AUTHENTICITY OF PROBIOTIC FOODS AND DIETARY SUPPLEMENTS

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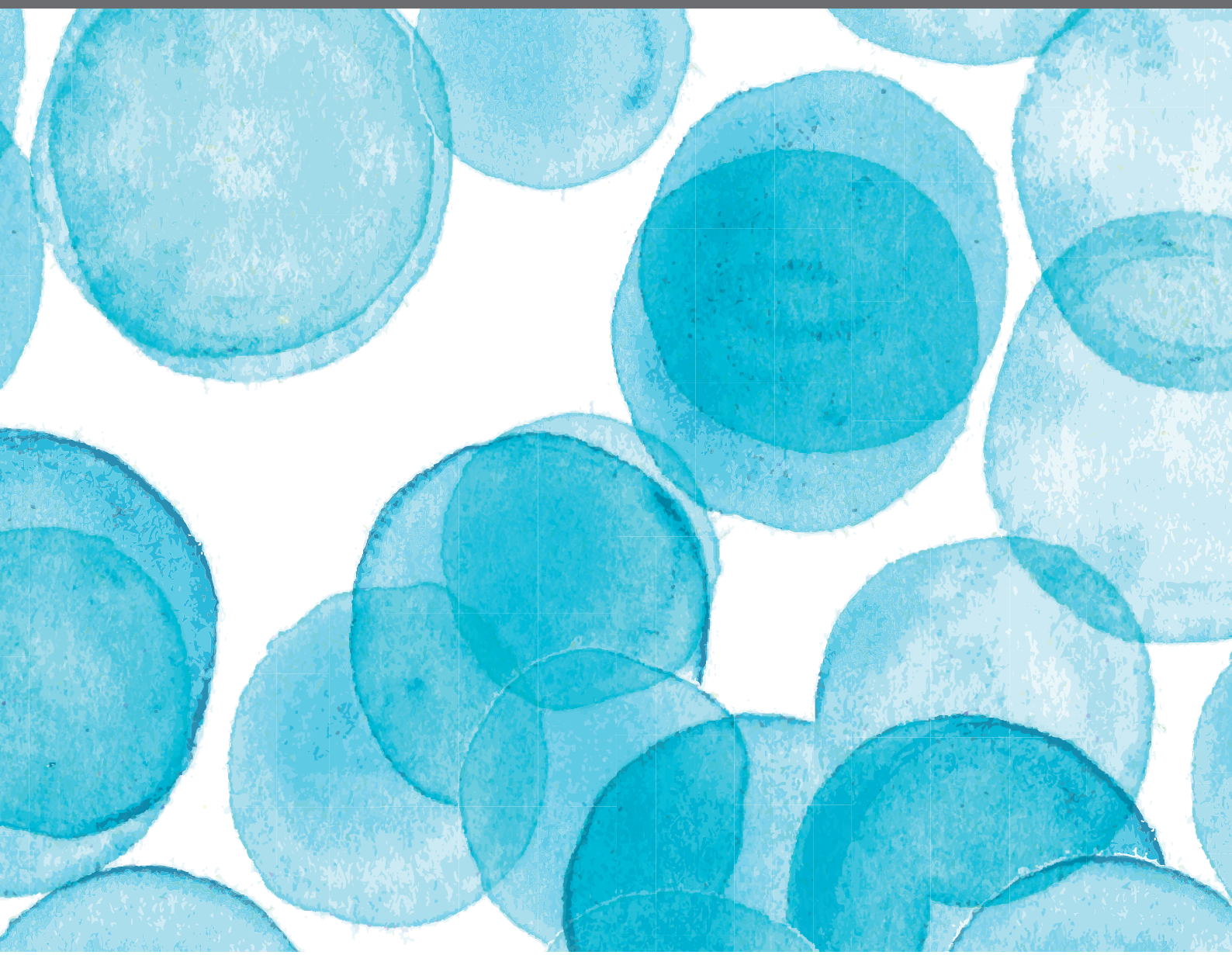

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AUTHENTICITY OF PROBIOTIC FOODS AND DIETARY SUPPLEMENTS

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EDITORIAL
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Editorial: Authenticity of Probiotic Foods and Dietary Supplements

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Editorial on the Research Topic

Authenticity of Probiotic Foods and Dietary Supplements

Probiotics are viable microorganisms, which, if ingested in adequate amounts, confer a health benefit to the host (Hill et al., 2014). An authentic probiotic food must contain the number of viable cells of the specific probiotic strain correctly cited on the label and provide the claimed beneficial health effects, which should not be deceptive for consumers (Di Lena et al., 2015).

While the production and the global market of probiotic foods and supplements is increasing worldwide, the indication of the probiotic microorganisms reported on the label might be misleading both at quali- and quantitative levels. Indeed, several studies have reported inconsistency between the actual content of probiotics in commercial foods and dietary supplements and their label information in terms of the dose of viable cells and type of microorganism (at genus, species, or strain level) (Fusco et al., 2021). This scenario is further complicated by the taxonomic amendments that have occurred in the last years mainly due to the availability of complete genomes of (probiotic) strains (Makarova et al., 2006; Briczinski et al., 2009; Loquasto et al., 2013; Holzapfel and Wood, 2014; Milani et al., 2014; Lugli et al., 2019; Zheng et al., 2020). As an example, the *Lactobacillus* genus has been recently reclassified into 25 genera including the amended genera *Lactobacillus*, *Paralactobacillus*, and 23 novel genera (Zheng et al., 2020). The scientific community as well as regulators and consumers must deal with these taxonomic revisions as soon as they occur.

All the above findings prompt the need to improve and standardize the methods to assess the authenticity of the probiotic foods and supplements and harmonize their regulation at the global level.

The most valuable methods are those able to distinguish among dead, viable, and viable but not cultivable (VBNC) cells, which might be present in probiotic foods due to the biotic and abiotic stresses that probiotics undergo during the production, storage, distribution, and consumption (Fusco and Quero, 2014; Fiocco et al., 2020; Fiore et al., 2020; Fusco et al., 2021). Nevertheless, in recent years, alternative methods including fluorescent *in situ* hybridization (FISH) (Babot et al., 2011), flow cytometry (Wilkinson, 2018), or combination of multi-omics approaches, such as the promising propidium monoazide (PMA)-metagenomics (Fusco et al., 2021) and culturomics, have been proposed.

This Research Topic aims to collect the latest research on the authenticity evaluation of probiotic foods and dietary supplements. It covers a total of six articles, including three original researches, two methods, and one review, with a focus on the legislation, assessment, development, and application of chemical, molecular, and omics methods to evaluate the authenticity of probiotic foods and supplements.

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Pammi et al. presented the characterization of a traditional Indian rural drink obtained by the Toddy Palm Nectar, indicating its probiotic potential by nutritional profiling and isolation of lactic acid bacterial strains, which could be exploited in developing therapeutic applications.

Lorbeg et al. evaluated the quality of dietary supplements containing viable bacteria available in Slovenian pharmacies using plate counting, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and species- or subspecies-specific PCR. Besides revealing mislabeling of *Lacticaseibacillus casei* in some products, the study confirmed that MALDI-TOF MS can be effectively used in the quality control of probiotic products, being as a faster and simpler alternative to PCR identification. It was also indicated that the generation of a dedicated in-house library may further improve the identification accuracy at the species and sub-species level.

Colom et al. performed a clinical trial to directly investigate, for the first time, the presence and germination of the probiotic strain *Bacillus subtilis* DE111® after the ingestion of commercially available capsules in the small intestine using a novel methodology involving healthy adults with an ileostomy. *B. subtilis* DE111® spores were able to retain their viability during the transit through the stomach and germinate in the small intestine of humans within 3 h of ingestion.

Deidda et al. described the first investigation of bifidobacterial strain typing using Fourier transform infrared (FTIR) spectroscopy. Compared to pulsed-field gel electrophoresis (PFGE), whole-genome sequencing (WGS), multilocus sequence typing (MLST), FTIR resulted more informative and able to differentiate strains within the *B. animalis* subsp. *lactis* group.

Weitzel et al. illustrated how the implementation of the analytical procedure lifecycle management (APLM) in plate counting can lead to lower variability and significantly impact the manufacturing process, reduce costs for industries and improve the quality evaluation of probiotic products, while supporting claims of dose and, therefore, health benefits.

A very comprehensive review by Mazzantini et al. described the many incongruences in the compositional quality of some probiotic formulations available on the worldwide market, highlighting the need of using recommended, standardized, and updated methodologies for analyzing and labeling probiotic products.

All the contributions presented in this topic confirm the need to urgently harmonize the regulation on probiotics worldwide, as promoted by the International Scientific Association for Probiotics and Prebiotics (ISAPP, https://isappscience.org) and the International Probiotics Association (IPA, https://internationalprobiotics.org), to develop updated, standardized, faster, and reliable methods to assess the authenticity of probiotics and ensure the criteria of taxonomy, viability, stability, and safety needed to characterize probiotic foods and supplements. These advances are expected to generate improvements in the manufacturing process and quality control to guarantee the development and validation of probiotic-based therapeutical strategies, as well as in the defense of the consumers' right of being correctly informed and aware of their choices.

AUTHOR CONTRIBUTIONS

All authors write, revised, and accepted the manuscript.

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Bioprospecting of Palmyra Palm (*Borassus flabellifer*) Nectar: Unveiling the Probiotic and Therapeutic Potential of the Traditional Rural Drink

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The present study investigates the therapeutic and probiotic attributes of traditional Toddy Palm Nectar (TPN). Glucose was found to be the highest with 4.37 mg/ml and arabinose was the least with 2.85 mg/ml. The average ethanol concentration of fresh TPN was found to be 0.3 mg/ml. The nutritional profile of TPN revealed 18 volatile fatty acids, the major one being hexadecenoic acid (M/Z 74). Amino acid profiling showed 26 amino acids, with OH-lysine-2 the highest (12.86%). About 120 morphologically distinct lactic acid bacteria (LAB) were isolated from 26 TPN samples, based on differential growth and *in vitro* probiotic characteristics. After 16S rRNA sequencing, four indigenous LAB strains were identified as *Lactobacillus plantarum* group OUBN1, *Enterococcus faecium* OUBN3, *Pediococcus acidilactici* OUBN4, and *Pediococcus pentosaceous* OUBN5 and their sequences were deposited to NCBI. Microbiological safety evaluation studies showed the absence of hemolytic, gelatinolytic and proteolytic activity. The bacterial isolate OUBN3 showed a maximum survival rate of 6.91 ± 0.04 log cfu/ml at acidic pH 2.5 and isolate OUBN5 showed 6.94 ± 0.02 log cfu/ml at pH 3.0. Similarly, the isolate OUBN5 showed 7.92 ± 0.03 log cfu/ml to 0.3% ox-bile after 4 h and 8.94 ± 0.03 log cfu/ml to simulated gastric juice after 3 h of treatments. OUBN1 expressed the highest autoaggregation (81.76 ± 1.25%), cell surface hydrophobicity (79.71 ± 3.42%), and displayed the maximum coaggregation with *E. coli* MTCC452 (76.96%), *K. pneumoniae* MTCC109 (75.62%), and *S. aureus* MTCC902 (70.69%). All strains showed significant antibiotic and antimicrobial activity. Isolate OUBN1 displayed hydroxyl radical scavenging activity (68.71 ± 1.0%) with an IC_{50} value of 75.62 μg/ml and the highest anti-cancer activity (percentage inhibition of 88.55) against HT-29 cells. Based on the characteristics observed, *L. plantarum* group OUBN1 and *P. pentosaceous* OUBN5 were found to be potential isolates to employ as probiotic microbiota in food and forage preparations. These findings reinforce the fact that LAB isolated from TPN could be exploited as an alternative means toward potential therapeutic applications.

Keywords: probiotics, lactic acid bacteria, toddy palm nectar, volatile fatty acids, amino acids

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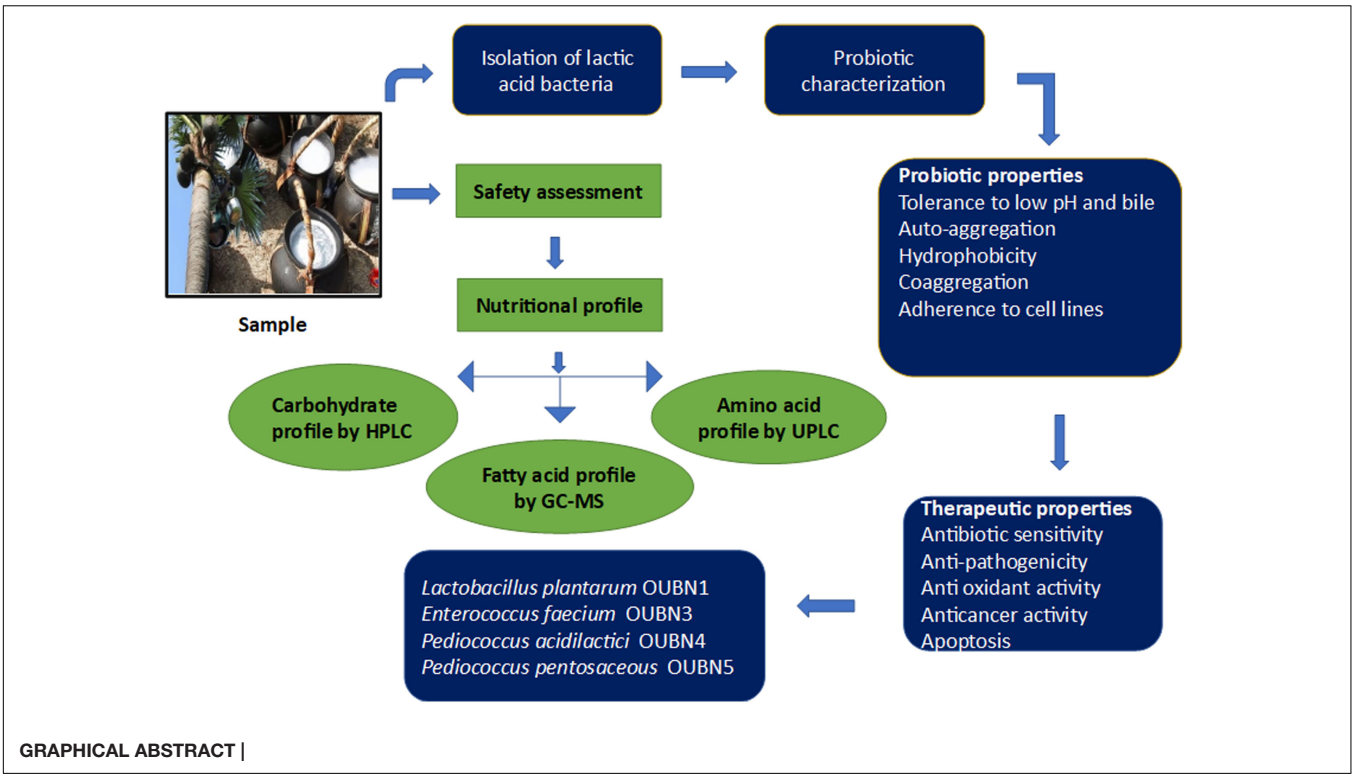

GRAPHICAL ABSTRACT |

INTRODUCTION

Fermented plant-based beverages have been persistent in human societies for ages (Lamba et al., 2019). Among them, toddy palm nectar (TPN) is one of the renowned naturally fermented seasonal traditional alcoholic beverages consumed in various regions of rural India. Due to growing lactose intolerance and allergy to milk and milk-based products, consumers are intensely demanding functional probiotic products from natural sources such as fruits, vegetables, and cereals (Sornplang and Piyadeatsoontorn, 2016).

Therefore, more attention toward fermented products of plant origin is a need of the hour (Coutiño et al., 2020). It is noteworthy that TPN represents a traditional rural drink of the Indian society for ages. Owing to the vital role it has occupied in traditional culture, it is necessary to understand the microbiological and biochemical nature of TPN, as not many studies have been conducted on probiotics and nutritional aspects of it.

TPN is a naturally fermented sap from young and matured inflorescences of *Borassus flabellifer* Linn. (Palmyra Palm), belongs to the family *Arecaceae,* and is commonly referred to as "toddy" (Zeid and FarajAlla, 2019). As a traditional energizing drink with significant health-promoting effects, TPN is enjoyed by people in parts of South America, Africa, and Asia (Reshma et al., 2017). Local names of the product include kallu in southern India, emu, and ogogoro in Nigeria, nsafufuo in Ghana, and tuba in Mexico. Fresh TPN is a colorless sweet-flavored drink with a pH between 6.0 and 7.0, which contains various carbohydrates and proteins. Generally, it contains reducing and non-reducing sugars, ethanol, and various nutrients including volatile fatty acids (VFAs), amino acids, and flavonoids (Lasekan and Abbas, 2010). It is also a good source of ascorbic acid, nicotinic acid, vitamin A, riboflavin, several minerals, and salts (Morton, 1988; Chinnamma et al., 2019). As a folk medicine, TPN obtained from the flower stalks of palm trees can be used as a tonic, stimulant, laxative, diuretic, anti-phlegmatic, and amebicide (Lim, 2012; Mariselvam et al., 2020).

Volatile fatty acids have several applications in the food, healthcare, and pharmaceutical industries and their derivatives are used as anticonvulsants in neurodegenerative diseases as neuroprotective agents (Lei et al., 2016). VFAs help to regulate insulin secretion and indirectly affect cholesterol synthesis. Plant-derived amino acids are important dietary bioactive components for human and animal nutrition. Essential amino acids have been recognized as nutrient regulators in muscle protein synthesis and tissue regeneration. Therefore, estimation of these nutritional attributes is essential for the development of plant-based foods (Tayade et al., 2017).

Fresh TPN collected under hygienic conditions during the early hours of a day is known to contain various probiotic strains of yeast and bacteria (Somashekaraiah et al., 2019). It is reported that TPN could be a potential source of probiotic LAB like *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Pediococcus, and Streptococcus,* etc. (Amoa-Awua et al., 2007; Somashekaraiah et al., 2019). Bacteria related to *Lactobacillus*, *Pediococcus,* and *Bifidobacterium* are predominant groups used in many dietary supplements and functional foods (Pandey et al., 2015). Ideal probiotics must exhibit *in vitro* characteristics, such as tolerance to low pH, bile, gastric juice, antibiotics and efficiently adhere to intestinal epithelial cells. It must also

obey other technical qualities such as auto-aggregation, co-aggregation, cell surface hydrophobicity, vigorous antimicrobial activity against enteric pathogens, and boosting the immune response (Saadat et al., 2019). Antioxidant and anticancer properties provide a potential platform for their therapeutic applications (Barigela and Bhukya, 2021). The influence of LAB against enteric pathogens, reducing toxicity and increasing nutritive value of fermented foods has been studied by Bartkiene et al. (2018). Numerous probiotic studies have shown promising results in improving health ailments such as anxiety, depression (Aslam et al., 2020), antibiotic-related diarrhea, irritable bowel syndrome (Sanders et al., 2019), and alleviation of lactose intolerance (Aspri et al., 2020).

The prime objective of the current study is to gain awareness and explore the nutritional and probiotic qualities of TPN. Selected LAB were analyzed for their *in vitro* probiotic attributes to confirm their potential health benefits and therapeutic applications.

MATERIALS AND METHODS

Sample Collection and Processing

Twenty-six fresh samples of toddy palm nectar (TPN) were collected from different geographical areas of Telangana, India in sterilized polythene bags under hygienic conditions and brought to the laboratory in an icebox at 4°C, without exposure to the sun. One set of samples was directly stored in the refrigerator and another set of samples were centrifuged at 10,000 rpm for 10 min, supernatants were filtered through a 0.22 μm membrane filter (Sartorius AG, Göttingen, Germany) and stored at –20∘C for further analysis. The pH of TPN samples was determined using a pH meter (Systronics, India).

Chemical Profile of Toddy Palm Nectar

Evaluation of Total Sugars and Ethanol by HPLC

Total sugars and ethanol from fresh TPN were analyzed on HPLC (Shimadzu Inc.) with the method described by Lefebvre et al., 2002. Analysis was performed under isocratic conditions using a platinum amino column (250 × 4.6 mm, 5 μm) and a photodiode array detector (PDA) maintained at 40°C. The mobile phase consisted of 0.001 N H_2SO_4 was pumped with a flow rate of 1 ml/min with an injection volume of 20 μl. The eluent and samples were filtered using 0.22 μm nylon membrane filters (Sartorius AG, Göttingen, Germany) prior to analysis. All chemicals used were of HPLC grade and supplied by Sigma-Aldrich. Elution was monitored at 215 nm. Ethanol and different sugars like sucrose, fructose, glucose, galactose, lactose, arabinose, raffinose were identified by comparing the retention times with authentic standards and their concentrations were determined.

Evaluation of Volatile Fatty Acids by GC-MS Analysis

Gas chromatography and mass spectrometry (GC-MS) analysis were carried out as described by Dos Santos et al., 2011 using GCMS-QP2010 PLUS (Shimadzu, Japan) with DB5 MS (0.25 × 30 × 0.25) column. Helium (carrier) was used at a rate of 1 ml/min and an injection volume of 1μl was used with an injector temperature of 280°C and ion-source temperature of 200°C. The temperature was maintained at 100°C (isothermal for 4 min), with an increase of 10°C/min, up to 200°C. The temperature was then raised at a level of 4°C until it reached 280°C and maintained for 12.95 min. The sample was prepared by mixing 500 μl of TPN with 200 μl chloroform, vortexed for 5 min and allowed to settle, followed by centrifugation at 4,000 rpm for 10 min. The chloroform layer was collected, and the above step was repeated three times After each step, chloroform layers were pooled and dried under vacuum. Derivatization was carried out for the speed vac dried pellet with methanol and heated at 60∘C for 20 min. Subsequently, 1 ml of chloroform was added and after a short spin, approximately 100 μl of chloroform layer was taken for injection. Mass spectra were recorded at two scans per second with a scanning interval of 50 –600 m/z. Compounds were identified based on GC retention times and compared with standard mass spectra using the Wiley and NIST (National Institute of Standards and Technology) Librarie 11.

Estimation and Quantification of Amino Acids by Ultra-Performance Liquid Chromatography (UPLC)

Amino acid profiling was carried out by UPLC (Waters Acquity) equipped with a PDA detector according to the previous protocol (Szkudzińska et al., 2017). The column temperature was maintained at 55∘C and 260 nm wavelength with a flow rate of 0.7 ml/min. In brief, 2 ml of 6 M HCL was taken into a 50 ml flat bottom tube containing internal standard. Hundred microliter of the test sample was taken into a separate clean glass vial and inserted into a flat bottom tube. The tube was sealed with parafilm and placed in a dry bath at 60°C under N_2 gas for 15 min, to maintain inertness. The temperature of the dry bath was gradually increased to 110°C and the incubation was extended till 24 h to get the pellet. Borate buffer (200 μl) was added to the pellet, vortexed, and centrifuged. Later 10 μl of sample from the supernatant was added with 70 μl of borate buffer and 20 μl of Accq Tag ultra-reagent and incubated for 10 min at 55°C for derivatization. After incubation, 1 μl was loaded into UPLC and quantified using amino acid standards (Sigma).

Isolation and Screening of LAB From TPN Samples

From 26 TPN samples, 1 ml each was added to de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37 ∘C for 24 h in aerobic conditions, and a further 10- fold dilution was made up to 10^{-7} by adding phosphate buffer saline (PBS). Aliquots of 0.1 ml of each dilution were seeded on MRS agar plates containing $CaCO_3$ by the spread plate method (Xiao et al., 2015) to distinguish acid-producing bacteria. The bacterial colonies forming clear zones due to the hydrolysis of $CaCO_3$ around them were considered as LAB and were individually picked and streaked on MRS agar for further screening. After incubation, 120 morphologically discrete colonies were randomly selected, obtained LAB isolates were subcultured on MRS agar plates and stored at 4°C for further characterization. All LAB isolates were examined by Gram staining, Catalase test, their tolerance to salts and temperature was evaluated and morphology was studied by microscopic observation. Isolates were cultured at different

temperatures (10–45°C) and different concentrations of NaCl (2, 4, 6% w/v) (Ni et al., 2015).

Molecular Identification of LAB Isolates

Extraction of bacterial genomic DNA was done using the MagGenome XpressDNA isolation kit (India). Amplification of 16S rRNA was performed using a primer set of 27F (5AGAGTTTGAYCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) (Macrogen, South Korea). Obtained sequences were compared with the data available in the GenBank database by NCBI-BLAST (Altschul et al., 1997). The phylogenetic tree was constructed using the Neighbor-joining method with Mega X software (Saitou and Nei, 1987) and sequences were submitted to NCBI.

Safety Evaluation

Hemolytic, proteolytic and gelatinase activities of TPN microflora was evaluated by inoculating the TPN and overnight grown cultures of LAB isolates in Columbia blood agar (BD, Difco), MRS agar supplemented with 1% skimmed milk powder and MRS agar (Himedia, India) supplemented with 5% (w/v) gelatin (HiMedia, India), respectively, and incubating at 37°C under aerobic conditions for 48 h. After incubation, plates were observed for hemolytic properties viz. α-hemolysis (greenish clear zone around the colony), β-hemolysis (clear zone around the colony) and, γ-hemolysis (no clear zone around the colony) (Yasmin et al., 2020). The presence of a clear zone around the colonies in skimmed milk supplemented medium was considered positive for proteolytic activity (Da Silva et al., 2019). Gelatinase activity was observed in gelatin supplemented medium after 72 h. of incubation and subsequently, plates were kept at 4°C for 4 h to observe the clear zone around the colonies as a positive result (Perin et al., 2014). A multiple tube fermentation test was carried out to check the coliform contamination (Mannapperuma et al., 2011). TPN samples were inoculated, incubated at 37°C aerobically for 48 h in MacConkey broth (Himedia, India) to activate coliforms. These active cultures were added to the Brilliant Green Lactose Bile broth (Himedia, India) embedded with Durhams tubes, then incubated at 37°C for 48 h. Coliforms can be confirmed by observing gas production in inverted Durhams tubes and the appearance of a red ring after adding 0.2 ml of Kovac's reagent to the Brilliant Green Lactose Bile broth.

Evaluation of Probiotic Properties

LAB isolates were evaluated *in vitro* for their probiotic potential.

Acid and Bile Tolerance

Tolerance to acidic pH was carried out with the methodology described by Zommiti et al. (2018). The overnight grown cultures of LAB isolates were inoculated in MRS broth with pH adjusted to 2.5 and 3.0 using 1N Hydrochloric acid (HCL) and incubated aerobically at 37○C for 3 h. The MRS broth with initial pH of 6.5 was considered as a control for acidic pH comparison. Samples were collected at every 1 h interval to check for the viability of isolates for 3 h and calculated the log cfu/ml. Bile salt tolerance of isolates was carried out using the protocol of de Albuquerque et al. (2018). The overnight grown LAB cultures were inoculated in MRS broth supplemented with 0.3% (w/v) Ox-gall and incubated at 37°C for 4 h. MRS broth without bile salt was used as a control for comparison. Samples were collected at every 1h interval, serially diluted, plated on MRS agar, and incubated at 37°C for 48 h and viable cell counts (log cfu/ml) were determined.

Survival in Simulated Gastric Juice

Subsequently, the selected isolates from each of the above experiments were further tested to determine their ability to survive in a gastric environment by inoculating them in simulated gastric juice as described by Singhal et al., 2019. Five ml of overnight grown cultures of LAB strains were centrifuged, and the bacterial pellet was washed and re-suspended in 4 ml of saline (0.8% NaCl). To the 1 ml of cell suspension, 9 ml of simulated gastric juice (pH 3) was added and vortexed for 15 s. After incubation for 1, 2, and 3 h, cells were harvested by taking 1 ml of sample broth into sterile 2 ml Eppendorf tube, then centrifuged at 10,000 rpm for 5 min. Viable counts were determined by growing on MRS agar incubated at 37°C for 24 h. Cell viability (log cfu/ml) was assessed using the plate count method. MRS medium without simulated gastric juice was used as a control. Survival (%) of the organisms was calculated as follows: % Survival = (log no of viable cells survived/log no of initially viable cells) × 100.

Auto Aggregation, Coaggregation and Cell Surface Hydrophobicity

Auto aggregation and coaggregation ability of LAB isolates were carried out by the method of Choi et al. (2018). For autoaggregation, overnight grown LAB cultures were harvested, washed, and re-suspended in PBS. The optical density of suspension was adjusted to 0.50 at 600 nm (OD_{600}) and incubated aerobically at 37°C without agitation. OD_{600} was measured after 24 h of incubation, and the percent aggregation was determined as follows: $A\% = (1 - At/A_0) \times 100$, where A0 and At refers to the OD_{600} at 0 h and at the indicated time, respectively.

The ability of LAB isolates to aggregate with enteric pathogens like *Escherichia coli* MTCC 452, *Salmonella enterica* ser. *paratyphi* MTCC 3216, *Enterococcus faecalis* MTCC 6845, *Proteus vulgaris* MTCC426, and *Klebsiella pneumoniae* MTCC 109 was studied. Equal volumes of the LAB cultures and selected pathogenic bacteria were mixed after adjusting the OD_{600} to 0.5. The co-aggregation was expressed as the percentage reduction in the absorbance of the mixed suspension compared to the individual suspensions by taking the OD_{600} after 6 and 24 h incubation. The growth rate of pathogenic bacteria without adding cell-free supernatant (CFS) was considered as 100% (control).

Further, LAB isolates were also assessed for cell surface hydrophobicity, by measuring adhesion capacity to hydrocarbons (Rokana et al., 2018). Overnight cultures were harvested by centrifugation at 8,000 rpm, 4○C for 10 min, and the pellet was washed twice with PBS and re-suspended in the same buffer followed by measurement of OD_{600}. Three milliliter of cell suspension was blended with 1 ml of hydrocarbon (xylene) and incubated without shaking at 37○C for 1 h to get aqueous and organic phases separately. One milliliter of the aqueous phase was removed carefully and the OD_{600} was measured. Cell surface

hydrophobicity was calculated using the following formula: Cell surface hydrophobicity % = $(1- A_1/A_0] \times 100$.

Antibiotic Sensitivity

The antibiotic sensitivity of selected LAB isolates was assessed by the disc diffusion method (Singhal et al., 2019). Antibiotic discs of Penicillin-G (10 units), ampicillin (AMP 10 μg), polymyxin-B (300 units), vancomycin (VA 30 μg), amoxicillin (AMX 10 μg), rifampicin (RIF 5 μg), trimethoprim (TR 10μg), norfloxacin (NX 10 μg), ciprofloxacin (CIP 5 μg), streptomycin (S 10 μg), chloramphenicol (C 30 μg), tetracycline (TE 30 μg), clindamycin (CD 2 μg), erythromycin (E 15 μg), lincomycin (L 10 μg), kanamycin (K 30 μg) and gentamycin (GEN 10 μg) were chosen based on the recommendations of European Security Food Authority (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012). Hundred microliter of overnight grown cultures of LAB were spread onto the MRS medium plates, allowed to dry and antibiotic discs were placed, then incubated aerobically at 37∘C for 24 h. The diameter of the clear zone of inhibition was measured using an antibiotic zone scale. Results obtained were expressed as sensitivity/resistance in mm.

Antimicrobial Activity Against Enteric Pathogens

The antimicrobial feature of LAB isolates against enteric pathogens was assessed using the agar well diffusion method (Yadav et al., 2016). Indicator organisms like *Escherichia coli* MTCC452, *Salmonella enterica* ser. *paratyphi* MTCC 3216, *Pseudomonas aeruginosa* MTCC424, *Enterococcus faecalis* MTCC 6845, *Proteus vulgaris* MTCC426, *and Klebsiella pneumoniae* MTCC109 were incubated in Luria-Bertani broth for 24 h, diluted until it gets the OD_{600} of 0.06, then spread on Muller Hinton agar. Neutralized cell-free supernatant (nCFS) was prepared by centrifuging overnight cultures of LAB isolates at 10,000 × g/10 min at 4°C, and the pH of the supernatant was adjusted to 6.5 using 5 M NaOH. Another set of CFS without neutralization was also prepared to differentiate the impact of pH. Subsequently, 30 μl of cell-free supernatant (CFS) of LAB cultures were placed in each well and incubated for 24 h at 37°C to determine the zone of inhibition around the well.

Antioxidant Assay

DPPH radical scavenging ability of LAB was assessed by the method described by Xing et al. (2015). Briefly, 100 μl of freshly prepared 0.2 mM DPPH solution (in methanol) was added to 96- well plate comprising different concentrations of sample, then made up to 200 μl with distilled water. The sample was vortexed and kept at 37∘C for 30 min in dark. Blank was prepared by replacing DPPH with methanol. Scavenged DPPH was examined by determining the absorbance at 517 nm against a blank on a microplate reader (Epoch Biotech). Ascorbic acid was used as standard and methanol along with DPPH served as control. IC_{50} values were calculated from the data to find out the concentration of sample required to eliminate DPPH free radicals by 50%. Percentage inhibition to scavenge the DPPH radicals was calculated using the following formula:

DPPH activity (μl/ml) = [(Ao-Ae)/Ao] × 100

where, Ao and Ae are the absorbance of the control and test samples, respectively.

Cell Culture and Adhesion Assay

HT-29 cell lines (human colon adenocarcinoma) were obtained from National Cell Repository at NCCS (Pune, India) and maintained in a Minimum Essential Medium (MEM) supplemented with 20% heat-inactivated Fetal Bovine Serum (FBS) and penicillin 10 U/ml, then incubated at 37°C in a 5% CO_2 incubator. Adhesion of LAB cells to HT-29 cells was carried out by the method described by Sharma and Kanwar (2017). HT-29 cells (5×10^5 cells/ml) were seeded in a six-well plate and cultured until the cells reached the required confluence. Overnight cultures of LAB isolates were harvested, washed twice, and re-suspended in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) at a concentration of 10^9 cfu/ml and added to HT-29 cells. Later 300 μl of methanol was added to each well, followed by incubation for 10 min at room temperature. Methanol was completely removed and cells were fixed by Giemsa staining (0.72% w/v; Sigma) for 30 min at room temperature. Plates were washed with ethanol, air-dried, and bacterial adhesion was examined under an inverted microscope (Olympus BX64, Japan) at a scale of 200 μm.

Anticancer Activity by MTT [-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] Assay

Effect of CFS of LAB on HT-29 colon cancer cell lines for anti-proliferation ability was evaluated by MTT assay according to the protocol of Chandel et al. (2019). Each well of the 96-well plate was seeded with 5×10^5 cells in 200 μl MEM. After 24 h, CFS of LAB were added to each well in a volume-dependent mode (20, 40, 60, 80, and 100 μl) and incubated for 24 h. After precise incubation, 50 μl of MTT solution (0.4 mg/ml) was added to each well and re-incubated for 4 h in a 5% CO_2 incubator. After incubation, the MTT solution was replaced with 100 μl of dimethyl sulfoxide to solubilize the formazan crystals and incubated for 30 min at 37∘C. The absorbance of each well was measured using an ELISA reader (Epoch Biotech microplate reader) at 570 nm. Results were expressed as % anti-cancer activity of LAB which was calculated as 1-(OD of test sample/OD of control)X 100. For comparison, MRS broth was taken as control. IC_{50} values were calculated to know the required CFS of LAB to obtain 50% anti-cancer activity.

Detection of Cell Apoptosis by DAPI (4′, 6-Diamidino-2-Phenylindole) Staining

Two ml of HT-29 cells (1.2×10^5 cells/ml) were added to each well of a six-well plate and incubated in a 5% CO_2 incubator at 37°C for 48 h. After achieving a confluence of 50 –60%, 500 μl of selected LAB-CFS was added to each well, and wells without CFS were considered as control. After 24 h, cells were carefully washed with DMEM and then 4% formaldehyde was added. After 5 min of incubation, the fixed cells were washed twice with PBS, permeabilized, and then again treated with PBS containing 0.1% Triton X-100 for 5 min at 37°C. Cells were stained with 50 μl of DNA-intercalating agent DAPI (1:2,000 dilution) and incubated

for 24 h at 37°C (Nami et al., 2015). Subsequently, the plates were washed with PBS and evaluated under an inverted microscope with a U-MWU2 fluorescence filter (Olympus BX64, Japan).

Statistical Analysis

Each experiment was carried out in triplicates. Values were statistically analyzed and expressed as mean ± standard deviation (SD). Significant differences in the results of each test were determined by comparing relative control values by ANOVA (Analysis of Variance) using Graph Pad Prism software. $P < 0.05$ was considered statistically significant.

RESULTS

Chemical Analysis of Palm Nectar

Sugar, Ethanol, Fatty Acid, and Amino Acid Profile

To understand the nutritional and therapeutic efficiency of TPN, sugar, ethanol, fatty acid (VFA), and amino acid profiling was done. Among sugars, glucose, arabinose, and galactose were detected at retention time (RT) 6.3, 6.4, and 6.2, respectively. Glucose was found to be the highest with 4.37 mg/ml and arabinose was the least with 2.85 mg/ml. The average ethanol concentration of fresh TPN was found to be 0.3 mg/ml. A total of 18 major VFAs were identified in the crude TPN chloroform extract on GC-MS. Among them, significant peaks were observed for hexadecenoic acid, methyl palmitate at RT 23.86 (M/Z 74), E-15-heptadecenal at RT 21.17 (M/Z55) and E-14-hexadecenal at RT 16.73(M/Z55) (**Table 1**). Similarly, a total of 26 primary amino acids were identified in fresh TPN. OH-lysine-2 (12.86% mole) was being the major one and alanine (12.52% mole) and leucine (6.08% mole) were identified as the second and third major amino acids, respectively, while histidine (0.30% mole) was the lowest one (**Table 2**).

TABLE 1 | Volatile fatty acid composition of toddy palm nectar detected by GC-MS (gas chromatography and mass spectrometry).

Name of the compound	RT	M/Z	Area	Biological importance
Triethyl ester (CAS) Boron ethoxide	1.578	73	5375199	Anti-microbial
Butanedioic acid, dimethyl ester	3.296	115	2810517	Flavoring agent
Dodecane, 2,6,11-trimethyl-	3.773	57	1598524	Anti-microbial
1-Dodecene (CAS) Adacene 12	6.62	55	2002846	Anti-bacterial
Permethylated and reduced globicide	4.607	115	896593	Anti-coagulant
Benzene, 1,3-bis(1,1-dimethylethyl)-	8.268	175	5198754	Adenocarcinoma
Benzaldehyde, 4-propyl-	8.755	91	1669626	Biological applications
Dodecane, 2,6,11-trimethyl-	8.901	57	922289	Anti-bacterial
1-Non-adecene (CAS)	9.808	69	333589	Anti-cancer
Hexadecane, 2,6,11,15-tetramethyl-	10.1	57	1879178	Flavoring agent
Tetratriacontane (CAS) n-	12.58	57	714879	Anti-cancer
Eicosane	14.43	57	5924526	Anti-bacterial
Eicosanoic acid, Arachidic acid	15.09	74	1879512	Anti-oxidant
E-14-Hexadecenal	16.73	55	6683918	Anti-inflammatory
Octadecane (CAS) n-Octadecane	19.42	57	5421797	Role in Pheramones
2-Butoxysulfonylhexadecane	20.35	57	1718274	Anti-microbial
E-15-Heptadecenal	21.17	55	7676234	Anti-oxidant
Hexadecanoic acid, Palmitic acid	23.86	74	11666977	Anti-oxidant

Bacterial Isolation and Characterization

A total of 120 bacterial isolates were obtained from twenty-six TPN samples on MRS agar with $CaCO_3$ then further subjected to physiological and biochemical screening. Gram-positive and catalase-negative isolates were presumptively identified as LAB. The growth of four selected isolates at various salinity and temperatures showed that the isolates displayed the ability to grow in presence of 6% NaCl and at 42 ∘C except for one isolate OUBN4 (**Table 3**). Based on the above, these four LAB isolates were further characterized at the species level by 16S rRNA sequencing analysis. Phylogeny of these four LAB isolates viz. OUBN1, OUBN3, OUBN4, and OUBN5 have shown maximum similarity with the *Lactobacillus plantarum* group (99.18%), *Enterococcus faecium* (99.85%), *Pediococcus acidilactici* (100%), and *Pediococcus pentosaceous* (98.33%), respectively (**Figure 1**). Sequences were submitted to the NCBI GenBank database and accession numbers were obtained for the gene sequences of isolate OUBN1 (MF992176), OUBN3 (MF992189), OUBN4 (MF992177), and OUBN5 (MF992178).

Safety Evaluation

TPN samples were analyzed for their microbiological safety to confirm their plausibility as a safe drink. In our study, TPN samples and four selected strains showed non-hemolytic, non-gelatinase, and non-proteolytic activity, indicating that the TPN and micro-organisms present in TPN are non-pathogenic. Results of the presumptive coliform test also revealed the absence of coliforms in the TPN samples.

Evaluation of Probiotic Properties

Survival in Acidic pH, Bile, and Simulated Gastric Juice

The tolerance of isolates to acidic pH shows their ability to survive in hostile conditions of the gastrointestinal tract. Selected LAB isolates showed maximum survival at pH 2.5 –3.0 at 37°C for 3 h (**Table 4A**). Tolerance to bile salts helps in evaluating the ability of isolates for their establishment in the gastric environment. All 4 isolates showed maximum survival with 0.3% ox bile after 1-3 h of treatment at 37°C (**Table 4B**). All isolates were found to maintain above 95% viability ($P < 0.001$) at pH 3 and have shown good viable count after 3 h exposure to 0.3% bile concentration ($P < 0.001$). Further, all isolates survived well in simulated gastric juice with above 90% viability after 1-3 h of incubation at 37°C. In particular, isolates OUBN3 and OUBN5 showed the highest viability of 8.94 log cfu/ml at 3 h incubation (**Table 4C**).

TABLE 2 | Amino acids profile from toddy palm nectar detected by UPLC (ultra-performance liquid chromatography).

Name	Conc in μ g/ml	%mole	Biological importance
Histidine	246.74	0.302	Protein interactions, Precursor of histamine
Aspargine	242.38	0.348	Protein synthesis
Serine	1566.74	2.833	Biosynthesis of purines and pyrimidines
Arginine	1275.58	1.391	Cardiovascular diseases, anti-aging
Glycine	692.69	1.754	Schizophrenia, anti-inflammatory
Aspartic acid	1248.02	1.782	Vital role in neuro endocrine system.
Citruline	3214.16	3.487	Alzheimer's, dementia, Sickle cell
Glutamic acid	2586.01	3.341	Important neurotransmitter
Sarcosine	1351.66	2.884	Used as adjunctive therapy in Schizophrenia
Threonine	2415.59	3.855	Immunostimulant, better livestock growth
Alanine	5870.45	12.52	Treat Hypoglycemia, prostatic hypertrophy
GABA	1722.08	3.175	Anxiety and improves mood, PMS
aAAA	617.403	0.7281	–
bAIBA	1156.95	2.132	Expression of brown adipocyte
Proline	1165.84	1.924	Proteinogenic
OH-Lysine-1	1573.41	1.844	Collagen formation
OH-Lysine-2	10974.76	12.86	Multifunctional enzyme
Ornithine	1253.58	1.802	Reduces stress and fatigue
Cystine	660.55	1.036	Antioxidant and anti-aging
Lysine	3797.71	4.937	Calcium absorption; proteinogenic
Tyrosine	3489.97	3.661	Stress reliever
Methionine	745.49	0.949	Growth and tissue repair.
Valine	2554.57	4.143	Improves dendritic cell function
Leucine	4202.03	6.088	Growth hormone production
Phenylalanine	2318.24	2.667	Neuro transmitter, anti-depressant
Trptophan	388.30	0.361	Sleep aid and psychiatric disorders

Auto Aggregation, Hydrophobicity, and Coaggregation

Isolate OUBN1 has shown higher autoaggregation activity (81.76%), while OUBN3 showed the lowest autoaggregation (69.26%) (**Figure 2A**). Also, the cell surface hydrophobicity of 79.71% was observed for OUBN1, which was significantly higher ($p < 0.05$) when compared to OUBN5 (70.15%). Whereas, there was no significant difference ($p > 0.05$) in cell surface hydrophobicity of LAB isolates OUBN3 and OUBN4 (**Figure 2A**). All four LAB isolates had good co-aggregation ability with pathogens after 24 h incubation. Of all these isolates, OUBN1 displayed highest percentage of coaggregation with *E. coli* MTCC452, *K. pneumoniae* MTCC109, and *S. aureus* MTCC902 (**Figure 2B**).

TABLE 3 | Morphological, biochemical, and physiological characteristics of the LAB Isolates from Toddy palm nectar.

Characteristics	OUBN1	OUBN3	OUBN4	OUBN5
Shape	Rod	Cocci	Cocci	Cocci
Catalase activity	–	–	–	–
Growth in 0.5% NaCl	+	+	+	+
1%NaCl	+	+	+	+
2%NaCl	+	+	+	+
4%NaCl	+	+	+	+
6%NaCl	+	+	-	+
Growth At				
20°C	+	+	+	+
30°C	+	+	+	+
37°C	+	+	+	+
40°C	+	+	+	+
42°C	+	+	–	+

Positive results (+), Negative results (–).

Antibiotic Sensitivity

The antibiotic sensitivity of LAB isolates against different antibiotics was determined and obtained results were equated with the interpretative chart of zone size provided in the catalog (**Table 5**). The four isolates were highly susceptible to antibiotics such as chloramphenicol, tetracycline, penicillin-G, clindamycin, erythromycin, rifampicin, and lincomycin; while resistant to trimethoprim, ampicillin, streptomycin, polymyxin-B, vancomycin, gentamycin, amoxicillin, norfloxacin, ciprofloxacin, and kanamycin.

Antimicrobial Activity of LAB Isolates

The antimicrobial activity of selected LAB isolates against common enteric pathogens was tested. The un-neutralized CFS of all 4 isolates inhibited the growth of all pathogens tested. Whereas, neutralized CFS (nCFS) inhibited the growth of only a few tested pathogens (**Table 6**). The nCFS of OUBN1 inhibited the growth of *P. aeruginosa* MTCC424 and *P. vulgaris* MTCC426, whereas nCFS of OUBN3 inhibited *E. coli* MTCC 452, *P. aeruginosa* MTCC424, *E. faecalis* MTCC 6845, and *K. pneumoniae* MTCC 109. It was also observed that nCFS of OUBN5 inhibited the growth of *P. aeruginosa* MTCC424 only while un-neutralized CFS and nCFS of OUBN4 inhibited the growth of all pathogens tested in this study.

DPPH Radical Scavenging and Adhesion Activity

Radical scavenging activity of LAB isolates ranged from 44.05 to 68.71% and IC_{50} was from 75.62 to 117.11 μg/ml (**Figure 3**). Among the four isolates, OUBN1 showed significantly higher ($p < 0.05$) hydroxyl radical scavenging activity of 68.71% with an IC_{50} of 75.62 μg/ml, followed by OUBN5 (62.48%) and OUBN4 (56.59%) with IC_{50} of 82.23 and 89.01 μg/ml, respectively. Whereas, OUBN3 was found to have lower scavenging activity of 44.05% with IC_{50} of 117.11 μg/ml compared to other LAB isolates.

Adhesion assay for four LAB isolates was done for their ability to adhere to HT-29 cell lines and considerable variation was observed as presented in **Figure 4**. Among all isolates,

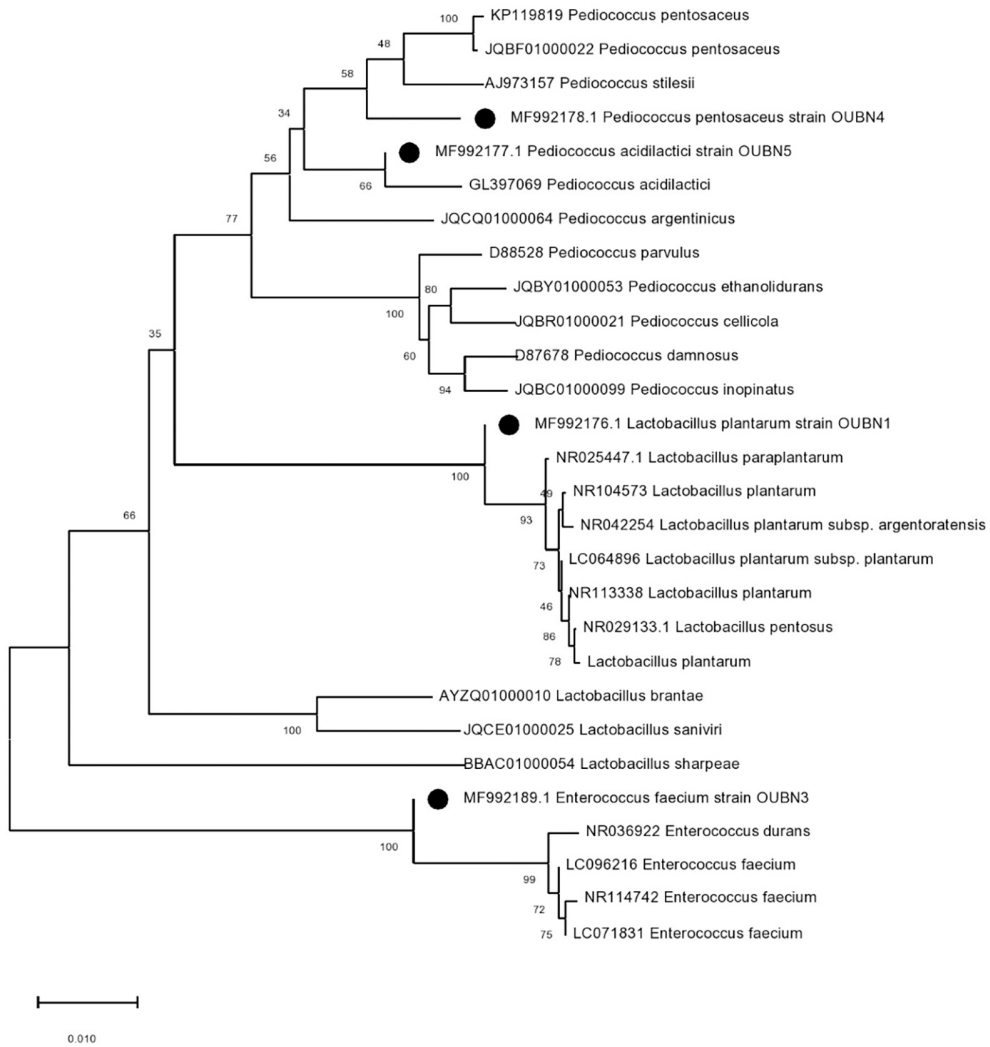

FIGURE 1 | Phylogenetic tree constructed by the neighbour-joining method showing the relative positions of LAB isolates (OUBN1, OUBN3, OUBN4, and OUBN5) based on 16S rRNA gene sequences from Palm toddy nectar samples. Evolutionary analyses were conducted in MEGA X. The tree is drawn to scale. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test of 1,000 replicates is shown next to the branches.

TABLE 4A | Viable cell count log cfu/ml of LAB survived in MRS broth at different pH and time intervals.

LAB strains	pH 2.5				pH 3.0			
	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h
OUBN1	7.01 ± 0.05	6.67 ± 0.04	6.62 ± 0.01	6.53 ± 0.04	6.93 ± 0.01	6.85 ± 0.02	6.78 ± 0.05	6.72 ± 0.05
OUBN3	6.98 ± 0.04	6.50 ± 0.04	6.40 ± 0.05	6.91 ± 0.04	6.90 ± 0.04	6.89 ± 0.04	6.86 ± 0.04	6.84 ± 0.05
OUBN4	7.05 ± 0.03	6.85 ± 0.02	6.81 ± 0.01	6.75 ± 0.03	6.96 ± 0.03	6.90 ± 0.05	6.85 ± 0.04	6.89 ± 0.02
OUBN5	7.03 ± 0.03	6.59 ± 0.03	6.56 ± 0.02	6.51 ± 0.02	6.99 ± 0.03	6.96 ± 0.03	6.88 ± 0.03	6.94 ± 0.02

The mean of three values is presented ± SD for each sample. Viable counts were expressed in log10 cfu/ml. P-value is significant (P < 0.001).

OUBN1 and OUBN5 showed strong adhesion, while OUBN3 and OUBN4 were moderate.

Anticancer Activity and Cell Apoptosis

The CFS of four LAB isolates were tested for their anti-cancer activity against HT-29 cells. Though all four isolates exhibited anti-cancer activity after treatment of cells with 100 μl/ml CFS in contrast to untreated cells, the highest anti-cancer activity of 88.55% was found with isolate OUBN1 while the least activity of 64.05% was found with OUBN3 (**Figure 5A**).

Subsequently, staining with DAPI was done to detect the visual indication of cell death in untreated and treated cells

TABLE 4B | Viable cell count log cfu/ml of LAB survived in MRS after 0, 1, 3, and 4 h in the presence of 0.3% bile salts.

	Viable cell count log cfu/ml				
LAB strains	**0 h**	**1 h**	**2 h**	**3 h**	**4 h**
OUBN1	7.90 ± 0.03	7.67 ± 0.05	7.78 ± 0.05	7.84 ± 0.05	7.85 ± 0.03
OUBN3	8.04 ± 0.01	7.71 ± 0.06	7.69 ± 0.07	7.82 ± 0.03	7.81 ± 0.03
OUBN4	7.99 ± 0.03	7.72 ± 0.04	7.84 ± 0.03	7.90 ± 0.02	7.87 ± 0.04
OUBN5	8.01 ± 0.03	7.71 ± 0.03	7.85 ± 0.08	7.98 ± 0.03	7.92 ± 0.03

The mean of three values is represented as ± SD for each sample. Viable counts were expressed in log10 cfu/ml. P-value is highly significant ($P < 0.0001$).

TABLE 4C | Viable cell count log cfu/ml of LAB survived in simulated gastric juice at different time intervals.

	Viable cell count log cfu/ml			
LAB strains	**0 h**	**1 h**	**2 h**	**3 h**
OUBN1	6.96 ± 0.05	6.89 ± 0.04	6.88 ± 0.03	6.82 ± 0.03
OUBN3	9.00 ± 0.02	8.94 ± 0.03	8.95 ± 0.02	8.94 ± 0.02
OUBN4	7.98 ± 0.01	7.94 ± 0.02	7.91 ± 0.06	7.90 ± 0.03
OUBN5	9.01 ± 0.03	8.93 ± 0.03	8.92 ± 0.05	8.94 ± 0.03

The mean of three values is presented for each sample ± SD. Viable counts wer expressed in log cfu/ml. No significant ($P < 0.17$) difference in the viability of the cells in the gastric conditions incubated for 3 h.

by fluorescent microscopy. Viable cells were observed to be blue intact, while dead cells were differentiated by bright blue with shrinking and blubbing along with condensed nucleus or fragments that ultimately degenerated, as shown by arrows in **Figure 5B**.

DISCUSSION

In the present study, a naturally fermented and easily available drink, toddy palm nectar (TPN) was selected to evaluate its nutritional value and probiotic attributes.

Among the volatile fatty acids (VFA) in such natural ferments, alcohols are the main components and are considered the final standard products of degradation of glucose and amino acid catabolism. Of all VFAs, N-hexadecanoic acid is the major one found in TPN, which is an anti-inflammatory compound and also a phospholipase A2 inhibitor. Hydrolysis of ester bond linkages by phospholipase A2 is the initiating step to start inflammation. Enzyme kinetics study proved that n-hexadecanoic acid inhibits phospholipase A2 in a competitive way (Aparna et al., 2012). Other fatty acids identified include hexadecanoic acid methyl ester (CAS), methyl palmitate, and E-15-heptadecenal, which are responsible for the antibacterial and antioxidant properties of TPN. Several other volatile compounds have been reported previously from different types of palm wines such as *Cocas nucifera* (Borse et al., 2007; Karthikeyan et al., 2014) *Nypa fruticans* (Nur Aimi et al., 2013), and *Elaeis guineensis* (Lasekan and Abbas, 2010). Identification of octadecanoic acid in *Lactobacillus helveticus* by GC–MS was reported by Sharma et al. (2014). Various organic acids and effective antimicrobial activity of *Lactobacillus* strains further supported the organic acid-mediated inhibitory effect (Bajpai et al., 2016). The use of medicinal oils rich in n-hexadecanoic acid for the treatment of rheumatic complications has been described in the traditional

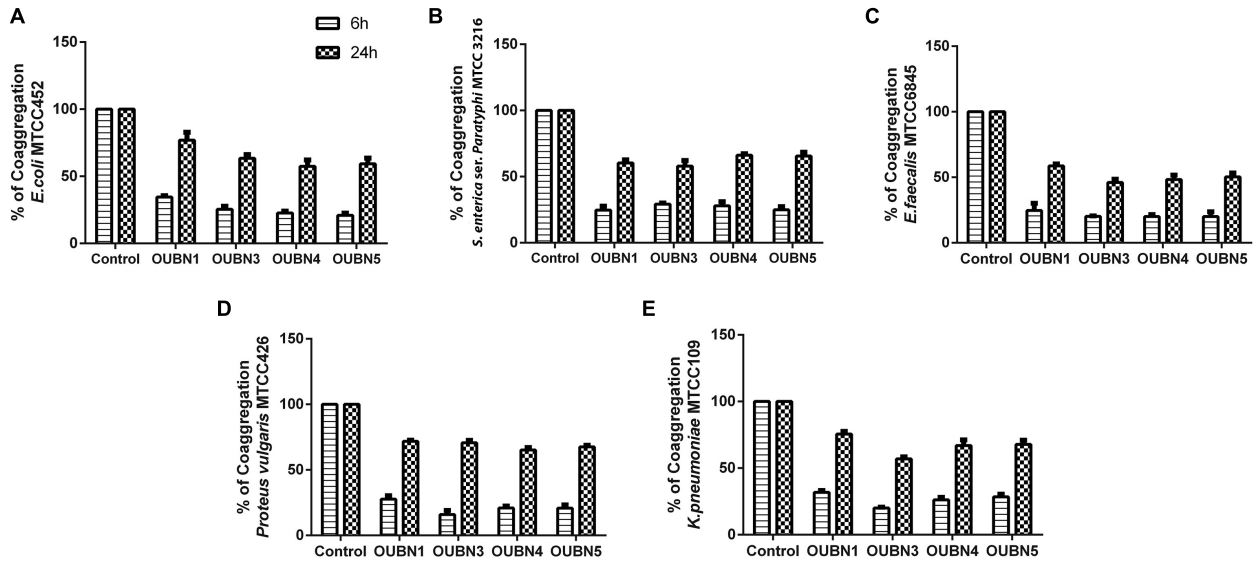

FIGURE 2 | (A) Assessment of Auto aggregation and hydrophobicity of LAB isolates. **(B)** Percentage of coaggregation by selected LAB isolates after 6 and 24 h against enteric pathogens **(A)** *Escherichia coli* MTCC 452. **(B)** *Salmonella enterica* ser. *paratyphi* MTCC3216. **(C)** *Enterococcus faecalis* MTCC 6845. **(D)** *Proteus vulgaris* MTCC 426. **(E)** *Klebsiella pneumoniae* MTCC 109. Bacterial growth was monitored at OD_{600}. The growth of enteric pathogens without CFS was considered as control (100%). Data shown are mean ± SD of triplicate values of independent experiments. $P > 0.005$.

medical system of Indian Ayurveda (Aparna et al., 2012). Previous studies confirm the anti-microbial and anti-oxidant properties exerted by the presence of organic acids in fermented foods (Bajpai et al., 2016).

In the present study, 26 amino acids were identified by UPLC, possibly of plant origin, but there is a possibility that microorganisms may also contribute to amino acid production. OH-lysine-2 is an essential amino acid and is found to be the primary amino acid in TPN. It plays a vital role in various biological processes, including the conversion of lipids into metabolic energy, synthesis of collagen fibers, connective tissues, and also participates in the regulation of calcium levels (Peter et al., 2010). Alanine was found as the second major amino acid in TPN tested. Alanine is involved in the metabolism of sugars and acids, which is known to boost immunity and provide energy to the brain, central nervous system, and muscle tissue (Sarah et al., 2013). Leucine was the third major amino acid, essential in protein metabolism, and plays a role in the initiation pathways of muscle protein synthesis. Alanine participates in reversible phosphorylation of proteins that control the binding of mRNA to the 40S ribosomal subunit (Li et al., 2011). GABA (γ-aminobutyric acid) is another important compound found in TPN which is an inhibitory neurotransmitter of the central nervous system. Production of GABA by *L. fermentum* isolated from palm wine was reported by Rayavarapu et al. (2019). Citrulline is also found in TPN and it is the primary precursor of L-arginine in the nitric oxide cycle. Citrulline is known to prevent neuronal cell death and protect against cerebrovascular damage. Therefore, it may provide a neuroprotective role to improve cerebrovascular dysfunction (Lee and Kang, 2018).

Of 26 samples, 120 morphologically different LAB were identified based on differential growth and *in vitro* probiotic characteristics. Previous studies have specified the presence of various LAB, non-LAB, and yeast in palm wine (Tapsoba et al., 2016; Astudillo-Melgar et al., 2019). In our preliminary studies, it was observed that LAB were more abundant than non-LAB and yeasts in TPN. One of our previous studies reported the functional probiotic and therapeutic potential of *Saccharomyces cerevisiae*, which was isolated from palm nectar (Srinivas et al., 2017). Tolerance of selected LAB isolates to various salinity and temperature ranges show that these LAB can endure growing in severe unfavorable environments which is an optimistic feature to select a probiotic.

Based on the above observations, four potential bacterial isolates were selected and subjected to 16S rRNA analysis, confirmed as LAB at the species level, and named as *Lactobacillus*

TABLE 5 | Antibiotic susceptibility of LAB strains isolated from toddy palm nectar samples.

	ZOI in mm			
Antibiotics	**OUBN1**	**OUBN3**	**OUBN4**	**OUBN5**
Trimethoprim (10μg)	–	–	+	+
Ampicillin (10 μg)	–	–	–	–
Streptomycin (10 μg)	–	–	–	–
Chloramphenicol (30 μg)	+ +++	+++ +	+++	+++ +
Polymyxin-B (300 units)	–	–	–	–
Tetracycline (30 μg)	+ +++	+++ +	+ +++	+++ +
Penicillin-G (10 units)	+ ++	+++	+ ++	+++
Vancomycin (30 μg)	–	–	–	–
Clindamycin (2 μg)	+ +++	+++ +	+ +++	+++ +
Gentamycin (10 μg)	+	+	–	–
Amoxycillin (10 μg)	+	–	+	+
Erythromycin (15 μg)	+ ++	+++ +	+ +++	
Rifampicin (5 μg)	++++	++++	++++	++++
Norfloxacin (10 μg)	–	–	–	–
Ciprofloxacin (5 μg)	–	–	–	–
Lincomycin (10 μg)	+ +	+++	+ ++	+++
Kanamycin (30 μg)	–	–	–	–

ZOI (zone of inhibition) - no effect detected, +, ++, +++, ++++ represents diameter of zone of inhibition between 1–2, 2–5, 5–10 and more than 10 mm, respectively.

TABLE 6 | Antimicrobial activity against enteric pathogens shown by LAB isolated from toddy palm nectar samples

Pathogens	**Zone of inhibition in cm**							
	OUBN1		**OUBN3**		**OUBN4**		**OUBN5**	
	CFS	**nCFS**	**CFS**	**nCFS**	**CFS**	**nCFS**	**CFS**	**nCFS**
E. coli MTCC 452	1.8	–	1.6	1.2	1.6	1.4	1.8	–
P. aeruginosa MTCC424	1.8	1.0	1.6	1.0	1.8	1.0	1.8	1.2
P. vulgaris MTCC426	1.6	0.6	1.8	–	1.6	0.6	1.6	–
S. para typhi enterica ser. MTCC 3216	1.4	–	1.4	–	1.4	0.6	1.6	–
E. faecalis MTCC 6845	1.6	1.6	1.6	1.6	1.8	1.2	1.6	–
K. pneumoniae MTCC 109	2.0	1.6	2.0	1.8	2.4	1.8	2.0	–

CFS (Cell-free supernatant), nCFS (Neutralized cell-free supernatant).

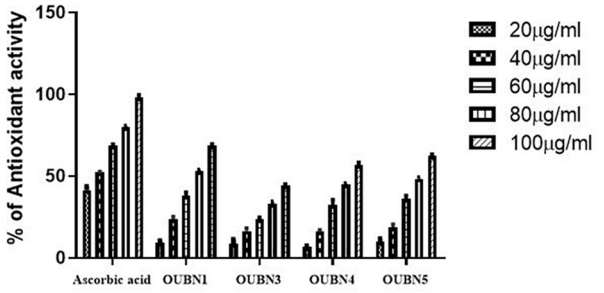

FIGURE 3 | Antioxidant abilities of LAB isolates by using CFS (cell free supernatant) of 20, 40, 60, 80, 100 μg/ml concentrations from Ascorbic acid, OUBN1, OUBN3, OUBN4, and OUBN5, respectively. Ascorbic acid was used as standard.

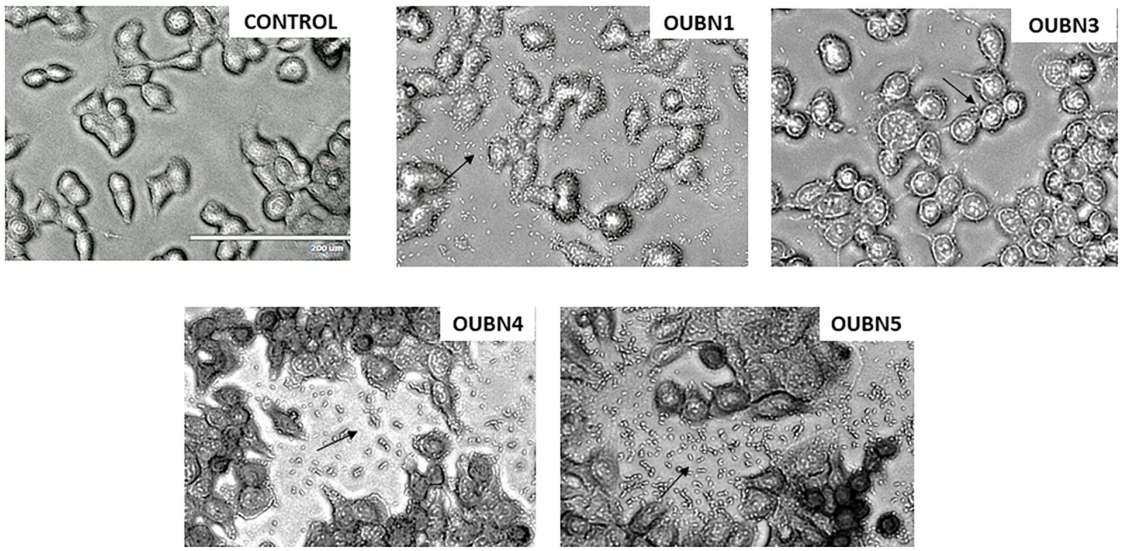

FIGURE 4 | Adhesion of LAB strains on HT-29 cell cultures observed under oil immersion microscope (100X) after staining with Giemsa strain. (Control)- HT-29 cell line without treatment and HT-29 cells treatment with OUBN1, OUBN3, OUBN4, OUBN5, respectively. Scale 200 μm.

plantarum group OUBN1, *Enterococcus faecium* OUBN3, *Pediococcus acidilactici* OUBN4, and *Pediococcus pentosaceo*us OUBN5. The 16S rRNA analysis revealed high efficacy and congruency for LAB species and selected LAB isolates may be candidates for further investigation as better probiotic strains.

The evaluation of hemolytic activity is regarded as a safety asset according to the European Food Safety Authority (EFSA), to consider the probiotic strains (Joint FAO/WHO Expert Committee on Food Additives Meeting and World Health Organization, 2006). Hemolytic activity of selected strains was evaluated on Columbia blood agar plates and the tested strains showed neither α- hemolytic nor β-hemolytic activity. Our findings were in agreement with the results of Oh and Jung (2015) and Wang et al. (2018), who evaluated the *Lactobacillus* species isolated from millet-based alcoholic beverages fermented by traditional methods and spontaneously fermented non-dairy foodstuffs for their hemolytic activity. Non-hemolytic activity is noteworthy during the selection of probiotic strains, as such strains are non-virulent and lack of hemolysin ensures their non-pathogenic nature (Joint FAO/WHO Expert Committee on Food Additives Meeting and World Health Organization, 2006). Gelatinase enzyme is considered a virulence factor as it may hydrolyze collagens that initiate an inflammatory response (Da Silva et al., 2019). However, in the present investigation, all the LAB were non-hemolytic and non-gelatinolytic. Non-hemolytic and non-gelatinase criterion is measured to be significant to use as starter cultures in the dairy industry (Marroki and Bousmaha-Marroki, 2014). None of the LAB of the present study is positive for proteolytic activity. No pathogenic organisms were detected in any of the toddy (TPN) samples studied, indicating the hygienic status of the tappers, the extraction method involved and the materials used. Results of, non-hemolytic, non-gelatinase, non-proteolytic properties, absence of coliforms, mild ethanol content of TPN indicate its microbiological safe nature.

Tolerance to low pH and high concentration of bile salts is one of the prerequisites for characterizing probiotic strains. Also, they should effectively pass through the gastric stomach and also remain in the small intestine (Anandharaj et al., 2015). Ilavenil et al. (2016) studied four LAB strains and reported a low pH (2.5) and bile salts (0.3%) tolerance, reflecting high survival and proliferation efficiency in hostile intestinal conditions. In our study, exposure to pH 2.5 dramatically reduced the count of different LAB isolates after 2 h of incubation, while efficient growth was observed after 3 h of exposure to pH 3.0. Overall, no significant reduction in CFU count was observed after exposure to pH 3.0. This may be due to a sudden drop in optimal pH and subsequent adaptation to harsh acidic conditions, which has been observed with a higher survival rate in our LAB isolates. Tolerance to 0.3% bile salts was a positive observation with selected LAB isolates which promote easy colonization to the host gut as previously described (Adesulu-Dahunsi et al., 2018). Few studies reported high survival rates of selected LAB strains at pH 2.5 and 0.3% (w/v) bile salts (Nami et al., 2019). Growth of LAB isolates has also been observed in the simulated gastric juice to assess their survival in hostile environments of the gastrointestinal tract. Isolates were able to resist simulated digestive tract conditions with a slight reduction in the number of viable cells, and these results are in agreement with previous findings of Nami et al. (2019).

After entering the intestine, probiotics must adhere to the intestinal mucosa, which accelerates the transient colonization and hinders its elimination by peristalsis. The current study showed a high auto-aggregation of selected LAB isolates, which is in agreement with observations of Somashekaraiah et al. (2019).

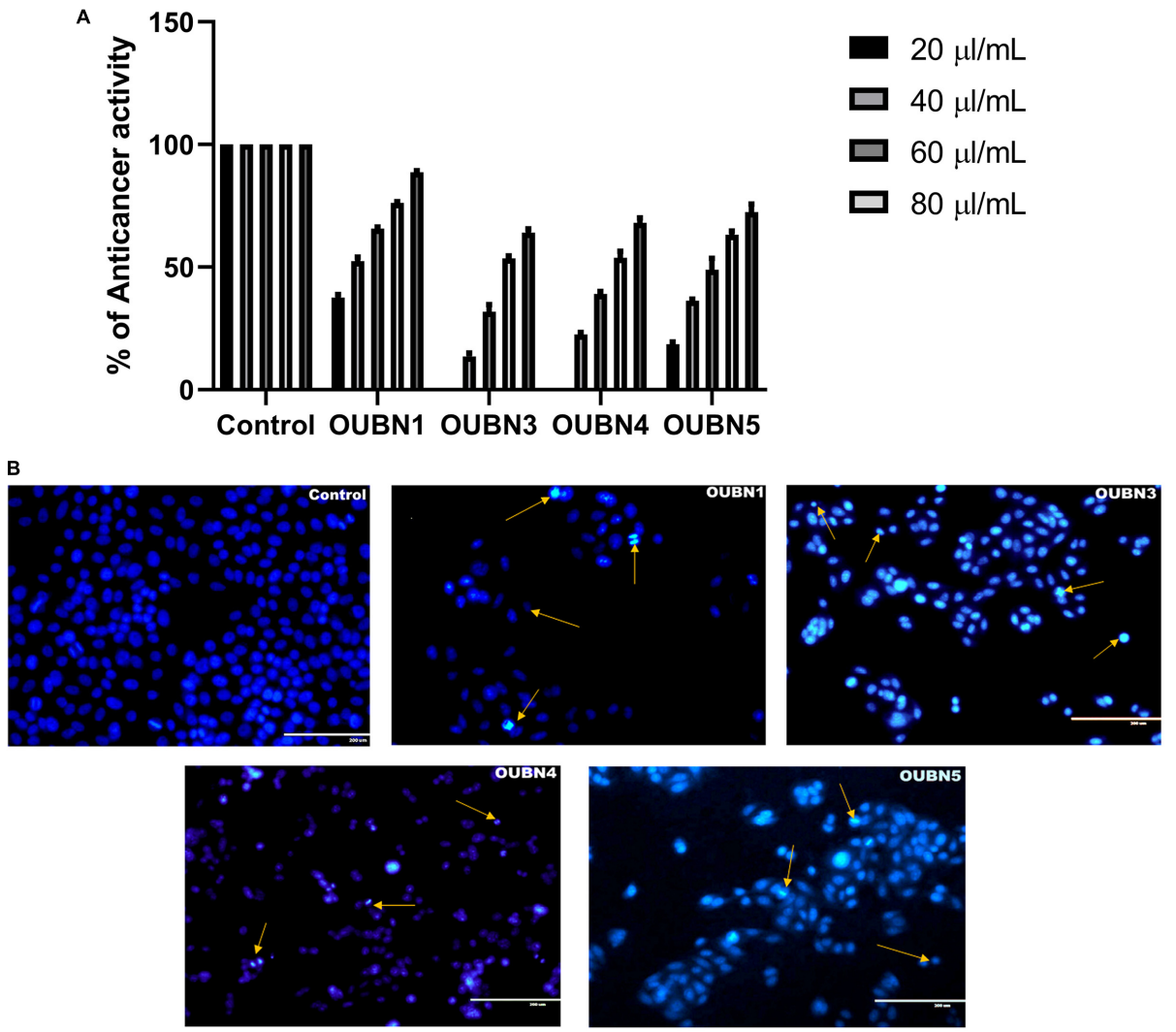

FIGURE 5 | (A) Anticancer activity determined by MTT [-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Experiments were done by using CFS of 20, 40, 60, 80, 100 µl/ml concentrations from CFS of OUBN1, OUBN3, OUBN4, and OUBN5, respectively. Data shown are triplicate values of mean ± SD of independent experiments. **(B)** Apoptosis determined for untreated and treated HT-29 cells with OUBN1, OUBN3, OUBN4, OUBN5 isolates by DAPI (4, 6-diamidino-2-phenylindole) staining. Scale 200 µm.

There is a strong association between auto-aggregation of probiotic strains and their ability to adhere to intestinal epithelial cells, signifying host defense, which is a prerequisite for effective colonization and better persistence in the gastrointestinal tract (Zommiti et al., 2018). Cell surface hydrophobicity has been carried out to determine the efficiency of strains to adhere to the gut region since it is one of the characteristic features of potential probiotic strains. In the present study, four isolates exhibited hydrophobicity greater than 70% and interestingly, strain OUBN1 showed the highest of 79.71%. The auto-aggregation and adhesion efficiency of our isolates are superior to the LAB strains isolated from plant-based fermented food and neera (Choi et al., 2018; Somashekaraiah et al., 2019). Co-aggregation ability of the strains could be a key factor, potentially inhibiting the adherence of pathogenic bacteria to the epithelial surface, which leads to hampering of surface colonization by pathogens. The Auto-aggregation ability of LAB plays a vital role in adhesion to intestinal epithelial cells and thus further prevents the colonization of pathogens. Among the strains, OUBN1 exhibited a maximum coaggregation of > 70% with *E. coli* MTCC452, *K. pneumoniae* MTCC109, and *S. aureus* MTCC902. The inhibitory impact of LAB strains can be associated with the co-aggregation of foodborne pathogenic bacteria. Since the auto-aggregation potential of LAB strains plays an important role in adhesion to intestinal epithelial cells, they further prevent the colonization of pathogens (Yadav et al., 2016). These results proved that the LAB strains assessed in the current study could tolerate and survive efficiently in the human intestinal environment.

To be accepted as an excellent probiotic, organisms must meet specific functional properties like sensitivity to antibiotics and antimicrobial activity against pathogens. The inherent resistance of LAB strains to antibiotics is not considered a risk to human and animal health but may promote therapeutic and preventive benefits when administered together with antibiotics (Al Kassaa et al., 2014). In our study, four LAB isolates were tested for sensitivity to 17 different types of antibiotics and found that they are susceptible to a specific group of antibiotics, ampicillin, erythromycin, chloramphenicol, and tetracycline. Present results are in agreement with the previous study by Caggia et al. (2015) who isolated LAB from kimchi and found it susceptible to penicillin G, erythromycin, and clindamycin which bind to ribosomes, further block protein synthesis, and are effective against Gram-positive microorganisms (Reuben et al., 2020). The LAB isolates of the present study showed resistance to aminoglycosides (gentamicin and streptomycin) sulfonamide (trimethoprim) and glycopeptide (vancomycin) has been reported in LAB, which is associated with its intrinsic resistance resulting from the permeability of its membrane, probably through a resistance flow mechanism that is not transferable (Gueimonde et al., 2013). Furthermore, the electron transport mediated by cytochrome responsible for the absorption of the drug is absent in most of the LAB (Monteagudo-Mera et al., 2012). The intrinsic resistance of LAB strains to antibiotics is not considered a risk to animal and human health but may promote therapeutic and preventive benefits when administered together with antibiotics (Al Kassaa et al., 2014). Natural resistance to ciprofloxacin has been observed in our study, which is consistent with the study conducted by Tang et al. (2018). Geographic location and source of LAB also determined the antibiotic susceptibility patterns of potential probiotic strains (Anandharaj et al., 2015). Antibiotic resistance of bacteria has progressively become an alarming medical problem. Multiple-drug resistance of pathogenic microorganisms against prophylactic antibiotics has become a risk and serious challenge to overcome with the purpose to treat infected persons. Therefore, susceptibility to antibiotics signifies a fundamental prerequisite for probiotics.

Antagonistic activity and production of antimicrobial compounds by LAB against enteric pathogens can be considered as the main probiotic attribute for maintaining the stability of intestinal microbiota. Previous studies have examined the role of LAB and their inhibition of various enteric and foodborne pathogens (Li et al., 2017; Reuben et al., 2020). In our experiments, un-neutralized CFS of LAB showed significant antagonism against applied enteric pathogens. This could be due to the activities of organic acids they produce (Choi et al., 2018). Various effects of nCFS on inhibition of pathogens suggesting the production of bacteriocins by LAB could play a crucial role in the abolition of potentially harmful gut microbes. The LAB strains also contribute to the quality improvement of fermented foods, through deterioration and control of pathogenic bacteria, thus extending the shelf life and improving sensory quality (Beganović et al., 2014).

Fermented palm nectar contains various phytochemicals and microorganisms, including LAB, which exhibit antioxidant activity (Ghosh et al., 2015). The DPPH assay should be considered as an easy and cost-effective spectrophotometric method to evaluate the antioxidant activity of natural compounds and fermented food products. In the present study, LAB isolates showed the dose-dependent scavenging potential of DPPH as the percentage of scavenging activity increased linearly in all samples with increased concentration of DPPH and the reported IC_{50} values suggested a defensive role of TPN against oxidative stress with favorable radical quenching activities. Strains OUBN1 and OUBN5 showed higher antioxidant activity compared to previous studies by Lin et al. (2018), who reported antioxidant activity of *L. plantarum* AR501 and *P. pentosaceus* AR243.

HT-29 cell lines were used to assess the adhesion ability and anti-cancer activity of LAB isolates. *L. plantarum* group OUBN1 and *P. pentosaceous* OUBN5 showed excellent adhesive properties. MTT assay is the most widely used technique for examining new components in a short period based on their level of toxicity to cancer cells (Vuotto et al., 2014). Srinivas et al. (2017) evaluated the cytotoxic effect of yeast isolates OBS1 and OBS2 using cancer cell lines MCF7 (breast cancer) and IMR32 (neuroblastoma). Apoptosis was observed by fluorescent microscopy, which is considered a primary strategy during chemotherapy of cancer. DAPI staining method was used to observe visual symptoms of apoptosis in treated cells. Viable cells were identified as intact blue cells, while apoptotic cells were characterized by morphological changes, such as blue cells contracted with a fragmented or condensed nucleus (Haghshenas et al., 2015). HT-29 cells treated with LAB strains for 48 h showed symptoms of apoptosis, including membrane blisters, cell narrowing, nucleus fragmentation, and apoptotic body formation (Elliott et al., 2007). In the present study, CFS of all four LAB isolates showed significant cell morphological changes, including cell contraction, damage, and degeneration of cells.

CONCLUSION

The present investigation involves the nutritional profiling and isolation of lactic acid bacterial strains from TPN. The nutritional profile of TPN confirmed the presence of 18 VFAs and 26 amino acids. Due to the presence of probiotic microbiota, with rich amino acid and volatile fatty acid profile, TPN the natural drink can be tried as a therapeutic agent. All the four LAB isolates viz. *L. plantarum* group OUBN1, *E. faecium* OUBN3, *P. acidilactici* OUBN4, and *P. pentosaceous* OUBN5 were found to be efficiently tolerant of low pH and bile conditions along with other severe intestinal parameters of the stomach. All four isolates showed good antimicrobial activity along with considerable antioxidant and anti-cancer activities. These strains exhibited ideal surface-binding properties, which are useful in colonizing the gastrointestinal tract and play a significant role in increasing healthy gut microbiota. The *Lactobacillus plantarum* group (reclassified as *Lactiplantibacillus plantarum*) OUBN1 and *Pediococcus pentosaceus* OUBN5 expressed potential probiotic characteristics. In conclusion, the LAB isolates from TPN established a probiotic attribute *in vitro*, thus revealing the ability to employ them as possible probiotic microbiota in food preparations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

AUTHOR CONTRIBUTIONS

All the experiments were carried out by NP with the help of KB. RL and BB designed the concept, monitored the work, and drafted the manuscript. All authors read and approved the final manuscript.

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METHODS
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Improving and Comparing Probiotic Plate Count Methods by Analytical Procedure Lifecycle Management

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Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts. This definition links probiotic efficacy to microbial viability. The current gold standard assay for probiotic potency is enumeration using classical microbiology plating-based procedures, yielding results in colony-forming units (CFU). One drawback to plating-based procedures is high variability due to intrinsic and extrinsic uncertainties. These uncertainties make comparison between analytical procedures challenging. In this article, we provide tools to reduce measurement uncertainty and strengthen the reliability of probiotic enumerations by using analytical procedure lifecycle management (APLM). APLM is a tool that uses a step-by-step process to define procedure performance based on the concept that the reportable value (final CFU result) must be fit for its intended use. Once the procedure performance is defined, the information gathered through APLM can be used to evaluate and compare procedures. Here, we discuss the theory behind applying APLM and give practical information about its application to CFU enumeration procedures for probiotics using a simulated example and data set. Data collected in a manufacturer's development laboratory is included to support application of the concept. Implementation of APLM can lead to reduced variability by identifying specific factors (e.g., the dilution step) with significant impact on the variability and providing insights to procedural modifications that lead to process improvement. Understanding and control of the analytical procedure is improved by using these tools. The probiotics industry can confidently apply the information and analytical results generated to make decisions about processes and formulation, including overage requirements. One benefit of this approach is that companies can reduce overage costs. More reliable procedures for viable cell count determinations will improve the quality evaluation of probiotic products, and hence manufacturing procedures, while ensuring that products deliver clinically demonstrated beneficial doses.

Keywords: probiotics, USP, colony-forming units, enumeration, analytical procedure lifecycle management, analytical target profile, target measurement uncertainty, methods comparison

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INTRODUCTION

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Probiotic preparations must meet strict criteria related to quality, safety, and functionality (Vankerckhoven et al., 2008; Binda et al., 2020). A key quality criterion is that they contain accurately defined numbers of live cells as expressed on the product label. Hence, it is critical to accurately enumerate the population of live microbes in the preparation and express this information to the consumer on the product label. This presents a major analytical challenge for the probiotics industry as enumeration becomes paramount to assessing the quality of commercial probiotic products. There are numerous approaches to the measurement of probiotic cell viability including measurement of colony-forming units (CFU) by plating, flow cytometry, viability quantitative PCR, and droplet digital PCR (Hansen et al., 2018, 2020; Kumar and Ghosh, 2019). These methods or approaches measure different aspects of cell viability.

Most recognized standards such as those published by the International Standards Organization (ISO), International Dairy Federation (IDF), and United States Pharmacopeia (USP) use plate count procedures for bacterial enumeration of beneficial bacteria as well as contaminants (ISO, 2003, 2006, 2010; USP, 2013). The current standard in the probiotics industry is to measure probiotic potency using traditional microbiological plate count procedures, which fulfill growth requirements (i.e., nutrients, temperature, atmosphere). The benefits of plate counts are technical simplicity and ease of implementation. The challenges associated with plate count procedures are mainly related to laborious manual handling and variables within the procedure. Culture-based procedures generate counts with large total error [15–30% coefficient of variation (CV); Corry et al., 2007] and with varying degrees of intermediate precision and reproducibility. Limits ranging from 0.2 to 0.5 Log_{10} for the critical difference between two tests at the 95% confidence interval can be found in international standards and national guidelines. Additionally, no single plating procedure is applicable to all probiotic organisms, as there are considerable differences in growth requirements between bacterial species and strains as well as their manufacturing conditions (Davis, 2014). Therefore, a means to reduce variation is needed to obtain accurate CFU counts of probiotic products. Estimation of measurement uncertainty (*MU*) provides a means to assess and compare the overall variability of an analytical procedure carried out within a single laboratory or within different laboratories. Uncertainty and procedure variability associated with CFU enumeration of probiotic strains call for qualified procedures that can be applied for robust enumeration of culturable cells. The use of such procedures and the data generated provides more reliable quality metrics for the industry.

The United States Pharmacopeia (USP) is an organization known for creating quality standards for drugs, excipients, dietary supplements, and foods. These quality standards include monographs for probiotic ingredients, dietary supplements, and finished products which cover the identification, purity, assay, and contaminants. In response to the rapid and wide-spread growth of probiotic usage and subsequent increasing requests for probiotic monograph development, the USP formed a Probiotics Expert Panel (PEP).

Initially, probiotic monographs were developed for individual strains of a probiotic species and organizations submitting data included detailed CFU enumeration information that followed qualified analytical procedures. The number of CFU enumeration methods increased as monograph submissions for different strains within the same species increased. Most of these methods varied in parameters and qualification procedures, which underlined the need for tool(s) for qualifying and comparing different CFU enumeration procedures.

The approach for qualification and comparison of analytical procedures for live bacterial products, needs to accommodate the diversity of probiotic products, throughput of analyses, procedure uncertainty, and most importantly, must be accepted by manufacturers and regulators. Actions undertaken to understand analytical procedures will provide considerable opportunities for improving data quality as well as overall probiotics quality from commercial, regulatory, and consumer perspectives.

In this paper, analytical procedure lifecycle management (APLM; Martin et al., 2013) combined with tolerance interval (*TI*) calculations is used to compare analytical CFU enumeration procedures and provide a framework for implementation of this approach. Lifecycle management has been used for diverse applications, e.g., monitoring and improving chemicals, biologicals, drugs, immunoassays, information technology systems, biotechnological processes, and product marketing. However, these tools have not previously been applied to the evaluation of analytical procedures for live bacterial products.

Here, steps are detailed for developing APLM for CFU enumeration of probiotics. An in-depth APLM analysis, in the form of results for a simulated probiotic powder example using randomly generated data sets and statistical comparisons demonstrates the approach. The information used to generate the data is based on known variability in probiotic CFU measurements. The example identifies, defines, evaluates, and applies basic APLM principles to enumeration procedures and is followed by statistical analysis using *TI* as described in "USP <1210> Statistical Tools for Procedure Validation" (USP, 2018). To further support the value of this approach, real-life data for a *Lactobacillus acidophilus* powder is included. Combined, APLM and *TI* calculations characterize procedure performance, furnish a basis for comparing procedures, and provide tools that the probiotics industry can use to improve the reliability of their decision-making data and increase product consistency.

MATERIALS AND METHODS

The theory and principles of APLM are presented. Consecutively and step-by-step, the theory and principles of APLM are applied to a general illustrative example, which demonstrates and further elaborates the potential of this approach. New terms introduced by APLM and others relevant to this paper

are defined in the glossary (**Supplementary Appendix 1**). The uncertainty classifications from ISO 19036:2019, Microbiology of the Food Chain – Estimation of Measurement of Uncertainty for Quantitative Determinations, are used for some statistical calculations (ISO, 2019).

DETAILS FOR THE EXAMPLE

The analytical procedure used for the general example determines culturable cells of *Lactobacillus* spp. as CFU/g in a powder. Data were randomly generated using the MS EXCEL (2016) to demonstrate performance of sample enumeration via agar plating. The function NORMINV was used to return numbers that were normally distributed around a mean, altered by a standard deviation and by a probability factor. The provided means, standard deviations, and probability applied for the example are based on empiric knowledge, expertise, and experience. In this example, the manufacturer states on the certificate of analysis (CoA) that the powder contains ≥91.67 billion CFU *Lactobacillus* spp./g or 10.962 Log_{10} CFU/g. In the example, the CoA claim is also referred to as the lower limit. Manufacturing overage was set at 0.500 Log_{10} above the planned CoA claim to ensure potency throughout product shelf life. The manufacturer's internal release specification, which accounts for overage, is 11.462 Log_{10} CFU/g. The laboratory plans to plate dilutions that will cover two Log_{10} CFU/g above and below internal release specification, i.e., the procedure will be applicable for CFU enumeration of 9.462–13.462 Log_{10} CFU *Lactobacillus* spp./g powder. Selected dilutions from each test sample will be plated in triplicate. Although various counting ranges exist, for this analysis the laboratory uses 25–250 colonies per plate.

Rounding and Significant Figures

Internal policies on rounding and significant figures may be followed. Generally, final uncertainty is given using two significant figures. The reportable value should be rounded to be consistent with the uncertainty. For this example, Log_{10} values are shown to three decimal points. In actual calculations all digits are used. This may result in minor discrepancies in values. The specification values, which are most often reported as arithmetic numbers, show two decimal points when referring to the CoA.

ANALYTICAL PROCEDURE LIFECYCLE MANAGEMENT (APLM)

Good manufacturing practices (GMP) require analytical procedures used for potency analysis of probiotics demonstrate fitness for intended use. APLM is a holistic model that encompasses the traditional approaches to procedure development, qualification, verification, and transfer rather than viewing these concepts as separate entities. Moreover, an analytical control strategy (ACS) is applied to ensure the analytical procedure remains in a stage of control throughout the lifecycle.

APLM is based on the reportable value, in this case the CFU concentration for CFU enumeration analytical procedures, being fit for its intended use. Hence, the intended use must be clearly defined and understood. In APLM, the intended use of an analytical, quantitative procedure is defined by developing an analytical target profile (ATP) as defined in USP PF 46(5) (USP, 2020), "Analytical Procedure Life Cycle":

> "The ATP is a prospective description of the desired performance of an analytical procedure that is used to measure a quality attribute, and it defines the required quality of the reportable value produced by the procedure."

The ATP provides the information needed to set procedure qualification criteria. As such, it can also provide criteria for comparing analytical procedures. The three steps used to develop an ATP will be discussed in detail:

1. Develop the measurand which describes what is being measured.
2. Develop the decision rule which describes the maximum acceptable measurement uncertainty (*MU*) or target measurement of uncertainty (*TMU*) and acceptable probability of being wrong.
3. Develop the ATP.

As seen in **Figure 1**, ATP and its components (measurand, decision rule, and *TMU*) are interactive. Therefore, the fitness for intended use needs to be evaluated and adjusted according to performance throughout the lifecycle of the procedure.

The Key Components in APLM and How to Use Them

Step 1 Develop the Measurand

The measurand, as defined in ISO 19036:2019 (ISO, 2019), is the quantity subjected to measurement. The measurand is developed using information from prior knowledge, ingredient manufacturing, product formulation, and analytical development. In this step, a complete statement of the item being analyzed is developed. It may include, but is not limited to, details such as the probiotic microorganism(s), matrix, product form, units of measure, possible contaminants, and/or impurities. Moreover, the entity for which the decision will be made (e.g., the lot or batch of bulk in the warehouse), and the entity used to make the decision (i.e., a representative sample, a laboratory sample, a composite, or single grab), are also defined.

The advantage of defining the measurand is that it provides a mechanism for communicating to all parties exactly what is being measured. **Table 1**, which includes a series of questions and example answers pertinent to probiotic ingredient powders, was compiled as an aid to developing measurand statements specifically for probiotics.

The initial definition of the measurand can be supplied by the department responsible for developing the probiotic product, often Research and Development. As a product development project advances, changes may be made to the development process and/or the formulation. For example, a new excipient may be added to the matrix. This change will trigger a revision

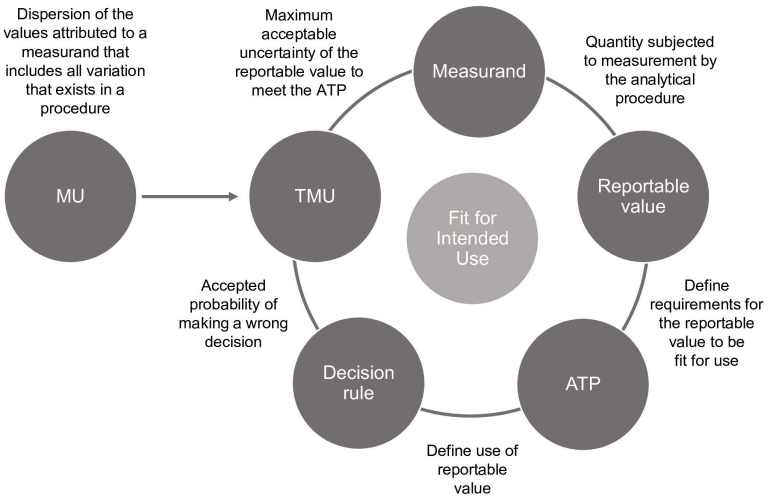

FIGURE 1 | The ATP and its components are related and interactive. If any component changes, another component may need to change also. MU, measurement uncertainty; TMU, target measurement uncertainty; ATP, analytical target profile.

TABLE 1 | Questions useful for identifying the information needed to define the measurand.

Question(s)	Answer and/or guidance for a specific product
What is the analyte? What is being detected? What is being counted?	The analyte is the entity measured by the analytical procedure. The analyte is culturable cells enumerated as colony forming units (CFU).
What is the matrix? Are there excipients? Stabilizers?	Matrix components are generally cryoprotectants, excipients, bulking agents for powder flow, etc.
Are there possible contaminants in the matrix?	Non-microbial contaminants: Carryover from fermentation media, leachables, and extractables from production systems. Microbial contaminants (both live and dead), remnants of cell-walls, cross-contaminants (from other strains produced in the same facility), and environmental contaminants.
Will the term "pure" be used to describe the ingredient?	Probiotic ingredients are often described as "pure" powders. The term "pure" is controversial but useful. The discipline of defining a measurand requires that the meaning of the controversial term, pure or purity, be defined if used. The probiotic ingredient (freeze dried cells) is usually a pure powder that may contain cryoprotectant and carryover of fermentation media. It does not contain excipients as do final formulated product.
Matrix: other components.	Is the probiotic ingredient a pure powder? Is the probiotic ingredient definition, above, used to describe the term pure? Is the probiotic ingredient in a solution or suspension? Include the solvent or suspension liquid in the measurand definition. Usually, there are no solvents in a freeze-dried product.
What is the decision unit (also known as parent body)? For what entity will the decision be made?	Options to consider for the decision unit: Laboratory sample, a batch of probiotic ingredient, a product lot. Composite sample or a single grab sample. Randomly selected from a bulk-capsule or finished capsule lot. The sample taken from the beginning, middle, or end of the batch, or at all three time points. The form of the sample is a bulk ingredient, formulated blend, capsules, sachets. R&D may conduct a study during process development to ensure the sample is representative, and that the uncertainty contribution from sampling is not of practical importance.
What is the physical form of the decision unit?	Powder, solution, etc. Describe the form.
Define the units for the quantity.	For example, the unit can be CFU/g or CFU/mL.
The information in the following two points is not needed to define the measurand; but is needed to complete the ATP. It is convenient to collect this information along with details for defining the measurand.	
What is the concentration range of test results that should be reported by the analytical procedure?	The laboratory may extend that range to include concentrations for potentially OOS values. This information is usually provided by the development team.
What is the counting range?	There are different standards for the counting range. The counting range depends on the size of the Petri dish, the applied agar, the probiotic strain, etc. It is up to the manufacturer to assess the counting range and the linearity for a specific ingredient and/or product with the applied CFU method.

The answer for each question is either information applicable to the analytical probiotic enumeration procedure or guidance for a company's specific product. These questions address both the probiotic ingredient and finished product.

to the definition of the measurand and everyone involved in developing the product and/or the relevant analytical procedures will be notified so the analytical procedure can be evaluated and adapted as needed.

Questions and Answers to Develop the Measurand for the Example Lactobacillus spp. Powder

Table 1 is presented as a tool for gathering relevant information used to define the measurand. The reader could copy **Table 1** and use, fill in, or adapt the responses. Information gathered for the *Lactobacillus* spp. example follows:

- The analyte consists of culturable *Lactobacillus* spp. cells enumerated as CFU.
- The matrix is cryoprotectant. The cells are freeze-dried.
- The product is not manufactured with wheat, gluten, soy, milk, egg, fish, shellfish, or tree nut ingredients. It is produced in a GMP facility that processes multiple probiotic strains and other ingredients containing these allergens. It is expected that cells from other probiotic strain and allergens, if present, are in very low concentrations and do not impact the measurement.
- The term pure or purity is not used.
- The decision unit is the laboratory sample. During product development, it was established that the laboratory sample was representative of the lot of probiotic powder.
- The physical form is a powder.
- The unit for quantity is the concentration CFU/g. The laboratory decided to report results as CFU/g on CoA, while using Log_{10} transformed data for conducting statistical analyses and trending.

Measurand for the Example Lactobacillus spp.

Culturable cells (live cells freeze-dried) of *Lactobacillus* spp., CFU/g, in powder with cryoprotectant.

Step 2 Develop the Decision Rule

The decision rule defines the fitness requirements for an analytical procedure in the context of using the reportable value. It describes how measurement uncertainty will be considered when deciding whether to accept or reject a product according to its specification and the result of a measurement. In other words, the decision rule is a prescription for the acceptance or rejection of a probiotic product based on the reportable value, its uncertainty, and the specification limit or limits, considering the acceptable level of the probability of making a wrong decision. Documentation of the decision rule is critical to ensuring clarity of these requirements.

Four components are included in the decision rule: (i) product specification (CoA claim) often with guard bands or coverage factors to set decision limits; (ii) the acceptable probability for making an incorrect decision, e.g., erroneously accepting a lot that does not meet specifications or rejecting a false out-of-specification (OOS) lot; (iii) a defined reportable value; and (iv) the standard uncertainty (u) associated with the reportable value. The decision rule can be formulated using information from sources external to the laboratory such as the customers, stakeholders, decision makers, and risk managers. **Figure 2** shows the elements of a decision rule for a specification with lower and upper limits. Use of an upper limit may depend on the country and regulatory classification of the final product. Overdosing or adding overage is a common practice within the industry, used to ensure and maintain label claims. It is expected that overage has been added to a level that will maintain concentrations greater than or equal to the CoA claim specification throughout the shelf life of the product.

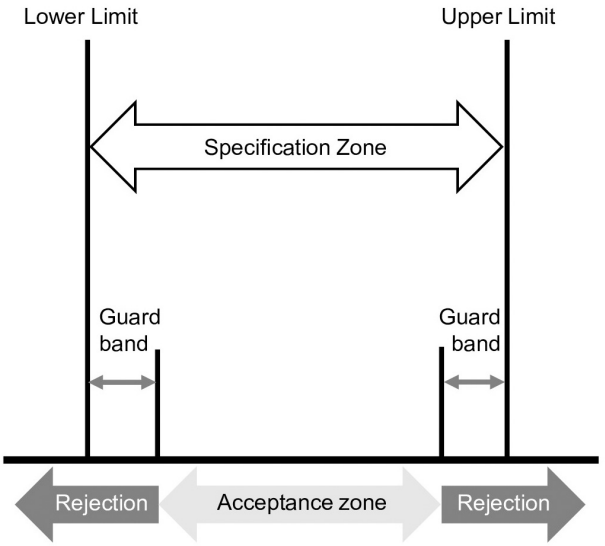

FIGURE 2 | The elements of a decision rule illustrated for a specification with upper and lower limits. A guard band is used to control the probability of making a wrong decision. In this case, the acceptance zone is smaller than the specification zone.

The decision rule defines the use of the reportable value and provides the acceptable probabilities for making a wrong decision with the reportable value. These acceptable probabilities are needed to set the *TMU*, defined in VIM (ISO/IEC, 2015) as "measurement uncertainty specified as an upper limit and decided on the basis of intended use of measurement results." The TMU for an analytical procedure must be consistent with the decision rule and the values specified within. Thus, the decision rule can provide an understanding of the maximum variability or *TMU* (see **Figure 3** for more information) that can be associated with a reportable value to allow the result to remain fit for its intended use. *TMU*, which considers intended use of the measurement, can become part of the ATP.

When there is a defined limit for the measurand, typically in legislation or a technical specification, there may also be guidance about the acceptable magnitude of the uncertainty. For probiotics, the potency specification is usually a minimum limit. When reference documents for the specification limit of the measurand do not include *TMU*, this requirement needs to be defined in another way. Empirical knowledge, data, and risk management can contribute to the determination of *TMU*.

The process for calculating *TMU* is described later in this paper. For detailed discussions of decision rules and *TMU*,

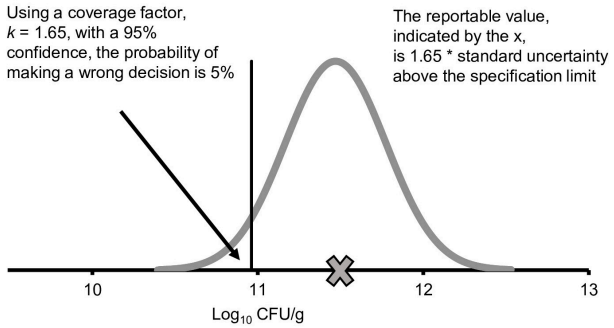

FIGURE 3 | Illustration and identification of the target measurement uncertainty (*TMU*). When the reportable value, shown by the **x**, is 1.65 × standard uncertainty above the lower limit, the probability of being wrong is 5%. [1]Lower specification limit from the example *Lactobacillus* spp. [2]X, the reportable value, is also the mean or mid-point of the distribution. [3]For determining the *TMU*, the MS EXCEL formula, = NORM.DIST, can be used. For a lower limit, the formula is = NORM.DIST(lower limit, X, TMU, TRUE). In this example, the reportable value must be above the label claim to release the product. Therefore, lower limits are designated by label claims. The value of the *TMU* is varied until the formula matches the desired probability of being wrong. [4]Probability of being wrong defined in the decision rule.

readers are directed to the following references: USP stimuli article, "Fitness for Use: Decision Rules and Target Measurement Uncertainty" (Burgess et al., 2016); Guidelines by Ellison and Williams (2012) and American Society of Mechanical Engineers (2019); and Weitzel and Johnson (2012).

Mathematical Considerations Pertaining to the Decision Rule

Log_{10} (CFU) and Normal Distributions. Probiotic CFU counts generally follow a Log_{10}-normal distribution. Log_{10} transformed data is used to calculate the *TMU*. Care needs to be taken when using and interpreting Log_{10} transformed data, e.g., Log_{10} numbers cannot be added or subtracted to calculate the difference between the numbers. More information on the Log_{10} transformation is available from the World Health Organization (WHO) guide *Statistical Aspects of Microbiological Criteria Related to Foods* (FAO/WHO, 2016).

If counts do not follow a Log_{10}-normal distribution, there are other techniques to use, but these are beyond the scope of this paper.

Expanded Uncertainty (U) and Coverage Factors (k). The expanded measurement uncertainty, or just expanded uncertainty (*U),* is an interval around a measurement result that is expected to encompass a large fraction of the distributed values that could reasonably be attributed to the measurand. The fraction may be regarded as the coverage probability or level of confidence of the interval (ISO, 2019). Guides on uncertainty recommend that laboratories report the expanded uncertainty because it provides an interval within which the true value is believed to lie with a higher level of confidence than for a standard uncertainty. Expanded uncertainty is calculated from the standard uncertainty (u) and a coverage factor (k_{pr}): $U = u \times k_{pr}$ (**Supplementary Appendix 2**). The coverage factor is chosen based on the acceptable probability of making a wrong decision (pr). The coverage factor is like the Z factor for a standard normal distribution. For a two-tailed distribution, at the 95% level of confidence, $k_{pr} = 1.96$, but the estimate 2 is often used in calculations.

An example to assess compliance with a lower limit only (a one-tailed distribution) is illustrated in **Figure 3**. To have 95% confidence that a reportable value is above the specification (company has decided that the acceptable probability for making a wrong decision is 5%), the standard uncertainty for the value is multiplied by a coverage factor, k_{pr}. The k_{pr} value is obtained from a Z factor table for confidence levels of one-tailed normal distributions (Devore and Beck, 2011). The coverage factor for the example is $k_{pr} = 1.65$. Under the condition of 95% confidence (probability of making wrong decision is 5%), the result must be 1.65 × standard uncertainty (u) above the lower specification limit for the reportable value to comply with the specification of 95% confidence. Since the expanded uncertainty (U) is calculated using k_{pr}, *TMU* can be calculated by dividing the desired expanded uncertainty range by k_{pr}:

$$TMU = U/k_{pr} \text{ (Eq. 1, } \textbf{Supplementary Appendix 2)}$$

Measurement Uncertainty

VIM (ISO/IEC, 2015) defines measurement uncertainty as a "non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used." It describes the range in which the true value is expected to be. Measurement uncertainty includes all random variation that exists in each step of the analytical procedure.

A detailed description of measurement uncertainty (*MU*) is provided in the USP 44(1) stimuli article, "Measurement Uncertainty for the Pharmaceutical Industry" (Weitzel et al., 2018). Guides on how to evaluate measurement uncertainty are provided by Eurachem (Ellison and Williams, 2012), ISO 19036:2019 (ISO, 2019), and MIKES (Niemelä, 2003).

Wording of Decision Rules

The decision rule can be written in different ways. A regulatory agency may not have specific information about a probiotic product, so it would write a general decision rule. The regulatory requirement states that a product is acceptable if the reportable value is within the specification range. In the United States, the specification range for a probiotic product is the label claim. When a value at the lower limit of specification (label claim) is obtained, the product is acceptable. The normal distribution curve (representing the expanded uncertainty or the range in which the true value may lie) is then centered over the limit. Hence, half of the normal curve is below the limit and half of the curve is above the limit. This means that there is a 50% probability the true value is below the limit and a 50% probability the true value is above the limit. A manufacturing company can apply the

regulatory decision rule or write a more conservative rule based on the company risk profile and inclusion of specific information for its product.

The regulatory decision rule could be:

The decision unit, which is the batch of powder (culturable cells or spores, freeze- or spray-dried) will be considered compliant with the specification (100% label claim) if the probability of being wrong is ≤50%. Otherwise, it will be considered non-compliant.

A general format for company decision rules could be:

The decision unit, which is the batch of powder (culturable cells or spores, freeze- or spray-dried) will be considered compliant with the specification of SPEC if the measurement uncertainty is less than the *TMU* and probability of being wrong is ≤5%. Otherwise, it will be considered non-compliant.

SPEC means the manufacturer's specification and *TMU* is the manufacturer's *TMU* value. The company includes its values for SPEC and *TMU* in its decision rule.

Developing the Decision Rule for the Example Lactobacillus spp. Powder

First, the components included in the rule were defined.

1. The product specification often with guard band(s) is used to set decision limits. The product specification becomes the acceptance zone.
    a. The specification is *Lactobacillus* spp. concentration ≥91.67 billion CFU/g which is 10.962 Log_{10} CFU/g.
    b. The company includes an overage of 0.500 log_{10} CFU/g. For this powder, the manufacturing variability has been well characterized and is much less than 0.500 Log_{10} CFU/g. This means the laboratory samples will have values close to 11.462 Log_{10} CFU/g.
    c. In this example, the label claim is applied as the specification. A more cautious approach would be to apply a higher release specification to compensate for loss of culturability throughout shelf life
2. The acceptable probability for making an incorrect decision, e.g., erroneously accepting a lot that does not meet specifications or rejecting a false OOS lot.
    a. The decision makers provide the acceptable probability of being wrong and releasing a lot that is below specification as 5%.
        i. The acceptable probability of being wrong can be any percentage the company chooses and is willing to accept. In many industries and in this example 5% is selected.
    b. In this example, it is not likely that the probability of erroneously accepting a lot that is manufactured below CoA claim will be significant because there is relatively low variation in the manufacturing process and the overage ensures that the *Lactobacillus* spp. concentration will be above the CoA claim.
3. A defined reportable value.
    a. The reportable value is that which is obtained for each lot in routine testing.
4. The standard uncertainty (u) associated with the reportable value.
    a. This uncertainty is the *TMU* to meet the requirement of the acceptable probability of being wrong, which is 5% for this example.

After defining the components of the decision rule, *TMU* is calculated. *TMU* can be determined using the equation in **Supplementary Appendix 2**, using available calculators, or by creating a MS EXCEL spreadsheet using the NORMDIST formula as shown in **Figure 3**. In the example *Lactobacillus* spp., *TMU* = 0.305 Log_{10} CFU/g.

The decision rule for the example *Lactobacillus* spp.:

The laboratory sample, taken from the batch of *Lactobacillus* spp. probiotic powder (culturable cells, freeze-dried) will be considered compliant with the specification of 10.962 Log_{10} CFU/g if the reportable value is ≥10.962 Log_{10} CFU/g, the *MU* is <0.305 Log_{10} CFU/g, and the probability of being wrong is ≤5%. Otherwise, it will be considered non-compliant.

Step 3 Develop the ATP

The ATP is essential to the APLM. It stipulates the required quality of the reportable value and provides clear, pre-defined objectives for performance of the analytical procedure. Components of the ATP should express the definition of the measurand, as well as the requirements specified in the decision rule where performance of the procedure and external factors have been considered. For further information on ATP development for analytical procedures that are in accordance with USP guidance refer to Martin et al. (2017); Barnett et al. (2016), and USP PF 46(5), "<1220> Analytical Procedure Lifecycle" (USP, 2020).

The wording of the ATP can follow the format provided in "Proposed New USP General Chapter: The Analytical Procedure Lifecycle <1220>" (Martin et al., 2017):

> "The procedure must be able to quantify [analyte] in the [description of test article] in the presence of [x, y, z] so that the reportable values fall within a *TMU* of ± C%. The probability of being wrong must be less than w%."

The ATP provides and informs the acceptance criteria for analytical procedure qualification. Moreover, the ATP can be applied for performance comparison of different analytical procedures.

Analytical Target Profile (ATP) for the Example Lactobacillus spp.

Using the information from the measurand and the decision rule, the ATP for the example *Lactobacillus* spp. was written following the proposed USP format (Martin et al., 2017).

> The procedure must be able to enumerate the *Lactobacillus* spp. culturable cell count in CFU/g of powder with cryoprotectant, formulated to 11.462 Log_{10} CFU/g, so the reportable values fall below a *TMU* = 0.305 Log_{10} CFU/g (i.e., the *TMU* associated with the reportable value is <0.305) and the probability of being wrong is ≤5%. The plating range used by the laboratory will cover 9.462–13.462 Log_{10} CFU/g, two Log_{10} above and below the internal release specification.

Analytical Procedure Development and Qualification

The ATP is used to guide analytical procedure development and qualification. This paper does not discuss these topics in detail; but does provide some experimental approaches useful for these activities.

Qualification activities consist of designing and conducting experiments that will demonstrate the procedure is performing as required and focus on evaluating the measurement uncertainty (ISO, 2019; Martin et al., 2017). Procedure variables and parameters that carry uncertainty need to be included in qualification experiments. Variables that carry uncertainty can be determined through a risk analysis of the analytical procedure. Risk analysis is discussed in detail under the section Quality Risk Management (QRM).

Bias

Bias (or accuracy) is not included for microbiological CFU enumeration procedures because, currently, there are limited CFU or proliferation-based reference standards with assigned, certified, or reference values available. Also, for experiments it is difficult to create test samples that have the same value because the analyte (culturable cells), changes with time. For these reasons, the experiments focus on determining the precisions: repeatability and intermediate precision.

ANOVA Experiments

There are many ways to determine the repeatability and intermediate precision. In our process, analysis of variance (ANOVA) experimental design is used to determine repeatability and intermediate precision. The actual performance of an analytical procedure in routine use is expressed by varying the conditions, including those identified through risk analysis (section "Risk Analysis for the Example *Lactobacillus* spp."), used in the experimental runs (replicates) of the ANOVA design. For example, multiple lots of agar media or different operators can be used. The experimental conditions should capture as many possible scenarios for operating the analytical procedure as is practical.

The impact of variables is evaluated in ANOVA experiments. It is acceptable to group variables into experimental "conditions" to streamline work. However, using conditions or grouping variables does not allow the uncertainty for each variable to be estimated. The conditions simulate scenarios that could be seen during routine use of the procedure. The ANOVA design for the example *Lactobacillus* spp. is included in section "ANOVA Design for the Example *Lactobacillus* spp."

For qualification of the analytical procedure, the ANOVA statistical tool is applied to the gathered data and ANOVA analysis of the values provides the uncertainty for the specified procedure and the probability of being wrong. Values determined through qualification activities are compared to those stated in the ATP. If the values determined through qualification are less than or equal to those stated in the ATP, the procedure is performing as required.

Precision From Replicating Steps in an Analytical Procedure

The uncertainty contributed from steps that are replicated in an analytical procedure can be estimated. A common replication is to duplicate steps. For example, multiple representative test portions can be taken from a sample sent to the laboratory. Here, assume that one test portion is used to prepare an initial suspension. The initial suspension is used to create two series of dilutions (technical replicates). The act of creating two series of dilutions, duplicates the step in the analytical procedure. Two technical replications (the dilution series) made from one test portion now exist. The technical replicates results can be analyzed to estimate the uncertainty created at the step in which the dilution series are prepared. A description of the use of duplicate or technical replicates is provided in Weitzel and Johnson (2013).

Another example of replication is seen in the use of triplicate plating for the CFU procedure in the example *Lactobacillus* spp. (**Figure 4**). One test portion of the laboratory sample is used to produce the initial suspension. The initial suspension is serially diluted, and three plates (diluted sample + agar medium) are generated from specified dilutions. Each plate is a replicate. The calculation of the uncertainty for the plating step from triplicate plate counts is demonstrated later in this article.

Quality Risk Management (QRM)

A cornerstone in the APLM approach is an analytical control strategy based on the process presented in the USP stimuli article, "Analytical Control Strategy" (Kovacs et al., 2016). The reader is referred to that paper for the explanation of the QRM theory and process. Briefly, QRM is a tool to obtain improved understanding of the link between procedure variables and the accuracy and precision of a reportable value, as well as the interdependencies of the different variables. The QRM process includes four major parts:

1. Risk assessment (comprised of the following three steps)
    a. Risk Identification
    b. Risk Analysis
    c. Risk Evaluation
2. Risk Control
3. Risk Communication
4. Risk Review

QRM identifies risks that need to be included in the evaluation of measurement uncertainty. These risks become the variables included in the ANOVA experiments to evaluate the intermediate precision, i.e., the intra-laboratory reproducibility (ISO, 2019).

Of specific interest for analytical procedures enumerating CFU and the comparison of such procedures, is identification of variables and potential risks involved with conducting an analytical procedure using plate count techniques producing CFU/g or CFU/mL as the reportable value. It is valuable to identify variables within the analytical procedure that may require control. Risk control may result in mitigation or understanding and acceptance of the risk without mitigation. Controlling risks has the potential to reduce uncertainty. These aspects are addressed in part 1 (risk assessment) and part 2 (risk control) of the QRM process.

Risk assessment starts with the risk (hazard) identification. This is when the question, "What might go wrong?" is asked. The laboratory identifies risks applicable to their specific analytical procedure. To gain additional insight into the enumeration procedure, the potential risks can be categorized as technical, matrix, or distributional as per ISO 19036:2019 (ISO, 2019). Technical uncertainty may also be called operational uncertainty. It is associated with the technical steps of the analytical procedure and covers items such as sampling, mixing, diluting, plating, and counting. Matrix uncertainty is related to how well the laboratory sample behaves when mixed, causing larger variability between test portions. Distributional uncertainty is intrinsic. It is an unavoidable variation associated with the distribution of the microorganisms in the sample, initial suspension, and subsequent dilutions.

Risk Analysis for the Example Lactobacillus spp

A risk analysis for the example *Lactobacillus* spp. is found in **Supplementary Appendix 3**. It includes a comprehensive list of potential risks that can serve as guidance for preparation of risk analyses associated with other probiotic enumeration analytical procedures. Risk control requires review of the potential hazards/risks associated with the analytical procedure and evaluation of the individual risks so that a mitigation strategy can be developed or to allow acknowledgment and acceptance of the risk without mitigation. Prior knowledge and experimental studies can be applied in making such decisions. It should be noted that in the work of this manuscript, microbiologists and probiotics enumeration specialists created both the extensive list of hazards/risks and the examples of mitigation strategies. However, neither the list of hazards/risks nor mitigations presented in **Supplementary Appendix 3** are considered definitive. Each laboratory must identify the risks applicable to its procedure, evaluate them, and design appropriate mitigation strategies.

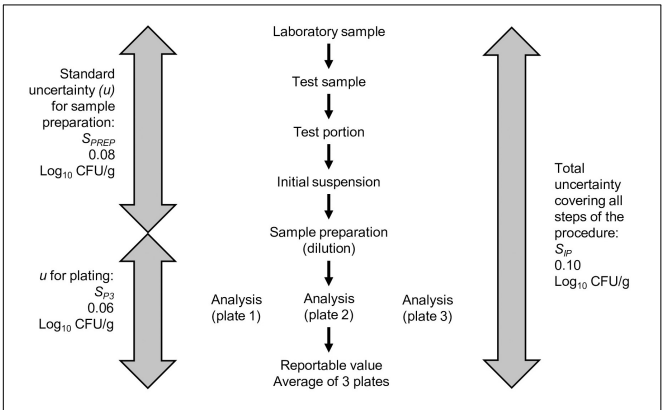

FIGURE 4 | Flow chart of the *Lactobacillus* spp. enumeration procedure. For each analysis, three plate counts are generated. The standard deviations for the plate count (S_{P3}) can be calculated from the triplicate plate count data to provide an estimate of the uncertainty from the plating and counting steps. The variance of the plate count can be subtracted from the variance covering the entire procedure to estimate the variance of the sample preparation (S_{PREP}). The values shown are for the example *Lactobacillus* spp.

Theoretically, every step in the analytical procedure, from sampling to the final reportable value, has the potential to contribute to the *MU* of the reportable value. There may be one or more variables in each step. Strategies for the analytical procedure controls can be designed to reduce input variation or to adjust for input variation to reduce its impact on the output, or a combination of both approaches. The systematic approach to risk management ensures that the performance of the analytical procedure can be explained logically and/or scientifically as a function of procedure parameters and inputs and is most effective when supported by solid knowledge base. Sources of knowledge include prior knowledge (public domain or internally documented), expertise (education and experience), experience with similar applications, and product or process specific knowledge developed and/or acquired with each application as it becomes available.

Some risks are related and thus may require related mitigation strategies. For example, a counting error could have many sources. Discussions of relationships are included in **Supplementary Appendix 3**. Other risks can be handled by complying with GMP. Mitigation by GMP follows several assumptions:

- The analytical procedure is used in a GMP laboratory.
- The equipment is properly qualified.
- Calibration and preventive maintenance programs are in place.
- Analysts are trained and competent.
- Instructions, such as Standard Operating Procedures (SOP), analytical procedure descriptions, work instructions, etc. are in place. These must be in place before any experiments are conducted.
- There are control programs for media and reagents.

Examples where GMP compliance could be used as the mitigation strategy include: (i) The potential risk of an incubator exceeding its maximum load. If the incubator qualification demonstrates a maximum load for the incubator to maintain the temperature specification, then a GMP compliant laboratory will mitigate the risk of overloading by stipulating the maximum load in their SOP, analytical procedures, and/or policies. (ii) Risk of pipetting errors. Pipettors must be qualified and

calibrated. (iii) Pipetting technique, which can cause a huge variance in results. Under GMP requirements, the laboratory should provide adequate instruction and training to ensure the analysts are competent and pipet consistently. When transferring analytical procedures, laboratories should consider and compare their pipetting techniques. The detailed instructions from the analytical control strategy assist with this comparison.

Analytical Procedure Qualification Activities for the Example *Lactobacillus* spp.

The purpose of analytical procedure qualification is to demonstrate that procedure performance meets the requirements outlined in the ATP. This is tested by an experimental design using variables determined to be significant through risk analysis of the procedure. The decision rule and ATP provide the goals and acceptance criteria for the qualification activities.

From the risk analysis of example *Lactobacillus* spp. (**Supplementary Appendix 3**), uncertainty components were identified as variables that should be included in the ANOVA experiments (**Table 2**). Most risks for this CFU enumeration procedure fell into the ISO category: technical uncertainty (ISO, 2019). The laboratory varies as many risks as practical to mimic routine experimental conditions.

ANOVA Design for the Example Lactobacillus spp.

For illustration of a procedure qualification experimental design, simulated data were generated for the example *Lactobacillus* spp. The data represents the impact of conducting the procedure while varying uncertainty components.

For this qualification, the experimental design included:

- Four conditions with variable uncertainty components.
    - The conditions cover variables encountered by plating to obtain a reportable value of 9.462–13.462 Log_{10} CFU/g powder.
- Ten replicates per condition.
    - The entire analytical procedure is performed on each replicate.
    - Each replicate represents one test portion of the laboratory sample.
    - A single dilution series is conducted per replicate.
    - Dilutions are plated in triplicate.
    - The reportable value is the mean value of three plates. Plates counts must fall within the specified counting range, e.g., 25–250 CFU/plate.

TABLE 2 | Uncertainty components for the simulated procedure qualification for the example *Lactobacillus* spp.

Uncertainty component	Condition			
	1	**2**	**3**	**4**
Days	A	B	C	D
Analyst	A	B	A	C
Lot of plating medium	1	2	1	2
Lot of suspension/rehydration medium	2	2	1	1
Lot of dilution buffer	1	2	3	4
Disposable serological pipettes	Lot 1	Lot 2	Lot 1	Lot 3
Pipettors with tips	Set A	Set B	Set A	Set C
pH meter	A	B	A	B
Analytical balance	1	2	2	1
Autoclave	1	2	3	2
Agar tempering water bath	2	1	1	2
Incubator	2	3	1	5

The components were identified through the risk analysis, and the individual variation during the ANOVA experiment are shown for each condition.

The standard deviation, variance, and average for each condition is calculated, followed by calculation of pooled standard deviation. For this ANOVA, the between condition variance is not considered because the true or reference values for each condition are not known and cannot be controlled. An overview of the experimental design and ANOVA results are compiled in **Table 3**.

Uncertainties Associated With Example Lactobacillus spp.

Using the data obtained from qualification experiments (**Table 3**) it is possible to evaluate the measurement uncertainty for the analytical procedure, as well as uncertainty contributions from individual procedure components. For the example *Lactobacillus* spp., measurement uncertainty (*MU)* for the entire procedure, as well as uncertainty from the triplicate plating component, alone, were estimated. Equations required to calculate both uncertainties are provided in **Supplementary Appendix 2**.

ANOVA data yields total uncertainty for the procedure. This is known as intermediate precision (S_{IP}) and consists of the pooled standard deviation from all conditions and replicates (**Table 3**).

The data for the triplicate plate counts informs the calculation of standard deviation for a single plate count and the standard error of mean (*SEM*) for the average of all three plate counts (**Table 3**). This is discussed above in section "Precision From Replicating Steps in an Analytical Procedure."

The uncertainty contribution from triplicate plating requires the use of all procedure qualification data for all conditions and replicates. For simplicity, **Table 4** shows only the values for condition 1, replicate 1. The headings in **Table 4** indicate values that are required for calculating the uncertainty from plating. **Supplementary Appendix 2** provide the steps to calculate triplicate plating uncertainty and the standard deviation for a single plate count. In this paper the average formula, rather than weighted, is used to determine the average plate count for one replicate. Other formulas for calculating averages in microbiology that weight the inputs according to dilutions or mass can be used when relevant. Weighted means divide the sum of all colonies counted by the sum of all volumes involved. This is a reasonable way to try to use all the information gathered to obtains the best possible density (ISO, 2020). For the example in this paper averages (unweighted means) are used because the manufacturing process is well-characterized and produces consistent product, and the analytical enumeration procedure reliably produces plates from one dilution with counts between 25 and 250. Here, weighted calculations are not needed,

TABLE 3 | The ANOVA experimental design and data for procedure qualification of the example, *Lactobacillus* spp.

Replicate	Counts in Log_{10} CFU/g															
	Condition 1				Condition 2				Condition 3				Condition 4			
	1	2	3	Average	1	2	3	Average	1	2	3	Average	1	2	3	Average
1	11.336	11.478	11.424	11.416	11.236	11.443	11.311	11.330	11.335	11.575	11.234	11.381	11.162	11.326	11.211	11.233
2	11.146	11.312	11.485	11.404	11.442	11.357	11.224	11.341	11.531	11.606	11.584	11.574	10.964	10.996	10.959	10.973
3	11.506	11.688	11.583	11.592	11.466	11.274	11.348	11.356	11.418	11.373	11.386	11.392	11.169	10.946	10.945	11.020
4	11.324	11.363	11.178	11.288	11.167	11.297	11.295	11.253	11.506	11.275	11.322	11.368	10.929	11.018	11.112	11.020
5	11.397	11.519	11.358	11.425	11.424	11.267	11.416	11.369	11.351	11.315	11.282	11.316	11.206	10.986	11.093	11.095
6	11.511	11.639	11.565	11.572	11.416	11.272	11.439	11.376	11.439	11.460	11.695	11.531	10.962	10.815	10.798	10.858
7	11.436	11.510	11.503	11.483	11.511	11.338	11.446	11.432	11.446	11.546	11.441	11.478	11.154	11.290	11.071	11.172
8	11.551	11.700	11.486	11.579	11.193	11.203	11.366	11.254	11.413	11.409	11.389	11.404	11.047	11.191	11.081	11.106
9	11.429	11.607	11.521	11.519	11.283	11.276	11.265	11.275	11.334	11.563	11.018	11.305	10.870	11.005	10.819	10.898
10	11.733	11.712	11.462	11.636	11.258	10.997	11.156	11.137	11.407	11.201	11.486	11.365	10.999	11.074	11.127	11.067
Std. Dev. (S_C)				0.1080				0.0841				0.0888				0.1160
Variance (S_C^2)				0.0117				0.0071				0.0079				0.0134
Average (C)				11.491				11.312				11.411				11.044
Intermediate precision = Pooled Std. Dev. (S_{IP})										0.1001						
Std. Dev. for single plate count (S_{P1})										0.1033						
SEM for average of three plate counts (S_{P3})				0.05964												
Std. Dev. for sample preparation (SPREP)										0.080393						

The design includes four conditions with 10 replicates each and three plates per replicate. The standard deviation, variance, and average are calculated (equations are provided in ***Supplementary Appendix 2****). The standard deviations for the four conditions are pooled to yield the intermediate precision. The standard deviation for an individual plate count is calculated. The SEM for the average of three plate counts is calculated, which allows the determination of the standard deviation for the sample preparation (see also* ***Table 4*** *and* ***Figure 4****).*

TABLE 4 | Determining the uncertainty of plating.

Condition	Replicate	Plate (p)	Plate count (y) Log_{10} CFU/g	Mean count($\bar{y}$) Log_{10} CFU/g	Squared differences from mean	Degrees of freedom	Sum of squared differences from mean	Sum of degrees of freedom	Standard deviation for one plate count Log_{10} CFU/g (S_{p1})	Contribution to combined uncertainty Log_{10} CFU/g (S_{p3})
1	1	1	11.336	11.416	0.0063468	2	0.853	80	0.1033	0.0596
		2	11.487		0.0050884					
		3	11.424		0.0000694					

Using data from the ANOVA experiment for the example Lactobacillus spp., uncertainty is determined for a single plate. Intermediate results for one replicate of one condition are shown in the white cells, while results for the entire experiment are shown in gray. Equations required to calculate the uncertainties are provided in ***Supplementary Appendix 2****. p, number of plates in each replicate; y, one plate count; S_{p1}, standard deviation for one plate count; S_{p3} standard error of the mean for the average of three plate counts.*

and the simplified approach helps to optimize workload in the laboratory. When all qualification data are included in the calculations, as required, the standard deviation for all plate counts equals 0.0962 Log_{10} CFU/g. Since the average of the three plate counts is used for ANOVA analysis, the standard error of mean ($\bar{y}$) for the triplicate plate counts is calculated by dividing by the square root of three ($\sqrt{3}$): $\bar{y} = 0.0962/\sqrt{3} = 0.0556$ Log_{10} CFU/g (Eq. 10, **Supplementary Appendix 2**). This value is an estimate for the uncertainty for the triplicate plating component reflected in the reportable value.

The pooled variance from the plating steps (S_{P3}) can subsequently be subtracted from the pooled variance of the total procedure (S_{IP}) to yield the variance for sample preparation (S_{PREP}). The square root of the variance yields the estimate of the uncertainty or standard deviation for S_{PREP} (**Table 3**). **Figure 4** illustrates the uncertainties discussed above in relation to the steps in the *Lactobacillus* spp. analytical procedure.

Completing the Qualification Procedure for Example Lactobacillus spp.

It is important to assess and summarize the alignment of qualification results with the requirements of the decision rule and ATP. In qualification experiments, the uncertainty of the total procedure (intermediate precision; S_{IP}) and the *MU* were determined. These qualification results, along with specifications set during development of the ATP (lower limit or CoA claim, center of normal distribution curve – lower limit plus overage, release limit, *TMU*) can be used to determine the actual probability of being wrong by adding the information to an MS EXCEL (2016) worksheet for calculating NORMDIST (**Supplementary Appendix 2**) or by following manual calculations in **Supplementary Appendix 2**. **Figure 5** represents the NORMDIST calculation. Using the example S_{IP}, the probability of being wrong is 0.00% This is much less than the decision rule requirement of 5%. Therefore, the company could consider increasing uncertainty (e.g., use two plates instead of three) or decreasing overage.

To complete the qualification of the analytical enumeration procedure used in the example *Lactobacillus* spp., a qualification statement must be documented. For example:

> In qualification experiments, the selected analytical procedure enumerated culturable *Lactobacillus* spp. cells as specified by the ATP. Therefore, the procedure is fit for intended use.

PROCEDURE COMPARISON

Development scientists are often called upon to judge whether an existing analytical procedure is fit-for-purpose

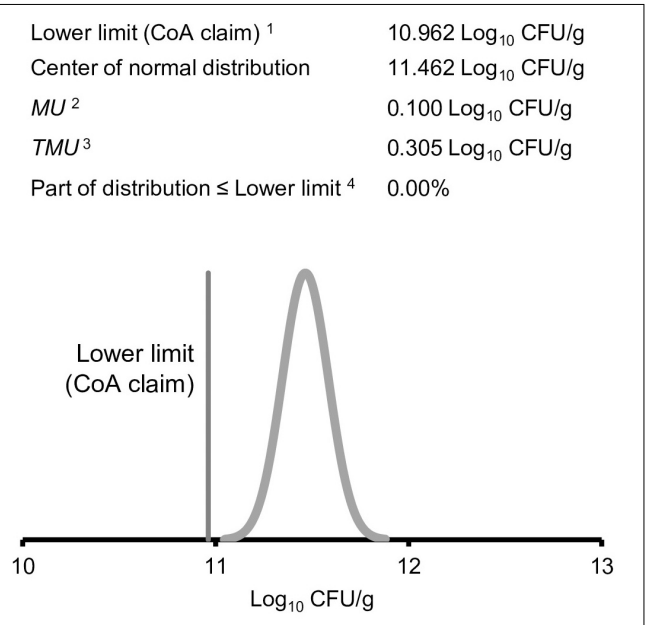

FIGURE 5 | The experimental intermediate precision, S_{IP}, is used to calculate the probability of being wrong, which is <0.0%. Data from the example *Lactobacillus* spp. [1]Certificate of analysis (CoA). [2]Measurement uncertainty (*MU*) determined from experimental intermediate precision (**Table 3**). [3]Target measurement uncertainty (*TMU*) obtained from **Figure 3**. [4]Probability of being wrong <0.0% is less than the decision rule requirement of 5.0%.

or whether an adaptation or development of a new analytical procedure is needed. The APLM process described in the proposed USP <1220> (Martin et al., 2017) provides tools to understand analytical procedures and generate and evaluate data needed to make these determinations. In the exercise of comparing two CFU analytical procedures, detailed information about enumeration procedures and data can be gathered by following the steps outlined in this manuscript. Additionally, the QRM question: "What might go wrong?" along with risk mitigations can be included in the comparison.

APLM can be used to evaluate whether both procedures in the comparison are fit for intended use. The acceptance criteria for fitness are found within the measurand, the decision rule, and the ATP.

It should be noted that available information for probiotic CFU enumeration analytical procedures that have been published or developed in a laboratory for proprietary use may be limited. In such cases, prior knowledge, expertise, literature information, or any documented information is used to select variables that should be investigated before the comparison is conducted. In all cases, a comparison should be considered carefully when full, detailed information is not available to ensure that the comparison is scientifically rational.

Tolerance Interval (*TI*) to Compare Procedures

The performance of each enumeration procedure can be demonstrated and evaluated using *TI* as described in USP <1210> (USP, 2018). The *TI* is the given range in which a specified proportion of all future reportable values will fall. The uncertainty and average value determined during qualification for each procedure to be compared can be used to calculate the *TI*. The interval calculated for each procedure can be compared as an individual piece of information and be evaluated against the ATP. The risk analysis performed during APLM can also inform the comparison.

Three possible outcomes from procedure comparison using the ATP and *TI* are illustrated in **Figure 6**. Both procedures have met the requirement of the ATP and the *TI* for both procedures are identical. It can be concluded that the procedures perform the same (**Figure 6A**). Both procedures fulfill the requirements of the ATP, but one procedure displays a narrower *TI* and hence a smaller measurement uncertainty compared to the other procedure (**Figure 6B**). Both procedures fulfill the requirements of the ATP, thus are fit for use, although the *TI* do not overlap (**Figure 6C**).

In the protocol to design the analytical procedure comparison, the acceptability of one or all three outcomes must be stated. The acceptability of outcome A, in which the procedures perform the same, is straightforward. Future values from both procedures can be compared directly. For outcome B in which one procedure is different from the other, but the *TI* overlap, the end-user must decide whether this difference in performance is acceptable. In outcome C, the analytical procedures perform differently. There is no overlap in the *TI*. The difference in future results needs to be accounted for.

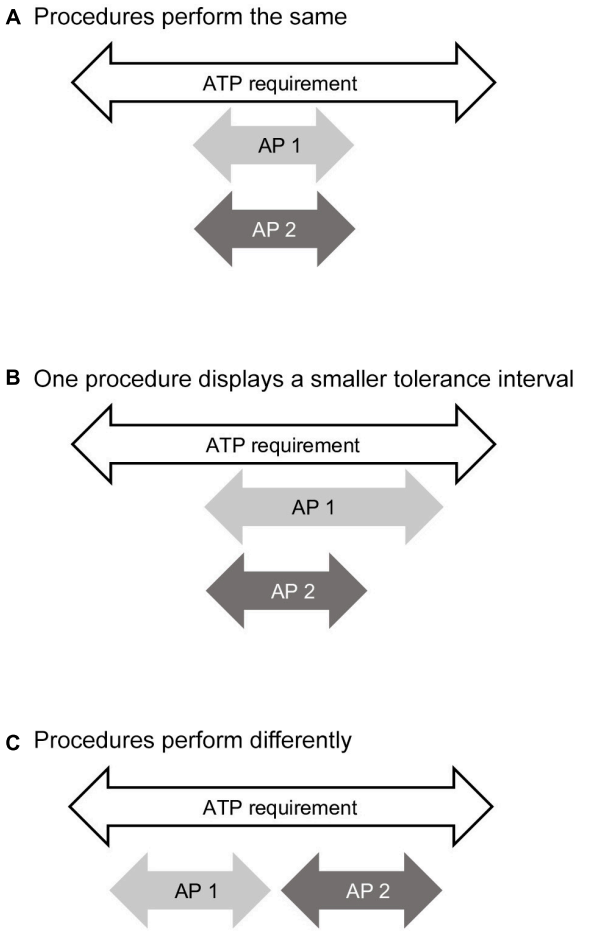

FIGURE 6 | Analytical procedure comparison using the analytical target profile (ATP) and tolerance intervals (*TI*). The white arrow represents the specification range required by the ATP for the analytical procedure to be fit for use. The light gray arrow (AP 1) represents the tolerance interval for one analytical procedure. The dark gray arrow (AP 2) represents the tolerance interval for the second analytical procedure. **(A)** Comparison showing both procedures perform the same. **(B)** Comparison showing the tolerance interval for procedure 1 is larger than procedure 2. **(C)** Comparison showing the two tolerance intervals of the two procedures do not overlap. In all situations both procedures are fit for use.

Bias and Tolerance Interval (*TI*)

The *TI* considers both accuracy (bias) and precision. For microbiological CFU procedures, bias may not be able to be determined, as discussed above. Therefore, the *TI* may not overlap, simply due to distributional uncertainty of the test samples used during qualification experiments. The protocol for comparing the methods must take this into account.

Tolerance Interval (*TI*) Comparison of Two Procedures for the Example *Lactobacillus* spp.

In this comparison, procedure A will represent information and data simulated for the example *Lactobacillus* spp. The second procedure, procedure B, uses a different plating agar and a different mixing technique for the initial sample suspension. To demonstrate the use of *TI*, values for procedure B were simulated.

First, the measurand, decision rule, and ATP for procedures A and B are compared to ensure the procedures are designed for the same purpose. Second, the results of the risk analyses are reviewed to confirm the procedures can be compared. If the outcome of these evaluations stipulates the two procedures are comparable, an actual comparison of the procedures performances is made based on data gathered during qualification experiments. If this data is not available, information used to inform the decision of fit for intended use must be closely scrutinized. It is possible, in some cases, to apply historical data to gather information and make calculations needed to develop ATP requirements. However, before proceeding, it may be necessary to design and conduct qualification experiments, such as the ANOVA experiments described in this paper.

Using data that is acceptable to informing the ATP, the *TI* for each procedure is calculated and compared. The data for procedure A was taken from condition one in the *Lactobacillus* spp. example (**Table 3**). Data for procedure B was simulated to represent one condition from qualification experiments. The *TI* calculations needed for comparison are shown in **Table 5**. It should be noted that the factor *K* used in *TI* calculations is not the *k*-factor used in uncertainty calculations. Both equations needed to calculate *TI* and *K* are found in **Supplementary Appendix 2**.

For this comparison, both procedures are deemed fit for intended use; both meet ATP requirements. The *TI* show a substantial overlap. Therefore, both procedures could be used; the end-user needs to decide whether the difference in performance is acceptable.

TABLE 5 | Comparing procedure performance by tolerance intervals (*TI*).

***TI* calculations for procedure A (Log_{10} CFU/g)**				
Condition	$\overline{C}$	S_C	***TI***	
			$\overline{C}-(K \times S_C)$	$\overline{C}\ (K + S_C)$
A-1	11.491	0.108	11.218	11.765
***TI* calculations for procedure B (Log_{10} CFU/g)**				
B-1	11.442	0.126	11.123	11.761

TI calculated for one ANOVA condition of procedure A (A-1) and B (B-1), respectively. TI = $\overline{C} \pm (K \times S_C)$, where $\overline{C}$ = mean value for the condition. K = coverage factor (90% confidence, 90% coverage, n = 10) = 2.535. S_c = Standard deviation for each condition. K values for TI calculations can be calculated as described in ***Supplementary Appendix 2****. $\overline{C}$, average for one condition; S_C, standard deviation for one condition; K, tolerance interval factor.*

REAL-LIFE DATA FOR *LACTOBACILLUS ACIDOPHILUS* POWDER

To illustrate the practical use of the APLM for CFU procedures, the tool was applied to the analysis of real-life data from a probiotic manufacturer's development laboratory. The laboratory was investigating the enumeration of a probiotic *Lactobacillus acidophilus* powder. The ANOVA design includes five conditions with 10 replicates each. Each replicate consists of three plates generated as per proprietary procedure. The conditions capture modifications to the procedure. Condition details and the ANOVA analysis table of are included in **Supplementary Appendix 4**.

Although study parameters used in the manufacturer's development laboratory and the simulation are different (analytical procedures; ANOVA designs—number of conditions, details varied), it is interesting that the standard deviations, variances, and standard errors determined via the ANOVA analysis are smaller for the real-life data than those of the simulation. This observation may align with conservative estimates knowingly used when designing the simulation. The data shows that variations occur when changes are made to the procedure. This underscores the need for qualification and comparison tools such as APLM and *TI*. For complete application and maximized impact to the probiotic industry, utilization of the complete APLM approach, including the ATP, measurand definition, risk analysis, and comparison with *TI* described in USP <1210> (USP, 2018) is recommended.

DISCUSSION

Through information acquisition, educated decision-making, and documenting key requirements, APLM creates a fluid knowledge base that becomes the cornerstone of communication for all discussions regarding an analytical procedure and the product(s) it supports. This type of qualification (validation/verification) management system ensures an analytical procedure is and remains fit for intended use throughout its lifecycle. Moreover, the APLM process enhances organizational communications regarding the status quo or changes to the procedure that will improve the quality assessment of the probiotic products it supports. Process transparency, efficient knowledge transfer, and routine use of appropriate analytical procedures makes authenticating label claims and meeting stakeholder expectations easier for all parties involved.

Both the qualification of procedures under APLM and comparison by *TI* hold the potential to add flexibility in determining the quality and release of probiotic products. The insights gained through *TI* comparison, taken along with APLM information, can inform decisions regarding the procedure itself (Is it performing as desired? Should it be modified or replaced?). Details captured while conducting

the procedure, such as the effects produced by selecting the product of one supplier over another, analysis errors caught midstream, or replacement of equipment used routinely for the procedure can also be observed. Changes made in the manufacturing process may or may not be reflected in the product quality and hence the reportable values of the analytical procedure used to test the end-product. Either way, changes are documented in the system and new results can be compared to prior results to evaluate the impact to product quality. The information compiled can point to equivalencies and differences; again, providing flexibility and improving product quality.

Other strategies, tools, and guidelines are available to meet GMP regulatory requirements surrounding the use of scientifically valid procedures. For example, the probiotics industry is aware of and uses quality by design, risk analysis, control strategies, and validations guided by the United States Food and Drug Administration (FDA), Health Canada, the World Health Organization (WHO), and others. These programs are predecessors or foundation blocks that have been applied to lifecycle management. When APLM is applied to CFU enumeration, elements of predecessor programs can be used to help detail steps within the approach, but the path forward and elements required to complete each step are streamlined. For companies that have already selected alternative options to meet regulatory requirements and fit their needs, APLM can be used as a complementary approach. Depending on experience with design and/or qualification of procedures, the combination of information gained through predecessor programs and APLM/*TI* can be used to build flexibility. If a company chooses to switch to APLM, much of their historical data would support the steps and requirements within the approach. For new companies, APLM and this manuscript provide a step-by-step procedure to meet regulatory requirements. APLM uses tools and practices of good science and metrological principles. The APLM process is streamlined, logical, and organized. It is focused on ensuring the reportable value is fit for intended and uses many documented and accepted practices expressed in recognized standards. Moreover, the lifecycle view of analytical development facilitates the analytical procedure to follow the development status of the product, which is especially important during the transition from development to final product.

High quality products require high quality analytical procedures and performance control to ensure efficient evaluation and monitoring of product quality. As outlined in this article, using the ATP, APLM, and *TI* comparison will allow probiotic manufacturers to define, control, monitor, and compare procedure performance. This is illustrated by revisiting the USP (2020) definition: "The ATP is a prospective description of the desired performance of an analytical procedure that is used to measure a quality attribute, and it defines the required quality of the reportable value produced by the procedure."

CONCLUSION

APLM ensures that an analytical procedure is fit for its intended use. Pertinent information must be gathered, understood, and experimentally explored before procedures are applied for product quality assessment. This includes a thorough risk analysis and complete control strategy. The APLM approach and understanding it provides can also be used to effectively document and compare analytical procedures using well-evaluated measurement uncertainty and statistical tools such as tolerance intervals. This type of comparison has a broad range of applications.

The comparison exercise using APLM information and *TI* calculations showed that *TI* can be used to identify agreements and anomalies. The *TI* set expectations for the usefulness of analytical procedures. If the procedure(s) are deemed fit as per the APLM ATP, then one needs only to compare TI limits or endpoints, when considering continued fitness, need for modification, or whether a change in procedure(s) may be beneficial. These comparisons can be conducted over the life cycle of the procedure.

Better understanding and control of analytical procedures will improve the quality of results that the probiotics industry uses to control and improve processes that lead to the delivery of quality products to consumers. It will also improve the quality of results used for making business decisions and supporting claims of dose and, therefore, health benefits. Accumulated, detailed knowledge gathered through APLM and *TI* comparisons will drive innovation in the probiotics industry.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BK, JS, MW, and VG wrote and edited drafts and revisions of the manuscript. CP, CV, MP, and PB edited the revisions. JS, MW, and MP drafted the initial risk analysis. MW contributed to all statistical analyses and developed the EXCEL workbook for the project. CV refined, formatted, and compiled all figures and tables. MP contributed to the data examples to enhance discussion of project topics and improve example parameters. JS compiled author contributions and edited to form the final manuscript. All authors contributed to the discussions of project topics, edited the abstract, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

ORIGINAL RESEARCH
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Evaluation of Dietary Supplements Containing Viable Bacteria by Cultivation/MALDI-TOF Mass Spectrometry and PCR Identification

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The insufficient quality of products containing beneficial live bacteria in terms of content and viability of labelled microorganisms is an often-reported problem. The aim of this work was to evaluate the quality of dietary supplements containing viable bacteria available in Slovenian pharmacies using plate counting, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and species- or subspecies-specific PCR with DNA isolated from consortia of viable bacteria, from individual isolates, or directly from the products. Twelve percent of the products (3 of 26) contained insufficient numbers of viable bacteria. Eighty-three of the labelled species (111 in total) were confirmed by PCR with DNA from the product; 74% of these were confirmed by PCR with DNA from viable consortium, and 65% of these were confirmed by MALDI-TOF MS analysis of colonies. Certain species in multi-strain products were confirmed by PCR with DNA from viable consortia but not by MALDI-TOF MS, suggesting that the number of isolates examined (three per labelled strain) was too low. With the exception of *Lacticaseibacillus casei* and closely related species (*Lacticaseibacillus rhamnosus* and *Lacticaseibacillus zeae*), PCR and MALDI-TOF identification results agreed for 99% of the isolates examined, although several MALDI-TOF results had lower score values (1.700–1.999), indicating that the species identification was not reliable. The species *L. zeae*, which appeared in 20 matches of the Biotyper analysis, was identified as *L. rhamnosus* by PCR. The MALDI-TOF MS analysis was also unsuccessful in detecting *Lactobacillus acidophilus* La-5 and *Bacillus coagulans* due to missing peaks and unreliable identification, respectively. Mislabelling was detected by both methods for two putative *L. casei* strains that turned out to belong to the species *Lacticaseibacillus paracasei*. PCR remains more successful in subspecies-level identification as long as the database of MALDI-TOF MS spectra is not expanded by building in-house databases. The lack of positive PCR results with viable consortia or colonies, but positive PCR results with DNA isolated directly from

the products observed in 10% (11/112) of the labelled strains, suggests the presence of non-culturable bacteria in the products. MALDI-TOF MS is a faster and simpler alternative to PCR identification, provided that a sufficient number of colonies are examined. Generation of in-house library may further improve the identification accuracy at the species and sub-species level.

Keywords: lactobacilli, bifidobacteria, dietary supplements, MALDI-TOF MS, identification, probiotic, PCR, viability

INTRODUCTION

Researchers from several countries have pointed out for many years the insufficient quality of products containing beneficial live bacteria in terms of the content and viability of the labelled microorganisms (Temmerman et al., 2003; Elliott and Teversham, 2004; Huys et al., 2006; Lin et al., 2006; Matijašić and Rogelj, 2006; Morovic et al., 2016). Until the European Union Regulation on nutrition and health claims [Regulation (EC) No. 1924/2006] came into force in Europe, these bacteria were called probiotic bacteria. However, the term probiotic has since been replaced in the EU by "live bacteria," "beneficial bacteria," or similar terms due to the lack of approved health claims, while it continues to be used in most other regions of the world. However, these restrictions only apply to foods, including dietary supplements, and not to medicinal products, medical devices, and other products with intentionally added microorganisms. In order to distinguish between food probiotic products, including dietary supplements, and those for therapeutic use, the term "live biotherapeutic products" was introduced by the FDA (2012) and since 2019 has also been recognised by the European Directorate for the Quality of Medicines and Healthcare (Cordaillat-Simmons et al., 2020).

In Slovenia, the quality of dietary supplements containing live bacteria from Slovenian pharmacies, marketed as "probiotic" until 2018, has been monitored for almost two decades (Matijašić and Rogelj, 2006; Bogovič Matijašić et al., 2010; Lorbeg and Matijašić, 2017). Our study published in 2017 showed that the overall quality of products sold in Slovenian pharmacies improved slightly, as 42% of them complied with the label, compared to only 25% in the previous study in 2009 (Lorbeg and Matijašić, 2017). The need for stricter control of orally administered probiotic formulations, which should contain the specified number of viable bacteria and be free of contaminating microorganisms, was also recently highlighted by the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics (Kolacek et al., 2017). Although significant improvements in the quality of these products have been observed in other markets in recent years, deficiencies in the content and viability of the declared microorganisms are still regularly reported (Lewis et al., 2016; Chen et al., 2017; Ansari et al., 2019; Di Pierro et al., 2019; Korona-Glowniak et al., 2019; Seol et al., 2019; Aldawsari et al., 2020; Dioso et al., 2020; Kesavelu et al., 2020; Shehata and Newmaster, 2020; Vermeulen et al., 2020).

Products containing multiple strains present a particular challenge, as not all strains need to be present in the same quantity at the time of production and can vary greatly in viability during storage. In addition to conventional cultivation-based and PCR-based methods for viability assessment and identification, more advanced methods are increasingly being used, such as next-generation sequencing, terminal restriction fragment length polymorphism, and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Angelakis et al., 2011; Lewis et al., 2016; Morovic et al., 2016; Patro et al., 2016; Čanžek Majhenič et al., 2018; Huang and Huang, 2018; Vecchione et al., 2018; Celandroni et al., 2019; Seol et al., 2019; Dioso et al., 2020; Vermeulen et al., 2020). Although some of them, such as viability PCR methods and advanced flow cytometry methods, are rapid and provide high-quality results, they may not be as useful for routine quality control because they require expensive equipment and chemicals as well as well-trained personnel.

Bacterial identification using MALDI-TOF is based on the comparison of protein mass spectra obtained from whole bacterial cells (Moore et al., 2011; Singhal et al., 2015). This method has already been recognised as an attractive tool for clinical isolate identification, microbial diversity studies, and other applications. With the improved databases, it is now increasingly used also for the analysis of milk and dairy products (Angelakis et al., 2011; Duskova et al., 2012; Nacef et al., 2017; Gantzias et al., 2020) but has not been widely used for the quality control of dietary supplements with added live beneficial bacteria (probiotic products) (Singhal et al., 2015; Čanžek Majhenič et al., 2018). A few reports on the use of MALDI-TOF MS for the analysis of dietary supplements include the identification of bifidobacteria, lactic acid bacteria (LAB), *Bacillus* species, and *Saccharomyces boulardii* in various products available on the EU market and in the United States (Bunesova et al., 2014; Vecchione et al., 2018; Ansari et al., 2019; Celandroni et al., 2019).

Previous studies in Slovenia and elsewhere have shown that there are inconsistencies between reported and observed data on the number and type of live bacteria in products. In this study, dietary supplements containing live bacteria available in Slovenian pharmacies were evaluated for compliance with the declared content of microbial species and amounts within the declared shelf-life of the product using conventional cultivation

Abbreviations: *B.*, *Bifidobacterium*; *Bac.*, *Bacillus*; *E.*, *Enterococcus*; *L.*, former *Lactobacillus* genus, now comprising new genera (*Lactobacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Lactiplantibacillus*, and *Ligilactobacillus*); *Lc.*, *Lactococcus*; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; *Sacch.*, *Saccharomyces*.

methods and identification of colonies by analysis of the mass spectra of cell proteins with MALDI-TOF MS. In addition, the main objective was to evaluate the applicability of MALDI-TOF MS in the identification of labelled bacteria in the products studied compared to the PCR approach.

MATERIALS AND METHODS

Products

Dietary supplements for the general population (n = 17; marked as 1–17) or children (n = 9; marked as 18–26) were purchased in Slovenian pharmacies in March 2019. They were stored under the conditions indicated on the labels until analysis.

Determination of Viable Counts (CFU/g)

The products in the powdered or oil suspension formulations were aseptically diluted and homogenised in the buffered peptone water (10% w/w) (Merck, Darmstadt, Germany). The products in tablet form were crushed to a fine powder before homogenization. The homogenised samples were allowed to stand at room temperature for 30 min to rehydrate. The appropriate dilution was spread on agar media plates as follows: TOS (Yakult, Tokyo, Japan) with mupirocin (50 mg/L) (AppliChem GmbH, Germany) for bifidobacteria, MRS (pH 6.2) for lactobacilli and *Bacillus*, ROGOSA (pH 5.5) with glacial acetic acid (0.13%) for lactobacilli in mixed formulations, M17 for lactococci and *Streptococcus* (*S.*) *salivarius* subsp. *thermophilus*, CATC for enterococci, and YGC for yeasts. Microbiological media were purchased from Merck (Darmstadt, Germany) except where otherwise indicated. Incubation followed for bifidobacteria and lactobacilli at 37°C for 72 h, anaerobically (GENbox anaer, Bio-Mérieux, Marcy l'Etoile, France); *S. salivarius* subsp. *thermophilus*, aerobically at 37°C for 24 h; lactococci, aerobically at 30°C for 72 h; *Bacillus*, aerobically at 37°C for 5 days; and yeasts, aerobically at 25°C for 72 h.

MALDI-TOF MS Identification

In general, the number of colonies examined by MALDI-TOF was three times the number of labelled species. Putative representatives of different species (three colonies per labelled strain) were isolated from different agar media selected based on label information. Colonies were selected based on the morphological characteristics of the colonies when observed, otherwise randomly from a portion of the agar plate.

Colonies were transferred onto a steel plate (MSP 96 target polished steel BC, Bruker, Germany) and air-dried (direct spotting method). One microlitre of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid; Bruker Daltonics, Bremen, Germany) was applied to each sample and further processed according to the manufacturer's instructions (Microflex LT system TM 1.1, Bruker Daltonics, Germany). Identification was performed using MALDI Biotyper 3.1 software and library (version 3.1.66; Bruker Daltonics). Calibration of the mass spectrometer was performed with the Bruker's bacterial test standard (*Escherichia coli* DH5α extracts with the additional proteins RNase A and myoglobin, Bruker Daltonics). The results of the comparative analysis of the obtained spectra with the reference spectra were expressed as confidence score values and associated colour codes. The score values $\geq$ 2.300 were considered as highly probable species identification. Score values ranging from 2.000 to 2.299 and from 1.700 to 1.999 meant confident genus identification/probable species identification and probable genus identification, respectively. In case of unreliable/unsatisfactory results (score values below 1.7), the extended direct transfer method with 70% formic acid was used.

Bacterial DNA Isolation

DNA was isolated directly from the products and from a mixture of colonies grown on selective media (i.e., viable consortia). To isolate DNA directly from the product, 50 mg of a sample was transferred to a microtube and resuspended in 0.4 ml of TE buffer containing 4 mg of lysozyme (Sigma Chemical, St. Louis, MO, United States) and 4 μl of mutanolysin (2,500 U/ml) (Sigma Chemical, St. Louis, MO, United States). After 1 h of incubation at 37°C, DNA was isolated using Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI, United States) according to the manufacturer's protocols. In addition, DNA was also isolated from the mixture of colonies washed from the selective media by pipetting 2 ml of physiological saline onto the surface of the media, then scraping the colonies from the surface with a disposable L-shaped cell spreader, and transferring 1 ml of the suspension to a microcentrifuge. The suspension was centrifuged at 12,000 rpm for 2 min, and DNA was isolated from the pellet as described above.

The colonies selected for MALDI-TOF MS analysis were first streaked onto appropriate selective media and incubated under the conditions described above. Grown colonies were scraped from the media, resuspended in 0.4 ml TE buffer, and stored in the freezer. Prior to isolation, 0.1 ml TE buffer containing 5 mg lysozyme and 5 μl mutanolysin (2,500 U/ml) was added and incubated for 1 h at 37°C. The suspension was then centrifuged at 12,000 rpm for 2 min, and the supernatant was removed. DNA was further isolated using the Isolate II Genomic DNA Kit (Bioline Reagents Ltd., London, United Kingdom) according to the manufacturer's instructions.

Species-Specific PCR

PCR specific for particular species or subspecies was performed with DNA isolated directly from the products or from viable consortia, i.e., colonies scraped from agar plates. The reaction mixtures (20 μl) contained GoTax flexi buffer (Promega), 1.5–7 mM $MgCl_2$, 0.5 μM oligonucleotide primers, 0.1 mM dNTP, 0.025 U/μl Taq polymerase, and 2 μl DNA. PCR reactions were performed on Simplism^{TM} Thermal Cycler (Thermo Fisher Scientific, United States) using specific primers as described previously: *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* (Ward and Timmins, 1999), *L. casei*, *L. paracasei*, *Lacticaseibacillus rhamnosus* (Bottari et al., 2017), *Lactobacillus plantarum*, *Lactobacillus salivarius* (Chagnaud et al., 2001), *L. rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus acidophilus* (Walter et al., 2000), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Torriani et al., 1999), *Lactobacillus gasseri* (Song et al., 2000), *Lactococcus lactis* (Barakat et al., 2000),

Bifidobacterium bifidum, *Bifidobacterium infantis* (Matsuki et al., 1999), *Bifidobacterium longum*, *Bifidobacterium animalis* subsp. *lactis* (Malinen et al., 2003), *B. animalis* (Junick and Blaut, 2012), *Bifidobacterium breve* (Kwon et al., 2005), *Saccharomyces cerevisiae* (Josepa et al., 2000), *Bacillus coagulans* (Liu et al., 2010), *Enterococcus faecium* (Dutkamalen et al., 1995), and *S. salivarius* subsp. *thermophilus* (Tilsala Timisjarvi and Alatossava, 1997). The primers and reaction conditions are listed in **Supplementary Table 1**. The presence and size of PCR amplicons were checked by agarose gel electrophoresis on 1.2% agarose gels (agarose Sigma) using TAE buffer (Sigma) and Gene Ruler, 1 kb DNA Ladder (Thermo Scientific). Gels were stained with SYBR Safe (Invitrogen, OR, United States), visualised under UV, and documented using the UVITEC system (Cambridge, United Kingdom).

16S rDNA Sequencing

The DNA of selected strains, extracted as described above, was amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′) (Yu et al., 2011). The reaction mixture was the same as described above for species-specific PCR. The amplification protocol included 2 min of denaturation at 95°C, 30 cycles of denaturation (95°C, 1 min), annealing (59°C, 1 min), elongation (72°C, 2 min), and final elongation for 5 min at 72°C. Sanger sequencing was performed by Microsynth (Balgach, Switzerland). The sequences were analysed using National Center for Biotechnology Information database and BLAST algorithm.[1]

RESULTS

The products examined comprised those for the general population (n = 17) and those for children (n = 9). Ten of them (38.5%) contained only one strain, two (7.7%) contained two strains, two (7.7%) contained three strains, and nine products (34.6%) contained more than three strains (4–14) belonging to different genera, i.e., *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, and newly introduced genera derived from previous *Lactobacillus*, i.e., *Lacticaseibacillus*, *Limosilactobacillus*, *Lactiplantibacillus*, and *Ligilactobacillus* (Zheng et al., 2020). It should be noted that the strains indicated on the labels (**Supplementary Table 2**) still bear the old species names, as the products were obtained before the publication of the new classification of the former genus *Lactobacillus* in 2020 (Zheng et al., 2020).

Determination of Viable Counts (CFU/g)

Compliance with the labelled number of viable bacteria was not related to the date of purchase, as eight products that were not fully adequate in terms of the number of viable bacteria were not close to the end of shelf-life, but at least 12 months remained until the end of shelf-life. For five products, the number of colony-forming units per gram (CFU/g) differed by less than 0.5 log units, while for three products the viable count was 0.5–1 log unit lower than indicated (**Figure 1** and **Supplementary Table 2**).

The results which were <0.5 log below the indicated CFU/dose were not considered a serious deviation from the indicated concentration since we should also take into account the usual deviations of the plate counting method and since the media and protocols of sample preparation may not have been optimal for certain strains. In plate counting, indeed up to about ±0.5 $\log_{10}$ is often considered as acceptable accuracy (ISO, 2003, 2010; Jackson et al., 2019). Overall, three products out of the 26 tested (12%) can be considered inadequate in terms of viability.

Assessment of the Presence of Labelled Species by Species-Specific PCR

To assess the presence of the species indicated on the labels, three approaches were used. First, individual colonies were collected from agar plates and analysed by MALDI-TOF MS and by species-specific PCR using primers specific to the species listed on the labels. Second, DNA was isolated from a mixture of colonies grown on specific agar media (DNA from viable consortia) and subjected to species-specific PCR. Third, total (metagenomic) DNA was isolated from the products and analysed by species-specific DNA.

Overall, 83% of the labelled species (111 total) were confirmed by PCR with DNA from the product, 74% of them were confirmed by PCR with DNA from viable consortium, and 65% of them were confirmed by MALDI-TOF MS analysis of colonies (**Table 1**). When specific species were detected in the products by PCR on isolates, the presence of these species was generally also detected in total DNA from the product and in DNA from viable consortia (**Table 1**). Two exceptions were *B. breve* in product 8 and *L. casei* species in product 3, which could not be detected by PCR in DNA from viable consortia or from the products, and *L. acidophilus* in product 17 which could not be detected in DNA from the product, Conversely, in some cases, such as *B. longum*, *L. paracasei*, *L. salivarius* in product 1, *B. bifidum* in product 2, etc., the species was confirmed only by PCR with total DNA, which may indicate that the representatives of these species

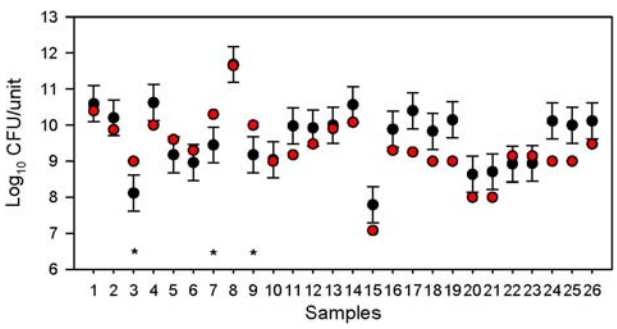

FIGURE 1 | Compliance of 26 dietary supplements with the labelled number of colony-forming units (CFU; bacteria). Black dots, number (CFU/unit of product) determined by plate counting; whiskers, 0.5 $\log_{10}$ acceptable accuracy; red dots, labelled number of CFU/unit of product; *, non-compliance with the labelled number of CFU.

[1] http://blast.ncbi.nlm.nih.gov/blast/

are present in the product in a non-culturable state or in low numbers. DNA from product 3 and DNA from viable consortia were also amplified with *L. paracasei* primers, which should not generate amplicons with *L. casei* and *L. rhamnosus* (Ward and Timmins, 1999), indicating that mislabelling of *L. casei* in this product is possible. Similarly, mislabelling for *L. casei* is likely in products 12 and 25, where the presence of *L. paracasei* was confirmed by PCR on individual isolates and metagenomic DNA.

Interestingly, in one of the products (sample 17), we could not amplify any of the target sequences by PCR with DNA isolated directly from the product, although DNA isolation was repeated three times and additional purification of DNA was performed to remove possible PCR inhibitors, while five labelled species were detected by PCR with DNA from viable consortia.

Comparison of MALDI-TOF MS and PCR on Individual Colonies

The best matches of the analysis of MALDI-TOF MS results by Biotyper together with the score values indicating the probability of correct identification are shown in **Supplementary Table 3** and the overview in **Tables 1,2** and **Supplementary Table 4**. Sixty-six percent (238/362) of the colonies were identified as highly probable or probable species (score value $\geq$ 2.000, highlighted in green) and 30% (107/362) were with probable genus identification (score value 1.700–1.999), while 17/362 (5%) of the samples could not be identified due to the absence of peaks (11 samples) or due to non-reliable identification (6 samples).

Overall, highly probable species identification (score value $\geq$ 2.300) was not frequently observed. Only 33 of 362 (9%) had a score value above 2.3, and the best match was not restricted to specific species. In general, not only the "green" results of MALDI-TOF MS but also most results with score values of 1.700–1.999 (highlighted in yellow) were confirmed by species-level PCR (**Supplementary Table 3**).

The matching of identification results for individual colonies with MALDI-TOF MS and PCR was generally good but with a few exceptions (**Tables 1, 2** and **Supplementary Table 3**). Two putative *L. casei* in product 3 (score values 2.042 and 1.847, respectively) were not confirmed as *L. casei* by PCR but were identified as *L. rhamnosus*. This species was also indicated on the label of product 3. Twelve isolates were identified as *Lacticaseibacillus zeae* by MALDI-TOF MS, but with score values of 1.700–1.999, indicating low reliability of results at the species level. Considering that *L. rhamnosus* species was not found among the best matches, it is likely that these isolates actually belong to *L. zeae* species but were mislabelled – which is not unusual considering that these two species are closely related and that the current type strain ATCC 15820/DSM 20178 of *L. zeae* (Liu and Gu, 2020) has been reclassified a few times. However, PCR with primers PrI and RhaII, which are specific for *L. rhamnosus* and do not amplify the DNA of *L. zeae* (Walter et al., 2000), yielded positive results for these 12 isolates. Furthermore, the *L. rhamnosus* species identity was confirmed also by PCR using the primers and conditions of Bottari et al. (2017) as well as by 16S rDNA sequencing (**Supplementary Table 3**).

Eleven isolates showed no peaks but could be identified by PCR. Among the isolates that could not be reliably identified by MALDI-TOF MS, while PCR analysis confirmed the labelled species, were strain *L. acidophilus* La-5 from product A16 and *B. coagulans* from product 8. In the case of strain La-5, no peaks were observed, while in the case of *B. coagulans*, the score value was less than 1.700.

The results of MALDI-TOF MS identification are not considered reliable at the subspecies level, so the best matches obtained by Biotyper with the presumed *B. animalis* ssp. *lactis* were shown as *B. animalis*. In fact, the spectra of the isolates of this species typically matched *B. animalis* subsp. *lactis* DSM 10140 (type strain) in the first place. All these isolates were confirmed by PCR to belong to the subspecies *B. animalis* subsp. *lactis* (**Tables 1, 2** and **Supplementary Table 3**). In the case of *S. salivarius* subsp. *thermophilus*, the best matches in all cases were presented as *S. salivarius* subsp. *thermophilus*. *B. longum* representatives belonging to the subspecies *B. longum* subsp. *longum* or *B. longum* subsp. *infantis* were labelled either at the subspecies level (correct only in product 4; in another five products as *B. infantis*) or at the species level (as *B. longum*) in nine products. In one of the products (sample 15) with *B. infantis* on the label, this subspecies was confirmed by MALDI-TOF MS as *B. longum* subsp. *infantis*, while in another product (sample 25), the result was shown as *B. longum* (**Supplementary Table 3**). Results for the presence of *B. longum* subsp. *longum* were reported exclusively as *B. longum* in those cases where they were positive, but in fact in all these cases one of the *B. longum* subsp. *longum* reference strains from the Biotyper database was reported as the best result, so we can assume that identification was indeed successful at the subspecies level.

The identity of *B. longum* subsp. *infantis* was also confirmed by a PCR assay that was able to distinguish between the subspecies *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* (Matsuki et al., 1999), while the *B. longum*, where labelled (including a *B. longum* subsp. *longum* strain), was analysed by PCR using only the primers that generated positive results in both subspecies (Malinen et al., 2003).

Fifty-six colonies were identified with higher or lower probability as *L. acidophilus*, with score values ranging from 1.903 to 2.494. In accordance with the Biotyper recommendations, it should be taken into account that the species *L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, and *Lactobacillus kitasatonis* of the genus *Lactobacillus* have very similar patterns, making it difficult to distinguish between these species. Among the best matches, *L. gallinarum* was found twice and *L. acidophilus* 70 times. Nevertheless, PCR confirmed the species *L. acidophilus* for all colonies tested and confirmed the presence of this species in 13 products in which it was labelled.

DISCUSSION

Viability assessment by plate counting showed certain improvements in the quality of these products available in Slovenian pharmacies compared to previous studies. While

in the 2017 study, which included 17 products, 29% of the products were found to have an insufficient number of CFU/dose and another 24% of the products had a slightly lower number (<0.5 CFU/dose) (Lorbeg and Matijašić, 2017), the present study showed an insufficient number in 12% (3/26) of the products and a partially insufficient number

TABLE 1 | Results of the assessment of the presence of labelled species or subspecies in 17 dietary supplements for adults (A1–A17) and of 9 dietary supplements for children (C1–C9) by PCR using DNA isolated from products and from viable consortia (colonies) and by MALDI-TOF MS analysis of individual colonies (three per labelled strain).

Product	Labelled species (as stated on the labels)	Species-specific PCR (DNA from product)	Species-specific PCR (DNA from viable consortia)	Species-specific PCR (DNA from single colonies)	MALDI-TOF MS (single colonies)
1	*L. acidophilus*	+	+	+	+
	L. plantarum	+	+	+	+
	L. rhamnosus	+	+	–	–
	B. breve	–	–	–	–
	L. salivarius	+	–	–	–
	B lactis	+	+	+	+
	L. casei	+	+	+	+ (also *zeae*+)
	L. paracasei	+	–	–	–
	S. thermophilus	+	+	+	+
	B. longum	+	–	–	–
2	*L. casei*	– (*para*+)	– (*para*+)	– (*para*+)	– (*para*+)
	L. acidophilus	+	+	+	+
	L. paracasei	+	+	+	+
	B. lactis	+	+	+	+
	L. salivarius	+	+	+	+
	Lc. lactis	+	+	+	+
	B. lactis	+	+	+	+
	L. plantarum	+	+	+	+
	B. bifidum	+	–	–	–
3	*L. acidophilus*	+	+	+	+
	L. casei	– (*para*+)	– (*para*+)	–	+
	L. plantarum	+	–	–	–
	L. reuteri	+	+	–	–
	L. rhamnosus	+	+	+	+
	B. longum	+	+	+	+
	S. thermophilus	+	+	–	–
4	*B. animalis* subsp. *lactis*	+	+	+	+
	L. paracasei	+	+	+	+
	B. breve	+	+	+	±
	L. gasseri	+	+	+	+
	L. rhamnosus	+	+	+	±
	L. acidophilus	+	+	+	+
	L. plantarum	+	+	–	–
	B. longum subsp. *longum*	+ (*longum*)	–	–	–
	B. bifidum	+	+	+	+
	L. casei	+	+	+	+
	L. reuteri	+	+	–	–
	Lc. lactis	+	+	+	+
	B. longum subsp. *infantis*	+	–	–	–
5	*L. acidophilus*	+	+	+	+
	B. lactis	+	+	+	+
	L. plantarum	+	–	–	–
	B. breve	–	–	–	–
6	*Bac. coagulans*	+	+	+	–

(Continued)

TABLE 1 | Continued

Product	Labelled species (as stated on the labels)	Species-specific PCR (DNA from product)	Species-specific PCR (DNA from viable consortia)	Species-specific PCR (DNA from single colonies)	MALDI-TOF MS (single colonies)
7	*L. acidophilus* DDS-1	+	+	+	+
	B. lactis	+	+	+	+
	L. plantarum	+	–	–	–
	L. rhamnosus	+	+	+	+
	L. casei	+	–	–	– (*zeae*±)
	B. longum	+	–	–	–
	S. thermophilus	+	+	+	+
8	*S. thermophilus*	+	+	+	±
	B. breve	–	–	+	±
	B. longum	+	–	–	–
	B. infantis	–	–	–	–
	L. acidophilus	+	+	+	+
	L. plantarum	+	+	+	+
	L. paracasei	+	+	+	+
	L. delbrueckii subsp. *bulgaricus*	–	–	–	–
9	*L. rhamnosus*	+	+	+	±
10	*L. reuteri*	+	+	+	+
11	*Sacch. boulardii*	– (*cer*+)	– (*cer*+)	– (*cer*+)	– (*cer*+)
	L. acidophilus	+	+	+	+
	B. breve	+	+	+	+
	B. infantis	+	+	–	–
	B. longum	+	+	–	–
12	*B. animalis*	+	+	+	+
	L. acidophilus	+	+	+	+
	L. salivarius	+	+	+	+
	E. faecium	+	+	+	+
	Lc. lactis	+	+	+	+
	L. casei	– (*para*+)	– (*para*+)	– (*para*+)	– (*para*+)
13	*L. acidophilus*	+	+	+	+
	L. paracasei	+	+	+	±
	L. rhamnosus	+	+	–	–
	E. faecium	+	+	–	–
	L. salivarius	+	+	+	+
	L. plantarum	+	+	+	+
	B. bifidum	–	–	–	–
	B. lactis	+	+	+	+
	B. longum	–	–	–	–
14	*L. rhamnosus*	+	+	+	±
15	*L. gasseri*	+	+	+	+
	B. infantis	+	+	+	+
	E. faecium	+	+	+	+
16	*L. acidophilus*	+	+	+	–
	B. animalis subsp. *lactis*	+	+	+	+
17	*L. plantarum*	–	+	+	+
	L. fermentum	–	+	–	–
	L. acidophilus	–	+	+	+
	L. reuteri	–	+	–	–
	L. rhamnosus	–	+	–	–
	B. bifidum	–	–	–	–

(Continued)

TABLE 1 | Continued

Product	Labelled species (as stated on the labels)	Species-specific PCR (DNA from product)	Species-specific PCR (DNA from viable consortia)	Species-specific PCR (DNA from single colonies)	MALDI-TOF MS (single colonies)
18	*Bif. animalis* subsp. *lactis*	+	n.d.	n.d.	+
19	*Bif. animalis* subsp. *lactis*	+	n.d.	n.d.	+
20	*L. reuteri*	+	n.d.	n.d.	+
21	*L. reuteri*	+	n.d.	n.d.	+
22	*L. rhamnosus*	+	n.d.	n.d.	+
23	*L. rhamnosus*	+	n.d.	n.d.	+
24	*L. rhamnosus*	+	n.d.	n.d.	+
	Bif. animalis subsp. *lactis*	+	n.d.	n.d.	+
25	*L. casei*	− (*para*+)	n.d.	n.d.	± (*para*+)(*zeae*±)
	L. rhamnosus	+	n.d.	n.d.	+
	Bif. breve	+	n.d.	n.d.	+
	Bif. longum subsp. *infantis*	+	n.d.	n.d.	+ (*B. longum*)
	L. delbrueckii subsp. *bulgaricus*	+	n.d.	n.d.	−
	S. thermophilus	+	n.d.	n.d.	+
	L. acidophilus	−	n.d.	n.d.	− (*gall*±)(*helv*±)
26	*Lc. Lactis*	+	n.d.	n.d.	+
	Bif. animalis subsp. *lactis*	+	n.d.	n.d	+ (*B. animalis*)
	Bif. bifidum	+	n.d.	n.d.	−

Bac., Bacillus; B., Bifidobacterium; E., Enterococcus; L., Lactobacillus [in accordance with the new taxonomic classification; Zheng et al. (2020); also Lacticaseibacillus, Lactiplantibacillus, Limosilactobacillus, Ligilactobacillus]; Lc., Lactococcus; S. thermophilus, Streptococcus salivarius subsp. thermophiles; + (MALDI-TOF MS), species confirmed with at least one isolate; − (MALDI-TOF MS), species not confirmed; ± (MALDI-TOF MS), score value 1.700–1.999; − (cer+), negative result for Sacch. boulardii, positive result for Sacch. cerevisiae; − (para+), negative result for Lacticaseibacillus casei, positive result for Lacticaseibacillus paracasei; − (zeae+), negative result for Lacticaseibacillus casei, positive result for Lacticaseibacillus zeae; − (gall+)(helv+), negative result for Lactobacillus acidophilus, positive result for Lactobacillus gallinarum and Lactobacillus helveticus; (longum), PCR was specific for B. longum species, not for B. longum subsp. longum; n.d., not determined.

(<0.5 CFU/dose) in 19% of products. The improvements are even more significant compared to the situation in 2010 when 65% (13 out of 20 tested) of dietary supplements or medicinal products did not contain sufficient CFU of the labelled bacteria (Matijašić and Rogelj, 2006; Bogovič Matijašić et al., 2010).

Despite some limitations and drawbacks of plate counting, such as lack of differentiation among species, counting a chain as 1 CFU, time-consuming and labour-intensive procedures, lack of standardised culture media, etc., this approach is still predominantly used in the control of products containing intentionally added viable microorganisms (Vinderola et al., 2019). In addition, advanced formulations or strain characteristics often require optimised protocols, and protocols developed by manufacturers are often not available. Nevertheless, several recent studies conducted in different parts of the world, such as China, United States–Canada, Philippines–Korea, Italy–France, and Poland, have demonstrated through plate counting that products that do not comply with labels are still regularly found in the market (Chen et al., 2017; Di Pierro et al., 2019; Korona-Glowniak et al., 2019; Dioso et al., 2020; Shehata and Newmaster, 2020). Chen et al. (2017) reported that viable cells could not be recovered from two (25%) probiotic supplements from the Chinese market. In four of seven dietary supplements (57%) from the Italian and French markets that claimed a beneficial effect on the urogenital tract, the number of total viable count did not match the label (57%) (Di Pierro et al., 2019). Five out of 10 (50%) tested products available in Poland did not reach the number of viable bacteria claimed by the manufacturer (Korona-Glowniak et al., 2019). One in 10 (10%) probiotic preparations available for consumption by children in the Republic of the Philippines and the Republic of Korea contained a lower number of viable microorganisms than claimed on the label (Dioso et al., 2020). The number of viable microorganisms determined in 72 samples of probiotic products from the United States and Canada was lower than labelled in 5 samples (6.9%), including one product that had no viable cells (Shehata and Newmaster, 2020).

Our observation that the adherence to the number of viable bacteria was not associated with the date of purchase is consistent with the study by Morovic et al. (2016) who found no significant correlation between the estimated number of viable bacteria relative to the declared number of viable bacteria and the time to expiration (R-square was 0.02547, and P-value was 0.1806) (Morovic et al., 2016).

In our previous studies (Bogovič Matijašić et al., 2010; Lorbeg and Matijašić, 2017), we also pointed out the absence of certain species among the viable microbiota from the products determined by PCR performed on DNA retrieved from individual colonies and from the mixture of colonies grown on dedicated agar media

TABLE 2 | Overview of the validation of MALDI-TOF MS (Biotyper) identification of 292 isolates (three isolates per labelled strain) from 17 dietary supplements (1–17) by PCR.

Result of identification by MALDI-TOF MS (Biotyper)	Score value ≥ 2.000	Score value 1.700–1.999	Species-specific PCR
B. animalis	45	12	1 n.d., all others *B. animalis* subsp. *lactis*
B. breve	8	6	All *B. breve*
B. longum	1	2	All *B. longum*
B. bifidum	1	/	All *B. bifidum*
B. infantis	3	/	All *B. infantis*
E. faecium	4	5	All *E. faecium*
L. acidophilus	54	5	1 n.d., all others *L. acidophilus*
L. casei	3 (one of them *L. casei* or *L. zeae*)	1	Two (one score value 2.203, the other score value 2.15 or 2.168, *L. casei* or *L. zeae*) *L. casei*; one (score value 2.042) *L. rhamnosus*; one (score value 1.847) *L. rhamnosus*
L. gasseri	6	/	1 n.d., all others *L. gasseri*
L. paracasei	26	2	All *L. paracasei*
L. plantarum	16	17	All *L. plantarum*
L. reuteri	6	/	5 *L. reuteri,* 1 n.d.
L. rhamnosus	27	8	All *L. rhamnosus*
L. salivarius	5	/	All *L. salivarius*
L. zeae (*L. rhamnosus* and *L. casei* on the labels)		12	12 *L. rhamnosus*
Lc. Lactis	9	/	All *Lc. Lactis*
Sacch. cerevisiae	1	5	All *Sacch. Cerevisiae*
S. thermophilus	3	5	All *S. thermophilus*
Not reliable identification (score values < 1.700)	6		3 *Bac. coagulans* 1 n.d. 1 *L. plantarum* 1 *L. rhamnosus*
No peaks detected	11		6 *L. acidophilus* 3 *B. lactis* 2 *E. faecium*

n.d., not determined.

(DNA from viable consortia). In contrast to previous studies, this study also determined the presence of labelled species by analysing individual colonies using MALDI-TOF MS. In addition, species-specific PCR of the same colonies and of the total DNA from product or viable consortia was performed.

The incorrect naming of *B. longum* subsp. *infantis* as *B. infantis* observed in this study still appears to be very common and has been reported previously. The two subspecies *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* are difficult to differentiate taxonomically, thus misidentifications and mislabelling are common (Lewis et al., 2016; Morovic et al., 2016; Patro et al., 2016; Shehata and Newmaster, 2020). In this study, *B. longum* has been examined by PCR, with the primers yielding positive results for both subspecies (Malinen et al., 2003), while *B. longum* subsp. *infantis* (products 4, 11, 15, 8) has been confirmed by PCR specific for this subspecies (Matsuki et al., 1999). However, it appears that both subspecies could also be distinguished by MALDI-TOF MS, considering the best matches. The MALDI-TOF MS results showed that all strains labelled as *B. longum* indeed had high similarity in their spectra to the reference strains of *B. longum* subsp. *longum* in the Biotyper database, indicating that the strains labelled as *B. longum* all belonged to the subspecies *longum* and that the MALDI-TOF MS analysis could distinguish between these two subspecies. In general, PCR identification still offers advantage over MALDI-TOF MS analysis in the ability of accurate identification down to

subspecies level, which was also evident in this study, i.e., in the case of *Streptococcus salivarius* subsp. *thermophilus*, *B. animalis* subsp. *lactis*, *B. longum* subsp. *longum*, and *B. longum* subsp. *infantis*.

Another problem observed in this study concerned the representatives of *L. casei* and *L. paracasei* in the products, as some colonies were identified as *L. paracasei* or *L. zeae*, although the latter species was not indicated on the labels. Misidentification of *L. casei* and related species has been reported previously (Morovic et al., 2016; Patro et al., 2016). A recent study showed that multiplex PCR and MALDI-TOF MS proved to be the most useful methods for species-level identification of bacteria belonging to the *L. casei* group (Jarocki et al., 2020).

The species *L. zeae*, which was one of the frequent matches in the analyses of colonies by MALDI-TOF MS, was not found among the bacteria listed on the labels of the products examined in this study. It is well known that *L. casei*, *L. paracasei*, and *L. rhamnosus*, species collectively referred to as the *Lactobacillus casei* group, are difficult to distinguish from each other and have been reclassified several times in the past (Hill et al., 2018). In 2008, the type strain of *L. casei* was confirmed as the original strain ATCC 393, and strain ATCC 334 was designated as *L. paracasei*. The species name *L. zeae* was reclassified as *L. casei* in 2008 (Tindall and Syste, 2008) and reassigned to distinct species status in 2020 (Liu and Gu, 2020). Therefore, the confusion around the species *L. zeae*, which is found in the Biotyper database but absent from the labels, is not surprising. Moreover, there are only two *L. zeae* strains and one *L. casei* strain in the Biotyper 1.3.66 database compared to 13 *L. rhamnosus* strains and 15 *L. paracasei* strains. Another fact that complicates the routine identification of *L. zeae* and closely related species is that PCR analyses based on 16S rDNA, as the most common taxonomic marker, are not always reliable due to the high sequence similarities between the three species (*L. casei*, *L. rhamnosus*, and *L. zeae*). Since many strains within the *L. casei* group are commonly used as starter cultures and probiotics, the possibility of improper labelling should be considered. In this study, colonies that showed similar MALDI-TOF MS spectra to *L. zeae*, but with lower score values (1.700–1.999), were identified as *L. rhamnosus* by using species-specific primers designed not to amplify DNA from *L. zeae* and *L. casei* (Walter et al., 2000) and also using primers from Bottari et al. (2017) that can distinguish between *L. casei*, *L. rhamnosus*, and *L. paracasei*. In addition, the affiliation to this species was confirmed also by 16S rDNA sequencing.

At least one strain, *L. casei* W56, in two products examined in this study (2, 12) was found to be mislabelled since the species *L. casei* in these products could not be confirmed by either species-specific PCR or MALDI-TOF MS, while the species *L. paracasei* was detected in both products. This is in line with the fact that the strain W56 is already available in GenBank (accession number HE970765.1) and indeed belongs to the species *L. paracasei*. Similar observations were reported by Kim et al. (2020) who examined 29 products containing members of the *L. casei* group using newly designed PCR primers based on the comparative genetics of whole-genome sequencing (Kim et al., 2020). They found that 23 products purchased in markets around the world (Korea, Canada, and the United States of America) were consistent with label claims, while the remaining products contained species different from those indicated in the label claims. They also confirmed that the 16S rRNA gene sequence was inadequate for distinguishing between species in the *L. casei* group. The misidentification of a commercial *L. casei* strain (DG, CNCM I-1572) found in an Italian pharmaceutical product was also reported by Blandino et al. (2016), who successfully used multiplex PCR based on tuf gene amplification (Blandino et al., 2016).

The absence of certain strains was observed in most of the products for general use (11 out of 17), all of which contained multiple strains, and only in one product for children (25). This problem has been previously reported in several studies based on PCR assessment, high-throughput next-generation sequencing, or denaturing gradient gel electrophoresis analysis (Morovic et al., 2016; Patro et al., 2016; Chen et al., 2017; Shehata and Newmaster, 2020). For 10% of the labelled strains (11 of 112 examined), presence was confirmed by species-specific PCR with viable consortia, but not by MALDI-TOF MS analysis of individual colonies, with three colonies per labelled species included in the analysis. This could be improved by analysing more colonies using MALDI-TOF MS analysis.

The absence of positive results with viable consortia or colonies, but positive results with DNA by PCR using DNA isolated directly from the products observed in 10% (11/112) of the labelled strains, indicates the presence of non-culturable bacteria in the products. However, the labelled bacteria are expected to be in a viable and culturable state since the amount of bacteria is expressed in CFU/g or CFU/unit of product. This is the main reason why quantitative PCR or other molecular methods cannot easily replace cultivation-based methods (Kramer et al., 2009).

Identification of LAB and bifidobacteria in probiotic products and starter cultures using MALDI-TOF MS has not been as widely used in the past, mainly because of the high cost of the equipment. Moreover, the identification of LAB by MALDI-TOF MS was not very satisfactory at the beginning because of the lack of high-quality databases containing the spectra of several reference strains of this group of bacteria. Nowadays, commercial databases such as the MALDI Biotyper (Bruker Daltonics), the SARAMIS^{TM} (BioMerieux), and the Andromas (Andromas SAS), as well as other open-source databases that are regularly updated, are available, which allows better discrimination between closely related species (Čanžek Majhenič et al., 2018). In the present study, the Biotyper database allowed the satisfactory identification of most species present in dietary supplements, with some exceptions such as *L. zeae* and *L. casei*, as well as those that could not be accurately distinguished by spectra comparisons. *L. paracasei* and *L. rhamnosus*, on the other hand, could be distinguished from *L. casei*, although the species are closely related and

difficult to distinguish based on 16S rDNA sequences. For example, Huang and Huang (2018) validated the accuracy of MALDI-TOF MS in identifying three related species belonging to the genus *Lacticaseibacillus*, i.e., *L. casei*, *L. paracasei*, and *L. rhamnosus*, and concluded that the in-house database containing the spectra of several reference strains grown under optimised culture conditions, in combination with ClinProTools, can improve identification by MALDI-TOF MS and enable the identification of subspecies (*L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*) (Huang and Huang, 2018). They were able to detect *L. paracasei* in two yoghurt drinks, *L. casei* in two probiotic preparations, and *L. rhamnosus* in three probiotic preparations. *L. zeae*, on the other hand, was not included in their study.

An interesting observation was that, even in the products containing only one strain, the probability of correct MALDI-TOF MS identification of *B. longum* varied among three colonies, i.e., one or two were identified to species level with score values indicating probable species identification, while another one or two showed only probable genus identification (score value between 1.700 and 1.999). The reason for this discrepancy can be found in the fact that the quality of the spectrum may be influenced by several factors, including the amount of bacteria spotted on the target and, in the case of anaerobic bacteria, the exposure of the bacterial cells to oxygen. Veloo et al. (2014) reported that *B. longum* required on-target extraction with 70% formic acid to obtain reliable species identification and that the quality of the spectrum was influenced by the amount of bacteria, the homogeneity of the smear, and the experience of the investigator (Veloo et al., 2014). As a too-low amount of bacteria can result in the absence of a peak, a too-high amount can result in an atypical spectrum due to saturation and the predominance of prominent peaks.

In our study, 30% of isolates were identified with score values ranging from 1.700 to 1.999, values considered too low for accurate species identification using the manufacturer's cutoff values. Nevertheless, species affiliation was confirmed in almost all cases by 16S rDNA sequencing and/or PCR, suggesting that the general criteria for the interpretation of MALDI-TOF MS results may be too stringent in the case of LAB and bifidobacteria. In a study by Gautam et al. (2017), MALDI-TOF MS-based identification of non-fermenting Gram-negative bacilli at the species level was successful when the cutoff value was decreased. A 4% increase in species identification rate was achieved when the cutoff of $\geq$1.9 was taken instead of $\geq$2.

Several other studies have shown that score values and identification accuracy can be significantly improved by using in-house-built reference database containing several MALDI-TOF MS spectra of reference strains and own isolates of interest, depending on the sample composition and scope of the analyses, and by using a more advanced analysis of the spectra (Singhal et al., 2015; Nacef et al., 2017; Gantzias et al., 2020; Jarocki et al., 2020). However, it should be kept in mind that, in this study, we intentionally used only the commercially available Biotyper library to demonstrate whether the MALDI-TOF MS approach is widely applicable in high-throughput routine laboratories for the control of probiotic products, without extensive expertise in this methodology.

CONCLUSION

In conclusion, this study showed a good quality of most products (88%) in terms of total CFU/g. Considering the results of our study, we can also conclude that MALDI-TOF MS could not accurately distinguish between *L. zeae* and *L. casei*, while *L. paracasei* and *L. rhamnosus* could be correctly identified. Some isolates that were identified as *L. zeae* by MALDI-TOF MS but had lower score values (1.700–1.999), indicating unreliable species identification, were most likely *L. rhamnosus* as indicated by species-specific PCR analysis. Moreover, all these isolates were obtained from the product containing *L. rhamnosus* on the labels. The inability of identification of *L. acidophilus* La-5 and *B. coagulans* with MALDI-TOF MS could be explained by the low number of reference spectra (three and two, respectively) for these two species in the Biotyper database or by the low-quality spectra obtained. Otherwise, e.g., 15 fingerprints of *L. paracasei*, 13 of *L. rhamnosus*, and 9 of *L. gasseri* are available for comparison.

PCR is still considered the more reliable of MALDI-TOF MS, especially for subspecies or strain-level identification, but compared to MALDI-TOF MS, PCR analysis is more labour intensive and requires more trained personnel. MALDI-TOF MS is easier to use, faster, cheaper, and allows testing of a large number of colonies if needed (in case of negative results for a particular species). Nevertheless, our results confirm that MALDI-TOF MS can be effectively used in the quality control of probiotic products considering some limitations, such as unsatisfactory identification of certain closely related species. In such cases, PCR confirmation can help. Furthermore, generation of a dedicated in-house library containing MALDI-TOF MS spectra of various reference strains and isolates of interest may further improve the accuracy of identification at the species and sub-species level.

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

PM, BB, and MK designed the experiments. PM and MG performed the experiments and collected the data. PM, PT, and BB conducted all analyses. PM, BB, PT, and MK prepared

the manuscript. All the authors have contributed to, seen, and approved the manuscript.

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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.2021.700138/full#supplementary-material

Supplementary Table 1 | Species-specific PCR primers and conditions.

Supplementary Table 2 | Compliance of 26 dietary supplements with the labelled number of colony-forming units.

Supplementary Table 3 | Results of the identification of isolates from nine dietary supplements for children (C1–A9) by MALDI-TOF MS analysis and by species-specific PCR (three isolates per labelled strain).

Supplementary Table 4 | Overview of the MALDI-TOF MS (Biotyper) identification of 70 isolates (three isolates per labelled strain) from 9 dietary supplements for children (products 18–26).

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REVIEW
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Spotlight on the Compositional Quality of Probiotic Formulations Marketed Worldwide

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On the worldwide market, a great number of probiotic formulations are available to consumers as drugs, dietary supplements, and functional foods. For exerting their beneficial effects on host health, these preparations should contain a sufficient amount of the indicated living microbes and be pathogen-free to be safe. Therefore, the contained microbial species and their amount until product expiry are required to be accurately reported on the labels. While commercial formulations licensed as drugs are subjected to rigorous quality controls, less stringent regulations are generally applied to preparations categorized as dietary supplements and functional foods. Many reports indicated that the content of several probiotic formulations does not always correspond to the label claims in terms of microbial identification, number of living organisms, and purity, highlighting the requirement for more stringent quality controls by manufacturers. The main focus of this review is to provide an in-depth overview of the microbiological quality of probiotic formulations commercialized worldwide. Many incongruences in the compositional quality of some probiotic formulations available on the worldwide market were highlighted. Even if manufacturers carry at least some of the responsibility for these inconsistencies, studies that analyze probiotic products should be conducted following recommended and up-to-date methodologies.

Keywords: probiotics, compositional quality, viable cells, microbial species, purity, label claims

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INTRODUCTION

Although the term probiotic made its first appearance in 1965, the first universally accepted definition was introduced by the Joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) in 2001 (FAO/WHO, 2001). For grammatical reasons, this definition was revisited in 2014 and definitively turned into "live microorganisms that, when administered in adequate amounts, confer health benefits on the host" (Hill et al., 2014).

A great number of studies highlight the ability of orally administered probiotics to improve the gut-barrier function, modulate the gut microbiota, enhance host immune response, and exert antimicrobial activities (Bermudez-Brito et al., 2012; Bron et al., 2017; Hu et al., 2017; Maldonado Galdeano et al., 2019). In addition, probiotics can be able to synthetize vitamins, food-degrading enzymes, and molecules contributing to cellular metabolism, thus ameliorating host health (LeBlanc et al., 2017; Oak and Jha, 2019).

Probiotics support physiological bodily functions and may reduce risk or shorten the duration or severity of many diseases. In particular, probiotic drugs are frequently used to prevent or help in the treatment of many gastrointestinal diseases due to infections, microbiome dysbiosis, and gut barrier perturbation. These include traveler, antibiotic-associated, and acute-infectious diarrhea, ulcerative colitis, necrotizing enterocolitis, inflammatory bowel diseases, irritable bowel syndrome, and *Clostridium difficile* and *Helicobacter pylori* infections (Wilkins and Sequoia, 2017). In the last decades, the use of probiotics has been extended to a variety of other disorders, including lactose intolerance, respiratory and urinary infections, asthma, atopic dermatitis, osteoporosis, allergy, metabolic syndromes, as well as liver, neurological, cardiovascular, and autoimmune diseases (Stavropoulou and Bezirtzoglou, 2020).

Microbes used as probiotics must be safe for consumption (FAO/WHO, 2002). To this regard, probiotic microbes intended for human use may have a "generally recognized as safe" (GRAS) notification for specific intended use to the US Food and Drug Administration (FDA, 2019) or the "qualified presumption of safety" (QPS) status (European Food Safety Authority: EFSA, 2007, 2018; Mattia and Merker, 2008). In addition, the antibiotic resistance profile of probiotics should be determined to highlight the presence of acquired resistance genes that can potentially be transmitted to pathogens (FAO/WHO, 2002; Cohen, 2018).

Several lactic acid bacteria (LAB) belonging to the *Lacticaseibacillus, Lactiplantibacillus, Lactobacillus*, *Lentilactobacillus, Levilactobacillus*, *Ligilactobacillus, Limosilactobacillus, Bifidobacterium*, and *Streptococcus* genera are traditionally used as probiotics, constituting the vast majority of commercial products available in the worldwide market (Williams, 2010; Hanchi et al., 2018; Zheng et al., 2020). Spore-forming bacteria of the genus *Bacillus* have progressively taken on, since they can be administered as spores that are extremely resistant to the harsh gastrointestinal conditions (Cutting, 2011; Elshaghabee et al., 2017; Jeżewska-Frąckowiak et al., 2018; Lee et al., 2019). Among yeast, the species *Saccharomyces cerevisiae* var. *boulardii* exhibited a variety of beneficial properties, being adopted as probiotic microbe for several decades (Sen and Mansell, 2020). Several other microorganisms isolated from the human gut (e.g., *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*) have been proposed as next generation probiotic candidates to face up specific diseases (Chang et al., 2019; Zhai et al., 2019).

CATEGORIZATION OF PROBIOTICS AND NOTES ON REGULATION

In different countries, both regulatory aspects that govern probiotics and probiotic categories profoundly differ (for detailed regulatory aspects see Arora and Baldi, 2015; Kumar et al., 2015; de Simone, 2019; Domínguez Díaz et al., 2020; Koirala and Anal, 2021). Probiotics are generally classified as drugs (i.e., medicinal products and pharmaceuticals), dietary supplements (also referred as food supplements), and functional foods depending on the intended use (Halsted, 2003; Sreeja and Prajapati, 2013; de Simone, 2019; Koirala and Anal, 2021). Probiotic drugs are used for the prevention, treatment, and mitigation of human diseases and are subjected to the stringent regulations applied to other drugs, thus requiring approval before marketing and continuous pre- and post-marketing safety and quality controls (Venugopalan et al., 2010; Sreeja and Prajapati, 2013; Kolaček et al., 2017).

Dietary supplements can be administered as tablets, capsules, liquid suspensions, or powders and are intended to complement diet by ensuring the intake of specific dietary components (Taylor, 2004; Domínguez Díaz et al., 2020). In Europe, probiotic food supplements fall under the Food Products Directive and Regulation and each health claim need to be accurately scrutinized by the EFSA before being authorized (de Simone, 2019; Fusco et al., 2021). Good Manufacturing Practice that manufacturers of dietary supplements should follow have been developed (Food Supplements Europe, 2014). In the US, dietary supplements are regulated by the FDA under the Dietary Supplement Health and Education Act (DSHEA) and the Federal Trade Commission (FTC) and need to comply the Good Manufacturing Practice guidelines (Brown, 2017; de Simone, 2019; Fusco et al., 2021).

Although a statutory definition of functional foods does not exist in several countries, these products are intended to provide health benefits being consumed as part of the usual diet (Gul et al., 2016; Domínguez Díaz et al., 2020). This category meets the general laws applicable to foods in many countries (Kumar et al., 2015; Domínguez Díaz et al., 2020; Koirala and Anal, 2021).

PROBIOTIC LABELING

Probiotic formulations possess specific label requirements that take into account the current laws and regulatory specifications of each country. FAO/WHO reported a list of information that manufacturers are recommended to state on the product label. In particular, labels should clearly indicate the minimum number of each strain until the expiry, the identification of each microorganism contained in the formulation based on the current nomenclature, and the claimed beneficial effects. According to the FAO/WHO guidelines, labels should also contain the suggested serving size to obtain health benefits, the appropriate storage conditions, and corporate contact details (FAO/WHO, 2002).

In 2017, the International Probiotics Association, in partnership with the Council for Responsible Nutrition, added new details to the FAO/WHO indications on the basis of the US regulation and proposed new guidelines for probiotic labeling that worldwide manufacturers were recommended to comply (Council for Responsible Nutrition and International Probiotics Association, 2017). These guidelines were aimed at improving transparency, consistency, and consumer understanding, thus promoting comparisons between different probiotic formulations and improved consumer awareness. Product labels should indicate the quantitative amount of alive microorganisms expressed as colony forming units (CFUs) and the expiration date. For formulations containing multiple species and/or

strains, information on the total amount of microorganisms was recommended and, when technically feasible, the amount of each species as well.

COMPOSITIONAL QUALITY

Although publications on probiotics rapidly expanded in the last years, most of the studies have been focused on the beneficial properties exhibited by probiotic microbes. A relatively low number of studies analyzed the real-time composition of probiotic formulations that consumers find on the market.

Some reports denouncing poor quality of commercial probiotic products already populated the scientific literature before general guidelines were developed (Gilliland and Speck, 1977; Hamilton-Miller et al., 1996; Yaeshima et al., 1996; Canganella et al., 1997; Hamilton-Miller and Gibson, 1999; Hamilton-Miller et al., 1999; Schillinger, 1999; Hoa et al., 2000). With the introduction of guidelines for probiotics in 2002 (FAO/WHO, 2002), some investigations were conducted to evaluate the compliance between label claims and effective microbial composition, amount of viable cells, and absence of contaminant microorganisms of probiotic formulations available on the market.

In this review, we took into consideration only studies that were published after the introduction of the FAO/WHO guidelines. The probiotic formulations analyzed in different studies were divided into the three category drugs, dietary supplements, and functional foods (**Tables 1–3**). For simplicity, probiotic formulations indicated as foods with specific medical purposes (FSMP) were included in the category dietary supplements. We decided to incorporate in the tables only products for which name, category, country of origin, and possibly, manufacturer were known. For formulations produced by unmentioned manufacturers, product name and country of origin were used to conduct a careful research for retrieving the missing data. In addition, we did not include commercial products for which the concordance with label claims of amount of living cells, contained species, and purity were not clearly stated by the Authors. For completeness, studies not clearly stating product names, manufacturers, categorization, or origin (further referred to as unidentified probiotics) will be separately discussed in the main text.

Microbial Composition

The identity of microbes (i.e., at genus, species, and strain level) included in commercial probiotic formulations should be stated by the manufacturer on the product label and follow the current accepted nomenclature (FAO/WHO, 2002; Council for Responsible Nutrition and International Probiotics Association, 2017). To this regard, Weese analyzed the labels of 21 dietary supplements intended for human use to verify label's accuracy (Weese, 2003). The author showed that nine products reported adequate label information in terms of microbial composition with only two indicating microbes at the strain level. On the other products, microorganisms were indicated with a name not concordant with the actual nomenclature or misspelled (Weese, 2003). Similarly, label analysis of the top 10 probiotic formulations sold on the Indian market revealed that seven did not specify the contained species, but only gave an unsuitable explanation (i.e., Lactic Acid *Bacillus*; Ghattargi et al., 2018).

In this study, we also evidenced that the labels of some formulations, particularly functional foods, did not comply general guidelines for probiotic labeling, since not indicating the contained microbes or only reporting a general and inaccurate description (e.g., "LAB," "probiotics," "lactic ferment," "lactic acid bacillus," "live probiotic AB cultures," "active bifidus," "special live cultures," "viable LAB," "bifidus essensis," "life yeast") (Fasoli et al., 2003; Temmerman et al., 2003a,b; Elliot and Teversham, 2004; Masco et al., 2005; Theunissen et al., 2005; Lin et al., 2006; Perea Vélez et al., 2007; Angelakis et al., 2011; Chen et al., 2014; Kesavelu et al., 2020). In addition, we found that some preparations only indicated the contained microbes at the genus level, thus resulting in disagreement with the FAO/WHO guidelines (Fasoli et al., 2003; Temmerman et al., 2003a,b; Masco et al., 2005; Theunissen et al., 2005; Lin et al., 2006; Perea Vélez et al., 2007; Chen et al., 2014; Yonkova Marinova et al., 2019).

Regarding probiotic drugs, the microbial composition of 31 products available in Europe (Italy, Belgium, and Poland), India, and Pakistan is reported in **Table 1** (column 4). Only four products among these 31 (Codex, Enterogermina, Lacidofil, Lakcid) were analyzed by independent studies, with three of them showing concordant results. In fact, *Lactobacillus acidophilus* declared to be contained in Lacidofil was detected only in one study by species-specific PCR (Korona-Glowniak et al., 2019) but not in the other, which used biochemical methods and matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Zawistowska-Rojek et al., 2016). Overall, our analysis highlighted that 17 formulations (56.67%) contained the species stated on the labels. *Bacillus coagulans* in two products (SPORLAC, Vizylac) was labeled with an obsolete nomenclature (i.e., *Lactobacillus sporogenes*). Thirteen formulations (43.33%) did not comply the label claims. In particular, six formulations (Darolac, Ecogro, Entromax, Pre Pro Kid L, Regutol, Tufpro) contained more species than those declared, while three (Combiflora, Reflora Z, Remune Al) lacked one or more species. In addition, Benegut, Bifilac, Pre Pro Kid, and Vibact possessed different species than those labeled (**Table 1**, column 4).

Regarding dietary supplements, the microbial composition of a total of 106 products marketed around the world is reported in **Table 2** (column 4). Byotik and Yovis were analyzed in two studies by using different methods, but the recovered species were found to be concordant with the label claims only in one study each. The discordant results obtained for Yovis could depend on the different years in which the studies were performed (Fasoli et al., 2003; Vecchione et al., 2018). In fact, it can not be excluded the product underwent changes in 15 years and the manufacturer's controls had increased over time. Aciforce, Asecurin, Bactisubtil, Dicoflor (Dicopharm), Proflora, and VSL#3 were also analyzed by independent studies with concordant results. Globally, among 104 products, 56 (53.85%) contained the same species declared on the labels, with the exception of *Lactobacillus crispatus* in Probiosan that was indicated with an

TABLE 1 | Compliance with the label claims of probiotic drugs marketed worldwide: focus on the microbial composition, amount of living cells, and presence of contaminant microorganisms.

Product	Manufacturer	Country	Compliance (composition)	Compliance (amount)	Contaminants	Reference
Benegut	Abbott	India	No	No	Yes	Kesavelu et al., 2020
Bifilac	Tablets India Ltd	India	No	Yes	Yes	Kesavelu et al., 2020
Bifilac GG	Tablets India Ltd	India	Yes	Yes	No	Kesavelu et al., 2020
Biogermin	Union Health S.r.l.	Italy	Yes	Yes	No	Celandroni et al., 2019
Codex	Zambon	Italy	Yes	Yes	No	De Vecchi et al., 2008
			Yes	Yes	No	Vecchione et al., 2018
Combiflora	Medopharm	India	No	No	No	Kesavelu et al., 2020
Cyfolac	Karnataka Antib & Pharm Ltd	India	Yes	Yes	No	Kesavelu et al., 2020
Darolac	Aristo Pharmaceuticals Pvt Ltd	India	No	No	No	Kesavelu et al., 2020
Ecogro	Akum Drugs & Pharma	India	No	Yes	Yes	Patrone et al., 2016
Econorm	Dr. Reddy's Laboratories Ltd	India	Yes	N.D.	No	Kesavelu et al., 2020
Entero Plus	Glaxo India Ltd	India	Yes	Yes	No	Kesavelu et al., 2020
Enterogermina	Sanofi	Italy	Yes	Yes	No	De Vecchi et al., 2008
		India	Yes	Yes	No	Vecchione et al., 2018
			Yes	Yes	No	Celandroni et al., 2019
			Yes	Yes	No	Patrone et al., 2016
			Yes	Yes	No	Kesavelu et al., 2020
Enterol capsules	Biodiphar	Belgium	Yes	Yes	No	Vanhee et al., 2010
Enterol sachets	Biodiphar	Belgium	Yes	Yes	No	Vanhee et al., 2010
Entromax	Mankind Pharma	India	No	Yes	No	Patrone et al., 2016
GNorm	Nouveau Medicament	India	Yes	N.D.	No	Kesavelu et al., 2020
GutPro	Riata Life Sciences Pvt Ltd	India	Yes	No	No	Kesavelu et al., 2020
Infloran	BERNA	Italy	Yes	No	No	Fasoli et al., 2003
Lacidofil	Merck	Poland	No	Yes	No	Zawistowska-Rojek et al., 2016
			Yes	Yes	No	Korona-Glowniak et al., 2019
Lakcid	Biomed	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
			Yes	Yes	No	Korona-Glowniak et al., 2019
Ospor	Matrix Pharma	Pakistan	Yes	No	No	Patrone et al., 2016
Pre Pro Kid	Fourrts India Laboratories	India	No	No	No	Kesavelu et al., 2020
Pre Pro Kid L	Fourrts India Laboratories	India	No	No	Yes	Kesavelu et al., 2020
Reflora Z	Sundyota Numandis	India	No	No	No	Kesavelu et al., 2020
Regutol	Alembic Pharmaceuticals Ltd	India	No	Yes	Yes	Kesavelu et al., 2020
Remune Al	Sundyota Numandis	India	No	No	No	Kesavelu et al., 2020
SPORLAC	Sanzyme Ltd	India	Yes*	No	No	Kesavelu et al., 2020
Super Flora GG	Sundyota Numandis	India	Yes	No	No	Kesavelu et al., 2020
Tufpro	Virchow Biotech Pvt. Ltd.	India	No	No	Yes	Patrone et al., 2016
ViBact	USV	India	No	Yes	Yes	Kesavelu et al., 2020
Vizylac	Torrent Pharmaceuticals Ltd	India	Yes*	No	No	Kesavelu et al., 2020

N.D., not definable.
**Incorrect nomenclature.*

obsolete nomenclature. The other 48 products (46.15%) were not compliant for the microbial composition, since lacking one or more species or containing more or different species than those declared on the labels (**Table 2**, column 4).

As regards products categorized as functional foods, a total of 37 formulations is reported in **Table 3** (column 4). For three products (Dannon, Imunele, Pomogayka), the correspondence between contained species and label information could not be established, since the identification of microbes isolated from these products was only performed at the genus level (Astashkina et al., 2014). Actimel, Activia, and Yakult were analyzed by independent studies, all showing concordant results with label information. Globally, the microbial composition was concordant with the label claims for 15 functional foods (44.12%), with 5 of them reporting an obsolete nomenclature (Actimel, Actimel Orange, Activia, Lactus Nature, Yakult). In the

TABLE 2 | Compliance with the label claims of probiotic dietary supplements marketed worldwide: focus on the microbial composition, amount of living cells, and presence of contaminant microorganisms.

Product	Manufacturer	Country	Compliance (composition)	Compliance (amount)	Contaminants	Reference
40 + Acidophilus	Solgar Laboratories	The Netherlands	No	N.D.	No	Temmerman et al., 2003a
4lacti	Norfarm	Poland	No	No	No	Korona-Glowniak et al., 2019
Acidolac#	Polpharma	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Acidophi Kidz	Nature's Plus	United States	No	No	No	Drago et al., 2010
Acidophilus Pearls	Enzymatic	United States	Yes	Yes	No	Drago et al., 2010
Acidophilus Plus	Quest Vitamins	United Kingdom	No	N.D.	No	Temmerman et al., 2003a
Acidophilus Probiotic	Nature's bounty	United States	No	No	Yes	Drago et al., 2010
Aciforce	Biohorma	Netherlands	No	N.D.	No	Temmerman et al., 2003a
			No	N.D.	No	Temmerman et al., 2003b
Align Digestive Care	Procter & Gamble	United States	Yes	No	No	Drago et al., 2010
Antedia	Will-Pharma	Belgium	Yes	Yes	No	Vanhee et al., 2010
Asecurin	Aflofarm	Poland	No	No	No	Zawistowska-Rojek et al., 2016
			No	No	No	Korona-Glowniak et al., 2019
Bacilac	THT	Belgium	No	N.D.	No	Temmerman et al., 2003b
Bactisubtil	Synthelabo	Belgium	Yes	N.D.	No	Temmerman et al., 2003a
	Belgium		Yes	N.D.	No	Temmerman et al., 2003b
Beneflora	ORTIS	Belgium	No	N.D.	No	Temmerman et al., 2003a
Bifidus complex	Biover	Belgium	No	N.D.	Yes	Temmerman et al., 2003a
Bififlor	Eko-Bio	Netherlands	No	N.D.	No	Temmerman et al., 2003b
Bifilact	Fidia Farmaceutici	Italy	No	No	No	Fasoli et al., 2003
BioGaia#	Ewopharma	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
BioGaia Protectis	Ewopharma	Poland	Yes	Yes	No	Korona-Glowniak et al., 2019
Biolactine family	Sella	Italy	No	No	Yes	Celandroni et al., 2019
BioPro Reuteri straws	Unknown	South Africa	Yes	Yes	No	Elliot and Teversham, 2004
BioPro Reuteri tablets	Unknown	South Africa	Yes	Yes	No	Elliot and Teversham, 2004
Biopron 9	Valosun	Poland	No	No	No	Korona-Glowniak et al., 2019
Biotyk	Lekam	Poland	No	No	No	Zawistowska-Rojek et al., 2016
			Yes	No	No	Korona-Glowniak et al., 2019
Boulardi-Sanifort	Sanifort Pharma	Belgium	Yes	Yes	No	Vanhee et al., 2010
Coloflor#	Oleofarm	Poland	Yes	No	No	Zawistowska-Rojek et al., 2016
Colon C	A-Z Medica	Poland	No	Yes	No	Zawistowska-Rojek et al., 2016
Combiforte capsules	Bioflora CC	South Africa	No	Yes	No	Elliot and Teversham, 2004
Culturelle	CAG Functional Foods	United Kingdom	Yes	N.D.	No	Temmerman et al., 2003a
Culturelle	Amerifit	United States	Yes	Yes	No	Drago et al., 2010
Culturelle sachets	Unknown	South Africa	No	Yes	Yes	Elliot and Teversham, 2004
Culturelle tablets	Unknown	South Africa	No	No	No	Elliot and Teversham, 2004
Daily Fiber and Prob.	Walgreens	United States	No	No	Yes	Drago et al., 2010
Diaclo	Sandoz	Germany	Yes	Yes	No	Vanhee et al., 2010
Dicoflor	Dicopharm	Italy	Yes	No	No	De Vecchi et al., 2008
			Yes	Yes	No	Vecchione et al., 2018
Dicoflor#	Vitis Pharma	Poland	Yes	Yes	No	Korona-Glowniak et al., 2019
Dicoflor 30 Kid#	Vitis Pharma	Poland	Yes	Batch dep.	No	Zawistowska-Rojek et al., 2016
Dicoflor 60#	Vitis Pharma	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Enterelle	Bromatech	Italy	Yes	Yes	No	Vecchione et al., 2018
Enteriphar	Teva	Israel	Yes	No	No	Vanhee et al., 2010
Enterofermenti family	SB Pharma C	Italy	Yes	No	No	Celandroni et al., 2019
Enterolactis	SOFAR	Italy	Yes	No	No	De Vecchi et al., 2008
Enterolactis Plus	SOFAR	Italy	Yes	Yes	No	Vecchione et al., 2018
Enterolife	Paladin Pharma	Italy	Yes	No	No	Celandroni et al., 2019
Ercéflora Supra	Sanofi Aventis	France	Yes	N.D.	No	Vanhee et al., 2010
Ferzym Plus	Specchiasol	Italy	No	No	Yes	Celandroni et al., 2019
Floraviva	Tredi Farmaceutici	Italy	No	No	No	Fasoli et al., 2003

(Continued)

TABLE 2 | Continued

Product	Manufacturer	Country	Compliance (composition)	Compliance (amount)	Contaminants	Reference
GiSol	Metagenics	Belgium	Yes	Yes	No	Vanhee et al., 2010
Ido-Form Kid	Ferrosan	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Iladian	Aflofarm	Poland	No	No	No	Zawistowska-Rojek et al., 2016
Imutis	Trenker Pharmaceutical	Belgium	Yes	Yes	No	Vanhee et al., 2010
Infantiforte capsules	Unknown	South Africa	Yes	Yes	No	Elliot and Teversham, 2004
LaciBios Femina	Asa	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Lacidar	Tantus	Poland	No	No	No	Zawistowska-Rojek et al., 2016
Lacidozone	Ozone Laboratories	Poland	No	No	No	Zawistowska-Rojek et al., 2016
Lacium Zdrovit	Zdrovit	Poland	Yes	No	No	Zawistowska-Rojek et al., 2016
Lactèol	Menarini Benelux	Belgium	No	N.D.	No	Temmerman et al., 2003a
Lacteol Forte capsules	Unknown	South Africa	No	No	No	Elliot and Teversham, 2004
Lacteol Forte sachets	Unknown	South Africa	No	No	No	Elliot and Teversham, 2004
Lactimum	Biorès	Belgium	No	N.D.	Yes	Temmerman et al., 2003a
Lactiv up	Farma-Projekt	Poland	Yes	No	No	Zawistowska-Rojek et al., 2016
Lactò Piu'	Recordati OTC	Italy	Yes	No	No	Celandroni et al., 2019
Lacto5	Biosan	Italy	No	No	No	Fasoli et al., 2003
Lactoflorene Plus	Montefarmaco OTC	Italy	Yes	No	No	Vecchione et al., 2018
Lactoral	Biomed	Poland	No	No	No	Zawistowska-Rojek et al., 2016
Lactovita capsules	Unknown	South Africa	No	No	No	Elliot and Teversham, 2004
Latopic#	IBSS Biomed	Poland	No	Yes	No	Zawistowska-Rojek et al., 2016
Life Top Straw	BioGaia Biologics	Sweden	Yes	N.D.	No	Temmerman et al., 2003a
Linex Forte	Lek Pharmaceuticals	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Multi-billion dophilus	Solgar Laboratories	Netherlands	No	N.D.	No	Temmerman et al., 2003a
MultiTab ImmunoKid	Ferrosan	Poland	Yes	Yes	No	Zawistowska-Rojek et al., 2016
Natrol Prob. Intest.	Natrol	United States	No	Yes	Yes	Drago et al., 2010
Neolactoflorene	Montefarmaco OTC	Italy	No	Yes	No	Fasoli et al., 2003
Novaflora	Pharmaphood	Belgium	No	N.D.	No	Temmerman et al., 2003a
Nucleogermina	Pharmaelle	Italy	No	Yes	Yes	Celandroni et al., 2019
Nutriplant	Agropharm	Poland	Yes	Batch dep.	No	Zawistowska-Rojek et al., 2016
Omniflora Akut	Novartis	Switzerland	Yes	Yes	No	Vanhee et al., 2010
Optibac-*S. boulardii*	Wren Laboratories	United Kingdom	Yes	Yes	No	Vanhee et al., 2010
Oslonka normal	Apotex	Poland	Yes	Yes	No	Korona-Glowniak et al., 2019
Phillip's Colon Health	Bayer	United States	Yes	Yes	No	Drago et al., 2010
Prévite acidophilus	Unknown	Unknown	No	N.D.	Yes	Temmerman et al., 2003a
Probiosan	Nutrisan	Belgium	Yes*	N.D.	No	Temmerman et al., 2003a
Probiotic Immunity	New Chapter Organics	United States	No	Yes	Yes	Drago et al., 2010
Proflora	Chefaro	Belgium	No	N.D.	No	Temmerman et al., 2003a
			No	N.D.	No	Temmerman et al., 2003b
Progermila	Chemist's Research	Italy	No	Yes	Yes	Celandroni et al., 2019
Progermila bambini	Chemist's Research	Italy	No	Yes	Yes	Celandroni et al., 2019
Prolife	Zeta Farmaceutici	Italy	Yes	Yes	No	Vecchione et al., 2018
Provag#	Biomed	Poland	No	Yes	No	Zawistowska-Rojek et al., 2016
Psyllium actif	Biover	Belgium	No	N.D.	Yes	Temmerman et al., 2003a
Reuflor	Italchimici	Italy	Yes	Yes	No	Vecchione et al., 2018
Reuterin/Reuflor	Noos/Italchimici	Italy	Yes	Yes	No	De Vecchi et al., 2008
S. boulardii	Supersmart	Portugal	Yes	Yes	No	Vanhee et al., 2010
Sacchiflora	3D Pharma	Belgium	Yes	N.D.	No	Vanhee et al., 2010
Sanprobi IBS	Sanum Poland	Poland	Yes	No	No	Zawistowska-Rojek et al., 2016
Super Acidophilus	GNC	United States	No	No	Yes	Drago et al., 2010

(Continued)

TABLE 2 | Continued

Product	Manufacturer	Country	Compliance (composition)	Compliance (amount)	Contaminants	Reference
Super Probiotic Comp.	GNC	United States	No	No	Yes	Drago et al., 2010
Superior Probiotics	BioGaia Biologics	Sweden	Yes	N.D.	No	Temmerman et al., 2003a
Sustenex	Ganaden Biotech	United States	Yes	Yes	No	Drago et al., 2010
Triflora	Farmapia	Poland	Yes	Batch dep.	No	Zawistowska-Rojek et al., 2016
Trilac[#]	Krotex	Poland	Yes	No	No	Korona-Glowniak et al., 2019
Ultra Prob. compl. 25	GNC	United States	No	Yes	Yes	Drago et al., 2010
Ultralevure	Biocodex	France	Yes	N.D.	No	Vanhee et al., 2010
VSL#3	Ferring	Italy	Yes	Yes	No	Vecchione et al., 2018
	Farmaceutici		Yes	Yes	No	Mora et al., 2019
Wapiti Darmbalans	Emonta b.v.	Netherlands	Yes	Yes	No	Vanhee et al., 2010
Yovis	AlfaSigma	Italy	No	No	No	Fasoli et al., 2003
			Yes	Yes	No	Vecchione et al., 2018
ZirFos	Alfa Wasserman	Italy	No	No	No	De Vecchi et al., 2008

N.D., not definable.
**Incorrect nomenclature.*
[#]Food for specific medical purposes.

other 19 products (55.88%), no concordance between microbial composition and labeled information was found.

As regards studies on unidentified probiotics, Weese reported concordant results with label claims in only 2 of 5 products intended for human use (Weese, 2002). Analysis of the microbial composition of 58 products obtained from 13 Countries indicated that many formulations were mislabeled (Masco et al., 2005). Similarly, a study conducted on 10 products sold in Europe indicated that three were not concordant with the label claims and four were concordant at the species level but contained different strains than those labeled (Coeuret et al., 2004). Aureli et al. (2010) verified the compliance to the label claims of 41 dietary supplements sold in Italy showing that 20 were constituted by the species reported on the labels. Toscano et al. (2013) investigated the microbial composition of 24 dietary supplements available on the Italian market by using PCR, pyrosequencing of the 16S rRNA gene, and biochemical methods. They showed that 21 products were compliant with the label claims. Nevertheless, seven of these supplements did not contain all the labeled microbes in the amount declared by manufacturers (Toscano et al., 2013). Di Pierro et al. (2019) evaluated the compositional quality of nine formulations declaring to contain alive *L. crispatus* alone or in combination with other species. The presence of *L. crispatus* in the formulations was investigated by using selective media and by species-specific PCR (Di Pierro et al., 2019). The authors found that all products contained *L. crispatus* by culture-independent methods, but only six formulations carried living cells (Di Pierro et al., 2019). The identification of species declared to be contained in five Italian oral products revealed concordant microbial composition to the label claims for four formulations (Blandino et al., 2016). Identification of species contained in 13 French functional foods by MALDI-TOF MS, PCR and sequencing of *tuf* and 16S rRNA genes revealed different species than those stated for two products (Angelakis et al., 2011). The analysis of different lots of a probiotic formulation intended for preterm infant in The Netherlands evidenced the absence of one or more species claimed on the label depending on the tested lot (Vermeulen et al., 2020). The microbial composition of five Polish medicinal products was analyzed by biochemical methods (Szajewska et al., 2004). This study showed that only three products completely complied the labeled information in terms of composition, while the other two contained other species than those declared (Szajewska et al., 2004). Investigation of 26 Bulgarian dietary supplements by two-step multiple PCR indicated that none contained all the species claimed on the labels (Yonkova Marinova et al., 2019).

By using PCR-based denaturing gradient gel electrophoresis (DGGE) and species-specific PCR to decipher the microbial composition of 20 products commercialized in South Africa, Theunissen et al. (2005) found that 54.6% of functional foods and 33.3% of dietary supplements contained the microorganisms stated on the labels.

In a study aimed at evaluating the composition of seven functional foods available on the Columbian market, the authors showed that the content of many dairy products did not comply the label claims (Perea Vélez et al., 2007). Compliance for microbial composition was found for 1 of 5 products available in the US (Drisko et al., 2005). The analysis of the microbial compositions of 14 dietary supplements commercialized in the US by terminal restriction fragment length polymorphism (T-RFLP) and species-specific PCR revealed that 12 products contained additional species than those declared (Marcobal et al., 2008). By studying the bacterial diversity of 16 *Bifidobacterium*-containing products available in the US market, Lewis et al. (2016) showed that only 1 product completely fulfill the label claims. The other formulations lacked one or more *Bifidobacterium* species and/or contained additional species. In addition, a pill-to-pill and lot-to-lot variation was highlighted by the authors for most formulations (Lewis et al., 2016). In a complex study aimed at evaluating the microbiological quality of 52 dietary supplements sold in North Carolina, the authors showed that 30 products effectively contained the labeled species

TABLE 3 | Compliance with the label claims of probiotic functional foods marketed worldwide: focus on the microbial composition, amount of living cells, and presence of contaminant microorganisms.

Product	Manufacturer	Country	Compliance (composition)	Compliance (amount)	Contaminants	Reference
ABC	Sitia YOMO	Italy	Yes	N.D.	No	Fasoli et al., 2003
Actimel	Danone	France	Yes*	N.D.	No	Temmerman et al., 2003a
			Yes	N.D.	No	Temmerman et al., 2003b
Actimel orange	Danone	France	Yes*	N.D.	No	Temmerman et al., 2003a
Active-più	Parmalat	Italy	Yes	N.D.	No	Fasoli et al., 2003
Activia	Danone	Italy	Yes*	N.D.	No	Fasoli et al., 2003
		France	Yes	N.D.	No	Temmerman et al., 2003b
		Russia	N.D.	Yes	No	Astashkina et al., 2014
Almighurt	Almighurt	Germany	Yes	N.D.	No	Temmerman et al., 2003a
B'A fruits	B'A	France	No	N.D.	No	Temmerman et al., 2003a
B'A vanille	B'A	France	No	N.D.	No	Temmerman et al., 2003a
Benecol	McNeil Cons Nutritionals	United Kingdom	No	N.D.	No	Temmerman et al., 2003a
BI'AC	TMA	Germany	No	N.D.	No	Temmerman et al., 2003a
BIO abricot	Danone	France	No	N.D.	No	Temmerman et al., 2003a
BIO framboise	Danone	France	No	N.D.	No	Temmerman et al., 2003a
Bio Snac'	Danone	France	No	N.D.	No	Temmerman et al., 2003a
Biogarde Hafvol Naturel	Strothmann	Germany	No	N.D.	No	Temmerman et al., 2003a
Biogarde plus (naturel)	Almhof	Netherlands	No	N.D.	No	Temmerman et al., 2003a
Biomild drink	Mona	Netherlands	No	N.D.	No	Temmerman et al., 2003a
Biospega	Spega	Italy	Yes	N.D.	No	Fasoli et al., 2003
Dannon	Dannon	Russia	N.D.	Yes	No	Astashkina et al., 2014
Fitness Quark	Onken	Germany	No	N.D.	No	Temmerman et al., 2003a
Fysiq	Mona	Netherlands	No	N.D.	No	Temmerman et al., 2003a
Gefilus	Valio	Finland	Yes	N.D.	No	Temmerman et al., 2003a
Imunele	Wimm Bill- Dan	Russia	N.D.	Yes	No	Astashkina et al., 2014
Joghurt Mild Gartenfrutch	Bremerland	Germany	No	N.D.	No	Temmerman et al., 2003a
Kinderyoghurt mild	J. Bauer KG	Germany	No	N.D.	No	Temmerman et al., 2003a
Kyr	Giglio	Italy	Yes	N.D.	No	Fasoli et al., 2003
Lactus Nature	Carrefour	France	Yes*	N.D.	No	Temmerman et al., 2003a
Lc1	Nestlè	Germany	Yes	N.D.	No	Temmerman et al., 2003a
Mio	Nestlè	Italy	Yes	N.D.	No	Fasoli et al., 2003
Natumild	Natuur Hoeve	Netherlands	No	N.D.	No	Temmerman et al., 2003a
Pomogayka	Nestle	Russia	N.D.	Yes	No	Astashkina et al., 2014
Procult Drink	Alois Müller	Germany	No	N.D.	No	Temmerman et al., 2003a
Provie	Skåne Mejerier	Sweden	Yes	N.D.	No	Temmerman et al., 2003b
Teddy	Fattoria Scaldasole	Italy	Yes	N.D.	No	Fasoli et al., 2003
Vifit Drink	Mona	Netherlands	No	N.D.	No	Temmerman et al., 2003a
Vitamel	Campina	Netherlands	No	N.D.	No	Temmerman et al., 2003b
Weight Watchers Bifidus	Senoble	France	No	N.D.	No	Temmerman et al., 2003a
Yakult	Yakult	Netherlands	Yes*	N.D.	No	Temmerman et al., 2003a
			Yes	N.D.	No	Temmerman et al., 2003b

N.D., not definable.
**Incorrect nomenclature.*

(Morovic et al., 2016). By culture-independent metagenomic sequencing of 10 dietary supplements marked in the US, Patro et al. (2016) showed that five products were mislabeled (Patro et al., 2016).

The analysis of 28 products available on the Chinese market revealed that some preparations respected the labeled species (Chen et al., 2014). Lastly, 4 of 17 products marketed in China were not compliant with the labeled information for microbial composition (Ullah et al., 2019).

Amount of Viable Cells

According to the guidelines for probiotics, manufacturers are required to declare the amount of microbes contained in commercial formulation by the expiration date. In the

analysis of labels of 21 products for human use conducted by Weese, 16 were shown to report the contained amount of cells (Weese, 2003). In this analysis of the published data, the amount of cells was not labeled for two drugs (Econorm, GNorm; **Table 1**, column 5) and three dietary supplements (Ercèflora Supra, Sacchiflora, Ultralevure; **Table 2**, column 5). Two dietary supplements (Acidophilus Probiotic Gold, Natrol Probiotic Intestinal) declared the total amount of microbes at the production time and not at the expiration date (**Table 2**, column 5).

As regards studies on unidentified probiotics, the microbial amount was not declared for one Italian dietary supplement, 4 European functional foods, 5 Indian products, 4 products sold in Taiwan, and 15 dairy products available on the Chinese market (Coeuret et al., 2004; Lin et al., 2006; Chen et al., 2014; Ghattargi et al., 2018; Di Pierro et al., 2019).

The majority of multispecies or multistrain products were found specifies on the labels only the total amount of microorganisms. In fact, species/strain amount declaration was only found for six drugs (Bifilac, Pre Pro Kid, Pre Pro Kid L, Reflora Z, Regutol, Vibact; **Table 1**) and five dietary supplements (Biolactine family, Infloran, Ferzym Plus, Floraviva, Yovis; **Table 2**). Three drugs and two dietary supplements sold in China, one functional food available in South Africa, as well as two drugs and nine supplements marketed in Italy indicated the specific amount of the contained species or strain (Theunissen et al., 2005; Aureli et al., 2010; Toscano et al., 2013; Chen et al., 2014; Di Pierro et al., 2019; Ullah et al., 2019).

Microbial counts in probiotic formulations were conventionally performed by the plate count method. This culture-based analysis may underestimate the real number of alive microbes if methodologies which are not up to current standards were used. Recommended methods for the analysis of probiotics in foods and supplements have been published (Champagne et al., 2011; Schoeni, 2015). In the analysis of probiotic formulations the use of an appropriate medium, period, and temperature for rehydration, homogenization procedure, plating medium, and incubation time, temperature, and redox level should be adopted (Champagne et al., 2011; Schoeni, 2015). Regarding the evaluation of the amount of viable cells compared with the number declared on the label, we found that 15 out of 29 probiotic drugs (51.72%) were compliant (**Table 1**, column 5). Among them, all the products investigated in different studies (Codex, Enterogermina, Lacidofil, Lakcid) provided similar results. The other 14 probiotic drugs (48.28%) contained less microbes than those stated on the labels.

As regards to dietary supplements (**Table 2**, column 5), microbial viability was not definable for 22 products, since the amount of cells was not declared. In addition, contrasting outputs were obtained for Dicoflor and Yovis in different studies (Fasoli et al., 2003; Vecchione et al., 2018) and some variations due to different product batches were observed for Dicoflor 30 Kid, Nutriplant, and Triflora (**Table 2**, column 5). Among the remaining 79 supplements, 44 (55.70%) were compliant for the number of cells. The total CFU amount obtained for 35 products (44.30%) was lower than the number declared on the labels. In particular, the analysis of Asecurin and Byotik in two independent studies led to the same incongruent results (Zawistowska-Rojek et al., 2016; Korona-Glowniak et al., 2019).

As shown in **Table 3** (column 5), the compliance with the labeled amount of microbes could not be established for 33 out of 37 functional foods, since the amount of cells was not declared (Fasoli et al., 2003; Temmerman et al., 2003a,b). The remaining four products analyzed for microbial viability (Activia, Dannon, Imunele, Pomogayka) contained the amount of cells declared by manufacturers on labels.

As regards studies on unidentified probiotics, Masco et al. (2005) showed that many of the investigated formulations did not contain viable cells. An incongruent microbial amount was found in 5 among 10 investigated European products (Coeuret et al., 2004). Thirty among 46 analyzed Italian products contained the amount of living microbes declared on the labels (Aureli et al., 2010; Blandino et al., 2016). Among 24 Italian products tested by Toscano et al. (2013), 10 were found to contain an incorrect amount of cells. On a total of nine Italian analyzed formulations, only two contained the amount of viable cells declared by manufacturers (Di Pierro et al., 2019). All the Polish probiotic formulations included in the study by Szajewska et al. (2004) complied with the label claims for content of viable cells. The analysis of the content of living microbes in 26 supplements sold in Bulgaria revealed that for 10 there was concordance with the label claims (Yonkova Marinova et al., 2019).

By analyzing the amount of viable cells of five dietary supplements available in South Africa, three of them resulted to contain a lower microbial load than that labeled (Brink et al., 2005). Determination of viable *Bifidobacterium* conducted in 58 yogurts from 7 manufacturers sold in North Carolina revealed that 14 did not contain viable cells (Ibrahim and Carr, 2006). Morovic et al. (2016) found that 17 of 52 supplements had a CFU count drastically below the labeled amount. On a total of 10 formulations analyzed, one was found to contain a lower amount of viable cells than that declared by the manufacturer (Patro et al., 2016).

In one study aimed at investigating the presence of LAB in eight probiotic products available in Taiwan, the authors found that two products did not contain the labeled amount of LAB since no viable cells were recovered from these formulations (Lin et al., 2006). For 28 Chinese products, concordance with the labeled number of total living microbes was evidenced for 13 products (Chen et al., 2014). Nevertheless, the authors showed that other 12 products in which the contained amount of cells was not labeled met the minimum quantitative requirement of living cells established in China (Chen et al., 2014). In addition, Ullah and coauthors showed that 5 among 17 tested Chinese dietary supplements contained a number of viable cells that was not concordant with the amount declared on the labels. In particular, four products were found to contain a lower microbial load and no viable cells were detected in one product (Ullah et al., 2019). Among four analyzed dietary supplements available in Bangladesh, none contained the labeled amount of cells (Begum et al., 2015).

Microbiological Purity

Commercial probiotic formulations should be free of pathogenic organisms (further reported as contaminants) that can constitute a health risk for humans. While all the analyzed functional foods were shown to lack contaminants (**Table 3**, column 6), 7 drugs (22.58%; **Table 1**, column 6) and 17 dietary supplements (16.04%; **Table 2**, column 6) contained potential pathogens.

As regards to drugs, the presence of *Bacillus thuringiensis* was revealed in Benegut and Regutol, while *Enterococcus hirae* was detected in Bifilac, Pre Pro Kid L, and Vibact (**Table 1**). Interestingly, while DGGE detected *Bacillus cereus, Alcaligenes faecalis*, *Xanthomonas* spp., and *Lysinibacillus* spp. in Tufpro, culture-dependent analysis identified only *B. cereus* in the product, thus indicating the presence of viable cells of this organism (**Table 1**). Culture-independent DGGE analysis of Ecogro (**Table 1**) revealed several contaminants, e.g., *B. cereus*, *Bacillus licheniformis*, *Acinetobacter* spp., *Xanthomonas*/*Pseudomonas* spp. *Staphylococcus*/*Lysinibacillus* spp. Regarding dietary supplements, high loads of *B. cereus* (about 1.0×10^{10} CFU/dose) and *Lysinibacillus fusiformis* (about 1.3×10^{11} CFU/dose) were detected in Progermila bambini (**Table 2**). In addition, *B. cereus* was found to contaminate 1 unidentified dietary supplement (4.5×10^{6} CFU/g) available on the Italian market (Aureli et al., 2010). The products Biolactine family and Ferzym Plus were found to contain more than 1.0×10^{3} CFU/dose of *B. licheniformis* and *Bacillus badius*, respectively (**Table 2**). Super Probiotic Complex was contaminated by *Bacillus lentus* (9.0×10^{9} CFU/dose) and *Bacillus* spp. (1.0×10^{2} CFU/dose). Contamination by *Bacillus* spp. was also found in Daily Fiber and Probiotics (3.5×10^{2} CFU/dose) and Natrol Probiotic Intestinal (1.3×10^{8} CFU/dose). Drago et al. (2010) showed the presence of 1.0×10^{2} CFU/dose of *Staphylococcus* spp. in Acidophilus Probiotic Gold and a mold in Ultra Probiotic Complex. *L. fusiformis* was detected in high amount (about 7.0×10^{11} CFU/dose) in Progermila and *Acinetobacter baumannii* (about 1.2×10^{11} CFU/dose) was isolated from Nucleogermina (**Table 2**).

Enterococcus faecium was the most common contaminant found in dietary supplements. The microorganism was found in Bifidus complex, Culturelle sachets, Lactimum, Prévite acidophilus, and Psyllium actif (**Table 2**). *E. faecium* was also detected in high amount in Probiotic Immunity (4.2×10^{9} CFU/dose), Super Acidophilus (4.1×10^{5} CFU/dose), and Super Probiotic Complex (9.9×10^{4} CFU/dose) (**Table 2**). Additionally, the microbe was found in several unidentified products. In particular, *E. faecium* was revealed in one US and two Italian dietary supplements, as well as in most of the Chinese products analyzed by Chen et al. (2014; Toscano et al., 2013; Patro et al., 2016). The presence of *E. faecium* and *Enterococcus faecalis* was denounced in some batches of a Dutch product for infants (Vermeulen et al., 2020).

Other potential pathogens such as *B. cereus*, *B. licheniformis*, *Enterobacter cloacae*, *Enterobacter* spp., *Staphylococcus epidermidis*, *Klebsiella* spp., and *Serratia* spp. were revealed by DGGE analysis in some Chinese probiotics (Chen et al., 2014). Nevertheless, none of these microbes were isolated from the products by using culture-dependent methods, thus suggesting that they could be present in a killed form (Chen et al., 2014). Microorganisms belonging to the genus *Weissella* were isolated from some products marketed in Bulgaria (Yonkova Marinova et al., 2019). Lastly, contamination of a dietary supplement for preterm newborns by *Rhizopus oryzae* was evidenced by culture-dependent methods (Vallabhaneni et al., 2015).

DISCUSSION

According to their definition, probiotics must be alive and administered in adequate amount to benefit host health (FAO/WHO, 2001, 2002; Hill et al., 2014). The administration of probiotic preparations containing lower amounts of living microbes may lead to reduced or even absent beneficial effect (Ouwehand, 2017). The daily amount of living probiotics should derive from *in vitro* and *in vivo* experiments and be opportunely determined for each strain and product. Therefore, a universal dose to administer is not established *a priori* for all products. However, some countries (e.g., Italy) require that the daily dose of probiotic formulations contains at least 10^{9} CFU (Ministero della Salute, 2018). Manufacturers are recommended to clearly state the total content of microbes on the product label and this amount should be guaranteed until the expiration date at the declared handling and storage conditions (FAO/WHO, 2002; Guarner et al., 2012; Council for Responsible Nutrition and International Probiotics Association, 2017; World Gastroenterology Organisation [WGO], 2017).

The analysis of commercial probiotic products included in this review highlighted a critical scenario. Many probiotics, particularly functional foods, do not indicate the contained amount of microbes on the label and discrepancies between label information and real content emerged. Even if manufacturers carry at least some of the responsibility for these incongruences, it should be mentioned that the included studies have adopted different methodologies that were sometimes not rigorous and not adherent to recommended methods for the analysis of probiotic products. In fact, papers published before 2015 could have suffered of the lack of guidelines for good practices (Schoeni, 2015) and the adopted methods led to erroneous (underestimated) or incomplete results. It should be mentioned that many studies analyzed products off the shelf, and did not store them until the expiration ("best before") date prior to analysis. It could be that some products judged to be "compliant" were not at the expiration date, particularly if the manufacturers allow storage at room temperature. Thus many conclusions in **Table 2** are only "presumptively compliant."

Among the formulations that declare the total CFU number, more than 40% included in the category drugs and dietary supplements carried a lower amount of viable cells than stated. In the analyzed studies, determination of the amount of living microbes was performed by the plate count method and the number of cells for dose or gram of product was generally provided (Jackson et al., 2019). Although some culture-independent technologies (e.g., flow cytometry, direct imaging and enumeration, nucleic acid amplification techniques) for

quantifying the total microbial content (i.e., alive and dead cells) of probiotic formulations are available, the plate count method currently represents the gold-standard applied in probiotic industry for quality controls (International Standards Organisation [ISO], 2006; International Standards Organisation [ISO], 2010; Davis, 2014; Jackson et al., 2019). However, some limits on the use of the plate count method for the analysis of probiotic formulations have been evidenced. In addition to the fact that not all microbes are culturable by traditional methods and that different microorganisms can require extremely variegate cultural conditions, this technique is unable to detect viable but non-culturable (VBNC) cells that can be generated by the stressful manufacturing procedures (Davis, 2014; Jackson et al., 2019). To obtain the correct enumeration of microbes contained in probiotic products, both plate-count and culture-independent techniques should be applied, as performed in a limited number studies included in this review (Vanhee et al., 2010; Morovic et al., 2016; Patro et al., 2016; Ullah et al., 2019). In culture-dependent analyses, enumeration of different species in multi-species formulations can be critical. To overcome this limit, selective media are commonly used to discriminate species with different metabolic features (Fasoli et al., 2003; Elliot and Teversham, 2004; Aureli et al., 2010; Drago et al., 2010; Toscano et al., 2013; Chen et al., 2014; Di Pierro et al., 2019; Korona-Glowniak et al., 2019; Yonkova Marinova et al., 2019; Kesavelu et al., 2020). Nevertheless, this aim remains challenging, particularly for *Lactobacillus* and *Bifidobacterium* species. In fact, some selective media can impair growth of the target microbe or allow growth of other microorganisms, thus resulting not completely selective (Roy, 2001; Davis, 2014; Hayek et al., 2019; Margolles and Ruiz, 2021).

The viability of probiotic microbes in the finished product is influenced by manufacturing, packaging, and handling. To this regard, overage amounts of microbes are commonly included by manufacturers in probiotic supplements to guarantee the presence of the labeled dose until the expiration date (Fenster et al., 2019). In addition, probiotic products are often produced in a dried or microencapsulated form to ensure stability of microbes over time (Fenster et al., 2019; Grumet et al., 2020). Adequate packing is also crucial to prevent humidification, which can affect cell viability (Fenster et al., 2019).

An additional factor that affects the stability of microbes is the duration of the product shelf life. In fact, while for functional foods the expiration date is generally measured in days or months, for dietary supplements and drugs it is commonly considerably longer (i.e., up to 24 months) (Fenster et al., 2019). Different studies demonstrated a progressive and time-related decrease in the number of viable cells in probiotics investigated at different times before the expiry (De Vecchi et al., 2008; Toscano et al., 2013; Blandino et al., 2016). Stability controls should be performed by manufacturers to guarantee the presence of living cells, since some probiotic organisms can enter in a viable but non-culturable status or lose viability (Forssten et al., 2011; Jackson et al., 2019).

Beneficial health effects of probiotics are generally species- and often strain-specific (Hill et al., 2014; Vitetta et al., 2017). Therefore, the correct identification of microorganisms contained in commercial probiotic formulations is of crucial importance (FAO/WHO, 2002; Council for Responsible Nutrition and International Probiotics Association, 2017).

In this work, we highlighted that more than 40% of the formulations included in the category drugs, dietary supplements, and functional foods do not comply the label claims for microbial composition. Analyzing all the investigated papers over time and looking at the percentages of compliant formulations, no substantial improvement in the quality of probiotic products was found (**Supplementary Table 1**). Nevertheless, due to the fact that many of the analyzed papers were published many years ago, we cannot exclude the current situation of marketed probiotics is improved.

The evidenced lack of concordance between label claims and microbial composition could be due to manufacturer-dependent biases and flaws in quality controls. The FAO/WHO guidelines recommended to identify microbes included in probiotic formulations by using molecular techniques, such as DNA-DNA hybridization or 16S rRNA gene sequencing (FAO/WHO, 2002). In a comprehensive study analyzing 213 microbial cultures intended for production of probiotic formulations, more than 28% were incorrectly identified for the application of unsuitable identification methods (Huys et al., 2006).

It is remarkable to underline that the use of different methods for the identification of microbes can lead to divergent results, particularly for multispecies products. Contrasting results in the composition of the same probiotic brands were evidenced in studies using different methods (Fasoli et al., 2003; Zawistowska-Rojek et al., 2016; Vecchione et al., 2018; Korona-Glowniak et al., 2019). In addition, we cannot exclude that some of the analyzed papers could have produced erroneous conclusions, since not applying rigorous and up-to-date methodologies in the analysis of probiotic formulations. In most of the included studies, the microbial composition of probiotic products was analyzed by a combination of culture-dependent methods (e.g., biochemical tests, MALDI-TOF MS, species-specific PCR, sodium dodecyl sulfate polyacrylamide gel electrophoresis, sequencing of target genes, microsatellite-based typing) applied to microbes isolated from the formulations (Temmerman et al., 2003a; Coeuret et al., 2004; Szajewska et al., 2004; Perea Vélez et al., 2007; De Vecchi et al., 2008; Aureli et al., 2010; Drago et al., 2010; Vanhee et al., 2010; Blandino et al., 2016; Zawistowska-Rojek et al., 2016; Vecchione et al., 2018; Celandroni et al., 2019; Korona-Glowniak et al., 2019; Vermeulen et al., 2020; Yonkova Marinova et al., 2019). However, culture-dependent methods are strictly bound to the ability of the investigators to grow *in vitro* all microbes contained in probiotic products and to phenotypically discriminate colonies belonging to different species. This aim is particularly challenging for multispecies formulations and can result in the inability to detect some species, as humbly reported in some studies (Temmerman et al., 2003a; Drago et al., 2010; Vecchione et al., 2018). In addition, culture-dependent methods fail in the detection of VBNC cells that do not grow on selective media and some biochemical tests (e.g., API system) have been shown to misidentify closely related species and identify *Bifidobacterium* only at the genus level (Blandino et al., 2016; Zawistowska-Rojek et al., 2016). Similarly,

sequencing of the 16S rRNA gene can result unable to distinguish closely related species that share an almost identical sequence of this gene (Fusco et al., 2021). On the other hand, culture-independent techniques (DGGE, T-RFLP, species-specific PCR, high-throughput sequencing, shotgun metagenomic sequencing) directly applied to the whole probiotic products were primarily adopted for microbial identification in some papers (Elliot and Teversham, 2004; Drisko et al., 2005; Theunissen et al., 2005; Marcobal et al., 2008; Chen et al., 2014; Morovic et al., 2016; Mora et al., 2019). These methods lead to detect all the species effectively contained in the products (including VBNC) but are unable to establish if the identified species are viable. Although some probiotic cells have been shown to maintain their beneficial properties also in a dead form (Taverniti and Guglielmetti, 2011; de Almada et al., 2016), probiotics are required to be alive to benefit host health (FAO/WHO, 2001). Considering the limits of culture-dependent and culture-independent techniques, we believe that a combination of both methods should be reasonably applied to definitely decipher the microbial composition of commercial probiotic products, as performed in some of the included studies (Fasoli et al., 2003; Temmerman et al., 2003b; Masco et al., 2005; Angelakis et al., 2011; Toscano et al., 2013; Lewis et al., 2016; Patro et al., 2016; Patrone et al., 2016; Di Pierro et al., 2019; Ullah et al., 2019; Kesavelu et al., 2020). Recently, whole genome sequence analysis has been proposed as a promising method for the identification of microbes contained in probiotic formulations (Fusco et al., 2021).

Purity of commercial probiotic formulations represents a major issue in product testing and quality controls. The introduction of potential pathogenic microorganisms in probiotics can accidentally occur during the entire manufacturing and handling steps and constitute a dangerous health threat, particularly for susceptible individuals. A case of fatal gastrointestinal mucormycosis in a preterm infant due to the assumption of probiotic dietary supplement contaminated by the mold *Rhizopus oryzae* has been reported (Vallabhaneni et al., 2015). Although some *Enterococcus* strains are conventionally used as probiotics, FAO/WHO recommended to keep careful attention in the inclusion of *Enterococcus* microbes in commercial formulations. In fact, some strains can exhibit transmissible vancomycin resistance, acquire other resistance genes, and show a certain degree of pathogenic potential (FAO/WHO, 2001, 2002; Ben Braïek and Smaoui, 2019).

Enterococcus hirae, *E. faecium*, and *E. faecalis* were found to contaminate some probiotic products, particularly dietary supplements. However, Temmerman et al. (2003a) declared than none of *E. faecium* strains isolated in their study as contaminant was resistant to vancomycin. Due to their marked antibiotic resistance and ability to cause urinary tract and wound infections, endocarditis, and bacteremia, *E. faecium* and *E. faecalis* are well known as important nosocomial pathogens (Madsen et al., 2017; Gao et al., 2018; Ben Braïek and Smaoui, 2019; Zhou et al., 2020). Differently, *E. hirae* is generally considered a zoonotic pathogen and is rarely isolated from clinical samples derived from humans. Nevertheless, some studies highlighted this microorganism as responsible with urinary tract infections, endocarditis, and bacteremia (Bourafa et al., 2015; Dicpinigaitis et al., 2015; Ebeling and Romito, 2019; Pinkes et al., 2019; Winther et al., 2020).

Other products resulted to be contaminated with high *Bacillus* loads. Although some *Bacillus* strains are traditionally used as probiotics, others are sufficiently equipped of virulence determinants and cause infections in humans (Cutting, 2011; Celandroni et al., 2016; Elshaghabee et al., 2017; Jeżewska-Frąckowiak et al., 2018; Lee et al., 2019). In fact, *B. cereus* is well known as causative agent of two-types of food poisoning diseases. While the emetic syndrome is due to the ingestion of food contaminated by the pre-formed cereulide, the diarrhoic syndrome follows the consumption of food containing high doses of *B. cereus* spores. Once reached the gut, spores germinate and vegetative cells produce some enterotoxins that are responsible of the symptomatology (Ehling-Schulz et al., 2019). In addition, many other opportunistic infections such as endophthalmitis, endocarditis, bacteremia, as well as wound, respiratory, urinary, and central nervous system infections have been associated with this organism (Bottone, 2010; Celandroni et al., 2016; Ehling-Schulz et al., 2019). The ability of *B. thuringiensis* to cause food-poisoning diseases, periodontitis, wound infections, and bacteremia in humans has occasionally been reported (Celandroni et al., 2014). *B. licheniformis* can be responsible of serious diseases such as peritonitis, food poisoning, and bacteremia, particularly in immunocompromised patients (Park et al., 2006; Celandroni et al., 2016). *B. badius* and *B. lentus* are generally not considered human pathogens, but some strains of *B. lentus* have been shown to possess many *B. cereus* virulence factors (Beattie and Williams, 1999).

Lysinibacillus fusiformis was also detected in some products. This species can behave as opportunistic pathogen in humans, particularly immunocompromised patients, being responsible of a variety of infections (Wenzler et al., 2015). Isolation of the opportunistic nosocomial pathogen *A. baumannii*, potentially causing severe infections, was also reported (Antunes et al., 2014; Harding et al., 2018; Celandroni et al., 2019).

In conclusion, in our review of the published data we found a great number of inconsistencies in the compositional quality of many probiotic formulations available on the worldwide market. In general, no improvement in the quality of products over time was evidenced (**Supplementary Table 1**). Several discrepancies were also observed for probiotic drugs, although this category is subjected to different quality controls compared to dietary supplements and functional foods (de Simone, 2019). As regards quality of probiotic formulations, the ESPGHAN Working Group for Probiotics and Prebiotics already published a society paper stressing the need to improve quality controls for commercial formulations (Kolaček et al., 2017). More recently, another international expert panel emphasized the requirement of more transparency by manufacturers on the quality of probiotics (Jackson et al., 2019). We also believe manufacturers should be imposed to perform more accurate quality controls and adopt innovative methods for producing preparations that are microbiologically pure and qualitatively and quantitatively compliant with the label claims. Scientists

who examine compliance of products should wait until the expiration date to carry out the analysis and at the storage temperature indicated on the label; if there is no mention of the need to refrigerate the product during storage, it should be tested after storage at room temperature. Furthermore, it should be stressed that the use of adequate methods for enumerating and identifying microbes contained in commercial formulations by the investigators is crucial to obtain an accurate and objective overview of the product quality. In addition, it should be underlined the urgent need for more specific and shared regulatory guidelines that govern the global market of these widely used products.

AUTHOR CONTRIBUTIONS

DM and EG reviewed the literature and wrote the manuscript. MC, FC, and AL contributed to the analysis and discussion. All the authors revised the manuscript and approved its submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.693973/full#supplementary-material

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Presence and Germination of the Probiotic *Bacillus subtilis* DE111® in the Human Small Intestinal Tract: A Randomized, Crossover, Double-Blind, and Placebo-Controlled Study

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Spore-based probiotics offer important advantages over other probiotics as they can survive the harsh gastric conditions of the stomach and bile salts in the small intestine, ultimately germinating in the digestive tract. A novel clinical trial in 11 ileostomy participants was conducted to directly investigate the presence and germination of the probiotic strain *Bacillus subtilis* DE111® in the small intestine. Three hours following ingestion of DE111®, *B. subtilis* spores ($6.4 \times 10^4 \pm 1.3 \times 10^5$ CFU/g effluent dry weight) and vegetative cells ($4.7 \times 10^4 \pm 1.1 \times 10^5$ CFU/g effluent dry weight) began to appear in the ileum effluent. Six hours after ingestion, spore concentration increased to $9.7 \times 10^7 \pm 8.1 \times 10^7$ CFU/g and remained constant to the final time point of 8 h. Vegetative cells reached a concentration of $7.3 \times 10^7 \pm 1.4 \times 10^8$ CFU/g at 7 h following ingestion. These results reveal orally ingested *B. subtilis* DE111® spores are able to remain viable during transit through the stomach and germinate in the small intestine of humans within 3 h of ingestion.

Keywords: probiotic, *Bacillus subtilis*, DE111®, germination, small intestine, ileostomy

INTRODUCTION

The predominant probiotic species on the market are strains of *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*. However, there is increasing interest in the use of a number of different *Bacillus* species as safe and effective probiotics for humans (Bader et al., 2012; Suva et al., 2016; Lefevre et al., 2017; Anaya-Loyola et al., 2019; Maher, 2019). In order to be efficacious, probiotics need to reach their target location and remain viable. With gastric pH ranging between near-neutral levels immediately after a meal and pH 1 to 2.5 sometime after meal consumption and in the fasted state (Dressman et al., 1990; Kalantzi et al., 2006), the stomach presents a significant barrier for most probiotics (Evans et al., 1988). *Bacillus* is spore-forming bacteria and confers many advantages over the lactic acid bacteria probiotic strains. In their spore form, they are able to survive the harsh gastric environment and reach the small intestine alive. The small intestine lies between the stomach and the large intestine. It is approximately 20 ft (6 m) in length and is

responsible for around 90% of digestion and absorption of nutrients from the diet (Korelitz and Janowitz, 1957; Aidy et al., 2015). There are three regions to the small intestine; the duodenum, the jejunum, and the ileum. In the duodenum, secretion of enzymes, bile salts, and bicarbonate allow for neutralization of pH and the digestion of carbohydrates, proteins, and lipids. The jejunum, which follows, is specialized for the absorption of the digested particles. The ileum absorbs the remaining nutrients, particularly vitamin B12 and bile salts, allowing the body to recycle them. In addition to digestion and nutrient absorption, the intestinal epithelium acts as the first barrier against external pathogens and plays a central role in the immune response, containing almost 70% of the entire immune system and the majority of immunoglobulin A-producing plasma cells (Vighi et al., 2008; Santaolalla et al., 2011).

Several *Bacillus* species have been reported to show probiotic potential, including *B. subtilis*, *B. coagulans, Bacillus licheniformis* and *Bacillus clausii* (Horosheva et al., 2014; Cuentas et al., 2017; Lakshmi et al., 2017). *Bacillus subtilis* DE111® is a commercially available probiotic that has been shown to support a healthy gut microbiome and to promote digestive and immune health in both adults and children (Cuentas et al., 2017; Maher, 2019; Paytuví-Gallart et al., 2020; Slivnik et al., 2020; Toohey et al., 2020). The beneficial effect of *B. subtilis* has been shown to be 2-fold, in that both the spore and vegetative forms can confer benefits to the host (Huang et al., 2008; Elshaghabee et al., 2017). Spores of *B. subtilis* can themselves modulate the immune response of the host (Huang et al., 2008); however, the full potential of spore-based probiotics can only be achieved if they also germinate and become active vegetative cells in the small intestinal tract. The vegetative form of *B. subtilis* is also able to modulate the immune response and, in addition, secretes enzymes, antioxidants, vitamins, peptides, and antimicrobial compounds, which help balance the gut microbiota and aid digestion (Elshaghabee et al., 2017). Vegetative *B. subtilis* has also been shown to have antiviral properties against avian influenza and adenovirus (Esawy et al., 2011; Starosila et al., 2017). Germination of *B. subtilis* spores is primarily triggered by nutritional signals (Moir and Smith, 1990). Following passage through the stomach, germination of a *B. subtilis* spore-based probiotic is triggered by the rich nutrient environment of the small intestine (Tam et al., 2006). Once in the vegetative form, the probiotic is able to exert its beneficial effects supporting a healthy gastrointestinal tract. The small intestinal tract has a dynamic microbiome with less diversity than that found in the large intestine (Kastl et al., 2020). The microbiota found in this environment quickly adapt to dietary influences and specializes on metabolism of simple carbohydrates, lipids, and bile salts (Kastl et al., 2020). *Bacillus subtilis* is known to have flexibility in its metabolism and is capable of digesting carbohydrates, proteins, and lipids (Liebs et al., 1988; Eymann et al., 2004). The environment of the small intestine is therefore ideal for germination and proliferation of *B. subtilis*.

Confirming the presence of *Bacillus* spores and vegetative cells in the small intestinal tract is challenging. While studies suggest that ingested *Bacillus* spores can germinate in the small intestinal tract of animals (Spinosa et al., 2000; Casula and Cutting, 2002; Leser et al., 2008), this information is lacking in humans. One yet unexplored approach to investigate the fate of probiotics in the human small intestine involves analyzing the ileal effluent of healthy participants with an ileostomy. An ileostomy is a surgical procedure during which the end of the ileum is passed through an opening in the abdomen known as an ileal stoma. Connected to the stoma is an ostomy bag where all the intestinal contents are collected. As needed throughout the day the contents of the bag are emptied.

The aim of this study was to investigate the survivability and germination of the *B. subtilis* DE111® strain of probiotic in the small intestine using a novel methodology involving healthy adults with an ileostomy.

MATERIALS AND METHODS

Study Design

The current study was performed on ileal effluent samples obtained from a wider 4-arm study evaluating the impact of meal properties and dietary supplementation on digestion. This randomized, crossover, double-blind, and placebo-controlled study was carried out between October 2020 and April 2021 at a single site in Ireland. Each of the four interventions was composed of one meal and one study product (placebo or active treatment). The results presented here correspond to two arms only: (1) Meal A + placebo; (2) Meal A + probiotic strain *Bacillus subtilis* DE111®. A flowchart of the study is depicted in **Figure 1**. The protocol was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review reference number: ECM 4 (d) 05/05/2020) and registered on clinicaltrials.gov (NCT04489810). Adults (aged 18–75) were recruited on the basis of inclusion (having an ileostomy stable for at least 3 months post-operation showing normal stoma function and were otherwise healthy) and exclusion (obstruction of the stoma in the previous 3 months, body mass index below 18 kg/m^2 or above 30 kg/m^2, being immunocompromised, history of bariatric surgery, history of drug and/or alcohol abuse, and concurrent participation in other research studies, not using an acceptable method of contraception and not pregnant) criteria. In addition, a current or past diagnosis of one or more of the following was also an exclusion criterion: coeliac disease, allergy to wheat and/or any other ingredients in the study meals; mouth, throat, or active gastrointestinal pathology (other than ileostomy) that may affect normal ingestion and digestion of food; pancreatic disease; and diabetes (both Type 1 and Type 2). Participants were asked not to use any proton pump inhibitors or anti-diarrheal medication during the week and day, respectively, preceding each study day. They were also asked to refrain from excessive alcohol consumption and intensive physical activity the day before the study sessions. All participants gave their written informed consent to participate after receiving oral and written information about the research and study protocol. The study was conducted following the principles of the WMA Declaration of Helsinki and ICH-Good Clinical Practice guidelines.

Eleven participants received either *B. subtilis* DE111® (5×10^9 CFU) or placebo in the morning at breakfast. Each participant received both interventions in this study, in a random sequence

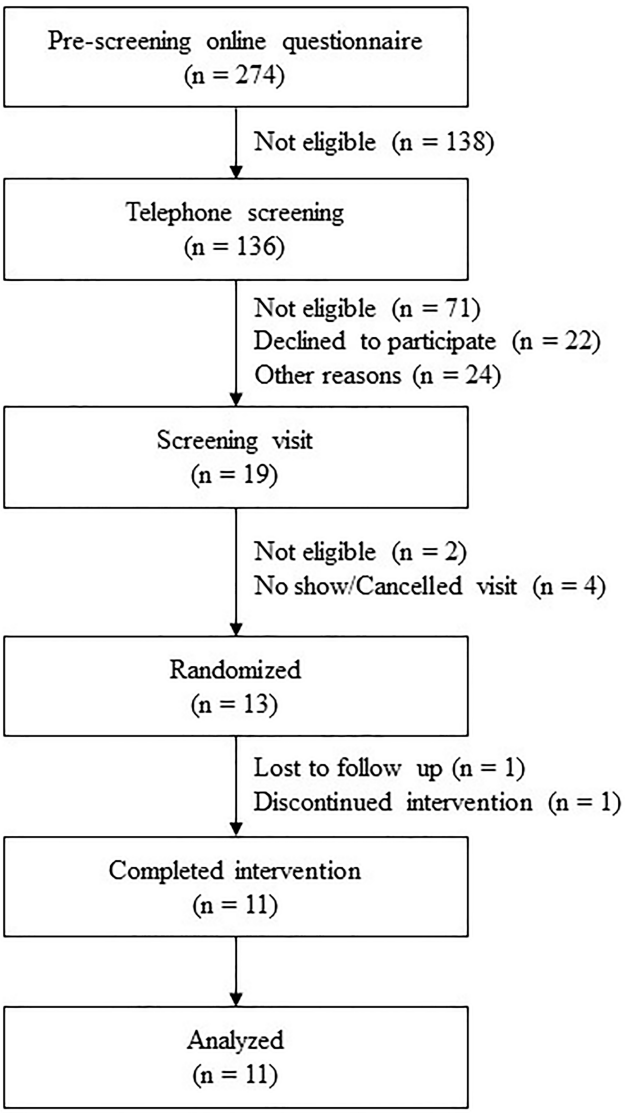

FIGURE 1 | Flowchart of the study profile.

and on different days scheduled at least 1 week apart. Participants consumed a standard gluten-free dinner no later than 21h00 the evening before each study session. Only water was allowed after dinner. The following morning, participants arrived at the study site in a fasted state and remained on-site for the duration of the study session. Participants emptied their ileostomy pouch into a sample collection bag prior to consumption of the investigational products and standard breakfast [two pots of porridge (Flahavan's Organic Original Porridge), one Weetabix, and one glass of water (125 ml)] at 09h00. A standard lunch, at 13h30, consisting of 400 g of a smooth soup (Cully & Sully, Cork, Ireland) and 150 g of jelly (Boyne Valley Group, Louth, Ireland) was consumed by all participants. Throughout the study session, participants' water intake was monitored but unrestricted (up to 1.5 L each session). Ileal effluent was collected at baseline and once every hour for 8 h after breakfast.

Study Product

The study products were provided in the form of capsules packaged in identical containers in single servings. The DE111® supplement was composed of *Bacillus subtilis* DE111® (5×10^9 CFU), medium chain triglycerides, and low-moisture rice maltodextrin. The placebo consisted of an identical capsule containing maltodextrin.

Ileal Effluent Collection and Processing

Ileum effluent sample collection was performed on-site by the participants, who emptied their ileostomy pouches into sterile bags (Buerkle™ SteriBag™ StandUp Polyethylene Sampling Bags). After sample collection, the participants placed the samples in a polystyrene box with frozen (−80°C) cooling packs prior to on-site processing. Samples were collected every hour for 8 h. Upon collection each sample was weighed, diluted 50:50 (w/w) with phosphate buffered saline (PBS) (7.2–7.4 pH) and thoroughly homogenized by vigorous shaking. Aliquots for bacterial enumeration were stored at −80°C in 40% (v/v) glycerol.

Dry Weight Determination

Samples (1–7 g) were placed in a sterile 20 ml universal container and placed in an oven at 60°C for 48 h to obtain effluent dry weight. All counts of CFU/g represent the g dry weight of the effluent.

Enumeration of *Bacillus subtilis*

Mannitol egg yolk polymyxin agar (MYP, Merck) was used as selective medium for detection of the *B. subtilis* DE111® probiotic (Ozkan et al., 2013). Polymyxin B and egg yolk supplements (Merck) were added as recommended by the manufacturer. Colonies of *B. subtilis* DE111® were identified based on morphology, mannitol fermentation (yellow color colonies and surrounding area), and absence of lecithinase activity (lack of white halo around the colonies). This identification was confirmed by 16S sequencing of random colonies isolated from several participants using primers 63f – 5'-CAGGCCTAACACATGCAAGTC-3' and 1387r – 5'-GGGCGGWGTGTACAAGGC-3. Total *B. subtilis* counts were done by performing serial 10-fold dilutions of each sample in PBS. Samples were plated on MYP plates, incubated for 18 h hours at 37°C and colonies counted. To obtain spore counts of *B. subtilis* DE111®, an aliquot of each sample was heat shocked by incubating at 75°C for 10 min to inactivate all vegetative cells. Using the effluent dry weights, counts are reported as CFU/g effluent. The number of vegetative cells was calculated using equation 1. Average calculations, including standard deviations, were performed using all participant data points.

$$\text{Original sample} - \text{Heat shock sample} = \text{Vegetative cells} \quad (1)$$

RESULTS

Baseline Characteristics of Participants

In total, 274 volunteers were screened for eligibility, of whom 13 were randomized as inclusion criteria were met.

One participant was lost to follow-up after the screening visit, another participant dropped out after the first study session. The baseline characteristics of the participants who completed the study are presented in **Table 1**.

Presence of *Bacillus subtilis* DE111® in the Small Intestine

Spores of *B. subtilis* DE111® ($6.4 \times 10^4 \pm 1.3 \times 10^5$ CFU/g) were detected in the small intestinal tract 3 h following ingestion of the probiotic capsule (**Figure 2; Supplementary Table 1**). An increase in the number of spores over time was seen and reached a peak at 6 h following ingestion ($9.7 \times 10^7 \pm 8.1 \times 10^7$ CFU/g). The same concentration of spores continued to be present in the ileal effluent at each time point assessed until end of the study session at 8 h following ingestion. Over the course of the 8-h study session, a total of $3.0 \times 10^9 \pm 6.8 \times 10^9$ CFU of the originally inoculated spores were recovered from the small intestinal effluent.

Vegetative cells of *B. subtilis* DE111® were also evident after 3 h ($4.7 \times 10^4 \pm 1.1 \times 10^5$ CFU/g; **Figure 2**), revealing germination of the spore in the small intestine. Vegetative *B. subtilis* DE111® concentrations in the ileal effluents reached a peak concentration 7 h after ingestion ($7.3 \times 10^7 \pm 1.4 \times 10^8$ CFU/g), with the final concentration of $1.2 \times 10^7 \pm 1.4 \times 10^7$ CFU/g at the final time point.

All participants had both spores and vegetative cells present in their ileal effluent although the rate at which they first presented and persisted varied among individuals (**Supplementary Table 1; Table 2**). Presence of spores and vegetative cells was seen from 3 h after ingestion, with spores identified in 36% of participants and vegetative cells in 27% of samples at this point (**Table 2**). Four hours following ingestion, 80% of participants had spores in their ileal effluents and 60% had vegetative cells. All participant samples had spores present 5 h after ingestion and spores remained present in the effluents until the end of the 8-hour study session (**Table 2**). Detection of vegetative *B. subtilis* DE111® in ileum effluents was 82% after 5 h, 91% at 6 h, and remained similar until the end of the study. All participants had vegetative cells present in their ileal effluent at some time throughout the session (**Table 2**).

TABLE 1 | Baseline characteristics of the participants.

Characteristics	Data
Gender, n (%)	
Female	4 (36.4%)
Male	7 (63.6%)
Age, years	48 ± 14 (24–75)
BMI[1], kg/m^2	27.1 ± 3.3 (21.4–29.9)
Fasting blood glucose[2], mmol/l	5.1 ± 0.7 (3.8–6.8)
Blood pressure (BP), mmHg	
Systolic BP	126 ± 17 (102–160)
Diastolic BP	77 ± 7 (63–87)
Pulse/Heart rate	76 ± 8 (56–90)

All values reported as mean ± SD [min-max] or absolute number (%).
[1]BMI, body mass index.
[2]Fasting blood glucose based on baseline concentrations on study days.

DISCUSSION

A majority of human intervention studies examining *Bacillus* probiotic behavior in the gut involve samples recovered from the end of the intestinal tract *via* feces (Hanifi et al., 2015). Confirming the presence of *Bacillus* vegetative cells in the

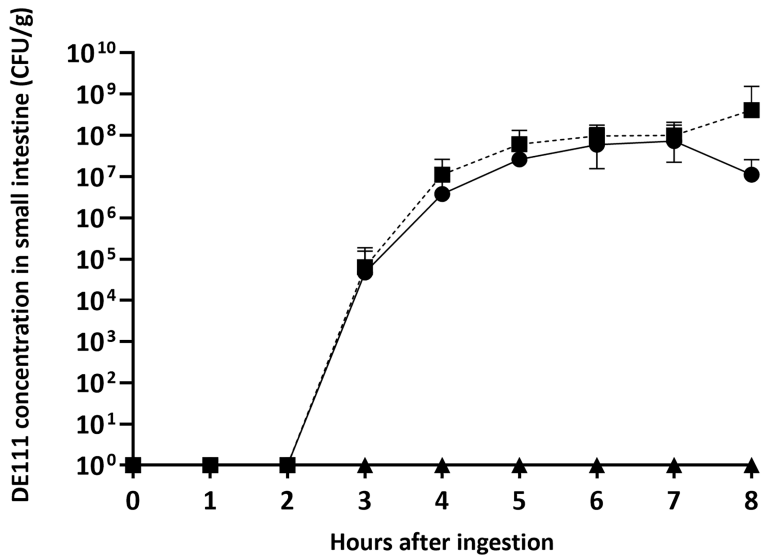

FIGURE 2 | *B. subtilis* DE111® concentration in the small intestinal tract of healthy individuals with an ileostomy stoma. Vegetative DE111® (●), DE111® spores (■), and placebo (▲). Values are average concentrations (n = 11) ± standard deviation.

TABLE 2 | *Bacillus subtilis* DE111® relative spore and vegetative cell concentration (% of total DE111® counts) in ileal effluents of individual participants (A–K) over the course of the study session (0–8 h).

Hours	Participant											Average abundance
	A	B	C	D	E	F	G	H	I	J	K	
0	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0 ± 0***
	0	0	0	0	0	0	0	0	0	0	0	0 ± 0
1	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0 ± 0***
	0	0	0	0	0	0	0	0	0	0	0	0 ± 0
2	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0***	***0 ± 0***
	0	0	0	0	0	0	0	0	0	0	0	0 ± 0
3	***13***	***0***	***0***	***35***	***0***	***0***	***0***	***0***	***0***	***53***	***0***	***9 ± 18***
	87	100	0	65	0	0	0	0	0	47	0	27 ± 40
4	***0***	***24***	***0***	***28***	***0***	***6***	***29***	***22***	***0***	***NS***	***35***	***20 ± 24***
	100	76	0	72	0	94	71	78	100	NS	65	60 ± 39
5	*21*	***67***	***55***	***62***	***16***	***7***	***31***	***0***	***19***	***0***	***27***	***28 ± 24***
	79	33	45	38	84	93	69	100	81	100	73	72 ± 24
6	***16***	***42***	***79***	***38***	***38***	***0***	***20***	***3***	***15***	***36***	***44***	***30 ± 23***
	84	58	21	62	62	100	80	97	85	64	56	70 ± 23
7	***15***	***73***	***73***	***47***	***28***	***0***	***16***	***2***	***31***	***47***	***8***	***31 ± 26***
	85	27	27	53	72	100	84	98	69	53	92	69 ± 26
8	***17***	***0***	***NS***	***59***	***3***	***16***	***44***	***17***	***21***	***0***	***25***	***20 ± 19***
	83	100	NS	41	97	84	56	83	79	100	75	80 ± 19

NS, no sample available. Average abundance (%) is shown as the average of all participants ± standard deviation. Values in the first row of each cell indicate vegetative counts ***(bold italics)****. Values in the second row of each cell indicate spore counts.*

small intestinal tract is challenging. To date, germination of *Bacillus* spores in the small intestine of humans has only been characterized in artificial gastrointestinal models (Bernardeau et al., 2017). A majority of studies examining the fate of *Bacillus* spores administered orally have been carried out in animal models and reveal a disparity of results. In one study, mice inoculated with spores of *B. subtilis* and *B. clausii* had no vegetative cells detected in the intestinal tract (Spinosa et al., 2000). In comparison, another mouse model study investigating the spore germination of two different strains of *B. subtilis* revealed the presence of vegetative cells in the jejunum 12–18 h following ingestion (Tam et al., 2006). Molecular approaches based on competitive reverse transcription-PCR, targeting a gene uniquely expressed by vegetative *B. subtilis* cells, detected 1–12% germination of spores in the jejunum and ileum of mice (Casula and Cutting, 2002). In contrast, 70–80% germination of *B. subtilis* and *B. licheniformis* spores was observed in the proximal intestinal tract of pigs (Leser et al., 2008). Taken together, these studies suggest the fate of spore-forming probiotics in the gut is strongly strain dependent and may differ dramatically depending on the model organism used for the study. This highlights the necessity of strain-specific studies that are performed in the target population to collect accurate data regarding the behavior of the probiotic strain in the gut.

This current study is, to the best of the authors knowledge, the first time in which the fate of a probiotic in the small intestine was investigated. A novel clinical intervention trial in healthy human participants with an ileostomy was developed, enabling real-time, direct access to effluent at the end of the ileum (small intestine). Using this method, the ability of the spore-based probiotic *B. subtilis* DE111® to survive gastrointestinal transit and germinate in the small intestine was evaluated. Three hours following the ingestion of a commercially available capsule of *B. subtilis* DE111®, spores and vegetative cells were found to be present in the ileum. The number of both spores and vegetative cells increased over the course of 6 h in ileum effluxes and remained constant through to the end of the time course (8 h). The counts in this study are representative of non-adhered cells, with in-situ numbers potentially being higher as *Bacillus* species are known to adhere to intestinal mucus (Elshaghabee et al., 2017) and specifically, DE111® has been seen to adhere to Caco2 cells (unpublished data). While spores of *B. subtilis* have been shown to enhance host immunity in the small intestine (Huang et al., 2008), additional host benefits are only possible if the vegetative form of the bacteria is also present. Detecting vegetative cells of *B. subtilis* DE111® in the small intestinal tract suggest metabolically active bacteria are present, producing key beneficial molecules and supporting a healthy microbiome and gut (Elshaghabee et al., 2017). This finding is significant, as for a probiotic to produce enzymes and small molecules to assist in digestion and exert maximal immune benefits it needs to be in the vegetative form and proliferate in the small intestinal tract (Vighi et al., 2008; Santaolalla et al., 2011; Aidy et al., 2015). The time after ingestion at which vegetative cells were first seen in the small intestinal tract varied between individuals, with a proportion having vegetative cells evident 3 h following ingestion of the probiotic. Widespread presence of vegetative cells across participants was observed after 5 h and remained reasonably constant until the end of the study session. It has been shown that in healthy adults transit time, from ingestion through to the end of the ileum, can range from 157 to 240.5 min (O'Grady et al., 2020). Therefore, the variations observed in the timing of initial presence of *B. subtilis* DE111 in

ileum effluxes may be attributed to inherent differences in transit times for each participant. The small intestinal microbiota is dynamic, reflecting the complexity of the environment (Kastl et al., 2020). Recent studies showed daily intake of *B. subtilis* DE111® results in subtle shifts in some key genera, ultimately supporting a healthy microbiome in children aged 2–6 years old (Paytuví-Gallart et al., 2020). Future investigations including metagenomic profiling specifically of the small intestinal microbiota during ingestion of the spore-based probiotic may help further elucidate the beneficial effects of *B. subtilis* DE111® in this region of the gastrointestinal tract.

In conclusion, a unique real-time intervention trial was developed which allowed proof of the survivability of the probiotic *B. subtilis* DE111® through the upper gastrointestinal tract and subsequent germination in the human small intestine. Interestingly, while germination of spores was seen in all participants, the timeline of when vegetative cells first emerged in the ileal effluent was individual dependent. Further studies examining the presence and vegetation of *B. subtilis* over an extended intervention period would be interesting and offer insight into efficacy, metabolic activity, colonization, and re-sporulation of this spore-based probiotic in the small intestinal tract.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review reference number: ECM 4 (d) 05/05/2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DF and AB were involved in protocol writing and study execution. JC and AS performed the sample analysis. MB was the medical advisor for the study and reviewed the manuscript. JC, DF, AB, JD, and AW contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.715863/full#supplementary-material

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METHODS
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Bifidobacteria Strain Typing by Fourier Transform Infrared Spectroscopy

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Fourier transform infrared (FTIR) spectroscopy, a technology traditionally used in chemistry to determine the molecular composition of a wide range of sample types, has gained growing interest in microbial typing. It is based on the different vibrational modes of the covalent bonds between atoms of a given sample, as bacterial cells, induced by the absorption of infrared radiation. This technique has been largely used for the study of pathogenic species, especially in the clinical field, and has been proposed also for the typing at different subspecies levels. The high throughput, speed, low cost, and simplicity make FTIR spectroscopy an attractive technique also for industrial applications, in particular, for probiotics. The aim of this study was to compare FTIR spectroscopy with established genotyping methods, pulsed-field gel electrophoresis (PFGE), whole-genome sequencing (WGS), and multilocus sequence typing (MLST), in order to highlight the FTIR spectroscopy potential discriminatory power at strain level. Our study focused on bifidobacteria, an important group of intestinal commensals generally recognized as probiotics. For their properties in promoting and maintaining health, bifidobacteria are largely marketed by the pharmaceutical, food, and dairy industries. Strains belonging to *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium animalis* subsp. *lactis* were taken into consideration together with some additional type strains. For *B. longum* subsp. *longum*, it was possible to discriminate the strains with all the methods used. Although two isolates were shown to be strictly phylogenetically related, constituting a unique cluster, based on PFGE, WGS, and MLST, no clustering was observed with FTIR. For *B. animalis* subsp. *lactis* group, PFGE, WGS, and MLST were non-discriminatory, and only one strain was easily distinguished. On the other hand, FTIR discriminated all the isolates one by one, and no clustering was observed. According to these results, FTIR analysis is not only equivalent to PFGE, WGS, and MLST, but also for some strains, in particular, for *B. animalis* subsp. *lactis* group, more informative, being able to differentiate strains not discernible with the other two methods based on phenotypic variations likely deriving from certain genetic changes. Fourier transform infrared spectroscopy has

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highlighted the possibility of using the cell surface as a kind of barcode making tracing strains possible, representing an important aspect in probiotic applications. Furthermore, this work constitutes the first investigation on bifidobacterial strain typing using FTIR spectroscopy.

Keywords: *Bifidobacterium*, probiotics, FTIR spectroscopy, strain typing, PFGE, MLST, live biotherapeutic products

INTRODUCTION

Fourier transform infrared (FTIR) spectroscopy is a technology traditionally used in chemistry to determine the molecular composition of a wide range of sample types, and it is based on the different vibrational modes of covalent bonds induced by the absorption of infrared (IR) radiation (Griffiths and De Haseh, 2007; Berthomieu and Hienerwadel, 2009). Starting from the 1990s, this methodology has been largely applied to the microbiology field for the discrimination, classification, and identification of microorganisms at different taxonomic levels, such as genera, species, and even strain level (Helm et al., 1991; Naumann et al., 1991; Novais et al., 2019). The power of FTIR consists of producing an IR spectrum composed of many different vibrational modes of all cellular components, allowing the discrimination of microbial cells in a non-destructive manner (Davis and Mauer, 2010). Each bacterial cell exhibits a unique FTIR spectrum, corresponding to its specific fingerprint signature and correlating with genetic information (Helm et al., 1991; Naumann et al., 1991; Lasch and Naumann, 2015).

Several studies using FTIR spectroscopy focused on foodborne, clinical, and epidemiological pathogens, e.g., *Escherichia coli*, *Salmonella enterica*, *Streptococcus pneumoniae*, and *Listeria monocytogenes* (Preisner et al., 2010; Fetsch et al., 2014; Nyarko et al., 2014; Novais et al., 2019), and have recently paved the way for outbreak investigation (Martak et al., 2019; Hu et al., 2020). Particularly, as FTIR was broadly explored for bacterial classification at the species level, it was also proposed for bacterial typing at the subspecies level, aiming to find an alternative to the established, but very expensive, time-consuming and not applicable on large-scale DNA-based techniques and other methods with high discriminatory power commonly used for epidemiological purposes. Among these, multilocus sequence typing (MLST) (Maiden et al., 1998; Enright and Spratt, 1999) and pulsed-field gel electrophoresis (PFGE) have been largely used for outbreak monitoring and examination (Neoh et al., 2019). In this context, whole-genome sequencing (WGS), which can provide consistent genetic information, has become the new gold standard for identifying, comparing, and classifying microorganisms (Gilchrist et al., 2015). However, besides the discriminatory power, it is necessary to consider the high-cost, laborious, and time-consuming laboratory work related to these technologies, which usually limits their routine application (Sabat et al., 2013).

Fourier transform infrared has been already successfully investigated for subtyping *Yersinia enterocolitica*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *L. monocytogenes*, and *E. coli* (Quintelas et al., 2018) and recently started to be implemented into the laboratory routine (Novais et al., 2019). The main advantages related to this method are the analysis time, cost, laboratorial simplicity, absence of chemical reagents, and very low sample amount per analysis, in addition to relevant information about the biomolecular content of the microorganisms including lipids, carbohydrates, proteins, and nucleic acids deriving from the IR spectrum (Quintelas et al., 2018). In 2018, Bruker commercialized an automated typing system based on FTIR technology, the IR Biotyper (Bruker GmbH, Bremen, Germany), whose application has gained large interest, becoming common in typing bacterial isolates especially in the field of clinical microbiology (Bruker GmbH Daltonics Division, 2018; Burckhardt et al., 2019; Martak et al., 2019; Hu et al., 2020).

All these features contribute to make the FTIR a fascinating and attractive technique not only for clinical and epidemiological investigation but also for other sectors related to the microbiology field, such as the probiotic industry. In the last 20 years, the attention for probiotic microorganisms has increased in both researchers and consumers, promoting the maintenance of health status and host wellness. Distinguishing probiotic products is challenging due to differences in their mechanisms of action, manufacturing processes, quality control, and efficacy of different strains. The production of good-quality probiotics is already fundamental in the early stages of the process, and it is not limited to the only biomass growth. As many properties can affect the development of probiotics, in order to guarantee quality, stability, and safety of the product, strain-specific verification is required. Moreover, in the clinical field, the efficacy of probiotics has been demonstrated to be clearly strain- and disease-specific (McFarland et al., 2018); therefore, the choice of the appropriate strain for the patient can be challenging. In these contexts, FTIR can constitute a quick and reliable technique for typing probiotic bacteria and an efficient tool for identifying a target probiotic product.

Bifidobacteria are considered key commensals in human–microbe interactions and are recognized to play an important role in maintaining a healthy gut (Turroni et al., 2017). These health-promoting bacteria are widely used as probiotics in preventive and therapeutic strategies for human diseases, especially in pediatric subjects, for their capability of reaching and colonizing the gastrointestinal tract, their long history of safe use, and their documented health benefits (Biavati et al., 2000; Leahy et al., 2005; Sanders et al., 2010; Di Gioia et al., 2014; Bozzi Cionci et al., 2018).

This study was aimed to test the potential discriminatory power of FTIR technology at strain level among members of the *Bifidobacterium* genus recognized as probiotics in order to

pave the way for the introduction of this new phenotyping-type method into routine process for the development of probiotic products. Specifically, the FTIR performance was compared with other assessed genotyping techniques, PFGE, WGS, and MLST, with a particular focus on the strains belonging to *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium animalis* subsp. *lactis*.

TABLE 2 | Additional type strains of *Bifidobacterium* included in the analyses.

Species	Code	Deposit code	Techniques used
B. bifidum	BB-CT	DSM20456^T	PFGE, FTIR
B. breve	BR-CT	DSM20213^T	PFGE, FTIR
B. adolescentis	BA-CT	DSM20083^T	PFGE, FTIR
B. longum subsp. *infantis*	BI-CT	DSM 20088^T	PFGE, FTIR

FTIR, Fourier transform infrared; PFGE, pulsed-field gel electrophoresis.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A total of four *B. longum* subsp. *longum* and four *B. animalis* subsp. *lactis* strains commonly used as probiotics were included in this study, and the type strains *B. longum* subsp. *longum* DSM20219^T and *B. animalis* subsp. *lactis* DSM10140^T were used as reference controls (**Table 1**). Further four species belonging to *Bifidobacterium* were included in the PFGE and FTIR analysis to evaluate the discriminatory power of the FTIR method at species level (**Table 2**).

The strains were maintained at −80°C and subcultured in MRS broth (Difco) anaerobically at 37°C for 72 h.

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

The protocol was described by Tynkkynen et al. (1999). Cell suspension was mixed with an equal volume of 2% agarose gel (Pulsed Field Certified Agarose; Bio-Rad) prepared in 0.125 M ethylenediaminetetraacetic acid (EDTA) (pH 7.6) and dispensed into disposable plug molds (10 mm × 5 mm × 1.5 mm; Bio-Rad). The plugs were incubated in 1 ml of 1 M NaCl, 6 mM Tris–HCl, 100 mM EDTA, 1% Sarkosyl buffer (pH 7.6; Sigma) with 10 mg/ml lysozyme (Sigma), and 500 units/ml mutanolysin (Promega) at 37°C for 18 h. The plugs were then incubated in fresh Sarkosyl buffer with 1 mg/ml proteinase K (Sigma) at 37°C for 48 h.

The plugs were washed twice with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) in 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) at 37°C for 60 min in a shaking water bath. Two slices (2-mm wide) were prepared from the plugs and washed three times in 1 ml of 10 mM Tris–HCl, 0.1 mM EDTA (pH 8.0) for 60 min at room temperature (r.t.). The slices were preincubated at r.t. for 30 min in 500 μl of the appropriate restriction endonuclease buffer. They were then transferred to 500 μl of a fresh restriction digest mixture containing 40 units of *Xba*I and incubated at 37°C for 18 h.

Electrophoresis (Briczinski and Roberts, 2006) was performed on 1.0% agarose gel (Bio-Rad) using 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). A lambda PFG ladder (BioLabs N.E.) was included as a molecular weight marker. Electrophoresis was performed using a CHEF III System (Bio-Rad). Switch times were increased linearly from 0.2 to 35.4 s for 14 h, with an angle of 120° at 6 V/cm and 14°C. Gels were stained with a solution of ethidium bromide (0.4 mg/L; Promega) for 1 h, then destained for 30 min. Restriction patterns were visualized on a UV transilluminator, and images were captured using a GelDocXR System (Bio Rad) and saved as TIFF files for future analysis.

Whole-Genome Sequencing (WGS) and OrthoANI Calculation

Total DNA was extracted from the strains using the Wizard genomic DNA purification kit (Promega, Mannheim, Germany) according to the manufacturer's recommendations. The concentration of the DNA was measured by a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, United States) and a Qubit 4.0 fluorometer (Life Technologies; Invitrogen, CA, United States). Furthermore, the quality of the DNA was assessed on agarose gel electrophoresis. The DNA was stored at −20°C prior to WGS.

TABLE 1 | Strains included in the PFGE, FTIR, WGS, and MLST analyses. *Bifidobacterium longum* subsp. *longum* BL-CT and *Bifidobacterium animalis* subsp. *lactis* BS-CT are type strains.

Species	Code	Deposit code	GenBank accession number	Techniques used
B. longum subsp. *longum*	BL03	DSM16603	JAGGDB000000000	PFGE, FTIR, WGS, and MLST
B. longum subsp. *longum*	W11	LMG P-21586	MRBG00000000.1	PFGE, FTIR, WGS, and MLST
B. longum subsp. *longum*	DLBL07	DSM25669	JAGGDA000000000	PFGE, FTIR, WGS, and MLST
B. longum subsp. *longum*	DLBL09	DSM25671	JAGGCZ000000000	PFGE, FTIR, WGS, and MLST
B. animalis subsp. *lactis*	BS01	LMG P-21384	JAGGCY000000000	PFGE, FTIR, WGS, and MLST
B. animalis subsp. *lactis*	BS05	DSM23032	JAGGCX000000000	PFGE, FTIR, WGS, and MLST
B. animalis subsp. *lactis*	MB2409	DSM23733	JAGGCW000000000	PFGE, FTIR, WGS, and MLST
B. animalis subsp. *lactis*	BB12	ATCC 27673	CP001853.1	PFGE, FTIR, WGS, MLST
B. longum subsp. *longum*	BL-CT	DSM20219^T	FNRW00000000.1	PFGE, FTIR, WGS, and MLST
B. animalis subsp. *lactis*	BS-CT	DSM10140^T	CP001601.1	PFGE, FTIR, WGS, and MLST

FTIR, Fourier transform infrared; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; WGS, whole-genome sequencing.

The WGS was performed for the strains reported in **Table 1**, except for the strains *B. longum* subsp. *longum* W11, *B. animalis* subsp. *lactis* BB12, and the type strains of both species, whose genome sequences were available at the time of the study in GenBank database (**Table 1**). Genome sequences were determined using Illumina MiSeq sequencing technology with a 300-paired-end library. The sequences of adapters were searched and removed from the reads using cutadapt tool (Martin, 2011) and applying BLASTn searches with a minimum evalue 1e-05. Subsequently, quality trimming was performed on the reads with Erne-filter (Del Fabbro et al., 2013) using default parameters, and only sequences passing the quality thresholds were assembled into contigs by CLC Workbench v7. The genome sequences thus obtained were deposited at DDBJ/ENA/GenBank under the accession reported in **Table 1**.

The whole genome similarity between the strains of the species *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis* was determined through the comparison of the OrthoANI values calculated by means of the OAT software (Lee et al., 2016).

Multilocus Sequence Typing (MLST) Analysis

The genome sequences of the strains reported in **Table 1** were used to retrieve the nucleotide sequence of the seven loci included in the MLST scheme described by Delétoile et al. (2010). The MLST sequences obtained by BLASTn searches were used for the construction of phylogenetic tree based on the concatenated sequence of the genes *clpC*, *fusA*, *gyrB*, *ileS*, *purF*, *rplB*, and *rpoB*. Multiple alignments were made through ClustalX 2.1, and the tree was reconstructed using the neighbor-joining method and Jukes–Cantor substitution model.

Fourier Transform Infrared (FTIR) Analysis

An IR Biotyper spectrometer (Bruker Optics-Daltonics GmbH) was used for this investigation.

An amount of 1 μl overloaded loop of bacterial colonies taken from the confluent part of the culture were resuspended in 50 μl of 70% ethanol solution in an IR Biotyper suspension vial. After vortexing, 50 μl of deionized water were added, and the solution was mixed by pipetting. Here, 15 μl of the bacterial suspension were spotted in four technical replicates onto the 96-spot silicon IR Biotyper target and let dry for 15–20 min at 35°C ± 2°C.

In each run, prior to sample spectra acquisition, quality control was performed with the Infrared Test Standards (IRTS 1 and 2) of the IR Biotyper kit. IRTS 1 and IRTS 2 were resuspended in 90 μl of deionized water; 90 μl of absolute ethanol were added and mixed. Here, 12 μl of suspension were spotted in duplicate onto the IR Biotyper target and let dry as previously described for the samples. After spectra acquisition and evaluation of IRBT1 and IRBT2, spectra of the samples were acquired, intercalating a background spectrum between each sample.

Spectra acquisition, visualization, and processing were performed in transmission mode in the spectral range 4,000–600 cm^{-1} (mid-IR). The second derivative of the spectra was calculated using Savitzky–Golay algorithm over nine data points. Spectra were then cut to 1,300–800 cm^{-1} (Van der Mei et al., 1993) and vector-normalized to amplify differences between isolates, and correct variations related to spectra acquisition.

First, the discriminatory power of IR Biotyper was investigated within the *Bifidobacterium* genus using six different *Bifidobacterium* species (*B. animalis* subsp. *lactis*, *B. longum* subsp. longum, *B. longum* subsp. *infantis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium adolescentis*) (**Table 2**). After that, the discriminatory power within the *B. animalis* subsp. *lactis* and *B. longum* subsp. *longum* species was evaluated (**Table 1**). Hierarchical cluster analysis (HCA) to assess the similarity of the samples was performed by means of the IR Biotyper Client software v1.5 using Euclidean metric and single linkage. For each dataset explored, the IR Biotyper software automatically calculated a clustering cutoff, which is the product of the Simpson's index of diversity and the mean coherence of the parameter defined by the user (individual strain, in this study) (Hunter and Gaston, 1988). For each dataset, a strain belonging to another species was included to have a reference of "non-relatedness."

RESULTS

Bifidobacterium longum subsp. *longum*

Four strains of *B. longum* subsp. *longum* (BL03, W11, DLBL07, and DLBL09) and a type strain (BL-CT) were examined. The MLST analysis confirmed that the strains belong to the species *B. longum* subsp. *longum*, clustering separately from the type strain *B. animalis* subsp. *lactis* BS-CT, used as reference control (**Figure 1A**). The MLST analysis revealed a very high relatedness between the strain DLBL07 and the strain DLBL09, which constituted a single phylogenetic cluster, while the strains W11 and BL03 and the type strain BL-CT were located in separated clusters (**Figure 1A**). Similarly to what was observed for the MLST analysis, strains DLBL07 and DLBL09 shared an OrthoANI value of 100%, while they exhibited values ranging from 98.58 to 98.71% when they were compared with the strains W11 and BL03 (**Figure 1B**). The comparison between the analyzed strains and the type strain BL-CT displayed OrthoANI values between 98.68 and 98.86%.

In accordance with genomic results, different PFGE patterns were evidenced for the strains W11 and BL03 and the type strain BL-CT, while strains DLBL07 and DLBL09 showed the same PFGE profile and were not discernible (**Figure 2B**).

Dendrograms built from FTIR spectra acquisition evidenced five different types corresponding to the five strains used in this study (cutoff, 0.223); in particular, FTIR spectroscopy was able to distinguish correctly the strains DLBL07 and DLBL09 (**Figure 2A**).

Bifidobacterium animalis subsp. *lactis*

Four strains of *B. animalis* subsp. *lactis* (BS01, BS05, MB2409, and BB12) and a type strain (BS-CT) were processed. The MLST analysis confirmed that the strains belong to the species

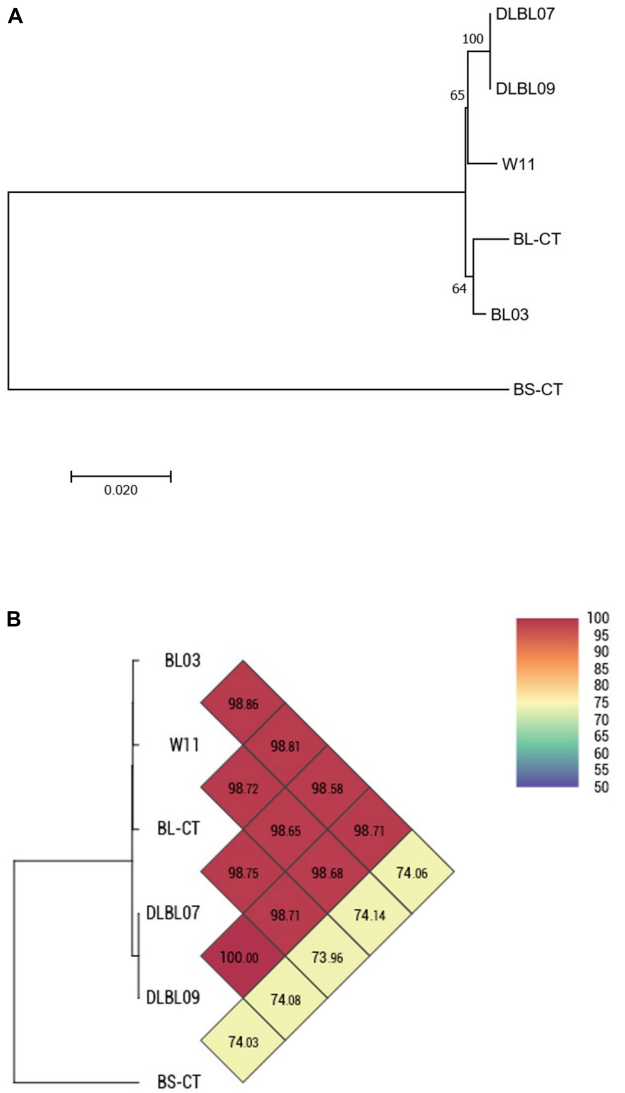

FIGURE 1 | Whole-genome comparison for *Bifidobacterium longum* subsp. *longum* strains based on the multilocus sequence typing (MLST) analysis and the OrthoANI values. **(A)** Phylogenetic tree based on the concatenated nucleotide sequences of the genes *clpC*, *fusA*, *gyrB*, *ileS*, *purF*, *rplB*, and *rpoB* included in the MLST scheme for *B. longum* subsp. *longum* strains. Bootstrap values (1,000 replicates) are shown as a percentage at the branching points. The scale bar represents the number of nucleotide substitutions per site. **(B)** Heatmap generated with OrthoANI values calculated from the OAT software for *B. longum* subsp. *longum* strains.

B. animalis subsp. *lactis*, clustering separately from the type strain *B. longum* subsp. *longum* BL-CT, used as reference control (**Figure 3A**). However, only BS05 was easily distinguished, while strains BS01 and MB2409 constituted a separate single phylogenetic cluster with strain BS-CT and probiotic strain BB12, as reported in **Figure 3A**. The high similarity between strain BS01 and type strain BS-CT was confirmed by the shared OrthoANI value that corresponds to 100% (**Figure 3B**). Moreover, within the strains examined for the species *B. animalis* subsp. *lactis*, BS05 exhibited the lowest OrthoANI values compared to the strains under analysis, which are between 99.33 and 99.40% (**Figure 3B**).

The same homogeneous trend was observed in PFGE patterns. In fact, BS05 was the only strain showing a discernible profile, while BS01, MB2409, and BB12 exhibited the same PFGE pattern (**Figure 4B**). Moreover, the type strain BS-CT shared the same PFGE profile of BS01, MB2409, and BB12.

Differently, dendrograms built from FTIR spectra acquisition classified the examined isolates in different clusters, distinguishing all the strains used as different types, corresponding to BS01, BS05, MB2409, and BB12 and type strain BS-CT (cutoff, 0.173) (**Figure 4A**).

Bifidobacterium Species/Subspecies

A total of six species or subspecies belonging to *Bifidobacterium* genus, BA-CT, BI-CT, BR-CT, BB-CT, BL-CT, and BS-CT, were subjected to PFGE and FTIR analysis in order to evaluate the discriminatory power of FTIR spectroscopy also at the species/subspecies level. Moreover, a comparison between FTIR and PFGE has been carried out. No contradictory results were evidenced from the two techniques: PFGE showed six different profiles, and FTIR distributed the isolates in six clusters, corresponding to the species/subspecies tested (cutoff, 0.206) (**Figures 5A,B**).

DISCUSSION

Microorganisms are known to play a crucial role in maintaining the human health status: some bacteria, invading the host, can cause a range of diseases, but several others, establishing a mutualistic relationship with the human body, contribute to the normal host physiology. The administration of beneficial microorganisms may represent a key determinant of the general health status and disease susceptibility. Therefore, bacterial typing at the strain level represents a great challenge to human health, considering not only factors associated with harmful microorganisms, such as increased virulence, transmissibility of pathogens, resistance to multiple antibiotics, but also the favorable side, including the mechanisms that lead to benefits deriving from probiotics administration (Fournier et al., 2004).

The discriminatory power of FTIR spectroscopy by typing at the species and strain level bacteria belonging to *Bifidobacterium* genus was assessed in this study. The procedure was successful in distinguishing the different species/subspecies tested (*B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. animalis* subsp. *lactis*) and, surprisingly, also the different strains belonging to *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis*. Although the functionality of FTIR method in species typing has been already verified for Gram-negative bacteria, such as *E. coli*, *Klebsiella oxytoca*, and *Y. enterocolitica* (Kuhm et al., 2009; Sousa et al., 2013; Dieckmann et al., 2016), and isolates responsible for hospital outbreaks, such as *Enterobacter cloacae*, *K. pneumoniae*, and *Pseudomonas aeruginosa* (Martak et al., 2019; Hu et al., 2020), its discriminatory

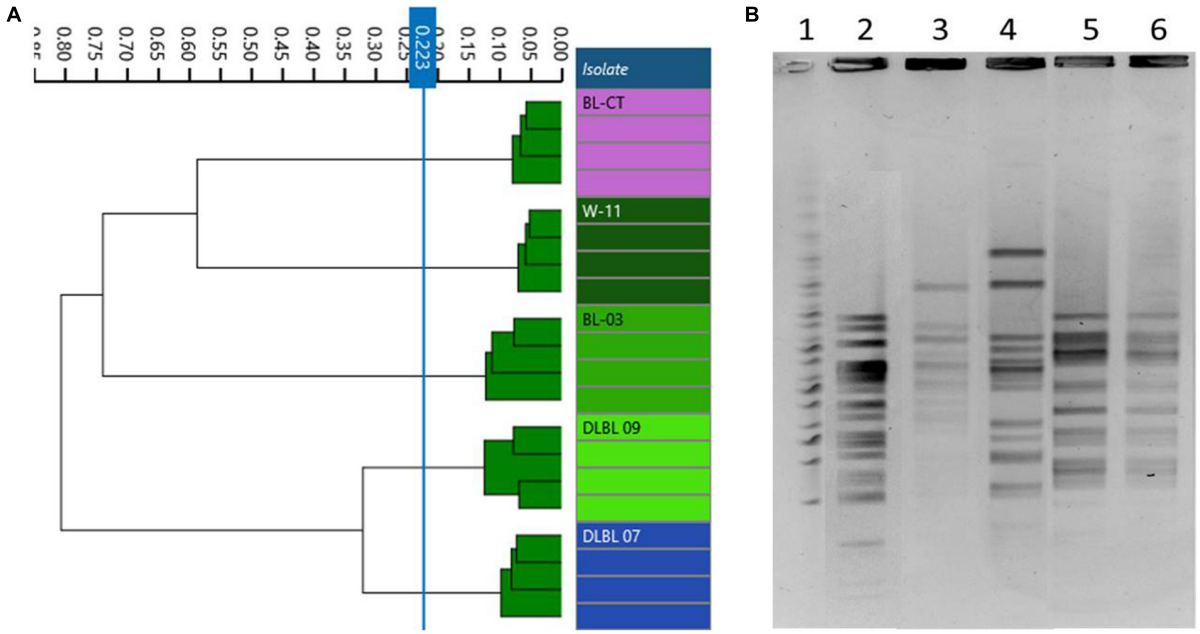

FIGURE 2 | Comparison of Fourier transform infrared (FTIR) and pulsed-field gel electrophoresis (PFGE) typing methods on *B. longum* subsp. *longum* strains. **(A)** Dendrogram obtained by clustering FTIR spectra for *B. longum* subsp. *longum* strains (cut-off, 0.223). **(B)** *B. longum* subsp. *longum* strain PFGE profiles with *Xba*I (1) Marker; (2) BL-CT; (3) BL03; (4) W11; (5) DLBL07; (6) DLBL09.

power has not been assessed for Gram-positive and probiotic bacteria until now.

Fourier transform infrared provided potentially equivalent data with respect to those resulting from the gold standard PFGE and MLST, which discriminated the strains belonging to the *B. longum* subsp. *longum* group, with the exception of DLBL07 and DLBL09, and the strain BS05 of the *B. animalis* subsp. *lactis* group. Surprisingly, FTIR technology was able to discriminate DLBL07 and DLBL09, which, on the contrary, clustered together in MLST and PFGE analyses, and therefore they were not discernible with these DNA-based methods. Another unexpected and relevant result was that FTIR revealed to be more informative for *B. animalis* subsp. *lactis* group with respect to the other two techniques, distinguishing all the strains belonging to this group, while MLST and PFGE did not reach this result, but they were only able to discriminate BS05. Moreover, the typing power of FTIR within the *Bifidobacterium* genus was demonstrated both at the species level and at the strain level.

It is important to consider that while gold standard methods that have been tested in this study are DNA-based techniques, FTIR technology acquires spectra deriving from the carbohydrate composition of the bacterial cell wall. Consequently, MLST and PFGE detect genomic variations that may lead to alterations in carbohydrate composition, but FTIR spectroscopy can highlight modifications in bacteria wall that probably are not always attributable to genetic differences.

Bifidobacterium longum subsp. *longum*, classified as subspecies together with *B. longum* subsp. *infantis* and *B. longum* subsp. *suis* of the species *B. longum* (Mattarelli et al., 2008), is considered one of the most important contributors to host health, and representative strains are frequently used as probiotics, fermented products, and pharmaceutical preparations. *B. longum* strains have been demonstrated to have a high genomic heterogeneity in PFGE analysis (Roy et al., 1996; Ventura et al., 2004; Ward and Roy, 2005), in accordance with our results.

Bifidobacterium animalis subsp. *lactis*, described by Masco et al. (2004) as subspecies belonging to the species *B. animalis*, is currently the most utilized probiotic species among the bifidobacteria (Mattarelli and Biavati, 2017). However, from a genetic point of view, this subspecies, exhibiting a huge sequence similarity among the different strains presently described, has been named "monomorphic" or "monophyletic" (Milani et al., 2013). It is possible that the large importance acquired in the probiotic industry led to a lack of diversity within *B. animalis* subsp. *lactis* strains. In fact, the intense focus on commercially relevant strains could have resulted in a reisolation of the same strains and assignment as new ones. Loquasto et al. (2013) supported this hypothesis demonstrating that *B. animalis* subsp. *lactis* ATCC 27673, isolated from sewage, constituted a genetically distinct strain with respect to other strains of *B. animalis* subsp. *lactis* isolated from human feces. Despite the lack of genetic variability, strains of *B. animalis* subsp. *lactis* have been shown to differ in phenotypic characteristics, such as in resistance to oxidative stress (Oberg et al., 2011). Therefore, FTIR spectroscopy, according to our results, which demonstrated the highest discriminatory power with respect to PFGE and MLST on *B. animalis* subsp. *lactis* strains, can overcome genetic limitations regarding this monomorphic subspecies, evidencing potential phenotypes that can be beneficial for industrial or human health purposes.

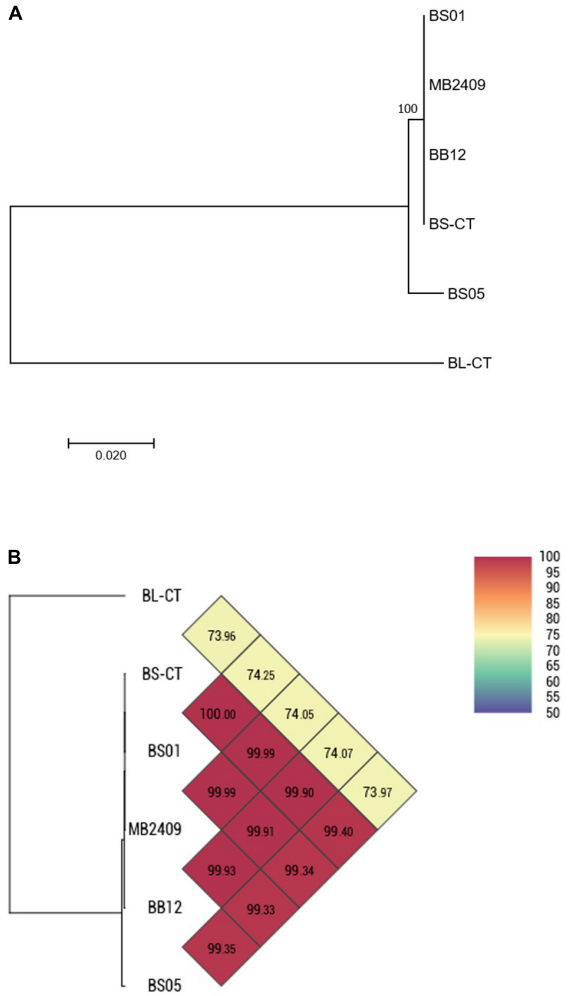

FIGURE 3 | Whole-genome comparison for *B. animalis* subsp. *lactis* strains based on the multilocus sequence typing (MLST) analysis and the OrthoANI values. **(A)** Phylogenetic tree based on the concatenated nucleotide sequences of the genes *clpC*, *fusA*, *gyrB*, *ileS*, *purF*, *rplB*, and *rpoB* included in the MLST scheme for *B. animalis* subsp. *lactis* strains. Bootstrap values (1,000 replicates) are shown as a percentage at the branching points. The scale bar represents the number of nucleotide substitutions per site. **(B)** Heatmap generated with OrthoANI values calculated from the OAT software for *B. animalis* subsp. *lactis* strains.

Probiotic products are unique in their properties to confer a health benefit, and they can present different challenges in design, development, scale-up, manufacturing, commercialization, and life cycle management (Jackson et al., 2019). Quality and safety assessment of probiotic food and supplements is a responsibility of the industry, and FTIR spectroscopy constitutes a method that can be inserted in the process for rapid biotyping of different strains belonging to the same species.

In the scenery of clinical application, it should be pointed out that matching the appropriate probiotic strain to patients who suffer from a certain disease can represent a challenging task. In this regard, McFarland et al. (2018) demonstrated the importance of considering both probiotic strain specificity and disease specificity. Based on this evidence, a technology that is able to guarantee a clear discrimination among probiotic strains can be considered essential for the design of clinical trials focused on the prevention or treatment of diseases. FTIR spectroscopy, with the potential highlighted from this study in discriminating bifidobacteria, can be successfully inserted in the process of the clinical choice of the suitable probiotic strain, accelerating and implementing the clinical strategy.

The *Bifidobacterium* genus has always gained high microbiological interest due to its potentially health-promoting effects and increasing use as a probiotic. The comparison of important characteristics of bifidobacterial species and strains, such as interactions with the host, gut colonization dynamics, or ecological distribution, is object of intensive studies in probiotic industries. As probiotic effects are species- and even strain-specific, the European Food Safety Authority requests a precise characterization of food constituents that are microorganisms, which are the subject of health claims (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2009).

In the perspective of growing demand on probiotic bacteria, industries need rapid and accurate identification of specific bacteria. Moreover, isolation of new microorganisms from various environments may lead to multiple isolations of the same strain. Only a few studies focused on the use of FTIR and vibrational spectroscopic techniques to investigate lactic acid bacteria and probiotics (Wenning et al., 2010; Santos et al., 2015), including *Bifidobacterium* at species level (Mayer et al., 2003), but the investigation on bifidobacteria at the subspecies level has been unexplored until the current study. Therefore, it is important to apply rapid, low-cost, and effective procedures able to differentiate bifidobacteria strains. In this context, the correct identification of probiotic strains can provide a useful framework for examining the evolutionary dynamics and phylogenetic distribution of significant strain properties. PFGE being quite laborious and MLST considerably expensive, and both time-consuming (2–3 days), particularly when a large group of new isolates is typed, their routine application for probiotic identification can be demanding. Instead, FTIR spectroscopy, as a quick, inexpensive, and high-throughput tool for bacterial typing, provides reliable discriminatory information, based on our results regarding bifidobacteria, and with the availability of well-composed databases, it can constitute a suitable and appropriate method for typing bifidobacteria in the frame of probiotic production. Although our investigation was limited to a restricted number of strains belonging to *Bifidobacterium*, the results obtained are extremely promising, supporting the application of this method also to other probiotic cultures.

CONCLUSION

We demonstrated that FTIR spectroscopy successfully discriminated a group of bacteria traditionally used as probiotics, bifidobacteria, at the species/subspecies and strain levels. The typing functionality was not only equivalent compared to two other consolidated techniques but also more informative especially for *B. animalis* subsp. *lactis* strains. In addition,

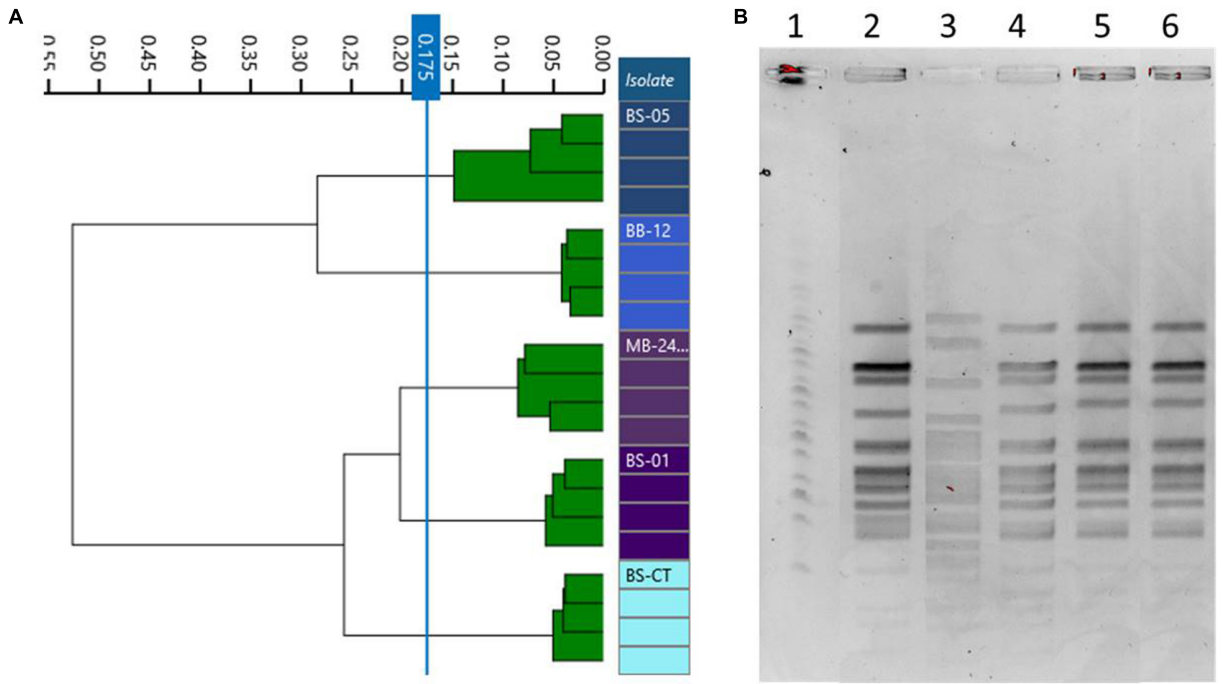

FIGURE 4 | Comparison of Fourier transform infrared (FTIR) and pulsed-field gel electrophoresis (PFGE) typing methods on *B. animalis* subsp. *lactis* strains. **(A)** Dendrogram obtained by clustering FTIR spectra for *Bifidobacterium animalis* subsp. *lactis* strains (cut-off, 0.175). **(B)** *B. animalis* subsp. *lactis* strain PFGE profiles with *Xba*I (1) Marker; (2) BS01; (3) BS05; (4) MB2409; (5) BB12; (6) BS-CT.

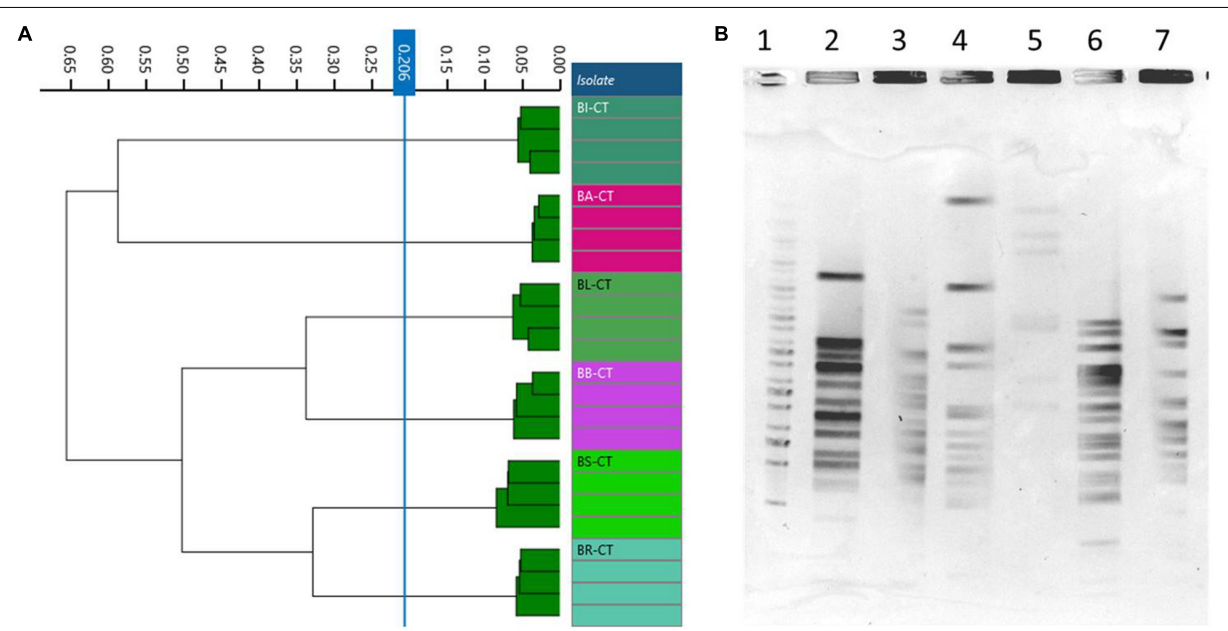

FIGURE 5 | Comparison of Fourier transform infrared (FTIR) and pulsed-field gel electrophoresis (PFGE) typing methods on *Bifidobacterium* species/subspecies. **(A)** Dendrogram obtained by clustering FTIR spectra for *Bifidobacterium* type strains (cut-off, 0.206). **(B)** Type strain PFGE profiles with *Xba*I (1) Marker; (2) BA-CT; (3) BI-CT; (4) BR-CT; (5) BB-CT; (6) BL-CT; (7) BS-CT.

FTIR technology can be suitable for the routine of probiotic industry laboratories—thanks to its huge advantages with respect to DNA- based techniques, such as the ease of use, the fast turnaround time, the user-friendly software, and the relatively low running costs. This study can pave the way for the use of FTIR in the probiotic industry for typing other species

belonging to *Bifidobacterium*, other genera/species traditionally used as probiotics, and novel strains with probiotic potential.

DATA AVAILABILITY STATEMENT

The dataset including the genome sequence of the strain BL03, DLBL07, DLBL09, BS01, BS05, and MB2409 analyzed in this study can be found in the BioProject PRJNA716495 at DDBJ/ENA/GenBank database, where the genome sequence are available under the accession JAGGDB000000000, JAGGDA000000000, JAGGCZ000000000, JAGGCY000000000, JAGGCX000000000, and JAGGCW000000000, respectively. The version described in this paper is the first version.

AUTHOR CONTRIBUTIONS

MP and FD conceived the study. FD performed the PFGE analysis. MC carried out the FTIR experiments. FF and IC performed the MLST and OrthoANI analyses. NBC interpreted the results and contributed to the writing of the manuscript. DDG, MP, and SA critically revised the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: MP and FD are employees of Probiotical Research S.r.L., Novara, Italy. MC is employee of Bruker Daltonik, Bremen, Germany. FF and IC are employees of Microbion S.r.L., San Giovanni Lupatoto, Verona, Italy.

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