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ORIGINAL ARTICLE

Comparison of real-time PCR assay and plate count for *Lactobacillus paracasei* enumeration in yoghurt

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Abstract *Lactobacillus paracasei* is a mesophilic lactic acid bacterium technologically active in food fermentation. Culture-independent methods have rapidly been recognized as a valuable alternative to culture-dependent methods for lactic acid bacteria enumeration. In the present work, the efficacy of different protocols to extract DNA from yoghurt were compared, real-time PCR (qPCR) targeting *tuf* gene for *L. paracasei* enumeration was evaluated, and qPCR and plate counts of *L. paracasei* in yoghurt samples were compared. Total DNA concentrations from commercial yoghurts were higher using DNAzol method 2 than using the other tested methods. Standard curves presented suitable mean efficiency values of 91 % (pure *L. paracasei* strain CTT 7501), 95 % (pure *L. paracasei* strain FNU), and 103 % (yoghurt with *L. paracasei* strain FNU). Limit of detection is 3 log DNA copy number, corresponding to 2.78 log CFU, a suitable range of CFU enumeration for probiotic bacteria in yoghurt samples, considering that they should be present in large amounts. The *L. paracasei* (CFU) enumerated by qPCR were compared to culturable *L. paracasei* enumerated by plate counts at 7, 14, 21, and 28 days of yoghurt manufacture. Differences between qPCR and plate counts were observed only 28 days after yoghurt preparation, counts were similar at 7, 14, and 21 days. In conclusion, this qPCR assay is a useful and rapid tool to enumerate *L. paracasei* in yoghurt, although it does not distinguish dead and viable cells.

Keywords *Lactobacillus paracasei* · Quantitative PCR · Bacterial enumeration · Yoghurt · *Tuf* gene

Introduction

Lactobacilli species are involved in both spontaneous fermentation and large-scale fermentation processes for the preservation and transformation of many raw materials such as milk (Furet et al. 2004; Rushdy and Gomaa 2013). Lactobacilli strains have been used commercially over the last years as they are believed to possess probiotic features (Francesca et al. 2013; Herbel et al. 2013). Probiotics are defined as "live micro-organisms that when administered in adequate amounts, confer a health benefit on the host". However, to exert health benefits, the concentration of live probiotic bacteria needs to be of approximately 6 log CFU g^{-1} of the product at the time of consumption (Roy 2005).

Among the various types of food products, yoghurt or similar products have been used as the most popular vehicles for the incorporation of probiotic microorganisms (Kristo et al. 2003). Yoghurt has long history of recognition as a dietary product with many desirable effects. It is made from the symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. These yoghurt starter bacteria might not survive the gastric passage or colonize the gut and consequently may not play a role in the human gut. Hence, the recent trend is to add probiotic bacteria to yoghurt (Ashraf and Shah 2011).

Lactobacillus paracasei is a mesophilic lactic acid bacterium technologically active in food fermentation. *L. paracasei* subsp. *paracasei* NTU 101 and its fermented products proved to be effective for the management of blood cholesterol and

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pressure, prevention of gastric mucosal lesion development, immunomodulation and alleviation of allergies, prevention of osteoporosis, and inhibition of fat tissue accumulation (Chiang and Pan 2012). *L. paracasei* M7 was shown to inhibit the adhesion of *Salmonella* to epithelial cells (Xue et al. 2015). *L. paracasei* FNU was shown to possess desirable in vitro resistance to low pH and bile salts (Ilha et al. 2014).

Currently, despite their economic impact, most of the assays that are used to identify and quantify lactobacilli are classical microbiological methods. Lactobacilli are often hard to be distinguished by classical microbiological techniques since most of them have similar nutritional and growth requirements (Kao et al. 2007; Poltronieri et al. 2008). Generally, dairy products, such as yoghurt and cheese, contain a population of several species of intimately related lactic acid bacteria. Furthermore, phenotypic characteristic analysis is always time-consuming and labor-intensive.

Culture-independent methods have rapidly been recognized as a valuable alternative to culture-dependent methods (Agrimonti et al. 2013; Ceccherini et al. 2013; De Medici et al. 2015; Ilabaca et al. 2014; Ke et al. 2014; Reitschuler et al. 2014; Rodriguez-Lazaro et al. 2015; Ruiz et al. 2014). These methods can be based on the direct analysis of DNA extracted from the food matrix (Achilleos and Berthier 2013; Rodríguez et al. 2012). DNA extraction is a crucial step for reliable DNA quantification by real-time PCR, called qPCR (Cankar et al. 2006; Garcia et al. 2013; Oliveira et al. 2013; Tian et al. 2013). Bacterial DNA amplification by PCR from milk samples can be affected by the presence of inhibitory substances such as Ca^{2+}, fat, and proteins (Machado et al. 2013), so it is a challenge to obtain good quality DNA from dairy products for PCR purposes (Pirondini et al. 2010; Quigley et al. 2012).

In order to monitor lactic acid bacteria on dairy products, molecular methods based on bacterial DNA amplification by real-time PCR have been successfully developed for *Lactobacillus acidophilus*, *L. brevis*, *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*, and *L. reuteri* in yoghurt (Herbel et al. 2013), and for *S. thermophilus*, *L. delbrueckii*, *Lactobacillus casei*, *L. paracasei*, *L. rhamnosus*, *L. acidophilus,* and *L. johnsonii* in fermented milk products (Furet et al. 2004). Several species-specific primer pairs were designed based on the variability of 16S rRNA sequences for differentiating five strains of lactobacilli that were added into probiotic products in Taiwan (Kao et al. 2007). It was simple to identify *L. acidophilus* and *L. delbrueckii* by species-specific primers, but it could not be used to distinguish *L. casei*, *L. paracasei*, and *L. rhamnosus* (Kao et al. 2007). The *yycH* gene was proposed as an additional molecular marker for *L. casei* group species discrimination that provides higher resolution than 16S rRNA (Huang et al. 2014). Recently, a real-time PCR assay to quantify *L. paracasei* in cheese was developed targeting an elongation factor gene (Achilleos and Berthier 2013). The elongation factor (*tuf*) gene was shown to be highly variable among lactic acid bacteria, especially among closely related species such as those of the *L. casei* group (Chavagnat et al. 2002; Yu et al. 2012).

In the present study, *L. paracasei* FNU was incorporated as a probiotic strain in yoghurt. This strain was previously characterized as being resistant to low pH and bile salts (Ilha et al. 2014). The objectives of the present study were to verify *L. paracasei* FNU growth and viability in yoghurt by plate count, to compare the efficacy of protocols to extract DNA from yoghurt, to evaluate qPCR targeting the *tuf* gene for *L. paracasei* enumeration, and to compare qPCR and plate counts of *L. paracasei* in yoghurt samples. The novel aspects of this work are the comparison of bacterial DNA extraction methods giving different extraction yields; the bacterial count to check survival of the added culture during 28 days; and the use of Tuf primers for *L. paracasei* DNA quantification in a new matrix, yoghurt containing high population of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, instead of cheese as proposed previously (Achilleos and Berthier 2013).

Material and methods

Bacterial strains and culture conditions

For the primer specificity test, bacterial strains *Lactobacillus paracasei* FNU, isolated from grape sourdough (Ilha et al. 2014); *Lactobacillus paracasei* CCT 7501 acquired from the collection of cultures of André Tosello Foundation (Campinas, São Paulo, Brazil); *Lactobacillus paracasei* LY0 750 provided by Danisco (Cotia, São Paulo, Brazil); and *Lactobacillus plantarum* (ATCC 8014) purchased from ATCC (Manassas, VA, USA), were grown in De Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) at 30 °C for 24 h. Furthermore, *Pseudomonas* spp. was grown in Luria-Bertani broth (USB, Cleveland, OH, USA) at 28 °C, *Escherichia coli* (ATCC 25922, Manassas, VA, USA) was grown in Brain-Heart Infusion broth (Himedia, PA, USA), while *Bacillus cereus* (ATCC 14579, Manassas, VA, USA) was grown in nutrient broth (Himedia, PA, USA) at 37 °C for 24 h. Optical density (OD) of bacterial cell culture was measured at 600 nm using Hitachi U2910 Spectrophotometer (IL, USA).

Lactobacillus acidophilus (LA-5, Chr. Hansen, Hónsholm, Denmark) and *Bifidobacterium animalis* subsp. *lactis* (BB-12, Chr. Hansen, Hónsholm, Denmark) were maintained in UHT whole milk at−20 °C and activated at 37 °C for 2 h. The thermophilic starter culture was prepared using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Yo-Flex® L812, Chr. Hansen, Horsholm, Denmark) according to the manufacturer's protocol.

Preparation of *L. paracasei* FNU culture for addition to the yoghurt

Cell suspension was prepared in MRS broth by growing the strain 24 h at 30 °C three times to reach 1 L of culture. Afterwards, cells were harvested by centrifugation (17,920*g*, 20 min, 4 °C), washed twice with sterile peptone water (0.01 g mL^{-1}), suspended in 500 mL whole UHT milk, and kept under−20 °C in sterile glass bottles. The viable cell count in culture suspended in UHT whole milk after freezing was carried out in MRS agar.

Yoghurt preparation

Yoghurt was prepared with UHT milk with added starter culture containing *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, according to manufacturer's instructions. The mixture was incubated at 45 °C, until reaching a pH of 4.6, followed by cooling to 4 °C and storage at 4 °C for 24 h. The prepared yoghurt was then divided into two lots: yoghurt without addition of *L. paracasei* FNU (control yoghurt) and yoghurt with addition of milk suspension containing *L. paracasei* FNU (8 log CFU mL^{-1} final concentration). Yoghurts were distributed in 200 mL sterile glass bottles, kept at 4 °C, and sampling was performed at days 1, 7, 14, 21, and 28 after yoghurt preparation. Samples were used immediately for plate counting or stored at−20 °C for DNA extraction.

Commercial probiotic yoghurts, called commercial yoghurt A and commercial yoghurt B, were acquired in local supermarkets and used only for evaluation of DNA extraction protocols.

Evaluation of DNA extraction protocols

DNA extraction protocols were evaluated: DNeasy Mericon Food Kit (Qiagen, Venlo, Netherlands), DNeasy Mericon Food Kit (Qiagen, Venlo, Netherlands) with some modifications (20 μL of proteinase K instead of 2.5 μL and incubation at 65 °C instead of 60 °C), DNAzol® method 1 (Villegas-Rivera et al. 2013), DNAzol® method 2 (Achilleos and Berthier 2013), and CTAB method (Lipp et al. 1999). Samples of milk, commercial yoghurt, and bacterial culture medium were used to evaluate DNA extraction protocols in duplicate.

Bacterial culture DNA extraction protocol

For genomic DNA extraction in duplicate from bacterial culture medium, 2 mL medium aliquots (OD 0.8) were centrifuged (6000*g*, 3 min, 4 °C) and pellets were stored at−80 °C until DNA extraction. Pellets were suspended in 100 μL of ultrapure water, frozen at−80 °C for 20 min, and immediately heated in boiling water (100 °C) for 10 min (Pereira et al. 2014). Cell suspension was cooled to room temperature, centrifuged (13,000*g*, 10 s) and supernatant was used for DNA extraction using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. DNA concentration and purity were determined on a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, DE, USA) with measurements at 260 and 280 nm.

DNA extraction protocol for yoghurt: DNAzol method 2

For genomic DNA extraction from yoghurt, starter culture and probiotic bacteria activated in milk, DNA was extracted in duplicate according to a method adapted from (Achilleos and Berthier 2013), named DNAzol® method 2. Ten milliliters of yoghurt or milk samples, 25 mL of 0.9 % NaCl, 8 mL of 25 % trisodium citrate, 2 g of polyethylene glycol 8000, and water, to a final volume of 50 mL, were homogenized for 5 min, separated into two aliquots of 25 mL, and centrifuged (9700*g*, 15 min, 4 °C). Pellets were stored at−20 °C until DNA extraction. Frozen pellets were thawed for 15 min at room temperature, suspended in 1 mL of DNAzol® reagent (Life Technologies, Carlsbad, CA, USA), and protocol was followed as described by (Achilleos and Berthier 2013).

Real-time PCR quantification

In order to detect and quantify by qPCR the presence of *L. paracasei* in yoghurt, Tuf primer pair was used, (TCCGGGAACTGCTCAGC and TGTTTCAC GAACAGGTG) (Achilleos and Berthier 2013), which amplifies a fragment of 161 bp of the elongation factor Tu (*tuf*) gene. Quantitative real-time PCR was performed in ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA). Amplification reactions were carried out in duplicate in a final volume of 25 μL containing 12.5 μL of 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM of TufF and 150 nM of TufR, water, and template DNA (10 ng). The amplification program was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence signal was measured at the end of each 60 °C step. Melting curve analysis was performed automatically by continuous heating from 65 °C to 95 °C. All real-time PCR runs were analyzed using automatic software settings.

Construction of standard curves

Standard curves were prepared with serial dilutions of genomic DNA isolated from *L. paracasei* FNU, *L. paracasei* CCT 7501 and yoghurt. The number of bacterial DNA copies were calculated on the basis of the size of the *L. paracasei* strain

8700:2 (GenBank accession number NC_022112.1) genome (3.025 Mbp) using Avogadro's constant (6.023×10^{23}) and the molecular weight of DNA (660 Da/bp). Genomic DNA was tenfold serially diluted in ultra-pure water to final concentrations ranging from 10^7 to 10^0 genome copies per 2 μL, equivalent to concentrations of 33.2 to 3.32×10^{-6} ng.

The Cq versus log CFU of *L. paracasei* was estimated using genomic DNA extracted from the bacteria culture grown until stationary growth phase (OD 0.8) or from yoghurt with *L. paracasei* day 1. Ten times serial dilutions of DNA extracted from *L. paracasei* was performed and the corresponding CFU was calculated based on plate counting of the same sample, bacterial culture or yoghurt with *L. paracasei* day 1.

Standard curves were generated by the plot cycle threshold (Cq) values versus logarithm of bacterial DNA copy number (pure culture) or CFU (pure culture and yoghurt). Amplification efficiencies were determined using the following equation: $E=10^{(-1/S)}-1$, where E is the efficiency and *s* is the slope obtained from the standard curve.

L. paracasei enumeration by qPCR

Genomic DNA was extracted from the yoghurt with *L. paracasei* from day 1 and serial dilutions were prepared, equivalent to concentrations of 33.2 to 3.32×10^{-6} ng of total DNA. Taking into account CFU obtained by plate counting from the same sample, CFU per reaction was calculated using the plot Cq versus CFU.

Bacterial count (CFU mL^{-1}) in control yoghurt and yoghurt with *L. paracasei* collected in different days were determined using the following equation: $bacterial\ \ count = \frac{A*B*C}{D*E}$, where A is CFU per reaction well obtained from Cq of the DNA sample using standard curve (Cq versus log CFU), B is the extracted DNA concentration (ng μL^{-1}), C is total volume of extracted DNA (μL), D is template DNA mass in reaction well (ng), and E is yoghurt volume (mL) used for DNA extraction.

L. paracasei enumeration by plate counting

The enumeration of *L. paracasei* FNU in yoghurt was carried out as previously described (Van de Casteele et al. 2006) with modifications. Control yoghurt was prepared with the addition of starter culture and yoghurt with *L. paracasei* was prepared with the addition of starter culture and *L. paracasei* FNU in a final concentration of 8 log CFU mL^{-1}. *Lactobacillus casei* (LC) medium (Ravula and Shah 1998), adjusted to pH 5.0 using 5 M HCL, and supplemented with a membrane-filtered sterile solution of 10 % (w/v) D (−) ribose (Sigma Aldrich; 1 % final concentration) and 0.2 % (v/v) bromocresol green solution (Sigma Aldrich; 0.04 % (v/v) final concentration) was used. The samples were tenfold diluted and aliquots were pour plated on LC medium with an overlay of LC medium and incubated at 30 °C for 3–4 days.

Enumeration of *L. paracasei* CCT 7501 and *L. paracasei* FNU in pure cultures were made by pour plate technique on MRS agar (Merck, Darmstadt, Germany) with an overlay of MRS agar and incubation for 48 h at 30 °C. Enumerations were performed in triplicate.

Results

Comparison of yield and purity of DNA extracted using five protocols

Protocols were tested to compare their relative efficiency with respect to extracting DNA on the basis of yield and purity from milk, commercial yoghurt, and bacterial culture medium. The first tested protocol was DNeasy Mericon Food Kit, but it provided a very low DNA yield from commercial yoghurt (Table 1), despite the modifications realized to this method. Using DNAzol method 1 (Villegas-Rivera et al. 2013) and CTAB method (Lipp et al. 1999), it was not possible to extract DNA from *L. paracasei* bacterial culture medium or *L. paracasei* activated in milk. Using DNAzol method 2 (Achilleos and Berthier 2013), total DNA concentrations from commercial yoghurts A and B were higher than DNA concentrations obtained using modified DNeasy Mericon kit from the same samples, although DNA purity was better using modified DNeasy Mericon kit. With respect to DNA yield from milk containing bacteria (*L. paracasei* FNU, *L. acidophilus* LA-5, *Bifidobacterium* BB-12, or starter culture), DNA concentrations varied from 27 to 371 ng μL^{-1} (Table 1).

Using DNAzol method 2, total DNA concentrations from yoghurt prepared with thermophilic starter culture, with or without *L. paracasei*, varied from 37 to 154 ng μL^{-1} (Table S1).

Genomic DNA was successfully extracted from pure bacterial culture medium using Wizard kit from *L. paracasei*, *L. plantarum*, *E. coli*, *B. cereus*, and *Pseudomonas* spp. (Table S2), and they were used to test primer specificity.

Primer specificity for *L. paracasei*

Specificity test was conducted using DNA extracted from *L. paracasei* strains and other bacterial genera to verify if the Tuf primer pair was able to detect exclusively *L. paracasei*. Specificity test was done using bacterial DNA extracted from medium cultures or milk (10 ng of template DNA). *L. paracasei* strains FNU, CCT 7501, and LYO750 presented mean Cq values±standard deviation equal to 16.7±0.7, 18.2±0.4, and 15.7±0.2, respectively, and amplicon presented Tm of 78.3±0.2, 78.2±0.4, and 78.2±0.2, respectively (Table 2). *Bifidobacterium* BB-12 and *L. plantarum* did not

Table 1 Yield and quality of total DNA obtained with DNA extraction methods from commercial yoghurt, milk, and bacterial culture medium samples

Extraction method	Sample	DNA concentration (ng μL^{-1})	Purity (A_{260}/A_{280})
Dneasy Mericon Food Kit (Qiagen)	Commercial yoghurt A	6.4	1.37
	Commercial yoghurt A	3.6	1.35
	Commercial yoghurt B	1.4	2.75
	Commercial yoghurt B	2.6	1.42
Modified Dneasy Mericon Food Kit (Qiagen)	Commercial yoghurt A	4.6	1.60
	Commercial yoghurt A	4.7	1.90
	Commercial yoghurt B	5.1	1.86
	Commercial yoghurt B	4.7	1.78
	L. paracasei FNU in culture medium	5.5	1.71
	L. paracasei FNU in culture medium	6.8	1.94
DNAzol® method 1 [a]	*L. paracasei* FNU in culture medium	nd	nd
	L. paracasei FNU in culture medium	nd	nd
	L. paracasei FNU in UHT milk	nd	nd
	L. paracasei FNU in UHT milk	nd	nd
CTAB method [b]	*L. paracasei* FNU in culture medium	nd	nd
	L. paracasei FNU in culture medium	nd	nd
	L. paracasei FNU in UHT milk	nd	nd
	L. paracasei FNU in UHT milk	nd	nd
DNAzol® method 2 [c]	Commercial yoghurt A	10.7	1.35
	Commercial yoghurt A	11.1	1.25
	Commercial yoghurt B	14.4	1.21
	Commercial yoghurt B	20.1	1.32
	L. paracasei FNU in UHT milk	27.0	1.07
	L. paracasei FNU in UHT milk	26.7	1.35
	L. acidophilus LA-5 in UHT milk	155.0	1.90
	L. acidophilus LA-5 in UHT milk	161.0	1.80
	Bifidobacterium BB-12 in UHT milk	110.5	1.70
	Bifidobacterium BB-12 in UHT milk	157.6	1.60
	Starter culture in UHT milk	355.0	1.80
	Starter culture in UHT milk	388.7	1.90

[a] (Villegas-Rivera et al. 2013)

[b] (Lipp et al. 1999)

[c] (Achilleos and Berthier 2013)

nd, not detected

present amplification as expected for all qPCR assays. Other negative samples presented some unspecific amplification, with late Cq and different Tm values compared to *L. paracasei* Tm value of 78.2. *Bacillus cereus* presented 2 positives out of 6 total repetitions, with Cq higher than 31.7. Starter culture (*L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*) presented three positives out of six total repetitions, with late Cq (Cq>38.4) and different Tm values. *E. coli*, *Pseudomonas* spp., and *L. acidophilus* also presented unspecific amplification at late Cq values (Table 2). *L. acidophilus* showed a late Cq (Cq>34.3) in three reactions out of six repetitions. *E. coli* and *Pseudomonas* presented late Cq (Cq>33.9 and Cq>32.7, respectively) for all six repetitions with different Tm values.

qPCR parameters for *L. paracasei* enumeration

The reaction parameters (efficiency and correlation coefficient) of the qPCR assay using Tuf primer pair were determined based on standard curves obtained from tenfold serial dilution of bacterial DNA isolated from pure culture of *L. paracasei* CCT 7501 (Fig. 1) and *L. paracasei* FNU (Fig. 2a), performed in three and five qPCR runs on different days, respectively. The standard curves presented suitable

Table 2 Cq and Tm obtained by qPCR assay using DNA extracted from *L. paracasei* (positive controls) and other bacterial species (negative controls)

Sample*	Cq	Tm1	Tm2	Tm3	Sample*	Cq	Tm1	Tm2	Tm3
Lactobacillus paracasei FNU	17.83	78.01			*Pseudomonas* spp.	32.91	76.88	67.37	
	16.61	78.20				32.65	75.78	86.75	67.73
	16.82	78.39				32.78	85.47	77.61	
	16.23	78.39				33.47	85.65	67.00	
	16.26	78.39				35.89	77.06	72.12	
	16.42	78.39				36.88	77.06	64.32	
Lactobacillus paracasei CCT 7501	19.00	77.63			*Bifidobacterium* BB12	nd	nd		
	24.02	77.82				nd	nd		
	16.74	78.16				nd	nd		
	16.63	78.34				nd	nd		
	16.50	78.52				nd	nd		
	16.47	78.52				nd	nd		
Lactobacillus paracasei LYO 750	15.94	78.01			*Lactobacillus acidophilus* LA-5	nd	nd		
	16.00	78.20				39.06	72.69		
	15.40	78.52				34.28	78.3	65.55	
	15.42	78.34				nd	nd		
	15.48	78.16				36.41	74.24	78.44	64.7
	15.69	77.97				36.04	72.91	79.59	
Bacillus cereus	33.98	77.82			*Lactobacillus plantarum*	nd	nd		
	nd	nd				nd	nd		
	31.68	78.01				nd	nd		
	nd	nd				nd	nd		
	nd	nd				nd	nd		
	nd	nd				nd	nd		
Escherichia coli	33.87	77.79	65.90		Starter culture	38.34	73.42		
	35.35	79.07	65.54	84.74		39.61	73.42		
	37.92	63.89				nd	nd		
	35.30	84.56	65.54			39.53	81.07		
	35.29	65.90	81.26	84.92		nd	nd		
	37.96	63.71				nd	nd		

* 10 ng of template DNA; nd, not detected with Cq>40.

linear correlation coefficient (R^2) and mean efficiency (E), R^2>0.93 and E of 91 % for *L. paracasei* CCT 7501 and R^2>0.95 and E of 95 % for *L. paracasei* FNU (Table 3).

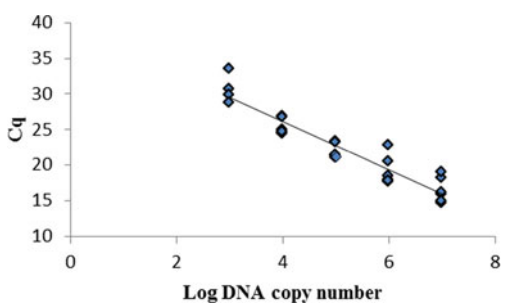

Fig. 1 qPCR assay standard curves for *L. paracasei* CCT 7501 performed in three qPCR runs in different days

The limit of detection (LOD) for both *L. paracasei* strains was 3 log DNA copy number, corresponding to 3.32 pg of DNA. For *L. paracasei* FNU, LOD corresponded to a mean Cq of 29.1. The Cq versus log CFU of *L. paracasei* FNU (Fig. 2b) was estimated using genomic DNA extracted from the *L. paracasei* FNU bacterial culture plate counted in parallel, so tenfold serial dilutions of bacterial DNA were performed and the corresponding CFU values were calculated based on plate counting. LOD corresponded to 2.78 log CFU of *L. paracasei* (Fig. 2b).

In order to evaluate qPCR enumeration of *L. paracasei* in yoghurt samples, amplification efficiency value was determined by the construction of standard curve of serial dilution of DNA extracted from yoghurt prepared with *L. paracasei* (day 1). In this case, the efficiency value was 103 % and R^2

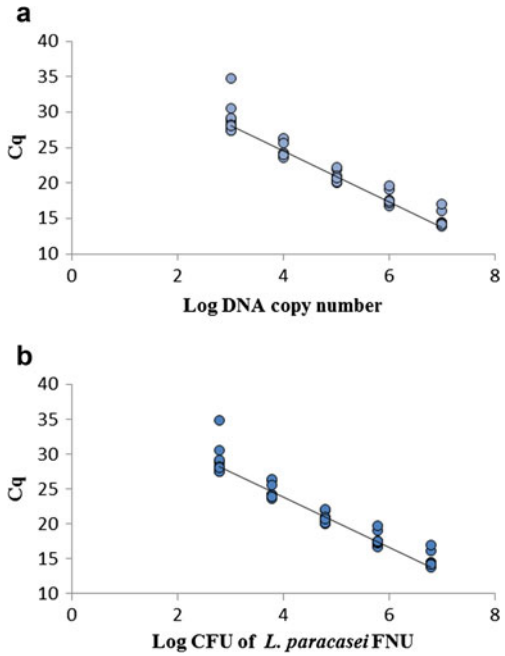

Fig. 2 qPCR assay standard curves for *L. paracasei* FNU performed in five qPCR runs in different days, DNA extracted from culture medium. (**a**) Cq versus log DNA copy number and (**b**) Cq versus log CFU for *L. paracasei* FNU

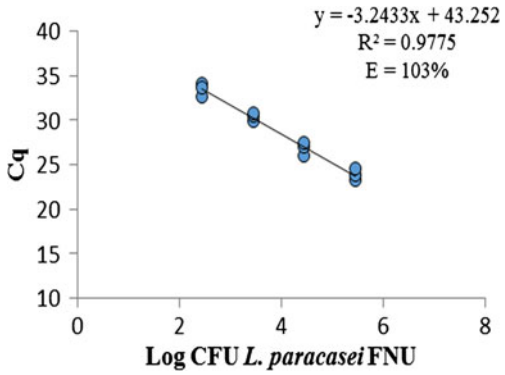

Fig. 3 qPCR assay standard curves for *L. paracasei* FNU performed in three qPCR runs in different days, Cq versus log CFU. DNA extracted from yoghurt with *L. paracasei* FNU day 1

was 0.98 (Fig. 3), while for yoghurt sample (day 1) the log CFU was calculated based on plate counting.

Enumeration of *L. paracasei* in yoghurt samples

The average count of *L. paracasei* FNU in frozen UHT culture used for yoghurt preparation was 9.13 log CFU mL^{-1}, obtained by plate counting. Yoghurt samples were collected 1, 7, 14, 21, and 28 days after preparation, and *L. paracasei* enumeration in these yoghurt samples was performed by plate counting and qPCR. DNA was extracted in duplicate for each sample using DNazol method 2 (Table S1) and submitted to qPCR assay (Table 4). Control yoghurt samples collected at 1, 7, 14, 21, and 28 days did not present amplification or

Table 3 qPCR parameters, efficiency values (E) and correlation coefficient (R^2), of standard curves for *L. paracasei* CCT 7501 and *L. paracasei* FNU performed in different days

	L. paracasei CCT 7501	*L. paracasei* FNU		
	E (%)	R^2	E (%)	R^2
Day 1	90	0.963	95	0.996
Day 2	93	0.937	116	0.954
Day 3	89	0.986	87	0.989
Day 4	-	-	81	0.958
Day 5	-	-	95	0.998
Mean	91	0.962	95	0.979
SD	2.08	0.02	13.24	0.02

Table 4 Mean Cq and Tm obtained by qPCR assay using DNA extracted from control yoghurt and yoghurt with *L. paracasei*

Sample	Cq	Tm1	Tm2	Sample	Cq	Tm1	Tm2
Control yoghurt				Yoghurt with *L. paracasei*			
Day 1	32.94	73.57		Day 1	23.22	78.78	
	31.9	73.38			22.14	78.78	
	nd	nd			21.84	78.81	
	37.05	80.12			22.28	78.81	
	34.10	71.76	83.40		24.22	78.72	74.14
	35.69	78.82	71.76		23.57	78.72	73.57
Day 7	31.87	73.57		Day 7	21.19	78.72	73.38
	33.20	73.38			20.83	78.72	
	38.54	62.31			19.16	78.59	
	32.52	78.39			18.70	78.59	
	37.52	72.95			17.85	78.23	
	37.90	64.34			18.22	78.23	
Day 14	32.35	73.57		Day 14	20.50	78.72	
	32.62	73.76			20.09	78.72	
	39.04	72.27			18.60	78.59	
	36.81	74.57			18.62	78.59	
	nd	nd			19.39	78.42	
	nd	nd			18.87	78.23	
Day 21	32.67	73.95		Day 21	23.87	78.72	
	32.24	73.57			24.36	78.53	73.38
	nd	nd			22.56	78.78	
	nd	nd			22.39	78.59	
	nd	nd			22.90	78.44	
	nd	nd			22.75	78.44	
Day 28	32.46	73.57		Day 28	21.25	78.16	
	33.27	73.57			20.35	78.16	
	nd	nd			19.77	78.59	
	35.38	74.37			19.83	78.59	
	nd	nd			19.51	78.44	
	nd	nd			19.74	78.44	

*nd, not detected with Cq>40.

presented unspecific amplification with late Cq (Cq>31.9). Tm values of control yoghurt samples were different for *L. paracasei* FNU mean Tm value of 78.3±0.2 (Table 2) and also different for yoghurt with *L. paracasei* mean Tm value of 78.6±0.2 (Table 4). Regarding yoghurt samples with *L. paracasei*, Cq values ranged from 18.9 to 23.4, and all replicates presented amplification with similar Tm value, mean Tm of 78.6±0.2 (Table 4).

The standard curve equation Cq versus log CFU of samples of yoghurt prepared with *L. paracasei* day 1 (Fig. 3) was used to calculate CFU per reaction well of all yoghurt samples from obtained Cq values. It was possible to obtain *L. paracasei* count (CFU $mL^{-1}$of yoghurt) of yoghurt samples by qPCR (Table 5) using the equation described in material and methods. Bacterial count was obtained from the same yoghurt samples by plate counting (Table 5) based on a plate count method previously described (Van de Casteele et al. 2006). *L. paracasei* counts were similar by qPCR and plate count for samples collected from 7, 14, and 21 days after yoghurt preparation (Table 5); however, *L. paracasei* enumeration was lower by plate count (7.75 log CFU mL^{-1}) than qPCR count (9.73 log CFU mL^{-1}) for sample collected 28 days after yoghurt preparation. *L. paracasei* FNU viable cells were presented in high quantity (7.75 log CFU mL^{-1}) even 28 days after yoghurt preparation as determined by plate count.

Discussion

The first requirement of a DNA-based method for bacterial quantification in food is an efficient DNA extraction method from that food (Garcia et al. 2013; Oliveira et al. 2013; Quigley et al. 2012). Bacterial DNA extraction from a dairy product is a special challenge to obtain DNA without PCR inhibitors such as calcium and fat (Pirondini et al. 2010). In the present study, different protocols were assessed to compare their relative success with respect to DNA extraction from commercial yoghurt. Using DNAzol method 2, total DNA concentrations from commercial yoghurts were higher than DNA concentrations obtained using the other tested methods (Table 1). DNAzol method 2 was chosen to extract DNA from yoghurt (Table S1) because it was used for qPCR enumeration assay of *Lactobacillus paracasei* and *Lactococcus lactis* in cheese (Achilleos and Berthier 2013). A qPCR assay targeting the *tuf* gene was successfully employed for *L. paracasei* enumeration in cheese (Achilleos and Berthier 2013). Several other lactic acid bacteria were tested as negative controls by other authors (Achilleos and Berthier 2013), and this qPCR assay using Tuf primers were specific enough for the identification of *L. paracasei*. We tested the specificity of these primers using other bacterial species (Table 2), and some bacterial species presented unspecific amplification; however, Cq values were always above the Cq value corresponding to LOD (Cq=29), and they presented amplicons with different Tm values compared to *L. paracasei* samples. The ΔCq observed between DNA samples (10 ng) of *L. paracasei* strains (Cq<24) and the other bacteria (Cq> 31) is sufficient to reinforce the use of this Tuf primer pair because these amplifications of other bacterial DNA are unspecific and they are easily distinguishable by their Tm. Unspecific amplification of other bacterial DNA with late Cq were also observed for qPCR assays targeting *tuf* gene developed for *L. helveticus* and *L. rhamnosus* even using hydrolysis probe (Desfosses-Foucault et al. 2012).

The choice of DNA extraction method can influence the quantification by real-time PCR and it is essential that the procedure results in an optimal yield of DNA and in removal of substances that could influence PCR efficiency (Cankar et al. 2006; Rodriguez-Lazaro et al. 2007). Comparing qPCR efficiency values obtained for this qPCR assay when target DNA is DNA extracted from *L. paracasei* pure cultures (Table 3 and Fig. 2) or DNA extracted from yoghurt prepared with *L. paracasei* FNU (Fig. 3), standard curves presented mean efficiency values of 91 % (pure strain CTT 7501), 95 % (pure strain FNU), and 103 % (yoghurt with strain FNU); suitable efficiency values shall be between 90 % and 110 % (Rodríguez et al. 2012). The qPCR efficiency values obtained by Achilleos and Berthier (2013) ranged from 81.1 % to 99.5 % for *L. paracasei* Tuf qPCR assay. The qPCR assay applied to one matrix may not be suitable for

Table 5 Comparison of *L. paracasei* FNU count (log CFU/mL) obtained by qPCR and plate count

Sample	qPCR count (log CFU/mL)[a]	Plate count (log CFU/mL)[b]
Yoghurt with *L. paracasei* – Day 1	-	8.17±0.09
Yoghurt with *L. paracasei* – Day 7	9.48±0.47	9.12±0.15
Yoghurt with *L. paracasei* – Day 14	9.40±0.27	8.81±0.72
Yoghurt with *L. paracasei* – Day 21	8.30±0.27	7.95±0.05
Yoghurt with *L. paracasei* – Day 28	9.73±0.30	7.75±0.15*

[a] mean value±SD calculated from three different qPCR runs

[b] mean value±SD of triplicate plate count

*The significance of differences ($P<0.05$) between qPCR count and plate count was assessed by Student's *t*-test.

other matrices. In order to verify applicability of this qPCR assay for bacterial DNA quantification in yoghurt samples, standard curves using total DNA extracted from yoghurt were performed and an efficiency value of 103 % was obtained, indicating the absence of inhibitors from yoghurt. This can be evidence that the matrix did not significantly affect the PCR (Agrimonti et al. 2013).

Limit of detection (LOD) is the lowest amount of sample that can be reliably detected; in this qPCR assay the LOD is 3 log DNA copy number. This *L. paracasei* qPCR assay ensured the reliably detection of *L. paracasei* DNA ranging between 7 log genome copies (33.2 ng) to 3 log genome copies (3.32 pg) in the reaction well. It is a suitable range of genome copy number enumeration for probiotic bacteria in yoghurt samples, once they should be presented in large amounts. Similar to our results, the qPCR assays targeting the Tuf gene developed for *L. helveticus* and *L. rhamnosus* presented a range of quantification from 8 log to 3 log copy number (Desfosses-Foucault et al. 2012).

The *L. paracasei* FNU were presented in the range 8.30–9.73 log CFU mL^{-1} of yoghurt as determined by plate count at 7, 14, 21, and 28 days after yoghurt preparation. This concentration of live probiotic bacteria is considered enough to exert health benefits (Roy 2005). The *L. paracasei* FNU quantity (CFU mL^{-1}) enumerated by qPCR was compared to *L. paracasei* enumerated by plate counts at different days of yoghurt manufacture (Table 5). Difference between qPCR and plate count was observed only 28 days after yoghurt preparation; counts were similar at 7, 14, and 21 days. A possible explanation on the statistical significant difference between qPCR and plate count at 28 days is the presence of dead cells that could not be distinguished from viable cells by qPCR, since it amplifies DNA from both dead and viable bacteria as DNA remains stable after the death of bacteria (Li et al. 2013). An alternative approach to detect only viable bacteria is viability qPCR using dyes that intercalate DNA of dead cells, such as ethidium monoazide and propidium monoazide (Barbau-Piednoir et al. 2014; Elizaquivel et al. 2014). Another strategy to detect viable bacteria is the analysis of 16S transcripts by RNAseq (Gosalbes et al. 2011), although it is an expensive strategy. In conclusion, this qPCR assay is a useful and rapid tool to enumerate *L. paracasei* in yoghurt, although it does not distinguish dead and viable cells.

Conflict of Interest Authors declare that they have no conflict of interest.

Compliance with Ethical Standards This article does not contain any studies with human or animal subjects.

Informed consent was obtained from all individual participants included in the study.

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