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QUANTIFICATION OF *LACTOBACILLUS HELVETICUS* IN A MIXTURE OF LACTIC ACID BACTERIA USING qPCR IN CHEESE

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Conventional quantification of *L. helveticus*, in presence of other lactobacilli species, using classical plate method employing low selective media is very inaccurate. Determination of *L. helveticus* using quantitative PCR (qPCR) was performed in six artisanal Kazakh soft cheeses made from cow's milk or from a mixture of cow's and goat's milk. *L. helveticus* was quantified by species-specific qPCR, monitoring the presence of genes encoding for peptidoglycan hydrolases. Quantification of *L. helveticus* based on qPCR ranged from 2.6×10^6 to 4.1×10^8 CFU·g^{-1} according to the type of the cheese. The microflora of cheese consisted of a mixture of starter and non-starter lactic acid bacteria.

Keywords: *L. helveticus*, cheese, qPCR, peptidoglycan hydrolases

National Kazakh cheese is an acid-rennet curd cheese ripened over a short period of time containing *L. helveticus*, a thermophilic lactic acid bacterium used traditionally for production of Gruyère, Emmental, Grana, and Parmesan cheeses (Beresford et al., 2001). This variety was first produced with a mixture of goat's milk, and currently is made from cow's milk. *L. helveticus* can be determined using culture-dependent or culture-independent methods. Culture-dependant methods are generally labour intensive, time consuming, and more or less specific of a microbial group (Achiellos & Berthiere, 2013). Only strains able to grow under the defined environmental conditions can be monitored, apart from viable but non-cultivable cells. Moreover, *L. helveticus* occurs in cheese in the presence of other lactobacilli; therefore, its quantification using classical plating method is difficult (Fortina et al., 2001). In a food matrix, such as cheese, culture-independent methods have rapidly been recognized as valuable alternatives to culture-dependant methods. These methods are based on the direct analysis of DNA extracted from the cheese matrix with no enrichment steps. Among culture-independent methods, qPCR represents a powerful tool for the quantification of microbial populations through the determination of targeted gene copies (Achiellos & Berthiere, 2013). Both dead and viable cells can be detected by qPCR (Garcia-Cayule et al., 2009). The design and use of specific primers has been proven to be a valuable tool for quantifying bacteria in cheeses like Emmental (Falentin et al., 2010, 2012), fresh cheese (Furet et al., 2004), and other cheese-types (Ladero et al., 2008, 2010). The qPCR has been used for quantification of technologically important bacteria such as *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Brevibacterium linens, Lactobacillus fermentum*, *Streptococcus thermophilus*,

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Lactobacillus delbrueckii, Lactobacillus casei, *Lactobacillus paracasei,* or *Lactobacillus acidophilus* (Achiellos & Berthiere, 2013). Many studies showed that *L. helveticus* has technological importance, especially regarding probiotic activity and bioactive peptides production by certain strains (Sadat-Mekmene et al., 2011). To our knowledge, a simple PCR method to quantify *L. helveticus* in a mixture of lactic acid bacteria in cheese has not been reported yet. Therefore, the aim of this work was to quantify *L. helveticus* in soft cheeses produced in Kazakhstan by a species specific qPCR method in a mixture of starter and non-starter cultures.

1. Materials and methods

1.1. Cheese manufacture

Six cheese samples were manufactured in a dairy pilot plant in Kazakhstan. Three cheeses were produced from cow´s milk and three cheeses were the new samples of Kazakh national soft cheese (irimshik). They were made from a mixture of goat's and cow's milk with the addition of skimmed cow's milk up to 15% mass fraction of dry matter in the milk. Milk for cheese production was treated with a low pasteurization (72±2 °C, 15–20 s). Christian Hansen FD-DVS DCC-260 was used as starter culture and contained these lactic acid bacteria: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis, Lactobacillus casei, Lactobacillus helveticus,* and *Streptococcus thermophilus*.

Two different cheese types were made following the traditional technology in three different moments to obtain the 7, 14, and 21 day-old cheeses: CCM (cheese made from cow's milk), and CGCM (cheese made from goat's milk and cow's milk, 1:1 ratio). The 7, 14, and 21 day-old cheeses were analysed at the same time. The pH was determined by using a pH-meter (expert-001, Russia).

1.2. Determination of bacterial composition

Serial dilutions of cheese were prepared in sterile 1% (w/v) peptone solution and plated on MRS agar medium (pH 5.4) for the determination of *Lactobacillus* spp., M17 agar medium for the determination of *Lactococcus* spp. and *Streptococcus thermophilus*, agar medium according to Nickels and Leesment (1964) for the determination of citrate-fermenting bacteria (ISO, 2006), and agar medium according to Nickels and Leesment (1964) with vancomycin supplementation for the determination of *Leuconostoc* spp. (ISO, 2006). Plates with MRS agar medium (pH 5.4) were incubated anaerobically at 37 °C for 72 h. Plates with M17 agar medium were incubated aerobically at 37 °C for 48 h. Plates with medium according to Nickels and Leesment (1964) with and without vancomycin were incubated aerobically at 25 °C for 5 days. Pure cultures of *L. helveticus* DPC 4571 were determined on MRS agar (pH 5.4) and incubated at 37 °C for 48 h.

1.3. PCR amplification

DNA was isolated from pure cultures and cheeses as published by Parayre and co-workers (2007). This method uses combination of mechanical and enzymatic lysis. DNA concentration was determined by using Nanodrop ND-1000 (NanoDrop Technologies, USA). *L. helveticus* DPC 4571, *L. acidophilus* LA5, *L. casei* L26, and *L. rhamnosus* CCM1825 were cultured in

culture tubes with MRS broth (pH 5.4). Total DNA from cell culture was extracted by using DNeasy Blood & Tissue kit (Qiagen, Germany) as described in the manufacturer´s instructions. All DNA samples were frozen before analyses.

Real-time PCR amplification and analysis were performed using Real-time PCR cycler CFX-96 (Bio-Rad, USA) with species-specific primers for *L. helveticus* for Lhv_0190 gene (Fwd: CAGTTGTGTTGACTTCCACAAT, Re: CAAATTGTGGCTGGTGATTCT) and Lhv_0191 gene (Fwd: GGGCTGATTACAGTGGCTAAT, Re: CTTGCCCTTTTCGGTGTAAA). The selected primers are highly selective and are able to discriminate accurately *L. helveticus* from other closely related homofermentative lactobacilli, especially *L. gallinarum*, *L. acidophilus,* and *L. delbruckeii* (Jebava et al., 2014). The initial composition of the PCR reaction mix was as follows: 2 µl genomic DNA, 10 µl iQSybr Green Supermix (BioRad), 7 µl demineralised sterile water, 1 µl of each primer (50 µM) in a final volume of 20 µl. The PCR conditions were: initial denaturation step at 95 °C for 5 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 45 s).

The PCR conditions and composition of the PCR reaction were the same for pure culture of *L. helveticus* DPC 4571 (described above) as well as for negative control strains (*L. acidophilus* LA5, *L. casei* L26, *L. rhamnosus* CCM1825). Non-template control was included in all PCR assays. Purification of the PCR product was carried out using commercially available QIAquick PCR Purification Kit (Qiagen, Germany) as described in the manufacturer´s instructions. The concentration of the purified PCR product was measured by spectrophotometry using Nanodrop ND-1000 and the corresponding copy number was calculated using the following equation (Lee et al., 2006).

$$\mathrm{DNA(copy)} = \frac{6.02\times10^{23}\,(\mathrm{copy/\,mol})\times \mathrm{DNAamount(g)}}{\mathrm{DNAlenght\ (dp)}\times 660\ (\mathrm{g\,/\,mol\,/\,dp})}$$

Decimal dilutions for the calibration curve were prepared according to a calculation of the copy number of DNA. Two calibration curves were prepared using two sets of primers (Lhv_0190, Lhv_0191). The same method for absolute quantification using qPCR as describe above was used to quantify counts of *L. helveticus* in pure cultures and in cheeses.

2. Results and discussion

2.1. Cheese manufacture and determination of bacterial composition

Characteristics of six Kazakh cheeses and results for culture-dependent plating methods used for the identification and quantification of microorganisms in cheeses are presented in Table 1. No significant differences in counts of bacteria were detected, and they ranged from 10^8 to 10^9 CFU g^{-1}. Only cheese N°6 showed lower numbers of citrate fermenting bacteria (10^6 CFU g^{-1}). The most important mesophilic lactic acid bacteria that ferment citrate belong to *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. Diagnostic characteristics of lactococci and leuconostoc were distinguishable by this method (ISO, 2006). Leuconostoc colonies are blue; lactococcus colonies are white with a zone of clearing. Present knowledge of microbial diversity and dynamics in cheese is mainly based on culture-dependent methods, involving traditional numeration followed by

identification of dominant microorganisms using phenotypic and molecular methods (Falentin et al., 2012). Cheese contains a complex combination of microorganisms that changes with time; initially containing large numbers of starter lactic acid bacteria and then with maturation, an increasing number of non-starter lactic acid bacteria (Karimi et al., 2012).

Table. 1. Cheese characterization, bacterial counts by plating method and quantification of *L. helveticus* using qPCR method with two primer sets

Sample	Used milk	Period of ripening (day)	pH	
Cheese 1	CGCM[a]	7	5.06	
Cheese 2	CGCM[a]	14	4.91	
Cheese 3	CGCM[a]	21	4.95	
Cheese 4	CCM[b]	7	4.86	
Cheese 5	CCM[b]	14	4.67	
Cheese 6	CCM[b]	21	4.70	
	Bacterial counts [CFU·g^{-1}]			
	Lactococcus spp., *St. thermophilus*	citrate-fermenting bacteria	*Leuconostoc* spp.	*Lactobacillus* spp.
Cheese 1	$2.4 \cdot 10^9$	$3.4 \cdot 10^8$	$2.5 \cdot 10^8$	$1.0 \cdot 10^9$
Cheese 2	$2.8 \cdot 10^9$	$2.0 \cdot 10^8$	$1.5 \cdot 10^8$	$1.9 \cdot 10^9$
Cheese 3	$1.2 \cdot 10^9$	$1.8 \cdot 10^9$	$1.7 \cdot 10^8$	$9.8 \cdot 10^8$
Cheese 4	$1.8 \cdot 10^9$	$2.5 \cdot 10^8$	$1.7 \cdot 10^8$	$9.4 \cdot 10^9$
Cheese 5	$2.1 \cdot 10^9$	$1.7 \cdot 10^8$	$1.7 \cdot 10^8$	$7.6 \cdot 10^8$
Cheese 6	$1.4 \cdot 10^9$	$5.0 \cdot 10^6$	$3.6 \cdot 10^8$	$6.3 \cdot 10^8$
	Number of *L. helveticus* cells [CFU·g^{-1}]			
	Lhv_0190		Lhv_0191	
Cheese 1	$3.7 \cdot 10^7 \pm 6.9 \cdot 10^3$		$8.9 \cdot 10^7 \pm 4.1 \cdot 10^3$	
Cheese 2	$4.3 \cdot 10^7 \pm 4.7 \cdot 10^3$		$1.0 \cdot 10^8 \pm 7.1 \cdot 10^3$	
Cheese 3	$2.5 \cdot 10^8 \pm 4.2 \cdot 10^4$		$4.1 \cdot 10^8 \pm 4.6 \cdot 10^3$	
Cheese 4	$3.9 \cdot 10^6 \pm 5.9 \cdot 10^2$		$1.0 \cdot 10^7 \pm 3.3 \cdot 10^4$	
Cheese 5	$4.8 \cdot 10^7 \pm 6.3 \cdot 10^3$		$1.2 \cdot 10^8 \pm 3.4 \cdot 10^3$	
Cheese 6	$2.6 \cdot 10^6 \pm 1.1 \cdot 10^2$		$6.5 \cdot 10^6 \pm 3.6 \cdot 10^2$	

[a]CGCM (cheese made from goat's milk and cow's milk, 1:1 ratio), [b]CCM (cheese made from cow's milk)

Because of the presence of other lactobacilli closely related to *L. helveticus* in the culture, and the deficiency in a reliable species selective agar medium, it was impossible to determine selectively *L. helveticus* using plating method based on MRS agar. Thus, total counts of lactobacilli were determined. Most plating methods are based on pure cultures of these organisms and fail to work in products because of the presence of multiple and closely related species making the differential or selective enumeration of probiotic and starter bacteria difficult due to similarity in growth requirements and overlapping biochemical characteristics of the species (Ashraf & Shah, 2011).

2.2. qPCR determination

First the absolute quantification method was verified in pure cultures of *L. helveticus* DPC 4571 in comparison with the plate count method. The results were as follows: plate count on MRS (pH 5.4) agar $8.5 \cdot 10^7 \pm 2.1 \cdot 10^1$ CFU·ml^{-1}, qPCR with Lhv_0190 $9.2 \cdot 10^7 \pm 1.2 \cdot 10^2$ CFU·ml^{-1}, qPCR with Lhv_0191 $1.1 \cdot 10^8 \pm 3.1 \cdot 10^2$ CFU·ml^{-1}. These results are averages of 10 assessments and do not differ substantially, and thus the suitability of chosen qPCR method is confirmed.

The soft cheese irimshik was selected for the first trial, because DNA isolation from this soft cheese was unambitious and defined starter culture was used for its production. The DNA concentration (quantified by Nanodrop ND-1000) extracted from 6 different cheeses varied from 4.7 to 56.7 μg·g^{-1} cheese. The DNA concentration was in agreement with previously published data quantifying DNA extracted from cheese matrix (Jacquin et al., 2001; Lee et al., 2006), reporting concentrations of DNA extracted from 10 different dairy products between 0.7 to 29.2 μg·g^{-1} using picogreen spectrofluorimetry, respectively, 4 to 19 μg DNA·g^{-1} of Emmental cheese using spectrophotometric method.

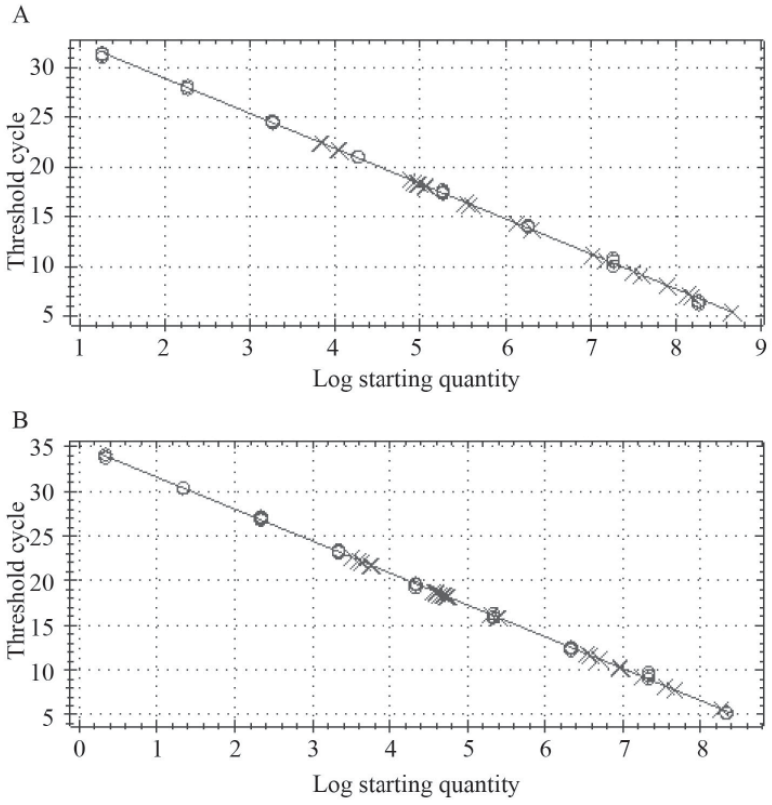

Fig. 1. Calibration curves for A: Lhv_0190 and B: Lhv_0191 from qPCR assay
o: standard – *L. helveticus* DPC 4571; X: unknown samples (cheeses)
–: SYBR (A: efficiency 91.9%, slope –3.53; B: efficiency 90.6%, slope –3.57)

Two qPCR assays targeting peptidoglycan hydrolases were developed for the absolute quantification of *L. helveticus* in food matrices. One pair of primers Lhv_0190 encoding *N*-acetylmuramidase and the other one Lhv_0191 encoding amidase. Two pairs of primers were used to observe possible differences. Ascertained differences between obtained results for these two pairs of primers were minimal and error is within the standard deviation. Quantification of *L. helveticus* using qPCR ranged from $2.6 \cdot 10^6$ to $4.1 \cdot 10^8$ CFU·g^{-1}, and particular results are shown in Table 1. The standard curves for Lhv_0190 and Lhv_0191 were linear in the range tested (R^2>0.999) by the triplicate reactions (Fig. 1).

Amplification was linear over the range of $1.8 \cdot 10^1$ to 1.8·108 for Lhv_0190 and $2.1 \cdot 10^0$ to 2.1 108 for Lhv_0191, respectively, of DNA copies. The slope of the standard curves for Lhv_0190 and Lhv_0191 were –3.53 and –3.57, respectively. Quantitation cycle (Cq) for positive control was 16.2 for Lhv_0190 and 15.1 for Lhv_0191, respectively, Cq for all three negative controls were above 34 for both primer sets, Cq for NTC was >35. Efficiencies of qPCR were 91.9% for Lhv_0190 and 90.6% for Lhv_0191, respectively. Intra-assay CV was 2.3% for Lhv_0190 and 0.9% for Lhv_0191.

Because both *L. helveticus* and *L. casei* were present in the used starter culture and cheese contains probably other lactobacilli of non-starter origin as well, the determination of *L. helveticus* was difficult using non-selective MRS (pH 5.4) agar. It was not possible to compare the results of plating methods to qPCR. Direct qPCR quantification has proven to be useful, providing additional information to plate counts. In addition, the use of species-specific primer pairs at the given PCR conditions proved to be a very rapid and effective method for quantification of the species. Our results confirmed the necessity of using the species-specific qPCR for the determination and quantification of lactobacilli in mixtures.

When we critically assess both the pros and cons of qPCR, we have to say that usage of qPCR for quantification of *L. helveticus* is an alternative to plating methods. More particularly, qPCR is considered as the first choice method, and its major advantage is that it is less-time consuming than conventional culture based methods. It is also highly sensitive, specific, enables simultaneous detection of different microorganisms, and requires no post-processing (Martinéz et at., 2011; Postollec et al., 2011). Method of qPCR has also some limitations, for example the precision of molecular quantification by PCR depends on the effectiveness of bacterial lysis of DNA extraction, and on the presence of PCR inhibitors in the DNA solution (Furet et al., 2004). Reliable quantification depends on optimized and carefully performed qPCR reactions. The accuracy of qPCR is influenced by primer design, the quality of the template DNA, and the handling and storage of samples, primers, and enzymes. With food samples, special attention must be paid to the possible presence of inhibitors and to the efficiency of DNA extraction. Provided appropriate control (e.g. positive PCR control, non-template control, control for environmental contamination, etc.) are included in the analyses, proposed qPCR appears to be highly accurate and reliable for quantification of targeted genes (Postollec et al., 2011).

3. Conclusions

In conclusion, our results suggest that application of simple, fast, and accurate qPCR method is eligible for species specific quantification of *L. helveticus* in cheese matrix containing other lactic acid bacteria including lactobacilli. In this work soft cheese was used as matrix. Other experiments with different cheeses and other dairy products will follow in the very near future to verify appropriateness of this method and its independence of food matrix and composition of microflora.

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