

ORIGINAL ARTICLE

Selective method for identification and quantification of *Bifidobacterium animalis* subspecies *lactis* BB-12 (BB-12) from the gastrointestinal tract of healthy volunteers ingesting a combination probiotic of BB-12 and *Lactobacillus rhamnosus* GG

D.D. Poutsiake¹ , I.J. Mahoney¹, L.A. McDermott¹, L.L. Stern³, C.M. Thorpe¹, A.V. Kane¹, C. Baez-Giangreco¹, J. McKinney³, L.E. Davidson¹, R. Leyva³, B. Goldin² and D.R. Snyderman¹

¹ Tufts Medical Center, Boston, MA, USA

² Tufts University School of Medicine, Boston, MA, USA

³ Pfizer Consumer Healthcare, Madison, NJ, USA

Keywords

enumeration, intestinal microbiology, isolation, *Lactobacillus*, probiotics.

Correspondence

Debra D. Poutsiake, Division of Geographic Medicine and Infectious Disease, Box 041, Tufts Medical Center, 800 Washington Street, Boston, MA 02111, USA.
E-mail: dpoutsiake@tuftsmedicalcenter.org

2016/1525: received 1 November 2016, revised 27 January 2017 and accepted 25 February 2017

doi:10.1111/jam.13436

Abstract

Aim: To develop a novel validated method for the isolation of *Bifidobacterium animalis* ssp. *lactis* BB-12 (BB-12) from faecal specimens and apply it to studies of BB-12 and *Lactobacillus rhamnosus* GG (LGG) recovered from the healthy human gastrointestinal (GI) tract.

Methods and Results: A novel method for isolating and enumerating BB-12 was developed based on its morphologic features of growth on tetracycline-containing agar. The method identified BB-12 correctly from spiked stool close to 100% of the time as validated by PCR confirmation of identity, and resulted in 97–104% recovery of BB-12. The method was then applied in a study of the recovery of BB-12 and LGG from the GI tract of healthy humans consuming ProNutrients[®] Probiotic powder sachet containing BB-12 and LGG. Viable BB-12 and LGG were recovered from stool after 21 days of probiotic ingestion compared to baseline. In contrast, no organisms were recovered 21 days after baseline in the nonsupplemented control group.

Conclusions: We demonstrated recovery of viable BB-12, using a validated novel method specific for the isolation of BB-12, and LGG from the GI tract of healthy humans who consumed the probiotic supplement.

Significance and Impact of the Study: This method will enable more detailed and specific studies of BB-12 in probiotic supplements, including when in combination with LGG.

Introduction

Probiotics are live micro organisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al.* 2014). They exert their effect by arriving in the gastrointestinal (GI) tract in a viable state. *Bifidobacterium* and *Lactobacillus* species, normal inhabitants of the human GI tract, are employed as exogenously administered probiotics (Turrone *et al.* 2014). Studies have demonstrated their viability within the GI tract after

ingestion as well as their health benefits either alone or in combination (Myllyluoma *et al.* 2005; Larsen *et al.* 2006; Mohan *et al.* 2006, 2008; Dommels *et al.* 2009; Laitinen *et al.* 2009; Francavilla *et al.* 2010; Hojsak *et al.* 2010; Morrow *et al.* 2010; Saxelin *et al.* 2010; Davidson *et al.* 2011; Wang *et al.* 2011; Rizzardini *et al.* 2012; Zhang *et al.* 2012; Foolad *et al.* 2013; Granata *et al.* 2013; Partty *et al.* 2013; Smith *et al.* 2013; Janvier *et al.* 2014; Chau *et al.* 2015; Hauser *et al.* 2015; di Cerbo *et al.* 2016). However, studies on the live recovery of

Bifidobacterium animalis ssp. *lactis* BB-12 (BB-12), one of the most commonly used probiotics, from the human GI tract are lacking. The need for selective, rapid and reliable methods for the enumeration of bifidobacteria to support probiotic science has been highlighted (Roy 2001). In addition, the recovery of live BB-12 and *Lactobacillus rhamnosus* GG (LGG), another commonly used and well-characterized probiotic (Goldin *et al.* 1992), from the GI tract after ingestion of a proprietary powder matrix has not been previously studied.

The resistance of BB-12 and LGG to physiological stress encountered in the GI tract accounts for their wide use and frequent study, either individually, in combination with one another or with other probiotics, in dairy products and supplements in North America and Europe (Bonaparte and Reuter 1997; Jacobsen *et al.* 1999; Matto *et al.* 2004).

Previous studies have demonstrated that the recovery from the GI tract after ingestion of viable probiotic organisms including *Bifidobacterium* species is often poor (Hamilton-Miller *et al.* 1999; Temmerman *et al.* 2003). As the beneficial effects of probiotics, BB-12 and LGG in particular, have become increasingly well established and their use becomes more widespread, there is a greater need for accurate identification, and enumeration of probiotic organisms to test the quality and utility of these products. The culture-dependent and independent methods for identifying and enumerating *Lactobacillus* species including LGG are well established (Rogosa *et al.* 1951; Ellis and Wb 1958; Sabine and Vaselekos 1965; Ahlroos and Tynkkynen 2009). There are established culture-independent methods for identifying and enumerating *Bifidobacterium* species using 16S ribosomal gene variable regions (Matsuki *et al.* 2003; Kwon *et al.* 2005). Identification of a highly conserved single-copy *tuf* gene provides a unique marker differentiating BB-12 from other *Bifidobacterium* species, and has been used to develop a highly specific quantitative PCR assay (Solano-Aguilar *et al.* 2008). However, most PCR-based assays do not demonstrate the presence of viable organisms.

Current culture-dependent methods are insufficiently selective for *Bifidobacterium* species and/or produce inaccurate quantitative results (Roy 2001). For example, a culture method incorporating mupirocin in the medium for the enumeration of bifidobacterial species from dairy products exists (Rada and Koc 2000) but does not discriminate between species. More recently, a method for the selective isolation of a specific species, *Bifidobacterium bifidum*, has been developed (Pecher *et al.* 2014). The relative resistance of BB-12 to tetracycline has been described (Masco *et al.* 2006; Saarela *et al.* 2007) and was used to develop a culture-dependent method for isolating BB-12 in human faecal samples (Larsen *et al.* 2006). That report,

however, did not include data directly validating the accuracy of the culture method using PCR methodology.

We observed that pure BB-12 cultured on agar formed colonies with a 'fried egg' morphology not observed in the culture of other bifidobacterial strains. This observation was the basis for the objectives of this study (i) to develop novel, selective and PCR-validated culture-dependent methods for isolating and enumerating BB-12 from human stool and (ii) to apply these methods, and published methods of LGG isolation, to examine the recovery of live BB-12 and LGG from the GI tract of healthy individuals after oral ingestion of Pronutrients™ Probiotic supplement.

Materials and methods

Laboratory methods

Preparation of Wilkins–Chalgren media with added antibiotics

Wilkins–Chalgren for *Bifidobacterium* Mupirocin agar (WCBM) (Ferraris *et al.* 2010) was prepared with the following: 48 g l⁻¹ of dehydrated Wilkins–Chalgren Agar (Difco, Detroit, MI), 10 g l⁻¹ glucose, 5 g agar (Difco), 5 ml l⁻¹ of Tween 80 (Sigma, St Louis, MO), 0.5 g l⁻¹ of L-cysteine HCL (Sigma) and 50 mg l⁻¹ mupirocin (Sigma). WCBM with tetracycline (WCBMT) was prepared as above with the addition of tetracycline to achieve final concentrations of 4, 8, 10, 12 and 16 µg ml⁻¹ in the agar plates (WCBMT). Stock concentrations of tetracycline and mupirocin were prepared in water and added to molten agar to achieve the desired concentrations.

BB-12 and LGG stocks

BB-12 and LGG were obtained in lyophilized form (Probio-Tec®BB-12® Blend-200 and Probio-Tec®LGG® Blend-200; Chr. Hansen Holding A/A, Hoersholm, Denmark).

Microbiological assay of BB-12

BB-12 was obtained in the lyophilized form (Probio-TecBB-12 Blend-200; Chr. Hansen Holding A/A). Working cultures of pure BB-12 from lyophilized stock were prepared by inoculation of stock into prereduced MRS broth supplemented with 0.05% of cysteine hydrochloride (Difco) and incubated in an anaerobic chamber (5% CO₂, 10% H₂, 85% N₂) at 37°C. These culture conditions were used throughout the study. The pure broth culture was streaked for isolation of BB-12 on WCBM and incubated for 72 h (Ferraris *et al.* 2010). Subsequently, a single BB-12 colony derived from the streaking of the working culture was inoculated into 25 ml of prereduced MRS broth. The culture was incubated until an OD₆₀₀ of approximately 1.0 was reached (18–20 h) to yield the

desired colony density of approximately 1×10^8 CFU per ml. This broth preparation was then used for all spiking experiments.

Collection of stool for isolation, enumeration and PCR experiments

For method development, fresh stool samples were collected from healthy volunteers, homogenized by mixing with spatulas and stored at 4°C under aerobic conditions. No more than 24 h elapsed between stool production and plating. Each stool used for method development and validation was determined to be free from endogenous BB-12 by PCR, as described below, and lacked colonies with a fried egg morphology after culturing on WCBMT agar. Similarly, stools were determined to be free of LGG using accepted culture methods as described below. In addition, stools used for method development and validation did not amplify in qPCR for LGG.

For the collection and processing of stool samples from clinical study subjects, stool samples were processed as above. For enumeration studies, samples were serially diluted in PBS and plated on the appropriate media within 24 h of stool production. For PCR studies, stool aliquots were stored at -80°C until batch thawed within 4 weeks for DNA isolation.

Selection and enumeration of potential BB-12 colonies after exogenous addition to stool

One gram of homogenized stool from healthy subjects not involved in the clinical supplementation study was suspended in 9 ml of sterile PBS. BB-12 grown in pure broth culture was added to the suspension to give a concentration of $\log_{10}$ 8.90 BB-12 per gram of stool ($\log_{10}$ 7.90 per ml suspension in a total of 10 ml). Aliquots (0.1 ml) of serial 10-fold dilutions of the suspension were plated onto WCBM plates with and without varying concentrations of tetracycline. In parallel, 0.1-ml aliquots of 10-fold dilutions of the pure broth culture (final concentration of $\log_{10}$ 8.90 BB-12 in 10 ml of PBS) were plated onto WCBM agar plates with and without tetracycline. After 72 h, plates were inspected for colonies with fried egg-like morphology (Fig. 1), which were then enumerated. Recovery of BB-12 from the stool spiked with BB-12 was assessed by comparing plate counts of spiked stool suspension to those of pure BB-12 broth culture. The average recovery of CFU per gram of stool for the spiked samples was considered adequate if within 70% of the broth culture count (US Pharmacopeia Convention, 2009).

Isolation of LGG

LGG was isolated from the same faecal specimens used for BB-12 method development or clinical supplementation study subjects by culture at 37°C in an anaerobic chamber

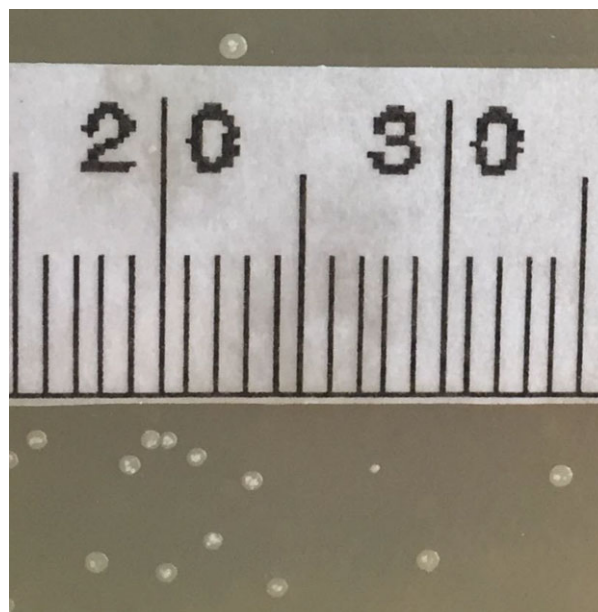


Figure 1 BB-12 in pure culture on WCBM agar containing $8 \mu\text{g ml}^{-1}$ tetracycline (WCBMT).

(5% CO_2 , 10% H_2 , 85% N_2) for 48 h on *Lactobacillus* selection agar (LBS; Becton Dickinson B11327, Becton Dickinson and Company, Franklin Lakes, NJ) supplemented with tomato juice to enhance the recovery of *Lactobacillus* species from stool (Sabine and Vaselekos 1965).

Culture and enumeration of BB-12 and LGG from stool

Six serial 10-fold dilutions of homogenized stool were prepared in phosphate-buffered saline and 100- μl aliquots were plated in duplicate onto the surface of LBS and WCBMT agar plates resulting in a countable range of 2.5×10^3 to 2.5×10^9 CFU per g of stool (Maturin and Peeler 2001). Colonies demonstrating morphologies consistent with BB-12 or LGG (white, creamy, regular appearance and distinct buttery smell) were enumerated and single colonies were transferred to the appropriate media for PCR confirmation of identity. The percentage of colonies that were identified by PCR as BB-12 or LGG was multiplied by the count based on presumptive morphology to determine CFU per g of stool. Colony counts below the lower limit of detection (LLD) of the linear portion of the standard curve were set to the LLD.

Preparation of template DNA for BB-12 and LGG PCR

Colonies with the fried egg morphology were picked to confirm their identification as BB-12 using *tuf* gene targeted PCR as follows. Presumptive BB-12 colonies were picked and reisolated onto WCBM agar. Individual colonies were picked, suspended in 100 μl of elution buffer (Qiagen, Valencia, CA) and boiled for 10 min at 100°C.

The crude extraction was stored at -20°C for future use as template DNA. LGG template DNA was produced and stored in a similar manner.

PCR for validation of BB-12 and LGG identification by culture

For BB-12, the PCR mixture contained a final concentration of 666 nmol l^{-1} each of forward primer 5'-GTGTCGAGCGCGCAA-3' and reverse primer 5'-CTCGCACTCATCCATCTGCTT-3' (Solano-Aguilar *et al.* 2008). The thermal profile used in the cycler (Model 2720 Thermal Cycler; Applied Biosystems, Foster City, CA) was as follows: 95°C for 15 min initially, then 30 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 1 min each. The identification of morphologically chosen colonies was confirmed as positive for BB-12 if all three *tuf* gene PCR triplicates produced amplicons consistent with the expected size of BB-12 colonies (~ 117 base pairs), and negative controls did not produce amplicons.

For LGG, the PCR mixture contained a final concentration of 666 nmol l^{-1} each of forward primer 5'-CGCCCTTAACAGCAGTCTTCAAAT-3' and reverse primer 5'-ACGCGCCCTCCGTATGCTTAAACC-3' (Ahlroos and Tynkkynen 2009). The thermal profile was as follows: 95°C for 15 min initially, then 95°C for 1 min, 65°C for 1 min, 72°C for 1 min, then five cycles of 95, 60, 72°C for 1 min each, 25 cycles of 95, 55, 72°C for 1 min each, then 72°C for 5 min. All samples were run in triplicate. Positive and negative controls were included in each run. Reaction mixtures were resolved on agarose gels to determine the presence or absence bands of appropriate size (117 base pairs for BB-12 and 762 base pairs for LGG, Fig. 2 for a representative gel of BB-12).

Extraction of DNA from stool

DNA was extracted from frozen stool samples using a QiaAMP DNA Stool MiniKit (Qiagen) according to the manufacturer's instructions with the following modification. Samples were reconstituted in 1.4 ml Qiagen ASL buffer to which was added 50.1 mg lysozyme (Sigma), 13.5 U lysostaphin (Sigma) and 13 μl UltraClean PCR water (MoBio, Carlsbad, CA). Following 10 min of incubation at 37°C , 20 mg of RNase A (Sigma), 200 mg proteinase K (Qiagen) and 5 mg sodium dodecyl sulphate (SDS) (Sigma) were added to each tube. They were then incubated at 70°C for 10 min, followed by 5 min of bead beating in a vortex adapter (MoBio) on a Vortex Genie 2 (Cole-Parmer, Vernon Hills, IL) with 0.5 g of 0.1 mm zirconium/silica beads (Biospec Products, Bartlesville, OK) at 4°C . The beads and insoluble debris were spun down at 16 800 g for 1 min and the supernatant was treated with a Qiagen InhibitEx tablet from the kit. From that point on the standard protocol was followed.

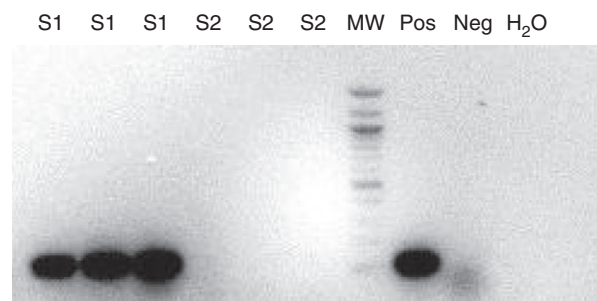


Figure 2 PCR was performed on DNA extracted from putative BB-12 (S1) and putative non-BB-12 (S2) colonies. Amplicons were resolved on a 1.25% agarose gel, then visualized with ethidium bromide under UV light. MW = 100 bp ladder. 'pos' = positive control; 'neg' = negative control, 'H₂O' = water control. The expected size of the *tuf* gene target BB-12 sequence is 117 base pairs (Solano-Aguilar *et al.* 2008).

Ethanol precipitation of DNA from stool samples

During the development phase, it was noted that some faecal samples contained inhibitory substances. These were removed by ethanol precipitation. Extracted DNA was stored at -70°C until analysed. The extract was thawed, and 25 μl was mixed with 2.5 μl of 3 mol l^{-1} sodium acetate (pH 5.2). 125 μl of 100% ethanol was added to precipitate the DNA, and the suspension was incubated at -20°C for 20 min. The suspension was then centrifuged at 13 200 g for 15 min, and the supernatant decanted. One millilitre of 70% ethanol was added to the pellet, and the suspension was centrifuged at maximum speed 13 200 g for 15 min. The supernatant was decanted, and the pellet was air-dried. Once dry, the pellet was resuspended in 25 μl Qiagen Buffer AE (Qiagen), and used as template DNA for qPCR.

Quantitative PCR

The qPCR mixture contained 500 nmol l^{-1} of forward and reverse primers as described for BB-12 colony PCR above, with 250 nmol l^{-1} of FAM-labeled probe ATC AAC ACG AAC GTC GAG. The thermal profile used in the cycler (Mx3000P qPCR System; Agilent Technologies, Santa Clara CA) was as follows: 5 min of initial denaturation at 95°C , followed by 40 cycles of 15 s at 95°C , 60 s at 60°C and 30 s at 72°C , with fluorescence measured at the end of each annealing step.

For LGG, the qPCR mixture contained SYBR green (Qiagen), and 500 nmol l^{-1} of forward and reverse primers as described for LGG colony PCR above. The thermal profile was as follows: 15 min of initial denaturation at 95°C , followed by 40 cycles of 15 s at 95°C , 60 s at 60°C , and 60 s at 72°C . Fluorescence was measured at the end of each chain extension step.

A standard curve using a plasmid with the appropriate target sequence was used in each run. For LGG and BB-12, the standard curve ranged in 10-fold dilutions from 5×10^7 copies of plasmid per 5 μ l to 50 copies of plasmid per 5 μ l. Each standard was run in triplicate, and the average cycle threshold (Ct) of each triplicate was determined, and used to create a line of best fit.

R^2 values ranged from 0.995–0.999. The standard deviations of standard triplicates ranged from 0.0023–1.347. Unknown samples were run in triplicate, and the cycle threshold was averaged. The standard deviations of unknown samples ranged from 0–1.518. The concentration in each unknown sample was calculated from the line of best fit.

To determine interassay variability, Ct for each standard in every qPCR run was averaged across all runs, and used to calculate a coefficient of variation for each concentration. Coefficients of variation ranged from 0.0144–0.0472.

Comparison of BB-12 enumeration by qPCR and quantitative culture

For quantitative culture, BB-12 was grown from lyophilized BB-12 to mid-log phase in pure broth culture and serially diluted. Aliquots of the pure broth culture and first four 10-fold dilutions of that broth culture were directly plated onto WCBM (no tetracycline) and WCBMT containing 4–16 μ g ml⁻¹ tetracycline for colony count. For qPCR, DNA from the broth and the four 10-fold serial dilutions was extracted using a QiaAMP DNA Stool Mini-Kit (Qiagen) according to the manufacturer's instructions except that samples were reconstituted in 1.4 ml Qiagen ASL buffer to which was added 50.1 mg lysozyme (Sigma), 13.5 U lysostaphin (Sigma), and 13 μ l UltraClean PCR water (MoBio). Following 10 min incubation at 37°C, 20 mg of RNase A (Sigma), 200 mg proteinase K (Qiagen) and 5 mg SDS (Sigma) were added to each tube and tubes were incubated at 70°C for 10 min, followed by 5 min of bead beating in a vortex adapter with 0.5 g of 0.1 mm zirconium/silica beads at 4°C. After removal of beads and insoluble debris by centrifugation, the supernatant was treated according to the manufacturer's protocol. The extract was stored at -70°C until the time of qPCR analysis as described above. The number of DNA copies of BB-12 in the DNA extract for the broth culture and dilutions were calculated from a plasmid-based standard best-fit curve. For the broth culture and each dilution, both the colony count on WCBMT agar and the qPCR assay were performed in triplicate and the results were averaged.

Clinical study methods

Study design

This study was a randomized, open-label, two-arm parallel group prospective study (clinicaltrials.gov ID NCT01757054).

Study population

Inclusion criteria for healthy volunteers were age 18–70 years, BMI 19–35 kg m⁻², normal baseline haematology and blood chemistry values and physical examination. Exclusion criteria were probiotic consumption within 30 days prior to the start of the study, unwillingness to comply with dietary restrictions, known or suspected allergy to milk, wheat or constituents of the supplement, receipt of antibiotics within 3 months prior to or at enrolment, lactose intolerance, immunosuppression, pregnancy, lack of use of contraception, structural heart disease, history of endocarditis or heart valve replacement, or the presence of an implanted cardiac device. Subjects with inactive or controlled chronic or past medical conditions such as cancer, collagen vascular disease, an implanted orthopaedic device, hypertension, coronary artery disease, depression or resolved hepatitis were allowed to participate in the study. Study schedule is shown in Fig. 3. This study was approved by the Tufts Medical Center Institutional Review Board.

Study procedures and intervention

Subjects who met inclusion criteria were instructed on study procedures such as dietary restrictions and faecal collection methods. Dietary restrictions included foods containing live micro organisms such as yogurt with live cultures, fermented foods and beverages, probiotic-containing supplements and food bars, and fibre-containing supplements.

Subjects were enrolled in the study by the study physician after a 2-week run-in period if their diary demonstrated adherence to dietary restrictions during the run-in period and they were able to provide a properly collected baseline faecal specimen (visit 3). During this enrolment visit, subjects were randomized to supplement or no supplement groups by a computer-generated randomization schedule in a 5 : 2 ratio. Laboratory personnel responsible for quantitative culture and PCR were blinded to assignment although investigators and subjects were not. The randomization allocation sequence was concealed from investigators and subjects until the subject qualified for randomization.

The Pronutrients™ Probiotic supplement produced and supplied by Pfizer contained BB-12 and LGG each in amounts of at least 1×10^9 CFU per organism for a total of at least 2×10^9 CFU per day. The product was stored at 4°C in the study pharmacy and at room temperature by study subjects. Stability studies performed on the supplement through the duration of the study demonstrated recovery of at least 1×10^9 CFU per dose of each organism when stored at either room or refrigerated temperature for up to 24 months (data not shown).

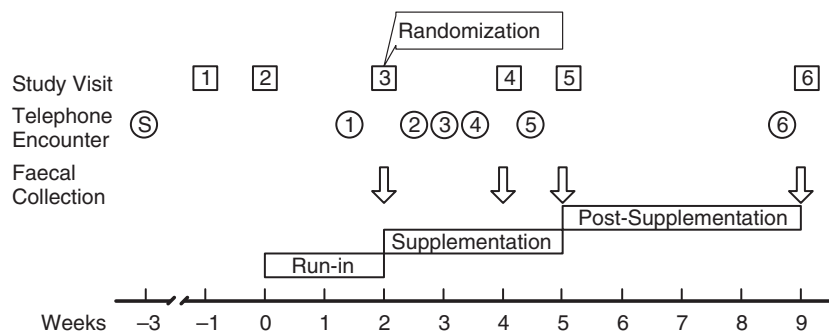


Figure 3 Squares—study visits; S within circle—screening telephone call. arrows - faecal specimen collection.

Subjects in both the probiotic and control groups maintained diaries similar to that used in the run-in period. Compliance, defined as taking at least 80% of doses per instructions, was assessed during weekly phone calls and through collection and counting of unused probiotic supplement sachets.

Outcomes

The primary outcome was quantitative culture (the number of CFU recovered per gram of stool) of BB-12 and LGG in specimens after 21 days of oral ingestion of probiotic as compared to baseline. Secondary outcomes were quantitative culture of BB-12 and LGG 14 days after randomization and 28 days after the end of the supplementation period, qPCR enumeration (number of genome copies per gram of stool) 14 and 21 days after randomization and 28 days after the end of the supplementation period, comparison of the quantity of LGG and BB-12 in the faecal specimens of subjects in the control and probiotic groups before and after the supplementation period and the occurrence of adverse events over the course of the study.

Sample size determination

Sample size determination was directed at the primary endpoint, the number of colonies of LGG or BB-12 per gram of stool isolated from specimens produced 21 days after randomization compared to baseline. Assumptions for the variability in recovery of LGG (2 CFU per g of stool on the $\log_{10}$ scale) were based on previously published data (Dommels *et al.* 2009). There was no available information regarding the variability in BB-12 using quantitative culture. Therefore, the variability in BB-12 recovery by quantitative culture was assumed to be equivalent to that of LGG. This assumption was based on data showing that the variability in quantifying BB-12 by qPCR was lower than that of LGG (Myllyluoma *et al.* 2007). Applying these assumptions, a sample of 15 subjects receiving probiotic and providing complete data would provide 99% power

to detect a difference from baseline of 2.3 CFU per g of faeces on the $\log_{10}$ scale and 90% power to detect a difference from baseline of 1.7 CFU per g of stool for BB-12. We assumed negligible recovery of LGG or BB-12 from subjects in the control group. Therefore, a control group of six subjects would provide 80% power to detect an effect size of 3.3 CFU per g of stool on the $\log_{10}$ scale between the probiotic and the control groups. Based on our prior experience, we estimated that 70% of enrolled subjects would provide complete data. Therefore, our enrolment target was 28 subjects in order to obtain 21 subjects with complete data.

Statistical analysis

Descriptive statistics were used to characterize the study population and groups. Quantitative culture and qPCR results were expressed as $\log_{10}$ scale CFU or copies, respectively, per gram of stool. Within-group changes were determined by paired *t*-test for continuous data. Differences between groups were determined by ANOVA with a treatment term for continuous data and by the Cochran–Mantel–Haenszel test for categorical data. *P*-values of <0.05 were considered statistically significant. All tests were two-sided. All analyses were performed by SAS 9.2 (SAS Institute, Inc, Cary, NC).

Results

Isolation and selective identification of BB-12

Plating stool specimens with exogenously added BB-12 on WCBM plates without tetracycline yielded plates crowded with colonies making it difficult to distinguish BB-12 colonies based on the fried egg morphology. Furthermore, the recovery of the BB-12 confirmed by PCR was poor (data not shown). Therefore, this approach was abandoned.

The addition of tetracycline to the agar (WCBMT) led to a reduced size and number of colonies from background flora on the plate, which allowed for easier

identification of colonies that gave the fried egg morphology of presumptive BB-12 colonies (Table 1a). On WCBMT plates containing tetracycline concentrations of 4, 8, and 10 $\mu\text{g ml}^{-1}$, all colonies selected as presumptive BB-12 by laboratory personnel based on the fried egg colonial morphology were confirmed to be BB-12 by PCR with a recovery close to 100%, thus validating the ability of the culture-based isolation method for BB-12. There was a decrease in recoverable BB-12 as the tetracycline concentration increased from 0 to 8 $\mu\text{g ml}^{-1}$ of 0.35 log. When using tetracycline concentrations of 12 $\mu\text{g ml}^{-1}$, 85.7% of presumptive colonies were confirmed as BB-12. In addition, it was more difficult to identify the 'fried egg' morphology. The remaining 14.3% of colonies not confirmed as BB-12 were presumed to be other bifidobacteria species but were not further characterized. On WCBMT plates containing 16 $\mu\text{g ml}^{-1}$ tetracycline, there was limited growth of all bacterial species reducing the ability to isolate and identify BB-12.

All subsequent isolation and enumeration of BB-12 from stool were performed on WCBMT (tetracycline 8 $\mu\text{g ml}^{-1}$) since further experiments showed that at this concentration of tetracycline, 94% of selected colonies were confirmed to be BB-12 by PCR and the recovery compared to pure broth culture was acceptable (Table 1b).

To determine the relationship between BB-12 *tuf* gene copy number and the number of BB-12 CFU, qPCR was performed on DNA extracted from pure BB-12 broth cultures or stool suspensions spiked with BB-12 and

compared with the number of enumerated BB-12 CFU obtained after culturing aliquots of the same broth cultures on WCBMT. Quantitative PCR detection of serial dilutions from the DNA of pure BB-12 culture or spiked stool suspensions was linear for bacterial copy numbers over a range of 1000–10 000 000 CFU for pure cultures (Fig. 4) or 100 00–10 000 000 CFU for spiked stool suspensions (data not shown).

Isolation of BB-12 and LGG from the GI tract of healthy volunteers

Subjects

Nineteen subjects and eight subjects were randomized to supplement and not supplement, respectively, between February 2013 and August 2013 with follow-up continuing until October 2013. Thirteen (48%) of the 27 subjects were male, 14 (52%) were White, 6 (22%) were Black and 3 (11%) were Asian. The mean age was 42.3 years (standard deviation 13.3), mean weight was 72.3 kg (standard deviation 17.7) and mean body mass index was 25.8 kg m^{-2} (standard deviation 4.5). There were no differences in baseline characteristics between the supplement and no supplement group. Of the supplement group, three were excluded from the per protocol analysis (two had entry criterion violations and one had a protocol violation). All subjects in the probiotic group met or exceeded the compliance criterion of consuming at least 80% of probiotic samples as directed during the supplementation period.

Table 1 Identification and recovery of BB-12 exogenously added to stool after culture on WCBM agar (a) with and (b) without tetracycline

	BB-12 colony counts		% of colonies obtained from spiked stool concordant for BB-12 by PCR	PCR-confirmed count†	% Recovery of BB-12 added to stool‡
Tetra cycline (µg ml ⁻¹)	Pure broth culture	Spiked stool (morphology-based count)			
(a)					
0	7.90*	8.91*	13.3	8.03*	102
4	7.81	7.56	100	7.56	97
8	7.55	7.72	100	7.72	102
10	6.98	7.28	100	7.28	104
12	6.59	5.90	85.7	5.84	89
16	ND	ND	—	ND	—
(b)					
8	7.00*	6.58*	92	6.54*	93
8	7.00	6.94	83	6.86	98
8	7.60	8.18	100	8.18	107
8	7.60	8.09	100	8.09	106

Each colony count represents the mean of duplicate samples at each concentration of tetracycline.

ND = not detected

*Log₁₀ CFU BB-12 recovered per 1.0 ml of pure broth suspension or spiked stool suspension that contained log₁₀ 7.90 BB-12.

†Log₁₀ (raw spiked stool morphology-based count × % of colonies concordant by PCR).

‡(PCR-confirmed log₁₀ CFU BB-12 colonies recovered from spiked stool/log₁₀ CFU BB-12 colonies recovered from pure broth culture) × 100%.

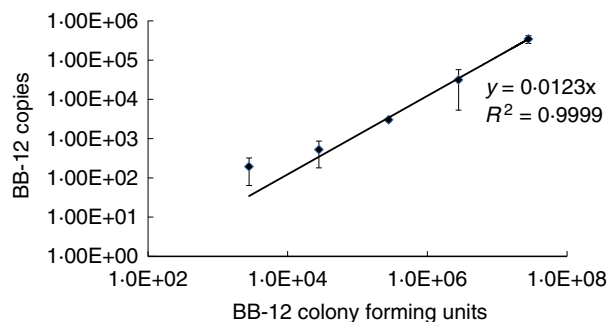


Figure 4 CFU of BB-12 (x-axis) compared to copies of BB-12 DNA (y-axis) as determined by qPCR following extraction of DNA from BB-12 grown to log phase in broth culture and then serially diluted. Mean data from three independent experiments are shown, with standard deviations. $R^2 = 0.99$.

Recovery of BB-12 and LGG after supplementation

We performed an intention to treat analysis of the primary aim, CFU of BB-12 or LGG isolated per gram of stool produced 21 days after randomization compared to CFU isolated from baseline samples from the same group. Viable BB-12 was detected in 16 subjects (84%) who ingested a probiotic compared to none in this group at baseline (Table 2, Columns A vs E, $P < 0.001$ baseline vs after 21 days of supplementation). Similar results were obtained for LGG although one subject had detectable LGG by quantitative culture at baseline (Table 2, Columns A vs E, $P < 0.001$). Upon further questioning, the potential exposure of this subject to LGG was the ingestion of a fermented milk product. In the control group, there was no viable BB-12 or LGG detected by quantitative culture above the LLD at baseline or at the end of the 21-day supplementation period (during which subjects did not ingest the supplement, Table 2, columns B vs F).

Secondary outcomes for quantitative culture analyses are also shown in Table 2. BB-12 and LGG were recovered at significantly higher amounts by quantitative culture of 21-day samples in the probiotic group compared to the control group (Table 2, Columns E vs F, $P < 0.001$ for BB-12 and $P = 0.006$ for LGG). No BB-12 or LGG was isolated by quantitative culture in either the probiotic or the control group 28 days after the end of the supplementation period (Table 2, Columns G and H).

When enumerated by qPCR, BB-12 and LGG were detected in the stool samples of 17 of 19 and 13 of 19 probiotic group subjects, respectively, after 21 days of supplementation compared to samples from the same subjects at baseline, when BB-12 was detected in one subject and no LGG was detected (Table 2, Columns A vs E). There was no difference in the detection of BB-12 or

LGG by qPCR in the control group at baseline compared to after 14 or 21 days of the supplementation period, during which control subjects did not ingest the probiotic combination. However, BB-12 was detected in the baseline and 14-day stool samples in each of two control subjects and the 21-day sample of one of these subjects. Similarly, LGG was detected by qPCR in the sample from one control subject at the end of the 21-day supplementation period during which the subject did not ingest probiotic.

In the samples obtained 28 days after the end of the supplementation period, no BB-12 or LGG was isolated by quantitative culture. However, when assessed by qPCR, three subjects who received a probiotic had detectable BB-12 at that time point. Additionally, two control subjects had BB-12 or LGG detected by qPCR during or after the supplementation period.

Overall, the probiotic supplement was well tolerated. Two mild AE were related to the receipt of the probiotic: one subject experienced abdominal distension and one subject experienced diarrhoea.

Discussion

We demonstrated that BB-12 colonies can be accurately identified based on fried egg colonial morphology from stool culture in modified Wilkins–Chalgren media with added tetracycline. This culture method was highly selective for isolating BB-12 colonies from BB-12-spiked human stool as validated by PCR confirmation and a high degree of recovery as compared to pure broth culture. This is contrasted with previous studies that found differences in quantitative culture of bifidobacteria among different methods and no standard best culture-dependent method for the identification and enumeration of *Bifidobacterium* species (reviewed in (Roy 2001)). In addition, we found that enumeration of BB-12 by the culture-dependent method described in this study was linearly related to enumeration of copy numbers of BB-12 as determined by qPCR on the same samples, consisting of either pure cultures in broth or of stool with exogenously added organisms.

Importantly, we demonstrated the utility of this isolation and enumeration method in assessing the recovery of viable BB-12 from the GI tract of healthy human subjects ingesting a combination BB-12-LGG probiotic in a previously unstudied matrix. BB-12 and LGG have each been recovered from the stool of both healthy subjects and patients after ingestion in a variety of vehicles, such as within capsules, milk products or yogurt (Myllyluoma *et al.* 2005, 2007; Larsen *et al.* 2006; Mohan *et al.* 2006; Ahlroos and Tynkynen 2009; Dommels *et al.* 2009; Saxelin *et al.* 2010; Granata *et al.* 2013). Strengths of this

Table 2 Recovery of BB-12 and LGG from the GI tract of healthy volunteers

Group Column	Baseline		14 days		21 days		28 days after cessation of supplementation period	
	P*	C†	P	C	P	C	P	C
	A	B	C	D	E	F	G	H
<i>BB-12 quantitative culture</i>								
Mean‡ (SD)	5.4 (0.0)	5.4 (0.0)	7.0 (1.3)	5.4 (0.0)	7.2 (1.1)	5.4 (0.0)	5.4 (0.0)	5.4 (0.0)
N above LLD§	0	0	13	0	16	0	0	0
<i>LGG quantitative culture</i>								
Mean (SD)	3.5 (0.6)	3.4 (0.0)	6.0 (1.6)	3.4 (0.0)	5.5 (1.7)	3.4 (0.0)	3.4 (0.0)	3.4 (0.0)
N above LLD	1	0	16	0	17	0	0	0
<i>BB-12 qPCR</i>								
Mean¶ (SD)	4.3 (0.2)	4.4 (0.3)	5.8 (1.0)	4.3 (0.1)	5.8 (1.0)	4.4 (0.2)	4.4 (0.4)	4.3 (0.0)
N above LLD**	1	2	17	2	17	1	3	0
<i>LGG qPCR</i>								
Mean (SD)	4.3 (0.0)	4.3 (0.0)	5.4 (0.8)	4.3 (0.0)	5.2 (1.0)	4.3 (0.0)	4.3 (0.0)	4.4 (0.2)
N above LLD**	0	0	15	0	13	1	0	1

*Probiotic, *N* = 19.†Control, *N* = 8.‡Mean (standard deviation) log₁₀ colony-forming units (CFU) per g of faecal specimen.§Lower limit of detection (log₁₀ CFU per g): LGG, 3.4; BB-12, 5.4.¶Mean (standard deviation) log₁₀ copies per g of faecal specimen.**Lower limit of detection (log₁₀ copies per g of faecal specimen) for both LGG and BB-12, 4.3.

study include a parallel control group and two different methods used to determine the amount of LGG and BB-12 in faecal specimens. We elected to use both colony counts and qPCR techniques to assess colonization of the GI tract with the strains present in the probiotic combination. The qPCR technique that we employed does not distinguish between viable and nonviable organisms. However, a PCR-based method that estimates viable organism count using propidium monoazide has been described (Desfosses-Foucalt *et al.* 2012).

Recovery of either probiotic from the GI tracts of subjects prior to supplementation was unusual (11% for BB-12 and 7% for LGG) and similar to or less than that found in most other studies (Myllyluoma *et al.* 2007; Kekkonen *et al.* 2008; Dommels *et al.* 2009; Saxelin *et al.* 2010). There might be several explanations to the lower number of subjects with BB-12 or LGG present prior to supplementation in this study compared to several others. First, a number of LGG and BB-12 studies were conducted in Europe, where probiotics, particularly LGG, are common supplements in food. Second, we used stringent methods for confirming the identity of both organisms isolated by quantitative culture from stool that potentially resulted in fewer false positive identifications. Finally, we included a 30-day period prior to randomization when probiotic products were not to be consumed.

In all but rare instances, supplementation with the BB-12-LGG probiotic combination resulted in detectable levels

of both organisms by quantitative culture after 21 days of supplementation. This high degree of recovery is similar to other studies, despite differences in study design (Larsen *et al.* 2006; Myllyluoma *et al.* 2007; Kekkonen *et al.* 2008; Dommels *et al.* 2009; Saxelin *et al.* 2010; Granata *et al.* 2013). Additionally, the failure to recover BB-12 or LGG from a minority of subjects has been observed in many of these studies. Thus, recovery of live LGG and BB-12 prepared in a previously unstudied matrix is confirmed and similar to that seen when different matrices are used.

Results were similar to quantitative culture when the detection method for LGG or BB-12 was qPCR. However, our study used a standard curve with known copy numbers of the relevant qPCR target, rather than DNA extracted from broth cultures, or DNA extracted from stool cultures spiked with bacteria. Thus, copy number per g stool from this study cannot be directly compared to other studies in which CFU per g stool is reported. In this study, LGG was detected by qPCR in fewer subjects who ingested the probiotic supplement than when detected by quantitative culture. The reason for this is unclear but it is possible that the faecal specimens contained substances that inhibited the PCR reaction.

BB-12 was detected by qPCR 4 weeks after the cessation of supplementation but no live colony-forming BB-12 were isolated from the same samples. The likely explanation is that qPCR detected DNA from nonviable organisms that remained in the GI tract after the clearance of

viable BB-12 upon cessation of probiotic supplementation. It is also possible that qPCR detected other strains of non-BB-12 *Bifidobacterium* although the specificity of the PCR target for BB-12 used in this study has been demonstrated (Solano-Aguilar *et al.* 2008). For LGG the clearance was complete in that no LGG was detected 4 weeks after stopping supplementation regardless of detection method used. Complete clearance has also been observed by others albeit after a 6-week washout period (Myllyluoma *et al.* 2005). Other studies have recovered viable LGG as well as LGG detected by qPCR 4 weeks after supplementation (Ahlroos and Tynkkynen 2009), whereas others have documented persistence of LGG and BB-12 by qPCR after a 3-week washout period (Saxelin *et al.* 2010; Granata *et al.* 2013). Possible explanations for these observations are unintended ingestion of the probiotic organism, differences in study design and in one study, the presence of additional probiotic organisms in the supplement might have conferred a survival benefit to LGG and BB-12 (Saxelin *et al.* 2010).

Overall, the probiotic supplement was well tolerated, which is consistent with a recent study of LGG and BB-12 in a similar dose and matrix which showed no difference in adverse effects between probiotic and placebo groups (Smith *et al.* 2013).

A potential limitation of this study was its small size. However, based on findings in the literature and our assumptions, the study was designed to provide 90% power to observe a statistically significant difference in the primary outcome, which was achieved. In addition, conducting the study at one centre might limit the ability to generalize findings. However, the agreement of the main study findings with those in the literature conducted under many differing conditions suggests that the findings are applicable to the broader population.

In summary, we have developed a novel method for the isolation and specific identification of BB-12 and have successfully utilized it in a clinical study of the recovery of viable BB-12 and LGG from the GI tracts of healthy human subjects ingesting a combination probiotic-containing BB-12 and LGG in a previously unstudied matrix.

Funding information

This study was funded by Pfizer. Pfizer (L.L.S., J.M. and R.L.) aided in study design and statistical analysis.

Acknowledgements

The authors thank Abigail Benudis and Emily O'Carroll for their diligent efforts. L.M., C.B.G., J.M. and B.G. developed and/or refined, validated and executed quantitative culture methods. C.M.T., I.J.M. and A.V.K. refined,

validated and executed PCR methods. J.M. and L.L.S. consulted in the development and validation of methods. D.D.P., L.E.D., L.L.S., R.L. and D.R.S. designed the clinical study. D.D.P. drafted the clinical research protocol and L.L.S., R.L. and D.R.S. reviewed and edited it. D.D.P. and D.R.S. consented study subjects and reviewed all clinical data. L.L.S. and R.L. provided essential materials. R.L. performed statistical analyses. D.D.P. and I.M. wrote the paper. D.D.P., L.L.S. and D.R.S. had primary responsibility for the final content. All authors provided content and/or critical revision of the manuscript and approved the final version.

Conflict of Interest

L.L.S., J.M. and R.L. are employees of Pfizer and own shares in the company. D.D.P., L.E.D., C.M.T., A.V.K., I.J.M., L.A.M., C.B.G., B.G. and D.R.S. received grant support from Pfizer.

References

- Ahlroos, T. and Tynkkynen, S. (2009) Quantitative strain-specific detection of *Lactobacillus rhamnosus* GG in human faecal samples by real-time PCR. *J Appl Microbiol* **106**, 506–514.
- Bonaparte, C. and Reuter, G. (1997) Bifidobacteria in commercial dairy products: which species are used? *Microecol Ther* **28**, 181–197.
- di Cerbo, A., Palmieri, B., Aponte, M., Morales-Medina, J.C. and Iannitti, T. (2016) Mechanisms and therapeutic effectiveness of lactobacilli. *J Clin Pathol* **69**, 187–203.
- Chau, K., Lau, E., Greenberg, S., Jacobson, S., Yazdani-Brojeni, P., Verma, N. and Koren, G. (2015) Probiotics for infantile colic: a randomized, double-blind, placebo-controlled trial investigating *Lactobacillus reuteri* DSM 17938. *J Pediatr* **166**, 74–78.
- Davidson, L.E., Fiorino, A.M., Snyderman, D.R. and Hibberd, P.L. (2011) Lactobacillus GG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. *Eur J Clin Nutr* **65**, 501–507.
- Desfosses-Foucalt, E., Dussault-Lepage, V., le Boucher, C., Savard, P., Lapointe, G. and Roy, D. (2012) Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification. *Front Microbiol* **3**, Article 350, 1–11.
- Dommels, Y.E., Kemperman, R.A., Zebregs, Y.E., Draaisma, R.B., Jol, A., Wolvers, D.A., Vaughan, E.E. and Albers, R. (2009) Survival of *Lactobacillus reuteri* DSM 17938 and *Lactobacillus rhamnosus* GG in the human gastrointestinal tract with daily consumption of a low-fat probiotic spread. *Appl Environ Microbiol* **75**, 6198–6204.
- Ellis, R. and Wb, S. (1958) Isolation of antibiotic-resistant lactobacilli. *J Bacteriol* **75**, 272–277.

- Ferraris, L., Aires, J., Waligora-Dupriet, A.J. and Butel, M.J. (2010) New selective medium for selection of bifidobacteria from human feces. *Anaerobe* **16**, 469–471.
- Foolad, N., Brezinski, E.A., Chase, E.P. and Armstrong, A.W. (2013) Effect of nutrient supplementation on atopic dermatitis in children: a systematic review of probiotics, prebiotics, formula, and fatty acids. *JAMA Dermatol* **149**, 350–355.
- Francavilla, R., Miniello, V., Magista, A.M., de Canio, A., Bucci, N., Gagliardi, F., Lionetti, E., Castellana, S. *et al.* (2010) A randomized controlled trial of *Lactobacillus* GG in children with functional abdominal pain. *Pediatrics* **126**, e1445–e1452.
- Goldin, B.R., Gorbach, S.L., Saxelin, M., Barakat, S., Gualtieri, L. and Salminen, S. (1992) Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Dig Dis Sci* **37**, 121–128.
- Granata, M., Brandi, G., Borsari, A., Gasbarri, R. and Gioia, D.D. (2013) Synbiotic yogurt consumption by healthy adults and the elderly: the fate of bifidobacteria and LGG probiotic strain. *Int J Food Sci Nutr* **64**, 162–168.
- Hamilton-Miller, J.M., Shah, S. and Winkler, J.T. (1999) Public health issues arising from microbiological and labelling quality of foods and supplements containing probiotic microorganisms. *Public Health Nutr* **2**, 223–229.
- Hauser, G., Salkic, N., Vukelic, K., Jajacknez, A. and Stimac, D. (2015) Probiotics for standard triple *Helicobacter pylori* eradication: a randomized, double-blind, placebo-controlled trial. *Medicine* **94**, e685.
- Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B. *et al.* (2014) Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**, 506–514.
- Hojasak, I., Abdovic, S., Szajewska, H., Milosevic, M., Krznaric, Z. and Kolacek, S. (2010) *Lactobacillus* GG in the prevention of nosocomial gastrointestinal and respiratory tract infections. *Pediatrics* **125**, e1171–e1177.
- Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandstrom, B., Tvede, M. *et al.* (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Appl Environ Microbiol* **65**, 4949–4956.
- Javier, A., Malo, J. and Barrington, K.J. (2014) Cohort study of probiotics in a North American neonatal intensive care unit. [Erratum appears in J Pediatr. 2014 Jun; 164 (6):1503]. *J Pediatr* **164**, 980–985.
- Kekkonen, R.A., Lummela, N., Karjalainen, H., Latvala, S., Tynkkynen, S., Jarvenpaa, S., Kautiainen, H., Julkunen, I. *et al.* (2008) Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World J Gastroenterol* **14**, 2029–2036.
- Kwon, H.S., Yang, E.H., Lee, S.H., Yeon, S.W., Kang, B.H. and Kim, T.Y. (2005) Rapid identification of potentially probiotic *Bifidobacterium* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol Lett* **250**, 55–62.
- Laitinen, K., Poussa, T. and Isolauri, E. (2009) Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. *Br J Nutr* **101**, 1679–1687.
- Larsen, C.N., Nielsen, S., Kaestel, P., Brockmann, E., Bennedsen, M., Christensen, H.R., Eskesen, D.C., Jacobsen, B.L. *et al.* (2006) Dose-response study of probiotic bacteria *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus paracasei* subsp. *paracasei* CRL-341 in healthy young adults. *Eur J Clin Nutr* **60**, 1284–1293.
- Masco, L., van Hoorde, K., de Brandt, E., Swings, J. and Huys, G. (2006) Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. *J Antimicrob Chemother* **58**, 85–94.
- Matsuki, T., Watanabe, K. and Tanaka, R. (2003) Genus- and species-specific PCR primers for the detection and identification of bifidobacteria. *Curr Issues Intest Microbiol* **4**, 61–69.
- Matto, J., Malinen, E., Suihko, M.L., Alander, M., Palva, A. and Saarela, M. (2004) Genetic heterogeneity and functional properties of intestinal bifidobacteria. *J Appl Microbiol* **97**, 459–470.
- Maturin, L. and Peeler, J. (2001) *Aerobic Plate Count. Bacteriological Analytical Manual*. Silver Spring, MD: U.S. Food and Drug Administration.
- Mohan, R., Koebnick, C., Schildt, J., Schmidt, S., Mueller, M., Possner, M., Radke, M. and Blaut, M. (2006) Effects of *Bifidobacterium lactis* Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. *J Clin Microbiol* **44**, 4025–4031.
- Mohan, R., Koebnick, C., Schildt, J., Mueller, M., Radke, M. and Blaut, M. (2008) Effects of *Bifidobacterium lactis* Bb12 supplementation on body weight, fecal pH, acetate, lactate, calprotectin, and IgA in preterm infants. *Pediatr Res* **64**, 418–422.
- Morrow, L.E., Kollef, M.H. and Casale, T.B. (2010) Probiotic prophylaxis of ventilator-associated pneumonia: a blinded, randomized, controlled trial. *Am J Respir Crit Care Med* **182**, 1058–1064.
- Myllyluoma, E., Veijola, L., Ahlroos, T., Tynkkynen, S., Kankuri, E., Vapaatalo, H., Rautelin, H. and Korpela, R. (2005) Probiotic supplementation improves tolerance to *Helicobacter pylori* eradication therapy – a placebo-controlled, double-blind randomized pilot study. *Aliment Pharmacol Ther* **21**, 1263–1272.
- Myllyluoma, E., Kajander, K., Mikkola, H., Kyrönpalo, S., Rasmussen, M., Kankuri, E., Sipponen, P., Vapaatalo, H. *et al.* (2007) Probiotic intervention decreases serum

- gastrin-17 in *Helicobacter pylori* infection. *Dig Liver Dis* **39**, 516–523.
- Partty, A., Luoto, R., Kalliomaki, M., Salminen, S. and Isolauri, E. (2013) Effects of early prebiotic and probiotic supplementation on development of gut microbiota and fussing and crying in preterm infants: a randomized, double-blind, placebo-controlled trial. *J Pediatr* **163**, 1272–1277.e1–2.
- Pechar, R., Rada, V., Parafati, L., Musilova, S., Bunesova, V., Vlkova, E., Killer, J., Mrazek, J. et al. (2014) Mupirocin-mucin agar for selective enumeration of *Bifidobacterium bifidum*. *Int J Food Microbiol* **191**, 32–35.
- Rada, V. and Koc, J. (2000) The use of mupirocin for selective enumeration of bifidobacteria in fermented milk products. *Milchwissenschaft* **55**, 65–67.
- Rizzardini, G., Eskesen, D., Calder, P.C., Capetti, A., Jespersen, L. and Clerici, M. (2012) Evaluation of the immune benefits of two probiotic strains *Bifidobacterium animalis* ssp. *lactis*, BB-12 and *Lactobacillus paracasei* ssp. *paracasei*, L. casei 431 in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. *Br J Nutr* **107**, 876–884.
- Rogosa, M., Ja, M. and Rf, W. (1951) A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J Bacteriol* **62**, 132–133.
- Roy, D. (2001) Media for the isolation and enumeration of bifidobacteria in dairy products. *Int J Food Microbiol* **69**, 167–182.
- Saarela, M., Maukonen, J., von Wright, A., Vilpponen-Salmela, T., Patterson, A.J., Scott, K.P., Hamynen, H. and Matto, J. (2007) Tetracycline susceptibility of the ingested *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic/probiotic intervention. *Int J Antimicrob Agents* **29**, 271–280.
- Sabine, D.B. and Vaselekos, J. (1965) Isolation of *Lactobacillus acidophilus* from faecal material. *Nature* **206**, 960–961.
- Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., Jarvenpaa, S., Korpela, R. et al. (2010) Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *Int J Food Microbiol* **144**, 293–300.
- Smith, T.J., Rigassio-Radler, D., Denmark, R., Haley, T. and Touger-Decker, R. (2013) Effect of *Lactobacillus rhamnosus* LGG super(registered) and *Bifidobacterium animalis* ssp. *lactis* BB-12 super(registered) on health-related quality of life in college students affected by upper respiratory infections. *Br J Nutr* **109**, 1999–2007.
- Solano-Aguilar, G., Dawson, H., Restrepo, M., Andrews, K., Vinyard, B. and Urban, J.F. Jr (2008) Detection of *Bifidobacterium animalis* subsp. *lactis* (Bb12) in the intestine after feeding of sows and their piglets. *Appl Environ Microbiol* **74**, 6338–6347.
- Temmerman, R., Pot, B., Huys, G. and Swings, J. (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol* **81**, 1–10.
- Turrone, F., Ventura, M., Butto, L.F., Duranti, S., O'Toole, P.W., Motherway, M.O. and van Sinderen, D. (2014) Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. *Cell Mol Life Sci* **71**, 183–203.
- US Pharmacopeia Convention (2009) *USP 32-NF27, General Notices. 1223 Validation of alternative microbiological methods* [Online]. Available at: http://www.drugfuture.com/pharmacopoeia/usp32/pub/data/v32270/usp32nf27s0_c1223.html (accessed 28 March 2016).
- Wang, Y., Kirpich, I., Liu, Y., Ma, Z., Barve, S., McClain, C.J. and Feng, W. (2011) *Lactobacillus rhamnosus* GG treatment potentiates intestinal hypoxia-inducible factor, promotes intestinal integrity and ameliorates alcohol-induced liver injury. *Am J Pathol* **179**, 2866–2875.
- Zhang, J.W., Du, P., Gao, J., Yang, B.R., Fang, W.J. and Ying, C.M. (2012) Preoperative probiotics decrease postoperative infectious complications of colorectal cancer. *Am J Med Sci* **343**, 199–205.