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Development of a Standardized Method For The Quantification of *Lactobacillus Paracasei* F19 In Stool Samples Of Various Ages

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Abstract

Several strains of *L. paracasei* sub sp. *paracasei*, among which strain F19, are actively marketed as functional microorganisms in food products due to their reported health benefits. A reliable detection and quantification method is essential for studying these bacteria in clinical studies. This study reports the development of strain-specific qPCR primers for *L. paracasei* F19 and a standardized method for its DNA extraction using the Qia Amp DNA Stool Mini Kit and quantification with SYBR green. Fecal samples were spiked with known amounts of the bacterium in order to make a calibration curve, where after two datasets of fecal samples from human subjects that daily consumed yoghurt containing 1E+9 *L. paracasei* F19or a placebo during the past fourteen days were used to determine the dynamic range of the method. A clear Ct cutoff of 29.81 was found, resulting in a dynamic range of adequate quantification from approximately 5 log CFU/g until 11 log CFU/g. Moreover, it was found that the bacterium can still effectively be quantified in fecal samples that are stored at -45°C for three years, which may reduce the analytical imprecision in a long-term clinical study by avoiding errors caused by multiple rounds of DNA extraction by different people and under different conditions.

Keywords: *Lactobacillus paracasei F19; qPCR; DNA extraction; feces; Clinical studies; long-term storage*

Abbreviation: CFU: Colony Forming Units; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Ct: Cycle threshold (number of cycles required for the fluorescent signal to cross a threshold); qPCR: Quantitative polymerase chain reaction

Introduction

Lactobacillus paracasei is a Gram-positive and facultative hetero fermentative bacterium that is commonly found naturally in dairy, on plants and in the human intestine. Several strains of *L. paracasei* subsp. *paracasei*, among which strain F19, are actively marketed as functional microorganisms in food products. Of *L. paracasei* F19, it was shown that the strain survives gastric transit, transiently colonizes parts of the intestine, does not perturb the population dynamics of other major populations of bacteria in the intestinal microbiota of healthy subjects [1] and that it is genetically stable, which renders its administration reliable and effective in immunocompromised people [2]. These properties make this bacterium a candidate to reduce or prevent colonization of the intestine by pathogens [2,3] or otherwise to solve intestinal dysbiosis, also in subjects with various diseases. For example, consumption of *L. paracasei* F19 relieved patients with intestinal discomfort and bloating related to symptomatic uncomplicated diverticular disease [4] and use of proton pump inhibiting medicines that foster intestinal dysbiosis [5]. Consumption of the bacterium was also shown to lead to reduced fat storage [6] and is therefore suggested for the prevention of obesity. It is, however, evident that the fat storage modulation does not last once the bacterium is out of the system [7]. Finally, it was found that *L. paracasei* F19 interacts with the host's immune system, as is exemplified by its protective effect against oxidative and metabolic hepatic injury [8].

In clinical studies where either a specific bacterium needs to be detected in fecal material or the microbiota of the intestine is studied, it is of key importance to apply a detection method that is optimized and standardized for the specific application, minimizing the analytical imprecision. That not only counts for culture-dependent methods [9], but also for molecular methods dependent on DNA extraction. Several studies compared DNA extractions with commercial kits from various suppliers and adaptations on the standard procedures. For the same type of stool samples, different methods give the highest microbial DNA yield [10-12], although this variation is also reported smaller then that effected by the exact isolation source such as the individual subjects [13]. It must also be noted that the total DNA yield is not necessarily related to the amount of PCR amplifiable DNA of a target strain [11], indicating that there is species to species or even strain to strain variation in the efficacy of DNA extraction [13]. This means that the choice of extraction method has implications for the dynamic range and the detection limit of bacteria when quantifying with quantitative PCR (qPCR) afterwards [14]. In order to reduce the effects of sample and bacterial community variation, a combination of the two main types of DNA extraction methods (mechanical and enzymatic) is proposed [15].

There can be many substances in feces that are not filtered out during DNA extraction and that may interfere with down-stream processes such as PCR [12,16,17]. With different commercial DNA extraction kits, the most optimal amount of fecal starting material was found to be between 10 and 50 mg in one study [18] and around 200 mg in another [19]. Moreover, it was noted that DNA extracts should be diluted at least 10 times when performing PCR in order to avoid effects of inhibitory compounds present in the extract [12] and that an internal control may be a good tool to monitor the efficacy of DNA extraction and subsequent qPCR reaction and to eliminate false negatives [17].

Additional to picking the optimal DNA extraction method, the methods for sampling and storage of fecal samples are important for optimizing the quantification of microorganisms in feces. If live bacteria are needed for downstream analyses, it is recommended to apply fecal samples to glycerol broth and store them at -80°C instead of -20°C as some bacterial species may reduce in viability at the higher temperature [20]. For long-term storage of samples meant for DNA extraction also -80°C is recommended, but short-term storage at higher temperatures (-20°C for 1 week and +4°C for 24 hours) is also possible without major changes in the general composition of the microbiota [21]. It is, however, unclear how individual species react to different storage times. Mathay and co-workers noted that for optimal DNA yield and purity, it is best to freeze fecal samples at -20°C or -80°C in standard tubes immediately after collection and without additional processing [19]. To the best of our knowledge, there is no study performed yet on the stability of bacterial DNA in fecal samples for a period of years.

The objectives of this study were to develop strain-specific qPCR primers for *Lactobacillus paracasei* subsp. *paracasei* F19 and to develop a method for its detection and quantification in fecal samples, which will simplify future clinical trials with this organism. Moreover, this study shows that the bacterium can still be identified in fecal samples that are stored at -45°C for up to three years, which will aid in reducing analytical imprecision in long-term clinical studies.

Materials and Methods

Sample harvesting

Of human subjects who had daily consumed 250g yoghurt containing1.5 x 109 colony-forming units per gram (CFU/g) *Lactobacillus paracasei* subsp. *paracasei* F19 cells or a placebo for 14 days, fecal samples were collected at day 14, and stored at -45°C until DNA extraction. As the bacterium only transiently colonizes the intestine, a two-week washout period was applied before starting the actual trial in order to reduce the number of possible false positives.

DNA extraction

DNA was extracted from approximately 200 mg (in practice ranging from 70 mg to 360 mg) of feces with the QiaAmp DNA Stool Mini Kit (Qiagen, Silkeborgvej 2, 8000 Aarhus C, Denmark) according to the manufacturer´s instructions with the following modification: after the addition of ASL buffer 0,1 mm glass beads were added to the tubes and the tubes were treated in a Bullet Blender (Next Advance,

Inc., 1548 Burden Lake Road, Averill Park, NY 12018-2818, USA) for 5 min at maximum speed. The DNA samples were stored at -20°C until analysis.

Primer design and testing

The genome of *L. paracasei* F19 was sequenced (Integrated Genomics, 2355 S Arlington Heights Rd #270, Arlington Heights, IL 60005, USA). To find a unique region in the genome, the sequence was aligned with the genome sequences of four closely related strains, being Lactobacillus casei ATCC334 and BL23 and *L. paracasei* 8700:2 and ATCC25302, which are available in the ERGO database (Integrated Genomics). A 7957 nucleotide long CRISPR region was found. Primers were designed using Primer Express (Applied Biosystems, Fisher Scientific, Industrivej 3, 3350 Slangerup, Denmark) on a 205 bp long region (Figure 1). The primers CRISPR2F (5´ -CGTGTGCCGATATAATGGGAACG-3´) and CRISPR2R (5´- CCAAAGATCATCAAGCGTGCCAT-3´) were tested for specificity using NCBI BLAST and with PCR amplifications of a total of 28 *L. paracasei* strains, 10 other *lactic acid bacteria* and 16 non *lactic acid bacteria* (Table 1). The PCR reactions were performed according to Björneholm., *et al*. [22] with the following modifications: the primer concentration was0.1µM, and the enzyme used was Tth polymerase (Roche Molecular Systems Inc., 4300 Hacienda Dr, Pleasanton, CA 94588, USA). The annealing temperature was set to 62°C.

GTTTTCCCCGCACATGCGGGGGTGATCCCTGGGGTAGCACAGGTAGGTGCGCTCGAAACAAGTTTTCCCCGCACATGCGGGGGTGATCCCTGCT-
GACCCAGCAATTGCATAGTAGTAGCTATGTTTTCCCCGCACATGCGGGGGTGATCCCAACTGCGGATATTTGTACGTTGCTTTGTCTAAGTTTTC-
CCCGCACATGCGGGGGTGATCCTCCACTGTAGCTTGTCTGTGGTGGCTTCTGGGGGTTTTCCCCGCACATGCGGGGGTGATCCCGTATGACCT-
TGCTTGGCTCGGTTTTTCAGCTAGTTTTCCCCGCACATGCGGGGGTGATCCCTATATTCTTCACCCTTTACCCCTCCCGAAGTAGTTTTCCCCG-
CACATGCGGGGGTGATCCCAGACTTTTGATGTTGTTGAGTGCCGTACTTTTGTTTTCCCCGCACATGCGGGGGTGATCCCGTCAATCTTTCGCG-
GTTGCTTGAGTCTGCTTTGTTTTCCCCGCACATGCGGGGGTGATCCTAAAGCTTGCCAGCCTTGCTTAGTTGGGACCTTGTTTTCCCCGCA-
CATGCGGGGGTGATCCTACACCCAAGACGATCCCCGCTTGCAACATGCTGTTTTCCCCGCACATGCGGGGGTGATCCTGATACCGACCGGAG-
TAGCTGAGGTACCGTGCCGTTTTCCCCGCACATGCGGGGGTGATCCCAGATCAGTGAAAATGACGCAAAGGCCGGCGATGTTTTCCCCGCA-
CATGCGGGGGTGATCCCGAATATCAAGCCCAGTGCGAGATTGAGAAAACGTTTTCCCCGCACATGCGGGGGTGATCCCGGCTACACCGTG-
GCAAAGGAGAAGAAATACAAGTTTTCCCCGCACATGCGGGGGTGATCCTGGACTAAGTAGCTAACTGGAGCTATTTAGTCCGTTTTCCCCGCA-
CATGCGGGGGTGATCCTGACCAAATCCAGCAAAGAAGTCGAAAAAATAGGTTTTCCCCGCACATGCGGGGGTGATCCCGTATTTTCGTCCACAT-
TCATTGTATAAGGTGTGTTTTCCCCGCACATGCGGGGGTGATCCTGAAGACTAAGAAAGGTAACCCGCTTCGGCGGGGTTTTCCCCGCACATGCG-
GGGGTGATCCCTTGCTGACGGTAGTAGTTTGGGTGAGACTATTGTTTTCCCCGCACATGCGGGGGTGATCCCTTGCTGACGGTAGTAGTTTG-
GGTGAGACTATTGTTTTCCCCGCACATGCGGGGGTGATCCTC**CGTGTGCCGATATAATGGGAACG****CCAGCATTGTTTTCCCCGCACATGCG-
GGGGTGATCCCAGAATTAAAATTAATGGCGCCAATCTTATCAAGTTTTCCCCGCACATGCGGGGGTGATCCCAACGCTAGACGACGCAACT-
CAAGAACAAGTCGGTTTTCCCCGCACATGCGGGGGTGATCCT**ATGGCACGCTTGATGATCTTTGG**CCAAACCAAGTTTTCCCCGCACAT-
GCGGGGGTGATCCCTGGTTCAGATGGATAGGTCCCAGCATTAGGCAGTTTTCCCCGCACATGCGGGGGTGATCCTGGTGTCGGTATAGTAT-
TCAGGATCAGCTGGGTGTTTTCCCCGCACATGCGGGGGTGATCCCTCAAACCGACCGACTTCAGGACCGCTCGTATCGTTTTCCCCGCACAT-
GCGGGGGTGATCCTATATACTGACTGATTGCCCACTGGTACATTTGGTTTTCCCCGCACATGCGGGGGTGATCCCAAGAAGATTACCGGTAAT-
GACTACGTCGCCAGGTTTTCCCCGCACATGCGGGGGTGATCCCTAAATCCATTCAGCAAAAACACGTCACCAGCGGTTTTCCCCGCACATGCG-
GGGGTGATCCCGCCAACTCACGGCCACTAGATCGTTGGACGGTGTTTTCCCCGCACATGCGGGGGTGATCCCGGTGTCGCAATGCCTAGCTG-
GGTTCAGGGCAAGTTTTCCCCGCACATGCGGGGGTGATCCTAATGTGCCAGTGGGAGCAATGGTGTTGTCGATGTTTTCCCCGCACATGCG-
GGGGTGATCCTGATCACCCCAGATCAATACAAGCAAATTACAGGTTTTCCCCGCACATGCGGGGGTGATCCTAATGCTAGTGTGGTTCTCGTAAA-
GACCATCTGGTTTTCCCCGCACATGCGGGGGTGATCCTGGTGTCGGTATAGTATTCAGGATCAGCTGGGTGTTTTCCCCGCACATGCGGGGGT-
GATCCCTCAAACCGACCGACTTCAGGACCGCTCGTATCGTTTTCCCCGCACATGCGGGGGTGATCCCGAAACCGTTGGCCCCTATAAAGCTC-
CACAAGCGTTTTCCCCGCACATGCGGGGGTGATCCCAAGCTAACCTTTCGCCTAGATCGTCTTGATTTGTTTTCCCCGCACATGCGGGGGT-
GATCCCAAAAGTCTGAACGCGTTTATCATCTCGATTTCAGTTTTCCCCGCACATGCGGGGGTGATCCCCATAGCGGCGTCAACATTAGTACGCT-
CAATCTGTTTTCCCCGCACATGCGGGGGTGATCCGGGGATGACTGTATGAACGAACGCAATGAAAAAGTTTTCCCCGCACATGCGGGGGT-

GATCCCTAATGTTACTGATATTTTTATCAAGCCATTTGGTTTTCCCCGCACATGCGGGGGTGATCCCACTTTGTATTTTCCAGCCATTTCCCA-
CTTCACGTTTTCCCCGCACATGCGGGGGTGATCCCTAATGTTACTGATATTTTTATCAAGCCATTTGGTTTTCCCCGCACATGCGGGGGTGATC-
CCACTTTGTATTTTCCAGCCATTTCCCACTTCACGTTTTCCCCGCACATGCGGGGGTGATCCCGCTGTCTTGCCTCACGCACAGTTTCAG-
CAGCCGTTTTCCCCGCACATGCGGGGGTGATCCTGGAGATCAAGATGAGTATTATGCATACGTTGGGTTTTCCCCGCACATGCGGGGGTGATC-
CCGACTGATACTTCTCTGAACCGGTTCATTTGTGGTTTTCCCCGCACATGCGGGGGTGATCCTGCCATTCCACAGTCCGCTGAAGAATGAAGC-
GAGTTTTCCCCGCACATGCGGGGGTGATCCAGCACAGACGCCGAAGGGAAGCATCGTTGCTTTGTTTTCCCCGCACATGCGGGGGTGATC-
CCAAGCCATGAAAAAAGCCGCTGGCCCGCATGCCGTTTTCCCCGCACATGCGGGGGTGATCCCACACGATGACGAGCTTGGCAGTCACTCG-
GTTAGTTTTCCCCGCACATGCGGGGGTGATCCCCTCTGCCCAGTCACCAATCGTGTATCCAAAAAAGTTTTCCCCGCACATGCGGGGGTGATC-
CTGATCTTCGCAACCGTGAAGGGATTTCAATCAGGTTTTCCCCGCACATGCGGGGGTGATCCCATTAGACGCCGGAACAGGCTGTGAATTT-
GTTGGTTTTCCCCGCACATGCGGGGGTGATCCCAAACAAGCGGTGACAACTAGCTTGCGTGTGGCGTTTTCCCCGCACATGCGGGGGTGATC-
CCGCGTTGGGATTGATCCATGGGAGCTTATTCGGGTTTTCCCCGCACATGCGGGGGTGATCCTAAAATCAGAGATCACCCGACACACACAG-
CACTGTTTTCCCCGCACATGCGGGGGTGATCCTATATACTGACTGATTGCCCACTGGTACATTTGGTTTTCCCCGCACATGCGGGGGTGATC-
CCAAGAAGATTACCGGTAATGACTACGTCGCCAGGTTTTCCCCGCACATGCGGGGGTGATCCCTAAATCCATTCAGCAAAAACACGTCACCA-
GCGGTTTTCCCCGCACATGCGGGGGTGATCCCGCCAACTCACGGCCACTAGATCGTTGGACGGTGTTTTCCCCGCACATGCGGGGGTGATC-
CCGGTGTCGCAATGCCTAGCTGGGTTCAGGGCAAGTTTTCCCCGCACATGCGGGGGTGATCCTAATGTGCCAGTGGGAGCAATGGTGTTGTC-
GATGTTTTCCCCGCACATGCGGGGGTGATCCTGATCACCCCAGATCAATACAAGCAAATTACAGGTTTTCCCCGCACATGCGGGGGTGATC-
CTAATGCTAGTGTGGTTCTCGTAAAGACCATCTGGTTTTCCCCGCACATGCGGGGGTGATCCTGGATTGAGCTGTACAAATCTGGTAAGCAT-
TCTGTTTTCCCCGCACATGCGGGGGTGATCCTGCCGATTTGCAAACGATCGAGCGGTTCTCCAAGTTTTCCCCGCACATGCGGGGGTGATC-
CCCGTGGTTCCCAAGCTTTTTTGTTTGCCATTTTGTTTTCCCCGCACATGCGGGGGTGATCCTCAAAGCCCACATTAACAAGGTTGCCAT-
GACGAGTTTTCCCCGCACATGCGGGGGTGATCCCAACAACCAACCTTGCCAAACTTGAAAAGCAGGGTTTTCCCCGCACATGCGGGGGT-
GATCCTTGCGCCCATGTCTTTGCAAGTTTTGTCATAGCGTTTTCCCCGCACATGCGGGGGTGATCCTAAGATTGTGACGTCCGGCATATTTAT-
GATCTGTTTTCCCCGCACATGCGGGGGTGATCCTGACAACAGTCAGGTGACGCATACCGAACTCAGGTTTTCCCCGCACATGCGGGGGTGATC-
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CACGTTTTCCCCGCACATGCGGGGGTGATCCCAAAAACCCCACAATGTCATCGTTTAACCAGTCGTTTTCCCCGCACATGCGGGGGTGATC-
CCGGGTAGTTGAACTGATTGACCGCCAGCAGTGAGTTTTCCCCGCACATGCGGGGGTGATCCCATCATCGGCAAGCTTGCTGACAAGAAC-
GAAGAGTTTTCCCCGCACATGCGGGGGTGATCCTGCCTTTTTACGGTTCAATGCGGCCATAGAGCGGTTTTCCCCGCACATGCGGGGGTGATC-
CCGGTCCCGCTAAGTTTACGTTCGACAAATGGCGGTTTTCCCCGCACATGCGGGGGTGATCCCATCTCTTCGAACCCCAAATACAGCGTCAT-
TACGTTTTCCCCGCACATGCGGGGGTGATCCTGACAAATAGATATTTGCAACGCTTGGACAAACGTTTTCCCCGCACATGCGGGGGTGATC-
CCCATCGCTTGCGCCAATCGTGATTTAATCGTCGGTTTTCCCCGCACATGCGGGGGTGATCCTTTATTAGAACTAGCTGAAGATGGAACAACT-
GAGTTTTCCCCGCACATGCGGGGGTGATCCTCACCAATGGTACGTTCTGCTTCATTCAATCCAGTTTTCCCCGCACATGCGGGGGTGATC-
CAACTCGGGGGAAATATAATCGTCACTGGCGTCAGTTTTCCCCGCACATGCGGGGGTGATCCCAATCAATTCGCCAACCGTTAATTCGCCAT-
TCAGTTTTCCCCGCACATGCGGGGGTGATCCCGAGCTGCAGGACTACTCACGATGGACGAATTAGTTTTCCCCGCACATGCGGGGGTGATCCT-
GGTTGATCCGGCCGCTTATGTATCCGCACGGAGTTTTCCCCGCACATGCGGGGGTGATCCCAGTTCCTACCTGACTGTCCCACCAAGGTGCCG-
GTTTTCCCCGCACATGCGGGGGTGATCCCGCAACCGCCGGGATTTGAACCCGATAAGGCCAGTTTTCCCCGCACATGCGGGGGTGATCCCCA-
TGCACGGGCAACGGCTACTTGGTTGTCAGCGTTTTCCCCGCACATGCGGGGGTGATCCCGAACGTAACAACGTGGCCACCATGTTTTCGCG-
GTTTTCCCCGCACATGCGGGGGTGATCCTCGAAAGAGGTAGAAAACATGAAAACAGCAAGCGTTTTCCCCGCACATGCGGGGGTGATC-
CTCCTAAGCTGGCTTTGTTGTTAGGCATTAAAAAGTTTTCCCCGCACATGCGGGGGTGATCCCAGGAGGAACCATCATGGCAGAAGAAACA-
CAAAGTTTTCCCCGCACATGCGGGGGTGATCCTACATCAACAAAGAGTATGACGTCAGCGGCACCGTTTTCCCCGCACATGCGGGGGTGATC-
CTGACATCATCACACACAAGGAGCCATACCAAGCGTTTTCCCCGCACATGCGGGGGTGATCCTTATTGTCAAAACCACCGTAGTTTTGGCCT-
GTTGTTTTCCCCGCACATGCGGGGGTGATCCTGAAATATCGTAAGAAGCCGGTTGAAATTGAAGGTTTTCCCCGCACATGCGGGGGTGATC-
CATCCTTACCTTTAGCAATGCTAATCAGCATAAGGTTTTCCCCGCACATGCGGGGGTGATCCCAACTGGCGTGCTCGCTCGTGCATGGTGG-
GAAGGTTTTCCCCGCACATGCGGGGGTGATCCCGACTTGGACGCGCTCAACTATGATCTTTTCCTGTTTTCCCCGCACATGCGGGGGTGATC-

CCCTTATCGCAAGTAATAGGTACCATATTAAGTTGTTTTCCCCGCACATGCGGGGGTGATCCCAGCTTGCGCACACAAATGACAAATGC-
CGAAGCGTTTTCCCCGCACATGCGGGGGTGATCCCAACTCGCCTATATTCTTCTACTATGGCGAAGAGTTTTCCCCGCACATGCGGGGGT-
GATCCCAGTGCGGCGAATAATAATACCAGTCCGGCAAGGTTTTCCCCGCACATGCGGGGGTGATCCCACCGTTTTTGCAAATGGTCAATGACG-
GTTTCAGTTTTCCCCGCACATGCGGGGGTGATCCCAACGTTTCGCGTAATCTTAGCAAACACTAGATGTTTTCCCCGCACATGCGGGGGT-
GATCCCTTATTATATTTTGGAATGGCGTGCCGAGGTTTGTTTTCCCCGCACATGCGGGGGTGATCCTGCGATTCACGAAGATTTTTATTTT-
GAATAGCAGTTTTCCCCGCACATGCGGGGGTGATCCTAACTTTCGTCCTATTTCTGTTCCTTCAGTCAAGTTTTCCCCGCACATGCGGGGGT-
GATCCCGAAACTGGTTGGTTGACGTTCTCCTGGTTTCCAGTTTTCCCCGCACATGCGGGGGTGATCCTGAGCTTGTTAAGGACGACCCAGT-
TACCATCAAGTTTTCCCCGCACATGCGGGGGTGATCCCAATCTGGTTAACTACACCATCTTGATTGATATGTTTTCCCCGCACATGCGGGGGT-
GATCCCTCAGGCAAGTTATCTGGCAAAAATCCAGATCGGTTTTCCCCGCACATGCGGGGGTGATCCTTTATGAGTATCAGCCAGCACAAAACG-
CAATGGGTTTTCCCCGCACATGCGGGGGTGATCCCAAGGCCGCTTCGCATTCCTTGACTTATGCACTGTTTTCCCCGCACATGCGGGGGT-
GATCCTGTAATCAATTGCTACTAAAAATATTCGGAGGTGTTTTCCCCGCACATGCGGGGGTGATCCCTGCTATCCGATCGTGGTCGACCCACG-
TACAAAGTTTTCCCCGCACATGCGGGGGTGATCCCAACAACCGAATGGAAACCCTGAACAACAACCGGTTTTCCCCGCACATGCGGGGGT-
GATCCTGAAATTTCAACGCTGTCCAGAACTTTGTCATTGTTTTCCCCGCACATGCGGGGGTGATCCCTCGCAAGCGACAACCGGCAAGAATCT-
TACATCGTTTTCCCCGCACATGCGGGGGTGATCCCGCAAAATAGGCCGCAAATATTTAGAATAAAGGGTTTTCCCCGCACATGCGGGGGT-
GATCCTTGTCGCCATTCTTTGACGGAGTTTGACCCCGAGTTTTCCGCGCACATGCGGGGGTGATCCTGTTACGTTCGATCCTGATGGTAACGT-
TGGTGAGTTTTCCCCGCACATGCGGGGGTGATCCTGCGAAAGCCAAAGCGAACAACCGCAAAAAAGGGTTTTCCCCGCACATGCGGGGGT-
GATCCTAAAGAATAAAGGAAAAAATATTTTGTGTTCTAGTTTTCCCCGCACATGCGGGGGT

Figure 1: *Sequence of a CRISPR region that was only found present in Lactobacillus paracasei F19. The bold region is the fragment that is amplified by PCR using the binding sites for primers CRISPR2F (5´-CGTGTGCCGATATAATGGGAACG-3´) (yellow) and CRISPR2R (5´- CCAAAGATCATCAAGCGTGCCAT-3´) (green).*

Organism	Strain	Used for alignment test*	CRISPR region
Bacillus cereus	SLV 056		-
Bacillus subtilis	SMR 772		-
Bifidobacterium breve	ATCC 15700		-
Bifodobacterium bifidum	ATCC 29521		-
Clostridium sporogenes	ATCC 19404		-
Enterococcus faecium	ATCC 19434		-
Enterocuccus faecalis	SLV 047		-
Escherichia coli	SLV 165		-
Lactobacillus acidophilus	NCFB 1748		-
Lactobacillus casei	ATCC334	x	-
Lactobacillus casei	BL23	x	-
Lactobacillus fermentum	353		-
Lactobacillus gasseri	ACA-DCD 242		-
Lactobacillus lactis	ATCC 11454		-
Lactobacillus paracasei ssp paracasei	8700:2	x	-
Lactobacillus paracasei ssp paracasei	ATCC 25302	x	-
Lactobacillus paracasei ssp paracasei	ATCC393		-
Lactobacillus paracasei ssp paracasei	CH 10008		-
Lactobacillus paracasei ssp paracasei	CH 10018		-

Lactobacillus paracasei ssp paracasei	CH 10034		-
Lactobacillus paracasei ssp paracasei	CH 10058		-
Lactobacillus paracasei ssp paracasei	CH 10060		-
Lactobacillus paracasei ssp paracasei	CH 10072		-
Lactobacillus paracasei ssp paracasei	CH 10090		-
Lactobacillus paracasei ssp paracasei	CH 10096		-
Lactobacillus paracasei ssp paracasei	CH 10114		-
Lactobacillus paracasei ssp paracasei	CH 10125		-
Lactobacillus paracasei ssp paracasei	CH 10145		-
Lactobacillus paracasei ssp paracasei	CH 10153		-
Lactobacillus paracasei ssp paracasei	CH 10163		-
Lactobacillus paracasei ssp paracasei	CH 10166		-
Lactobacillus paracasei ssp paracasei	CH 10180		-
Lactobacillus paracasei ssp paracasei	CH 10181		-
Lactobacillus paracasei ssp paracasei	CH 10184		-
Lactobacillus paracasei ssp paracasei	CH10030		-
Lactobacillus paracasei ssp paracasei	F19		+
Lactobacillus paracasei ssp paracasei	LMG 13087		-
Lactobacillus paracasei ssp paracasei	VTT 78078		-
Lactobacillus paracasei ssp paracasei	VTT 90377		-
Lactobacillus paracasei ssp paracasei	VTT 91466		-
Lactobacillus paracasei ssp paracasei	VTT 91467		-
Lactobacillus paracasei ssp paracasei	VTT 97949		-
Lactobacillus paracasei ssp paracasei	VTT E981006		-
Lactobacillus plantarum	43364		-
Lactobacillus reuteri	DMS 20016		-
Lactobacillus rhamnosus	ATCC 7469		-
Lactobacillus salivarius	43321		-
Listeria innocua	ATCC 33076		-
Listeria monocytogenes	SLU 2615		-
Proteus mirabilis	ATCC 14153		-
Pseudomonas aeruginosa	SLV 079		-
Pseudomonas fluorescens	SMR 216		-
Salmonella enteritidis	SLV 397		-
Staphylococcus aureus	SLV 350		-
Stretococcus thermophilus	STH14		-

Table 1: *Bacterial strains used for testing the specificity of the L. paracasei F19 CRISPR primers.*

**The genome sequence of these organisms were found in ERGO database (Integrated Genomics).*

Quantitative PCR

Extracted DNA was diluted 10-fold and 100-fold.Both dilutions and the undiluted sample were used for qPCR in triplicates. PCRs were done in 96-wells plates (Applied Biosystems) in a 7500 Real time PCR system (Applied Biosystems). Each sample of 50 µL contained 25 µL SYBR green master mix (Applied Biosystems), 150 nM of both primers (DNA Technology A/S, Voldbjergvej 16, 8240 Risskov, Denmark) and 5 µL of DNA template.

Standards and quantification

A feces-based standard series of *L. paracasei* F19 was obtained as follows. Suitable amounts of a bacterial culture were added to fecal dilutions in triplicates, resulting in standards that contained approximately 10^1 to 10^9 CFU/g. DNA was extracted from 200 µl of the suspensions. qPCRs were performed five times with each sample in triplicates. The exact number of *L. paracasei* F19 in each suspension was determined by plating dilutions of the pure culture onto MRS pH 5.4-plates (Oxoid, Wade Road, Basingstoke, Hampshire, RG24 8PW, UK). The plates were incubated anaerobically at 37°C for 3 days. Feces without added *L. paracasei* F19 was used as negative control.

Statistical analysis

The significance of differences between the Ct values derived from samples acquired from subjects that consumed either *Lactobacillus paracasei* F19 or a placebo and between true and false discovered samples was determined with a two-tailed Student's T-test with two-sample unequal variance. Since our results confirmed a previous study [12] showing that 10-fold diluted DNA gave more reliable results in terms of quantification (see below), the statistical analyses were performed on the Ct values acquired with the diluted samples. Significance is defined as P<0,001.

Results and Discussion

Method development

A calibration curve of known amounts of *L. paracasei* F19 added to stool and subsequent DNA extraction and qPCR was prepared. The log concentrations of *L. paracasei* F19 from plate counts were correlated to the mean Ct values of triplicate PCR quantifications from 18 separate calibration series. These were found to have a best fit linear curve with a slope of -0.2741*Ct and an intercept of 12.645 log CFU (Figure 2). The maximum Ct value that represents a reliable estimation of cells counts was initially set as follows. Of triplicate PCR quantifications from 14 separate experiments the Ct values were determined of the no template controls (37.00±1.27). The maximum Ct was set two standard deviations lower, i.e. 34.46, which is slightly higher than the highest Ct from the calibration series (34.26±0.54, corresponding to 3.22 log CFU). A Ct above 34.46 was therefore considered as absence of *L. paracasei* F19. The lowest reliable Ct was determined as the highest concentration of bacteria tested (15.89±0.20, corresponding to 8.22 log CFU), as this still fitted the linear curve. Therefore, Ct values below 15.89 were considered positive, but unreliable in terms of quantification. It was also noted that the 10-fold diluted DNA in most cases gave the most reliable results due to the possible presence of impurities in the undiluted sample, confirming a previous study [12].

Method challenging

The efficacy of the developed method was tested by challenging it with two separate datasets. First, stool samples from 47 people that consumed yoghurt containing *L. paracasei* F19 and from 48 people that received placebo yoghurt were subjected to DNA extraction and qPCR within a few weeks after collection. It was possible to determine only 73% of the samples correctly (Table 2), mostly due to a high number of false positives (24%). Therefore, it was decided to lower the highest Ct cutoff to minimize the total number of false positives, at the same time making sure that there was no substantial increase in false negatives. It was found that lowering the Ct cutoff to 29.81 gave the lowest number of false determinations with only five false negatives (5%). Herewith, the average Ct of the true positives (24.82±1.87) was significantly different from that of the true negatives (34.81±2.08, P=2.70E-40) and the false negatives (35.06±2.24, P=2.71E-4), but the two latter were similar (P=0.82).

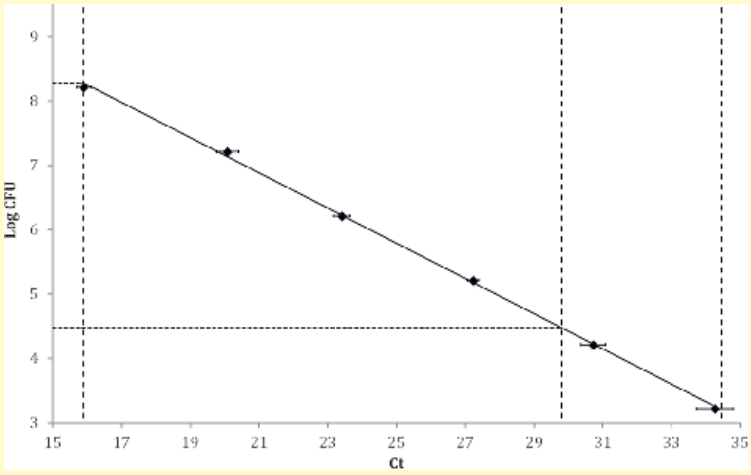

Figure 2: *Relationship between cell counts and Ct values for stool samples spiked with fixed amounts of L. paracasei F19. The curve follows a straight linefollowing the formula y = -0,2741x + 12,645 and has an R2 of 0,9992. The dashed lines from left to right indicate the minimal Ct cut-off for reliable quantification, the maximal Ct cut-off after minimizing the number of false discoveries and the maximal Ct cut-off before minimizing. The space between the horizontal dashed lines indicates the quantifiable range in Log CFU.*

	High Ct cut-off 34,26		**High Ct cut-off 29,81**	
Treatment	Positive	Negative	Positive	Negative
F19	44 (94%)	3 (6%)	42 (89%)	5 (11%)
Placebo	23 (48%)	25 (52%)	0 (0%)	48 (100%)

Table 2: *Number of true and false positive and negative qPCR measurements of 47 stool samples containing L. paracasei F19 and 48 stool samples containing placebo.Percentages are in numbers of F19 treatments and placebos that are detected correctly and wrongly.*

Supplementary material

See file: Standard sheet F19 qPCR.xlsx – standard Excel sheet for re-calculating Ct values to CFU/g feces.

The above explained experimental set-up allows the comparison of both study (containing *L. paracasei* F19) and control (placebo) samples from two datasets within one experiment, where the second set of control samples comes from the same human subjects that consumed *L. paracasei* F19 during the first trial. In addition, the second set of study samples in principle had a six-week washout period (two times two weeks washout with two weeks placebo consumption in between) before consumption of *L. paracasei* F19, where those from the first trial only had a two-week washout period. The average Ct values of the first (34.98±1.69) and the second (34.30±1.25) set of control samples were not significantly different (P=0.12), indicating that there is no influence of having consumed *L. paracasei* F19 during the first trial on the second trial. This is confirmed by the comparison of the average Ct values of the first (27.14±1.42) and second (27.59±1.95) set of study samples, which are also considered similar (P=0.35). From these results, it can be concluded that a washout period of two weeks is sufficient.

Of the previous dataset, atotal of 39 true positive samples ranging in Ct for from 24.29 to 29.77 and two of the false negatives, having Ct values of just over 30, were subjected to another DNA extraction and quantification after three years of storage at -45°C in order

to assess the stability of the samples during prolonged storage. Here, all the samples were found to be true positives. This result not only confirms a previous study [19] showing that directly frozen and further unprocessed stool samples are good for quantification of microbes by qPCR, but also that long storage at -45°C does not negatively affect the detection. In fact, all the new quantifications had a significantly (P=1.94E-19) lower Ct (22.04±2.13) than the initial quantifications (27.04±1.44), corresponding to a 26-fold higher amount of DNA. As *L. paracasei* F19 is not expected to grow at -45°C, the difference must come from changes in procedures or materials. Moreover, although there was a clear correlation between the Ct of the initial quantifications and those of the later ones, there was a high variation, as is indicated by a standard deviation of 1.97 on the average difference, corresponding to a 3.5-fold difference in DNA level. This may all be a result of small optimizations in the DNA extraction kit, the qPCR materials or the person performing the exactions [23]. In many clinical studies, it is essential that the analytical imprecision is minimized, i.e. a study is standardized and used materials - and in best case researchers - stay the same throughout the study. In that respect, it is positive that microbes still can be detected effectively after years in case of a long-term clinical trial. Freezing samples during the course of the trial and extracting DNA of all samples simultaneously will reduce the analytical imprecision by avoiding random errors caused by multiple rounds of DNA extraction by different people under different conditions.

Conclusions

This paper presents a standardized method that allows the detection and quantification of *L. paracasei* F19 in fecal samples. The dynamic range for adequate detection is from approximately 5 log CFU/g until 11 log CFU/g, depending on the exact amount of fecal material that is used for extracting DNA. Using the developed standard series and the optimization procedures, a standard Excel sheet was designed to automatically recalculate Ct values to number of cells per g. This Excel sheet is available as supplementary material. Moreover, it was shown that the bacterium still can be identified and quantified in fecal samples that are stored at -45°C for up to three years, indicating that prolonged storage of fecal samples at -45°C does not negatively affect DNA sample quality and that it can be a good way to reduce analytical imprecision in long-term clinical studies.

Conflict of interest

The authors declare no conflict of interest.

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