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Novel Method for Enumeration of Viable *Lactobacillus plantarum* WCFS1 Cells after Single-Droplet Drying

Jimmy Perdana,[a] Ludmila Bereschenko,[b,c]* Mark Roghair,[a] Martijn B. Fox,[c] Remko M. Boom,[a] Michiel Kleerebezem,[b,c,d] and Maarten A. I. Schutyser[a]

Food Process Engineering Group, Wageningen UR, Wageningen, The Netherlands[a]; Laboratory of Microbiology, Wageningen UR, Wageningen, The Netherlands[b]; NIZO Food Research, Ede, The Netherlands[c]; and Host-Microbe Interactomics Group, Wageningen UR, Wageningen, The Netherlands[d]

Survival of probiotic bacteria during drying is not trivial. Survival percentages are very specific for each probiotic strain and can be improved by careful selection of drying conditions and proper drying carrier formulation. An experimental approach is presented, comprising a single-droplet drying method and a subsequent novel screening methodology, to assess the microbial viability within single particles. The drying method involves the drying of a single droplet deposited on a flat, hydrophobic surface under well-defined drying conditions and carrier formulations. Semidried or dried particles were subjected to rehydration, fluorescence staining, and live/dead enumeration using fluorescence microscopy. The novel screening methodology provided accurate survival percentages in line with conventional plating enumeration and was evaluated in single-droplet drying experiments with *Lactobacillus plantarum* WCFS1 as a model probiotic strain. Parameters such as bulk air temperatures and the carrier matrices (glucose, trehalose, and maltodextrin DE 6) were varied. Following the experimental approach, the influence on the viability as a function of the drying history could be monitored. Finally, the applicability of the novel viability assessment was demonstrated for samples obtained from drying experiments at a larger scale.

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Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (11). Health benefits are usually related to the influence of probiotic bacteria on the microbial balance in the hosts' intestine or via modulation of the gut-associated immune system (6, 12, 19, 21, 25). Probiotics are delivered to the gastrointestinal tract as food products or dietary supplements and supplied on the market as fermented food commodities, freeze-dried cultures, or frozen cultures, which enhance their stability and facilitate their implementation in appropriate product formulations (1, 16, 30). Compared to freeze drying and freezing, spray drying could be an interesting alternative for providing shelf life to probiotic ingredients. Spray drying is more energy and cost efficient and can be operated continuously at higher production capacities (2, 26, 34). The major drawback of spray drying is the limited survival of probiotics (31, 33). Several studies have successfully explored approaches to increase the survival percentages after spray drying (8, 10, 13, 23). However, most of the results so far are very specific and difficult to translate between different species. It was, for example, found that a high variability exists between different strains of the same species (14, 24). In addition, the process conditions applied during spray drying (2, 28) and the protective carrier materials added (10, 31) have strong influence on the final viability. Since these parameters need to be optimized for each specific case (different process parameters, different strains or species, and different carrier formulations used during drying), many cost-, time-, and labor-intensive experiments are required.

Here we present an experimental approach to assess probiotic survival during drying, starting with the drying of small single droplets deposited on a flat hydrophobic surface (29), followed by cell rehydration on Anopore chips (17), and subsequent evaluation of the microbial viability by fluorescence microscopy.

A recently developed single-droplet drying method is used to produce powder particles dried under well-defined conditions to investigate the influence of drying process conditions and carrier formulations (29). Specifically, drying parameters, such as droplet size, temperature, relative humidity, and flow rates of the air, can be varied effectively and systematically over wide ranges.

The availability of a rapid and reliable live/dead assay is critical to the assessment of the viability after drying. The assay developed here employs a microporous aluminum oxide chip (Anopore) (17, 18). Following rehydration of the droplets, fluorescence probes are used for live/dead enumeration using fluorescence microscopy. This assay is compatible with medium- to high-throughput techniques, especially compared to more conventional enumeration by plating. Moreover, the proposed method allows direct visualization of live and dead populations after drying without the requirement for growth. It has the potential to measure the viability in small sample volumes, i.e., the viability of bacteria present in a single powder particle. Subsequently, the method is applied just as well to assess the microbial viability in the particles obtained from single-droplet drying experiments and from stabilization experiments at a larger scale. In our experiments, *Lactobacillus plantarum* WCFS1 was selected as the model bacterium. This selection is based on the fact that this *Lactobacillus* strain has been the topic of extensive studies before (20), without *per se L. plantarum* being a probiotic bacterium.

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Address correspondence to Maarten A. I. Schutyser, maarten.schutyser@wur.nl.
* Present address: Ludmila Bereschenko, KWR Watercycle Research Institute, Nieuwegein, The Netherlands.
J.P. and L.B. contributed equally to this article.
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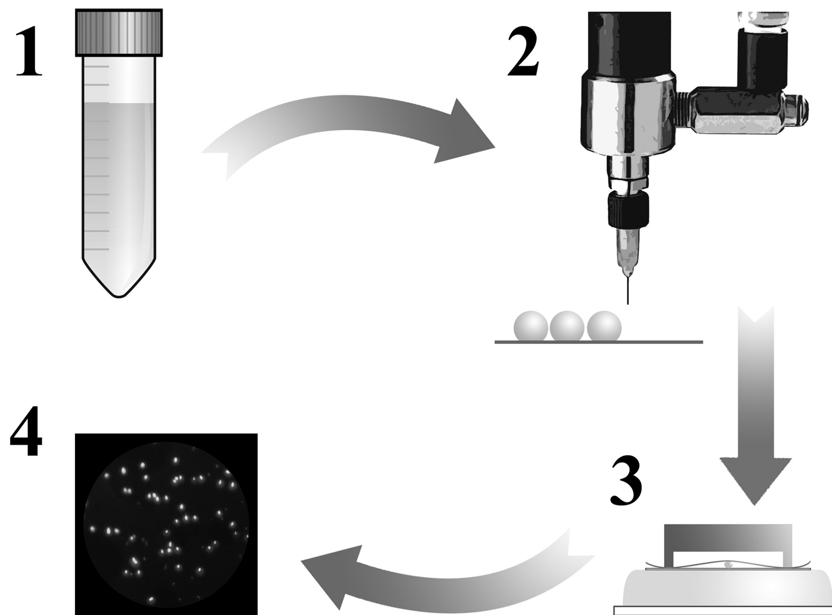

FIG 1 Schematic overview of the experimental procedure, including microbial culture preparation (1), drying of single droplets (2), particle rehydration and fluorescence probe staining (3), and fluorescence microscopy analysis and automated relative viable cell enumeration (4).

MATERIALS AND METHODS

The entire experimental procedure includes the following steps: (i) microbial culture preparation, (ii) drying of single droplets, (iii) particle rehydration and fluorescence probe staining, and (iv) fluorescence microscopy analysis and automated relative viable cell enumeration (Fig. 1).

Culture preparation and washing. A culture with high viability (>99%) was acquired by growing *L. plantarum* WCFS1 in 10 ml sterilized lactobacillus culture medium (MRS; BD Difco) at 30°C for 16 h. The final cell density approximated 109 CFU per ml.

The overnight culture was centrifuged using Eppendorf Centrifuge 5804R with an F-34-6-38 rotor at 13.5 × *g* at 4°C for 10 min. The resulting pellet was washed twice with phosphate-buffered saline (PBS) solution (BD Difco). To minimize washing stress, the pH of the PBS solution was adjusted to a pH of 4, which is similar to the pH of the culture at the end of growth.

Single-droplet drying experiments. Directly before the drying experiment was initiated, the washed bacterial cells were suspended in a carrier matrix, consisting of 20% (wt/wt) maltodextrin DE 6 (Glucidex 6, Roquette, France), trehalose (Sigma-Aldrich, Germany), or glucose (Sigma-Aldrich, Germany). These carbohydrates are known to provide protection to bacterial cells (4, 7).

The drying experiments were performed using the same equipment as described by Perdana et al. (27). The droplets were generated using a pneumatic dispenser of the Microdot 741 MD-SS series (Engineered Fluid Dispensing; Nordson). The droplet deposition on the flat surface (5 by 20 by 0.2 mm) was automated, using an XYZ positioning platform Ultra 525 TT automation series (Engineered Fluid Dispensing; Nordson). The dispensing needle AKA740TK precision tips (Engineered Fluid Dispensing; Nordson) were coated with DOW Corning 340 heat sink compound (Dow Chemical) to prevent the droplets from creeping up along the outside of the needle.

Prior to the deposition of the droplets, the microdispensing system was flushed with sterilized MilliQ water (Millipore), followed by 70% (vol/vol) ethanol solution (VWR International, France), and then rinsed again with MilliQ water. The droplets were deposited on a flat hydrophobic membrane, Accurel type PP 2E HF (Akzo Nobel Faser AG, The Netherlands). To prevent microbial contamination, the membrane was sterilized with 70% (vol/vol) ethanol solution and dried under aseptic conditions at room temperature.

The droplets were dried using preconditioned, filtered (dust/microbes and oil-free) drying air, which was heated to the desired temperature by leading it through a coil that was submerged in an oil bath (Julabo EH-5, Germany). The heated air was then fed to an insulated tunnel which acted as the drying chamber. The air temperature in the tunnel was monitored using a thermocouple type K (NiCr-NiAl) (RS Component, United Kingdom), with a probe diameter of 500 μm. The airflow velocity was monitored (type 1355; Brooks Instruments, The Netherlands). The tunnel was filled with a highly porous medium to develop airflow with a uniform velocity. Meanwhile, the temperature was maintained by insulating the tunnel with heating oil. After the deposition of the droplets, the samples were placed in the drying chamber and dried using various drying times and regimes. The setup was equipped with a μEye 1480ME charge-coupled-device (CCD) camera with a lens magnification of ×9 (Imaging Development Systems GMBH, Germany) to monitor the droplet geometry evolution during drying.

The single-droplet drying experiments were performed using dry air (rH = 0.0%), preheated to a temperature between 25 and 70°C and a bulk air velocity of 0.12 to 0.52 m/s. For each experiment, three identical droplets were dispensed and dried simultaneously. Biological duplicates were performed by repeating the experiments twice.

Rehydration and staining chip. The substrate for cell rehydration and staining was prepared by applying an Anopore chip (17) on a low-melting point (LMP) agarose gel (Sigma, The Netherlands). The gel was prepared by dissolving 1 g of agarose in 100 ml of MilliQ water (Millipore) and autoclaved (121°C) for 20 min. The agarose solution was allowed to cool; after the temperature reached approximately 40°C, 2 μl of a fluorescence staining probe, Live/Dead BacLight bacterial viability kit (Invitrogen), was added to 10 ml of the agarose solution. The agarose solution was then spread on microscope slides (76 by 26 mm) and allowed to solidify for 30 min in a dark environment. Afterwards, a sterilized Anopore chip (8 by 35.6 mm; thickness, 60 μm; pore size, 0.2 μm; 3×10^9 pores per cm^2) (Microdish BV, The Netherlands) was carefully placed on the agarose gel. The Anopore chips were sterilized by submerging them into 70% (vol/vol) ethanol solution for 2 h in Falcon tubes. Subsequently, ethanol was decanted, the chips were dried in a sterile flow chamber for 12 h, and then the tubes were tightly closed for storage. The Anopore chips were positioned on the agarose gel for at least 30 min prior to the rehydration of the dried particles. All preparations were carried out under aseptic conditions.

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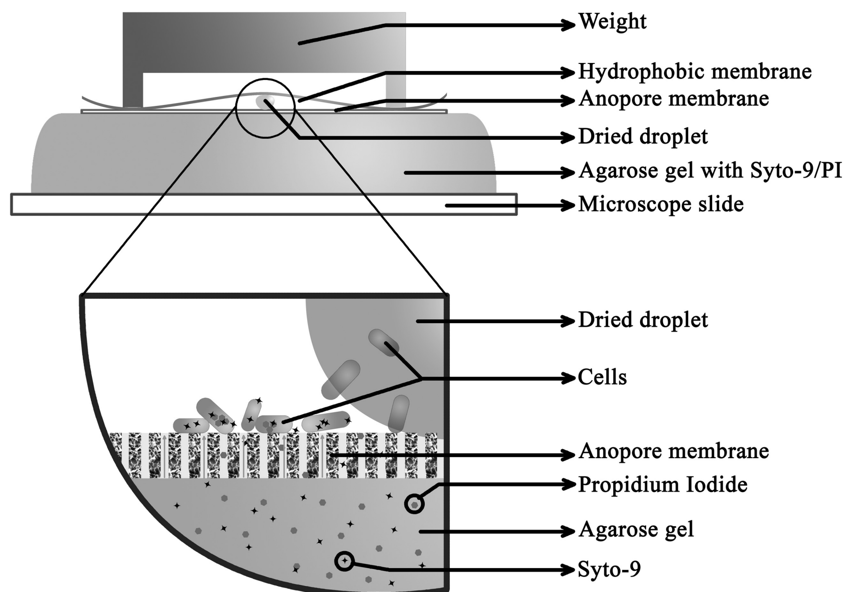

FIG 2 Schematic drawing of the rehydration and staining chip.

Cell rehydration and enumeration. After drying, the powder particles were directly transferred to the rehydration and fluorescence staining substrate. The powder particles were transferred to the Anopore chip by turning the hydrophobic membrane upside down on the Anopore chip (Fig. 2). A small weight providing 1.25 g/cm^2 pressure was put on top of the membrane to ensure contact between the membrane and the Anopore surface for the entire membrane surface. Therefore, rehydration could be considered universal and complete. After rehydration, the weight and hydrophobic membrane were removed carefully from the Anopore chip.

The rehydrated samples on the Anopore chip were analyzed using a fluorescence microscope, Axioskop 40FL (Carl Zeiss, Germany). Fluorescence images were captured with a magnification of ×630 using an Olympus XC30 camera (Olympus, Japan) and Cell^B imaging software (Olympus, Japan). Due to decrease of fluorescence signal of the sample in time because of bleaching, the sample was not exposed to the light source for more than 5 min.

The BacLight bacterial viability kit (Invitrogen) is composed of two nucleic acid-binding stains: Syto 9 and propidium iodide. Syto 9 can be internalized in cells depending on their membrane integrity and membrane potential, resulting in green fluorescent staining of cells that are alive. In contrast, propidium iodide can passively penetrate cells that have damaged membranes and therefore can be considered to be dead. The presence of propidium iodide within the cell can override the green signal of Syto 9, resulting in red fluorescent cells (5). The total viability is therefore calculated by differential enumeration of green and red cells. This method can be automated using an image processing routine as shown in Fig. 3.

An in-house coded routine in Matlab 7.10.0 was developed to perform automated image processing and extract live/dead enumerations. First, the red and green channels of the resulting image from microscopy analysis were selectively split. Subsequently, the routine counted the number of cells for red and green channels separately (Fig. 3). The package was able to evaluate multiple images at once, and the resulting live and dead enumerations were automatically summarized.

Cell enumeration through plating. The results of the rehydration and enumeration method developed here were compared to the conventional enumeration of viable cells using plating on MRS agar. For this method, powder particles were suspended and dissolved in a 1-ml PBS solution for 30 min and subsequently diluted further to an appropriate dilution (10^{-1} and 10^{-2}). Subsequently, CFU enumeration was performed in duplicate by plating 50 μl of the suspension on MRS agar plates using an Eddy Jet 2 spiral plater (IUL Instruments, Spain). The plates were incubated at 30°C for 48 h, following the colony counting of dilutions having between 30 and 300 colonies. The viability fraction was determined by dividing the number of the colony for each treatment by the colony of the untreated sample, assuming 100% viability in the untreated sample.

Spray-drying experiments. Spray-drying experiments were carried out in a Buchi B-190 spray dryer (Buchi Labortechnik AG, Switzerland). The outlet temperatures of the spray dryer were set between 50 and 90°C, and the inlet temperatures were set 30°C higher than the applied outlet temperature. The resulting powder was further dried using contact air drying (ambient temperature, rH = 0%) for 24 h and was analyzed for viability before and after drying. The viability analyses were done by spreading a small amount of the powder (approximately 1 mg) on the rehydration and fluorescence staining substrate. Subsequently, a similar treatment was applied to the droplets dried using the single-droplet drying method.

Moisture content analysis. The moisture content of the samples was analyzed using the Sartorius MA 30 (Sartorius Mechatronic, Germany) gravimetric analyzer. Approximately 1 g of the sample was spread on an aluminum pan and put in the moisture content analyzer, which heated the sample up to 130°C for approximately 5 min.

RESULTS AND DISCUSSION

Method development: optimum cell density for microscopic evaluation. The microbial enumeration is affected by too-high or too-low cell densities. High cell densities may induce flocking or lead to visual overlap of cells. Conversely, low cell densities may not provide sufficient numbers of cells for statistically significant enumeration. Therefore, an optimum cell density for the enumeration needs to be established. The current method allowed for determination of viability percentages for maximally ~500 cells per image (2,080 by 1,544 pixels). For higher cell densities, the enumeration routine may not provide accurate results due to the frequent overlapping fluorescence signals from different individual cells. Additionally, the viability fraction was evaluated as a function of the number of counted cells within an image, showing

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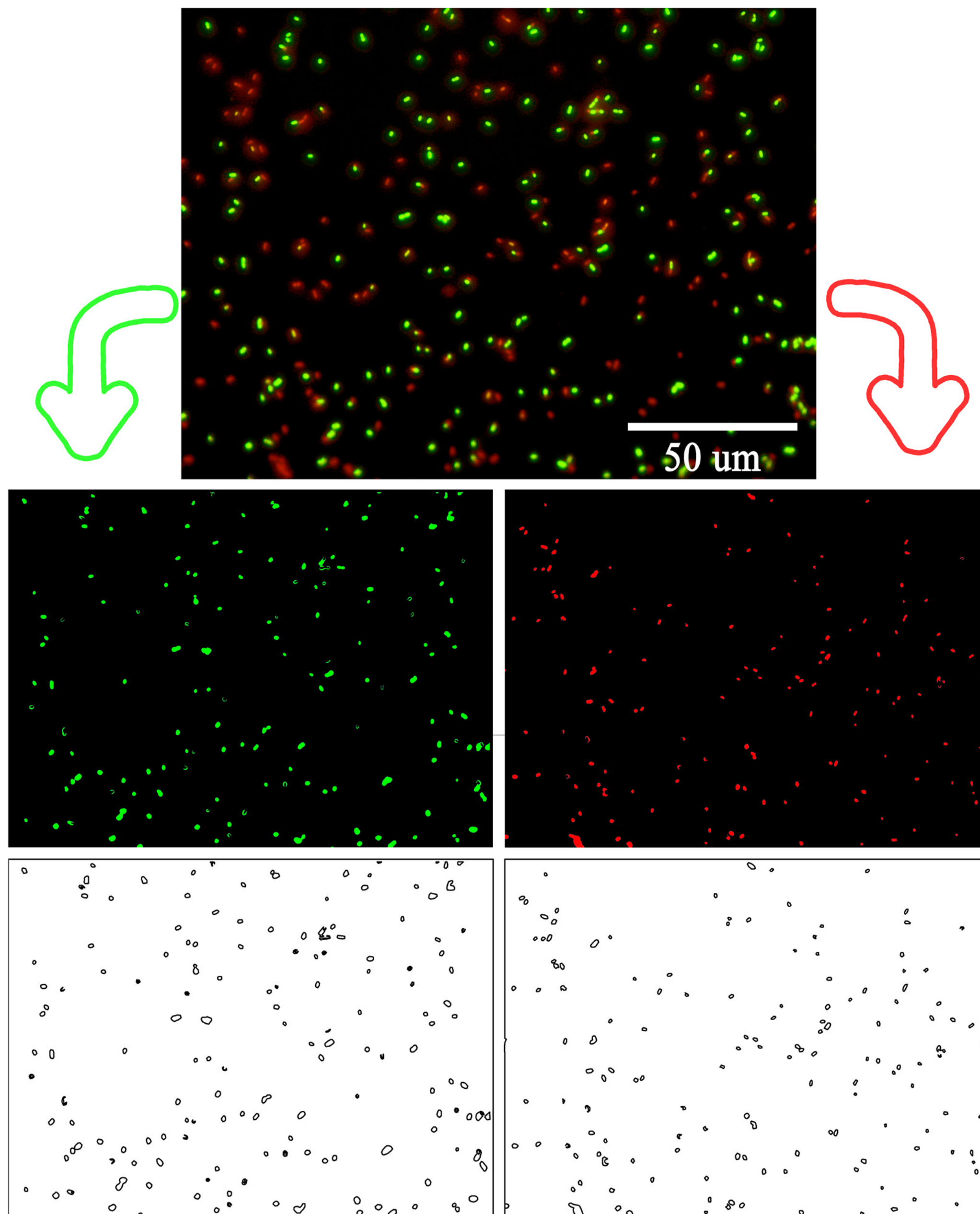

FIG 3 Live/dead counting. (Top) Original fluorescence microscopy image of *L. plantarum* WCFS1 cells rehydrated on an Anopore chip observed with ×630 magnification. (Middle) Red and green channels are separated. (Bottom) Cell counting to determine the number of live (left) and dead (right) cells.

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a stable viability fraction between 200 and 500 cell densities per image.

Rehydration time. The rehydration time is defined as the time between the transfer of the dried powder particle from the hydrophobic membrane to the Anopore chip and the subsequent removal of the hydrophobic membrane. The rehydration time is critical, since too-short rehydration times may lead to the transfer of only a small percentage of the cells to the Anopore surface.

We found that more than 90% of the cells were transferred to the Anopore chip using rehydration times between 15 and 90 min. Additionally, the fluorescent dyes could be detected immediately following the transfer. During further experiments, 15 min of rehydration time was used for the transfer of cells.

Time period between rehydration and microscopic analysis. For practical purposes, it was evaluated whether a delay time between rehydration and fluorescence microscopy would be of influence on the final viability assessment. The major advantage of this delay time is that it facilitates the combination of the results from multiple drying experiments with a single subsequent viability assessment run. The delay time was evaluated for a time period between 0 and 3 h. The delay time did not have a significant impact on the viability assessment outcome (Fig. 4C).

However, the microscopic images obtained directly after re-

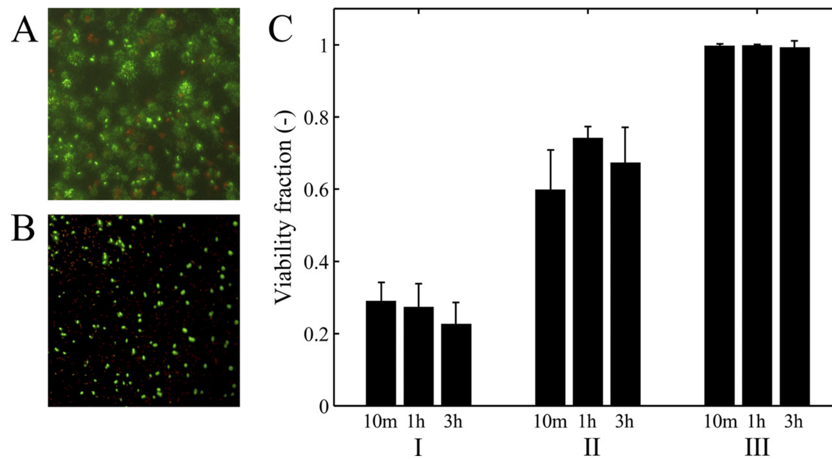

FIG 4 (A) Image of a rehydrated particle observed under the microscope after 0 min of delay time. (B) Image of a rehydrated particle observed under the microscope after 10 min of delay time. (C) Residual microbial viability of particles dried under three different circumstances and varying delay times of 10 min, 1 h, and 3 h. The error bars represent the standard deviations.

moval of the hydrophobic membrane were substantially less sharp than the images obtained 10 min after membrane removal (Fig. 4A and B). This might be explained by the presence of a thin water layer on the surface of the Anopore chip after the removal of the hydrophobic membrane, which disappears upon exposure to air, either by evaporation or reabsorption into the agarose gel. The presence of a water layer may negatively affect the sharpness of the image due to the focus depth of the microscope. Therefore, a minimum delay time of 10 min was used between membrane removal and microscopy analysis, while the maximum delay time was fixed at 3 h.

Final evaluation of the viability assessment method. The enumeration of live and dead cells was carried out with a Matlab routine. These results were compared to manual enumeration. Eighty images were selected randomly; the viability fraction for every image was determined manually and compared to the result from the Matlab enumeration routine. The two methods generated similar counts (Fig. 5A), which validates the automated enumeration routine. The total number of counted cells should be such that an accurate value of the viability fraction is obtained. A total number of 200 cells was found sufficient to obtain an accurate value. From this and previous results, it can be concluded that the ideal cell density lies between 200 and 500 per image.

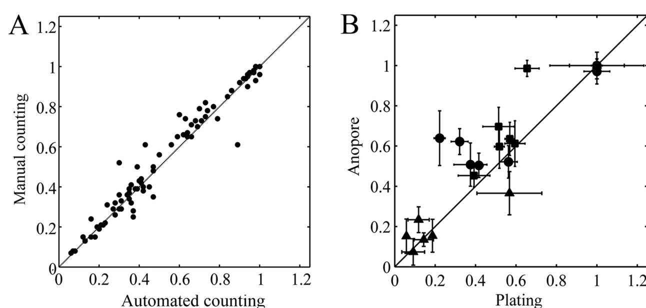

FIG 5 (A) Parity plot of the viability fractions obtained via the Matlab enumeration routine (*x* axis) and the manual enumeration method (*y* axis). (B) Parity plot of the viability fraction of *L. plantarum* WCFS1 assessed by conventional plating (*x* axis) and the new enumeration method (*y* axis). The samples were obtained by drying *L. plantarum* WCFS1 in 20% maltodextrin for 10 min with hot dry air temperatures of 25°C (●), 50°C (■), and 70°C (▲) and with a droplet size of 600 μm and an air velocity of 0.52 m/s.

Finally, the fluorescence microscopy-based viability enumeration was compared to results obtained via conventional plating. The two enumeration methods generate similar viability counts (Fig. 5B), which validates the fluorescence microscopy method.

Application of the viability assessment method. (i) Inactivation during single-droplet drying. The novel and validated assessment method was subsequently applied to evaluate the effect of the drying history on the viability loss of *L. plantarum* WCFS1. A single-droplet drying method was used to generate droplets with the bacteria suspended in different carbohydrates (20% [wt/wt]).

The residual viability decreased with drying time at different bulk air temperatures (Fig. 6A). A higher bulk air temperature led to a significantly increased loss in viability. Almost all loss in viability occurs in the first minutes of the process ($t < 5$ min), after which the residual viability remains more or less constant. This loss in viability corresponds to a period of rapid drying, while the subsequent period with constant viability shows little or no further decrease in moisture content. The small decrease in residual viability during the latter period can be explained by the immobilization of the bacteria in a rigid matrix, which slows down the inactivation process (4).

Subsequently, the influence of the carrier matrix on survival was examined. Low-molecular-weight carbohydrates (glucose and trehalose) provided better protection to the cells during drying than maltodextrin DE 6 (Fig. 6B).

Two hypothetical mechanisms exist on how sugar molecules protect the cell membrane from leakage by minimizing the chance of membrane phase transition (9). The first mechanism is proposed to act via water replacement. In fully hydrated conditions, the cell membrane lipids are in undisturbed liquid-crystalline form. According to this hypothesis, sugar molecules replace water in the hydration shell of the cell membrane, thereby maintaining the spacing between the phospholipid molecules (15). In this case, lower-molecular-weight sugars, e.g., glucose and trehalose, can provide better protection because of their better ability to enter the spacing between phospholipid molecules compared to higher-

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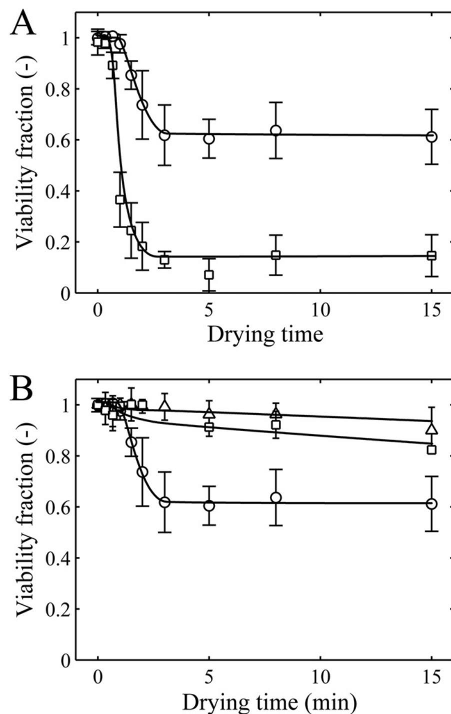

FIG 6 (A) Viability fractions after drying with air temperatures of 25 (○) and 70°C (□) and an air velocity of 0.52 m/s. (B) Viability fractions after drying with different carrier formulations of glucose (△), trehalose (□), and maltodextrin DE 6 (○) dried with an air temperature of 25°C and an air velocity of 0.52 m/s. Initial concentrations of the formulations were 20% (wt/wt). The average droplet size was 600 μm. The error bars represent the standard deviations, and the solid lines are guidelines.

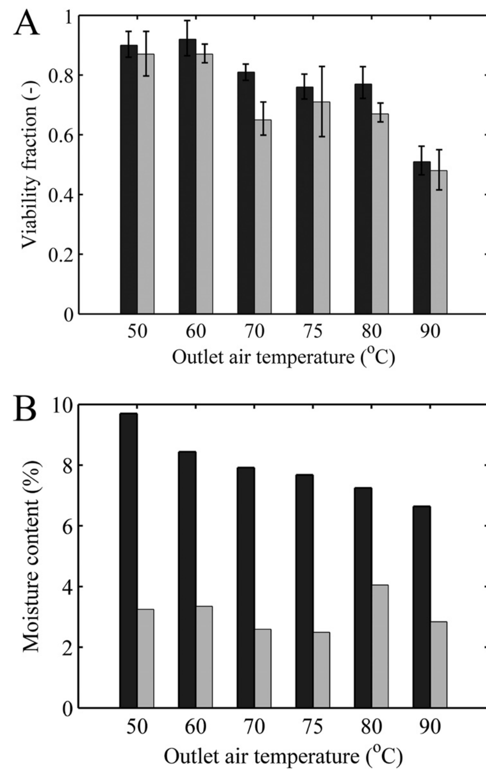

FIG 7 (A) The viability fraction of *L. plantarum* WCFS1 after spray drying in 20% (wt/wt) maltodextrin DE 6 at various outlet air temperatures (black bars). Subsequently, samples were dried for 24 h in a desiccator at ambient temperature and viability was assessed again (gray bars). The inlet temperatures of the spray-drying air were fixed 30°C above the outlet temperatures. (B) The corresponding moisture contents of the powder after spray drying (black bars) and after subsequent drying for 24 h (gray bars).

molecular-weight molecules or polymers, in this case maltodextrin (22). The second mechanism is proposed to act via the hydration force (vitrification hypothesis) (32). During drying, the presence of sugar molecules hinders water removal from the surface of the cell membrane due to the high osmotic pressure. At the same mass concentration, lower-molecular-weight molecules are able to provide a higher osmotic pressure and thus provide more protection than larger molecules or polymers. In the glassy state, the rigidity and the mechanical properties of the sugar matrix limit the possibility for the cell membrane to collapse. Therefore, the protective ability of different sugars can be related to their glassy forming ability, where higher glass transition temperature of the sugar is found to contribute to increased protection. Based on this, trehalose could be expected to provide more protection than glucose due to its higher glass transition temperature. However, the viability results do not confirm this effect (Fig. 6B), although the differences in (high) viability were not significant.

(ii) Inactivation in a laboratory-scale spray dryer. Since the viability assessment method allows more rapid detection compared to conventional plating, it was applied to check the viability loss of *L. plantarum* WCFS1 during spray drying at a larger scale than single droplets. The method selected was spray drying with a laboratory-scale spray dryer at various inlet-outlet temperature combinations, followed by (ambient temperature) air drying.

L. plantarum WCFS1 was found to be susceptible to inactivation even with a mild spray drying process. Although drying in a laboratory-scale spray dryer was very fast (less than 1 s residence time), more than 10% reduction in viability was observed, which increased considerably with applied air temperatures (Fig. 7). Subsequent additional drying for 24 h at ambient temperature reduced the residual viability only slightly, which again can be related to vitrification due to glassification (3, 32).

Outlook on the application of the new viability enumeration method. The enumeration method developed here is generic, as the stains applied in the method are widely applied to detect viability of microorganisms. Furthermore, powder particles formulated with different drying or stabilization methods at different scales or the impact of storage conditions on microbial viability can be assessed using this new method.

The proposed enumeration method is complementary to the traditional plating method. Traditional plating provides information on cell numbers of living bacteria on a $\log^{10}$ scale, whereas the new method provides a live-dead ratio, i.e., 1 to 100% viability. The new method can distinguish, for example, between 20% and 60% viability, while traditional plating is able to distinguish between living cell concentrations, for example, 10^7 and 10^5 CFU/ml. For optimization of microbial viability during drying processes, the proposed enumeration method may be preferred over traditional plating, as it is most sensitive and accurate near the maximum percentage of living cells. A possible drawback of the new method is that it is less sensitive at small living cell numbers, e.g., less than 1% viability.

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Conclusion. A novel method was developed to determine the microbial viability of probiotics in small samples, such as single powder particles. The method employs Anopore carrier chips for rehydration of semidried particles followed by live/dead staining using fluorescence probe methodology. The enumeration was carried out by microscopy and automated image analysis using a Matlab routine. The robustness of the method was evaluated and validated with conventional plating. In combination with a single-droplet drying approach, the influence of the drying air temperature and carrier formulation on viability loss of *L. plantarum* WCFS1 was mapped as a function of drying time. The latter approach can also be of major support in determining optimal drying conditions and formulations for spray drying of probiotics.

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REFERENCES

1. **Anal AK, Singh H.** 2007. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci. Technol. **18**:240–251.
2. **Ananta E.** 2005. Impact of environmental factors on viability and stability and high pressure pretreatment on stress tolerance of *Lactobacillus rhamnosus* GG (ATCC 53103) during spray drying. Technischen Universität Berlin, Berlin, Germany. http://opus.kobv.de/tuberlin/volltexte/2005/1119/.
3. **Ananta E, Volkert M, Knorr D.** 2005. Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. Int. Dairy J. **15**:399–409.
4. **Augustin MA, Hemar Y.** 2009. Nano- and micro-structured assemblies for encapsulation of food ingredients. Chem. Soc. Rev. **38**:902–912.
5. **Boulos L, Prévost M, Barbeau B, Coallier J, Desjardins R.** 1999. Live/Dead BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods **37**:77–86.
6. **Bron PA, van Baarlen P, Kleerebezem M.** 2012. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat. Rev. Microbiol. **10**:66–78.
7. **Chávez BE, Ledeboer AM.** 2007. Drying of probiotics: optimization of formulation and process to enhance storage survival. Dry Technol. **25**: 1193–1201.
8. **Corcoran BM, Ross RP, Fitzgerald GF, Stanton C.** 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. **96**:1024–1039.
9. **Crowe JH, Crowe LM, Hoekstra FA.** 1989. Phase transitions and permeability changes in dry membranes during rehydration. J. Bioenerg. Biomembr. **21**:77–91.
10. **Desmond C, Ross RP, O'Callaghan E, Fitzgerald G, Stanton C.** 2002. Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. J. Appl. Microbiol. **93**:1003–1011.
11. **FAO/WHO.** 2002. Report of a joint FAO/WHO expert consultation on guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization, London, Ontario, Canada.
12. **Fuller R.** 1991. Probiotics in human medicine. Gut **32**:439–442.
13. **Gardiner GE, et al.** 2000. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Appl. Environ. Microbiol. **66**:2605–2612.
14. **Guilbaud M, Zagorec M, Chaillou S, Champomier-Vergès M-C.** 2012. Intraspecies diversity of *Lactobacillus sakei* response to oxidative stress and variability of strain performance in mixed strains challenges. Food Microbiol. **29**:197–204.
15. **Hoekstra FA, Golovina EA, Buitink J.** 2001. Mechanisms of plant desiccation tolerance. Trends Plant Sci. **6**:431–438.
16. **Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U.** 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. **73**:365S–373S.
17. **Ingham CJ, et al.** 2007. The micro-petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proc. Natl. Acad. Sci. U. S. A. **104**:18217–18222.
18. **Ingham CJ, van den Ende M, Pijnenburg D, Wever PC, Schneeberger PM.** 2005. Growth and multiplexed analysis of microorganisms on a subdivided, highly porous, inorganic chip manufactured from Anopore. Appl. Environ. Microbiol. **71**:8978–8981.
19. **Jankovic I, Sybesma W, Phothirath P, Ananta E, Mercenier A.** 2010. Application of probiotics in food products—challenges and new approaches. Curr. Opin. Biotechnol. **21**:175–181.
20. **Kleerebezem M, et al.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U. S. A. **100**:1990–1995.
21. **Kleerebezem M, Vaughan EE.** 2009. Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu. Rev. Microbiol. **63**:269–290.
22. **Koster KL, Lei YP, Anderson M, Martin S, Bryant G.** 2000. Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. Biophys. J. **78**:1932–1946.
23. **Mauriello G, Aponte M, Andolfi R, Moschetti G, Villani F.** 1999. Spray-drying of bacteriocin-producing lactic acid bacteria. J. Food Prot. **62**:773–777.
24. **Molenaar D, et al.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187**:6119–6127.
25. **Parvez S, Malik KA, Ah Kang S, Kim HY.** 2006. Probiotics and their fermented food products are beneficial for health. J. Appl. Microbiol. **100**: 1171–1185.
26. **Peighambardoust SH, Tafti AG, Hejazi MA, Hesari J.** 2011. Application of spray drying for preservation of lactic acid starter cultures: a review. Trends Food Sci. Technol. **22**:215–224.
27. **Perdana J, Fox M, Schutyser M, Boom R.** Mimicking spray drying by drying of single droplets deposited on a flat surface. Food Bioprocess Technol. doi:10.1007/s11947-011-0767-4.
28. **Perdana J, Fox MB, Schutyser MAI, Boom RM.** 2012. Enzyme inactivation kinetics: coupled effects of temperature and moisture content. Food Chem. **133**:116–123.
29. **Perdana J, Fox MB, Schutyser MAI, Boom RM.** 2011. Single-droplet experimentation on spray drying: evaporation of a sessile droplet. Chem. Eng. Technol. **34**:1151–1158.
30. **Ranadheera RDCS, Baines SK, Adams MC.** 2010. Importance of food in probiotic efficacy. Food Res. Int. **43**:1–7.
31. **Rokka S, Rantamäki P.** 2010. Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. Eur. Food Res. Technol. **231**:1–12.
32. **Santivarangkna C, Higl B, Foerst P.** 2008. Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. Food Microbiol. **25**:429–441.
33. **Santivarangkna C, Kulozik U, Foerst P.** 2008. Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. J. Appl. Microbiol. **105**:1–13.
34. **Schutyser MAI, Perdana J, Boom RM.** 2012. Single droplet drying for optimal spray drying of enzymes and probiotics. Trends Food Sci. Technol. [Epub ahead of print.] http://dx.doi.org/10.1016/j.tifs.2012.05.006.

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