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*Article*

# A Rapid Method to Evaluate Bacterial Content in Food

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**Abstract:** The importance of detecting bacteria in various food products is ever-increasing, due to recent food trends that lend themselves to food contamination. Additionally, the detection of probiotics in food products is of increasing importance to consumers, who realize the benefits of probiotics on one's diet. Existing technologies for detection of bacteria in food are accurate, but most are slow, increasingly costly and unsuitable for applications outside of research laboratories. Optic approaches have recently emerged as an alternative, allowing rapid detection of bacterial presence. This study employs a portable kinetics fluorometer, fabricated in-house, in conjunction with NADH sensitive fluorescence reporter for analysis of various food products. The presence of bacteria is detected in 5 minutes. Both pathogenic and probiotic bacteria were detected in food products, such as raw chicken and beef, spoiled lettuce and contaminated water, yogurt, and kombucha tea. The cellular activity of two probiotic pills was also verified. All samples displayed varying levels of bacterial activity. The study indicates the viability of biosensors being used as an alternate method to detect bacteria in food products – and the viability of a fluorescence-based biosensor to detect viable bacteria. The approach is suitable for both laboratory and field determinations.

**Keywords: optic biosensor; fluorescence; bacterial detection;**

## 1. Introduction

In 2018, an E. coli outbreak due to contaminated romaine lettuce sickened nearly 200 people across the United States, and led to the deaths of five people [1]. Food contamination most often occurs at various stages at the production level; this, coupled with the rising popularity of raw foods and fresh produce [2], makes the detection of pathogens in food increasingly important. Ready-made meals also have the potential to host pathogenic bacteria [3,4], and should therefore be a source of concern. As long as these food products remain popular with buyers, the detection of potentially harmful bacteria should be a primary concern in order to protect consumers and ensure food quality.

On the other hand, there are types of beneficial bacteria, or probiotics, that are present in many types of food, and consumers are increasingly aware of the benefits presented by these probiotics. However, "there is no FDA regulatory definition for the term *probiotic*" and thus the labeling of foods that may contain them is somewhat ambiguous [5]. It is of increasing interest to be able to analyze these foods for the presence of probiotics. Despite improvements in bacterial detection technology, there is still a need for universal bacterial detection processes [6].

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There are various widely accepted methods for microbial detection that have been traditionally employed in the past. One of the oldest techniques is the culture-based approach. What this method offer in terms of accuracy, reliability, and cost-effectiveness, it lacks in efficiency; most culture-based procedures require 2 to 3 days for preliminary identification of pathogens [7].

Another slightly more advanced category of approaches are immunoassays, which rely on the binding of antibodies to antigens [8]. These tests are easier to perform, produce more timely results, and are generally more cost-effective than methods that require bacterial culture. However, they are not always accurate, as false positive results are possible. Additionally, it is favorable to use monoclonal antibodies as opposed to polyclonal antibodies for immunoassays because they are more sensitive and return more precise results; however, they are also much more costly [9].

A slightly different approach involves the use of nucleic acid-based detection methods such as polymerase chain reaction (PCR), nucleic-acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP); these methods focus on targeting and amplifying specific gene sequences of pathogens for identification. These techniques are popular because they are accurate, sensitive, and increasingly time-efficient [10]. However, there are disadvantages to these methods as well. Not only are they a much more expensive alternative [7], but they present more procedural difficulties and are not as easily transferrable to on-site work, as opposed to a research setting [8].

The most recently adopted method of detection is biosensors, generally comprised of a bioreceptor and a transducer, which translates the reactions within the sensor into electrical signals [7]. The optic biosensor has proven to be a highly specific type of biosensor in terms of detecting pathogens. Optic biosensors utilize transducers to detect the physical changes of an analyte after bonding with a biological recognition element; this change is then converted into qualitative or quantitative units in order for easy interpretation by the end user [11]. The use of biosensors in the evaluation of food quality is limited; however, there are many advantages that make them a viable option. Along with their high sensitivity and specificity, they require much less extensive sample preparation, making it a simpler and more time-efficient method [11]. They are also smaller in size, and their portability makes them an attractive option for on-site work. The drawbacks of optical biosensors, and all biosensors, are the high cost and necessary quality assurance that is often associated with the increased efficiency of the product [7].

The focus of this study is rapid identification of viable bacterial cell activity in a variety of food sources using an optical sensor that monitors the change in fluorescence of resazurin dye. Resazurin, which is blue and non-fluorescent, is reduced to resorufin, which is pink and highly fluorescent [12]. Resazurin dye is commonly used to indicate cell viability, and was first used to detect the presence of bacteria in spoiled milk by Pesch and Simmert in 1929 [12].

## 2. Materials and Methods

This study examined the presence of both beneficial and harmful bacteria in various food samples. The tests were conducted under laboratory conditions using sterile equipment and aseptic techniques. In order to estimate the activity of the bacteria, the results were compared to a 1000 cfu/mL of E.coli in lysogeny broth (LB) media. The samples were measured using a fluorescence-based microfluidic device that was fabricated in-house [13,14]. The device automates

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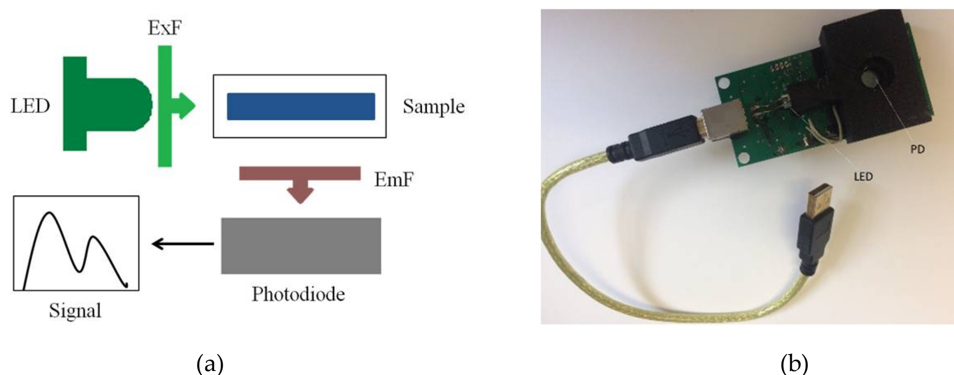
83 the detection of the change in fluorescence of resazurin dye in the presence of viable cells. The device
84 operation and sample preparation are briefly discussed below.
85 The kinetics fluorometer is a single-excitation, single emission photometer that can detect
86 and plot the change in fluorescence of a sample. The block schematics of the device are presented in
87 Figure 1(a). The green denotes the excitation light path (light source, filter, aperture, direction of the
88 excitation light), while the red denotes the emission light path. The excitation and emission light
89 paths are oriented perpendicularly to each other [13]. A prototype of the portable device is shown in
90 Figure 1(b).




91
92 (a) (b)
93 **Figure 1. (a)** Block schematic of the portable kinetic fluorometer, detailing the function of the device. **(b)**
94 Prototype of the device, labeled with the cassette holder, photodiode (PD), emission and extraction filters
95 (EmF and ExF).
96 The microfluidic cassette acts as a sample holder for the device. It is fabricated using three
97 poly (methyl methacrylate) (PMMA) sheets; one 1.5 mm in thickness, and two 0.2 mm thick. A laser
98 cutter is used to cut the 1.5 mm sheets, as well as to engrave the channels. The cassette channel is set
99 to hold 350 µL of a sample. The reading chamber was designed in a way that allows the most
100 efficient filling of the cassette when the sample is injected.
101 After the slides have been cut, each side is sanded for 5 seconds using wet, fine sandpaper.
102 Two holes are drilled into the side of the slide using a 1/32 carbide drill bit. Then they are coated
103 with 70% ethanol and bonded in a conventional oven. This process requires a vice clamp, two metal
104 plates, and two rubber sheets. One side of the cassette is bonded at a time, using the setup described
105 in Figure 2. The pressure from the clamp presses the acrylic sheets onto the slide, allowing them to
106 bond. The rubber sheets and metal plates serve to protect the slide and maintain constant application
107 of heat. The clamp setup is left in the oven for 4 minutes to allow the 0.2 mm sheet to bond to the
108 slide. After one side is finished, two rubber septa are placed in the cassette channel, as demonstrated
109 in Figure 3. After this, the other side of the cassette is bonded in the same way as the first.

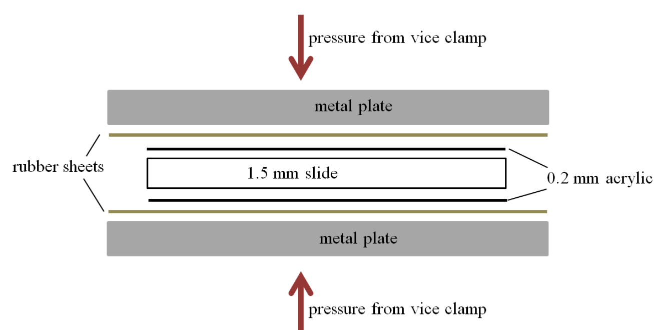



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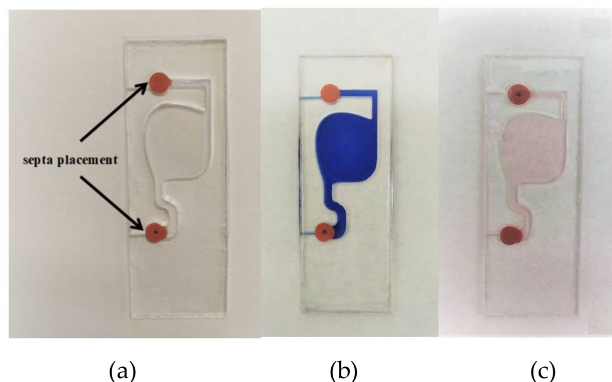
**Figure 2.** The setup, within the vice clamp, that is placed in the oven. The rubber sheets protect the slide from direct contact with the metal plates.




(a) (b) (c)

**Figure 3. (a)** An empty microfluidic cassette showing septa placement, **(b)** a cassette with a non-fluorescent sample, **(c)** and a cassette with a fluorescent sample.

One traditional method of binding acrylic is utilizing high temperatures; however, coating the acrylic in ethanol, then using heat and pressure, removes the internal stresses from the acrylic slide and allow it to bond to two sheets of 0.2 mm acrylic at a lower temperature, forming the microfluidic cassette [15]. PMMA cassettes enhance the accuracy of the device due to their optical characteristics that ensure a high sensitivity to changes in fluorescence.

10 mg of resazurin dye powder was mixed with 1.0 mL of filtered water to create a master mix in a 1.5 mL centrifuge tube. This mixture was then shaken to ensure a homogeneous solution.

This experiment tested several food samples for viable cells. Contaminated water, chicken, and beef were tested for pathogenic bacteria. The presence of probiotics was tested in commercial and homemade yogurt, kombucha tea, and two types of probiotic pills.

Three milliliters of creek water was spiked with $10^3$ cfu/mL E.coli. The mixture was added to 47 mL of LB media. This mixture was then incubated at 37 °C in a shaker at 150 rpm for 2 hours. 1 mL of the resulting cell culture was mixed with 5.0 μL of resazurin dye. The meat samples were tested in a similar way; 1 $cm^3$ of each meat was dipped in 50 mL of respective media, and then quickly removed. It was then incubated under the same conditions as the water sample. Lettuce samples were tested by dropping one piece of lettuce in 50 mL of clean LB media, then incubating for 2 hours at 37°C and 150 rpm.

Both commercially produced and homemade yogurts were tested twice: once when they were fresh and again one week later, after being left in a refrigerator. The yogurts, along with the kombucha tea, were each tested using a standard set of procedures, shown in Figure 4. First, 3 mL of the sample was added to 47 mL of LB media. This mixture was then incubated at 37 °C in a shaker at 150 rpm for 2 hours. 1 mL of the resulting cell culture was mixed with 5.0 μL of resazurin dye, 350 μL of which is injected into the microfluidic cassette and then tested for cell activity.

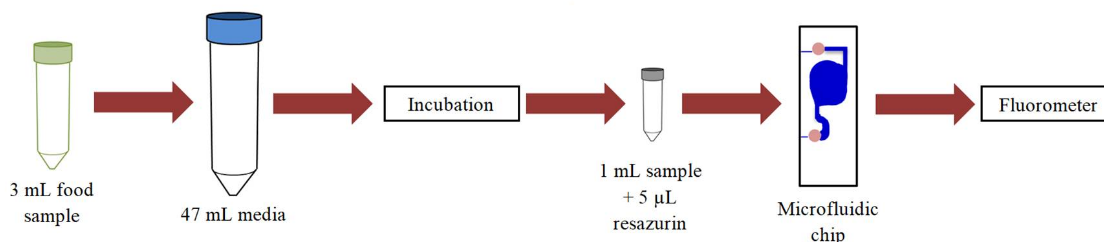



**Figure 4.** Flowchart detailing the process used to test various food samples for probiotic activity.

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Additionally, two types of probiotic pills were tested: bifidobacillus and lactobacillus, both of which have the same colony forming units per mL (cfu/mL). The bifidobacillus pills were added to 50 mL of LB media, and then incubated at 37°C in a shaker at 150 rpm for 2 hours. 1 mL of the resulting culture was then mixed with 5.0 μL of dye, and 350 μL was injected into the microfluidic cassette. This process was repeated for a second trial. The lactobacillus pills were tested twice; one trial was conducted the same way as the bifidobacillus. The lactobacillus pills were also tested after going through stomach-like conditions, simulated by placing the pills in a solution of pH 2.0 until they dissolved. The bacteria pellets were then washed with LB media five times to get rid of the acidic solution, then placed in LB media and incubated according to the procedure previously described. The full procedure is detailed in Figure 5.

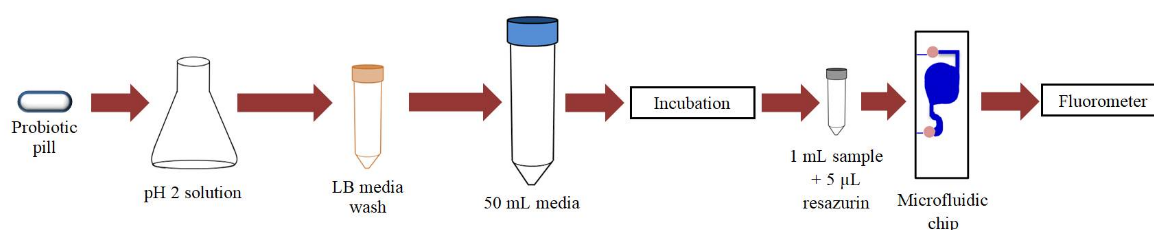


**Figure 5.** Flowchart detailing the process used to test probiotic pills after exposure to a stomach-like environment.

After the samples were prepared and mixed with resazurin dye, they were injected into the microfluidic cassette. Once the cassette was inserted into the device, the control program was initiated. The program continuously measured and displayed the fluorescence intensity every three seconds. The control program also calculated the running value of the slope of the fluorescence intensity change. A linear relationship exists between the amount of bacterial activity in a sample and the change in fluorescence of resazurin dye; therefore, the steeper the slope, the more active bacteria the sample contains.

## 3. Results

The metabolic activity of the various samples was evaluated in duplicates. Negative controls were used for each individual sample. The contaminated water was compared against water filtered through 0.2 micron filter, the yogurts against milk, the kombucha against filtered kombucha (black tea), and the media containing probiotic pills against plain media. Both meats were tested multiple times over a several-day time period, with fresh raw beef/chicken used as a negative control. Lettuce was tested after being left in a sealed refrigerated bag for 2 weeks, and was compared to fresh lettuce. All tests were performed at room temperature for 5 minutes and the activity was compared to a 1000 cfu/mL concentration of E.coli in media. Figure 6 demonstrates the dynamics of the test.

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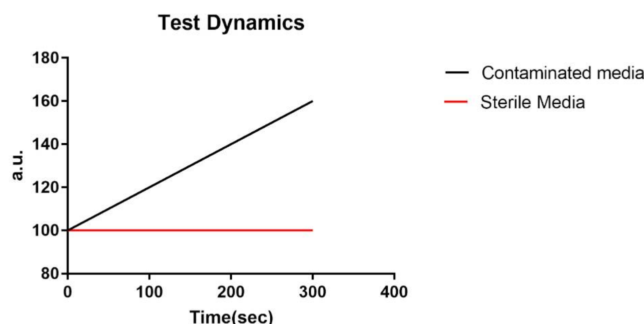
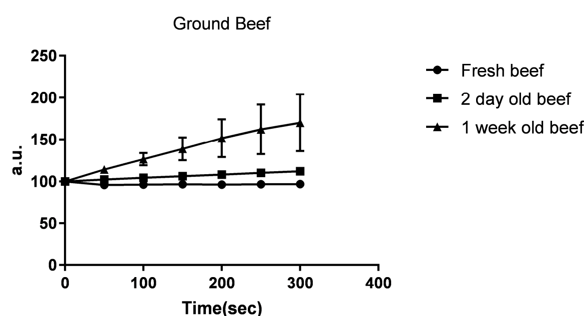



171

172 **Figure 6.** A hypothetical test run demonstrating the difference between sterile media and media
173 containing bacteria. Contaminated media should result in a positively sloped line, while sterile media
174 should have a slope of near-zero.

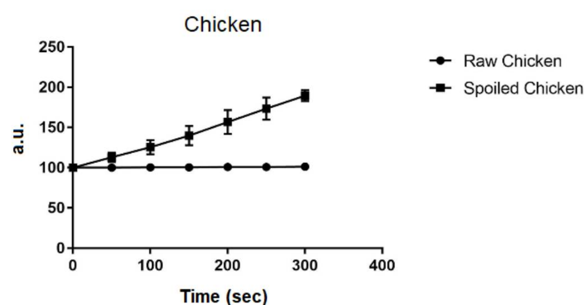
175 *3.1. Pathogen Results*

176 Ground beef and chicken, both raw, were tested and compared to their negative controls.
177 The ground beef was tested three times: once fresh, again after being left at room temperature for 2
178 days, and finally after 7 days at room temperature. As shown in Figure 7, the fluorescence intensity
179 increased significantly on Day 2 and Day 7 when compared to the low change in fluorescence of the
180 negative control (fresh beef).




181
182 **Figure 7.** The fluorescence intensity (a.u.) of beef at different time intervals.

183 The chicken was tested twice: once fresh, to act as the negative control, and again two days
184 later, after having been left at room temperature. The fluorescence intensity increased when the
185 chicken was measured on the second day; the difference in the change in fluorescence intensity
186 between these two samples is indicated in Figure 8.




187

188 **Figure 8.** The fluorescence intensity (a.u.) over time of spoiled chicken as compared to raw chicken.

189 Contaminated lettuce was also tested; lettuce was left in a sealed plastic bag in a refrigerator
190 for 2 week, then the bacteria was cultured and tested. This lettuce was compared to a fresh piece of

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lettuce. The spoiled lettuce displayed much more bacterial activity than the fresh lettuce. Figure 9 displays the changes in fluorescence of both samples.

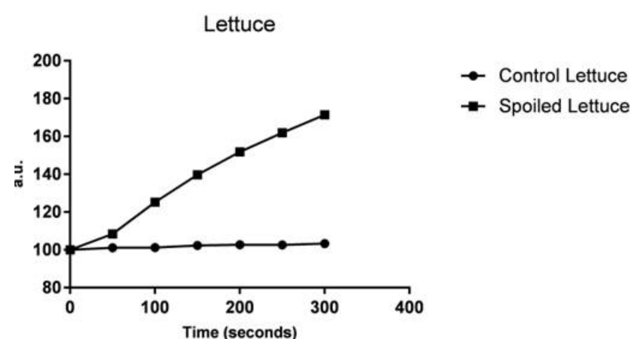


**Figure 9.** The fluorescence intensity (a.u.) over time of spoiled lettuce was compared to fresh lettuce.

Contaminated creek water was also tested, against filtered water. As expected, the contaminated water displayed much more bacterial activity than the filtered water, which displayed almost no activity. Figure 10 compares the slopes of fluorescence change of the two samples.

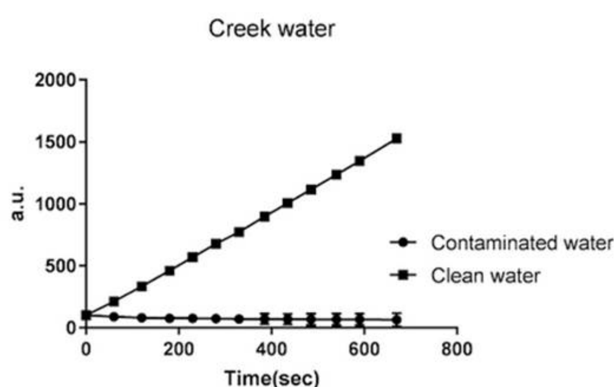


**Figure 10.** The fluorescence intensity (a.u.) of contaminated creek water over time compared to that of filtered water.

*3.2. Probiotic Results*

Kombucha tea, homemade yogurt, and commercial yogurt were tested and compared to each other; the resulting slopes of the change in fluorescence for each test are shown in Table 1. Different yogurts vary in activity levels because they have distinctive recipes that lead to the use of different strains. Although most commercial yogurts are enhanced with probiotics, the commercial yogurt tested initially displayed less activity when compared to the homemade yogurt, with slopes of 2.1 and 4.8, respectively. One week later, however, the homemade yogurt dropped in activity while the commercial yogurt did not display this same decrease in activity; the slopes decreased by 4.4 and 0.4, respectively.

**Table 1.** All tested probiotic sources and the slope of the resulting absorbance values, representative of the activity of the probiotic.

| Sample Type | Slope (a.u.) |
|---|---|
| Fresh homemade yogurt | 4.8 +/- 0.40 |
| One week old homemade yogurt | 0.4 +/- 0.01 |
| Fresh commercial yogurt | 2.1 +/- 0.10 |
| One week old commercial yogurt | 1.7 +/- 0.10 |
| Milk | 0.03 +/- 0.001 |
| Plain media | 0.0001 |
| Kombucha tea | 1.4 +/- 0.1 |

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| | |
|---|---|
| Filtered Kombucha tea | 1.0 +/- 0.1 |
| Plain media | 0.0001 |
| $10^3$ cfu/mL E. coli (control) | 0.2 +/- 0.01 |

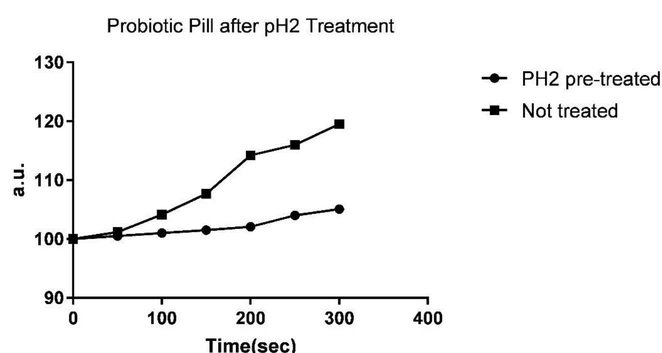
212 Similarly, kombucha tea also showed high cell activity, with a slope of 1.4. Upon testing the 213 filtered kombucha (black tea), it was noticed that the tea behaved similarly to a contaminated 214 sample. Kombucha tea is made by adding what is commonly referred to as a SCOBY, a symbiotic 215 culture of bacteria and yeast, to green or black tea. The bacteria and yeast both aid in the 216 fermentation of the tea, and some of the added bacteria are similar to the probiotics found in 217 yogurts, although they are not as prevalent in the tea [16]. Table 1 shows the activity of the probiotics 218 in various food sources, represented by their slopes.

219 In addition to yogurt and kombucha tea, probiotic pills of different strains were tested 220 against each other in different concentrations. In order to demonstrate the device sensitivity, the 221 probiotic pills samples were diluted. Table 2 shows the different concentrations of bifidobacillus and 222 lactobacillus pills and their corresponding slopes. It was shown that bifidobacillus was more active 223 than lactobacillus. Differences in initial bacterial concentration, bacterial generation time, or 224 environmental tolerance could all have contributed to this distinction. The fluorometer clearly 225 distinguished between the two different probiotics, as well as the different concentrations. The 226 largest standard error was 0.5, calculated for a 25% concentration of lactobacillus.

227 **Table 2.** Various concentrations of probiotic pills, and the corresponding slopes of the absorbance values.

| | Slope (a.u.) | |
|---|---|---|
| Percent Concentration (%) | *Bifidobacillus* | *Lactobacillus* |
| 50 | 2.1 +/- 0.11 | 1.4 +/- 0.2 |
| 25 | 1.5 +/- 0.06 | 1.0 +/- 0.5 |
| 12.5 | 1.0 +/- 0.02 | 0.4 +/- 0.0 |

228 The lactobacillus probiotic pills were also tested after being pre-treated in a pH 2 229 environment. This was done to simulate the activity of the probiotic after passing through 230 stomach-like conditions. Figure 11 shows the results of this trial; the pH pre-treated probiotic 231 exhibited much less cell activity than its non-treated counterpart.




232

233 **Figure 11.** The fluorescence intensity (a.u.) of probiotic pills over time. One trial was performed after the 234 pill was exposed to an acidic environment, to simulate stomach-like conditions.

235 **4. Discussion**

236 The optical biosensor was indeed able to detect the presence of both pathogenic and 237 probiotic bacteria. Between each test for pathogenic bacteria, both the beef and chicken were stored 238 at room temperature; bacteria is known to grow most rapidly between 40°F and 140°F, therefore it is 239 expected that the amount of bacteria in the sample would continue to increase as time progressed

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[17]. The results obtained from the kinetic fluorometer support this statement. The fresh beef, which served as a negative control, showed no bacterial activity. When tested seven days later, however, the fluorescence had increased significantly, indicating an increase in bacterial activity. This principle also holds true for chicken: when tested two days later, the fluorescence of the chicken had increased much more than that of the fresh, raw chicken.

The results of the probiotic testing give insight into the cell activity in probiotic-heavy foods such as yogurt and kombucha. Some commercial yogurts are subjected to heat treatment, a process meant to prolong shelf life that also kills most of the live cultures that were used to produce the yogurt. Yogurts that bear the "Live and Active Culture" seal, such as the yogurt that was tested in this study, must contain 100 million cultures per gram when the yogurt is manufactured, a standard set by the National Yogurt Association [18]. However, there is no set way to determine how much bacteria is present in homemade yogurt, because the amount of starter culture used is variable. Additionally, most homemade yogurts are not heat-treated after fermentation because they do not need to have a very long shelf life.

Commercially produced yogurt maintained a steadier level of bacterial activity, most likely because of the additional steps in the manufacturing process that retain bacterial cultures or provide a more suitable environment for their preservation. This phenomenon could be further expanded upon by testing various types of commercially produced yogurt, some heat-treated, as well as homemade yogurts with different concentrations of starter cultures. Further testing may elucidate the reasons behind commercial yogurt's apparent ability to sustain probiotic cultures more efficiently than homemade yogurt.

Testing kombucha tea for bacterial activity revealed a less-than-expected difference between kombucha, which contains probiotics, and regular black tea. This indicates that the mechanisms of the sensor may not have accounted for interference due to caffeine or other compounds present in regular tea.

When the probiotic pills were tested, both pills displayed high levels of bacterial viability. Additionally, testing the pill after exposure to a strongly acidic environment resulted in a large decrease in activity. The pH range for optimal lactic acid bacteria activity, including lactobacillus activity, is 6.3-6.9 [19], and very acidic environments have been shown to greatly impact the viability of these bacteria [20]. This indicates that the passing of probiotic bacteria, unprotected, through the stomach would significantly reduce the activity of the bacteria.

## 5. Conclusions

In this study, we showed that our approach allows for field detection of the presence of bacteria, either probiotic or pathogenic, in various food products based on viable cell activity. To study the bacterial activity, the fluorescence change in resazurin dye over time was examined by a portable microfluidic based kinetics fluorometer. The use of a portable biosensor such as the one described in this study would be beneficial in the food industry, as an on-site rapid way to test the quality and safety of food products.

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writing—original draft preparation, Mustafa Al-Adhami and Marisa Patsy.; writing—review and editing, Yordan Kostov.; visualization, Mustafa Al-Adhami.; supervision, Yordan Kostov.; project administration, Yordan Kostov and Govind Rao.; funding acquisition, Yordan Kostov.

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