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

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Abstract

Several probiotic products containing species of the genus *Lactobacillus* are available on the market. Usually, these are fermented dairy products such as yoghurts 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 *Gro*EL 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 10^4 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: Reuflor® chewable tablets (Italchimici, Pomezia, Italy) containing approximately 10^8 cfu/tablet of viable *L. reuteri* DSM 17938 [14] and Milchsäure-Kulturen Bifido-Flor Kapseln® (dm-drogeriemarkt, Karlsruhe, Germany) containing *Bifidobacterium animalis* subsp. *lactis* BB-12® and *L. acidophilus* – both in an amount of 2.0×10^6 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

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Species	Designation	Origin
Lactobacillus acidophilus	LA-11, DGCC 9353, IMT 22354	Danisco, Kantvik, Finland
Lactobacillus acidophilus	IMT 30260	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

Abbreviations
IMT: Institut für Mikrobiologie & Tierseuchen strain collection number
DSM: Deutsche Sammlung von Mikroorganismen strain collection number

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

Species	Primer[a]	Target	Sequence (5' to 3')	Accession-No.[b]	qPCR annealing temperature and time	Size of Amplicon (bp)	Species-specific amplification, detection limit
Universal primerpair	UniLactoHsp60FOR1 UniLactoHsp60REV1	*Gro*EL[c] *Gro*EL	ATGGAAAAGGTTGGCCA [220-236] TCAGTTACCATGTATTGTGACA [323-344]	- -	53° C, 30 s	124	-
L. acidophilus	LAcidophTaq3FOR	*Gro*EL	FAM[d]TCGAAGATTCACGTGGTATCAATAC[254-278] Dabcyl[e]	AY424311	-	-	√[f], n.t.[g]
L. reuteri	LReuteriTaq1FOR	*Gro*EL	FAMCTCGTGGTGTTGACACTAGCG [263-283]Dabcyl	AF429714	-	-	√, >10⁴

[a]FOR, Forward primer, REV, Reverse primer
[b]Accession-No., NCBI-Accession-No. in NCBI database
[c]*Gro*EL, heat shock protein
[d]FAM, 6-carboxyfluorescein reporter
[e]Dabcyl, Dabcyl quencher
[f]√, species-specific amplification was possible using this primer in combination with universal primer set
[g]n.t., not tested

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

of the genera *Lactococcus* 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). 200 µl of each serial dilution was plated on MRS broth (Roth, Karlsruhe, Germany [16]), LBS agar (BD, Heidelberg, Germany [17]), COL and CHOC plates (Sarstedt, Nümbrecht, Germany). After incubation for 48 h (37° C, 5 % CO_2) colonies were sub-cultured and species identification was performed as described below. The same procedure had been used to isolate *L. acidophilus* used in Milchsäure-Kulturen Bifido-Flor Kapseln® (dm-drogerie markt, Karlsruhe Germany).

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 databasis (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 *Gro*EL (synonymes *hsp*60, *cpn*60, *gro*L) 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 *Gro*EL 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 melting 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 10^8 cfu/ml using 0.5 McFarland Standard in a Sensititre® Inoculator (Thermo Scientific, Dreieich, Germany) and diluted from 10^8 cfu/ml to 10^3 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 % CO_2). 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 10^8 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 (10^6 or 10^9 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_t value the C_t 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_t value allowed a distinct 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 10^4 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 (10^9 cfu/tablet) was $R^2 = 0{,}99458$ and its equation of the linear regression line is $y = -3.6571x + 35.467$ which is comparable to the SYBR® Green real-time PCR ($R^2 = 0.99576$; $y = -4.7429x + 38.993$) (Supplement 2). The theoretical number of genome equivalents (GE) was calculated to be $7.23x10^7$ GE/µl for 10^8 cfu/ml pure culture and $8.23x10^7$ GE/µl for the DNA isolated from Reuflor® tablets (10^9 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 10^9cfu/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 $6.34x10^9$cfu/tablet. However, in another run using a different batch of tablets (2TSA156)

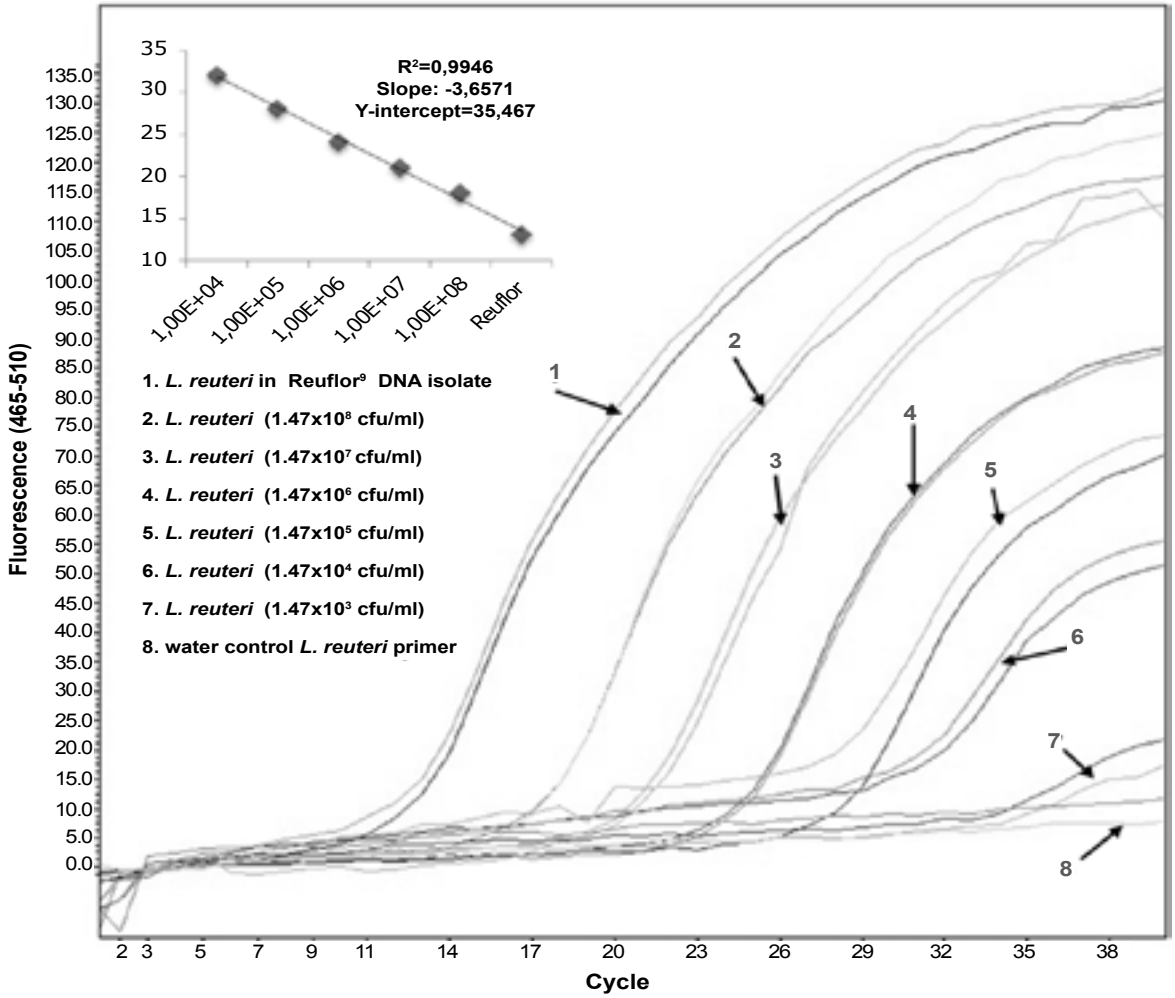

Figure 1: Quantification of *L. reuteri* in DNA extracted from Reuflor® tablets using TaqMan® labeled real-time PCR assay. Amplification curves of *L. reuteri* reference strains's serial dilution DNA isolates, DNA isolate from a tablet and negative controls containing universal primers and *L. reuteri* TaqMan® labeled primer.
1. *L. reuteri* detected in a Reuflor® tablet DNA isolate,
2. *L. reuteri* (IMT 21493, 1.47 x 10^8 cfu/ml),
3. *L. reuteri* (IMT 21493, 1.47 x 10^7 cfu/ml),
4. *L. reuteri* (IMT 21493, 1.47 x 10^6 cfu/ml),
5. *L. reuteri* (IMT 21493, 1.47 x 10^5 cfu/ml),
6. *L. reuteri* (IMT 21493, 1.47 x 10^4 cfu/ml),
7. *L. reuteri* (IMT 21493, 1.47 x 10^3 cfu/ml),
8. water control containing universal primers and *L. reuteri* TaqMan® labeled primer.
Experiments were repeated with three biological and two technical repeats.

we detected 10^8 cfu/tablet (batch number: 2TSA156, both data not shown). As Reuflor® tablets are designated to contain 10^8cfu/tablet of *L. reuteri* both batches seem to carry an adequate number of probiotic bacteria. In addition, one package of Reuflor® tablets (batch number: 1TSA051) 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 *Gro*EL 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 10^6 to 10^{10} 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 10^8 and 10^9 cfu/tablet actually. However, in case of probiotic strains this does not seem to be problematic as health beneficial effect for the consumer could be expected (10^6 to 10^8) [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 ($6.34x10^9$ 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, according to the package insert there should be 10^8 cfu/tablet. There are several possible reasons for this low number of probiotic bacteria such as a loss or varying numbers of *L. reuteri* cells during lyophilization or the long storage time including DNA extraction within the expiration date of the product [31]. It seems rather likely that this lower number of bacteria was already present when the tablets were manufactured. A loss of viable bacteria due to the long storage time seems to be unlikely as bacterial DNA is relatively stable under dry conditions such as in a tablet and should have been detected by real-time PCR as well. Indeed a detected amount of 10^6 cfu/tablet is of high importance as recent studies have shown that differing doses of probiotic cells have different effects in the host [32,33]. Thus, our TaqMan® probe based real-time PCR was highly specific and sensitive in the singleplex assay we had difficulties in establishing a multiplex real-time PCR approach. This might have been caused by primer-primer interactions [35].

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 rapidness 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.

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