Bacterial cell recovery after hollow fiber microfiltration sample concentration: Most probable bacterial composition in frozen vegetables

Peter Irwin, Yiping He, Ly Nguyen, Matthew Gehring, Andrew G. Gehring, Chin-Yi Chen, Joseph Capobianco

Molecular Characterization of Foodborne Pathogens, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA, 19038, USA

ABSTRACT

A commercial “concentrating pipette” was scrutinized from the perspective of sample volume reduction to augment native bacterial quantitation and identification within food samples. Aspects related to the elution protocol were evaluated for their impact on bacteria recovery. The results demonstrated there was no significant effect from different diluents/eluents on the relative recovery rate. Further, results from a complementary technique, dynamic light scattering, imply that recoveries less than unity are likely due to an inability to completely elute the bacterial cells from the capillary filter as opposed to bacterial injury caused by sample processing. When concentrating PBS washes from thawed frozen vegetables and enumerating after culturing at either 30 or 37°C, the recovery rates were only slightly lower than the simplified experimental conditions. However, in food wash samples, significantly more mass-normalized bacteria were recovered at the lower culture temperature. Nevertheless, the vegetable wash concentration process did not change the most probable isolate composition relative to that of the controls at either culture temperature. The study demonstrates the potential for the system to serve as an effective tool for bacterial recovery and concentration within water-based food systems such as equipment rinses, process wash water, and other samples coming from food processing plants.

1. Introduction

Manifold technologies for bacterial detection and quantitation would be useful if bacteria from foods could be easily separated and concentrated (Brewster, 2003, 2009; Fukushima, Katsube, Hata, Kishi, & Fujiwara, 2007; Li et al., 2013; Stevens & Jaykus, 2004). For example, in theory PCR is the most sensitive analytical method and should be able to detect 1 DNA copy per sampled volume. However, PCR’s low theoretical limit of detection (LOD) is not experimentally attainable due to a number of factors (Irwin, Nguyen, Chen, Uhlich, & Paoli, 2012; Irwin et al., 2014; Schrader, Schielke, Ellerbroek, & Johne, 2012). The current standardized laboratory protocols for pathogen detection utilized by food industry and regulatory laboratories employ “cultural enrichment” steps in order to reduce the number of false negative responses (Shaw & Esteban, 2019, p. 49). Cultural enrichment is necessary to ensure a sufficient number of targets, $10^5$-$10^6$ (Koyuncu, Andersson, & Häggblom, 2010; Stevens & Jaykus, 2004) are present within the small subsample that can be assayed using PCR. Common sample cleanup procedures rely on physical mechanisms, such as size and density, to facilitate filtration and sedimentation approaches or they can also utilize bioaffinity to more specifically separate different microorganisms using extracellular phenotypic markers (Armstrong et al., 2019; Bain et al., 2012; Brehm-Stecher, Young, Jaykus, & Tortorello, 2009). These separation techniques are generally necessary in order to improve detection sensitivity by sample size reduction (Brewster, 2003, 2009; Fukushima et al., 2007; Irwin, Damert, Brewster, Gehring, & Tu, 2002; Irwin, Damert, & Tu, 2004; Irwin, Fortis, Chau, & Tu, 2003). Thus, systems that can efficiently separate bacteria from sample matrices and reduce the total sample volume can relax or eliminate the need for cultural enrichment and reduce the errors associated with subsampling.

For quantitative meta-genomic analysis of various environmental samples (i.e., food processing plant: process water, equipment rinses, produce wash water, water/buffer-suspended surface or air samples) the standards for minimal sampling error are exacerbated due to the relatively high concentration of background organisms (Irwin, Nguyen, Chen, & Paoli, 2008) which occur simultaneously with low levels of

* Corresponding author.
E-mail address: Joseph.Capobianco@usda.gov (J. Capobianco).

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pathogens (Brehm-Stecher et al., 2009; Brewster, 2003; Brewster, 2009). For such samples, “cross-flow” capillary microfiltration (Li et al., 2013) has shown promise inasmuch as a food wash or liquid environmental sample can be concentrated and then thoroughly washed leaving behind only particles greater than the pore size of the filtration material and these trapped particles are not compressed as they would be with centrifugation and are, therefore, readily re-suspended for analysis. These trapped particles are not compressed as they would be with only particles greater than the pore size of the filtration material and sample can be concentrated and then thoroughly washed leaving behind sample that cannot contaminate the space outside the filter apparatus. The total concentration process we used involved drawing up the sample (Fig. 1B and C) into the filter tip, washing the concentrated sample (with at least 100 mL of buffer [for bacteria] or 18 mL–cm water [for beads]), and followed with elution (Fig. 1D and E; elution buffers, in canister form, either TBS (25 mM Tris +2.7 mM KCl + 137 mM NaCl; pH 7.4 ± 0.2; Boston BioProducts, Ashland, MA 01721) or PBS (10 mM Na2HPO4 + 2 mM NaH2PO4 + 137 mM NaCl; pH 7.4 ± 0.2; Boston BioProducts) ± 0.075% Tween 20 [for bacteria] or 18 mL–cm water [for beads]) of the concentrated bacteria and bringing the volume back up to a volume equivalent to the Control (C = that half of the paired sample not concentrated). The entire concentration process takes no more than ~5 min and was immediately followed by drop-plating the samples as discussed below. Non-commercial systems which are similar in principle take substantially longer (Li et al., 2013).

2.2. Bacterial growth conditions

Our test E. coli strain (isolate “C13B” from chicken) was routinely grown overnight in either Luria–Bertani (LB) broth or BHI and enumerated on solid media (same broth with 2% [w/v] agar) at 30 °C. All work with naturally occurring bacteria on pre-thawed frozen vegetables was performed using BHI broth and enumerated on BHI solid media grown at 30 and 37 °C. Two temperatures were used for bacterial growth inasmuch as any pathogens present would be more likely to show up from the background at 37 °C (Irwin et al., 2008).

2.3. Food sampling protocol

For each food sampling ~25–70 g of pre-thawed (~15 min at room temperature) frozen vegetables were washed with a volume of filter-sterilized PBS equivalent to double the mass of the sample. In order to assist in the detachment of tissue-bound cells, 0.075% [w/v] Tween-20 (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103) was added to the PBS. All washing was performed in ethanol-rinsed, dried and UV-irradiated plastic zip-lock bags after which the food matrix and buffer wash was gently agitated at 80 rpm for approximately 20 min and immediately passed through a 40 μm nylon cell strainer (BD Falcon; Becton Dickinson Biosciences, Bedford, MA) to remove large food particles. The food washes typically had a slightly green hue.

Directly sampled frozen food washes (the 5 mL control) as well as hollow fiber microfilter-concentrated (each 5 mL sample was diluted to ~ 100 mL, concentrated, then washed with another 100 mL buffer ± Tween-20, and eluted with ~ 4–5 mL of the diluent) samples were collected and enumerated using a 6 x 6 drop plate method (Chen, Nace, & Irwin, 2003) taking advantage of 1:2 serial dilutions (i.e., dilution factor = 0.5) for colony selection on BHI plates (2% [w/v] agar). When necessary, the protocol was changed from 1:10 to 1:2 dilutions in order to improve the probability of attaining an average of ca. 3 (± 3√s) colonies per drop (7 μL which diminishes the likelihood of colonies being close together (Chen et al., 2003; Irwin, He, & Chen, 2019; Irwin, Nguyen, & Chen, 2007, 2010).

Briefly, this drop plate method involved loading 400 μL of each wash (either control or concentrated samples brought back to the control sample’s original volume ~ 5 mL) into the first well (row A) of a 96-well microtiter plate. Two-fold serial dilutions were made by transferring 200 μL (multichannel pipette, Rainin, Emeryville, CA) from the first row (row A; dilution 0) into 200 μL of diluent (PBS) in the 2nd row (row B; dilution 1), mixing 10 times while continuously stirring, and repeating the process until five 1:2 dilutions were produced; pipette tips were changed between rows. Based on an analysis of 6 x 6 drop plate observed and calculated sampling error (Irwin et al., 2019; Irwin et al., 2007), sixteen to eighteen 7 μL (ideal volume for the spacing between the tips of a multichannel pipette with minimal spreading) drops from
each of the 6 selected dilutions (dilutions 0–5; overall dilution factors of 0.50 = 1 to 0.5^5 = 0.03125) were plated onto BHI agar using a multi-channel pipette: 6 drops/dilution, 6 dilutions/plate, 3 plates = 18 drops for each dilution. After drop plating, the Petri dishes were allowed to dry, inverted and then incubated at two temperatures (30 and 37 °C). Colonies were counted after 16–32 h. Colony collection for the bacterial isolation and identification involved selecting all colonies from dilution 2 (0.5^2 = 0.25 dilution).

2.4. Bacterial isolation and identification

Across the 4 treatment combinations (Control 30 °C, InnovaPrep 30 °C, Control 37 °C, InnovaPrep 37 °C) the total number of collected colonies was n = 55, n = 49, n = 41 and n = 41, respectively. These colonies were carefully removed from the agar plate’s surface using Rainin L20 micropipette tips and dispersed in 25 μL PCR quality water (Fisher Scientific Sterile Water; Lot # 027417–24) in a separate 96-well PCR plate. Approximately 5 μL of this suspension was used to inoculate 150 μL of BHI in a separate microtiter plate. This latter growth plate was incubated (covered) at 30 °C – 24 h after which 150 μL of 30% (v/v) glycerol in PBS was added to each well, mixed and sealed and stored at –20 °C. The remaining 20 μL of each dispersed colony was spun down and 25 μL of PrepMan Ultra (Applied Biosystems, Foster City, CA) was added to each pellet, mixed, and heated in a thermocycler at ca. 100 °C for 15 min. PrepMan Ultra is not very efficient at extracting DNA (less than 1% for Gram-positive and 14–44% for Gram-negative bacteria: Irwin et al., 2014) but since we collected an entire colony there was enough DNA available for amplification and sequencing. Upon cooling, each sample was centrifuged to separate the DNA solution from the cell debris. The supernatant was then transferred to a clean 96-well microtiter plate and stored temporarily at 5 °C until the DNA amplification step (PCR) was performed. 16 S rRNA gene was amplified using EubA and EubB primers (Cottrell & Kirchman, 2000) and sequenced using 4 primers as previously described (Irwin PL, Nguyen L-HT, Chen C-Y, Paoli G, 2008) on a ABI 3730 Sequencer (Applied Biosystems, Foster City, CA). DNA sequences were edited and contigs assembled using Sequencher as explained in detail previously (Irwin et al, 2008). Bacterial identifications were based upon rDNA sequence matching 1000–1400 base pair contigs searching against NCBI’s GenBank 16 S database. All results presented here gave 99–100% sequence identity with the organisms most closely matching experimentally-determined rDNA contigs.

2.5. Latex particle recovery and size distribution after InnovaPrep sample concentration

Latex-coated polystyrene beads of variable diameters were purchased from Sigma Aldrich (Milwaukee, WI). The beads were supplied in the form of 10% (w/v) aqueous suspensions with small amounts of surfactant and inorganic salts. The particle sizes used in this trial were approximately: 0.3 μm (Sigma Aldrich LB3), 0.6 μm (Sigma Aldrich LB6), 0.8 μm (Sigma Aldrich L88) and 1.1 μm (Sigma Aldrich LB11). Dynamic light scattering (DLS) was used to investigate the effect of the InnovaPrep (IP) concentration treatment on latex bead suspensions of various diameters (recovery and size distribution). The DLS measurements were conducted using a Mastersizer™ 3000 (Malvern Instruments, Worcestershire, UK) equipped with two light sources, red (λ = 633 nm) and blue (λ = 470 nm) and small volume dispersion unit (SVDU). The values for refractive index of the particles (1.590, red light; 1.600 blue light), density of the particles (1.05 g mL^-1) and refractive index of the solvent, water (1.33) were constants selected from a database contained in the software provided by Malvern Instruments. DLS methods depend upon the analysis of the diffraction light produced when a beam of light passes through a dispersion of particles whereupon the angle of diffraction increases as particle size decreases. Particle size distributions were estimated by measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample and Mie scattering theory was used to simulate the observed angular scattering intensity data. The sample’s particle concentration was measured by “obscurcation” which is the percentage loss of laser light through the sample. In order to ensure the obscurcation values were in the range suggested by the manufacturer (5–15%), the stock latex bead suspensions were diluted by a factor of 2500 ×. Specifically, 120 μL of latex bead suspension was added to 300 mL of 18 M2·cm water. This 300 mL sample was split into two 150 mL aliquots; one aliquot was tested without being subjected to any further treatment, while the other was tested immediately following IP concentration to about 5 mL which was then brought back up to 150 mL with water.

One hundred twenty-five mL of the latex bead diluent (18 M2·cm water) was added to the SVDU and circulated through the system using a stirring rate of 1500 rpm. The system was aligned, and a background measurement was collected using a 10 s duration for both blue and red light conditions. The water was completely drained from the system and the 125 mL of experimental sample (either C or IP) was loaded into the unit. Similar to the background measurements, the suspension was recirculated through the system using a stirring rate of 1500 rpm, and the sample measurements were collected using a 10 s duration for both blue and red light conditions. The number of measurements collected for each sample was set to 10 with a 1 s delay in between measurement cycles. General purpose analysis for spherical particles (Mie theory) was utilized in order to generate a number-based size frequency distribution for particle sizes ranging from 10 nm to 3500 μm. Concentration was also reported by the Mastersizer 3000 on a per volume basis.

Following the measurement of each experimental sample, the unit was cleaned. The cleaning cycle entailed circulating 125 mL of diluent through the system for 2 min, and then completely draining the rinse water from the system. The process is repeated three times, and the background measurement is collected using the 125 mL of water retained in the system during the final cleaning cycle.

2.6. Statistics and computations

Analysis of variance (Steel & Torrie, 1960) was performed on most results. In all such experiments either a randomized complete block (all E. coli-spiked experiments) or a completely randomized experimental design (all frozen vegetable experiments) was utilized. All frozen vegetable sampling replication was performed on different days. The probability that two treatment comparisons were equivalent (P) was calculated in Microsoft Excel as “ = F.DIST.RT (F-statistic, treatment degrees of freedom, error degrees of freedom).” When more than two means were compared they were statistically separated based upon a “Tukey Multiple Range” analysis which is also known as the “Honestly Significant Difference” (HSD) Test (Zar, 1999, pp. 210–214). All curve-fitting was based upon a modified Gauss-Newton algorithm using least squares minimization performed on a Microsoft Excel spreadsheet (Hartley, 1961; Irwin, Damert, & Doner, 1994). When appropriate, confidence limits (CL) have also been calculated (Irwin et al., 2019) which were based upon the propagation of error method (Bevington & Robinson, 1992) for estimating the standard error associated with each kth point of the fitting function (f_k) and the diagonal components of the variance-covariance matrix (Salter, 2000).

3. Results and discussion

3.1. Diluent and eluent effects on recovery of bacteria from the hollow fiber microfilter

Table 1 demonstrates that using either E. coli-spiked TBS or PBS there was no substantial advantage (P = 0.257) in recovery (R) between the two dilution systems (either ± Tween 20). The pressurized (canister which fits into the pipette apparatus, Fig. 1) IP “Elution Fluid” used in these particular experiments always matched the two diluents utilized
and always contained TWEEN 20. Comparing the TBS- and PBS-based diluent experiments (i.e., effects of diluent ± TWEEN 20) there was not much of an impact using a surfactant in these diluents (PBS ± TWEEN 20, P = 0.646; TBS ± TWEEN 20, P = 0.867); when the diluents contained TWEEN 20, the R values varied between 0.802 and 0.979 (T ± s: R = 0.908 ± 0.0684); for these same diluents without surfactant, the R values ranged between 0.839 and 0.957 (T ± s: R = 0.924 ± 0.0434).

Table 2 shows that there was also no notable advantage (P = 0.353) using InnovaPrep’s PBS-based Elution Fluid (0.075% TWEEN 20) compared with flushing a pre-loaded (and 100 mL diluent washed) hollow fiber microfiltration tip with PBS which was manually fed through the top (Fig. 1D) and collected from the bottom (Fig. 1E) utilizing a 50 mL sterile syringe. Combined, these R values varied between a low of 0.855 and a high of 0.957 (T ± s: R = 0.920 ± 0.0512). Contrariwise, when the PBS eluent was forced through the top with a 50 mL syringe there was a small, but significant (P = 0.0207), lessening in relative recovery as well as a substantial increase in error (T ± s: R = 0.746 ± 0.0894) when compared to the standard IP eluent process.

The syringe-based, bottom collection results in Table 2 have been confirmed using latex beads wherein we observed that R values level off between 0.767 and 0.924 (T ± s: R = 0.846 ± 0.111) for beads of a size similar to E. coli’s long axis (Fig. 2C; T ± s: μ = 0.630 ± 0.00368 and 1.02 ± 0.0581 μm; 10 measurements for each). The latex bead diameter probability density results displayed in Fig. 2A were experimentally obtained by fitting DLS-based latex bead optical data (normalized for scaling purposes) to a Log-normal probability (P_k; k = 1, 2, ..., 200) density fitting function

\[ P_k = A \cdot \exp \left( -\frac{(\ln[D_k] - \mu_{\ln[D]})^2}{2\sigma_{\ln[D]}^2} \right) \]  

where the fitting parameters, \( \mu_{\ln[D]} \) and \( \sigma_{\ln[D]} \), represent the mean and standard deviation, respectively, associated with the Log-normal form (x-axis = \( \ln[D_k] \)) and \( D \) is diameter; \( A \) is the amplitude (height at the center of the distribution) and the Area under the fitted (i.e., \( k = 1, 2, \ldots, K = 200 \)) equation is solved for as a parameter in the fitting algorithm but should be approximately equal to

\[ \text{Area} \approx \frac{1}{K} \cdot \sum_{k=1}^{K} \frac{2^\mu \cdot (\ln[D_k] - \ln[D_{\text{ref}}])}{\sigma_{\ln[D]}^2} \times \sum_{k=1}^{K} P_k \]  

The converted mean diameters or most probable diameters (non-Log) in Fig. 2A are based upon the relation

\[ \mu' = \exp \left( \mu_{\ln[D]} + \frac{\sigma_{\ln[D]}^2}{2} \right) \]  

(http://mathworld.wolfram.com/LogNormalDistribution.html). The latex bead diameter results were determined experimentally from 10 repeated measurements of particle size distribution of the four size classes (Sigma Aldrich: LB3, LB6, LB8, and LB11) based upon Mie scattering theory (Ye, Jiang, & Wang, 2012). It should also be noted that the smallest latex particle size (Fig. 2A; T ± s; \( \mu = 0.260 ± 0.0409 \) μm) was not very well recovered (Fig. 2C; R ≈ 16%). The results in Fig. 2A–B also show a size-related shift in the probability densities wherein the
shifts \( \Delta \mu = [\bar{\mu} - \mu_{IP}] \) from the IP concentration process alone caused as much as a 20% change in \( \bar{\mu} \), and \( \Delta \mu \) was inversely proportional to \( \bar{\mu} \). \( \Delta \mu \) was not apparent in optical density, these results imply that the larger sizes in each of the distributions are tightly adhering to the filters. The latex bead results argue that E. coli cell recoveries, which are usually less than unity, are likely due to an inability to completely elute the bacterial cells from the capillary filter matrix albeit there is a slight advantage (not significant) using InnovaPrep’s canister-based elution buffer (Table 2; \( P = 0.353 \)). In summary, the small inefficiencies in bacterial recovery we observe (Tables 1 and 2) are not likely due to cell damage but rather cell loss in the capillary filter itself.

### 3.2. Bacterial cell recovery and elution volume

The cumulative CFU and recovery rates with aggregate elution volume (R [f1], R [f1-f2], R [f1-f2-f3], and R [f1-f2-f3-f4]) experiments depicted in Fig. 3A and B utilized \( n \sim \) seventy-two 7 μL samplings for CFU enumeration. Due to the much higher concentrations in Fig. 3C, quantitation involved taking \( n = 16-18 \) samplings each of 7 μL from at least two of the five dilutions. All cumulative recovery data (R [f1] to R [f1-f2-f3-f4]) were fit to a saturation curve equation (solid smooth lines) which provides a projected R\(_{max}\) asymptote ± ASE (Irwin et al., 1994). The fitting equation

\[
\mu = \frac{\exp[\mu_{IP}] + \sigma_{IP}^2}{2}
\]

**Fig. 2.** Recovery of latex beads from aqueous suspension based upon dynamic light scattering (DLS) results. A: Dependency of an apparent shift in Log-normal population size probability distributions on bead diameter (D) as related to the InnovaPrep concentration process. Averaged (Control and InnovaPrep-treated) number fraction probability distribution means (average ± s.d) = 0.260 ± 0.0409 (blue circles), 0.477 ± 0.0404 (red triangles), 0.632 ± 0.00368 (green diamonds), 1.02 ± 0.0581 (purple squares). Equation (4) repeated. B: Demonstration that the population coefficients of variation (\( \sigma_p / \mu_p \)) were approximately equal even after the D-shifts (\( \Delta \mu \)) in particle distribution. C: Establishment of latex particle recovery as a function of measured average population particle size.

**Fig. 3.** A: Cumulative recovery of \( 5 \times 10^3 \) CFU E. coli (native chicken isolate) in 5 mL (\( 10^3 \) CFU mL\(^{-1}\)) PBS after concentrating using the InnovaPrep Concentrating Pipette system as a function of aggregate elution volume. B: Cumulative recovery of \( -5 \times 10^3 \) CFU E. coli in 100 mL (50 CFU mL\(^{-1}\)) PBS after concentrating using the InnovaPrep Concentrating Pipette as a function of aggregate elution volume. In Fig. 3A and B, \( n \sim \) seventy-two samplings (7 μL each) were used to enumerate both the Control and InnovaPrep-concentrated samples. C: Cumulative recovery of \( -5 \times 10^3 \) CFU E. coli in 100 mL PBS after concentrating using the InnovaPrep Concentrating Pipette as a function of aggregate elution volume. In Fig. 3C, \( n = 16-18 \) samplings (7 μL each) from at least two of the dilutions were used to enumerate both the Control and InnovaPrep-concentrated samples. All recovery data were fit to an equation (\( P = 0.05 \) CL: dotted lines) which provides projected R\(_{max}\) asymptotes ± asymptotic standard error (Irwin et al., 1994). Dashed lines (triangular symbols) represent cumulative CFU. Recovery values are all provided with associated HSD-based letters of significance.
The following components: $R_{\text{max}}$, the maximum recovery asymptote at a large cumulative volume ($V_{c}$); $V_{c, \text{trans}}$ is an $x$-axis translation correction factor; $r$ (mL$^{-1}$) is the rate of change in $R$ with respect to $V_{c}$. The $P = 0.05$ confidence limits associated with these non-linear regression curve-fits are displayed as dotted lines and are very sensitive to small variations in the fitting function. The dashed lines associated with triangular symbols in Fig. 3 represent the cumulative CFU recovered at various aggregate volume fractions.

Fig. 3 shows results related to the aggregate recovery (as well as total CFU recovered) of the *E. coli* isolate diluted in either 5 ($\sim 10^{5}$ CFU mL$^{-1}$; Fig. 3A) or 100 ($\sim 50$ CFU mL$^{-1}$; Fig. 3B) mL PBS. In these experiments, the concentrated samples were always washed with $\sim 100$ mL PBS buffer before elution with InnovaPrep’s pressurized, canister-based elution buffer (PBS + 0.075% Tween 20). Four fractions (1.27 $\pm$ 1.5 mL each; averaged across all experiments: $x \pm s$; $f_1 = 1.40 \pm 0.0714$ mL; $f_2 = 1.52 \pm 0.0375$ mL; $f_3 = 1.23 \pm 0.0050$ mL; $f_4 = 1.07 \pm 0.0494$ mL) and all procedures replicated thrice in a randomized complete block experimental design (each “block” represents each colony used to inoculate the overnight culture). Fig. 3A displays the cumulative recovery of approximately 5 $\times$ 10$^5$ CFU *E. coli* (native chicken isolate; in 5 mL PBS) after microfiltration-based concentration as a function of aggregate elution volume. After the elution of only 1.27 $\pm$ 0.112 mL, 81.3 $\pm$ 2.25% of the CFUs had been recovered with an infinite volume $R_{\text{max}}$ asymptote of about 90% ($R_{\text{max}} = 0.915 \pm 0.000205; \pm$ ASE) in a total volume of 5.25 $\pm$ 0.158 mL with only small changes in $R$ in between: R ($f_1$,$f_2$) through R ($f_1$,$f_2$,$f_3$,$f_4$) were not significantly different at the $P = 0.05$ level based upon the aforementioned HSD test. Another result was observed in experiments depicted in Fig. 3B (cumulative recovery of approximately 5 $\times$ 10$^5$ CFU *E. coli* in 100 mL PBS). In this set of results 80.7 $\pm$ 1.83% recovery was achieved after elution of 1.51 $\pm$ 0.149 mL and the cumulative $R$-values flattened out at around 94% recovery ($R_{\text{max}} = 0.943 \pm 0.000464$). As was observed in Fig. 3A, the cumulative R data (R ($f_1$,$f_2$) through R ($f_1$,$f_2$,$f_3$,$f_4$)) in 3 B were also not significantly different at the $P = 0.05$ level indicating that it is probably more efficient to collect no more than a single 1.5-2 mL fraction. Working with $\sim 1000$-fold more bacteria, similar results (Fig. 3C) were seen: 85.8% of the CFUs were recovered in $f_1$ (1.14 $\pm$ 0.151 mL) and dropped off to $\sim$ 98% (cumulative volume of 5.14 $\pm$ 0.238 mL; $R_{\text{max}} = 0.984 \pm 0.000464$). There was no significant differences seen except between R ($f_1$) and R ($f_1$,$f_2$,$f_3$,$f_4$).

### 3.3. Recovery of native bacterial contaminants on frozen vegetables

Table 3 shows that the level of bacterial contaminants on frozen vegetables on a per gram basis varied greatly between random samplings: 1490–3900 CFU g$^{-1}$ (30 °C) and 357–2744 CFU g$^{-1}$ (37 °C). These results also show that the R values associated with sub-sampling 5 times (over a 77 day period) from one bag of frozen vegetables (sampling and plating protocol in Materials and Methods section) were fairly constant but they were slightly lower with greater variability ($x \pm s$; $R_{30} = 0.739 \pm 0.113$; $R_{37} = 0.791 \pm 0.149$) than similar results from the *E. coli*-spiked buffers ($x \pm s$; $R_{30} = 0.948 \pm 0.0464$).Randomly (in *Mathematica*: Table [1 = Random [Integer, {1, 5}], {i, 3}] which provides 3 random numbers between 1 and 5) selecting 3 out of the 5 experimental recovery rates for the frozen vegetable experiments (30 °C only) and comparing them with those associated with above *E. coli*-spiked buffers we found that these differences were only significant at the 0.1 level ($P = 0.0956$). It is likely that the native foodborne microbiome is innately more susceptible to IP-associated damage than our *E. coli* test strain due to the harsh environment upon which they reside. There was no significant effect ($P = 0.767$, Table 3) of growth/recovery temperature on R. Nevertheless, the frozen vegetable wash concentration process did not appear to perturb the most probable isolate composition (Table 4; colonies from Sampling #1, 28.6 g) relative to that of the Control inasmuch as the two major isolates (80–95% of the total isolates were *Leuconostoc* and *Lactococcus*) were in about the same proportions for either culture temperature for either the directly plated samples (C) or the IP concentrated samples. This finding argues that these major isolates were equally susceptible to damage from the concentration process. The minor fractions (<10% in *toto*) displayed an appreciably higher variability which was probably due to errors associated with random sampling. Thus, the two *major* species sampled were relatively repeatable because of their abundance, adequate sampling (n = 16–18), and very little treatment effect. However, the *minor* constituents would have had to be sampled at least 2.77 $\pm$ 0.647-fold more (for various $x$ counts $< \sqrt{\sum_{x \text{major}} + \sqrt{\sum_{x \text{minor}}}$; Irwin et al., 2019) for an equivalent precision to the *Luconostoc* and *Lactococcus* fractions since the requisite number of samples for the low count fractions, above, is proportional to the inverse cubed root (Irwin et al., 2010; Irwin, Reed, Brewer, Nguyen, He, 2013) of the number of counts per sampled volume.

### 4. Conclusions

In this work we have gauged the performance of a “concentrating pipette” equipped with a 0.2 μm polysulfone hollow fiber filter “tip” from the perspective of the sample volume reduction so as to improve quantitative metagenomics study of native foodborne bacteria. Results were presented concerning both the relative recovery rate of bacteria in *E. coli*-spiked buffers as well as the perturbation in the bacterial composition due to the concentration process. We found that there was
Table 4
Comparison of most probable 16 S rDNA-based composition of control and Innovaprep-concentrated bacteria from frozen vegetables (based upon Sampling #1 from Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Most Probable Organisms(^b) from 16 S Sequence Analysis</th>
<th>Occurrence</th>
<th>CFU/g ± s/√d(^c)</th>
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<td>30 °C</td>
<td>37 °C</td>
<td>30 °C</td>
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<td>directly Plated</td>
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<tr>
<td>Leuconostoc (+)</td>
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<td>Lactococcus (+)</td>
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<td>41%</td>
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<td></td>
<td>8%</td>
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<td>Streptococcus (+)</td>
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</tr>
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<td>n = 55(^f)</td>
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<tr>
<td>Innovaprep Concentrated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuconostoc (+)</td>
<td>49%</td>
<td>32%</td>
<td></td>
</tr>
<tr>
<td>Lactococcus (+)</td>
<td>43%</td>
<td>63%</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter [-]</td>
<td></td>
<td></td>
<td>4%</td>
</tr>
<tr>
<td>Streptococcus (+)</td>
<td></td>
<td></td>
<td>2%</td>
</tr>
<tr>
<td>n = 49</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Sampling #1 from Table 3: 28.6 g of frozen vegetables + 57.2 mL PBS.

\(^b\) Average of d dilutions (n = 16–18 for each dilution).

\(^c\) + = Gram-positive; - = Gram-negative.

\(^d\) \(R = \frac{\{\text{CFU}/g\} + \{\text{CFU}/g\}C = \{I\}}{\{C\}}\).

\(^e\) Propagated error = \(\sqrt{\frac{S^2}{n} + \frac{S^2}{C} + \frac{C^2}{n}}\).

\(^f\) n: number of colonies collected.

no significant effect of different diluents/eluents on the relative recovery of our test strain. Cumulative recoveries from 4 sequential fractions (100 mL, 50.2 CFU mL\(^{-1}\)) were also examined from both 100 mL (above data) as well as 5 mL E. coli-spiked PBS (5 mL, 1015 CFU mL\(^{-1}\)) and we found that the first fraction collected contained about 80% of the total. We also demonstrated that the small inefficiencies in bacterial recovery rates were not likely due to damage to cells. Upon concentrating PBS washes of pre-thawed frozen vegetables and enumerating after incubation at 30 and 37 °C, the recovery rates were only somewhat lower than for spiked buffers. Lastly, the frozen vegetable wash concentration process did not much change the most probable isolate composition relative to that of the control. We thus show that the Innovaprep concentration pipette system should work efficiently for water-based food systems such as process water, vat rinses, food processing-related equipment rinses, produce wash water, and water/buffer-suspended surface/air samples coming out of food processing plants.

5. Glossary of common terms

\(\bar{x}_j\): mean of variable \(x_{ij}\); \(\text{AVERAGE (x-array)}\); \(\frac{1}{n} \sum_{i=1}^{n} x_{ij}\); \(i\) replications and \(j\) experiments.

\(n\): number of replicates in each experiment \(j\th\)th.

\(s\): standard deviation; \(\text{STDEV.S (x-array)}\).

\(S_p\): estimate of the standard deviation of a population of \(\bar{x}\); \(S_p = s / \sqrt{n}\) whereupon \(n\) is the number of replicates used to calculate \(\bar{x}_j\).

\(D\): particle diameter in units of \(\mu\)m as measured by dynamic light scattering (DLS).

\(\mu\): for any probability density function, the population’s average of any real-valued, randomly changing variable.

\(\mu_{\phi(x)}\): the population mean for the Log-normal Gaussian probability density function; all particle diameter (\(D\)) distributions are Log-normal (https://en.wikipedia.org/wiki/Log-normal_distribution).

\(\mu\): linear conversion from \(\mu_{\phi(x)}\) when using the Log-transformed x-axis;

\[\exp \left[ \mu_{\phi(x)} + \frac{\sigma^2_{\phi(x)}}{2} \right]\]

\(V_c\): cumulative volume in recovery experiments

\(R = [R_{\text{max}} \{1 - \exp\{V_c k + V_{c,\text{trans}} r\}\}]\); fitting function for recovery data as a function of \(V_c\); \(V_{c,\text{trans}}\) is the x-axis translational correction and \(r\) is the rate of change in R with respect to \(V_c\).

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CRediT authorship contribution statement

Peter Irwin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

Yiping He: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing.

Joseph Capobianco: Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing.

Matthew Gehring: Methodology, Investigation, Writing - original draft, Writing - review & editing.

Andrey G. Gehring: Methodology, Validation, Writing - original draft, Writing - review & editing.

Chin-Yi Chen: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing.

Andrew G. Gehring: Methodology, Validation, Writing - original draft, Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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