Original Article

TIPS Trends in Pharmaceutical Sciences 2016: 2(4): 265-276.

Selective detection, isolation, and enumeration of *Bifidobacterium animalis* subsp. *lactis* BB-12 from Iranian commercial probiotic yoghurts

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Abstract

Fermented dairy products are one of the most prevalent vehicles for delivering probiotic bacteria to the consumer. A minimal concentration of 10^6 CFU/g or mL of a product is required to exert probiotic effects. In this study, we first evaluated the selectivity of WCM 50 and WCM 100 (Wilkins-Chalgren agar supplemented with 50 mg/L and 100 mg/L mupirocin), as well as mMRS (De Man Rogosa Sharpe agar supplemented with 0.1 mg/L clindamycin plus 10 mg/L ciprofloxacin) media, using pure cultures of prevalent Bifidobacterial and Lactobacilli probiotic strains. For each strain, the selectivity and cell recovery rate on each medium was compared statistically with that obtained on the non-selective media. Afterwards, one *tuf* gene-based specific primer set was designed for the detection of *Bifidbacterium animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts. The specificity of the designed primer set was evaluated by operation of PCR reactions with extracted DNAs from reference strains and commercial probiotic yoghurts. Finally, strain BB-12 was detected, enumerated, and confirmed through *tuf* gene-based PCR, selective plate count and fructose-6-phophate-phosphoketolase assay (F6PPK), respectively, during shelf life and after the expiry date of commercial probiotic yoghurts. The results showed that WCM 100 was completely selective for Bifidobacteria, with the recovery of about 100%. However, mMRS was not completely selective for Lactobacilli. The PCR assays confirmed the specificity of *tuf* gene-based primer set for strain BB-12. Although the counts of strain BB-12 showed a significant decrease during shelf-life, these counts didn't fell below the CODEX standard (10^6 CFU/mL) until the expiry date of products.

Keywords: BB-12, Detection, Enumeration, PCR, Probiotic Yoghurt, *tuf* gene.

1. Introduction

Probiotics are live bio-therapeutic agents that can serve as an alternative to antibiotic therapy, especially with regard to the development of resistant populations of pathogenic bacteria (1).

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The most acceptable definition of probiotics is "live microorganisms that when administered in adequate amounts confer a health benefit on the host beyond inherent general nutrition" (2-4).

Most human probiotic species are members of two genera of lactic acid bacteria (LAB): *Lactobacillus* and *Bifidobacterium* (3, 5, 6).

Recieved:06/8/2016; Accepted:21/08/2016

In recent years, fermented milk and yoghurt have been used as a popular vehicle for delivering probiotic bacteria in food (2, 7-10).

In these products, probiotic strains of *Bifidobacterium* and *Lactobacillus* are present in combination with other lactic acid and starter bacteria (2, 5, 8).

From the microbial aspect, the therapeutic effects or health benefits of probiotic bacteria depend on three fundamental criteria: viability, metabolic activity, and the quantity of probiotic bacteria in products. The CODEX standard for fermented milks (CODEX STAN 243-2003) confirms that the counts of probiotic bacteria in the fermented milks should be at least $\geq 10^6$ CFU/g or mL (CFU: colony forming units) at the end of product shelf-life (2, 8-14).

However, some studies have reported low or variable counts of these bacteria in commercial probiotic yoghurts, as well as wrong labeling of probiotic species (2, 6, 9, 15, 16).

Therefore, appropriate procedures are needed for accurate qualitative and quantitative control of probiotic products (11, 13, 17-20).

Conventional culture-dependent methods that identify bacterial populations to the genus level involve 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 (18, 21-24).

In practice, the presence of multiple and closely related species of lactic acid bacteria in probiotic products makes the differential or selective enumeration of probiotic and yoghurt starter bacteria difficult, due to similarity in growth requirements and overlapping biochemical characteristics of the species (2, 8-10, 14).

Recently, alternative culture-independent (molecular) methods were introduced for rapid, accurate, sensitive, and efficient identification and quantification of probiotic bacteria (18,19, 22-25).

In terms of quantification, the most widely used method is quantitative real-time PCR (qPCR) (18,19, 21, 22, 26, 27).

However, some molecular approaches are more applicable for taxonomic or epidemiological objectives, some of these methods are expensive and difficult to handle in a routine manner (23). Therefore, traditional culture-based methods are still preferable for routine enumeration purposes, and are frequently used as a satisfactory gold standard for molecular approaches (10, 13, 18, 19, 28).

Several media have been developed for selective and differential enumeration of probiotic bacteria (2,13, 29).

Wilkins-Chalgren agar containing mupirocin is claimed to be selective for the isolation and enumeration of Bifidobacteria in fecal and probiotics samples that contain mixed populations of lactic acid bacteria. On this selective medium, mupirocin completely inhibits the growth of Lactobacilli, Lactococci, Leuconostocs, and Streptococci strains (2, 30-35).

International Organization for Standardization (ISO, 20128/IDF 192: 2006) has recommended De Man Rogosa Sharpe (MRS) agar supplemented with clindamycin (and also ciprofloxacin in some references) as the preferred medium for the selective isolation and enumeration of *Lactobacillus acidophilus* strains in dairy probiotics products containing other lactic acid bacteria and Bifidobacteria (2, 10, 11, 14, 29, 36).

In the present study, we assessed the selectivity of Wilkins-Chalgren agar containing mupirocin (WCM) and De Man Rogosa Sharpe (MRS) agar containing clindamycin/ciprofloxacin culture media in different antibiotic concentrations for probiotic *Bifidobacterium* spp. and *Lactobacillus* spp. Afterwards, one *tuf* gene-based specific primer set was designed for *Bifidobacterium animalis* subsp. *lactis* BB-12, and its specificity was determined by PCR assays whit DNAs extracted from prevalent Bifidobacterial and Lactobacilli probiotic strains. Finally, strain BB-12 was detected and enumerated using *tuf* gene-based PCR and selective plate count, during shelf-life and after the expiry date of Iranian commercial probiotic yoghurts.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and probiotic products

Pure lyophilized bacterial strains used for verification and validation of designed primer set and culture media are listed in Table 1. Bifidobacterial and Lactobacilli strains were cultured

Table 1. Bacterial reference strains used in this study.

Species	Strain[a]	Source[b]
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

[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).

in Wilkins-Chalgren broth (WCB), (Oxoid, Dardilly, France) and De Man Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), respectively, both supplemented with 0.05% L-cysteine hydrochloride (L-cysteine-HCl) (Sigma-Aldrich, Steinheim, Germany) as a reducing agent to provide a more strict anaerobic conditions. All cultures were incubated overnight at 37 °C under anaerobic conditions with a gas mixture of 5% CO_2, 5% H_2, and 90% N_2 (Anoxomat, Kelvinlaan, Netherlands). Commercial probiotic yoghurts, containing *B. animalis* subsp. *lactis* BB-12, *L. acidophilus* LA-5 and *L. delbrueckii* subsp. *bulgaricus*, were obtained from Pegah Fars Dairy Industries Company (Shiraz, Iran), and stored at 4 °C during the experiments.

2.2. Preparation of antibiotic stock solutions

Clindamycin and ciprofloxacin stock solutions (1000×) were prepared by dissolving sufficient amounts of pure powders of clindamycin (Alborz Darou Pharmaceutical Company, Tehran, Iran) and ciprofloxacin (Excir Pharmaceutical Company, Tehran, Iran) in double-distilled deionized water followed by filter sterilization (0.2 µm). For preparation of mupirocin stock solutions (1000×), sufficient amounts of pure powder of mupirocin (Pars Darou Pharmaceutical Company, Tehran, Iran) were dissolved in double-distilled deionized water containing Tween 80 (1:10 v/v) followed by filter sterilization (0.2 µm). Tween 80 was 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 (31, 37).

2.3. Selectivity of media

An optical density at 600 nm of 1.00 (OD600:1.0) was prepared from an overnight broth culture of *Bifidobacterium* and *Lactobacillus* strains (Table 1) using photobiometer (Ependdorf, Hamburg, Germany). WCB and MRS broth, both supplemented with 0.05% L-cysteine-HCl, served as blanks and diluents. Afterwards, 10-fold serial dilutions were obtained from OD600:1.0 solution of *Bifidobacterium* spp. and *Lactobacillus* spp. using sterile Ringer solution (Merck, Darmstadt, Germany). Aliquot of each dilution (1 mL) was pure-plated on selective and non-selective media (Table 2). Colonies were counted after 72 h of anaerobic incubation at 37 °C, as described above.

2.4. Specific primer set for *B. animalis* subsp. *lactis* BB-12

The elongation factor Tu gene (*tuf*) sequences for all *Bifidobacterium* species, were retrieved from the GenBank database of the Nation-

Table 2. Non-selective and selective media tested in this study and their composition.

Type of media	Medium abbreviation	Medium name	Base	Final concentration of additives
Non-selective media	MRSA	De Man Rogosa Sharpe Agar	MRS broth	L-cystein HCL 0.05% (w/v) Agar 1.5% (w/v)
	WCA	Wilkins-Chalgren Agar	Wilkins-Chalgren broth (WCB)	L-cystein HCL 0.05% (w/v) Agar 1.5% (w/v)
Selective media	mMRS	Modified MRS	MRSA	Clindamycin (0.1 mg/L) Ciprofloxacin (10 mg/L)
	WCM 50	Wilkins-Chalgren Mupirocin	WCA	Glacial acetic acid (1 mL/L) Mupirocin (50 mg/L)
	WCM 100	Wilkins-Chalgren Mupirocin	WCA	Glacial acetic acid (1 mL/L) Mupirocin (100 mg/L)

al Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), and aligned using the ClustalW program (http://workbench.sdsc.edu). Afterwards, the overall non-conserved regions of these sequences were used to design a new primer set for the detection of *B. animalis* subsp. *lactis* BB-12, using Allele ID6 software. The oligonucleotide sequences of the designed primer set (Table 3) were compared with all the sequences retrieved from the GenBank database via blast program. Finally, specificity of the designed primer set was confirmed by PCR assay with the genomic DNAs extracted from *Bifidobacterium* and *Lactobacillus* strains (Table 1), as well as commercial probiotic yoghurts.

2.5. DNA extraction, PCR reactions, and gel electrophoresis

Reference bacterial strains (Table 1) were cultured in the appropriate broth media under the conditions described above. Genomic DNAs were extracted from 1 mL of bacterial broth cultures. In order to extract DNA from yoghurt, 1 mL of yoghurt was homogenized thoroughly in 9 mL of sterile Ringer solution (Merck, Darmstadt, Germany) and further centrifuged (4000×g, 10 min). Pellete was then resuspended in 1 mL double-distilled water. DNA extraction carried out using CinnaPure-DNA Kit (for Gram positive bacteria) (SinaClon, Tehran, Iran) according to the manufacturer's instructions. 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 µL of each primers (10 picomole), 0.15 µL Taq DNA polymerase, and 5 µL template DNA. Amplifications were performed using a DNA thermocycler (BIO-RAD, Hercules, CA, USA) with the following temperature profiles: initial template denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The final extension step was 5 minutes 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 GelDoc system (wave length, 260 nm).

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

Probiotic yoghurts were analyzed using PCR and selective plate count during shelf-life (day 1, day 7, day 14, and day 21) and after the

Table 3. Oligonucleotide sequences of the tuf gen-based primer set used in this study.

Primers name	Primers sequence (5′-3′)	Length (bp)	Tm (°C)	Reference
Forward: FEF bif	5′-ACA AGC AGA TGG ATG AGT G-3′	19	51.6	This study
Reverse: REF bif	5′-AGA AGA ACG GCG AGT GAC-3′	18	53.5	This study
PCR product	-	186	82.5	-

expiry date (day 28 and day 35). For selective plate count, 1 mL of sample yoghurts was10-fold serially diluted in sterile Ringer solution. One mL of each dilution was pure-plated on WCM 100 medium. Colonies were counted after 72 h of anaerobic incubation at 37 °C. PCR reactions were carried out with extracted DNA from 1 mL of yoghurts under the conditions as described above.

2.7. Microbiological and enzyme-based colorimetric assays for confirmation of Bifidobacteria

In case of probiotic yoghurts, 10 presumptive *Bifidobacterium* colonies developed on WCM 100 medium were picked up and subsequently tested for identity as a member of the genus *Bifidobacterium* based on the following criteria: (i) they were Gram positive, pleomorphic rods with characteristic bifurcated *Bifidobacterium* cellular morphology, (ii) they were unable to grow under aerobic conditions, and (iii) they were catalase negative. After confirmation of these criteria, the detection of fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22) activity (an indicator enzyme assay for *Bifidobacterium* spp.) was also performed with colonies arising on WCM 100 medium from probiotic yoghurts, as well as with broth cultures of reference strains (Table 1). In case of probiotic yoghurts, developed colonies on WCM 100 medium were cultivated anaerobically in 10 mL WCB at 37 °C for 42 h. The F6PPK assay was carried out mainly according to Orban and Patterson (2000) (38). Cells were harvested by centrifugation at 14000×g for 5 minutes. The pellet was washed twice with a phosphate buffer solution (0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/L), and the cells were suspended in 1 mL of buffer. Cells were disrupted by 0.4 mL of a cetridium bromide solution (0.45 mg CTAB in 1 mL of distilled water; Sigma). The samples were sonicated on ice for 5 minutes. Then, 0.25 mL of reagents solution (3 mg/mL NaF, 5 mg/mL sodium iodoacetate, 80 mg/mL fructose-6-phosphate) was added. The solution was vortexed and then incubated at 37 °C for 30 minutes. Following incubation, 1.5 mL of hydroxylamine•HCl (13 g/100 mL) was added. After 10 minutes incubation at room temperature, 1 mL trichloroacetic acid (TCA,15%),and1mL$FeCl_3$•$6H_2O$(5%)wereadded. A positive reaction was recorded upon development of red-violet color.

2.8. Statistical analysis

All the experiments were carried out in triplicate, and the results are shown as mean±standard deviation (SD). The significance of differences was determined by analysis of variance using Statistical Package for Social Sciences (SPSS) program for Windows, version 6.1.2. All

Table 4. Bacterial counts expressed as $\log_{10}$ CFU/mL (X±SD)[a] of culture (OD600:1.0) on the tested media after 72 hours of incubation at 37 °C.

Strains	Non-selective media		Selective media		
	MRSA	WCA	mMRS	WCM 50	WCM 100
B. animalis subsp. *lactis* BB-12	9.12±0.09[b]	9.06±0.06[b]	3.07±0.53[c]	9.04±0.10[b]	9.18±0.27[b]
B. animalis subsp. *animalis*	8.98±0.17[b]	9.02±0.20[b]	3.67±0.15[c]	9.17±0.60[b]	9.36±0.40[b]
B. breve	9.21±0.23[b]	8.95±0.37[b]	3.15±0.52[c]	8.90±0.33[b]	8.89±0.10[b]
B. bifidum	9.05±0.08[b]	8.99±0.09[b]	2.69±0.16[c]	9.00±0.80[b]	9.08±0.12[b]
B. longum subsp. *longum*	8.79±0.11[b]	9.06±0.11[b]	2.74±0.14[c]	9.09±0.08[b]	8.97±0.09[b]
B. longum subsp. *infantis*	8.77±0.24[b]	9.02±0.12[b]	3.31±0.34[c]	9.11±0.13[b]	9.14±0.12[b]
L. acidophilus LA-5	8.92±0.21[b]	9.05±0.13[b]	9.01±0.15[b]	3.07±0.23[c]	<4[c,d]
L. paracasei subsp. *paracasei*	9.17±0.11[b]	9.21±0.09[b]	9.14±0.15[b]	2.57±0.36[c]	<4[c,d]
L. delbrueckii subsp. *bulgaricus*	9.20±0.11[b]	9.15±0.09[b]	8.93±0.07[b]	3.02±0.08[c]	<4[c,d]

[a]Mean±standard deviation (SD) of three trials.
[b]CFU values don't significantly different from other media in the same row (*P* value>0.05).
[c]CFU values significantly different from other media in the same row (*P* value≤0.05).
[d]No growth was observed on the triplicate experiment.

other calculations were performed using Microsoft Excel, statistical functions, version 5 (Microsoft Corp., Redmond,WA, USA). Differences were considered significant at the *P* value≤0.05 level.

3. Results

3.1. Selectivity of culture media

The related results to selectivity of culture media are shown in Table 4. All examined *Bifidobacterium* spp. and *Lactobacillus* spp. could grow on non-selective media (WCA and MRSA), and the counts of Bifidobacterial and Lactobacilli strains in optical density at 600 nm of 1.00 (OD600: 1.0) were calculated about 10^9 CFU/mL on non-selective media (*P* value>0.05). When comparing non-selective (WCA, MRSA) and selective (mMRS, WCM 50, WCM 100) media, the counts of Bifidobacterial strains (OD600: 1.0) on WCA, MRSA, WCM 50, and WCM 100 didn't have significant difference (*P* value>0.05). In case of Lactobacilli enumeration, there was no significant difference between obtained counts from WCA, MRSA, and mMRS media (*P* value>0.05). However, *Bifidobacterium* and *Lactobacillus* strains could grow somewhat on mMRS and WCM 50, respectively (*P* value≤0.05, in comparison whith non-selective media). This means that the mMRS medium was not completely selective for Lactobacilli, as well as WCM 50 for Bifidobacteria. The absolutely selective medium was WCM 100, since only *Bifidobacterium* strains could grow on this medium, and no growth was observed for *Lactobacillus* strains. On the other hand, mupirocin as a selective agent (in concentration of 100 mg/L and not 50 mg/L) could inhibit the growth of *Lactobacillus* strains (*P* value≤0.05, in comparison whith non-selective media), whereas the growth of *Bifidobacterium* strains was not affected by mupirocin in this concentration (*P* value>0.05, in comparison whith non-selective media).

3.2. Specificity of designed primer set

Based on the *tuf* gene sequences, a novel specific primer set was designed for the detection of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts (Table 3). 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 nonconserved regions of these sequences were used for primer designing (data not shown). Comparison of the primer set sequences with all sequences from the GenBank database using blast program, revealed its specificity for *B. animalis* subsp. *lactis* (data not shown). PCR assay with genomic DNAs extracted from the reference strains (Table 1) confirmed the specificity of the designed primer set for *B. animalis* subsp. *lactis* BB-12 (Table 5), since no amplification was observed for all reference strains, except strain BB-12 that produced an expected 186 bp amplicon (data not shown). In *tuf* gene-based PCR assay

Table 5. Specificity of designed tuf gene-based primer set.

Template DNAs	**PCR reactions with FEF bif/ REF bif primer set (amplicon size:186 bp)**
B. animalis subsp. *lactis* BB-12	+
B. animalis subsp. *animalis*	-
B. breve	-
B. bifidum	-
B. longum subsp. *longum*	-
B. longum subsp. *infantis*	-
L. acidophilus LA-5	-
L. paracasei subsp. *paracasei*	-
L. delbrueckii subsp. *bulgaricus*	-
Commercial probiotic yoghurts	+

+:positive result (with expected amplicon).
-:negative result (without any amplicon).

Figure 1. Agarose gel electrophoresis of PCR products from 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* LA-5 genomic DNA.
L6: *B. animalis* subsp. *lactis* BB-12 genomic DNA.

whith genomic DNAs extracted from commercial probiotic yoghurt and strains that existed 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 the correlated amplicon in commercial probiotic yoghurt (Figure.1). Therefore, the designed primer set can be appropriate for PCR-based specific detection of *B. animalis* subsp. *lactis* BB-12 in probiotic products.

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

B. animalis subsp. *lactis* BB-12 cells were enumerated in commercial probiotic yoghurts using confirmed WCM 100 selective medium (Table 6). Totally, the counts of *B. animalis* subsp. *lactis* BB-12 cells were significantly decreased during shelf-life (P value≤0.05). But at the expiry date, this count was 6.42 log CFU/mL, which was according to the CODEX standard. However, after the expiry date it decreased rapidly. As shown in Figure 2, *B. animalis* subsp. *lactis* BB-12 was detectable using *tuf* gene-based PCR assay in commercial probiotic yoghurts, even after the expiry date.

3.4. Identification via microbiological and enzymatic assays

In analysis of commercial probiotic yoghurts during mentioned intervals, all colonies developed on WCM 100 medium were Gram positive rods, with bifurcated cellular morphology, catalase negative, and couldn't grow under aerobic conditions. These criteria are correlated to *Bifidobacterium* genus. The phosphoketolase assay (F6PPK) was also performed to confirm the identity of isolated colonies. All Bifidobacterial reference strains exhibited distinctive positive F6PPK activity and all tested Lactobacilli strains were F6PPK negative. In case of commercial probiotic yoghurts, all isolated colonies had positive F6PPK activity (Table 7).

4. Discussion

Due to the great potential of probiotics bacteria in prevention and treatment of diseases, these microorganisms can be used as an alternative therapeutic strategy to conventional therapies, such as antibiotics (5, 39, 40). Minimal concentration of 10^6 cells/g(mL) and viability are two vital prerequisites for optimal probiotic

Table 6. *B. animalis* subsp. *lactis* BB-12 counts (log CFU/mL) in the commercial probiotic yoghurts.

Strain	During shelf-life				After the expiry date	
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35
B. animalis subsp. *lactis* BB-12	10.10±0.10	8.11±0.08	7.13±0.12	6.42±0.34	5.11±0.33	3.53±0.16

Table 7. Fructose-6-phosphate phosphoketolase assay.

Samples	F6PPK[a] activity
B. animalis subsp. *lactis* BB-12	+
B. animalis subsp. *animalis*	+
B. breve	+
B. longum subsp. *longum*	+
B. longum subsp. *infantis*	+
B. bifidum	+
L. acidophilus LA-5	-
L. paracasei subsp. *paracasei*	-
L. delbrueckii subsp. *bulgaricus*	-
probiotic yoghurts (day 1)	-
probiotic yoghurts (day 7)	+
probiotic yoghurts (day 14)	+
probiotic yoghurts (day 21)	+
probiotic yoghurts (day 28)	+
probiotic yoghurts (day 35)	+

[a]Fructose-6-phosphate phosphoketolase.

functionality. Therefore, the monitoring of these criteria in probiotic products is indispensable (2, 5, 8-12, 14, 16).

In terms of enumeration and quantification, traditional culture-dependent methods based on the selective microbiological media and confirmatory phonotypical/biochemical tests are still frequently used (10, 18, 19, 21-24). Alternative molecular methods have been developed for identification and quantification of probiotic bacteria (18, 19, 22-24).

The most widely used method for culture independent quantification is quantitative real-time PCR (qPCR) with species-specific primers (12, 18, 21, 22, 26, 27). The majority of qPCR methods are based on the quantification of the 16S rRNA gene (24). Nonetheless, some factors may compromise the interpretation of quantitative data obtained by qPCR: 1) 16S rRNA genes can be present in multiple copies in the bacterial chromosome, resulting in an overestimation of the number of bacteria in a sample (19). 2) Bacterial quantification by real-time PCR can be affected by differences in the number of rRNA operons, sequence heterogeneity or differential amplification of different DNA molecules (20, 41). 3) In qPCR reaction both living and dead bacterial DNA are detectable, resulting in an overestimation of the number of target bacteria (12, 19, 42). 4) In RNA-based real-time PCR (RT-qPCR), although RNA tends to degrade relatively rapidly after cell death, there are some RNA molecules that can also persist in cells for extended time periods after loss of viability and this persistence of RNA can lead to false positive results (43).

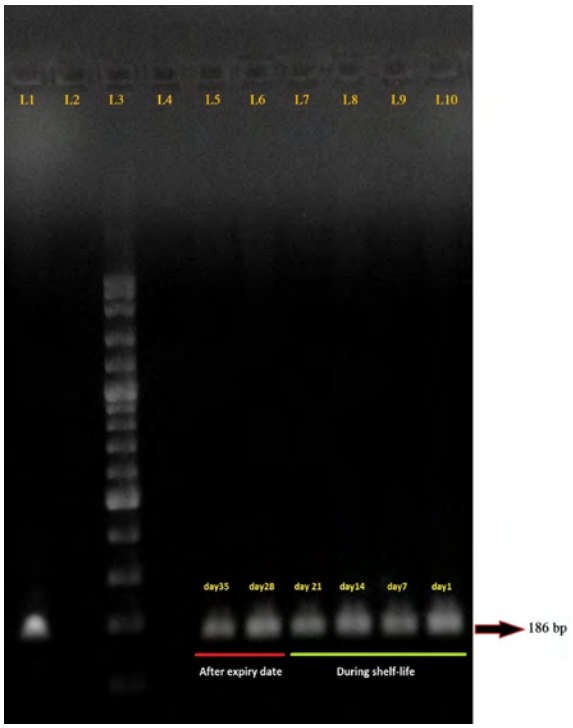

Figure 2. The *tuf* gene-based PCR detection of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts.

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.

Because of the limitations and relatively high costs of quantitative molecular methods, most manufacturers still rely on culture-based enumeration procedures as a satisfactory golden standard (10, 28).

Several media for selective enumeration of probiotic bacteria have been previously proposed and reviewed (2, 29, 44, 45). The need for accurate quality and quantity control of probiotic products prompted us to evaluate the selectivity of Wilkins-Chalgren agar containing mupirocin (WCM) and

De Man Rogosa Sharpe (MRS) agar containing clindamycin/ ciprofloxacin culture media with different antibiotic concentrations for selective detection and quantification of Bifidobacterial and Lactobacilli probiotic strains. In some studies, Wilkins-Chalgren agar containing mupirocin (in concentration of 50 mg/L or 100 mg/L) is reported as a selective medium for isolation and enumeration of Bifidobacteria in probiotic products and feces samples, since the growth of Lactobacilli, Lactococci, Leuconostocs, and Streptococci were completely inhibited on this selective medium (30-32, 37, 46).

The results of the present study (Table 4) indicated that WCM 100 containing mupirocin (in concentration of 100 mg/L and not 50 mg/L) was completely selective for Bifidobacteria, since only 100 mg/L concentration of mupirocin could inhibit the growth of *Lactobacillus* strains (P value≤0.05), whereas the growth of *Bifidobacterium* strains was not affected by mupirocin in this concentration (P value>0.05).

We used a pure powder of mupirocin, dissolved in sterile water and tween 80, instead of antimicrobial discs used by RADA et al. (31, 32). Based on our results, we recommend the use of Wilkins-Chalgren agar medium supplemented with 100 mg/L mupirocin for the selective enumeration of Bifidobacteria in dairy products. This medium is easy to prepare, does not need pH adjustment, and the recovery of Bifidobacteria was found to be about 100%. As shown in Table 4, the counts of Bifidobacterial strains at OD600:1.0 on WCA, MRSA, WCM 50, and WCM 100 didn't have significant difference (P value>0.05).

Although in some studies MRS agar containing clindamycin (10, 14), and or clindamycin plus ciprofloxacin (11) was reported as selective medium for Lactobacilli, in this study mMRS (containing clindamycin plus ciprofloxacin) was not completely selective for Lactobacilli, because Bifidobacterial strains could grow partly on mMRS (Table 4) (P value≤0.05).

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 5, Figure 1). We suggest that the designed primer set can be use for PCR-based specific detection of *B. animalis* subsp. *lactis* BB-12 in probiotic products. Moreover, since this gene is common to all previously described Bifidobacteria and to date has only been detected as a single copy on the Bifidobacterial genome (47), the designed primer set can be applicable for qPCR reactions targeting single copy gene.

The obtained results from the enumeration of *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts (Table 6) indicated that *B. animalis* subsp. *lactis* BB-12 counts decreased during shelf-life considerably (P value≤0.05). However, these counts were equivalent to the recommended standard of International Dairy Federation (IDF) for Bifidobacterial counts in dairy products, until the expiry date of products (6.42±0.34 log CFU/mL in the expiry date). We hypothesize that this count-retaining ability can be due to very high initial inoculum during manufacturing, since in the first day of shelf-life (day 1), the count of *B. animalis* subsp. *lactis* BB-12 was 10.10±0.10 log CFU/mL. The product shelf-life was 21 days, which is fairly short in comparison with similar products in other countries. We guesstimate that incorporation of prebiotics can enhance the shelf-life period of dairy probiotic products.

After the expiry date, the counts of *B. animalis* subsp. *lactis* BB-12 decreased rapidly, and fell below the CODEX standard (10^6 CFU/mL). It is important that Bifidobacteria can survive in fermented dairy products until consumption. The viability of Bifidobacteria depends on the degree of acidification, bacterial strains, fermentation conditions, storage temperature, and preservation methods, and is mainly limited by their sensitivity to high acidity (13). *B. animalis* subsp. *lactis* BB-12 was detected using PCR with designed primer set, even after the expiry date (Figure 2).

Moreover, by means of fructose-6-phosphate phosphoketolase (F6PPK) assay we confirmed that all colonies arising on WCM 100 medium from probiotic yoghurts were belong to

Bifidobacterium genus (Table 7). This activity was detected during shelf-life and even after the expiry date.

It is important to keep in mind that laboriousness and time consuming are two obvious disadvantages of culture-based quantitative methods. In addition, the viable plate count method quantifies 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 (11, 12, 18-20, 22-24, 48).

In conclusion, our results showed that WCM 100 medium (with 100 mg/L mupirocin) is a credible medium for the selective enumeration of Bifidobateria in commercial probiotic yoghurts, with a very good cell recovery. WCM 100 medium can be a very useful alternative to already described media that are less efficient and/or have complex compositions, which makes them unsuitable for routine work. WCM 100 medium is prepared from a commercially available base (Wilkins-Chalgren broth), does not need pH adjustment, can be autoclaved with its additives, does not require long incubation times for high bacterial recovery levels, and allows pleiomorphic rod Y-shape of Bifidobacteria. Neither WCM 50 medium (containing 50 mg/L mupirocin) and mMRS (containing 0.1 mg/L clindamycin plus 10 mg/L ciprofloxacin) were selective for Bifidobateria and Lactobacilli, respectively. The Iranian commercial probiotic yoghurt analyzed in this study, has an acceptable level of *B. animalis* subsp. *lactis* BB-12 during shelf-life and at the expiry date. The relatively high reduction in viable cell counts during this period could be attributed to possible presence of factors inhibitory to Bifidobacteria. The *tuf*-gene based PCR reaction developed in this study could effectively detect *B. animalis* subsp. *lactis* BB-12 in commercial probiotic yoghurts during shelf-life and afterwards.

Acknowledgements

This work was supported by Research Council of Shiraz University of Medical Sciences, Shiraz, Iran. We are grateful Pegah Fars Dairy Industries Company (Shiraz, Iran), for their assistance in sample collection and disclosing product microbial information to us.

Conflict of Interest

None declared.

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