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Rapid detection and enumeration of aerobic mesophiles in raw foods using dielectrophoresis

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ABSTRACT

The concept of dielectrophoresis (DEP), which involves the movement of neutral particles by induced polarization in nonuniform electric fields, has been exploited in various biological applications. However, only a few studies have investigated the use of DEP for detecting and enumerating microorganisms in foodstuffs. Therefore, we aimed to evaluate the accuracy and efficiency of a DEP-based method for enumerating viable bacteria in three raw foods: freshly cut lettuce, chicken breast, and minced pork. The DEP separation of bacterial cells was conducted at 20 V of output voltage and 6000 to 9000 kHZ of frequency with sample conductivity of 30–70 μS/cm. The accuracy and validity of the DEP method for enumerating viable bacteria were compared with those of the conventional culture method; no significant variation was observed. We found a high correlation between the data obtained using DEP and the conventional aerobic plate count culture method, with a high coefficient of determination ($R^2 > 0.90$) regardless of the food product; the difference in cell count data between both methods was within 1.0 log CFU/mL. Moreover, we evaluated the efficiency of the DEP method for enumerating bacterial cells in chicken breasts subjected to either freezing or heat treatment. After thermal treatment at 55 °C and 60 °C, the viable cell counts determined via the DEP method were found to be lower than those obtained using the conventional culture method, which implies that the DEP method may not be suitable for the direct detection of injured cells. In addition to its high accuracy and efficiency, the DEP method enables the determination of viable cell counts within 30 min, compared to 48 h required for the conventional culture method. In conclusion, the DEP method may be a potential alternative tool for rapid determination of viable bacteria in a variety of foodstuffs.

1. Introduction

Constant monitoring and rapid identification of microbial contamination in each process of food manufacturing are required to optimize manufacturing conditions and improve food quality (Salam et al., 2013). The traditional method of microbiological testing, primarily based on culture methods, is used as a gold standard in the food industry. However, conventional culturing processes require complicated laboratory techniques and are often time consuming, which potentially delays quick and appropriate actions against potential hazards, thereby resulting in loss of product quality (Lazcka et al., 2007; Leonard et al., 2003). In addition, conventional methods are limited in sensitivity; false-negative results may be obtained owing to viable but non-culturable bacteria (Law et al., 2015). Based on this perspective, methods that are reliable, rapid, and effortless are required to enable the detection and monitoring of microorganisms in food products (Hindson et al., 2005; Lim et al., 2005; MicroVal Secretariat, 1998; Miyamoto, 2000).

Several approaches have been employed to improve existing methods and develop rapid alternatives to traditional techniques, which are based on immunology, biochemistry, and molecular biology, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), near-infrared (NIR) spectroscopy, and Raman spectroscopy (Garcia-Gonzalez et al., 2010; Hiwaki et al., 2009; Levi et al., 2003; Madrigal et al., 2016; Barreiro et al., 2017; Alexandrakis et al., 2011; Meisel et al., 2012). Nevertheless, every developed method for rapid microbial detection has certain limitations. Immunoassays, biosensors, and nucleic acid-based methods such as the polymerase chain reaction (PCR) method, for instance, are novel detection methods with high sensitivity and specificity and are rapid; however, they require

Abbreviations: dielectrophoresis, (DEP); polymerase chain reaction, (PCR); phosphate-buffered saline, (PBS); flow cytometry, (FCM).

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sample pre-enrichment to enhance the sensitivity of methods and skilled personnel. In particular, PCR-based methods cannot distinguish viable cells from dead cells (Park et al., 2014).

An alternative microbial detection system relying on the principle of dielectrophoresis (DEP), which involves the movement of neutral particles by induced polarization in nonuniform electric fields, has demonstrated its potential for effective manipulation, separation, and characterization of microorganisms and bioparticles. A DEP force is mainly depended on particle sizes, electric field strength and frequency, and dielectric properties of particles and media. The phenomenon was first described by Pohl in the 1950s and has since been utilized for the concentration and separation of microbes and the characterization of bacterial properties (Gascoyne and Vykoukal, 2002; Pethig, 2010; Pethig and Markx, 1997; Pohl et al., 1978; Pohl and Kaler, 1979; Pohl, 1951). DEP-based approaches for separation have been demonstrated using lab-on-a-chip devices comprising microchannels and microelectrodes, along with the improvement of miniaturization techniques and microfluidics. Various improved devices have been designed and developed to perform highly efficient DEP manipulation of biological matter (Tuval et al., 2005; Whitesides, 2006). Some devices enabled to distinguish between live and dead cells (Hakoda et al., 2010; Lapizco-Encinas et al., 2004). Kikkeri et al. (2018) used a microfluidic platform for monitoring bacterial viability. Although DEP manipulation of biological cells in healthcare applications has had the greatest impact, DEP techniques demonstrate great potential for applications in other areas such as environmental research and drug development (Abdul Razak et al., 2013; Becker et al., 1995; Martinez-Duarte, 2012; Abd Rahman et al., 2017). We previously used a DEP-based instrument for the rapid enumeration of aerobic bacteria in drinking water (Wakizaka et al., 2020).

The potential of DEP-based approaches as a practical tool to rapidly detect the presence of bacteria in food has attracted attention in the food industry. Betts suggested that the application of DEP techniques is theoretically possible despite the complex nature of food materials being an obstacle (Betts, 1995). Brown et al. (1999) reported the potential of a real-time enumeration method for suspended bacteria using a continuous-flow DEP system. In the 2000s, the interest in using DEP techniques for the identification of foodborne pathogens increased, based on an urgent need for novel approaches for microbial identification (Fernandez et al., 2017). Although several studies have focused on various aspects of bacterial detection, few studies have focused on the enumeration of microorganisms in actual foodstuffs by DEP-associated tests.

To facilitate the use of DEP techniques as practical alternatives in industrial food safety measures, the effectiveness and robustness of DEP-based methods for bacterial detection using various food types need to be evaluated. Therefore, we evaluated the efficiency of a DEP method using microfluidic devices for the enumeration of viable bacteria in certain types of raw foods, compared to a conventional culture method that is commonly used for microbiological testing. The purpose of this study was to demonstrate the effectiveness of the DEP method for enumerating viable microbial cells that contaminate food. In addition, to evaluate the applicability of the DEP method in physically processed food products, viable bacterial cells in frozen and heated foods were enumerated.

2. Materials and methods

2.1. Comparison between DEP-based and conventional culture methods for identifying viable bacteria

2.1.1. Sample collection

Cut lettuce, raw chicken breast, and minced pork were purchased from local supermarkets in Sapporo, Japan, because these items were relatively high sales volume and rapid determination of bacterial contamination level is important for quality control. Some food samples were stored at 3 ± 1 °C from 24 h to 72 h to increase the bacterial concentration.

2.1.2. Sample preparation

A portion of 5 g of food sample was placed in a storage bag along with 45 g of dilute phosphate buffer (ELESTA Buffer, ELB100N, AFI Corp., Kyoto, Japan), which was then homogenized using a Stomacher (Seward, Worthing, United Kingdom) for 2 min at the normal speed. Since we confirmed the equivalence of the viable bacterial cell numbers between 5 g, 10 g, and 25 g samples in a preliminary experiment, we employed 5 g sample to test for many samples in the present study.

2.1.3. Enumeration of viable bacteria via conventional culture method

One milliliter of the homogenized sample was serially diluted 10-fold with 0.1% peptone water (Merck, Darmstadt, Germany). To determine the aerobic plate count (APC), an aliquot of 100 μL of sample solution, which was diluted at the range from 10^{-1} to 10^{-4}, was spread onto tryptic soy agar (Merck) plates. The plates were then incubated at 35 °C for 48 h, and the colonies were enumerated (Langston et al., 1993; Fang et al., 2003; Koseki and Isobe, 2006). Triplicate plates were subjected at each dilution degree, and the mean values of plate counts for the triplicate plates were transformed to log CFU/g.

2.1.4. Enumeration of viable bacteria via DEP method

2.1.4.1. DEP instrumentation setup. A schematic representation of the experimental setup is shown in Fig. 1. The system consists of PixeeMo™ (AFI Corp.), which was the main operation apparatus, a microfluidic device, and a dedicated computer for image analysis. PixeeMo™, a compact instrument that enables rapid enumeration of bacterial cells using microfluidic devices containing micro-electrodes for attracting microbes (ELESTA chips, ELC121N, AFI Corp.), was used to identify viable bacteria using the DEP method (Wakizaka et al., 2020). Operation of the system comprised four steps: feeding liquid into the microchannel of the device, capture of microbes on electrodes, microscopic observation, and enumeration of microbial cells. The devices are based on fluid, electric filtering, and sorting technology and enable the separation of microorganisms from other components in heterogeneous particle mixtures such as food products. An alternating current electric field is generated by the electrodes, and a DEP force is subsequently exerted to attract bacteria from the sample that is fed into the microchannel. The device contains two functional parts of micro-electrode rows with each voltage output channel (channel 1: CH1, channel 2: CH2). CH1 selectively captures bacteria from other particles at a relatively high flow speed, and CH2 is used to recapture the bacteria. The installed software controls the constant fluidic flow rate and voltage supply, whereas the

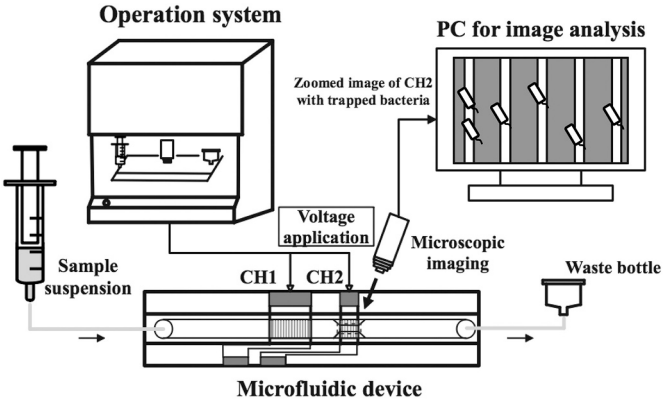

Fig. 1. Schematic representation of the DEP system. The system consists of a main operation apparatus, a microfluidic device, and a dedicated computer for image analysis. DEP, dielectrophoresis.

apparatus operates the liquid feed and voltage application. When the sample liquid was expelled from the syringe and voltage was applied to the electrodes, the bacteria were captured onto the edges of the electrodes. After a specified feeding time period, digital images of electrodes were captured and analyzed using an optical microscope, and then the concentration of the bacteria was calculated.

2.1.4.2. Procedure for DEP-based microbial detection

2.1.4.2.1. Sample preparation. The homogenized suspension obtained as described in Section 2.1.2. was added to two 50 mL conical tubes (5 mL each), and the two samples were centrifuged with a refrigerated centrifuge (Model 3700, Kubota, Tokyo, Japan) at 8000 ×*g* for 5 min. The supernatant (4.5 mL) in each tube was carefully discarded and replaced with the same amount of ELESTA buffer. This process was repeated twice (cut lettuce) or thrice (raw chicken breast and minced pork) to ensure that all bacterial cells were collected, and to lower the conductivity of the suspension to the required extent. After a final centrifugation and displacement step, the two homogenized suspensions were mixed into a single tube. If necessary, an appropriate amount of phosphate-buffered saline (PBS) was subsequently added to the mixture to adjust its conductivity to an appropriate value (Table 1). The prepared solution (3 mL) was drawn into a 10 mL syringe, which was then connected to the instrument via the syringe holder. The syringe containing the sample was attached to the microchannel device installed on the stage of the apparatus, and then a definite amount of the liquid sample was fed into the microchannel of the device.

2.1.4.2.2. Sample feeding into the microchannel. The value of the output voltage and frequency applied to CH1 and CH2 was shown in Table 1. The bacterial suspension was fed into the microchannel at a flow rate of 60 μL/min for 90 s before applying voltage to CH1. After feeding 1 mL of the sample during voltage application to CH1, the flow rate was reduced to 5 μL/min. An additional sample was fed at 5 μL/min for 45 s at the voltage applied to CH1 to stabilize the flow rate in the microchannel. Subsequently, the applied voltage was switched from CH1 to CH2, and the bacterial cells trapped in CH1 were recaptured in CH2. After 4 min, the feeding step was completed.

2.1.4.2.3. Enumeration of the bacteria. After feeding the sample, we used an optical microscope, a Coaxial Vertical Zoom Lens CX-10C (Hirox, Tokyo, Japan) attached to the main apparatus to observe the electrodes and slits in the line of CH2 containing trapped bacterial cells, and obtained a zoomed-in digital image. If the photographing area did not include the right position or the electrode edge appeared obscure, an additional image was taken after readjusting the position and focus. Subsequently, the bacterial cells in the image were automatically enumerated via image analysis with the installed software (PixeeMo™ counter, Ver. 3.0 Rev. 3, AFI Corp.), and the number of bacterial cells/g of food sample (cells/g) was obtained. Cell counting data were used to calculate the log cells/g. The detection limit of the DEP method was 10^2 cells/g which was equivalent to the conventional plate count method.

2.2. Application of the DEP method for physically processed food products

Samples of raw chicken breast treated via either heating or freezing were used to determine the applicability of the DEP method to physically processed food products.

Table 1
Electrical parameters applied to each sample for DEP separation.

Sample	Sample conductivity (μS/cm)	Output voltage (Vpp)	Frequency (kHz)
Cut lettuce	30	20	9000
Chicken breast	50	20	6000
Minced pork	70	20	9000

DEP, dielectrophoresis.

2.2.1. Freezing treatment of samples

A portion of 5 g of raw chicken breast was placed in sealed bags and stored in a freezing chamber at −20 °C for 24 h. The frozen samples were thawed at 15–20 °C for 30 min. Finally, the defrosted samples were subjected to the aforementioned bacterial determination test.

2.2.2. Thermal treatment of samples

A portion of 5 g of raw chicken breast was placed in sealed bags and heated at 50, 55, and 60 ± 1 °C in a water bath for 5 min. Subsequently, the samples were immediately cooled in ice water and were subjected to the aforementioned bacterial determination test.

2.3. Statistical analysis

Regression analysis was performed to assess the correlation between the DEP method and the conventional method by coefficients of determination. In addition, triplicate data of cell counts was obtained with the DEP method sampling one cut lettuce, and a value of the coefficient of variation was evaluated. All statistical analyses were performed with Microsoft Excel Ver. 16.43 (Microsoft, Washington, US).

3. Results

3.1. Comparison of bacterial enumeration using the DEP and conventional methods

The reproducibility of the DEP method was determined as <2.0% of the coefficient of variation as shown in Table 2.

The results showed a high correlation between both procedures, with coefficients of determination (R^2) of 0.93, 0.97, and 0.93 for cut lettuce, raw chicken breast, and minced pork, respectively (Fig. 2). The general cell counts for cut lettuce samples obtained using the DEP method were clearly biased toward lower values than those obtained using the culture method. The regression line implying the value of the intercept was 0.62. The difference in cell counts between the two methods ranged from −0.03 to −0.91 log (median value = −0.43). The difference in cell counts of raw chicken breast and minced pork between the methods ranged from −0.12 to 0.82 log (median value = 0.31) and −0.62 to 0.66 log (median value = 0.24), respectively. There was no bias, which meant one-sidedness of the observed data on the y = x line in the figure and defined as the average difference between the DEP and plate count of the respective number of samples, in the results of raw chicken breast (bias = 0.20) and minced pork (bias = 0.17) cell counts between both methods, contrary to that observed in cut lettuce (bias = −0.46).

3.2. Application of the DEP method to physically processed food products

The difference in cell counts of chicken breast subjected to freezing treatment between the DEP method and the conventional culture method ranged from 0.11 to 0.67 log (Fig. 3).

The difference in cell counts of chicken breast subjected to thermal treatment at 50, 55, and 60 °C for 5 min between the DEP and

Table 2
Repeatability accuracy in triplicate measurement of the same sample by DEP method.

	Viable cell numbers (log Cells/g)			
Sample	1st	2nd	3rd	Coefficient of variation (%)
Lettuce 1	4.02	4.12	4.18	1.93
Lettuce 2	6.46	6.46	6.51	0.38
Chicken 1	5.31	5.40	5.46	1.38
Chicken 2	5.91	5.97	6.05	1.20
Pork 1	4.58	4.70	4.72	1.59
Pork 2	5.38	5.40	5.30	0.98

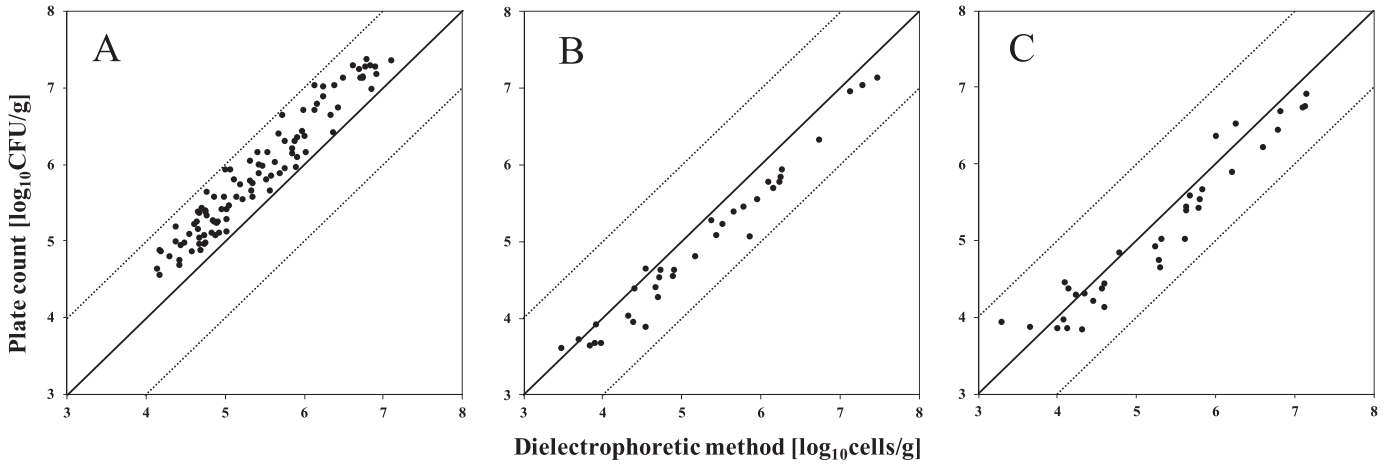

Fig. 2. Comparison of viable bacterial cell counts between the DEP method and the culture method. Each single dot means the relationship between the estimated viable bacterial cell number by conventional plate count method and that by DEP method of each tested sample. Three food products were sampled: (A) cut lettuce, (B) raw chicken breast, and (C) minced pork. Solid and black lines indicate y = x; dashed lines indicate y = x ± 1.0 log. DEP, dielectrophoresis.

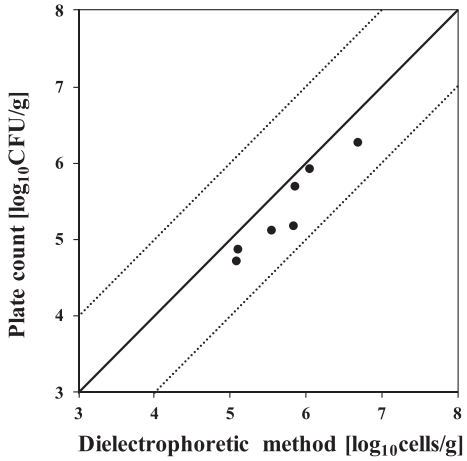

Fig. 3. Comparison of viable bacterial cell counts between the DEP method and the culture method for chicken breast samples subjected to freezing and thawing treatment. Each single dot means the relationship between the estimated viable bacterial cell number by conventional plate count method and that by DEP method of each tested sample. Solid and black lines indicate y = x; dashed lines indicate y = x ± 1.0 log. DEP, dielectrophoresis.

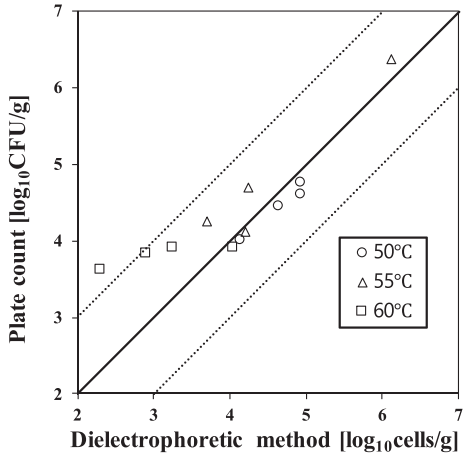

Fig. 4. Comparison of viable bacterial cell counts between the DEP method and the culture method for chicken breast samples subjected to heat treatment at (○) 50 °C, (△) 55 °C and (□) 60 °C for 5 min. Each symbol means the relationship between the estimated viable bacterial cell number by conventional plate count method and that by DEP method of each tested sample. Solid and black lines indicate y = x; dashed lines indicate y = x ± 1.0 log. DEP, dielectrophoresis.

conventional plate count methods ranged from 0.15 to 0.33 log for 50 °C, from −0.55 to 0.10 log for 55 °C, and from −1.31 to 0.14 log for 60 °C (Fig. 4).

4. Discussion

We found a high correlation between the DEP method and the conventional APC/TPC culture method ($R^2 > 0.90$) for each food sample. In addition to raw foods, when physically processed chicken breast samples were tested, the difference in cell counts between both methods was within ±1.0 log, excluding one sample subjected to thermal treatment at 60 °C. However, in the case of cut lettuce, the total viable counts obtained using the DEP method were clearly biased to be approximately 0.5 log lower than those obtained via the culture method, whereas there was no bias in the cell counts of raw chicken breast (bias = 0.20) and minced pork (bias = 0.17) between both methods. The bias associated with cut lettuce samples may be attributed to the presence of bacteria in subvital states. Sanitization procedures using disinfectants such as sodium hypochlorite and acidic electrolyzed water are usually performed during production of cut vegetables (Gil Muñoz et al., 2010; Sun et al., 2012). Since the set value of the output voltage and frequency of the DEP method for cut lettuce analysis was largely focused on detecting healthy vital bacteria, any subvital bacterial cells injured via the sanitization process might not have been determined appropriately via the DEP method. In contrast, the culture method, which takes 48 h for incubation, could have enabled the recovery of sublethally injured bacteria to form colonies of healthy cells. Therefore, the bias may be associated with bacteria that are injured during production, which can recover under favorable conditions.

Furthermore, the potential of the DEP method for the rapid detection of injured bacteria was demonstrated in the experiment using chicken breast subjected to thermal treatment. Notably, when heat treatment was performed at 60 °C, the cell counts obtained using the DEP method were lower than those obtained via the conventional culture method. It is suspected that the number of injured bacteria increased with an increase in treatment temperature, and these heat-injured bacteria were not detected via the DEP method, largely owing to membrane damage and dielectric properties (Amako et al., 2011). Although this suggests that the DEP method may be suitable for only detecting healthy bacteria,

the number of injured bacteria can be estimated via comparison with the total cell count obtained using the culture method. We need further investigation for clarifying the effect of injured cells on the enumeration by DEP method in the future study.

The conventional culture method is widely used for the detection and enumeration of injured bacteria, and a few cultivation-free methods have been recently proposed as rapid alternatives. Culture methods generally use conventional growth media, where both healthy and injured bacteria can grow to form colonies, and selective media such as deoxycholate hydrogen sulfide lactose agar, in which only healthy bacteria can grow to form colonies, because the surfactant action of sodium deoxycholate exacerbates membrane damage and inhibits the recovery of injured bacteria (Cui et al., 2018; Espina et al., 2016). Therefore, the number of injured bacteria is determined based on the difference between viable counts of different media.

The proposed rapid methods include flow cytometry (FCM) and real-time PCR. Fluorescent techniques in combination with FCM use several fluorescent reagents containing different fluorescent wavelengths to distinguish injured cells based on the extent of fluorescent dyeability (Zhao et al., 2011). Amor et al. (2002) reported that the FCM method overestimates the viability of bacteria compared to that estimated using the culture method. Recent approaches using PCR methods for biological analysis have focused on distinguishing between live and dead cells using nucleic acid-binding agents such as ethidium bromide monoazide and propidium monoazide. These agents cannot penetrate healthy cell membranes; however, they react to inactivate DNA leaking from dead cells (Magajna and Schraft, 2015). The efficiency of the PCR method in combination with such agents has been demonstrated; however, various factors such as the concentration of agents and cells, reaction time, and DNA chain length for amplification via PCR have to be optimized to improve the efficiency of the agents for inactivating DNA derived from dead cells (Takahashi et al., 2018).

As described above, conventional methods are time-consuming and complex for identifying injured bacteria and determining the extent of injury. In the present study, the applied parameters of the DEP method, such as sample conductivity and frequency, were overall appropriate for detecting only healthy bacterial cells. *Re*-adjustment of the parameter values is required to optimize the valid measurement conditions. However, since the sample liquid processed via the DEP system that is eluted into a waste bottle during voltage application is expected to contain injured and/or dead microorganisms, an efficient separation and collection of only injured bacterial cells from other particles by reusing the sample may be attainable. However, a more fundamental investigation into the distribution of the dielectric properties of injured bacterial cells is required. Cottet et al. (2019) developed software that enables analysis of the DEP behavior of particles and cells in a suspended medium according to different conditions, and have also provided a database containing published information on particle properties. By combining data with improvement of the software and modifying the set parameters, the DEP method may develop into a useful tool for detecting individual bacterial cells showing different metabolic states.

In the present study, the bacterial contamination level of food samples ranged from approximately 3.0 to 8.0 log CFU/g; the efficiency of the DEP method with low bacterial concentration samples (<2.0 log) has been demonstrated in a previous study (Wakizaka et al., 2020). This suggests that the DEP method can be used for foods containing a wide range of bacterial concentrations. In addition, the total determination time including sample preparation (< 30 min) and analysis (< 30 min) for the DEP method was <60 min. Although sample preparation steps are still needed, the DEP method provides a much more rapid procedure than that of the conventional culture method, which requires 2 days for result acquisition. Thus, the DEP method is arguably superior to other current rapid detection methods.

Nucleic acid-based, biosensor-based, and immunological-based methods have recently been developed as representative rapid methods. Nucleic acid-based methods use PCR, microarrays, and loop-mediated isothermal amplification to enable bacterial detection at a high level of sensitivity and specificity within 24 h (Bhunia et al., 2020; Zhao et al., 2014). However, such methods cannot efficiently distinguish between viable and dead cells, and pre-enrichment steps are labor-intensive (Foddai and Grant, 2020; Gracias and Mckillip, 2004). Biosensors for bacterial detection are generally divided into four groups: optical, mass, electrochemical, and thermal biosensors (Law et al., 2015; Zhao et al., 2014). The duration of the assay for every approach is approximately several hours, and detection limits for bacterial cell concentrations and the presence of certain food components may decrease the sensitivity (Ivnitski et al., 1999; Law et al., 2015). Immunological methods relying on antigen-antibody binding enable the detection of target bacteria within 24 h by using suitable antibodies (Law et al., 2015). However, these methods require pre-enrichment and may show cross-reactivity for closely related antigens (Park et al., 2014; Zhao et al., 2014). Regarding the duration, the DEP method that provides results within 30 min is a remarkable approach to enumerate viable bacteria in foods compared to other conventional methods.

5. Conclusion

We evaluated the efficiency of the DEP method for enumerating viable bacterial cells in several foods in comparison with the conventional culture method. It was confirmed that the results obtained using the DEP methods were equivalent to those obtained using the conventional culture method, indicating a high correlation between these two methods for a wide range of bacterial cell concentrations. Moreover, the DEP method enabled bacterial enumeration within 60 min from sample preparation to the acquisition of results, whereas the conventional method required more than 48 h including incubation period to provide results. When injured bacterial cells are subjected to physicochemical treatments such as heating and disinfection, the DEP method tended to underestimate the viable bacterial cell counts compared to those obtained via the conventional culture method. Nevertheless, the DEP method has the potential to be a useful alternative tool for rapid enumeration of viable bacteria in a variety of foodstuffs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106251.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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