Probiotics and Antimicrobial Proteins
https://doi.org/10.1007/s12602-026-11100-z

RESEARCH

Multi-Analytical Approach for the Quality and Functional Evaluation of Probiotic Formulations: The Case of *Lactobacillus acidophilus* LA-5®Commercial Products

Margherita Bottaioli[1] · Viola Termine[2] · Aurora Botti[3] · Beatrice Vitali[3] · Diego Mora[2] · Luca Laghi[4] · Lorenzo Morelli[1] · Loris Pinto[5] · Alessia Marzulli[5] · Federico Baruzzi[5] · Alessandra Fontana[1]

Received: 18 March 2026 / Accepted: 8 June 2026

Abstract

The increasing use of probiotics in dietary supplements and functional foods highlights the need for robust analytical strategies to assess microbial viability, functional performance, and safety. Conventional culture-based methods may underestimate probiotic potential, particularly in multi-strain formulations. In this study, a multi-analytical framework was applied to evaluate four commercial formulations containing *Lactobacillus acidophilus* LA-5®, differing in composition and delivery technology. A combination of culture-dependent and culture-independent approaches was employed, including plate counting, flow cytometry (FC), whole-genome sequencing (WGS), digital droplet PCR (ddPCR), antimicrobial activity assays, in vitro gastrointestinal digestion, and metabolomic profiling. WGS confirmed the taxonomic identity of all isolates as *L. acidophilus* and the absence of genes of concern. FC revealed that viable cells accounted for 59–88% of total fluorescent units, exceeding culturable counts and indicating the presence of viable but non-culturable populations. ddPCR enabled strain-level quantification of taxonomic and selected functional genes. Functional assays demonstrated formulation- and dose-dependent antimicrobial activity against selected enteropathogens, with up to 40–50% growth inhibition at higher probiotic cell densities. In vitro digestion experiments showed marked differences in gastrointestinal resistance, with lyophilized products undergoing reductions of up to 5–6 log units during the gastric phase, whereas fresh cells and fermented matrices retained viable populations after intestinal digestion. ^{1}H-NMR-based metabolomic analysis of post-digestion samples further revealed formulation-dependent metabolic fingerprints. This integrated approach provides a biologically relevant framework linking probiotic viability, functionality, and metabolic behavior, supporting improved quality assessment of commercial products and highlighting the role of formulation and matrix effects in determining probiotic performance.

Keywords Taxonomic Identity · Flow Cytometry · ddPCR · Antimicrobial Activity · Gastrointestinal Resistance · Metabolomics

Margherita Bottaioli and Viola Termine Co–first authors.

✉ Alessandra Fontana
alessandra.fontana@unicatt.it

[1] Department for Sustainable Food Process (DiSTAS), Catholic University of the Sacred Heart, Via S. L. Bissolati 74, Cremona 26100, Italy

[2] Department of Food Environmental and Nutritional Sciences (DeFENS), University of Milan, Via G. Celoria 2, Via Mangiagalli 25, Milano 20133, Italy

[3] Department of Pharmacy and Biotechnology (FaBiT), University of Bologna, Via P. Gobetti 87, Bologna 40129, Italy

[4] Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Piazza G. Goidanich 60, Cesena 47521, Italy

[5] Institute of Sciences of Food Production, National Research Council of Italy (CNR-ISPA), Via G. Amendola 122/O, Bari 70126, Italy

Published online: 13 June 2026

Introduction

The growing popularity of probiotic products has led to a significant expansion of the global market, with a wide range of dietary supplements and functional foods available to consumers. However, this rapid growth has raised important concerns about the quality and safety of such products. The lack of standardization in production and quality assessment has made it difficult for consumers and health professionals to distinguish between high-quality products and those that are potentially ineffective [1].

According to the definition provided by the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [2]. However, the quality of probiotic products is determined by several critical factors, including accurate strain identification, genetic and phenotypic stability, survival during gastrointestinal transit, and the ability to exert functional effects at the target site [3]. Notably, strain-specific variability, even within the same species, can substantially influence these properties and, consequently, clinical efficacy.

Safety assessment represents an additional cornerstone of probiotic evaluation and requires verification of strain identity, purity, and the absence of harmful traits, including transferable antibiotic resistance. This aspect is particularly relevant for vulnerable populations, where host-related factors may increase susceptibility to adverse effects. Although international guidelines [4] and EFSA guidance documents provide a framework for the quality and safety assessment of microorganisms used in the food chain [5], probiotic evaluation still requires particular attention, as safety, technological, and functional traits are often strain-specific. Accurate strain identification and detection are consequently critical, particularly in complex food matrices and multi-strain formulations. The International Dairy Federation (IDF) has also emphasized the significance of reliable methods for probiotic strain-level identification [6], even though current methodological guidelines may quickly become outdated due to the continuous advancement of molecular and genomic technologies [7].

Despite increasing regulatory attention, the absence of harmonized analytical frameworks remains a major limitation in the probiotic field. Current evaluation strategies often focus on isolated parameters and rely heavily on conventional culture-based methods, which may underestimate viable cell populations, and fail to provide information on genomic stability, safety-related traits, and functional attributes [1, 8, 9]. Indeed, bacterial populations may include cells that remain viable and metabolically active but are unable to form colonies on laboratory media due to the viable but non-culturable (VBNC) status [10]. Since live/dead cell counting by flow cytometry relies on cell membrane integrity, while plate counting relies on cell growth on agar plates, this can result in counting differences between the two methods, usually with higher quantifications in flow cytometry. Although these limitations are well recognized, fewer studies have simultaneously evaluated how commercial formulations containing the same probiotic strain may differ in viability status, functional activity, gastrointestinal resistance, and metabolic signatures. This limitation is further amplified by the growing complexity of probiotic products, including multi-strain formulations and incorporation into diverse food matrices, where interactions between microorganisms and formulation components can significantly affect stability and functionality [11]. These considerations underscore the need for integrated and standardized evaluation frameworks that combine scientific validation with quality control criteria and industrial performance metrics. Rather than representing a routine analysis to be applied in full to every commercial batch, such frameworks may serve as targeted tools for product development, advanced quality control, and regulatory assessment, particularly when conventional enumeration alone is insufficient to characterize complex probiotic formulations.

In response to these challenges, advanced analytical tools have been increasingly adopted to support probiotic characterization, viability assessment, and safety evaluation. Molecular approaches, viability profiling techniques, functional assays, metabolomics, and in vitro digestion models have contributed to a more comprehensive understanding of probiotic performance under conditions relevant to production, storage, and gastrointestinal transit. Among these approaches, metabolomics represents a valuable complement to microbial taxonomic, functional, and viability assessments, as metabolite profiles reflect the biochemical activity and physiological state of a system and provide insight into the microbial phenotype and its response to environmental conditions [12, 13]. Untargeted metabolomic approaches, in particular, allow the parallel detection of a broad range of metabolites, enabling the identification of subtle biochemical differences and functional signatures that may remain undetected using exclusively taxonomic or targeted methods. Previous studies have shown that metabolomics can reveal strain-dependent metabolic signatures in fermented milk products, as well as metabolic differences among multi-strain probiotic formulations related to manufacturing conditions [14, 15]. However, these tools are often applied independently, and their integration into a coherent quality assessment framework remains limited.

Species belonging to the genus *Lactobacillus* have a long history of probiotic applications, including *Lactobacillus acidophilus* [16]. Accordingly, *L. acidophilus* LA-5®

was selected as a case study. This strain is widely used in commercial probiotic formulations and has been extensively characterized for its safety, metabolic adaptability, and tolerance to gastrointestinal conditions, supporting its relevance as a representative model for probiotic quality assessment [17].

The primary aim of this work was to establish a proof of concept for a targeted and modular workflow supporting the quality and functional assessment of probiotic formulations. To this end, four key quality criteria (QC) were evaluated: (QC-1) taxonomic identity, functional potential, and safety to ensure correct labeling, assessed by whole-genome sequencing (WGS) and digital droplet PCR (ddPCR); (QC-2) cell viability and quantification by flow cytometry; (QC-3) antimicrobial activity profiling in relation to product formulation and production technology; and (QC-4) culture-dependent assessment of probiotic viability and metabolic changes following co-administration with foods possessing their own microbial populations, using in vitro digestion assays and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Materials and Methods

Sample Collection

Four commercial probiotic products containing *Lactobacillus acidophilus* LA-5® were selected to represent different formulation types, delivery matrices, and production technologies (Table 1). Product 1 was a multi-strain orodispersible supplement marketed for supporting intestinal microbiota balance. Product 2 was a single-strain capsule supplement positioned for the maintenance of intestinal microbiota balance. Product 3 was a single-strain granulated preparation intended for use in the dairy sector and associated with digestive and intestinal microbiota support. Product 4 was a multi-strain fermented milk product marketed as a probiotic yogurt for supporting intestinal microbiota balance. Overall, the selected products were primarily positioned for intestinal microbiota and digestive support, providing the rationale for assessing viable cell status, gastrointestinal survival, and functional activities potentially related to probiotic performance. Antimicrobial activity was not intended to reflect a specific anti-infective claim but was included as a functional screening assay commonly used to explore potential inhibitory interactions associated with probiotic strains and formulations.

Isolation, Identification, and Enumeration of Viable Cells by Plate Counting

Selective media, including Homo-fermentative Hetero-fermentative Differential (HHD) medium (Biolife Italiana Srl), Transgalactosylated Oligosaccharide Propionate (TOS-propionate) agar (Merck Millipore, Billerica, MA, USA) supplemented with mupirocin (100 mg/L), de Man, Rogosa and Sharpe (MRS) medium (Difco™, BD, Sparks, MD, USA) supplemented with vancomycin (10 µg/mL), and MRS medium supplemented with sodium acetate (25 g/L) and oxgall bile (1.5 g/L), pH 5.4, were used for the isolation, identification, and enumeration of viable cells of the different bacterial strains present in the four commercial products. These media were used in combination with specific dilution buffers (Table 2 and Table S1) designed to minimize osmotic stress and cell aggregation, as previously reported [18]. Each product was left at room temperature for 30 min prior to serial ten-fold dilutions. The initial dilution was prepared by suspending 1 g of sample in 9 mL of Mitsuoka buffer at pH 6.5 (Tale S1) [19], followed by a 30 min incubation at room temperature and homogenization using a stomacher (2 min, 230 rpm). Subsequent dilutions were prepared in Buffered Peptone Water (BPW, Table S1) (Oxoid, Basingstoke, UK) at pH 7.0. Both buffers were pre-heated at

Table 1 Commercial products evaluated in the study and their microbial composition as retrieved by the product label

Commercial product	Type of formulation	Microbial species included	Claimed daily dose (log CFU)	Claimed concentration (log CFU g^{-1} or mL^{-1})
Product 1	Orodispersible, lyophilized, multi-strain	*Lactobacillus acidophilus* LA-5®	9.00	8.91
		Heyndrickxia coagulans	7.60	7.50
		Bifidobacterium animalis subsp. *lactis*	9.00	8.91
		Lacticaseibacillus paracasei	7.60	7.50
Product 2	Capsule, lyophilized, single-strain	*Lactobacillus acidophilus* LA-5®	9.00	8.92
Product 3	Granules, lyophilized, single-strain	*Lactobacillus acidophilus* LA-5®	NA*	11.00
Product 4	Fermented milk product, viable cells, multi-strain	*Lactobacillus acidophilus* LA-5®	8.00	6.00
		Bifidobacterium animalis subsp. *lactis*	9.30	7.20
		Lactobacillus delbrueckii subsp. *bulgaricus*	NA**	9.19
		Streptococcus thermophilus	NA**	7.15

*Not available: business-to-business product. **Not available: strains employed as starter cultures in the production of the fermented product

Table 2 Culture media and incubation conditions used for the cultivation of all the microorganisms present in each commercial product

Culture medium	Incubation conditions	Cultivable microorganisms
HHD agar	37 °C, 72 h, anaerobiosis 50 °C, 24 h, anaerobiosis***	*L. acidophilus* *L. paracasei* *H. coagulans*
TOS-propionate agar supplemented with mupirocine (100 mg/L*)	37 °C, 72 h, anaerobiosis	*Bifidobacterium* spp.
MRS agar supplemented with vancomycin (10 µg/µL)	37 °C, 72 h, anaerobiosis	vancomycin-resistant lactobacilli
MRS agar supplemented with sodium acetate (25 g/L) and oxgall bile (1.5 g/L), pH 5.4**	37 °C, 72 h, anaerobiosis	bile-resistant lactobacilli

* [22]; ** [23]; ***spores and vegetative cells of *H. coagulans* (Fig. S1)

40 °C for 30 min, prior to use. All analyses were performed in triplicate on the same batch of each product within the declared shelf life.

Colonies grown on selective media were first identified at the species level by Sanger sequencing of PCR amplicons targeting the 16 S rRNA gene and selected species-specific genetic markers, following total DNA extraction as previously described [19–21], and subsequently enumerated. Primer sequences and amplification conditions are reported in Table S2.

Absolute Quantification by Flow Cytometry

Flow cytometry (FC) was used for the absolute quantification of viable and non-viable cells in the four commercial probiotic products, following the ISO 19344:2015 [24] procedure with minor modifications. Samples were diluted in BPW (pH 7.0) to maintain an event rate between 2,000 and 5,000 events per second in unstained conditions. Staining was performed using 0.1 µM SYTO24™ (Thermo Scientific, Italy) and 0.2 µM propidium iodide (PI) (Sigma-Aldrich, Italy) in a final volume of 1 mL with PBS (pH 7.4), followed by incubation in the dark at 37 °C for 15 min. After incubation, 50 µL of CountBright™ Plus Absolute Counting Beads (49,000 beads/50 µL) were added prior to the quantification of active fluorescent units (AFU) and non-active fluorescent units (nAFU).

FC analysis was performed using a C6 Plus flow cytometer (BD Biosciences, Milan Italy) equipped with a 488 nm excitation laser. Thresholds were set at 3,000 for FSC and 1,000 for SSC. Green and red fluorescence signals were collected in channels FL1 (530 nm ± 30 nm) and FL3 (> 670 nm), respectively. All parameters were recorded on a logarithmic scale. Samples were acquired at a medium flow rate (35 µL/min), and the number of events in a total volume of 50 µL was recorded for each sample.

Data were analyzed using BD Accuri™ C6 Plus software version 1.0 (BD Biosciences, Milan, Italy). AFU/µL and nAFU/µL values were calculated according to the manufacturer instruction of CountBright™ Plus Absolute Counting Beads (Thermo Scientific, Italy).

All analyses were performed in triplicate on the same batch of each product within the declared shelf life. Statistically significant differences between absolute quantification and cultivability data for each product were evaluated using unpaired t-tests.

DNA Extraction from Isolated Pure Cultures, Whole-Genome Sequencing and Bioinformatic Analyses

Pure cultures of *L. acidophilus* LA-5®, isolated from each investigated product, were used to inoculate MRS broth and incubated overnight at 37 °C. Genomic DNA was extracted from 1 mL of liquid culture using the MasterPure™ Gram Positive DNA Purification Kit (Biosearch Technologies, Novato, CA, USA). DNA concentration was measured using a Qubit™ 4 Fluorometer with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and DNA quality was assessed by electrophoresis on a 0.8% agarose gel.

WGS was performed using both short- and long-read technologies, namely the Illumina NovaSeq 6000 platform (paired-end 250 bp) and the PacBio Sequel II (CLR mode), respectively. Raw short reads were quality-filtered and adapters were removed using Trimmomatic (v0.39) [25]. Raw long reads in.*bam* format were converted to.*fastq* using SAMtools [26]. Hybrid genome assembly was performed using Unicycler (v0.5.1) [27] to obtain complete genome sequences. Assembly quality was evaluated using QUAST (v5.3.0) [28].

Taxonomic identification was performed using average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) through OAT [29] and TYGS [30], respectively. The presence of plasmids and genes of concern, including antibiotic resistance genes (ARGs) and virulence factors (VFs), was assessed using ABRicate (v1.0.1) [31], as previously described [32], applying EFSA thresholds (%identity ≥ 80% and %coverage ≥ 70%) [33].

DNA Extraction from Commercial Products, Primer Design and ddPCR Assay Development

One gram of each sample was suspended in 9 ml of BPW (pH 7.0) and left at room temperature for 30 min, followed by vortex mixing. From this suspension, 1 mL was collected for DNA extraction using a bead-beating FastPrep-24®

system (MP Biomedicals, USA) with Lysing Matrix E tubes (6.5 m/s for 50 s). Samples were then centrifuged at 12,000 × g for 15 min, and the supernatant was purified using the Genomic DNA Clean & Concentrator™−10 kit (Zymo Research, Irvine, CA, USA). DNA concentration and quality were assessed as previously described.

The six primer pairs used for the taxonomic and functional assessment of *L. acidophilus* LA-5® in the four probiotic products are reported in Table 3. New primers were designed using Geneious Prime software (version 2025.2.1) based on the four complete genome sequences. All primer sets targeted single-copy genes.

ddPCR was performed using the QX200™ Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). Primer concentrations and annealing temperatures are reported in Table 3. Thermal cycling conditions included an initial enzyme activation step at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s and annealing at the optimized primer-specific temperature. The amplification ended with a signal stabilization step at 4 °C for 5 min and 90 °C for 5 min.

All reactions were carried out using QX200™ ddPCR™ EvaGreen Supermix (1x) (Bio-Rad, Hercules, CA, USA) in a final volume of 20 µL. Singleplex assays were performed for the 16–23 S region and *bsh*2 gene, whereas duplex assays were performed for *bsh*1/*cIII* and *cAsor*/*slpA* gene pairs. For each assay, *L. acidophilus* DSM 20,079^T was used as a positive control, while nuclease-free water was used as a no-template control (NTC). For multi-strain products (Products 1 and 4), DNA from non-target strains was included as additional negative controls (Product 1: *B. animalis* subsp. *lactis*, *L. paracasei* subsp. *paracasei*, *H. coagulans*; Product 4: *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *B. animalis* subsp. *lactis*). Each assay was performed in triplicate, and a threshold of 10,000 droplets was set for statistically reliable quantification. Droplets were analyzed using a QX200 Droplet Reader and QuantaSoft™ Software (version 2.3.1). Results were expressed as gene copies × μL^{-1}.

Antimicrobial Activity Testing

The antimicrobial activity of the four probiotic products containing *L. acidophilus* LA-5® was evaluated using cell-free supernatants (CFS) obtained from the samples. CFS were prepared following the protocol described by Parolin et al. [35], with minor modifications. Briefly, probiotic formulations were resuspended in 10 mL of either sterile peptone water (1 g L^{-1} peptone, 8.5 g L^{-1} NaCl) or simulated intestinal fluid (SIF; 0.1% pancreatin, 0.15% oxgall, pH 7), and incubated under anaerobic conditions at 37 °C for 0, 5, and 15 h at two initial concentrations of *L. acidophilus* LA-5® (10^8 and 10^5 CFU mL^{-1}). All reagents, unless otherwise specified, were purchased from Merck (Milan, Italy). Peptone water was sterilized by autoclaving at 110 °C for 30 min, while SIF was sterilized by filtration through 0.22 µm polyethersulfone (PES) membrane filters (Membrane Solutions, LLC, Auburn, WA, USA).

Based on the microbial load declared on product labels (confirmed by plate counting), an equivalent of 10^9 CFU was used for each sample; for multi-strain products, the content of *L. acidophilus* LA-5® was used as a reference. For Product 4, containing 10^6 CFU mL^{-1} of LA-5®, only the lower concentration (10^5 CFU mL^{-1}) was tested. For all samples prepared at the lower concentration, appropriate dilutions were performed in physiological saline supplemented with L-cysteine.

Samples were incubated in anaerobic jars with GasPak™ EZ (BD, Milan, Italy). At each time point, CFS were collected by centrifugation at 5,000 × g for 10 min at 4 °C (Sartorius Centrisart® D-16 C, Sartorius, Goettingen, Germany) and filtered through 0.2 µm membrane filters. Supernatants were stored at −20 °C until use.

Table 3 Primers pairs and ddPCR assays characteristics

Name primer	Target gene	Sequence (5'−3')	Primer concentration (nM)	Amplicon (bp)	Annealing temperature (°C)	ddPCR assay	Reference
AcidophilusF AcidophilusR	16–23 S	CCTTTCTAAGGAAGCGAAGGAT ACGCTTGGTATTCCAAATCGC	220	129	58	Singleplex	[34]
bsh2F bsh2R	Choloyglycine Hydrolase bsh2	GCGGACGTTACTCCACATA GGAGTGTGTGCCAAGACAA	135	152	55	Singleplex	This study
cIIIF cIIIR	Class III Bacteriocins	CCGGTCATTACTAAGTGAGG GATGCACCATGTAGTAGCAC	45	156	59	Duplex*	This study
bsh1F bsh1R	Choloyglycine Hydrolase bsh1	GAAGAGAGGAGGCTTGCATT GTATGGCCGGACTCAACTAT	135	195			
slpAF slpAR	Pore forming S-layer protein	ACTGTTAGCGCTGCTACTAC CCAGTAAGGTTACCGGCAAT	35	152	59	Duplex**	This study
cAsorF cAsorR	Class A sortase	GGCCTGCAAGTGGATAATTC CTGCACGATCACAGGTTAAG	135	174			

*_bsh_1 and _cIII_ genes were both quantified in the first duplex assay; **_cAsor_ and _slpA_ genes were both quantified in the second duplex assay

Antimicrobial activity was assessed against three intestinal pathogens: enterotoxigenic *Escherichia coli* H10407, *Salmonella choleraesuis* serovar Typhimurium, and *Yersinia enterocolitica*, following Giordani et al. [36–39]. All strains were obtained from the Department of Pharmacy and Biotechnology, University of Bologna.

Pathogens were pre-cultured aerobically at 37 °C for 24 h in Tryptic Soy Broth (TSB; BBL, Milan, Italy) for *E. coli* and *Salmonella*, or Brain Heart Infusion (BHI; BBL, Milan, Italy) for *Yersinia*. Working suspensions were prepared by calibrating bacterial density based on OD_{600} measurements to obtain final concentrations of 2×10^5 CFU mL^{-1} for *E. coli* and Salmonella, and 2×10^6 CFU mL^{-1} for *Yersinia*.

Antimicrobial assays were performed in 96-well flat-bottom plates (Corning Inc., Pisa, Italy). Aliquots of 50 µL of pathogen suspension was mixed with 50 µL of CFS (in triplicate). No pH adjustment or buffering was applied to the pathogens growth media during incubation. Controls included sterile medium blanks (50 µL of SIF or peptone water plus 50 µL of pathogen growth medium) and pathogen growth controls (50 µL of pathogen suspension plus 50 µL of sterile culture medium). Plates were incubated aerobically at 37 °C for 24 h (*E. coli* and *Salmonella*) or 48 h (*Yersinia*). Bacterial growth was measured by OD_{600} using a microplate reader (EnSpire Multimode Plate Reader, PerkinElmer Inc., Waltham, MA, USA). Reduction in OD_{600} relative to growth controls was considered indicative of antimicrobial activity. Statistical analysis was performed using two-way ANOVA followed by Bonferroni correction.

In Vitro Static Digestion Assays

The four probiotic formulations were subjected to in vitro static digestion according to the INFOGEST protocol [40]. Lyophilized formulations were resuspended in phosphate-buffered saline (PBS) or in two food model systems (commercial UHT apple juice and yogurt drink) used as probiotic carriers. Preliminary tests showed that the viability of *L. acidophilus* LA-5® in lyophilized formulations resuspended in PBS or food model systems did not change after a rehydration time of 30 min (data not shown). Therefore, lyophilized formulations (Products 1, 2, and 3) were directly resuspended in PBS or in the food model systems prior to digestion. The multi-strain fermented milk product (Product 4) was as such, since *L. acidophilus* LA-5® was already present in the corresponding food matrix. Fresh cells (48 h cultures) of *L. acidophilus* LA-5® suspended in sterile saline solution (9 g L^{-1} NaCl) were used as control in each digestion experiment using PBS or food model systems as carriers.

Based on label information, confirmed by viability assays, probiotic formulations were prepared to obtain an initial *L. acidophilus* LA-5® cell density of 8.00 ± 0.50 log CFU mL^{-1}.

The viability of presumptive lactobacilli, including the LA-5® strain, was evaluated by plate counting on MRS agar (VWR International, Darmstadt, Germany) under anaerobic conditions (AnaeroGen™ 2.5 L Sachet, Oxoid), before digestion and after the gastric and intestinal phases. Cell density of total lactobacilli and *L. acidophilus* LA-5® was expressed as log CFU mL^{-1}. Each digestion experiment was performed in duplicate.

To discriminate the LA-5® strain among biotypes from MRS agar plates, colonies collected at each digestion step were analyzed by cluster analysis using IR-biotyping (IR Biotyper spectrometer, Bruker Optics-Daltonics GmbH, Bremen, Germany), as previously described [41, 42]. *L. acidophilus* LA-5®, *L. acidophilus* CNRZ 216, *L. acidophilus* ATCC 4355, and *L. acidophilus* ATCC 4356 were used as reference strains. Hierarchical clustering analysis (HCA) was performed using IRBT Client Software v4.1.1.59 (Bruker Daltonik GmbH, Bremen, Germany). Dendrograms were constructed using Euclidian distance and the unweighted pair group method with arithmetic mean (UPGMA).

Post-Digestion Metabolomics

Digestates obtained at the end of the intestinal phase were analyzed by metabolomics using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Samples were centrifuged at 18,630 × *g* for 10 min at 4 °C to separate a semi-solid pellet and a supernatant. Supernatants were directly analyzed, whereas pellets (160 mg) were resuspended in 1 mL of bidistilled water prior to analysis.

For metabolomic analysis, an NMR solution containing TSP (3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt, 10 mmol L^{-1}) and NaN_3 (2 mmol L^{-1}) in D_2O was prepared. TSP was used as an internal standard for chemical shift calibration, while NaN_3 served as an antimicrobial agent. For each sample, 0.7 mL of supernatant was mixed with 0.1 mL of the D_2O solution and centrifuged as described above.

^{1}H-NMR spectra were acquired using an AVANCE™ III spectrometer (Bruker, Milan, Italy) operating at 600.13 MHz and 298 K, controlled by TopSpin software (v.3.5). Water signal suppression was achieved by presaturation, and CPMG filter (total duration 330 ms, 400 echoes, τ = 400 µs, 180° pulse of 24 µs) was applied to minimize broad signals from macromolecules. Spectra were recorded with 256 scans, a relaxation delay of 5 s, 32,000 data points, and a spectral window of 7184 Hz. After phase-adjustment in TopSpin, spectra were exported to the R environment in ASCII format. Signal assignment was performed using Chenomx software (v10; Chenomx Inc., Edmonton, AB,

Canada), by comparing multiplicity, shape, and chemical shifts with reference libraries and the Human Metabolome Database [43]. Metabolite quantification was performed using TSP as internal standard, and probabilistic quotient normalization (PQN) was applied to correct for differences in sample dilution [44]. Signal integration was carried out using Global Spectra Deconvolution (GSD) implemented in MestReNova software (v14.2.0–26256; Mestrelab research S.L., Santiago De Compostela, Spain), using a line broadening of 0.3 and a limit of quantification (LOQ) of 5. Baseline correction was performed using the Whittaker smoother algorithm.

For data analysis, mean metabolite concentrations from duplicate digestions were used. To isolate the contribution of probiotic formulations, metabolite profiles of non-inoculated controls were subtracted from the corresponding profiles of samples containing probiotic products. Differences between commercial formulations and fresh *L. acidophilus* LA-5® cells were evaluated by identifying metabolites consistently higher or lower than the corresponding from fresh cells.

Metabolite datasets (apple juice and yogurt matrices, pellet and supernatant fractions) were used to build robust principal component analysis (rPCA) models [45]. Results were visualized as score plots, representing sample distribution in the PCA space, and loading plots, indicating the contribution of each metabolite to the model.

Results

Cell Viability Assessment

The viability of the four probiotic formulations containing *L. acidophilus* LA-5® was assessed using both plate counting and flow cytometry.

Plate counts were compared with label claims for both total viable counts and LA-5®-specific CFU g^{-1} (Table 4). For all products, the quantified LA-5® content was consistent with label declarations, with ratios of measured to claimed CFUs exceeding 1. However, discrepancies were observed for other strains in multi-strain formulations. In particular, Product 4 met LA-5® CFU specifications but showed an approximately 1-log lower CFU than declared, mainly due to a threefold reduction in *B. animalis* subsp. *lactis* counts (Table S3). A similar trend was observed for Product 1, where overall viability was consistent with label claims (Table 4), but species-specific counts of *B. animalis* subsp. *lactis* were approximately four-fold lower than expected (Table S3).

FC enabled the discrimination between viable cells (AFU) and non-viable cells (nAFU), as illustrated in PI versus SYTO24™ fluorescence density plots (Fig. 1).

In terms of relative abundance, viable cells accounted for approximately 75% of total fluorescent units in Products 1 and 3. Among the formulations, Product 4 showed the highest viability (88.33 ± 3.15% AFU and 11.67 ± 3.15% nAFU), whereas Product 2 exhibited the lowest proportion of viable cells (58.54 ± 1.52% AFU). Comparison between plate counts (log CFU g^{-1}) and FC data (log AFU g^{-1}) revealed significant differences for all products ($p < 0.001$), indicating the presence of VBNC cell populations.

WGS-Based Taxonomic and Safety Evaluation of the Isolated *L. Acidophilus* LA-5®

In silico taxonomic analysis of the four *L. acidophilus* LA-5® complete genomes revealed ANI and dDDH values of 99.92% and 99.80%, respectively, compared to the *L. acidophilus* type strain DSM 20,079^T, confirming their assignment to the *L. acidophilus* species (Tables S4 and S5). No plasmids were detected. In addition, in silico safety assessment showed the absence of genes of concern, with no hits above the applied thresholds (%identity ≥ 80% and %coverage ≥ 70%) against the investigated databases (CARD, NCBI-AMRFinderPlus, ARG-ANNOT, ResFinder, VFDB).

ddPCR Assays for Taxonomic and Functional Assessment

The developed ddPCR assays, including singleplex (targets: 16–23 S region and *bsh*2) and duplex (targets: *bsh*1/*cIII* and *slpA*/*cAsor*), showed a clear separation between positive and negative droplets across all commercial products and

Table 4 Results of overall and LA5 cultivability together with absolute quantification' results of each product by flow cytometry

Commercial product	Overall CFU (Log CFU g^{-1})	Claimed overall CFU (Log CFU g^{-1})	CFU of L. acidophilus LA-5® (Log CFU g^{-1})	Claimed CFU of L. acidophilus LA-5® (Log CFU g^{-1})	Active fluorescent units (Log AFUg^{-1})**	Non-active fluorescent units (Log nAFU g^{-1})	Total fluorescent units (Log FU g^{-1})
Product 1	9.31 ± 0.02	9.30	9.26 ± 0.02	9.00	10.03 ± 0.02	9.60 ± 0.02	10.17 ± 0.01
Product 2	9.84 ± 0.04	9.00	9.84 ± 0.04	9.00	10.08 ± 0.01	9.93 ± 0.02	10.31 ± 0.01
Product 3	11.03 ± 0.01	11.00	11.02 ± 0.01	11.00	11.13 ± 0.02	10.66 ± 0.02	11.26 ± 0.02
Product 4	9.20 ± 0.01	9.56*	6.20 ± 0.02	6.00	9.37 ± 0.05	8.49 ± 0.09	9.43 ± 0.04

* Including the plate-counts of starter microorganisms (Table S5); **Significant difference (p-value < 0.001) between overall CFU and AFU according to unpaired t test

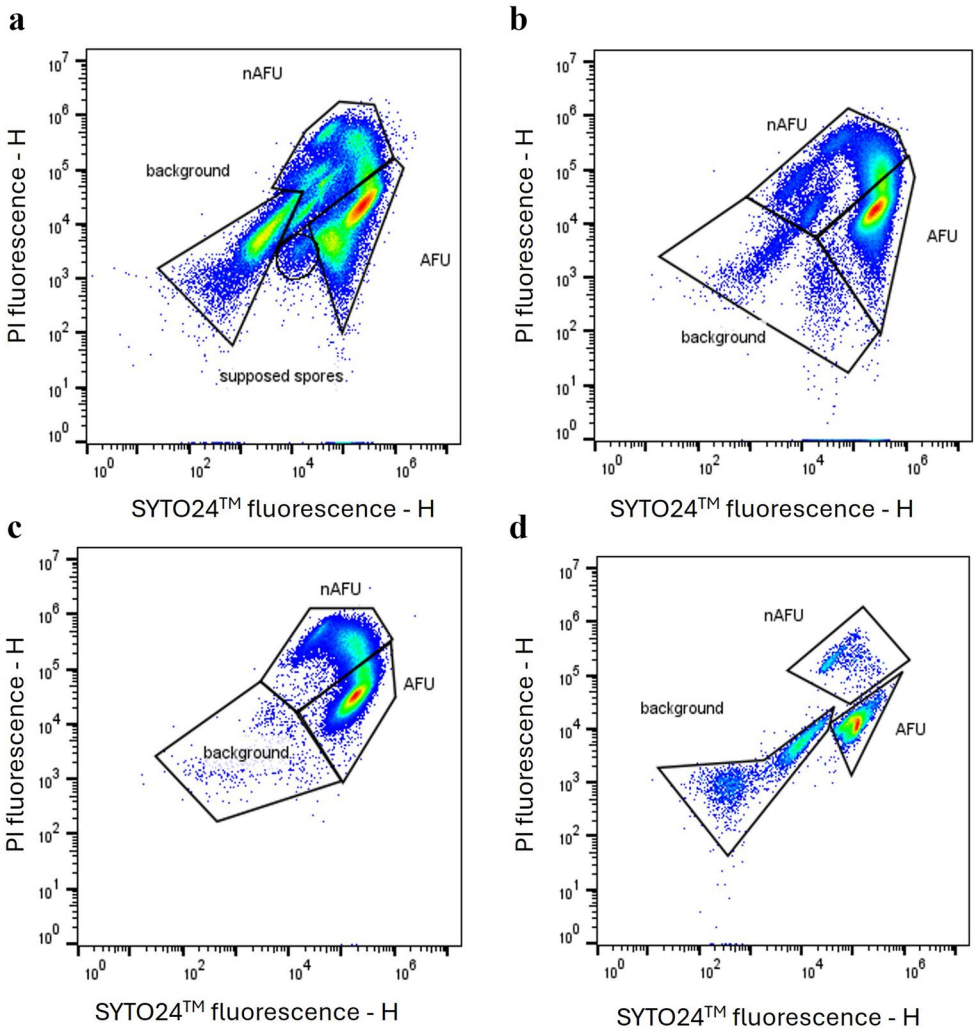

Fig. 1 Visualization of viable (AFU) and damaged/dead (nAFU) cells of Product 1 (**a**), Product 2 (**b**), Product 3 (**c**), and Product 4 (**d**) by flow cytometry using PI and SYTO24 ™ as fluorescent probes

dilution levels (Figs. S2-S5). Quantification of LA-5® target genes obtained by ddPCR was compared with plate counts and label claims, expressed as log CFU g^{-1} (Fig. 2). Among the analyzed products, Product 2 consistently showed approximately a 1-log higher quantification (10.62 ± 0.08 log CFU g^{-1}), while Product 4 exhibited an approximately 2-log increase (6.19 ± 0.02 log CFU g^{-1}) compared to both plate counts and label values. In contrast, Products 1 and 3 showed good agreement between ddPCR quantification, plate counts, and label claims, with average values of 9.68 ± 0.24 and 11.15 ± 0.12 log CFU g^{-1}, respectively. The positive control included in each assay yielded results consistent with plate counts (Fig. S6). No amplification was observed for non-target strains used as negative controls in multi-strain products (Products 1 and 4), confirming assay specificity (Fig. S7).

Antimicrobial Activity of Probiotic Cell Free Supernatants

No significant antimicrobial activity was observed for probiotic cell-free supernatants (CFS) prepared in simulated intestinal fluid (SIF) against the three tested enteropathogens (*E. coli*, *S. choleraesuis*, and *Y. enterocolitica*), regardless of probiotic cell density, incubation time, or pathogen (Table S6). In contrast, CFS prepared in peptone water showed detectable antimicrobial activity. The strongest effects were observed for the single-strain capsule formulation (Product 2) and the multi-strain orodispersible formulation (Product 1). At the higher probiotic cell density (10^8 CFU mL^{-1}), both products exhibited significant inhibitory activity against *E. coli* and *S. choleraesuis*, but not against *Y. enterocolitica*. This effect was consistently observed in CFS collected after 5 h and 15 h of anaerobic incubation, resulting in a 40–50% reduction in pathogen growth. At time zero (T0), antimicrobial activity was detected only against *E. coli* (Fig. 3).

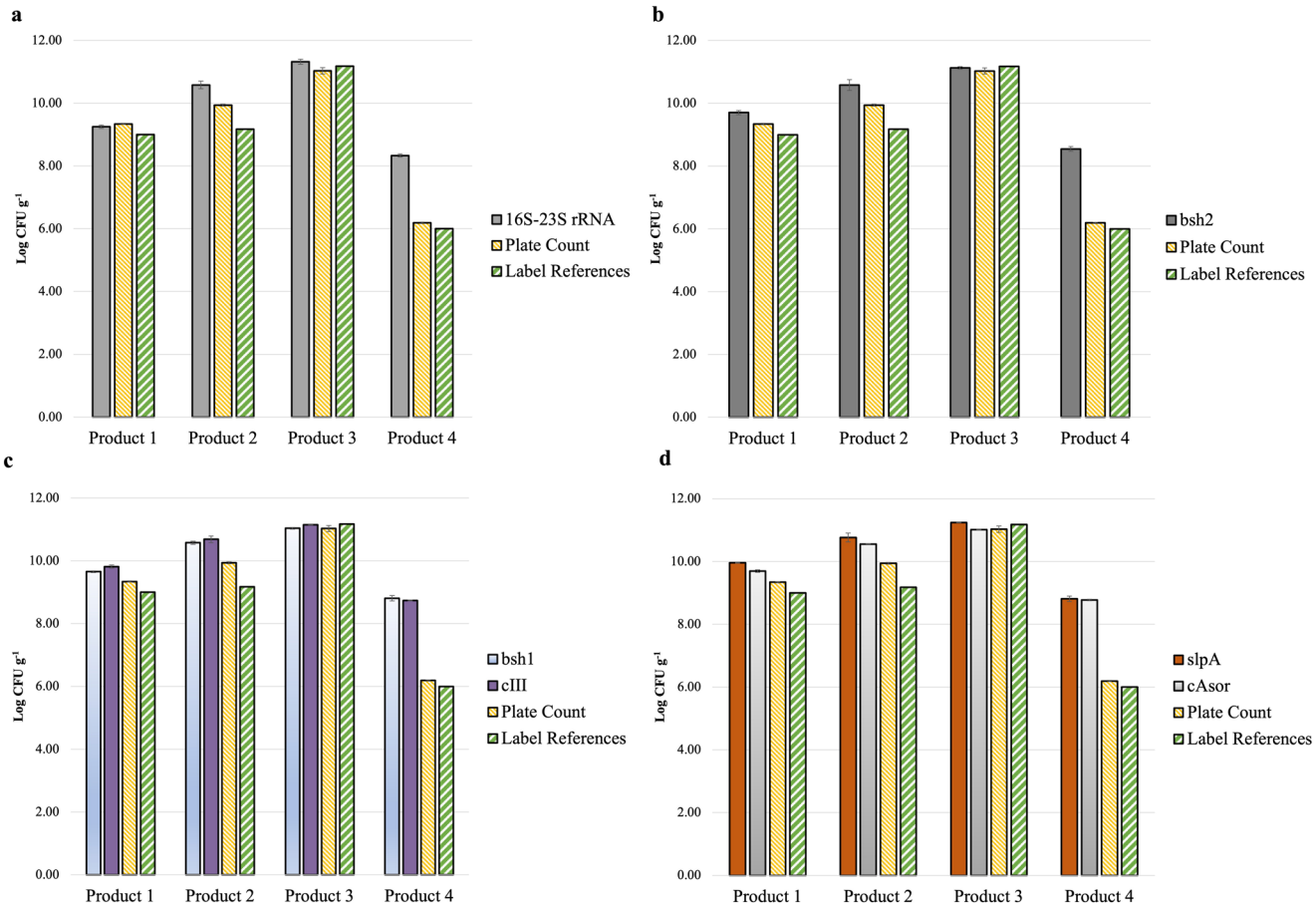

Fig. 2 ddPCR assays quantification of the taxonomic and functional genes in the four commercial products. Singleplex ddPCR assays for (**a**) 16–23 S and (**b**) *bsh2* genes; duplex ddPCR assays for (**c**) *bsh1/cIII* and (**d**) *slpA/cAsor*

For both products, the higher cell density (10^8 CFU mL^{-1}) corresponded to the recommended intake dose reported on the product label. Notably, only CFS derived from this higher inoculum exhibited significant antimicrobial activity.

In Vitro Static Digestion Test

The in vitro digestion of lyophilized formulations (Products 1–3) resuspended in PBS showed a complete loss of viable *L. acidophilus* LA-5® after the intestinal phase for Products 2 and 3, whereas fresh cells survived at approximately 4 log CFU mL^{-1} (Table S7). In Product 1, no viable LA-5® cells were detected by IR-biotyping, although total lactobacilli decreased from approximately 7 to 5 log CFU mL^{-1} during digestion (Table S7).

When digestion was performed in food model systems, different survival patterns were observed. In apple juice (Table 5), initial LA-5® counts ranged between 8 and 9 log CFU mL^{-1}, except for Product 1, where the strain was detected only in one replicate. The gastric phase caused reductions of 1.9–4.2 log CFU mL^{-1} for fresh cells and Product 3, whereas a reduction of approximately 5 log units was observed for Product 2. After the intestinal phase, fresh cells showed the highest survival, while Product 2 retained approximately 4 log CFU mL^{-1} in some replicates. In contrast, no viable cells were detected for Product 3 or Product 1.

In yogurt drink (Table 6), initial LA-5® counts were close to 8 log CFU mL^{-1}, except for Product 1, where no viable cells were detected. The gastric phase reduced viability by approximately 4 log units for fresh cells and by 4–5.5 log units for Products 2 and 3. After the intestinal phase, only fresh cells remained detectable, whereas no viable LA-5® cells were recovered from Products 1–3. Total lactobacilli showed higher survival than LA-5®across all conditions.

The digestion of the multi-strain fermented milk product (Product 4) showed a different pattern. After an initial reduction of 5–6 log units during the gastric phase, LA-5® cells remained detectable after the intestinal phase at approximately 2.8 log CFU mL^{-1} (Table 6), confirming the reproducibility of this result across replicates.

IR-biotyping confirmed the identity of LA-5® colonies recovered from digestion experiments (Figs. S8–S14). In apple juice and yogurt drink, colonies from Products 2 and

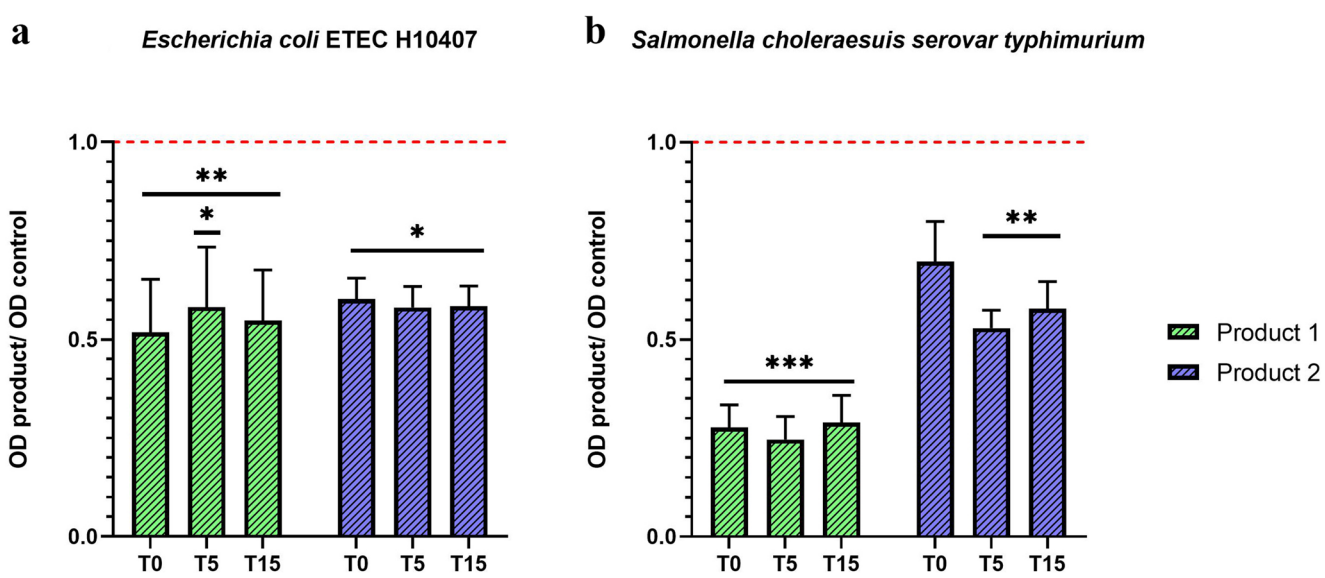

Fig. 3 Antimicrobial effect of the CFS produced by Products 1 and 2 in peptone water, against *E. coli* (**a**) and *Salmonella* (**b**). CFS were collected after 0, 5 and 15 h of anaerobic incubation at 37 °C, using the initial concentrations of 10^8 CFU mL-1. Bacterial growth was assessed by measuring turbidity (OD_{600}) expressed as the ratio of the treated sample over the untreated control (dashed red line represents untreated controls). Statistical analysis was performed using two-way ANOVA followed by Bonferroni correction ($^{*}p<0.033$, $^{**}p<0.002$, $^{***}p<0.001$)

Table 5 Mean values of viable cell counts (log CFU mL^{-1}) of presumptive *L. acidophilus* LA-5® in apple juice inoculated with broth culture, Product 1, 2, or 3 formulations during the INFOGEST digestion process (R1, R2: digestion replicates)

Product	Digestion phase	Total lactobacilli		*L. acidophilus* LA-5®	
		R1	R2	R1	R2
Product 1	Oral	7.17±0.05	7.13±0.03	6.01±0.03	n.d.*
	Gastric	5.87±0.05	5.57±0.07	n.d.	n.d.
	Intestinal	5.83±0.04	5.43±0.04	n.d.	n.d.
Product 2	Oral	8.28±0,18	8.26±0.18	8.28±0,18	8.26±0.18
	Gastric	3.54±0.07	3.05±0.07	3.54±0.07	3.05±0.07
	Intestinal	4.05±0.11	n.d.	4.05±0.11	n.d.
Product 3	Oral	8.72±0.13	8.80±0.24	8.72±0.13	8.80±0.24
	Gastric	4.03±0.11	3.62±0.52	4.03±0.11	3.62±0.52
	Intestinal	n.d.	n.d.	n.d.	n.d.
Broth culture	Oral	8.13±0.28	7.92±0.28	8.13±0.28	7.92±0.28
	Gastric	6.21±0.27	3.69±0.05	6.21±0.27	3.69±0.05
	Intestinal	5.15±0.29	2.55±0.11	5.15±0.29	2.55±0.11

*n.d.: not detected; limit of detection of 2.2 log CFU mL^{-1}

Table 6 Mean values of viable cell counts (log CFU mL^{-1}) of total lactobacilli and presumptive *L. acidophilus* LA-5® in a yogurt drink inoculated with broth culture, Product 1, 2, and 3, formulations, and Product 4 as received, during the INFOGEST digestion process (R1, R2: digestion replicates)

Product	Digestion phase	Total lactobacilli		L. acidophilus LA-5®	
		R1	R2	R1	R2
Product 1	Oral	7.93±0.21	7.67±0.18	n.d.*	n.d.
	Gastric	5.39±0.03	5.48±0.10	n.d.	n.d.
	Intestinal	5.68±0.03	5.92±0.05	n.d.	n.d.
Product 2	Oral	8.69±0.22	8.55±0.31	8.29±0.31	8.20±0.57
	Gastric	2.70±0.05	n.d.	2.70±0,05	n.d.
	Intestinal	3.25±0.03	n.d.	n.d.	n.d.
Product 3	Oral	8.58±0.06	8.52±0.25	7.48±0.01	7.77±0.49
	Gastric	5.12±0.03	5.22±0.11	n.d.	3.97±0.03
	Intestinal	4.04±0.12	3.50±0.12	n.d.	n.d.
Product 4	Oral	8.00±0.29	8.02±0.22	8.00±0.29	8.02±0.22
	Gastric	3.04±0.08	n.d.*	3.04±0.08	n.d.
	Intestinal	2.86±0.13	2.76±0.07	2.86±0.13	2.76±0.07
Broth culture	Oral	8.57±0.33	8.49±0.32	8.37±0.37	8.29±0.41
	Gastric	4.77±0.43	4.68±0.33	4.34±0.43	4.22±0.21
	Intestinal	4.55±0.36	4.80±0.29	3.80±0.25	4.24±0.21

*n.d.: not detected; limit of detection of 2.2 log CFU mL^{-1}

3 and fresh cells consistently clustered with the LA-5® reference strain. In contrast, Product 1 showed high variability, with multiple lactic acid bacteria clusters and limited detection of LA-5®. Additional clusters observed in yogurt drink samples likely corresponded to starter cultures present in the matrix.

Overall, comparison between PBS and food model systems showed a limited protective effect of the food matrix on LA-5® survival during digestion.

Metabolomic Profiling of Food Matrix Digestates

^{1}H-NMR spectroscopy enabled the identification and quantification of 43 and 45 metabolites in the pellet and supernatant fractions of apple juice digestates, respectively. The detected compounds mainly belonged to amino acids, organic acids, carbohydrates, and alcohols, and were associated with central metabolic pathways, including glycolysis, pyruvate metabolism, and the tricarboxylic acid (TCA) cycle.

In the pellet and supernatant fractions, 16 and 27 metabolites, respectively, showed consistent differences between samples containing probiotic formulations and non-inoculated controls. These metabolites were used to build two distinct rPCA models (Fig. 4). In both models, samples inoculated with fresh cells clustered at negative values along PC1, whereas samples containing commercial formulations were distributed on the right side. Among these, Product 1 (P1) showed the most pronounced divergence from fresh-cell samples, while Products 2 (P2) and 3 (P3) exhibited intermediate profiles. In yogurt drink digestates, ^{1}H-NMR spectroscopy identified and quantified 44 and 43 metabolites in the pellet and supernatant fractions, respectively, with similar metabolic classes as observed in apple juice. A total of 12 (pellet) and 7 (supernatant) metabolites were consistently different between probiotic samples and controls. These variables were used to generate two rPCA models (Fig. 5), which showed a similar clustering pattern: samples inoculated with fresh cells were located at negative PC1 values, while those containing commercial formulations were positioned on the right side. Product 1 (P1) again showed the greatest divergence from fresh-cell samples, followed by Product 4 (P4), whereas Products 2 (P2) and 3 (P3) clustered closer to fresh cells.

These results highlight formulation-dependent differences in metabolic profiles under simulated gastrointestinal conditions.

Discussion

Since probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" [2], the evaluation of probiotic formulation quality must primarily consider microbial viability, with particular attention to the target strain *Lactobacillus acidophilus* LA-5®.

Traditionally, cultivability has been used as the standard parameter to assess microbial viability. However, this approach excludes cells that remain structurally intact and metabolically active but have lost the ability to replicate due to stresses associated with industrial processes such as fermentation, biomass concentration, cryopreservation, lyophilization, powder grinding, and storage [1]. In this study, viability was therefore assessed by combining plate counting and flow cytometry, enabling a more comprehensive evaluation of probiotic cell status.

Flow cytometry analysis revealed that viable cells accounted for more than 50% of total fluorescent units in all formulations, with clear differences among products. In particular, Product 3 showed the highest proportion of viable cells among single-strain formulations, whereas Product 2 exhibited lower viability despite being within its shelf life. Among multi-strain products, Product 4 displayed the highest viability, likely due to its fermented matrix, refrigerated storage, water activity, and shorter shelf life compared to lyophilized formulations.

Comparison between flow cytometry and plate counts showed significantly higher viable cell numbers than culturable cells ($p < 0.001$) in all products, indicating the presence of viable but non-culturable (VBNC) populations. This observation is consistent with previous studies reporting that loss of cultivability occurs faster than loss of membrane integrity [10] and highlights the importance of integrating culture-independent approaches in probiotic quality assessment.

From a regulatory perspective, the declared bacterial species and corresponding concentrations were confirmed in all products, except for *B. animalis* subsp. *lactis* in the multi-strain formulations. In all products, *L. acidophilus* LA-5® counts consistently exceeded label claims. This overage is likely intentional to ensure that viable cell numbers remain above declared levels throughout shelf life [46]. Conversely, *B. animalis* subsp. *lactis* counts were significantly lower than declared, in agreement with the known sensitivity of bifidobacteria to environmental stresses, including oxygen exposure and freeze-drying, which may compromise their survival in commercial products [21].

The genomic and molecular analyses further confirmed the robustness of the proposed approach. Whole-genome sequencing verified the taxonomic identity of all isolates as *L. acidophilus* and confirmed the absence of genes of concern, supporting the safety of the investigated formulations. Although *L. acidophilus* LA-5® is a well-known probiotic strain with established use in food and supplement applications, the inclusion of WGS in the proposed workflow was not intended to re-assess its safety status, but rather to serve as a verification step for commercial probiotic products. In this context, WGS provides added value by confirming

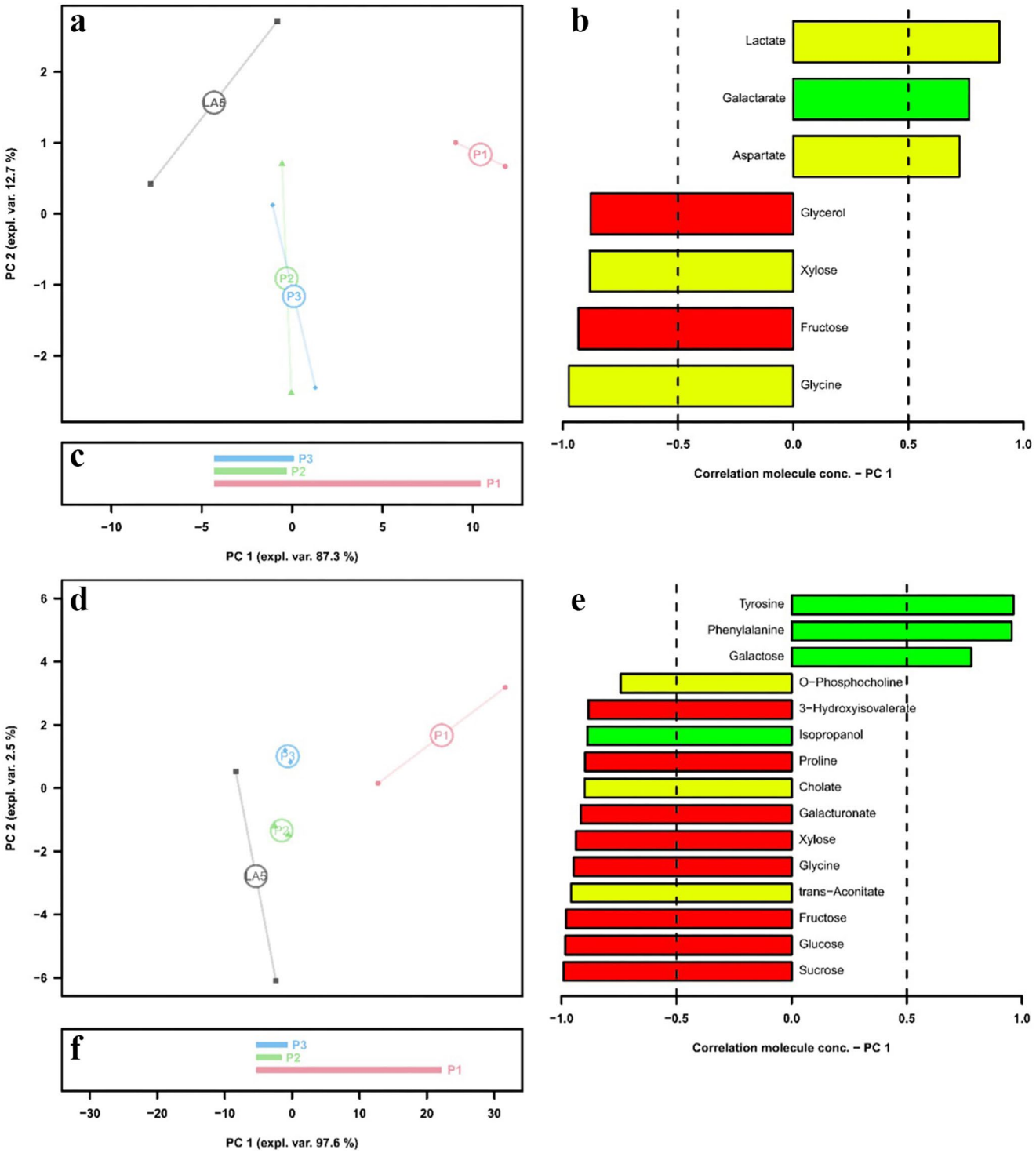

Fig. 4 rPCA models based on the molecules quantified in the pellet (**a-c**) and in the supernatant (**d-f**) of apple juice digestates. Panels **a** and **d** show the score plots, while panels **c** and **f** show their summaries. Panels **b** and **e** show the loading plots, only for the molecules with a significant correlation concentration-importance. Bars point right when molecules are more concentrated in the samples located on the right side of the score plot. Green (or red) bars represent molecules that are, more (or less) concentrated in all samples added with commercial products compared with the non-inoculated counterparts. Yellow bars indicate mixed trends relative to the non-inoculated counterparts. Concentrations of the metabolites in panels **b** and **e** are reported in Figs. S15 and S16, respectively

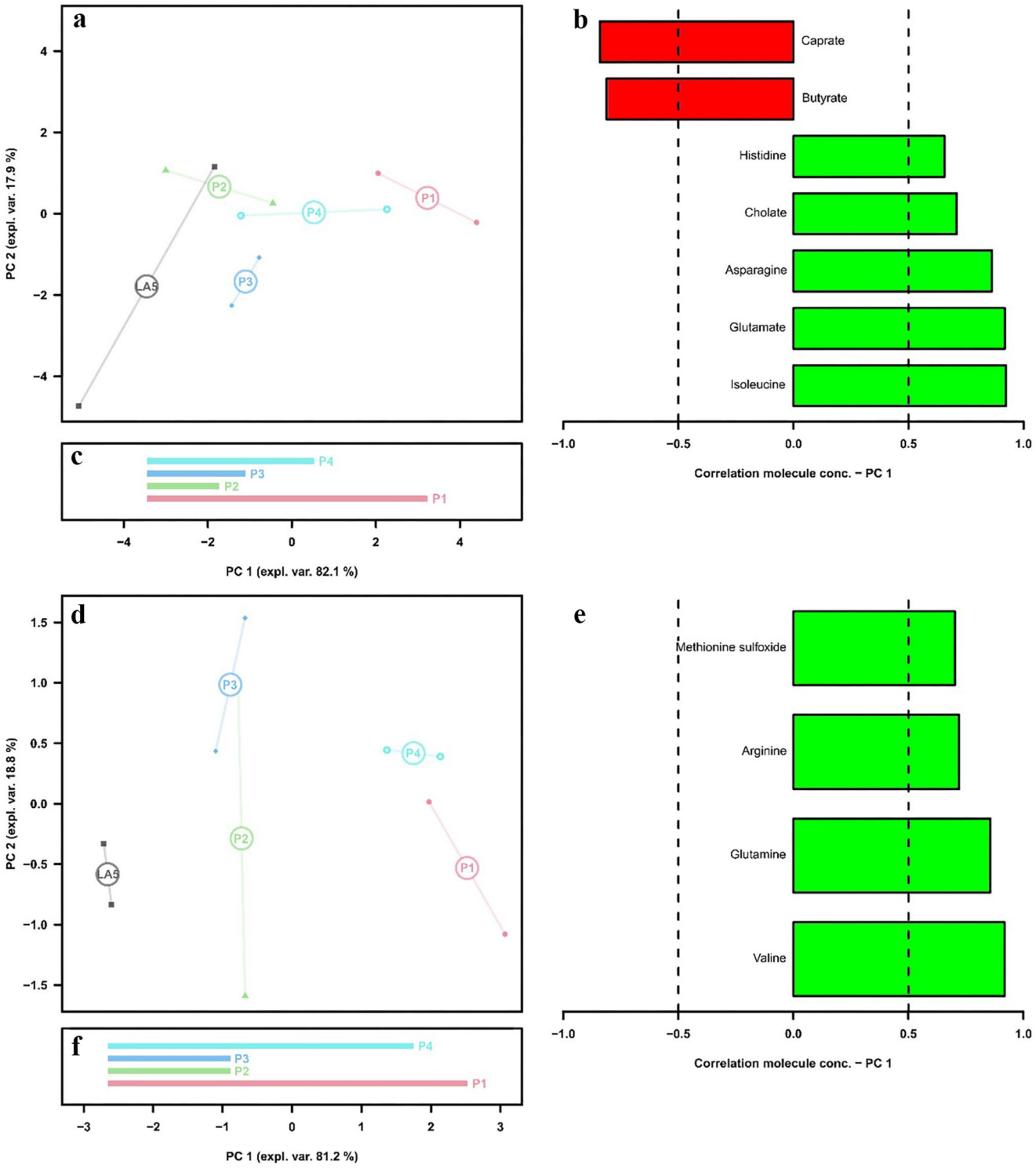

Fig. 5 rPCA models based on the molecules quantified in the pellet (**a-c**) and in the supernatant (**d-f**) of yogurt drink digestates. Panels **a** and **d** show the score plots, while panels **c** and **f** show their summaries. Panels **b** and **e** show the loading plots, only for the molecules with a significant correlation concentration-importance. Bars point right when molecules are more concentrated in the samples located on the right side of the score plot. Green (or red) bars represent molecules that are, more (or less) concentrated in all samples added with commercial products compared with the non-inoculated counterparts. Concentrations of the metabolites in panels **b** and **e** are reported in Figs. S17 and S18, respectively

taxonomic identity, excluding possible misidentification or contamination, and verifying the absence of unexpected safety-related traits, including acquired antibiotic resistance genes, virulence-associated determinants, or mobile genetic elements of concern. In addition, genomic analysis can also help identify genetic features potentially contributing to probiotic functionality, thus providing information that extends beyond basic safety requirements. Moreover, advances in WGS technologies now allow comprehensive characterization of probiotic strain genomic structures through in-depth microbial analysis, providing valuable support for food safety assessment and regulatory evaluation [5, 47]. In this context, EFSA has recognized the value of WGS for the assessment of microorganisms intentionally used in the food chain, particularly for taxonomic identification, detection of genetic modifications, and identification of genes of concern [5].

The developed ddPCR assays enabled accurate and reproducible quantification of target taxonomic and functional genes, with results generally consistent with plate counts and label claims. Minor overestimations observed in one product likely reflect the detection of DNA from non-viable cells, a known limitation of DNA-based methods. This highlights the potential of ddPCR as a reliable tool for strain-specific monitoring in complex probiotic formulations.

Beyond viability, probiotic functionality may involve different functional traits. In this context, antimicrobial activity assays revealed a strong dependence on both the medium and probiotic concentration. No antimicrobial effect was observed in simulated intestinal fluid (SIF), whereas significant inhibition of *E. coli* and *S. choleraesuis* was detected in peptone water, particularly for Products 1 and 2 at higher cell densities (10^8 CFU mL^{-1}). These findings suggest that the antimicrobial activity of probiotic supernatants is strongly dependent on the experimental matrix and pH conditions. While SIF represents a physiologically relevant environment, its neutral pH and the presence of bile salts and digestive enzymes may attenuate or mask acid mediated antimicrobial effects observed in less buffered systems such as peptone water.

The lack of inhibition against *Y. enterocolitica*, a pathogen known for increased acid tolerance, further supports the contribution of pH dependent and pathogen specific susceptibility to probiotic-derived bioactive compounds. This variability has been previously reported [16] and underscores the importance of evaluating antimicrobial activity against multiple targets to define the functional spectrum of probiotic formulations.

The gastrointestinal resistance of probiotic formulations further emphasizes the impact of formulation and physiological state. In this context, IR-biotyping confirmed the strain-level recovery of LA-5® after digestion. This information complemented the species-level identification and quantification provided by the ddPCR assays developed in the first phase of the workflow proposed in this study, further supporting the integrated approach used for probiotic characterization. Lyophilized products showed substantial viability losses during the gastric phase, often leading to complete loss of culturable LA-5® cells after intestinal digestion. In contrast, fresh cells and the fermented matrix exhibited higher survival rates, indicating that metabolic activity and matrix composition play a crucial role in probiotic resilience. These results are consistent with previous studies demonstrating improved survival of probiotics in fermented matrices and reduced resistance in freeze-dried formulations [48–52].

Among the analyzed products, the multi-strain fermented milk (Product 4) showed the highest proportion of viable cells (active fluorescent units, AFU) and the lowest proportion of non-viable cells, together with the highest ratio of measured to declared CFUs. This feature likely contributed to the improved gastrointestinal survival of *L. acidophilus* LA-5® in this formulation compared to lyophilized products. Pawlos et al. showed that milk matrix exerted a protective effect on the viability of probiotic bacteria during gastrointestinal digestion, like an oat-based beverage [53]. In contrast, lyophilized formulations, particularly Product 1, showed a high proportion of viable but non-culturable (VBNC) cells, which may explain the low cultivability of LA-5® observed at the beginning of digestion. These observations are consistent with previous studies reporting higher proportions of damaged cells in freeze-dried probiotic formulations compared to fermented matrices [54]. The variability of the cell counts values found in few cases among different digestion replicates has been already reported in literature [55], and it could be linked with the methodology, the distribution of the cells within the sample, and the physiological state of the cells during digestion. However, overall, the gastric phase emerged as the most critical step for probiotic survival, whereas cells surviving gastric stress generally tolerated intestinal conditions. This behavior has been previously reported for *L. acidophilus* LA-5® and may be related to its ability to adapt to acidic environments and recover under favorable intestinal conditions [55]. In line with previous findings, lactic acid bacteria present in fermented matrices or starter cultures also showed enhanced resistance during simulated digestion [56, 57].

Metabolomic analysis further provided insights into probiotic functionality under simulated gastrointestinal conditions. ^{1}H-NMR profiling revealed that metabolic signatures were strongly influenced by both the formulation and the food matrix, reflecting the metabolic activity of the strain during digestion.

The metabolites detected in the digestates of apple juice and yogurt drink were mainly associated with central carbon metabolism, particularly glycolysis and pyruvate metabolism, with minor contributions from intermediates of the tricarboxylic acid (TCA) cycle. This metabolic profile reflects the characteristic fermentative behavior of *L. acidophilus*, which predominantly utilizes carbohydrates through glycolysis, with pyruvate acting as a key metabolic hub [58, 59].

In apple juice digestates, rPCA models clearly discriminated samples containing fresh LA-5® cells from those containing commercial formulations, indicating a formulation-dependent modulation of the metabolic fingerprint. In particular, the lyophilized multi-strain formulation (Product 1) showed the most pronounced divergence from fresh cells, mainly in terms of carbohydrates, organic acids, and amino acids, suggesting differences in substrate utilization and metabolic activity. The observed depletion of simple sugars, such as glucose and fructose, together with the accumulation of fermentation-related metabolites (e.g., lactate), reflects the characteristic glycolytic metabolism of lactic acid bacteria, in agreement with previous NMR-based studies in plant-based matrices [60]. The low buffering capacity and high sugar content of apple juice likely amplify these metabolic changes, making them more detectable after digestion. Moreover, it is worth noting that increased tyrosine levels were observed in the supernatants of apple juice digestates for all tested products. This finding may be relevant because dietary tyrosine is involved in the synthesis of brain neurotransmitters, particularly catecholamines, through precursor availability [61].

In yogurt drink digestates, metabolomic differences were less pronounced, likely due to the intrinsic complexity of the dairy matrix and the presence of metabolites produced by starter cultures. However, multi-strain formulations, particularly Product 1 and Product 4, clustered separately from mono-strain formulations and fresh cells, suggesting that interactions among co-occurring microbial species contribute to shaping the metabolic output. In this matrix, variations in amino acids and nitrogen-containing compounds were more relevant than changes in carbohydrates, indicating a shift toward proteolytic and amino acid metabolism. Similar patterns have been reported in metabolomic studies of fermented dairy products, where probiotic-driven effects are partially masked by the background metabolic activity of starter cultures [62]. Additionally, the increased levels of cholates observed in yogurt digestates may reflect changes in bile salt metabolism, a process related to probiotic tolerance and resistance to bile salts under gastrointestinal conditions [63]. This observation may be relevant for food matrices, as cholates and related bile acid derivatives have been reported to influence intestinal barrier function and epithelial permeability through the regulation of tight junction organization and epidermal growth factor receptor (EGFR) signaling [64]. The detection of metabolites related to pyruvate metabolism and branched-chain amino acid turnover further supports the involvement of central metabolic pathways in the adaptive response of LA-5® to digestion-related stress. Notably, the distinct clustering of samples inoculated with commercial formulations compared to fresh cells suggests that technological processing, formulation matrix, and the physiological state of the bacteria influence post-digestion metabolic outcomes, as previously reported for freeze-dried and encapsulated probiotics [58].

The ^{1}H-NMR metabolomic approach thus represents a powerful complementary tool to viability and functional assays, enabling the characterization of probiotic–matrix interactions and their contribution to the metabolic signature of digested foods.

Conclusion

This study demonstrates that the quality and functional evaluation of probiotic products cannot be reliably addressed using single analytical approaches, particularly in complex and multi-strain formulations. The integration of culture-dependent methods with molecular, cytometric, and functional analyses enabled a comprehensive characterization of *Lactobacillus acidophilus* LA-5® commercial products. The main contribution of this study is not the confirmation that culture-based methods may underestimate viable probiotic cells, but the demonstration that different commercial formulations containing the same strain can display distinct viability profiles, gastrointestinal recovery, antimicrobial activity, and metabolic signatures when assessed through an integrated workflow.

The combined use of flow cytometry and plate counting revealed discrepancies between viability and cultivability, highlighting the presence of viable but non-culturable populations not captured by conventional CFU-based methods. In parallel, whole-genome sequencing and ddPCR supported taxonomic identification, safety assessment, and label compliance, while IR-biotyping confirmed the recovery of LA-5® at the strain level after digestion.Functional outcomes, including antimicrobial activity, gastrointestinal survival, and metabolomic profiles, were strongly influenced by formulation, concentration, and delivery matrix, even when the same strain was used. Differences between lyophilized products, fresh cells, and fermented matrices underline the critical role of technological and physiological factors in probiotic performance. These findings reinforce the importance of considering both formulation and matrix effects when evaluating probiotic functionality under gastrointestinal conditions.

Overall, this multi-parameter approach provides a proof of concept for a targeted and modular workflow to improve probiotic quality and functional assessment. By integrating complementary quality criteria, the proposed framework may support manufacturers in product development, validation, and advanced quality control, and may also provide regulatory bodies, trade associations, and research institutions with a structured basis for harmonized evaluation strategies, transparent labeling, and consumer protection.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-026-11100-z.

Acknowledgements The authors acknowledge Chr Hansen Italia S.p.A., part of Novonesis Group, for providing "Product 3", which was used in this study.

Author Contributions Conceptualization: A.F., L.M., D.M., B.V., F.B.; Methodology: A.F., D.M., B.V., F.B., L.P., L.L.; Formal analysis and investigation: M.B., V.T., A.B., A.F., L.P., A.M., L.L.; Writing - original draft preparation: M.B., V.T., A.B., L.P.; Writing - review and editing: A.F., D.M., B.V., F.B.; Funding acquisition: A.F., D.M., B.V., L.P.; Supervision: L.M.

Funding Open access funding provided by Università Cattolica del Sacro Cuore within the CRUI-CARE Agreement. This work was supported by the Decree n.104 of 2 February 2022 of Italian Ministry of University and Research (Projects of Relevant National Interest - PRIN 2022) funded by the European Union–NextGenerationEU, Award Number: Project Code 20227J3THJ, Concession Decree No. 1048 of 14 July 2023 adopted by the Italian Ministry of University and Research, CUP J53D23009650008, Project Title "Guidelines and protocols for quality and functional assessment of probiotic formulations (Prob-ID)".

Data Availability The datasets supporting the conclusions of this article are included within the article and its additional file. Data will be made available on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

References

1. Jackson SA, Schoeni JL, Vegge C, Pane M, Stahl B, Bradley M, Goldman VS, Burguière P, Atwater JB, Sanders ME (2019) Improving End-User Trust in the Quality of Commercial Probiotic Products. Front Microbiol 10:739. https://doi.org/10.3389/fmicb.2019.00739
2. Hill C, Guarner F, Reid G et al (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514. https://doi.org/10.1038/nrgastro.2014.66
3. Sarita B, Samadhan D, Hassan MZ, Kovaleva EG (2025) A comprehensive review of probiotics and human health-current prospective and applications. Front Microbiol 15:1487641. https://doi.org/10.3389/fmicb.2024
4. Probiotics in food (2006) Health and Nutritional properties and Guidelines for Evaluation. Food and Agriculture Org
5. EFSA (European Food Safety Authority) (2024) EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. EFSA J 22(8):e8912. https://doi.org/10.2903/j.efsa.2024.8912
6. International Dairy Federation (2021) *Identification of probiotics at strain level: Guidance document.* Bulletin of the IDF No. 513/2021. International Dairy Federation
7. Sanders ME, Akkermans LM, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, Huys G, Levy DD, Lutgendorff F, Mack D, Phothirath P, Solano-Aguilar G, Vaughan E (2010) Safety assessment of probiotics for human use. Gut Microbes 1(3):164–85. https://doi.org/10.4161/gmic.1.3.12127
8. Postollec F (2014) Evolution of microbiological analytical methods for dairy industry needs. 2014 annual meeting. Front Microbiol 5:16. https://doi.org/10.3389/fmicb.2014.00016
9. Wetzel MLJ, Vegge CS, Pane M, Goldman VS, Koshy B, Porsby CH, Burguière P, Schoeni JL (2021) Improving and comparing probiotic plate count methods by analytical procedure lifecycle management. Front Microbiol 12:693066. https://doi.org/10.3389/fmicb.2021.693066
10. Foglia C, Allesina S, Amoruso A, De Prisco A, Pane M (2020) New insights in enumeration methodologies of probiotic cells in finished products. J Microbiol Methods 175:105993. https://doi.org/10.1016/j.mimet.2020.105993
11. Ayyash M, Abdalla A, Alhammadi A, Ranadheera CS, Baig MA, Al-Ramadi B, Chen G, Kamal-Eldin A, Huppertz T (2021) Probiotic survival, biological functionality and untargeted metabolomics of the bioaccessible compounds in fermented camel and bovine milk after in vitro digestion. Food Chem 363:130243, 130243. https://doi.org/10.1016/j.foodchem.2021.130243
12. Patti G, Yanes O, Siuzdak (2012) Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 269:263–269. https://doi.org/10.1038/nrm3314
13. Laghi L, Picone G, Capozzi F (2014) Nuclear magnetic resonance for foodomics beyond food analysis. TrAC Trends Anal Chem 59:93–102. https://doi.org/10.1016/j.trac.2014.04.009
14. Sun Y, Guo S, Wu T et al (2023) Untargeted mass spectrometry-based metabolomics approach unveils biochemical changes in compound probiotic fermented milk during fermentation. npj Sci Food 7:21. https://doi.org/10.1038/s41538-023-00197-z
15. Trinchieri V, Laghi L, Vitali B, Parolin C, Giusti I, Capobianco D, Mastromarino P, De Simone C (2017) Efficacy and safety of a multistrain probiotic formulation depends from manufacturing. Front Immunol 8:1474. https://doi.org/10.3389/fimmu.2017.01474
16. Bull M, Plummer S, Marchesi J, Mahenthiralingam E (2013) The life history of *Lactobacillus acidophilus* as a probiotic: a tale of revisionary taxonomy, misidentification and commercial success.

FEMS Microbiol Lett 349(2):77–87. https://doi.org/10.1111/1574-6968.12293
17. Papadimitriou K, Zoumpopoulou G, Foligné B, Alexandraki V, Kazou M, Pot B, Tsakalidou E (2015) Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. Front Microbiol 6:58. https://doi.org/10.3389/fmicb.2015.00058
18. Muto M, Abe F, Yaeshima T, Iwatsuki K (2010) Effect of enumeration method on *Bifidobacterium* cell counts in commercial powder products. Biosci Microflora 29(3):143–148. https://doi.org/10.12938/bifidus.29.143
19. Mora D, Fortina MG, Parini C, Daffonchio D, Manachini PL (2000) Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains The GenBank accession numbers for the sequences determined in this work are given in Table 4 T4 . Microbiology (Reading) 146(8):2027–2038. https://doi.org/10.1099/00221287-146-8-2027
20. Stahl B, Barrangou R (2013) Complete genome sequence of probiotic strain *Lactobacillus acidophilus* La-14. Genome Announc 1:e00376–e00313. https://doi.org/10.1128/genomeA.00376-13
21. Oberg TS, Steele JL, Ingham SC, Smeianov VV, Briczinski EP, Abdalla A, Broadbent JR (2011) Intrinsic and inducible resistance to hydrogen peroxide in *Bifidobacterium* species. J Ind Microbiol Biotechnol 38:1947–1953. https://doi.org/10.1007/s10295-011-0983-y
22. ISO 29981 :2010 [IDF 220:2010]. Milk Products—Enumeration of Presumptive Bifidobacteria—Colony Count Technique at 37° C
23. Gilliland S, Speck M (1977) Enumeration and identity of lactobacilli in dietary products. J Food Protect 40(11):760–762. https://doi.org/10.4315/0362-028X-40.11.760
24. ISO 19344 2015 [IDF 232:2015] Milk products - Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry
25. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114–2120. https://doi.org/10.1093/bioinformatics/btu170
26. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H (2021) Twelve years of SAMtools and BCFtools. GigaScience 10(2) https://doi.org/10.1093/gigascience/giab008
27. Wick RR, Judd LM, Gorrie CL, Holt KE (2017) Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13(6):e1005595. https://doi.org/10.1371/journal.pcbi.1005595
28. Gurevich A, Saveliev V, Vyahhi N, Tesler G (2013) QUAST: quality assessment tool for genome assemblies. Bioinformatics 29(8):1072–1075. https://doi.org/10.1093/bioinformatics/btt086
29. Lee I, Kim YO, Park S, Chun J (2015) OrthoANI: An improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol 66(2):1100–1103. https://doi.org/10.1099/ijsem.0.000760
30. Meier-Kolthoff JP, Göker M (2019) TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat Commun 10(1):2182. https://doi.org/10.1038/s41467-019-10210-3
31. Seemann T, Abricate Github https://github.com/tseemann/abricate
32. Fanfoni E, Bellassi P, Fontana A, Sinisgalli E, Rocchetti G, Piccinini S, Morelli L, Cappa F (2025) Metagenomics and untargeted metabolomics reveal antibiotic resistance dynamics in an anaerobic digestion-composting system treating organic fraction of municipal solid waste. Environ Microbiome 20(1):106. https://doi.org/10.1186/s40793-025-00769-4
33. Authority EFS (2021) EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. EFSA J 19(7):e06506. https://doi.org/10.2903/j.efsa.2021.6506
34. Kim E, Yang S, Lim B, Park SH, Rackerby B, Kim H (2020) Design of PCR assays to specifically detect and identify 37 *Lactobacillus* species in a single 96 well plate. BMC Microbiol 20(1):96. https://doi.org/10.1186/s12866-020-01781-z
35. Parolin C, Marangoni A, Laghi L, Foschi C, Palomino RAÑ, Calonghi N, Cevenini R, Vitali B (2015) Isolation of vaginal lactobacilli and characterization of Anti-Candida activity. PLoS ONE 10(6):e0131220. https://doi.org/10.1371/journal.pone.0131220
36. Giordani B, Naldi M, Croatti V, Parolin C, Bartolini M, Vitali B (2023) Exopolysaccharides from vaginal lactobacilli modulate microbial biofilms. Microb Cell Fact 22(1):45. https://doi.org/10.1186/s12934-023-02053-x
37. Giordani B, Parolin C, Abruzzo A, Foschi C, Marangoni A, Luppi B, Vitali B (2023) Limosilactobacillus vaginalis Exerts Bifidogenic Effects: A Novel Postbiotic Strategy for Infant Prebiotic Supplementation. Nutrients 15(20):4433. https://doi.org/10.3390/nu15204433
38. Alessandro D', Parolin C, Patrignani S, Sottile G, Antonazzo P, Vitali B, Lanciotti R, Patrignani F (2022) Human breast milk: a source of potential probiotic candidates. Microorganisms 10(7):1279. https://doi.org/10.3390/microorganisms10071279
39. Giordani B, Monti F, Corazza E, Gasperini S, Parolin C, Abruzzo A, Foschi C, Marangoni A, Lenzi M, Luppi B, Vitali B (2025) Functional and Safety Profile of Limosilactobacillus vaginalis and Development of Oral Fast-Disintegrating Tablets for Gut Microbiota Modulation. Pharmaceutics 17(8):1011. https://doi.org/10.3390/pharmaceutics17081011
40. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carrière F, Boutrou R, Corredig M, Dupont D, Dufour C, Egger L, Golding M, Karakaya S, Kirkhus B, Le Feunteun S, Lesmes U, Macierzanka A, Mackie A, Marze S, McClements DJ, Ménard O, Recio I, Santos CN, Singh RP, Vegarud GE, Wickham MSJ, Weitschies W, Brodkorb A (2014) A standardised static in vitro digestion method suitable for food–an international consensus. Food Funct 5(6):1113–1124. https://doi.org/10.1039/C3FO60702J
41. Deidda F, Cordovana M, Cionci NB, Graziano T, Di Gioia D, Pane M (2022) In-process real-time probiotic phenotypic strain identity tracking: The use of Fourier transform infrared spectroscopy. Front Microbiol 13:1052420. https://doi.org/10.3389/fmicb.2022.1052420
42. Li X, Zhu L, Wang X, Li J, Tang B (2022) Evaluation of IR biotyper for *Lactiplantibacillus plantarum* typing and its application potential in probiotic preliminary screening. Front Microbiol 13:823120. https://doi.org/10.3389/fmicb.2022.823120
43. Wishart DS, Tzur D, Knox C et al (2007) HMDB: the Human Metabolome Database. Nucl Acids Res 35:D521–D526. https://doi.org/10.1093/nar/gkl923
44. Dieterle F, Ross A, Schlotterbeck G, Senn H (2006) Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in ^{1}H NMR metabonomics. Anal Chem 78(13):4281–4290. https://doi.org/10.1021/ac051632c
45. Hubert M, Rousseeuw PJ, Vanden Branden K (2005) ROBPCA: A new approach to robust principal component analysis. Technometrics 47(1):64–79. https://doi.org/10.1198/004017004000000563
46. Fiore W, Arioli S, Guglielmetti S (2020) The neglected microbial components of commercial probiotic formulations. Microorganisms 8:1177. https://doi.org/10.3390/microorganisms8081177
47. Shrivastava S, Sharma N, Sharma A, Gupta S (2025) Comprehensive genomic assessment of available probiotics in India: A

framework for safety and functionality evaluation. Bioinformation 21(05):1163–1168. https://doi.org/10.6026/973206300211163
48. Dodoo CC, Wang J, Basit AW, Stapleton P, Gaisford S (2017) Targeted delivery of probiotics to enhance gastrointestinal stability and intestinal colonisation. Int J Pharm 530(1–2):224–229. https://doi.org/10.1016/j.ijpharm.2017.07.068
49. Fredua-Agyeman M, Gaisford S (2014) Comparative survival of commercial probiotic formulations: tests in biorelevant gastric fluids and real-time measurements using microcalorimetry. Benef Microbes 6(1):141–152. https://doi.org/10.3920/bm2014.0051
50. Vecchione A, Celandroni F, Mazzantini D, Senesi S, Lupetti A, Ghelardi E (2018) Compositional quality and potential gastrointestinal behavior of probiotic products commercialized in Italy. Front Med 5:59. https://doi.org/10.3389/fmed.2018.00059
51. Talearngkul R, Sae-Tan S, Sirivarasai J (2023) Effect of yogurt ice cream on the viability and antidiabetic potential of the probiotics *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus*, and *Bifidobacterium animalis* subsp. *lactis* after in vitro digestion. Foods 12(23):4373, 4373. https://doi.org/10.3390/foods12234373
52. Da Silva MN, Tagliapietra BL, Flores VDA (2021) In vitro test to evaluate survival in the gastrointestinal tract of commercial probiotics. Curr Res Food Sci 4:320–325. https://doi.org/10.1016/j.crfs.2021.04.006
53. Pawlos M, Szajnar K, Znamirowska-Piotrowska A (2024) Probiotic Milk and Oat Beverages with Increased Protein Content: Survival of Probiotic Bacteria Under Simulated In Vitro Digestion Conditions. Nutrients 16(21):3673. https://doi.org/10.3390/nu16213673
54. da Rodrigues C, da Silva VC, Simabuco LGS, Venema FM, Antunes K AEC (2019) Survival, metabolic status and cellular morphology of probiotics in dairy products and dietary supplement after simulated digestion. J Funct Foods 55:126–134. https://doi.org/10.1016/j.jff.2019.01.046
55. Soares MB, Martinez RCR, Pereira EPR, Balthazar CF, Cruz AG, Ranadheera CS, Sant'Ana AS (2019) The resistance of *Bacillus*, *Bifidobacterium*, and *Lactobacillus* strains with claimed probiotic properties in different food matrices exposed to simulated gastrointestinal tract conditions. Food Res Int 125:108542, 108542. https://doi.org/10.1016/j.foodres.2019.108542
56. Vinderola C, Reinheimer J (2003) Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. Food Res Int 9969(03):895–904. https://doi.org/10.1016/s0963-9969(03)00098-x
57. Arioli S, Koirala R, Taverniti V, Fiore W, Guglielmetti S (2018) Quantitative recovery of viable *Lactobacillus paracasei* CNCM I-1572 (*L. casei* DG®) after gastrointestinal passage in healthy adults. Front Microbiol 9:1720, 1720. https://doi.org/10.3389/fmicb.2018.01720
58. Papadimitriou K, Alegría Á, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, van Sinderen D, Varmanen P, Ventura M, Zúñiga M, Tsakalidou E, Kok J (2016) Stress physiology of lactic acid bacteria. Microbiol Mol Biol Rev. https://doi.org/10.1128/mmbr.00076-15
59. Kleerebezem M, Hugenholtz J (2003) Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol 14(2):232–237. https://doi.org/10.1016/s0958-1669(03)00033-8
60. Ramos A, Neves AR, Santos H (2002) Metabolism of lactic acid bacteria studied by nuclear magnetic resonance. Antonie Van Leeuwenhoek 82:249–261. https://doi.org/10.1023/A:1020664422633
61. Fernstrom JD, Fernstrom MH (2007) Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. J Nutr 137(6 Suppl 1):1539S-1548S. https://doi.org/10.1093/jn/137.6.1539S
62. Trimigno A, Bøge Lyndgaard C, Atladóttir GA, Aru V, Balling Engelsen S, Harder Clemmensen LK (2020) An NMR Metabolomics Approach to Investigate Factors Affecting the Yoghurt Fermentation Process and Quality. Metabolites 10(7):293. https://doi.org/10.3390/metabo10070293
63. Remagni MC, Paladino M, Locci F, Romeo FV, Zago M, Povolo M, Contarini G, Carminati D (2013) Cholesterol removal capability of lactic acid bacteria and related cell membrane fatty acid modifications. Folia Microbiol 58(6):443–449. https://doi.org/10.1007/s12223-013-0228-8
64. Raimondi F, Santoro P, Barone MV, Pappacoda S, Barretta ML, Nanayakkara M, Apicella C, Capasso L, Paludetto R (2008) Bile acids modulate tight junction structure and barrier function of Caco-2 monolayers via EGFR activation. Am J Physiol Gastrointest Liver Physiol 294(4):G906–G913. https://doi.org/10.1152/ajpgi.00043.2007