Specific TaqMan® Probes for the Identification and Quantification of Lactobacilli in Pharmaceuticals

Herbel SR1,2,*, Von Nickisch-Rosenegk M3, Kuhn M4, Murugaiyan J5, Wieler LH1 and Guenther S1

1Freie Universität Berlin, Centre for Infection Medicine, Institute of Microbiology and Epizootics, Robert-von-Ostertag–Str.7-13, 14163 Berlin, Germany
2Freie Universität Berlin, Department of Biology, Chemistry, Pharmacy, Takustr. 3, 14195 Berlin, Germany
3Fraunhofer Institute, IBMT Potsdam-Golm, Am Mühlenberg 13, 14476 Potsdam, Germany
4CONGEN Biotechnologie GmbH, Robert-Rösle-Straße 10, 13125 Berlin, Germany
5Freie Universität Berlin, Centre for Infection Medicine, Institute of Animal and Environmental Hygiene, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany

Abstract

Several probiotic products containing species of the genus Lactobacillus are available on the market. Usually, these are fermented dairy products such as yoghurt and vegetables. Additionally, probiotic bacteria are used in pharmaceuticals, which are also believed to have beneficial effects on human health. Common pharmaceutical application forms to deliver probiotics are tablets, drops or granulate formulations for oral administration. They should contain sufficient numbers of viable probiotics to assure active health benefits. Despite the successful commercialization of lactobacilli, their traditional species identification methods are time-consuming and labor-intensive and do not allow quantification of the species. Therefore, the objective of the present work was to develop a culture independent, fast identification and quantification method for two commercially important species of the genus Lactobacillus (L. acidophilus and L. reuteri). We used a TaqMan® real-time PCR assay based on the GroEL heat shock protein region. Therefore, universal lactobacilli primers and species-specific TaqMan® primers have been developed. The assay allowed an unambiguous species-specific detection of L. acidophilus and L. reuteri from bacterial cultures as well as directly from tablets. Using this assay, we were able to detect lactobacilli strains to a level of 10^6 cfu/ml, which is a sufficient detection limit as commercial pharmaceuticals usually contain 10^8–10^10 cfu/tablet of probiotic strains.

Introduction

Within the last decades species of the genus Lactobacillus have been widely commercially used as they are believed to possess probiotic features and thereof resulting beneficial health effects. These strains are utilized in manufacturing fermented food from milk such as yoghurt and cheese [1]. As an example L. reuteri is producing Reuterin (3-hydroxypropionaldehyde [3-HPA]), which is water soluble, effective in a wide pH range and resistant to proteolytic and lipolytic enzymes [2,3]. Therefore, L. reuteri is used in therapeutic treatment being bioactive against bacteria, viruses and fungi [2-4].

Conclusively, these probiotic bacteria have also been used in a wide range of pharmaceuticals such as tablets, drops and granulate [1,5]. Other vehicles used are lozenges, powder, gelatin or straws [1]. As an example Klayraung et al. (2009) found that probiotic bacteria administered in liquid or semisolid formulations showed low cell viability after oral administration caused by harsh conditions in the stomach [6]. Thus, development of dry dosage forms, matrix forming excipients and attuned compression forces used for tablet preparation increases bacterial survival rates in low pH conditions [6].

The viability of the strains has to be assured for having beneficial effects on the host according the FAO/WHO guidelines (“live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”) [7,8]. However, these guidelines focused food; concluding biotherapeutic agents [9]. Nevertheless, the basic principles of this definition also apply for probiotic pharmaceuticals [10].

The adequate number of probiotic bacteria having health beneficial effects has to be accurately assessed, when used in food or pharmaceuticals [11]. As an example, the amount of probiotic bacterial cells needed to induce immune defensive benefits, reduction of cholesterol levels and preventing diarrhea and food allergies ranges between 10^6 and 10^8 cfu/ml [7,12,13].

Since the usage of lactobacilli is increasing steadily in medical treatment, two examples of commercially available probiotic species were included in the TaqMan® labeled real-time PCR assay: Reulfor® chewable tablets (Italchimici, Pomezia, Italy) containing approximately 10^8 cfu/tablet of viable L. reuteri DSM 17938 [14] and Milchsäure-Kulturen Bifidoflor-Kapseln® (dm-drogeriemarkt, Karlsruhe, Germany) containing Bifidobacterium animalis subsp. lactis BB-12® and L. acidophilus – both in an amount of 2.0x10^9 cfu/capsule.

Therefore, a rapid identification tool would be useful to identify and quantify probiotic bacteria in different products. As conventional PCR or MALDI-TOF MS lack the capacity to quantify bacterial loads in products, real-time PCR based approaches such as TaqMan® probes are necessary.

Materials and Methods

Lactobacilli strains and isolates

In total, 77 different strains of the genera Lactobacillus (L.) and Bifidobacterium (B.) were used as positive and negative controls to confirm primer specificity (primer pairs: Table 2). In addition, species

*Corresponding author: Herbel SR, Freie Universität Berlin, Centre for Infection Medicine, Institute of Microbiology and Epizootics, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany, Tel: 00493083851901; E-mail: Stefan.Herbel@fu-berlin.de
Received December 20, 2013; Accepted January 28, 2014; Published February 04, 2014

Citation: Herbel SR, Von Nickisch-Rosenegk MV, Kuhn M, Murugaiyan J, Wieler LH, et al. (2014) Specific TaqMan® Probes for the Identification and Quantification of Lactobacilli in Pharmaceuticals. J Prob Health 2: 115. doi: 10.4172/2329-8901.1000115

Copyright: © 2014 Herbel SR, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
of the genera *Lactobacillus* and *Streptococcus* were also chosen for comparison as they are often used as probiotics. A complete list of all tested species is published in Herbel et al. (2013) [15].

Reuflor® tablets (Italchimici, Pomezia, Italy) were serially diluted from 10^8 to 10^1 using 0.9 % sodium chloride (Roth, Karlsruhe, Germany).

**Table 1:** Reference *Lactobacillus* strains and field isolates obtained from tablets in this study.

| Species                          | Designation          | Origin                                      |
|---------------------------------|----------------------|---------------------------------------------|
| *Lactobacillus acidophilus*      | LA-11, DGCC 9355, IMT 22354 | Danisco, Kantvik, Finland                   |
| *Lactobacillus Acidophilus*      | IMT 30280            | Proviact® yoghurt                           |
| *Lactobacillus reuteri*          | K1 D287 Botazzi v. 2/91, IMT 21493 | Institute of Meat Hygiene and Technology, Freie Universität Berlin, Germany |

**Sample isolates**

- *Lactobacillus acidophilus* IMT 32362 dm Milchsäure-Kulturen Bifido-Flor® capsules
- *Lactobacillus reuteri* DSM 17938, IMT 32017 Reuflor® chewable tablets

**Table 2:** *Lactobacillus* species-specific primers based on GroEL gene.

| Species               | Primera,b | Target | Sequence (5' to 3') Accession-No.b | qPCR annealing temperature and time | Size of Amplicon (bp) | Species-specific amplification, detection limit |
|-----------------------|-----------|--------|-----------------------------------|-----------------------------------|----------------------|------------------------------------------------|
| *Lactobacillus acidophilus* | UniLactoHsp60FOR1, UniLactoHsp60REV1 | GroEL | ATGGAAAAGGTGGCCGCA [220-236] | - | 53° C, 30 s | 124 | - |
| *Lactobacillus acidophilus* | LAcidophTaq3FOR | GroEL | DMYCTGAGGATCTAGGTTGACAT[254-278] Dabcyl® | n.t. | - | √ | >10^4 |
| *Lactobacillus reuteri* | LRACm02F, LRACm03R | GroEL | ATGGAAAAGGTGGCCGCA [220-236] | - | 53° C, 30 s | 124 | - |

*FOR, Forward primer, REV, Reverse primer
*Accession-No., NCB-Accession-No. in NCBI database
*GroEL, heat shock protein
*FAM, 6-carboxyfluorescein reporter
*Dabcyl, Dabcyl quencher
√, species-specific amplification was possible using this primer in combination with universal primer set
n.t., not tested

**Confirmation of species identification**

Reference strains and strains isolated from Reuflor® tablets and Milchsäure-Kulturen Bifido-Flor Kapseln® are listed in Table 2. In parallel to real-time PCR assay classical microbiological methods had been used to assure species identity [18]. In addition, species identification was confirmed by MALDI-TOF MS measurements carried out using a Microflex LT instrument (Bruker Daltonics, Bremen, Germany). The real-time classification tool of the Biotyper 3.0 software tool (Bruker Daltonics, Bremen, Germany) was utilized for culture-independent species identification [19]. Additionally, all amplicons processed by real-time assay utilizing universal primer pairs were fully sequenced (LGC Genomics, Berlin, Germany) and compared to public available sequence databases (Supplement 1).

**DNA extraction from pure *lactobacilli* cultures and tablets**

DNA extraction from pure cultures was performed as described by Herbel et al. (2013) [15,20]. The protocol for tablets followed the one given for yoghurt. In brief, tablets were dissolved in 500 μl 0.4 M sodium hydroxide (Roth, Karlsruhe, Germany) and 150 μl of 40 % tri-sodium citrate di-hydrate (Roth, Karlsruhe, Germany). The mixture was carefully shaken, incubated for 15 min at room temperature and centrifuged for 2 min (15,000 x g). Following, supernatant was removed, the pellet was washed and treated with mutanolysin (Roth, Karlsruhe, Germany) as described by Lick et al. (1996) [21]. In addition, sonication was done using UP100H Hielscher Ultrasound Technology (Teltow, Germany) followed by DNA isolation as described in Herbel et al. (2013) [15].

**Primer design**

The heat shock protein region GroEL (synonyms hsp60, cpn60, groL) is a single copy target gene in the genome of all *lactobacilli* [22-24]. The principal usability of this region as a target for species identification has been already shown in several publications [15,24,25]. Partial sequences of this region were retrieved from the NCBI database. In addition, the heat shock protein gene regions of all reference strains and the *lactobacilli* isolated from products were sequenced (Table 1). Sequence alignments were performed by Megalign® alignment suite (Lasergene DNA Star, Madison, Wisconsin, USA) applying the ClustalW algorithm [26]. The alignments were used to design lactobacilli universal primer pairs and to identify species-specific primers within the universal primer pair region. Furthermore, all designed primers were screened for biophysical similarities and dimer formations using BLAST algorithm and Oligoanalyzer 3.0 software (Integrated DNA Technologies, Coralville, Iowa, USA) [27]. Ensuring their specificity for the GroEL region of the mentioned species, all primers were verified using the BLAST algorithm of the NCBI database [27-29].
The predicted amplicon size for the universal primer pair was 124 bp and each primer set was evaluated by melt curve analysis on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Darmstadt, Germany) using Power SYBR® Green (Applied Biosystems, Darmstadt, Germany) (data not shown). The primers listed in Table 2 showed the best specificity of all evaluated oligonucleotides of this study and were therefore used.

Quantitative Real-time PCR

All universal primer pairs and specific TaqMan® probes developed during this study were tested with DNA samples (20 ng/µl) of the genera Lactobacillus, Bifidobacterium, Lactococcus and Streptococcus, which were used as positive and negative controls.

Real-time PCR was performed using a Light Cycler® 480 (Roche, Mannheim, Germany) based on TaqMan® detection. Each singleplex real-time PCR sample contained 5 µl of DNA template, 10 µl Taq polymerase, 1 µl of each universal primer (10 µmol, Thermo Scientific, Dreieich, Germany), 1 µl of TaqMan® labeled specific primer (10 µmol, each 6-FAM®/Dabcyl-labeled, TIB MOLBIOL, Berlin, Germany) and 2 µl of PCR grade water (Roche, Mannheim, Germany). A single initial denaturation step of 10 min at 95°C was followed by 40 cycles of 95°C for 1 min (denaturation), 53°C for 30 s (annealing) and 73°C for 30 s (elongation). The fluorescence signal was measured at the end of each 73°C elongation step.

Each real-time PCR included two technical repeats and the results were analyzed by using the Roche® Light Cycler 480® software.

Determination of the real-time PCR detection limit using pure lactobacilli DNA

To assess the sensitivity of the assay and to compare real-time PCR results to Colony Forming Units (CFU) of bacteria obtained by plating, DNA was isolated from serial dilutions of lactobacilli, which were plated on agar, respectively. Therefore, liquid cultures (three technical repeats) of each strain utilizing 0.9% sodium chloride solution (Roth, Karlsruhe, Germany) were set to a concentration of 10⁹ cfu/ml using 0.5 McFarland Standard in a Sensititre® Inoculator (Thermo Scientific, Dreieich, Germany) and diluted from 10⁹ cfu/ml to 10¹² cfu/ml. 200 µl of each dilution were plated on MRS, LBS, COL and CHOC agar plates (origin as described above) and incubated for 24 h to 48 h (37°C; 5% CO₂). Colonies were counted and used for extrapolating the cfu/ml.

One milliliter of each serial dilution of the strains was used for parallel DNA isolation using a protocol published by Herbel et al. (2013) [15,20,22].

The theoretical number of genome equivalents (GE) was calculated based on the DNA isolated from 10⁹ cfu/ml L. reuteri (160 ng/µl) and the published genome size of L. reuteri (1,969,869 bp) [30].

Results

Species-specific amplification of DNA isolated from lactobacilli

By using the BLAST algorithm we were able to confirm that the primer sequences solely targeted the genome of the lactobacilli species of interest. No other genes were found which showed a comparable DNA sequence to the used primers, which could have led to false positives.

First, the universal primer set was evaluated using DNA from reference strains and the strains isolated from tablets. A set of two diverse forward and eight reverse universal primers had been tested. Finally, the universal primer set of UniLactoHsp60FOR1 and UniLactoHsp60REV1 had been chosen, because it was amplifying the highest number of different species belonging to the genus Lactobacillus (L. acidophilus, L. brevis, L. helveticus and L. reuteri) (Table 2). Following, species-specific TaqMan® probes targeting regions within the universal primer amplicon region were established. Evaluation of the specific TaqMan® probes revealed no amplification by using the DNA of the wide range of negative controls. However, the TaqMan® probes were able to specifically amplify positive control DNA and therefore detect the species L. acidophilus and L. reuteri in a singleplex real-time PCR run. Unfortunately, we were not able to perform a duplex detection assay of both species utilizing the same universal primers and specific TaqMan® probes.

In parallel, we always confirmed these results with an established SYBR® Green real-time PCR including melting curve analysis. As shown in supplement 2 TaqMan® and the SYBR® Green real-time PCR using L. reuteri standard curves and DNA isolated from two batches of Reuflor® tablets (10⁹ or 10¹² cfu/tablet) showed comparable results. Besides melting curve analysis standard curves had been generated for all shown real-time PCRs (Figure 1, Supplement 2). Additionally to assure the real-time PCR results L. reuteri had been isolated from tablet material and was confirmed by MALDI-TOF MS analysis (data not shown). Furthermore, the amplicon of the universal primer pair has been sequenced resulting in a 124 bp long sequence, which confirmed L. reuteri (Supplement 1). In this study, the C value the C values of each real-time PCR run in this study were ranging between 10 to 32 threshold cycles and declared as unspecific signals if they started from the 34th cycle onwards. Thus, analysis of C value allowed to distinguish species-specific identification of the tested lactobacilli species. So summing up to test the specificity of the assay, we tested DNA isolated from pure cultures as well as from probiotic Reuflor® tablets containing L. reuteri. We were able to identify L. reuteri within the DNA obtained from cultures and tablets using our assay in a singleplex real-time TaqMan® PCR approach (Figure 1).

Detection limit, identification and quantification of Lactobacillus isolates from tablets

Using standard curves of DNA of known concentrations and correlate these results to the colony forming unit counting in the parallel plating experiments we observed a detection limit of 10⁴ cfu/ml for our TaqMan® approach and SYBR® Green assay (Figure 1).

The calculated correlation coefficient of TaqMan® real-time PCR of DNA isolated from Reuflor® tablets (10⁹ cfu/tablet) was R² = 0.99458 and its equation of the linear regression line is y = -3.6571x + 35.467 which is comparable to the SYBR® Green real-time PCR (R² = 0.99576; y = -4.7429x + 38.993) (Supplement 2). The theoretical number of genome equivalents (GE) was calculated to be 7.23x10⁴ GE/µl for 10⁹ cfu/ml pure culture and 8.23x10⁴ GE/µl for the DNA isolated from Reuflor® tablets (10¹² cfu/tablet, batch number: 2TSA122, Figure 1). We also used this standard curves to estimate the number of bacteria present in a tablet. By correlating of the amplification curves of DNA extracts from Reuflor® tablets, we quantified the number of L. reuteri at 10⁴ cfu/tablet (batch number: 2TSA122, Figure 1). The parallel plating and counting of serial dilutions derived from tablets of the same batch revealed that viable cell number of L. reuteri was 6.34x10⁵ cfu/tablet. However, in another run using a different batch of tablets (2TSA156)
we detected 10^8 cfu/tablet (batch number: 2TSA156, both data not shown). As Reuflor® tablets are designated to contain 10^8 cfu/tablet of L. reuteri both batches seem to carry an adequate number of probiotic bacteria. In addition, one package of Reuflor® tablets (batch number: ITSA051) was stored at ambient temperature for a duration of two years, which was still within the expiring date of the product, when DNA had been isolated. Using our real-time PCR assay based on DNA isolated from the stored tablets, we quantified the number of bacteria in the stored tablet to 10^6 cfu/tablet only (data not shown).

Discussion
Herein we describe a singleplex TaqMan® labeled real-time PCR approach for the rapid and culture independent detection and quantification of probiotic lactobacilli directly from tablets. The heat shock protein GroEL has been demonstrated being a suitable target region for DNA based species identification [15,24]. Additionally, we used this region for establishing a TaqMan® real-time PCR system and it turned out to be highly specific in our assay as well as we obtained no false positive results while evaluating a wide spectrum of reference strains. The species-specificity of the assay was evaluated in parallel based on classic culture methods and culture independent using MALDI-TOF MS.

One of the advantages of this assay is its rapidity, allowing a species-specific identification of lactobacilli species within 7 hours without any
prior cultivation step. The detection limit measured by SYBR Green and TaqMan real-time PCR assay was at a level of 10^4 cfu/ml, which is adequate to quantify strains used in tablets, capsules or granulate formulations containing 10^5 to 10^6 cfu per dosage in order to exert probiotic activity [7].

In case of the Reuflor tablets the stated number of bacteria in the package insert was 10^8 cfu/tablet, however, by evaluating tablets from two different batches we detected a cell number between 10^6 and 10^7 cfu/tablet actually. However, in case of probiotics strains this does not seem to be problematic as health beneficial effect for the consumer could be expected (10^6 to 10^7) [7,12]. The actual number of cells in a batch of tablets might differ due to manufacturing reasons causing a cell loss by freeze-drying or incorporating the cells in tablet matrix. Additionally, DNA extraction method used in our assay might contribute to these findings as the calculated gene equivalents DNA from the tablets (8.23x10^7 GE) was lower than the actual colony forming units we obtained by plating (6.34x10^7 cfu/ml). In contrast to that for pure cultures the numbers fitted well (7.23x10^7 GE vs 1.47x10^8 cfu/ml).

As we detected more or less comparable numbers of cells culture depended (plating) and culture independent (real-time PCR) this point toward a high viability of the lactobacilli in the tablets. In case of high rates of dead bacteria in the tablets the numbers detected in the PCR assay should have been higher than the results from plating and counting colony-forming units.

The evaluation of tablets that have been stored for two years at room temperature led to the detection of 10^6 cfu/tablet in total, however, as we detected more or less comparable numbers of cells culture depended (plating) and culture independent (real-time PCR) this point toward a high viability of the lactobacilli in the tablets. In case of high rates of dead bacteria in the tablets the numbers detected in the PCR assay should have been higher than the results from plating and counting colony-forming units.

In conclusion, our TaqMan® labeled real-time PCR system was useful for the detection of L. acidophilus and L. reuteri with high specificity and sensitivity directly in tablets without prior cultivation. This, in contrast to other methods such as physiological or morphology testing, contributes to the rapidity of the assay by working culture-independent. Additionally, besides an identification real-time PCR also allows a proper quantification of Lactobacillus sp. directly from tablets within seven hours. Therefore, this TaqMan® real-time PCR assay could be a useful tool for the detection of Lactobacillus sp. strains in drugs and food for regulation and quality management purposes.

Acknowledgments

This project was financed by the ZIM fond (Zentrals Innovationsprogramm Mittelstand, K2267401MD9) of the Federal Ministry of Economics and Technology of the Federal Republic of Germany in cooperation with the IBMT Fraunhofer Institute (Potsdam-Golm, Germany) and the CONGEN Biotechnologie GmbH (Berlin-Buch, Germany).

The project was also supported by the SFB 852 (grant no. SFB852/1).

Special thanks to Dr. Ouwenhart (Danisco, Finland), Prof. Dr. Schmidt (Universität Hohenheim, Germany) and Prof. emer. Dr. Dr. h.c. Reuter (Freie Universität Berlin, Germany), Dr. Loh (Deutsches Institut für Ernährungsforschung, Germany), Dr. Vahjen (Freie Universität Berlin, Germany) and Prof. Dr. Schillinger (Max-Rubner-Institut, Germany) for providing different probiotic strains (Table 1). Thanks to Mr. Offert Landt, executive director of TIB MOLBIOL, Berlin, Germany for providing the used TaqMan® labeled primer for L. reuteri.

References

1. Caglar E, Ciltir SK, Ergeneли S, Sandall N, Twetman S (2006) Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium Lactobacillus reuteri ATCC 55730 by straws or tablets. Acta Odontol Scand 64: 314-318.
2. Cleusis V, Lacroix C, Vollenweider S, Duboux M, Le Blay G (2007) Inhibitory activity spectrum of reuterin produced by Lactobacillus reuteri against intestinal bacteria. BMC Microbiol 7: 101.
3. Schaefer L, Autschlag TA, Hermens KE, Whitehead D, Borhan B, et al. (2010) The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. Microbiology 156: 1589-1599.
4. Langa, S., et al., In situ reuterin production by Lactobacillus reuteri in dairy products. Food Control, 2013. 33: p. 200-206.
5. Kaur IP, Chopra K, Saini A (2002) Probiotics: potential pharmaceutical applications. Eur J Pharm Sci 15: 1-9.
6. Klaryaaung S, Vienriewhten H, Okonogi S (2009) Development of tablets containing probiotics: Effects of formulation and processing parameters on bacterial viability. Int J Pharm 370: 54-62.
7. G. Silva JP, Sousa SC, Colb P, Cerdeira E, Amaral MH, et al. (2013) Development of probiotic tablets using microfluidics: viability studies and stability studies. AAPS PharmSciTech 14: 121-127.
8. Reid G, Bocking A (2003) The potential for probiotics to prevent bacterial vaginosis and preterm labor. Am J Obstet Gynecol 189: 1202-1208.
9. Morelli L (2013) Probiotics: Definition and Taxonomy 10 Years After the FAO/WHO Guidelines, in Probiotic Bacteria and Their Effect on Human Health and Well-Being. A. Guarino, E.M.M. Quigley, and W.A. Walker, Editors. Karger: Basel. 1-6.
10. N.N (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, a joint FAO/WHO expert consultation, WHO, Editor, FAO/WHO Working Group: Cordoba, Argentina.
11. Karapetasia A, Vavoulidisa E, Galanis A, Sandzalidzopoulous R, Kourkoutas Y (2010) Rapid detection and identification of probiotic Lactobacillus casei ATCC 393 by multiplex PCR. J Mol Microbiol Biotechnol 18: 156-161.
12. Shah NP (2000) Probiotic bacteria: selective enumeration and survival in dairy foods. J Dairy Sci 83: 894-907.
13. Robinson RK (1987) Survival of Lactobacillus acidophilus in fermented products. Suid Afrikaanse Tydskrif Vir Suikerkunde 19: 25-27.
14. Maranielli C, Cifani N, Pasqual P (2010) Evaluation of antimicrobial activity of probiotic bacteria against Salmonella enterica subsp. enterica serovar typhimurium 1344 in a common medium under different environmental conditions. Research in Microbiology, 161: 673-680.
15. Herbel SR, Lauztz B, von Nickisch-Rosenegk M, Kuhn M, Murugaiyan J, et al. (2013) Species-specific quantification of probiotic lactobacilli in yoghurt by quantitative real-time PCR. J Appl Microbiol 115: 1402-1410.
16. Danner H, Holzer M, Maynhuber E, Braun R (2003) Acetic acid increases stability of silage under aerobic conditions. Appl Environ Microbiol 69: 562-567.
17. Song Y, Kato N, Liu C, Matsumiya Y, Kato H, et al. (2000) Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol Lett 187: 167-173.
18. Xiong T, Song S, Huang X, Feng C, Liu G, et al. (2013) Screening and identification of functional Lactobacillus specific for vegetable fermentation. J Food Sci 78: M84-89.
19. Murugaiyan J, Artholdt J, Kowbel V, Roesler U (2012) Establishment of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry database for rapid identification of infectious achlorophyllous green micro-
algae of the genus Prototheca. Clinical Microbiology and Infection 18: 461-467.

20. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, et al. (2000) Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl Environ Microbiol 66: 297-303.

21. Lick S, Keller M, Bockelmann W, Heier KJ (1996) Optimized DNA extraction method for starter cultures from yoghurt. Milchwissenschaft-Milk Science International 51: 183-186.

22. Claesson MJ, van Sinderen D, O'Toole PW (2008) Lactobacillus phylogenomics—towards a reclassification of the genus. Int J Syst Evol Microbiol 58: 2945-2954.

23. Hill JE, Penny SL, Crowell KG, Goh SH, Hemmingsen SM (2004) cpnDB: a chaperonin sequence database. Genome Res 14: 1669-1675.

24. Biaiotta, G., et al., Lactobacillus strain diversity based on partial hsp60 gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. Applied and Environmental Microbiology, 2008. 74: 208-215.

25. Jie Y, Zhihong S, Wenjun L, Qiuhua B, Jiachao Z, et al. (2012) Phylogenetic study of Lactobacillus acidophilus group, L. casei group and L. plantarum group based on partial hsp60, phES and tuf gene sequences. European Food Research and Technology 234: 927-934.

26. Higgins DG, Thompson JD, Gibson TJ (1996) CLUSTAL: a multiple sequence alignments. Methods Enzymol 266: 383-402.

27. McGinnis S, Madden TL (2004) BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32: W20-25.

28. Bressanini D, Stefan A, Piaz FD, Cianchetta S, Reggiani L, et al. (2009) Proteolysis of the proofreading subunit controls the assembly of Escherichia coli DNA polymerase III catalytic core. Biochim Biophys Acta 1794: 1606-1615.

29. Junick J, Blaut M (2012) Quantification of human fecal bifidobacterium species by use of quantitative real-time PCR analysis targeting the groEL gene. Appl Environ Microbiol 78: 2813-2822.

30. Heavens D, Tailford LE, Crossman L, Jeffers F, Mackenzie DA, et al. (2011) Genome sequence of the vertebrate gut symbiont Lactobacillus reuteri ATCC 53608. J Bacteriol 193: 4015-4016.

31. Chávarri M, Marañón I, Ares R, Ibáñez FC, Marzo F, et al. (2010) Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int J Food Microbiol 142: 185-189.

32. Liu F, Wen K, Li G, Yang X, Kocher J, et al. (2014) Dual Functions of Lactobacillus acidophilus NCFM as Protection Against Rotavirus Diarrhea. J Pediatr Gastroenterol Nutr 58: 2613-2622.

33. Wen K, Li G, Bui T, Liu F, Li Y, et al. (2012) High dose and low dose Lactobacillus acidophilus exerted differential immune modulating effects on T cell immune responses induced by an oral human rotavirus vaccine in gnotobiotic pigs. Vaccine 30: 1198-1207.

34. Westin L, Xu X, Miller C, Wang L, Edman CF, et al. (2000) Anchored multiplex amplification on a microelectronic chip array. Nat Biotechnol 18: 199-204.