dependent effect, where increasing the number of blocking layers decreases uncontrolled antibody flow through the system (Fig. 2B). The 5 μm membrane-based setup with 6 active blocking layers showed lower enzymatic activities in the absorption layer, similar to background levels, and this configuration was adopted for further experiments.

The next step in the optimization procedure was to examine the effect of media composition on the HRP enzymatic reactions (Fig. 3). Enzymatic activities of the anti-*E. coli*–HRP antibodies at two dilutions (1:5000 and 1:10,000) in 10% (v/v) and pure milk or PBST solutions were determined. For both tested concentrations, the enzymatic activities were similar for both solvents. Pure milk did not have any effect on the light intensity compared to the clear PBST. With 10% milk, both

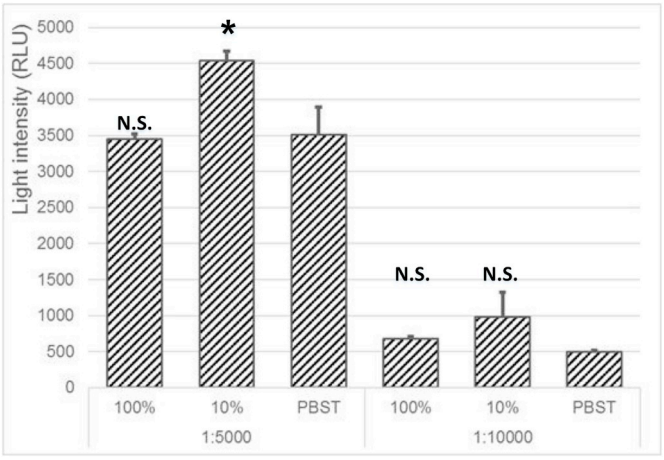

Fig. 3. Effect of milk composition (100%, 10% (v/v) and 0% (v/v) dissolved with PBST) on the anti-*E. coli*–HRP antibodies' enzymatic reaction. Antibodies were diluted (A) 1:5000 and (B) 1:10,000. N.S.- not significant. *P < 0.001 by pair *t*-test between the enzyme light activities in milk and PBST.

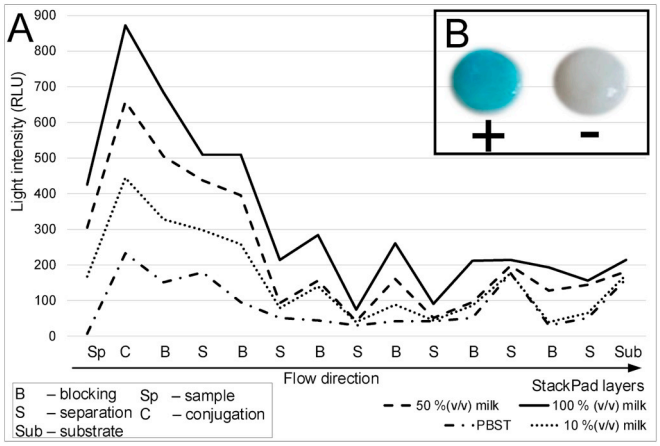

Fig. 4. (A) Effect of milk concentration (100%, 50% (v/v), 10% (v/v) and 0% (v/v) dissolved with PBST) on antibody-diffusion efficiency through the stack pad system. (B) Proof of concept of the optimized immunoassay to detect target bacteria in milk samples.

antibody concentrations showed higher enzymatic activities. This might be due to antibody aggregation in the PBTS solution, and to minor disruptions in light scattering due to the opaqueness of milk. Thus, the 10% dilution rate is not only clear enough to provide more efficient light emission but may prevent molecule aggregation in the liquid. Fig. 3 showed that at a 1:10,000 dilution, the differences between solutions were much lower. At this antibody concentration, the aggregation rates were much lower and would not have a significant role in signal generation. The results showed the ability of the stack pad system to operate effectively at different milk concentrations.

Fig. 4Ashows the effect of milk concentration on the diffusion patterns of the anti-*E. coli*–HRP antibodies through the stack pad setup. Similar response patterns were observed with all conditions, with the highest luminescence in the conjugation pads, followed by a decrease in light intensity through the stack pad system. The light strength, correlated to the enzyme concentration in the membrane, provided information on the antibodies' location in the system at the end of the measurement steps. The blocking processes of the stack pad system are reflected in stronger light intensities in the blocking vs. separation layers. For all variations, the strongest responses were observed in the first two blocking membranes, whereas the output values were similar for the other blocking membranes (Fig. 4A). Changing the milk concentration not only affected antibody distribution, but also enhanced the light responses in the pads. The highest light intensity observed in the first (i.e., conjugation and blocking) stack pad layers was produced by the antibodies bound to those pads. Under all tested conditions, the PBST samples showed the lowest light activity, with no peaks in the blocking layers. This might be due to the speed of the antibodies' diffusion through the system. In this case, rapid release and diffusion rates cannot provide long conjugation/interaction times for the antibodies, and therefore most of them pass through the blocking layers too quickly. In the milk sample, changes in analyte viscosity decreased antibody-diffusion rates, thereby providing longer interaction times. In addition, the complexity of the milk composition may decrease the sample's flow rate because of fouling events in the system [25]. Indeed, increasing the milk concentration enhanced the system output values in all setup layers. Fig. 4 shows these enhancing effects with changes in diffusion patterns. The strongest effect of milk concentration on antibody migration was observed in the first layers, where decreasing milk dilution increased enzymatic activity. This might be due to slower, and therefore more efficient diffusion rates of the antibodies from the conjugation layer. Stronger enzymatic activities in the next 4 blocking layers also reflected slower diffusion processes and therefore higher blocking activity. Nevertheless, the setup showed similar light values in

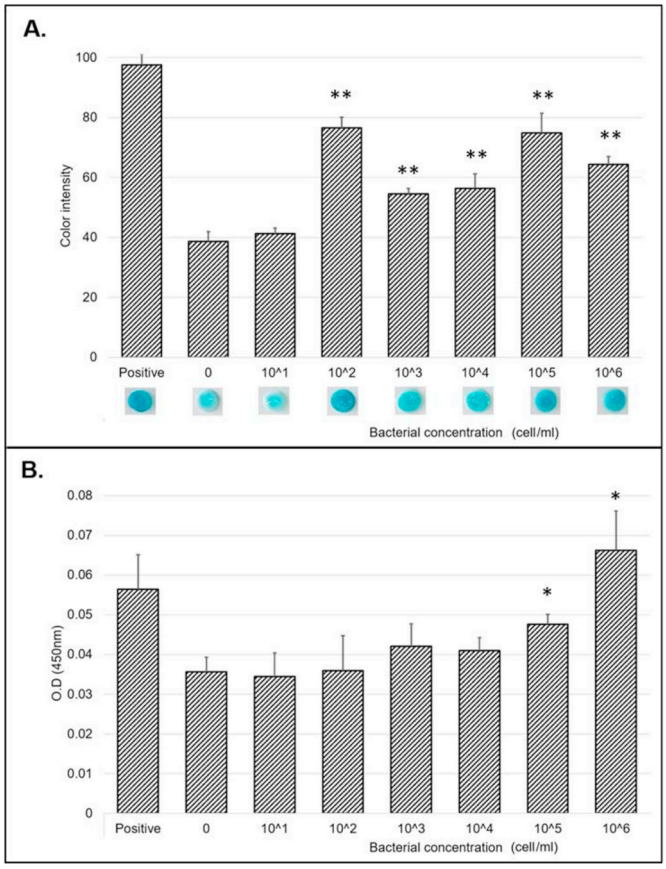

Fig. 5. The response of the stack pad system vs. ELISA to different DH5α *E. coli* strain concentrations. (A) Pad image and JPEG signal analysis based on ImageJ software. (B) Colorimetric ELISA. *$P < 0.005$, **$P < 0.001$.

the last (substrate) layer for all tested samples, despite the different response mechanisms. In the PBST samples, fast diffusion of the liquid through the system concentrated antibodies in the first and last layers of the setup. Thus, after setup deconstruction and substrate addition, enzymatic activities in the layers were expected to be proportional to the antibody concentrations. However, the lowest responses were observed with the PBST sample. The possible reason for such weak responses is a light-quenching process in the layers. The efficiency of quenching is proportional to the extinction coefficient of the molecules

and their concentration in the layer. Thus, in pads with intensive enzymatic reactions, a high concentration of luminescence molecules might increase quenching rates and therefore reduce luminescence activity. Such effects have been observed in other biosensor systems [26]. The system's response to the milk samples supported these assumptions. For example, with 10% milk, the light intensity in the last layers was similar to that of the PBST samples, indicating approximately the same quenching activity. However, the differences in the light activities in the first layers indicated higher antibody-release rates, supported by the observed higher enzymatic activity throughout the setup. Thus, lower antibody concentrations reduced the quenching effects and therefore increased light responses. Further increasing the milk concentration enabled much more effective release and conjugation processes, and therefore more antibodies were stopped in the middle of the stack, reducing enzyme concentrations at the top and bottom of the system and the possible quenching effects. The main conclusion from this stage was that pure milk contributes to the setup's operation conditions and may be used as a sample.

Finally, this modified stack pad system was tested for the ability to detect bacterial infection in milk samples. The final stack pad setup (1:5000 diluted anti-*E. coli*-HRP antibodies in the conjugation pad) was exposed to milk samples with and without DH5α bacteria (10^6 cell/mL). Fig. 4B shows the proposed system's ability to effectively detect bacterial contamination in the milk samples.

3.2. Correlation curve

The stack pad's sensitivity was compared to the colorimetric ELISA, the accepted standard in pathogen detection [27,28]. The proposed system threshold sensitivity (1×10^2 cfu/mL) (Fig. 5A) was higher than that of the ELISA (1×10^5 cfu/mL) (Fig. 5B). Fig. 5A demonstrates a nonlinear correlation between bacterial concentrations and signal generation. The possible reason behind this phenomenon is the effect of the bacterial concentration on cell diffusion efficiency through the setup layers. At higher concentrations, bacteria may stack in the membranes and produce obstacles to other cells migration. Thus, at a lower bacterial concentration (1×10^2 cfu/mL), most of the bacterial will be diffused to the absorption layer to produces higher output signals. The sensitivity of the conventional (commercial) ELISA for the detection of foodborne bacteria is usually relatively low (about 10^5 to 10^7 cfu/mL) [12,29–31], that is 1000-fold lower than that of the stack pad technology, and not useful for pathogen detection in many cases. Recent advancements in nanotechnology improved the detection limit of commercial ELISA by amplifying the assay signals with different nanoparticles. For example, incorporation of gold nanoparticles or carbon nanotubes increases ELISA sensitivity to 10^2 cfu/mL and 10^3 cfu/mL, respectively [29,32]. Other approaches, such as PCR and various bioassays (e.g., applications based on nanocomposites, low-fouling surface plasmon resonance techniques or immune or magnetic gold nanoparticles) have shown similar or better sensitivity [33,34]. However, all of those techniques are much more expensive and time-consuming, requiring a laboratory environment and complicated equipment, and have not yet been validated for use in the field [12]. In addition, they have long assay times and an inability to measure more than one pathogen/test. The proposed application, on the other hand, together with operation simplicity, portability and sensitivity, may be easily adapted to measure many target analytes in the same sample.

3.3. Setup specificity

The stack pad immunoassay's specificity was determined by comparing different *E. coli* (e.g., DHα, K-12) and *S. typhimurium* (e.g., R306, SA2386, TA10104) bacterial strains (Fig. 6). As in the other experiments, pure milk spiked with different bacterial strains was layered on top of the stack in the plastic holder, allowing the bacteria to diffuse from the top to bottom layers. All bacterial strains expressed much less

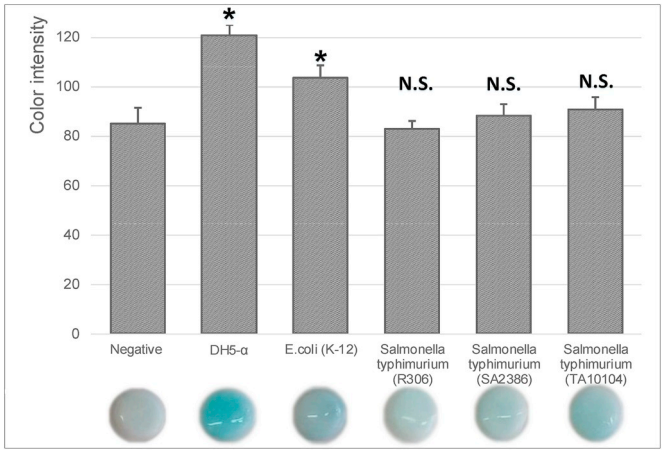

Fig. 6. The specificity of the stack pad assay for different bacterial strains. N.S.-not significant. *P < 0.001 by pair *t*-test between the sensor response to bacteria and negative controls.

intense color changes than the target DH5α strain, confirming the specificity of the antibodies. The low signal generation of the nontarget *E. coli* strain K-12 and *S. typhimurium* strain TA10104 can be explained by cross-reactivity. These results support the general limitations of immunoassays that depend on the specificity of the antibodies used, which may or may not produce cross-reactivity. Thus, to eliminate cross-reactivity, the appropriate single-epitope monoclonal antibodies should be used for future commercial kits.

3.4. Different milk types

The measurement procedure (similar to the previous experiments) with different milk types with and without DH5a cell (10^3 cfu/mL), shows the effective operation of the stack pad system with a variety of milk types (Fig. 7A). The results also shows variance among the 4 samples, probably due to the fact that each sample underwent different modifications during the production process and therefore have different textures, viscosities and compositions [35], which may affect the system. Among the factors influencing milk characteristics, animal feeding, and particularly the nature of the pastures, have been cited as most important for milk quality [35,36]. In parallel, the stack pad application was compared to that of the ELISA (Fig. 7B). Similar to the stack pad results, each type of milk affected the ELISA's differently. What is interesting that the response patterns of both measuring applications were similar. Similar to the stack pads, the lowest responses and differences between negative and positive concentrations were observed with Tnuva and the highest with Tara milk. These results not only validate the stack pads' ability to measure the presence of bacteria in the various types of milk, but also suggested that the source of the signal differences between samples is not a problem in the setup operation or construction. The similarity of the results suggested possible milk effects on antibody–bacteria conjugation or signal-generation processes. In general, despite the differences in the stack pad responses to the different milk types, it still was able to differentiate between all positive and negative samples. These results showed that the proposed system may be used as a fast and simple tool for monitoring bacterial presence in milk.

4. Conclusion

An immunoassay systems for bacterial identification in milk was developed. This technology is based on lateral flow membranes, but the membranes are stacked, instead of in the more common lateral setup. This modified lateral flow immunoassay uses multiple membrane biosensors (from top to bottom): a sample layer, a conjugation layer (with

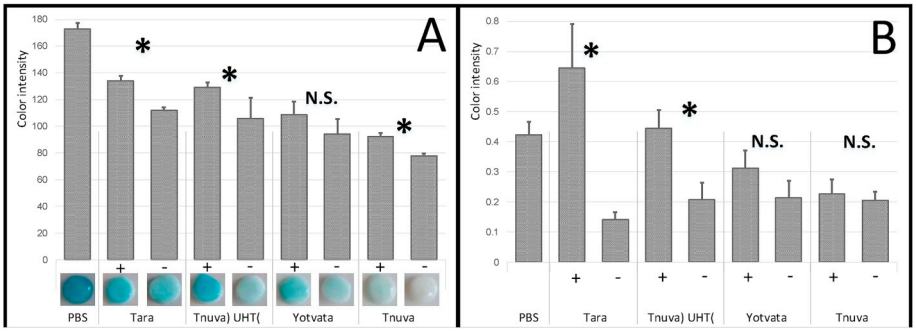

Fig. 7. The response of (A) the stack pad and (B) ELISA to the different commercial milk brands. Both measurement approaches were exposed to clear and bacterium-spiked (DH5α cells, 10^3 cfu/mL) milk samples. N.S.- not significant. *P < 0.001 by pair *t*-test between the sensor with and without bacteria.

anti-*E. coli*–HRP antibodies), 6 blocking pads with DH5α bacteria and an absorption layer with dry substrate. The special feature of this technology is the modified target-capturing nitrocellulose blocking layer, which prevents the unbound HRP-conjugated target antibodies from migrating to the lower layer with the substrate. Thus, the color change is only observed in the presence of the target microorganisms. This method was suitably optimized and partially validated. Aside from being a reliable assay, with 1000-fold higher sensitivity than the standard laboratory ELISA, the colorimetric stack pad immunoassay for bacterial identification in milk can also provide relevant information in a short time and at minimal cost, without the need for a clinical diagnostic laboratory.

5. Future perspectives

Detection of pathogens in milk at different manufacturing and storage stages is important to avoid toxicity and food loss issues. This paper proposes a concept and setup for a rapid, low-cost, simple and field-operational tool based on a color reaction that is not only observable with the naked eye, but can also be photographed for semi-quantitation with a cellphone. This miniaturized system can be operated by unskilled personnel for rapid determination of pathogen presence in milk, at each step during milk processing. The fast diagnostics will facilitate rational decision-making in the management of dairy production by decreasing response times. Multiply measuring strategy will be proceeded by testing the same sample with a bundle of the stacks, each sensitive to a different target analyte.

Author contribution statements

Eltzov Evgeni (E.E.) developed the theory and performed a proof of concept studies. E.E. encouraged Reuven Afriat (R.A.) to investigate the use of this concept for the determination capability of the system to detect microbiological content of milk. R.A. and Daniel Chalupowicz (D.C.) carried out the biosensor experiments. All authors discussed the results and commented on the manuscript and contributed to the design and implementation of the research to the analysis of the results and the writing of the manuscript.

Declaration of competing interest

The authors claim that there are no conflicting interests either from the funding source or their institutional affiliations. We thus state that there are no conflict of interest from the authors.

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