Responses of *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* to Simulated Food Processing Treatments, Determined Using Fluorescence-Activated Cell Sorting and Plate Counting[▽]

Deirdre Kennedy, Ultan P. Cronin,[†] and Martin G. Wilkinson^{*}

Food Science Research Centre, Department of Life Sciences, University of Limerick, Limerick, Ireland

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Three common food pathogenic microorganisms were exposed to treatments simulating those used in food processing. Treated cell suspensions were then analyzed for reduction in growth by plate counting. Flow cytometry (FCM) and fluorescence-activated cell sorting (FACS) were carried out on treated cells stained for membrane integrity (Syto 9/propidium iodide) or the presence of membrane potential [DiOC₂(3)]. For each microbial species, representative cells from various subpopulations detected by FCM were sorted onto selective and nonselective agar and evaluated for growth and recovery rates. In general, treatments giving rise to the highest reductions in counts also had the greatest effects on cell membrane integrity and membrane potential. Overall, treatments that impacted cell membrane permeability did not necessarily have a comparable effect on membrane potential. In addition, some bacterial species with extensively damaged membranes, as detected by FCM, appeared to be able to replicate and grow after sorting. Growth of sorted cells from various subpopulations was not always reflected in plate counts, and in some cases the staining protocol may have rendered cells unculturable. Optimized FCM protocols generated a greater insight into the extent of the heterogeneous bacterial population responses to food control measures than did plate counts. This study underlined the requirement to use FACS to relate various cytometric profiles generated by various staining protocols with the ability of cells to grow on microbial agar plates. Such information is a prerequisite for more-widespread adoption of FCM as a routine microbiological analytical technique.

In the food industry, the bacterial membrane is known to be a primary target for the inactivation treatments which are applied during processing to guarantee food safety (29). Such treatments involve chemical preservatives, heating, or chemical disinfection procedures. The antimicrobial effect of a number of these treatments appears to be due to their ability to alter microbial cell permeability, which in turn causes a loss of macromolecules from the interior (3, 18). Chemical agents in particular may interfere with membrane organization (12, 28) and membrane proteins, causing deformation in structure and functionality (2). Traditionally, the methods used to assess the effects of various food processing treatments on bacterial survival involve subsequent enumeration of viable cells using a standard plating technique. However, this methodology generates information limited to the ability of a cell to recover and grow on a particular medium under defined conditions. Plate counting does not permit any assessment of the physiological heterogeneity within a microbial population, including the existence of subpopulations in various viable and nonviable states. However, the emergence of microbial flow cytometry (FCM) as a rapid and sensitive technique allows analysis of cells at rates of >50,000 per min, and when combined with

differential staining, it can discriminate various subpopulations based on physiological characteristics of individual cells. Multiparameter FCM can provide data on a range of structural and functional properties of a cell, which then can enable quantification of viability beyond the traditional reproductive capacity, such as on agar and other microbiological media (16, 26). The more-widespread application of FCM to the study of heterogeneity within microbial populations can now provide us with a greater insight into the functional strain-related responses to various industrial applications, such as food processing treatments (1, 6). Assessment of membrane integrity using FCM is often done by measuring a cell's ability to exclude compounds (4, 24) such as the membrane-impermeant dye propidium iodide (PI). Generally, the degree of PI staining is used to interpret cell damage, and because of this, it has found broad applications with FCM analysis. Syto 9 and PI dyes are often used in combination to assess viable and nonviable cells based on their ability to selectively penetrate viable bacterial cells. Syto 9 penetrates all bacterial membranes and stains the cells, resulting in green fluorescence at 530 nm, while PI penetrates only cells with damaged membranes, displacing Syto 9 with red fluorescence (25).

Detection of the presence or absence of membrane potential (MP) is often used as an indicator of vitality in microbial FCM. The transmembrane electrical potential gradient in microorganisms is typically of the order of 100 mV, and changes in this can be reported by staining with what are referred to as "distributional probes." An example of such a probe is the lipophilic carbocyanine dye DiOC₂(3) (3,3'-diethyloxacarbocya-

^{*} Corresponding author. Mailing address: Food Science Research Centre, Department of Life Sciences, University of Limerick, Limerick, Ireland. Phone: 353 61 213440. Fax: 353 61 331490. E-mail: martin.wilkinson@ul.ie.

[†] Present address: CNIO, C/ Melchor Fernández Almagro, Madrid, Spain.

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TABLE 1. Typical MoFlo cytometer settings^a used for analysis of bacterial species

Parameter	Setting for stain(s) and species			
	PI/Syto 9		DiOC ₂ (3)	
	<i>E. coli</i>	<i>S. aureus</i> /L. <i>monocytogenes</i>	<i>E. coli</i>	<i>S. aureus</i> /L. <i>monocytogenes</i>
FSC	Log A ^c	Log A	Log A	Log A
SSC	Log A	Log A	Log A	Log A
FL1 (530 ± 30 nm)	Log A; 500 V	Log A; 480 V	Log A; 500 V	Log A; 470 V
FL3 (670 ± 30nm)	Log A; 460 V	Log A; 460 V	Log A; 460 V	Log A; 460 V
Compensation	0	0	NA ^b	NA

^a FSC, forward-scattered light; SSC, side scattered light; FL1, detector for green fluorescence; FL3, detector for red fluorescence.

^b NA, not applicable.

^c Log A, logarithmic amplification.

nine iodide), which can readily enter the cell membrane and accumulate according to charge (17, 20). DiOC₂(3) exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self associate at the higher cytosolic concentrations caused by larger membrane potentials. Indeed, the outer membrane of Gram-negative organisms has been shown to exclude lipophilic or hydrophobic molecules, such as cyanine dyes (20). The lipopolysaccharide structure of Gram-negative bacteria can be destabilized by the addition of chelating agents which extract Ca²⁺ and Mg²⁺ cations, allowing permeabilization of their outer membrane (10), and sonication is commonly used to break up aggregates (16, 21). This pretreatment may have the ability to remove structural or spatial barriers to the uptake of stain for this particular microbial strain. Hence, the extent of green-to-red fluorescence shift is an indicator of differential physiological states of the cell. However, the nature of the relationship between cytometric profiles generated using various staining regimes and the extent to which they reflect the ability of cells to grow on microbial agar plates are unclear. Fluorescence-activated cell sorting (FACS) allows cells from various stained cytometric subpopulations arising from exposure to cell stressors to be plated onto selective and nonselective media and subsequently evaluated for growth under standard plate counting conditions. In this study, we utilized the above technique to investigate the effects of various simulated food processing treatments on the viability and membrane damage of various food pathogenic bacteria and the relationship between the various identified subpopulations and their ability to grow on various enumeration media following cell sorting.

MATERIALS AND METHODS

Preparation of pure suspensions of bacterial cells. *Listeria monocytogenes* type strain ATCC 19115, *Escherichia coli* type strain ATCC 17775, and a food isolate of *Staphylococcus aureus* from the University of Limerick collection were used in all experiments. Monthly working stocks of cultures were kept on nutrient agar (NA) (Oxoid, Basingstoke, United Kingdom) slants, and weekly stocks were maintained on NA. From the weekly stock, a single pure colony was inoculated into 10 ml of nutrient broth (NB) (Oxoid) and incubated overnight at 37°C. Five milliliters of the suspension was added to 95 ml of NB in a conical flask and incubated with shaking at 37°C. Depending on the species, the suspension was grown for 1 to 2 h or until cells had reached an optical density at 600 nm (OD₆₀₀) of approximately 0.2 with a cell density of >10⁷ CFU/ml. From previous growth experiments, it was found that cells were in exponential phase at this time.

Stressors applied to bacterial cell suspensions. The following stressors were applied to all three strains of bacteria before testing: heat at 60°C for 20 min, heat at 80°C for 10 min, 70% isopropyl alcohol (IPA), 0.1% (wt/vol) potassium

sorbate, 10% (wt/vol) NaCl, 0.1% (wt/vol) Tween 80, 0.5 mM cetyl trimethylammonium bromide (CTAB), and pH 4 (acidulant HCl).

Heat treatments were applied using an Eppendorf Thermomixer comfort system (Eppendorf Ltd., Cambridge, United Kingdom) into which 1-ml aliquots of cells were placed and subjected to the heat treatments. For all other treatments, cells were exposed to treatments for 30 min at room temperature. In the case of exposure to pH 4, relevant quantities of hydrochloric acid were added to NB before autoclaving, and this solution was used as described above. Samples exposed to chemical stressors were washed by resuspension of cell pellets obtained after centrifugation (2,500 × g for 5 min) in 1 ml of sterile phosphate-buffered saline (PBS) (pH 7.2). Subsequently, samples were analyzed by FCM analysis and plate counting.

Enumeration of viable cells by plate counting. Plate counts to determine total viable cells were carried out on solidified NA, a general-purpose medium which supports the growth of all three bacterial species. Serial dilutions of each treated sample and control were performed in 0.1% buffered peptone water (CM1049, pH 7.0; Oxoid), where 100 µl was added to tubes containing 900 µl of peptone water. One hundred microliters of each relevant dilution was spread on NA plates and allowed to dry. Plates were then inverted in an incubator overnight at 37°C. Plate counts were carried out in triplicate, and the process was repeated again with separate prepared samples. Plates with colonies in the range of 30 to 300 were then used to calculate the average number of CFU/ml.

Flow cytometric analysis of bacterial cells. Two staining regimes were applied following exposure to the conditions described above for all three bacterial species in order to assess the effect on cell physiology. The first regime involved staining cells with the dyes Syto 9 (Life Technologies, Carlsbad, CA) and PI (Sigma-Aldrich, Dublin, Ireland). Syto 9 solution (in dimethyl sulfoxide [DMSO]) was diluted in sterile water and used at a final concentration of 10 µM for staining of bacterial suspensions. PI was first made up as a stock solution of 15 mM by dissolving 10 mg in 1 ml DMSO and then immediately diluted (1/30) in sterile water and stored as 1-ml aliquots of a 0.5 mM concentration. Stock solutions were stored at -80°C, and working stocks were kept at 4°C in the dark for 1 to 2 weeks. A final concentration of 5 µM was used for staining of cells.

The second staining regime involved using the Invitrogen BacLight bacterial membrane potential kit (Life Technologies, Carlsbad, CA). The main component, DiOC₂(3), emits fluorescence at both red (613-nm) and green (520-nm) wavelengths when excited at 488 nm. The kit was used according to the manufacturer's instructions, where a final concentration of 5 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used to provide a depolarized control and 30 µM DiOC₂(3) was added to all samples except the unstained control. To facilitate DiOC₂(3) uptake by *E. coli*, 1 mM EDTA was added to 1-ml cell aliquots, followed by sonication at 6 W for 15 s using a 1/4-in. microtip attachment of a Branson Sonifier 150 cell disruptor (Heineman, Gmünd, Germany). Prior to use, the tip was sterilized by immersion in 70% (vol/vol) ethanol and rinsed three times with sterile distilled H₂O. The probe tip was immersed in the center of the 1-ml sample in sterile Eppendorf tubes for the time stated, and this was strictly repeated for all samples. During sonication, samples were maintained on ice. Sonication was performed prior to dye addition and subsequently appeared to result in facilitated dye entry. This sonication was previously determined to have no negative effect on viability (data not shown). After staining, samples were vortexed and incubated at room temperature in the dark for 15 min. All stained samples were analyzed in triplicate on a MoFlo cell sorter (Beckman Coulter, Fullerton, CA) using settings described in Table 1. All equipment and sheath fluid (PuraFlow 8x; Beckman Coulter) were maintained within an air-condi-

tioned class II laboratory set to 18°C. The trigger was set on the forward scatter (FSC) channel. Prior to analysis, compensation and voltage settings were maximized by running unstained samples, Syto 9- and PI-only-stained samples, dually stained samples, and DiOC₂(3)-stained exponential-phase cells.

Ten thousand events were collected for each sample, and results were analyzed using the Summit v4.3 analysis software program (Dako Colorado Inc.). Instrument standardization involved daily laser alignment and calibration with Flow-Check fluorospheres (Beckman Coulter) and particle size and alignment using 1- μ m Partec calibration beads (Partec GmbH, Munster, Germany). Microbial populations were discriminated from debris and noise using FSC and side scatter (SSC) height and area parameters and doublet discrimination using pulse width, and physiologically distinct subpopulations were identified on the basis of fluorescence detected in FL1 and FL3 channels (see Fig. 1).

Sorting of subpopulations onto agar media. Subpopulations were determined by first drawing gates around controls generated for each microbial species corresponding to “intact” (Syto 9 positive), “permeabilized” (PI positive), “polarized,” or “depolarized” cells. Sorting of populations within these regions was then carried out using the “single” sort mode, which is triggered to sort single cells, in conjunction with a three-drop envelope, which guarantees deflection of a cell even in the event of drop delay drift. Sorting was carried out through a 70- μ m nozzle. Typically, sorting was performed at a drop drive frequency of 94,630 Hz, a drop drive A of 15.00 V, phase 53.4°, defanning of 15%, and drop delay of 40 1/16. Using these settings and an acquisition rate ranging from 2,000 to 9,000 events per second (eps), the sort rate was generally 2,500 to 8,000 eps and the abort rate was 180 to 200 eps, and a sort efficiency of ~90% was generally achieved. The CyCLONE function of the MoFlo sorter was used to prepare and perform sorting onto agar plates. Polygons were drawn around regions of interest, and a sort procedure was set up to sort one event per drop within these regions from the right-hand stream. Using the sorting function, 25 single cells were sorted in a 5-by-5 matrix onto selective and nonselective agar for each species. Ten plates with 5-by-5 matrices were seeded for each region and each agar type and carried out in triplicate. This entire process was repeated again on a separate day to check sorting consistency of the procedure. Typically, results were calculated as a percentage of 500 cells (incorporating results from two separate days). Gates were drawn according to controls, and cells present in these regions were sorted onto the various agars. In some cases, only a single main population was detected following a particular treatment. However, when two distinct regions were detected following a particular treatment, then 500 cells were sorted from each region of interest onto agars and all results were expressed as follows: (average no. of colonies counted [day 1] + average no. of colonies counted [day 2]/500) $\times$ 100.

Sorting of cells of all species after various treatments was carried out on both nonselective NA and MacConkey, Baird-Parker, or *Listeria* selective agar for *E. coli*, *S. aureus*, and *L. monocytogenes*, respectively.

A schematic of the cell selection and sorting process is shown in Fig. 1.

Statistical analysis and presentation of data. Data were analyzed using the Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA) and SPSS 16 (SPSS Corporation, Chicago, IL) software programs.

RESULTS

Plate counting. Data from plate counts on NA for the various treatments were converted to their base 10 logarithm (log), and log reductions for all three bacterial species were compared against untreated controls (Fig. 2). Three of the treatments (80°C heat, 70% IPA, and 0.5 mM CTAB) resulted in the complete absence of growth for all three species when they were plated on NA. Exposure to 60°C for 20 min resulted in a reduction of 4 to 6 log units for each species, while the remaining treatments caused a population reduction of the order of ~2 to 4 log units.

FCM analysis. Analysis of the Syto 9- and PI-stained cells was carried out using scatter plots of green versus red fluorescence. Discrete “intact” or “permeabilized” population controls were generated for each bacterial species as described above (Fig. 3). The presence of membrane potential in treated cells stained using DiOC₂(3) was also analyzed by scatter plots of green versus red fluorescence. CCCP-treated or heat-killed

cells were used to generate “depolarized” control populations. Subsequently, gates were drawn based on these controls with subpopulations present in “polarized” or “depolarized” regions and expressed as a percentage of the total profile in Tables 2, 3, and 4.

Fluorescence-activated cell sorting of subpopulations. The percentage growth of cells sorted onto the various agars before and after treatments is shown in Tables 2 to 4. An example of where cells from two distinct regions (subpopulations) resulted from a particular treatment and were sorted is shown in Fig. 4b. Generally, the growth of sorted cells on nonselective NA was not significantly lower than that on selective agar ($P > 0.05$; paired t test, 2-tailed). In only one case (sorting of DiOC₂(3)-stained cells of *S. aureus* onto nonselective agar) was there significantly lower growth than that on selective agar ($P < 0.05$; paired t test, 2-tailed, $df = 13$).

Heating to 80°C for 10 min resulted in a complete reduction in bacterial numbers recovered as CFU/ml on solid agar for all species. However, for this treatment, FCM data indicated that 81 to 97% of all species became PI positive (permeabilized), which does not correlate well to the complete kill or absence of growth detected on solid medium. Exposure to both of the heat treatments appeared to significantly affect membrane potential for all three bacterial species, with 70 to 100% of populations becoming depolarized.

In agreement with previous reports of the poor uptake of DiOC₂(3) by Gram-negative bacteria, *E. coli* staining using this dye required additional optimization, with various levels of EDTA being evaluated for their ability to promote maximal uptake. Data for heat treatments of all species were generally mirrored closely by the absence of growth of viable cells after FACS onto selective and nonselective media. The single exception for the three microbial species subjected to both heat treatments was *E. coli* heated to 60°C. At this temperature, ~11% of the cells originating from the “intact” region labeled with PI/Syto 9 formed single colonies on MacConkey agar from the original 500 sorted cells, indicating a degree of survival under these conditions for this particular species.

Treatment with Tween 80 or exposure to pH 4 resulted in ~2 log reductions in population for all three bacterial species. Exposure to the acidic conditions resulted in an FCM profile where the majority of Syto 9/PI-labeled cells appeared to be within the “intact” region, with 0 to 6% or 5 to 18% within the “permeabilized” or “depolarized” regions, respectively. For *E. coli*, growth of sorted cells stained using PI/Syto 9 on MacConkey selective agar was higher than for those sorted onto nonselective NA. However, for sorted *E. coli* cells stained with DiOC₂(3), similar growth was noted on both media. For *S. aureus*, the growth of sorted DiOC₂(3)-stained cells onto Baird Parker selective agar was higher than that on nonselective NA but was generally similar on either medium after staining with PI/Syto 9 (98 or 91%).

In the case of exposure to Tween 80, following Syto 9/PI staining, a single main population was detected for *E. coli*. When sorted, 27% of cells gave rise to colonies on NA, with 88% of cells growing on MacConkey. In the case of *S. aureus*, sorted cells from the main population appeared to give rise to similar levels of growth on either medium (~95%), while for *L. monocytogenes* a complete absence of viability was noted for both media. Tween 80 treatment appeared to have a major

FIG. 1. Flow cytometric analysis and sorting strategy used for various microbial species.

membrane-depolarizing effect on *E. coli* or *L. monocytogenes*, with 93 and 63% of cells, respectively, located within depolarized regions. Sorting of cells from these regions, in the case of *E. coli*, resulted in 96 to 98% growth on either medium, while for *L. monocytogenes* growth was absent on both media. For *S. aureus*, depolarized cells exhibited a major difference, with the

number of outgrowing cells on selective and nonselective agars being 89 or 43%, respectively.

In this study, treatment with potassium sorbate resulted in a 2 to 4 log reduction in growth for all species. FCM data indicated that the majority (87 to 96%) of cells for all three species were located within the “intact” region when stained using

FIG. 2. Mean log reduction of exponential-phase cells of *S. aureus*, *E. coli*, and *L. monocytogenes* exposed to various simulated food processing treatments compared with controls. For all samples, $n = 6$. Black bars are results for *S. aureus*, light-gray bars are results for *E. coli*, and dark-gray bars indicate results for *L. monocytogenes*. Treatments were as follows: 80°C, cells heated to 80°C for 10 min; 60°C, cells heated to 60°C for 20 min; pH4, cells exposed to broth at pH 4; 0.1% Tween 80, 0.1% (wt/vol) Tween 80 solution; 0.1% Sorbate, 0.1% (wt/vol) potassium sorbate solution; 10% NaCl, 10% (wt/vol) sodium chloride solution; 70% IPA, 70% isopropyl alcohol; 0.1% SDS, 0.1% (wt/vol) sodium dodecyl sulfate solution; 0.5 mM CTAB, 0.5 mM cetrimonium bromide.

PI/Syto 9. However, DiOC₂(3) staining revealed that 27 to 40% of the cells for each of the microbial species exhibited depolarized membranes. Treatment with sorbate resulted in higher growth of sorted *E. coli* cells stained with PI/Syto 9 on selective agar (93%, compared with 57% on nonselective agar). In the case of *L. monocytogenes*, sorting of DiOC₂(3)-stained cells resulted in the absence of growth on either selective or nonselective media following sorbate exposure; this was also noted for other treatments.

FCM staining for all three bacterial species exposed to 10% (wt/vol) NaCl displayed various responses. Using plate count enumeration, generally 2 to 4 log reductions were noted for all species on NA. In the case of FCM profiles generated for *E. coli* exposed to NaCl, clearly distinguishable intact or permeabilized regions were absent, and therefore 500 cells were sorted from the main population, which was both PI and Syto 9 positive. Of this main sorted population, 65 or 85% of *E. coli* cells grew on nutrient or selective agars, respectively. For *S. aureus*, Syto 9/PI-stained cells from both the distinct “intact” and “permeabilized” regions were sorted onto either medium without any resultant growth. *L. monocytogenes* cells were also sorted from “intact” or “permeabilized” regions; the “intact” sorted cells

FIG. 3. Cytometric profiles of *E. coli* cells stained with PI/Syto 9 after heat treatment at >95°C (a) or treatment with 0.5 mM CTAB (b).

TABLE 2. FCM analysis and sorting results for *E. coli* cells using two staining regimes after exposure to various simulated food processing treatments^a

Treatment	Population	% cells detected using FCM	% recovery	
			Nutrient agar	MacConkey agar
Unstained control	Intact	NA ^c	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	NA	NA	NA
	Depolarized	NA	NA	NA
	Other			
	Unstained population	NA	100 ± 0.00	99.6 ± 0.57
Live control	Intact	99.09 ± 0.75	70.4 ± 2.3	85.2 ± 3.4
	Permeabilized	0.13 ± 0.02	NA	NA
	Polarized	87.40 ± 0.91	98.2 ± 0.85	92.4 ± 6.8
	Depolarized	2.51 ± 0.65	NA	NA
Dead control	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	97.96 ± 0.61	0.00 ± 0.00	0.00 ± 0.00
	Polarized	1.16 ± 0.14	NA	NA
	Depolarized	86.29 ± 2.53	0.00 ± 0.00	0.00 ± 0.00
Depolarized control	Intact	NA	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	23.38 ± 1.23	NA	NA
	Depolarized	67.97 ± 1.47	88.8 ± 1.69	92.4 ± 6.79
80°C	Intact	0.41 ± 0.26	NA	NA
	Permeabilized	96.84 ± 0.38	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.45 ± 0.42	NA	NA
	Depolarized	96.74 ± 0.52	0.00 ± 0.00	0.00 ± 0.00
60°C	Intact	4.87 ± 0.55	0.00 ± 0.00	11.4 ± 1.41
	Permeabilized	79.67 ± 0.68	0.00 ± 0.00	0.00 ± 0.00
	Polarized	11.68 ± 0.71	NA	NA
	Depolarized	72.77 ± 1.49	0.00 ± 0.00	0.00 ± 0.00
pH 4	Intact	91.08 ± 0.66	17.46 ± 6.51	78.6 ± 2.55
	Permeabilized	3.31 ± 0.76	0.00 ± 0.00	21.0 ± 4.81
	Polarized	70.55 ± 4.38	97.6 ± 2.82	98.4 ± 0.57
	Depolarized	14.14 ± 1.67	NA	NA
0.1% Tween	Intact	86.21 ± 0.96	27.6 ± 4.53	88.0 ± 1.69
	Permeabilized	4.23 ± 0.27	NA	NA
	Polarized	3.44 ± 0.79	NA	NA
	Depolarized	92.85 ± 1.93	96.8 ± 3.96	98.6 ± 0.85
0.1% sorbate	Intact	96.88 ± 1.25	57.6 ± 2.83	93.8 ± 1.98
	Permeabilized	0.96 ± 0.17	NA	NA
	Polarized	55.31 ± 1.14	95.6 ± 2.26	96.8 ± 1.70
	Depolarized	30.48 ± 1.77	NA	NA
10% NaCl	Intact	88.52 ± 1.13	65.2 ± 3.96	84.8 ± 2.83
	Permeabilized	0.10 ± 0.08	NA	NA
	Polarized	61.37 ± 2.47	97.8 ± 1.98	97.0 ± 3.68
	Depolarized	30.47 ± 1.06	NA	NA
70% IPA	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	98.83 ± 0.44	0.00 ± 0.00	0.00 ± 0.00
	Polarized	1.72 ± 1.01	NA	NA
	Depolarized	86.89 ± 2.74	0.00 ± 0.00	0.00 ± 0.00
0.1% SDS	Intact	6.31 ± 0.32	88.0 ± 4.52	84.4 ± 2.82
	Permeabilized	75.94 ± 1.97	54.4 ± 3.39	55.4 ± 3.11
	Polarized	91.29 ± 1.78	91.2 ± 2.26	96.2 ± 3.11
	Depolarized	1.93 ± 0.16	NA	NA
0.5 mM CTAB	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	14.02 ± 1.68	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	96.25 ± 1.32	0.00 ± 0.00	0.00 ± 0.00
	Other			
	"CTAB" permeabilized region ^b	NA	0.00 ± 0.00	0.00 ± 0.00

^a Live control, exponential-phase cells that were untreated; dead control, cells heated to >95°C for prolonged periods to ensure loss of viability; CCCP control, cells treated with 5 μM CCCP, which acts as a depolarizing agent; 80°C, cells heated to 80°C for 10 min; 60°C, cells heated to 60°C for 20 min; pH 4, cells exposed to broth at pH 4; 0.1% Tween, 0.1% (wt/vol) Tween 80 solution; 0.1% sorbate, 0.1% (wt/vol) potassium sorbate solution; 10% NaCl, 10% (wt/vol) sodium chloride solution; 70% IPA, 70% isopropyl alcohol; 0.1% SDS, 0.1% (wt/vol) sodium dodecyl sulfate solution; 0.5 mM CTAB, 0.5 mM cetrimonium bromide.

^b Most CTAB-treated cells appeared in a region not denoted by the live or dead controls.

^c NA, not applicable.

TABLE 3. FCM analysis and sorting results for *S. aureus* cells using two staining regimes after exposure to various simulated food processing treatments^a

Treatment	Population	% cells detected using FCM	% recovery	
			Nutrient agar	Baird-Parker agar
Unstained control	Intact	NA ^c	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	NA	NA	NA
	Depolarized	NA	NA	NA
	Other			
	Unstained population	NA	98.0 ± 2.83	100 ± 0.00
Live control	Intact	93.01 ± 1.43	92.4 ± 5.10	95.0 ± 2.55
	Permeabilized	3.36 ± 1.22	NA	NA
	Polarized	89.91 ± 0.79	30.6 ± 5.94	85.6 ± 3.39
	Depolarized	4.98 ± 0.59	NA	NA
Dead control	Intact	0.45 ± 0.51	NA	NA
	Permeabilized	95.07 ± 1.57	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.03 ± 0.06	NA	NA
	Depolarized	95.96 ± 1.58	0.00 ± 0.00	0.00 ± 0.00
Depolarized control	Intact	NA	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	3.61 ± 0.32	NA	NA
	Depolarized	72.17 ± 1.58	98.2 ± 2.55	99.4 ± 0.85
80°C	Intact	9.66 ± 2.19	NA	NA
	Permeabilized	81.82 ± 3.27	0.00 ± 0.00	0.00 ± 0.00
	Polarized	1.27 ± 0.16	NA	NA
	Depolarized	92.49 ± 0.49	0.00 ± 0.00	0.00 ± 0.00
60°C	Intact	10.88 ± 1.29	0.00 ± 0.00	0.00 ± 0.00
	Permeabilized	77.44 ± 3.05	0.00 ± 0.00	0.00 ± 0.00
	Polarized	2.75 ± 0.57	NA	NA
	Depolarized	83.52 ± 1.91	0.00 ± 0.00	0.00 ± 0.00
pH 4	Intact	93.54 ± 1.54	91.7 ± 8.91	98.2 ± 0.85
	Permeabilized	0.84 ± 0.35	NA	NA
	Polarized	75.98 ± 5.58	50.2 ± 2.55	71.2 ± 3.96
	Depolarized	18.52 ± 0.88	NA	NA
0.1% Tween	Intact	11.43 ± 1.11	NA	NA
	Permeabilized	70.39 ± 3.52	97.8 ± 0.85	95.4 ± 4.81
	Polarized	83.37 ± 0.66	43.2 ± 4.53	89.0 ± 5.37
	Depolarized	11.80 ± 0.71	NA	NA
0.1% Sorbate	Intact	96.41 ± 1.04	99.8 ± 0.28	100.0 ± 0.00
	Permeabilized	1.26 ± 0.94	7.0 ± 3.68	7.6 ± 1.70
	Polarized	48.77 ± 0.39	42.1 ± 4.10	69.8 ± 5.94
	Depolarized	39.36 ± 1.45	NA	NA
10% NaCl	Intact	89.72 ± 2.14	0.00 ± 0.00	0.00 ± 0.00
	Permeabilized	2.77 ± 1.36	0.00 ± 0.00	0.00 ± 0.00
	Polarized	77.23 ± 2.65	70.8 ± 6.22	75.3 ± 2.40
	Depolarized	21.44 ± 2.18	NA	NA
70% IPA	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	95.83 ± 0.27	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	96.63 ± 1.84	0.00 ± 0.00	0.00 ± 0.00
0.1% SDS	Intact	0.85 ± 0.27	0.00 ± 0.00	0.00 ± 0.00
	Permeabilized	97.63 ± .42	0.00 ± 0.00	0.00 ± 0.00
	Polarized	59.96 ± 2.33	0.00 ± 0.00	0.00 ± 0.00
	Depolarized	18.51 ± 5.72	NA	NA
0.5 mM CTAB	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	22.96 ± 3.04	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	89.08 ± 1.49	0.00 ± 0.00	0.00 ± 0.00
	Other "CTAB" permeabilized region ^b	NA	0.00 ± 0.00	0.00 ± 0.00

^a Live control, exponential-phase cells that were untreated; dead control, cells heated to >95°C for prolonged periods to ensure loss of viability; CCCP control, cells treated with 5 µM CCCP, which acts as a depolarizing agent; 80°C, cells heated to 80°C for 10 min; 60°C, cells heated to 60°C for 20 min; pH 4, cells exposed to broth at pH 4; 0.1% Tween, 0.1% (wt/vol) Tween 80 solution; 0.1% sorbate, 0.1% (wt/vol) potassium sorbate solution; 10% NaCl, 10% (wt/vol) sodium chloride solution; 70% IPA, 70% isopropyl alcohol; 0.1% SDS, 0.1% (wt/vol) sodium dodecyl sulfate solution; 0.5 mM CTAB, 0.5 mM cetrimonium bromide.

^b Most CTAB-treated cells appeared in a region not denoted by the live or dead controls.

^c NA, not applicable.

TABLE 4. FCM analysis and sorting results for *L. monocytogenes* cells using two staining regimes after exposure to various simulated food processing treatments^a

Treatment	Population	% cells detected using FCM	% recovery	
			Nutrient agar	<i>Listeria</i> selective agar
Unstained control	Intact	NA ^c	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	NA	NA	NA
	Depolarized	NA	NA	NA
	Other			
	Unstained population	NA	93.5 ± 2.97	91.8 ± 5.94
Live control	Intact	95.49 ± 0.73	98.8 ± 1.70	97.4 ± 1.98
	Permeabilized	1.23 ± 0.31	NA	NA
	Polarized	89.94 ± 1.73	8.6 ± 1.98	10.4 ± 2.83
	Depolarized	4.43 ± 0.84	NA	NA
Dead control	Intact	0.13 ± 0.11	NA	NA
	Permeabilized	96.14 ± 1.01	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.26 ± 0.15	NA	NA
	Depolarized	96.14 ± 1.01	0.00 ± 0.00	0.00 ± 0.00
Depolarized control	Intact	NA	NA	NA
	Permeabilized	NA	NA	NA
	Polarized	27.24 ± 2.55	20.8 ± 3.39	16.2 ± 2.55
	Depolarized	60.12 ± 1.08	85.6 ± 4.53	85.4 ± 3.11
80°C	Intact	0.90 ± 0.06	NA	NA
	Permeabilized	95.59 ± 0.86	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	95.65 ± 1.94	0.00 ± 0.00	0.00 ± 0.00
60°C	Intact	1.93 ± 0.49	0.00 ± 0.00	0.00 ± 0.00
	Permeabilized	77.87 ± 2.79	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	94.99 ± 1.05	0.00 ± 0.00	0.00 ± 0.00
pH 4	Intact	84.93 ± 0.93	41.5 ± 1.84	37.2 ± 2.83
	Permeabilized	5.78 ± 0.56	32.6 ± 3.11	33.6 ± 1.13
	Polarized	88.38 ± 2.03	0.00 ± 0.00	0.00 ± 0.00
	Depolarized	5.54 ± 0.38	NA	NA
0.1% Tween	Intact	86.79 ± 0.29	0.00 ± 0.00	0.00 ± 0.00
	Permeabilized	7.24 ± 0.17	NA	NA
	Polarized	23.11 ± 4.32	NA	NA
	Depolarized	63.23 ± 3.60	0.00 ± 0.00	0.00 ± 0.00
0.1% Sorbate	Intact	87.59 ± 0.56	34.0 ± 4.53	27 ± 3.67
	Permeabilized	3.32 ± 0.49	NA	NA
	Polarized	54.88 ± 1.86	0.00 ± 0.00	0.00 ± 0.00
	Depolarized	27.52 ± 2.19	NA	NA
10% NaCl	Intact	90.84 ± 0.41	11.8 ± 1.98	20.2 ± 2.55
	Permeabilized	1.78 ± 0.08	0.6 ± 0.85	1.0 ± 1.41
	Polarized	84.14 ± 1.46	0.00 ± 0.00	0.00 ± 0.00
	Depolarized	5.66 ± 0.63	NA	NA
70% IPA	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	96.32 ± 0.64	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	97.53 ± 1.16	0.00 ± 0.00	0.00 ± 0.00
0.1% SDS	Intact	0.05 ± 0.06	NA	NA
	Permeabilized	92.23 ± 1.18	0.00 ± 0.00	0.00 ± 0.00
	Polarized	64.66 ± 4.82	0.00 ± 0.00	0.00 ± 0.00
	Depolarized	27.88 ± 1.99	0.00 ± 0.00	0.00 ± 0.00
0.5 mM CTAB	Intact	0.00 ± 0.00	NA	NA
	Permeabilized	13.83 ± 1.74	0.00 ± 0.00	0.00 ± 0.00
	Polarized	0.00 ± 0.00	NA	NA
	Depolarized	86.06 ± 318	0.00 ± 0.00	0.00 ± 0.00
	Other "CTAB" permeabilized region ^b	NA	0.00 ± 0.00	0.00 ± 0.00

^a Live control, exponential-phase cells that were untreated; dead control, cells heated to >95°C for prolonged periods to ensure loss of viability; CCCP control, cells treated with 5 μ M CCCP, which acts as a depolarizing agent; 80°C, cells heated to 80°C for 10 min; 60°C, cells heated to 60°C for 20 min; pH 4, cells exposed to broth at pH 4; 0.1% Tween, 0.1% (wt/vol) Tween 80 solution; 0.1% sorbate, 0.1% (wt/vol) potassium sorbate solution; 10% NaCl, 10% (wt/vol) sodium chloride solution; 70% IPA, 70% isopropyl alcohol; 0.1% SDS, 0.1% (wt/vol) sodium dodecyl sulfate solution; 0.5 mM CTAB, 0.5 mM cetrionium bromide.

^b Most CTAB-treated cells appeared in a region not denoted by the live or dead controls.

^c NA, not applicable.

FIG. 4. Cytometric profiles of *S. aureus* cells stained with DiOC₂(3) after exposure to heating at 80°C (a) or heating to 60°C (b).

exhibited low growth, with ~11% or ~20% growth on nutrient or selective agars, respectively. Cells sorted from the “permeabilized” region did not grow on either medium. In the case of NaCl-treated DiOC₂(3)-stained cells sorted onto the various media, almost all sorted *E. coli* cells (~97%) grew on both types of media; *S. aureus* cells had 70 to 75% growth on both agars, and unsurprisingly, the sorted *L. monocytogenes* cells exhibited an absence of growth (Fig. 5).

Treatment of all three bacterial strains with 70% IPA resulted in almost complete migration to the PI-positive (permeabilized) region, with DiOC₂(3) staining indicating that cells appeared completely depolarized. Subsequent sorting of cells indicated the complete absence of growth on either selective or nonselective media for all species. These data indicated the highly bactericidal nature of exposure of food-borne pathogens to IPA, with resultant damage to cell membranes and lack of survival and growth.

Treatment of the various microbial species with 0.1% (wt/vol) SDS resulted in ~4 to 6 log population reductions. Generally, FCM profiles of the various species indicated that cells had become permeable to PI; however, this treatment did not appear to have a depolarizing affect on cell membranes as revealed by DiOC₂(3) staining. For Syto 9/PI staining, discrete populations of intact and permeabilized cells were noted. Sorting of cells from these populations indicated major differences in growth on either medium, with cells originating from the

“intact” region having 54 or 84% growth on NA or MacConkey agar, respectively. For *E. coli* cells sorted from the “permeabilized” region, a similar growth recovery was noted on both agars (~55%). In the case of sorted *S. aureus* or *L. monocytogenes* cells from “intact” or “permeabilized” regions, treatment with SDS resulted in the absence of growth on either medium.

Two discrete “polarized” and “depolarized” regions were generated following treatment of *L. monocytogenes* with 0.1% SDS, which was not noted for the other two bacterial species, where a single main DiOC₂(3)-stained profile was generated. However, none of the *L. monocytogenes* cells sorted from these regions exhibited growth on either medium. For *E. coli*, sorted cells from the DiOC₂(3)-stained region exhibited high viability (~91%) on either medium. Conversely, sorted cells from the general population of DiOC₂(3)-stained *S. aureus* did not grow on either medium. The FCM profile obtained for all species stained with PI/Syto 9 after CTAB treatment generated a discrete region of fluorescence not related to the previously determined controls (Fig. 3). The majority of cells for all species were depolarized after CTAB treatment, with a complete reduction in population and no growth on various media for sorted cells.

DISCUSSION

The heterogeneity of the microbial response to various stressors, such as those encountered during food manufac-

FIG. 5. Cytometric profiles of live *Listeria monocytogenes* control cells stained with Syto 9/PI (a), *Listeria monocytogenes* treated with IPA and stained with Syto 9/PI (b), or *Listeria monocytogenes* treated with NaCl and stained with DiOC₂(3) (c).

ture and/or cleaning treatments, has been previously demonstrated using FCM (5–7). However, few microbiological studies of such treatments have utilized fluorescence-activated cell sorting, which allows selective staining of bacterial

populations combined with sorting of individual cells or subpopulations of various physiological states onto selective and nonselective agars. In this article, we report the application of fluorescence-activated cell sorting to evaluate the

responses of various food borne pathogens to stressors and to relate various degrees of membrane damage with bacterial growth on microbiological media. In the case of important food pathogens, such as *L. monocytogenes* or *S. aureus*, the degree to which membrane damage caused by various stressors impacts the ability of individual cells or subpopulations within a culture to grow on traditional enumeration agars has not yet been determined.

A structurally intact cytoplasmic membrane and a functioning transmembrane potential are recognized as being essential for metabolic activity and the ability of the cell to reproduce and grow (16). However, with FCM the drawback which often arises is the inability of Gram-negative bacteria, such as *E. coli*, to take up many of the frequently used fluorescent stains. In this study, membrane potential measurement using DiOC₂(3) was used to analyze both Gram-positive and Gram-negative bacteria by gating of regions following preparation of appropriate control samples. However, while unstained *L. monocytogenes* bacteria were capable of growth after sorting, the corresponding DiOC₂(3)-stained cells were rendered nonviable after the sorting procedure. This stain-related effect was generally absent for *E. coli* and *S. aureus* and indicates the importance of investigation of the suitability of particular dyes for the study of different bacterial species and the optimization of staining protocols, as noted by other workers (14). It would appear that sorting of DiOC₂(3)-stained *L. monocytogenes* onto agar may not be a reliable indicator of cell viability following exposure to various stressors.

Heat is the most common treatment involved in food manufacture, causing a reduction in bacterial cell populations to levels considered safe for consumption (16). In this study, a range of heat treatments reflecting commercial pasteurization/heat inactivation processes were evaluated. A uniform lethal homogenous population response to exposure of cells to the highest temperature (80°C) was generally obtained for both FCM analysis and cell counts. However, at lower-heat treatments (60°C), the three species tested exhibited a range of tolerances to heat, and for *E. coli* a number of sorted intact cells were capable of growth on selective media after this treatment. This highlights clear differences between cytometric data, which represent a heterogeneous profiling of a culture into various subpopulations, and data obtained from traditional plate counting, where the presence/absence of growth and cell numbers are the main data outputs. This correlation also appears to be both species and stain specific, as shown for *S. aureus*, where ~81% became PI permeable after heating to 80°C while plate count data indicated a far greater killing effect.

The mechanism by which certain preservatives inhibit bacterial growth is of interest to the food industry. Potassium sorbate is widely recognized as being a useful food preservative, especially against yeasts and molds. The method of inhibition of this compound has been implicated as an ability to permeate or interfere with the cell membrane (8). In this study we have shown that the exposure of various microbial species to sorbate appeared to have a predominant effect on membrane potential rather than on structural membrane integrity. Preservation by NaCl is widely employed by the food industry at both high and low levels. The

main method of microbial inhibition by NaCl is primarily that it induces plasmolysis, which results in growth inhibition and possibly cell death (19). FCM data in this study indicated low-level depolarization on exposure to NaCl, while PI/Syto 9 staining of *S. aureus* or *L. monocytogenes* NaCl-treated cells rendered them completely unculturable after sorting. This particular finding for the effect of NaCl on these two microbial species may indicate a synergistic interaction between NaCl and the PI/Syto 9 staining procedure to render cells incapable of growth on solid media. Additionally, this finding highlights the necessity of developing standardized staining protocols before FCM/FACS analysis can find more-widespread use in the food industry. A potential application of FCM is also to assess bacterial physiological states after chemical disinfection treatments. Highly red fluorescent profiles were found after exposure of various microbial species to IPA or CTAB following staining with PI/Syto 9, indicating extensive cell membrane damage. The commercial use of IPA is as a disinfection agent in the food or process industries, and data in this study would support its efficacy in sterilizing surfaces where bacteria may be present. Cetyltrimethylammonium bromide (CTAB) is a quaternary ammonium compound (QAC) that appears to rupture the cell membrane and consequently causes cell lysis as a secondary effect (23). CTAB, as noted by our group (6, 9), appears to cause a distinct, highly permeabilized subpopulation, which was also found in this study.

Hayouni et al. (11) showed that multiparametric FCM was a convenient and rapid tool for evaluating the viability of lactic acid bacteria, with good correlation with plate count data. Muñoz et al. (15) used FCM as a rapid method to determine the antibacterial effect of supercritical extracts of plant essential oils and the physiological state of *L. monocytogenes*. These workers evaluated bacterial viability by dual staining of *L. monocytogenes* with Syto 9 and PI and subsequently revealed three different cell populations, specifically, living, dead, and compromised cells, which were mirrored by plate count data which suggested these compromised cells had the ability to recover and grow. In this study, we sorted representative cells from various cytometric subpopulations and related their growth on various media. Overall, the resulting data confirmed that growth on various media is not always directly related to cytometric profiles, especially when basing cell damage on cytometric membrane potential staining. An obvious example of this is *E. coli* treated with Tween, where 96 to 98% recovery was noted on both agar types from a population which was labeled depolarized. It has been previously noted that depolarization is a marker only of a decrease of cell functionality but does not indicate cell death (16). In this study, differences in viability and recovery of sorted subpopulations on various media were noted after exposure to stressors. Microbiologists are currently confronted with an emerging analytical technology with an ever-expanding range of stains purporting to identify a range of subpopulations differing in physiological states. However, the first question a microbiologist will ask is how these profiles relate to viability on agar plates. It is clear from this study that prior to adoption of microbial cytometry, each species must be checked systematically to correlate the relationship be-

tween cytometric profiles and traditional agar-based methods. In this study, we demonstrated the complexity of this relationship by FCM profiling combined with plate counting of subpopulations by FACS. This relationship was clearly shown to be affected by interactions arising from staining protocols and associated strain-specific responses. Hence, direct FACS-generated data on microbial viability and recovery require careful protocol development.

In this report, the application of FCM using optimized staining protocols provides an additional insight into the mechanism by which certain food processing treatments affect food-borne pathogenic bacterial species. Treatments that may impact cell membrane permeability do not necessarily have a comparable effect on membrane potential, and cells with damaged membranes may still be able to replicate and grow under the right conditions, for example, those *E. coli* cells labeled dead by PI/Syto 9 after treatment with SDS and exposure to pH 4. This brings into perspective the differences between cytometric data which represent a heterogeneous profiling of a culture into various subpopulations but which have variable correlation to data obtained from traditional plate counting, where the presence/absence of growth and cell numbers are the main data outputs.

Multiparametric FCM, with stains for different cellular targets, can be used to define subpopulations of interest (5, 9, 15). Accordingly, this approach yields data based on measurements of individual cells within a large population and generates a more comprehensive profile of the heterogeneity existing within a population. For the food industry, FCM offers the combined advantages of rapidity with the simultaneous analysis of large numbers of cells (16, 19). The traditional plate count is still the method used for the enumeration of microorganisms (1, 13, 29). The potential FCM offers is that it can reveal previously unknown levels of heterogeneity within a culture arising as a result of various degrees of cell injury. Cells with various physiological states within these populations can be analyzed, sorted, and simultaneously assessed. However, before a more widespread acceptance of cytometric profiling as a measure of cell viability occurs, studies such as this must be undertaken to determine the extent to which various FCM profiles reflect the ability of cells to grow on microbial agar plates. Studies have previously shown these limitations (22, 27). Hence, FCM data must be related to those obtained by traditional microbiological methods, and therefore FACS studies are a key component in understanding the relationship between FCM and plate count enumeration techniques.

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