Comparison of *tuf* gene-based qPCR assay and selective plate count for *Bifidobacterium animalis* subsp. *lactis* BB-12 quantification in commercial probiotic yoghurts

[1]Odooli, S., [1,2]Khalvati, B., [1]Safari, A., [1]Mehraban, M.H., [4]Kargar, M. and [1,2,3]*Ghasemi, Y.

[1]Pharmaceutical Sciences Research Center, School of Pharmacy, ShirazUniversity of Medical Sciences, Shiraz 713451583, Iran

[2]Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz 7146864685, Iran

[3]Department of Medical Biotechnology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz 7134814336, Iran

[4]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom 74135355, Iran

Abstract

Yoghurt is one of the most prevalent vehicles for the delivering of probiotic bacteria to the consumer. A minimal concentration of 10^6 colony forming units (CFU)/ml of a product is required for optimal probiotic functionality. In this study, a new quantitative real-time PCR (qPCR) assay based on Bifidobacterial single-copy *tuf* gene was developed for the detection and quantification of *Bifidobacterium animalis* subsp. *lactis* BB-12. The specificity of designed primer set was evaluated by operation PCR reactionswith DNAs from common probiotic Bifidobacteria and Lactobacilli strains presented in probiotic yoghurts. Finally,BB-12 was detected and enumerated through *tuf* gene-based PCR, tuf gene-based qPCR and selective plate count during shelf life and after the expiry date of commercial probiotic yoghurts. Statistical comparison of enumeration by selective plate count and qPCR methods was also investigated. The PCR assay confirmed the specificity of tuf gene-based primer set for BB-12. The obtained standard curve of tuf gene-based qPCR reactions from 10^4-10^9CFU/ml was linear (R^2=0.98) with the efficiency of 90.4%. Significant differences were observed among BB-12 counts measured in yoghurts with the qPCR and selective plate count. Total bacterial count averages were higher with the qPCR method compared to selective plate count. Although the counts of *B.animalis* subsp.*lactis* BB-12 had a significant decrease during shelf-life, but these counts didn't fall below CODEX standard10^6CFU/ml) until the expiry date of the products. In conclusion,despite the fact that the new qPCR assay developed here is a specific, rapid and easy method for quantification of both cultivable and dormant BB-12 cells, but it does not distinguish dead and viable cells. Moreover, selective plate count method doesn't detect dormant bacterial populations. We deducethat the choice of enumeration method for probiotic bacteria may have a significant effect on the results of the analysis.

Introduction

Probiotics are live and non-pathogenic microorganisms which when consumed in adequate amounts confer some health benefits on the host and are able to prevent or improve a variety of gastrointestinal diseases (Reimann *et al*., 2010; Kadooka *et al.,* 2011; Rodrigues *et al*., 2012; Bunešová *et al*., 2014; Davis, 2014; Jungersen *et al.*, 2014). Several health benefits have been claimed for probiotic bacteria e.g., anti-mutagenic and anti-carcinogenic properties, antimicrobial activity, anti-diarrheal, prevention of eczema and atopy, reduction in blood pressure, reduction in serum cholesterol concentration, modulation of the immune system, growth stimulation, improvement ingastroenteritis/inflammatory bowel disease, increased resistance to infectious diseases, reduction of lactose intolerance and maintenance of balanced flora (Ashraf and Shah, 2011; Rodrigues *et al.*, 2012; Jungersen *et al.*, 2014; Mianzhi and Shah, 2015).The majority of the species used as human probiotics are belong to *Lactobacillus* and *Bifidobacterium* genera (Fotiadis *et al*., 2008; Davis, 2014; Jungersen *et al*., 2014; Quinto *et al*., 2014).

Fermented milk and yoghurts are one of the

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*Corresponding author.
Email: *ghasemiy@sums.ac.ir*
Tel/Fax: (+98) 713 242-6729

most popular carriers for delivering probiotic bacteria in food (Van de Casteele *et al.*, 2006; Ashraf and Shah, 2011; Rodrigues *et al.,* 2012). *Bifidobacterium animalis* subsp. *lactis* BB-12 is one of the most documented probiotic *Bifidobacterium*, which is commonly used in the dairy products and infant formulas. This strain remarkably survivesin the gastrointestinal tract and is capable of adhering extraordinarily to enterocytes. Furthermore, BB-12 is a technologically appropriate strain, since it does not affect the flavor and appearance of the foods in which it is used and remain viable over the shelf-life of probiotic products (Nilsson *et al.*, 2006; Savard *et al.*, 2011; Solano-Aguilar *et al.*, 2008; Jungersen *et al.*, 2014).

Viability, metabolic activity, and count are three basic criteria for probiotic bacteria to exert expected health positive effects. According to the CODEX standard for fermented milk (CODEX STAN 243-2003), the minimum counts of probiotic bacteria in the fermented milk should be atleast $\geq 10^6$CFU/g or ml at the end of the shelf-life of the product (Roy, 2001; Talwalkar and Kailasapathy, 2004; Van de Casteele *et al.*, 2006; Tabasco *et al.*, 2007; García-Cayuela *et al.*, 2009; Kramer *et al.,* 2009; Reimann *et al.*, 2010; Saccaro *et al.*, 2012; Lv *et al.,* 2015; Mianzhi and Shah, 2015).However, low (variable) counts of these bacteria and improper labeling of probiotic species have been reported in commercial probiotic product (Biavati *et al.*, 2000; Yeung *et al.*, 2002; Talwalkar and Kailasapathy, 2004; Ashraf and Shah, 2011; Raeisi *et al.*, 2013; Davis, 2014).

There is an increasing interest in the development of easy, accurate and rapid methods for qualitative and quantitative control measurements of probiotic products(Ward and Roy, 2005; Gueimonde *et al.*, 2007; Masco *et al.*, 2007; Sheu *et al.*, 2010; Rodrigues *et al.*, 2012; Sheu *et al.*, 2013; Bunešová *et al.*, 2014).Such methods are also required to routinely determine the initial inoculum, accurate species labelling, surveillance the possible physiological or biochemical changes in the probiotic bacterial population during the storage of products and to estimate the storage time period these organisms remain viable (Roy, 2001; Van de Casteele *et al.*, 2006; Tabasco *et al.*, 2007).Another application of accurate species and strain identification methods is in any public health-related monitoring, as well as clinical studies involved in monitoring the viability and distribution of cells during passage through the human gastrointestinal tract, to select potential probiotic strains (Ventura *et al*, 2001; Mathys *et al.*, 2008; Savard *et al.*, 2011; Mianzhi and Shah, 2015).

Conventional culture-dependent methods such as plate count, are based on the isolation of pure cultures using selective media, followed by the Gram-staining, morphological observations, analysis of carbohydrate fermentation, detection of specific enzymes and other biochemical tests (Ward and Roy, 2005; Youn *et al.*, 2008; Matsuda *et al.,* 2009; Sheu *et al.*, 2010; Rodrigues *et al.*, 2012;Bunešová *et al.*, 2014; Davis, 2014; Mianzhi and Shah, 2015). However, traditional culture-dependent techniques are along with some disadvantages, briefly including laboriousness, poor accuracy, lack of suitable selective media and time consuming. The viable plate count method counts only the more numerous and easily cultivable organisms in the sample. These methods, on the other hand, can be frustrated by clumping, inhibition by neighboring cells,and composition of the growth media used(Matsuki *et al.*, 1998; Mullié *et al.*, 2003; Matsuki et al., 2003; Ward and Roy, 2005; Lahtinen *et al.*, 2006; Masco *et al.*, 2007; Gueimonde *et al.*, 2007; Youn *et al.*, 2008; García-Cayuela *et al.*, 2009; Kramer *et al.*, 2009; Davis, 2014). Furthermore, differential or selective enumeration of probiotic and yoghurt starter bacteria is difficult to achieve, due to the presence of multiple and closely related species of lactic acid and probiotic bacteria in probiotic products with similarity in growth requirements and overlapping biochemical characteristics of the species(Talwalkar and Kailasapathy, 2004; Van de Casteele *et al.*, 2006; Tabasco *et al.*, 2007; Ashraf and Shah, 2011; Saccaro *et al.,* 2012).

Alternative culture-independent methods (molecular procedures) such as quantitative

real-time PCR (qPCR), were introduced recently for rapid, accurate, sensitive and efficient identification and quantification of probiotic bacteria (Matsuki *et al.*, 2003; Mullié *et al.*, 2003; Matsuki *et al.*, 2004; Ward and Roy, 2005; Mathys *et al.*, 2008; Youn *et al.*, 2008; Matsuda et al., 2009; Turroni *et al.*, 2009; Rodrigues *et al.*, 2012).The majority of qPCR methods are based on the quantification of the 16S rRNA gene (Sheu *et al.*, 2010).Since 16S rRNA genes can be present in multiple copies in the bacterial genome, the quantitative data obtained by 16S rRNA-based qPCR can be imprecise (an overestimation of the number of target bacteria) (Masco *et al.*, 2007; Rodrigues *et al.*, 2012; Mianzhi and Shah, 2015). This problem can be solved by determining the copy number of the 16S rRNA gene through southern hybridization with a ribosomal probe, or by targeting a single-copy gene (Masco *et al.*, 2007; Cleusix *et al.*, 2010). In addition to the divergent 16S rDNA sequences among rrn operons of a single organism, the high similarities of Bifidobacterial 16S rRNA genes

Table 1. Bacterial strains used in this study and the specificity of designed
tuf gene-based primer set.

Species or Template DNA	Strain[a]	Source[b]	PCR reactions with FEF bif/ REF bif primer set (amplicon size:186 bp)
Bifidobacterium			
B. animalis subsp. *lactis*	BB-12	Chr. Hansen	+
B. animalis subsp. *animalis*	25527[T]	ATCC	—
B. breve	15700[T]	ATCC	—
B. longum subsp. *longum*	15707[T]	ATCC	—
B. longum subsp. *infantis*	15697[T]	ATCC	—
B. bifidum	15521[T]	ATCC	—
Lactobacillus			
L. acidophilus	LA-5	Chr. Hansen	—
L. paracasei subsp. *paracasei*	LC-01	Chr. Hansen	—
L. delbrueckii subsp. *bulgaricus*	LBA-40	Chr. Hansen	—
Commercial probiotic yoghurts	—	Pegah Fars	+

[a]T, T type strain
[b]Chr. Hansen strains were obtained from the Chr. Hansen Collection (Hørsholm, Denmark).
All other strains were obtained from ATCC (American Type Culture Collection, Manassas,Virginia, USA).
Commercial probiotic yoghurts were obtained from the Pegah Fars Dairy Industries Company(Shiraz, Iran).
＋ : positive result (with expected amplicon).
― : negative result (without any amplicon).

(92% to 99%.), as well as the relatively small size of 16S rRNA gene (ca. 1,500 bp) impose difficulty in the primer designing and differentiation between these species, which reflects on some designed species-specific primers based on the 16S rRNA gene that in fact are not species-specific(Sheu *et al.*, 2010; Junick and Blaut, 2012; Sheu *et al.*, 2013;Mianzhi and Shah, 2015).

In the present study, we designed one specific primer set based on the highly conserved sequence of single-copy elongation factor Tu gene (*tuf* gene) for *Bifidobacterium animalis* subsp. *lactis* BB-12, and its specificity was determined by operating PCR assay whit DNAs extracted from prevalent Bifidobacterial and Lactobacilli probiotic strains. Afterwards, a *tuf* gene-based quantitative real-time PCR (qPCR) assay was established for quantification of *B. animalis* subsp. *lactis* BB-12. Finally, *B. animalis* subsp. *lactis* BB-12 was detected and enumerated through *tuf* gene-based PCR, *tuf* gene-based qPCR and selective plate count during shelf life and after the expiry date of commercial probiotic yoghurts.

Materials and Methods

Bacterial strains, growth conditions and probiotic products

Pure lyophilized Bifidobacteria and Lactobacilli strains used for verification and validation of designed primer set are listed in Table 1. All strains were cultured in Wilkins-Chalgren broth (WCB) (Oxoid, Dardilly, France) supplemented with 0.05% L-cysteine hydrochloride (L-cysteine-HCl) (Sigma-Aldrich, Steinheim, Germany) as a reducing agent to provide more strict anaerobic conditions. All cultures were incubated overnight at 37°C under anaerobic conditions with a gas mixture of 5% CO_2, 5% hydrogen, and 90% nitrogen (Anoxomat, Kelvinlaan, Netherlands). Commercial probiotic yoghurts, containing *Bifidobacterium animalis* subsp. lactis BB-12, *Lactobacillus acidophilus* LA-5 and *Lactobacillus delbrueckii* subsp. *bulgaricus* were obtained from the Pegah Fars Dairy Industries Company(Shiraz, Iran), and stored at 4°C during the experiments.

Preparation of selective medium

Wilkins-Chalgren Mupirocin 100 (WCM 100) containing Wilkins-Chalgren broth (WCB), Agar (1.5% w/v), Glacial acetic acid (1 ml/l), L-cysteine-HCl (0.05%) and mupirocin (100 mg/l) served as the selective medium for enumeration of *B. animalis* subsp. *lactis* BB-12 (Rada *et al.*, 1999; Rada and Petr, 2000;Grand *et al.*, 2003; Thitaram *et al.,* 2005; Ferraris *et al,* 2010). Mupirocin stock solution (1000×) was prepared by dissolving sufficient amount of pure powder of mupirocin (Pars Darou Pharmaceutical Company, Tehran, Iran) in double-

distilled deionized water containing Tween 80 (1:10 v/v) followed by filter sterilization (0.2 μm). Tween 80 used as mupirocin dissolvent and allowed us to eliminate the need for soaking and extracting paper sensitivity discs or water-soluble pharmaceutical grade polyethylene glycol base as previously reported(Rada and Petr, 2000; Thitaram et al., 2005). Filter sterilized mupirocin aseptically added to the autoclaved base medium previously cooled to 50°C at the final concentration of 100 mg/l.

Designing specific primer set for B. animalis *subsp.* lactis *BB-12*

The sequences of elongation factor Tu gene (*tuf* gene) for all *Bifidobacterium* species, were retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI), and aligned using the Clustal W program *(http://workbench. sdsc.edu)*. The overall non-conserved regions of these sequences were used to design a new primer setfor the detection and quantification of *B. animalis* subsp. *lactis* BB-12 using Allele ID6 software. Oligonucleotide sequences of the designed primer set were compared with all sequences retrieved from the GenBank database *(http://www.ncbi.nlm.nih.gov)* via the BLAST program. Afterwards, specificity of designed primer set was confirmed by PCR assay with genomic DNAs extracted from Bifidobacteria and Lactobacilli strains (Table 1) as well as commercial probiotic yoghurts. The oligonucleotide sequences of designed primer set are as follow:

Forward primer (FEF bif);5/- ACA AGC AGA TGG ATG AGT G - 3/ and
Reverse primer (REF bif); 5/- AGA AGA ACG GCG AGT GAC - 3/ .

DNA extraction, PCR reactions and gel electrophoresis

Reference bacterial strains (Table 1) were cultured in the WCB under the conditions as described above. Genomic DNAs were extracted from 1 ml of bacterial broth cultures using CinnaPure-DNA Kit (for Gram positive-bacteria) (SinaClon, Tehran, Iran) according to the manufacturer's instructions. In order to extract genomic DNA from yoghurt, 10 ml of yoghurt samples, 25 ml of 0.9% NaCl, 8 ml of 25% trisodium citrate, 2 g of polyethylene glycol 8000, and waterto a final volume of 50 ml, were homogenized for 5 min, and centrifuged (9700 ×g, 15 min, 4°C). Pellets were suspended in 1 ml of DNAzol® reagent (Life Technologies, Carlsbad, CA, USA), and protocol was followed as described by Achilleos and Berthier(2013) (Achilleos and Berthier, 2013). Prepared genomic DNAs were used as templates for PCR amplifications.

The reaction mixture in 25 μl volume contained: 14.1 μl sterile distilled water, 2.5 μl PCR buffer, 0.75 μl MgCl$_2$, 0.5 μl dNTPs, 1 μlof each primers (10 picomoles), 0.15 μlTaq DNA polymerase and5 μl template DNA. Amplifications were performed using a DNA thermo cycler (BIO-RAD, Hercules, CA, USA) with the following temperature profiles: initial template denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The final extension step was 5 min at 72°C. The amplicons were then electrophoresed on a 1% agarose gel, visualized by staining with Ethidium Bromide (0.5 μg/ml) and photographed under UV light by the GelDoc system (wave length: 260 nm).

Creation of standard curve

For quantification of *B. animalis* subsp. *lactis* BB-12 in an unknown sample (commercial probiotic yoghurts) a standard curve was generated to make correlations between bacterial counts determined by qPCR and plate counts. An optical density at 600 nm of 1.00 (OD$_{600}$: 1.0 ~ about 10^{9}CFU/ml) was prepared from an overnight broth culture of *B. animalis* subsp. *Lactis* BB-12 in WCB, using photobiometer (Ependdorf, Hamburg, Germany). DNA was extracted from 1 ml of OD$_{600}$: 1.0 of *B. animalis* subsp. *lactis* BB-12 broth culture as described above. Afterwards, 10-fold serial dilutions of extracted DNA were prepared using double-distilled deionized water. The serially diluted DNAs served as template in real-time PCR reactions to generate the standard curve by the plotting cycle threshold values versus log CFU/ml. Amplification efficiency was 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.

Detection and quantification of B. animalis *subsp.* lactis *BB-12 in commercial probiotic yoghurts*

Probiotic yoghurtswere analyzed using PCR, qPCR, and selective plate count during shelf-life (day 1, day 7, day 14 and day 21) and after the expiry date (day 28 and day 35). In each phase, two independent packages of the product (A and B) belong to different serial production were analyzed in three trials. For selective plate count, 1 ml of yoghurts was 10-fold serially diluted in sterile Ringer buffer (Merck, Darmstadt, Germany). One ml of each dilution was pure-plated on WCM 100 selective medium. Colonies were counted after 72 h of anaerobic incubation at 37°C. For PCR-based detection and qPCR assays, respectively PCR andreal-time PCR reactions were carried out with extracted DNA from yoghurts under

the described conditions.

Quantitative real-time PCR (qPCR) reactions

Real-time PCR reactions were performed with the serially diluted DNAs (extracted DNA from1 ml of OD$_{600}$: 1.0 of *B. animalis* subsp. *lactis* BB-12) in order to create the standard curve, as well as with DNAsamples obtained from probiotic yoghurts during mentioned intervals for enumeration of *B. animalis* subsp. *lactis* BB-12 cells. PCR amplifications were carried out with real-time PCR detection system Cycler (BIO-RAD, Hercules, CA, USA). The 25 μl reaction mixture contained: 12.5 μl of SYBR Green Supermix (Fermentase), 0.6 μlof each primer

(10 picomoles), 8.8 μl sterile distilled water and 2.5 μl of template DNA. The amplification program consisted of initial template denaturation step for 1 min at 95ºC, followed by 45 cycles of denaturation for15 s at 95ºC, annealing for 30 s at 58ºC, and elongation for 30 s at 72ºC. The fluorescent product was detected at the last step of each cycle. Following amplification, melting curve analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 1°C/s increments from 50°C to 90°C, with continuous fluorescence collection.

Statistical analysis

All the experiments were carried out in triplicate and the results are shown as the mean ± standard deviation (SD). The Student's t-test (GraphPad Prism v6.07 for Windows, GraphPad Software, La Jolla, California, USA) was used to determine statistically significant differences between the counts derived from qPCR assays and selective plate counts. Differences were considered significant at the P value≤0.05 level. All other calculations were performed using Microsoft Excel, statistical functions, version 5 (Microsoft Corp., Redmond, WA, USA).

Results

Specificity of designed primer set

Based on the *tuf* gene sequences, a novel specific primer set (which amplifies a fragment of 186 base pairs of the elongation factor Tu gene), was designed for the detection and quantification of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts. For this purpose, the *tuf* gene sequences for all *Bifidobacterium* species, available in the GenBank database were retrieved and aligned using the Clustal W program and then the overall non-conserved regions of these sequences were used for primer
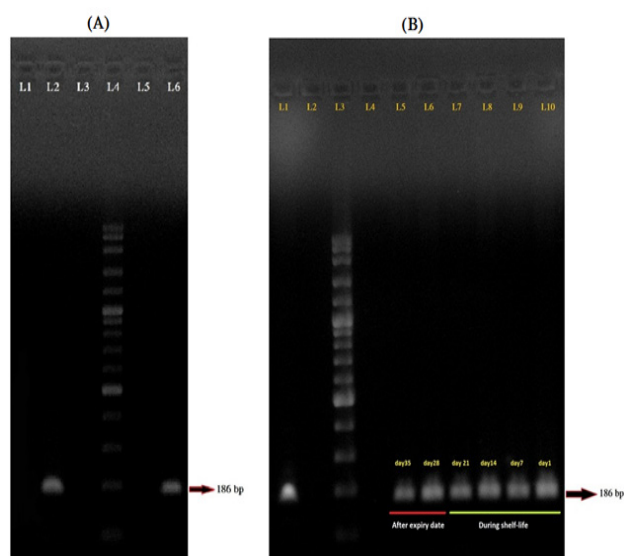

Figure 1.
(A): Agarose gel electrophoresis of PCR assays using tuf gene-based primer set.L1: Negative control, L2: Genomic DNA directly extracted from probiotic yoghourt, L3: *L. delbrueckii* subsp.*bulgaricus* genomic DNA, L4: DNA ladder marker (100 bp), L5: *L. acidophilus* subsp. LA-5 genomic DNA, L6: B. *animalis* subsp.*lactis* BB-12 genomic DNA.
(B): Agarose gel electrophoresis of the *tuf* gene-based PCR detection of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts.L1: Positive control: B. animalis subsp. lactis BB-12 genomic DNA, L2: Negative control, L3: DNA ladder marker (100 bp), L4: Empty well, L5-L10:Genomic DNAs directly extracted from probiotic yoghourts during mentioned intervals.

designing. Comparison of primer set sequences with all sequences from the GenBank database using BLAST program, revealed its specificity for *B. animalis* subsp. *lactis*. PCR assay with genomic DNAs extracted from the reference strains confirmed the specificity of designed primer set for *B. animalis* subsp. *lactis* BB-12, since no amplification was observed for all reference strains, except strain BB-12 that produced an expected 186 bp amplicon (Table 1). In *tuf* gene-based PCR assay whit genomic DNAs extracted from commercial probiotic yoghurt and strains that attended in commercial probiotic yoghurt (*B. animalis* subsp. *lactis* BB-12, *L. acidophilus* LA-5 and *L. delbrueckii* subsp. *bulgaricus*), one specific amplicon (186 bp) was generated only for strain BB-12, as well as correlated amplicon in commercial probiotic yoghurt (Figure1A).

Standard curve

The efficiency of amplification for *tuf* gene-based specific primer set in the individual real-time PCR assays was calculated from the standard curve obtained by plotting the CT values against the 10-fold
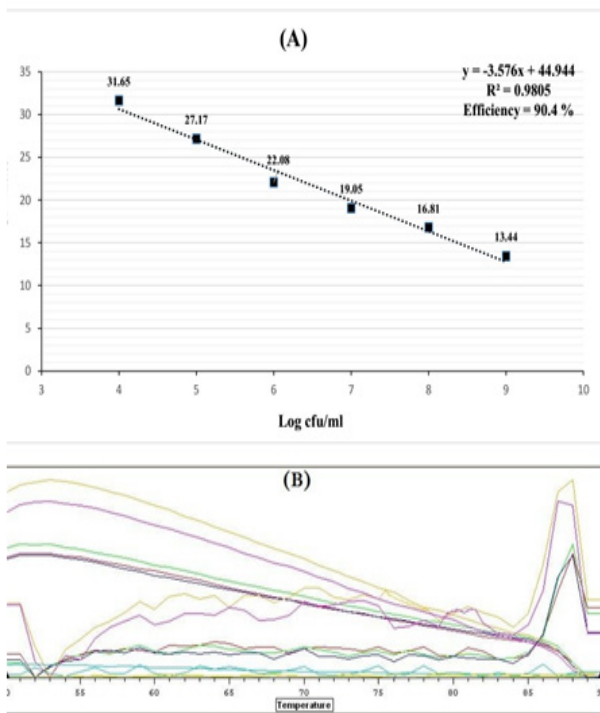
Figure 2.
(A): Standard curve between CFU/ml of standard samples and the CT values detected with the real-time PCR.
(B): Melting curve obtained from standard samples real-time PCR reactions.

Figure 3. Schematic representation of *B. animalis* subsp. *lactis* BB-12 counts obtained by qPCR and selective plate countmethods during shelf-life and after the expiry date of package A commercial probiotic yoghurts (A), package B commercial probiotic yoghurts (B), and comparison of *B. animalis* subsp. *lactis* BB-12 counts in package A and B commercial probiotic yoghurts using selective plate count (C) and qPCR (D) methods. All values are presented as Mean±SD(log CFU/ml) of triplicate trials.

serial dilution of target DNA from known amount of *B. animalis* subsp. *lactis* BB-12 (10^9CFU/ml). The generated standard curve (Figure 2A) presented suitable correlation coefficient value(R^2: 0.98) and mean efficiency (E: 90.4%), indicating that the results were linear over the tested range of bacterial concentrations (10^4–10^9CFU/ml). Analysis of the melting curve(Figure 2B) obtained for each reaction did not reveal the formation of either non-specific fragment or primer-dimers that could interfere during the fluorescence reading, especially when using SYBR Green as the issuer of fluorescence, because all double chain DNAs are detectable.

Qualitative and quantitative analysis of probiotic yoghurts by PCR, qPCR and selective plate counts

As shown in Figure 1B, *B. animalis* subsp. *lactis* BB-12 was detectable using *tuf* gene-based PCR assay in commercial probiotic yoghurts during shelf-life and even after the expiry date. On all analyzed days, one specific 186 bp amplicon was only detected. The Table 2 and Figure 3 show the enumeration results of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts via selective plate count and qPCR. Bacterial counts derived from both methods were compared to each other. Significantly decrease in *B. animalis* subsp. *lactis* BB-12 count was observed through both selective plate count
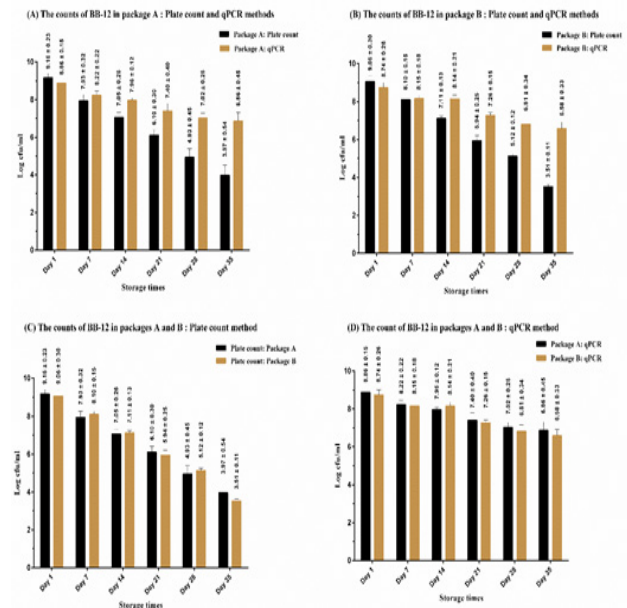
and qPCR methods (P value≤0.05).However, in the expiry date, this count was according to CODEX standard and decreased rapidly after the expiry date.

Discussion

The great potential of probiotics bacteria in prevention and treatment of diseases, make these microorganisms as an alternative therapeutic strategy(Corr *et al*., 2009; Ng *et al*., 2009).Minimal concentration of 10^6 cells/g or ml is required for probiotic bacteria to exert health-promoting effects. Therefore, it is absolutely necessary to enumerate these bacteria in probiotic products(Talwalkar and Kailasapathy, 2004; Van de Casteele *et al*., 2006; Tabasco *et al*., 2007; García-Cayuela *et al*., 2009; Kramer *et al*., 2009; Ashraf and Shah, 2011; Saccaro *et al*., 2012; Sohier *et al*., 2014; Mianzhi and Shah, 2015).

Although culture-dependent enumeration methods are stillfrequently used(Mullié *et al*., 2003; Ward and Roy, 2005; Van de Casteele *et al*., 2006; Masco *et al*., 2007; Youn *et al*., 2008;Matsuda *et al*., 2009;Davis, 2014),but these techniques suffer from several drawbacks such as lack of suitable selective media, time-consuming, limiting microbial recovery and sensitivity and labor-intensive(Matsuki *et al.,* 2003; Lahtinen *et al.*, 2006; Youn *et al.*, 2008;

Table 2. The countsof *B. animalis* subsp. *lactis* BB-12 performed by
the selective plate count and real-time PCR methods.

strain	Storage time		package	methods		Student's t-test
				Plate count[a]	qPCR[b]	(significance level p≤0.05)
			A	9.16 ± 0.23	8.86 ± 0.15	*p*=0.146; NS
		Day 1	B	9.06 ± 0.30	8.74 ± 0.26	*p*=0.229; NS
	During shelf-life	Day 7	A	7.93 ± 0.32	8.22 ± 0.22	*p*=0.287; NS
			B	8.10 ± 0.15	8.15 ± 0.18	*p*=0.713; NS
B. animalis subsp. *lactis* BB-12		Day 14	A	7.05 ± 0.26	7.96 ± 0.12	*p*=0.014; S
			B	7.11 ± 0.13	8.14 ± 0.21	*p*=0.003; S
		Day 21	A	6.10 ± 0.30	7.40 ± 0.40	*p*=0.012; S
			B	5.94 ± 0.25	7.26 ± 0.15	*p*=0.003; S
	After expiry date	Day 28	A	4.93 ± 0.45	7.02 ± 0.26	*p*=0.004; S
			B	5.12 ± 0.12	6.81 ± 0.34	*p*=0.007; S
		Day 35	A	3.97 ± 0.54	6.86 ± 0.45	*p*=0.002; S
			B	3.51 ± 0.11	6.58 ± 0.33	*p*=0.001; S

NS: no significant difference, S: significant difference.
[a]Mean±SD values (log CFU/g) obtained by selective plate counting on triplicate plates.
[b]Mean±SD values (log CFU/g) as calculated from CT values; based on three parallel DNA extracts from which three real-time PCR cycles have been run.

García-Cayuela *et al.*, 2009; Sheu *et al.*, 2010; Davis, 2014; Mianzhi and Shah, 2015). Moreover, another important aspect related to culture method is matrix effect. Indeed, protocols developed to enumerate microbes in a given food product may not be reliable with another food product, so variable bacterial counts were reported depending on the matrix used (Sohier *et al.*, 2014).

Among developed alternative molecular procedures (Ward and Roy, 2005; Youn *et al.*, 2008; Davis, 2014; Mianzhi and Shah, 2015),quantitative real-time PCR (qPCR) with species-specific primers is the most widely used method for culture-independent enumeration of probiotic bacteria (Matsuki *et al.*, 2004; Ward and Roy, 2005; Mathys *et al.*, 2008; García-Cayuela *et al.*, 2009; Matsuda *et al.*, 2009; Rodrigues et al., 2012; Merenstein *et al.*, 2015).The majority of qPCR methods are performed with 16S rRNA specific primers (Youn *et al.*, 2008; Sheu *et al.*, 2010; Lv *et al.*, 2015; Mianzhi and Shah, 2015). Quantitative data obtained by16S rRNA-based qPCR can be overestimated, since 16S rRNA genes can be present in multiple copies in the bacterial genome. It is possible to obtain more accurate quantitative data by targeting a single-copy gene in qPCR assay (Masco *et al*., 2007; Cleusix *et al.*, 2010; Sheu *et al.*, 2013; Mianzhi and Shah, 2015).

The need for accurate quality and quantity control of probiotic products containing Bifidobacteria prompted us to develop a rapid and sensitive qPCR for specific detection and quantification of B. animalis subsp. lactis BB-12, which is one of the most commonly used Bifidobacterium strains

in commercial probiotic yoghurts (Vitali *et al.*, 2003; Solano-Aguilar *et al.*, 2008). In contrast to the previously published 16S rRNA-based qPCR methods, we used the Bifidobacterial *tuf* gene, encoding the elongation factor Tu, which facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA during translation. The *tuf* gene has only been detected as a single-copy on the Bifidobacterial genome and is highly useful for the differential detection of *Bifidobacterium* species (Solano-Aguilar *et al.,* 2008; Sheu *et al*., 2013; Mianzhi and Shah, 2015).

The specificity of designed *tuf* gene-based primer set was confirmed by operation of PCR reactions with extracted DNAs from reference strains and commercial probiotic yoghurts. PCR reactions generated one specific amplicon (186 bp) for *B. animalis* subsp. *lactis* BB-12, as well as correlated amplicon in commercial probiotic yoghurt, and no amplification was observed for other examined Bifidobacterial and Lactobacilli strains (Table 1, Figure 1A). We propose that designed primer set can be appropriate for PCR-based specific detection of *B. animalis* subsp. *lactis* in probiotic products. A new *tuf* gene-based qPCRwas developed for enumeration of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts. The standard curve obtained from104-109 fold serial dilution of target DNA from known amounts of bacteria was linear (R^2=0.98) with the amplification efficiency of 90.4% (Figure 2B).

The obtained results from quantitative analysis of different commercial probiotic yoghurt packages by selective plate count and qPCR (Table 2, Figure

3) indicated that *B. animalis* subsp. *lactis* BB-12 counts considerably decreased during shelf-life(P value≤0.05). However, these counts were equivalent to the recommended standard of the International Dairy Federation (IDF) for Bifidobacterial counts in dairy products, until the expiry date of the products. We hypothesize that this count-retaining abilitycan be due to very high initial inoculum during manufacturing, since on the first day of shelf-life (day 1), the counts of *B. animalis* subsp.*lactis* BB-12 were nearly10^9CFU/ml. The products shelf-life was 21 days, which is some deal short in contrast to similar products in other countries. We guesstimate that incorporation of prebiotics can enhance the shelf-life period of dairy probiotic products.Totally, packages A and B (in compare to each other) didn't have significant difference in the obtained counts fromselective plate count and qPCR(P value>0.05).

Several qPCR methods have been described already for quantification of *Bifidobacterium* spp. in different samples (Gueimonde *et al*., 2004; Matsuki *et al*., 2004; Penders *et al*., 2005; Haarman and Knol, 2005; Gueimonde *et al*., 2007; Delroisse *et al*., 2008; Matsuda *et al*., 2009; Reimann *et al*., 2010; Rodrigues *et al*., 2012).However, in most systems, the target gene is 16S rRNA, which can be imprecise for quantification since it can be present in multi-copies in one single bacterial cell (Cleusix *et al*., 2010; Sheu *et al*., 2013; Mianzhi and Shah, 2015).Reimann *et al*. (2010) developed reverse transcription real-time PCR (RT-qPCR) to quantify viable *B. longum* NCC2705 cells exhibiting different morphologies by measuring the mRNA expression of two housekeeping genes *cysB* and *purB*. The 400- bp fragment of *purB* has been shown as a suitable biomarker of cell viability by comparing the results obtained fromRT-qPCR and selective plate count (Reimann *et al.,* 2010). Junick and Blaut (2012) showed that thesingle-copy *groEL*gene can be a suitable molecular marker for the specific and accurate quantification of human fecal *Bifidobacterium* species by qPCR (Junick and Blaut, 2012).Cleusix *et al*. (2010) developed a new qPCR method based on the Bifidobacterial single-copy xfp gene for detection of Bifidobacteria in faecal samples. Detection limit of their assay was $2.5×10^3$cells per g faeces(Cleusix *et al*., 2010).Masco *et al*. (2007) used qPCR targeting the multi-copy16S rRNA and the single-copy *recA* genes for enumeration of Bifidobacteria in probiotic products and compared the results of both assays. They discovered that the quantification of the 16S rRNA gene turned out to be more sensitive than the recA-based assay, especially in probiotic products containing low amounts of Bifidobacteria. On the other hand, in such samples

the detection limit of 16S rRNA gene-based qPCR assay is lower than recA gene-based qPCR assay (Masco *et al*., 2007).Gloria Solano-Aguilar *et al*. (2008) evaluated the use of qPCR assaybased on thesingle-copy *tuf* gene as a marker for detection of *B. animalis* subsp. *lactis* BB-12 in the gastrointestinal track (GIT) of pigs orally treated with BB-12 and differentiate it from *B. animalis* subsp.*animalis,* since only the *tuf* gene-based qPCR assay was able to differentiate the *B. animalis* subsp. *lactis* from *B. animalis* subsp. *animalis* (Solano-Aguilar *et al*., 2008). Sheu *et al*. (2010) designed *tuf* gene-based primer sets for detection and quantification of Bifidobacteria in probiotic products. They found similar bifidobacterial cell counts for both cultural and qPCR methods, indicating that within 15 days storage (4∘C) after manufacture, all bifidobacterial cells originally present in yogurt products were viable and culturable during the storage (Sheu *et al*., 2010).

According to the selective plate count data (Table 2), the counts of *B. animalis* subsp. *lactis* BB-12 decreased rapidly after the expiry date and fell below CODEX standard (10^6CFU/ml). The viability of Bifidobacteria depends on the bacterial strains, fermentation conditions and preservation methods, degree of acidification, storage temperature, oxygen, and is mainly limited by their sensitivity to the high acidity (Roy, 2001; Rodrigues *et al.*, 2012). As shown in Table 2 and Figure 3, the bacterial counts obtained by selective plate count on days 1 and 7 of shelf-life were similar to qPCR (P value>0.05), but on days 14, 21, 28 and 35 the bacterial counts obtained by selective plate count were lower than bacterial counts derived from qPCR, especially after the expiry date(P value≤0.05).

There are two rational interpretations for such significant differences between quantitative data obtained from selective plate count and qPCR targeting a single-copy gene: First, the viable plate count method quantifies only the cultivable organisms in the sample and dormant bacteria (VBNC: viable but non-cultivable) are not quantified by plate count. (Matsuki *et al*., 1998; Mullié *et al*., 2003; Matsuki *et al*., 2003; Ward and Roy, 2005; Lahtinen et al., 2006; Gueimonde *et al*., 2007; Masco *et al*., 2007; García-Cayuela *et al*., 2009; Kramer *et al*., 2009; Youn *et al*., 2008; Sohier *et al*., 2014; Davis, 2014).In response to some physiological stresses (bile, pH, temperature and etc.) and prolonged storage, some fraction of the live probiotic bacteria in dairy probiotic products may enter a dormant state (Lahtinen *et al*., 2006; Davis, 2014).Unlike plate count approaches, qPCR methods can detect both cultivable and non-cultivable populations of bacteria (Requena *et al*.,

2002; Davis, 2014).This ratiocination confirms that the quantitative data obtained from qPCR are more accurate than selective plate count.

Second, due to this fact that DNA molecules can remain intact hours or even days after cell death, qPCR does not enable to discriminate between live and dead cells, because both DNA arising from live and dead cells participate and amplify in reaction, which may result in an overestimation of the number of bacterial cells (Lahtinen *et al.*, 2006; Delroisse *et al.*, 2008; García-Cayuela *et al.,* 2009; Kramer *et al.*, 2009; Mianzhi and Shah, 2015).This ratiocination confirms that the quantitative data obtained from selective plate count are more precise than qPCR. Therefore, traditional culture-based methods are still preferable for routine enumeration purposes, and frequently used as complementary or satisfactory gold standard for molecular approaches (Roy, 2001; Requena *et al.*, 2002; Ward and Roy, 2005; Van de Casteele *et al.*, 2006; Masco *et al*., 2007; Sohier *et al*., 2014; Mianzhi and Shah, 2015).

One promising way to distinguish between viable and dead bacteria is the use of unstable mRNA as a target molecule in real-time PCR (RT-qPCR). However, this technique requires special attention to minimizing losses during RNA extraction and purification(Reimann *et al*., 2010; Davis, 2014; Sohier *et al.*, 2014). Furthermore, although RNA tends to degrade relatively rapidly after cell death, but there are some RNA molecules that can also persist in cells for extended time periods after the loss of viability and this persistence of RNA can lead to false positive results(Nocker and Camper, 2009; Sohier *et al.,* 2014).Propidium monoazide (PMA)is a DNA-intercalating dye that can selectively enter dead cells with damaged membranes and covalently bind to DNA, inhibiting the PCR. Therefore, subjecting a bacterial population to PMA treatment before PCR results in selective amplification of DNA from live cells with intact membranes only (Nocker *et al.,* 2006; Nocker and Camper, 2009; Nocker *et al*., 2009; Yang *et al*., 2011; Elizaquível *et al*., 2012; Zhu *et al.*, 2012). Although PMA is an expensive reagent, but for the sake of equitable judgment, we propose the use of PMA in combination with single-copy based qPCR (PMA-qPCR) and subsequently comparing obtained results with respective single-copy based qPCR and selective plate count.

Conclusion

In conclusion, the use of specific primer based on the single-copy *tuf* gene in the assay allows the more accurate and precise identification and enumeration of the Bifidobacteria in complex mixtures present in fermented milk products. Other advantageous features of the procedure include simple, rapid, sensitive and easy to perform. The time needed to yield results with real-time PCR can be performed in about 3 h compared with 72 h required with plate count methods. Since qPCR allowed the enumeration of both cultivable and uncultivable cells, it can serve as a promising alternative or complementaryprocedure for culture-based methods, which considered only the cultivable part of the probiotic organism's population. Our results showed that The Iranian commercial probiotic yoghurts analyzed in this study has an acceptable level of *B. animalis* subsp. *lactis* BB-12during shelf-life and at the expiry date. The relatively high reduction in viable cell counts during this period could be attributed to the possible presence of Bifidobacterial inhibitory factors.The choice of the enumeration method for probiotic bacteria should depend on theproperties of theprobiotic strains, as well as the objectives of the analysis. This study provides a comparison between potentialmethods of enumeration for probiotic bacteria, emphasizes the need for further assessment of the methods applied to determine the viability of probiotic products. Two important probabilities, dormant and dead cells, should be considered when suitable enumeration methods are selected.

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