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Emerging technologies for viability enumeration of live microorganisms

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Emerging technologies for viability enumeration of live microorganisms

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Editorial: Emerging technologies for viability enumeration of live microorganisms

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Editorial on the Research Topic
Emerging technologies for viability enumeration of live microorganisms

Introduction

The live microorganism industry is rapidly growing, producing probiotics and live biotherapeutic products (LBPs) designed to deliver health benefits. Ensuring these products contain viable, strain-specific microorganisms at effective levels is essential, but accurately measuring viability and potency remains challenging.

Colony-forming unit (CFU) enumeration, the traditional gold standard, relies on a cell's ability to form colonies on culture media. While widely used, it has significant limitations. CFU methods fail to account for viable but non-culturable (VBNC) cells, which maintain metabolic activity but cannot grow on culture media. Moreover, CFU enumeration often falls short for probiotic blends, as strains with varying growth requirements or interactions may not form colonies under standardized conditions.

With growing consumer awareness and stricter regulatory demands, more accurate and comprehensive enumeration techniques are needed. Emerging methods such as flow cytometry, real-time PCR, digital PCR, and advanced imaging assess viability based on cellular activity rather than replication alone. These approaches offer reliable assessments of complex probiotic formulations, ensuring higher product quality and efficacy.

Adopting advanced techniques is critical to meet regulatory standards, enhance product reliability, and build consumer trust, marking a significant step forward in ensuring the health benefits of live microorganism products.

"*Emerging Technologies for Viability Enumeration of Live Microorganisms*," focuses on advanced techniques and how researchers have adapted them to fit their needs. The Research Topic includes two reviews, five reports detailing successful development and use of real-time PCR (qPCR) assays for probiotics, two articles highlighting the adaptability of flow cytometry, one extending understanding of microbial activity and viability using isothermal microcalorimetry, and one utilizing Cell Counting Kit-8.

Reviews

Boyte et al. reviewed available enumeration methods for probiotics and postbiotics, including plate counting (culture dependent) and alternative, culture-independent methods: flow cytometry, real-time PCR (qPCR), and digital PCR (dPCR). Advantages, limitations, viability determination, and the potential of each technique for use in the probiotics industry, including newer categories such as next generation probiotics and tyndallized/heat-killed bacteria were discussed.

Noting that maintaining cell viability is essential to the therapeutic functionalities of probiotic foods, Sibanda et al. reviewed viability challenges encountered from manufacturing through consumption of fermented dairy foods. The authors emphasized the critical nature of viability enumeration for quality assurance and discussed flow cytometry, propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR), next-generation sequencing, and single-cell Raman spectroscopy (SCRS) approaches to reduce quality assurance challenges.

Polymerase chain reaction

Several PCR based methods were developed for the enumeration of probiotic targets. Four articles described the development of eight species-specific enumeration methods and one article described the development of one strain-specific enumeration method. Researchers successfully demonstrated that PMA-qPCR could be used for species-specific viability enumeration of well-known probiotics: *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Catone et al.), *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium* spp. (Marole et al.), *Lacticaseibacillus rhamnosus* (Marole et al.; Guo et al.), and *Lacticaseibacillus paracasei* (Guo et al.). All methods showed PMA efficiently inhibited counting dead cells and were highly specific to target species. These methods were applied to various matrices. This demonstrates the flexibility of PCR and makes the collection useful to those working in research and development, quality assurance, and manufacturing.

Shehata et al. developed a strain-specific PMAxx-qPCR method for strain *B. longum* subsp. *longum* UABl-14. High specificity, reaction efficiency, and precision were demonstrated. The method enabled stability monitoring of the target strain in multi-strain finished products during storage, which cannot be achieved using plate count methods.

Flow cytometry

Jordal et al. conducted a ring test for fluorescence flow cytometry (FCC) and a study comparing the ring test results to those of impedance flow cytometry (IFC) to address challenges presented by traditional plate counting methods. It appears to be the first peer-reviewed comparison of FCC and IFC. Both methods evaluate the presence of intact membranes for single cells in solutions. The FCC ring test demonstrated robustness across changes in equipment, procedures, materials, and operators. After a one-time per strain optimization, the IFC method showed good agreement with FCC results. Combined, the ring test and comparison results indicated that these culture-independent flow cytometry methods saved time, were reliable, precise, adaptable to bacterial enumeration, and allowed exploration of viability.

A 2.0–2.5 h flow cytometry and fluorescence *in situ* hybridization (Flow-FISH) protocol specific for Gram-positive bacteria in probiotic products was presented by Snaidr et al.. Individual probiotics and three-species blends were evaluated by Flow-FISH protocol alone or in combination with live/dead (L/D) staining and/or plate counting. Data showed: (1) Flow-FISH and L/D staining outperformed standard plate counting in quantification. (2) Flow-FISH surpassed plate counting and L/D staining in repeatability and uncertainty. (3) Unlike plating and staining, Flow-FISH was capable of species-specific quantification in blended products. (4) Flow-FISH performed linearly and demonstrated robustness between two flow cytometry instruments. The authors suggested their study established the use of Flow-FISH for comprehensive quality control.

Isothermal microcalorimetry

ICM captures changes in heat produced by living organisms (e.g., metabolic processes). Morazzoni et al. contributed a proof-of-concept study featuring the application of IMC to determine viability and growth dynamics and its correlation to the plate counts for *Lacticaseibacillus rhamnosus* and *Limosilactobacillus fermentum*. Experiments established suitability of ICM for viability assessment and enumeration of probiotic products. Relationships between ICM and plate counting were determined via standard curves and linear regression analyses. Method robustness was observed through the maintenance of correlations between time-to-peak (TTP) heat detected in ICM and CFU/mL from plate counting across various culture conditions. Finally, IMC, flow cytometry, and acidification measurement experiments were conducted under diverse conditions to demonstrate how IMC can be used as a complementary approach that extends understanding of microbial activity and viability.

Cell counting Kit-8

Yang et al. (2021) introduced the application of tetrazolium-based colorimetric cell counting kits (CCK-8) to live bacteria. Health and clinical scientists have adopted CCK-8 to enumerate viable probiotics (Chang et al., 2024; Sudan et al., 2022; Xu et al., 2023; Yue et al., 2022). Here, Shang et al. investigated the role of *B. longum* in the prevention and treatment of colorectal cancer (CRC). CCK-8 was used to optimize the concentration of viable *B. longum* cells and time of coculturing with CRC. The optimized conditions were applied to various assays to demonstrate the inhibitory effects of *B. longum*.

Conclusions

This Research Topic highlights the need to improve viability enumeration methods for live microorganisms. The enumeration technologies presented have provided innovative approaches for the enumeration of live microorganisms that

are faster, with higher specificity and precision, and lower uncertainties than plate counting. These are characteristics needed in research, manufacturing, and clinical settings. As innovations are adopted, new insights and understanding generated will drive improvements from product conception to consumer confidence.

Author contributions

HS: Conceptualization, Writing – original draft, Writing – review & editing. MP: Writing – review & editing. EB: Writing – review & editing. BK: Writing – review & editing. CV: Writing – review & editing. JS: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

HS is employed by Purity-IQ Inc. EB is employed by University of Pretoria. BK is employed by United States Pharmacopeia. MP is employed by Probiotical Research S.r.l. CV is employed by Probi AB. JS is employed by Eurofins Microbiology Laboratory, Inc. This Editing Team recused themselves in any instance where a conflict of interest might be construed.

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Collaborative cytometric inter-laboratory ring test for probiotics quantification

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Introduction: Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. From this definition, accurate enumeration of probiotic products is a necessity. Nonetheless, this definition does not specify the methods for assessing such viability. Colony forming units is the *de facto* gold standard for enumerating viable in probiotic products. The notion of microbial viability has been anchored in the concept of cultivability, which refers to a cell's capacity to replicate and form colonies on agar media. However, there is a growing consensus that the term "viability" should not be exclusively tied to the ability to cultivate cells. For example, bacterial cells can exist in a Viable But Non-Culturable (VBNC) state, characterized by the maintenance of characteristics such as membrane integrity, enzymatic activity, pH gradients, and elevated levels of rRNA, despite losing the ability to form colonies.

Methods: Herein we present the results of a collaborative inter-laboratory ring test for cytometric bacterial quantification. Specifically, membrane integrity fluorescence flow cytometry (FFC) method and the newer impedance flow cytometry (IFC) method have been used. Both methods interrogate single cells in solution for the presence of intact membranes. FFC exploits fluorochromes that reflect the presence or absence of an intact membrane. IFC probes membrane integrity in a label-free approach by detecting membrane-induced hindrances to the propagation of electricity.

Results: A performance ring-test and comparison design on the FFC method showed that the method is robust against the exchange of equipment, procedures, materials, and operators. After initial method optimization with assessments of rehydration medium, wake-up duration, and phase shift gating on the individual strains, the IFC method showed good agreement with the FFC results. Specifically, we tested 6 distinct species of probiotic bacteria (3 *Lactobacillus* and 3 *Bifidobacterium* strains) finding good agreement between FFC and IFC results in terms of total and live cells.

Discussion: Together, these results demonstrate that flow cytometry is a reliable, precise, and user-friendly culture-independent method for bacterial enumeration.

KEYWORDS

probiotics, culture-independent, fluorescence flow cytometry, impedance flow cytometry, electrical impedance spectroscopy flow cytometry (EIS-FC), dormant, viable but non-culturable

Introduction

The concept of probiotics necessitates administering a specific quantity of viable bacteria to the consumer to yield health benefits (Hill, 2014). However, this quantity depends strongly on the type of analysis used. For over 125 years, the colony forming unit (CFU) has been the favored method for microbial enumeration (USP, 2021). In fact, CFUs are regarded as the probiotic industry's analytical quantification gold standard (Weitzel et al., 2021).

Key advantages of the CFU method include its wide acceptance, technical simplicity, and ease of implementation. Yet, its limitations include low throughput, lengthy time-to-result (often exceeding 72 h of incubation), and low precision (Jackson et al., 2019). Using this method for certain bacterium types, like strict anaerobes, can also prove challenging. Furthermore, the CFU method has inherent blind spots. For instance, it requires bacterial proliferation for colony formation. Stressors known to potentially induce viable but non-culturable (VBNC) states in the production of probiotic bacteria are largely ignored in CFU analysis results (Emerson et al., 2017; Fiore et al., 2020; Foglia et al., 2020; Wendel, 2022). Another disadvantage is its inability to detect dead bacteria, making it inapplicable for postbiotic products constituted by inactivated bacteria.

Traditionally, viability has been gauged by cultivability, i.e., the ability to divide and form colonies, a principle originating from Robert Koch's initial landmarks. However, recent suggestions advocate for a broader viability definition to include all metabolically active microbes or those with intact membranes (Breeuwer and abee, 2000). This extended definition permits the use of culture-independent techniques such as flow cytometry, and PCR- or FISH-based methods for viability assessment (Davis, 2014; Wendel, 2022).

Flow cytometry is emerging as a more extensive microbiological characterization method, capable of detecting and quantifying colony-forming, VBNC, and dead bacterial states (Chiron and Tompkins, 2017). It overcomes the CFU method's limitations by offering real-time results and improved precision, detecting up to millions of objects with high sensitivity (Chiron and Tompkins, 2017; Fiore et al., 2020). As such, flow cytometry is gaining popularity as a speedy alternative for profiling microorganisms, including probiotics (Lahtinen et al., 2005; ISO, 2015; Jackson et al., 2019).

Flow cytometry relies on the premise of studying individual cells within a heterogeneous population. In Fluorescence Flow Cytometry (FFC), membrane integrity stains are commonly used to discern the live/dead status of bacteria (Figure 1). Certain dyes, such as propidium iodide (PI), can enter bacteria with compromised membranes, while other membrane-diffusible stains, like SYTO-9, SYTO-24, and thiazole orange, can permeate bacteria independent of membrane status. These fluorophores change their emission properties when bound to bacterial DNA. The concentration of active (or viable) bacteria is calculated by deducting the dead proportion from the total cells (ISO, 2015; Wilkinson, 2018).

While FFC holds clear advantages over CFUs, it also has limitations, such as complex staining procedures, the use of potentially carcinogenic substances, high initial investment costs, and the need for skilled operators (Davey, 2011; Zand et al., 2021). Furthermore, there is a concern that stains, solvents, and penetration enhancers could affect the bacteria's membranes (Chitemerere and Mukanganyama, 2014; Nescerecka et al., 2016; Deng et al., 2020).

Recently, Impedance Flow Cytometry (IFC) or Electrical Impedance Spectroscopy (EIS-FC) has been introduced as a label-free technique for enumerating and characterizing viable bacteria (Clausen et al., 2018; Bertelsen et al., 2020; Modena and Hierlemann, 2021). IFC, an adaptation of the Coulter counter principle, is a multiparametric method to analyze cells in suspension (Coulter, 1953; Modena and Hierlemann, 2021). Briefly, IFC uses a narrow microfluidic channel and electrode sets in contact with the liquid. The passage of an object through the electric field results in a slight impedance change, which is used to obtain information on the object's size, membrane integrity, and intracellular content (Figure 1). By analyzing the change in impedance, one can determine whether the bacterium's membrane is intact or compromised (Sun and Morgan, 2010; Clausen et al., 2018; Bertelsen et al., 2020; Bertelsen, 2021).

In this study, we aimed to use a ring-test design to establish robust FFC-based active and total bacterial concentrations for six probiotic bacteria and subsequently compare these results with the label-free IFC technique. To our knowledge, the present study represents the first peer-reviewed comparison of FFC and IFC for bacteria enumeration.

Materials and methods

Species information

Probiotic bacteria used for the present study were provided by Probiotical and are referenced with their internal identifiers. *Lactobacilllus* species were *Lactiplantibacillus plantarum* (ID 091), *Lactocaseibacillus rhamnosus* (ID 1697), *Lacticaseibacillus casei* (ID 1872), *Bifidobacterium breve* (ID 1747), *Bifidobacterium longum* (ID 1152), and *Bifidobacterium animalis* subsp. *lactis* (ID 1518). In addition, *Lactocaseibacillus rhamnosus* PB01 (DSM 14870) was provided from Deerland probiotics. This strain was not part of the ring test experiments but was included in this publication because it showed the most profound changes in phase shift and amplitude distributions during the wake-up experiments.

Cultivation

To optimize live/dead classification of the given strains, cultures were maintained in an incubator (37°C, 200 RPM) throughout the lag-, exponential-, stationary-, and death phase. Inoculation was done by transferring 1 µL of 1:10 (g/g) stomacher homogenate to a ready-made MRS broth vial (Bio-Rad laboratories inc, cat. no. ##3554488). Tubes were placed horizontally for efficient agitation.

Plate counts (see Supplementary Material) were performed in accordance with existing ISO methods: ISO 29981 IDF 220 for *Bifidobacteria* spp. and 27,205 IDF 149 – ISO 7889 IDF 117 for *Lactobacillus* spp. Briefly, an amount of 4.0–5.0 g of sample was serially diluted in peptone saline water solution. The appropriate dilutions were plated by inclusion technique on TOS-propionate agar medium or De Man, Rogosa and Sharpe agar according to the genera and relative ISO method. Plates were then incubated in anaerobic jar at 37°C and colonies counted after 72 h of incubation.

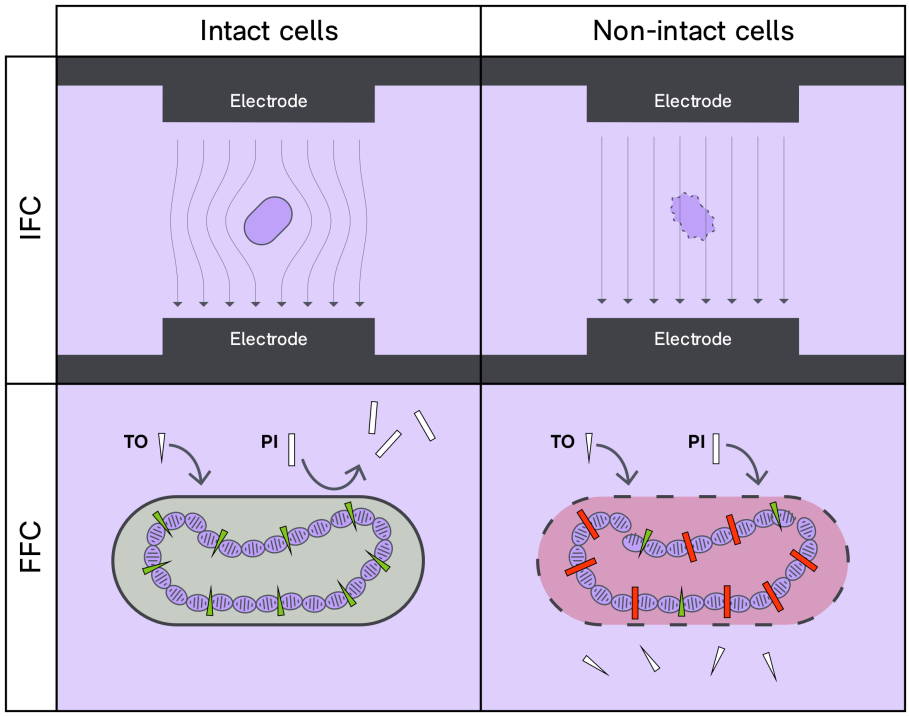

FIGURE 1
Interrogation of membrane intactness status by IFC and FFC techniques. With the IFC technique an intact lipid membrane will impose a pronounced hindrance to electricity that differs from that of non-intact bacteria. For FFC, membrane intactness is probed by differential penetrance to charged DNA-binding fluorophores. Thiazole orange (TO) can penetrate intact cell membranes and upon binding to DNA, TO will fluoresce green when excited at 488 nm. Contrarily, propidium iodide (PI) enters cells and bind to DNA if the membrane is compromised; PI will fluoresce red when excited at 488 nm.

Sample preparation

All freeze-dried samples were stored at −20°C in sealed aluminum sachets until the time of analysis. Samples were reconstituted at 1:10 (g/g) in either buffered peptone water or PBS (for the FFC technique) or MRS broth (for the IFC technique) and subjected to stomacher homogenization (Seward stomacher model 400, 260 RPM, 4 min). For the IFC technique, the sample was re-homogenized after 30 min (stomacher, 260 RPM, 1 min).

Fluorescence flow cytometry

FFC was performed in a ring test design at three different companies: Probiotical QC laboratory conducted experiments with 2 different operators in 2 different QC laboratories using a FACS Calibur instrument (Becton Dickinson). Probiotical R&D laboratory conducted experiments with 2 different operators in 2 different R&D laboratories using a Cytoflex instrument (Beckman Coulter). AAT-Advanced Analytical Technologies conducted experiments in the same laboratory with two different operators using an Attune NxT instrument (Thermo Fisher). The FACS Calibur uses analogue technology with hydrodynamic focusing and reference beads as internal standard to calculate absolute concentrations of bacteria. The Beckman Cytoflex is a digital instrument with hydrodynamic focusing and absolute concentrations based on volumetric counting instead of internal standards. Finally, the Thermo Fisher Attune NxT instrument uses acoustic focusing and reference beads as internal standard to calculate absolute concentrations of bacteria.

The BD Cell Viability Kit with liquid counting beads (BD Biosciences, Cat. no. 349483) was used. Cell staining was performed according to ISO 19344: IDF 232 (2015). Briefly, 100 µL of a diluted suspension containing approximately 10^5–10^6 cells/mL in buffered peptone water was added to 835 µL of PBS. Then 10 µL of PI (prior diluted in water at 0.2 mmol/L) and 5 µL of TO (42 µmol/L) were added to the dilution and the sample was vortexed. Stained sample was incubated for 15 min at 37°C in the dark. For the flow cytometers without volumetric counting, the counting beads suspension was gently vortexed for 30 s and then 50 µL was added to the cell suspension for a final volume of 1 mL. For Cytoflex instruments no counting beads were added because the concentration is based on the defined sample volume taken from the needle. In this case the volume of PBS for the final dilution was 885 µL instead of 835 µL.

For the Attune NxT instrument, cell staining was performed according to the ISO 19344:2015 IDF 232:2015, protocol B. Briefly, cells were diluted in decimal serial dilutions in PBS to obtain about 10^5 cells/mL: 100 µL of this final dilution was added to 880 µL of PBS. Then 10 µL of PI (prior diluted in H_2O at 0.2 mmol/L) and 10 µL of Syto24 (prior diluted in H_2O at 0.1 mmoL/L) were added to the dilution and the sample was vortexed. The stained sample was incubated for 15 min at 37°C in the dark. Before analysis, the counting beads were used as internal control (ThermoFisher Scientific, Cat. No. C36950) was gently vortexed for 30 s and then 50 µL was added to the cell suspension for a final volume of 1,050 µL.

FACSCalibur acquisition settings

The FACScan FACSCalibur cytometer (BD FACSCalibur Software; Becton Dickinson, San Jose, CA) was equipped with 488 nm argon laser excitation and CellQuest software. An SSC-H (Side Scatter) threshold was used for microbial cells. Cells were gated using forward versus side scatter (FSC-H vs. SSC-H). Thiazole Orange (TO) fluoresces primarily in the FL1 channel and Propidium iodide (PI) fluoresces primarily in the FL3 channel. The best discrimination of live and dead populations was on an FL1 versus FL3 plot. To exclude any false positive and negative results, reference control gating was generated on a fresh culture of *L. rhamnosus* GG; the fresh culture was representative of live population while the same culture after isopropanol treatment was used as reference for dead cell population. Live sample was stained only with TO while dead sample was stained with PI.

Attune NxT acquisition settings

The Attune NxT Acoustic Focusing Cytometer (Thermo Fisher) was equipped with 488 nm laser excitation. An SSC-H (Side Scatter) and FSC-H (Forward Scatter) thresholds were used for microbial cells. Cells were gated using forward versus side scatter (FSC-H vs. SSC-H). Syto 24 fluoresces primarily in the BL-1 channel and Propidium iodide (PI) fluoresces primarily in the BL-3 channel. The best discrimination of live and dead populations was on an BL-1 versus BL-3 plot.

Cytoflex acquisition settings

The CytoFLEX cytometer (Beckman Coulter srl) was equipped with 488 nm laser excitation and CytExpert software. An SSC-H (Side Scatter) and FSC-H (Forward Scatter) thresholds were used for microbial cells. Cells were gated using forward versus side scatter (FSC-H vs. SSC-H). The best discrimination of live and dead populations was on an FL1 versus FL3 plot.

Impedance flow cytometry

IFC was done in a single laboratory (SBT Instruments) by a single operator using a BactoBox® HW version 7.4, SW version 2023.04. To be within the linear measurement range, each 1:10 (g/g) stomacher homogenate was diluted by two consecutive DF 201 dilutions in BactoBox diluent (50 µL sample added to 10,000 µL 1:9 PBS). Samples were analyzed immediately after preparation of each dilution series. Replicates were based on fresh dilution series of the stomacher homogenates. At least three replicates with fresh dilution series were prepared for each species.

Microscopy

An LS620 fluorescence microscope (Etaluma) equipped with an Olympus 60× long-working distance objective was used to obtain information on the presence of single-cell suspensions as well as live/dead information based on membrane integrity. Samples were prepared by depositing 2 µL sample on an objective glass and subsequently pressing the droplet flat with a cover slip and the posterior end of a plastic Pasteur pipette. Membrane intactness was evaluated using a combination of phase contrast (total objects), SYBR-green I (for total bacterial, Thermo Fisher cat. No. #S7563) and thiazole red, TO-PRO-3 (for impaired membrane, Biotium cat. No. #40087). Bacteria were stained in the dark in 1× PBS using 1:10,000 dilution of the stock concentration of SYBR-green I and 10 µM TO-PRO-3 for 15 min. Live/dead fluorescence thresholds were adjusted by analyzing 100% live samples and samples killed with 70% denatured alcohol for 15 min.

Statistical analysis

The "Data Analysis" plug-in for excel was used to assess the statistical significance of variance of the mean with the single-factor and Nested ANOVA tool using an alpha value of 0.05. Probability values (*p*-values) below 0.05 were considered statistically significant. Scatter charts were prepared in Graphpad prism.

Results

With membrane integrity as a proxy for bacterial viability we set out to investigate two flow cytometry platforms for enumeration and live/dead characterization of freeze-dried probiotic bacteria. Membrane-integrity FFC is already an established technique for bacteria and the method parameters are well-defined for routine in-house quality as outlined in ISO 19344. Contrarily, for the IFC technique, the present study on freeze-dried probiotics is the first of its type and therefore method optimization was needed prior to determining actual bacterial concentrations and live/dead ratios. Four major learnings were realized for the IFC technique:

i. Concentrations exceeding 500,000 total particles/mL are required to obtain reliable live/dead ratios.
ii. Some probiotic bacteria do not adhere to the default IFC rulesets and require custom gating for accurate live/dead classification.
iii. Nutrient-rich media such as MRS broth are required as rehydration medium before conducting measurements.
iv. A wake-up period of 30–60 min is necessary to rehydrate and obtain normal impedance fingerprints before IFC measurements can be made.

IFC analyses should be performed at concentrations exceeding 500,000 total particles/mL

It is generally recommended to conduct analyses at a particle concentration ranging between 500,000 to 5,000,000 total particles per mL. This recommendation is based on two fundamental reasons.

Firstly, precision in particle detection statistics tends to improve with the detection of a larger number of events, provided that the detector element does not become oversaturated. This means that gathering as many data points as possible helps to ensure that the analysis is accurate and reliable.

Secondly, the operation of the IFC instrument involves a peristaltic pump, whose head rollers can occasionally release

microplastic particles from the peristaltic tubing. At low bacterial concentrations, longer measurement times, such as three minutes, are typically employed. Because the sample is constantly recirculated during this process, the microplastic particles can be detected as non-conductive objects, artificially inflating the concentration of non-bacterial objects. These microplastic objects are typically observed at approximately 1 radian, a range outside that of the dead bacterial phase shift. However, they are still included in total concentrations, which can potentially lead to an underestimation of the live/dead ratio. To avoid this, maintaining a high bacterial concentration is necessary to reduce the proportion of these non-conductive, non-bacterial objects in the sample.

Custom gating is needed for some probiotic bacteria

The IFC method typically employs a default ruleset for live/dead classification, specifically defining the intact cell region with a lower and upper value for the 7 MHz phase shift angle. Our initial analysis using these default parameters yielded an excellent agreement between FFC and IFC for two strains, *L. plantarum* and *B. lactis*, as will be discussed later. However, for the remaining strains, the Intact Cell Concentration (ICC) determined by IFC were frequently about 50% lower than the Active Fluorescent Units (AFU) determined by the FFC method.

Recognizing that the default IFC gating thresholds were suboptimal for these four strains, we sought to customize the phase shift thresholds. To achieve this, we created sample sets composed almost entirely of live bacteria and other sample sets consisting of almost entirely dead bacteria, as illustrated with *L. casei* (Figure 2).

Both live and dead sample sets were derived from a straightforward batch inoculation growth curve experiment conducted in MRS broth. Spectra representing 100% live bacteria were obtained by analyzing the bacteria during the mid- to late-exponential growth stage. For *L. casei*, these "100% live" timepoints occurred between 3.6–9.0 h (Figure 2A). At these points, the bacteria had yet to enter the death stage, making the initial presence of dead cells negligible. Fluorescence microscopy with SYBR Green I (total stain) and TO-PRO-3 (impaired membrane) confirmed the presence of 100% intact bacteria at these timepoints (results not shown).

Similarly, impedance spectra of 100% dead cells were obtained by analyzing the cultures when the bacteria had fully entered the death stage, for *L. casei* this corresponded to 6 and 8 days of incubation, i.e., 144 and 191 h, respectively (Figure 2A). TO-PRO-3 staining clearly indicated that these cultures consisted almost entirely of dead bacteria.

Upon examining the phase shift distributions for the 100% live incubation times, we found a significant proportion of the objects consistently in the non-intact range from 1.60 rad to 1.83 rad. This resulted in an intact:total plateau at 75% (Figure 2D, violet curve). The intersection between the 100% live and 100% dead cultures was found at 1.6 rad. By reducing the upper phase shift threshold to 1.6 rad, the intact:total ratio now approached 100% for the high viability incubation times, and remained close to 0% when the culture had entered the death stage (Figure 2D, green curve).

Similar growth curve experiments were conducted to optimize gating for *L. rhamnosus*, *B. breve*, and *B. longum*. As will be demonstrated later, these refinements significantly improved the correlation between Active Fluorescent Units (AFU) and Intact Cell Concentrations (ICC), moving from a roughly 50% correlation to a near 1:1 agreement.

MRS medium is needed for proper rehydration of *Lactobacillus* species

The ISO FFC method for probiotic bacteria (ISO, 2015) advises the use of phosphate-buffered saline (PBS) as a diluent in the membrane-integrity assay protocol (assay B). In contrast, for the IFC method, it quickly became apparent that MRS broth was necessary, particularly for *Lactobacillus* strains (Figure 3). Upon reconstitution in MRS, over time, a portion of the bacteria demonstrate a noticeable

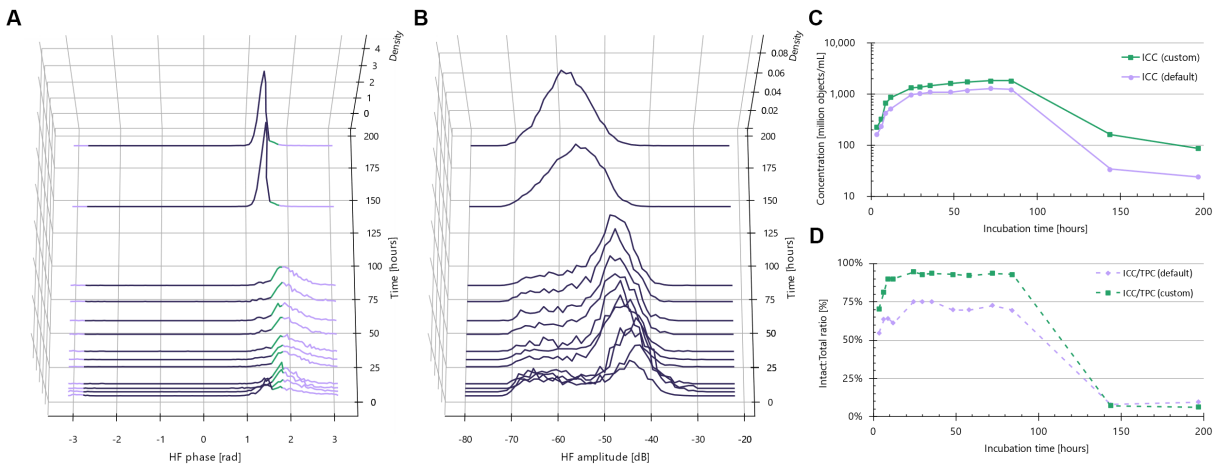

FIGURE 2
Custom gating is needed for *Lacticaseibacillus casei*. **(A)** Normalized phase shift distributions plotted as a function of incubation time. Lavender color indicates default outside phase shift limits from −2.72 to +1.83 rad. Green color indicates lowering of default limits to +1.60 rad and violet color indicates other particles than intact cells, e.g., dead cells. **(B)** Normalized amplitude distributions plotted as a function of incubation time. **(C)** Growth curve for default intact cells (lavender) and custom-gated intact cells (green). **(D)** Ratio between intact and total cells as a function of incubation time.

shift to the right, indicating a trend towards more electrically conductive properties. In other words, the primary non-conductive population at approximately 1.8 rad after 5 min of 'wake-up' time gradually decreases, while the proportion of objects in the lavender region expands (Figure 3A). Concurrently, the high frequency (HF) amplitude rises, suggesting an increase in object size and/or enhanced electrically conductive properties (Figure 3B). This change leads to an increase in intact cell concentrations over time (Figure 3C), likely attributable to the rehydration of freeze-dried, anhydrous cells. Comparatively, the total particle concentration remains relatively stable over time, suggesting no cellular division. Viewed as a proxy for the live/dead ratio, the 'wake-up' period appears to stabilize after approximately 35 min.

When the same rehydration experiment is conducted with buffered peptone water (BPW), the phase shift populations and amplitudes (Figure 4) do not display the same morphological and/or electrical changes observed with rehydration in MRS broth. Instead, the phase shift distributions remain unchanged over time, barring a broadening of the left shoulder at the 66 min mark (Figure 4A). Furthermore, more low-amplitude objects appear at later time points (Figure 4B). Cumulatively, these observations indicate a time-dependent increase in presumable dead cells with low conductivity and smaller amplitudes. The decline in intact cell concentrations over time is evident (Figure 4C), particularly after about 40 min of incubation. This trend is further supported by the decreasing intact-to-total ratio (Figure 4D); rehydration in BPW results in a drop from approximately 25 to 20%, compared to an increase from roughly 25 to 60% when rehydrated in MRS broth. Therefore, MRS not only prevents lysis of freeze-dried *Lactobacillus* species but also allows the cells to kickstart their metabolism.

In summary, for reliable live/dead assessments using the IFC method, it is crucial to use MRS broth as a rehydration medium and to allow for a 'wake-up' time of 30–60 min. The suitable rehydration duration varies depending on the specific species. As the total particle concentration remains stable throughout this period, there's no risk of overestimation due to cell division. An advantage of the IFC method is that the results from 'wake-up' experiments are instantly available. This allows for the real-time monitoring of morphological and membrane-related changes.

FFC and IFC results are in good agreement

Following the initial optimization of the IFC method, the results were compared with the findings from the FFC technique. It's crucial to note that while the IFC analyses were conducted in a single laboratory, the FFC analyses utilized a ring test design, with tests performed at three distinct companies each using a different type of cytometer: Becton Dickinson's FACS Calibur (using beads as an internal standard), Beckman Coulter's Cytoflex (which performs volumetric absolute counts), and Thermo Fisher's Attune (which uses an acoustic focusing method). Consequently, greater variability is observed in the FFC with an uncertainty of 0.24 and 0.20 for Active Fluorescent Units (AFU) and Total Fluorescent Units (TFU) respectively, making it more meaningful to compare the averages of the two types of membrane-integrity flow cytometry.

Moreover, it was possible to evaluate the ring-test performances across three different labs using substantially different FFC equipment which differentiate according to the references and sampling technologies (volumetric absolute counts vs. beads used as reference and hydrodynamic focusing versus acoustic focusing methods) with an overall reproducibility (S_R) of 0.12 and 0.10 for AFU and TFU respectively, that are lower than those defined in the ISO 19344:2015 (S_R reported as 0.16 and 0.134 for AFU and TFU respectively). The *Z*-scores calculated for the different laboratories that performed the FFC analysis were all lower than 2.

Broadly, the results in this article from FFC and IFC techniques align well (Figure 5). This consistency is seen when comparing the active fluorescent units (AFU) from the FFC technique with the intact cell concentration (ICC) from the IFC technique with an estimated overall uncertainty of 0.12 – all tested species did not show statistically significant difference. Similarly, a good agreement is also evident when

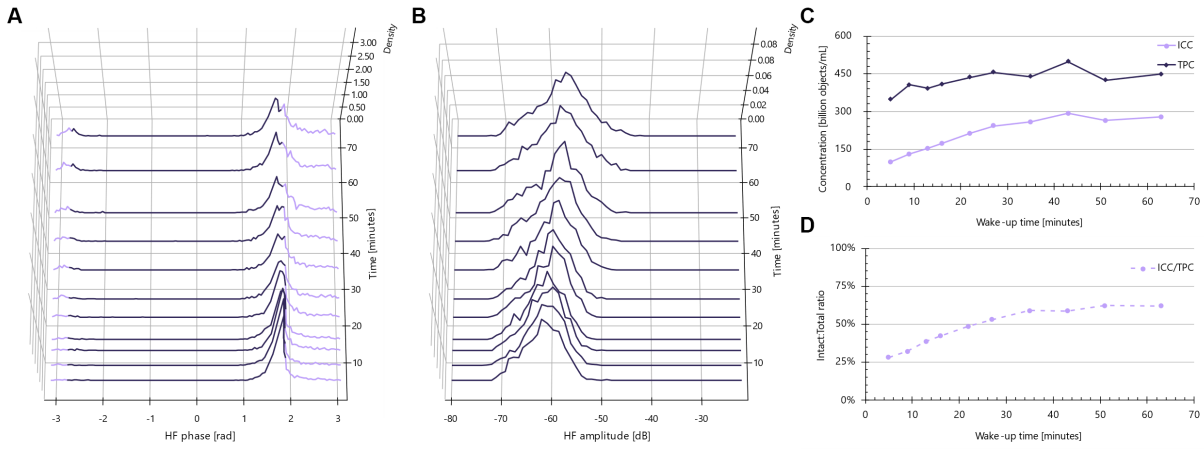

FIGURE 3
Impedance signatures for *Lacticaseibacillus rhamnosus* PB01 change during wake-up in MRS medium. **(A)** Normalized phase shift distributions plotted as a function of wake-up time. Lavender color indicates default intact cell limits. Violet color indicates other particles than intact cells, e.g., dead cells. **(B)** Normalized amplitude distributions plotted as a function of incubation time. **(C)** Intact cell and total particle concentration plotted as a function of wake-up time. **(D)** Ratio between intact and total cells as a function of incubation time.

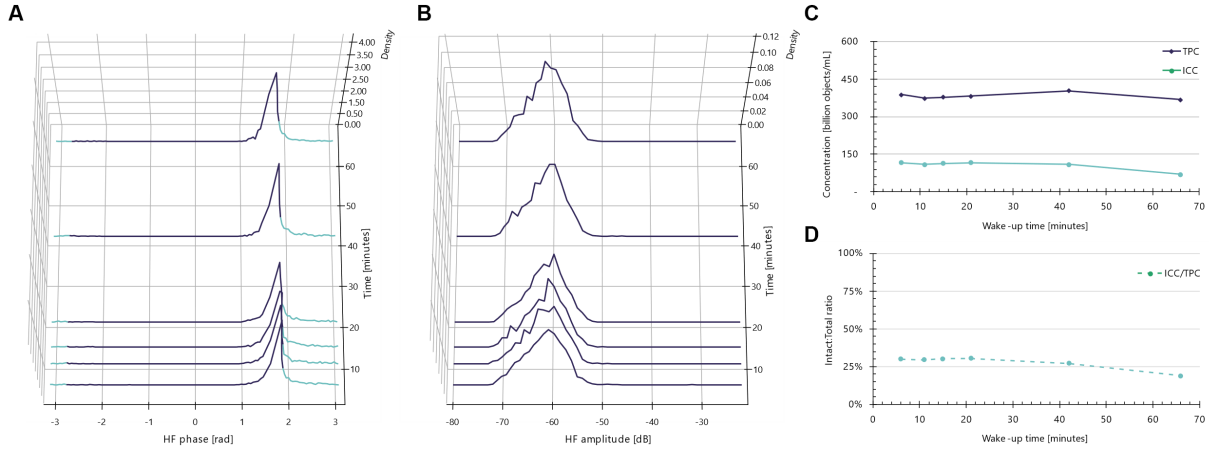

FIGURE 4
Impedance signatures for *Lacticaseibacillus rhamnosus* PB01 do not change in BPW. **(A)** Normalized phase shift distributions plotted as a function of wake-up time. Teal color indicates default intact cell limits. Violet color indicates other particles than intact cells, e.g., dead cells. **(B)** Normalized amplitude distributions plotted as a function of incubation time. **(C)** Intact cell and total particle concentration plotted as a function of wake-up time. **(D)** Ratio between intact and total cells as a function of incubation time.

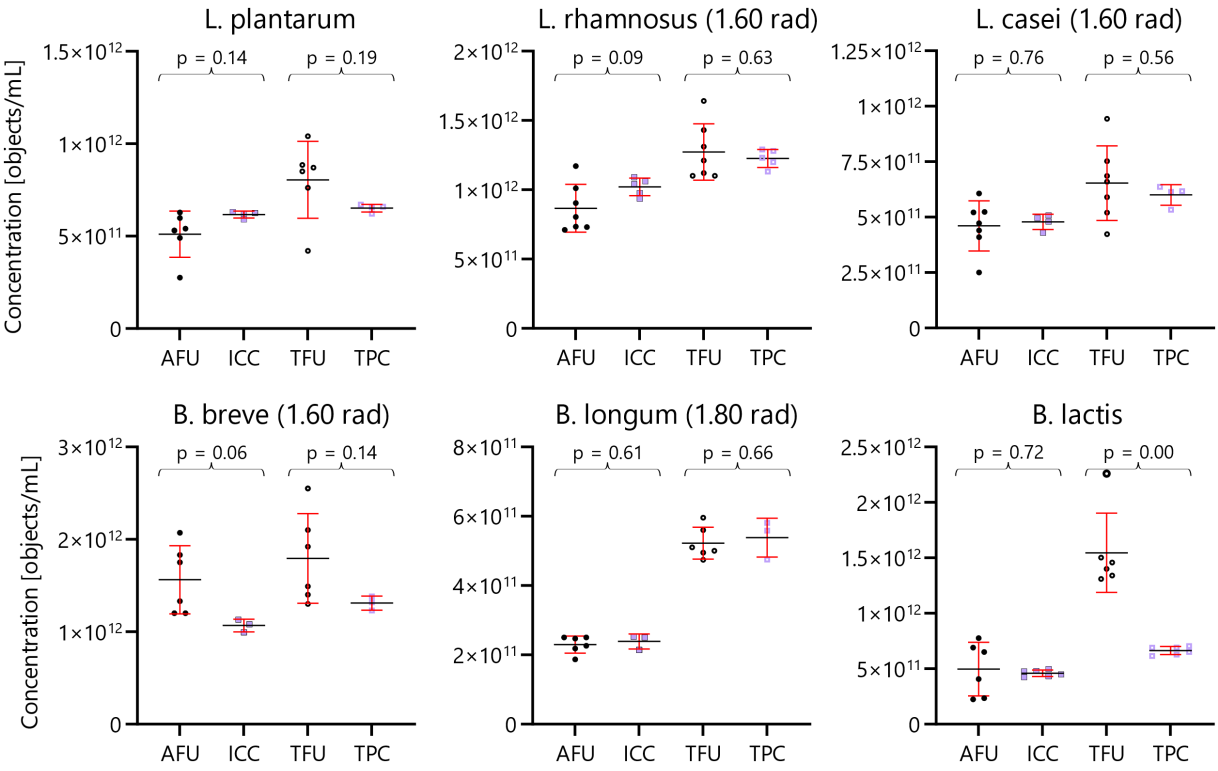

FIGURE 5
Head-to-head comparison of FFC and IFC techniques. Scatter charts for three *Lactobacillus* and three *Bifidobacterium* species. FFC measurements are shown with black circles, while IFC measurements are shown with lavender boxes. Active fluorescent units (AFU) and intact cell concentration (ICC) are depicted with filled data points, while total fluorescent units (TFU) and total particle concentrations (TFU) are shown with hollow data points. Average concentrations are depicted with black, horizontal bars while standard deviation is depicted with red error bars. Results of ANOVA tests for each AFU:ICC and TFU:TPC comparison is shown with brackets and *p*-values above each set of data points. Note that the FFC results were performed with a ring test design, while IFC analyses were done in a single lab.

comparing total fluorescent units (TFU) and total particle concentration (TPC) derived from the FFC and IFC methods with an estimated overall uncertainty of 0.23 – five out of six tested species did not show statistically significant difference.

The average concentrations of *L. casei* and *B. longum* show exceptional agreement, being virtually identical. The comparison does not reveal statistically significant differences ($p < 0.05$) except for one instance, involving *B. lactis* where the TFU is approx. Twice the

result of TPC. For this species, the amplitude was low (centered at −65 dB). Typically, dead cells shrink in size and potentially these exceedingly small objects could be hidden by the background noise. Another potential explanation for the discrepancy between TFU and TPC for *B. lactis* might be that the FFC gating includes objects other than dead cells.

In summary, the six species of probiotic bacteria analyzed yielded highly comparable bacterial concentrations for live and dead cells when comparing the FFC and IFC methods. Concentrations from the plate count technique are available in the Supplementary Material. Plate counts differ in the detection principle as it relies on cultivability opposed to membrane integrity probed by FFC and IFC. Nonetheless, agreement between AFU, ICC and CFU is within 50% for all species except for *Bifidobacterium animalis* subsp. *lactis*.

Discussion

In this study, our goal was to evaluate the concordance between two flow cytometry methods, FFC and IFC. Both techniques assess bacterial viability by examining the integrity of the lipid membrane, serving as a key indicator of the live/dead status of bacteria.

The need for custom IFC classification parameters

In this study, four out of the six examined probiotic bacterial strains required tailored classification parameters for precise live/dead determination. The standard IFC classification ruleset is predicated on the analysis of five actively growing cultures, with diverse Gram statuses and morphologies, including *E. coli* ATCC 8739 (rod-shaped, Gram-negative), *A. baumannii* ATCC 12457 (coccobacillus, Gram-negative), *S. epidermidis* ATCC 12228 (coccoid, Gram-positive), *K. aerogenes* ATCC 16048 (rod-shaped, Gram-negative), and *L. innocua* ATCC 33090 (rod-shaped, Gram-positive) (SBT Instruments, 2023). When cultured under ideal growth conditions, all these species are approximately 0.5 µm wide.

Conversely, the four probiotic bacteria requiring customized classification parameters appeared thinner when inspected using phase-contrast microscopy. This observation implies a larger membrane surface area relative to their cytoplasmic volume. Considering the hydrophobic, non-charged properties of the membrane's interior, which hinders the propagation of current at 7 MHz, coupled with the cytoplasm's ion-rich nature that excellently propagates electricity, it can be concluded that thin bacteria will typically propagate current less effectively than the default classification IFC ruleset accounts for.

As a result, all four bacteria needing custom gating required a decrease in the upper phase shift threshold to account for their less electrically conductive properties. Importantly, the optimization of gating is a one-time engineering effort per strain, which means routine post-analysis gating is not necessary. In general, we recommend investigating the need for custom gating by performing IFC measurements in conjunction to a simple shake flask experiment. Subsequently gating parameters can be established by comparing the phase shift distribution for the late-stage exponential culture (approx. 100% live) with an extensively aged or alternatively boiled culture (approx. 100% dead).

Wake-up in growth medium

Significant shifts in the phase and amplitude distributions, particularly for *Lactobacillus* species, were observed when the lyophilized powders were rehydrated using a rich MRS broth. To the best of our knowledge, this study is the first to monitor this reactivation phenomenon in real time using the IFC technique. This phenomenon was not seen when buffered peptone water was used. A review of scientific literature reveals that this methodology aligns with the USP's recommendation for the cultivation-based enumeration of probiotic bacteria. As per the USP, the sample should be dissolved in MRS broth, homogenized using a blender or stomacher, pre-incubated at room temperature, and re-homogenized before analysis (USP, 2019).

Furthermore, the ISO 19344 protocol for DiOC2 favors the use of a rich rehydration medium like MRS or M17 broth (in the case of *S. thermophilus*) to activate the cells. It also suggests a 30 min pre-incubation or "wake-up" period at 30°C for mesophilic bacteria and 37°C for thermophilic strains (ISO, 2015).

During the 0–60 min wake-up phase, no significant cell division was observed according to the total particle concentration. Increases in amplitude and shifts towards more conductive objects could be attributed to changes in object size and alterations in membrane or cytoplasm constitution. Microscopic evaluation of the *L. rhamnosus* PB01 strain during wake-up suggested an increase in object size over time (results not shown), thus the most plausible explanation for the changes in impedance properties is that the dehydrated bacteria are swelling in the nutrient-rich medium. Most wake-up intact:total curves stabilized within 30–40 min, making it feasible to complete the procedure within the 45 min permitted by the ISO 6887-1 method between sample rehydration and plating.

Curiously, the same wake-up effect was not observed for the *Bifidobacterium* species in MRS. Bifidobacteria are less oxygen-tolerant than *Lactobacillus* species (Charteris et al., 1997). The rehydration procedures were carried out in an ambient atmosphere where oxygen was present, which, we hypothesize, could have inhibited the activation of the bifidobacterial species. This theory aligns with the observation that *Lactobacillus* species demonstrated a short lag phase of a few hours, while the bifidobacterial lag phase extended to about 24 h. Currently, studies are underway to compare the wake-up effects of Bifidobacteria in rich MRS medium under both aerobic and anaerobic conditions.

Comparative overview of FFC and IFC from a helicopter perspective

Flow cytometry significantly improves repeatability over traditional plate count methods, offering the additional benefit of distinguishing between live and dead bacteria and analyzing heterogeneous bacterial samples (Foglia et al., 2020; Michelutti et al., 2020; Tracey et al., 2023). The robustness of the FFC technique was reaffirmed in our ring test design, demonstrating its reliability against variations in operators and analytical instruments.

Both FFC and Impedance Flow Cytometry (IFC) deliver similar outcomes in assessing membrane integrity. However, differences are evident in the methods' versatility and the requisite skill-level for instrument operation. FFC is versatile yet complex, with the ability to select from a wide range of stains and excitation parameters. This

versatility is advantageous when exploring various aspects of viability, as exemplified in the ISO 19344 standard, which describes three proxies for viability (ISO, 2015). Moreover, FFC allows for fluorescence-assisted cell sorting and species-level discrimination using techniques like Fluorescent *in-situ* Hybridization (FISH) or strain-level discrimination with antibody-probes (Chiron and Tompkins, 2017). However, FFC's precision and versatility come with higher costs and the need for extensive operator training, making it a common choice for end-point analysis in centralized quality control and research laboratories (Modena and Hierlemann, 2021).

In contrast, IFC is cost-effective, easy to implement, and practically operator-independent. Its compact size (30 × 30 × 20 cm) allows it to fit in a Laminar Air Flow (LAF) cabinet or an anaerobic chamber. IFC's robustness is evident in its label-free technique, which only requires dilution of the primary sample. This simplicity contributes to its tight standard deviations with low coefficients of variations (CVs) ranging from 2 to 10%. Additionally, its quick sample preparation enables real-time measurements, as demonstrated with the wake-up data for *L. rhamnosus* PB01. However, IFC's potential limitations include limited capacity to discriminate bacterial species in multispecies samples. Consequently, IFC is best suited for total counts or monoculture applications, where the primary requirements are to ascertain the concentration of viable bacteria and to determine the sample quality based on the live/dead ratio.

Conclusion

Six single probiotic strains (3 lattobacilli and 3 bifidocateria) were evaluated using both Fluorescent Flow Cytometry (FFC) and Impedance Flow Cytometry (IFC) techniques, using membrane integrity as a proxy of bacterial viability. The ring-test design for the FFC technique included three different labs with substantially distinct FFC equipment differing with respect (i) volumetric absolute concentrations versus beads as a reference and (ii) with or without acoustic focusing. All laboratories have *Z*-scores less than 2 with data within the uncertainty defined by ISO 19344.

Once custom gating parameters were established for the IFC technique, a good agreement was observed between the two methods: 6 out of 6 strains did not show statistically significant difference when comparing FFC active fluorescence units to IFC intact cell concentrations. When comparing the FFC total fluorescent units with IFC total particle concentrations 5 out of 6 strains did not show statistically significant difference. In addition, this research represents the pioneering effort in utilizing impedance flow cytometry to observe the initial stages of rehydration kinetics. In line with the established procedures for membrane-potential-sensitive dyes such as $DiOC_2$, a preliminary activation period of approximately 30 min in a nutrient-rich medium is essential to activate bacterial metabolism. Once this phase is completed, the proportions of live-to-dead cells can be accurately determined thereby improving assessment of the heterogeneity of bacterial populations in the sample. These results are extremely promising and further analysis on a bigger panel of different bacterial species is required to confirm these findings.

Probiotics quality is a key credibility factor for health care professionals and for consumers. Recently probiotics products have diversified to novel products containing, e.g., strictly anaerobic bacteria and inactivated bacteria (postbiotics). The emergence of these challenges poses significant analytical obstacles for the conventional gold standard plate counts used in probiotic product testing. Therefore, instrumentation and procedures need to be improved to assure reliable characterization and quantification of bacteria. Beside metagenomics and qPCR, flow cytometry proposes analysis opportunities of real and deeper efficiency as alternative or complementary to conventional microbiology (Warzée et al., 2021). This innovative and collaborative inter-laboratory approach for bacterial quantification is truly unique and plays a pivotal role in driving advancements in instrumentation within this field. Finally, we do reiterate that careful evaluation was done defining the measurand (Weitzel et al., 2021) which in our setting was an industrial derived sample of freeze-dried single strain of lactobacilli or bifidobacteria, which viability was assessed probing membrane integrity. Present approach and results cannot be generalized to multi-strain products (since FFC would need the development of specific markers, and IFC is label free) nor correlated to plate count methodologies since the measurand is different and based on cellular replication.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. MD: Data curation, Software, Visualization, Writing – review & editing. CM: Formal analysis, Writing – review & editing. SA: Conceptualization, Data curation, Validation, Writing – review & editing. DZ: Investigation, Writing – review & editing, Methodology. DC: Methodology, Writing – review & editing. SG: Methodology, Writing – review & editing. EG: Methodology, Writing – review & editing. J-PW: Supervision, Writing – review & editing. MP: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

PJ and MD were employed by SBT Instruments A/S. MP, CM, SA, and DZ were employed by Probiotical Research. DC, SG, and EG were

employed by AAT – Advanced Analytical Technologies, Fiorenzuola d'Arda. J-PW was employed by European Scientific League for Probiotics.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1285075/full#supplementary-material

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Probiotic and postbiotic analytical methods: a perspective of available enumeration techniques

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Probiotics are the largest non-herbal/traditional dietary supplements category worldwide. To be effective, a probiotic strain must be delivered viable at an adequate dose proven to deliver a health benefit. The objective of this article is to provide an overview of the various technologies available for probiotic enumeration, including a general description of each technology, their advantages and limitations, and their potential for the future of the probiotics industry. The current "gold standard" for analytical quantification of probiotics in the probiotic industry is the Plate Count method (PC). PC measures the bacterial cell's ability to proliferate into detectable colonies, thus PC relies on cultivability as a measure of viability. Although viability has widely been measured by cultivability, there has been agreement that the definition of viability is not limited to cultivability. For example, bacterial cells may exist in a state known as viable but not culturable (VBNC) where the cells lose cultivability but can maintain some of the characteristics of viable cells as well as probiotic properties. This led to questioning the association between viability and cultivability and the accuracy of PC in enumerating all the viable cells in probiotic products. PC has always been an estimate of the number of viable cells and not a true cell count. Additionally, newer probiotic categories such as Next Generation Probiotics (NGPs) are difficult to culture in routine laboratories as NGPs are often strict anaerobes with extreme sensitivity to atmospheric oxygen. Thus, accurate quantification using culture-based techniques will be complicated. Another emerging category of biotics is postbiotics, which are inanimate microorganisms, also often referred to as tyndallized or heat-killed bacteria. Obviously, culture dependent methods are not suitable for these products, and alternative methods are needed for their quantification. Different methodologies provide a more complete picture of a heterogeneous bacterial population versus PC focusing exclusively on the eventual multiplication of the cells. Alternative culture-independent techniques including real-time PCR, digital PCR and flow cytometry are discussed. These methods can measure viability beyond cultivability (i.e., by measuring cellular enzymatic activity, membrane integrity or membrane potential), and depending on how they are designed they can achieve strain-specific enumeration.

KEYWORDS

digital PCR, real-time PCR, enumeration, quantification, viable count, flow cytometry, plate count, culture-independent

Introduction

Probiotics, which represent the largest category of non-herbal/traditional dietary supplements worldwide, are experiencing significant growth. The global market size for probiotics was valued at USD 58.17 billion in 2021 and is anticipated to grow at a compound annual growth rate (CAGR) of 7.5% from 2021 to 2030 (Grand-View-Research-Inc, 2022).

The World Health Organization in 2002 initially defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The definition was later refined in 2014 to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), a statement that has gained broad acceptance within both the scientific community and the industry. According to this definition, a probiotic strain must be viable in an appropriate quantity to confer a health benefit to the consumer. However, this definition does not provide any specific standards to identify or quantify this viability, but the common practice is to measure viability using direct plate count (PC) enumeration which expresses results in Colony Forming Units (CFUs).

Breeuwer and Abee in 2000 proposed a broader definition of bacterial viability as having an "intact cytoplasmic membrane, protein and other cell components synthesis (nucleic acids, polysaccharides, etc.) and energy production necessary to maintain cells metabolism; and, eventually, growth and multiplication" (Breeuwer and Abee, 2000). Building on Breeuwer and Abee's definition, a variety of methodologies can provide a more comprehensive view of the viability of a heterogeneous bacterial population than the traditional PC method, which focuses solely on growth and multiplication potential of a subset of the bacterial population. Moreover, the emergence of a new generation of probiotics comprising strictly anaerobic bacteria presents significant challenges for enumeration using traditional PC methods, making it necessary to explore alternative techniques that can assess their viability and provide a more accurate cell count.

This paper will delve into the most widely used methods for quantifying and assessing the viability of probiotic strains, discuss their limitations, and explore alternative techniques that overcome these challenges. The paper will also introduce the concepts and applications of culture-dependent and culture-independent enumeration methods. To provide a general overview of the status of viability acceptance across different regulations and guidelines we did provide a summarized table as a reference (Table 1).

Culture-dependent enumeration methodologies

The traditional microbiological PC method is the most common choice for enumerating viable beneficial microorganisms and contaminants in international standards. These standards are issued by bodies such as the International Organization for Standardization (ISO), the International Dairy Federation (IDF), Bacteriological Analytical Manual (BAM), and the United States Pharmacopeia (USP). The PC method measures the ability of bacterial cells to proliferate into detectable colonies on agar media, presenting results in Colony Forming Units (CFUs). The Colony Forming Unit (CFU) has been the unit for microbial enumeration for at least 125 years (USP, 2018). This method's popularity arises from its technical simplicity, ease of implementation, and wide acceptance, marking it the 'gold standard' in the probiotic industry for the analytical quantification of probiotics (Weitzel et al., 2021). The PC method, however, has multiple disadvantages such as laborious workload and lengthy periods of incubation (USP, 2019). Additionally, it should be noted that a CFU count has always been an estimation of the number of viable microorganisms present and not a true cell count (Davey, 2011; USP, 2018). The viable counts estimated using culture-dependent methods rely on the suitability of the growth media and incubation conditions for the strain to be quantified (Wendel, 2022). Furthermore, the applied method will likely change the qualitative and/or quantitative properties of the original sample since the selective pressure may alter its native composition and state. This is specifically true for probiotic blends where the additional variable of the interaction between strains during the incubation time can shift the relative abundances of the original sample (Sielatycka et al., 2021).

The variability between species and between strains in response to plating procedures also means that no single methodology can be universally applied to all probiotic organisms (Davis, 2014). This complexity extends to enumerating species or strains in a complex blend. In response, probiotic strain manufacturers have developed PC methods that utilize chemical components to promote or inhibit growth of specific bacterial taxa (Davis, 2014). For example, MRS (deMan Rogosa, Sharpe) agar is commonly used for *Lactobacilli* enumeration (Champagne et al., 2011). However, when supplemented with raffinose and lithium chloride, it enables the growth of *Bifidobacteria* (Hartemink et al., 1996). Another example is adding 0.5 ppm of clindamycin to MRS medium to allow the enumeration of heterofermentative *Lactobacillus* genus (Van de Casteele et al., 2006; Davis, 2014). It is well recognized that the high number of variables that can affect PC enumeration generates a continuous debate on which methodology to correctly apply. Recently, the USP probiotic panel working group published a comprehensive overview of the Analytical Procedure Lifecycle Management (APLM) for comparing PC methods. This approach is universal as it is a process to define procedure performance based on the concept that the reportable value must fit its intended use; therefore, information gathered through APLM can be used to evaluate and compare any procedure (Weitzel et al., 2021).

The emergence of novel dosage forms of probiotics, such as gummies and oils, and their blending with other active ingredients like herbs, fruits and vegetable extracts, vitamins, and minerals, adds another layer of complexity when using PC methods or any alternative enumeration method. For example, bacteria can remain trapped within gummy particles, resulting in underestimation of the total count, or the cell growth in culture media may be inhibited by other ingredients in the products. Consequently, with every new active ingredient and delivery form, testing laboratories need to validate the method to ensure scientific validity and fitness for purpose, thereby requiring additional financial investment, time, and human resources.

Given the numerous variables that can affect PC enumeration, the industry has accepted a variability range between 20–30% or a Relative Standard Deviation (RSD) of 10–15% (Hansen et al., 2018). The Italian Ministry of Health and the European Scientific League for Probiotics (ESLP) have also provided guidelines and, the latter, quality seals based on scientific evaluation and control of the CFU content, respectively with a variability of 0.5 and 1 log at the end of the product

TABLE 1 Overview of the status of viability acceptance across different regulations and guidelines.

No.	Country	Comments	References
1	Italy	The recommended product serving for daily consumption shall contain a quantity of 10^9 live cells of at least one of the strains. It is pointed out that the most suitable analysis methods to quantify live micro-organisms may vary according to each species.	Ministero-Della-Salute-Italy (2018)
2	France	The recommended product serving for daily consumption shall be between 10^7 and 10^9 viable cell per day from one strain.	DGCCRF (2023)
3	Australia	The quantity (potency) of each strain must be expressed in CFU/g, CFU/mL or CFU per metric unit or dose; or as the number of viable cells per mL based on a viable-cell assay.	TGA (2023)
4	Europe	For live biotherapeutics, the potency of each strain expressed in CFU/mL, CFU/g, CFU/unit or viable cells/mL. For food or food supplements, there is no specific legislation that regulates the use of probiotics in human nutrition, therefore the EU legislation does not specify any specific labeling provisions for probiotic enumeration reporting other than for the approved claim which must be reported in CFU.	EDQM (2019) and IPA (2022)
5	Codex alimentarius	From a labeling side, the product label should contain the amount of viable cells of total probiotic microorganisms (CFU/g). Although, from an enumeration side, traditionally, plating has been used and endorsed as the "standard way" to evaluate microbial viability and it has been determined through counting "colony-forming units," CFU. The plate count method is based on the premise that a single bacterium can grow and divide to give an entire colony. This method is historically and currently, the most broadly used method to demonstrate the activity of the microorganisms. Now, other methods such as flow cytometry (ISO 19344 IDF 232) are coming to be used widely and a standardized method has been developed and used as a way to evaluate total probiotic microorganisms. All work will be coordinated with the applicable general subject Codex Committee to ensure the appropriate application of Codex. Expertise and resources.	CCNFSDU (2019)
6	Norway	The number of viable probiotic bacterial cells in the product within the time frame of its shelf life should be clearly given including a proviso that recommended storage conditions have been upheld. The numbers may be expressed as log Colony Forming Units (CFU) per gram of product or per serving of a specified size.	Yazdankhah et al. (2014)
7	USA	For dietary supplements, it is mandatory to declare the quantitative amount of live microbial ingredients in terms of weight in the Supplement Facts label. The concentration can be declared in CFU as long as it is done in a manner that clearly separates and readily distinguishable from the weight. However, the FDA believes that CFUs provide a useful description of the quantity of live microbial dietary ingredients and is aware that researchers are currently evaluating other methods and units of measure for live microbial dietary ingredients and that such alternative methods have the potential to more accurately and more efficiently quantify the number of viable cells. For food containing microorganisms, such as yogurt, the product label may be indicate "contains live and active cultures" or another appropriate descriptor if the food contains a minimum level of live and active cultures of 10^7 colony forming units per gram (CFU/g) at the time of manufacture with a reasonable expectation of 10^6 CFU/g through the manufacturer's assigned shelf life of the product.	FDA (2018) and FDA (1977)
8	Brazil	The product must be labeled with the quantity to be consumed in CFU/day to obtain the desired effect.	ANVISA (2021)
9	India	Minimum viable number of added probiotic organisms in food shall be $\geq 10^8$ CFU in the recommended serving size per day.	FSSAI (2022)
10	Canada	All individual strain quantities of live microorganisms must be indicated in Colony Forming Units (CFU) per dosage unit.	Health-Canada (2023)
11	Colombia	The food should contain a number of viable cells $\geq 1 \times 10^6$ CFU/g in the finished product until end of shelf life	Ministry-of-Health-and-Social-Protection-of-Colombia (2011)
12	IPA	The quantitative amount(s) of probiotics in a product should be expressed in Colony Forming Units (CFUs).	CRN-IPA (2017)

shelf-life (Warzée, 2016). Despite these efforts, the question remains as to the best methodology for microbial enumeration, given the high variability and lack of precision inherent in PC methods. The challenges associated with this evaluation highlight the need for both standard PC enumeration methods and alternative techniques to ensure accurate quantification and enumeration of probiotics.

In addition to technical difficulties in enumerating probiotics belonging to traditional probiotic taxa like *Bifidobacterium* spp. and, *Lactobacillus* spp., the industry is confronted with additional challenges when enumerating novel microorganisms, often referred to as Next-Generation Probiotics (NGPs) (O'Toole et al., 2017; Saarela, 2019; Singh and Natraj, 2021; Torp et al., 2022). NGPs are "live microorganisms identified on the basis of comparative microbiota analyses that, when administered in adequate amounts, confer a health benefit on the host" (Martín and Langella, 2019). An alternate term that is proposed for NGP is Live Biotherapeutic Product (LBP) (Martín and Langella, 2019). Many of these organisms, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Prevotella copri*, *Bacteroides* spp., *Roseburia* spp. (Meehan and Beiko, 2014), *Bacteroides uniformis* (Gomez-Arango et al., 2016), *Christensenella minuta* (Goodrich et al., 2014), *Oxalobacter formigenes* (Stewart et al., 2004), and *Alistipes putredinis* (Png et al., 2010), are highly adapted to the gastrointestinal environment or other human body niches. These NGPs are often strict anaerobes, highly sensitive to atmospheric oxygen, thus necessitating specific growth conditions and advanced culturing techniques to grow them in a laboratory setting (O'Toole et al., 2017; Saarela, 2019; Singh and Natraj, 2021; Torp et al., 2022). Achieving appropriate growth conditions that mimic their native environments is far from a trivial task and often involves intricate adjustments (O'Toole et al., 2017; Saarela, 2019). Thus, quantification of these NGPs using traditional culture-based techniques proves complex, and the use of culture-independent methods becomes highly advantageous as they can provide a more accurate assessment of viability, addressing a critical need where traditional culture-based methods may fall short (Chang et al., 2019; Saarela, 2019; Singh and Natraj, 2021; De Filippis et al., 2022; Torp et al., 2022).

Importance of strain specificity

The concept of bacterial strain identity has undergone considerable transformation with the advent and progression of molecular methodologies that offer precise and distinct identification of bacterial genomes. Traditionally, bacterial strains have been identified through laborious culture-based methods, with the definition rooted in taxonomic practices and phenotypic traits.

According to the first edition of Bergey's Manual of Systematic Bacteriology, 'a strain is made up of the descendants of a single isolation in pure culture and usually made up of a succession of cultures ultimately derived from an initial single colony' (Staley and Krieg, 1984). This definition inherently ties a bacterial strain to the process of *in vitro* culturing and isolation of a bacterial colony. This implies that the existence of a strain, as defined within the scientific context, is tied to the human act of isolation, and not as a natural entity within the ecosystem it was derived from Achtman and Wagner (2008).

However, the narrative has gradually evolved, largely owing to advancements in genomic technology. The strain, as we refer to it in the current context, is often more closely associated with a human-operated setting, an artifact of the laboratory environment and techniques used to isolate and culture it, rather than a naturally occurring, distinct entity within its ecological niche (Gevers et al., 2005). According to Thea Van Rossum et al., 2020, the biological basis for strain definition is not well established and may not exist (Van Rossum et al., 2020).

This shift in perspective opens up important dialogs on the biological relevance and ecological roles of bacterial strains as we have defined them (Doolittle and Papke, 2006). It also underscores the potential discrepancies that may arise when translating laboratory findings to a more complex, real-world context (Polz et al., 2013). Given these considerations, it becomes increasingly important to re-evaluate and contextualize the concept of strains within the broader framework of bacterial ecology and evolution. This is an area where continued advancements in genomics and related fields can contribute significantly to our understanding of microbial diversity and function (Koeppel and Wu, 2013).

A modern definition by Ghazi et al. (2022) proposes a strain as "a collection of cells or genomes within a relatively small range of phylogenetic variation (i.e., a very narrow subspecies clade)." With species identity often defined by approximately 95–97% of whole-genome nucleotide sequence similarity, a strain could represent even greater sequence similarity, up to >99% or > 99.9% whole-genome sequence similarity. Theoretically, even one single nucleotide polymorphism (SNP) could delineate strain identity, although no concrete rules have been established on how many SNPs define a unique strain or whether such SNPs need to result in phenotypic changes to justify strain discrimination (Ghazi et al., 2022). This leads to the consideration that SNPs alone may not be sufficient for strain discrimination and suggests the need to employ multiple methodologies to fully comprehend a strain's uniqueness, also including factors such as clinical and intellectual property backgrounds of the strain.

The concept of strain-specificity in probiotics has traditionally been considered the cornerstone of probiotic science. To meet the World Health Organization's definition of probiotics, a probiotic microorganism must exhibit a health benefit, and any claims of a specific health benefit must be supported by strain-level clinical evidence. It is generally accepted that a probiotic's beneficial effects on the host will be specific to a particular strain, and that the characteristics and efficacy of a certain strain cannot be generalized to other strains within the same species, or to strains of other species (Lee et al., 2013). A systematic review of the literature and various meta-analyses conducted in 2018 suggests that there is strong evidence showing that the efficacy of probiotics is both strain-specific and condition-specific (McFarland et al., 2018). The strain specificity of probiotic health benefits highlights the importance of methods that enable strain-specific identification and enumeration of probiotics in both research and production settings to confirm product efficacy.

While culture-dependent PC methods and their corresponding CFU counts are still considered the gold standard for quantification of probiotic bacteria, they lack the specificity required to quantify individual strains in a multi-strain blended material. Therefore companies will often rely on a combination of assays to confirm both identity and quantity as respective datasets. It usually involves a total

count of CFUs present or a quantification to the genus-level and a separate confirmation of identity using a genomic application as described above often at species level resolution (Jackson et al., 2019).

Alternatively, a company may rely on raw material concentration information and formulation targets to determine a theoretical number of probiotic bacteria present in the finished product (Quantification by Input); but this approach lacks the confirmation of cellular viability in the final product as ingredients are subjected to manufacturing processes and potentially negative interactions with other active ingredients. Since strain-level quantification in a blend cannot be achieved using traditional PC techniques, methods based on real-time quantitative PCR (rtPCR or qPCR), digital chip-based or droplet PCR (cdPCR or ddPCR) (Hansen et al., 2018; Shehata et al., 2023), or antibody-coupled flow cytometry (Chiron et al., 2018) have been developed to combine identification with quantification to enumerate probiotics at the strain level within a coherent methodological validation setting. Keep into account that the concept of strain specificity is fluid and that if product design implies one micro-organism or different species or genus (and not different strains of the same species) any methodologies that discriminate at the species and genus level shall be considered valid, especially with the broader definition of the strains as the sum of the genetic, phenotypic, productive, pre-clinical, clinical and intellectual proprieties evidences.

Alternatives in viability definition

Although the concept of viability was primarily gauged by cultivability, i.e., the ability of a cell to replicate and form a colony on agar media (USP, 2018; Fiore et al., 2020), there was agreement that the definition of viability should not be constrained to cultivability alone (Wendel, 2022). For instance, bacterial cells may exist in a viable but not culturable (VBNC) state, where cells lose the ability to form colonies – yet maintain membrane integrity, enzyme activity, a pH gradient, and high levels of rRNA (Lahtinen et al., 2006b, 2008; Fiore et al., 2020; Wendel, 2022) – as a survival strategy for microorganisms under various environmental stresses (İzgörd et al., 2022). This distinguishes VBNC cells from dead cells, which exhibit irreversibly damaged cell membranes and no metabolic activity (Li et al., 2014).

The concept of Viable But Non-Culturable (VBNC) cells has garnered increasing attention in the realm of probiotic enumeration as well as in the broader context of microbial ecology. Traditional methods like Plate Count (PC) often underestimate the actual number of viable cells, as they do not account for cells in the VBNC state. These cells, although not cultivable can exhibit probiotic properties (Kiepś et al., 2023).

While VBNC cells cannot grow and form colonies on agar without resuscitation, they are nonetheless viable (Davis, 2014), thus challenging the conventional association between viability and cultivability (Wendel, 2022). This discrepancy has led to scrutiny of the accuracy of culture-dependent enumeration methods for evaluating all viable cells in probiotic products (Foglia et al., 2020; Visciglia et al., 2022). Consequently, a cell count obtained through culture-dependent methods is now considered an estimate rather than an accurate viable cell count (USP, 2018). This is because PC methods may potentially underestimate viable cell numbers, as they fail to detect VBNC cells (Jackson et al., 2019; Fusco et al., 2022). Recent advancements in enumeration techniques, such as Imaging Flow Cytometry (IFC),staining-based flow cytometry and viability qPCR, have shown promise in capturing the VBNC population more accurately (Ma et al., 2023; Pereira et al., 2023; Shehata et al., 2023).

Interestingly, VBNC probiotic populations may contribute to health benefits within the host (Wendel, 2022), as VBNC cells can resuscitate, regain the ability to divide, and interact with the host upon encountering favorable conditions in the gut (Pinto et al., 2015; Fiore et al., 2020; Puntillo et al., 2022). This phenomenon mirrors that of pathogenic bacteria in a VBNC state (Li et al., 2014; Zhao et al., 2021, 2022), which have been found to regain pathogenicity and virulence after resuscitation (Li et al., 2014). Resuscitation from the VBNC state has been widely studied, especially for risk control of recovered pathogenic or spoilage bacteria. The phenomenon of resuscitation is crucial for proving the existence of the VBNC state and has potential applications in the food industry (Pan and Ren, 2022). One of the major advances in resuscitating VBNC cells is the discovery of bacterial cytokine proteins like resuscitation-promoting factor (Rpf), which have potential applications in environmental bioremediation (Xie et al., 2021). Moreover, short-chain fatty acids (SCFAs) have been identified as potential resuscitation factors that can break the dormancy of certain marine bacteria within 5 days (Sun et al., 2023). Metabolomic studies have revealed significant differences between VBNC and recovered cells, particularly in *Lacticaseibacillus paracasei* Zhang, a probiotic and starter strain. Levels of specific amino acids like L-cysteine, L-alanine, L-lysine, and L-arginine notably increased in revived cells, suggesting altered physiology in the VBNC state (Wang et al., 2023).

This has led to requests to extend the probiotic viability definition beyond cultivability to probiotic activity, which can be measured based on membrane integrity, metabolic activity, membrane potential, or RNA content (Davey, 2011).

Understanding the physiology and metabolism of VBNC cells is essential for both risk control and the exploration of beneficial microbial resources (Pan and Ren, 2022).

Given the potential role VBNC cells may play within the host, it is crucial to consider enumeration methods that account for cells in this state. Culture-independent methods could potentially count both culturable and VBNC cells, yielding more accurate viable counts (Figure 1). This is particularly important for finished probiotic products and during shelf life, as probiotic cells undergo a dynamic shift to enter a VBNC state during shelf life and upon exposure to stresses during storage (Davis, 2014; Foglia et al., 2020). This shift to a VBNC state results in a disparity between CFU counts and actual viable counts (Wendel, 2022), thus it has been observed as a gap between counts determined using culture-dependent and culture-independent methods (Foglia et al., 2020; Visciglia et al., 2022; Shehata et al., 2023).

Unlike VBNC cells, dead cells that have the capability to interact with the host eliciting a potential health benefit do not qualify as probiotics according to the WHO definition (Binda et al., 2020). They are instead referred to as "postbiotics" (Aguilar-Toalá et al., 2018).The emerging category of postbiotics refers to a preparation of inanimate microorganisms and/or their components that confer a health benefit on the host (Salminen et al., 2021). These inanimate microorganisms, often referred to as tyndallized or heat-killed bacteria, need to be characterized before inactivation (Salminen et al., 2021). There are many inactivation methods, but currently heat treatment is the preferred method in the industry and the most historical (Piqué et al.,

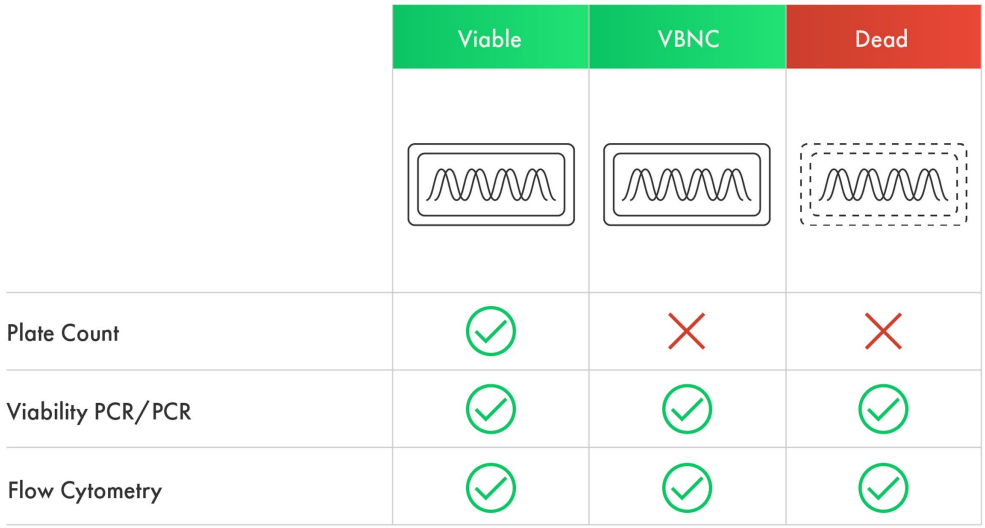

FIGURE 1
The ability of culture-dependent and culture-independent methods to detect viable, VBNC, and dead cells.

2019; Rabiei et al., 2019; Vallejo-Cordoba et al., 2020). Interest in postbiotics is increasing due to factors such as their higher stability during industrial preparation, longer shelf life compared to probiotics, ease of transport and storage, and compatibility with products where viability is a challenging parameter (Salminen et al., 2021). However, this class of products cannot be enumerated by culture-dependent methods, and alternative quantification methods are needed. A bacterial counting chamber could be used, where cells are treated with dyes like propidium iodide that stain bacteria with damaged membranes (dead bacteria) only (Lahtinen et al., 2006b; Sugahara et al., 2017). Culture-independent methods would also be useful for enumerating postbiotics. For instance, the recent approval of *Akkermansia muciniphila* as a Novel Food in Europe (Turck et al., 2021) pursuant to Regulation (EU) 2015/2283, is a notable example, where the dose was enumerated in Total Fluorescent Units (TFU) by flow cytometry with a safety target of <10 CFU/g.

Flow cytometry (FCM), a modern method to measure different viability parameters

Flow cytometry has emerged as an advanced tool in probiotic viability assessment, capable of extracting detailed information on individual cells including their size, granularity, and morphology through the analysis of laser light scattering. This technique leverages the ISO 19344 IDF 232 lactic acid bacteria enumeration method, utilizing three different staining protocols to evaluate cellular enzymatic activity, membrane integrity, and membrane potential, providing comprehensive insights into bacterial viability (ISO, 2015).

The membrane integrity protocol for example, employs a DNA binder colorant that penetrates all bacterial cells (SYTO 24) regardless of their viability (thereby identifying Total Fluorescent Units, TFU: bacteria that are alive, damaged, and dead) and another colorant which only enters bacterial cells with a compromised membrane (Propidium Iodide) (Figure 2). The difference between the two groups is expressed as Active Fluorescent Units (AFU) which represents the viable cells (cells with intact membranes) based on this protocol. Total Fluorescent Units (TFU) provide information on the total number of cells in the sample, whereas the difference between TFU and AFU (TFU-AFU), termed as non-AFUs (n-AFUs), represents the dead, likely irreparable, bacterial population (Fallico et al., 2020; Ma et al., 2023). The enzymatic activity protocol is based on fluorescence generated by the non-fluorescent dye Carboxylfluorescein diacetate succinimidyl ester (cFDA) when it is cleaved by cellular esterase (a proxy of cellular viability), meanwhile the membrane potential is based on $DiOC_2$ that binds the membrane with a green fluorescence emission; when cells are activated the maximum fluorescence is then red-shifted.

Apart from the fluorescence techniques, ISO 19344 has been validated using a broad array of probiotic species, including *Lactobacillus* spp., *Bidifobacterium* spp., and *Streptococcus* spp., which emphasizes the method's capability to be utilized for enumerating any strain belonging to the validated species, thereby offering a generalized approach which overcomes the intrinsic limitations of cultivability methods that rely on specific protocols that can vary according to the taxonomical species under examination. These technical advantages make FCM a more accurate and faster technique compared to PC enumeration, as FCM directly enumerates each single cell in a given sample and provides information on the bacterial population heterogeneity based on the fluorochrome used (Sielatycka et al., 2021; Ma et al., 2023).

It is important to note, ISO 19344 has been validated on fresh samples, and no data have been provided on aging samples and/or stability data. It is generally accepted that AFU and CFU data tend to correlate for fresh probiotic products (Sielatycka et al., 2021). However, recent studies have compared the performance of flow cytometry during long-term probiotic stability studies to PC enumeration using predictive microbiology (Foglia et al., 2020; Visciglia et al., 2022). The studies revealed that as the storage temperature increases, the CFUs decrease faster than AFUs, suggesting that the loss of cultivability is

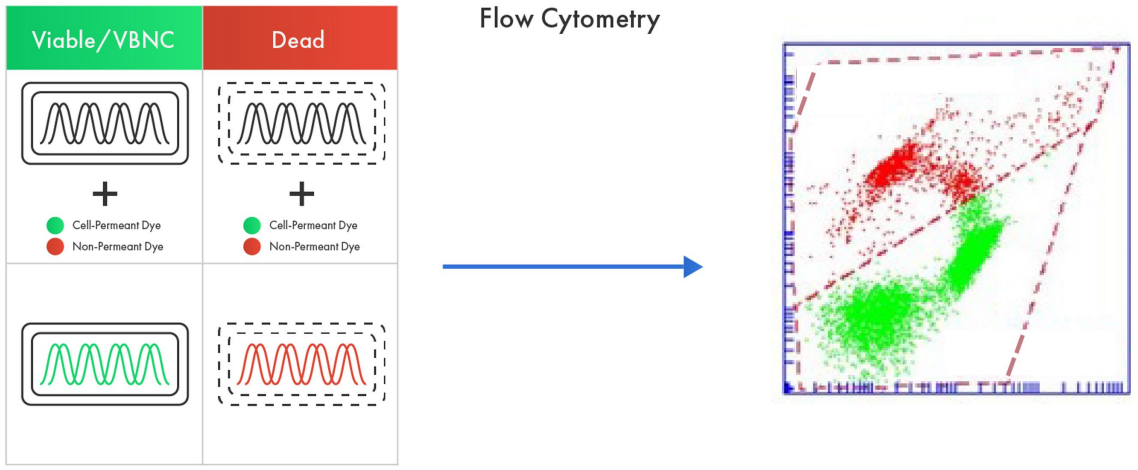

FIGURE 2
Flow cytometry as an advanced tool in probiotic viability assessment based on membrane integrity. The method utilizes a cell permeant dye that penetrates all bacterial cells regardless of their viability (live, damaged, and dead) and a non-permeant dye that only enters dead bacterial cells. The difference between the fluorescence from two dyes represents viable cells.

quicker than the loss of membrane integrity. Yet, the metabolic potential of the probiotic products was maintained, observed as the ability to acidify a fermentation broth and hinting that the product would be able to exhibit its beneficial effects under appropriate circumstances (Visciglia et al., 2022).

The difference between AFU and CFU readings might be attributed to cells that exist in a VBNC state, bacterial populations exhibiting metabolic activity but loss of cultivability (Lotoux et al., 2022). This situation is often observed in probiotic products due to the numerous unavoidable stressful conditions that probiotic cultures undergo during industrial production and the shelf-life of the finished product (Wendel, 2022).

Discrepancies between AFU and CFU counts can also be observed in multi-strain probiotic formulations where several issues may arise and impede PC effectiveness. For one, interactions between the different strains in a blend, such as competition for nutrients or the production of inhibitory compounds, could underestimate the total count of probiotic bacteria determined using PC compared to counts determined using a cytometric method (Sielatycka et al., 2021). Method suitability factors such as growth enhancers, incubation time, incubation temperature, and oxygen conditions (aerobic, microaerophilic, anaerobic) also play a critical role (Sielatycka et al., 2021).

Beyond the ISO 19344 IDF 232 protocol, other fluorescent dyes with alternative properties can be used, such as carboxyfluorescein diacetate succinimide ester (CFDA), which binds to intracellular proteins of intact cells (Ma et al., 2023), or CellROX® Green Reagent, a DNA-binding cell-permeant dye that exhibits bright green fluorescence when oxidized by reactive oxygen species (ROS) (Fallico et al., 2020).

FCM cell counting is based either on a standard reference microsphere counting method or an absolute enumeration (volume method), where the actual number of target cells in a sample is determined using the optical characteristics of the cells and the sample volume. Advances in FCM have further improved volume methods by using acoustic focusing, which generates ultrasonic waves to transport particles to the center of the sample stream, reducing analysis time (Ward and Kaduchak, 2018).

Impedance Flow Cytometry (IFC) is a less well-known but promising label-free, non-invasive technology. It relies on the electrical characteristics of the cell. Since viable bacteria have lipid membranes that resist electricity propagation, IFC uses microfluidic channels where bacteria pass through electric fields one cell at a time. Each bacterium results in a small perturbation, and by analyzing the change in electricity, it's possible to determine if a cell's membrane is intact or compromised (Clausen et al., 2018; Bertelsen et al., 2020).

A notable limitation of both ISO 19344 FCM enumeration and PC methodologies is their inability to discriminate different species or strains within a blend. Only a few ISO methods, such as ISO 20128:2006 for *Lactobacillus acidophilus* group and ISO 29981:2010 for *Bifidobacterium* genus, provide selective enumeration of probiotic microorganisms using PC. Hence, enumerating individual strains in a multi-strain blend using either PC or FCM methods remains a significant challenge.

However, there have been some interesting attempts to enumerate and identify multi-strain blends using FCM, notably through the use of strain-specific antibodies. Chiron et al. (2018) managed to produce custom polyclonal antibodies against five commercial probiotic strains, successfully enumerating and differentiating closely related strains within three different probiotic food supplements. Bellais et al. (2022) employed flow cytometry and cell sorting to detect, separate, isolate, and then cultivate novel anaerobic strains from human fecal matter, demonstrating the potential of this approach for handling complex bacterial microbiota. Meanwhile, Yang et al. (2017) developed a polyclonal antiserum against the recombinant pilus protein of *L. rhamnosus* GG strain, which is essential for its adherence to the intestinal epithelium. These studies collectively show the feasibility of developing strain-specific antibodies, even those specific to functional traits like strains' pili, for identifying and enumerating strains in complex bacterial communities, such as commercial blends.

Furthermore, the complexity and cost of such developments should not be underestimated. The success in obtaining strain

specificity, hence the development of antibodies, hinges on checking against antibody cross-reactivity. This is technically possible but conceptually complex as commercial probiotic products can comprise strains from various producers and in different quantities. Therefore, the absence of cross-reactivity should ideally be validated against the largest possible number of different commercial strains. However, this is not realistic as each producer has its cell bank, and not all strains are available from culture collections. Further, the Limit of Quantification (LoQ) and Limit of Detection (LoD) must be validated in a relative abundance experiment. Such validation should answer the question, "Am I able to discriminate and quantify each single strain in a blend with strains from different suppliers and in different quantities?"

It is widely accepted that the FCM Method is not only faster but also more accurate than the PC method. Notably, the interpretation of results from these two methods – Total Fluorescent Units (TFU) and/or Colony Forming Units (CFU) for FCM and PC, respectively, – should be separated from their biological significance and their correlation. It has been reiterated that a close to 1:1 correlation between Active Fluorescent Units (AFU) and CFU data is typically seen in fresh, non-stressed, single-strain probiotic products. However, this correlation diminishes over time and is affected by variables such as temperature, humidity, and the presence of additional strains. Consequently, attempts to correlate FCM and PC are bound to falter under these conditions.

The value of FCM is found in its rich output, providing a comprehensive overview of the heterogeneity within a bacterial population: total cells, dead cells, live cells, and potential Viable But Nonculturable (VBNC) cells. Coupled with PC, it also provides information on cells capable of replicating under specific cultural conditions. CFU data informs only on the sub-population capable of replicating under given experimental conditions, but it provides no information on the VBNC fraction. Many pathogenic microorganisms that are food-borne, such as *Campylobacter jejuni*, *Campylobacter coli*, *Enterococcus faecalis*, *Escherichia coli*, *Helicobacter pylori*, *Salmonella*, *Shigella*, *Vibrio cholerae*, among others, are known to enter VBNC states (Ramamurthy et al., 2014). For this reason, FCM enumeration is now officially recommended for all freshwater analysis in Switzerland for the detection of pathogens (Egli and Kötzsch, 2015; Van Nevel et al., 2017). If VBNC pathogens pose a risk and need to be managed using FCM because they can thrive when they find themselves in a conducive ecosystem, it is not plausible to presume that probiotics, which also have enteric origins, would behave similarly?

Answering these questions propels us into a new perspective, as FCM results can be expressed as the total number of cells present in a product (TFU) and their heterogeneity in compliance with the staining protocol (ISO 19344: membrane integrity, membrane potential, and enzymatic activity) as AFUs.

FCM methods provide the opportunity to also explore postbiotics, specifically when the cell is of interest, and not its degree of "viability." This is particularly relevant for applications where probiotic microorganisms may not easily or at all survive (for instance in food ingredients that require cooking, beverages such as tea, coffee, sports drinks, and even water, aggressive industrial processes, and product categories like cosmetics). Nevertheless, quantifying the total number of cells present in a given product becomes functionally relevant if the efficacy is associated with the total number of cells (TFUs).

Finally, Live Biotherapeutics are gaining interest and traction with many novel developments, however most of the bacterial candidates are strictly anaerobic and difficult to propagate, as amply demonstrated by Bellali et al. (2019). An interesting use of FCM in novel strain applications belong to the recent Novel Food approval by EFSA of *Akkermansia municiphila,* which has been approved as a novel ingredient based on the data provided in TFUs since it is a pasteurized ingredient (Turck et al., 2021), which functionality resides not on the cellular metabolism but on a specific membrane protein (Plovier et al., 2017).

Real time PCR based methods

Another culture-independent probiotic enumeration strategy involves DNA-based methods such as Polymerase Chain Reaction (PCR) methods. PCR is a lab technique that amplifies a particular DNA sequence region, creating millions of copies that are easy to detect. This reaction is driven by two primers (short, single-stranded nucleic acid sequences that serve as DNA synthesis starting points), which create the two ends of the sequence to be amplified, and DNA polymerases that build a new DNA strand based on the complementary strand's information (van Pelt-Verkuil et al., 2008). PCR runs use thermal cycling to heat and cool the DNA, with each cycle composed of three steps: denaturation at around 95°C, which separates the template DNA double helix into two single strands; annealing at roughly 50–65°C, enabling the primers to bind to a complementary template sequence; and extension or elongation at approximately 72°C, allowing the polymerase enzyme to synthesize a new complementary DNA strand. Hence, the number of copies of the target sequence region theoretically doubles after each cycle (Mullis and Faloona, 1987). There are different platforms to conduct PCR, such as conventional end-point PCR, real-time PCR, and digital PCR. Both real-time PCR and digital PCR can be used for probiotic quantification.

In real-time PCR (qPCR), PCR product accumulation after each cycle can be monitored in real-time using fluorescence signals (Holland et al., 1991; Higuchi et al., 1992). The fluorescence intensity increases as the number of DNA copies increases after each qPCR cycle. Once the fluorescence signal crosses a threshold, fluorescence becomes discernible from the background, marking the quantification cycle (Cq). The Cq is the output from a qPCR run and reflects the initial amount of target DNA in a sample. DNA quantification is achieved by constructing a calibration curve using the initial target DNA amounts and the corresponding Cq values (Kralik and Ricchi, 2017). Fluorescence signals can be measured using non-specific fluorescent DNA dyes such as SYBR Green I or a fluorescently labeled oligonucleotide probe (hydrolysis probe) (Holland et al., 1991; Higuchi et al., 1992; Wilhelm and Pingoud, 2003). The hydrolysis probe chemistry enhances specificity and enables simultaneous detection of multiple targets in one PCR reaction (multiplexing) when multiple primer pairs and a combination of probes with different fluorophores are used (Elnifro et al., 2000).

Real-time PCR methods are targeted methods that can identify specific analytes, such as a particular probiotic species or strain. The capacity to detect a specific species or strain depends on the primers and hydrolysis probe used. Carefully designed primers can identify minimal genetic variations between strains, like single nucleotide

polymorphisms. High-quality genome sequences and bioinformatic tools are required to design species- or strain-specific primers and probes. This process can be especially challenging for very closely related targets, like different strains of *Bifidobacterium animalis* subsp. *lactis* (Milani et al., 2013). Notably, strain-specific assays are designed based on sequences available in public databases such as GenBank at the time of assay design. Thus, frequent updates in sequence databases with new sequence deposits may impact the specificity of strain-specific assays and may require designing new methods or modifying existing methods by targeting additional sequence regions to ensure strain level specificity.

Real-time PCR methods have been developed for probiotic strain-specific identification, such as *L. rhamnosus* GG (Ahlroos and Tynkkynen, 2009; Shehata and Newmaster, 2020), *B. animalis* subsp. *lactis* Bb12 (Solano-Aguilar et al., 2008), *B. animalis* subsp. *lactis* DSM 15954 and Bi-07™ (Shehata et al., 2021a), *L. gasseri* BNR17, and *L. reuteri* LRC (NCIMB 30242) (Shehata et al., 2021b).

Real-time PCR methods can also offer species-specific or strain-specific enumeration of probiotics (Furet et al., 2004; Achilleos and Berthier, 2013; Herbel et al., 2013). To count only viable cells, viability qPCR (v-qPCR) is used, in which probiotic cells are pretreated with a viability dye like ethidium monoazide (EMA), propidium monoazide (PMA), or modified forms of PMA (Figure 3). These viability dyes render DNA from dead cells unresponsive in a PCR reaction, achieved by their ability to enter dead and membrane-damaged cells and intercalate with their DNA (Fittipaldi et al., 2012; Shehata and Newmaster, 2021). The viability dye treatment must be optimized for each target strain because the effectiveness in inactivating DNA from dead cells differs among targets (Kiefer et al., 2020). After viability dye treatment, bead beating is typically used for DNA liberation, as commercial DNA purification kits do not yield 100% DNA recovery (Mumy and Findlay, 2004; Hansen et al., 2018), and this loss in DNA recovery can lead to an underestimation of the target quantity (Kralik and Ricchi, 2017). Viability qPCR-based methods include methods for enumerating *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 (Kramer et al., 2009; Dias et al., 2020), *Lactococcus* sp., *L. helveticus*, *L. rhamnosus*, and *B. animalis* subsp. *lactis* (Desfossés-Foucault et al., 2012), *L. plantarum* 564 and *L. paracasei* Z-8 (Radulović et al., 2012), *B. bifidum* BF-1 (Fujimoto and Watanabe, 2013), *L. paracasei* (Scariot et al., 2018), *L. rhamnosus* GG (Shehata and Newmaster, 2021), and *L. paracasei* 8,700:2 (Shehata et al., 2023).

Several studies have compared how v-qPCR counts align with PC, the standard enumeration method. Most studies found an agreement between counts determined using both methods. For instance, PC and v-PCR methods yielded relatively similar results when quantifying *L. acidophilus* LA-5 and *B. animalis* ssp. *lactis* BB-12 in lyophilised products (Kramer et al., 2009). In another study, bacterial counts of spray-dried *L. plantarum* 564 and *L. paracasei* Z-8 determined using v-PCR were not significantly different from results determined using PC methods (Radulović et al., 2012). Scariot et al. (2018) found that plate counts were comparable to v-PCR counts for *L. paracasei* viable cells in yogurt. Another study reported similar cell counts for *B. animalis* subsp. *lactis* by v-qPCR and PC on a selective media during 30 days of storage at 4°C (Dias et al., 2020). On the other hand, the counts of *B. bifidum* BF-1 determined by v-PCR method was approximately 50 times higher than plate counts on selective agar supplemented with antibiotics, which was attributed to the use of antibiotics leading to underestimation of viable cells (Fujimoto and Watanabe, 2013). The viable counts of *L. paracasei* 8,700:2 by v-qPCR were higher than the PC method, which may be attributed to cells in a VBNC state (Shehata et al., 2023).

Despite the numerous benefits of v-qPCR, it is important to acknowledge its limitations, as well as the workarounds that have been developed to address them. One inherent challenge in the v-qPCR methodology is the necessity to design specific primers and probes for each target species or strain to be quantified. The level of bioinformatic

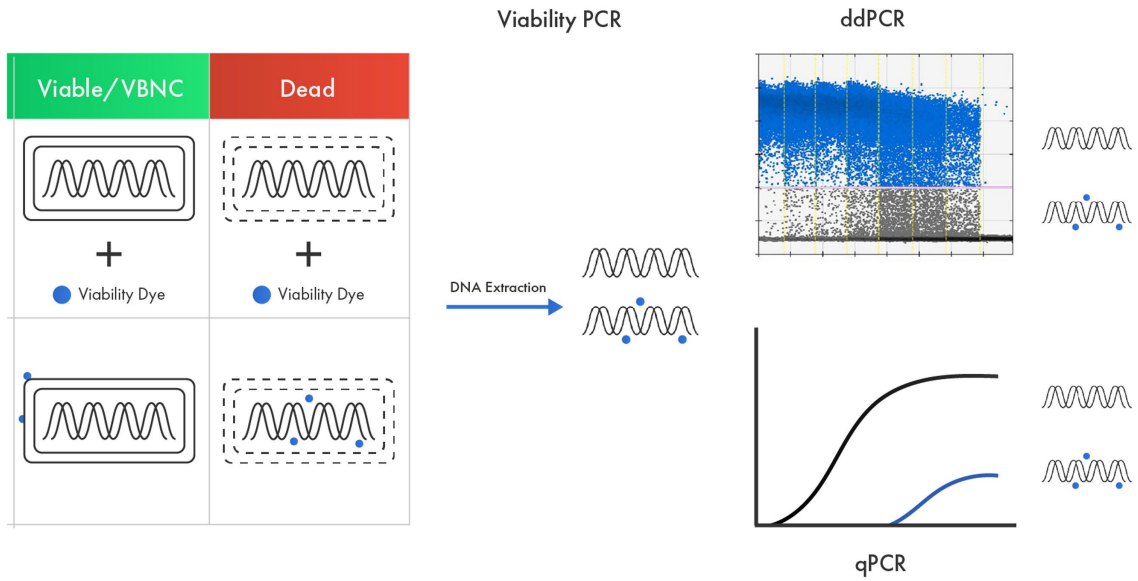

FIGURE 3
v-qPCR as an advanced tool in probiotic viability assessment based on membrane integrity. A viability dye that can enter only dead cells and membrane-damaged cells and intercalate with their DNA is used to render DNA from dead cells unresponsive in a PCR.

analysis required can increase substantially when the target strain is highly genetically similar to other strains, making precise identification more difficult.

Moreover, every v-qPCR method needs to be meticulously optimized and validated for several key parameters: specificity, sensitivity, repeatability, reproducibility, and practicability (Bustin et al., 2009; Broeders et al., 2014). This includes optimizing the viability dye treatment to ensure the detection of live cells. If not thoroughly validated, the v-qPCR method will not yield accurate quantification results. For instance, an assay that is not fully specific to the target could lead to an overestimation of the quantity, as it may inadvertently pick up other targets present in the test sample. Likewise, an assay with a reaction efficiency outside the ideal range of 90–110% could either underestimate or overestimate the target quantity.

Additionally, each assay must be validated for various sample matrices to assess their performance and confirm the absence of inhibitory effects from other components in the sample. Despite these challenges, robust assay design and comprehensive validation can effectively mitigate these limitations, enabling reliable results.

Nevertheless, v-qPCR remains a compelling choice for probiotic enumeration due to its distinct advantages over traditional culture-based methods. These benefits include higher precision, higher throughput, and a significantly shorter time to results (approximately 10 times faster), and the ability to achieve quantifications that are not possible with culture-based methods. For instance, v-qPCR can enumerate individual strains in multi-strain blends (Jackson et al., 2019; Shehata and Newmaster, 2021; Shehata et al., 2023), which is particularly valuable when evaluating product stability during shelf life. Furthermore, v-qPCR methods can quantify viable but non-culturable (VBNC) cells (Kell et al., 1998; Lahtinen et al., 2006a; Davis, 2014; Wilkinson, 2018; Gorsuch et al., 2019), NGPs and potentially some types of postbiotics. For example, v-qPCR is applicable to heat-killed cells where DNA is expected to be present, but not applicable to purified components or metabolites. Therefore, despite the complexity of the optimization and validation processes, v-qPCR offers promising potential for comprehensive and efficient probiotic enumeration.

Digital PCR based methods

Digital PCR represents a powerful technique for probiotic enumeration, building on the core principles of real-time PCR, and includes several distinct characteristics (Table 2). Like real-time PCR, digital PCR uses species-specific- or strain-specific primers along with fluorescent dyes or probes to amplify and identify specific genomic regions. What sets digital PCR apart is the unique approach it takes: it distributes the target molecules individually into many small partitions and runs PCR on each single molecule across thousands of simultaneous reactions (Vogelstein and Kinzler, 1999). This yields a positive fluorescent signal for each positive reaction, which a fluorimeter then reads. By applying Poisson's law of small numbers, the ratio of positive to negative signals can be calculated, thereby producing a quantitative value, typically in copies per microliter (Jacobs et al., 2017).

Different partitioning technologies have been developed for digital PCR, with chip-based systems (cdPCR), plate-based systems, and oil droplet-based systems (ddPCR) being among the most common. In chip-based platforms, microfluidics are used to partition individual molecules into thousands of microscopic wells on a chip or plate. These platforms then perform end-point PCR detection with fluorescence on the chip (Zhang and Xing, 2007; Sanders et al., 2011). Plate-based platforms, meanwhile, are scalable, with plates housing up to 96 individual wells partitioned in a manner similar to chip-based systems. Droplet-based platforms create thousands of microscopic droplets using a droplet generator in a process involving an immiscible fluid in oil. The target nucleic acid is randomly encapsulated in the droplets, and end-point PCR is then performed. Positive signals are processed and analyzed using Poisson statistics, yielding an absolute count of the DNA copies present (Gobert et al., 2018). The evolution of instrumentation has facilitated the ability to multiplex up to five targets in a single reaction.

Similar to real-time PCR, the digital PCR method requires thorough optimization and validation to ensure specificity, sensitivity, repeatability, reproducibility, and practicability of the reaction (Broeders et al., 2014). ddPCR has shown greater sensitivity than real-time PCR in detecting low bacterial concentrations in dairy products spiked with bacteria (Kim et al., 2023). Additionally, digital PCR has demonstrated a higher tolerance to PCR inhibitors, which makes it a preferred choice for detecting low levels of target organisms in complex matrices like soil and wastewater (Rački et al., 2014).

Studies comparing the performance characteristics of real-time PCR and digital PCR have shown good linearity and high coefficients of determination for both platforms when quantifying *Lactiplantibacillus plantarum* subsp. *plantarum* in raw material and food matrices. Digital PCR displayed a 10-fold lower limit of detection, suggesting superior sensitivity, but demonstrated limitations when quantifying high probiotic concentrations (Choi et al., 2022). Comparative analyses of v-qPCR and v-ddPCR on *L. paracasei* 8700.2 revealed very high correlation and no significant differences (Shehata et al., 2023).

One key advantage of dPCR over qPCR in probiotic enumeration is that the results generated are not influenced by reaction efficiency or standard curve calibration, leading to enhanced precision (Hindson et al., 2013; Raurich et al., 2019). Several studies have reported improved accuracy and reproducibility with dPCR compared to qPCR (Pinheiro et al., 2012; Nshimyimana et al., 2019). The advantages of v-qPCR, including better precision, reduced labor, higher throughput, species- or strain-specific enumeration based on primer specificity, and the ability to detect and quantify VBNC states, can be directly translated to dPCR as both techniques share similar principles.

However, some of the same limitations apply to dPCR as well, such as the need for primer specificity, which entails comprehensive bioinformatic analysis. Each method must be individually optimized and thoroughly validated to ensure confidence in the results produced. Despite these challenges, the promise of dPCR for reliable and accurate enumeration of probiotics continues to generate interest.

Culture-independent methods in the probiotic industry

New culture-independent methods are proving to be particularly beneficial in research and development stages of product design, especially in experimental settings where a multitude of microorganisms are examined for specific functions (Supplementary Table S1). Clinical

TABLE 2 Comparing real-time PCR and digital PCR for probiotic enumeration.

	Real-time PCR	Digital PCR
Taxonomic resolution	Strain-specific or species-specific	Strain-specific or species-specific
Primer design	Required	Required
Robust validation	Required	Required
Multiplexing	Yes	Yes
Throughput	Up to 384-well	Up to 96-well
Calibration curve	Required	Not required
Inhibitors	Prone to inhibitors	High tolerance to inhibitors
Effect of PCR efficiency on results	Results are affected by PCR efficiency	Results are not affected by PCR efficiency
Real-time monitoring	Yes	No
Dynamic range	Broad dynamic range	Narrower dynamic range
Equipment and running costs	Economical	Less economical

studies play a pivotal role in demonstrating the efficacy of probiotics, and accurate probiotic cell count, or concentration, is fundamental to correlate the health benefit with the delivered dose. However, only about 42% of global clinical trials on probiotics accurately reported the dosage of the tested products, generally in CFUs (Dronkers et al., 2020). Furthermore, most studies do not specify the point at which the product concentration is measured: at manufacturing (Quantity by Input), point of consumption, or end of shelf life (Goldman, 2019).

Unfortunately, CFU count is inherently flawed when it comes to standardization and comparison between various biological isolates and experiments. CFUs provide insight into a potentially viable subgroup of micro-organisms capable of forming a colony, but do not offer a complete picture of the entire bacterial population within a sample. It's important to consider that humans consume the entire spectrum of bacterial population heterogeneity (Fiore et al., 2020). This varied heterogeneity of a given probiotic or potential probiotic may contribute to diverse functional characteristics. This raises compelling questions: Should consumers be informed about this complexity?

Furthermore, the post-experimental, not real-time, correlation with CFU makes comprehensive analysis and comparison of published literature rather complex (Davey, 2011). Even at the industrial production level, real-time monitoring of cell number and heterogeneity can fine-tune the process, aiming for the highest possible viability in the finished product (Supplementary Table S1). However, achieving a one-to-one correlation between plate count (CFUs) and other viability proxies is challenging and unlikely, especially when the product is composed of multiple microorganisms, each with its specific characteristics and industrial process, or when monitored for its shelf-life (Foglia et al., 2020; Visciglia et al., 2022).

From a commercial perspective, the insistence on strain-level identity poses challenges for companies formulating probiotic products, especially when dealing with multi-strain blends or complex formulations. Ensuring that each strain included in a product is individually characterized and that their combined effects have been clinically validated can be a daunting and costly task. From this perspective, it seems easier to formulate products with a single strain or a few easily identifiable strains. The validation process would realistically be more suited to the producers rather than the Contract Manufacturing Organization (CMO), which potentially formulates sourcing from different producers.

The ever-evolving probiotic market and growing interest from regulators and large companies put pressure on the need for more robust quality controls. However, lack of harmonized regulations and market diversification results in a multitude of products, with the only quality information often being the label details. In a future scenario where strain discrimination and enumeration become mandatory, considerations must be given to smaller companies with limited resources and contract manufacturing organizations (CMOs) that primarily work with blends of various strains. In fact, with a requirement to demonstrate the qualitative-quantitative composition at the end of the shelf-life, there might be a progressive move away from multi-strain products toward simpler formulas. A balanced approach might be to promote species level identification and enumeration while encouraging the achievement of strain specificity. The key is to consider product design within the available methodologies.

Culture-independent methods also offer alternative solutions for enumerating heat-killed bacteria, which can provide significant insights into the process of tyndallization and its potential improvements to achieve higher yields without damaging cells (Supplementary Table S1). By using dyes to differentiate live from dead cells based on membrane integrity, these methods allow for a more accurate yet rapid approach similar to the counting chamber.

Culture-independent methods have the potential to facilitate market access, especially for hot climate zones (Zone IVa and Zone IVb with 30°C and 65 and 75% Relative Humidity (RH) respectively) such as Asia, India, Latin America, and North Africa. To ensure the guaranteed concentration at the end of the product's shelf life, manufacturers often increase the initial concentration two to three-fold, or even higher. This increase, often referred to as "overage," is typically determined through stability studies conducted on the final product under recommended storage conditions (Roe et al., 2022). In fact, designing products with a CFU target at the end of the shelf-life compared to alternative methods based on membrane integrity implies higher overages than in temperate markets (Foglia et al., 2020; Visciglia et al., 2022). These overages are inherently limited by factors such as space, homogeneity, etc., and notably, price – a factor that significantly impacts market access in less affluent countries.

It appears that rather than debating the correlation between methods, especially the ones that probe viability by different means, the focus should be on improving the procedures to track and report experimental and clinical data. Enhancing these procedures and providing regulatory framing for them is critical. This approach would ensure that accurate, reliable data is available for all stakeholders in the probiotic industry. Harmonizing these procedures globally could also provide a standard against which all probiotic products are measured, enhancing the probiotic industry's credibility and fostering trust in these products' efficacy and safety.

Conclusion

In conclusion, the field of probiotics research and production has seen remarkable advances over the past decades. However, challenges still exist in the methods used for quantifying and characterizing these beneficial microorganisms. Traditional culture-dependent methods, such as CFU enumerations and optical density measurements, lack the precision and the comprehensiveness required for standardization and comparison across various studies and strains. Culture-independent methods, including flow cytometry and PCR-based techniques, have emerged as promising alternatives that provide real-time, strain-specific data and offer a deeper understanding of the heterogeneity and viability of bacterial populations. The principles of culture-independent methods align with the official probiotic definition (Hill et al., 2014), defined as: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.," as they produce outputs indicative of cellular viability.

These methods, however, are not without their challenges, particularly when it comes to the development of strain-specific markers, antibodies for flow cytometry and primers and probes for qPCR and dPCR. Creating a central depository for commercial strains, physical materials and whole genome sequences, would be of great benefit when evaluating the strain specificity of the developed strain-specific markers. As the probiotics field continues to mature, it is critical that the scientific community and industry stakeholders work collaboratively to further refine these methods, champion their adoption, and work toward the establishment of global, harmonized standards. This will not only enhance the reproducibility and comparability of research data, but also ensure the delivery of high-quality, well-characterized probiotic products to consumers, underpinning their confidence in the market and driving the growth of this important sector. The advancement and refinement of these techniques have potential implications far beyond the probiotics field, heralding a new era in microbial research and its numerous applications across various domains of human health.

Author contributions

M-EB: Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing. AB: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. MP: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. HS: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

M-EB is the president of Nutrapharma Consulting Services and was involved with external laboratories as a consultant. AB was employed by Eurofins USA. MP was employed by Probiotical Research s.r.l. HS was employed by Purity-IQ Inc.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1304621/full#supplementary-material

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Identification and quantification of viable *Lacticaseibacillus rhamnosus* in probiotics using validated PMA-qPCR method

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The identification and quantification of viable bacteria at the species/strain level in compound probiotic products is challenging now. Molecular biology methods, e.g., propidium monoazide (PMA) combination with qPCR, have gained prominence for targeted viable cell counts. This study endeavors to establish a robust PMA-qPCR method for viable *Lacticaseibacillus rhamnosus* detection and systematically validated key metrics encompassing relative trueness, accuracy, limit of quantification, linear, and range. The inclusivity and exclusivity notably underscored high specificity of the primers for *L. rhamnosus*, which allowed accurate identification of the target bacteria. Furthermore, the conditions employed for PMA treatment were fully verified by 24 different *L. rhamnosus* including type strain, commercial strains, etc., confirming its effective discrimination between live and dead bacteria. A standard curve constructed by type strain could apply to commercial strains to convert qPCR C_q values to viable cell numbers. The established PMA-qPCR method was applied to 46 samples including pure cultures, probiotics as food ingredients, and compound probiotic products. Noteworthy is the congruity observed between measured and theoretical values within a 95% confidence interval of the upper and lower limits of agreement, demonstrating the relative trueness of this method. Moreover, accurate results were obtained when viable *L. rhamnosus* ranging from 10^3 to 10^8 CFU/mL. The comprehensive appraisal of PMA-qPCR performances provides potential industrial applications of this new technology in quality control and supervision of probiotic products.

KEYWORDS

probiotics, *Lacticaseibacillus rhamnosus*, identification, viable cell quantification, PMA-qPCR method, validation

1 Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (The World Health Organization, 2001). Accurate identification and quantification of live probiotics are essential to ensure production process control and quality. *Lacticaseibacillus rhamnosus*, as one of the most popular *Lactobacillus* strains, has been widely studied because of its safety profile and desirable features of conventional probiotics

(Kalliomäki et al., 2001; Mathipa-Mdakane and Thantsha, 2022; Xavier-Santos et al., 2022). *L. rhamnosus* species, e.g., LGG, HN001 etc., possess great market value in food industry attributed to their excellent fermentation performance and probiotic effect. Characteristics of tolerance to acid and bile as well as good growth ability allow them to survive and thrive within the gastrointestinal tract (De Champs et al., 2003). *L. rhamnosus* is able to form biofilms displaying as an excellent mucus-adhering *Lactobacillus* strain that enhance its ability to protect and strengthen the cytoskeleton integrity to inhibit pathogen colonization (Segers and Lebeer, 2014; Martín et al., 2019). Additionally, *L. rhamnosus* has been well documented for its clinical benefits. Many studies have reported on the use of *L. rhamnosus* GG for the prevention and treatment of gastrointestinal infections and diarrhea in children (Szajewska et al., 2007, 2011).

Compound probiotics have been applied to food, dietary supplements, infant formula, medical food, cosmetics and pharmaceuticals fields due to their generally recognized health benefits (Quin et al., 2018). Presently, lots of studies demonstrate that the efficacy of probiotics is strain-specific and disease-specific (McFarland et al., 2018). Campana et al. (2017) indicated that individual Lactic Acid Bacteria (LAB) strains showed strain-specific probiotic properties to inhibit the invasion of intestinal pathogens to Caco-2 cells. Kekkonen et al. (2008) studied a milk-based drink or a placebo drink containing *L. rhamnosus* GG (LGG), *Bifidobacterium animalis* ssp. *Lactis* Bb12 (Bb12), or *Propionibacterium freudenreichii* ssp. JS (PJS) and found that probiotics exhibited strain-specific anti-inflammatory effects in healthy adults. Additionally, the health benefits of probiotics are closely related to the amount of viable cells intake. However, viability of probiotic bacteria mostly depends on the bacterial strains, preservation methods, fermentation, and storage conditions (temperature, oxygen) (Odooli et al., 2018). Thus, it is necessary to monitor and selectively enumerate specific viable cells to ensure the stable quality of probiotic products. Currently, quantification of LAB is mainly by heterotrophic plate count methods. However, culture-based technologies are usually time-consuming (Odooli et al., 2018) and difficult to distinguish or selectively enumerate probiotics due to similar growth requirements and biochemical characteristics of multiple probiotic species in products (Ashraf and Shah, 2011). Therefore, development of species-specific detection methods for probiotic identification and enumeration are great meaningful for manufacturers to speeding up products releasing time, government product supervision and consumer rights protection.

Nucleic acid-based methods such as quantitative PCR (qPCR) have been widely applied to fields of biology, food science, environmental science for microorganisms detection as it is rapid, specific, and highly sensitive (Ceuppens et al., 2010; Portilho et al., 2018; Guo et al., 2020). However, its inability to distinguish between viable and dead cells limits its application. Fortunately, a novel dye named propidium monoazide (PMA) could be coupled with qPCR (PMA-qPCR) for viable cells quantification through selective staining based on membrane integrity (Nocker et al., 2006). The PMA dye can only penetrate membrane damaged cells and covalently cross-link with DNA during photolysis, thus preventing PCR amplification of the DNA. Consequently, DNA from membrane-intact cells could be selectively amplified by the following PCR procedure (Chiao et al., 2014; Scariot et al., 2018). The PMA-qPCR shows its advantages for selectively detecting individual strains in compound probiotic products based on species specific primer design. Several crucial factors could affect the accurate numeration of viable cells by PMA-qPCR method, such as DNA extraction method (McOrist et al., 2002; García-Cayuela et al., 2009), PMA treatment conditions (Miotto et al., 2020), construction of standard curves (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018), bacterial density (Zhu et al., 2012; Tantikachornkiat et al., 2016; Lu et al., 2019), etc. All these factors should be considered and confirmed its suitability to the target strains to ensure accurate results. Presently, the PMA-qPCR method has been applied to monitor viable cells of specific LAB during fermentation process or shelf life (Berezhnaya et al., 2021; Gagnon et al., 2021; Yang et al., 2021).

Microbiological methodologies necessitate the comprehensive evaluation and validation of their performance parameters, as recommended by established standards such as ISO 16140-6 (2019); United States Pharmacopeia (2023). Notably, the PMA-qPCR method offers a dual capability of enabling specific microbial identification at the genus, species, or strain level, along with the precise enumeration of viable cells. Ensuring the precision of detecting target microorganisms necessitates the rigorous validation of primer inclusivity and exclusivity. It is worth noting, however, that numerous studies frequently referenced primer sequences from existing literatures, yet often omit subsequent validation steps or inadequately encompass a comprehensive spectrum of strains, thereby leading to erroneous outcomes, such as false positives or negatives. Quantitative methodologies, including the PMA-qPCR method, demand meticulous assessment of performance parameters such as accuracy, precision, specificity, quantification limit, linearity, and ruggedness (Broeders et al., 2014). These metrics hold undeniable significance in gauging the robustness and dependability of the established methods. Although the PMA-qPCR technique has garnered widespread application across diverse sectors, encompassing fields such as food, environment, and clinical analysis, a conspicuous void remains regarding the comprehensive evaluation of its efficacy in accurately quantifying specific target species.

In this study, we developed and systematically evaluated a precise PMA-qPCR method for quantifying viable *L. rhamnosus*. Validation of the *L. rhamnosus*-specific primer included comprehensive inclusivity and exclusivity assessments through whole-genome sequence blast and strain collection at various taxonomic levels. The efficacy of PMA treatment conditions was confirmed using 24 *L. rhamnosus* strains, ensuring non-interference with viable cell PCR amplification while effectively inhibiting non-viable cells. A standard curve relating qPCR C_q values to viable bacteria numbers was established. The established PMA-qPCR method was then applied to diverse samples, revealing relative trueness, accuracy, linear, limit, quantification range. This study successfully established a robust PMA-qPCR tool for quantifying viable *L. rhamnosus* in heterogeneous samples, with implications for assessing probiotic product viability and quality.

2 Materials and methods

2.1 Inclusivity and exclusivity of primer tests

The *L. rhamnosus* specific primer sequence was Lrh-F: TGC TTG CAT CTT GAT TTA ATT TTG; Lrh-R: GGT TCT TGG ATY TAT GCG GTA TTA G (Byun et al., 2004; Mansour and Ismail, 2016). Strains of *L. rhamnosus* CICC 6224^T, *L. rhamnosus* HN001, *L. rhamnosus*

UALr-06, *Bifidobacterium animalis* subsp. *lactis* Bi-07, *B. lactis* HN019, *B. lactis* UABLa-12, *Bifidobacterium breve* M-16 V, *Bifidobacterium longum* UABL-14, *Lactobacillus acidophilus* DDS-1, *Limosilactobacillus fermentum* CECT5716, *Lactiplantibacillus plantarum* 299 V, and three products that contain *L. rhamnosus* were firstly used to validate the specificity of the primer through PCR conduction and gel electrophoresis. Positive amplification was observed on the DNA template from the three *L. rhamnosus* and the three products. No amplification occurred on the non-target strains. These results initially demonstrated the specificity of the primer to *L. rhamnosus*. Then, systemic inclusivity and exclusivity validation were performed.

Inclusivity, defined as the detection of target strains (ISO 16140-2, 2016), was firstly assessed *in silico* using the Basic Local Alignment Search Tool (BLAST®)[1]. The whole-genome sequences (WGS) of 35 *L. rhamnosus* were downloaded from NCBI website[2]. All these WGS are from bacteria including type strain, commercial strains, and others. Then, primers of *L. rhamnosus* were aligned with WGS through Primer-BLAST on NCBI[3].

Inclusivity of primers was further tested by PCR amplification using the DNA template from 24 different *L. rhamnosus* strains (Supplementary Table S1). All the strains were firstly identified by MALDI-TOF (MBT Smart, Bruker) or 16S rRNA sequencing method to ensure the correct classification. For PCR assay (PCR system 9,700, ABI, USA), thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 32 s and 72°C for 25 s, followed by a final extension step of 72°C for 10 min. The amplification products were analyzed with electrophoresis on 1% agarose gel and examined under UV light (Bio-Rad Laboratories Pte. Ltd., Singapore).

Exclusivity, is defined as the non-detection of non-target strains (ISO 16140-2, 2016). Similar with inclusivity, exclusivity was also firstly assessed *in silico* using the Basic Local Alignment Search Tool. The 80 WGS of 25 *Lacticaseibacillus* on species level, 281 WGS of 30 *Lactobacillaceae* on genus level, and 72 WGS of the 35 strains in Chinese list of cultures that can be used for food were downloaded from NCBI website. Then, primers of *L. rhamnosus* were aligned with WGS through Primer-BLAST on NCBI.

Thirty-five strains in Chinese list of cultures that can be used for food were collected and further identified by MALDI-TOF or 16S rRNA sequencing method (Supplementary Table S2). The PCR amplification was conducted using the primer and the DNA templates of these strains. Then, PCR products were identified by 1% agarose gel electrophoresis.

2.2 Propidium monoazide treatment

Twenty-four pure culture strains of *L. rhamnosus* were chosen to verify the applicability of the PMA treatment conditions used in this study. When employing PMA, qPCR amplification of DNA from viable cells should remain largely unaffected, while DNA from dead cells should be completely inhibited. Consequently, live, and dead bacterial groups of *L. rhamnosus* were obtained for each strain, respectively. All *L. rhamnosus* strains were initially revived on MRS solid medium at 37°C for 48 h under anaerobic conditions. Subsequently, they underwent an additional 48 h of incubation after being inoculated onto MRS solid medium. Given that all *L. rhamnosus* strains were incubated twice on MRS solid medium under optimal culture conditions, most of the bacteria were presumed to be highly active. The resulting cultures were resuspended and diluted using a 0.85% sodium chloride solution. Concentrations of the resuspended bacteria were adjusted to an optical density at 620 nm (OD_{620}) of 0.3–0.5, corresponding to approximately 10^8 CFU/mL, a measure further validated by plating on MRS agar plates. Subsequently, the bacteria were categorized into live and dead groups. For the dead group, the bacteria at 10^8 CFU/mL underwent a 20-min heat treatment at 80°C. Validation on MRS solid medium revealed no observable growth of viable cells, thus confirming the successful generation of the dead group bacteria. Both live and dead bacterial suspensions with approximate 10^8 CFU/mL were divided into PMA treatment and non-treatment groups.

PMA (Biotium, USA) solution was dissolved in ddH_2O to create a 20 mmol/L stock solution and 1.25 μL of that was added to 500 μL of cell suspensions to achieve final concentrations of 50 μM. The mixed samples were then placed in the dark for 5 min to allow PMA to penetrate dead cells and bind to the DNA. The treated samples were exposed to a 60 W LED light source (Biotium, USA) for 15 min. Then, both the bacterial suspensions of PMA treatment and non-treatment group were centrifuged at 12,000 *g* for 15 min. The harvested bacterial pellets were subjected to DNA extraction.

2.3 Genomic DNA extraction

In this study, total genomic DNA were extracted using the bead-beating (BB) method. The BEAD RUPTOR 12 (OMNI International, USA) was used as a mechanical cell disruptor. The (Zirconia/Silica) 0.1 mm beads (0.25 g) were placed in a screw-cap 2.0 mL sample tubes and both were then autoclaved. Bacterial suspension within 200 μL ddH_2O were added into the tubes. Then, samples were lysis for 12 s at the 6.0 m/s speed setting using the bead mill homogenizer and centrifuged (15 min, 12,000 g). Fifty microliter supernatants containing DNA were taken and added into 1.5 mL sterile tubes for qPCR assay.

2.4 Quantitative PCR assay

The qPCR assays were performed on an ABI 7500 Fast real-time PCR system. Each 25 μL reaction mixture contained 12.5 μL of 2× SYBR Green premix (TaKaRa, Japan), 1 μL of 10 μM each primer, 0.5 μL ROX, 5 μL DNA template, and 5 μL ddH_2O. DNA samples, negative DNA control (sterile water) was included in triplicate in each qPCR run. The thermal cycle program was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 55°C for 32 s, 72°C for 25 s.

2.5 Construction of PMA-qPCR standard curves

The standard curve between viable cell numbers and qPCR C_q values was made. Type strain of *L. rhamnosus* CICC 6224^T was initially

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2 https://www.ncbi.nlm.nih.gov/genome/

3 https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

revived on MRS solid medium at 37°C for 48 h under anaerobic conditions. Subsequently, they underwent an additional 48 h of incubation after being inoculated onto MRS solid medium. Samples were resuspended and then diluted to approximate 10^8 CFU/mL in 0.85% sodium chloride solution. On one hand, viable cell numbers were enumerated by culture-based method. On the other hand, the 10^8 CFU/mL bacteria solution were treated by PMA and DNA were extracted as described above. DNA solutions were diluted 10-fold in series. The diluted DNA was used to run the qPCR assay and C_q values were obtained of each dilution. Then, the standard curve between C_q values and viable cell numbers were constructed (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018).

2.6 Quantification of viable *Lacticaseibacillus rhamnosus* in a variety of samples using PMA-qPCR method

The established PMA-qPCR method was applied to detect viable *L. rhamnosus* in pure cultures, probiotics as food ingredients, and probiotic products to validate the performance of this method. The concentrations of 24 fresh cultured *L. rhamnosus*, including CICC 6224^T, CICC 6142, CICC 20253, CICC 25096, CICC 6155, CICC 20257, CICC 20255, CICC 6143, CICC 20258, CICC 20259, CICC 21769, CICC 20061, R0011, HN001, UALr-06, MP108, GR-1, NJ551, TR08, Lr-G14, FloraActive32550, NCC 4007, FloraActive19070, LGG, were adjusted to approximate 10^8 CFU/mL followed by PMA treatment, DNA extraction, and qPCR amplification. Meanwhile, numbers of these pure cultures were detected by the culture-based method to get the theoretical values of each strain.

The 11 probiotics as food ingredients consisted of singular *L. rhamnosus* strains (e.g., HN001, R0011, MP108, etc.) or combinations with other probiotics and lactic acid bacteria, with simple excipients like maltodextrin. The 11 compound probiotic products typically featured a more complex microbial composition, incorporating one or more *L. rhamnosus* strains in combination with one or more other probiotics and lactic acid bacteria. These compound products typically featured more intricate formulations, incorporating complex excipients such as common additives (e.g., maltodextrin, resistant dextrin, etc.), prebiotics (e.g., fructooligosaccharides, erythrosis, stachyose, etc.), and botanical ingredients (e.g., blueberries, cranberry powder, etc.). In the context of *L. rhamnosus* probiotics as food ingredients and products, a quantity of 25 g was amalgamated with 225 mL of a 0.85% sodium chloride solution to yield a bacterial suspension. Subsequently, the overall bacterial concentration was meticulously adjusted to approximately 10^8 CFU/mL. This prepared suspension then underwent the PMA treatment protocol. Following a centrifugation step at 12,000 *g* for a duration of 15 min, the resultant pellet underwent a DNA extraction process and qPCR amplification to get the measured values of viable *L. rhamnosus*. Theoretical values of viable *L. rhamnosus* in these samples were obtained according to products claims.

Moreover, the PMA-qPCR method was applied to detect samples encompassing a wide range of concentrations, spanning from low to high levels of *L. rhamnosus*. This experimental approach involved the creation of samples by combining viable *L. rhamnosus* cells with nonviable cells of *Bifidobacterium longum* subsp. *infantis*. In each sample, a consistent count of nonviable *B. infantis* cells was maintained at approximate 10^8 CFU/mL, while varying concentrations of viable *L. rhamnosus* cells were introduced, namely 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/mL. The quantification of viable *L. rhamnosus* cells in all samples was conducted using the established PMA-qPCR method. To assess the precision of the PMA-qPCR method, 10 replicates were performed using distinct aliquots from the same sample. Simultaneously, the culture-based method was employed to enumerate viable *L. rhamnosus* cells, obtaining the theoretical values for each sample. A comparative analysis involving linear regression was employed to examine the relationship between the theoretical and PMA-qPCR measured values, thus elucidating their linear correlation. Notably, this analysis facilitated the determination of both the quantification limit and the range of the PMA-qPCR method, further enhancing its practical applicability.

2.7 Statistical analysis

The *T*-test method, conducted using Excel (Microsoft Office 16), was employed to assess the significance of PMA concentrations on viable cells between the treated and non-treated groups. Additionally, the *T*-test was applied to analyze the significance between the theoretical and the measured values of all 46 samples. A significance level of $p < 0.05$ was considered to indicate a significant difference. The Bland–Altman method was applied to assess the trueness of PMA-qPCR method by R software (R version 4.2.2).

3 Results

3.1 Inclusivity and exclusivity of primer

The evaluation of the inclusivity and exclusivity was firstly assessed *in silico* by performing a BLAST analysis. Based on this initial test, no significant similarity with non-target microorganisms was observed. Subsequently, the specificity of the primer for detecting *L. rhamnosus* was evaluated using a PCR assay in which 24 target strains of *L. rhamnosus* and 35 non-target strains were tested. The results demonstrated that only *L. rhamnosus* strains produced a positive amplification signal, indicating that the primer was highly specific for *L. rhamnosus* and did not cross-react with other bacteria (Figure 1). The specific primer enables targeted detection of *L. rhamnosus* within multi-strain products resulting positive identification.

3.2 Evaluation of PMA treatment conditions on live and dead *Lacticaseibacillus rhamnosus*

The effectiveness of PMA treatment is associated with the bacterial density (Zhu et al., 2012; Tantikachornkiat et al., 2016; Lu et al., 2019), indicating that there is a specific range of bacterial density corresponding to an optimal PMA treatment conditions. In terms of experimental practicality, a total bacterial concentration of 10^8 CFU/mL is convenient for centrifugation to acquire bacteria and facilitate subsequent DNA extraction operations. Therefore, the choice of a bacterial concentration of 10^8 CFU/mL was made to determine the

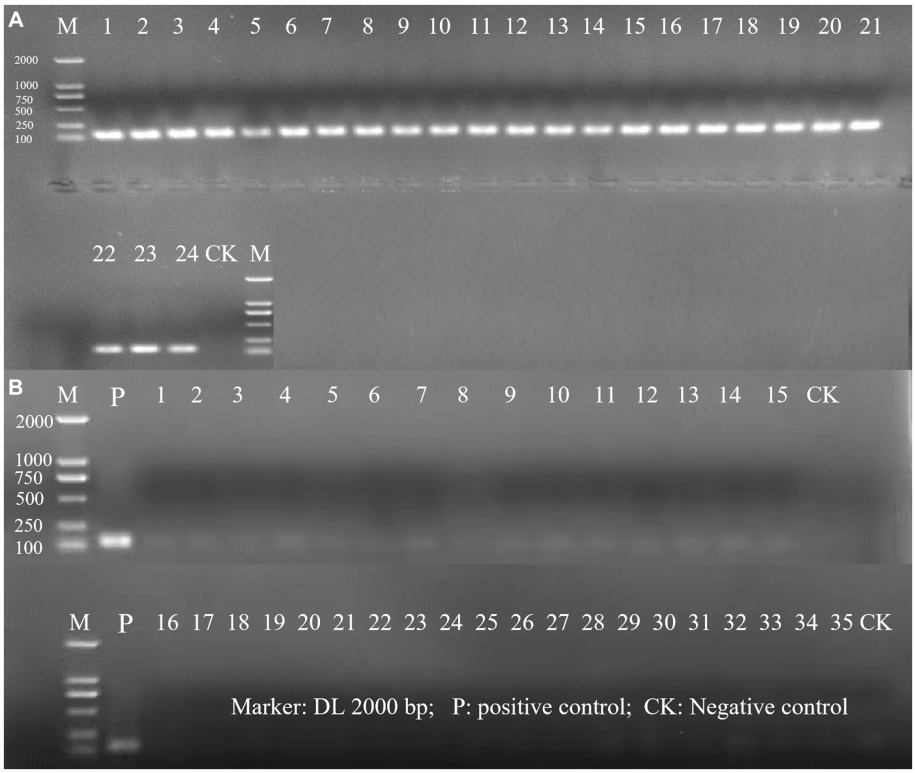

FIGURE 1
The PCR amplification of inclusivity and exclusivity assay visualized on an agarose gel. **(A)** Inclusivity assay with 24 target strains. The strain serial number is the same as in Supplementary Table S1; **(B)** Exclusivity assay with 35 non-target strains. The strain serial number is the same as in Supplementary Table S2.

optimal PMA treatment conditions corresponding to this specific bacterial density.

To evaluate the impact of PMA treatment on viable *L. rhamnosus* cells, 24 different strains were treated with and without PMA, with each strain containing an approximate concentration of 10^8 CFU/mL. The resulting C_q values in both groups were statistically analyzed using the *T*-test method. The *p* values ranged from 0.065 to 0.676 ($p > 0.05$) indicating no significant difference between the treated and non-treated groups for each strain (Figure 2A).

The PMA treatment efficiency was further evaluated when the total 10^8 CFU/mL bacteria are all dead cells. The 24 different strains of *L. rhamnosus*, each with a concentration of 10^8 CFU/mL, underwent heat inactivation and were subsequently divided into PMA treatment and non-treatment groups. The inhibition efficiencies of PMA treatment on qPCR amplification of dead cells from each *L. rhamnosus* strain were calculated. As shown in Figure 2B, the inhibition efficiency of each strain ranged from 99.764 to 99.994% (nearly 100%), indicating that qPCR amplification of DNA from dead cells was almost inhibited.

The PMA treatment conditions, involving a final concentration of 50 μM, a dark incubation period of 5 min, followed by light exposure for 15 min, have been identified as optimal for distinguishing between viable and dead cells of *L. rhamnosus* including the type strain, commercial strains, etc. These conditions are specifically designed to be effective under a total bacterial density of approximate 10^8 CFU/mL. When applying this PMA treatment conditions to actual probiotic samples, it is recommended to use the optical density at 620 nm (OD_{620}) method to adjust the total bacterial concentration to $OD_{620} = 0.3–0.5$, corresponding to an approximate concentration of 10^8 CFU/mL.

3.3 Establishment of a standard curve

A standard curve was generated by performing 10-fold serial dilutions of DNA extracted from viable *L. rhamnosus* CICC 6224^T cells, with culturable numbers precisely quantified at a concentration of 10^8 CFU/mL (Figure 3) (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018). The generation of the standard curve involved obtaining a minimum of five concentration gradient points following the aforementioned qPCR procedure. Notably, the standard curve for DNA demonstrated a robust linear correlation ($R^2 = 0.998$) within the approximate range of 10^3–10^8 genome equivalents per reaction. The high R^2 value (> 0.99) indicated exceptional linearity of the qPCR assay (Elizaquível et al., 2012). Moreover, a slope of −3.17, falling within a reasonable theoretical range, was derived, and the amplification efficiency (*E*) was calculated as 107.01% using the formula $E = 10^{(-1/\text{slope})} - 1$ (Fricker et al., 2007). This efficiency value is considered acceptable as it falls within the range of 90–110% (Li and Chen, 2013). These outcomes further validate the sensitivity and suitability of the primer employed for detecting *L. rhamnosus*. By utilizing the standard curve, it became feasible to convert the C_q values of *L. rhamnosus* samples into CFU equivalent cells.

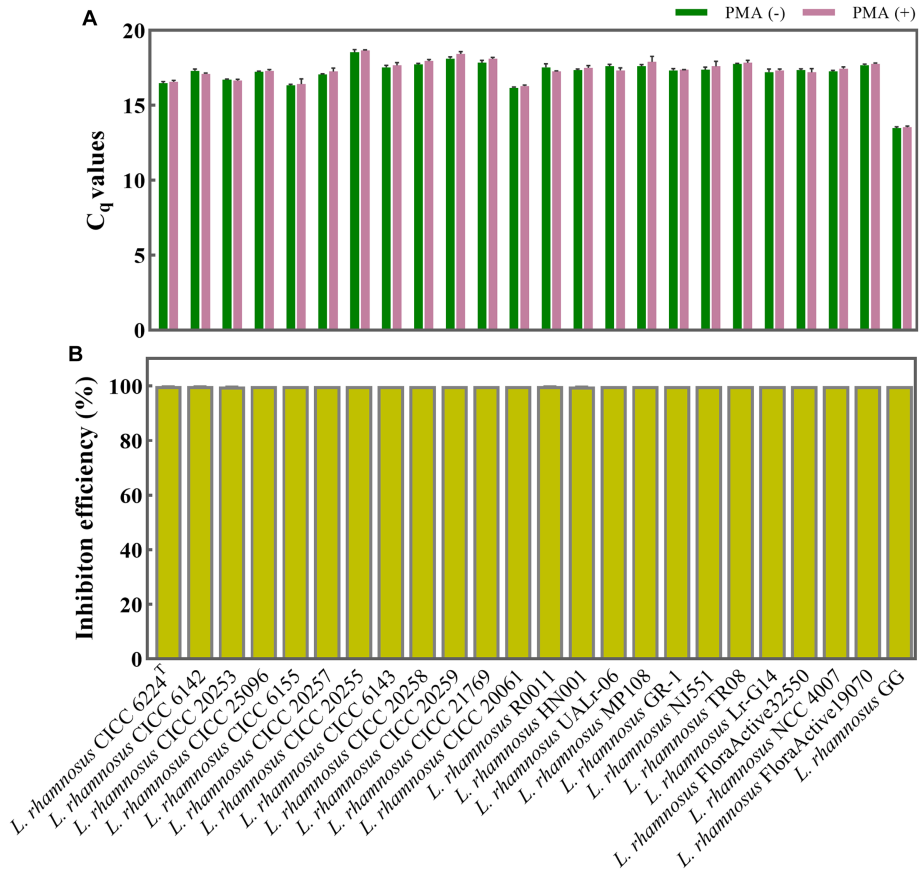

FIGURE 2
Evaluation of PMA treatment conditions for discriminating between viable and dead *L. rhamnosus* cells. **(A)** Assessment of the impact of PMA treatment on qPCR amplification of viable *L. rhamnosus* cells from 24 different strains. PMA (+) and PMA (–) represent samples treated with and without PMA, respectively. **(B)** Determination of the inhibition efficiency of PMA on dead *L. rhamnosus* cells.

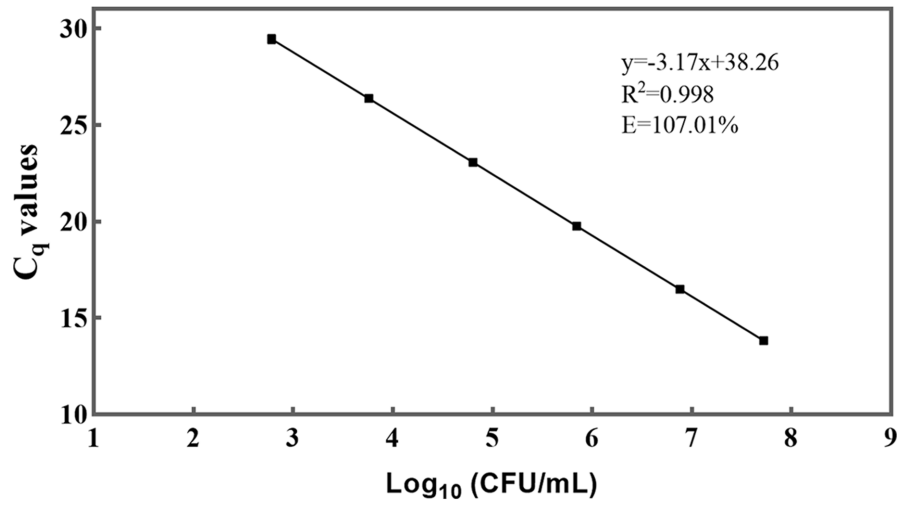

FIGURE 3
A standard curve was generated for the PMA-qPCR assay targeting *L. rhamnosus*. The plotted values on the curve represent the mean values and standard deviations obtained from three replicate tests. The C_q = Quantification Cycle.

3.4 Performance evaluation of the established PMA-qPCR method

3.4.1 Applications of PMA-qPCR method to different sample types

Initially, a line was plotted using the data obtained from each sample, allowing for a visual assessment of the level of agreement between the theoretical and measured values (Figure 4A). Most data points closely aligned with the line for each analyzed sample, indicating a high level of concordance between the theoretical and measured values (Figure 4A). Then, the results obtained were further analyzed using the Bland–Altman method according to ISO 16140-2:2016 (E). The average of each pair of theoretical and measured values were determined and the difference (D) between the values were also calculated. Compute the average difference $\bar{D}$ for each sample, the standard deviation of differences S_D and the limits of agreement using the formula $\left[\bar{D} \pm T \cdot S_D \sqrt{1+\frac{1}{n}}\right]$, Where n is the number of data pairs, T is the percentile of a student-t distribution for β the chosen probability of the interval and (n-1) degree of freedom, that is: $T_{\left(\frac{1-\beta}{2}\right);(n-1)}$. The individual sample differences against the mean values were plotted on a graph that shows the line of identity (zero difference), the line of bias, and the upper and lower 95% confidence limits of agreement (CLs) of the bias (Figure 4B).

The mean bias of the 46 samples was −0.003 Log_{10}. The lower and upper limit of agreement were −0.442 and 0.447 Log_{10}. When considering the 95% confidence limits, they were −0.558 and −0.327 Log_{10}, 0.331 and 0.562 Log_{10}, respectively (Figure 4B). The differences between the measured and theoretical values were −0.356 ~ 0.555, −0.425 ~ 0.500, and −0.413 ~ 0.087 Log_{10} in pure cultures, probiotics as food ingredients, and probiotic products, respectively. Evident is the remarkable coherence between the measured and theoretical values, consistently falling within the 95% confidence interval demarcated by the upper and lower limits of agreement. Furthermore, a T-test was employed to analyze the significance between the theoretical and measured values of all 46 samples. The resulting p value of 0.79 ($p > 0.05$) suggests no significant difference between the theoretical and measured groups within the 46 samples. These compellingly underscores the precision and reliability inherent in the PMA-qPCR method for the detection of viable cells across a diverse range of applications, including pure cultures, probiotics as food ingredients, and composite probiotic products.

3.4.2 Applications of PMA-qPCR method to samples with different concentrations of *Lacticaseibacillus rhamnosus*

Samples with different concentrations of viable *L. rhamnosus* were prepared and detected to validate the accuracy of the established PMA-qPCR method. The accuracy profile serves as a valuable tool for assessing whether the PMA-qPCR method satisfies the criterion of generating results for a sample that deviates from theoretical values by a specific acceptability criterion. This profile facilitates the assessment of both accuracy and precision by comparing the measured values with their corresponding theoretical values. According to ISO 16140-2:2016 (E), the accuracy profile provides a comprehensive understanding of the method's performance and its ability to meet the predefined criteria by examining the extent of agreement between these values. Typically, an acceptability limit (AL) of ±0.5 Log_{10} units is used to define the allowable difference between the measured and theoretical values (ISO 16140-2, 2016). This AL expresses the maximum acceptable deviation of the method from the theoretical values.

The data for each sample were subjected to a statistical analysis following the guidelines outlined in Step 1 to Step 9 of the Accuracy Profile study in ISO 16140-2:2016 (E). Firstly, a Log_{10} transformation was applied to the results. For each sample (i), various parameters were calculated, including the central value (Xi) representing the theoretical values, the central value (Yi) representing the PMA-qPCR results, the bias (Bi), the upper β-ETI (expected tolerance interval), and the lower β-ETI, as shown in Supplementary Table S3. The bias (Bi) was determined as the absolute difference between the medians of the theoretical and measured values (Bi = Yi − Xi). The β-ETI represents the interval within which the expected proportion of future results will fall, with β set at 80% in accordance with ISO 16140-2:2016 (E) for this study.

A graphical representation of computed results was made, in which the horizontal axis is for theoretical values Xi in Log_{10} units and the vertical axis is for the bias (Figure 5). The upper and lower tolerance-interval limits are connected by straight lines to interpolate the behavior of the limits between the different levels of the validation samples. The horizontal line represents the theoretical values. The differences between theoretical values and average concentration levels of *L. rhamnosus* are represented by black dots. Whenever no biases exist, these recovered values are located on the horizontal theoretical line. In addition, AL are represented by two dashed horizontal lines and β-ETI limits as broken full lines.

In this study, samples were prepared by combining *L. rhamnosus* with *B. infantis* cells to achieve a total bacterial density of approximately 10^8 CFU/mL, with viable *L. rhamnosus* numbers ranging from 10^3 to 10^8 CFU/mL. The bias between theoretical and measured values for each viable cell concentration was −0.09, 0.07, −0.03, 0.08, −0.14, and 0.12 Log_{10} units (Figure 5), respectively. Importantly, all these biases were found to fall within the acceptable limits (± 0.5 Log_{10} units) (ISO 16140-2, 2016), providing evidence for the accuracy of the PMA-qPCR method in quantifying viable *L. rhamnosus* at different bacterial densities, including low, intermediate, and high levels. Furthermore, the coefficient of variation (CV) was calculated for the Log_{10}-transformed viable cell counts of the 10 replicates of each sample, yielding values of 1.22, 1.75, 2.00, 0.90, 1.60, and 3.09%, respectively. These low CV values demonstrated the precision and robustness of the PMA-qPCR method in accurately quantifying viable cell counts.

3.4.3 Limit of quantification, linear, and range of the established PMA-qPCR method

In this study, the limit of quantification of the PMA-qPCR method for detecting low concentrations (10^2 and 10^3 CFU/mL) of *L. rhamnosus* was determined. It was observed that when the *L. rhamnosus* concentration was 10^2 CFU/mL, some of the samples exhibited C_q values higher than 30, which closely resembled the C_q values obtained from the negative controls. However, when the *L. rhamnosus* concentration was increased to 10^3 CFU/mL, the C_q values fell within the range of the standard curve (Figure 3), indicating

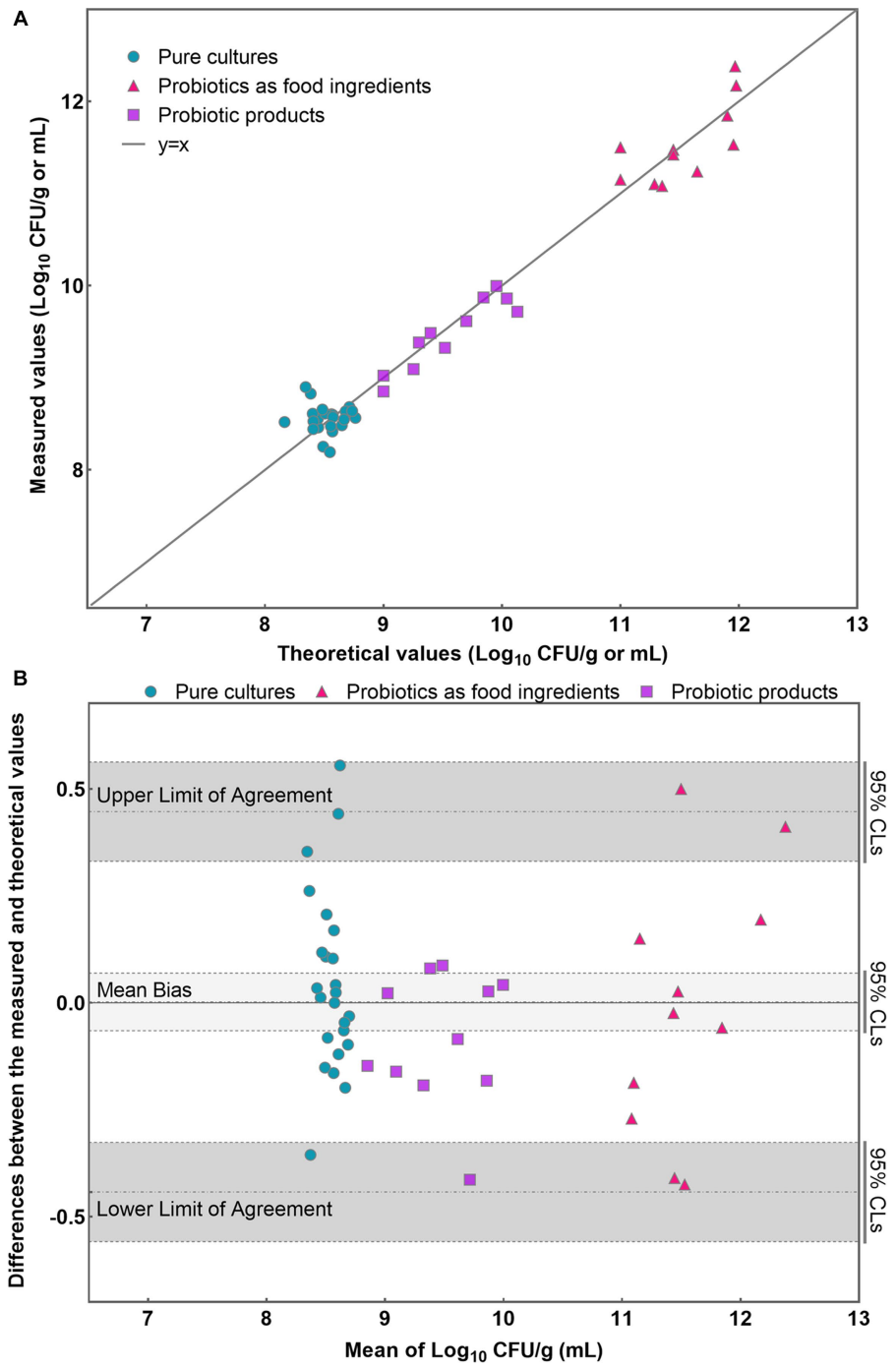

FIGURE 4
Application of PMA-qPCR method to detect viable *L. rhamnosus* in pure cultures, probiotics as food ingredients, and compound probiotic products. **(A)** Scatter plot of measured-values versus theoretical values for three different sample types; **(B)** Bland–Altman difference plot for different sample types detected by PMA-qPCR method.

the validity of the data. As a result, the limit of quantification for the PMA-qPCR method was established as 10^3 CFU/mL.

A linear regression analysis was performed to fit the theoretical values against the measured values (Figure 6). The resulting correlation coefficient (R^2) of the fitted curve was determined to be 0.994, indicating a strong linear relationship between the measured values and the theoretical values within 10^3–10^8 CFU/mL (Figure 6). Based on the findings from the accuracy profile study, limit of quantification study, and assessment of linear properties, it was determined that the quantitative range for accurate detection of *L. rhamnosus* using the PMA-qPCR method is 10^3–10^8 CFU/mL. These results indicate that the method can provide reliable and accurate quantification within this range of bacterial concentrations. However, it is worth noting that the upper limit of the quantitative range was set at 10^8 CFU/mL

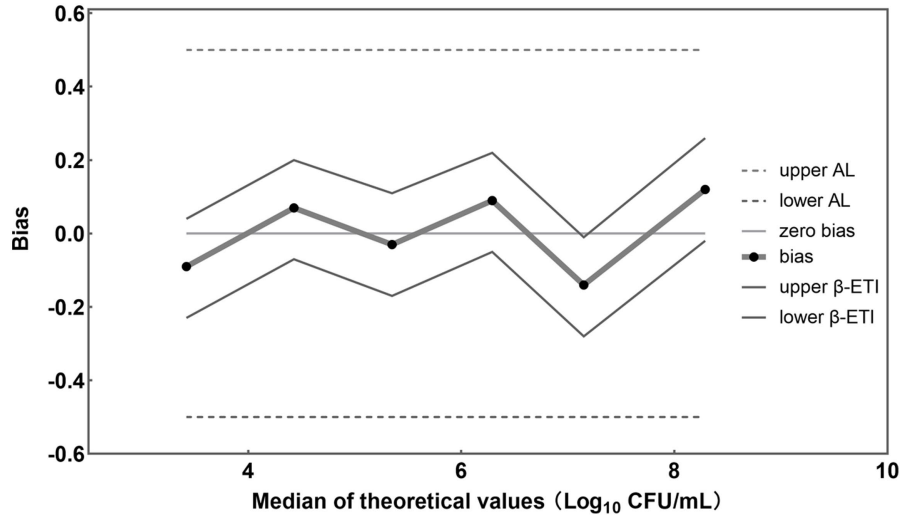

FIGURE 5
Accuracy profile for different concentrations of *L. rhamnosus* detected by the established PMA-qPCR method.

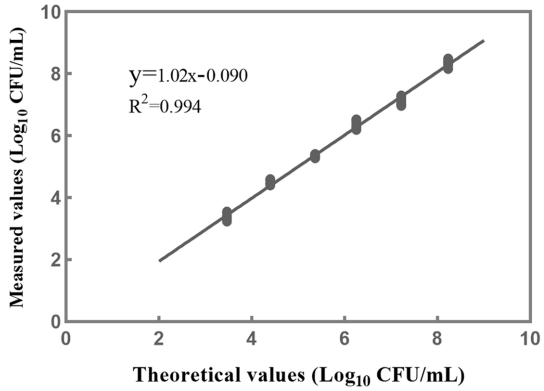

FIGURE 6
The linear fitting relationship between the theoretical and measured results when the concentrations of *L. rhamnosus* are within 10^3–10^8 CFU/mL. Each data point represents 10 replicates.

because this was the upper limit examined, and it does not necessarily represent the true upper limit of the developed method. In cases where bacterial density exceeds this concentration, the total bacterial density can be adjusted to 10^8 CFU/mL, and the optimal PMA treatment conditions can then be applied to the samples.

4 Discussion

Probiotics have garnered significant attention in academic research and have found widespread usage in various food products, primarily due to their potential health benefits and their capacity to enhance gut health. Nevertheless, accurately quantifying viable cell counts in probiotic formulations containing multiple strains presents a substantial challenge (Berezhnaya et al., 2021; Yang et al., 2021). The PMA-qPCR technique has gained considerable traction for detecting viable lactic acid bacteria (Desfossés-Foucault et al., 2012; Dias et al., 2020). A critical consideration in achieving precise detection of the target microbiota through the qPCR method lies in the sensitivity and specificity of the employed primers (Broeders et al., 2014; Garrido-Maestu et al., 2018). In the current study, a comprehensive assessment of inclusivity and exclusivity effectively demonstrated the specificity of the selected primers to *L. rhamnosus* (Figure 1). Notably, these species-specific primers were meticulously designed from the V1-V2 variable regions of the 16S ribosomal DNA sequence, as denoted by its GenBank accession number AF243146 (Byun et al., 2004). This strategic design ensures the exclusivity of the primers against non-*L. rhamnosus* species (Byun et al., 2004). Furthermore, the amplification efficiency of the established standard curve was impressively high, recording a value of 107.01%. This result underscores the inherent sensitivity and reliability of the primers to accurately quantify *L. rhamnosus* (Li and Chen, 2013; Bustin and Huggett, 2017). Moreover, the substantial R^2 value of 0.998 signifies a robust linear correlation between C_q values and viable bacterial counts (Figure 3). Consequently, the standard curve acquires the essential capability to translate DNA quantities into viable cell numbers (Ilha et al., 2016; Yang et al., 2021).

Effective DNA extraction is a pivotal determinant in facilitating the reliable qPCR detection of target DNA molecules within a given sample. A myriad of DNA isolation methods have been harnessed to extract DNA from bacterial source, including commercial kits (McOrist et al., 2002; Dorn-In et al., 2019), phenol-chloroform: isoamyl alcohol extraction (Vieira et al., 2021), heat treatment (Dashti et al., 2009), mechanical cell disruption (e.g., bead-beating) (Plotka et al., 2017), etc. The commercial DNA extraction kits are now mostly commonly used, which usually following by DNA purification steps. However, DNA loss during column purification has been a commonly observed phenomenon, predominantly attributed to the competitive binding of humic substances to silica membranes (Lloyd et al., 2010; Natarajan et al., 2016; Plotka et al., 2017). In the current study, DNA was extracted by only one-step lysis of cells using a bead mill homogenizer, which is rapid and easy to operate. Bead beating have been proved to effectively lysis not only Gram-negative but also

Gram-positive bacteria, which have a thick cell wall (Fujimoto et al., 2004). Of paramount importance, the simplification of operational steps serves as a pivotal factor in mitigating potential DNA loss, while the consistent fixation of lysis conditions (speed and time) ensures both the stability and reproducibility of DNA quality. The effective extraction of DNA assumes primary significance as it lays the groundwork for establishing a robust correlation between C_q values and viable cell numbers, as delineated in Figure 3. High degree of consistency between theoretical and PMA-qPCR measured values of viable *L. rhamnosus* in pure cultures, probiotics as food ingredients, and compound probiotic products demonstrated the effectiveness of the DNA extraction method to different sample types (Figure 4).

Suitable PMA conditions should effectively inhibit the subsequent DNA amplification of dead bacteria without inhibiting the DNA amplification of live bacteria (Nocker et al., 2006; Fujimoto and Watanabe, 2013; Yang et al., 2021). In this study, a commonly used PMA treatment condition to LAB (Desfossés-Foucault et al., 2012; Villarreal et al., 2013) was applied to *L. rhamnosus*. One issue should be mentioned is that the effect of bacterial density on the PMA treatment efficiency should not be underestimate, as it can impact the accuracy of the test results (Zhu et al., 2012; Tantikachornkiat et al., 2016; Lu et al., 2019). Papanicolas et al. (2019) found the PMA could not fully exclude DNA amplification from dead cells with high total bacterial density, especially with high ratios of dead cells, and accurate counting of viable cells was achieved by sample dilutions (Papanicolas et al., 2019). For a defined PMA treatment condition, it applies to an appropriate cell density range (; Nkuipou-Kenfack et al., 2013; Papanicolas et al., 2019). In this study, the defined PMA treatment condition corresponds to an approximate 10^8 CFU/mL total bacteria density, under which the PMA is enough to be very effective in modifying dead cell DNA without interfering with living cells (Figure 2). Moreover, Figure 2 confirms the wide suitability of the chosen PMA condition to commercial *L. rhamnosus* strains. These results provide reality for industrial application of the PMA-qPCR method to quantify viable *L. rhamnosus*.

The established PMA-qPCR method was used to detect viable *L. rhamnosus* in pure cultures, probiotics as food ingredients, and compound probiotic products to assess its suitability to different sample types. Figure 4 showed high consistency between theoretical and measured values of these samples, demonstrating the established PMA-qPCR method could accurately quantify viable *L. rhamnosus* in different sample types. For cells quantification by qPCR method, C_q values versus log CFU of standard curves were usually plotted using CFU by plate counting of one certain bacteria grown in culture medium (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018). The suitability of the standard curve made by one strain to other different strains including commercial ones within one species was not mentioned in the previous studies. The type strain CICC 6224^T (=ATCC 7469^T) of *L. rhamnosus* was used to made the standard curve in this study. The commercial strains of *L. rhamnosus* in probiotics as food ingredients included HN001, R0011, MP108, UALr-06, M9, LG12-2, and in probiotic products included one or more target strains such as HN001, Lr-32, LGG, UALr-06, R0011, M9 etc. The accurate results in Figure 4 demonstrated the standard curve made by type strain could be used to quantify an unknown commercial strains of *L. rhamnosus* in probiotic samples. These results make real sense for PMA-qPCR industrial application to quantify viable *L. rhamnosus* for unknown samples or samples containing multiple *L. rhamnosus* strains. Sample matrix play an important role in the applicability of the PMA-qPCR method (Zhu et al., 2012; Miotto et al., 2020). Corresponding to probiotic products, where there are more complex matrices, e.g., excipients, prebiotics, botanical ingredients, the results showed that PMA was not significantly affected by these matrices (Figure 4). On the other hand, probiotic products contain multiple bacterial species, and PMA-qPCR can accurately target and detect viable *L. rhamnosus*, which fully demonstrates the specificity of this method. Figure 5 illustrated that the PMA-qPCR method could effectively detect viable *L. rhamnosus* cells within a range of 10^3–10^8 CFU/mL with high accuracy and precision, exhibiting a satisfactory linear relationship between the measured and theoretical results (Figure 6). The above results showed that the PMA-qPCR conditions established in this work can be applied to count viable *L. rhamnosus* in actual compound probiotic products, providing technical support for product quality control and supervision.

5 Conclusion

In this study, a PMA-qPCR method was established and validated for viable *L. rhamnosus* detection in probiotics. The inclusivity and exclusivity of the primers demonstrated its high specificity to *L. rhamnosus*, which allows accurate identification of the target bacteria. The 24 *L. rhamnosus* strains including type strain, most known commercial ones etc., confirmed the selected PMA treatment conditions could effectively distinguish between viable and dead cells. The construction of a standard curve using known quantities of type strain viable cells proved effective in converting C_q values to viable bacterial counts and it can be applied to commercial strains. The established PMA-qPCR method could quantify viable *L. rhamnosus* in pure cultures, probiotics as food ingredients, and probiotic products with high accuracy and precision. The quantitative range of the PMA-qPCR method spanned from 10^3 to 10^8 CFU/mL, and a strong linear relationship was observed between the theoretical and measured values within this range. The results of this study provide possible application of the PMA-qPCR method to industry for viable cell numeration of *L. rhamnosus* in compound probiotic products.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

LG: Data curation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. XLZ: Data curation, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing. HF: Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. YL: Data curation, Methodology, Writing – review & editing. YG: Data curation, Methodology, Writing – review & editing. XZ: Data curation, Methodology, Writing – review & editing. CS: Methodology, Writing – review & editing. YJ: Methodology, Writing – review & editing. JL: Methodology, Writing –

review & editing. SM: Software, Visualization, Writing – review & editing. SY: Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

LG, HF, YL, YG, CS, YJ, JL, SM, and SY was employed by the company China National Research Institute of Food and Fermentation Industries Co., LTD.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1341884/full#supplementary-material

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Bifidobacterium species viability in dairy-based probiotic foods: challenges and innovative approaches for accurate viability determination and monitoring of probiotic functionality

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Bifidobacterium species are essential members of a healthy human gut microbiota. Their presence in the gut is associated with numerous health outcomes such as protection against gastrointestinal tract infections, inflammation, and metabolic diseases. Regular intake of *Bifidobacterium* in foods is a sustainable way of maintaining the health benefits associated with its use as a probiotic. Owing to their global acceptance, fermented dairy products (particularly yogurt) are considered the ideal probiotic carrier foods. As envisioned in the definition of probiotics as "live organisms," the therapeutic functionalities of *Bifidobacterium* spp. depend on maintaining their viability in the foods up to the point of consumption. However, sustaining *Bifidobacterium* spp. viability during the manufacture and shelf-life of fermented dairy products remains challenging. Hence, this paper discusses the significance of viability as a prerequisite for *Bifidobacterium* spp. probiotic functionality. The paper focuses on the stress factors that influence *Bifidobacterium* spp. viability during the manufacture and shelf life of yogurt as an archetypical fermented dairy product that is widely accepted as a delivery vehicle for probiotics. It further expounds the *Bifidobacterium* spp. physiological and genetic stress response mechanisms as well as the methods for viability retention in yogurt, such as microencapsulation, use of oxygen scavenging lactic acid bacterial strains, and stress-protective agents. The report also explores the topic of viability determination as a critical factor in probiotic quality assurance, wherein, the limitations of culture-based enumeration methods, the challenges of species and strain resolution in the presence of lactic acid bacterial starter and probiotic species are discussed.

Finally, new developments and potential applications of next-generation viability determination methods such as flow cytometry, propidium monoazide–quantitative polymerase chain reaction (PMA-qPCR), next-generation sequencing, and single-cell Raman spectroscopy (SCRS) methods are examined.

KEYWORDS

***Bifidobacterium*, viability, yogurt, probiotic, gut microbiota, stress response, viability improvement, next-generation methods**

1 Introduction

The genus *Bifidobacterium* consists of Gram-positive bacteria belonging to the family Bifidobacteriaceae (Biavati and Mattarelli, 2015). Members of this genus are anaerobic or sometimes aerotolerant, non-spore-forming pleomorphic bacteria (Eckel et al., 2020). The taxonomy of the genus has been steadily changing over time due to advances in genomic characterization techniques, with the discovery of new species and subspecies in recent years (Lugli et al., 2018, 2021; Neuzil-Bunesova et al., 2020; Chen et al., 2021). As of the end of 2021, there were 98 documented species of the *Bifidobacterium* genus (Turroni et al., 2021). The primary ecological niche of *Bifidobacterium* species is the gastrointestinal tract (GIT) of mammals, birds, and insects (Alessandri et al., 2021). Within this diverse genus, some *Bifidobacterium* species were historically assumed to colonize only the GIT of specific animal host species (Turroni et al., 2011). However, advanced metagenomic approaches, such as bifidobacterial internally transcribed spacer (ITS) profiling, revealed that several of the *Bifidobacterium* species are ubiquitous within the GIT of different host animal species (Alessandri et al., 2021). Based on the core genome analysis, the genus is divided into 10 phylogenetic groups (*B. adolescentis*, *B. boum*, *B. pullorum*, *B. asteroides*, *B. longum*, *B. psychraerophilum*, *B. bifidum*, *B. pseudolongum*, *B. bombi* and *B. tissieri*) that partially correlate with animal host ecological niche (Alessandri et al., 2021; Duranti et al., 2021). Four of these phylogenetic groups are typical colonizers of the human GIT (Duranti et al., 2021). These include, the *B. adolescentis* group (*B. adolescentis* and *B. catenulatum* strains), the *B. longum* group (*B. breve* and *B. longum* strains), the *B. pseudolongum* group (*B. animalis* strains) and the *B. bifidum* group (*B. bifidum* strains) (Alessandri et al., 2021; Duranti et al., 2021).

Bifidobacteria are an integral component of the human gut microbiota, and their presence is associated with several health benefits (Sharma et al., 2021; Li et al., 2023). Due to their strong association with breast milk, *Bifidobacterium* species are among the earliest and most dominant colonizers of the GIT of neonates, making up to 90% of the microbiota of infants (Wong et al., 2020; Yang et al., 2021). Their relative abundance decreases in adult humans but remains stable at about 10–40% of the microbiota (Arboleya et al., 2016). In old age, the proportion decreases to about 5% (Arboleya et al., 2016). In addition to the decline in the relative abundance, there is also a change in the species diversity with age. The species, *B. breve*, *B. longum* subsp. *infantis*, and *B. bifidum* are the most dominant in infants, while *B. longum*, *B. catenulatum* and *B. adolescentis* dominate in adults (Derrien et al., 2022). Moreover, variations have also been reported among the elderly populations (Wang et al., 2015; Kato et al., 2017). For instance, the microbiota of Chinese centenarians was found to comprise of unique species, such as *B. minimum*, *B. gallinarum*/*B. pullorum*/*B. saecularmay*, and *B. mongoliense* which were absent in younger elderlies of 80–90 years (Wang et al., 2015). Due to dietary and stress factors such as antibiotic use, bifidobacterial levels in the GIT may be depleted, resulting in a dysbiosis of gut microbiota (Derrien et al., 2022). Evidence has shown that their reduction in the GIT is correlated with adverse health outcomes such as an increased risk of obesity, type 2 diabetes, allergic pathologies, irritable bowel syndrome, colorectal cancer and infections due to enteric viruses and bacterial pathogens (Xu et al., 2012; Akay et al., 2014; Taverniti and Guglielmetti, 2014; Gao et al., 2015; Kosumi et al., 2018; Wei et al., 2018; Li L. et al., 2021; Colston et al., 2022).

A sustainable approach to mitigate dysbiosis of the human gut microbiota and the attendant adverse health effects is the supplementation of bifidobacteria in foods as probiotics (He et al., 2023). According to the Food and Agriculture Organization of the United Nations [FAO], and World Health Organization [WHO] (2001) definition, probiotics are "live micro-organisms, which when consumed in adequate amounts confer a health benefit on the host." Among the foods used as potential probiotic carriers, fermented dairy products, especially yogurt, are the most consumed. However, the sustenance of probiotic viability during processing and shelf-life of foods like yogurt is challenging. Viability is a prerequisite for probiotic functionality and therapeutic benefits (Terpou et al., 2019). The significance of viability has been demonstrated by the fact that probiotic functionalities such as antimicrobial effects, lactose intolerance relief and immune stimulation depend on cell viability (Terpou et al., 2019). Thus, for any therapeutic effects associated with probiotic intake, it is recommended that the levels of viable cells must be at least 10^6 CFU/g of food product at the time of consumption (Nyanzi et al., 2021). Several process factors such as homogenization, mixing, heating, fermentation, and cooling in the manufacturing of fermented dairy products potentially influence *Bifidobacterium* viability.

Physicochemical stresses such as dissolved oxygen, acidic pH, homogenization pressure and storage temperature constitute the main inhibitory factors (Meybodi et al., 2020). Moreover, antagonistic effects of the starter cultures, such as the production of H_2O_2, could also negatively impact *Bifidobacterium* viability (Meybodi et al., 2020). Due to the poor technological robustness,

many potentially beneficial *Bifidobacterium* species with superior probiotic functionalities in the human GIT cannot be effectively incorporated into fermented dairy foods. Although most of the identified *Bifidobacterium* species are from the GIT of animals, some species and strains have recently been shown to be endogenously present in fermented foods (Laureys et al., 2016; Eckel et al., 2020). So far, a few species and strains, such as the moderately aerotolerant *B. animalis* subsp. *lactis* are used in the commercial production of probiotic foods (He et al., 2023). The non-aerotolerant strains cannot be used due to their susceptibility to oxygen exposure.

Accurate enumeration of viable *Bifidobacterium* spp. in dairy products is a critical factor in probiotic quality assurance. Specific enumeration of *Bifidobacterium* viability in yogurt is complicated by the co-occurrence of lactic acid bacteria (LAB) starter cultures. In mixed species products with *Lacticaseibacillus rhamnosus* and *Lactobacillus acidophilus* strains, the widely used medium for enumeration of *Bifidobacterium* spp. [De Man, Rogosa and Sharpe (MRS) agar supplemented with neomycin, nalidixic acid, lithium chloride and paromomycin (MRS-NNLP agar)] could not select for *Bifidobacterium* spp. (Van de Casteele et al., 2006; Ashraf and Smith, 2015). Moreover, probiotic functionality is strain and species-specific, yet culture-based methods are unable to selectively differentiate between individual species and strains of bifidobacteria (Hagen and Skelley, 2019; Yoon et al., 2021). This paper reviews the subject of *Bifidobacterium* spp. viability and its significance in probiotic functionality. Given that yogurt is a widely consumed dairy product that is globally accepted as a delivery vehicle for probiotics, the paper focuses on the factors that influence *Bifidobacterium* spp. viability during the manufacturing as well as the strategies for viability retention. Furthermore, the article explores the next-generation methods for *Bifidobacterium* spp. viability determination and their applications in industrial probiotic viability quality assurance. The review was primarily based on literature published in the past 15 years, However, some earlier key studies with an enduring relevance and impact on the subject were selectively incorporated.

2 Probiotic functionality of *Bifidobacterium* species

A significant quantity of *in vitro* and *in vivo* evidence has demonstrated the probiotic functionalities of *Bifidobacterium* spp. (Konieczna I. et al., 2012; Groeger et al., 2013; Turroni et al., 2014; Din et al., 2020; Shang et al., 2020; Zhang et al., 2020; van der Hee and Wells, 2021; Schiweck et al., 2022; Álvarez-Mercado et al., 2022; He et al., 2023). These diverse probiotic functionalities include the enhancement of the host immune system, protection against communicable and non-communicable diseases, as well as improvement of nutritional metabolism (He et al., 2023). The immunomodulatory properties of *Bifidobacterium* species include the stimulation of both innate and adaptive immune defense systems (He et al., 2023). Some of the compelling empirical evidence for the immunostimulatory effects was demonstrated in immunosuppressed mice gavaged with *B. bifidum* strains, in which the oral administration of the probiotic resulted in increased secretion of immunoglobulin A and enhanced production and activity of lymphocytes, natural killer cells and macrophages (Turroni et al., 2014; Shang et al., 2020). In addition, experimental evidence in mice models, epithelial cell lines and human trial studies has shown that species such as *B. longum* subsp. *infantis*, *B. animalis* subsp. *lactis* and *B. infantis* can offer protection against chronic gastrointestinal inflammatory diseases such as inflammatory bowel disease and other non-enteric inflammatory diseases like autoimmune hepatitis (Konieczna I. et al., 2012; Groeger et al., 2013; Din et al., 2020; Zhang et al., 2020; Álvarez-Mercado et al., 2022). The protective mechanism is attributed to the ability of *Bifidobacterium* species to inhibit the release of proinflammatory cytokines while stimulating the release of anti-inflammatory cytokines (Konieczna I. et al., 2012; Zhang et al., 2020; Álvarez-Mercado et al., 2022; He et al., 2023). A further beneficial function of *Bifidobacterium* species relates to their role in the metabolism of dietary and human-derived heteroglycans (Li et al., 2023). Dietary heteroglycans such as arabinoxylans, pectin, and inulin are plant-derived components of dietary fiber that are not metabolized by human digestive enzymes (Kelly et al., 2021). Human-derived heteroglycans include mucin and human milk oligosaccharides (HMOs) (Luo et al., 2021). Except for a few other genera, *Bifidobacterium* species are the most prominent part of the gut microbiota capable of metabolizing heteroglycans (Li et al., 2023). The fermentation of heteroglycans has profound implications for health-promoting functions (Li et al., 2023). Among the fermentation products, short-chain fatty acids (SCFAs) such as acetate, propionate, butyrate and valerate that are linked to numerous beneficial effects (Parada Venegas et al., 2019; Schiweck et al., 2022). Using a murine model, Zhang et al. (2020) showed that oral intake of *B. animalis* subsp. *lactis* increased the concentration of fecal butyric acid in mice with experimentally induced autoimmune hepatitis. An abundance of evidence has shown that SCFAs are central to the regulation and induction of the immune system (van der Hee and Wells, 2021; Schiweck et al., 2022). They function as signaling molecules through cell surface G-protein coupled receptors (GPCRs) to control immune and metabolic functions (van der Hee and Wells, 2021; Li et al., 2023).

3 Viability as a necessity for probiotic functionality

As enunciated in the original definition, viability is a primary criterion for describing an organism as a probiotic (Food and Agriculture Organization of the United Nations [FAO], and World Health Organization [WHO], 2002). However, there have been many scientific reports and reviews indicating that many of the known health benefits previously ascribed to live probiotics can also be exhibited by their metabolites and/or non-viable cells (Geraldo et al., 2020; Vallejo-Cordoba et al., 2020; Martorell et al., 2021). Previous experiments comparing the physiological functionalities of live and heat-killed *B. breve* in murine models concluded that while both heat-killed and live cells exhibited similar activities in the suppression of pro-inflammatory cytokine secretion, live cells had a more significant effect on the regulation of intestinal metabolism (Sugahara et al., 2017). Similar comparisons

of protective properties of heat-inactivated and live *B. longum* subsp. longum and *B. animalis* subsp. *lactis* on ovalbumin-sensitized mice and cultured Caco-2 cells, respectively, showed that live cells exhibited a stronger inflammation-suppressing effect and increased barrier-integrity of epithelial cells (Castro-Herrera et al., 2020; Pyclik et al., 2021). Despite this recent evidence, it is undeniable that the requirement for viability continues to be the standard for the incorporation of probiotics into functional foods. While some physiological effects of probiotics, such as immunomodulatory properties, can be elicited by bacterial cell components such as lipoteichoic acids and peptidoglycan, some functions are dependent on metabolic activity and thus are a product of viable cells (Castro-Herrera et al., 2020). Several aspects of *Bifidobacterium* spp. probiotic functionality depend on their metabolic activities and secretion of enzymes and bioactive metabolites. A quintessential illustration of the viability-dependent function of *Bifidobacterium* spp. in gut health is their fermentation of dietary heteroglycans and human-derived oligosaccharides (Luo et al., 2021; Li et al., 2023). Apart from the direct benefits of such metabolism of complex carbohydrates, the unique carbohydrate-active enzymes of bifidobacteria enable them to support the growth and survival of other members of the gut microbiota through cross-feeding (An et al., 2014). *In vitro* experiments with co-cultures of *Bifidobacterium* and other genera of the human gut microbiota, such as *Faecalibacterium*, *Eubacterium* and *Anaerostipes* on fructooligosaccharide substrates, showed an enhanced production of butyrate, a bioactive SCFA (Belenguer et al., 2006; Rios-Covian et al., 2015; Kim H. et al., 2020). Thus, through synergistic metabolic interactions with other members of the gut microbiota, viable *Bifidobacterium* spp. can result in the amplification of biological signals that lead to enhanced probiotic functionality.

3.1 Stress factors affecting viability during yogurt processing and shelf-life

Fermented dairy products such as yogurts, cheeses, acidified milks and kefir are the most known category of food-based probiotic carrier systems for the human intake of *Bifidobacterium spp*. (Terpou et al., 2019; González-Orozco et al., 2022). Above all, the ability to survive the fermentation and associated processes during the production of these foods ultimately determines the number of viable *Bifidobacterium* spp. that reach the GIT, and the therapeutic benefit derived therefrom (Meybodi et al., 2020). This paper focuses on the viability of *Bifidobacterium* spp. in yogurt as an archetypal dairy-based probiotic carrier. Several physical and chemical stress factors associated with the yogurt manufacturing process and the subsequent storage period of the shelf-life impose adverse effects on *Bifidobacterium* viability (Meybodi et al., 2020). The sources of the physical and chemical stress factors associated with the yogurt manufacturing process are summarized in **Figure 1**. The ensuing subsections of the paper explore the physiological and genetic responses of *Bifidobacterium* spp. to the main stress factors associated with yogurt production (acid, osmotic, heat, oxidative and cold stress). A summary of the elucidated and postulated mechanisms is illustrated in **Figure 2**.

3.1.1 Acid stress

In most cases, probiotic *Bifidobacterium* spp. are incorporated into yogurt together with the starter culture at the onset of fermentation. As a neutrophile, the typical optimum pH for *Bifidobacterium* spp. growth is 6.5–7.0 (Biavati and Mattarelli, 2015). Given the low pH conditions in yogurt, the survival of *Bifidobacterium* spp. depend on the ability to activate an acid tolerance response needed to maintain intracellular pH homoeostasis. The mechanisms of acid stress response have been well elucidated and involve the increased expression of the proton-translocating F_1F_0-ATPase (Sánchez et al., 2007; Waddington et al., 2010; Jin et al., 2012). The F_1F_0-ATPase is an active proton pump whose activity results in the exclusion of protons using energy derived from the hydrolysis of ATP (Fiocco et al., 2020). In addition to the F_1F_0-ATPase-dependent acid tolerance response, other mechanisms include the alkalization of the cytoplasm through processes that consume intracellular H^+ protons (Fiocco et al., 2020). These include the glutamate decarboxylase (GAD) and branched-chain amino acid metabolism pathways, that generate ammonia (Schöpping et al., 2022a). The GAD system comprises of the enzyme glutamate decarboxylase (GadB) and an antiporter (GadC) encoded by the *GadB* and *GadC* loci, respectively (Yunes et al., 2016). In this pathway, glutamate is converted by the activity of GadB to γ-aminobutyrate (GABA), with the consumption of H^+ protons and the release of CO_2 (Duranti et al., 2020). Ammonia production is thought to play a crucial role in maintaining intracellular pH equilibrium by functioning as a proton scavenger (Wei et al., 2019). When exposed to acidity, acid-sensitive strains such as *B. longum* exhibit increased production of enzymes responsible for branched-chain amino acid (BCAA) biosynthesis (Wei et al., 2019). Furthermore, sulfur-containing amino acids, such as cysteine and methionine, are hypothesized to play a role in the acid stress response of *Bifidobacterium* spp. (Sánchez et al., 2007; Schöpping et al., 2022b). An analysis of the proteome of low pH adapted mutants of *B. longum* identified a higher constitutive presence of methionine synthase, cystathionine gamma-lyase and cystathionine gamma-synthase compared to the unadapted wild type (Sánchez et al., 2007). All these enzymes are involved in additional pathways of NH_3 generation and alkalization of the cytoplasm (Schöpping et al., 2022b). Furthermore, the acid tolerance response in *B. longum* strains has also been linked to an overall increase in cell envelope components under sub-lethal and lethal acid environments (Jin et al., 2015). Higher transcription rates were reported for genes associated with the synthesis of peptidoglycan, exopolysaccharides, and undecaprenyl-PP (UND-PP) in *B. breve* adapted at pH 3.2 (Jin et al., 2015). The high synthesis of peptidoglycan under acid stress conditions is postulated to strengthen the cell wall structure and provide protection against acid stress-induced cell damage (Jin et al., 2015). Moreover, acid stress induces changes in the fatty acid profiles of the cell membrane with a shift toward long-chain fatty acids (C16:0 and C18:0) (Wei et al., 2019).

3.1.2 Oxidative stress

Processes that allow oxygen diffusion into the milk or yogurt during manufacturing include stirring, homogenization, mixing and agitation (**Figure 1**). In addition, the packaging of yogurt in oxygen-permeable plastic containers can lead to an increased

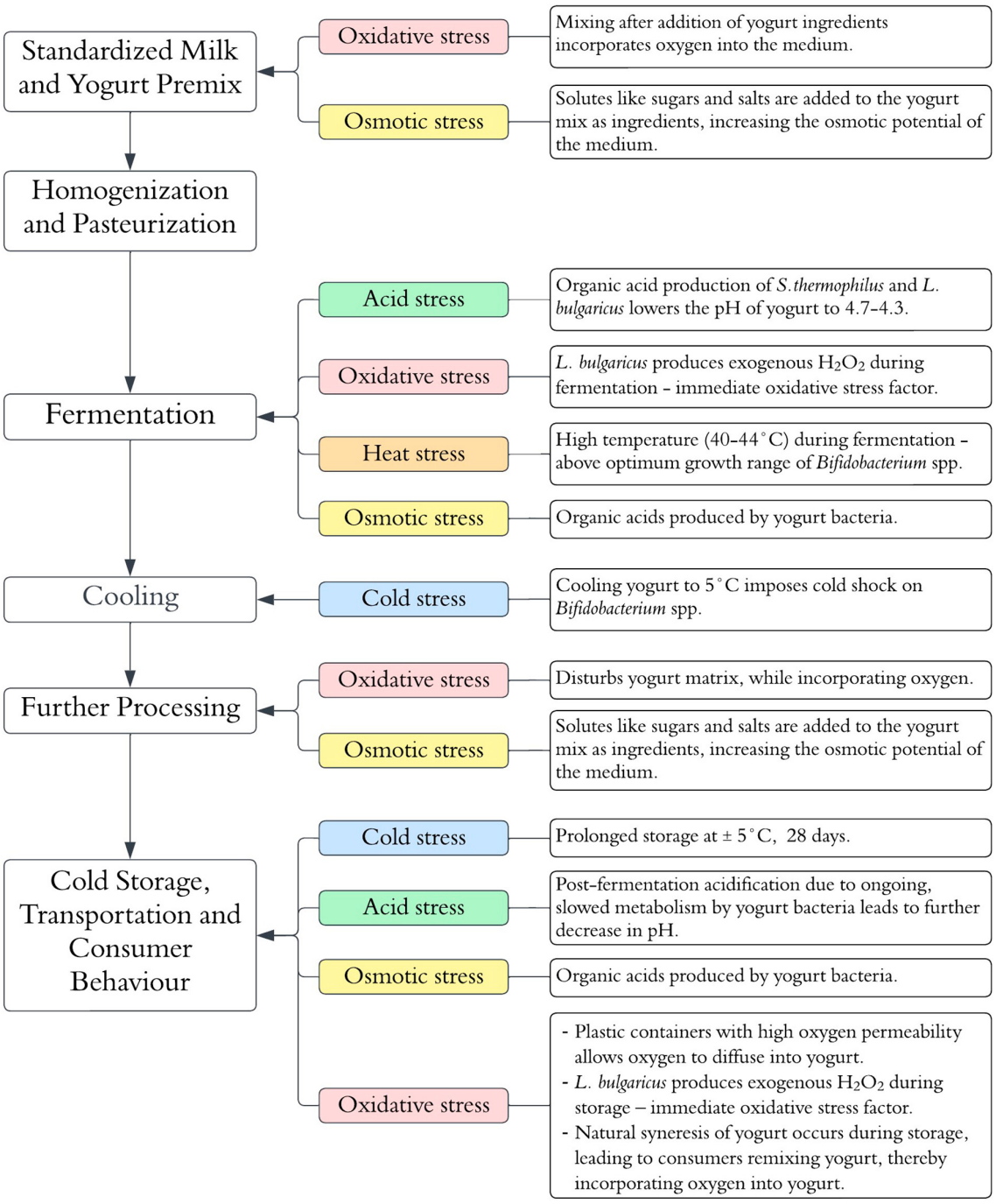

FIGURE 1
Stress factors potentially influencing *Bifidobacterium* spp. viability during yogurt manufacturing and shelf-life.

dissolved oxygen content over the storage period of the shelf life (Cruz et al., 2013). As anaerobes, *Bifidobacterium* spp. are intrinsically sensitive to oxygen and its derived reactive oxygen species (ROS). However, the sensitivities differ within the genus. Species such as *B. animalis* subspp. *lactis*, *B. asteroides*, *B. minimum* and *B. indicum*, are considered aerotolerant, while species such as *B. boum* and *B. thermophilum* are considered hyper-aerotolerant (Kawasaki et al., 2018). The primary response mechanisms to oxidative stress are based on the production of enzymes that can detoxify ROS. Except for a few aerotolerant species like *B. asteroides* and *B. indicum*, *Bifidobacterium* spp. generally lack the genes for the primary antioxidant enzymes like catalase and superoxide dismutase (Zuo et al., 2018). However, some *Bifidobacterium* spp. contain some inducible enzymes that can detoxify ROS (Schöpping et al., 2022b). One such enzyme is alkyl hydroperoxide reductase catalytic subunit C (AhpC) (Zuo et al., 2014). AhpC is a peroxidase component of alkyl hydroperoxide reductase enzymes systems that are found in many prokaryotes (Zuo et al., 2014). It functions together with the flavoprotein disulfide reductase (AhpF) to covert H_2O_2 to alcohol and water (Zuo et al., 2014).

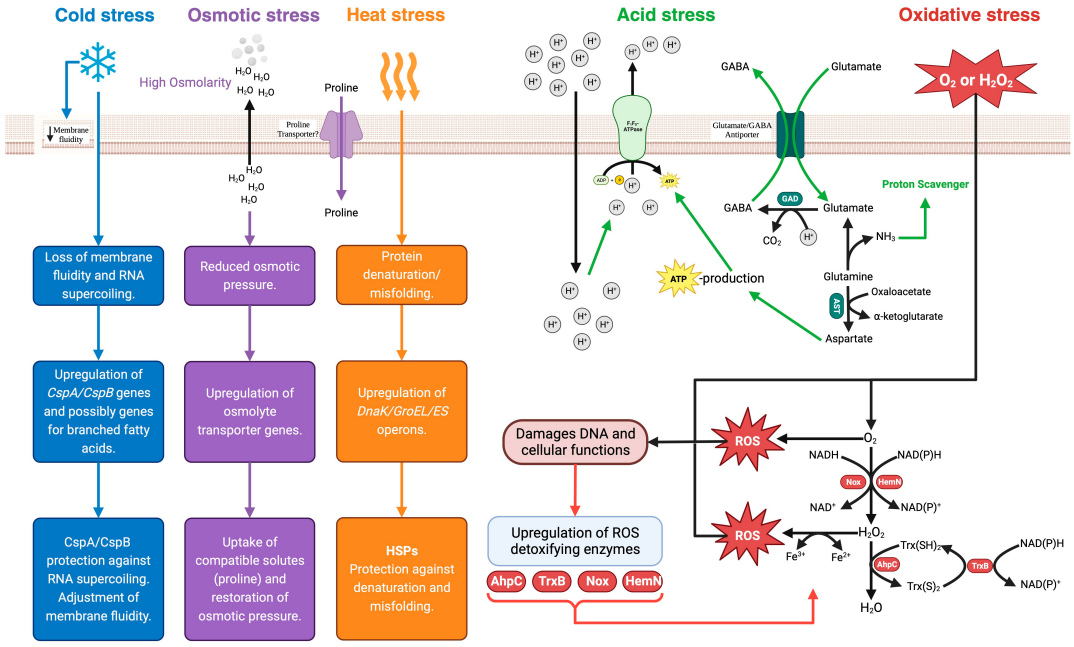

FIGURE 2
Bifidobacterium spp. stress response mechanisms. Csp, cold shock protein; Hsp, heat shock protein; GAD, glutamate decarboxylase; AST, aspartate transaminase; GABA, γ-amino butyric acid; EPS, exopolysaccharide; Nox, NADH oxidase; HemN, oxygen-independent coproporphyrinogen III oxidase; AhpC, Alkyl hydroperoxide reductase C-subunit; $Trx(S)_2$, oxidized thioredoxin; $Trx(SH)_2$, reduced thioredoxin; TrxB, thioreductase-like protein. Created with BioRender.com.

In some facultative anaerobes such as *E. coli*, AhpC is the main ROS detoxifying enzyme instead of catalase (Ma and Payne, 2012). In support of the hypothesis of inducible oxidative response systems in bifidobacteria, Xiao et al. (2011), observed that in aerobically grown *B. longum*, AhpC was one of the upregulated proteins together with DNA oxidative damage-protective proteins such as pyridine nucleotide-disulfide reductase (PNDR) and ribonucleotide reductase (NrdA). Similarly, Satoh et al. (2019), identified a thioredoxin reductase (TrxR) whose expression was induced by oxygen exposure in *B. bifidum*. Subsequently, TrxR was identified to be a reductase homologue of AhpF that interacted with AhpC *in vitro* to achieve H_2O_2 reduction. Hence, in *B. bifidum*, the oxidative stress response possibly involves an increased expression of thioredoxin reductase and the AhpF-AhpC H_2O_2 degradation system (Satoh et al., 2019).

3.1.3 Osmotic stress

To date, only a few studies have tried to elucidate the bifidobacterial osmoregulatory system (Cui et al., 2022; Zhang et al., 2022). Cui et al. (2016) experimentally determined the osmolarity limits for the growth of *Bifidobacterium spp*. to range from 850 to 1,300 mosM kg^{-1}. The presence of solutes in yogurt (**Figure 1**) results in a hyperosmotic extracellular environment that can lead to the loss of water from bacterial cells, and consequently a loss of turgor pressure and plasmolysis (Brauer et al., 2023). Based on the established osmoregulatory systems in other probiotic bacterial genera that involve the intracellular accumulation of compatible solutes such as trehalose, glycine betaine and amino acids such as proline, Zhang et al. (2022) and Cui et al. (2022) sought to characterize the osmoregulatory mechanisms in *B. bifidum* and *B. longum*, respectively. Using a genome and metabolome analysis, hyper-osmotolerant mutants of *B. bifidum* were observed to have an increased accumulation of amino acids, especially proline, compared to non-osmotically adapted wild types (Zhang et al., 2022). As no potential proline transporter proteins were identified in the organism, the proposed osmotic protection mechanism was that proline accumulation occurred through an endogenous synthesis from glutamic acid (Zhang et al., 2022). In contrast, Cui et al. (2022) observed that when the osmotic pressure of a culture environment was increased, the addition of proline substantially improved the survival of *B. longum*, suggesting the presence of proline transporter proteins in this organism.

3.1.4 Heat stress

Except for a few thermophilic species like *B. thermacidophilum*, bifidobacteria are generally mesophilic organisms with an optimum growth temperature range of 37–41°C (Biavati and Mattarelli, 2015). Hence, a yogurt fermentation, which typically occurs at 40–44°C, can impose a mild heat stress on probiotic *Bifidobacterium* species. In general, the physiological effects of mild heat challenges on bacterial cells include the destabilization of non-covalent molecular bonds, ribosome dysfunctionality, and protein denaturation (Fiocco et al., 2020). Notably, apart from the earlier work by Ventura et al. (2004) 2005 and Rezzonico et al. (2007), there has not been any studies on the bifidobacterial heat stress response systems in recent years. Like other prokaryotes, the heat stress response of *Bifidobacterium* spp. involves the increased production of heat shock proteins (HSPs) (Ventura et al., 2011). HSPs are ubiquitous and conserved proteins across the prokaryotic kingdom. They are encoded in two operons (*dnaK* and *groEL-groES*) and function as chaperones that protect physiological proteins against misfolding under conditions of heat

stress (Hu et al., 2022). A transcriptomic analysis of *B. longum* exposed to heat shock treatment at 50°C found that the *dnaK* operon-encoded molecular chaperones (DnaK, GrpE, DnaJ) were the main HSPs produced (Rezzonico et al., 2007). The second class of molecular chaperones (GroEL and GroES) encoded by the *groEL-groES* operon were less expressed in *B. longum* in response to mild heat stress (Rezzonico et al., 2007). Studies on *B. breve* heat stress response showed that the GroEL and GroES chaperones are required for mild heat shock survival while dnaK, GrpE, DnaJ chaperones are necessary for survival under extreme heat stress (Ventura et al., 2004, 2005).

3.1.5 Cold stress

At the end of fermentation, yogurt is typically cooled to approximately 5°C, to allow for gel setting and inhibit the growth and metabolic activities of the yogurt bacteria (Sfakianakis and Tzia, 2014). In addition to a drastic drop in temperature after fermentation, the yogurt is further stored at cold temperatures for the duration of the shelf-life, typically 28 days. Except for a few species, such as *B. mongoliense* and *B. psychraerophilum*, which can grow under cold conditions, most *Bifidobacterium* spp., minimum growth temperature is 25–28°C (Biavati and Mattarelli, 2015). The physiological effects of cold stress in mesophilic bacteria include the changes in the cell membrane from an elastic liquid crystalline state to a rigid gel-phase state that impairs nutrient uptake, and the stabilization of nucleic acid secondary structures that impede DNA replication and protein synthesis (Phadtare, 2004). So far, the molecular mechanisms behind the cold stress response of *Bifidobacterium* spp. have not specifically been elucidated. However, based on knowledge from other Gram-positive organisms, the adjustment of membrane fluidity through the incorporation of unsaturated anteiso-branched-chain fatty acids (BCFA) is one of the mechanisms of bacterial cold stress adaptation (Yoon et al., 2015). A second mechanism of bacterial cold stress adaptation involves the increased production of cold shock proteins (CSPs) that act as RNA chaperones to prevent supercoiling and facilitate effective translation under cold conditions (Phadtare, 2004). CPSs belong to a family of small, highly conserved, structurally related proteins widely distributed in the prokaryotic kingdom (Phadtare, 2004). So far, a few studies have observed the presence of CSP gene homologs (*CspA* and *CspB*) in bifidobacterial genomes (Ventura et al., 2004; Rezzonico et al., 2007; Schöpping et al., 2022b). Interestingly, *CspA* expression in *B. breve* was activated by heat stress exposure together with the *groEL-groES* operon, suggesting a heat-induced co-transcription of both genes (Ventura et al., 2004).

4 Strategies for *Bifidobacterium* viability retention and enhancement in dairy probiotic foods

4.1 Process modification

The dissolved oxygen content of milk is a crucial factor influencing *Bifidobacterium* viability in yogurt. Hence, processes targeted at reducing the dissolved oxygen content of milk before fermentation may present some rational chances of viability retention. A comparative summary of these methods is given in **Table 1**. Previous studies have investigated the use of gasses like nitrogen to achieve milk deaeration (Bolduc et al., 2006; Ebel et al., 2011). The bubbling of pasteurized milk with a gas mixture of N_2 and 4% (v/v) H_2 (N_2-H_2) for 4 h at a flow rate of 20 mL/min decreased the redox potential of milk from +440 mV to +350 mV and −300 mV, respectively (Ebel et al., 2011). When the de-aerated milk was fermented by yogurt starter cultures together with *B. bifidum*, the fermented products made from milk treated with N_2H_2 had higher survival of *B. bifidum* during storage, and the treatment had no adverse effects on the fermentation kinetics and starter cultures (Ebel et al., 2011). In a similar deaeration treatment with N_2, the dissolved oxygen concentration of milk was reduced from an average of 6.7 ppm to 0.3 ppm (Bolduc et al., 2006).

An alternative method of lowering redox potential is electroreduction. This is a physical treatment involving voltage application to reduce the redox potential by electrolysis (Roussel et al., 2022). It is an efficient process of decreasing the redox potential of milk. Through the application of a voltage of −1.55 V through a milk sample for 40 minutes, Bolduc et al. (2006) reported a reduction in redox potential of milk from > + 200 mV to < −300 mV and a decrease in dissolved oxygen concentrations to between 2–3 ppm from 6.7 ppm in untreated milk. When treated by electroreduction, it is possible to maintain a negative redox potential in milk for up to 7 days (Bazinet et al., 2009).

Another technological process modification considered a potential method for viability improvement is high-pressure homogenization (HPH). HPH is a non-thermal milk preservation method in which the milk is exposed to pressure above 100 MPa (Massoud et al., 2016). Experiments with *B. lactis* have shown that an increase in homogenization pressure from 100 to 200 MPa, combined with increasing temperature from 50 to 70°C, led to a significant improvement in the viability of the probiotic in the resultant yogurt (Massoud et al., 2015). The improvement in viability has been attributed to the increase in free amino acids needed for probiotic nutrition (Massoud et al., 2016). Most significantly, the release of cysteine has a positive effect on *Bifidobacterium* viability.

An additional approach to reducing the redox potential of milk and yogurt is using *Lactococcus lactis* as an oxygen scavenger (Tachon et al., 2009). *Lac. lactis* is known for its strong ability to decrease the redox potential of milk to as low as −220 mV (Tachon et al., 2009). Its co-inoculation with probiotics in milk remarkably improved the viability of *Bifidobacterium* spp. (Yonezawa et al., 2010; Odamaki et al., 2011). Interestingly, *Lac. lactis* is a dairy starter culture used extensively in cheese fermentations and as a probiotic (Ruggirello et al., 2016; Jaskulski et al., 2020). The incorporation non-starter, adjunct lactic acid bacterial cultures in yogurt is a worthwhile proposition in the production of yogurt with enhanced health benefits (Ayivi and Ibrahim, 2022). However, the potential benefits must be balanced against any potential negative effects on yogurt fermentation kinetics, flavor and texture (Ayivi and Ibrahim, 2022). Generally, the combination of *Bifidobacterium* spp. with other probiotic lactic acid bacterial species have shown positive outcomes in terms of bioactive metabolites and therapeutic benefits (Pápai et al., 2021; Peng et al., 2022).

In addition, processes targeting the control of post-fermentation acidification in yogurt have been proposed for

TABLE 1 Methods for reducing the redox potential of milk and yogurt for the enhancement of *Bifidobacterium* spp. viability.

Category	Method	Description	Strengths and limitations	References
Process modification	Deaeration	Use of N_2 and H_2 gas to purge O_2 from pasteurized milk for yogurt making	Effective at reducing the redox potential of milk (as low as −300 mV). No negative effects on fermentation kinetics. No effect on milk odor, color and taste.	Ebel et al., 2011; Roussel et al., 2022
	Electroreduction	Lowering of milk redox potential through electrolysis	Can lower milk redox potential to −300 mV. Redox potential is unstable. The low redox potential can only last up 7 days	Bazinet et al., 2009; Roussel et al., 2022
Reducing agents	L-Cysteine	Reducing agent	Strong reducing agent capable of maintaining a negative redox potential in yogurt for 30 days Sulfur taste effect limits use in yogurt	Dave and Shah, 1997a; Meybodi et al., 2020
	Ascorbic acid	Reducing agent	Instability limits the antioxidant potential	Dave and Shah, 1997b
	Oxygen-scavenging *Lactococcus lactis* strains	*Lac. lactis* has a strong reducing ability through cell surface thiol groups and membrane NADH dehydrogenases.	*Lac. lactis* is a dairy starter culture and probiotic Potential for enhanced probiotic benefit as it can complement *Bifidobacterium* spp.	Tachon et al., 2009; Michelon et al., 2010; Ayivi and Ibrahim, 2022
Redox enzymes	Glucose oxidase/Catalase system	Glucose oxidase achieves the removal of O_2 from milk through oxidation of glucose with the resultant H_2O_2 removed by catalase	Glucose oxidase/Catalase system is the compatible with many food applications	Cruz et al., 2012a; Dubey et al., 2017

managing *Bifidobacterium* viability in yogurt. The H^+-ATPase defective mutants of *L. delbrueckii* subsp. *bulgaricus* cannot exclude H^+ protons and are sensitive to acidity due to their inability to maintain cytoplasmic pH homeostasis (Wang et al., 2013). As the primary organism responsible for post-fermentation acidification, the use of such mutants in yogurt fermentation can reduce the accumulation of acidity post-fermentation (Wang et al., 2013). Ongol et al. (2007) reported an enhanced viability of *B. breve* during storage in yogurt fermented with H^+-ATPase defective mutants.

4.2 Stress adaptation

Stress adaptation involves the pre-exposure of an organism to sub-lethal stress conditions that induce the development of tolerance to subsequent lethal stress exposure (Fiocco et al., 2020). The process involves several repetitive generations of exposure to mild stress, punctuated by incremental stress intensification (Jiang et al., 2016). A typical stress adaptation process can involve up to 50 generations of repeated stress exposures in which stress-resistant variants are isolated every few generations (Jiang et al., 2016). Variants that exhibit a stable stress resistance phenotype are subsequently preserved as genetically adapted mutants (Berger et al., 2010). Some successful adaptation experiments have been reported for different *Bifidobacterium* species. An acid-resistant mutant strain of *B. longum* subsp. *longum* was isolated after a 50-generation successive subculturing in MRS broth adjusted to pH 2.5 (Jiang et al., 2016). Similarly, thermal stress- and oxidative stress-adapted bifidobacteria cells were isolated after successive exposures to heat and hydrogen peroxide, respectively (Berger et al., 2010; Mozzetti et al., 2010). The genetic basis of sustained stress adaptation is the evolutionary development of mutants that overexpress stress response genes. Using transcriptomic profiling of induced oxidative stress adaptation in *B. longum* subsp. *longum*, Xiao et al. (2011) observed an upregulation of genes encoding essential proteins involved in the protection or repair mechanisms of damaged cell components, such as alkyl hydroperoxide reductase C22 (AhpC), DNA-binding ferritin-like protein (Dps), ribonucleotide reductase (NrdA), and enolase. After successive heat shock treatments, Berger et al. (2010) also observed that heat-adapted mutants overexpressed the *dnaK* operon and the *clpB* gene in *B. longum*. Apart from the adaptive tolerance to the same stress factor used to induce adaptation (homologous adaptation), cross-protection to different stress factors (heterologous adaptation) can also occur (Chen et al., 2017). Central to the cross-tolerance is the role of the transcriptional general stress response regulators (Averina et al., 2012). In *Bifidobacterium* species, the WhiB-like proteins encoded by the *whiB* gene are the universal transcriptional regulators of stress response genes (Averina et al., 2012). As pleiotropic regulators, the effect of any induced stress adaptation can likely overlap among different stress factors (Averina et al., 2012).

4.3 Microencapsulation

Microencapsulation involves the use of biopolymers that entrap bacterial cells in a polymer matrix prepared into microgel spheres (Frakolaki et al., 2021). The entrapment within the microgel particles protects probiotics against environmental stress and aids their survival during food processing and storage (Yeung et al., 2016). While several biopolymers can be used in the preparation of probiotic microcapsules, alginate is by far the most widely used food-grade biopolymer (Liu et al., 2019; Abbas et al., 2022). A naturally occurring polysaccharide extracted from brown algae, alginate consists of a linear polymer of α-L-guluronic acid (G) and β-D-mannuronic acids (M) units in repetitive blocks (G-blocks and M-blocks) (Alba and Kontogiorgos, 2019). Solutions of alginates readily form gels in the presence of divalent cations such as Ca^{2+} through their interaction with the G-blocks in an egg-box model (Alba and Kontogiorgos, 2019). The microgel preparation process usually involves a solution of alginate mixed with a bacterial suspension that is subjected to extrusion to form droplets that are instantaneously hardened by treatment with $CaCl_2$ solution into three-dimensional gel spheres entrapping the probiotic bacteria (Liu et al., 2019; Abbas et al., 2022). Another commonly used method in the preparation of alginate gel droplets is emulsion. This method involves the alginate-bacterial mixture suspended in an oil bath with a surfactant to produce a water-in-oil emulsion (Frakolaki et al., 2021). The emulsion is subsequently treated with a $CaCl_2$ solution, and the formed beads are harvested by centrifugation (Liu et al., 2019). The gel spheres prepared by either of these methods are subsequently freeze-dried (Liu et al., 2019). Several studies have shown that encapsulation enhances the survival ability under environmental and simulated GIT stress conditions (Yeung et al., 2016; Ji et al., 2019; Cedran et al., 2021; Zhang et al., 2021). For instance, alginate- and chitosan-coated/alginate-encapsulated *B. longum* showed a 0.20–1.72 $\log_{10}$ CFU/g viability loss at 55–65°C compared to unencapsulated cells that had a viability loss of 3.0–5.0 $\log_{10}$ CFU/g loss (Ji et al., 2019). When subjected to simulated gastric conditions (pH 2.5), the viability of chitosan-coated/alginate-encapsulated *B. longum* decreased by 1.27 $\log_{10}$ CFU after 120 min compared to the viability of unencapsulated cells that had declined to undetected levels after the same time period (Ji et al., 2019). Besides the observed enhancement of survival under simulated stress conditions, the benefit of encapsulation has also been demonstrated in alginate-encapsulated *Bifidobacterium* spp. incorporated into yogurt and other foods. Cedran et al. (2021) observed a two-fold loss of viability of unencapsulated *B. lactis* compared to the alginate-encapsulated cells in jam after 6 days of storage. Similarly, Mousa et al. (2023) reported a higher viability of *B. bifidum* encapsulated in a double layer of whey protein and alginate in set yogurt during a 14-day storage at 4°C than free cells. Pradeep Prasanna and Charalampopoulos (2019) observed a > 3.0 $\log_{10}$ CFU/g viability decline for unencapsulated *B. animalis* subsp. *lactis* in goat milk yogurt over 28 days while the viability of alginate-encapsulated cells remained stable. However, it is worth noting that while the benefits of encapsulation indicate a better survival compared to unencapsulated cells, a general decline in viability especially over the duration of shelf life still occurs (Yeung et al., 2016; Mousa et al., 2023). Moreover, a disproportionately large number of encapsulation studies have been based on *B. animalis* subspp. *lactis*, an intrinsically acid and oxidative stress tolerant strain. Hence, there is still need for viability retention methods for the more stress-sensitive species like *B. bifidum*. Besides the viability retention benefits, the effects on microencapsulation on yogurt texture are as important. Mousa et al. (2023), observed an increase in viscosity, gumminess, chewiness, and adhesiveness in yogurt with alginate-encapsulated *B. bifidum*. Similarly, Li H. et al. (2021) observed an increase in water holding capacity and cohesiveness of yogurt with microcapsules of *L. paracasei*. Importantly most of the changes in texture are beneficial in yogurt quality and the microcapsule incorporated yogurt have been reported to be acceptable by sensory panels (Dimitrellou et al., 2019; Mousa et al., 2023).

4.4 Protective agents

Among the physicochemical stress factors associated with yogurt processing and storage, oxidative stress has the most significant effect on viability of *Bifidobacterium* spp. (Bolduc et al., 2006). Hence, the ability to control the dissolved oxygen content and the redox potential of yogurt can provide a sustainable approach to preserve viability. The incorporation of oxygen-scavenging compounds, such as ascorbic acid and cysteine, has been shown to improve *Bifidobacterium* spp. survival in yogurt (Norouzbeigi et al., 2021). At 500 mg/l, cysteine can maintain a negative redox potential in yogurt for 30 days (Dave and Shah, 1997a). However, despite its strong reducing capacity, cysteine is considered unsuitable for use in yogurt, as it impacts a sulfur taste (Meybodi et al., 2020). Regarding sensory effects, ascorbic acid is more compatible with dairy products (van Aardt et al., 2005). However, the antioxidant capacity of ascorbic acid is hindered by the gradual loss of its stability over the storage shelf life of yogurt (Dave and Shah, 1997b). From the highest concentration of 250 mg/Kg, only 15–20% of the ascorbic acid was retained in yogurt after 35 days of storage at 4°C (Dave and Shah, 1997b). An additional approach to protect *Bifidobacterium* spp. from the effects of oxidative stress is the addition of glucose oxidase and glucose in yogurt (Cruz et al., 2012a). Glucose oxidase utilizes oxygen as it oxidizes D-glucose to gluconic acid and hydrogen peroxide, thus causing a reduction in the dissolved oxygen content of yogurt (Afjeh et al., 2019). Cruz et al. (2012a) reported a significant increase in the viable population of *B. longum* in yogurts added with glucose oxidase and glucose. While the activity of glucose oxidase reduces the oxygen content of the yogurt, the release of hydrogen peroxide as a by-product has a negative impact on *Bifidobacterium* spp. Hence, the protective effect of the glucose oxidase + glucose system requires the addition of catalase as an accessory enzyme to eliminate the toxic effects of hydrogen peroxide (Cruz et al., 2012b). The use of protective agents in preserving probiotic viability is a technique that is already extensively used in spray-drying and freeze-drying processes for probiotic microencapsulation (Fiocco et al., 2020). A diverse range of substances have shown to be effective as protective agents against the osmotic, heat and cold stresses associated with spray- and freeze-drying processes (Fiocco et al., 2020). Some of these substances, such as skim milk powder, are milk by-products that are readily acceptable as ingredients in yogurt making. When used as a protectant, skim milk solids

stabilize the bacterial cell membrane by forming a protective coating on cell wall proteins (Terpou et al., 2019). This prevents cell damage due to thermal and osmotic stress associated with spray drying (Terpou et al., 2019). Other substances, such as sugars, sugar alcohols and complex carbohydrates, have proven to be effective cryoprotectants during freeze-drying (Rockinger et al., 2021). Although they are used as protective agents against freezing, during freeze drying, cryoprotective agents can be valuable in preserving *Bifidobacterium* viability during the long period of cold stress during yogurt shelf-life. Among the cryoprotective agents, trehalose and glycerol are the most favorable for use in yogurt. These compounds lower the phase transition temperature of the cell membrane under cold conditions, thus maintaining it in a flexible liquid crystalline state while also keeping the cell membrane hydrated through their hydrogen bond interactions with phospholipid heads (Rockinger et al., 2021). Furthermore, due to their water-binding abilities, the compounds can suppress ice nucleation and prevent the damaging effect of ice crystal formation (Rockinger et al., 2021).

5 *Bifidobacterium* viability determination methods

5.1 Culture-based methods

In the last 20 years, a broad range of culture media have been proposed for the enumeration of *Bifidobacterium* spp. in dairy products (Van de Casteele et al., 2006; Lima et al., 2009; Ashraf and Smith, 2015). Among them, is MRS-NNLP agar, a widely used selective media (Dave and Shah, 1996; Ashraf and Shah, 2011; Karimi et al., 2012). In such media, the use of selective supplements may lead to an underestimation of viability as some live *Bifidobacterium* spp. cells may be sensitive to the selective agents (Dave and Shah, 1997a). For example, Van de Casteele et al. (2006), reported a lower recovery of *Bifidobacterium* spp. on MRS-NNLP agar than on MRS agar. Secondly, the selectivity of the medium depends on the type of non-target species present in the product (Van de Casteele et al., 2006; Ashraf and Smith, 2015). In mixed species products with *L. rhamnosus* and *L. acidophilus* strains, MRS-NNLP agar could not select for *Bifidobacterium* spp. (Van de Casteele et al., 2006; Ashraf and Smith, 2015). Currently, the International Organization of Standardization (ISO) and International Diary Federation (IDF) recommended culture-based method for the enumeration of *Bifidobacterium* spp. in dairy products (ISO 29981:2010/IDF 220:2010) is based on *trans*-galactosylated oligosaccharides (TOS) propionate agar containing lithium mupirocin as a selective agent (TOS-Mup media) (International Organization for Standardization [ISO], and International Dairy Federation [IDF], 2010). Like MRS-NNLP agar, TOS-Mup media has a low recovery for some *Bifidobacterium* spp. (Bunesova et al., 2015). **Table 2** summarizes the recent applications of the recommended media for selective enumeration of *Bifidobacterium* spp. in yogurt and other dairy-based products. It is evident that despite all the intensive work, there is still a need for a medium that could be used as a standard for the quantification of all *Bifidobacterium* spp. Moreover, species and strain-specific physiological requirements affect quantification efficiency (Van de Casteele et al., 2006; Bunesova et al., 2015). In addition to the drawbacks mentioned above, culture-based methods are laborious and have long results turnaround time of up to 72 h as agar plates need to be incubated under specific growth conditions (Davis, 2014; Geng et al., 2014). Since these methods are based on the cultivability of the cells, they cannot quantify cells that are in a viable but non-culturable (VBNC) state (Jackson et al., 2019; Vinderola et al., 2019). Hence, culture-based methods may underestimate viable counts of beneficial probiotic bacteria (Davis, 2014).

5.2 Flow cytometry

Flow cytometry is a single-cell analysis technique that is used to explore the physical and physiological characteristics of microbial cells as they pass through a beam of light (usually blue laser, 488 nm) (Davis, 2014). When used with fluorescent staining, the technique can distinguish between live and dead cells based on viability markers such as membrane integrity and intracellular enzyme activity (Wendel, 2022). Cell integrity is often determined by double staining with the DNA binding dyes such as diamidinophenylindole (DAPI), acridine orange and the SYTO dye series, which emits green fluorescence after excitation with 488 nm laser and propidium iodide (PI), which emits red fluorescence after excitation at the same wavelength (Veal et al., 2000). The exclusion of PI by cells with intact membranes gives viable cells a green fluorescence, while non-viable cells with damaged membranes fluoresce red (Veal et al., 2000). Intracellular enzyme activity is often determined using membrane-permeant fluorogenic substrates such as 5,6-carboxyfluorescein diacetate (5,6-cFDA), which, upon enzymatic hydrolysis by intracellular esterases from live cells, release a green, fluorescent carboxyfluorescein (Hoefel et al., 2003). Following a gating for live- (green fluorescent) and dead-cell (red fluorescent) subpopulations, viability determination is then based on the enumeration of cells from an appropriately diluted sample that falls within the live-cell region (Foglia et al., 2020). Although FCM has been available for a long time as a high throughput method of studying bacterial cell viability, its use in the enumeration of probiotic viability in foods has been limited. So far, one protocol by the ISO and IDF (ISO 19344 – IDF 232: 2015) is available for the flow cytometric enumeration of starter cultures and probiotics in fermented products (International Organization for Standardization [ISO], and International Dairy Federation [IDF], 2015). However, the non-specificity of the method due to its inability to selectively enumerate viable probiotics in the presence of the starter cultures, is a significant limitation. Some studies have attempted to improve the species selectivity of flow cytometry by incorporating antibody labeling in conjunction with membrane integrity and enzyme activity fluorescent probes (Geng et al., 2014; Chiron et al., 2018). The immuno-flow cytometry assay utilizes the specific binding of a primary polyclonal antibody to ligands on the bacterial cell, which is subsequently bound to a secondary antibody conjugated to a fluorescent tag and further stained with a viability probe (Wilkinson, 2018). Using this concept of dual labeling with polyclonal antibodies and 5,6-cFDA, only viable *B. lactis* were enumerated from mixed cultures and fermented products containing *L. bulgaricus*, *S. thermophilus* and *Lac. lactis* (Geng et al., 2014). Similarly, polyclonal antibodies specific for *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*,

TABLE 2 Summary of the recent applications of the recommended media used for the selective enumeration of Bifidobacterium spp. in yogurt, lyophilized cultures, and other dairy-based products.

Base	Selective supplement	Species mixture	Target *Bifidobacterium* spp.	Product	References
MRS	NNLP (Nalidixic acid, neomycin sulfate, Lithium chloride and paromomycin sulfate)	*L. acidophilus, B. animalis subsp. lactis* BB-12, and *S. thermophilus*	*B. animalis* subsp. *lactis* BB-12	Fruited yogurt	Erkaya-Kotan, 2020; Najgebauer-Lejko et al., 2021
MRS	NNLP (Nalidixic acid, neomycin sulfate, Lithium chloride and paromomycin sulfate), 0.3% v/v L-cysteine HCl	*L. delbrueckii subsp. bulgaricus, B. animalis subsp. lactis* BB-12, and *S. thermophilus*	*B. animalis* subsp. *lactis* BB-12	Yogurt	Akalin A. S. et al., 2018; Frakolaki et al., 2022
MRS	NNLP (Nalidixic acid, neomycin sulfate, Lithium chloride and paromomycin sulfate), and L-cysteine	*Debaryomyces hansenii, Lactococcus cremoris, L. lactis, L. diacetylactis, Leuconostoc spp., S. thermophilus* and *B. bifidum* BB-11	*B. bifidum* B-11	Kefir	Buran et al., 2021
MRS	5% *v/v* NNLP (15 mg Nalidixic acid, 100 mg neomycin sulfate, 3 g Lithium chloride and 200 mg paromomycin sulfate) and 3% *v/v* L-cysteine HCl	*L. acidophilus* La-5, *B. animalis subsp. lactis* BB-12, *S. thermophilus and L. delbrueckii subsp. bulgaricus*	*B. animalis* subsp. *lactis* BB-12	Plain and Flavored Yogurts	Tsevdou et al., 2020
MRS	NNLP (Nalidixic acid, neomycin sulfate, Lithium chloride and paromomycin sulfate), and L-cysteine HCl	*B. bifidum* PTCC 1644 and ATCC 29521, *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* ST-20Y	*B. bifidum* PTCC 1644 and ATCC 29521	Yogurt	Ghaderi-Ghahfarokhi et al., 2021
TOS Propionate	MUP (Mupirocin)	*B. animalis subsp. lactis* BB-12 and *Propionibacterium shermanii subsp. freudenreichii*	*B. animalis* subsp. *lactis* BB-12	Dairy Drink	Yerlikaya et al., 2020
MRS	LP (0.3% Lithium chloride, 0.05% L-cysteine HCl and 0.9% sodium propionate)	*L. acidophilus, B. longum,* and *S. thermophilus*	*B. longum*	Yogurt	Zhang et al., 2019
MRS	MUP (Lithium Mupirocin)	YF-L812 starter cultures *(L. delbrueckii subsp. bulgaricus* and *S. thermophilus) and B. animalis subsp. lactis* BB-12	*B. animalis* subsp. *lactis* BB-12	Yogurt	He J. et al., 2019
Bifidobacteria Selective Media (BSM)		*L. acidophilus* DSMZ 20079, *B. bifidum* DSMZ 20456, *L. bulgaricus,* and *S. thermophilus*	*B. bifidum* DSMZ 20456	Flavored Yogurt	Turgut and Cakmakci, 2018
MRS	NNLP (Nalidixic acid, neomycin sulfate, Lithium chloride and paromomycin sulfate)	*L. acidophilus* and *B. animalis subsp. lactis*	*B. animalis* subsp. *lactis* BB-12	Ice Cream	Akalin A. et al., 2018
MRS	0.05 mg/mL MUP and, 0.05% L-Cysteine	*L. paracasei* PC-01 and *B. animalis subsp. lactis* Probio-M8	*B. animalis* subsp. *lactis* Probio-M8	Fermented Milk Beverage	Hao et al., 2023

L. helveticus and *L. rhamnosus* in combination with SYTO® 24 and PI staining were used to selectively enumerate the viable individual strains in multi-strain probiotic products (Chiron et al., 2018). Another immuno-flow cytometry concept with a potential application in probiotic enumeration is the immunomagnetic separation of specific probiotic strains from a mixed species using antibody-coated magnetic beads (Wilkinson, 2018). This technique, commonly used for the recovery and enrichment of pathogens, was recently used to isolate *L. paracasei* from human feces (Takada et al., 2023). When applied for probiotic viability enumeration, recovered cells from immunomagnetic separation can be analyzed by flow cytometry after staining with viability

TABLE 3 Summary of culture-independent techniques that have been used for the determination of Bifidobacterium spp. viability in yogurt and other dairy-based products.

Dairy-based probiotic food samples					
Method	Product	Species mixture	Target species	Findings	References
PMA-qPCR	Fermented milk	*S. thermophilus, L. delbrueckii subsp. bulgaricus, L. casei, L. acidophilus and B. lactis*	*B. lactis* (BB-12)	Viable counts comparable to plate count (Pearson correlation coefficient = 0.995). Rapid (results obtained within 3 h).	García-Cayuela et al., 2009
EMA-qPCR	Yogurt	*S. thermophilus, L. delbrueckii subsp. bulgaricus and B. longum* ATCC 15707	*B. longum* ATCC 15707	Viable counts slightly lower than plate counts. Good correlation between the two methods (R^2 = 0.9948). Rapid (results obtained within 4 h).	Meng et al., 2010
PMA-qPCR	Cheddar cheese	*Lactococcus* spp., *L. rhamnosus* RO011, *L. helveticus* RO052, and *B. animalis* subsp. *lactis* BB-12	*B. animalis* subsp. *lactis* BB-12	Viable counts higher than plate counts during cheese manufacturing.	Desfossés-Foucault et al., 2012
Flow Cytometry (FCM)	Fermented milk	*L. lactis* CNCM I-1631., *L. delbrueckii* subsp. *bulgaricus* CNCM I-1519, *S. thermophilus* CNCM I-1630, and *B. animalis* subsp. *lactis*	*B. animalis* subsp. *lactis*	Viable counts comparable to plate counts (correction coefficient = 0.954). Uses species-specific polyclonal antibody. Rapid (results obtained within 2 h).	Geng et al., 2014
PMA-qPCR	Synbiotic ice-cream	*L. acidophilus* LA-5, and *B. animalis* subsp. *lactis* BB-12	*B. animalis* subsp. *lactis* BB-12	Application of PMA-qPCR. Reliable method for quantification of probiotics under stressful environments.	Matias et al., 2016
PMA-qPCR	Petit-suisse cheese	*L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *S. thermophilus*	*B. animalis* subsp. *lactis* BB-12	Application of PMA-qPCR.	Padilha et al., 2016
PMA-qPCR	Synbiotic table spread	*Bifidobacterium* BB-12	*Bifidobacterium* BB-12	Good correlation between PMA-qPCR with plate counts (r = 0.92 to 0.97). Results of the two methods generally comparable	Dos Santos et al., 2018
Lyophilized Culture Samples					
Chip-Based dPCR coupled with PMA	Lyophilized cultures	*L. acidophilus* NCFM and *B. animalis* subsp. *lactis* BI-04	*B. animalis* subsp. *lactis* BI-04	Counts slightly lower but comparable to plate counts. Low variation between results compared to plate count method. Rapid (results obtained within 1 h) and enumeration at strain level.	Hansen et al., 2018
PMA-qPCR	Lyophilized capsules	*L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12	*B. animalis* subsp. *lactis* BB-12	Viable counts comparable to plate count.	Kramer et al., 2009
Flow Cytometry (FCM)	Freeze-dried probiotic cultures	*L. rhamnosus* R0011, *L. helveticus* R0052, B. *longum* subsp. *longum* R0175 and *Saccharomyces cerevisiae* var *boulardii* CNCM I-1079	*B. longum* subsp. *longum* R0175	Counts generally higher than plate counts (in 73% cases). Good correlation (R^2) of 0.8222 between the two methods. Uses species-specific polyclonal antibody. Rapid (results obtained within 2 h)	Chiron et al., 2018
		L. helveticus R0052, *B. longum* subsp. *infantis* R0033 and B. *bifidum* R0071	*B. longum* subsp. *infantis* R0033 and *B. bifidum* R0071		

(Continued)

TABLE 3 (Continued)

Dairy-based probiotic food samples					
Method	Product	Species mixture	Target species	Findings	References
Droplet dPCR coupled with PEMAX	Freeze-dried probiotic powders	*B. animalis* subsp. *lactis* BI-04	*B. animalis* subsp. *lactis* BI-04	Relative difference of 15% between the ddPCR and plate count. Good correlation (r) of 0.76 between the two methods. Low variation between results compared to plate count method	Hansen et al., 2020
		B. animalis subsp. *lactis* HN019	*B. animalis* subsp. *lactis* HN019		
		B. animalis subsp. *lactis* Bi-07	*B. animalis* subsp. *lactis* Bi-07		
Droplet dPCR coupled with PE51	Freeze-dried probiotic cultures	*B. animalis* subsp. *lactis* Bi-07	*B. animalis* subsp. *lactis* Bi-07	Used PE51 dye made from a combination of EMA and PMA. Good correlation between PE51-ddPCR with plate counts ($r = 0.762$), and better than PMA-ddPCR and EMA-ddPCR	Kiefer et al., 2022
		B. animalis subsp. *lactis* BI-04	*B. animalis* subsp. *lactis* BI-04		

probes (Wilkinson, 2018). Apart from its value in viability determination, flow cytometry offers other benefits in studying some of the physical and physiological characteristics of viable cells that could relate to their stress responses and probiotic functionalities (Wendel, 2022). The additional element of flow cytometry, fluorescence-activated cell sorting (FACS), allows for the isolation and recovery of different subpopulations from a flow cytometry assay (viable, dead, and injured cells) for further analysis of metabolic, physiological, and genetic characteristics relating to probiotic functionality (Wendel, 2022).

5.3 Molecular and next generation methods

5.3.1 qPCR

Real-time quantitative polymerase chain reaction (qPCR)-based methods are premised on the detection and amplification of DNA of target organisms using fluorescent DNA intercalating dyes (e.g., SYBR green) or sequence-specific fluorogenic probes (e.g., TaqMan probes) (Agrimonti et al., 2019; Ruijter et al., 2021). Sequence-specific oligonucleotide primers are used to flank specific fragments of a target gene to be amplified. As the amount of PCR amplicon increases during PCR, the fluorescent signal accumulates. The quantification cycle (Cq) is then measured in the exponential phase of qPCR when the fluorescence signal has accumulated above the background fluorescence (Davis, 2014). During exponential phase, the amount of PCR amplicon is directly proportional to the DNA template (Davis, 2014). Hence, using standard curves established by plotting the Cq values against DNA copies, the number of copies of target species in the food sample can be determined (Davis, 2014). While the 16S rRNA gene has frequently been used in experimental qPCR-based methods (Zhang and Fang, 2006; Kim H. B. et al., 2020), its use poses challenges for quantification as it may exist as more than one copy in some bacterial genomes and it has a high sequence similarity between the *Bifidobacterium* spp. (Kim H. B. et al., 2020; Fan et al., 2021; Shi et al., 2022). For reliable quantification, the target gene should be a single copy within the bacterial genome (Shi et al., 2022). Recently, protein-encoding housekeeping genes such as the translation elongation factor EF-TU (*tuf*) and phenylalanine tRNA ligase subunit alpha (*pheS*) genes have been successfully used in qPCR methods for probiotic quantification in dairy products (Scariot et al., 2018; Fan et al., 2021; Shi et al., 2022). Alternatively, comparative genomics can be used to find unique and specific genetic markers for primer design and selective detection of closely related species and subspecies, especially of *Bifidobacterium*, where the use of housekeeping genes may be limited (Kim H. B. et al., 2020). A recent study by Kim H. B. et al. (2020) successfully designed species and subspecies-specific primers for 22 *Bifidobacterium* species and subspecies based on the genetic markers identified using comparative genomics. The main challenge of qPCR-based methods, however, is their inability to differentiate between DNA from live and dead cells (Scariot et al., 2018; Shehata and Newmaster, 2021; Guo et al., 2022). This implies that the use of qPCR methods may overestimate counts. Therefore, its application in probiotic viability determination is limited unless coupled with another technique that allows selective quantification of viable counts.

5.3.2 Propidium monoazide qPCR for quantification of *Bifidobacterium* spp. in yogurt

The challenge of distinguishing between live and dead cells encountered with general qPCR methods can be circumvented with the inclusion of viability dyes (Scariot et al., 2018; Shi et al., 2022; Shehata et al., 2023). At present, there are three types of viability dyes used for the selective quantification of viable

bacterial cells, namely ethidium monoazide (EMA), propidium monoazide (PMA), and PMAxx, an improved version of PMA (Lv et al., 2021; Shehata and Newmaster, 2021; Mu et al., 2022). PMA is a next-generation viability dye developed in 2006 to overcome the challenges of EMA, which was found to penetrate cell membranes of live cells of some bacterial species (Nocker et al., 2006).

5.3.2.1 PMA mechanism of action

Propidium monoazide was produced through the chemical modification of propidium iodide by replacing the amino group on the phenanthridine ring with the azide group that can form a covalent crosslink with the DNA (Nocker et al., 2006; Cangelosi and Meschke, 2014). Recently, an improved and more effective version of PMA with the same spectral properties, PMAxx, was developed by Biotium Inc. PMAxx is a new generation DNA intercalating and membrane impermeant dye that can only penetrate the cell membranes of dead cells (Guo et al., 2022; Kallastu et al., 2023). The procedure for the PMAxx-based method involves an initial stage of incubation of the food sample with about 25–150 μM of PMAxx in the dark to allow the dye to penetrate compromised cell membranes and intercalate with the DNA (Nocker et al., 2006; Mu et al., 2022). Upon exposure to bright light, the azide group of PMAxx produces nitrene, a highly photo-reactive molecule, that forms a covalent crosslink with DNA or reacts with water to form hydroxylamine (an inactivated form of PMAxx) (Nocker et al., 2006; Fittipaldi et al., 2012). The PMAxx-DNA conjugate is insoluble and, therefore, is removed with cell debris during DNA extraction, while the remaining conjugates are not amplified during PCR (Nocker et al., 2006). Hence, only DNA from live cells is amplified during qPCR.

5.3.2.2 Application of PMA-qPCR method for quantification of *Bifidobacterium* spp. in yogurt

A few studies have reported the application of PMA-qPCR methods for quantification of *Bifidobacterium* spp. in fermented dairy products (**Table 3**). PMA-qPCR methods can provide insight into the physiological state of probiotics due to their ability to detect cells in the VBNC state (Dong et al., 2020). Kibbee and Örmeci (2017) reported the application of PMA-qPCR for quantification of VBNC cells of *E. coli* in wastewater effluents, while Guo et al. (2021) used the same method for VBNC cell enumeration in drinking water. *Bifidobacterium* spp. can exhibit the VBNC state as a protective mechanism when under stressful environments (Lahtinen et al., 2008). For example, *B. longum* and *B. animalis* subsp. *lactis* were found to exhibit the VBNC state under acidic conditions in fermented products (Lahtinen et al., 2008). The presence of VBNC *Bifidobacterium* spp. in fermented dairy products, can lead to an underestimation of viability, a key parameter in probiotic quality assurance. Several factors must be considered for the effective application of PMA-qPCR methods in yogurt. A review by Fittipaldi et al. (2012) identified factors that can affect the efficiency of PMA-qPCR methods. These factors include probiotic species type, dye concentration, pH and turbidity of the sample. The authors recommended a pH adjustment and dilution for highly acidic (pH $\leq$ 4) and turbid ($\geq$10 Nephelometric Turbidity Units, NTU) samples, respectively. Several PMA-qPCR studies have included the pretreatment step of fermented dairy products before PMA treatment to disperse the casein micelles and to adjust the pH to 6.5 (Scariot et al., 2018; Yang et al., 2021). In addition, PMA treatment in these studies was carried out in clear media such as water or phosphate-buffered saline (PBS). However, the inclusion of this pretreatment step is not consistent with the PMA-qPCR methods for the quantification of probiotics in dairy-fermented products. For example, Shi et al. (2022) added PMA directly to the fermented milk sample (1 mL) without including a pretreatment step. In all these studies, PMA-qPCR methods selectively quantified live probiotic cells in fermented dairy products (Scariot et al., 2018; Yang et al., 2021; Shi et al., 2022). This shows that PMA may be applied directly to fermented dairy milk. However, additional PMA-qPCR studies on fermented dairy milk are necessary to support this conclusion and to comprehend the impact of fermented milk product pH, such as yogurt, on PMA efficacy.

5.3.3 Digital PCR

Digital PCR (dPCR) is a third-generation PCR and an emerging technology for microbial quantification (Agrimonti et al., 2019; Kiefer et al., 2022). Unlike qPCR, dPCR does not require standard curves for absolute quantification, and its sample preparation and amplification confirmation methods are different (Salipante and Jerome, 2020). In dPCR, a PCR reaction mixture containing the target sequence is partitioned randomly into thousands of small individual microreactors, such as oil droplets and chip wells (Hansen et al., 2020; Salipante and Jerome, 2020; Lv et al., 2021). A signal fluorescence from partitions containing a single copy of the target sequence is measured at the end of a PCR run (Hansen et al., 2018, 2020; Salipante and Jerome, 2020). Poisson statistics based on comparing the number of positive (with signal fluorescence) and negative reactions is used to determine the absolute quantity of the target sequence (Hansen et al., 2020; Salipante and Jerome, 2020). Like normal qPCR, dPCR cannot distinguish between live and dead cells; hence, it is coupled with viability dyes for selective quantification of live cells (Kiefer et al., 2022). Studies have reported the application of dPCR methods for quantification of *Bifidobacterium* spp. and other lactic acid bacterial species, mainly in freeze-dried products, indicating a good correlation with plate counts (Summarized in **Table 3**). In addition, dPCR methods hold several advantages over culture-based methods. These include short results turnaround time, low variation between results and the ability to quantify probiotics at strain levels (Hansen et al., 2020). However, high DNA concentrations can affect probiotic quantification by dPCR (Kim et al., 2023). For example, a recent study quantifying probiotic *L. casei* in milk showed that quantification at high DNA concentrations was not possible as dPCR was saturated and resulted in a narrow linear dynamic range (Kim et al., 2023). However, this can be solved by diluting the sample (Kim et al., 2023).

5.3.4 Next generation sequencing methods

Although the application of next generation sequencing (NGS) methods in the study of food microbiomes has been widely reported (Cao et al., 2017; Jagadeesan et al., 2019),

their use as quality assurance tools for probiotic viability in foods is still limited. NGS applications in foods have mainly been limited to metagenomic analysis that provides data on relative abundance of different taxa (Cao et al., 2017). Despite the high-resolution ability of NGS methods, the inability to provide information on the absolute quantities and viability status of the organisms in the food has limited its use in viability enumeration. Interestingly, some recent studies have shown the potential application of NGS in viability determination when the technology is coupled with a viability dye (Kallastu et al., 2023). Using the 16S rRNA gene amplicon sequencing coupled with PMAxx reagent, Kallastu et al. (2023) elaborated a workflow for the determination of absolute numbers of viable organisms in *kimchi* and sauerkraut. The developed workflow involved the addition of a spike-in control (standard) into the sample following PMAxx treatment. After the 16S rRNA gene amplicon sequencing, the number of viable bacteria was determined from the relative abundance and the ratios of the spike-in reads (Kallastu et al., 2023).

5.4 Single-cell Raman spectroscopy (SCRS)

Culture-independent, label-free, non-invasive, rapid single-cell Raman spectroscopy (SCRS)-based techniques that give a collective insight into the organism's phenome and genome are emerging as potential methods of microbial characterization (Jayan et al., 2022; Zhang et al., 2023). Recently, a novel automated SCRS-based technique that combines single-cell identification, viability, vitality and sequencing (SCIVVS) for the characterization of probiotics including *Bifidobacterium* spp., was described by Zhang et al. (2023). In the SCIVVS technique, probiotic characterization is a stepwise process where cells are first harvested from the probiotic sample, treated with a stable isotope probing (SIP), namely, deuterium oxide (D_2O), which can only be taken up by live cells and subjected to Raman spectroscopy at single-cell resolution (Zhang et al., 2023). Other isotopes, such as ^{13}C and ^{15}N, respectively, can also be used for SIP in SCRS-based techniques (Jayan et al., 2022). SIP is based on the principle that the Raman spectra shift when an atom is substituted with its heavier isotope (Jayan et al., 2022). Hence, in the case of H_2O and D_2O (heavy water), the uptake of H_2O in live and metabolically active cells is in the silent region (2040–2300 cm^{-1}) of SCRS (He Y. et al., 2019). The uptake of D_2O by the cells results in the partial replacement of the hydrogen (H) atom with the deuterium atom (D) (He Y. et al., 2019; Jayan et al., 2022). This results in the production of C – D bands, which can be modeled to deduce the metabolic state and viability of the cell (He Y. et al., 2019; Zhang et al., 2023). Hence, the SCRS of D_2O-treated cells can be used to determine probiotic viability, vitality, and species-level identification (based on a compiled SCRS database of reference species) (Zhang et al., 2023). SCIVVS is also coupled with a single-cell assembly genome sequencing and thus gives comprehensive analysis from genome to phenome (Zhang et al., 2023). However, more studies are needed on applying and assessing the suitability of SCRS-based techniques for probiotic quantification.

6 Conclusion and future perspectives

Bifidobacterium species are one of the most important members of the GIT of healthy humans, with a substantial body of evidence showing their beneficial probiotic functionalities in experimental models and human trials. Notably, while evidence has shown that some physiological effects of probiotics, such as immunomodulatory properties, can be elicited by bacterial cell components like lipoteichoic acids and peptidoglycan, which can be constituents of both live and dead cells, some probiotic functionalities are dependent on viability and are species and strain specific. Therefore, as envisioned in the definition, viability is a critical quality assurance parameter for probiotics and probiotic foods. Unlike other common probiotic organisms such as lactic acid bacteria, the incorporation and sustained survival of bifidobacteria in probiotic carrier foods like yogurt is a significant challenge. Yogurt process stress factors such as acidity, oxygen, heat, osmotically active solutes, and cold storage impede *Bifidobacterium* spp. survival and result in viability decline over the product shelf life. Although several studies have investigated the phenotypic responses of different *Bifidobacterium* spp. to these stress factors, molecular stress response mechanisms still need to be fully elucidated. Response mechanisms to osmotic and cold stress particularly, are yet to be deciphered. Understanding the bifidobacterial stress response mechanisms is critical to the development of strategies to preserve cell viability. Hence, approaches such as stress adaptation, process modifications, microencapsulation and the use of stress protective agents have been investigated, with varying levels of success, as viability retention methods. In addition to viability retention approaches, viability measurement is the ultimate quality assurance requirement for probiotic foods. However, the available standard culture-based methods have proved inadequate for accurately determining *Bifidobacterium* viability in probiotic yogurt.

To a limited extent, flow cytometry has been considered an alternative method for the viability determination of yogurt cultures. However, the method lacks specificity and has limited application for *Bifidobacterium* viability. New innovations in immuno- flow cytometry, where fluorescent viability staining is linked to monoclonal antibodies, are expected to improve the applicability of the technology. Moving forward, molecular-based methods such as PMA-qPCR, digital PCR and sequencing, represent the future generation of methods for viability determination. Already, metagenomic sequencing, is used in microbiome analysis and relative quantification in foods. Innovative approaches to adapt the next generation sequencing for absolute microbial and viability quantification by coupling the sequencing with viability dyes and spike-in controls represents novel methods for the future. The adaptation of a recently described novel next-generation method called

single-cell identification, viability and vitality tests, and source-tracking (SCIVVS) utilizes a D_2O-probed single-cell raman spectrum (SCRS) that can accurately quantify cell viability at the species level based on the C–D band. Crucially, these alternative methods with the accompanying techno-economic assessments, should be developed into standardized and validated protocols for industrial probiotic viability quality assurance applications.

Author contributions

TS: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing. TM: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing. UT: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing. MT: Conceptualization, Funding acquisition, Project administration, Supervision, Writing—review and editing. EB: Conceptualization, Funding acquisition, Project administration, Supervision, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Assessing probiotic viability in mixed species yogurt using a novel propidium monoazide (PMAxx)-quantitative PCR method

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Viability is a prerequisite for any therapeutic benefits associated with the ingestion of probiotic bacteria. Current culture-based techniques are inadequate for the enumeration of probiotics in mixed-species food products. This study utilized a quantitative PCR (qPCR) method coupled with propidium monoazide (PMAxx), and novel species-specific *tuf* gene primers to selectively enumerate *Lacticaseibacillus rhamnosus*, *Bifidobacterium* spp., and yogurt starter cultures in mixed-species probiotic yogurt. The method was optimized for PMAxx concentration and specificity and evaluated for efficiency and applicability. PMAxx-qPCR showed high specificity to the target organisms in mixed-species yogurt, quantifying only viable cells. The linear dynamic ranges were established over five to seven orders of magnitude. The assay was reliable with an efficiency of 91–99%, R^2 values > 0.99, and a good correlation to the plate count method (r = 0.882). The results of this study demonstrate the high selectivity, improved lead time, and reliability of PMAxx-qPCR over the culture-dependent method, making it a valuable tool for inline viability verification during processing and improving probiotic quality assurance for processors and consumers.

KEYWORDS

probiotic, viability, quantitative PCR, *tuf* gene, *Bifidobacterium* species, *Lacticaseibacillus rhamnosus*, yogurt cultures, propidium monoazide

1 Introduction

Fermented dairy products are considered excellent carriers of probiotics due to their consumers' general acceptance as health-promoting foods (Nyanzi et al., 2021; Sakandar and Zhang, 2021). Among the fermented dairy products, yogurt is the most popular and consumed probiotic product, with a market share of around 37% (Sakandar and Zhang, 2021). Probiotics are associated with several health benefits, such as gut microbiota stabilization, antimicrobial activity against pathogens, improved antioxidant activity, and therapeutic effects against allergies, inflammatory bowel diseases, and diarrhea (Roobab et al., 2020; Nyanzi et al., 2021). The recommended minimum dosage required for probiotics to impart therapeutic benefits to the host is 10^6-10^7 colony-forming units (CFU) per gram (g) or milliliter (ml) at the time of product consumption (Ranadheera et al., 2017; Fazilah et al., 2018; Yao et al., 2020). This corresponds to 10^8-10^9 CFU per 100 g or 100 ml serving. Hence, it is a prerequisite to determine probiotic viability during product manufacturing and storage to ensure that the minimum therapeutic dosage is maintained and consumer's expectations of probiotic quality are met. The current

methods of probiotic viability determination are based on standardized culture-based techniques (Davis, 2014; Jackson et al., 2019; Vinderola et al., 2019). However, these methods have many limitations (Davis, 2014; Vinderola et al., 2019), and their use for specific quantification of closely related probiotics and starter cultures in mixed-species fermented dairy products such as yogurt is challenging due to possible similarity in growth conditions and shared biochemical properties (Tabasco et al., 2007). Hence, there is a need for alternative methods that can overcome the limitations of culture-based methods for probiotic quantification. Several culture-independent methods such as flow cytometry, real-time quantitative PCR (qPCR), digital PCR (dPCR), and Next Generation Sequencing (NGS) have been considered as alternatives for probiotic enumeration (Wilkinson, 2018; Jackson et al., 2019; Hansen et al., 2020; Nyanzi et al., 2021). Among these methods, qPCR-based methods are commonly used for microbial quantification in fermented dairy products (García-Cayuela et al., 2009; Scariot et al., 2018; Fan et al., 2021; Yang et al., 2021; Shi et al., 2022). These qPCR-based methods are highly selective, sensitive and have short results turnaround time (Fan et al., 2021; Shehata et al., 2023). Quantitative PCR methods use sequence-specific oligonucleotide probes with fluorophores or fluorescent DNA intercalating dyes for real-time continuous detection and amplification of the target DNA from a food sample (Zhang and Fang, 2006; Davis, 2014; Agrimonti et al., 2019). As the target DNA sequence is amplified during qPCR, fluorescence from the intercalating dye or probes increases. The cycle threshold (*Ct*) values are measured during the exponential phase of the amplification curve when fluorescence has accumulated above the background noise (Davis, 2014). Plotting the *Ct* values against known DNA copies allows for direct determination of probiotic quantity in the sample (Zhang and Fang, 2006). The main challenge with qPCR-based methods is their inability to differentiate between DNA from dead and live cells (Fittipaldi et al., 2012; Huang et al., 2018). This limitation can be solved using viability DNA intercalating dyes (Nocker et al., 2006; Nyanzi et al., 2021). At present, there are three types of viability dyes used to prevent the amplification of DNA from dead cells, namely, ethidium monoazide (EMA), propidium monoazide (PMA, next-generation dye) and PMAxx (new generation dye), an improved version of PMA (Shehata and Newmaster, 2021; Kallastu et al., 2023). These dyes are membrane impermeant DNA intercalating dyes that only penetrate the cell membranes of dead cells (Nocker et al., 2006; Fittipaldi et al., 2012). When exposed to bright light, the azide group of the dye releases a highly reactive nitrene molecule, which forms a covalent crosslink with the DNA of dead cells (Nocker et al., 2006; Fittipaldi et al., 2012). The resulting DNA-dye complex is insoluble and is removed with the cell debris during the DNA isolation process (Nocker and Camper, 2006).

While PMA-qPCR-based methods are regarded as sensitive and reliable in quantifying probiotic viability in foods, the methods currently available focus on quantifying a single probiotic species (Scariot et al., 2018; Shehata and Newmaster, 2021; Shehata et al., 2023). Only a few studies have reported the application of PMA-qPCR in mixed-species probiotic fermented dairy products (García-Cayuela et al., 2009; Yang et al., 2021; Shi et al., 2022). However, none of the studies have reported the use of PMA-qPCR to quantify probiotic *Lacticaseibacillus rhamnosus*, *Bifidobacterium* spp., and starter cultures in mixed-species probiotic yogurt. Secondly, the application and validation of PMAxx, a new generation viability dye, has never been reported for quantifying mixed species probiotics in yogurt. Hence, the objective of this study was to develop a qPCR method coupled with PMAxx and novel species-specific primers targeting the translation elongation factor EF-TU (*tuf*) gene with the aim of selectively quantifying viable *L. rhamnosus*, *Bifidobacterium* spp., and starter cultures in mixed-species probiotic yogurt.

2 Materials and methods

2.1 Bacterial reference strains and growth conditions

Streptococcus thermophilus NCIMB 8510 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIMB 11778 were obtained from the NCIMB Ltd., (Aberdeen, Scotland). While *Lacticaseibacillus rhamnosus* ATCC 53103, *Limosilactobacillus fermentum* ATCC 9338, *Lactiplantibacillus plantarum* ATCC 14917, *Bifidobacterium breve* ATCC 15700 and *Bifidobacterium bifidum* ATCC 11863 were obtained from KWIK-STIK™, Microbiologics (MN, USA). The lyophilized bacterial reference strains were cultured twice in 10 ml sterile de Man, Rogosa, and Sharpe (MRS) broth (Neogen, Lansing, MI, USA) for non-*Bifidobacterium* spp. and MRS broth supplemented with 0.05% L-cysteine (Sigma-Aldrich, St. Louis, USA) (MRSc), for *Bifidobacterium* spp. The MRS broth cultures were incubated at 37°C for 24 h under aerobic conditions, while MRSc broth cultures were incubated at 48 h−72 h under anaerobic conditions using anaerobic gas generating sachets (AnaeroGen™ 2.5L, Oxoid Ltd, Basingstoke, UK). Stock cultures in 25% (v/v) sterile glycerol and cryo-beads, were stored at −80°C until needed for use. In addition to reference cultures, *S. thermophilus* and *L. bulgaricus* were also isolated from a commercial yogurt starter culture (LYOFAST Y 259 A, SACCO, Como, Italy). The identity of the isolates from the commercial starter cultures and the reference species used in this study was confirmed using matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) Biotyper (Bruker, Bremen, Germany).

2.2 Species-specific primer design

The target gene selection for primer design was based on the multiple comparisons between the *tuf* and 16S rRNA gene sequences retrieved from the NCBI GenBank database.[1] The gene sequences of typical representative reference strains, namely *B. bifidum* ATCC 29521, *L. rhamnosus* ATCC 53103, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. thermophilus* ATCC 19258 were aligned and analyzed using the Multiple Sequence Comparison by Log Expectation (MUSCLE) program.[2] The alignment results were viewed using the Jalview software

1 https://www.ncbi.nlm.nih.gov/

2 https://www.ebi.ac.uk/Tools/msa/muscle/

(Waterhouse et al., 2009). Species-specific primers (Table 1) for *Bifidobacterium* spp., *L. rhamnosus,* and *L. delbrueckii* were designed using the free online software primer 3 plus.[3] The primers were designed using the *tuf* gene sequences of *B. bifidum* BCRC 11844 (Accession Number: FJ549340.1), *L. rhamnosus* strain W6 (Accession Number: JN694773.1), and *L. delbrueckii* strain A23 (Accession Number: JN694768.1) retrieved from the NCBI GenBank database. *S. thermophilus*-specific primers were obtained from Fan et al. (2021). The designed primers were synthesized by Integrated DNA Technologies (IDT, Brussels, Belgium).

2.3 Primer specificity and PCR conditions

2.3.1 DNA extraction

The total genomic DNA was extracted from the cell pellets obtained from the pure bacterial cultures and yogurt samples using NucleoSpin® Microbial DNA and NucleoSpin® Food DNA isolation kits (Macherey-Nagel Gmbh & Co. KG, Düren, Germany) respectively. DNA concentration was determined using Qubit™ 4 Fluorometer and dsDNA High Sensitivity (HS) working solution (1×) (Invitrogen™, Thermo Fisher Scientific, Waltham, USA). The DNA quality was determined using NanoDrop ND-1000 UV/Vis spectrophotometer V 3.8.1 (Peqlab, Erlangen, Germany) at A260/A280.

2.3.2 Primer specificity verification

The specificities of the designed primers for *L. delbrueckii* subsp. *bulgaricus*, *L. rhamnosus* and *Bifidobacterium* spp. were checked *in silico* using the Basic Local Alignment Search Tool (BLAST) program from the NCBI website[4] against the nucleotide collection (nt) and Refseq representative genomes database. Experimental primer specificity verification was performed using DNA isolated from the monocultures and five mixed-species samples (MRS broth) containing an equal concentration of 10^8 Cells/ml of each reference species. The mixed-species sample compositions were as follows: Sample A: *S. thermophilus, L. subsp. bulgaricus, L. rhamnosus, L. plantarum, L. fermentum, B. bifidum,* and *B. breve* (All species). Samples B to E were the negative controls and contained all the species minus *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. rhamnosus*, and *Bifidobacterium* spp., respectively. The DNA was diluted to 5 ng using PCR-grade ultra-pure water prior to the qPCR assay. The non-specific amplification and primer dimers were checked using melt curve analyses and gel electrophoresis as described in the section 2.3.3 (Real-time qPCR conditions).

2.3.3 Real-time qPCR conditions

Quantitative PCR reactions were conducted in duplicate, and each reaction contained 5.0 µL of 2× TB Green® Advantage® qPCR Premix consisting of TB Green dye, full-length Taq DNA Polymerase, hot-start antibody, dNTPs, and buffer (Takara Bio Inc, Mountain View, CA, USA), 0.2 µL of forward primer (10 µM), 0.2 µL of reverse primer (10 µM), 1.0 µL of template DNA (0.5 ng) and 3.6 µL of nuclease-free water in a final qPCR volume of 10 µL. Each qPCR run included no template control (NTC), and 1.0 µL of nuclease-free water was used as a template. The qPCR assay was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Quantitative PCR cycle conditions were as follows: initial denaturation at 95°C for 30 s followed by 35 cycles of denaturation at 95°C for 5 s for all species, annealing at 62°C for 20 s (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) or 62°C for 15 s (*L. rhamnosus*) and extension at 72°C for 6 s (*L. delbrueckii* subsp. *bulgaricus*), 72°C for 15 s (*L. rhamnosus*) and 72°C for 20 s (*S. thermophilus*). Annealing and extension were carried out as a combined step for *Bifidobacterium* spp. at 60°C for 20 s. Each reaction was held at 4°C for 5 s, followed by the melting curve analysis at 45 to 95°C with an increment of 0.5°C. The cycle threshold (Ct) was calculated automatically using a single threshold mode based on the point at which the threshold has crossed the background levels and at which the exponential phase of the qPCR reaction was reached. The qPCR products were electrophoresed with an ethidium bromide (Invitrogen, Carlsbad, USA) stained 3% agarose gel running at 90 V for 60 min in 0.75× TAE buffer. The gel was analyzed using a gel documentation system (Gel Doc™ EZ imager, Bio-Rad, California, USA).

2.4 PMAxx-qPCR

2.4.1 PMAxx treatment

The effective concentration of PMAxx was determined by treating heat-killed *L. rhamnosus* cells with different concentrations of PMAxx ranging from 50, 75 and 100 µM. Overnight cultures of *L. rhamnosus* in MRS broth (1.5 ml) were heat-treated in a water bath at 95°C for 5 min. PMAxx treatment was performed following a method described by Scariot et al. (2018) and PMAxx supplier protocol but with modifications. The heat-killed bacterial cells were centrifuged at 6000 × *g* for 2 min using a microcentrifuge (Ortoalresa, Madrid, Spain), then washed twice with sterile phosphate-buffered saline (PBS), pH 7.3 (Oxoid Ltd, Basingstoke, UK). The cell pellets were resuspended in 400 µl of ultra-pure water, and a PMAxx™ dye (Biotium Inc., Hayward, CA, USA) stock solution (20 mM) was added to give final concentrations of 50, 75, and 100 µM. All the samples were incubated in the dark for 10 min at room temperature and were subjected to mixing every 1 min. The samples were then placed on ice and exposed to a 500 W halogen light source at a distance of 12 cm (Shao et al., 2016) for 15 min to create a covalent link between PMAxx and DNA. The samples were turned frequently to ensure maximum light exposure was achieved. Upon PMAxx treatment, the samples were centrifuged at 6000 × *g* for 10 min. The obtained cell pellets were subjected to DNA extraction and qPCR.

2.4.2 Determination of PMAxx effectiveness and its effect on live cells

The effect of PMAxx at the concentration of 100 µM was tested on all the target bacterial species used in the study. Aliquoted

3 https://www.primer3plus.com/

4 https://www.ncbi.nlm.nih.gov/tools/primer-blast/

TABLE 1 Species-specific primers for probiotics and yogurt cultures.

Organism	Primer sequence		Primer name	Location within a gene	References
Bifidobacterium spp.	F	5′-AAGCCGTTCCTGATGCCTATC-3′	Bb-1F	398–418	This study
	R	5′-GAGGTAACGGTGGTGGTCTG-3′	Bb-1R	527–546	
L. delbrueckii	F	5′-AGACTCTTGACTTGGGTGAAGC-3′	Ldb-1F	112–133	This study
	R	5′-GTTCTGTGGGTCTTGATTGAGC-3′	Ldb-1R	211–232	
L. rhamnosus	F	5′-ATCGATCGTGGTACGGTTAAGG-3′	Lcr-1F	12–33	This study
	R	5′-ACCAAGATCCAAGGTCTTACGG-3′	Lcr-1R	107–128	
S. thermophilus	F	5′-CGTGGTGTTGTTCGTGTTAATGA-3′	ST-F		Fan et al. (2021)
	R	5′-CGGCAATACCTTCATCAAGTTGT-3′	ST-R		

F, Forward Primer; R, Reverse Primer.

samples containing bacterial cultures (1.5 ml) of each target species were used to determine the effect of PMAxx on live cells. Samples were divided into two groups, namely control and test samples, which contained live untreated cells and live PMAxx-treated cells, respectively. All samples were 10-fold serially diluted in a 7-point dilution and were spread plated (100 μL) on M17-glucose (*S. thermophilus*), MRS (*L. rhamnosus* and *L. bulgaricus*), and MRSc (*Bifidobacterium* spp.) agars.

The effectiveness of PMAxx in inhibiting the amplification of DNA from dead cells was determined by calculating the percentage of dead cell DNA removal based on the equations 1–4 recommended by the dye manufacturer but with slight modifications.

$$\Delta Ct_{(dead)} = Ct_{(dead\ PMAxx\ -\ treated)} - Ct_{(dead\ untreated)} \quad (1)$$

$$\text{Fold decrease by PMAxx} = 2^{\Delta Ct\ (dead)} \quad (2)$$

$$\%\ \text{Dead cell DNA remaining} = \frac{100}{\text{Fold decrease by PMAxx}} \quad (3)$$

$$\%\ \text{Dead cell DNA removed} = 100 - \%\ \text{dead cell DNA remaining} \quad (4)$$

2.4.3 Determination of linear dynamic range, efficiency, slope, correlation and limit of quantification

The standard curves were created using the genomic DNA isolated from PMAxx-treated pure cultures of *L. delbrueckii subsp. bulgaricus* NCIMB 11778, *S. thermophilus* NCIMB 8510, *L. rhamnosus* ATCC 53103, *B. breve* ATCC 15700, and *B. bifidum* ATCC 11863 on two different days. The genomic DNA was 10-fold serially diluted in PCR-grade ultra-pure water to the final copy number ranging from 10^7 to 10^0 per reaction. The linear dynamic range (LDR), efficiency (E), slope (K), and correlation coefficient (R^2) were determined from the standard curves created by plotting Ct values vs. log DNA copy number. The limit of quantification (LOQ) was determined using the standard curves created by plotting the Ct values vs. log CFU/ml. To plot Ct vs. log CFU/ml, the bacterial cultures of the target species were 10-fold serially diluted in a 7-point dilution and spread-plated (100 μl) as previously described. The qPCR amplification efficiencies were determined using the equation 5 (Broeders et al., 2014):

$$E = 100 \times (10^{-1/S} - 1) \quad (5)$$

Where E is the qPCR amplification efficiency, S is the slope obtained from the standard curve.

The DNA copy number was calculated using the equation 6 and the genome of *B. bifidum* ATCC 11863 (2,211,767 bp),[5] *B. breve* ATCC 15700 (2,275,660 bp),[6] *L. delbrueckii subsp. bulgaricus* ATCC 11842 (1,864,998 bp), NCBI: txid 390333 (Van De Guchte et al., 2006); *S. thermophilus* ATCC 19258 (2,102,268 bp), GenBank: CP038020 (Cho et al., 2021), and *L. rhamnosus* GG (3,010,111 bp) GenBank: FM179322.1 (Kankainen et al., 2009).

$$\text{DNA copy number} = \frac{\text{DNA amount (ng)} \times \text{Avogadro's constant } (6.022 \times 10^{23})}{\text{DNA template length (bp)} \times \text{MW} \times \text{CF}} \quad (6)$$

Where MW is the average molecular weight of double stranded DNA (660 Da) per base pair and CF is the conversion factor (1×10^9).

2.4.4 Comparison of PMAxx-qPCR method to standard plate count method

The target species' viable counts were determined using the plate count method and PMAxx-qPCR. The culture samples of the target species were prepared on two different days ($n = 10$) in MRS broth. The plate count method described in the section 2.4.2 (Determination of PMAxx effectiveness and its effect on live cells) was used to quantify the target species. To perform PMAxx-qPCR, the bacterial cultures were subjected to PMAxx treatment, DNA extraction, and qPCR.

5 https://genomes.atcc.org/genomes/0900f1128a5a4e56

6 https://genomes.atcc.org/genomes/efcf0d5d0df5440a

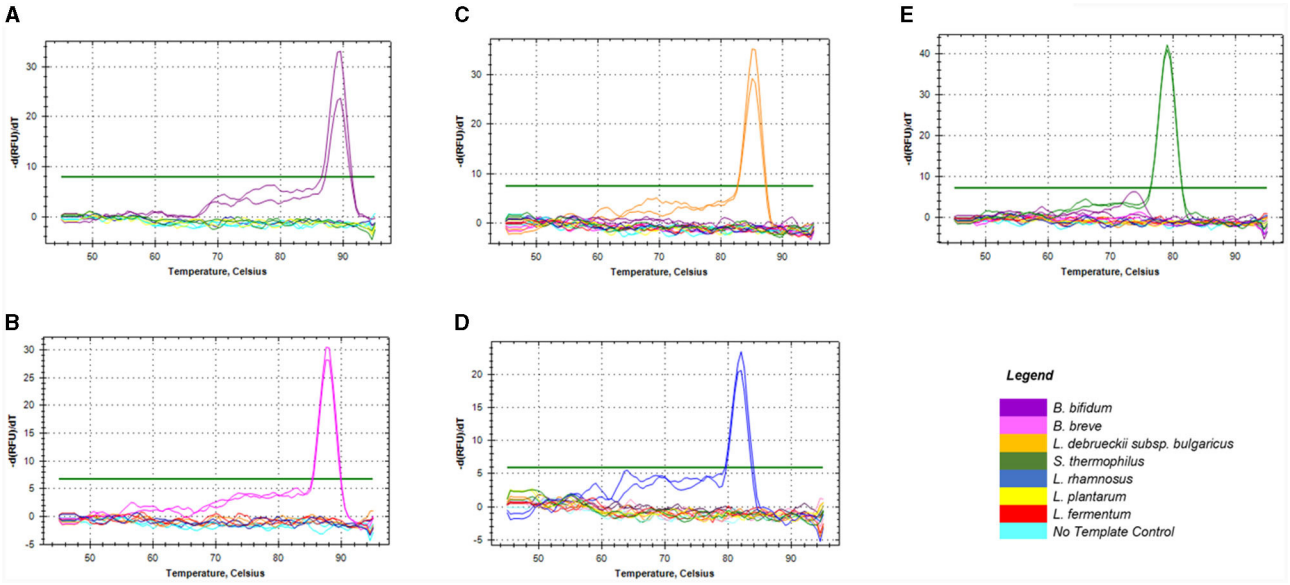

FIGURE 1
Melt curves showing the specificity of the four sets of primers against DNA of non-target species commonly used in dairy products. Graphs **(A, B)** represented primers for *Bifidobacterium* spp. namely; *B. bifidum* **(A)** & *B. breve* **(B)**, **(C)** for *L. delbrueckii* subsp. bulgaricus, **(D)** for *L. rhamnosus*, and **(E)** for *S. thermophilus*.

2.5 PMAxx-qPCR method application for viability determination in mixed-species probiotic yogurt during storage

Raw cow's milk collected from the University of Pretoria research farm (Pretoria, South Africa) was pasteurized at 85°C for 30 min. The milk was cooled to 40°C, and inoculated with the bacterial cultures at the final concentration of 1.5×10^9 Cells/ml each. Species mixtures used for fermentation were as follows: Yogurt mixture I: *L. rhamnosus* ATCC 53103, *L. delbrueckii* subsp. *bulgaricus* NCIMB 11778, *S. thermophilus* NCIMB 8510, *B. bifidum* ATCC 11863, *L. plantarum* ATCC 14917, and *L. fermentum* ATCC 9338 and yogurt mixture II: Similar to yogurt mixture I, except *B. bifidum* which was replaced with *B. breve*. The mixtures were incubated at 40°C until pH 4.5 was reached, then cooled and kept at 4°C. The yogurt was aliquoted (3 g) into two (Control sample and PMAxx-treated sample) on days 1 and 30 of storage, and its pH was adjusted to 6.5 with 1 M NaOH (García-Cayuela et al., 2009). Casein micelle was dispersed by adding 1 M tri-sodium citrate (3 ml) followed by centrifugation at 10,000 × *g* for 10 min at 4°C (García-Cayuela et al., 2009). Cell pellets were washed with sterile PBS (Yang et al., 2021) and resuspended in 400 µl ultra-pure water or MRS broth before the PMAxx treatment at 100 µM (except for non-treated yogurt). Cells were then subjected to DNA extraction and qPCR. The bacterial count in pure cultures and in the yogurt were calculated as described by Ilha et al. (2016).

2.6 Statistical analysis

GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) was used to analyse data. The *t*-test was used to determine the statistical difference between the viable counts of untreated and PMAxx-treated cells. Simple linear regression and Bland-Altman method of comparison were used to find the correlation between PMAxx-qPCR and plate count methods. All analyses were conducted in duplicates. P values < 0.05 were considered statistically significant.

3 Results

3.1 Similarity comparison of *tuf* and 16S rRNA gene sequences

The gene sequence identity based on the number of matched nucleotide bases between the representative target species was lower in the *tuf* gene compared to the 16S rRNA gene (Supplementary data). The overall identity of *tuf* gene sequences between the species was 69.31%, which accounted for 685 base matches within a gene length of 988 bp. Whereas, the overall gene sequence identity of 16S rRNA was 81.73%, which accounted for 1,098 base matches within a gene length of 1344 bp. In addition, the 16S rRNA gene copy number within the genomes of the target species ranged from 3 to 9 copies (supplementary data). In contrast, the *tuf* gene copy within the five target species was 1.

3.2 Primer specificity verification

The *in silico* specificity verification showed that the designed primers were specific to the target species and could amplify different strains within the same species. Although, *L. rhamnosus* primers could amplify three non-target species during *in silico* PCR, they were still suitable for this study. All the primers used in this

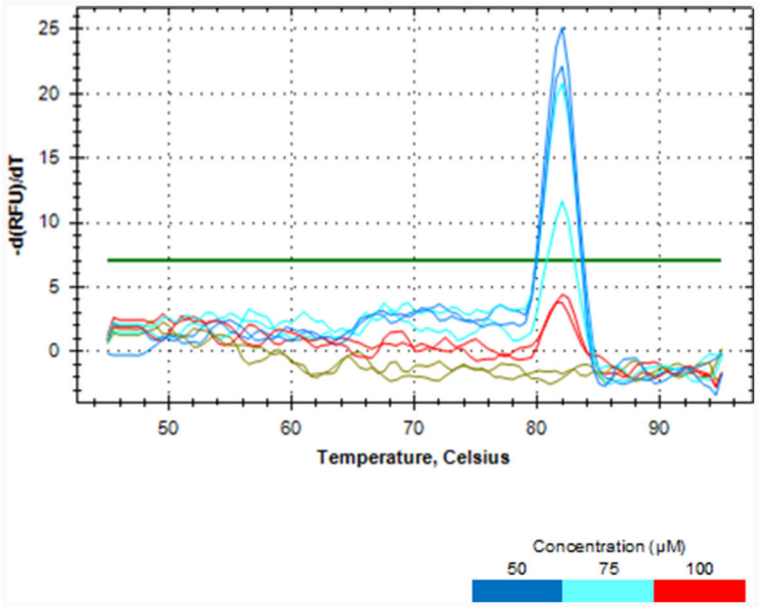

FIGURE 2
Melt curve analysis showing the effectiveness of different PMAxx concentrations of 50, 75 and 100 µM in removing DNA from dead cells of *L. rhamnosus* ATCC 53103.

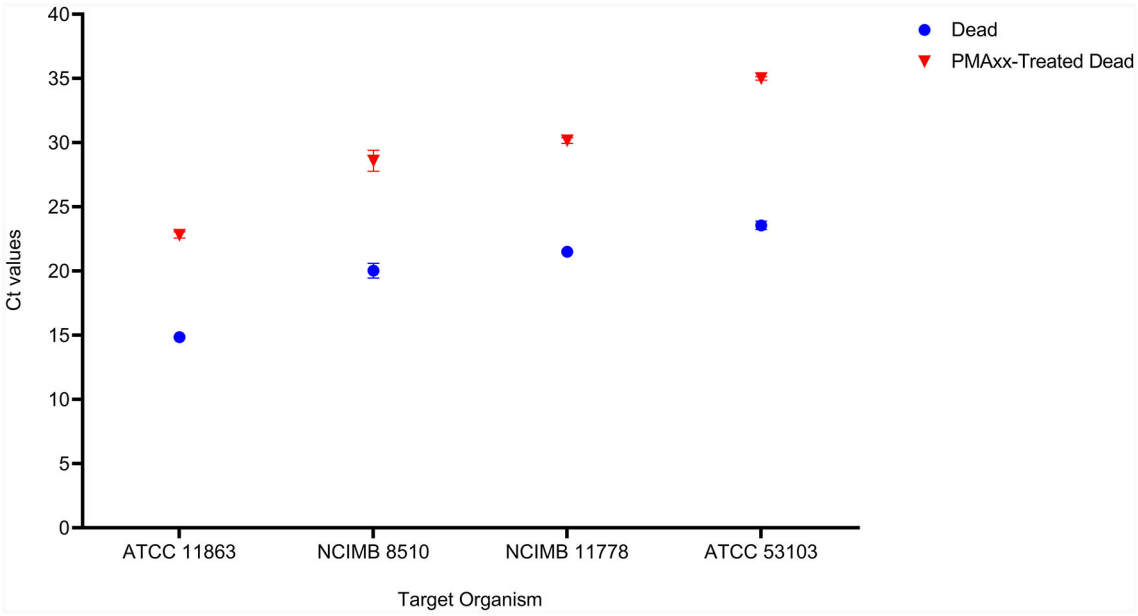

FIGURE 3
The effectiveness of PMAxx at the concentration of 100 µM on the removal of DNA from dead cells of *B. bifidum* (ATCC 11863), *S. thermophilus* (NCIMB 8510), *L. delbrueckii* subsp. *bulgaricus* (NCIMB 11778), and *L. rhamnosus* (ATCC 53103). Blue points – DNA from dead-untreated cells, and Red points – DNA from dead PMAxx-treated cells.

study only amplified the target fragment of the bacterial genome during the empirical specificity verification, producing only one melt peak for the target organism in monocultures (Figures 1A–E) and mixed species (Supplementary data). The amplicons with the melt temperatures (Tm) of 89, 87.5, 85, 79, and 82°C corresponding to *B. bifidum* ATCC11863, *B. breve* ATCC 15700, *L. delbrueckii* subsp. *bulgaricus* NCIMB 11778, *S. thermophilus* NCIMB 8510 and *L. rhamnosus* ATCC 53103 were produced, respectively. In addition, single bands with the expected sizes of 149, 121, 118 and 117 bp were produced on gel electrophoresis (data not shown) for *Bifidobacterium* spp., *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *L. rhamnosus*, respectively. There was no formation of artifacts or non-specific products during the qPCR melt curve and gel electrophoresis analyses.

3.3 PMAxx concentration optimization and treatment

3.3.1 Effective PMAxx concentration

Melt curve analysis showed that PMAxx, at a concentration of 100 μM, completely removed DNA from the dead cells of *L. rhamnosus* as no qPCR product was produced, as depicted in Figure 2. PCR amplicons were produced at concentrations of 50 and 75 μM. Hence, the 100 μM PMAxx concentration was chosen as the working concentration for this study.

3.3.2 Effectiveness of PMAxx concentration (100 μM) and its effect on live cells

The effectiveness of PMAxx was affected by the type of media or solution used for treatment. *S. thermophilus* NCIMB 8510 and *B. bifidum* ATCC 11863 cells were observed to be sensitive (viability declined, data not shown) to PMAxx when treated in ultra-pure water (ddH_2O) at 100 μM. On the contrary, when the two species were treated in MRS broth, PMAxx did not affect their viability. The effectiveness of PMAxx in removing DNA from dead cells was reduced when *L. delbrueckii* subsp. *bulgaricus* cells were treated in MRS broth (data not shown). The Ct values of dead untreated cells were 14.86 ± 0.44, 23.56 ± 0.32, 20.04 ± 0.57, and 20.62 ± 0.14 for *B. bifidum, L. rhamnosus, S. thermophilus*, and *L. delbrueckii* subsp. *bulgaricus*, respectively (Figure 3). The treatment of dead cells with PMAxx at 100 μM resulted in a significant shift in Ct values to 22.81 ± 0.24, 35.01 ± 0.14, 28.59 ± 0.82, and 28.89 ± 0.01, respectively. Hence resulting in a delta Ct >7 for all four species. In general, PMAxx at 100 μM effectively removed 99.6, 100.0, 99.7, and 99.6% (Table 2) of DNA from the dead cells of *B. bifidum* ATCC 11863, *L. rhamnosus* ATCC 53103, *L. delbrueckii* subsp. *bulgaricus* NCIMB 11778 and *S. thermophilus* NCIMB 8510, respectively.

In addition, PMAxx at 100 μM did not affect the viability of live cells of the target LAB species (Figure 4). There was no significant difference ($p > 0.05$) between the viable counts of untreated live and PMAxx-treated live cells for all the target species.

TABLE 2 Removal (%) of dead cells DNA at PMAxx™ concentration of 100 μM.

Species	Removed dead cells DNA (%)
S. thermophilus NCIMB 8510	99.6
L. delbrueckii subsp. bulgaricus NCIMB 11778	99.7
B. bifidum ATCC 11863	99.6
L. rhamnosus ATCC 53103/GG	100.0

3.4 Standard curves: linear dynamic range, efficiency, slope, and correlation determination

The standard curve parameters, namely slope (K), efficiency (E), and correlation coefficient (R^2) of two independent qPCR runs for the five target species, are summarized in Table 3. The overall mean of the Ct values and DNA copy numbers obtained from the two independent qPCR runs were used to establish the standard curve parameters for this study (Figure 5). There was a good linear fit ($R^2 > 0.99$, $p < 0.0001$) between the Ct values and log DNA copy number for all the target species. The replicate test for lack of

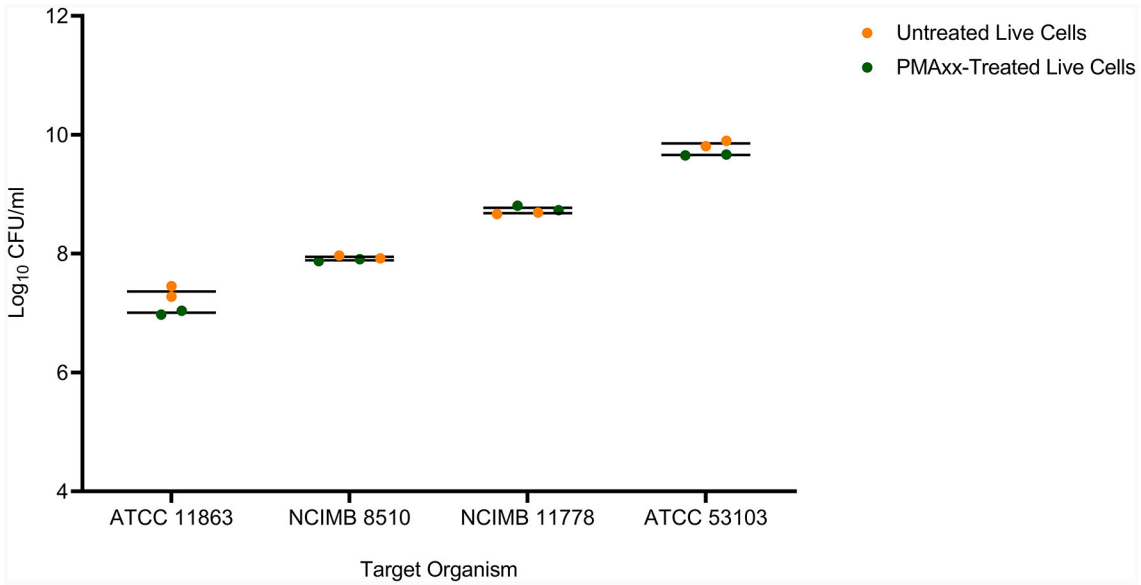

FIGURE 4
The effect of PMAxx at the concentration of 100 μM on live cells of *B. bifidum* (ATCC 11863), *S. thermophilus* (NCIMB 8510), *L. delbrueckii* subsp. *bulgaricus* (NCIMB 11778), and *L. rhamnosus* (ATCC 53103).

TABLE 3 Quantitative PCR efficiency, slope, correlation coefficient obtained by plotting Ct values against log DNA copy number.

Species	qPCR efficiency (E)	Slope (K)	Correlation coefficient (R^2)
S. thermophilus NCIMB 8510	97%	3.3975	0.9997
L. delbrueckii subsp. bulgaricus NCIMB 11778	99%	3.3455	0.9963
B. bifidum ATCC 11863	98%	3.3670	0.9995
B. breve ATCC 15700	92%	3.5470	0.9983
L. rhamnosus ATCC 53103/GG	95%	3.4623	0.9980

Data represent the mean values ($n = 4$) of two independent qPCR assays and DNA extractions.

fit showed that the linear model for all the five species adequately fits the data ($p > 0.05$). The PMAxx-qPCR assays for the five species were efficient (E = 91%−99%) with a slope ranging from −3.55 to −3.35. The linear dynamic ranges (LDR) were determined between 10 and 10^5 genome copies for *Bifidobacterium* spp. and *L. rhamnosus*, 10 and 10^6 genome copies for *L. delbrueckii* subsp. *bulgaricus*, and 1 and 10^6 genome copies for *S. thermophilus*. The LOQ was 10^2 CFU/ml for *B. bifidum* and *S. thermophilus*, 10^3 CFU/ml for *B. breve* and *L. delbrueckii* subsp. *bulgaricus*, and 10^4 CFU/ml for *L. rhamnosus* (Supplementary data).

3.5 Comparison of PMAxx-qPCR method to standardized plate count method

There was a high correlation between the viable counts of PMAxx-qPCR and the plate count method [Pearson correlation coefficient (r) = 0.882 and p = 0.0007], Figure 6A. The p-value showed that the true value of the coefficient (0.6303) was significantly different from zero. Hence, confirmed a relationship between the two methods. PMAxx-qPCR counts were generally significantly higher ($p < 0.0001$, two-tailed paired t-test) compared to plate count with a relative difference of 17% (range: 9%−26%) (Figure 6B, Bland-Altman method of comparison).

3.6 Applicability of PMAxx-qPCR method in mixed-species probiotic yogurt

Quantitative PCR without PMA quantifies all the genomic DNA from live and dead cells. Comparing the qPCR counts of PMAxx-treated and non-treated yogurt, therefore, gives information on the ability of the designed method to quantify viable cells in mixed-species yogurt during storage selectively (Figure 7). *S. thermophilus* NCIMB 8510 counts in PMAxx-treated and non-treated yogurts throughout storage were comparable ($p > 0.05$). This showed that only viable *S. thermophilus* cells were in the yogurt during storage. The qPCR counts for *L. delbrueckii* subsp. *bulgaricus* NCIMB 11778 in PMAxx-treated yogurt were lower than in non-treated yogurt counts ($p > 0.05$) by 1.66 and 0.96 log CFU/ml on days 1 and 30, respectively. Similarly, there was a difference ($p > 0.05$) of 0.80 log CFU/ml on day 1 and 1.02 log CFU/ml on day 30 in *L. rhamnosus* ATCC 53103 counts between PMAxx-treated and non-treated yogurts. The developed PMAxx-qPCR method showed that *Bifidobacterium* spp. have different survival abilities in mixed-species yogurt during storage. There was a significant reduction ($p < 0.05$) in *B. bifidum* ATCC 11863 cell viability on days 1 and 30 by 1.57 and 1.90 log CFU/ml in PMAxx-treated yogurt, respectively. In contrast, *B. breve* ATCC 15700 exhibited better survival ability than *B. bifidum* ATCC 11863. *B. breve* counts in PMAxx-treated and non-treated yogurts were comparable ($p > 0.05$) with no observable difference during storage. In general, qPCR without PMAxx overestimated cell counts by 13% on day 1 and 12% on day 30 (Bland-Altman method of comparison) between the target species.

4 Discussion

In our study, species-specific *tuf* gene primers were successfully designed or selected and validated for the selective quantification of *Bifidobacterium* spp., *L. rhamnosus*, and yogurt starter cultures in mixed-species yogurt. In qPCR-based methods, the 16S rRNA gene is commonly used as the target gene for quantifying mixed-species probiotics (García-Cayuela et al., 2009; Yang et al., 2021). However, the copy number of this gene varies between the genomes of LAB probiotic species (Lee et al., 2008; Fan et al., 2021). In addition, the resolution and discriminatory power of the 16S rRNA gene is low compared to that of protein-encoding genes such as *pheS* gene encoding the phenylalanine tRNA ligase subunit alpha, *hsp60* gene encoding the 60-kDa heat shock protein, and *tuf* gene encoding the elongation factor Tu (Yu et al., 2012). Hence, the *tuf* gene, which has high interspecific sequence difference, occurs as a single copy within the bacterial genome (Fan et al., 2021) and evolves at a faster rate than the 16S rRNA gene (Valiunas et al., 2019), was chosen as the target gene in this study.

Ideally, to ensure reliable quantification, qPCR primers should not exhibit sequence homology to the nucleotide sequences of non-target species in the *in silico* and empirical specificity evaluation. In our study, sequence homology was found between the *L. rhamnosus* primers and the sequences of *Lacticaseibacillus* spp., *Lactococcus lactis*, *Schleiferilactobacillus harbinensis,* and *Lactobacillus coryniformis*. The latter two species are not commonly used as starter cultures or probiotics in the production of yogurt. *S. harbinensis* is associated with non-dairy food products such as fermented cereals, tomato pomace, and spoiled soft drinks (Zheng et al., 2020). It was first isolated from the Chinese fermented vegetable "Suan cai" (Miyamoto et al., 2005). Similarly, *L. coryniformis* is commonly found in fermented vegetable products (Martín et al., 2005). Barring very poor manufacturing practices, the presence of these species in yogurt is unexpected. Similarly, *L. lactis* is primarily used in cheese, buttermilk and sour cream production (Cavanagh et al., 2015; Laroute et al., 2017; Fusieger et al., 2020). Its unintended presence in yogurt is unexpected. Despite the ability of *L. rhamnosus* primers to detect *L. lactis*, the specificity of all the primers designed in

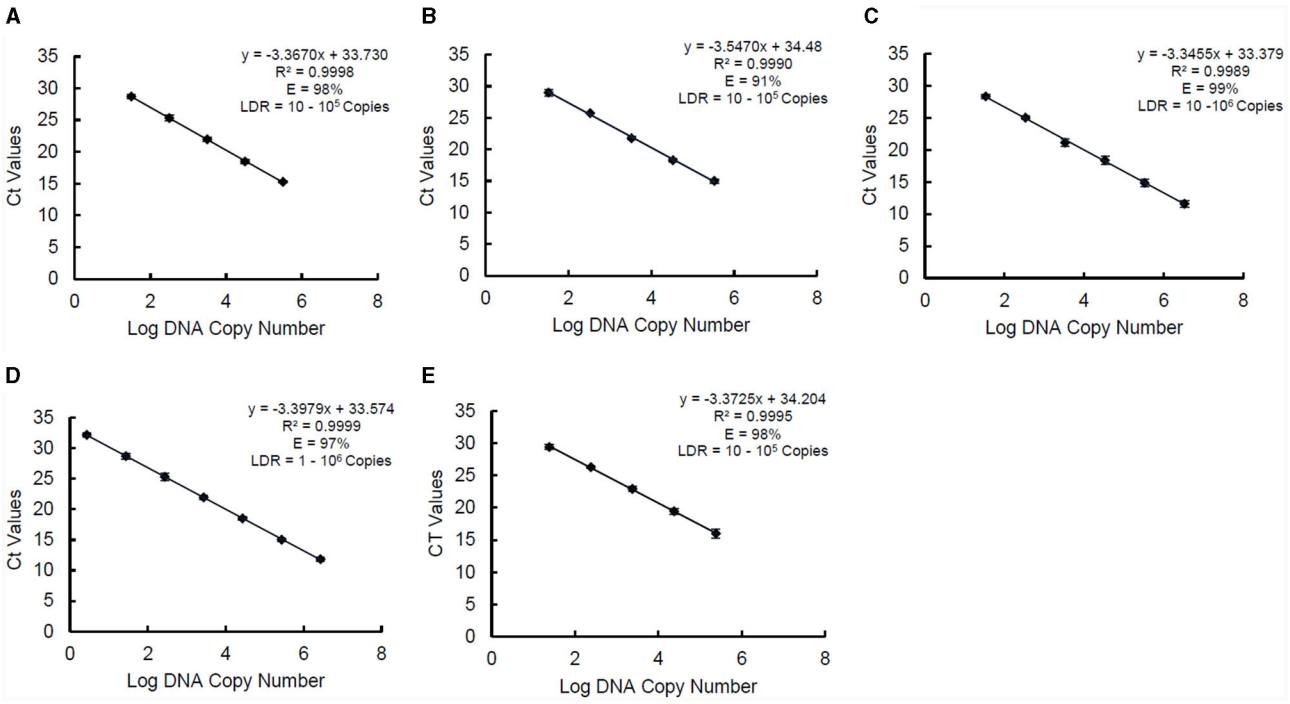

FIGURE 5
Standard curves of PMAxx-qPCR assay created and used for determining linear dynamic range (LDR), efficiency (E), and slope (K) for *B. bifidum* ATCC 11863 **(A),** *B. breve* ATCC 15700 **(B)**, *L. delbrueckii* subsp. *bulgaricus* **(C)**, *S. thermophilus* **(D)**, and *L. rhamnosus* **(E)**. Each point represents the mean ± standard deviation of CT values of two independent runs. Each run was carried out in duplicates ($n = 4$).

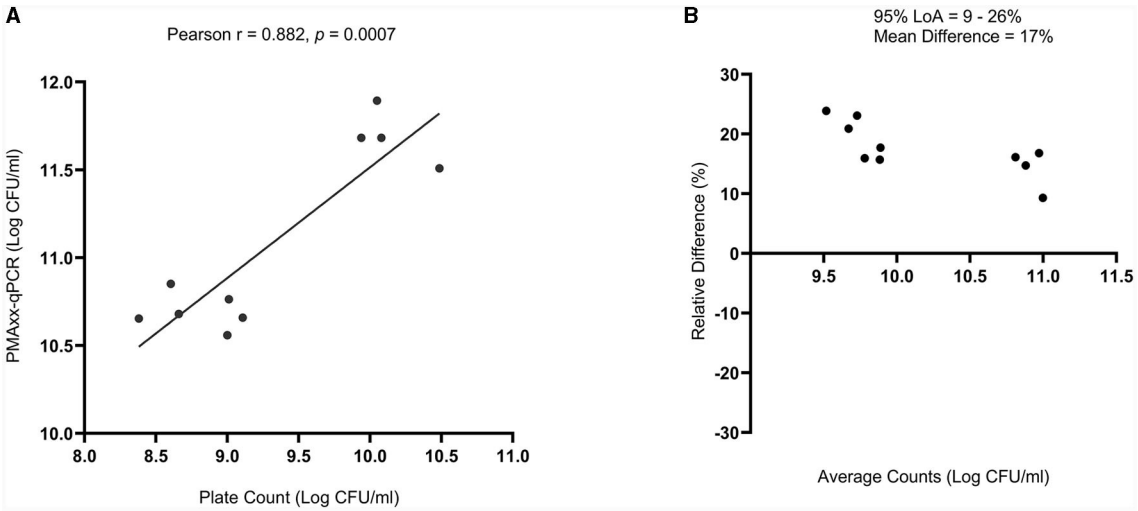

FIGURE 6
Comparison of PMAxx-qPCR method with standardized plate count method ($n = 10$) using **(A)** simple linear regression and **(B)** Bland-Altman method of comparison (%Difference vs. Average). The Bland-Altman comparison results are expressed as a percentage relative difference [100 × (PMAxx-qPCR count − Plate count)/average] vs. average. LoA means Limits of Agreement.

this study was generally acceptable. Hence, the primers were suitable for this study as they did not match genome sequences or amplify DNA fragments of commonly used starter cultures and probiotic species in yogurt. It is worth noting that, the use of *L. rhamnosus* primers designed in this study limits the application of this method to the species and combinations used for validation. Comparative genomics can be considered in future studies to identify unique genetic markers and design subspecies and strain-specific primers (Hyeon-Be et al., 2020; Lee et al., 2022). This will enable broad application of PMAxx-qPCR methods for quantifying probiotics in different mixed-strain yogurt products.

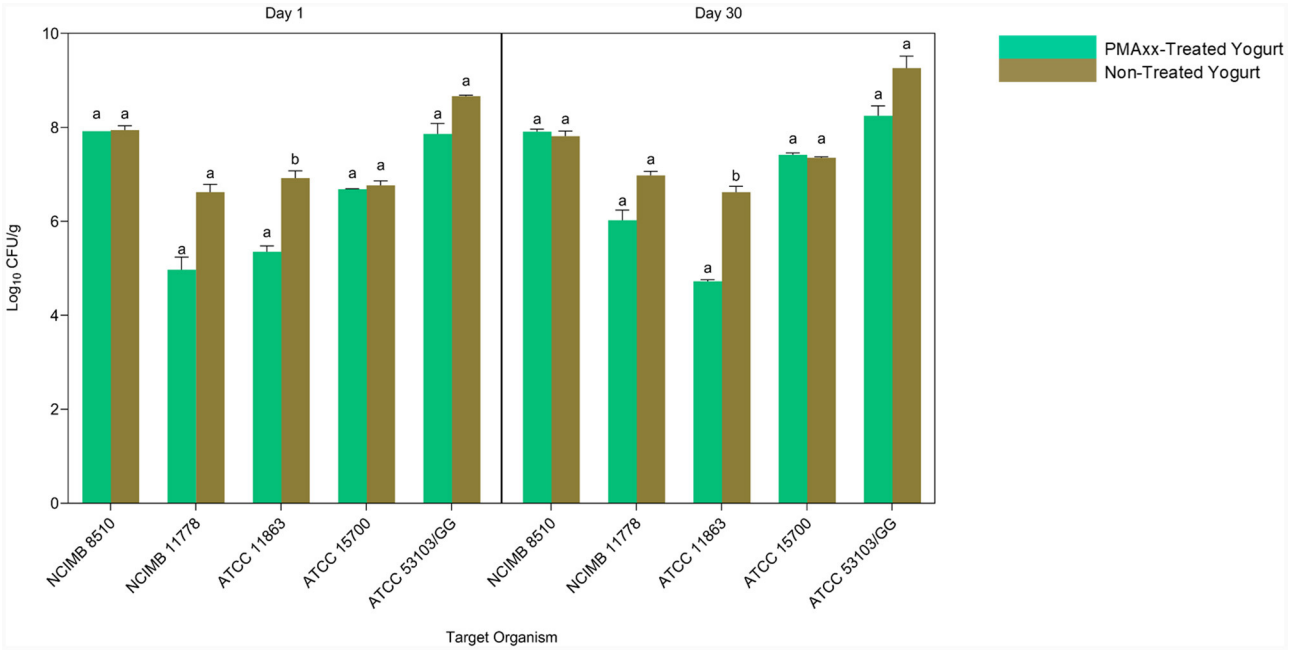

FIGURE 7
The Applicability of PMAxx-qPCR method in mixed-species yogurt during storage (day 1 and 30). Each bar represents the mean ± SD of duplicate qPCR reactions. DNA extractions from yogurt samples were done in duplicates. Mean values with different letters (a, b) were significantly different ($p < 0.05$).

Previous studies have shown that the PMA-DNA complex formation is dependent on the PMA concentration. For example, Shao et al. (2016), Shehata and Newmaster (2021), and Shehata et al. (2023) showed that qPCR signal from dead cells is reduced with increasing PMA concentration. These studies showed that a saturation point could be reached, resulting in no further effect if PMA concentration is increased beyond the optimum. Hence, finding an optimum PMA concentration to inhibit qPCR signal from dead cells effectively is essential. In our study, 50 μM was chosen as the starting concentration during PMAxx optimization as it was previously reported to be effective on other probiotic species (Scariot et al., 2018; Shehata and Newmaster, 2021; Shehata et al., 2023). However, in this study, 100 μM was an optimum concentration that effectively removed DNA from dead cells. In agreement with our findings, a recent study showed that PMAxx at high concentration completely removed DNA from high counts of dead cells of *Salmonella* Enteritidis (Thilakarathna et al., 2022). The PMAxx-DNA cross-linkage can be affected by different factors such as conditions of light exposure (light source, time, distance), bacterial species, the target gene (Shao et al., 2016), killing treatment (Yang et al., 2021), sample pH and turbidity (Fittipaldi et al., 2012). Since these factors are inconsistent in PMA-qPCR methods, they may have contributed to the difference in optimum PMA concentrations between this study and the literature.

At higher concentrations, PMAxx tends to adversely affect the counts of live cells (Thilakarathna et al., 2022). We observed a similar effect of PMAxx on live cells of *B. bifidum* and *S. thermophilus* when treated in a transparent medium (ultra-pure water). Hence, to overcome this, *Bifidobacterium* spp. and *S. thermophilus* were treated in MRS broth, while *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* were treated in ultra-pure water. Thilakarathna et al. (2022) attributed the ability of PMAxx to affect live cells at low counts to possible inactivation post-photoactivation step, thus allowing active PMAxx to be carried over to the lysis tube where it can form a crosslink with DNA from live cells post lysis. In addition, PMAxx treatment possibly modifies the surface charge (to less negative) of live cells with compromised cell membranes, enabling their attachment to the polypropylene tube wall (negatively charged) (Thilakarathna et al., 2022). Hence, the transfer of cells to the next tube in the subsequent step leaves the attached cells behind, resulting in a loss of viable cell counts (Thilakarathna et al., 2022).

Ideally, the qPCR assay should have an efficiency of 100%, signifying a doubling of the DNA template per cycle (Svec et al., 2015). However, practically, this is rare to achieve (Svec et al., 2015). Hence, the efficiency of a suitable qPCR method should be 90%−110% (Broeders et al., 2014). Factors such as target sequence and designed primers (primer dimers and hairpin formation) may lead to low qPCR efficiency (Svec et al., 2015; Langlois et al., 2021). The efficiency of the PMAxx-qPCR method designed in this study was within the generally acceptable range. This shows that the primers used in this study were efficient, and the assay is reliable for quantifying probiotics and starter cultures. In general, the qPCR assay was highly sensitive. However, the LOQ for *L. rhamnosus* was high. Notwithstanding this constraint, the method is still suitable for probiotic quality control, given that the LOQ falls below the minimum probiotic standard or

therapeutic levels. The high sensitivity of this protocol makes it suitable for quantifying target species appearing in low amounts in complex and mixed species products (Shehata et al., 2023), such as yogurt.

Furthermore, our findings show that the PMAxx-qPCR method can be used as a predictor of standardized plate counts, as indicated by a high Pearson correlation coefficient. Other studies have reported similar findings (Hansen et al., 2020; Shehata and Newmaster, 2021; Shehata et al., 2023). The discrepancy between the viable counts of the two methods, favoring qPCR assay, aligns with the findings of a previous study (Hansen et al., 2020). This can be attributed to the high counts of the PMAxx-qPCR method due to its ability to detect and quantify viable but non-culturable (VBNC) cells (Kibbee and Örmeci, 2017; Liu et al., 2018). Plate count methods cannot detect cells in the VBNC state (Shao et al., 2016; Jackson et al., 2019; Shehata et al., 2023). Cells in this state are still viable and metabolically active but have lost their culturability (Jackson et al., 2019; Hu et al., 2022). Several harsh conditions, such as fermentation, cryopreservation, lyophilisation, and storage, can induce a VBNC state as a protective mechanism in probiotics (Davis, 2014; Jackson et al., 2019). The underestimation of viable counts by plate count method can lead to inadvertent rejections of probiotic products whose counts fall below the minimum standard levels when, in fact, the actual number of viable cells in the product could be higher due to VBNC cells that remain uncounted. This will have cost implications for probiotic manufacturers. On the contrary, the ability of the PMAxx-qPCR method to detect VBNC cells will improve probiotic quality assurance and efficacy for processors and consumers. Compared to the plate count method, this PMAxx-qPCR assay is rapid, with quantification results obtained within a few hours (~8 h). However, further optimisation of this assay for simultaneous detection and amplification of all target species is possible and will reduce results turnaround time significantly.

Ideally, the use of standard curves obtained from the food matrix inoculated with the target species is recommended for a reliable quantification of viable cells in a food product (Postollec et al., 2011). However, as was done in this study, standard curves constructed from pure cultures give a measure of the efficiency of the qPCR reactions.

Probiotic viability determination in yogurt throughout storage is crucial for adherence to regulatory requirements and probiotic quality assurance in the dairy industry. The PMAxx-qPCR method developed in this study can selectively quantify viable cells of probiotics and starter cultures in mixed-species yogurt during storage. The relative viability loss of the five target species during yogurt storage, as indicated by higher counts of qPCR than PMAxx-qPCR, was in agreement with other studies (Scariot et al., 2018; Shi et al., 2022). The designed PMAxx-qPCR assay further showed that while some probiotics and starter cultures can fully maintain viability during processing and storage, other lost viability at different rates. This shows that some species are susceptible to yogurt processing stress while others are resilient. The findings of this study demonstrate the selectivity, sensitivity, and reliability of this PMAxx-qPCR method, which can detect VBNC cells and viability loss in mixed-species yogurt during storage.

5 Conclusion

This study outlines a real-time qPCR protocol for viability enumeration of probiotics in mixed species yogurt. The method which is based on newly developed species-specific primers for *L. rhamnosus*, *Bifidobacterium* spp. and *L. delbrueckii* subsp. *bulgaricus* and an optimized PMAxx-qPCR reaction protocol has a high sensitivity and is reliable. Moreover, the method has very high correlation with the standard viability plate counts, albeit with a consistently higher prediction rate, presumably due to its ability to enumerate cells in the VBNC state. With such a high sensitivity and short turnaround time, the qPCR protocol will be a good proposition for the inline viability quality assurance in probiotic yogurt processing. However, it must be emphasized that the protocol is applicable only to yogurt incorporated with probiotic species and yogurt starter cultures used in this study. Hence, products containing different species of probiotics would require optimization. Moreover, it would be necessary to do a detailed cost comparison analysis with other available methods of viability quantification, considering that industries could be at different levels of capitalization.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

TM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. TS: Conceptualization, Methodology, Supervision, Writing – review & editing. EB: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1325268/full#supplementary-material

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Bifidobacterium longum suppresses colorectal cancer through the modulation of intestinal microbes and immune function

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Colorectal cancer (CRC), one of the most common malignancies in the world, urgently requires more treatment strategies. Although there has been much research on probiotics, limited research has been done in treating cancer. The purpose of this study was to investigate the role of *Bifidobacterium longum* (*B. longum*) in the prevention and treatment of CRC. Through Cell Counting Kit-8 and Colony Formation Assays, 8 h and a *B. longum* count of 1×10^8 CFU/ml were chosen as the best cocultivation conditions with CRC cells. The role of *B. longum* in inhibiting the progression of CRC cells was verified by a series of functional and immunofluorescence assays. For instance, *in vivo* assays have verified that *B. longum* could alleviate CRC progression. In addition, according to the results of *in vivo* assays and clinical statistical analysis, *B. longum* could reduce diarrhea symptoms. Mechanistically, by 16S and RNA sequencing, it was found that *B. longum* could affect the development of CRC by regulating the composition of gut microbes and enhancing immune function. The *B. longum* might inhibit the occurrence and development of CRC and relieve diarrhea symptoms by regulating intestinal microbes and immune function.

KEYWORDS

CRC, *Bifidobacterium longum*, AOM/DSS, proliferation, invasion and migration

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the world, with a high mortality rate and low cure rate (Bray et al., 2018). It is believed to stem from interactions between the host and the microbiota in the long term and is caused by mutations, activations, and

deletions of oncogenes and tumor suppressor genes, which lead to adenoma-carcinoma (Coker et al., 2019). The majority of epithelial surfaces of our body are colonized by a vast number of microorganisms, especially the intestinal mucosa. The gut microbiota is a commensal, symbiotic and pathogenic microorganism that interacts with each other and with the host, which can affect their health (Song et al., 2020). Among many intestinal microorganisms, probiotics have undoubtedly been the focus of attention in recent years. Increasing evidence from epidemiological studies and *in vivo* models suggests that probiotic bacterial species can modulate the imbalance of gut microbiota composition, reduce the risk of cancer, and enhance the efficacy of tumor drugs (Behrouzi et al., 2022; Samanta, 2022).

In vitro, *in vivo* and gut microbiome studies have provided sufficient evidence of the preventive effects of probiotics for CRC. In particular, the role of *Bifidobacterium longum* (*B. longum*) has been mentioned many times. *In vitro* studies by Worthley et al. (2009) found that symbiotic supplementation comprising *B. longum* and resistant starch could induce specific beneficial changes in fecal microflora. NK and DC cells are very important in the prevention and control of autologous tumors. Fink et al. (2007) demonstrated that *B. longum* could initiate NK/DC interactions via DC maturation and the catalytic potential of NK cells to produce interferon-γ (IFN-γ), which proved the contribution of *B. longum* to tumor prevention and treatment at the cellular level (Song et al., 2018). Sivan et al. (2015) showed that *B. longum* could improve tumor control as well as anti-PD-L1 therapy. What was more noteworthy was that the combination treatment nearly abolished tumor outgrowth and increased the efficiency of the PD-L1 blocking antibody against tumors. *In vivo* studies have provided a basis for the antitumor activity of *B. longum* by using animal models (Sivan et al., 2015). Many animal models have already successfully provided insights to comprehend the link between gut microbes and CRC, such as genetic knockout, germ-free and chemical mouse models (Uttam et al., 2020). It would be interesting to employ animal models of inflammation-induced CRC for *B. longum* research to elaborate its potential benefits and to elucidate the molecular mechanism involved in their probiotics. Many articles have already used azomethane (AOM) and sodium dextran sulfate (DSS) mouse models to prove the benefit of *B. longum* (Yassin et al., 2019). It is also worth noting that in the study of probiotics, the relief of diarrhea by *B. longum* has also been a concern of many scholars, and many articles have reported the good relief of diarrhea by *B. longum* (Andresen et al., 2020).

The administration of probiotics has become a particular interest in the prevention and treatment of CRC. It should be emphasized that not all probiotics from a particular species have the same properties and will show the same effect in the organism because the probiotic strain itself is the main influencing factor (Suez et al., 2019). Fortunately, we isolated a new strain of *B. longum* from infant feces. The objective of this study was to verify the relationship between *B. longum* and the occurrence and development of CRC. Additionally, we evaluated the preliminary relationship between *B. longum* and CRC through cell function experiments and a mouse CRC model induced by AOM/DSS. Then, a small sample clinical study was conducted to explore the effect of *B. longum* on diarrhea diseases.

Materials and methods

Probiotic treatment

Freeze-dried living *B. longum* was kindly offered by Hebei Yiran Biotechnology Co., Ltd. for free (food production certificate No: SC13113012300078, Hebei, China). In cell assays, before *B. longum* was used in cell coculture, the culture medium was used by cell gradient dilution, rendering the probiotics at the experimental concentration. In animal experiments, PBS is used to configure the expected concentration of the probiotics.

Cell culture

The human CRC cell lines LOVO, SW480, and SW1463 were obtained from the First Hospital of Hebei Medical University and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY) and 1% penicillin (100 U/ml) with streptomycin (100 mg/ml) at 37°C in a humidified atmosphere of 5% CO2.

Cell proliferation assay

The growth of cells was measured with Cell Counting Kit-8 (CCK-8, Boster, Wuhan, Hubei, China). The cells were plated into 96-well culture plates (2.0×10^3/well) and incubated for 2 or 4 days at 37°C in a humidified incubator with 5% CO2. Every 24 h, the number of *B. longum* cells was quantified by adding 10 μl of CCK-8 to each well, followed by incubation for another 2 h. The absorbance of each well at 450 nm was measured by a microplate reader (Promega, Madison, WI, USA).

Colony formation assay

One thousand cells were plated in culture dishes and cultured for 14 days. The culture medium was replaced on the seventh day of the experiment. The cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 30 min. Then, fixed-sized colonies were selected as the standard for counting before counting the number of colonies.

Wound-healing assay

Colorectal cancer cell lines were divided into two groups: con and *B. longum*, which were inoculated in 6-well plates at a cell quantity of 5×10^5 cells per well. After 12 h, the *B. longum* group was cocultured with *B. longum* (1×10^8 CFU/well) for 8 h. After that, a wound was created by a 200 μl micropipette tip before serum-free DMEM was used to wash the cells three times. The scratch width was observed and photographed at 0 h, 24 h, and 48 h after wounding by microscopy.

Transwell assay

The migration and invasion of cells were measured by using an 8 μm Transwell chamber in 24-well plates (Corning, Waltham, MA, USA). First, a total of 650 μl of DMEM supplemented with 20% FBS was added to the lower chamber. Then, 100 μl of serum-free medium with 2×10^5 tumor cells were placed into the upper chamber. The cells were incubated for 24 h at 37°C. To measure invasion, a chamber containing Matrigel (Corning, Waltham, MA, USA) was used. The remaining steps were performed according to the above method. After 24 h, the chamber was stained by diff-quick staining (BASO, Taiwan, China) and counted in five random fields. It is important to note that we selected 20% FBS because probiotics cocultured with CRC cells contain 10 FBS.

Immunofluorescence staining

A slide was placed in each well of the 6-well plate before commencing the assay. Then, the CRC cell lines were divided into two groups: con and *B. longum*, which were inoculated in 6-well plates at a cell quantity of 5×10^5 cells per well. After 12 h, the *B. longum* group was cocultured with *B. longum* (1×10^8 CFU/well) for 8 h. All wells were fixed with 4% paraformaldehyde, blocked in 5% BSA, and washed with PBS for 5 min twice. The primary antibody against Ki-67 was diluted with PBS (1:250). Slides were incubated in a humidified chamber at 4°C overnight. After incubation, the slides were soaked in PBS for 2 min, which was repeated 3 times. The secondary antibodies were diluted with PBS (1:200) and were added to slides and incubated for 2 h at RT. Slides were rinsed 3 times with PBS for 2 min each. After washing, the slide was removed and placed on the fragment. Then, the cells on the slides were cultured in DAPI solution for 30 min and analyzed via an Olympus fluorescence microscope (×40).

H&E

Mouse colorectal tissues were formalin-fixed and then paraffin-embedded, and five-micrometer sections were cut for H&E staining.

Immunohistochemical tissue staining

Immunostaining was performed on 5-μm formalin-fixed, paraffin-embedded tissue sections using an immunoperoxidase method with rabbit anti-Ki-67 (1: 100; Sunbiote, Shanghai, China) monoclonal antibodies. Protein was visualized using PV and DAB chromogenic kits (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer's instructions.

Animals

Male BALB/c mice (4 weeks old, purchased from Hebei Medical University) were maintained in large group houses under 12-h dark and light cycles and were given access to food and water. The procedures were in accordance with the guidelines for the care and use of laboratory animals from Hebei Medical University (No: 17733). After 1 week of adaptation to the environment, the animals were randomly assigned to three experimental groups. First, animals in groups 1–2 (Normal and AOM + DSS Control, $n = 5$) received only PBS; group 3 (*B. longum*, $n = 5$) received *B. longum* at 1×10^9 CFU/mouse. The corresponding treatments were prepared daily and were gavaged every afternoon (0.2 mL total volume) to all mice throughout the 12 weeks. On the first day, animals in groups 2–3 were treated with a single intraperitoneal injection of azoxymethane (AOM, Sigma, St. Louis, MO, USA) 10 mg/kg, dissolved in NaCl 0.9%; then, in weeks 2, 5 and 8, 2.0% dextran sulfate sodium (DSS, Sigma, St. Louis, MO, USA) was administered *ad libitum* for 7 days. All animals were sacrificed at the 12th week after the corresponding drug was administered. Animal experiments were reviewed and approved by the Animal Committee of the First Hospital of Hebei Medical University (License number 20200326), and guidelines for the care and use of animals were followed. To analyze the inhibitory effects of the tested substances on tumor growth, tumor length (L) and width (W) were measured, and tumor volume (mm3) was calculated as $[V = (L \times W2)/2]$.

16S rRNA sequencing

16S rRNA can be used as the characteristic nucleic acid sequence of biological species, and it is considered to be the most suitable index for bacterial phylogeny and taxonomy (Caporaso et al., 2011).

Extraction of genomic DNA

Total genomic DNA from mouse fecal samples was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

Amplicon generation

Primers used for A. *lwoffii* were as follows: forward, 5′-TGGCTCAGATTGAACGCTGGCGGC-3′; reverse, 5′-TACCTTG TTACGACTTCACCCCA-3′. Primers used for *B. longum* were as follows: forward, 5′-TTCCAGTTGATCGCATGGTC-3′; reverse, 5′-GGAAGCCGTATCTCTACGA-3′. All polymerase chain reactions (PCRs) were conducted in 30-μl reactions with 15 μl of GoTaq® Green Master Mix (Promega, Madison, WI, USA), with 0.2 μM forward and reverse primers and approximately 10 ng of genomic DNA. Thermal cycling for amplification of A. *lwoffii* DNA began with the initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 67°C for 45 s, and elongation at 72°C for 60 s, and finally at 72°C for 7 min. Thermal cycling for the amplification of *B. longum* DNA began with the initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and elongation at 72°C for 50 s, and finally at 94°C for 15 s.

Agarose gel electrophoresis for PCR products

Agarose gel electrophoresis was performed on 0.7% agarose gel (SeaKem® LE Agarose; Lonza, Morristown, NJ, USA) with 0.5 × Tris-acetate-EDTA as an electrophoresis buffer. Prior to cool-down of the boiled agarose, EtB"Out" Nucleic Acid Staining Solution (5 μl; YB Biotech, Taipei City, Taiwan) was added to liquid agarose (100 ml) for visualization of the separated DNA bands under ultraviolet light after electrophoresis. The DNA sample was loaded into the wells with bromophenol blue dye. The power condition was set as 130 V and 400 mA, and electrophoresis proceeded for 20 min. The DNA bands were finally photographed under ultraviolet light.

RNA sequencing

Transcriptome sequencing is based on the Illumina sequencing platform, which plays an important role in understanding the development and disease of organisms (Wang et al., 2009). Clinical experiment. This study was conducted at the First Hospital of Hebei Medical University. The inclusion criteria of patients were as follows: patients whose IBS-SSS score was more than 175 points. The exclusion criteria included psychiatric disorders, pregnancy or breastfeeding, ingestion of probiotics or antibiotics < 2 weeks before inclusion, and unwillingness to sign the informed consent form. The primary end point was a reduction of ≥ 50 points on the IBS-SS scale. This was considered adequate to detect symptom improvement by the IBS-SS validated scoring system (Francis et al., 1997). Secondary endpoints included daily stool frequency and stool form (Bristol Stool Scale) (Plasse et al., 2020). The clinical trial was approved by the Ethics Committee of the First Hospital of Hebei Medical University (License number 20200326).

Statistical analysis

The results of normally distributed data are expressed as the mean ± SD, while those of non-normally distributed data are expressed as the median and interquartile range. Student's t test, one-way ANOVA and two-way ANOVA were used in this study. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, USA) and SPSS Statistics 21 (IBM, NY, USA). $P < 0.05$ was considered statistically significant.

Results

Basic information of *B. longum*

B. longum was isolated from the intestinal tract of healthy infants. This strain has 99% similarity to *B. longum* JCM 1217 DNA, so it is classified as Lactobacillus *longum* and belongs to the food catalog strain. **Supplementary Figure 1A** clearly shows that the colony diameter is approximately 1–2 mm and that the edge of the colony is neat and opaque. Scanning electron microscopy (SEM) showed that the average length of *B. longum* was 1.86 μm and the width was 0.42 μm (**Supplementary Figure 1B**).

The concentration and time for the coculture system of *B. longum* with CRC cells

When the five different concentrations of *B. longum* were cocultured with the SW1463 cell line for 4 h, the cell proliferation was greater than the IC85 within 24 h and 48 h, indicating that the cells could grow normally with *B. longum* under this time condition (**Figure 1A**). The results of the colony formation assay indicated that *B. longum* had a restraining effect on the long-term survival of the SW1463 cell line compared with the control group and inhibited the proliferation and growth of colon cancer tumors when the *B. longum* count was greater than 1×10^8 CFU/ml ($P < 0.05$, **Figure 1B**).

When the coculture time was set to 8 h, the CCK-8 assay proved that tumor cells could cogrow with *B. longum* without being affected by other factors when the *B. longum* count was between 1×10^6 CFU/ml and 1×10^8 CFU/ml (**Figure 1C**).

The results of the colony formation assay showed that when the *B. longum* count was greater than 1×10^7 CFU/ml, the long-term survival of the SW1463 cell line was significantly inhibited compared with the control group ($P < 0.05$, **Figure 1D**).

When the coculture time was adjusted to 12 h, the CCK-8 assay showed that cells could cogrow with *B. longum* at 1×10^6-1×10^8 CFU/ml (**Figure 1E**). The results of the colony formation assay indicated that there was a statistically significant difference between the long-term survival of the CRC cell line SW1463 and the control group when the number of bacteria was greater than 1×10^7 CFU/ml, which had an inhibitory effect on the growth and proliferation of tumor cells ($P < 0.05$, **Figure 1F**).

In summary, the short-term survival of the SW1463 cell line was not affected when the culture time was 8 h and the count of *B. longum* was 1×10^8 CFU/ml, indicating that it was not caused by other factors, such as the lack of nutrients or the effects of pH value.

Inhibitory effect of *B. longum* on the proliferation, migration and invasion of CRC cells

With the optimum conditions we found, we carried out a 96-h CCK-8 test and colony formation test in SW480, LOVO, and SW1463 cell lines. The results of the three groups of cell lines showed that the inhibitory proliferation of the *B. longum* group was significantly better than that of the control group ($P < 0.05$, **Figures 2A–F**).

Cell migration and invasion were assessed in LOVO, SW480, and SW1463 cell lines by wound-healing and Transwell assays, respectively. Through continuous scratching detection for 48 h, it was found that *B. longum* reduced the migration ability of cells. Through transwell migration and invasion results, it could be clearly seen that *B. longum* attenuated the migration of cells. In addition, *B. longum* also had the same effect on the invasive ability of cells ($P < 0.05$, **Figures 2G–I**).

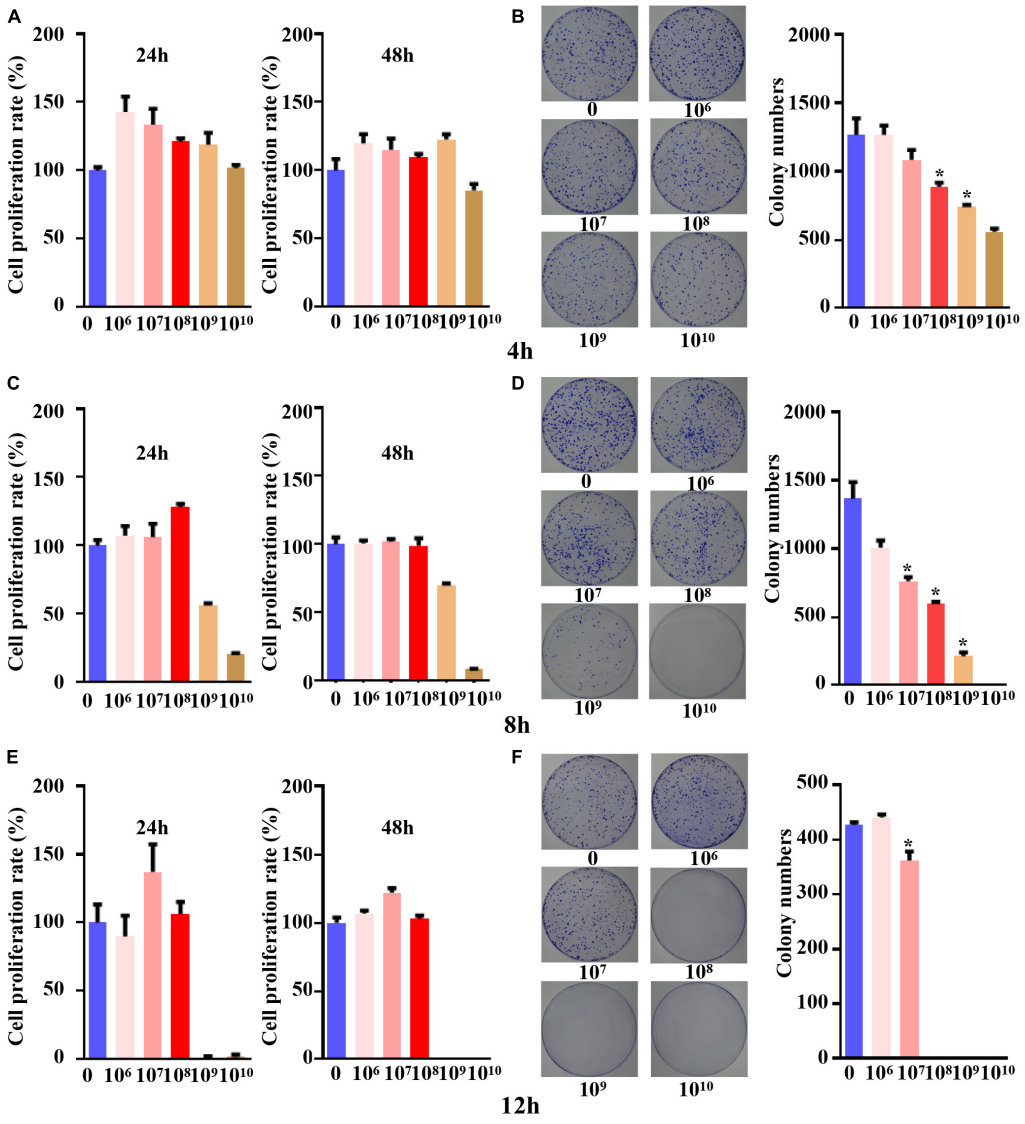

FIGURE 1
The concentration and time for co-culture system of *B. longum* with CRC cells. **(A,C,E)** Cell viability at 24 h and 48 h after co-culture for 4 h, 8 h, and 12 h was detected by CCK-8 assay. **(B,D,F)** After 4 h, 8 h and 12 h of co-culture, the colony formation at different concentrations (1×10^6-1×10^{10} CFU/mL) was detected. *$P < 0.05$. Similar results were obtained from three independent experiments.

Ki-67, a nuclear protein associated with proliferation, is often used as a mitotic index. We used immunofluorescence to observe Ki-67 expression in LOVO, SW480, and SW1463 cell lines. After the coculture of CRC cells and *B. longum*, we clearly found that Ki-67 was inhibited (**Supplementary Figures 2A–C**).

In summary, *B. longum* had an inhibitory effect on tumor cells at the cellular level, which also laid a good foundation for the *in vivo* assays.

Effect of *B. longum* on the AOM/DSS CRC mouse model

We induced colitis-associated CRC using an intraperitoneal injection of AOM followed by three cycles of DSS (**Figure 3A**). To evaluate the chemopreventive efficacy of *B. longum* supplementation in an AOM-DSS model, *B. longum* was added on the first day and performed once per day for the duration of this study, and body weight was determined weekly. The change in mouse body weight in the *B. longum* group was alleviated compared with that in the AOM/DSS group ($P < 0.05$, **Figure 3B**), which witnessed a decline in body weight after each round of DSS treatment.

The number of tumors and the length of colorectal tumors are considered to be two important evaluation indexes of the AOM/DSS mouse model (Ren et al., 2018). The length and tumor numbers in the AOM/DSS mouse model are visually reflected in **Figure 3C**. Compared with the AOM/DSS group, the AOM/DSS + *B. longum* group presented a significantly fewer number of tumors, tumor size and tumor volume ($P < 0.05$, **Figures 3D–F**).

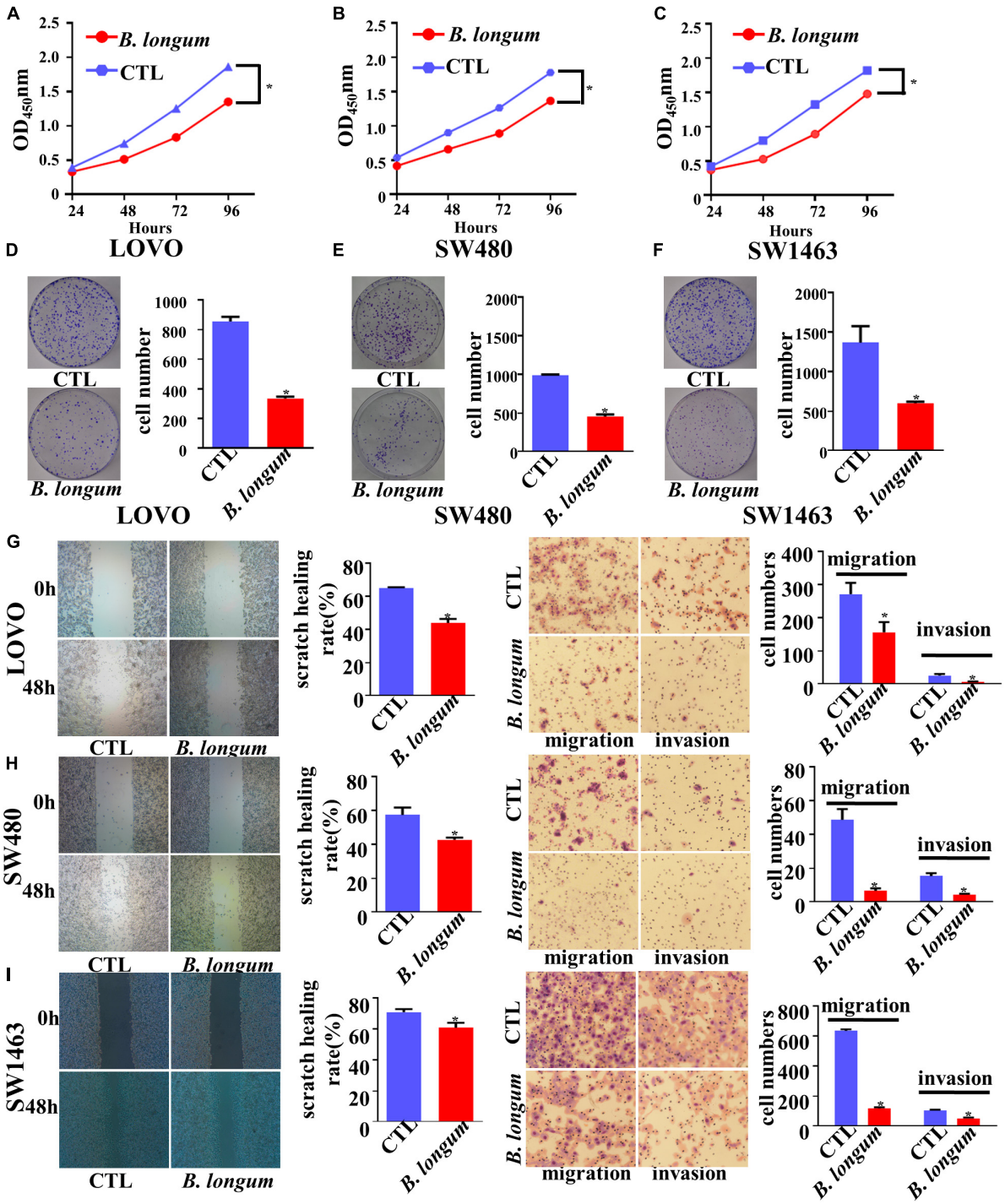

FIGURE 2
Inhibitory effect of *B. longum* on proliferation, migration, and invasion of CRC cells. **(A–F)** CCK-8 and colony formation assays comparing the effects of cell growth between the CTL and *B. longum* group in LOVO, SW480, and SW1463. **(G–I)** Wound-healing and Transwell assays comparing the effects of cell invasion and migration between the CTL and *B. longum* group in LOVO, SW480, and SW1463. *$P < 0.05$. Similar results were obtained from three independent experiments.

B. longum significantly relieved colon shortening and splenomegaly ($P < 0.05$, Figures 3G, H). During the observation, we also found that the recovery of diarrhea caused by DSS in the *B. longum* group was faster than that in the AOM/DSS group; meanwhile, the total days of diarrhea were significantly reduced ($P < 0.05$, Figures 3I, J).

Representative photomicrographs of H&E colorectal tissue sections and Ki-67 immunohistochemistry of each group of mouse models are shown in Supplementary Figure 3. However, improved histological injury was exhibited in the *B. longum* group, which had a low level of inflammatory cell infiltration, a better mucosal architecture and shaped crypts compared with

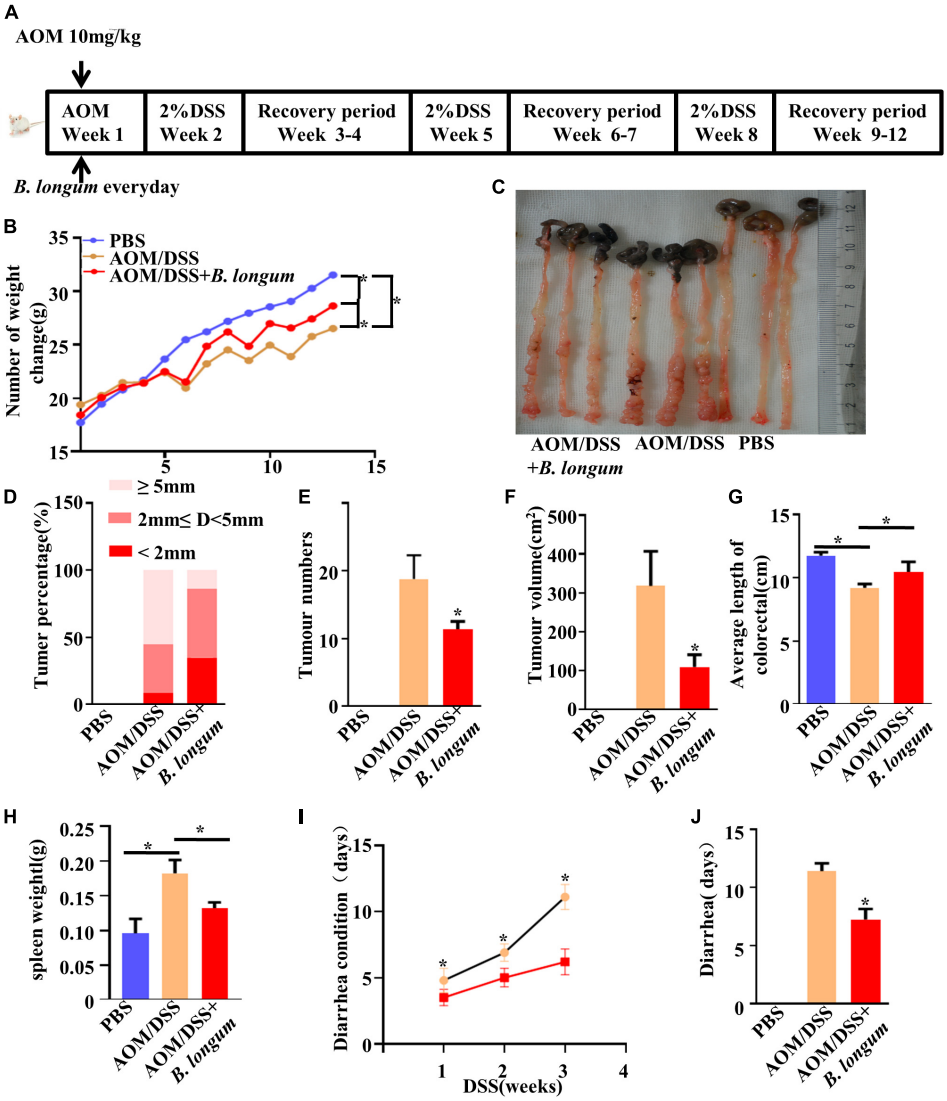

FIGURE 3
Effect of *B. longum* on AOM/DSS CRC mouse model. **(A)** Schematic representation of AOM/DSS model establishment and the Probiotics administration. **(B)** Changes in body weight of male BALB/c mice in three different treatment groups. **(C)** Tumor development, number. **(D)** Percentage of tumors in each group. **(E)** Tumor number. **(F)** Tumor volume. **(G)** Colon length in three different treatment groups. **(H)** Spleen weight. **(I)** Days of diarrhea after drinking DSS for three times. **(J)** Diarrhea days per group. *$P < 0.05$. Similar results were obtained from three independent experiments.

the AOM/DSS group. In addition, we evaluated the effects of *B. longum* on the expression of Ki-67 in male mouse CRC cells using immunohistochemical methods. CRC cells from the AOM/DSS group stained strongly with Ki-67, indicating a large number of highly proliferative cells. Conversely, those treated with AOM/DSS + *B. longum* showed significantly fewer Ki-67-positive cells (**Supplementary Figures 3A–I**).

B. longum altered gut microbiota dysbiosis in AOM/DSS mice

Fecal samples were collected from the PBS, AOM/DSS and *B. longum* groups, and the fecal flora was analyzed by 16S rRNA high-throughput sequencing. The comparison of the OTUs among the three groups revealed 1839 OTUs in the PBS group, 1970 OTUs in the AOMDSS group, and 2188 OTUs in the *B. longum* group, and a total of 1298 OTUs were shared by the different samples (**Figure 4A**). Afterward, a key analysis was conducted at the genus level, in which it was found that there were significant differences between Alipipes and Lachnospiraceae in the three groups (**Figures 4B–E**).

Mechanism of *B. longum* inhibition of AOM/DSS in an animal model

To investigate the mechanism of *B. longum* in CRC, RNA sequencing was performed in the AOM/DSS and *B. longum* groups. A total of 431 DEGs were identified, of which 236 were

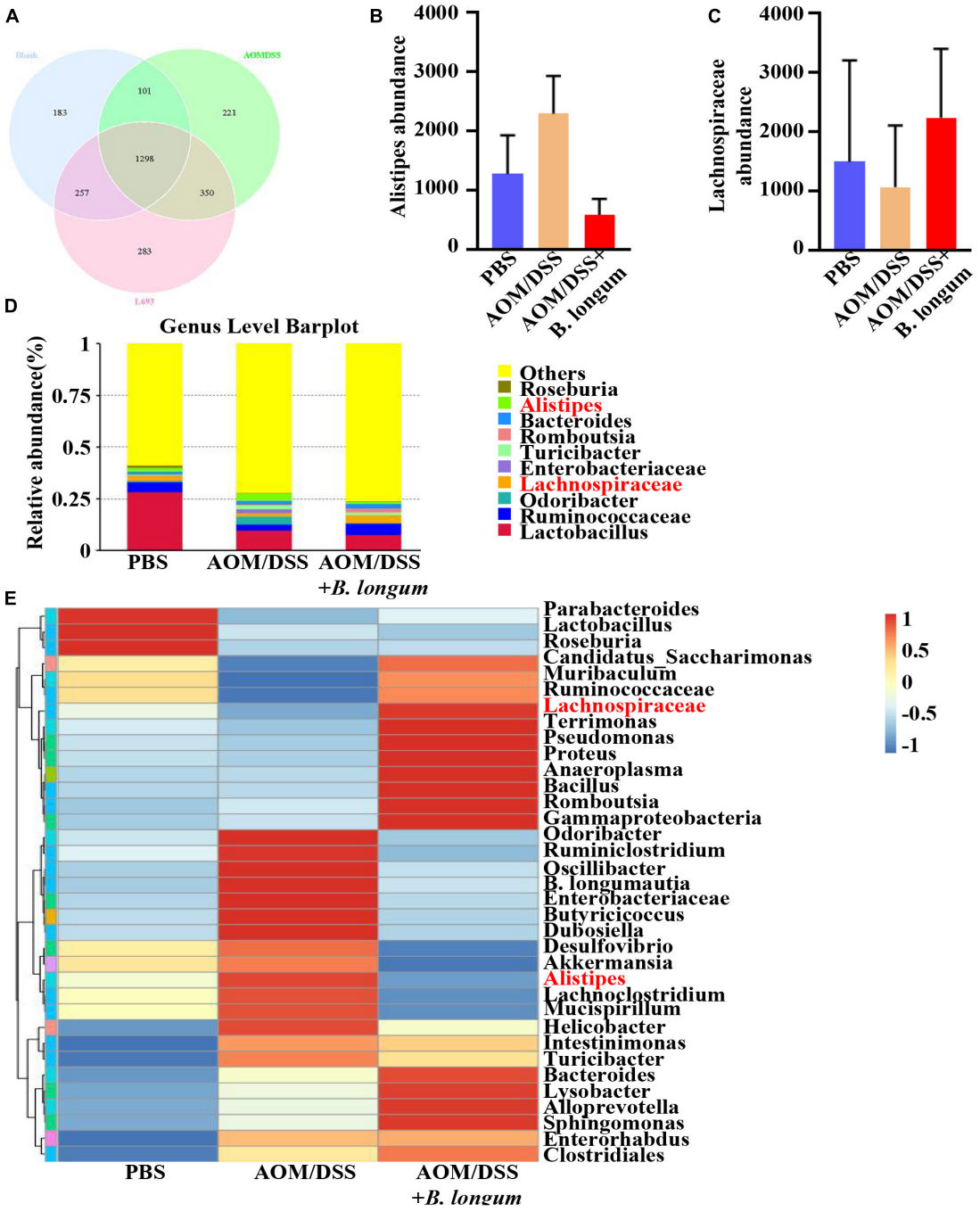

FIGURE 4
B. longum altered the gut microbiota dysbiosis in AOM/DSS mice. **(A)** Venn diagrams of bacterial OTUs. **(B)** The content of Alistipes in three groups. **(C)** The content of unidentified_Lachnospirace in three groups. **(D)** Bar charts at the Genus level of gut microbiota in the three groups (top 10). **(E)** The heat map of taxa in three groups. Similar results were obtained from three independent experiments.

upregulated and 195 were downregulated ($P < 0.05$; Figure 5A). Detailed information on the top 20 upregulated mRNAs (log2-fold change > 1; $P < 0.05$) and top 20 downregulated mRNAs (log2-fold change < -1; $P < 0.05$) is shown in Supplementary Tables 1 and 2. Through the analysis of KEGG pathways of upregulated and downregulated genes, it was found that *B. longum* affects several classical tumor pathways, as well as immune pathways and hormone pathways (Figures 5B, C). GO functional analysis of DEGs showed that the biological processes they were significantly enriched in were metabolic processes, developmental processes, signaling, growth, and biological adhesion (Figures 5D, E).

Clinical improvement of diarrhea patients after oral administration of *B. longum*

Through the observation of diarrhea in AOM/DSS model mice, it was found that *B. longum* could relieve diarrhea symptoms

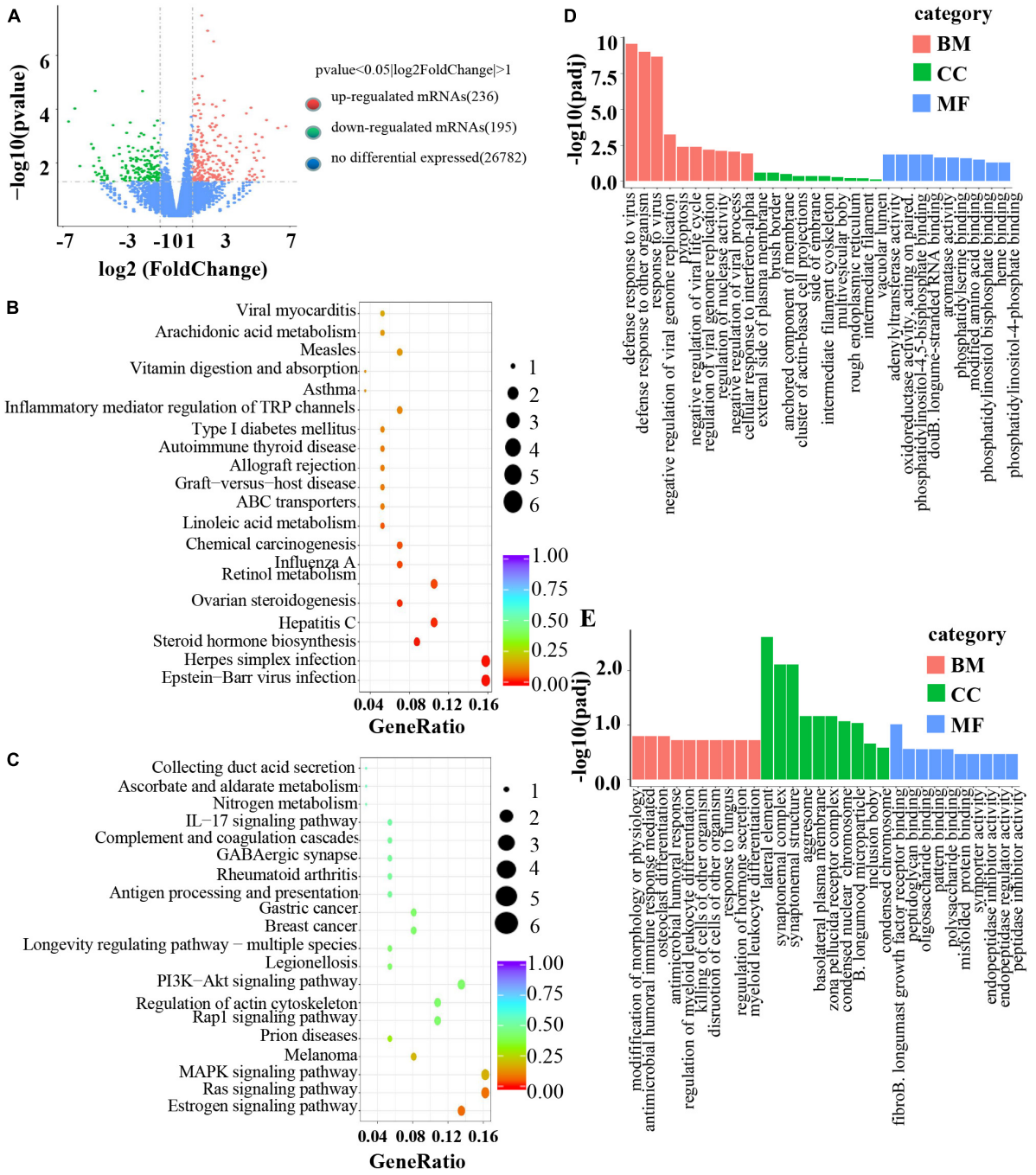

FIGURE 5
Mechanism of *B. longum* inhibiting AOM/DSS in animal model. **(A)** Volcano plot of upregulated (red spots, *n* = 236) and downregulated mRNAs (green spots, *n* = 195) from RNA sequencing data of *B. longum* vs. AOM/DSS. **(B,C)** KEGG pathway analysis based on DEGs upregulated and downregulated by *B. longum* from RNA sequencing data of *B. longum* vs. AOM/DSS. **(D,E)** GO pathway analysis based on DEGs upregulated and downregulated by BL from RNA sequencing data of *B. longum* vs. AOM/DSS.

very well, so we conducted a 4-week clinical comparative experiment of oral probiotics to relieve diarrhea. In this study, 20 patients with an average age of 31.05 ± 3.663 were included, including 15 males and 5 females. In the experiment, changes in the weekly IBS-SSS score, daily defecation frequency and Bristow stool score were observed (Supplementary Table 3). All patients were recorded in detail and completed the experiment.

Based on the statistics of the number of defecations in 21 days, it was found that although the number of defecations tended to decrease, it was not statistically significant (Figure 6A). Regarding the change in the Bristow score, we observed that there was a significant difference in the Bristow score from the 8th day compared with that from the first day, and the intake of *B. longum* improved the fecal traits (Figure 6B). Based on the individual statistics of the total IBS-SSS score and the five questions covered

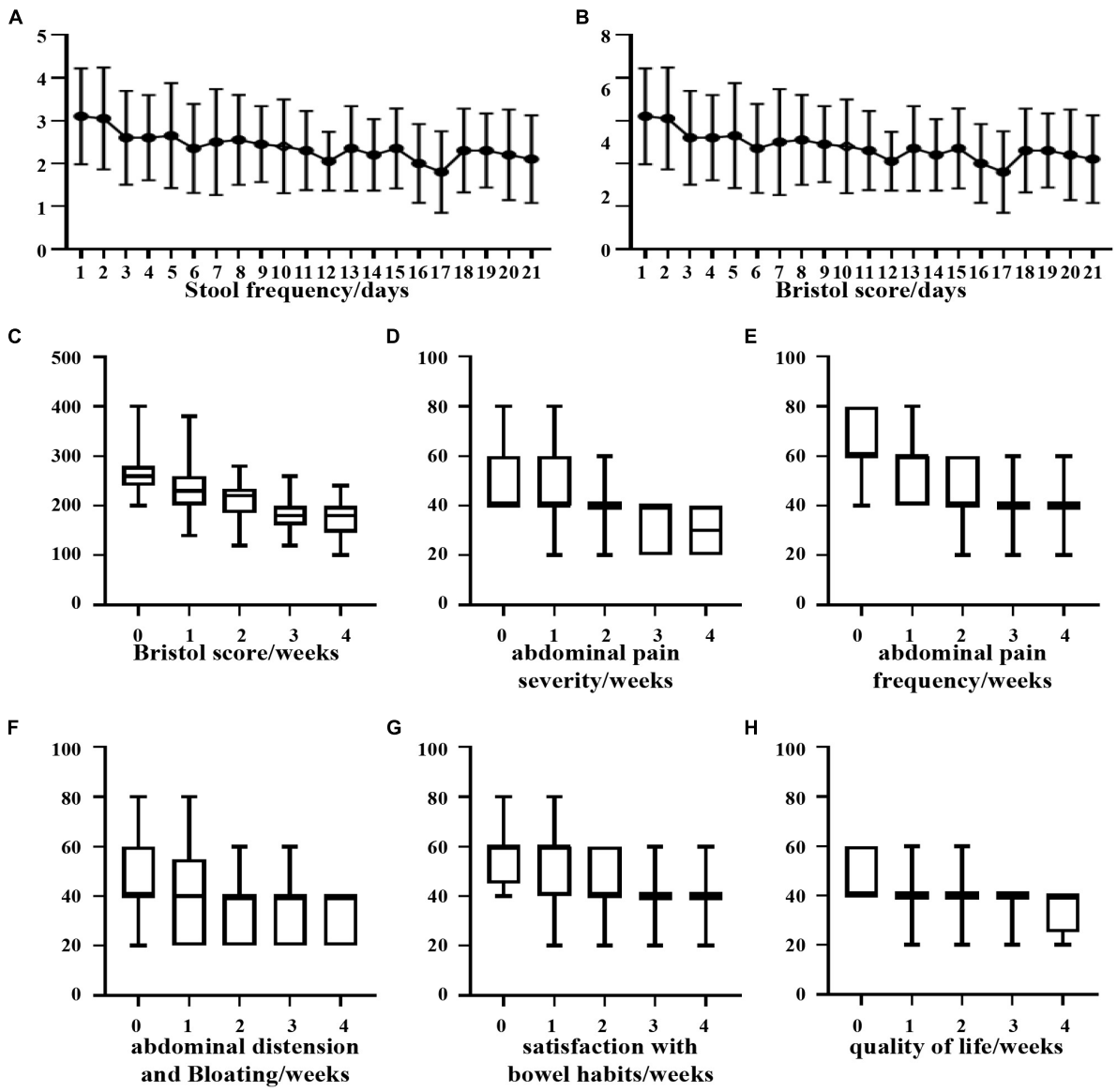

FIGURE 6
Clinical improvement of diarrhea patients after oral administration of *B. longum*. **(A)** Daily stool frequency. **(B,C)** Daily or weekly Bristol score. **(D)** Weekly abdominal pain severity. **(E)** Weekly abdominal pain frequency. **(F)** Weekly bloating frequency. **(G)** Weekly satisfaction with bowel habits. **(H)** Weekly quality of life.

by IBS-SSS, we found that supplementation with *B. longum* could improve each score, and the difference between the total IBS-SSS score of the first week and that of the fourth week was more than 50 points ($P < 0.05$; **Figures 6C–H**).

Discussion

With growing evidence being confirmed, it is now certain that probiotics possess numerous beneficial properties. The World Health Organization (WHO) and the Food and Drug Administration (FDA) have both reached a consensus that probiotics can be trusted as desirable dietary supplements (Ma et al., 2010). Despite previous extensive research on probiotics, their potential impact on cancer remains relatively unexplored. Therefore, it is crucial that we focus on gathering more evidence to determine the effectiveness of probiotics as a treatment and prevention method for cancer.

Ma et al. (2010) conducted a study using human colon cancer cells HT-29, DLD-1, and Caco-2 cells to investigate the potential of probiotic *Bacillus polyfermenticus* in reducing the impact of carcinogens and shrinking tumor size. Another recent report demonstrated that Streptococcus thermophilus inhibits colorectal tumorigenesis by secreting β-galactosidase. The authors employed various assays, including cell proliferation, necrosis, apoptosis, migration, invasion, and Ki-67, to support their conclusion using human colon cancer cells (Li et al., 2021). After establishing the effects of probiotics on CRC cells, the focus shifted to their impact on tumors. Tumor cells require oxygen for survival, while *B. longum* is an anaerobe. This prompted the exploration of the

optimal conditions to achieve a chemical equilibrium between them. The chosen condition involved co-culturing for 8 h at a concentration of 1×10^8 CFU/ml, aiming to inhibit tumor growth without inducing widespread tumor cell death. Subsequently, a series of functional assays were conducted. The results of CCK-8, colony formation, and immunofluorescence assays clearly demonstrated the inhibitory effect of *B. longum* on CRC cell growth. Similarly, the results of wound-healing and transwell assays indicated that *B. longum* also suppressed the migration and invasion of CRC cells. These findings align with the previously reported literature.

Foo et al. (2011) utilized a mouse model induced by 1,2-dimethylhydrazine to investigate the effects of *B. longum* and Lactobacillus gasser consumption. The study observed significant inhibitions of aberrant crypt foci and reductions in the number and size of tumors (Foo et al., 2011). Another study by *B. bifidum* and *Lactobacillus acidophilus* demonstrated tumor reduction through the modulation of IFN-γ and IL-10, as well as the activation of CD4 + and CD8 + cells. The researchers also assessed clinical tumor indicators such as CEA and CA199, finding that probiotics can reduce these tumor markers (Agah et al., 2019). Valadez-Bustos et al. (2019) showed that *B. longum* BAA-999 significantly reduced inflammation grade, tumor incidence, and adenocarcinoma incidence compared to the AOM + DSS group. AOM/DSS and 1,2-dimethylhydrazine are commonly used chemical models of colon cancer in mice, and the AOM/DSS model was chosen for the animal assays in this study. Our experimental results revealed that the tumor size in the *B. longum* group was smaller than that in the AOM/DSS group, which is consistent with previous experiments using the same animal models. Additionally, researchers fed mice with L. Acidophilus for 14 consecutive days and then induced murine colon adenocarcinoma CT-26 cells. After 28 days of observation and histological analyses, they found that the tumor size was nearly 50% constricted (Chen et al., 2012).

We conducted an analysis of the microbial composition and gene pathways in three different treatment groups. Our findings revealed that the microbial taxa were more abundant in the PBS and *B. longum* groups. Specifically, we observed a higher abundance of Lachnospiraceae in the *B. longum* group compared to the AOM/DSS group. Previous studies have shown that a high abundance of Lachnospiraceae is negatively associated with CRC (Flemer et al., 2018). Additionally, it has been reported that patients with colon cancer have a depletion of Lachnospiraceae compared to normal individuals (Peters et al., 2016). On the other hand, we found that the AOM/DSS group had a significantly higher abundance of Alistipes compared to the *B. longum* group. Alistipes has been identified as one of the key floras in promoting the occurrence and development of CRC (Dai et al., 2018; Parker et al., 2020). Notably, the intake of *B. longum* not only reduces cancer-promoting bacteria but also increases cancer-suppressor bacteria, demonstrating its preventive effect on CRC. Our sequencing results indicate that *B. longum* can influence various pathways, including hormone, metabolism, and immunity. Of particular interest is its impact on common tumor pathways such as the PI3K-Akt signaling pathway and MAPK signaling pathway. In future studies, we aim to further investigate the specific mechanism of action of *B. longum*.

In our animal experiments, we have observed that *B. longum* effectively alleviates diarrhea caused by DSS. This led us to investigate whether *B. longum* can improve clinical symptoms of diarrhea in patients. Our results indicate a significant positive effect. Previous studies have also reported the beneficial impact of probiotics on alleviating diarrhea symptoms. Additionally, research has shown that probiotics, particularly lactic acid bacteria and *B. longum*, have significant advantages in enhancing immunity, activating T cells, and improving the intestinal environment, thereby improving diarrhea symptoms (do Carmo et al., 2018). Chemotherapy and radiotherapy are commonly used treatments for cancer patients, often resulting in diarrhea as a side effect. Probiotics play a crucial role in alleviating these effects (Garczyk et al., 2022). Our study primarily focuses on individuals without underlying health conditions experiencing diarrhea symptoms. Although we only compared patients before and after using *B. longum*, we observed significant improvements in our main outcome measures. Our future research will delve deeper into the effects of *B. longum* on various types of diarrhea, with the hope of yielding even better results to benefit more patients.

In conclusion, *B. longum* has been found to impact the functions of CRC cells. Animal assays have shown that *B. longum* can influence the occurrence and development of CRC by regulating the composition of intestinal microbes and enhancing immune function. Furthermore, *B. longum* has demonstrated its effectiveness in regulating patients with diarrhea by restoring the balance of intestinal flora and promoting overall intestinal health. These findings highlight the significant role of *B. longum* as a probiotic in maintaining intestinal balance and promoting health.

Data availability statement

The 16S and RNA data presented in this article have been deposited to the NCBI Repository, under accession numbers PRJNA1083191 (16S) and PRJNA1084078 (RNA)

Ethics statement

The animal study was approved by the First Hospital of Hebei Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FS: Writing—original draft. XJ: Conceptualization, Methodology, Writing—original draft. HW: Data curation, Methodology, Writing—review and editing. SG: Data curation, Methodology, Writing—review and editing. SK: Methodology, Writing—review and editing. BX: Data curation, Methodology, Writing—review and editing. XW: Data curation, Methodology, Writing—review and editing. SC: Data curation, Methodology, Writing—review and editing. NL: Data curation, Writing—review and editing. BL: Conceptualization, Funding acquisition, Supervision, Writing—review and editing. ZZ: Funding acquisition, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1327464/full#supplementary-material

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Real-time PCR methods for identification and stability monitoring of *Bifidobacterium longum* subsp. *longum* UABl-14 during shelf life

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Bifidobacterium longum subsp. *longum* UABl-14™ is an important probiotic strain that was found to support digestive health. Here we present the development and validation of real-time PCR methods for strain-specific identification and enumeration of this important strain. The identification method was evaluated for specificity using 22 target samples and 30 non-target samples. All target samples successfully amplified, while no amplification was observed from any non-target samples including other *B. longum* strains. The identification method was evaluated for sensitivity using three DNA dilution series and the limit of detection was 2 pg. of DNA. Coupled with a viability dye, the method was further validated for quantitative use to enumerate viable cells of UABl-14. The viability dye treatment (PMAxx) was optimized, and a final concentration of 50 μM was found as an effective concentration to inactivate DNA in dead cells from reacting in PCR. The reaction efficiency, linear dynamic range, repeatability, and reproducibility were also evaluated. The reaction efficiency was determined to be 97.2, 95.2, and 95.0% with R^2 values of 99%, in three replicates. The linear dynamic range was 1.3×10^2 to 1.3×10^5 genomes. The relative standard deviation (RSD%) for repeatability ranged from 0.03 to 2.80, and for reproducibility ranged from 0.04 to 2.18. The ability of the validated enumeration method to monitor cell counts during shelf life was evaluated by determining the viable counts and total counts of strain UABl-14 in 18 multi-strain finished products. The viable counts were lower than label claims in seven products tested post-expiration and were higher than label claims in products tested pre-expiration, with a slight decrease in viable counts below label claim in three samples that were tested 2–3 months pre-expiration. Interestingly, the total counts of strain UABl-14 were consistently higher than label claims in all 18 products. Thus, the method enables strain-specific stability monitoring in finished products during shelf life, which can be difficult or impossible to achieve using the standard plate count method. The validated methods allow for simultaneous and cost-effective identification and enumeration of strain UABl-14 and represent an advancement in the quality control and quality assurance of probiotics.

KEYWORDS

real-time PCR, probe-based assay, strain specific PCR assay, probiotics, viability PCR, PMAxx, *Bifidobacterium longum* subsp. *longum* UABl-14, viable but non culturable

Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Delivering the correct probiotic strains at the correct dose of viable cells is essential to achieve their health benefits (Tripathi and Giri, 2014; Kolaček et al., 2017; Sánchez et al., 2017). However, several studies reported variable rates of non-compliance in probiotic products, more specifically, failure of probiotic products to meet declared strain contents and/or viable counts (Morovic et al., 2016; Shehata and Newmaster, 2020a,b). Thus, reliable, and accurate methods for probiotic strain identification and viable count determination are essential for probiotic authentication and quality assessment.

PCR based methods are widely used for probiotic identification including species-specific and strain-specific methods (Morovic et al., 2016; Kim et al., 2020; Shehata et al., 2021a,b; Kim et al., 2022). For probiotic enumeration, plate count methods are currently the most commonly used methods for probiotic quantification (Davis, 2014; Weitzel et al., 2021; Boyte et al., 2023), however, other culture-independent methods such as flow cytometry and PCR based methods are also emerging for probiotic enumeration (Boyte et al., 2023).

Plate count methods have several limitations such as the low specificity, i.e., inability to enumerate individual strains in multi-strain blends as these methods enable enumeration at the genus level or species level only if using selective growth media. This is a huge limitation since the health benefits of probiotics are strain specific (Klein et al., 2010; Sánchez et al., 2017; Mcfarland et al., 2018). Furthermore, plate count methods are culture-dependent methods which measure viability as cultivability, and thus these methods do not detect cells that exist in a viable but non culturable (VBNC) state (Wilkinson, 2018; Gorsuch et al., 2019; Wendel, 2022).

Alternative enumeration methods such as flow cytometry and viability PCR based methods are culture-independent methods that measure viability beyond cultivability (ISO, 2015; Hansen et al., 2018, 2020; Foglia et al., 2020; Kim E. et al., 2023; Ma et al., 2023; Shehata et al., 2023). Thus, these methods are able to count VBNC cells, hence, more accurate viable count determination. Additionally, PCR based methods can be designed to achieve strain specific viable count determination (García-Cayuela et al., 2009; Kramer et al., 2009), which is a huge improvement from the traditional plate count methods. PCR methods can be used with viability dyes in what is called viability PCR to quantify viable cells only (Hansen et al., 2020; Shehata et al., 2023). PCR methods are less laborious, high throughput, and offer shorter time to results (~6 h). Given the advantages of PCR methods over the traditional plate count methods, and their wide use for probiotic species and strain identification, PCR methods represent an attractive alternative method for probiotic enumeration, as they enable simultaneous strain-specific qualitative and quantitative detection.

Bifidobacterium longum subsp. *longum* is a common bacterium in the gut microbiome of both infants and adults (Oki et al., 2018; Díaz et al., 2021), and strains of this sub species were found to have health benefits such as improving chronic constipation in elderly individuals (Takeda et al., 2023), alleviating glucose intolerance in Type 2 diabetic mice (Kim W. J. et al., 2023), improving cognitive functions in healthy elderly adults (Shi et al., 2023), and reducing perceived stress in healthy adults (Boehme et al., 2023).

Strain *Bifidobacterium longum* subsp. *longum* UABl-14™ is a common probiotic strain in probiotic products that was found to support digestive health, modulate bowel functions and increase fibrolytic microbiota in participants with functional constipation when used in combination with other strains (Martoni et al., 2019). Here we present the development and validation of real-time PCR (qPCR) methods for strain specific identification and viable count determination of this important probiotic strain, *Bifidobacterium longum* subsp. *longum* UABl-14™.

Materials and methods

Reference materials and DNA extraction

In this study, 22 samples of *Bifidobacterium longum* subsp. *longum* strain UABl-14™ were used. Four of these samples were mono-strain samples and 18 were multi-strain samples acquired directly from manufacturers (Table 1). Additionally, reference samples from 30 probiotic strains were included in this study as non-targets (Table 1). The samples were collected from various probiotic manufacturers in USA and Canada. DNA extraction was performed using NucleoSpin Food kit (740945.50, Macherey Nagel, Germany), followed by DNA quantification using Qubit 4.0 FLuorometer (Q33238, Life technologies).

Strain-specific real-time PCR oligo design and real-time PCR protocol

UABl-14 strain-specific oligos were designed to amplify a strain specific sequence region that was identified using the sequence-based comparison function in Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015). Initially, the genome sequence of UABl-14 was compared to three other *B. longum* strain. The target sequence region identified from RAST was then searched on NCBI GenBank nucleotide collection database using the Basic Local Alignment Search Tool nucleotide function (BLASTn) to confirm the specificity of the identified target region to strain UABl-14. The oligos were designed using PrimerQuest Tool [Integrated DNA Technologies (IDT), Coralville, IA, United States] and were ordered from IDT (Table 2).

Each real-time PCR reaction consisted of 10 µL of 2x SensiFast Probes Master Mix (BIO-86020, Bioline), 1.8 µL of forward primer (10 µM working solution), 1.8 µL of reverse primer (10 µM working solution), 1.0 µl of hydrolysis probe (5 µM working solution), 1 µL of DNA, and up to 20 µL of molecular biology grade water. The thermal cycling program was denaturation for 5 min at 95°C followed by 40 amplification cycles (for 10 s at 95°C, and for 20 s at 60°C). Positive controls (DNA extracted from a reference sample of UABl-14 and diluted to 1 ng/µl) and negative controls (No Template Controls, NTC) were included in each run and samples were tested in triplicate on Hyris bCUBE.

Evaluating the specificity and sensitivity of UABl-14 strain-specific assay

To evaluate the specificity of the developed method, real-time PCR was run using 22 target samples (4 mono-strain and 18 multi-strain samples) and 30 non-target samples which included closely related

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TABLE 1 Target and non-target samples used to confirm the analytical specificity and analytical specificity results of *Bifidobacterium longum* subsp. *longum* UABl-14 strain-specific identification method.

Sample ID	Sample type	Strain	Mean Cq ± SEM *, #
T-1	Target (Mono-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	22.89 ± 0.08
T-2	Target (Mono-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	22.87 ± 0.12
T-3	Target (Mono-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	23.28 ± 0.07
T-4	Target (Mono-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	22.47 ± 0.04
T-5	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	22.55 ± 0.02
T-6	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	25.81 ± 0.03
T-7	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	26.00 ± 0.04
T-8	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	26.14 ± 0.07
T-9	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	26.27 ± 0.14
T-10	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	25.99 ± 0.08
T-11	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	26.39 ± 0.04
T-12	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	25.17 ± 0.21
T-13	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	25.03 ± 0.23
T-14	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	26.06 ± 0.25
T-15	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	27.59 ± 0.16
T-16	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	25.80 ± 0.04
T-17	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	27.79 ± 0.05
T-18	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	24.40 ± 0.06
T-19	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	23.41 ± 0.15
T-20	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	23.47 ± 0.10
T-21	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	23.58 ± 0.02
T-22	Target (Multi-strain)	*Bifidobacterium longum* subsp. *longum* UABl-14	28.16 ± 0.03
NT-1	Non-target	*Bifidobacterium animalis* subsp. *lactis* Bi-07	NA
NT-2	Non-target	*Bifidobacterium animalis subsp. lactis* UABla-12	NA
NT-3	Non-target	*Bifidobacterium bifidum* Bb-06	NA
NT-4	Non-target	*Bifidobacterium bifidum* HA-132	NA
NT-5	Non-target	*Bifidobacterium bifidum* UABb-10	NA
NT-6	Non-target	*Bifidobacterium breve* Bb-03	NA
NT-7	Non-target	*Bifidobacterium breve* HA-129	NA
NT-8	Non-target	*Bifidobacterium longum* subsp. *infantis* Bi-26	NA
NT-9	Non-target	*Bifidobacterium longum* subsp. *infantis* HA-116	NA
NT-10	Non-target	*Bifidobacterium longum* subsp. *infantis* R0033	NA
NT-11	Non-target	*Bifidobacterium longum* subsp. *longum* Bl-05	NA
NT-12	Non-target	*Bifidobacterium longum* subsp. *longum* HA-135	NA
NT-13	Non-target	*Bifidobacterium longum* subsp. *longum* R0175	NA
NT-14	Non-target	*Lacticaseibacillus casei* Lc-11	NA
NT-15	Non-target	*Lacticaseibacillus casei* UALc-03	NA
NT-16	Non-target	*Lacticaseibacillus paracasei* Lpc-37	NA
NT-17	Non-target	*Lacticaseibacillus paracasei* UALpc-04	NA
NT-18	Non-target	*Lacticaseibacillus rhamnosus* HN001	NA
NT-19	Non-target	*Lacticaseibacillus rhamnosus* Lr-32	NA
NT-20	Non-target	*Lactiplantibacillus plantarum* Lp-115	NA
NT-21	Non-target	*Lactiplantibacillus plantarum* UALp-05	NA
NT-22	Non-target	*Lactobacillus acidophilus* DDS-1	NA

(Continued)

TABLE 1 (Continued)

Sample ID	Sample type	Strain	Mean Cq ± SEM *, #
NT-23	Non-target	*Lactobacillus acidophilus* La-14	NA
NT-24	Non-target	*Lactobacillus gasseri* BNR17	NA
NT-25	Non-target	*Lactobacillus gasseri* Lg-36	NA
NT-26	Non-target	*Lactobacillus helveticus* R0052	NA
NT-27	Non-target	*Levilactobacillus brevis* Lbr-35	NA
NT-28	Non-target	*Ligilactobacillus salivarius* Ls-33	NA
NT-29	Non-target	*Limosilactobacillus reuteri* 1E1	NA
NT-30	Non-target	*Limosilactobacillus reuteri* LRC	NA

*SEM: The standard error of the mean.
#NA, No amplification.

TABLE 2 *Bifidobacterium longum* subsp. *longum* UABl-14 strain-specific primer and probe sequences.

Primer/probe	Sequence
Primer F	5′-CATCACACGAGAGAGCACAT-3′
Primer R	5′-CATAGAGAAGCTATCGCCGTATT-3′
Probe	5′-CGCCATCACATGTGCCAATCACAT-3′ (56-FAM and ZEN – 3IABkFQ)

strains such as other *Bifidobacterium longum* strains (Table 1). The same amount of DNA was used from all target and non-target samples. All DNA samples were quantified using Qubit 4.0 Fluorometer, then diluted to 1 ng/μl in molecular biology grade water (Shehata et al., 2019).

To evaluate the sensitivity or limit of detection (LOD), three 10-fold dilution series of DNA, with five dilution points each were used. The dilutions were 10 ng/μl to 0.001 ng/μl, 5 ng/μl to 0.0005 ng/μl and 2 ng/μlto 0.0002 ng/μl (Shehata et al., 2019; Shehata and Newmaster, 2020c). Each dilution point was tested in triplicate using real-time PCR as described above.

Optimization of viability pre-treatments

A viability dye treatment was used to enumerate viable cells only (Gobert et al., 2018). A viability dye has the ability to cross cell membranes of dead or membrane damaged cells only, and to irreversibly intercalate to DNA upon photoactivation, rendering DNA from dead or membrane damaged cells unreactive in PCR. Multiple concentrations of the viability dye were evaluated to find an effective concentration to inactivate DNA from dead cells as previously described (Shehata and Newmaster, 2021; Shehata et al., 2023). The heat-killed cells were prepared by heating the cells at 95°C for 20 min. PMAxx (40069, Biotium Inc., Hayward, CA, United States) at final concentrations of 0 μM, 50 μM, 100 μM, and 150 μM were tested. The cells and PMAxx were vortexed, followed by incubation at room temperature in the dark for 5 min. Tubes were then incubated in a PhAST BLUE Photoactivation System (GenIUL, Barcelona, Spain) for 15 min. DNA was liberated using bead beating in BeadBug™ prefilled tubes (Z763764, Sigma-Aldrich, St. Louis, MO, United States) for 5 min at 3,000 rpm (Hansen et al., 2018; Shehata et al., 2023). The integrity of the DNA from non-heated and heat-killed cells was evaluated by running the DNA from three reference samples T-1, T-2, and T-3 for 10 min on 2% E-gel with SYBR Safe DNA Gel Stain (G720802, Invitrogen), followed by visual inspection of the gel. E-Gel™ 1 Kb Plus DNA Ladder (10488090, Invitrogen) was used as a marker. The concentrations of DNA from the same samples were measured using Qubit 4.0 Fluorometer (Q33238, Life technologies). The effectiveness of the viability dye treatment in removing DNA from heat-killed cells was then calculated based on the shift in the Cq values observed with the treatment (Marole et al., 2024).

Evaluating the reaction efficiency and precision of UABl-14 strain-specific assay

Reaction efficiency, limit of quantification (LOQ), and linear dynamic range were evaluated. Ten-fold serial dilutions were prepared from reference samples at five dilution points each. Each dilution point was tested in triplicate using real-time PCR as described above. Standard curves were established between quantification cycle (Cq) and log genome number. Slopes were calculated from the standard curves using Prism 10 (GraphPad Software, San Diego, CA, United States) and were used to calculate reaction efficiency (Shehata and Newmaster, 2021; Shehata et al., 2023).

Repeatability and reproducibility were evaluated using 3 samples (samples T-1, T-2, and T-3) tested at five dilutions as previously described (Shehata and Newmaster, 2021; Shehata et al., 2023). The analysis was repeated on a different day for repeatability, and on a different bCUBE machine for reproducibility, and the variance was calculated as the relative standard deviation (RSD%).

Assessing the ability of UABl-14 strain-specific assay in monitoring strain stability in multi-strain finished products during shelf life

The viable counts of strain UABl-14 in 18 multi-strain finished products were determined using UABl-14 strain-specific assay by interpolation from the standard curve. The products were at different expiration dates with 7 products tested post-expiration and 11 products tested pre-expiration dates. All products were stored at room temperature. The viable counts were compared to label claims of viable counts. Additionally, the total counts (viable and dead) of strain UABl-14 were determined using UABl-14 strain-specific assay but eliminating the use of PMAxx.

Statistical analysis

Prism 10 (GraphPad Software, San Diego, United States) was used for statistical analyses and graphical displays.

Results

Strain-specific real-time PCR oligo design

RAST identified a target sequence region in the genome sequence of strain UABl-14, which codes for a hypothetical protein. To confirm that this target sequence region was unique to strain UABl-14, the target sequence region was BLASTn searched on NCBI GenBank in December 2020 and no similarity was found to any sequence in the Nucleotide collection (nr/nt) database. PrimerQuest Tool was used to design primers and a probe to amplify a 94 bp amplicon.

Evaluating the specificity and sensitivity of UABl-14 strain-specific assay

To confirm the strain specificity of the method, 22 target samples and 30 non-target samples were tested using the developed method (Table 1). All target samples successfully amplified with mean Cq value between 22.47 and 28.16. No amplification was observed from any of non-target samples including other *B. longum* strains (Table 1).

The LOD was determined from standard curves established from three DNA dilution series (5 dilution points each). The LOD was 0.002 ng of DNA or 755 copies (Figure 1).

Optimization of viability pre-treatments

The integrity of the extracted DNA was examined by running the DNA on a gel. DNA extracted from both non-heated and heat-killed cells of samples T-1, T-2, and T-3 showed high integrity (Figure 2A). The DNA concentrations from non-heated cells of samples T-1, T-2, and T-3 were 7 ng/μl, 8 ng/μl, and 8 ng/μl, and from heat-killed cells were 6 ng/μl, 7 ng/μl, and 7 ng/μl. Different concentrations of PMAxx viability dye (0 μM, 50 μM, 100 μM, and 150 μM) were evaluated using non-heated and heat-killed cells to find a concentration that achieved effective inactivation of DNA from dead cells. At 0 μM of PMAxx, non-heated and heat-killed cells showed similar Cq values (19.60 and 19.48, respectively). At 50 μM of PMAxx, non-heated and heat-killed cells showed different Cq values (20.90 and 31.29, respectively). Similar results were observed at 100 μM and 150 μM of PMAxx. At 100 μM of PMAxx, Cq values were 21.47 and 33.27 from non-heated and heat-killed cells, respectively. At 150 μM of PMAxx, Cq values were 21.62 and 32.49 from non-heated and heat-killed cells, respectively (Figure 2B). 50 μM of PMAxx was effective in inactivating dead cells' DNA from reacting in PCR. This viability dye treatment resulted in a significant shift in Cq value (11.8 cycles), achieving 99.97% removal of DNA from heat-killed cells.

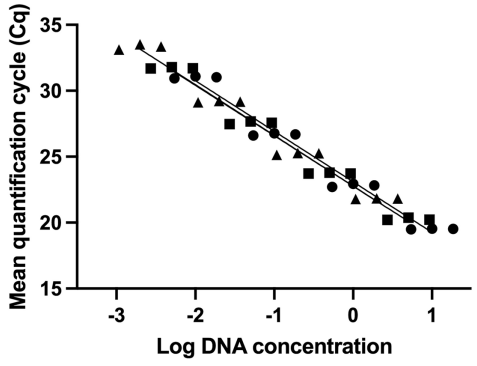

FIGURE 1
Evaluating the sensitivity of UABl-14 strain-specific assay. Three 10-fold dilution series of DNA were used to establish standard curves. The LOD was 0.002 ng of DNA or 755 copies.

Evaluating the reaction efficiency and precision of UABl-14 strain-specific assay

Reaction efficiency of the UABl-14 strain-specific assay was determined to be 97.2, 95.2, and 95.0% with R^2 values of 99% and p value of 0.0004, 0.0005, and 0.0005 in three replicates (Figure 3). The linear dynamic range was 1.3×10^2 to 1.3×10^5 genomes (Figure 3).

Repeatability and reproducibility were evaluated using 3 samples tested at five dilutions. The RSD% for repeatability ranged from 0.71 to 2.36, 0.03 to 1.51, and 0.43 to 2.80, and RSD% for reproducibility ranged from 0.06 to 0.61, 0.10 to 1.20, and 0.04 to 2.18 for the 3 samples (Figure 4).

Assessing the ability of UABl-14 strain-specific assay in monitoring strain stability In multi-strain finished products during storage

The viable counts of strain UABl-14 were determined in 18 multi-strain finished products at different expiration dates. The viable counts were lower than label claims in all 7 products tested post expiration dates (Figure 5). The viable counts were higher than label claims in products tested pre-expiration dates except for samples that were within 3 months to expiration (Figure 5). Interestingly, the total counts (viable and dead) of strain UABl-14 were consistently higher than label claims in all 18 products (Figure 6).

Discussion

Probiotics are sold in food format such as fermented food products as well as in pharmaceutical dosage forms such as capsules and tablets as natural health products or dietary supplements (Health Canada, 2003). The global probiotic market size is growing rapidly, valued at USD 58.17 billion in 2021, and anticipated to reach USD 111.21 billion in 2030 (Grand-View-Research-Inc, 2022). With the expanding market size, multiple reports have shown failure of probiotic products to meet label claims, observed as strain substitution, missing strains, presence of undeclared strains or lower viable counts compared to label claims during shelf life and before expiration dates (Morovic et al., 2016; Patro

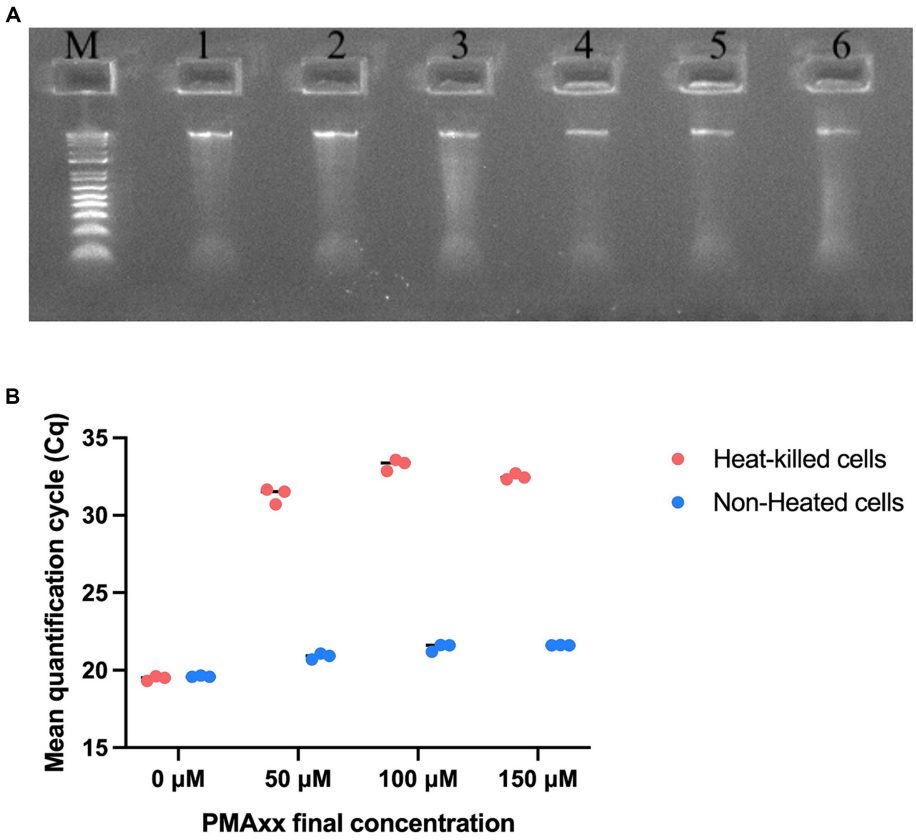

FIGURE 2
Optimization of viability pre-treatments of UABl-14 strain-specific assay. **(A)** Agarose gel electrophoresis to examine the integrity of the DNA from non-heated and heat-killed cells. M is E-Gel™ 1 Kb Plus DNA ladder. Samples 1–3 are the DNA from samples T-1, T-2, and T-3 (non-heated) and samples 4–6 are the DNA from samples T-1, T-2, and T-3 (heat-killed). **(B)** PMAxx viability dye treatments at 0 µM, 50 µM, 100 µM, and 150 µM were evaluated. PMAxx at 50 µM was used as an effective concentration in inactivating DNA from dead cells.

Replicate 1	Replicate 2	Replicate 3
Y = -3.390*X + 51.95	Y = -3.443*X + 52.27	Y = -3.448*X + 52.33

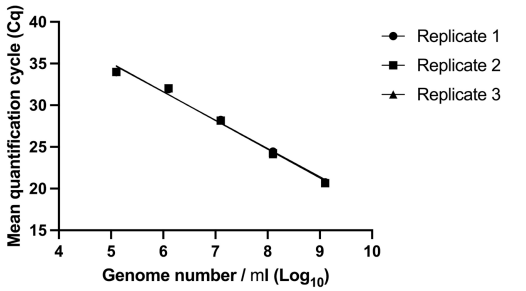

FIGURE 3
Evaluating the reaction efficiency and precision of UABl-14 strain-specific assay. Reaction efficiency of the UABl-14 strain-specific assay was determined to be 97.2, 95.2, and 95% with R^2 values of 99% and p value of 0.0004, 0.0005, and 0.0005 in three replicates.

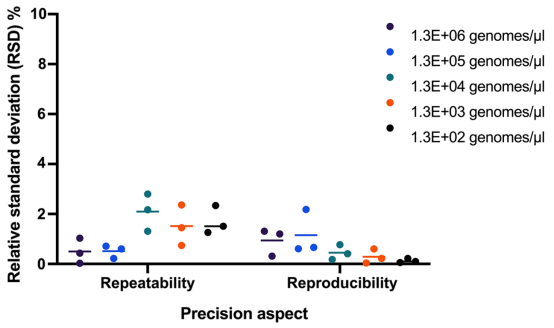

FIGURE 4
Evaluating the precision of UABl-14 strain-specific assay. Repeatability and reproducibility were evaluated using 3 samples tested at five dilutions. The RSD% for repeatability ranged from 0.71 to 2.36, 0.03 to 1.51, and 0.43 to 2.80, and RSD% for reproducibility ranged from 0.06 to 0.61, 0.10 to 1.20, and 0.04 to 2.18 for the 3 samples.

et al., 2016; Kolaček et al., 2017; Shehata and Newmaster, 2020a,b). This label non-compliance can result in partial or complete loss of efficacy (Tripathi and Giri, 2014; Kolaček et al., 2017; Sánchez et al., 2017; Jackson et al., 2019). Thus, analytical methods that support product authentication via confirming label information about product content is extremely important (Fusco et al., 2023).

B. longum subsp. *longum* UABl-14 is a common probiotic strain in probiotic products that was proven to support digestive health

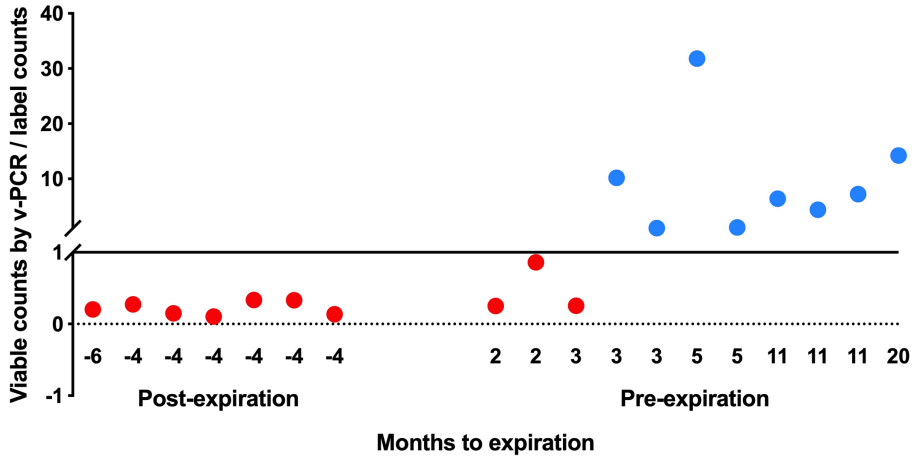

FIGURE 5
Assessing the ability of UABl-14 strain-specific assay in monitoring strain stability in 18 multi-strain finished products during shelf life. The viable counts were lower than label claims in all 7 products tested post expiration dates and were higher than label claims in products tested pre-expiration dates, with the exception of samples that were within 3 months of expiration.

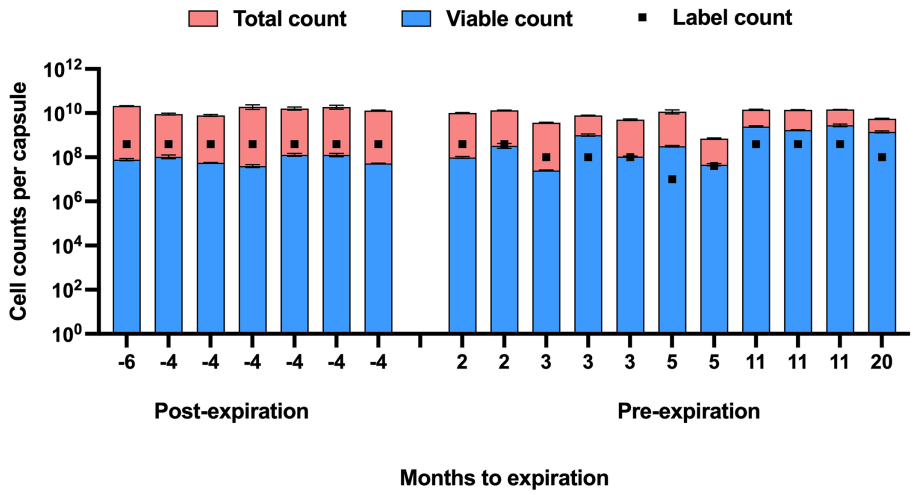

FIGURE 6
Total counts (viable and dead) and viable counts of strain UABl-14 versus label counts in 18 multi-strain finished products during shelf life. Unlike the viable counts of strain UABl-14, the total counts of strain UABl-14 were consistently higher than label claims in all 18 products.

(Martoni et al., 2019). However, to the best of our knowledge, there are no available methods to achieve strain-specific identification and enumeration of this strain. In this study, real-time PCR based methods for strain-specific identification and enumeration of strain UABl-14 were developed and validated to facilitate the quality assurance of probiotic products that contain this strain.

A strain-specific identification and/or enumeration method requires robust bioinformatic analyses of genome sequences to confirm strain specificity, as well as extensive validation to ensure accurate and precise performance. Bioinformatic analyses identified a unique sequence region in the genome of strain UABl-14. The sequence region showed no similarity to any sequence in the Nucleotide collection database in NCBI GenBank. Primers and a hydrolysis probe were designed to target this unique sequence region. The primers and probe were validated for use in strain-specific identification and enumeration methods. The specificity of the UABl-14 strain-specific assay was evaluated in qPCR where the assay successfully amplified all 22 target samples, which included mono-strain and multi-strain samples. Thirty non-target samples were used in specificity evaluation which included multiple strains of lactobacilli and *Bifidobacterium,* and included, other strains of *B. longum* such as *Bifidobacterium longum* subsp. *infantis* strains Bi-26, HA-116, and R0033 and *Bifidobacterium longum* subsp. *longum* strains Bl-05, HA-135, and R0175 to confirm strain level specificity (Table 1). No amplification was observed from any non-target strains. It is important to note that these non-target strains are commercialized probiotic strains available and common in the market in finished probiotic products. The results confirmed that the assay is strain specific to strain UABl-14 which means the assay will correctly identify strain UABl-14 only. The results also confirmed that the assay works well with both mono-strain and multi-strain samples.

The sensitivity of the UABl-14 strain-specific assay was also evaluated in qPCR. Sensitivity or the LOD is the lowest amount of the target that an assay can detect (Bustin et al., 2009). Standard curves were established and the LOD was determined to be 0.002 ng of DNA (Figure 1). Thus, the assay proved to be highly sensitive, which means the assay is applicable to multi-strain blends and products in which strain UABl-14 is present at low abundance.

The UABl-14 strain-specific assay was further validated for quantitative use for the enumeration of strain UABl-14. To enumerate viable cells only, the assay was used with PMAxx viability dye, a DNA-intercalating dye that inactivates DNA from dead cells. The viability dye treatment is known to vary between strains and thus optimization for each target strain is required (Kiefer et al., 2020). Optimization of PMAxx viability dye treatment with strain UABl-14 showed that 50 µM of PMAxx was effective in inactivating DNA from dead cells from reacting in PCR, achieving 99.97% removal of DNA from heat-killed cells (Figure 2B). Previous studies reported optimal final concentrations of PMA that ranged from 25 µM to 100 µM (Gobert et al., 2018; Hansen et al., 2018; Scariot et al., 2018; Shehata and Newmaster, 2021; Shehata et al., 2023).

A very important parameter to be considered when evaluating a quantitative assay is the reaction efficiency, with the ideal reaction efficiency ranging between 90 and 110% with R^2 values $\geq$ 0.98 (Broeders et al., 2014). Reaction efficiency values of the UABl-14 strain-specific assay were 97.2, 95.2, and 95.0% and R^2 value was 99% in all three replicates (Figure 3). The linear dynamic range covered four dilutions points (Figure 3). An ideal dynamic range covers 5 to 6 dilutions, with a minimum of three dilutions (Bustin et al., 2009). Thus, the UABl-14 strain-specific assay has high efficiency and adequate linear dynamic range.

The repeatability and reproducibility of the UABl-14 strain-specific assay were evaluated. The RSD% for repeatability using three samples tested at five dilutions was below 2.80, and RSD% for reproducibility using three samples tested at five dilutions ranged was below 2.18 (Figure 4). The results indicate that the UABl-14 strain-specific assay is highly precise, since the acceptable value for repeatability and reproducibility is below 25% (Broeders et al., 2014).

The UABl-14 strain-specific assay was evaluated for the ability to monitor strain stability in multi-strain finished products during storage by testing 18 multi-strain finished products at different expiration dates. The methods showed variable viable and total (viable and dead) counts of strain UABl-14 in finished products tested at different expiration dates (Figures 5, 6). Viability of probiotic strains is expected to decline during storage, the decline rate varying with storage conditions such as temperature and moisture levels (Tripathi and Giri, 2014). Improving strain stability during shelf life of probiotic products is a major challenge in the probiotic industry (Morovic et al., 2016). Probiotic products are expected to meet label claims of viable count until expiration dates to maintain efficacy. Thus, methods that enable strain-specific monitoring of stability during shelf life is of great importance.

Because the probiotic products that were used in the stability monitoring experiment were multi-strain products, it was not possible to compare the viable counts to plate counts. Nonetheless, plate count and viability PCR measure viability differently where plate count methods rely on cultivability while viability PCR relies on membrane integrity as a measure of viability (Boyte et al., 2023). Previous studies have reported discrepancies in viable counts determined using culture-dependent versus culture-independent methods, especially following storage (Fiore et al., 2020; Wendel, 2022; Shehata et al., 2023). This may be attributed to the fact that cell cultivability declines faster than membrane integrity, and to the portion of cells that exist in a VBNC state (Foglia et al., 2020). Since VBNC cells are considered probiotics, viable counts determined using culture-independent methods would be more accurate compared to culture-dependent methods (Foglia et al., 2020).

Conclusion

The real-time PCR methods developed and validated for strain-specific identification and viable count determination of strain UABl-14 are strain-specific, highly sensitive and enable the enumeration of VBNC cells. Thus, the methods offer a significant advancement in viable count determination over the traditional plate count method. The methods allow for simultaneous and cost-effective analyses, serving the dual purpose of identification and enumeration of strain UABl-14 in mono-strain as well as in multi-strain finished products to facilitate quality control measures for efficacious and compliant probiotic products.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

HS: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Conceptualization. BH: Writing – review & editing, Methodology, Investigation. SN: Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

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Conflict of interest

HS and BH were employed by Purity-IQ Inc.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Specific cultivation-independent enumeration of viable cells in probiotic products using a combination of fluorescence *in situ* hybridization and flow cytometry

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This study introduces an optimized integration of flow cytometry and fluorescence *in situ* hybridization (Flow-FISH) as an approach for the specific enumeration of gram-positive bacteria in probiotic products, overcoming the limitations of conventional methods. The enhanced Flow-FISH technique synergizes the rapid and automated capabilities of flow cytometry with the high specificity of FISH, facilitating the differentiation of viable cells at the species level within probiotic blends. By analyzing lyophilized samples of *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, and *Bifidobacterium animalis* subsp. *lactis*, and a commercial product, the study highlights the optimized Flow-FISH protocol's advantages, including reduced hybridization times to 1.5 h and elimination of centrifugation steps. Comparative evaluations with the widely accepted enumeration methods plate count and Live/Dead (L/D) staining were conducted. The study revealed that Flow-FISH produces higher viable cell counts than plate count, thereby challenging the traditional "gold standard" by highlighting its predisposition to underestimate actual viable cell numbers. Against L/D staining, Flow-FISH achieved comparable results, which, despite the different foundational premises of each technique, confirms the accuracy and reliability of our method. In conclusion, the optimized Flow-FISH protocol represents a significant leap forward in probiotic research and quality control. This method provides a rapid, robust, and highly specific alternative for the enumeration of probiotic bacteria, surpassing traditional methodologies. Its ability to enable a more detailed and reliable analysis of probiotic products paves the way for precise quality control and research insights, underscoring its potential to improve the field significantly.

KEYWORDS

Flow-FISH, enumeration, specific count, viability count, multi-species blend, FISH, probiotics, plate count

1 Introduction

Over the past few decades, research interest in microbiomes has increased significantly. It is now widely accepted that the human gut microbiome is not only crucial for proper digestion but also plays a vital role in a functioning immune system and is implicated in the development of various diseases such as irritable bowel syndrome and diarrhea (Menees and Chey, 2018; Li et al., 2021). Evidence also suggests its influence on neurodevelopmental and neurodegenerative diseases like Autism Spectrum Disorder, Alzheimer's, and Parkinson's Disease (Cryan et al., 2019; Loh et al., 2024).

This has generated considerable interest in the composition of a healthy microbiome and how it might be effectively influenced. Although understanding the microbiome has proven to be a complex endeavor, as the microbiomes of healthy people are not homogeneous in their composition (Eckburg et al., 2005; Arumugam et al., 2011; Pasolli et al., 2019), it has been discovered that certain types of bacteria, known as probiotics, positively impact human health when consumed alive and in sufficient quantities (Kerry et al., 2018; Ranjha et al., 2021; Latif et al., 2023).

The World Health Organization defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). This definition raises the question about methods for accurately quantifying not just the cells but specifically the viable cells.

Davis (2014) proposed that not only probiotic cells capable of reproducing but also all cells that are metabolically active and/or have an intact membrane should be considered alive. Hence, one of the major challenges posed by the plate count method is the so-called viable but non-culturable (VBNC) state. Those cells can enter a dormant state, during which they remain metabolically active but do not replicate, thus losing their ability to be cultured. However, they might effectively recover in the human gut, as it provides the appropriate environment (Lahtinen et al., 2006b; Davis, 2014; Fiore et al., 2020).

Since probiotic cells are often exposed to a stressful environment during industrial production as well as during storage, VBNC cells are frequently found in probiotic products (Mills et al., 2011; Wendel, 2022).

The most commonly utilized bacteria in probiotics are lactic acid bacteria, specifically *Lactobacillus* and *Bifidobacterium* species (Williams, 2010). Jackson et al. (2002) confirmed among other studies that the culture-based method does not always provide accurate insights into lactic acid bacteria (Moreno et al., 2006; Pereira et al., 2023).

An alternative method of great interest in the field of probiotics is flow cytometry (Adan et al., 2017; Pane et al., 2018; Sielatycka et al., 2021; Boyte et al., 2023; Pereira et al., 2023; Tracey et al., 2023) due to its rapid and automatic results. Staining the cells with fluorescent dyes prior to measurement can provide valuable information. The applications of fluorescent dyes in this context are diverse, including the determination of nucleic acid content, enzyme activities, and apoptotic cells, as well as the identification of cell surface receptors and diverse cell populations (Adan et al., 2017).

Additionally, DNA intercalating fluorescent dyes, which can penetrate the cell differently depending on the integrity of the membrane, are commonly used for probiotic cell labeling to determine their viability state, thus making flow cytometry suitable for quantifying the number of live cells in a sample. However, this method has severe limitations when applied to multiple species blends, which are often found in practice, as it is a non-specific method (ISO 19344:2015, Tracey et al., 2023).

Consequently, the combination of flow cytometry with a technique that permits the specific identification of microorganisms is not merely advantageous but essential for advancing the capabilities of flow cytometric analysis. Fluorescence *in situ* hybridization (FISH) is a suitable technology for this purpose as it facilitates the differentiation not only between viable and non-viable cells but also among distinct species.

The principle of FISH is based on the phylogeny of microorganisms (Woese, 1987) and utilizes fluorescently labeled oligonucleotide probes that target specific sites at the ribosomal RNA (rRNA) of the microorganisms. It enables the precise detection of viable microbial populations ranging from broad taxonomic groups to individual species and involves fixing microbial cells to stabilize and permeabilize them, followed by hybridization with fluorescently labeled oligonucleotide probes and subsequent analysis via epifluorescence microscopy (Amann et al., 1990a, 1995; Wagner et al., 1993; Snaidr et al., 1997). FISH has proven to be a powerful tool for the simultaneous visualization and characterization of multiple bacterial populations in the same sample (Amann et al., 1996; Lukumbuzya et al., 2019).

For probiotics, the FISH method has been successfully applied multiple times to identify and quantify various probiotic species in fecal and lyophilized samples (Langendijk et al., 1995; Rinne et al., 2005; Bezirtzoglou et al., 2011; Pasulka et al., 2021).

Although the classical FISH method offers some significant advantages, such as rapid results, specificity, and differentiation between live and dead cells, the standardization of microscopic evaluation is challenging due to its dependence on the performer. Consequently, the combination of FISH with methods enabling automated, objective, and thus standardized quantification is required. The first combination of flow cytometry and FISH, the so-called Flow-FISH dates back about 30 years (Amann et al., 1990a; Wallner et al., 1993; Snaidr et al., 1999).

Since then, both FISH and flow cytometry techniques have significantly advanced, and Flow-FISH has been successfully demonstrated for fecal microorganisms (Rigottier-Gois et al., 2003; Rochet et al., 2004; Vaahtovuo et al., 2005; Dinoto et al., 2006; Collado and Sanz, 2007; Cleusix et al., 2010).

However, the protocols required extended hybridization times of more than 10 hours, which is far from a rapid method. Moreover, they require several centrifugation steps which might lead to cell loss and by this negatively influence quantitative data.

In this study, we present an optimized and advanced combination of flow cytometry and FISH, demonstrating its suitable application, especially in the field of probiotics. The objective of the study was to demonstrate and validate the efficacy of the Flow-FISH method in accurately and specifically enumerating gram-positive bacteria species in both single and mixed blends of probiotics. For this purpose, we analyzed different lyophilized probiotic samples consisting of *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, and *Bifidobacterium animalis* subsp. *lactis*, and a commercial product, comparing the outcomes of this advanced Flow-FISH methodology with those of established techniques such as Live/Dead (L/D) measurement via flow cytometry and plate count analysis.

2 Materials and methods

2.1 Sample preparation

Lyophilized strains of *Lacticaseibacillus rhamnosus* SP1 (*L. rhamnosus*), *Lactiplantibacillus plantarum* LP-115 (*L. plantarum* LP-115) and 14D (*L. plantarum* 14D), and *Bifidobacterium animalis* subsp. *lactis* BLC1 (*B. lactis*), as well as a mix of these strains, were rehydrated according to ISO 19344:2015. In detail, 100 mg of each lyophilized strain, as well as from the self-mixed sample, was diluted at a 1:20 w/v ratio using a 0.1% peptone salt solution. Rehydration was done by shaking the samples at 100 rpm for 60 min at room temperature. For analyzing the commercially available product "IberoBiotics Pro" (Lot number: 69974, expiry date: February 2025; Bayer Vital GmbH, Leverkusen, Germany), the entire capsule content was transferred into a 50 mL tube and rehydrated in 50 mL of 0.1% peptone salt solution. The sample was shaken at 100 rpm for 60 min at room temperature. Immediately after rehydration, the samples were further processed.

2.2 Quantification via plate count

For the cultivation of the strictly anaerobic *B. lactis*, an anaerobic environment was established utilizing an airtight container in combination with anaerobic packs (Sigma Aldrich, Darmstadt, Germany) to ensure the absence of oxygen. *B. lactis* was cultured on DSM Medium 58 agar at 37°C. The microaerophilic *L. rhamnosus* and *L. plantarum* 14D were cultured on MRS agar at 37°C in a suitable atmosphere, utilizing microaerobic packs (BioMérieux SA, Marcy-l'Étoile, France). Each sample underwent a serial 10-fold dilution. Adequate dilutions were plated and incubated for 48 to 96 h. Grown colonies were counted and results reported as colony forming units (CFUs) per gram.

2.3 Analysis by Live/Dead staining

The LIVE/DEAD BacLight Bacterial ViabilityKit (Thermo Fisher Scientific Inc., Waltham, Massachusetts) was used to differentiate between live and dead cells by differential staining following the ISO 19344:2015 standard. The two dyes utilized were SYTO 9 and propidium iodide (PI), which differ in their spectral characteristics and ability to penetrate intact cells. SYTO 9, a green fluorescent nucleic acid stain, enters all cells regardless of membrane integrity. In contrast, PI is selective in entering cells with compromised membranes only, i.e., dead or damaged cells, thereby causing a reduction in SYTO 9 fluorescence within these cells. The extent of PI penetration and subsequent fluorescence reduction is dependent on the level of membrane damage, leading to either a very low signal of green fluorescence in presumed dead cells or a partial decrease in green fluorescence in damaged cells. In summary, this staining technique enables differentiation between living, damaged, and dead cells.

To examine the cells, 1.5 µL of each dye was added to a tube containing 997 µL of PBS buffer and mixed well. 990 µL of this dying solution was mixed with 10 µL of diluted rehydrated sample and incubated for 15 min at room temperature in the dark. The sample was then immediately measured on a Cytek Northern Lights flow cytometer (Cytek Bioscience Inc., Fremont, CA, United States). Results were reported as total fluorescence units (TFUs) and active fluorescence units (AFUs) per gram.

2.4 Analysis by Flow-FISH

Rehydrated sample was mixed in a 1:1 v/v ratio with lysozyme (Sigma Aldrich, Darmstadt, Germany), at a species-specific optimized concentration of 400,000 Units/mL for *L. rhamnosus* or 833,000 Units/mL for *L. plantarum* 14D and *B. lactis*, and incubated for 30 min (*L. rhamnosus* and *L. plantarum* 14D) or 5 min (*B. lactis*), at 40°C. For hybridization, 40 µL of a double strength hybridization buffer (40% formamide, 40 mM Tris HCl, 1800 mM NaCl, and 0.02% SDS) with 200 ng/µL of the specific, fluorescently labeled oligonucleotide probe was added to 40 µL of the lysozyme-treated sample. Hybridization was carried out in a heating block at 40°C for 90 min. In experiments involving a multi-species blend, the hybridization buffer was prepared with 200 ng/µL of each required fluorescently labeled oligonucleotide probe. Blend was treated with 400,000 Units/mL of lysozyme for 15 min at 40°C. For analyzing the commercial product, the rehydrated product was processed with the same lysozyme treatment as the lyophilized strains for *L. rhamnosus* and *B. lactis*, and with 83,000 units/mL for 1 min at 40°C for *Lactobacillus acidophilus*.

In this study, fluorescent dye was consistently attached to the 5′ end of each oligonucleotide probe. In single species experiments, the respective specific probe was labeled with the fluorescent dye 6-Carboxyfluorescein (6-FAM). To detect multiple species within a blend, the EUB338 probe (Amann et al., 1990b) was used for total bacterial count, and labeled with 6-FAM. For specific detection, *L. rhamnosus* probe was labeled with DY-415, *L. plantarum* specific probe was labeled with Cy3, and *B. lactis* specific probe was labeled with DY-631, allowing for the differentiation and quantification of these species in mixed cultures based on their unique 16S rRNA signatures (Table 1).

To mitigate non-specific signals arising from potential unspecific oligonucleotide binding, 40 µL of triple strength washing buffer was added post-hybridization. Washing buffer (60 mM Tris HCl, 645 mM NaCl, and 15 mM EDTA) contained 300 ng/µL oligonucleotide quencher probes complementary to the specific probes used for hybridization, linked with a corresponding quencher at the 3′ end. Washing was carried out in a heating block at 40°C for 15 min. For 6-FAM, BMN-Q535 was used as a quencher, for DY-415, BMN-Q460, and for Cy3 and DY-631, BMN-Q620 (Table 1).

To evaluate the linearity of the Flow-FISH method, a rehydrated sample was serially diluted three times in 10-fold steps. The undiluted sample and the four subsequent dilutions were then processed according to the protocol. Before being analyzed with the Cytek flow cytometer, the samples were further diluted to achieve an optimal event rate for measurement. Results were measured in "viable cells"/g (Figure 1).

2.5 Flow cytometer measurement

Flow cytometry measurements for this study were conducted using a Cytek Northern Lights flow cytometer (Cytek Biosciences Inc., Fremont, CA, United States), equipped with a three-laser system. The

TABLE 1 List of labeled oligonucleotides used in this study.

Probes	Sequences 5`-3`	Target organisms	16S/23S rRNA	Lysozyme treatment for Flow-FISH	Probe sequence references
EUB338	GCT GCC TCC CGT AGG AGT	All bacteria	16S	Dependent on the target organism	Amann et al. (1990b)
EUB338_Quencher	ACT CCT ACG GGA GGC AGC	–	–	–	This study
S-S-Lrham-1586-a-A-23	AGC ACC TTT CAA TAA TCA GAA CT	*Lacticaseibacillus rhamnosus*	16S	400.000 Units/mL, 30 min, 40°C	Goldberg et al. (2000)
S-S-Lrham-1586-a-A-23_Quencher	AGT TCT GAT TAT TGA AAG GTG CT	–	–	–	This study
Lbpla462	CCG TCA ATA CCT GAA CAG TTA C	*Lactiplantibacillus plantarum*	16S	833.000 Units/mL, 30 min, 40°C	This study
Lbpla462_Quencher	GTA ACT GTT CAG GTA TTG ACG G	–		–	This study
Biflac65	CAA GCT GCC AGG GAT CCC GT	*Bifidobacterium animalis* subsp. *lactis*	16S	833.000 Units/mL, 5 min, 40°C	This study
Biflac65_Quencher	ACG GGA TCC CTG GCA GCT TG	–		–	This study
Lbaci1872	TCG AAC CTT CGC TTT CGC	*Lactobacillus acidophilus*	23S	83.000 Units/mL, 1 min, 40°C	This study
Lbaci1872_Quencher	GCG AAA GCG AAG GTT CGA	–		–	This study

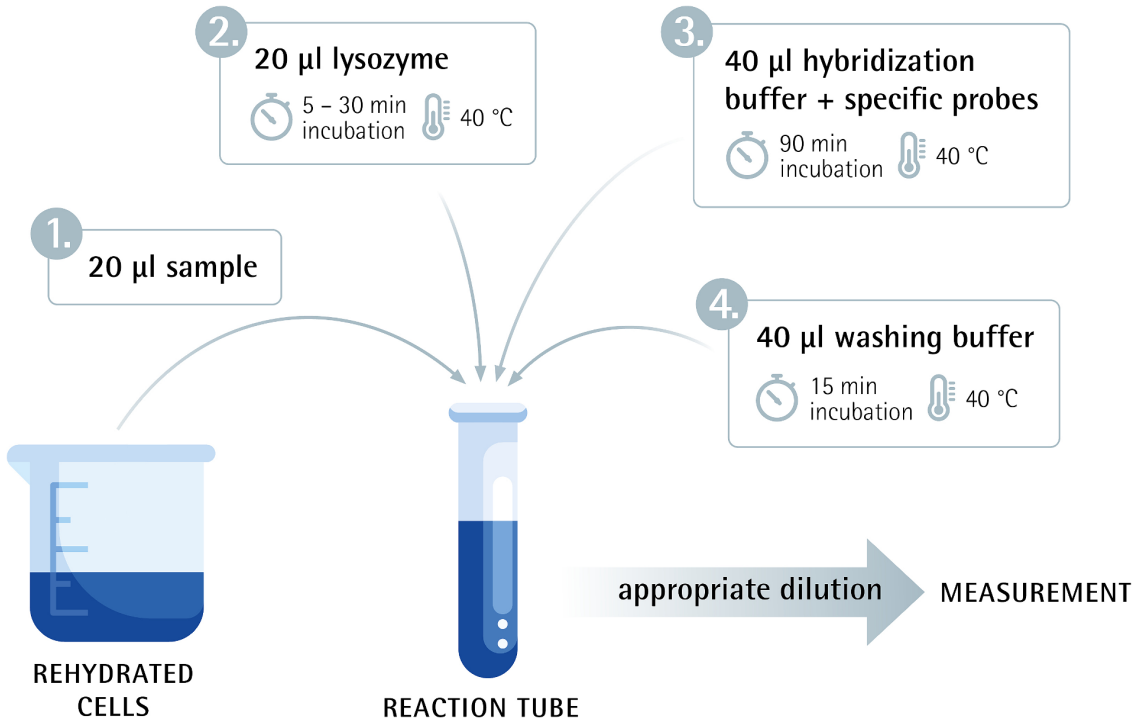

FIGURE 1
Flow-FISH method overview combining flow cytometry and fluorescence *in situ* hybridization.

cytometer's lasers configuration, featuring 405 nm (100 mW), 488 nm (50 mW), and 640 nm (80 mW) lasers. This setup facilitated the measurement of forward scatter (FSC) and side scatter (SSC) alongside fluorescence detection across a wide emission spectrum (420–829 nm) without the necessity for filter changes. Data analysis was performed using SpectroFlo® Software Version 3.2.1 (Cytek Biosciences, Inc.).

For L/D staining analysis, cells were discriminated from the background by gating on a positive SYTO 9 fluorescence signal. Discrimination between living, dead and damaged was achieved by correlation plots between SYTO 9 and PI intensity. Living cells were selected in an area with high SYTO 9 fluorescence, whereas dead cells were selected in a region of high PI fluorescence and lower SYTO 9

fluorescence. Damaged cells exhibit intermediate levels of both SYTO 9 and PI fluorescence. Flow rate was maintained at 30 µL/min.

For Flow-FISH analysis, cells were discriminated from the background by gating on a positive 6-FAM signal for single species analysis. For analyzing the multi-species blend, cells were quantified by gating on a positive signal of the respective fluorescent dye of the specific probe or by gating on 6-FAM to quantify all viable cells. Flow rate was maintained at 30 µL/min.

Experiments to rule out device differences were additionally measured using the CyFlow Cube 6 (Sysmex, Görlitz, Deutschland), with a single 488 nm (50 mW) laser and 5 detectors: forward scatter (FSC), side scatter (SSC) and 3 fluorescence channels (FL1 536/40 nm, FL2 590/50 nm and FL3 RG630 nm). Data analysis was performed using the FCS Express software (*De Novo* Software, FCS Express V5.01.0082).

Quality controls were performed daily before the instruments were used according to the manufacturer's specifications.

2.6 Statistical analysis

Statistical analyses (Dunnett's test, Wilcox test, R^2) were performed using RStudio https://www.rstudio.com/ Posit team (2024). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL http://www.posit.com/.

3 Results

3.1 Comparison of methods

The accuracy of the Flow-FISH method was evaluated through comparison with Live/Dead (L/D) staining, and conventional plate count for the commonly used probiotic species *Lacticaseibacillus rhamnosus* SP1, *Lactiplantibacillus plantarum* 14D and *Bifidobacterium animalis* subsp. *lactis* BLC1. To compare results, viable cells, active fluorescence units (AFUs) and colony forming units (CFUs) were extrapolated to 1 g of lyophilizate and presented as mean ± standard deviation (SD).

In our comparison of the Flow-FISH method with L/D staining, we assessed the average of three rehydrated samples, each with five technical replicates, for each organism. For *L. rhamnosus*, the Flow-FISH-detected viable cells numbered at $4.79 \times 10^{11} \pm 1.42 \times 10^{10}$ per gram and were similar to the count of AFUs detected by L/D staining at $4.92 \times 10^{11} \pm 1.58 \times 10^{10}$ per gram, and lower than the total fluorescence units (TFUs) count from L/D staining, which was $5.58 \times 10^{11} \pm 1.23 \times 10^{10}$ (Figure 2).

A similar trend was observed for *L. plantarum* 14D, with the count of viable cells detected by Flow-FISH at $8.32 \times 10^{11} \pm 2.67 \times 10^{10}$, closely matching the AFUs detected by L/D staining at $8.35 \times 10^{11} \pm 3.78 \times 10^{10}$, and lower than the TFUs, which were $1.06 \times 10^{12} \pm 3.21 \times 10^{10}$ (Figure 3).

Analysis of *B. lactis* revealed a largely comparable observation, with the exception that the count of viable cells detected by Flow-FISH, at $4.18 \times 10^{11} \pm 1.60 \times 10^{10}$, was higher than the number of AFUs by L/D staining, which was $3.64 \times 10^{11} \pm 1.41 \times 10^{10}$, and lower than TFUs of $5.31 \times 10^{11} \pm 1.02 \times 10^{10}$ (Figure 4).

The CFUs determined by plate count, at $3.55 \times 10^{11} \pm 6.85 \times 10^{10}$ for *L. rhamnosus*, $1.21 \times 10^{11} \pm 6.00 \times 10^{9}$ for *L. plantarum* 14D, and $1.15 \times 10^{11} \pm 1.14 \times 10^{10}$ for *B. lactis*, were lower than those determined by the other two methods. CFUs were determined by the mean value of three rehydrated samples, from which two dilution series were plated.

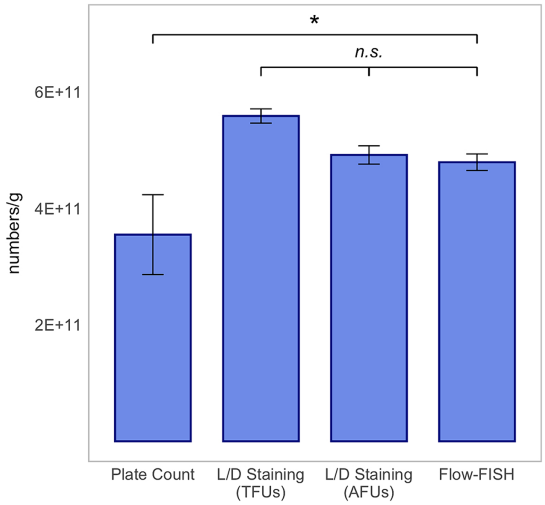

FIGURE 2
Method comparison for *Lacticaseibacillus rhamnosus* SP1. This analysis includes colony forming units (CFUs) derived from plate count, total fluorescence units (TFUs) and active fluorescence units (AFUs) from L/D staining, and viable cell counts as determined by the Flow-FISH method. Displayed data include the mean values and standard deviations (SDs) for each method. Statistical differences between the Flow-FISH method and the other techniques were assessed using Dunnett's test ($n = 3$). n.s., not significant, $p > 0.05$ and * = $p < 0.05$.

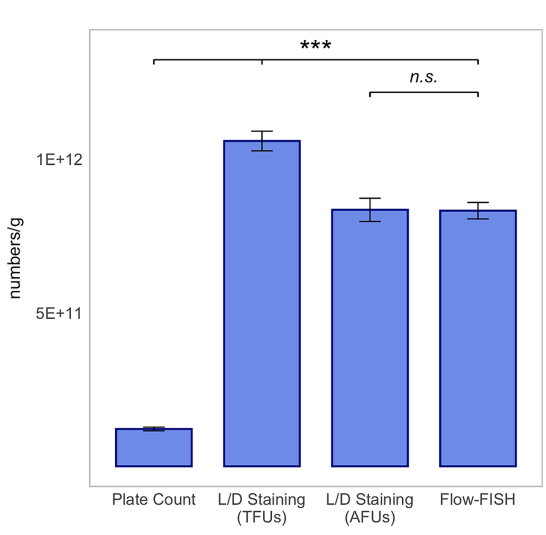

FIGURE 3
Method comparison for *Lactiplantibacillus plantarum* 14D. This analysis includes colony forming units (CFUs) derived from plate count, total fluorescence units (TFUs), and active fluorescence units (AFUs) from L/D staining, and viable cell counts as determined by the Flow-FISH method. Displayed data include the mean values and Standard Deviations (SDs) for each method. Statistical differences between the Flow-FISH method and the other techniques were assessed using Dunnett's test ($n = 3$). n.s., not significant, $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

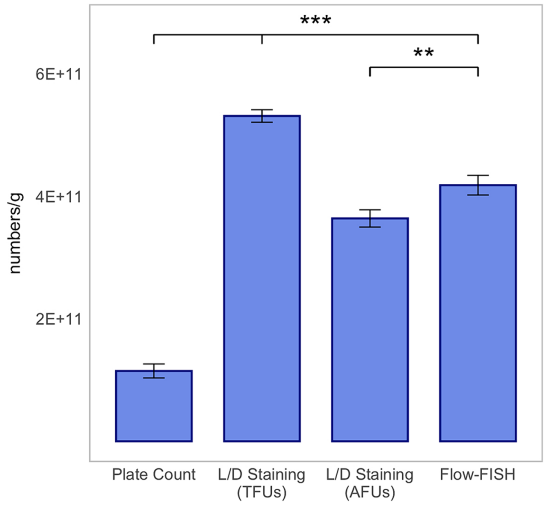

FIGURE 4
Method comparison for *Bifidobacterium animalis* subsp. *lactis* BLC1. This analysis includes colony forming units (CFUs) derived from plate count, total fluorescence units (TFUs), and active fluorescence units (AFUs) from L/D staining, and viable cell counts as determined by the Flow-FISH method. Displayed data include the mean values and standard deviations (SDs) for each method. Statistical differences between the Flow-FISH method and the other techniques were assessed using Dunnett's test ($n = 3$). n.s., not significant, $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

In summary, the count of viable cells detected by Flow-FISH aligned with the counts determined by L/D staining. Plate count quantified merely 74, 28% or 15% of CFUs compared to Flow-FISH (Figures 2–4).

3.2 Precision/repeatability

According to the definition of probiotics as: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host," the actual amount of viable cells in the probiotic product is relevant (Hill et al., 2014). Consequently, both manufacturers and consumers need to have a trustworthy method for enumeration that ensures reliable examination of probiotics by demonstrating high repeatability.

To assess the precision, i.e., repeatability, of the different methods, the Flow-FISH and L/D staining procedures were conducted three times with five independently diluted and measured technical replicates. For plate count, two dilution series were prepared, and two different dilutions were plated. The measure of repeatability chosen was the relative standard deviation (RSD).

For Flow-FISH, RSD among technical replicates ranged from 3.13 to 6.91% for *L. rhamnosus*, 2.71 to 6.48% for *L. plantarum* 14D and 5.90 to 6.98% for *B. lactis*. In case of L/D staining, TFUs count showed an RSD from 1.23 to 3.62% for *L. rhamnosus*, 5.14% to 6.80% for *L. plantarum* 14D and 2.79 to 4.69% for *B. lactis*. The RSD for AFUs was between 0.82 and 3.57% for *L. rhamnosus*, 5.83 to 6.48% for *L. plantarum* 14D, and 2.66 and 5.65% for *B. lactis*. The plate count method exhibited quite high RSD values, ranging from 8.56 to 31.07% for *L. rhamnosus*, 4.62 to 17.76% for *L. plantarum* 14D, and 9.97 to 21.97% for *B. lactis*.

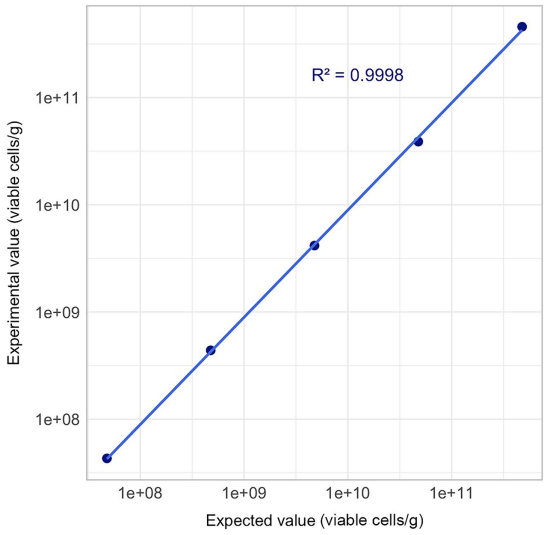

FIGURE 5
Linearity assessment of Flow-FISH method. Mean quantification results derived from three technical replicates are plotted across a five-point concentration gradient, extending from 4.79×10^7 to 4.79×10^{11} viable cells/g. Acceptance limit: $R^2 \geq 0.95$.

In conclusion, the molecular methods, Flow-FISH and L/D staining, demonstrate better repeatability and lower measurement uncertainty compared to the conventional gold standard, the plate count method, within the context of this study.

3.3 Linearity

Cell concentrations differ among probiotic products and across various stages of the manufacturing process. Consequently, a method's capability to accurately analyze different cell concentrations is essential. Accordingly, a linearity analysis of the Flow-FISH method was performed in this study to address this requirement.

Five different concentrations of viable cells per gram were evaluated. The rehydrated *L. rhamnosus*, characterized by a concentration of 4.79×10^{11} viable cells per gram as determined through comparative Flow-FISH method experiments previously described, underwent a series of 10-fold serial dilutions in triplicate. This procedure was meticulously conducted until the concentration achieved the theoretical target of 4.79×10^7 viable cells per gram.

Each dilution step was diluted and measured in triplicates, and mean values were used for linearity evaluation.

Linearity was confirmed with an R^2 value of 0.9998 ($R^2 \geq 0.95$), thereby validating the Flow-FISH method within a range between 10^7 and 10^{11} (Figure 5).

3.4 Specific enumeration of strains in a multi-species probiotic blend

Probiotic products are typically composed of multiple species, making the Flow-FISH method, with its capability to enumerate each species specifically, highly advantageous. To illustrate the

effectiveness of the Flow-FISH method, a mix containing equal amounts of *L. rhamnosus*, *L. plantarum* LP-115, and *B. lactis* lyophilizates was analyzed. Specific oligonucleotide probes were used for each species, each linked to a distinct fluorescent dye (see Materials and Methods). Additionally, the EUB338 probe (Amann et al., 1990b), universally binding to organisms of the kingdom *Bacteria*, enabled the determination of the total viable bacterial count.

Initially, the instruments' capability to differentiate between the chosen fluorescent dyes was validated through a similarity test.

The mixed blend was then processed using the Flow-FISH method and hybridized with a mixture of specific oligonucleotide probes. Each species targeted by a specific probe formed a population of viable cells that could easily be distinguished from background noise and other labeled cells in the sample (Figure 6).

Analysis via the EUB338 probe indicated the presence of 4.54×10^{11} viable cells per gram in the mixture. The breakdown of species-specific counts revealed 1.72×10^{11} viable cells for *L. rhamnosus*, 1.25×10^{11} viable cells for *B. lactis*, and 1.83×10^{11} viable cells for *L. plantarum* LP-115 per gram. The discrepancy of 5.31%

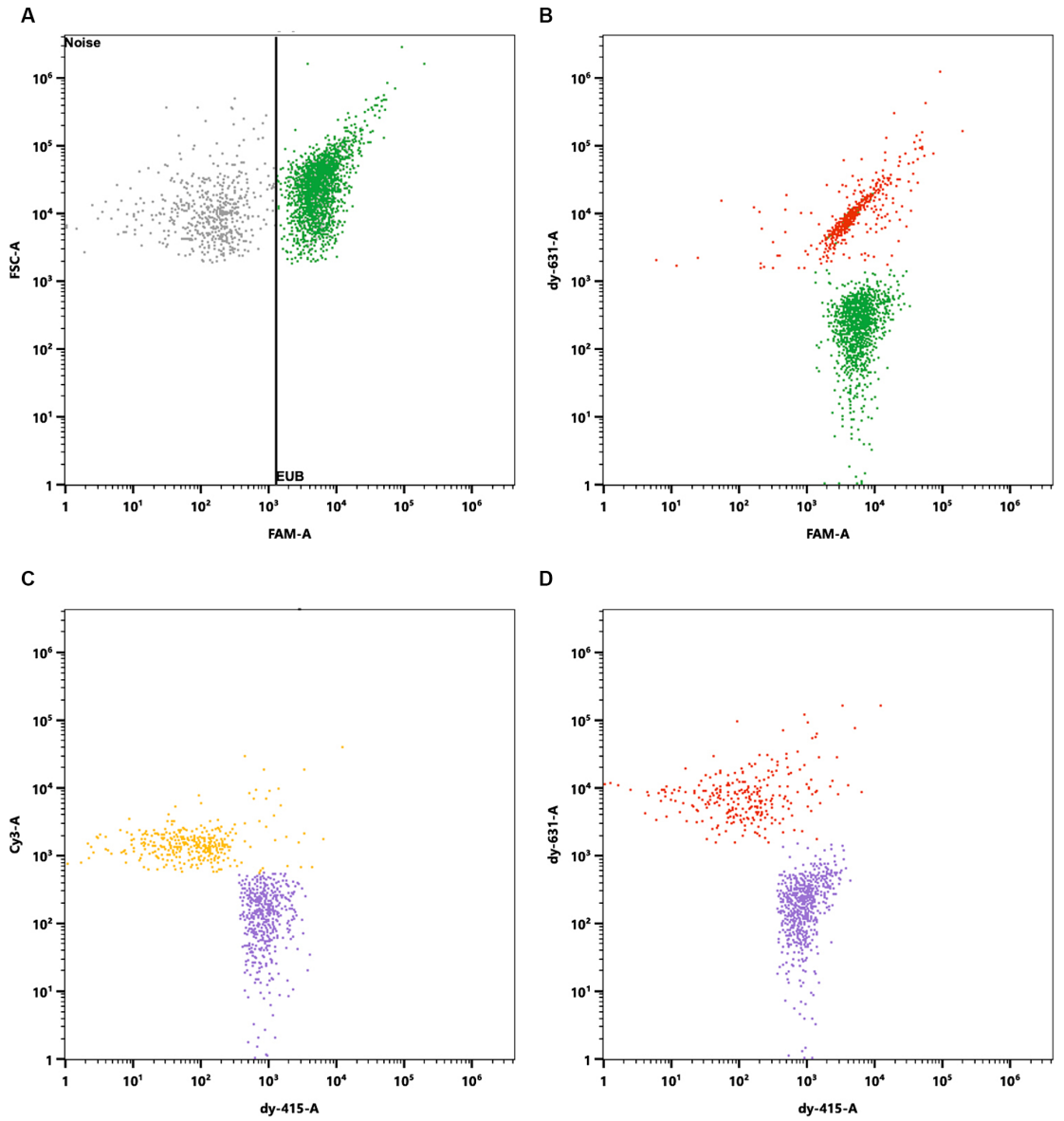

FIGURE 6
Specific enumeration of bacterial strains in a multi-species probiotic blend via flow cytometry coupled with fluorescence *in situ* hybridization (Flow-FISH). A universal bacterial probe, EUB338 (Amann et al., 1990b), tagged with 6-FAM, facilitated enumeration of total viable bacteria. Specific detection employed probes labeled with distinct fluorophores: *Lacticaseibacillus rhamnosus* SP1 with DY-415, *Lactiplantibacillus plantarum* LP-115 with Cy3, and *Bifidobacterium animalis* subsp. *lactis* with DY-631. **(A)** Signals were plotted against FAM and Forward Scatter (FSC) to distinguish all cells targeted by EUB338 (in green) from background noise. Cells positive for EUB338 were further gated to analyze the distinct strains within the blend. **(B)** FAM signals plotted against DY-631 to separate all cells (green) from *B. lactis* (red), which showed both FAM and DY-631 signals. **(C)** Cy3 signals plotted against DY-415 to distinguish *L. plantarum* (yellow) from *L. rhamnosus* (purple). **(D)** DY-631 signals plotted against DY-415 to differentiate *B. lactis* (red) from *L. rhamnosus* (in purple).

between the total cell count and the cumulative species-specific count is attributed to the methodological uncertainty, validating the species-specific detection capability of Flow-FISH.

3.5 Robustness: device comparison

For the validation of the Flow-FISH method destined for quantification of probiotic products, robustness, particularly its independence from specific equipment, is necessary.

Therefore, five technical replicates of rehydrated *L. rhamnosus* were measured using two different flow cytometers: Cytek Northern Lights and Sysmex CyFlow Cube 6. Results are presented as mean ± SD.

The same dilution was measured on each instrument. With the Cytek Northern Light measuring an extrapolated average of $4.36 \times 10^{11} \pm 1.38 \times 10^{10}$ viable cells/g and the Sysmex CyFlow Cube 6 measuring $4.60 \times 10^{11} \pm 2.54 \times 10^{10}$ viable cells/g, there was no significant difference between the results of the two instruments (Figure 7). This proves that the Flow-FISH method is not dependent on the device used.

3.6 Analysis of a commercial end product

Since bacterial strains undergo often various stressors during their processing into probiotic end products (Fenster et al., 2019; Kiepś and Dembczyński, 2022), it was necessary to demonstrate that the Flow-FISH protocol is also effective when analyzing a commercial end product. Therefore, the "IberoBiotics Pro" (Bayer Vital GmbH, Leverkusen, Germany) was analyzed using the Flow-FISH method and L/D staining for comparison. According to the manufacturer, it contains 6×10^9 CFUs per capsule, with each capsule containing approximately 300 mg of powder. The viable cells determined by Flow-FISH were $2.28 \times 10^{11} \pm 1.39 \times 10^{10}$ per gram, which was comparable to the AFUs detected by L/D staining at $2.24 \times 10^{11} \pm 1.22 \times 10^{10}$ per gram. The TFUs were higher, with a total count of $2.86 \times 10^{11} \pm 1.70 \times 10^{10}$ per gram. In detail, the viable cell count determined by Flow-FISH for *L. rhamnosus* was $9.16 \times 10^{10} \pm 6.80 \times 10^9$, for *L. acidophilus* was $6.13 \times 10^{10} \pm 4.35 \times 10^9$, and for *B. lactis* was $7.46 \times 10^{10} \pm 3.99 \times 10^9$ per gram (Figure 8).

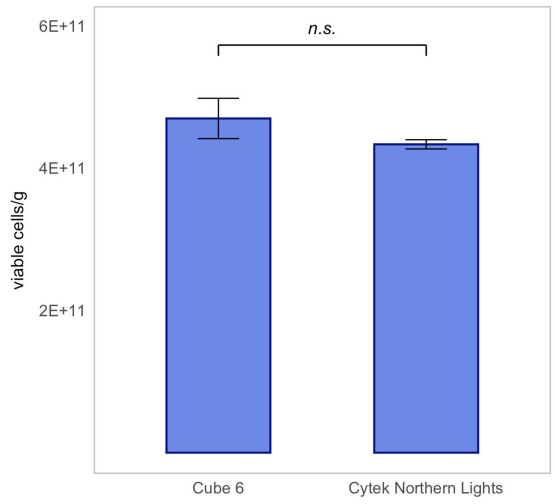

FIGURE 7
Device-independent validation of Flow-FISH measurements for *Lacticaseibacillus rhamnosus* SP1. Results obtained from lyophilized bacterial samples quantified on two different flow cytometers, illustrating the method's consistency across instruments, were compared. Presented are the mean values and their corresponding standard deviations (SD). The Wilcoxon test was applied to assess statistical differences between the devices ($n = 5$). n.s., not significant, $p > 0.05$.

4 Discussion

In this study, it was demonstrated that the optimized Flow-FISH method for gram-positive probiotic species represents a rapid, robust, and easily implementable technique. We compared the optimized Flow-FISH method with Live/Dead (L/D) flow cytometry and with the conventional plate count technology.

The Flow-FISH method showed that it yields results comparable to those of the established L/D staining but is additionally capable of specifically quantifying viable single species in multi-species blends. Compared to the gold standard plate count, the Flow-FISH method and likewise the L/D staining flow cytometry revealed higher and more precise results.

4.1 Comparison Flow-FISH vs. Live/Dead staining

An established method for enumerating probiotics is the L/D staining. It differentiates living from dead cells based on the integrity of the cell wall, performing under the assumption that non-viable cells lack an intact one. Fluorescent nucleic acid dyes are used, which have different capabilities for cell penetration depending on membrane integrity (Díaz et al., 2010; ISO 19344:2015). In contrast to the plate count method, it is significantly faster and easier to execute, as it does not require consideration of the various growth conditions of the cells. Moreover, cells are analyzed based on the criterion of cell permeability rather than their ability to grow, which leads to a more accurate count of viable cells (Lahtinen et al., 2005; Foglia et al., 2020; Visciglia et al., 2022). However, strict adherence to the staining times of the cells with the dye requires effective time management in the laboratory. This may result in periods of personal inactivity because the precision required during these stages prevents the concurrent performance of other tasks.

In our study, L/D staining effectively quantified living cells, yielding counts similar to those obtained via the Flow-FISH method. This congruence occurred despite the distinct assumptions underlying each technique regarding cell viability. Our results are in accordance with those of Lahtinen et al. (2008), who demonstrated similar findings when comparing cell membrane permeability to 16S rRNA content.

Given that probiotic cells are not only exposed to lyophilization as a stressor but also to thermal, osmotic, and oxidative stress factors, this may lead to variations in lysozyme stability and increased susceptibility (Fenster et al., 2019; Kiepś and Dembczyński, 2022). In this study, we demonstrated that the advanced Flow-FISH protocol is applicable not only to lyophilized probiotic gram-positive bacteria but also to a commercial end product, showing a

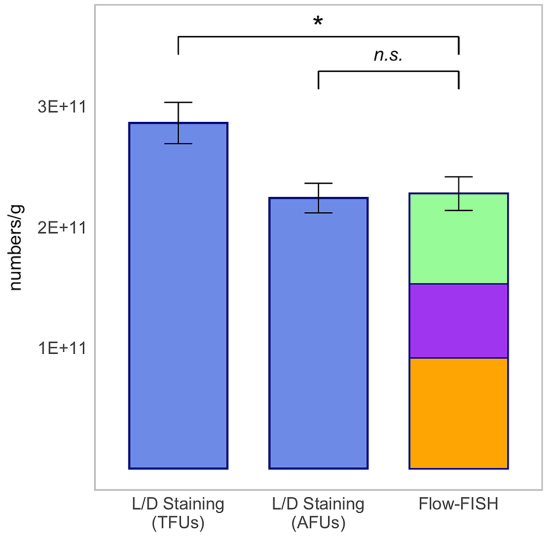

FIGURE 8
Method comparison for the commercial product "IberoBiotics Pro" (Lot number: 69974, expiry date: February 2025; Bayer Vital GmbH, Leverkusen, Germany). This analysis includes total fluorescence units (TFUs) and active fluorescence units (AFUs) derived from L/D staining, and viable cell counts as determined by the Flow-FISH method. Displayed data include the mean values and standard deviations (SDs) for each method. The Flow-FISH bar is color-coded to represent the mean values of individual species detected: *Lacticaseibacillus rhamnosus* in orange, *Lactobacillus acidophilus* in purple, and *Bifidobacterium animalis* subsp. *lactis* in green. Statistical differences between the Flow-FISH method and the other techniques were assessed using Dunnett's test ($n = 3$). n.s., not significant, $p > 0.05$ and * = $p < 0.05$.

comparable number of viable cells according to Flow-FISH and AFUs as determined by L/D staining, thereby confirming the reliability of the method without requiring adjustments in lysozyme concentration.

Flow-FISH, similar to L/D staining, can also determine the ratio of live to dead cells by employing a cell wall-permeable DNA dye that penetrates all cells, irrespective of their viability status. This approach enables the quantification of living cells relative to the total cell count, offering a comprehensive assessment of cell viability within a sample.

4.2 Comparison Flow-FISH vs. plate count

FISH accurately detects cells by using fluorescent probes that bind to specific rRNA sequences, forming stable RNA–DNA hybrids (Amann et al., 1990a). This method's sensitivity is greatly enhanced by the high number of rRNA molecules present in viable cells, which ranges from a few hundred to 100,000 per cell, integral to ribosomal function (Amann and Fuchs, 2008). The abundant rRNA, when labeled with these probes, produces a strong cumulative fluorescence signal upon excitation with high-energy light. This signal is detectable, enabling precise cell identification and localization based on rRNA expression (Amann et al., 1990a).

In contrast, plate count measures colony forming units (CFUs). These numbers are often underestimated because the indirect nature of the method does not guarantee that a colony derives from one single cell (Davis, 2014; Pereira et al., 2023). Moreover, in blends, which represent the majority of probiotic products, different species may inhibit each other and thus interfere with growth, leading to an underestimation and not reflecting the actual quantitative composition of the product (Avonts et al., 2004; Sielatycka et al., 2021).

Additionally, the plate count method is impractical for manufacturers' quality control, as it can involve long incubation steps that extend over several days. Also, plate count is laborious since it cannot be uniformly applied to all probiotic organisms, as distinct growth conditions, such as optimal temperatures, atmospheric oxygen levels, and particular nutrients are required for different species. Moreover, optimal growth conditions remain unknown for many microorganisms. Furthermore, this methodology lacks the ability to differentiate between closely related organisms and there is no guarantee that a colony results from a single cell rather than from an aggregate or chain of cells (Lahtinen et al., 2006a; Davis, 2014; Jackson et al., 2019; Vinderola et al., 2019).

Moreover, due to stress during manufacturing and storage, cells in probiotic products often enter the so-called viable but non-culturable (VBNC) state, where they are metabolically active and contain still high levels of rRNA, but might be not capable of replication (Bao et al., 2023). Nevertheless, they may have probiotic properties (Breeuwer and Abee, 2000; Lahtinen et al., 2006b, 2008; Fiore et al., 2020; Wendel, 2022).

For these reasons, plate count is nowadays considered more of an estimate than an actual quantification of viable cells (Davey, 2011; Boyte et al., 2023).

Therefore, the combination of flow cytometry with FISH, the so-called Flow-FISH method, offers a faster, more accurate and specific alternative. The results of this study, show more viable cells detected by Flow-FISH as well as with L/D staining compared to CFUs, indicate that Flow-FISH offers a more accurate representation compared to plate count. This is further supported by the fact that the Flow-FISH method is significantly more reproducible than plate count, as could be shown by our data, highlighting the issues with cultivation methods.

4.3 Advanced Flow-FISH protocol

The Flow-FISH protocol developed in this study presents significant advantages over existing protocols for analyzing gram-positive bacteria, addressing a longstanding challenge in the field. The concept of merging flow cytometry and FISH originated around three decades ago (Amann et al., 1990a; Wallner et al., 1993; Snaidr et al., 1999), and various probiotic and fecal samples have been analyzed using this combination (Rigottier-Gois et al., 2003; Rochet et al., 2004; Vaahtovuo et al., 2005; Dinoto et al., 2006; Collado and Sanz, 2007; Cleusix et al., 2010). However, such studies often proved to be challenging, especially because probiotics are typically lactic acid bacteria, which are gram-positive (Williams, 2010). The complexity arises from their cell wall structure, particularly the thicker peptidoglycan layer, which impedes the penetration of labeled oligonucleotide probes (Chapot-Chartier and Kulakauskas, 2014). Enzymatic treatments are mostly used to overcome these challenges and enable the effective diffusion of the probes into ethanol or paraformaldehyde fixed cells (Beimfohr et al., 1993). In our protocol, we combined highly concentrated lysozyme treatment of unfixed cells with a quenching step.

Therefore, previous FISH/flow cytometric protocols were not only time-consuming, requiring hybridization times of more than ten hours, but also labour-intensive because of necessary centrifugation steps.

The protocol optimized in this study has significantly reduced the hybridization time to just 1.5 h, resulting in a total protocol duration of merely 2 to 2.5 h. At the same time, it decreased the workload and minimized the risk of cell loss by eliminating the need for centrifugation steps. The required signal-to-noise ratio was achieved through a final dilution series and the inclusion of complementary quenchers in the washing buffer. These quenchers, designed to be complementary to the oligonucleotide probes, bind to any unbound probes, effectively suppressing their free signal and enhancing the specificity and clarity of the detection (Beimfohr et al., 2010).

In summary, the optimized Flow-FISH method protocol is effective for the reliable enumeration of probiotics, yielding results comparable in quantification and precision to the established L/D staining method. Moreover, due to its optimization, the protocol is user-friendly, necessitating minimal handling time, with a total duration, including incubation steps, of 2 to a maximum of 2.5 h.

In addition to its speed and practicality, the Flow-FISH method is also a robust technique. This study demonstrated that there is no significant difference in the results when technical replicates of the same sample are measured on different devices, indicating that the method's accuracy and reliability are independent of the specific device used. Furthermore, it was shown that cells can be reliably quantified across a wide range, from approximately 5×10^7 to 5×10^{11} cells per gram, demonstrating the method's broad applicability for analyzing samples with vastly different cell densities. Given that this range of cell counts is typical in both the manufacturing process and the final probiotic product, the Flow-FISH method proves suitable for quality control at in-process and end-process stages. This adaptability ensures accurate monitoring and validation of probiotic concentrations, which is crucial for maintaining product efficacy and regulatory compliance. Moreover, unlike L/D staining, the Flow-FISH method does not require immediate measurement of samples after staining. Our findings indicate that a delay between staining and measurement does not alter the results (data not shown), providing greater flexibility in daily laboratory operations. This characteristic enhances workflow efficiency, allowing for better planning and resource allocation without compromising the accuracy of the quantification.

Recently, there has been an increase in the prevalence of probiotic products containing spore-forming bacteria (Elshaghabee et al., 2017). However, since the majority of probiotic products on the market are composed of lactic acid bacteria, which are not spore formers (Chapot-Chartier and Kulakauskas, 2014), the protocol is optimized for lyophilized products without special consideration for spores. Spore-forming bacteria are particularly resilient due to their ability to form endospores (Elisashvili et al., 2019). Endospores possess a thick cell wall, composed of multiple layers including the cortex and spore coat, which provides substantial protection against environmental stresses but also makes them less accessible to FISH oligonucleotide probes (Filion et al., 2009). According to Chambon et al. (1968), endospores contain comparable rRNA content to vegetative cells, and Filion et al. (2009) successfully stained *Bacillus* spores with FISH using an optimized procedure to effectively penetrate the spores. For the specific detection of spores, the Flow-FISH protocol would require modifications. As spore detection was not within the scope of this study the Flow-FISH protocol described is optimized for the current market situation. It should also be noted that the Flow-FISH method was specifically developed for the reliable enumeration of probiotic viable cells and is not intended for the identification or control of contaminants.

In summary, our refined Flow-FISH protocol offers significant improvements over earlier methods that integrate FISH with flow cytometry. We chose not to use ethanol or paraformaldehyde for bacterial fixation (Manz et al., 1992), achieving cell wall permeability but high lysozyme and fluorescently labeled oligonucleotide probes concentrations instead. Through meticulous dilution of the hybridized sample and the use of quencher probes, our approach ensures precise and reliable outcomes. By reassessing the foundational principles of FISH, we have developed a method that enables the robust, rapid and specific identification of gram-positive probiotic bacteria of the genera *Lactobacillus* and *Bifidobacterium* through a synergistic combination with flow cytometry.

4.4 Analysis of multi-species probiotic blends

Probiotic products typically contain a mixture of different species, each with varying survival rates within lyophilized products (Drago et al., 2004). Given this variability, it is inadequate to merely calculate the initially added proportion of each species relative to the total number of living cells. A significant advantage of the Flow-FISH method over L/D staining and the plate count method lies in its specificity (Jackson et al., 2002). This specificity is crucial for accurately quantifying the individual species within a probiotic blend, ensuring the product's efficacy. Flow-FISH combines this specificity with the measurement of viable cells, offering a distinct advantage in the precise quantification of individual species within probiotic blends.

In the experiment conducted in this study, which aimed at specific enumeration within a multi-species blend, it was demonstrated that the Flow-FISH method is capable of identifying not only the total count of living bacterial cells but also the proportions of the three different species, *L. rhamnosus*, *L. plantarum*, and *B. lactis*.

Using different fluorescent labels linked to the specific oligonucleotides, the three species, as well as the total population of all living cells, could be detected within a single hybridization and measurement step.

This method enables a highly efficient process, where just a single analysis of the sample is sufficient to determine the total count of all living cells, their percentage share, and the precise quantification of each individual species. Given that gram-positive bacteria do not all require the same lysozyme treatment for effective probe penetration (Beimfohr et al., 1993), variations in the species blend may necessitate conducting more than one analysis. By categorizing species according to their similar treatment needs, a comprehensive evaluation of the probiotic blend can be achieved. This approach ensures that the unique cell wall characteristics of different gram-positive species are adequately addressed, allowing for accurate and effective quantification of each species within the blend, as shown by the analysis of the commercial product. Due to the specificity of the oligonucleotide probes, accurate quantification also remains feasible when different analytical approaches are used on the same sample.

This highlights the method's utility in accurately quantifying complex probiotic formulations.

4.5 Summary and outlook

This study has shown that the Flow-FISH method, refined with our protocol, excels in analyzing probiotic products. It outperforms both the L/D staining and traditional plate count methods by offering the combination of rapidity and specificity together with robustness and a better suitability for laboratory workflows.

Its proficiency in evaluating additional probiotic blends further establishes its utility for comprehensive quality control, making it an invaluable asset for both in-process and final product assessments, thereby ensuring product quality and efficacy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

LS: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. PM: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. CB: Writing – review & editing, Supervision, Conceptualization. CK: Writing – review & editing, Methodology. CR: Writing – review & editing. JS: Writing – review & editing, Supervision, Conceptualization.

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Conflict of interest

LS, PM, CB, CK, CR, and JS were employed by vermicon AG.

Publisher's note

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Proof of concept: real-time viability and metabolic profiling of probiotics with isothermal microcalorimetry

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Isothermal microcalorimetry (IMC) is a potent analytical method for the real-time assessment of microbial metabolic activity, which serves as an indicator of microbial viability. This approach is highly relevant to the fields of probiotics and Live Biotherapeutic Products (LBPs), offering insights into microbial viability and growth kinetics. One important characteristic of IMC is its ability to measure microbial metabolic activity separately from cellular enumeration. This is particularly useful in situations where continuous tracking of bacterial activity is challenging. The focus on metabolic activity significantly benefits both probiotic research and industrial microbiology applications. IMC's versatility in handling different media matrices allows for the implementation of viability assessments under conditions that mirror those found in various industrial environments or biological models. In our study, we provide a proof of concept for the application of IMC in determining viability and growth dynamics and their correlation with bacterial count in probiotic organisms. Our findings reinforce the potential of IMC as a key method for process enhancement and accurate strain characterization within the probiotic sector. This supports the broader objective of refining the systematic approach and methods used during the development process, thereby providing detailed insights into probiotics and LBPs.

KEYWORDS

viability assessment, viability enumeration, metabolic activity, beneficial microbes, probiotics, real-time, isothermal microcalorimetry

1 Introduction

In recent years, the precise assessment and quantification of microbial viability has become increasingly important, especially in the microbial biotechnology sector. This sector encompasses various activities such as research and development, product formulation, and quality control of probiotics and Live Biotherapeutic Products (LBPs). Although considered the gold standard for viability assessment, traditional methods such as plate count enumeration present significant drawbacks. These methods are laborious

and time-consuming, requiring incubation times that can extend from 2 to 5 days, depending on the microbial strain and the sample matrix (Fredua-Agyeman and Gaisford, 2015). Moreover, plate counts are susceptible to intrinsic variability, often within a range of 20–30%, which can significantly impact the accuracy of results (Jongenburger et al., 2010; Hansen et al., 2018). An additional concern is that certain microorganisms, despite being metabolically active, may fail to form colonies on standard agar plates, thus potentially leading to an underestimation of viable cell count (Staley and Konopka, 1985; Kell et al., 1998).Given these limitations, it has become evident that bacterial cultures exhibit heterogeneity, where viability is not strictly a function of the ability to replicate. The conventional plate count method may not effectively capture the entire spectrum of microbial activity, particularly for those cells termed "viable but non-culturable" (VBNC). This has driven the exploration of alternative methods that can provide a more comprehensive and nuanced picture of the microbial viability (Boyte et al., 2023).

For real-time monitoring of bacterial activity, pH monitoring has traditionally played an important role, especially as a complementary method for viability assessment. Acidification rates serve as a metric for real-time bacterial growth monitoring, particularly applicable for organic acid-producing organisms like lactic acid bacteria (LABs) (Visciglia et al., 2022).

Most commercially available probiotic strains trace their origins to the food and dairy industry and fall within the fermenting organic acid producers. The probiotics market is undergoing rapid growth, introducing new strains with beneficial probiotic properties that are used as Next Generation Probiotics (NGPs) or LBPs (O'Toole et al., 2017; Martín and Langella, 2019). Many of these strains are difficult to evaluate using conventional methods because they require strict anaerobic conditions or have proteolytic properties that interfere with methods like pH monitoring, optical density (OD) measurements, and plate counting (Wendel, 2021; Boyte et al., 2023). Two-step approaches, combining a less precise solution with a high-resolution method, offer a balance between economic constraints and higher throughput (Berninger et al., 2018). For instance, culture-based methods such as isothermal microcalorimetry (IMC) can serve as an initial screening step, enabling the evaluation of various formulations with minimal restrictions and higher throughput. This approach goes beyond a time or endpoint assay by providing kinetic information in addition to viability quantification, aiding in the selection of optimal culture conditions and formulations.

This study explores the complexities of microbial viability assessment, spotlighting the often overlooked technique of IMC (Braissant et al., 2015b; Garcia et al., 2017; Berninger et al., 2018; Nykyri et al., 2019). IMC monitors heat generation by a sample over time while maintaining a constant temperature (Figure 1). This heat originates primarily from the metabolic processes of microorganisms, which are essentially biochemical exothermic reactions. IMC is a highly sensitive method that captures even the slightest changes in heat produced by living organisms. The resulting heat flow curve (μW/s) directly reflects the metabolic rate of the microorganisms, making it an effective approach for viability assessment (Mihhalevski et al., 2011; Braissant et al., 2015a; Garcia et al., 2017; Fredua-Agyeman and Gaisford, 2019; Nykyri et al., 2019).

Relying on the total heat flow generated by the entire population in a sample, IMC is applicable to diverse sample types and media matrices. Notably, it remains unaffected by turbidity and viscosity and is insensitive to cell clumping (Braissant et al., 2010, 2015b). Despite the method's versatility and the valuable insights IMC can offer into microbial viability, the lack of case studies and the scarcity of comparative data have been hindering factors in implementing IMC in the probiotics industry.

This article introduces a novel method for viability assessment that decouples microbial cellular quantification from metabolic activity assessment using IMC. Emphasizing its wide applicability beyond cellular enumeration, this method includes assessing metabolic activity and growth under specific conditions by comparing it to complementary data from other methods, such as flow cytometry. IMC's versatility positions it as a valuable tool for developing new live bacteria products, growth media compositions, formulations, and stress tolerance.

2 Materials and methods

2.1 Microorganisms and culture conditions

Lactobacillus plantarum 299v (Probi AB) was utilized to evaluate method suitability, with inoculation commercial product powder formulation containing lyophilized cells and cryostocks of the isolated strain. Two inoculation methods were employed. First, the full capsule content of a commercial product (approximately 370 mg, claimed to contain at least 10 billion bacteria) was resuspended in 10 ml of peptone water (casein peptone 10 g/L, sodium chloride 5 g/L) and revived at room temperature for 30 min. Second, fresh colonies from de Man-Rogosa-Sharpe (MRS) agar plates were used to inoculate 2 ml of MRS (Millipore) in 15 ml plastic conical tubes. These tubes were capped and incubated overnight at 37°C and rotated at 180 r.p.m.

For the metabolic activity study using acidification kinetics, cytofluorimetric enumeration, and IMC in parallel, two strains, *Lacticaseibacillus rhamnosus* GG ATCC 53103 and *Limosilactobacillus fermentum* LF10 DSM 19187, both from the Probiotical SpA collection, were used. The probiotic strains were activated overnight at 37°C in MRS (Difco, BD, MD) broth and then sub-cultured, using at least two passages over the mid-log phase.

2.2 Microcalorimetric analysis for viability enumeration

A 10-fold dilution series of the bacterial suspensions of *L. plantarum* in peptone water was prepared. The isothermal microcalorimeter plate, equipped with titanium vials and plastic inserts, was prepared by adding 30 μl of the individual sample dilutions to 270 μl of sterile MRS broth. The vials were sealed and placed into a calScreener^{TM} isothermal microcalorimeter (Symcel AB, Sweden) in accordance with the manufacturer's guidelines. Kinetic heat flow was monitored using the calView 2.0 software (Symcel AB, 2023), which recorded heat flow curves over a period

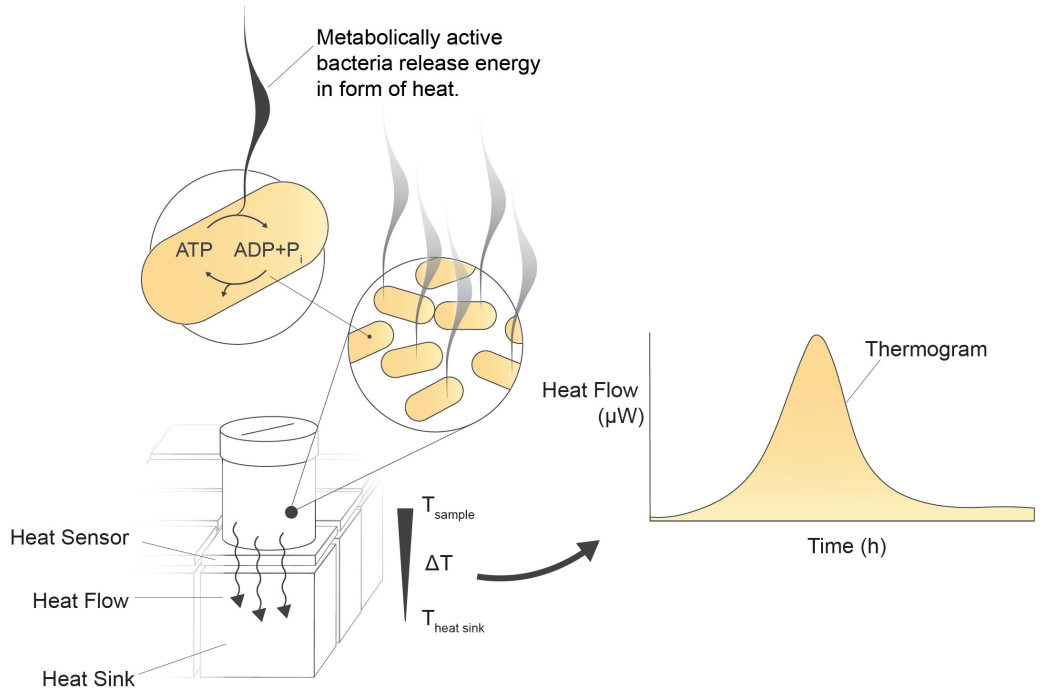

FIGURE 1
Isothermal microcalorimetry (biocalorimetry) principle. Isothermal incubation of a microbial culture in a vial positioned above a heat sensor on a heat sink. As metabolically active cells release energy, a byproduct of metabolic pathway reactions, the generated heat disperses over the heat sensor toward the heat sink. The heat sensor detects and converts these minute heat changes into an analog signal in μW. Heat flow is continuously measured every 2 s, enabling real-time monitoring of the sample's activity.

of 48 h. Various parameters were extracted using the online analysis tool calData.[1]

To assess the impact of turbid media viability counts, commercial plant-based milk (pasteurized oat drink, Oatly Barista Edition) was used. For culturing, 1:1 dilutions of MRS/oat drink were inoculated with serially diluted *L. plantarum* overnight culture in MRS, and 300 μl of the dilutions were added to vials for calorimetric measurements, as described above.

2.3 Viable counts using plate count assay

de Man-Rogosa-Sharpe agar plates were used to determine counts of viable colony-forming units (CFUs). Samples were serially diluted in peptone water in 10-fold steps. Of the diluted sample, 50 μl were spread on top of the agar of the plates, and subsequently incubated at 37°C for 48 h. Following incubation the colonies were counted and the viability value (CFU/ml) was calculated.

2.4 Cytofluorimetric counts

For strains GG and LF10, the BD Cell Viability Kit (BD Biosciences, Milan, Italy) was utilized to quantify viable cells (AFU/ml) and total cells (TFU/ml). Cell staining procedures were conducted in accordance with ISO 19344: IDF 232 (2015). Briefly, 100 μl of a diluted suspension containing approximately 10^5–10^6 cells/ml in buffered peptone water was mixed with 885 μl of PBS. Subsequently, 10 μl of propidium iodide (previously diluted in water at 0.2 mmol/L) and 5 μl of thiazole orange (42 μmol/L) were added to the dilution, followed by vortexing. The CytoFLEX cytometer (Beckman Coulter SRL), equipped with 488 nm laser excitation and CytExpert software, was employed for analysis. Thresholds for side scatter (SSC-H) and forward scatter (FSC-H) were established for microbial cells, which were gated using forward versus side scatter (FSC-H vs. SSC-H). The optimal discrimination between live and dead populations, used for the enumeration of AFU and TFU, was achieved on an FL1 versus FL3 plot. No counting beads were added to the diluted samples as internal standards as the instrument is designed for volumetric counting, whereby the concentration of events (cells) is determined based on the defined sample volume drawn by the needle.

2.5 Analysis of metabolic activity

The overnight cultures of GG and LF10 were inoculated in 30 ml of three growth media of different formulations (medium A, medium B, and medium C), with a final concentration of 10^7 AFU/ml.

For medium A, we employed MRS as the standard reference for the growth of Lactobacilli. Media B and C had two distinct formulations, each intentionally designed with differences in salt composition and primarily focusing on variations in carbon

1 https://symcel.com/analysis-tools/calorimetric-growth/

source concentration and nitrogenous source composition. The standardized cultures were placed at 37°C in a water bath for 24 h and pH variation during fermentation was measured at intervals of 4 min with the iCinac pH monitoring system (KPM Analytics, USA). At fixed time points (t = 0, 3, 5, 7, and 24 h) an aliquot of sample was taken and analyzed by CytofFLEX cytometer, as described above.

Simultaneously, microcalorimetric analysis was performed by adding 10^7 AFU in each vial provided with a plastic insert and 300 µl of the three different media. As described in section "2.2 Microcalorimetric analysis for viability enumeration," kinetic heat flow was monitored over a period of 48 h and heat flow curves were generated using the calView 2.0 software. Various parameters were extracted using the online analysis tool calData.

2.6 Statistical analysis for standard curve base viability enumeration

Standard curves were generated using both freshly prepared and freeze-dried cells. The initial cell concentration for fresh cells was approximately 3×10^9 CFU/ml. For rehydrated freeze-dried cells the concentration was approximately 10^{10} CFU/ml. The time to peak (TTP) values for each sample (liquid and freeze-dried) were calculated as a viability marker, and GraphPad Prism 10.0.2 (232) was employed for further analysis. The regression curves were constructed by plotting TTP against the logarithm of the cell concentration (CFU/ml). To analyze the reproducibility of IMC for viability assessments an unpaired two-tailed t-test was used in the same software.

3 Results

3.1 Assessing reproducibility of isothermal microcalorimetry for viability assessment

To examine the suitability of IMC for viability assessment and enumeration of probiotic products, we utilized a 48-channel isothermal microcalorimeter, the calScreener™, to test the reproducibility of the method.

In this assessment, a freeze-dried commercial probiotic product containing *L. plantarum* was diluted in fresh MRS medium and evenly distributed into 32 titanium vials. This plate was then introduced into the microcalorimeter, where over 24 h the instrument continuously monitored the heat flow from the samples and generated heat flow curves (thermograms). Notably, the readings from the individual sample vials gave consistent results, as indicated by the minimum SD (range 2.906–0.03 µW for the collected data points) of the thermograms. The same experiment was repeated on a different day, using a different instrument and a new batch of freeze-dried bacteria from the same product (Figure 2). An unpaired two-tailed t-test revealed no statistically significant difference [$t(582) = 0.0258$, $p = 0.9794$] between the two groups. This indicates that the observed difference in the means of the two thermograms is likely due to random chance and not a true underlying effect.

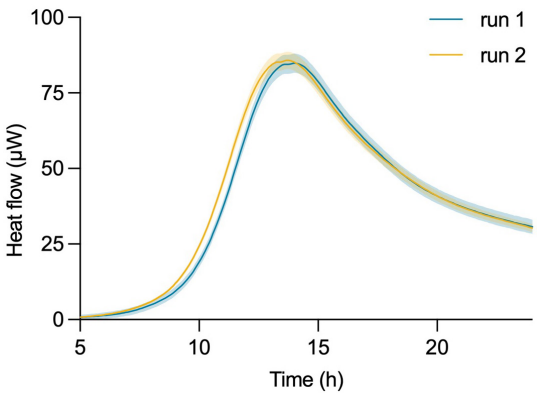

FIGURE 2
Robustness and reproducibility of isothermal microcalorimetric measurements using the calScreener™. Thermograms of two independent experiments on different days of *L. plantarum* started from independent batches of commercial probiotic product. Shading of each curve shows the SD of the 32 individual replicates per experiment.

3.2 Establishing a standard curve for viable cell enumeration

In our efforts to quantify the viability of *L. plantarum*, we conducted a series of experiments involving serial dilutions from overnight cultures, which were then inoculated into fresh MRS medium for isothermal calorimetric measurements at 37°C. The resulting thermograms consistently showed the thermal fingerprint of *L. plantarum* emerging at predicted intervals, confirming the method's suitability. There was a linear relationship between the initial inoculum size and the appearance of the thermograms (Figure 3A). We chose to analyze the data using the time to metabolic peak parameter to illustrate the principle. Time to signal detection, another calorimetric parameter extracted from the thermograms, exhibited a linear relationship with an initial number of microbial cells. Determining this parameter involved applying a threshold value set at a certain µW value to the data.

In parallel, the initial cell number was determined by plate count assay and a calibration curve was plotted. To create a standard curve for inoculum estimation, we applied a linear regression model to the data.

This approach allowed us to obtain a standard curve specific to strain under specified conditions, such as medium (here MRS), volume, and temperature (Figure 3B).

To further challenge the method's robustness, we investigated whether the linear relationship between inoculum size and TTP held true under different pre-culture and incubation conditions. We first used a dilution series of *L. plantarum* in a one-to-one MRS medium-oat drink mixture. The thermogram profiles remained consistent under these defined conditions, exhibiting the expected rightward shift with lower initial bacterial loads (Figure 4). Importantly, the strong linear relationship between inoculum size and TTP was maintained (Table 1).

Secondly, we explored the impact of pre-culture conditions by using three independently processed, freeze-dried samples. The standard curves generated from these samples closely resembled those obtained with the original liquid culture (Figure 5).

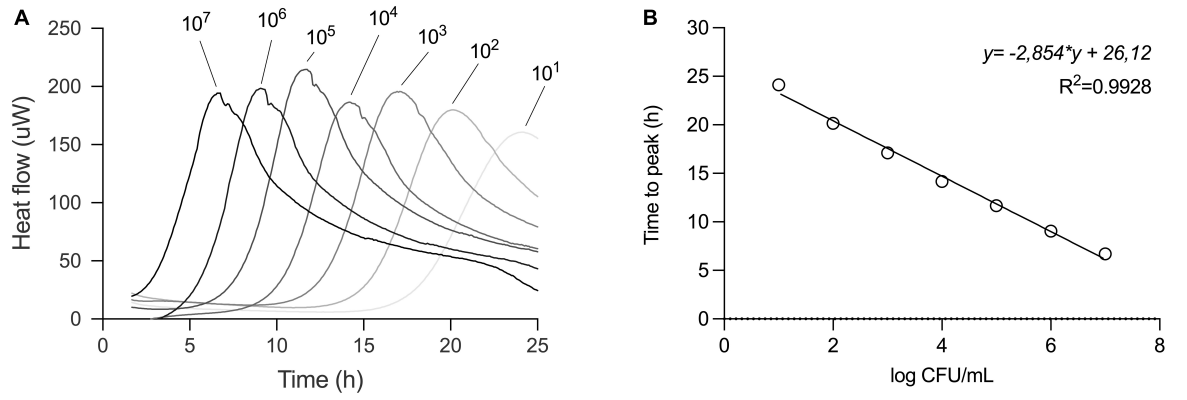

FIGURE 3
Serial dilution of *L. plantarum* to build a standard curve. **(A)** Thermograms of serial dilution of overnight culture of *L. plantarum* in MRS broth. Initial inoculum sizes are depicted above the thermograms. **(B)** For standard curve, the time to peak (TTP) of each dilution from the thermogram was determined and values were plotted against CFU/ml of respective dilution as confirmed by plate count. Relationship between TTP and CFU was described in a linear regression model.

As expected, the linear regression models for all three independent experiments again demonstrated a strong linear relationship between inoculum size and TTP (Table 1). To further solidify this finding, an F-test revealed no statistically significant difference between the slopes of the five linear regressions ($F = 1.569$, DFn = 4, DFd = 27, $P = 0.2111$), reinforcing the method's robustness across culture conditions.

Generalizing this standard curve across a broader range of strains and growth conditions may require further validation.

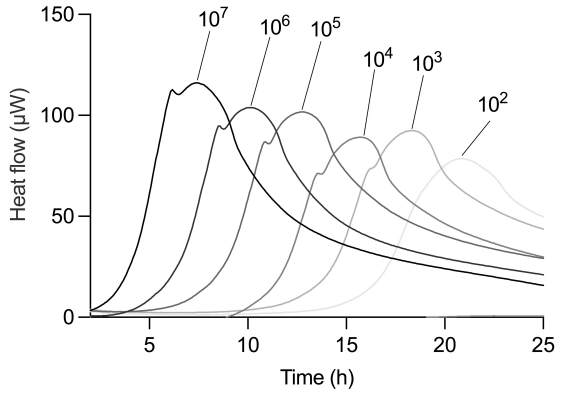

FIGURE 4
Correlation of TTP and CFU is universal independent from medium type. Metabolic activity of serial dilution of an overnight culture of *L. plantarum* in 1:1 MRS and oat drink. Dilution factors are indicated above thermograms.

TABLE 1 Summary of linear regression results of the five independent standard curve experiments.

L. plantarum suspension	Regression equation (*Y* value)	R^2
MRS broth		
Fresh culture	−2.854x + 26.12	0.9928
Freeze dried 1	−2.736x + 25.92	0.9977
Freeze dried 2	−2.722x + 26.05	0.9988
Freeze dried 3	−2.941x + 27.38	0.9900
MRS: oat drink		
Fresh culture	−2.702x + 26.33	0.9997

3.3 Beyond viability enumeration: IMC as a complementary approach

Empirical data involving various media formulations has shown that combining biomass quantification using time point measurements and metabolic activity measurements provide a comprehensive view of microbial activity and viability under diverse conditions. Especially during the substrate or media optimization process, it is relevant to consider various parameters.

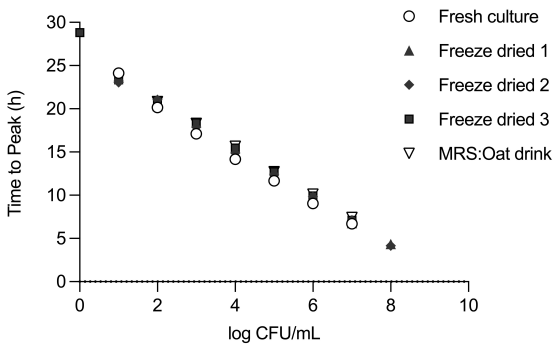

FIGURE 5
Reproducibility of standard curve under different pre-culture and incubation conditions of *L. plantarum*. Step-wise dilutions of *L. plantarum* (10^9 CFU/ml) overnight culture in MRS or a one-to-one MRS medium-oat drink mixture or step-wise dilution of reconstituted powder of commercial freeze-dried formulation of *L. plantarum* in MRS. Measurement by IMC and confirmation of initial cell number by plate counting. For the standard curves, TTP and CFU values were plotted on *x*–*y* axes.

Here, we executed three different experiments – IMC, flow cytometry for viability enumeration, and acidification measurements – in parallel on two representative probiotic strains, *L. rhamnosus* and *L. fermentum* (Figure 6). We demonstrated how information obtained by isothermal calorimetry, such as metabolic activity (heat flow over time) and peak metabolic activity gives an indication of viability, with information on growth, metabolic dynamics, and biomass formation during medium fermentation.

Unlike IMC, flow cytometry does not permit kinetic measurements and samples are taken at different time points. For the measurements, cells were sampled at t = 0, 3, 5, 7, and 24 h and viable counts expressed as active fluorescent units (AFU) (Figures 6B, D). When compared to thermograms, there was a correlation between metabolic activity profiles and biomass data obtained by flow cytometry (Figure 6). In particular, it is evident how the formulation of medium B, properly designed for strain activation, proves to be more active in stimulating and supporting growth compared to the standard medium for *lactobacilli* (MRS) and the third formulation, medium C, which is less effective in reaching high cell counts. For *L. fermentum*, we see a first, smaller metabolic peak at around 3–4 h when cultivated in medium B or C. This indicates a metabolic adaptation phase with a metabolic shift as indicated with arrows in Figure 6C. No such information was revealed in acidification curves, nor from the time point biomass measurements.

Additionally, we see that low biomass formation, early acidification, and higher acidification are associated with lower overall metabolic activity and a reduced metabolic peak. Figures 6C, D illustrate that IMC accurately reflects growth dynamics. For instance, the delayed and lower biomass formation measured by flow cytometry of *L. rhamnosus* in medium C is mirrored in the delayed metabolic activity signal, a nuance not captured by the acidification curves.

4 Discussion

Traditional viability assessment in microbial cultures or formulations relies on the gold standard method of plating serial dilutions and determining CFU. However, this method has inherent variability and may overlook aspects of bacterial population heterogeneity. To address these limitations, alternative techniques such as flow cytometry and PCR methods have been introduced, offering the ability to distinguish between viable and dead cells. In this context, IMC stands out as a potent method for viability assessment, offering an innovative and versatile solution that is both sensitive and rapid.

Isothermal microcalorimetry distinguishes itself by measuring the total metabolic activity of the bacterial population even in complex matrices. This has been demonstrated in studies involving *Lactobacilli* and the direct viability assessment of bacterial-coated seeds (Garcia et al., 2017; Nykyri et al., 2019). The real-time monitoring distinguishes IMC from other methods like plate count enumeration and flow cytometry, which provide data at specific time points (Braissant et al., 2010; Fredua-Agyeman and Gaisford, 2019). In our experiments, TTP values were used for standard curve-based enumeration. The time to detect a signal at a chosen detection threshold can also be used, allowing for more

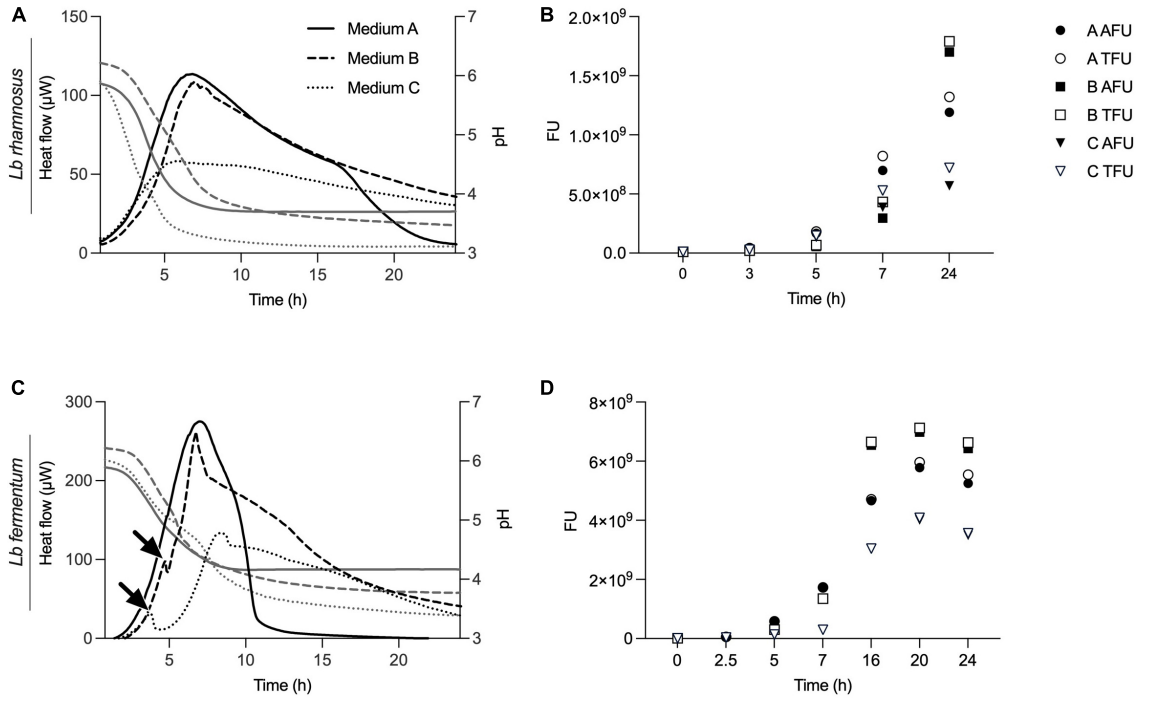

FIGURE 6
Real-time metabolic activity measurements to complement acidification and cell count data. Three growth medium formulations, medium A, B and C, were evaluated using acidification curves, isothermal microcalorimetry (plotted in panels **A**,**C**) and time point measurements using flow cytometry (shown in panels **B**,**D**). Two different strains, *L. rhamnosus* and *L. fermentum*, were used for the experiments. IMC data are presented as heat flow in µW, acidification curves depict pH values and for flow cytometry fluorescent units (FU) with total FU (TFU) and alive FU (AFU) are displayed.

rapid detection and making it independent from the thermogram fingerprint (Garcia et al., 2017; Fricke et al., 2019).

In the probiotics industry, strains belonging to *Bifidobacteria* spp. or *Lactobacillus* spp. taxa may pose technical challenges that traditional plate counts cannot easily address. This is particularly true for NGPs or LBPs where the emergence of novel strains presents new challenges, emphasizing the need for improved viability assays. These challenges include difficulties in growth, sensitivity, intolerance to oxygen, and clumping tendencies, which can impact industrial production and viability determination.

Isothermal microcalorimetry's sensitivity registers minimal changes in heat released from metabolically active cells. Metabolically active bacterial cells produce heat at an approximate rate of 2 pW per cell (Braissant et al., 2010). Operating within the micro-Watt range, IMC can detect as few as approximately 2×10^4–10^5 actively growing cells at the point of detection (Braissant et al., 2010). The method has also been used to follow microorganisms in different media matrices, such as ground meat, milk, juice, and urine (Gram and Søgaard, 1985; Gunasekera et al., 2000; Alklint et al., 2005; Bonkat et al., 2012; Maskow et al., 2012; Fricke et al., 2019; Nykyri et al., 2019).

Incorporating IMC into process and product development can ease the integration of newly identified beneficial microbes into the development pipeline. This is particularly beneficial for more delicate microbial species that are sensitive to environmental perturbations, obligate anaerobes, or those not engaged in acid fermentation. The minimal sample preparation required, the quick results, and the robustness of the technique against different matrices make IMC an attractive choice (von Ah et al., 2009; Braissant et al., 2010; Garcia et al., 2017). Moreover, IMC can discern the impact of active ingredients on bacterial activity, which may either stimulate or inhibit metabolic processes, a detail that may escape traditional methods like plate counting and modern approaches such as PCR methods and flow cytometry.

The usefulness of IMC is further demonstrated in its ability to quantify the effects of stress conditions encountered by bacteria during manufacturing processes or transit through the gastrointestinal tract, similar to the viability enumeration methods discussed here (Fredua-Agyeman and Gaisford, 2015). A notable application of this was in the study of clinical isolates of *Staphylococcus aureus* subjected to dehydration stress; IMC proved to be a direct and effective tool for quantifying effects on viability. In this study, *S. aureus* isolates were coated on PVDF coupons and then introduced into calorimetric vials for measurement (Baede et al., 2022).

While IMC offers valuable insights into microbial viability, its integration into the probiotics industry is hindered by a relative scarcity of comparative data compared to established techniques such as quantitative PCR, flow cytometry, and plate counting methods. This information gap can pose a deterrent for new users who may find it difficult to validate and trust IMC without a robust dataset. Additionally, the high sensitivity of IMC requires careful consideration of strain variations, media lot differences, and temperature fluctuations, which can significantly impact measurement outcomes. However, these challenges can be effectively mitigated by implementing appropriate controls. Additionally, IMC provides precise metabolic fingerprint for each experiment, readily indicating any deviations from expected patterns. In comparing IMC, flow cytometry, and acidification curves for assessing microbial viability, it can be seen that each method offers distinct advantages. Parallel measurements of flow cytometry and IMC revealed a positive correlation between increasing viable counts and metabolic activity in time. While flow cytometry offers a high resolution in distinguishing live and dead cells at sampled time points, IMC excels in continuous measurement of the overall metabolism of samples.

5 Conclusion

In summary, IMC introduces an innovative approach to viability assessment that brings knowledge beyond pure cellular enumeration. Its adaptability and continuous monitoring make it a versatile medium-throughput tool in many steps of the product development journey. Further investigation is needed to fully explore this novel concept.

Data availability statement

The original contributions presented in this study are included in this article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

CM: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MS: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SA: Investigation, Methodology, Writing – original draft, Writing – review & editing. MV: Writing – original draft, Writing – review & editing. KK: Writing – original draft, Writing – review & editing. MP: Conceptualization, Writing – original draft, Writing – review & editing. KB: Conceptualization, Project administration, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

CM, SA, and MP were employed by Probiotical Research s.r.l. MS, MV, KK, and KB were employed by Symcel AB.

Publisher's note

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Viability-PCR for the selective detection of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in live bacteria-containing products

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To exert their beneficial effects, microorganisms used in live bacteria-containing products must be viable and present in certain amounts. In this study, we developed a viability assay based on quantitative PCR coupled with propidium monoazide for the identification and enumeration of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. In order to optimize the protocol, the thermal inactivation conditions for the two target microorganisms and the PMA concentration inhibiting DNA amplification from the dead cells while allowing it from the live cells were first determined. The viability-PCR protocol was then applied to analyze a commercial product containing the two microorganisms. The quantities of both microorganisms determined using viability-PCR in the tested product were significantly higher than those obtained using the standard plate count, suggesting the presence of bacteria in a viable but non-culturable physiological state. Moreover, lower amounts of the two microorganisms were detected using viability-PCR compared to those achieved using quantitative PCR, possibly because of the presence of dead cells in the samples. Our results suggest that the viability-PCR method proposed here is a suitable alternative for rapid and accurate quantification and assessment of the viability of *L. acidophilus* and *B. bifidum* and could be easily adopted in the quality control screening of live bacteria-containing products.

KEYWORDS

viability-PCR, plate count enumeration, qPCR, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, live bacteria-containing products

1 Introduction

The qualitative and quantitative compositions of the human gut microbiota change in health and disease status. Therefore, maintaining a balanced gut microbiota or restoring it from perturbation may significantly maintain and improve health (Laudes et al., 2021; Afzaal et al., 2022).

Microorganisms in spontaneously fermented foods have been empirically used for these purposes (Leeuwendaal et al., 2022). Subsequent studies have revealed that only certain live microbial strains in abundant quantities can confer

health benefits to the host, mainly by enhancing metabolic functions, strengthening the mucosal intestinal barrier, protecting against pathogens, and stimulating the immune system (Campaniello et al., 2023; Skoufou et al., 2024). Scientific advances in the field have promoted the expansion and diversification of products containing live bacteria, including probiotic food supplements commonly used to ameliorate intestinal and general health, live biotherapeutic products intended to prevent or treat several diseases, and fecal microbiota that, once transferred from healthy donors to individuals with intestinal disorders, can restore the gut microbial balance (Mcilroy et al., 2019; Cordaillat-Simmons et al., 2022; Franciosa et al., 2023).

While the fecal microbiota consists of undefined microbial communities (Kump et al., 2018), both probiotic products and biotherapeutics (hereinafter collectively referred to as live bacteria-containing products, LBCP) include single or multiple microorganisms that must unequivocally be identified, viable, and administered in adequately high numbers to be effective (Hill et al., 2014; Campaniello et al., 2023). Therefore, for the manufacturing and regulation of LBCP, the three quality criteria—identification, viability, and quantity—should be reported in their labels and fulfilled throughout the product shelf life (FAO/WHO, 2002; Council for Responsible Nutrition International Probiotics Association, 2017; European Pharmacopoeia Commission, 2019).

Culture-dependent methods are typically applied for monitoring the production of LBCP and verifying label compliance. However, culture techniques have disadvantages that affect each of the above specified criteria requirements: (i) microbial identification may be challenging when multiple strains are used in the same product, especially if they have similar physiological properties and growth requirements; (ii) microbial viability may not always be detected by culturing, as some microbial cells may enter a viable but non-culturable (VBNC) physiological status in response to environmental stresses, losing cultivability while retaining metabolic activity and membrane integrity; and (iii) culture-based microbial quantification presents high coefficients of variation (Davis, 2014; Bagheripoor-Fallah et al., 2015; Boyte et al., 2023).

Alternative methods have been developed for testing LBCP, such as flow cytometry, mass spectrometry, or molecular approaches including whole-genome and next-generation sequencing; however, most fail to concomitantly provide the identification and absolute quantification of viable microorganisms (Angelakis et al., 2011; Pane et al., 2018; Sharma et al., 2020; Zawistowska-Rojek et al., 2022).

Quantitative PCR (qPCR) in combination with propidium monoazide (PMA), a method also referred to as viability-PCR (vPCR), has been used to detect and enumerate viable microorganisms, including lactic acid bacteria, in different matrices (García-Cayuela et al., 2009; Shao et al., 2016; Lai et al., 2017; Scariot et al., 2018; Shehata and Newmaster, 2021; Shi et al., 2022; Shehata et al., 2023; Marole et al., 2024). PMA can distinguish between live and dead cells as it enters dead bacteria with damaged membranes while being excluded from intact living bacteria; once in the compromised cells, PMA covalently binds the genomic DNA in the presence of strong visible light, thus preventing subsequent DNA amplification from dead bacteria and eliminating overestimation of the bacterial counts in qPCR assays (Nocker et al., 2007).

Here, we developed a vPCR protocol for rapid and accurate identification and enumeration of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, two microbial species frequently used in LBCPs. After optimization, the vPCR protocol was verified using different mixtures of live and dead cells obtained using thermal inactivation. Finally, a commercial LBCP was analyzed using vPCR, and the results were compared with those obtained using traditional plate count and qPCR.

The goal of the study was to provide the regulatory bodies and the manufacturers with a rapid and high-throughput method for the microbiological quality assessment of LBCPs.

2 Materials and methods

2.1 Bacterial strains and growth conditions

All strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), except for *Enterococcus faecium* SF68 which was from the Istituto Superiore di Sanità culture collection. The strains were stored at −80°C in cryogenic vials (Prolab Diagnostics).

L. acidophilus (DSM 20079) and *B. bifidum* (DSM 20456) were used as reference strains for vPCR. *L. delbrueckii* subsp. *bulgaricus* (DSM 20081); *L. delbrueckii* subsp. *lactis* (DSM 20072); *Lactiplantibacillus plantarum* (DSM 20174); *Lacticaseibacillus paracasei* (DSM 5622); *B. animalis* subsp. *lactis* (DSM 10140); *B. breve* (DSM 20213); *Bacillus clausii* (DSM 8716); and *Streptococcus thermophilus* (DSM 20617), which are the most frequently found in LBCPs available in Italy, and *E. faecium* SF68 were used to confirm the specificity of the primers/probe sets used in qPCR and vPCR.

Bacteria were grown in de Man, Rogosa, Sharpe (MRS) broth or agar (Oxoid), supplemented with 0.05% L-cysteine HCl for culturing the more strictly anaerobic bifidobacteria strains, and incubated at 37°C under aerobic or anaerobic conditions, depending on the bacterial species requirements. Anaerobic jars and gas generating kits (Oxoid) were used to simulate the anaerobic conditions.

2.2 Enumeration of viable *L. acidophilus* and *B. bifidum* using standard plate count

Plate count (PC) enumeration of *L. acidophilus* and *B. bifidum* in pure broth cultures was performed as previously described (Aureli et al., 2008). Briefly, the test samples were 10-fold diluted in 0.9% saline, and 100 µl of three consecutive dilutions were spread in duplicates on MRS agar or MRS agar supplemented with 0.05% L-cysteine HCl for enumerating *L. acidophilus* and *B. bifidum*, respectively. The plates were then incubated at 37°C for 48–72 h under anaerobic conditions.

Subsequently, the total number of *L. acidophilus* or *B. bifidum* was enumerated in plates containing 30–300 presumptive colonies, and counts were recorded as colony-forming units (CFU) per milliliter of broth culture. All experiments were repeated three

TABLE 1 Oligonucleotide primers ad probes used in this study.

Microbial species	Primer or probe name	Sequence (5′–3′)	Target region	Reference
L. acidophilus	F_acid R_acid Probe_acid	GAAAGAGCCCAAACCAAGTGATTCTTCCCAGATAA TTCAACTATCGCTTATACCACTTTGCAGTCCTACA	16S-23S intergenic spacer region	Haarman and Knol (2006)
B. bifidum	F_bifid R_bifid Probe_bifid	ACCGAATTCGCCTGTCACTTACGGCGCGGATTCGT CCGCTGGATGTGAAC	*oppD* gene*	Singh et al. (2013)

*Oligopeptide transport ATP-binding protein.

times for each reference microorganism, and the results expressed as mean ± standard deviation.

2.3 qPCR reactions and conditions

Genomic DNA was isolated from pure microbial broth cultures grown overnight using the Qiagen DNEasy Blood and Tissue kit, following the manufacturer's instructions. The quality and quantity of isolated DNA were estimated using an ultraviolet spectrophotometer (Biophotometer, Eppendorf). The DNA samples were stored at −20°C until use.

The two primer-probe sets used in separate qPCR reactions to detect *L. acidophilus* and *B. bifidum* were selected from the literature (Haarman and Knol, 2006; Singh et al., 2013) and their nucleotide sequences are reported in Table 1. The probes were labeled with the reporter molecule 6-carboxyfluorescein (FAM) and quencher tetramethylrhodamine (TAMRA) at the 5′-end and 3′-end respectively.

The qPCR reaction mixtures (20 µl final volume) were prepared in duplicates and consisted of 10 µl of 2X TaqPath qPCR Master Mix (Applied Biosystems), 1 µl of 20X primer-probe set (Integrated DNA Technologies, IDT), 5 µL of template DNA, and 4 µl of DNAse/RNAse-free water (Bioline). Two control replicates without a DNA template were included in each run.

Real time qPCR amplification was performed in a MicroAmp optical 96-well reaction plates sealed with optical adhesive covers (Applied Biosystems), using a 7,500 Real-Time PCR system (Applied Biosystems). The thermal cycling conditions included pre-incubation at 50°C for 2 min, an incubation step at 95°C for 10 min to activate the AmpliTaq Gold polymerase, 45 cycles at 95°C for 15 s and 60°C for 30 s, and a final incubation step at 60°C for 1 min. The fluorescence signal was measured at the end of each 60°C step. The threshold cycle (Ct) value, corresponding to the PCR cycle number at which fluorescence was detected above the threshold, was calculated using the 7,500 System software (Applied Biosystems). All the above assays were performed twice.

The specificity of the two primer-probe sets for *L. acidophilus* and *B. bifidum* was tested using genomic DNA isolated from all the strains described above.

To determine the absolute quantities of *L. acidophilus* and *B. bifidum* in unknown samples, the Ct value of each sample was compared to the corresponding standard curves, which were constructed using 10-fold serial dilutions of genomic DNA at known concentrations, isolated from the reference strains *L. acidophilus* DSM 20,079 and *B. bifidum* DSM 20,456. The number of microorganisms in the original broth cultures was determined using the PC method and expressed as CFU/ml. The DNA dilutions used for the standard curves were selected to represent at least five bacterial concentrations, ranging from 10^2 to 10^7 CFU/ml. The DNA extracts were aliquoted undiluted and stored at −20°C before subsequent single use for standard curve construction.

2.4 vPCR set up

2.4.1 Determination of the thermal inactivation conditions for L. acidophilus and B. bifidum

Bacterial pellets from overnight broth cultures of the reference strains *L. acidophilus* DSM 20,079 and *B. bifidum* DSM 20,456 were collected via centrifugation, washed with 0.9% NaCl, and resuspended in the same saline solution to achieve a density at 600 nm (OD600) of ∼ 1. Viable cell concentrations in the bacterial suspensions were determined using PC, as described above. Each strain suspension was then subjected to the following thermal inactivation treatments: 75°C for 30 min, 80°C for 20 min, 90°C for 15 min, or 100°C for 10 min. Lethality was verified by culturing on MRS agar plates. The untreated controls for each strain were included in the experiments. To test whether heating caused any DNA modification that affected the qPCR results, total DNA was isolated from both thermally-treated and -untreated samples using a Mag-Bind cfDNA kit (Omega Bio-Tek) and subjected to qPCR, as described above.

2.4.2 Optimization of the PMA concentration for sample pretreatment before qPCR

Reference strain suspensions with OD600 ∼ 1 were prepared as previously described, and cell concentrations were determined using PC. Each suspension was then split into two equal volumes, one was left untreated (live cells) and the other thermally inactivated at the conditions defined in the previous experiment to obtain dead cells. The absence of viable cells in the heat-treated samples was verified using PC.

Prior to use, PMA 20 mM (Biotium) was diluted to 2.5 mM with sterile water and stored on ice in the dark. The diluted PMA was then added to duplicate aliquots (250 µl) of live and dead cells, to achieve the final concentrations of 25 µM, 50 µM, and 100 µM. The resulting suspensions were incubated in the darkness for 10 min under gentle agitation. Aliquots of live and dead cells that were not mixed with PMA were used as controls.

All samples, with and without PMA, were placed on ice and photoactivated for 5 min using a 500 W halogen light source located at 20 cm distance from the samples.

After photoactivation, DNA was isolated from the samples using a Mag-Bind cfDNA kit and subjected to qPCR (two replicates per sample).

Moreover, to assess PMA cytotoxicity, bacterial counts in PMA-treated live cell samples were determined using PC and compared with those in PMA-untreated live cell samples.

The assays were repeated four times for each reference microorganism.

2.5 Verification of optimal PMA pretreatment for distinguishing between live and dead microbial cells

For each reference strain, live and dead cells in known amounts were prepared as described above and subjected to qPCR, either separately or in different combinations, with or without PMA pretreatment.

Regarding the preparation of bacterial mixtures, aliquots (250 µl) of live cells from broth cultures at OD600 ~ 1 (concentrations of ~10^8 CFU/ml and ~10^7 CFU/ml for *L. acidophilus* and *B. bifidum*, respectively) were placed in 1.5 ml tubes. Subsequently, equal volumes of dead cells, which were thermally treated under optimal conditions as described above to ensure zero viability, were added to each 1.5 ml tube containing the live cells at decreasing concentrations (i.e., 10^8, 10^6, and 10^4 CFU/ml for *L. acidophilus* and 10^7, 10^5, and 10^3 CFU/ml for *B. bifidum*).

A complementary experiment was performed using the same approach, except that decreasing concentrations of live cells were added at fixed amounts of dead cells.

Individual dead and live cells were used as controls. The cell mixtures and individual cells were treated then with 25 µM PMA as previously described. Two replicates were used for each cell mixture and control.

Finally, DNA was isolated from all samples using a Mag-Bind cfDNA kit and subjected to qPCR. All the experiments were repeated twice for each reference microorganism.

2.6 Identification and quantification of *L. acidophilus* and *B. bifidum* in a commercial product using PC, qPCR, and vPCR

Commercial LBCP capsules containing at least 10^9 cells of both *L. acidophilus* and *B. bifidum* per capsule, according to the product label, was purchased from the market, stored at 4°C, and analyzed within the expiration date. Five LBCP capsules were analyzed. Before analysis, each LBCP capsule was dissolved in 10 ml of a 0.9% NaCl solution.

PC enumeration of *L. acidophilus* and *B. bifidum* was performed as described previously. The two microorganisms were differentiated based on colony morphology on MRS agar plates and representative colonies were confirmed at the species level using 16SrRNA sequencing, as previously described (Boye et al., 1999). For *B. bifidum*, selective counting was also performed on Bifidum Selective Medium (BSM) agar plates (Millipore).

For vPCR, 1 ml of the capsule suspensions were mixed with 25 µM PMA and photoactivated at the conditions above described. A DNeasy kit was used to isolate DNA from 1 ml of PMA-untreated and -treated capsule suspensions. DNA samples were 100-fold diluted and subjected to qPCR using *L. acidophilus* and *B. bifidum* specific primer/probe sets in separate reactions. Negative controls without templates were included in each run. Each reaction was performed in duplicate. Serial dilutions of the DNA standards were performed in duplicate for each qPCR run. Concentrations of the individual species were plotted against the corresponding standard curve, with the slope and linear correlation of the curves automatically calculated using the AB 7,500 system software.

2.7 Statistical analyses

Statistical analysis was performed using the GraphPad Prism 10 software (GraphPad Software). Student's *t*-test and analysis of variance (ANOVA) were used to compare treatment pairs. Differences between treatments were considered statistically significant at $p < 0.05$.

3 Results

3.1 Specificity of primers and probes, and qPCR standard curves

The specificity of each primer/probe set used in this study has already been assayed, with positive qPCR reactions using *L. acidophilus* and *B. bifidum*, and no cross-reactions reported using several non-target bacteria (Haarman and Knol, 2006; Singh et al., 2013). Here, we confirmed the specificity of the primer/probe sets by testing 11 non-target microorganisms other than those previously evaluated (data not shown).

The standard curves of each microorganism showed a strong linear correlation ($r^2 = 0.9983$ and $r^2 = 0.9770$ for *L. acidophilus* and *B. bifidum*, respectively) between the Ct values and cell counts in the tested range (10^2-10^7 CFU/ml) (95% confidence interval), confirming the high accuracy of the qPCR assays. The amplification efficiencies (E) calculated using the formula $E = 10^{(-1/\text{slope})} - 1$ (Rasmussen, 2001) were 98% for *L. acidophilus* and 97% for *B. bifidum*.

3.2 Thermal inactivation conditions for *L. acidophilus* and *B. bifidum*

Two of the applied thermal treatments (i.e., at 90°C for 15 min and 100°C for 10 min) efficiently inactivated both *L. acidophilus* and *B. bifidum*, as confirmed by the absence of bacterial growth on MRS agar plates following treatments. Although exposures to 75°C and 80°C for 25 min and 20 min, respectively, were also lethal for *B. bifidum*, they did not ensure 100% mortality of *L. acidophilus*, as indicated by the growth of a few colonies on solid media.

For both microorganisms, none of the heat treatments modified the qPCR Ct values compared with the corresponding untreated samples (data not shown).

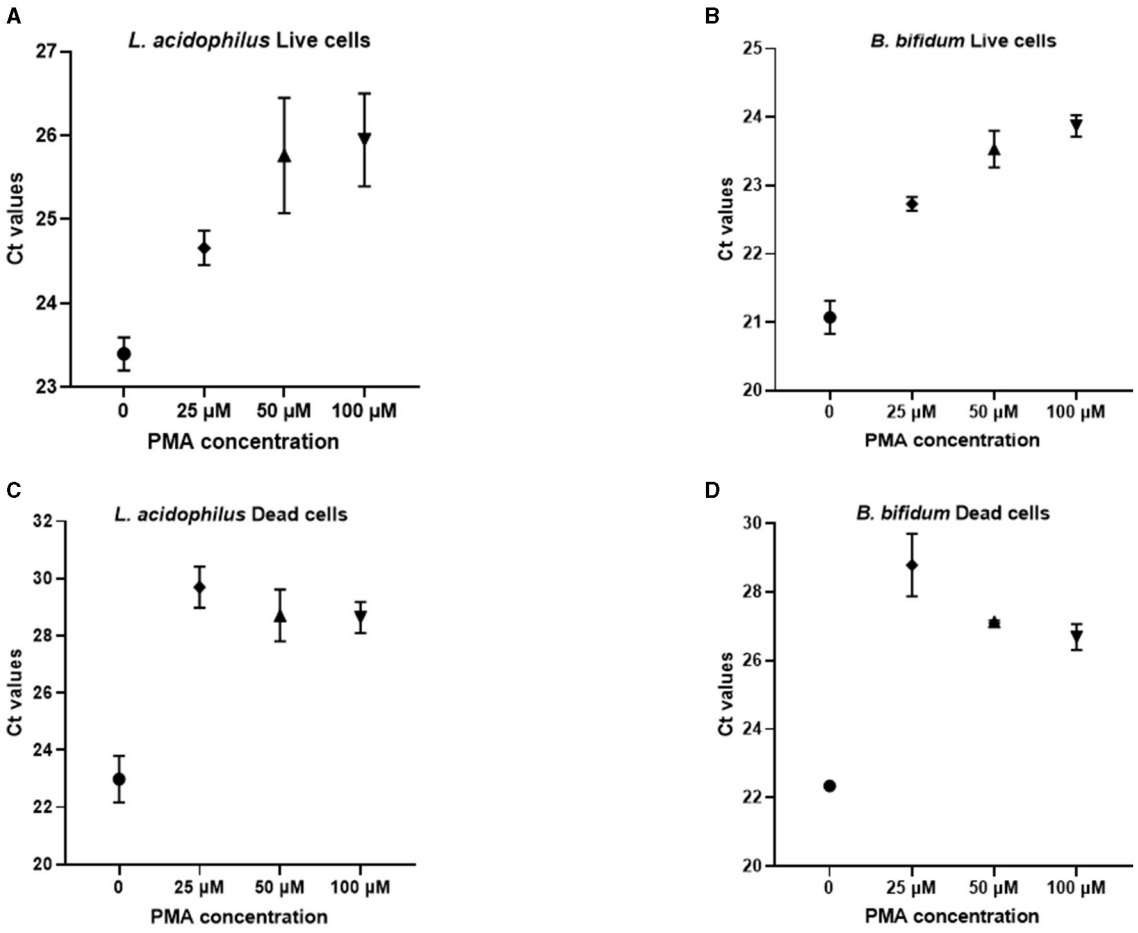

FIGURE 1
Ct values obtained from qPCR experiments after pretreatment of *L. acidophilus* and *B. bifidum* live cells **(A, B)** and dead cells **(C, D)** with different PMA concentrations.

Based on the above results, heat treatment at 100°C for 10 min was selected as the ideal thermal inactivation treatment to ensure zero viability of both target bacteria.

3.3 Optimization of PMA concentration

An optimal PMA concentration should allow the exclusive detection of viable microbial cells, while causing the minimal cytotoxic effects.

For selecting the PMA concentration that adequately distinguished between viable and non-viable target bacteria, the Ct values generated from PMA-treated live and dead cells after qPCR were compared to those of the corresponding PMA-untreated controls (Figure 1). For both microorganisms, treatment with 25 µM PMA concentration caused the lowest inhibition of qPCR from viable cells, as indicated by the minimum increase in the Ct value of DNA derived from PMA-treated live cells compared to the PMA-untreated controls (Figures 1A, B); and the highest inhibition from non-viable cells, as deduced by maximum increase in the Ct value of DNA from PMA-treated dead cells compared to the PMA-untreated controls (Figures 1C, D).

Hence, for both *L. acidophilus* and *B. bifidum*, the 25 µM PMA concentration allowed better detection of live cells with the lowest interference from dead cells.

Concerning the cytotoxic effects of PMA, the proportion of viable cells of both *L. acidophilus* and *B. bifidum* decreased as the PMA concentration increased (Figure 2). Compared to the PMA-untreated controls, the cytotoxic effects of PMA were significant at 50 µM ($p = 0.0008$) and 100 µM ($p = 0.0026$) for *L. acidophilus* (Figure 2A), and 100 µM for *B. bifidum* ($p = 0.0101$) (Figure 2B). The 25 µM PMA produced the least cytotoxic effects on both microorganisms, with no significant differences observed compared to the PMA-untreated samples.

Based on the above results, 25 µM PMA was selected to pretreat samples in the vPCR method.

3.4 qPCR of live and dead cells for verification of optimal PMA treatment

Figure 3 shows the effects of pretreatment using the optimal PMA concentration (25 µM) on live and dead cells of *L. acidophilus* and *B. bifidum*, respectively. For both microorganisms,

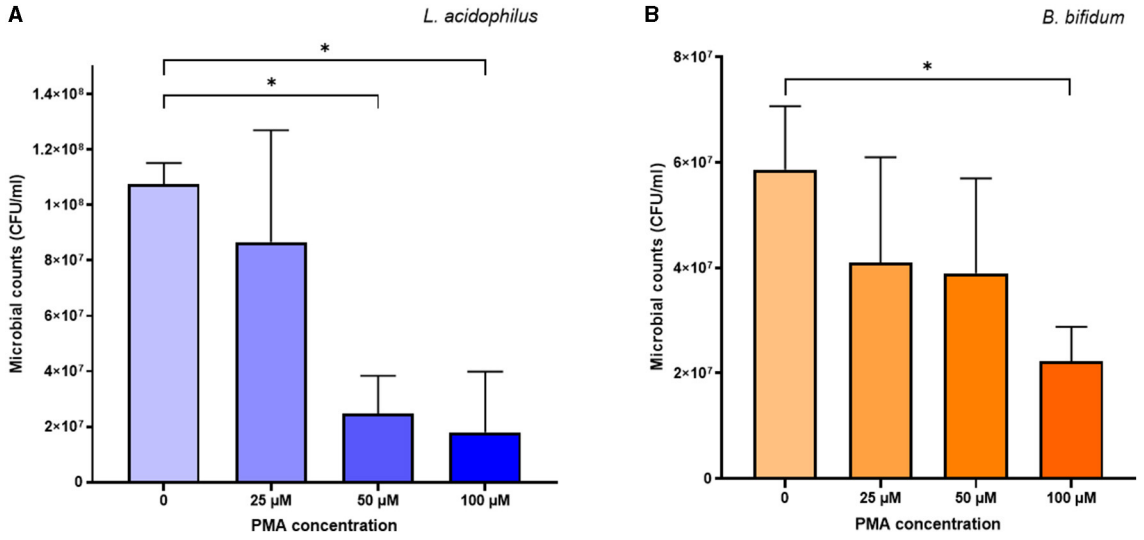

FIGURE 2
Cytotoxic effects of different PMA concentrations on *L. acidophilus* **(A)** and *B. bifidum* **(B)**. Bars depict the mean values of four experiments for each microorganism, with error bars representing the standard deviations. * $p < 0.05$.

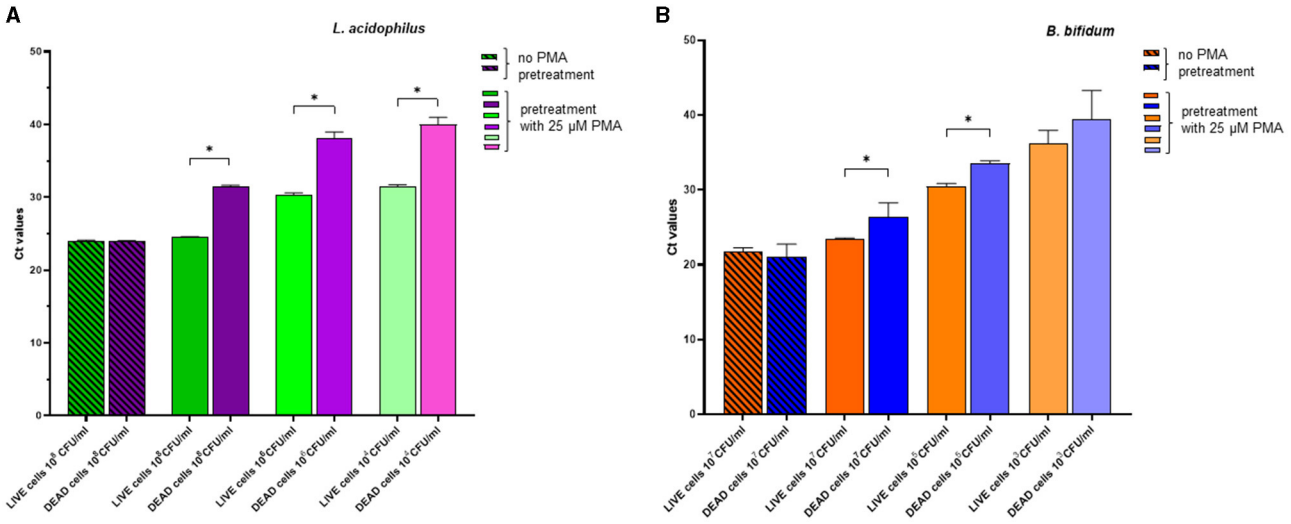

FIGURE 3
Effects of pretreatment with 25 µM PMA of different dilutions of live cells and dead cells of *L. acidophilus* **(A)** and *B. bifidum* **(B)** on the Ct values after qPCR. For each microorganism, the experiments were repeated twice with two replicates used in each experiment. Bars depict mean values with error bars representing the standard deviations. * $p < 0.05$.

in the absence of PMA pretreatment, the Ct values of DNA derived from live and dead cells were comparable, indicating that DNA was amplified irrespective of cell viability. In contrast, PMA pretreatment resulted in a significant increase in the Ct values of DNA from dead bacteria compared with those from live bacteria ($p < 0.0001$ for *L. acidophilus* and $p = 0.0210$ for *B. bifidum*). The reduction in the qPCR signal indicated effectively inhibited DNA amplification from dead cells. This result was confirmed when the live and dead cells of both microorganisms were serially diluted (Figures 3A, B).

In Figure 4, the effects of PMA pretreatment on qPCR of DNA derived from dead cells at decreasing concentrations, either alone or in combination with a fixed quantity of live cells, are shown for *L. acidophilus* and *B. bifidum*. For both microorganisms, the addition of live cells to different amounts of dead cells always produced significantly lower Ct values ($p < 0.0001$), as expected, because PMA should allow PCR amplification from live cells while suppressing amplification from dead cells. The fact that, for both microorganisms, the Ct values of all tested dead/live bacterial mixtures were very similar, regardless of the different dead cell concentrations, further demonstrates that DNA was essentially

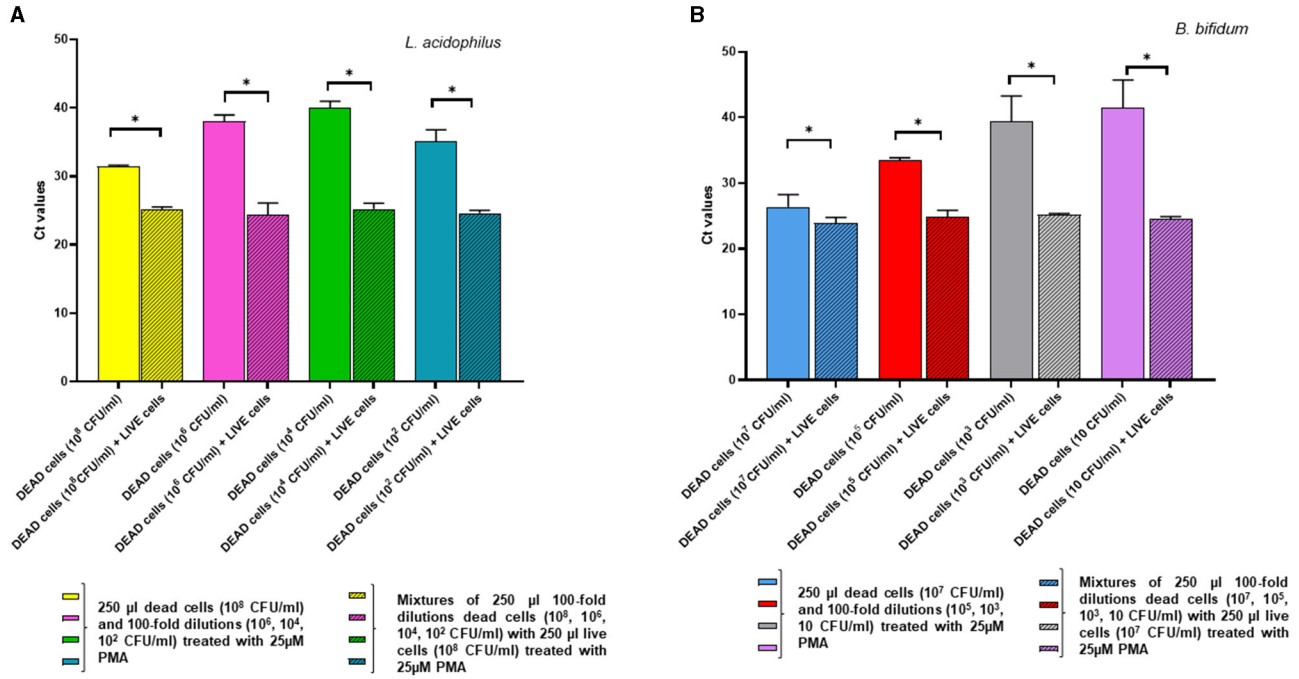

FIGURE 4
Effects of pretreatment with 25 uM PMA on qPCR of DNA derived from dead cells at decreasing concentrations, either alone or added with a fixed amount of live cells. Two replicates were used for each cell mixture and control, and each experiment was repeated twice. Bars depict mean values with error bars representing the standard deviations. **(A)** *L. acidophilus* and **(B)** *B. bifidum*. * $p < 0.05$.

amplified from the live cells present in all mixtures in the same amount (Figures 4A, B).

In the complementary experiment, for *L. acidophilus* the addition of a fixed quantity of dead cells to decreasing numbers of live cells followed by PMA pretreatment did not affect the Ct values compared to those of the live cells alone, confirming that PMA efficiently inhibited qPCR amplification from the dead cells. However, inhibition of DNA amplification by PMA treatment was not evident at the lowest *L. acidophilus* live cell concentration tested at 10^2 CFU/ml, as they generated a significantly higher Ct value compared to that of the relative mix with dead cells ($p = 0.0055$) (Figure 5A).

For *B. bifidum*, PMA inhibition of qPCR from dead cells was apparent when live cells in the live/dead cell mixtures were present at relatively high levels (i.e., 10^7 CFU/ml and 10^5 CFU/ml); in fact, at these concentrations there was no significant difference between the Ct values derived from live cells alone and the live/dead cell mixtures (Figure 5B). As for *L. acidophilus*, PCR inhibition by PMA pretreatment was not observable when the *B. bifidum* live cells quantities in the live/dead cell mixtures decreased ($\leq 10^3$ CFU/ml), as demonstrated by the significant increase in the Ct values of the live cells alone compared to those of the relative mixtures with dead cells ($p < 0.0001$) (Figure 5B). This result could be due to the fact that, when the live cell concentration decreased in the mixtures containing high dead cell ratios, the 25 µM PMA pretreatment reduced the amplification from the viable cells while not fully inhibiting amplification from the dead cells, in accordance with what observed by other authors (Papanicolas et al., 2019).

Thus, our overall data indicate that the live cells quantification limits for the proposed vPCR assay, consisting of qPCR preceded by 25 µM PMA treatment, were approximately 10^2 CFU/ml for *L. acidophilus* and 10^3 CFU/ml for *B. bifidum*.

3.5 Application of the optimized vPCR protocol to the analysis of a commercial LBCP

Figure 6 shows *L. acidophilus* and *B. bifidum* contents in a commercial LBCP, as determined by PC, qPCR, and the newly developed vPCR protocol.

Plate counts confirmed the presence of at least 10^9 CFU/capsule of *L. acidophilus*, which was consistent with the declared labeled amount for that microorganism. For *B. bifidum*, whose stated label claim was also $\geq 10^9$ CFU/capsule, both counts on MRS agar supplemented with cysteine and on BSM agar plates yielded $\sim 10^8$ CFU/capsule (Figure 6). Because *B. bifidum* is a "fastidious" microorganism to grow, being strictly anaerobic and nutrient-demanding, its concentration may have been underestimated using PC (Modesto, 2018). The production of inhibitory substances by *L. acidophilus* (the other microorganism present in the product formulation), such as organic acids and bacteriocins, or competition for nutrients on agar plates may also have contributed to the quantitative inconsistency between the product label and PC results for *B. bifidum*. Alternatively, it is possible that VBNC *B. bifidum* cells were present in the test samples and escaped culture detection.

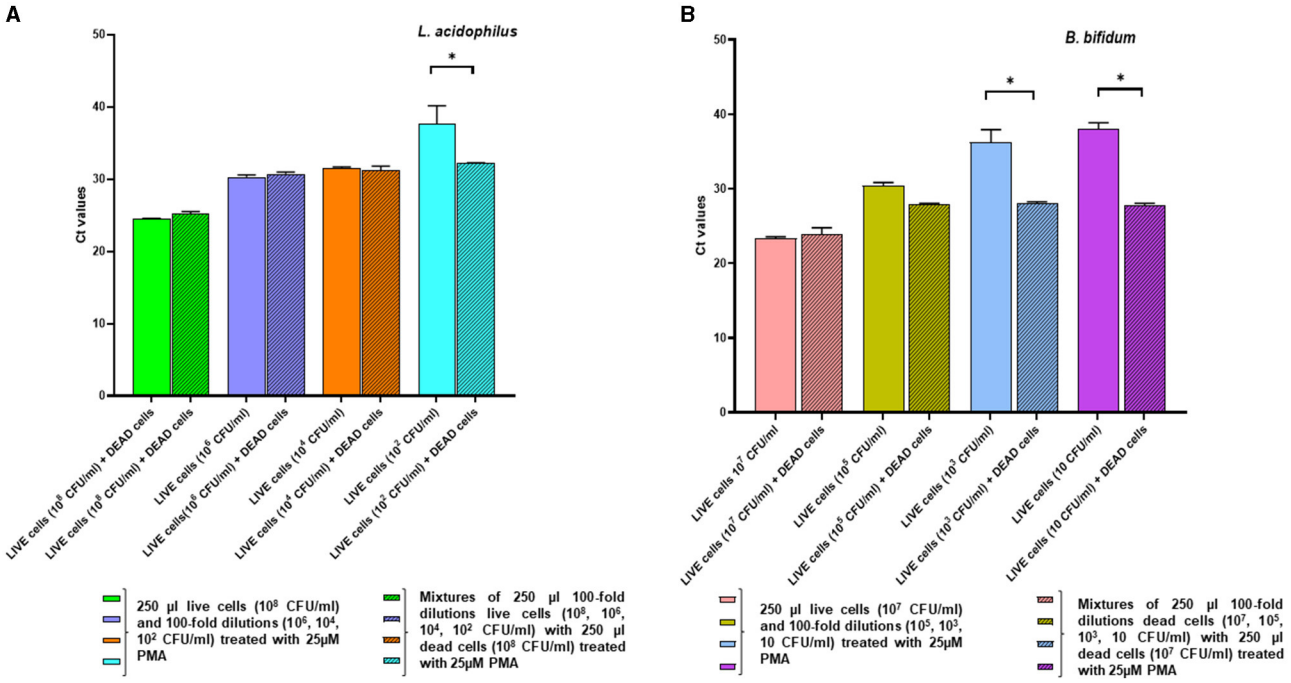

FIGURE 5
Effects of pretreatment with 25 uM PMA on qPCR of DNA derived from live cells at decreasing concentrations, either alone or added with a fixed amount of dead cells. Two replicates were used for each cell mixture and control, and each experiment was repeated twice. Bars depict mean values with error bars representing the standard deviations. **(A)** *L. acidophilus* and **(B)** *B. bifidum*. * $p < 0.05$.

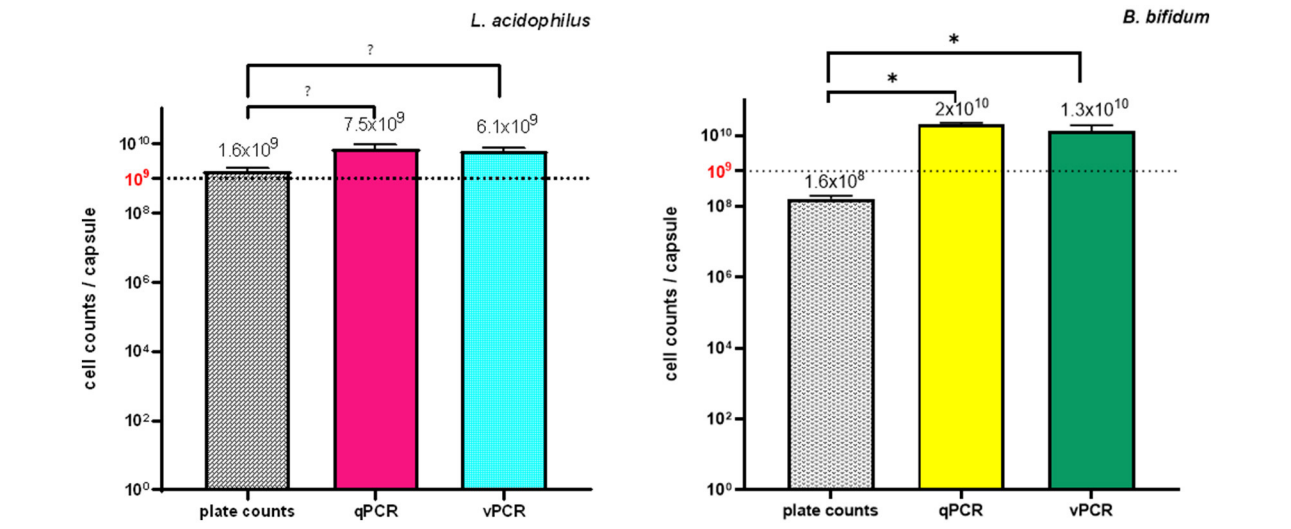

FIGURE 6
Quantities of *L. acidophilus* and *B. bifidum* in a commercial LBCP as determined by PC, qPCR and vPCR. Five LBCP capsules were analyzed. Each qPCR and vPCR reaction was performed in duplicate using *L. acidophilus* and *B. bifidum* specific primer/probe sets in separate reactions. Bars depict mean values with error bars representing the standard deviations. **(A)** *L. acidophilus* and **(B)** *B. bifidum*. * $p < 0.05$.

The presence of VBNC microbial cells might also account for the significantly lower quantitative values obtained for both microorganisms using PC compared to those estimated using qPCR and vPCR (all p values < 0.001) (Figure 6): in fact, while VBNC microbial cells fail to grow in culture media, their DNA can be amplified using PCR.

The quantity estimates of both bacteria using vPCR were lower than those determined using qPCR, as expected, since amplification of DNA from dead cells in the samples should be prevented by the PMA pretreatment step of the vPCR protocol. However, the lack of a statistically significant difference between the quantitative values obtained using qPCR and vPCR for both

microorganisms suggests that the tested LBCP contained a few dead cells (Figure 6).

4 Discussion

Qualitative and quantitative estimations of viable microorganisms deliberately added to LBCPs are essential to guarantee product efficacy and are required before marketing (FAO/WHO, 2002; Council for Responsible Nutrition International Probiotics Association, 2017; European Pharmacopoeia Commission, 2019).

The vPCR method described in this study allowed the identification and quantification of viable *L. acidophilus* and *B. bifidum* with adequate specificity, accuracy, and sensitivity of detection for testing LBCPs, which typically contain $>10^6$-10^7 CFU/g of live microorganisms to provide effective daily intake (Dinkçi et al., 2019; Marco et al., 2020; Boyte et al., 2023).

Once applied to the analysis of a LBCP containing both *L. acidophilus* and *B. bifidum*, the vPCR method showed better performance compared to both "gold standard" culture-dependent PC enumeration and the molecular approach of qPCR, which is also frequently used for routine microbiological testing purposes.

Although traditional PC enumeration relies on the ability of live microorganisms to multiply and form colonies on agar plates, vPCR uses membrane integrity as a viability criterion, thus including VBNC cells that are unable to grow on culture media (Davis, 2014; Bagheripoor-Fallah et al., 2015).

Indeed, our results from the LBCP analysis showed that the quantitative estimates of viable *L. acidophilus* and *B. bifidum* using vPCR were significantly higher than colony counts, suggesting that VBNC cells of both microorganisms were present in the product, likely because bacteria can easily enter the VBNC state in response to the manufacturing process (Oliver, 2005; Kumar and Ghosh, 2019).

Notably, VBNC cells in LBCPs can still exert beneficial effects on the host (Adams, 2010), can be resuscitated, depending on environmental factors, and restore full metabolic activity and the ability to multiply (Oliver, 2005; Kumar and Ghosh, 2019). Therefore, detecting VBNC microbial cells is not only essential for pathogens because of the risk that they can regain virulence upon resuscitation, but is equally significant for obtaining a more reliable quantification of the total viable beneficial bacteria in a product.

Our study confirms that, being able to detect VBNC cells, vPCR can provide more accurate quantitative estimates of viable microorganisms in a sample compared to classic microbiological culture-based methods and in a shorter time, considering the relatively long incubation periods required for bacterial cultivation. In addition, the advantage of detecting VBNC cells outweighs any disadvantages caused by the higher economic costs and sophisticated systems required to perform vPCR vs. PC enumeration. The potential applicability of the method to the detection of multiple microorganisms in a single test might reduce the overall costs if large numbers of samples per day are to be analyzed, as in routine control screening.

Compared with qPCR, which cannot distinguish between DNA from live and dead microbial cells, vPCR enables the selective detection of viable microbial cells (Kumar and Ghosh, 2019; Boyte et al., 2023). Accordingly, our results of the LBCP analysis using the vPCR method showed lower quantitative amounts of both tested microorganisms than those estimated using qPCR.

In conclusion, the vPCR assay proposed here allowed for the accurate identification, quantification, and viability determination of both *L. acidophilus* and *B. bifidum* in approximately 5 h, thus representing a reliable high-throughput molecular test for the microbiological quality assessment of LBCPs containing these microorganisms.

A potential limitation of this method is that it is species specific rather than strain specific, whereas the potential health benefits of the microorganisms to be included in LBCPs, as well as any potential concerns, should be demonstrated at the strain level (European Pharmacopoeia Commission, 2019; EFSA Panel on Biological Hazards, 2023). Nevertheless, although a consensus definition of microbial strain based on more recent genomic knowledge is still needed, species-specific methods may be considered acceptable for the analysis of products that do not contain individual strains of the same species (Boyte et al., 2023), currently representing the majority of LBCPs available on the market. Availability of the whole genome sequences from strains used in LBCPs would be necessary in order to be able to detect and quantify viable bacteria at the strain level by a molecular method.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SC: Data curation, Formal analysis, Supervision, Writing – review & editing. SI: Data curation, Formal analysis, Investigation, Writing – review & editing. DG: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. CVH: Writing – review & editing. GF: Conceptualization, Methodology, Supervision, Writing – original draft, Formal analysis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development and validation of a PMA-qPCR method for accurate quantification of viable *Lacticaseibacillus paracasei* in probiotics

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The effectiveness of probiotic products hinges on the viability and precise quantification of probiotic strains. This study addresses this crucial requirement by developing and validating a precise propidium monoazide combination with quantitative polymerase chain reaction (PMA-qPCR) method for quantifying viable *Lacticaseibacillus paracasei* in probiotic formulations. Initially, species-specific primers were meticulously designed based on core genes from the whole-genome sequence (WGS) of *L. paracasei*, and they underwent rigorous validation against 462 WGSs, 25 target strains, and 37 non-target strains across various taxonomic levels, ensuring extensive inclusivity and exclusivity. Subsequently, optimal PMA treatment conditions were established using 25 different *L. paracasei* strains to effectively inhibit dead cell DNA amplification while preserving viable cells. The developed method exhibited a robust linear relationship ($R^2 = 0.994$) between cycle threshold (C_q) values and viable cell numbers ranging from 10^3 to 10^8 CFU/mL, with an impressive amplification efficiency of 104.48% and a quantification limit of 7.30×10^3 CFU/mL. Accuracy assessments revealed biases within ± 0.5 Log_{10} units, while Bland–Altman analysis demonstrated a mean bias of 0.058 Log_{10}, with 95% confidence limits of −0.366 to 0.482 Log_{10}. Furthermore, statistical analysis ($p = 0.76$) indicated no significant differences between theoretical and measured values. This validated PMA-qPCR method serves as a robust and accurate tool for quantifying viable *L. paracasei* in various sample matrices, including pure cultures, probiotics as food ingredients, and composite probiotic products, thereby enhancing probiotic product quality assurance and contributing to consumer safety and regulatory compliance.

KEYWORDS

probiotics, *Lacticaseibacillus paracasei*, PMA-qPCR, identification, viable cell quantification, method validation

1 Introduction

Probiotics, live microorganisms beneficial to human health when consumed in appropriate quantities, offer diverse advantages such as alleviating lactose intolerance, reducing obesity, and enhancing gut microflora (The World Health Organization, 2001; Principi et al., 2018; Son et al., 2018; Jang et al., 2019; Song et al., 2023). Their extensive utility spans various sectors including food, cosmetics, dietary supplements, and pharmaceuticals, underscoring their importance in promoting human well-being (Kumar et al., 2015; Quin et al., 2018; Song et al., 2023). However, the efficacy of probiotics relies heavily on the specific strains used and their viability, which are influenced by factors like manufacture method, fermentation processes, and storage conditions (Fenster et al., 2019; Beck et al., 2022; Congjie et al., 2024; Hellebois et al., 2024; Wang and Zhong, 2024). Therefore, accurate quantification of viable cells, particularly in compound probiotic products, is critical for ensuring product quality, regulatory compliance, and consumer safety. This quantification not only verifies promised health benefits but also fosters market competitiveness and drives scientific innovation within the probiotic industry.

Currently, culture-based methodologies face inherent challenges in differentiating or selectively enumerating probiotics in compound products, failing to meet the demands of the probiotic industry (Boyte et al., 2023; Sibanda et al., 2024). Nucleic acid-based methods, particularly quantitative PCR (qPCR), have gained widespread acceptance across various disciplines such as biology, food science, and environmental science due to their rapidity, specificity, and exceptional sensitivity (Guo et al., 2020; Boyte et al., 2023; Shehata et al., 2023). When combined with propidium monoazide (PMA) dye, PMA-qPCR facilitates the quantification of viable cells through selective staining based on membrane integrity (Nocker et al., 2006; Guo et al., 2024; Marole et al., 2024). The PMA dye selectively penetrates membrane-damaged cells, forming covalent cross-links with DNA upon photolysis, preventing subsequent PCR amplification of DNA from dead cells. Consequently, DNA from membrane-intact cells is selectively amplified in the subsequent PCR procedure (Figure 1) (Nocker et al., 2006; Scariot et al., 2018; Shehata et al., 2023).

When applying PMA-qPCR, it is essential to consider several crucial factors that would impact the results, depending on the target strains and sample types. Firstly, the design of specific primers is fundamental in qPCR, as it ensures detection accuracy, enhances sensitivity, and minimizes false positives and negatives (Kwon et al., 2005; Fujimoto and Watanabe, 2013; Zhao et al., 2022; Kiousi et al., 2023). The efficiency of DNA extraction is another critical factor, as it directly affects how accurately the qPCR results reflect the biomass in the samples. Therefore, selecting an appropriate DNA extraction method based on the sample type is vital for obtaining accurate and stable results (Douglas et al., 2020; Shetty and Mariyam, 2020). Additionally, the PMA treatment conditions must be optimized to fully inhibit the amplification of DNA from dead cells without significantly affecting the detection of viable cells, which is crucial for accurate viable cell counts (Zhang et al., 2020; Latka et al., 2022). Another important factor is the qPCR amplification program, which impacts the amplification efficiency of the primers and affects the standard curve. This, in

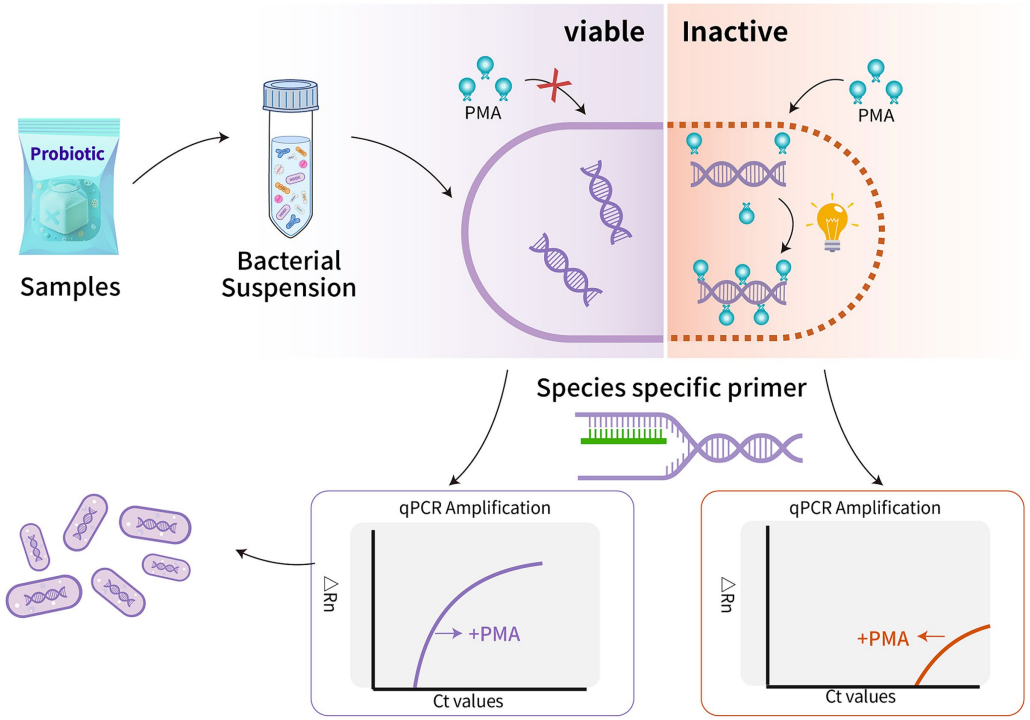

FIGURE 1
It illustrates the principle of viable cell counting using the PMA-qPCR method. This technique relies on cell membrane integrity and the use of specific primers to selectively enumerate viable cells of targeted probiotics in compound products. Viable cells with intact membranes are distinguished from non-viable cells, allowing for accurate quantification of viable probiotics present.

turn, influences the relationship between the C_q values and gene copies or viable cell numbers. Finally, the selection of strains used to construct the standard curve and its applicability to different strains within a species should be carefully considered (Svec et al., 2015; Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018; Ruijter et al., 2021).

The performance parameters of microbiological methodologies are recommended to be evaluated and validated to ensure that they are suitable for their intended use. Quantitative techniques like PMA-qPCR demand meticulous assessment of accuracy, precision, specificity, quantification limit, linearity, and ruggedness (Broeders et al., 2014). These metrics are crucial for determining the method's robustness and reliability across diverse applications. The PMA-qPCR method, renowned for its ability to differentiate between live and dead cells based on membrane integrity, holds significant promise for accurately quantifying viable cells, especially in complex matrices like compound probiotic products. However, despite its widespread application in various sectors, comprehensive evaluation of its efficacy in quantifying specific target species is often lacking. Thorough validation of the PMA-qPCR method is essential to ensure its precision and reliability across different applications, supporting scientific research, quality control, and regulatory compliance. Through method validation, the effectiveness of PMA-qPCR can be improved in real-world scenarios, ensuring that probiotic products meet their intended health benefits and maintain high standards of quality and safety. This, in turn, enhances public health and fosters consumer trust in these products.

Lacticaseibacillus paracasei, recognized as a pivotal probiotic resource, assumes a prominent role within the global health food industry. At present, several commercially available strains of *L. paracasei* find widespread application in the production of dairy items, solid beverages, and health supplements (Zhang et al., 2010; Falfán-Cortés et al., 2022; Pérez Martínez et al., 2023; Beverage et al., 2024). Furthermore, *L. paracasei* manifests commendable physiological effects, exerting a pivotal role in modulating the equilibrium of the human intestinal microbiota (Chuang et al., 2011) and serving as a probiotic in disease prevention (Chiang and Pan, 2012). Notably, it demonstrates the capability of maintaining stable viability within the human intestinal tract, positioning it as a promising candidate for incorporation into functional foods. Particularly, the domain of dairy product development stands out as an area with substantial potential for future advancement. In this study, a precise PMA-qPCR method for quantifying viable *L. paracasei* was developed and rigorously evaluated. A species-specific primer pair was meticulously designed based on core genes identified in the whole genome sequence of *L. paracasei*. The validation process for these primers encompassed comprehensive inclusivity and exclusivity testing, conducted through whole-genome sequence blasts and a thorough analysis of strains collected at various taxonomic levels. The efficacy of the PMA treatment conditions was verified using 25 different *L. paracasei* strains, ensuring that the method did not interfere with the PCR amplification of viable cells while effectively suppressing the amplification of non-viable cells. A standard curve correlating qPCR C_q values with viable bacterial counts was constructed. The PMA-qPCR method was then applied to a variety of samples, demonstrating its relative trueness, accuracy, linearity, limit, and quantification range. This study successfully established a robust PMA-qPCR method for accurately quantifying viable *L. paracasei* in heterogeneous samples, offering valuable implications for evaluating the viability and quality of probiotic products.

2 Materials and methods

2.1 Specific primer design

After executing data quality control and conducting an analysis of average nucleotide identity (ANI), we acquired 176 publicly available genomes of *L. paracasei* from the National Center for Biotechnology Information (NCBI). These genomes underwent re-annotation utilizing Prokka v1.14.6 to identify protein sequences, and the format was standardized to align with that of the 15 self-sequenced genomes. Subsequently, a gene presence/absence analysis was conducted based on the annotated protein sequences. Gene families of *L. paracasei* were individually constructed using the CD-HIT rapid clustering of similar proteins software (v4.6), applying a threshold value of 50% pairwise identity and a 0.7 length difference cutoff in amino acids (Li and Godzik, 2006; Li et al., 2008). The genes only present in all the infraspecific strains were preliminarily identified as the core genes in *L. paracasei*. In consideration of the gene presence/absence analysis being carried out at the protein level, the nucleotide specificity of these conserved genes was subsequently verified through BLASTN against the NCBI Nucleotide collection (NT) (Altschul et al., 1990). The species-specific gene for Alkaline shock protein 23 was identified for primer design. Subsequently, the corresponding PCR primer pairs for *L. paracasei* were meticulously crafted using Primer Premier v6.0, with adherence to various design principles (Singh et al., 1998; Elsalam, 2003). The primer, designed with a length of 180 bp (Lpa-F: 5'-ACGCTGGCATCAATAAGGAATT-3′; Lpa-R: 5'-CATCGCTCA GGTCTACATCCA-3′), was synthesized by Sangon Biotech (Shanghai, China).

2.2 Inclusivity and exclusivity validation of primer specificity

Inclusivity was conducted to assess primer ability to detect target strains (ISO 16140-2, 2016). Firstly, the primer was assessed *in silico* through aligning with 38 whole-genome sequences (WGS) of *L. paracasei* through Primer-BLAST on NCBI[1] (Ye et al., 2012; Lawley et al., 2017; Guo et al., 2024). The WGS sequences were downloaded from NCBI website[2] including type strain, commercial strains, and others. Then, the primer was further validated by PCR test using the DNA templates extracted from 25 different *L. paracasei* strains (Table 1). The thermal cycling of PCR assay consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 34 s and 72°C for 25 s, followed by a final extension step of 72°C for 10 min. The electrophoresis on 1% agarose gel was used to examine the amplification products using Gel Doc EZ System (Bio-Rad, California, USA).

The exclusivity of primer characterizes the non-detection of non-target strains (ISO 16140-2, 2016). Seventy whole-genome

1 https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

2 https://www.ncbi.nlm.nih.gov/genome/

TABLE 1 Bacterial strains used in this study.

Inclusivity and exclusivity study	Genus	Strains
Inclusivity	*Lacticaseibacillus*	*L. paracasei* CICC 6263^T, 6264^T, CICC 6028, CICC 6110, CICC 6138, CICC 6227, CICC 20241, CICC 20266, CICC 22165, CICC 22829, CICC 22830, CICC 22709, CICC 24700, CICC 24825, Z-022, 8130T, ET-22, K56, LC01, Shirota, LPC-37, 431, LC-37, Zhang, 207–27
Exclusivity	*Lacticaseibacillus*	*L. casei* CICC 6117^T, *L. rhamnosus* CICC 6224^T, *L. zeae* CGMCC 1.2442
	Bifidobacterium	*B. animalis* subsp. *lactis* CICC 24210^T, *B. animalis* subsp. *animalis* CICC 6250^T, *B. adolescentis* CICC 6070^T, *B. breve* CICC 6079^T, *B. longum* subsp. *longum* CICC 6186^T, *B. longum* subsp. *infantis* CICC 6069^T, *B. bifidum* CICC 6071^T
	Lactobacillus	*L. acidophilus* CICC 6081^T, *L. crispatus* JCM 1185^T, *L. delbrueckii* subsp. *bulgaricus* CICC 6103^T, *L. delbrueckii* subsp. *lactis* CGMCC 1.2625^T, *L. gasseri* CICC 24878^T, *L. helveticus* CICC 24208^T, *L. johnsonii* CICC 6252^T, *L. kefiranofaciens* subsp. *kefiranofaciens* CGMCC 1.3402^T
	Limosilactobacillus	*L. fermentum* CICC 24209^T, *L. reuteri* CICC 6132^T
	Lactiplantibacillus	*L. plantarum* CICC 6240^T
	Ligilactobacillus	*L. salivarius* CGMCC 1.1881^T
	Latilactobacillus	*L. curvatus* JCM 1096^T, *L. sakei* CICC 6245^T
	Streptococcus	*S. salivarius* subsp. *thermophilus* CICC 6222^T
	Lactococcus	*L. lactis* subsp. *lactis* CICC 6246^T, *L. cremoris* CICC 24337^T
	Propionibacterium	*P. freudenreichii* subsp. *shermanii* CGMCC 1.2231^T, *P. acidipropionici* CICC 24923^T
	Leuconostoc	*L.* subsp. *mesenteroides* CICC 25070^T, *L. mesenteroides* subsp. *cremoris* CICC 22181
	Pediococcus	*P. acidilactici* CGMCC 1.2696^T, *P. pentosaceus* CGMCC 1.2695^T
	Weizmannia	*W. coagulans* CGMCC 1.2009^T
	Staphylococcus	*S. vitulinus* CICC 10850, *S. xylosus* JCM 2418^T, *S. carnosus* ACCC 01657

sequences (WGS) of 25 strains of *Lacticaseibacillus* at the species level, 281 WGS of 30 strains within the family *Lactobacillaceae* at the genus level, and 73 WGS of the 36 strains listed in the Chinese catalog of food-safe cultures were downloaded from the NCBI. The primer was assessed *in silico* by aligning with these WGS through Primer-BLAST on NCBI (Ye et al., 2012; Lawley et al., 2017). Then, 36 strains in Chinese list of cultures that can be used for food and *L. zeae* were collected (Table 1). The DNA templates of these strains were isolated and PCR products were imaged by 1% agarose gel electrophoresis under UV lights.

2.3 Genomic DNA extraction

The bead-beating methods were demonstrated effectiveness for DNA extraction (Fujimoto et al., 2004; Guo et al., 2024). Briefly, the screw-cap 2.0 mL sample tubes containing 0.25 g of Zirconia/Silica beads with 0.1 mm were autoclaved. Bacterial suspensions within 200 μL of ddH_2O were aspirated into these tubes. The BEAD RUPTOR 12 (OMNI International, USA) served as the mechanical cell disruptor for 12 s at a speed setting of 6.0 m/s. Supernatants containing DNA were obtained by centrifuged at 12,000 rpm for 15 min and 50 microliters of that were transferred into 1.5 mL sterile tubes for subsequent qPCR assays.

2.4 Verification of optimal PMA treatment conditions

Accurate quantification of viable bacteria is closely linked to the appropriate conditions of PMA treatment. A commonly used PMA treatment condition, involving a concentration of 50 μM/L, followed by 5 min of dark incubation, and finally, 15 min of exposure to light, was selected (Desfossés-Foucault et al., 2012; Villarreal et al., 2013; Guo et al., 2024). To validate the suitability of the chosen PMA conditions across various *L. paracasei* strains, we deliberately selected 25 distinct strains (Table 1) for further investigation. Firstly, the bacteria were initially resuscitated on MRS solid medium at 37°C for 48 h. Subsequently, the cells were inoculated onto MRS solid medium and cultured for another 48 h. Having undergone dual cultivation on MRS solid medium under optimal conditions, the majority of the bacteria were considered highly active. The resuspended bacteria within 0.85% sodium chloride solution were adjusted to OD_{620} = 0.3–0.5 with approximate 10^8 CFU/mL, which was further validated by plating counts. Each strain was categorized into live and dead groups. To obtain the dead groups, the bacteria were subjected to heating at 90°C for 10 min. Subsequently, both live and dead bacterial suspensions, each containing approximately 10^8 CFU/mL, were divided into PMA treatment and non-treatment groups. The PMA solution from BIORIGIN (China) was dissolved in ddH_2O to create a 20 mmol/L stock solution. Subsequently, 1.25 μL of this stock solution was added to 500 μL of cell suspensions, resulting in a final PMA concentration of 50 μM. The mixed samples were then placed in the dark for 5 min to allow PMA to penetrate dead cells and bind to their DNA. Following this incubation, the treated samples were exposed to a 60 W LED light source (Biotium, USA) for 15 min. Subsequently, both the bacterial suspensions from the PMA treatment group and the non-treatment group were centrifuged at 12,000 rpm for 15 min. The harvested bacterial pellets were then subjected to DNA extraction.

2.5 Quantitative PCR amplification

The total qPCR volume was 20 μL per reaction, including 10.0 μL of 2× SYBR Green premix (TaKaRa, Japan), 0.4 μL of each 10 μM forward and reverse primers, 0.08 μL of ROX reference dye, 2 μL of bacteria genomic DNA, and 7.12 μL ddH_2O. The thermal cycle program was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s. The qPCR reactions were carried out in an ABI 7500 Fast real-time PCR system. Triplicates were performed for target DNA and sterile water (negative control).

2.6 Construction of standard curves between viable cell numbers and C_q values

In order to achieve viable cell counting by PMA-qPCR method, a standard curve between viable cell numbers and qPCR C_q values was performed (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018). Fresh cultures of *L. paracasei* CICC 6263^T was obtained and then diluted to 10^8 CFU/mL that further confirmed by culture plating. The bacteria with 10^8 CFU/mL were treated by PMA to filter dead cells and then DNA was extracted as described above. The DNA series with 10-fold dilutions was amplified to obtain the C_q values. Then, the standard curve between C_q values and viable cell numbers were constructed.

2.7 Linear and quantification limits of the PMA-qPCR method

Samples were prepared by combining viable *L. paracasei* cells with nonviable cells of *L. rhamnosus*. In each sample, a consistent count of nonviable *L. rhamnosus* cells was maintained at approximately 10^8 CFU/mL, while varying concentrations of viable *L. paracasei* cells were introduced, namely 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/mL. The 10^3 CFU/mL were further diluted to obtain lower *L. paracasei* concentration for quantification limits detection. Viable *L. paracasei* were quantified using the culture-based method to obtain the theoretical values. To obtain the linear characteristics, the PMA-qPCR measured values and theoretical values were linearly fitted.

2.8 Quantification of viable *Lacticaseibacillus paracasei* by PMA-qPCR method

The wide applicability of the established PMA-qPCR method was firstly confirmed using 25 strains of *L. paracasei* (Table 1). These strains included type strains CICC 6263^T, CICC 6264^T, and commercial strains Z-022, 8130T, ET-22, K56, LC01, Shirota, LPC-37, 431, LC-37, Zhang, 207–27, as well as CICC 6110, CICC 22165, CICC 20241, CICC 22830, CICC 6138, CICC 6028, CICC 24700, CICC 24825, CICC 6227, CICC 20266, CICC 22709, and CICC 22829. Each bacterial suspension was adjusted to approximately 10^8 CFU/mL, followed by PMA treatment, DNA extraction, and qPCR amplification. Viable cell number of each strain were further determined by plate counting.

As probiotics used in food ingredients typically contain high concentrations of bacteria, a selection of six probiotic formulations comprising singular *L. paracasei* strains (e.g., zhang, LPB-27, etc.) or combinations with other probiotics and lactic acid bacteria, along with simple excipients such as maltodextrin, were collected. Initially, the total bacteria of each sample were diluted to approximately 10^8 CFU/mL, and viable numbers of *L. paracasei* were detected using the established PMA-qPCR method. Theoretical values of *L. paracasei* in each sample were provided by the producer.

To further validate the PMA-qPCR method's capacity to accurately quantify viable *L. paracasei* within composite bacterial flora and withstand interference from the matrix, eight compound probiotic products were collected. Each compound probiotic product contained typically featured intricate formulations. These formulations incorporated complex excipients, including common additives such as maltodextrin and resistant dextrin, as well as prebiotics like fructooligosaccharides, erythrosis, and stachyose. Additionally, botanical ingredients such as cranberry, peach, and hawthorn powder were included in these probiotic formulations. Then, the established PMA-qPCR method was used to detect viable *L. paracasei* in these compound probiotics. Theoretical values of viable *L. paracasei* in these samples were obtained according to products claims. To enhance the analysis of probiotic products, qPCR was used on PMA-untreated samples to identify and measure dead or damaged bacteria, offering a complete view of the total bacterial count, encompassing both living and dead cells. The principles of how PMA-qPCR quantifies the number of viable target cells in compound probiotics are illustrated in Figure 1.

2.9 Statistical analysis

The statistical analysis comprised two key methodologies. Firstly, the *T*-test method, executed in Excel (Microsoft Office 2021), was employed to determine the significance of PMA treatment conditions on viable cells, comparing treated and non-treated groups. Additionally, the *T*-test assessed the significance between theoretical and measured values across all 39 samples, with a significance threshold of $p < 0.05$. Secondly, to ensure rigorous analysis and scientific validity, the Bland–Altman method was utilized. Implemented using R software (version 4.2.2), this method evaluated the agreement between theoretical and PMA-qPCR measured results. It involved plotting individual differences against mean values, incorporating the line of identity, line of bias, and upper and lower 95% confidence limits of agreement.

3 Results

3.1 Specificity of the newly designed primer

The specificity of the designed primer for *L. paracasei* was initially validated through a Primer-BLAST analysis on NCBI. In this preliminary test, no significant similarity with non-target

microorganisms was detected. Then, inclusivity and exclusivity of the primer were evaluated using DNA templates from 25 target strains of *L. paracasei* and 37 non-target strains by PCR amplification. Positive results for the 25 strains of *L. paracasei* were obtained, while other 37 strains were all negative (Figure 2). The results demonstrated the highly specificity of the newly designed primer to *L. paracasei* and target detection of *L. paracasei* within multi-strains would be achievable.

3.2 Evaluation of the optimal PMA treatment conditions

The PMA treatment conditions involving a concentration of 50 μM/L was selected. This was followed by 5 min of dark incubation and, finally, 15 min of exposure to light. To further validate the optimality and broad applicability of these chosen PMA treatment conditions, 25 strains of *L. paracasei* were employed with and without PMA treatment. For the viable group, the C_q values obtained from the PMA treatment and non-treatment groups underwent statistical analysis using the *T*-test method. No significant differences ($p = 0.057–0.993$) (Figure 3A) were observed between the treated and non-treated groups for each strain, indicating that the chosen PMA conditions would not inhibit the qPCR amplification of viable cells. The 25 different strains of *L. paracasei*, each with a concentration of 10^8 CFU/mL, underwent heat inactivation to obtain total dead cells. Subsequently, the efficiency of PMA treatment was further evaluated. The inhibition efficiencies of PMA treatment on qPCR amplification of dead cells from each *L. paracasei* strain were calculated. As depicted in Figure 3B, the inhibition efficiency of each strain ranged from 99.96 to 100.00%. This remarkable inhibition indicates that qPCR amplification of DNA originating from dead cells was nearly completely suppressed. These results demonstrate that the chosen PMA conditions are optimal for distinguishing between viable and dead cells of *L. paracasei*, including the type strain, commercial strains, etc.

3.3 Conversion of C_q values to viable cell numbers

For the qPCR method, C_q values are the direct results obtained. To determine viable cell numbers, a relationship between C_q values and viable cell numbers should be established (Figure 4). The slope of the linear equation between the C_q values of individual strains and the logarithm of the number of viable bacteria is −3.22, and R^2 is 0.997. The amplification efficiency (*E*) was calculated as 104.48% using the formula $E = 10^{(-1/\text{slope})} - 1$ (Rogers-Broadway and Karteris, 2015; Svec et al., 2015). This efficiency value is deemed acceptable as it falls within the range of 90 to 110% (Ruijter et al., 2009), indicating that the newly designed primer also exhibits good sensitivity and can be utilized for the detection of *L. paracasei* in probiotic products. Through the utilization of the standard curve, it became feasible to convert the C_q values of *L. paracasei* samples into CFU equivalent cells.

3.4 Limit of quantification of the established PMA-qPCR method

To ascertain the limit of quantification of the established PMA-qPCR method, three composite samples were prepared, each containing viable *L. paracasei* and non-viable *L. rhamnosus*. In each sample, the concentration of non-viable *L. rhamnosus* remained approximately 10^8 CFU/mL, while viable *L. paracasei* concentrations were 2.57×10^3 CFU/mL, 7.30×10^3 CFU/mL, and 1.54×10^4 CFU/mL, respectively. Five replicates were run for each sample. The average C_q values corresponding to 7.30×10^3 CFU/mL and 1.54×10^4 CFU/mL were 29.54 ± 0.21 and 28.49 ± 0.04, respectively, both falling below 30 and within the range of the standard curve (Figure 4). When the concentration of *L. paracasei* were 2.57×10^3 CFU/mL, the average C_q value was 31.01 ± 0.23, which was close to the negative control and beyond the range of the standard curve. Therefore, the limit of quantification for the PMA-qPCR method was established as 7.30×10^3 CFU/mL.

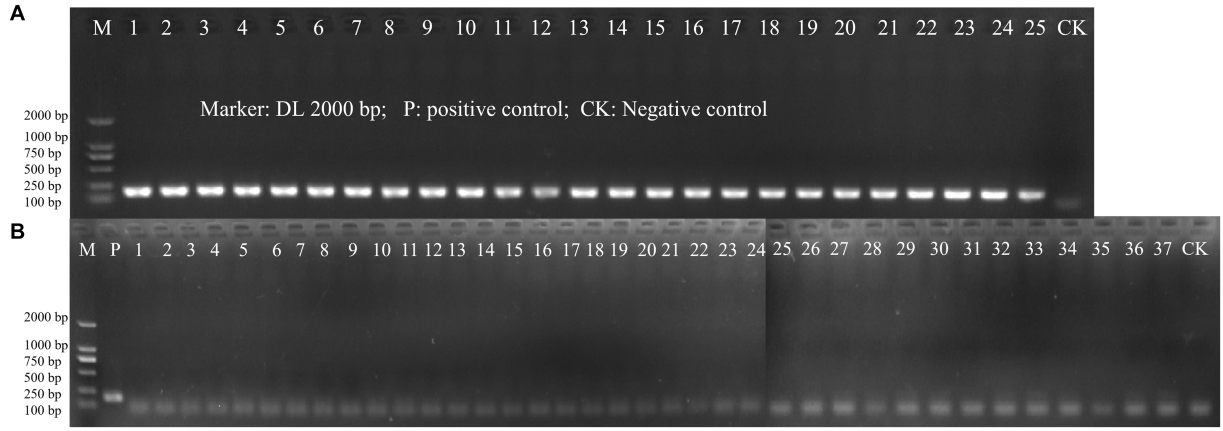

FIGURE 2
The PCR amplification of inclusivity and exclusivity assay visualized on an agarose gel. **(A)** Inclusivity assay with 25 target strains (Table 1); **(B)** Exclusivity assay with 37 non-target strains (Table 1).

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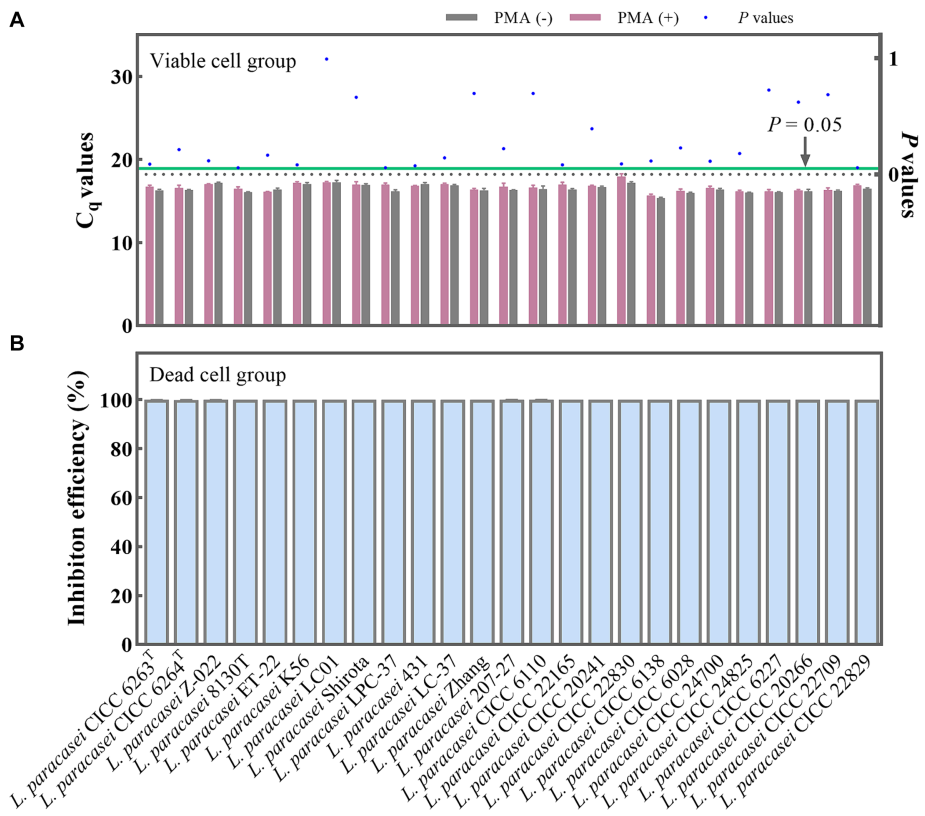

FIGURE 3
Optimal PMA treatment conditions evaluation. **(A)** Assessment of the impact of PMA treatment on qPCR amplification of viable *L. paracasei* cells from 25 different strains. PMA (+) and PMA (–) represent samples treated with and without PMA, respectively. **(B)** Determination of the inhibition efficiency of PMA on dead *L. paracasei* cells.

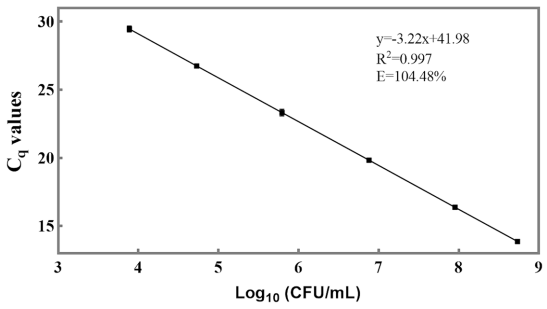

FIGURE 4
Sensitivity of the newly designed primer for quantification of viable *L. paracasei* by qPCR. The standard curve was constructed using the average C_q values derived from 10-fold serial dilutions of target DNA extracted from an equal proportion of *L. paracasei* and the logarithm of the concentration of culturable *L. paracasei*.

3.5 Linear, and range of the established PMA-qPCR method

In this study, compound samples comprising viable *L. paracasei* and deceased *L. rhamnosus* were prepared, with total bacterial concentrations of approximately 10^8 CFU/mL, while viable *L. paracasei* numbers ranged from 10^3 to 10^8 CFU/mL. Firstly, the accuracy of the established PMA-qPCR method within these range was validated. The accuracy profile facilitates the assessment of both accuracy and precision by comparing the measured values with their corresponding theoretical values (ISO 16140-2, 2016). Typically, an acceptability limit (AL) of ±0.5 Log_{10} units is employed to delineate the permissible difference between the measured and theoretical values. This AL represents the maximum allowable deviation of the method from the theoretical values.

The results obtained from PMA-qPCR detection were statistically analyzed according to ISO 16140-2 (2016) (*E*). A graphical representation of computed results was created, with the horizontal axis depicting theoretical values in Log_{10} units and the vertical axis illustrating the bias (Figure 5). Straight lines connect the upper and lower tolerance-interval limits to interpolate the behavior of the limits across different levels of the validation samples. The horizontal line denotes the theoretical values, while any disparities between theoretical values and average concentration levels of *L. paracasei* are depicted by black dots. In the absence of biases, these recovered values align with the horizontal theoretical line. Additionally, AL is indicated by two dashed horizontal lines, and β-ETI (expected tolerance interval) limits are shown as broken full lines. According to Figure 5, the bias between theoretical and measured values for each viable cell concentration was 0.05, 0.04, 0.06, −0.09, −0.05, and 0.27 Log_{10} units. Importantly, all these biases are all within the acceptable limits (± 0.5 Log_{10} units). This demonstrated the compelling evidence for the accuracy of the PMA-qPCR method in quantifying viable *L. paracasei* within 10^3–10^8 CFU/mL. Furthermore, five replicates were conducted

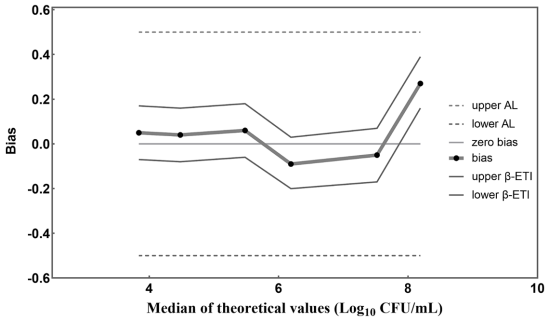

FIGURE 5
Accuracy profile for different concentrations of *L. paracasei* detected by the established PMA-qPCR method. The β-ETI represents the interval within which the expected proportion of future results will fall, with β set at 80% in accordance with ISO 16140-2:2016 (E) for this study. The bias (Bi) was determined as the absolute difference between the medians of the theoretical and measured values.

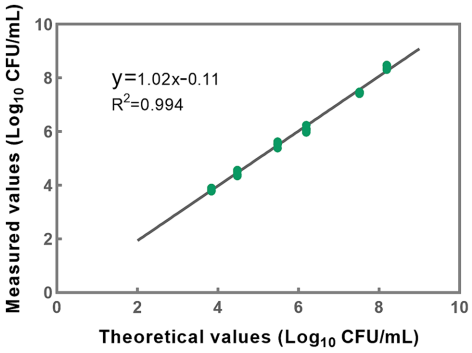

FIGURE 6
A linear correlation between theoretical and measured values was effectively established within the concentration range of 10^3 to 10^8 CFU/mL for *L. paracasei*. Each data point presented herein is derived from the analysis of five replicates.

for each sample to evaluate the precision of the established PMA-qPCR method. The coefficient of variation (CV) for the Log_{10}-transformed viable cell counts were calculated. Low CV values of 0.97, 0.39, 1.66, 1.61, 2.11, and 1.74%, underscored the robustness of the PMA-qPCR method in accurately quantifying viable cell counts.

Based on the accuracy results, a linear regression analysis was conducted to correlate the theoretical values with the measured values (Figure 6). The resulting correlation coefficient (R^2) of the fitted curve was determined to be 0.994, indicating a strong linear relationship between the measured and theoretical values within the range of 10^3 to 10^8 CFU/mL (Figure 6). These findings substantiate the method's reliability and accuracy in quantifying bacterial concentrations within the specified range. However, it is noteworthy that the upper limit of the quantitative range is set at 10^8 CFU/mL, reflecting the limit of detection rather than necessarily delineating the genuine upper threshold of the developed methodology. In cases where bacterial densities surpass this concentration, the total bacterial density can be adjusted to 10^8 CFU/mL, following which the optimal conditions for PMA treatment can be applied to the sample.

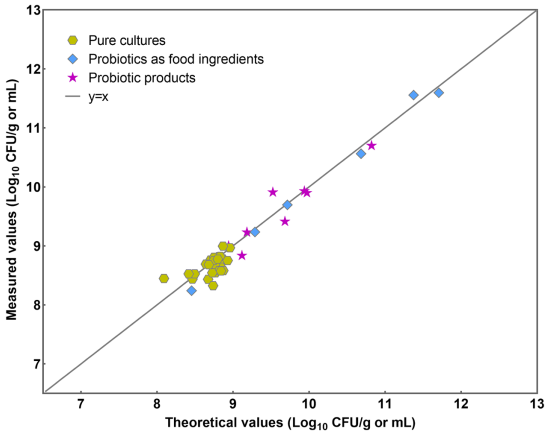

FIGURE 7
Scatter plot of theoretical values versus measured results.

3.6 Applications of PMA-qPCR method to different sample types

The implemented PMA-qPCR method was employed across three distinct sample categories: pure cultures, probiotics as food constituents, and probiotic products. Pure cultures denote samples exclusively containing bacteria devoid of any matrix influence. Probiotic as food ingredients encompass samples containing either single strains of *L. paracasei* or multiple bacterial strains, with little matrix influence. Probiotic products encompass samples containing multiple bacterial strains along with complex matrix effects. Initially, a y = x line was plotted to visualize the level of agreement between the theoretical and measured values (Figure 7). Most data points closely conformed to the line for each analyzed sample, indicating a high level of concordance between the theoretical and measured values.

The results were further analyzed using the Bland–Altman method in accordance with ISO 16140-2 (2016) (*E*). Individual sample differences against the mean values were plotted, showing the line of identity (zero difference), the line of bias, and the upper and lower 95% confidence limits (CLs) of agreement for the bias (Figure 8). The mean bias of the 39 samples was 0.058 Log_{10}, demonstrating high agreement between PMA-qPCR measured and theoretical values. The lower and upper limits of agreement were −0.272 and 0.388 Log_{10}. The 95% confidence limits were −0.366 to −0.178 Log_{10} and 0.294 to 0.482 Log_{10}, respectively (Figure 8). The differences between the measured and theoretical values of 38 samples consistently fell within the 95% confidence interval defined by the CLs. Only one probiotic sample exceeded the CLs, aligning with ISO 16140, which allows no more than 1 out of 20 data points to exceed the CLs. For this outlying sample, the difference between the measured and theoretical values was −0.385 Log_{10}, still within ±0.5 Log_{10}. This demonstrates the accuracy and suitability of the PMA-qPCR method for quantifying viable *L. paracasei*. A *T*-test was utilized to assess the significance of differences between the theoretical and measured values of all 39 samples. The resulting *p* value of 0.76 ($p > 0.05$) indicates no significant difference between the theoretical and measured groups within the

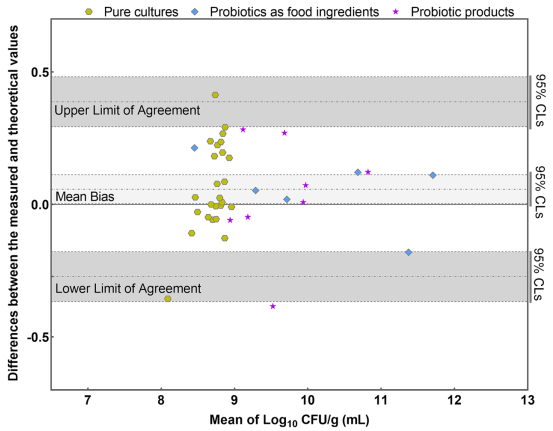

FIGURE 8
Bland–Altman plots comparing the quantitative values of PMA-qPCR measured values with those of theoretical values for 39 samples. (mean difference = 0.058 Log_{10}; SD = 0.168 Log_{10}).

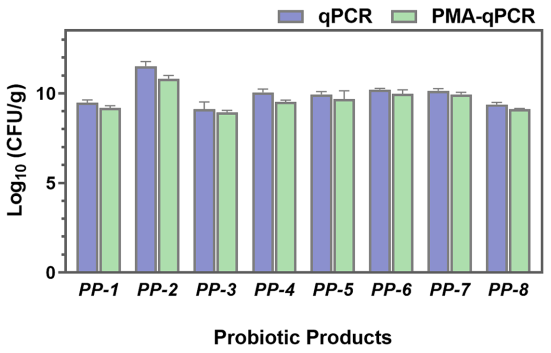

FIGURE 9
The total cell numbers (qPCR) and viable cell numbers (PMA-qPCR) of *L. paracasei* in eight probiotic products (PP-1 to PP-8) were assessed.

sample set. This underscores the precision and reliability of the PMA-qPCR method for detecting viable cells across various applications, including pure cultures, probiotics as food ingredients, and composite probiotic products.

To investigate the total, viable, and dead cells in probiotic products, both PMA-treated and untreated samples were analyzed using qPCR. As shown in Figure 9, the total cell numbers (qPCR) of *L. paracasei* were higher than the viable cell numbers (PMA-qPCR). Significant differences were particularly observed in PP-2 and PP-4 probiotic products, with p values lower than 0.05. These findings indicate the presence of dead or membrane-damaged cells in the probiotic products. The PMA-qPCR method effectively excluded dead or membrane-damaged cells, providing an accurate count of viable *L. paracasei* cells in composite probiotics.

4 Discussion

The specificity of the newly designed primer for *L. paracasei* is paramount for ensuring precise detection and quantification of this probiotic strain, particularly within complex sample matrices (Broeders et al., 2014; Garrido-Maestu et al., 2018), significantly enhancing probiotic product manufacturing and quality control. Our primer design methodology is founded on a meticulous analysis of genomic data, leveraging 176 publicly available *L. paracasei* genomes subjected to rigorous re-annotation to ensure data consistency. This comprehensive approach facilitated an accurate gene presence/absence analysis, crucial for identifying core genes specific to *L. paracasei*, ensuring the target sequence is present across all strains while absent in non-target organisms. Utilizing CD-HIT software for gene presence/absence analysis at the protein level enabled precise clustering of similar proteins, identifying conserved gene families with high confidence. Stringent thresholds (50% pairwise identity and 0.7 length difference) ensured the inclusion of genuinely conserved genes, enhancing the specificity of the target. Following identification of potential core genes, their nucleotide sequences underwent BLASTN analysis against the NCBI Nucleotide collection to validate their specificity to *L. paracasei* at the nucleotide level, eliminating significant similarities with non-target species. This dual-level verification, examining both protein and nucleotide levels, provided a robust foundation for designing highly specific primers. The selection of the gene encoding Alkaline Shock Protein 23 was based on its consistent presence across *L. paracasei* strains and absence in related species, with its stability and crucial role in stress response mechanisms contributing to its conservation as an ideal marker for species-specific detection. Validation processes further reinforced the primer's specificity, with *in silico* tests using Primer-BLAST against extensive whole-genome sequences from target and non-target strains confirming the absence of significant similarity with non-target organisms. Practical inclusivity and exclusivity tests involving 25 *L. paracasei* strains and 37 non-target strains provided empirical evidence of the primer's accurate discrimination between target and non-target DNA (Figure 2), underscoring the primer's reliability and specificity for diverse applications in probiotic research and product development.

The efficiency of DNA extraction is pivotal for accurately quantifying target microorganisms, with minimizing DNA loss being a crucial aspect in maintaining precision. Although commercial DNA extraction kits are widely used, concerns regarding DNA loss during column purification have been frequently documented, often attributed to the competitive binding of humic substances to silica membranes (Lloyd et al., 2010; Natarajan et al., 2016; Plotka et al., 2017). In this study, a streamlined approach to DNA extraction was employed, utilizing a one-step cell lysis method with a bead mill homogenizer due to its rapidity and ease of operation. This simplification of procedures significantly reduces the potential for DNA loss, thereby enhancing the reliability of downstream analyses. Moreover, maintaining consistent lysis conditions, including speed and duration, is essential for ensuring the stability and reproducibility of DNA quality. Effective DNA extraction is paramount as it lays the groundwork for establishing a robust correlation ($R^2 = 0.997$) between C_q values and viable cell numbers, as depicted in Figure 4. Consequently, the resulting standard curve facilitates the translation of DNA quantities into viable cell numbers (Ilha et al., 2016; Yang et al., 2021). Furthermore, the efficacy of this method extends across various sample types, including pure cultures, probiotics as food

ingredients, and compound probiotic products, as evidenced by the high degree of consistency between theoretical and PMA-qPCR measured values of viable *L. paracasei* (Figures 7, 8). Previous research has also demonstrated the accuracy of the bead-beating method in quantifying viable *L. rhamnosus* cell numbers (Guo et al., 2024), further affirming the advantages of this DNA extraction approach. Therefore, the bead-beating method emerges as a highly recommended tool for obtaining DNA followed by qPCR amplification, facilitating accurate viable cell counts and enhancing the reliability of microbial analysis in diverse sample matrices.

The PMA treatment condition is a crucial parameter for accurate viable cell counting, as it directly impacts the efficiency of dead cell or extracellular DNA filtration while leaving live cells unaffected (Nocker et al., 2006; Fujimoto and Watanabe, 2013; Yang et al., 2021). In this study, the chosen PMA treatment conditions (50 μM/L, 5 min, 15 min) (Desfossés-Foucault et al., 2012; Villarreal et al., 2013) were validated as optimal for distinguishing between viable and dead cells under bacterial concentrations of 10^8 CFU/mL (Figure 3). A notable aspect of this study is the comprehensive collection of 25 distinct strains of *L. paracasei* to confirm the PMA treatment conditions, which is rare in previous PMA-qPCR studies. The results presented in Figure 3 demonstrate the wide applicability of the optimal PMA treatment conditions across various strain types, including both laboratory strains and commercial ones. This highlights the robustness and versatility of the selected PMA treatment protocol for accurately distinguishing viable cells from dead ones, regardless of strain origin or source.

The developed PMA-qPCR method demonstrates high accuracy in quantifying viable *L. paracasei* across a broad range of concentrations and sample types. The strong correlation coefficient ($R^2 = 0.994$) observed in the linear analysis within the concentration range of 10^3 to 10^8 CFU/mL (Figure 6) underscores the method's reliability in quantifying viable cell numbers. This high degree of linearity indicates the precise ability of the PMA-qPCR method to maintain accuracy and consistency across varying levels of viable cells. Furthermore, the established PMA-qPCR method underwent validation across three dimensions of sample types: pure cultures, probiotics as food ingredients, and compound probiotic products (Figures 7, 8). For instance, in compound probiotic products containing multiple bacterial strains, such as *B. animals* subsp. *lactis*, *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. fermentum*, *L. gasseri*, *B. breve*, *L. delbrueckii* subsp. *bulgaricus*, *L. reuteri* and others, the difference between theoretical and PMA-qPCR measured values was −0.059 Log_{10} (Figure 8), demonstrating the high accuracy of the method in quantifying viable *L. paracasei* within complex probiotics. The accurate results obtained in this study further validate the specificity of the primers, affirming their capability to detect *L. paracasei* without interference from other non-target bacteria. Additionally, the compound probiotic products contained various matrix components such as resistant dextrin, erythritol, maltitol, polydextrose, or fructooligosaccharides. The successful quantification of viable *L. paracasei* within these complex matrices highlights the robustness and tolerance of the PMA treatment conditions and qPCR reaction to diverse sample compositions. Although utilizing the DNA of the target strain for preparing the standard curve theoretically enhances accuracy by tailoring the qPCR amplification process (Ilha et al., 2016; Odooli et al., 2018; Scariot et al., 2018), practical scenarios often involve unknown or unobtainable target bacterial strains. The high accuracy demonstrated in this study suggests the feasibility of applying a standard curve derived from the type strain to other strains within the same species. The comprehensive validation process and results provide a thorough overview confirming the suitability of the key parameters chosen, including DNA extraction, PMA treatment conditions, and standard curve preparation, for establishing the PMA-qPCR method. This underscores the method's versatility and suitability for assessing bacterial viability in real-world samples with diverse compositions.

This study further demonstrated the presence of dead/damaged cells in probiotic products, as illustrated in Figure 9. During production, storage, and distribution, probiotic products are subjected to various biological, physical, and chemical stresses. These stresses can damage the probiotic cells, resulting in a microbial population comprising viable, dead, and stressed/damaged cells, including those in a viable but non-culturable (VBNC) state (Fiore et al., 2020; Fusco et al., 2021). The presence of dead and VBNC cells may impact the quality and efficacy of probiotic products (Foglia et al., 2020; Fusco et al., 2021). Therefore, accurate quantification of viable cells in probiotic products is crucial to ensure their effectiveness. The PMA-qPCR method represents a significant advancement in the accurate identification of probiotics and the quantification of viable bacteria. Both the findings of this study and previous research endeavors have unequivocally demonstrated the method's accuracy and stability in achieving precise identification of target strains and enumeration of viable bacteria (Berezhnaya et al., 2021; Yang et al., 2021; Guo et al., 2024). Furthermore, the method's versatility allows for the simultaneous detection of multiple bacteria under the same PMA-qPCR conditions, thereby enhancing efficiency. This capability holds profound implications for consistency control in enterprise production processes and market supervision of compound probiotics. The simplicity and rapidity of the PMA-qPCR method make it highly conducive to standardized research and application. Its feasibility in routine use provides invaluable technical support for ensuring the quality and safety of probiotic products. By offering a reliable means of quantifying viable bacteria, the method contributes to enhancing the transparency and accountability of probiotic product labeling, thereby bolstering consumer confidence.

5 Conclusion

In conclusion, this study has successfully developed and validated a precise PMA-qPCR method for quantifying viable *L. paracasei* in probiotics. The specificity of the newly designed primers was rigorously evaluated, demonstrating high specificity for *L. paracasei* detection across various strains. Optimal PMA treatment conditions were established to effectively distinguish between viable and dead cells, ensuring accurate quantification of viable *L. paracasei*. The method exhibited a strong linear relationship between C_q values and viable cell numbers, with high amplification efficiency and a quantification limit of 7.30×10^3 CFU/mL. The accuracy and precision of the method were confirmed, with biases within acceptable limits across various concentrations of viable cells. Moreover, the method demonstrated robustness and reliability across different sample types, including pure cultures, probiotics as food ingredients, and compound probiotic products. Its simplicity, speed, and consistency make it indispensable for standardized research, offering vital technical

support for quality assurance in probiotic product manufacturing. Its implementation in routine testing procedures can enhance transparency and accountability in the probiotics industry, ultimately bolstering consumer confidence and satisfaction.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

LG: Visualization, Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. XZe: Writing – original draft, Writing – review & editing, Project administration, Methodology, Funding acquisition. YJia: Writing – review & editing, Writing – original draft, Methodology. CS: Writing – review & editing, Methodology, Data curation. XZh: Writing – review & editing, Data curation. ZS: Writing – review & editing, Software, Methodology, Data curation. SM: Visualization, Writing – review & editing, Software, Methodology. YL: Writing – review & editing, Project administration, Methodology. YG: Writing – review & editing, Project administration, Methodology. YJin: Writing – review & editing, Methodology. SY: Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Conflict of interest

LG, YJia, CS, ZS, SM, YL, YG, YJin and SY were employed by China National Research Institute of Food and Fermentation Industries Co., LTD.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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