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Highly accurate and sensitive absolute quantification of bacterial strains in human fecal samples

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Abstract

Background Next-generation sequencing (NGS) approaches have revolutionized gut microbiome research and can provide strain-level resolution, but these techniques have limitations in that they are only semi-quantitative, suffer from high detection limits, and generate data that is compositional. The present study aimed to systematically compare quantitative PCR (qPCR) and droplet digital PCR (ddPCR) for the absolute quantification of *Limosilactobacillus reuteri* strains in human fecal samples and to develop an optimized protocol for the absolute quantification of bacterial strains in fecal samples.

Results Using strain-specific PCR primers for *L. reuteri* 17938, ddPCR showed slightly better reproducibility, but qPCR was almost as reproducible and showed comparable sensitivity (limit of detection [LOD] around 10^4 cells/g feces) and linearity ($R^2 > 0.98$) when kit-based DNA isolation methods were used. qPCR further had a wider dynamic range and is cheaper and faster. Based on these findings, we conclude that qPCR has advantages over ddPCR for the absolute quantification of bacterial strains in fecal samples. We provide an optimized and easy-to-follow step-by-step protocol for the design of strain-specific qPCR assays, starting from primer design from genome sequences to the calibration of the PCR system. Validation of this protocol to design PCR assays for two *L. reuteri* strains, PB-W1 and DSM 20016^T, resulted in a highly accurate qPCR with a detection limit in spiked fecal samples of around 10^3 cells/g feces. Applying our strain-specific qPCR assays to fecal samples collected from human subjects who received live *L. reuteri* PB-W1 or DSM 20016^T during a human trial demonstrated a highly accurate quantification and sensitive detection of these two strains, with a much lower LOD and a broader dynamic range compared to NGS approaches (16S rRNA gene sequencing and whole metagenome sequencing).

Conclusions Based on our analyses, we consider qPCR with kit-based DNA extraction approaches the best approach to accurately quantify gut bacteria at the strain level in fecal samples. The provided step-by-step protocol will allow scientists to design highly sensitive strain-specific PCR systems for the accurate quantification of bacterial strains of not only *L. reuteri* but also other bacterial taxa in a broad range of applications and sample types.

Keywords DNA extraction, qPCR, ddPCR, Strain-specific primers, *Lactobacillus*, *Limosilactobacillus reuteri*

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Introduction

The human gut microbiota is a complex microbial community dominated by bacteria [1, 2] which plays an important role in host physiology and health, including the development of the immune system [3], colonization resistance against pathogens [4], nutrition utilization [5], and neural development [6]. Altered microbiota configurations (often termed dysbiosis) have been associated with not only intestinal diseases (e.g., colorectal cancer, inflammatory bowel disease) [7, 8] but also a complex range of chronic diseases (e.g., obesity, cardiovascular disease, diabetes, allergies) [9, 10]. Therefore, characterizing the gut microbial composition is a crucial step to explore its role in host physiology and to develop strategies aiming at microbiome modulation to improve health. As many microbial functional capacities are strain specific [11] due to strain-level genomic variations [12, 13], determining and quantifying individual strains are essential to establish connections between a certain group of gut microorganisms and host physiological status.

The vast majority of microbiome studies rely on next-generation sequencing (NGS; e.g., 16S rRNA gene sequencing and whole metagenome sequencing [WMS]) to characterize microbial communities, which has revolutionized the field over the last two decades. WMS not only allows a community-wide analysis but also can achieve strain-level resolution [14]. However, NGS data has limitations in that it is compositional (and thus only semi-quantitative) and suffers from a limited dynamic range and low sensitivity. Many studies have implicated alterations in the absolute abundance of specific species or strains, sometimes at very low levels, in effects of host physiology [15–18], demonstrating the importance of quantitative and sensitive detection methods. The inclusion of quantitative methods such as quantitative PCR (qPCR) [19] and flow cytometry [20] can be used to make NGS data more quantitative, but the data remains compositional, and detection limits are high. Therefore, there is a clear need for quantitative techniques that allow the accurate and sensitive detection and absolute quantification of specific microbial species or strains, such as studies that use probiotics or live biotherapeutics, which track target microbes with lower abundance (e.g., after vertical transmission, fecal microbiota transplantation, or translocation to host tissues), as well as confirming the presence of bacterial species or strains in low-biomass samples.

qPCR has been widely used to quantify members of the gastrointestinal microbiota (e.g., *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis* subsp. *lactis*, *Limosilactobacillus reuteri*, *Lacticaseibacillus casei*, and *Limosilactobacillus fermentum*) at the strain level [21–25]. However, qPCR has several limitations: (i) it is potentially affected by PCR efficiency and relies on external standards [26], and (ii) it is susceptible to inhibitors existing in the environmental or fecal samples [27–29]. Compared to qPCR, droplet digital PCR (ddPCR) is regarded as a more accurate and sensitive approach that does not require a calibration curve. ddPCR is based on individual amplification of targets in thousands of nanoliter-scale PCR reactions [30]. It has been applied to detect trace nucleic acid targets from clinical samples (e.g., blood and tissue) [30–32] and to quantify microorganisms from environmental and animal samples [33–35]. However, the performance of qPCR and ddPCR in terms of detection and quantification for target microorganisms from human fecal samples has not been systematically evaluated and compared. In addition, detailed and standardized protocols for strain-specific PCR primer design and accompanying validation workflows that are easily applicable have not been published to date.

The overall objective of this study was to design an optimized PCR-based approach for the quantitative detection of bacterial strains in human fecal samples in terms of sensitivity (limit of detection [LOD]), accuracy, reproducibility, time, and cost. To achieve this, we systematically compared qPCR and ddPCR in combination with three well-established DNA extraction methods for the strain-specific quantification of an *L. reuteri* strain in human fecal samples. Based on these comparisons and information from previous studies [21–23], we developed an easy-to-follow, step-by-step protocol for strain-specific qPCR assays that includes the identification of strain-specific marker genes and designing and validating the primers. We applied this protocol in designing strain-specific qPCR assays for two *L. reuteri* strains, PB-W1 and DSM 20016 ^{T}, and validated the PCR assays using spiked fecal samples as well as samples collected from human subjects who received live *L. reuteri* PB-W1 or DSM 20016 ^{T} as a part of a human trial, allowing direct comparisons between qPCR and NGS approaches.

Materials and methods

Growth conditions of bacteria

L. reuteri strains were grown on MRS agar plates (BD Difco Microbiology, Houston, TX, USA) for 48 h in an anaerobic chamber at 37 °C. Single colonies were picked and transferred to MRS broth (BD Difco Microbiology) and subcultured twice (24 h for the first subculture and 8 h for the second subculture to ensure bacterial cells are in the late exponential phase or early stationary phase and therefore highly active and alive) [36, 37]. Bacteria were harvested and used to spike human fecal samples (see the section below). Cell numbers in 8-h cultures

were determined by quantitative plating on MRS agar plates.

Fecal samples spiked with *L. reuteri* DSM 17938

Human fecal samples were collected from nine healthy individuals at the University of Alberta Human Nutrition Research Unit (Edmonton, Canada). All procedures were approved by the Health Research Ethics Board — Biomedical Panel of the University of Alberta (protocol no. Pro00077565). The absence of *L. reuteri* DSM 17938 was confirmed using qPCR as described previously [38], and all samples were *L. reuteri* DSM 17938 negative (Table S1). To evaluate and compare the performance of qPCR and ddPCR, three *L. reuteri*-negative fecal samples were selected, and aliquots of each sample were spiked with known quantities of *L. reuteri* DSM 17938: serial dilutions of the 8-h subcultured *L. reuteri* DSM 17938 with cell numbers were prepared with ice-cold phosphate buffered saline (PBS: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, Milli-Q Water 1 L, pH 7.0), resulting in fecal aliquots with *L. reuteri* DSM 17938 of 9.3×10^7, 9.3×10^6, 9.3×10^5, 9.3×10^4, 4.7×10^4, 2.3×10^4, 1.2×10^4, and 5.9×10^3 cells/g (Fig. S1). These spiked aliquots were stored at − 80 °C until DNA isolation.

DNA extraction from fecal samples

Three well-established protocols for isolating total DNA from human fecal samples were tested and compared in this study: a phenol–chloroform-based method [39], a modified method based on the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) [22, 40], and an optimized kit-based method based on the protocol Q described previously [41]. The purity of DNA was determined spectrophotometrically. Detailed procedures of these DNA isolation methods, including recipes for solutions and equipment used, are provided in Supplementary File 2.

Phenol–chloroform-based method (PC)

One gram of stool sample was weighted and diluted tenfold in ice-cold PBS buffer. Samples were vortexed vigorously, and 1 ml of the solution (i.e., 0.1 g of raw sample) was centrifuged (8000 × g for 5 min at 4 °C) and washed for three times with ice-cold PBS buffer. After centrifugation, cell pellets were resuspended in 750-µl lysis buffer and incubated at 37 °C for 20 min. After 85 µl of 10% SDS solution and 30-µl proteinase K (20 mg/ml) were added, the mixtures were incubated at 60 °C for another 30 min. Then 500 µl of phenol–chloroform-isoamyl alcohol (25:24:1) was added, and the DNA isolation was conducted following the procedures as described before [39].

QIAamp fast DNA stool mini kit-based method (QK)

This method was adapted from our previous publications [22, 40] with minor modifications. Fecal samples were washed with ice-cold PBS as described above for the method PC. Cell pellets were resuspended in 100 µl of lysis buffer and incubated at 37 °C for 30 min. Then 1 ml of buffer InhibitEX was added, and samples were homogenized thoroughly by vortexing and bead beating. After that, DNA was extracted with the use of QIAamp Fast DNA Stool Mini Kit (Qiagen).

Protocol Q-based method (PQ)

We followed the procedures of protocol Q developed previously [41] but further optimized it through adding two pre-treatment steps. Specifically, prior to conducting the original protocol Q, fecal samples were washed in ice-cold PBS as described for the method PC and incubated with 100 µl of lysis buffer at 37 °C for 30 min.

Quantification of *L. reuteri* DSM 17938 in fecal samples using ddPCR

Cell numbers of *L. reuteri* DSM 17938 were determined for the spiked fecal aliquots using the strain-specific primers developed previously [38]. This primer pair (1694f: 5′-TTAAGGATGCAAACCCGAAC-3′ and 1694r: 5′-CCTTGTCACCTGGAACCACT-3′) targets a chromosome-located surface protein gene that has a single copy on the genome, and the length of the target region is 177 bp [38]. ddPCR was performed using EvaGreen intercalating DNA dye to detect positive droplets. Each ddPCR reaction contained 1 µl of DNA (given the high original concentration, DNA extracted using PC and PQ was treated with a tenfold and threefold dilution, respectively), 12.5 µl of 2 × EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 200-nM primer each, and ddH_2O to bring the per-reaction volume to 25 µl in each well of a 96-well plate. The plate was put into a QX200 Auto Droplet Generator (Bio-Rad Laboratories Inc.), and EvaGreen droplet generation oil (Bio-Rad Laboratories Inc.) was added according to the manufacturer's manual. PCR reactions were conducted in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc.) as follows: (Step 1) 95 °C for 5 min, (Step 2) 95 °C for 30 s, (Step 3) 62 °C for 1 min, (Step 4) repeat steps 2 and 3 for 39 cycles, (Step 5) 4 °C for 5 min, (Step 6) 90 °C for 5 min, and (Step 7) hold at 4 °C. After the reaction, the plate was placed in the block of a Bio-Rad QX200 Droplet Reader (Bio-Rad Laboratories Inc.). Droplets were read one at a time, and data were analyzed using QuantaSoft Analysis Pro 1.0 (Bio-Rad Laboratories Inc.).

Quantification of *L. reuteri* DSM 17938 in fecal samples using qPCR

L. reuteri DSM 17938 was quantified by qPCR in the same samples and with the same primers as used for ddPCR [38]. The standard curve was constructed based on serial dilutions of *L. reuteri* DSM 17938 genomic DNA. The original copy number of the standard material was calculated based on the DNA concentration determined spectrophotometrically using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). This generated a standard curve ranging from 4×10^5 to 4 gene copies per microliter (µl), which covered a dynamic range of 4×10^8 to 4×10^3 copy numbers of *L. reuteri* per gram of fecal samples and showed excellent linearity ($R^2>0.99$) and acceptable efficiency ($E=89\%$; Fig. S2). The 20 µl of PCR mixture consisted of 1 µl of DNA, 10 µl of SensiFAST SYBR Hi-ROX Mix (FroggaBio, Vaughan, ON, Canada), 0.6 µl of each primer (10 µM), and 8.4 µl of nuclease-free water. The PCR program was composed of an initial denaturation step for 3 min at 95 °C, followed by 40 cycles of denaturation for 5 s at 95 °C, annealing and extension for 30 s at 62 °C, and fluorescent signal acquisition. PCR was performed using a HT 7900 machine (Thermo Fisher Scientific). After 40 cycles of PCR amplification, melting curves were evaluated to verify the correct product. The reaction program for melting curve analysis included a denaturation for 15 s at 95 °C, lowered to 60 °C for 1 min, and increased to 95 °C for 15 s with continuous fluorescence readings. The melting curve analysis showed one specific peak at around 87.5 °C (Fig. S3), confirming the good quality of the amplification, and there is nonspecific amplification or primer dimers.

Comparison between ddPCR and qPCR

For both approaches, data for gene copies per reaction were transformed to absolute copy numbers per gram feces following the formula $(C\times V_T)/(V_U\times M)$, where C is gene copies measured per reaction, V_T is the elution volume of extracted DNA, V_U is the volume of DNA used, and M is the amount of fecal sample used in DNA extraction. To systematically compare the two assays, parameters representing reproducibility, linearity, sensitivity, and accuracy, as well as the required time and costs, were evaluated. Specifically, the coefficient of variation (CV; the ratio between the standard deviation and the mean of three samples at each spiking concentration) was calculated to determine the reproducibility. Linearity measures how well the copy numbers evaluated by PCR assays related to the actual spiked cell series, which was assessed by calculating the R^2 of the linear regression model with spiked cell numbers as the dependent variable and copy numbers of PCR as the independent variable. To evaluate the sensitivity, the limit of detection (LOD; i.e., the lowest cell number at which target bacteria can differentially detected over the background noise) was determined. More specifically, the LOD was defined as the lowest amount of spiked cells that could be detected in more than 95% of replicates ($n=9$ for each spiked concentration, including both technical and experimental replicates in this study) [42]. For qPCR, as we observed the noise amplification from background (blank fecal samples without spiked *L. reuteri*) after 35 cycles, a threshold Cq value > 35 was considered undetected. For ddPCR, single well thresholding was used to distinguish positive and negative reactions through grouping droplets based on software's default setting. The accuracy of the two PCR assays was evaluated by determining the recovery rate, calculated as the ratio between the copy numbers per gram of feces measured from PCR and the cell numbers spiked [43]. Costs for both assays were compared on the basis of being performed on 96-well plates. Each 96-well plate accommodates 25 samples (in triplicate, excluding 6 serial dilutions as standards and 1 non-template-negative control) for qPCR and 30 samples (in triplicate, excluding 1 positive control and 1 negative control) for ddPCR. Cost for labor was not considered in this estimation.

Design and in silico evaluation of strain-specific primers

Strain-specific primers were designed based on gene sequences identified to be unique to particular strains using the Single Genes tool in IMG/MER phylogenetic profiler function from Joint Genome Institute (JGI; https://jgi.doe.gov/). The general workflow for developing and evaluating the strain-specific primers in silico is summarized in Fig. 1, and the in-detail protocol is summarized in Supplementary File 3. Validation of this protocol was performed using two *L. reuteri* strains, PB-W1 and DSM 20016 ^{T}. *L. reuteri* strain PB-W1 was isolated from a fecal sample of a rural Papua New Guinean [44]. DSM 20016 ^{T} is the type strain of *L. reuteri* that clusters in the phylogenetic lineage II of the species [13, 45, 46], which was recently described as subspecies *L. reuteri* subsp. *reuteri* [47]. Due to the high genetic homogeneity of lineage II [46], strain-specific genes could not be identified for the strains within this lineage, and thus, lineage-specific primers were designed. As the prevalence of *L. reuteri* in the human gastrointestinal tract in industrialized societies is low [46, 48, 49], lineage-specific primers were considered specific enough for the detection of DSM 20016 ^{T} in human fecal samples. Primer sets were designed by the Primer3Plus program [50]. The quality of primers was evaluated in silico using OligoAnalyzer (Integrated DNA Technologies, Coralville, IA, USA). The specificities of designed primers were verified in silico using Primer BLAST. Sequences and amplicon size of

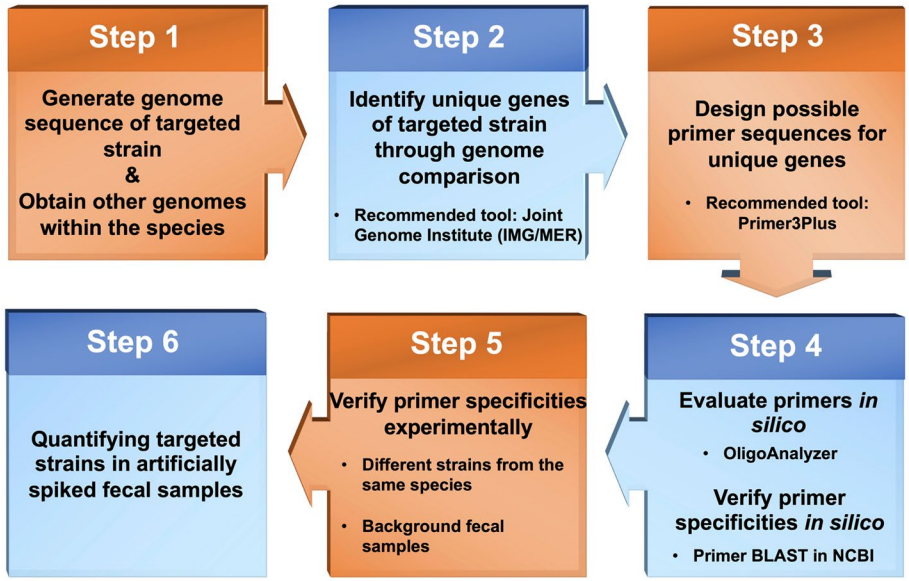

Fig. 1 A flow chart of main steps for designing and validating strain-specific primers

Table 1 Strain- and lineage-specific primers designed for *L. reuteri* PB-W1 and DSM 20016 ^{T}

Strain	Primer name	Nucleotide sequence (5′-3′)	Amplicon size
PB-W1	PLD-F	TCGTGCTCCTAGAGATGGGA	174 bp
	PLD-R	ACTTCTCCAGCTTTTACTGACGA	
DSM 20016 ^{T}	DHP-F	GTGTTAAAGAGGTTGCTAGAAAGTATT	139 bp
	DHP-R	GCCAGCTTAAATTCCTTTGAATAGC	

the primers that resulted from this process are listed in Table 1.

Experimental validation of strain-specific qPCR systems

The specificity of primer pairs was established by qPCR with genomic DNA from each of 19 human-origin *L. reuteri* strains (Table 2) and then further assessed against the background DNA of the nine fecal samples (Table S1). The absence of *L. reuteri* in these fecal samples was confirmed by plating on *L. reuteri* selective isolation medium (LRIM), as described previously [12]. To evaluate the performance of primers in quantifying *L. reuteri* from human stools, three fecal samples without detectable *L. reuteri* were selected and spiked with PB-W1 and DSM 20016 ^{T}, respectively, following the same procedure as described for DSM 17938 (Fig. S1) with specific bacterial concentrations (2.2×10^{8}, 2.2×10^{7}, 2.2×10^{6}, 2.2×10^{5}, 8.9×10^{4}, 3.5×10^{4}, 1.4×10^{4}, 5.6×10^{3}, 2.2×10^{3}, 9.1×10^{2} cells/g feces for PB-W1 and 1.0×10^{8}, 1.0×10^{7}, 1.0×10^{6}, 1.0×10^{5}, 1.0×10^{4}, 4.1×10^{3}, 1.6×10^{3}, 6.5×10^{2} cells/g feces for DSM 20016 ^{T}). After extracting DNA using the method PQ, cell numbers of *L. reuteri* PB-W1 and DSM 20016 ^{T} were determined using qPCR with the respective primer pairs. The 20 µl of PCR mixture consisted of 1 µl of DNA, 10 µl of SensiFAST SYBR Hi-ROX Mix (FroggaBio), 0.8 µl of each primer (at a concentration of 0.8 µM), and 8.2 µl of nuclease-free water. The PCR cycling was composed of an initial denaturation for 3 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 63 °C for 10 s, and extension at 72 °C for 15 s. Standard curves for *L. reuteri* PB-W1 and DSM 20016 ^{T} were generated with serial dilutions of genomic DNA of PB-W1 and DSM 20016 ^{T} from standardized cultures from which cell numbers were determined by quantitative plating.

Validation of strain-specific qPCR assays in human subjects who received live L. reuteri

To further validate the specificity and accuracy of our strain-specific qPCR assays, we conducted an analysis using a subset of fecal samples obtained from a recently completed human clinical trial (ClinicalTrials.gov Identifier: NCT03501082). These samples were collected at seven different time points over a period of

Table 2 Validation of the specificity of designed primer sets against other human-origin *L. reuteri* strains

Strain	Lineage[a]	Genome accession number	Host origin	Cq mean	
				PLD-F + PLD-R[b] (PB-W1)	DHP-F + DHP-R[b,d] (DSM 20016[T])
PB-W1	VII	GCA_020785355.1	Human	**10.0**	-
DSM 20016[T]	II	GCA_000016825.1	Human	-	**9.5**
FJ3	II	GCA_020785495.1	Human	-	NT
LMS11-1	II	GCA_020785575.1	Human	-	NT
LMS11-3	II	GCA_020785585.1	Human	-	NT
ME-261	II	GCA_020785505.1	Human	-	NT
ME-262	II	GCA_020785535.1	Human	-	NT
MM2-3	II	GCA_000160715.1	Human	-	NT
MM4-1a	II	GCA_000159475.2	Human	-	NT
SR11	II	GCA_020785595.1	Human	-	NT
SR14	II	GCA_020785545.1	Human	-	NT
CF48-3A	VI	GCA_000159615.1	Human	-	> 33[d]
Cor124_1_1	VI	GCA_020785655.1	Human	-	-
Cor137_1_1	VI	GCA_020785635.1	Human	> 35[b,d]	> 34[d]
Cor137_3_1	VI	GCA_020785735.1	Human	> 36[b,d]	> 34[c,d]
DSM 17938	VI	NA[c]	Human	-	-
M81-R43	VI	GCA_020785435.1	Human	-	-
MM34-4A	VI	GCA_002112805.1	Human	-	-
MM36-1a	VI	GCA_020785455.1	Human	-	> 34
MV4-1a	VI	GCA_020785395.1	Human	> 37[d]	-
PB-W2	VI	GCA_020785295.1	Human	-	-

[a] Lineage information was obtained from our previous studies [12, 13, 44–47]

[b] Dash, no amplification

[c] *NA*, not available

[d] *NT*, not tested because strains from the same lineage have low genetic homogeneity and are identical at the target site as DSM 20016[T]

21 days during the control phase with no dietary intervention, including baseline and days 4, 6, 8, 12, 16, and 21. On day 4 of the trial, a single dose of approximately 2.25×10^{10} viable cells of either *L. reuteri* strain PB-W1 or DSM 20016 [T] was administrated to 8 and 11 participants, respectively. The respective strain-specific qPCR assay was used in the two groups as described above; selective culture and NGS approaches (i.e., 16S rRNA gene sequencing and whole metagenome sequencing [WMS]) were also applied.

Microbial genomic DNA of each fecal sample was extracted using method QK, and the absolute abundance of PB-W1 and DSM 20016 [T] was estimated as described above. To determine the viable cells of *L. reuteri* in the fecal samples, we performed quantitative culture on the LRIM under the anaerobic condition at 45 °C for 48 h, which has been validated to be sufficiently specific for the quantification of *L. reuteri* [12]. 16S rRNA gene sequencing was performed for all the fecal samples, targeting the V5–V6 region using the primer pair 784F (5′-RGGATTAGATACCC-3′) and 1064R (5′-CGACRRCCATGCANCACCT-3′). This protocol has been shown to efficiently detect *L. reuteri* in human fecal samples, with sufficient taxonomic resolution in the V5–V6 region [48]. Amplicons were sequenced on the Illumina MiSeq PE300 platform (2 × 300 bp paired-end sequencing) at the University of Minnesota Genomics Center (Minneapolis, MN, USA). Baseline and day 8 (4 days after probiotic administration) samples were subjected to WMS using the Illumina NovaSeq6000 S4 PE150 platform (2 × 150 bp paired-end sequencing) at the Génome Québec Innovation Centre (Montréal, QC, Canada). The relative abundance of *L. reuteri* at the species level in the 16S rRNA gene sequencing and WMS datasets was determined using QIIME2 (based on the SILVA database, version 138) [51] and MetaPhlAn 4.0 [52], respectively. After denoising, merging paired-end reads, and eliminating chimeras, an average of 31,060 ± 1316 (mean ± SEM) 16S rRNA gene reads per sample were retained for subsequent analysis. For the WMS dataset, an average of

Table 3 Comparison of performance between qPCR and ddPCR based on three DNA extraction methods

Method	*L. reuteri* DSM 17938 spiked (Log_{10} cells/g)	Phenol–chloroform-based method (PC)		QIAamp Fast DNA Stool Mini Kit-based method (QK)		Protocol Q-based method (PQ)	
		Mean ± SD[b] (Log_{10} cells/g)	CV[c] (%)	Mean ± SD (Log_{10} cells/g)	CV[c] (%)	Mean ± SD (Log_{10} cells/g)	CV[c] (%)
qPCR	7.97	7.53 ± 0.12	1.58	7.62 ± 0.13	1.77	7.89 ± 0.12	1.53
	6.97	6.53 ± 0.17	2.59	6.63 ± 0.14	2.12	6.91 ± 0.11	1.55
	5.97	5.61 ± 0.24	4.23	5.63 ± 0.21	3.66	5.90 ± 0.04	0.60
	4.97	4.92 ± 0.34	6.85	4.71 ± 0.16	3.44	5.04 ± 0.11	2.20
	4.67	4.86 ± 0.30	6.17	4.46 ± 0.18	3.96	4.75 ± 0.10	2.18
	4.37	-	-	4.31 ± 0.16	3.67	4.54 ± 0.14	2.98
	4.07	-	-	4.08 ± 0.23	5.61	4.28 ± 0.07	1.67
	3.77	-	-	3.95 ± 0.17	4.22	4.11 ± 0.15	3.67
	Blank[a]	4.72 ± 0.49	10.38	3.72 ± 0.30	8.09	3.89 ± 0.15	3.81
ddPCR	7.97	7.43 ± 0.04	0.59	7.80 ± 0.03	0.34	7.86 ± 0.08	1.07
	6.97	6.41 ± 0.07	1.03	6.72 ± 0.02	0.30	6.87 ± 0.11	1.66
	5.97	5.40 ± 0.12	2.17	5.66 ± 0.04	0.74	5.89 ± 0.11	1.79
	4.97	4.88 ± 0.15	3.01	4.66 ± 0.05	1.10	4.85 ± 0.09	1.92
	4.67	4.76 ± 0.09	1.93	4.26 ± 0.16	3.69	4.54 ± 0.05	1.17
	4.37	-	-	4.06 ± 0.05	1.25	4.29 ± 0.10	2.33
	4.07	-	-	3.74 ± 0.20	5.38	4.21 ± 0.23	5.41
	3.77	-	-	3.62 ± 0.21	5.91	3.86 ± 0.20	5.28
	Blank[a]	4.28 ± 0.22	5.20	3.57 ± 0.19	5.34	3.66 ± 2.21	60.26

[a] Blank, fecal samples without *L. reuteri* DSM 17938 spiked

[b] Dash, beyond the limit of detection (LOD)

[c] *CV* Coefficient of variation

30.2 ± 2.0 (mean ± SEM) million read pairs per sample remained for our analysis after quality control, removing sequences identified as bacteriophage phiX174, and filtering out human DNA reads.

Statistical analysis

All the statistical analyses (e.g., descriptive statistics, R^2, and linear regression) of this study were conducted using R 3.4.2 [53].

Results

Comparison of three DNA extraction methods

The DNA concentrations varied substantially among three protocols. PC generated the highest DNA concentration (1525 ± 290 ng/μl; mean ± SD), followed by PQ (694 ± 76 ng/μl) and QK (259 ± 41 ng/μl) (Table S2). All DNA solutions showed adequate $A_{260/280\ nm}$ values (range, 1.95–2.12), with PQ slightly higher, indicating a more efficient removal of protein from DNA (Table S2). Meanwhile, $A_{260/230\ nm}$ value, indicating contaminations of phenol, carbohydrate, or humic acid, showed excellent values for the kit-based DNA isolation methods (2.10 ± 0.15 for PQ and 1.99 ± 0.15 for QK), while PC did not perform well (1.74 ± 0.16) (Table S2). Overall, although all three DNA isolation protocols produced nucleic acids with acceptable quantity for downstream PCR-based methods, DNA isolated with the kit-based protocols (QK and PQ) showed higher quality.

Comparison between qPCR and ddPCR

Performance of qPCR and ddPCR in detecting and quantifying *L. reuteri* strain DSM 17938 in fecal samples, in aspects of reproducibility, linearity, sensitivity, and accuracy, was evaluated and compared based on DNA isolated from human stools of three subjects spiked with established cell numbers of DSM 17938.

Reproducibility

The coefficient of variation (CV) among three aliquots at each spiking concentration was calculated as a measure of the reproducibility, and a CV ≤ 25% has been suggested previously as the threshold of acceptable reproducibility [54]. ddPCR had a higher reproducibility as its CV values were consistently lower than qPCR for DNA isolation methods QK and PC (QK: 0.30–5.91% of ddPCR vs. 1.77–5.61% of qPCR; PC: 0.59–3.01% of ddPCR vs. 1.58–6.85% of qPCR; Table 3), which is consistent with

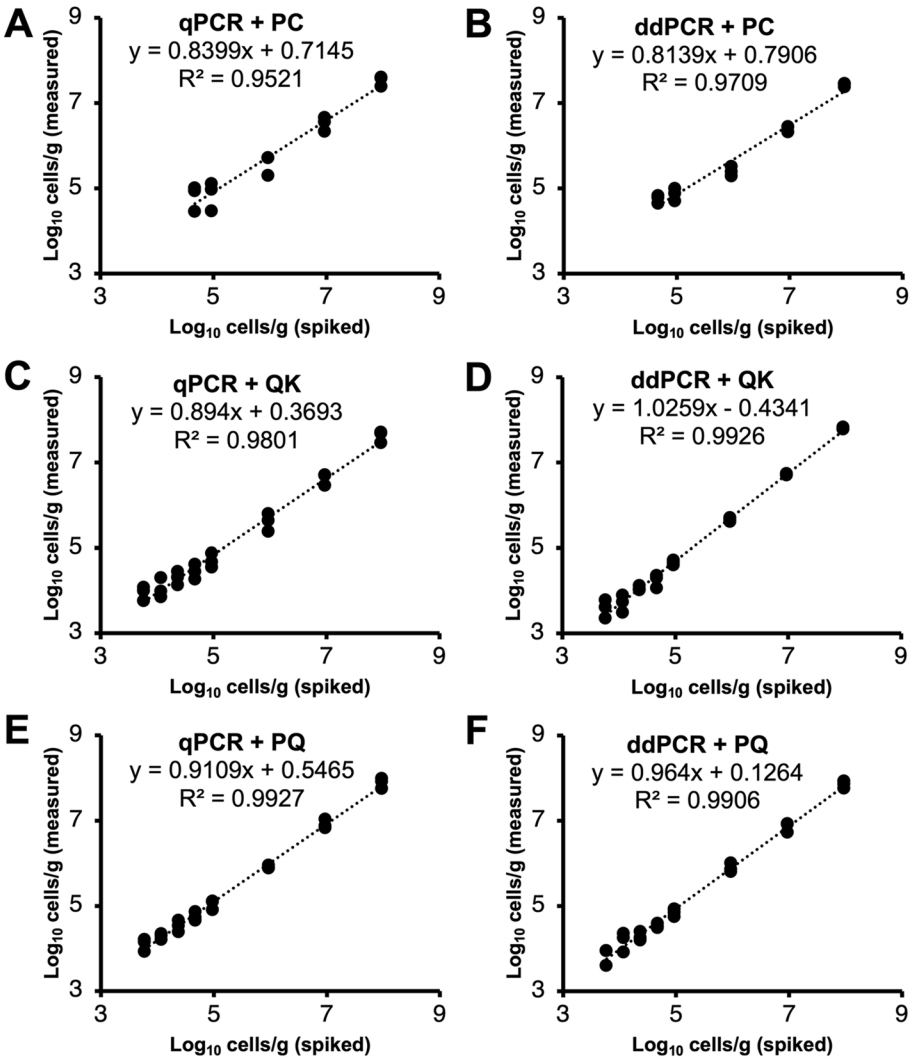

Fig. 2 Relationships between actual spiked cells/g of *L. reuteri* DSM 17938 and measured cells/g from qPCR and ddPCR assays, based on three different DNA extraction methods. Phenol–chloroform-based method (PC) combined with qPCR (**A**) and ddPCR (**B**). QIAamp fast stool DNA kit-based method (QK) combined with qPCR (**C**) and ddPCR (**D**). Protocol Q-based method combined with qPCR (**E**) and ddPCR (**F**). Spiked cells/g of *L. reuteri* DSM 17938 was estimated from quantitative culture on the MRS plate. Each bacterial concentration was conducted in biological triplicates

the literature [55–57]. However, when the DNA isolation method PQ was applied, CVs were comparable between ddPCR and qPCR (range of CV 1.07–5.41% for ddPCR vs. range of CV 0.60–3.67% for qPCR) (Table 3). With lower cell numbers of *L. reuteri* spiked into the stool aliquots, the CV values of ddPCR and qPCR increased, meaning their precision decreased. However, CV values among three fecal aliquots at each spiking cell density of *L. reuteri* were all lower than 25%, demonstrating a high reproducibility across the tested dynamic range for both ddPCR and qPCR.

Linearity

Linearity is a measure of how well the copy numbers of the target strain estimated by PCR relate to the actual spiked cell serial dilution. Both qPCR (R^2 = 0.9801 and 0.9927 for QK and PQ, respectively) and ddPCR (R^2 = 0.9926 and 0.9906 for QK and PQ, respectively) exhibited high linearities based on kit-based DNA isolation approaches (QK and PQ) (Fig. 2), which are all above the acceptable R^2 threshold of 0.98 [58]. This indicates that both qPCR and ddPCR accurately quantify *L. reuteri* from fecal samples if kit-based DNA isolation protocols are chosen. We also examined the consistency between qPCR and ddPCR based on the DNA isolated using PQ

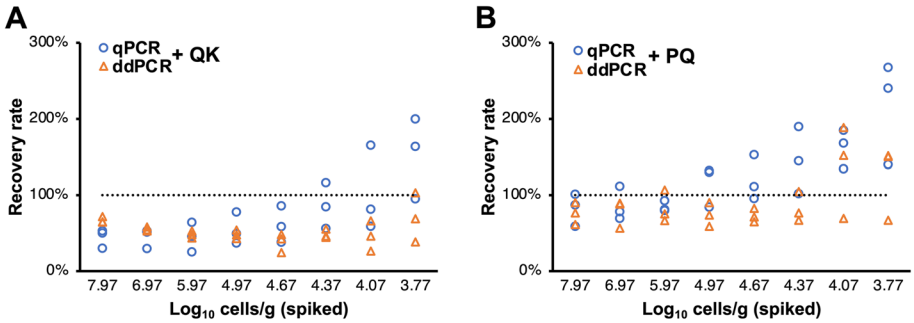

Fig. 3 The accuracy (represented by the recovery rate) of qPCR and ddPCR. DNA extracted from fecal samples (biological triplicates) spiked with *L. reuteri* DSM 17938 using QK (**A**) and PQ (**B**) were analyzed by qPCR and ddPCR. The recovery rate is defined as the ratio between the cell numbers per gram feces measured and the actual cell numbers spiked, as suggested previously [43]. Results were compared with 100% recovery which is indicated by the dotted line. QK, QIAamp Fast Stool DNA Kit-based method and PQ, protocol Q-based method

and confirmed that these two PCR techniques had a high level of agreement with each other ($y = 1.0607x - 0.4746$, $R^2 = 0.9913$; Fig. S4). The linearities of qPCR and ddPCR were both declined when PC was used ($R^2 = 0.95$ and 0.97 for qPCR and ddPCR, respectively), which are lower than the acceptable R^2 threshold of 0.98.

Sensitivity

The limit of detection (LOD) was determined to reflect the sensitivity of qPCR and ddPCR. By using the DNA extraction methods QK and PQ, *L. reuteri* DSM 17938 could still be detected at the input of 3.77 Log_{10} cells/g feces in both qPCR and ddPCR analyses, with no differences between the two methods (Table 3). LOD of both PCR approaches was 4.67 Log_{10} cells/g feces when the method PC was applied (Table 3). Therefore, the LOD were equal for both qPCR and ddPCR (Table 3 & Fig. 2), while the sensitivity mainly depended on the DNA isolation approach. Better performance of detection and quantification was observed when methods QK and PQ were used comparing with PC. Given that DNA isolated with PC performed consistently poorer in terms of linearity and sensitivity in both ddPCR and qPCR, this method was excluded from further analyses.

Accuracy

The accuracy of qPCR and ddPCR was evaluated by determining the recovery rate, which is defined as the percentage of cells detected out of the theoretical spiking concentration [43]. PQ resulted in higher accuracy for both qPCR and ddPCR compared to QK (Fig. 3), indicating a higher efficiency of PQ in the harvest of DNA from *L. reuteri*. When the sample contained a high density of *L. reuteri* (4.97–7.97 Log_{10} cells/g), ddPCR and qPCR showed comparable accuracy, regardless of PQ or QK was used (Fig. 3). However, when *L. reuteri* was spiked at a low cell density (from 3.77 to 4.37 Log_{10} cells/g), the choice of DNA isolation approach and PCR assay affected the accuracy of quantification. Specifically, within this *L. reuteri* spiking range, qPCR had a higher accuracy than ddPCR when QK was applied to isolate DNA, while both qPCR and ddPCR overestimated the cell numbers of *L. reuteri* when PQ was used (Fig. 3). Nevertheless, the discrepancy caused by this overestimation was < 0.34 Log_{10} cells/g, and therefore, this bias may be negligible when quantifying gut bacteria in fecal samples.

Costs

Assuming that all samples, standard materials, and controls are run in triplicate, as shown in Table 4, the cost per sample for ddPCR was almost four times as high as that for qPCR (CAD $9.6 vs. CAD $2.5). The time spent conducting ddPCR (6.5 h) was also higher when compared to qPCR (2.5 h). Therefore, when additionally considering labor cost, the costs of ddPCR become much higher than qPCR.

Summary

Our systematic comparison of qPCR and ddPCR showed that both approaches, combined with kit-based DNA extraction methods (PQ and QK), allowed a highly reproducible, accurate, and sensitive quantification of a bacterial strain (*L. reuteri* DSM 17938) in human fecal samples. ddPCR is slightly better in terms of reproducibility, but the performance of qPCR is comparable when appropriate DNA isolation methods were used and is much cheaper and faster. Therefore, after the outcomes mentioned above are all considered, we recommend the combination of the DNA extraction method PQ and qPCR as the optimal strategy to quantify *L. reuteri* in human stool, although method QK is almost as good and less time-consuming when compared to PQ.

Table 4 Cost estimation for qPCR and ddPCR

Method	Specifications	Items and time	Cost	Cost per sample
qPCR	• A standard curve (six serial dilutions, in triplicate) • Twenty-five unknown samples and a no template control (all in triplicate)	Reagents Consumables 2.5 h	CAD $60 CAD $20	CAD $3.2
ddPCR	• A positive control (in triplicate) • Thirty unknown samples and a no template control (all in triplicate)	Reagents Consumables 6.5 h	CAD $275 CAD $100	CAD $12.5

For qPCR, 25 samples (in triplicate) can be analyzed on a 96-well plate excluding no template controls. For ddPCR, 30 samples (in triplicate) can be analyzed on a 96-well plate excluding calibrators and controls. Cost for labor was not considered in this estimation

A step-by-step protocol for highly accurate and sensitive quantification of bacterial strains in human fecal samples

Based on the findings obtained above and our previous studies that used qPCR [21–23], we developed a step-by-step protocol for the design of qPCR assays for the accurate quantification of bacterial strains in human fecal samples. The protocol starts from the design of strain-specific primers, followed by the in silico and experimental validation of the primer specificity. The protocol is available as Supplementary File 3.

Validation of the protocol of strain-specific qPCR assays

The protocol was validated using PCR assays developed for two *L. reuteri* strains (PB-W1 and DSM 20016 ^{T}):

Design of strain-specific primers

Genome sequences of 31 *L. reuteri* strains available in public databases, representing six reported phylogenetic lineages, were obtained from JGI genome portal (Table S3). Genome comparisons using the IMG/MER phylogenetic profiler function from JGI identified 96 genes that were unique in PB-W1, and 86 genes were specific to lineage II that contains DSM 20016 ^{T}. The competence factor transport accessory protein (*ComB*) gene (IMG gene ID 2880791457) was selected for PB-W1, and a gene encoding a transposase-like protein (IMG gene ID 2760858907) was selected for DSM 20016 ^{T}, as the basis for primer design, respectively. Primers were designed for each of these two genes using Primer3Plus [50], and primer pairs PLD-F + PLD-R (for PB-W1) and DHP-F + DHP-R (for DSM 20016 ^{T}) were chosen (Table 1).

In silico evaluation of strain-specific primers

The technical characteristics of these two primer pairs were evaluated using OligoAnalyzer. The melting temperatures of hairpin structures were determined at 48.8 °C, 45.3 °C, 25.2 °C, and 37.2 °C, respectively, which were much lower than the annealing temperature (63 °C), making hairpins unlikely. The self-dimer and hetero-dimer analyses revealed that delta G values of these two primer pairs (> − 6.34 kcal/mole and − 6.53 kcal/mole, respectively) were higher than the OligoAnalyzer recommended threshold of − 9 kcal/mole for forming dimers, indicating that the designed primers are not likely to induce primer dimers. BLAST analysis against the entire NCBI NR database resulted in no hits from microbial genomes for primers PLD-F and PLD-R (primers for PB-W1), while hits for DHP-F and DHP-R (primers for DSM 20016 ^{T}) were only from strains belonging to lineage II of *L. reuteri*. Thus, in silico analysis indicated sufficient specificity for both primer pairs.

Experimental validation of primer specificity in vitro

The specificity of designed primer sets was tested using qPCR against genomic DNA of the 2 target strains and 19 *L. reuteri* strains of human origin (Table 2). Most strains other than those for which the primers were designed showed no amplification (Table 2). The primer set for PB-W1 was highly specific for PB-W1 (Cq of qPCR = 10), while the Cq for all other strains were over 35. The primer set for DSM 20016 ^{T} amplified the genomic DNA of this strain with a Cq of 9.5; other strains of lineage II were not tested as they are identical at this target site, while strains not belonging to lineage II all showed Cq over 33. The difference of Cq between target and non-target strains was therefore at least 25 and 23.5 cycles for PB-W1 and DSM 20016 ^{T}, respectively (Table 2), which relates to a cell number difference of more than 10^{7} cells/g feces. Therefore, with the same amount of DNA, non-target strains would only have a negligible impact on the quantification of target strains.

The primer specificity was further validated using qPCR against complex microbial communities of nine human fecal samples (Table S1). No positive amplification was observed with the primer set for PB-W1, while

Table 5 Evaluation of the qPCR assay for quantifying *L. reuteri* PB-W1 and DSM 20016 ^{T} from spiked human fecal samples[a]

***L. reuteri* PB-W1**			***L. reuteri* DSM 20016 ^{T}**		
Cells spiked (Log_{10} cells/g)	**Quantification from qPCR[d] (mean ± SD; Log_{10} cells/g)**	**CV (%)**	**Cells spiked (Log_{10} cells/g)**	**Quantification from qPCR[d] (mean ± SD; Log_{10} cells/g)**	**CV (%)**
8.34	8.15 ± 0.06	0.69	8.01	7.74 ± 0.04	0.54
7.34	7.19 ± 0.09	1.24	7.01	6.71 ± 0.07	1.00
6.34	6.15 ± 0.06	1.03	6.01	5.61 ± 0.05	0.91
5.34	5.17 ± 0.09	1.70	5.01	4.50 ± 0.07	1.60
4.95	4.68 ± 0.09	1.88	4.01	3.80 ± 0.11	2.96
4.55	4.35 ± 0.09	2.06	3.61	3.59 ± 0.18	5.09
4.15	3.98 ± 0.14	3.59	3.21	3.25 ± 0.15	4.53
3.75	3.53 ± 0.13	3.60	2.81[b,c]	2.96 ± 0.21	7.17
3.35[c]	3.05 ± 0.12	3.97	Blank	-[c]	-
2.96[b]	3.08 ± 0.34	11.10			
Blank	-[c]	-			

[a] The method PQ was applied to extract DNA from the spiked human fecal samples, and the cell numbers of *L. reuteri* PB-W1 and DSM 20016 ^{T} were determined using designed strain- and lineage-specific primers.

[b] Limit of detection (LOD)

[c] Dash, no amplification

three fecal samples showed positive readings when the primer set for DSM 20016 ^{T} was tested. For these three DSM 20016 ^{T} positive samples, two of them were merely present at low densities (3.90 and 4.15 Log_{10} cells/g feces). The only stool sample showing high numbers (5.53 Log_{10} cells/g feces) (Table S1) contained culturable *L. reuteri* which could explain the positive PCR reading. Therefore, we concluded that our designed primer sets for PB-W1 and DSM 20016 ^{T} showed high specificity to detect and quantify target *L. reuteri* strains in fecal samples.

Evaluation of strain-specific qPCR assays using spiked fecal samples

The qPCR assays were evaluated with three human fecal samples negative for *L. reuteri* that were then spiked with known cell numbers of either PB-W1 (2.96 to 8.34 Log_{10} cells/g feces) or DSM 20016 ^{T} (2.81 to 8.01 Log_{10} cells/g feces), and DNA isolation was based on method PQ. The qPCR amplification efficiency was 94.2% for the PB-W1 assay and 93.7% for the DSM 20016 ^{T} assay, both laying in the recommended efficiency range of 90–110% [58]. The LOD was 2.96 and 2.81 Log_{10} cells/g feces for the

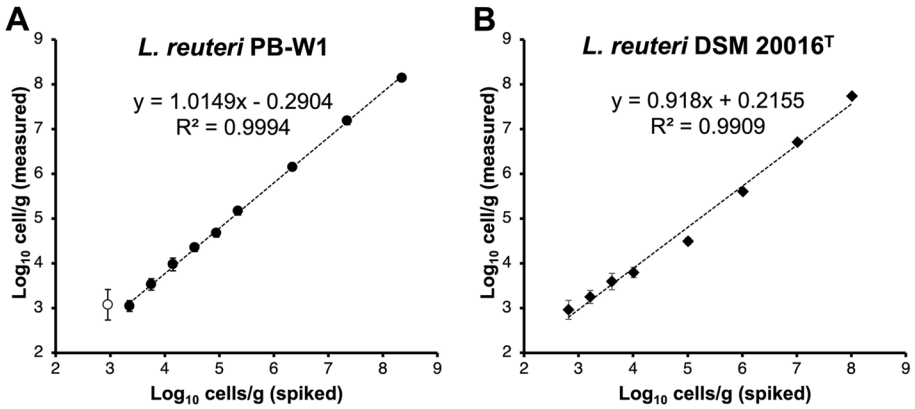

Fig. 4 Linearity between the number of *L. reuteri* cells spiked to stool samples and the cell number measured using our designed qPCR assays for PB-W1 (**A**) and DSM 20016 ^{T} (**B**). The regression line was created between the spiking of 3.35 and 8.34 Log_{10} cells/g feces for PB-W1 and between 2.81 and 8.01 Log_{10} cells/g feces for DSM 20016 ^{T}, respectively, within which detection was reliable. As amplification products were still detectable but lacked reproducibility and linearity with an input of PB-W1 (2.96 Log_{10} cells/g feces), it was shown in open round to highlight the difference. Each error bar displays the standard deviation (SD) from three replicates

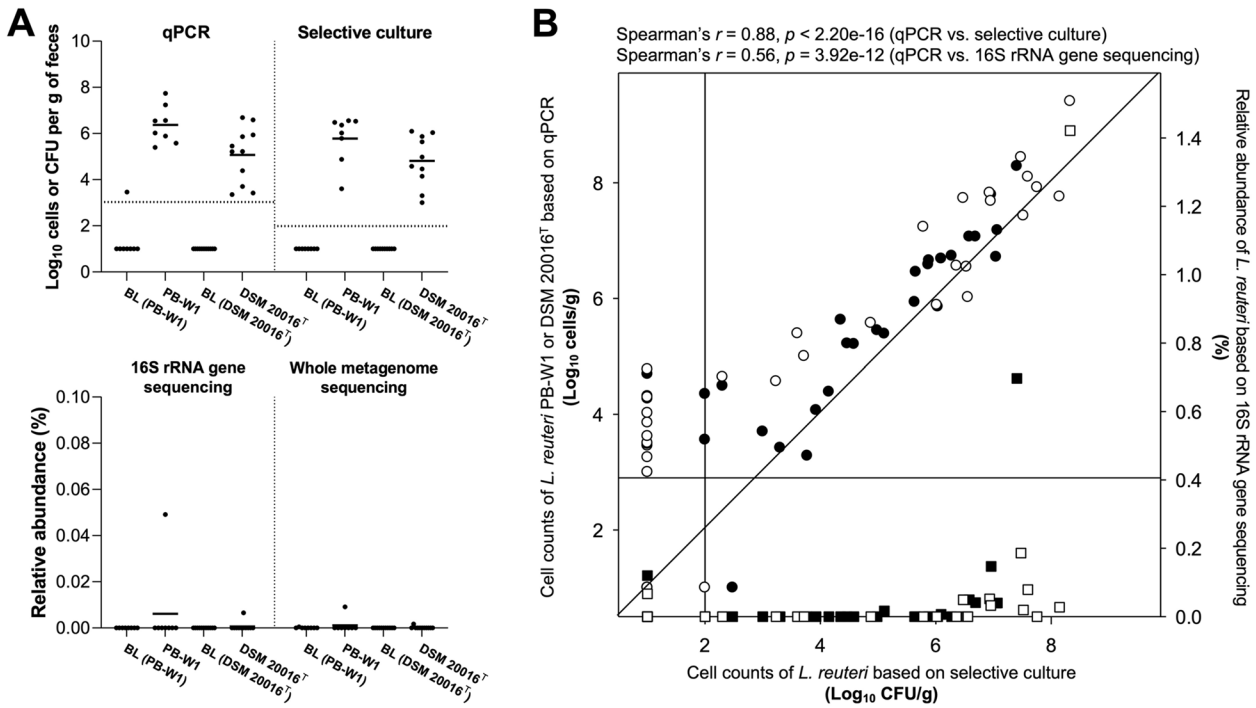

Fig. 5 **A** Quantification of *L. reuteri* in human fecal samples collected at baseline (BL; prior to *L. reuteri* administration) and after treatment (4 days after receiving a single dose of *L. reuteri* PB-W1 or DSM 20016 ^{T}). Quantification was performed using strain-specific qPCR, selective culture, 16S rRNA gene sequencing, and whole metagenome sequencing (WMS). The horizontal dotted lines denote the detection limits of qPCR and selective culture, at around three and two Log_{10} cells/g feces, respectively. Values below the detection limit were plotted with a numerical value of one. **B** Parity plot comparing viable cell counts of *L. reuteri* matching the respective inocula in individuals receiving PB-W1 (open symbols) or DSM 20016 ^{T} (black symbols) to the absolute abundance of the two strains as determined by qPCR (circles) or to relative abundance of *L. reuteri* determined by 16S rRNA gene sequencing (squares). The diagonal line denotes unity, while the horizontal line denotes the detection limit of qPCR at around three Log_{10} cells/g and the vertical line denotes the detection limit of selective culture at two Log_{10} cells/g feces. The detection limit of 16S rRNA gene sequencing is not shown, as it differs from sample to sample depending on the total cell count and the number of reads per sample. The symbols represent samples obtained from 19 individuals who received either *L. reuteri* PB-W1 ($n = 8$) or DSM 20016 ^{T} ($n = 11$)

PB-W1 and DSM 20016 ^{T} assays, respectively (Table 5 & Fig. 4), thus outperforming the qPCR assay for *L. reuteri* DSM 17938 described above. Both qPCR assays showed remarkably high reproducibility (CV of PB-W1 assay ranged from 0.69 to 3.97%, and CV of DSM 20016 ^{T} assay ranged from 0.54 to 7.17%) and high linearities between the numbers of cells spiked and the copy numbers measured by qPCR ($R^2 > 99\%$; Table 5 & Fig. 4). To evaluate the accuracy of the qPCR assays, we compared the discrepancy between the copy numbers estimated by qPCR and the actual cell numbers spiked. The differences ranged from − 0.30 to − 0.15 Log_{10} cells/g (equal to 50.1–70.8% of recovery rate) for PB-W1 and from − 0.51 to 0.15 Log_{10} cells/g (equal to 30.9–141.3% of recovery rate) (Table 5), which is negligible when quantifying gut bacteria in human stools.

Evaluation of strain-specific qPCR assays in fecal samples collected during a human trial

To validate our qPCR assays in a real-world setting and compare findings with those obtained with NGS approaches (i.e., 16S rRNA gene sequencing and whole metagenome sequencing [WMS]), we capitalized on fecal samples collected during the control phase (no dietary intervention) of a human trial that involved the administration of a single dose of *L. reuteri* PB-W1 or DSM 20016 ^{T} to different groups of individuals. Fecal samples before (baseline) and after the administration of the strains were included.

We first compared findings of qPCR, culture, 16S rRNA gene sequencing, and WMS at baseline and 4 days after administration of the live *L. reuteri* cells (Fig. 5A), as we had data of all four approaches available at these

time points. All baseline samples except one were negative in qPCR assays, with the one positive sample for PB-W1 being just slightly about the detection limit (3.46 Log_{10} cells/g). Four days after administration of the strains, *L. reuteri* becomes detectable in all subjects by qPCR and culture, with a broad dynamic range ranging from 3.35 to 7.74 Log_{10} cells/g feces for qPCR and 3.00 to 6.56 Log_{10} cells/g feces for culture. In contrast, *L. reuteri* was only detectable, at very low relative abundance, by 16S rRNA gene sequencing and WMG in one sample per group (Fig. 5A).

We then characterized findings from qPCR, culture, and 16S rRNA gene sequencing for all samples from baseline to the end of the monitoring period (day 21). Cell numbers of *L. reuteri* ranged from 9.41 Log_{10} cells/g to below the detection limit of 3 Log_{10} cells/g for qPCR and from 8.33 Log_{10} cells/g to below the detection limit of 2 Log_{10} cells/g based on selective culture. Correlation between qPCR and selective culture data revealed a strong correlation (Spearman's $r = 0.88$, $p < 2.20e\text{-}16$; Fig. 5B), although qPCR showed consistently higher cell numbers (Fig. 5B), likely due to qPCR detecting DNA from inactive and/or dead cells that are not cultivable. The relative abundance of *L. reuteri*, as determined by 16S rRNA gene sequencing, ranged from 0 to 1.42%, with most of the samples, especially those with cell numbers of $< 10^6$ as per qPCR, showing no detectable *L. reuteri*.

Discussion

In this study, we systematically compared the performance of qPCR and ddPCR in combination with three DNA extraction methods (two kit-based methods [QK and PQ] and a phenol–chloroform-based method [PC]). Based on these findings, we provided an easy-to-follow, step-by-step protocol for strain-specific qPCR assay development and validated this protocol by designing two PCR assays for two *L. reuteri* strains. The qPCR assays not only enabled absolute quantification with high accuracy but also exhibited a much lower LOD and a broader dynamic range for detecting *L. reuteri* in human fecal samples, especially when compared to NGS approaches (i.e., 16S rRNA gene sequencing and WMS). These findings demonstrate the feasibility of our approach to achieve the accurate and sensitive absolute quantification of bacterial strains in human stools, and the protocol will provide a resource for scientists to design strain-specific qPCR for their own applications and target bacterial strains.

Among the three DNA extraction methods tested in this study, PQ is the most robust protocol for human stool samples with the highest yield and purity of DNA, and it is therefore compatible with not only sequencing-based approaches, for which it was developed [41], but also PCR-based quantification. However, PQ requires more than twice as much time as QK. Although slightly less robust, QK produced DNA with sufficient quality and purity that performed well with both PCR approaches, which makes QK a good compromise between the time required and DNA quality. PC harvested a higher amount of DNA than PQ and QK but with much lower quality and purity. This might be due to a large amount of DNA extracted from the background and the insufficient removal of co-extracted PCR inhibitors using phenol–chloroform extractions, while protocols using silica columns (QK and PQ) substantially enhanced the separation of DNA and potential PCR inhibitors [59].

Our findings demonstrate that ddPCR had higher reproducibility than qPCR, and it therefore could improve the inter-study comparability among different laboratories. However, we did not find higher sensitivity of ddPCR than qPCR. In addition, qPCR is more practical than ddPCR for most applications for several reasons: first, we found that qPCR exhibited equal sensitivity (LOD) as ddPCR for quantification of *L. reuteri*. Second, given that ddPCR cannot detect more than 5 Log_{10} gene copies per reaction due to the limited number of droplets generated [30], qPCR has a broader dynamic range than ddPCR. This gives qPCR more flexibility when implemented in studies where samples of large microbial loads are detected. Specifically, with the method PQ, the Cq value of qPCR at the concentration of 7.97 Log_{10} cells/g feces was around 18 (data not shown), and therefore, the upper limit of qPCR reaches 10 Log_{10} cells/g feces. But ddPCR was nearly 80% saturated at the same amount of input and is thus theoretically unable to detect over eight Log_{10} cells/g feces, leading to a two Log_{10} difference between qPCR and ddPCR. Third, with respect to economic aspects, qPCR is less expensive and less time- and labor-intensive compared with ddPCR. Accordingly, we consider qPCR a superior approach to ddPCR for quantifying bacteria in human fecal samples.

Constructing an unbiased external standard curve is critical for achieving an accurate quantification using qPCR. We attempted two different strategies for the standard curve construction. To quantify *L. reuteri* DSM 17938, we made the standard curve by determining the amounts of nucleic acids in reference materials (genomic DNA of this strain) spectrophotometrically. We observed a higher number of gene copies determined via qPCR than ddPCR, which is consistent with other studies [29, 60]. The reason is that spectrophotometers indistinguishably quantify compounds with absorbance at 260 nm, resulting in an overestimation of DNA in reference materials. Apart from this strategy, cell numbers quantified by plating, flow cytometry, or microscopy could also be used as the calibrator for qPCR

[23, 29, 61]. When we developed the strain- and lineage-specific qPCR assays for PB-W1 and DSM 20016 ^{T}, we constructed the standard curves using serial dilutions of genomic DNA of standardized cultures with known cell counts of *L. reuteri* as determined by plating on MRS media. For this approach, only viable cells in reference materials are taken into consideration. Cells of *L. reuteri* used for calibration of the PCR assays were harvested at 8 h of growth, i.e., before the dead cells account for a relevant proportion of bacterial cells [36]. Standard curves constructed using both strategies showed high linearity ($R^2 > 0.99$) and acceptable efficiency (89–115%), and no apparent difference was observed. Therefore, both strategies could be applied for qPCR quantification.

Our primary motivation for the development of an easy-to-follow, step-by-step protocol for strain-specific qPCR (Supplementary File 3) was to provide a resource that scientists can follow even if they are not experts in comparative genomics. The protocol starts from the design of strain-specific primers using software that are easy to apply, followed by an in-detail description of both in silico and experimental validation procedures to ensure the primer specificity. When combined with appropriate DNA extraction methods, this approach results in highly accurate qPCR systems with high sensitivity. Our validation of qPCR assays designed with this approach using human fecal samples demonstrated a much lower LOD and a wider dynamic range for detecting and quantifying target bacteria strains, especially when compared to NGS approaches. The protocol we described is based on our previous successful work that used qPCR to quantify various target bacterial strains from the microbial communities, including *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis* subsp. *lactis*, *L. reuteri*, *Lacticaseibacillus case*, and *Limosilactobacillus fermentum* [21–25]. This confirms the wide applicability of the protocol presented in this study: it can easily be adapted to other bacterial taxa and sample types, but it is important that appropriate modifications are made to the DNA extraction method, which has to be compatible with both the specific sample type and the bacterial isolate.

Conclusions

We describe a strain-specific qPCR assay with high reproducibility, linearity, and accuracy, which further outperformed ddPCR in terms of dynamic range, cost, and time. Considering all these factors, we suggest the combination of qPCR with kit-based DNA extraction methods as the best option to quantify gut microbial members in human fecal samples at the strain level. To our knowledge, our study is the first to successfully push the LOD of a target strain to around three Log_{10} cells/g feces. The step-by-step protocol for strain-specific primer design and in silico and experimental validation described here will have a broad range of applications for scientists to detect and quantify various bacterial strains from a variety of circumstances (not only fecal samples but also other gut samples, such as biopsy and swab samples), including but not limited to evaluating the persistence of probiotics and live biotherapeutics, detecting pathogens or other disease-associated microbes, tracking bacterial strains during vertical transmission and fecal microbiota transplantation, and confirming and establishing the bacterial load in low-biomass samples.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01881-2.

Supplementary Material 1: Supplementary figures. Figure S1. Preparation of fecal samples spiked with known numbers of bacteria. *L. reuteri* DSM 17938 was grown on MRS agar for 48 hours in an anaerobic chamber at 37 °C. After that, single colonies were picked and transferred to MRS broth, and sub-cultured twice (24 hours for the first sub-culture, and 8 hours for the second sub-culture to ensure bacterial cells stay at the late exponential phase or early stationary phase). Cell numbers in cultures were determined by plating a ten-fold serial dilution on MRS agar. Then serial dilutions of bacteria with exact cell numbers were prepared by dilution, and mixed with 0.1 g stool aliquots. Figure S2. qPCR standards were established by a 10-fold serial dilution of genomic DNA of *L. reuteri* DSM 17938, and each dot represent the average Cq of six replicates from three independent runs. Figure S3. Melt curves for stool samples spiked with *L. reuteri* DSM 17938 in qPCR. (A-C), results obtained from spiked samples of three individuals and there was one specific peak at around 87.5℃, which indicated the amplification of target sequence for all reactions. Figure S4. Correlation between qPCR and ddPCR for fecal samples spiked with *L. reuteri* DSM 17938 when the method PQ was used. Solid line, regression line; dash line, y = x. All dots are results obtained in qPCR and ddPCR. Supplementary tables. Table S1. Screen the existence of *L. reuteri* DSM 17938 among nine human stools and test the specificities of the designed primers for *L. reuteri* PB-W1 and DSM 20016^{T} based on the fecal background DNA. Table S2. Quantities and qualities of DNA extracted using different DNA isolation methods. Table S3. *L. reuteri* genomes available in Joint Genome Institute (JGI) genome portal selected for the present study.

Supplementary Material 2. Procedures of the three DNA extraction methods tested in the present study. Phenol chloroform-based method (PC). QIAamp fast stool DNA kit-based method (QK). Protocol Q-based method (PQ).

Supplementary Material 3. A step-by-step protocol for the design of strain-specific qPCR assays for the strain-level detection and quantification of bacteria in fecal samples.

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Authors' contributions

FL and JW designed this study; FL and JL performed bacteria growth, artificial spiking of stool samples, DNA isolation, ddPCR, qPCR, identification of strain- and lineage-specific genes, primer design and validation, and statistical analyses; FL and JL collected fecal samples from the human clinical trial, and conducted qPCR, selective culture, 16S rRNA gene sequencing, and whole metagenome sequencing; FL, JL, MXM, SF, MGG, and JW were responsible for protocol development and optimization, data interpretation, and manuscript writing.

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Availability of data and materials
The original sequencing data of 16S rRNA gene sequencing and whole metagenome sequencing have been uploaded to the NCBI Sequence Read Archive (SRA), and the accession number is PRJNA1000186. All the other data and materials have been all described in the manuscript.

Declarations

Ethics approval and consent to participate
The present study received research ethics approval from the Health Research Ethics Board — Biomedical Panel of the University of Alberta (no. Pro00077565), and fecal samples were collected from health human subjects with written informed consent at the University of Alberta Human Nutrition Research Unit (Edmonton, Canada).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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