



A comparison of methods for enumerating bacteria in direct fed microbials for animal feed

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ARTICLE INFO

Keywords:

Bacterial enumeration
Viable but not culturable
Plate counts
Flow cytometry
qPCR

ABSTRACT

Aerobic plate counts are the standard enumeration method for probiotic-containing products. This counting method is limited by the ability of many cells to enter a viable but non-culturable (VBNC) state upon exposure to stressful conditions like dehydration and heating commonly used in probiotic product preparation. Alternative enumeration methods are available including flow cytometry (FC) which counts total live/dead cells by assessing cellular integrity and/or metabolic activity, and quantitative polymerase chain reaction (qPCR) in which enumeration is correlated with the quantity of a nucleic acid target. These three methods were compared for enumerating three lactic acid bacteria (LAB): *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*, and a *Bacillus subtilis* related strain in twenty samples of a mixed probiotic product ranging in age from one to 825 days post-production. Flow cytometry and qPCR enumerations were similar and much higher compared to plate counts at later storage times, suggesting that some strains in the population were entering the VBNC state and were only countable by FC and qPCR. We propose the use of FC and/or qPCR as an alternative to plate counts for more accurate enumeration of bacteria in probiotic products.

1. Introduction

A variety of methods exist for determining bacterial concentrations in commercial product preparations. These include traditional plating assays (Maturin and Peeler, 2001), measurements of optical density (turbidity) (Brown, 1980), direct counting methods such as flow cytometry (FC) (Gunasekera et al., 2000), cell mass determination (Hobbie et al., 1977), measurements of cellular activity (Rodriguez et al., 1992), and quantitative polymerase chain reaction (qPCR) (Davis, 2014). Depending on the specific product matrix, one assay technique may be preferable for accuracy and reproducibility.

Global labeling and regulatory mandates require accurate enumeration of viable microbes in a given product preparation. Most regulatory bodies rely on standard plating methods to determine compliance with product label claims. However, the most significant challenge to accurate counts via plating is that not all bacteria grow well on a synthetic medium (Davis, 2014). The preparation of products containing bacteria can be stressful to the microbes, and may cause up-

regulation and expression of stress-response genes in some bacterial strains (Mills et al., 2011). This can result in poor bacterial recovery via traditional plating methods. In response to environmental stress, many bacteria enter a unique bacterial hibernation stage referred to as the ‘viable but non-culturable’ (VBNC) state (Xu et al., 1982; Oliver, 2005) in which metabolic activity is measurable, but culturability is decreased (Ramamurthy et al., 2014). In this state, a significant portion of the bacterial population does not grow on traditional plating media. However, these cells can reestablish functionality and the ability to replicate in a more hospitable environment (Lahtinen et al., 2008). Furthermore, quantification of bacteria via standard plating methods gives a downward-skewed estimate because only colonies forming under the specific conditions of the plating assay are counted. In addition, a colony may arise from one cell or from thousands of cells in a clump or floc. As a result, plate counting typically underestimates bacterial concentrations (Davis, 2014).

Direct Fed Microbials (DFMs or Probiotics) are routinely administered to animals in commercial agricultural production (Krehbiel et al.,

Abbreviations: VBNC, viable but not culturable; qPCR, quantitative polymerase chain reaction; FC, flow cytometry; DFM, direct fed microbial; LAB, lactic acid bacteria; SFC, spore former count; CFU, colony forming unit; TO, thiazole orange; PI, propidium iodide

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<https://doi.org/10.1016/j.mimet.2019.04.003>

Received 23 February 2019; Received in revised form 3 April 2019; Accepted 3 April 2019

Available online 05 April 2019

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2003). They provide a range of benefits including improved feed conversion, faster weight gain, improved resistance to gut pathogens, and improved response to stress (McAllister et al., 2017). A key quality criterion in the manufacture, regulation, and marketing of DFM's is the accurate definition of the number of viable cells in the product according to the label. As noted above, there is growing evidence that the use of traditional plate counting methods does not fully articulate the activity and viability of many bacteria. This is particularly true for bacterial strains commonly used in DFM's like *Lactobacillus lactici* and *Lactobacillus plantarum* (Liu et al., 2017; Oliver, 2005).

Davis compared various methods for enumerating probiotics and concluded that alternative techniques such as qPCR, fluorescent microscopy, MALDI-TOF mass spectrometry, and Flow Cytometry are viable alternatives to traditional plate counting (Davis, 2014). With that in mind we undertook a study to quantify bacterial concentrations in a DFM product, a mixture of *Pediococcus acidilactici* (PA), *Pediococcus pentosaceus* (PP), *Lactobacillus plantarum* (LP) and a *Bacillus subtilis* related strain (BS), as a function of storage time. Enumeration of the lactic acid bacteria (LAB) and *Bacillus* in this DFM was compared using three different techniques: traditional plate counting using selective media and plating conditions to enumerate the LABs as a group and *Bacillus* independently, FC to count all cells, and a qPCR method using taxon-specific primers for each organism in the DFM product.

2. Materials and methods

2.1. DFM formulation and individual strains investigated

The Direct Fed Microbial product (DFM1) investigated in this study contained a mixture of lyophilized *Bacillus* and Lactic Acid Bacteria (LAB) in a dextrose matrix. This formulation contained four unregistered strains belonging to the following species: *Bacillus subtilis* related strain (BS), *Pediococcus acidilactici* (PA), *Pediococcus pentosaceus* (PP), and *Lactobacillus plantarum* (LP) originally isolated from a solid substrate fermentation on a rice bran/soy flour mixture. The individual strains were each produced by deep tank liquid fermentation under conditions optimized for each organism, and were then mixed in specific concentrations with the DFM matrix. BS was added to the product as spores. DFM1 has a label claim of 3×10^8 CFU/g for total LAB (10^8 CFU/g each) and 10^7 CFU/g for BS spores. The published shelf life for this product is 3 years when stored out of direct sunlight in a cool (below 40 °C) dry location.

Twenty samples of DFM1 were obtained from a commercial warehouse, where they had been stored at ambient temperature (about 25 °C) since their respective dates of manufacture (Table 1). Subsamples of each product were collected for plate counting assays, flow cytometry, and qPCR with BS, LP, PA and PP specific primers. Plate counting and DNA extraction were performed on the same day for all samples, and flow cytometry was performed four days later. The product's final age was defined to be the number of days elapsed between the date of manufacture and the date of assay for each method. Thus, although the samples tested with each method were identical, FC analysis was performed on samples four days older than samples subjected to plating and qPCR analysis.

Table 1

Number of samples tested from each period of storage. Samples from each storage day were replicates from the same manufacturing lot, with the exception of days 54 (2 lots) and 825 (4 lots).

Storage days	1	54	156	279	433	506	702	825	Total
Samples	5	4	1	2	1	2	1	4	20

2.2. Viable plate counts

To selectively enumerate the LAB and *Bacillus* endospores within the DFM1 preparation, each sample was subjected to Lactic Acid Bacteria (LAB) and Spore-Former Count (SFC) plating assays. Ten grams of DFM1 was diluted in 90 mL of sterile, 0.1% peptone blank. This tenfold dilution was shaken vigorously by hand for one minute, at which point a 10 mL sample was collected and transferred to a new bottle of 0.1% peptone blank. This step was repeated until a set of serial dilutions was achieved of one log above and one log below where the expected diluted microbial titer would yield a countable plate containing 25–250 colonies. For LAB assays, each of the three dilution bottles were shaken for 30 s, then triplicate 100 µL aliquots were removed and spread on cured DeMan, Rogosa and Sharpe (MRS) agar plates (EMD Millipore, Burlington, Massachusetts). This media is selective for lactic acid bacteria, and axenic cultures of BS plated on MRS showed no growth (data not shown).

For the SFC assay, a 10 mL subsample was collected from each of the three dilution bottles in the targeted dilution range, and transferred into a sterile, 15 mL conical tube for pasteurization in a Benchmark MyBath 8.0 L water bath at 80 °C for 15 min. This treatment kills vegetative cells, leaving endospores as the only colony-forming units (CFUs) in the sample. After heating, each tube was shaken for 30 s, and triplicate 100 µL aliquots were spread on Tryptic Soy Agar (TSA) plates (BD Difco, Franklin Lakes, NJ) for colony growth. All plates were incubated in a 35 °C incubator for 48 h. Only plates containing 25–250 colonies were used for data analysis.

2.3. Flow cytometry

DFM1 was suspended in a 10 fold volume of distilled water and homogenized using a Stomacher 400 (Seward, Ltd., United Kingdom). Dilutions into 0.1% peptone were performed to achieve a nominal concentration of 1×10^5 cells/mL based on product label claims. The cell permeant dye, thiazole orange (Sigma-Aldrich, Saint Louis, MO), was added at a concentration of 125.9 µM and the cell impermeant dye, propidium iodide (Sigma-Aldrich, Saint Louis, MO), was added at 14.9 µM. Samples were immediately loaded and run in triplicate on an Acea Novocyte flow cytometer (Acea Biosciences, Inc., San Diego, CA). Multiparametric data were collected using a scatter threshold for excluding small particles and cellular debris. Gates for live, injured, and dead cell populations were set up using a blank excipient control. Both vegetative cells and spores were counted. Flow cytometry data were analyzed using NovoExpress software (Acea Biosciences, Inc., San Diego, CA). Bacterial populations were enumerated by categorization based on variations in cluster patterns and fluorescence.

2.4. Genomic DNA isolation

Genomic DNA was obtained from either 1.5 mL of LAB liquid cultures (PA, PP, or LP) grown with shaking in MRS media (EMD Millipore, Boston, MA) for 16–20 h at 35 °C, or 1.5 mL of a liquid culture of BS grown in Tryptic Soy Broth (Carolina Biological Supply, Burlington, NC) media for 16–20 h at 37 °C, or from 0.1 g of DFM1 using the MoBio PowerSoil DNA Extraction Kit (MoBio, Carlsbad, CA; now available from QIAGEN, Inc. as the DNeasy PowerSoil Kit, Germantown, MD) with modifications to the standard protocol. Specifically, cell disruption was achieved using the FastPrep bead beater (MP Biochemicals, Solon, OH) at 6.5 m/s for 135 s.

2.5. Whole genome sequencing and identifying reference genomes

Whole genomic DNA from each of the four DFM strains was sent out to MR-DNA Labs (Shallowater, TX) for shotgun sequencing using an Illumina MiSeq protocol. The 16S rRNA gene sequences from each strain were identified in the genome sequence and subject to blastn

analysis using the National Center for Biotechnology Information (NCBI) database to determine probable species identity, and to find reference genomes or genomic scaffold sequences for each strain.

2.6. Identifying strain-specific sequences

To find unique DNA sequences in the strains, genomic sequences were compared to the appropriate reference genomes using the CLC Genomics Workbench (QIAGEN, Inc.). To find sequence variations, particularly insertions/deletions or large genome rearrangements, between the strains of interest and their respective reference genomes, 10,000 base pair sequence fragments were mapped to the reference genome. Test sequences that did not match to the reference genome were subjected to blastn analysis using the NCBI database to confirm sequence specificity.

2.7. Selection of primer/probe sequences

Unique genomic regions from each strain were subjected to the TaqMan Primer Design feature of the CLC Genomic Workbench. Primer/probe sets were selected based on specificity of the amplified regions (as determined by blastn analysis), a target size between 80 and 260 base pairs, and an approximately 10 °C difference between the T_m of the probe and the primers. Each forward/reverse primer set was also tested for specificity using the NCBI Primer BLAST program. Primer sets resulting in non-specific products close to the size of the intended target were not used. If the non-specific product was much larger than the intended target, the primer scheme was retained because amplification parameters could be adjusted to favor the smaller size of the intended target.

2.8. Primer specificity and sensitivity

The ability of each primer set (Table 2) to amplify its target sequence was determined by PCR amplification using 1 ng of PA, PP, LP or BS genomic DNA in a 20 µL reaction also containing NEB 2 × OneTaq Quickload Mastermix (New England Biolabs, Ipswich, MA) and 500 nM each of forward and reverse primer (Eurofins Genomics, Louisville, KY). All PCR reactions were performed at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 68 °C for 30 s. The strain-specific PCR primer schemes for each organism were also tested against an array of other closely related organisms to evaluate specificity, and against a dilution of DNA template from the target organism to test for primer sensitivity. The most specific and sensitive primer schemes were selected for further analysis (Table 2).

Table 2

Primer/Probe Schemes for Bacterial Strains present in DFM1 product. PA, PP, LP and BS refer to the bacterial species targeted by each primer set. F = forward primer, R = reverse primer, TP = Taqman probe.

Primer name	Primer sequence	T _m (°C)	Fragment size
PA.F	CGGTTGAGAAGTGAAGTTA	52.62	138
PA.R	GGTTGAAGCTTATGATGG	52.90	
PA.TP	TTTAGGGAAGTCGGTGCGG	60.33	199
PP.F	TCACTCTTTACGCCCTTC	53.95	
PP.R	GCGGGAGCATTACTATTT	54.44	
PP.TP	ACCGCCACGCTAGTTTCA	60.93	
LP.F	CCCGTAAACGCAAAGATAA	52.65	155
LP.R	TTCAATATGCTCTCCGTC	52.19	
LP.TP	CGATGATTAATCGGTGACAAATTTGGTC	62.15	256
BS.F	CCAACATATAAGACCTCTAC	52.66	
BS.R	TTATTTTCATCCCATCCTGAC	52.65	
BS.TP	CCCAACCAGCGATCCATAC	60.98	

2.9. CFU-specific standard curves for qPCR

The qPCR assays in this study were calibrated to colony forming units per milliliter (CFU/mL). An overnight broth culture was prepared for each species of interest. BS was cultured in Tryptic Soy Broth (Carolina Biological Supply) at 37 °C, while LP, PA and PP were cultured in MRS broth (Millipore Sigma) at 35 °C. All isolates were shaken at 200 RPM in a Thermo Scientific MaxQ 8000 Incubator/Shaker for 24 h. After incubation, each culture was subjected to three, tenfold serial dilutions. A 1.0 mL and a 10 mL sample were collected from each original broth culture as well as from each respective serial dilution for a total of four samples per isolate. The 10 mL samples were subjected to serial dilution and plate counting as described in section 1.2 above to determine CFU/mL. The 1.0 mL samples were pelleted at 12,000 × g for 15 min, and the resultant cell pellet was subjected to DNA extraction using a DNEasy PowerSoil DNA extraction kit (QIAGEN) following manufacturer's instructions.

2.10. qPCR on standard curve and test DNA

TaqMan probes were obtained through Life Technologies Corp (Carlsbad, CA). qPCR reactions for PP, LP, and BS included 1 × TaqMan Universal MasterMix II without uracil-N glycosylase (no UNG) (Applied Biosystems, Foster City, CA), 900 nM each of the forward and reverse primers, 250 nM of the appropriate TaqMan probe, and 4 µL of DNA in a 20 µL total reaction volume. DNA concentration was irrelevant since DNA isolation was from a given number of cells. PA qPCR reactions included 1 × TaqMan Universal MasterMix II (no UNG), 300 nM forward primer, 900 nM reverse primer, 50 nM TaqMan probe, and 4 µL DNA in a 20 µL reaction volume. qPCR reactions were performed on a QTower3 Real-Time PCR Thermal Cycler (Analytik Jena, Jena, Germany). Reaction conditions for PA and BS were 10 min at 95 °C, and 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 60 °C for 60 s. Reaction conditions for PP and LP were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 52 °C for 30 s and 57 °C for 60 s. A standard curve was generated by plotting threshold cycle (C_t) values (x-axis) versus log CFU/mL (y-axis) as determined by the plate counts described in section 1.9. This standard curve was then used to determine cell concentration in unknown samples based on obtained C_t values.

2.11. MIQE analysis of standard curves

The efficiency of the qPCR technique was evaluated according the method of Bustin (Bustin et al., 2009) using the equation:

$$\text{PCR Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100\%$$

where “slope” is the slope of the best-fit line on a semi log plot of log (CFU/mL) on the x-axis versus C_t value on the y-axis. An acceptable range for efficiency is typically 90% – 105%.

2.12. DFM dilutions for qPCR

Ten grams of DFM1 was suspended in 90 mL 0.1% peptone, and was vigorously shaken for 30 s. One mL of the solution was centrifuged at 12,000 × g for 15 min, and 900 µL of supernatant removed. The remaining pellet in 100 µL supernatant was subject to DNA extraction using a Qiagen DNEasy PowerSoil Kit, following modified instructions from the manufacturer intended to optimize recovery in low-biomass soils.

3. Results

Twenty samples of Direct Fed Microbial product (DFM1), produced by the same manufacturer and in the same facility, were collected on different dates and of different lots (Table 1) but with the same label claim for concentrations of three lactic acid bacterial (LAB) species,

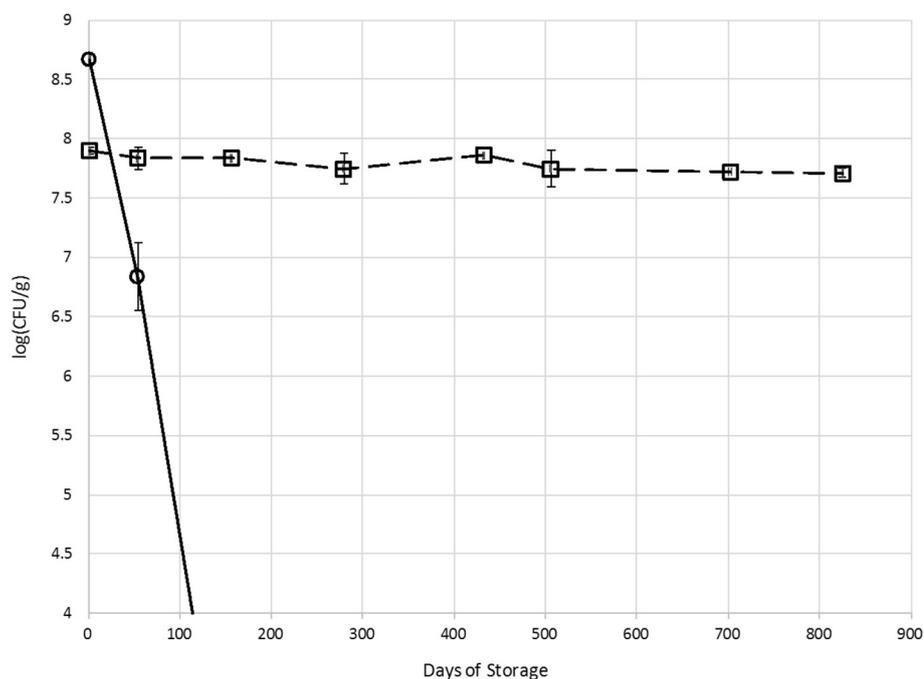


Fig. 1. Log LAB (circles) and BS (squares) plate counts (log(CFU/g)) in DFM1 versus days of storage. Error bars indicate standard deviation of a geometric mean when more than one sample was processed from a single storage time (Table 1). Detection limit was 10^4 CFU/g. Product label claims are: 3×10^8 CFU/g for LAB [8.48 log(CFU/g)], and 10^7 CFU/g for BS (SFC count).

Pediococcus acidilactici (PA), *Pediococcus pentosaceus* (PP), and *Lactobacillus plantarum* (LP), and a *Bacillus subtilis* related strain (BS). The samples were stored in sealed, metallized polyethylene bonded film bags at ambient temperature (roughly 25 °C) in a commercial warehouse prior to being sampled for microbial enumeration. Enumeration was accomplished using plating, flow cytometry (FC), and qPCR to compare enumeration across these three methods.

3.1. Direct plate count

Populations of BS and the three LAB species, PA, PP, and LP, were enumerated using the SFC assay for BS endospores and MRS agar for the LABs. The LABs are not easily distinguishable by colony morphology, so they were counted as a group in the plating assay. LAB plating data shows a drop below label claim by day 54 of product storage (average recovery 8.0×10^6 CFU/g) and a loss of any detectable titer ($< 1.0 \times 10^4$ CFU/g) by day 156 of storage. In contrast, SFC plating data shows stable recovery above the label claim for BS spores for all tested samples (Fig. 1).

3.2. Flow cytometry enumeration and evaluation of cell health

Flow cytometry (FC) was used to enumerate cells and evaluate cell viability based on differential membrane permeability to distinct fluorescent dyes. Using a combination of cell permeant and impermeant dyes, three populations were observed in each sample: healthy cells, injured/stressed cells, dead cells (Massicotte et al., 2017; Wilkinson, 2018). However, this form of FC is unable to distinguish between bacterial species; thus, total cell and spore counts in DFM1 were determined for each of the tested samples. By this method, DFM1 cell populations were relatively stable until Day 702 when a $> 50\%$ decrease is observed in the live cell population (Fig. 2). Prior to Day 702, total cell titers compare favorably to the DFM1 product's label claim. While there are slight increases in injured and dead cells after Day 54, those levels remain constant until the end of the study. They do not account for the 30–35% loss in total cells observed at days 702 and 825.

3.3. Enumeration by qPCR

Strain specific primers and probes were designed for each bacterial

species present in the DFM1 (Table 2), and tested for specificity to the correct strain compared to the other DFM1 strains by endpoint PCR. The PA, LP and BS primer schemes amplified only the appropriate PCR product for their respective strains (data not shown). The primer scheme for PP amplified the expected PP fragment, but also a high molecular weight product in PA and BS DNAs. However, the non-specific products were not detected in, and did not interfere with, qPCR in the PP-specific primer/probe scheme. Therefore, the primer schemes were sufficiently specific to distinguish between the bacterial species in DFM1.

Bacteria in DFM1 products were enumerated in qPCR assays by comparison of sample C_t values to cell count-based standard curves for each bacterial species and thus reported as CFU/g of DFM1 (Fig. 3). Calculated PCR efficiencies of the PA, PP, LP and BS standard curves ranged from 92.8–101.5%. PA showed reduced cell counts at days 433 and 506, but older samples showed cell numbers consistent with newer samples suggesting that the observed loss of viability could be due to factors other than product age. LP began to drop at 825 days of storage, but never went below the label claim of 10^8 CFU/g. In contrast, a sharp drop in enumeration to $< 10^5$ CFU/g was observed in PP at days 702 and 825. This is consistent with the 30% loss of total cells observed by flow cytometry, and suggests that PP is the most sensitive to long term storage. Enumeration of BS showed it was stable in DFM1 over 825 days of storage.

4. Discussion and conclusions

Many regulatory agencies rely only on aerobic plate counts or similar plating assays to evaluate the microbial activity of probiotic products and ensure the accuracy of label claims. However, these assays have a well-known propensity for underestimation of microbial titer for a number of reasons including entry of cells into the VBNC state. Therefore, other enumeration techniques should be considered to confirm advertised cell counts in probiotic products. In this study, the stability of a dextrose-based DFM product was monitored by three common methods for enumeration – traditional plating, FC, and qPCR.

Plate counts underestimated total cell numbers in DFM1 to varying degrees based on the age of the product in question. Bacterial cell counts obtained by plating compared favorably with values obtained from qPCR and FC only for one-day-old product samples. By 54 days,

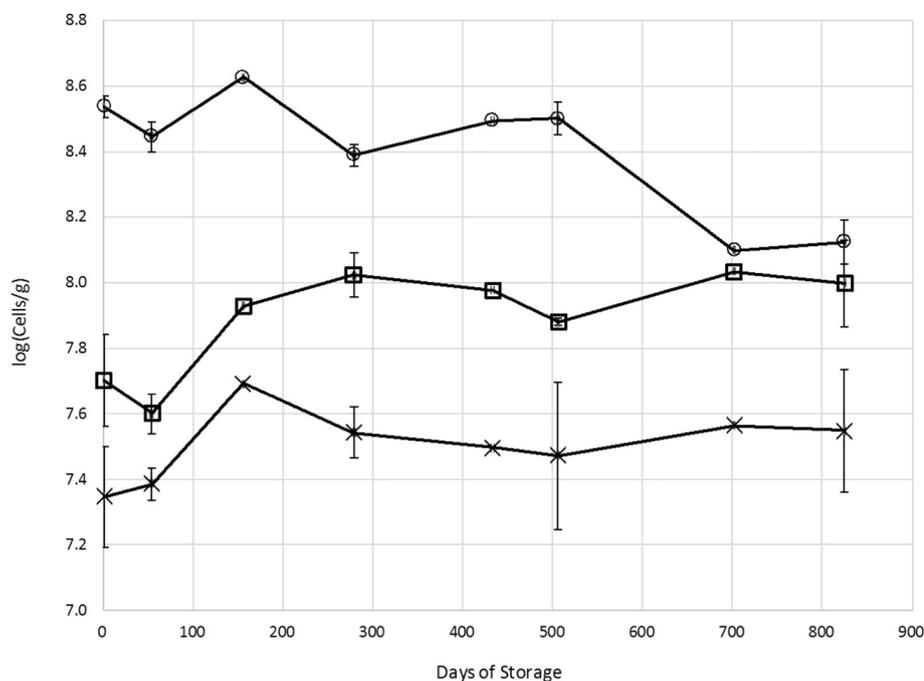


Fig. 2. Flow cytometry enumeration of total cell population in DFM1 versus days of storage. Live cells (circles), injured cells (squares) and dead cells (X) were tracked separately. Error bars indicate standard deviation of a geomean when more than one sample was processed from a single storage time (Table 1). Product label claim is 3.1×10^8 total active cells/g [8.49 log(cells/g)].

plating titer had fallen below the product's label claim, while qPCR and FC continued to produce readings which were at or above specification. Conceivably, plating may only accurately reflect an “immediately viable” cell count. The significant drop in recovered LAB activity in DFM1 using the plating method could theoretically be interpreted as a loss of cell viability, an increase in cell clumping, or an increase in the number of VBNC cells. Enumeration of the bacteria in these samples by qPCR did not show as drastic a drop, which could be because qPCR can detect intact DNA from dead cells. However, because FC analysis did not show a corresponding significant increase in injured or dead cell populations over the storage period it seems more likely to reflect a significant fraction of bacteria entering the VBNC state (Xu et al., 1982). Cells in this state maintain low level activity and would thus maintain an intact, functional cell membrane (Ramamurthy et al., 2014) making them

indistinguishable from culturable cells by FC and qPCR.

While following the same lots of DFM1 product over several years could have provided more detailed data, time constraints did not allow this. However, the comparison of different enumeration methods was not limited by analysis of different lots, considering the manufacturing source did not vary. Each enumeration method tested here provided different information regarding bacterial population viability over time. The plating and qPCR assays were more discriminatory in revealing the effects of prolonged storage on individual bacterial species than was FC while FC can distinguish live, injured, and dead cells. Differential plating distinguished *Bacillus* from LAB, and species-specific primers and probes uniquely identified each species in the DFM product. Plating and qPCR revealed the relative stability of the *Bacillus subtilis* related strain compared to the LAB over time, which is likely attributable to the

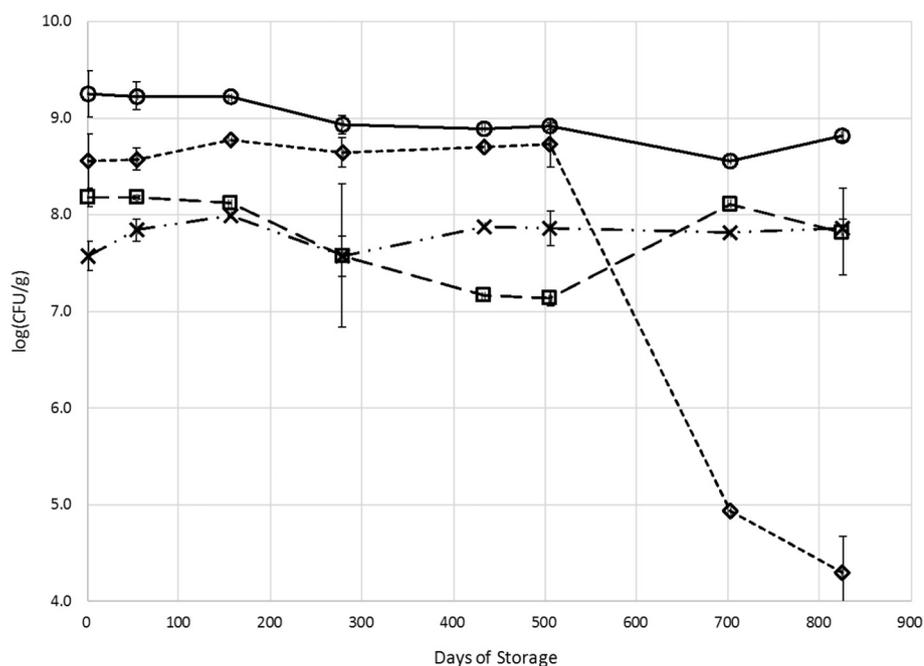


Fig. 3. Log(CFU/g) (via qPCR) for the species LP (circles), PP (diamonds), PA (squares), and BS (X), in DFM1 versus days of storage. Error bars indicate standard deviation of a geomean when more than one sample was processed from a single storage time (Table 1). Detection limit was 10^4 CFU/g. Product label claims are: 10^8 CFU/g for each LAB and 10^7 CFU/g for BS.

well-documented stability of the *Bacillus* endospore.

The DFM1 product tested here was used in animal feeding studies and resulted in improved weight gains and feed utilization independent of product storage time (Barnes et al., 2016) This further supports the hypothesis that CFU counts from traditional plating assays may not accurately reflect product efficacy or quality. It may be advantageous for regulatory agencies to consider alternative enumeration approaches when evaluating the viability of bacterial cells in probiotic formulations to ensure effective products of an appropriate titer are not erroneously denied entry into the market.

Funding

This work was supported by BiOWiSH Technologies, Inc. through research contracts with California State Polytechnic University, San Luis Obispo and Bioform Solutions.

Declaration of interest/funding source declaration

J. VanderKelen, C. Kitts, and D. Buckman are funded through research contracts with BiOWiSH Technologies, Inc. J. Gorsuch and D. LeSaint are BiOWiSH employees. Funds for this research were entirely from BiOWiSH Technologies, Inc.

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