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Detection and enumeration of *Lactobacillus helveticus* in dairy products

Aline Moser [a, b], Hélène Berthoud [a], Elisabeth Eugster [a], Leo Meile [b], Stefan Irmler [a, *]

[a] Agroscope, 3003 Bern, Switzerland
[b] Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, ETH Zürich, 8092 Zurich, Switzerland

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ABSTRACT

Lactobacillus helveticus, a lactic acid bacterium, is an important species in food fermentation, e.g., cheesemaking, and is considered beneficial to human health. We developed a quantitative real-time polymerase chain reaction (qPCR) method for the detection and quantification of *L. helveticus* in dairy products. The method uses a set of target-specific PCR primers and a fluorogenic probe and amplifies a part of the *pheS* gene that encodes the alpha subunit of the phenylalanine-tRNA synthetase. All 24 *L. helveticus* strains tested were qPCR positive; no signal was observed for 23 strains belonging to closely related species. The limit of detection was ten copies per reaction and the assay covered a linear dynamic range of eight logs. The method was used to detect and enumerate *L. helveticus* in milk and cheese during ripening; therefore it can be used to study the temporal and spatial distribution of *L. helveticus* during cheese manufacturing and ripening.

1. Introduction

Lactobacillus helveticus is a member of the group of lactic acid bacteria (LAB) that metabolises lactose and other carbohydrates predominantly into lactic acid (Hammes & Hertel, 2006). LAB are found in various habitats in which they encounter complex nutritional resources, such as in soil, sewage, and fermented food, as well as on the body surface and in the intestinal tract of humans and animals.

The species *L. helveticus* is commonly used as thermophilic starter in the manufacturing of cheeses, such as Swiss-type and Italian hard cheeses (Slattery, O'Callaghan, Fitzgerald, Beresford, & Ross, 2010). *L. helveticus* possesses cell-envelope bound proteases and intracellular peptidases, the latter of which can be released into the cheese matrix upon autolysis. Consequently, *L. helveticus* has also been used as adjunct in Cheddar cheeses to accelerate protein degradation and enhance flavour development during cheese ripening (Hannon et al., 2003; Kenny, FitzGerald, O'Cuinn, Beresford, & Jordan, 2006).

Because milk proteins are a source of peptides with various bioactivities and, can be, for instance, released by the proteolytic activity of *L. helveticus*, extensive research has been conducted on the use of *L. helveticus* strains as probiotics (see Griffiths & Tellez, 2013; Taverniti & Guglielmetti, 2012, for reviews). Well-known examples include the development and commercialisation of the antihypertensive milk drinks Evolus (Valio Ltd, Valio, Finland) and Calpis (Calpis Food Industry Co. Ltd. Tokyo, Japan), which contain *L. helveticus* strains. Based on this potential, the detection and enumeration of *L. helveticus* are essential for many applications. A simple and rapid quantification method for *L. helveticus* enables, e.g., the tracking of this species in milk production and fermentation.

We are interested in better understanding the microbiology of Gruyère PDO cheese and its influence on cheese ripening and flavour development. Gruyère PDO is manufactured with natural whey cultures (NWCs) (http://www.gruyere.com/en/specifications/). Preliminary microbiological investigations have shown that *L. helveticus* is one of the dominant species present (data not shown). A species-specific and easy-to-use method of detecting and enumerating *L. helveticus* is a valuable tool with which to study the temporal and spatial distributions of representatives of this species in the cheese manufacturing process, including those in the milk.

In this report, we present the development of a hydrolysis-probe-based real-time PCR assay targeting the phenylalanyl-tRNA synthase gene (*pheS*). This method allows both the detection and enumeration of *L. helveticus* in dairy products. The assay developed

* Corresponding author. Tel.: +41 58 463 81 55.
E-mail address: stefan.irmler@agroscope.admin.ch (S. Irmler).

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here was found to be specific for *L. helveticus* and was used to quantify this species in dairy products.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

All strains used in this study are listed in Table 1. Strains were stored at −80 °C in sterile reconstituted skim milk powder (10%, w/v). Strains were reactivated and cultivated in 10 mL MRS broth (de Man, Rogosa, & Sharpe, 1960) at 37 °C.

2.2. DNA extraction

For the broth cultures, a bacterial pellet that had been harvested from a 1 mL culture via centrifugation [10,000 × *g*, 5 min, room temperature (RT)] was used for DNA extraction. In case of NWC samples, the pellet (10,000 × *g*, 5 min, RT) obtained from 10 mL NWC, to which 50 μL 10% sodium dodecyl sulphate (SDS) was added, was used for DNA extraction.

With regard to cheese samples, 10 g grated cheese were added to 90 mL modified peptone water (10 g L^{-1} peptone from casein, 5 g L^{-1} sodium chloride, 20 g L^{-1} trisodium citrate dihydrate, pH 7.0). After incubation for 10 min at 40 °C, the sample was homogenised for 3 min in a stomacher (Masticator, IUL Instruments, Königswinter, Germany). After an additional incubation at 40 °C for 10 min, 50 μL 10% (w/v) SDS were added to 10 mL of the homogenate which was then centrifuged (4000 × *g*, 21 °C, 30 min). The pellet containing the bacteria was used for DNA extraction.

To improve DNA extraction, the bacterial pellets were treated with the following pre-lysis steps. First, the bacterial cells were incubated in 1 mL 50 mM sodium hydroxide for 15 min at RT. Next, the cells were treated with lysozyme (2.5 mg mL^{-1} dissolved in 100 mM Tris(hydroxymethyl)aminomethane, 10 mM ethylenediaminetetraacetic acid, 25% (w/v) sucrose, pH 8.0) for 1 h at 37 °C. Finally, the bacteria were collected via centrifugation at 10,000 × *g* at RT for 5 min. The genomic DNA (gDNA) was then extracted using the EZ1 DNA Tissue kit and a BioRobot EZ1 workstation (Qiagen, Hombrechtikon, Switzerland). The final volume of the eluted DNA was 100 μL.

To determine the DNA from the whole and lysed cells in the cheese, a protocol described by Gatti et al. (2008) was applied. Briefly, the bacterial pellet – called the whole-cell fraction – was treated with the Turbo DNA-free Kit (ThermoFisher Scientific, Switzerland) according to the manufacturer's instructions before DNA extraction. The lysed-cell fraction was obtained by passing the supernatant of the cheese homogenate through a sterile filter with a pore size of 0.22 μm (Whatman, Dassel, Germany) and extracting the DNA from the filtrate.

DNA from the raw milk was extracted as described by Turgay, Schaeren, Wechsler, Bütikofer, and Graber (2016).

2.3. Oligonucleotide primer and probe design

For various LAB, the nucleotide sequences of the *pheS* gene that encode the alpha subunit of the phenylalanine-tRNA synthetase were aligned (Supplementary Fig. S1) using CLC Main Workbench version 7.5.1 (CLC bio, Aarhus, Denmark). Thereby, an 87-bp region from nucleotide position 318 to 404 within the *pheS* gene was found to be a potential target site because it was identical in all analysed *L. helveticus* strains but showed variability in other LAB species. Primer3 software v.0.4.0 (Untergasser et al., 2012) was used to design the primers and the hydrolysis probe for this gene region. The primers and hydrolysis probe were synthesised by Microsynth (Balgach, Switzerland).

2.4. Real-time PCR assay

Quantitative real-time PCR (qPCR) assays were carried out on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) in a final volume of 12 μL containing 2 μL DNA extract, 300 nM of LbhelvF1 (5′-AGGTTCAAAGCATCCAATCAATATT-3′), 300 nM of LbhelvR1 (5′-TCGGGACCTTGCACTACTTTATAAC-3′), 100 nM of hydrolysis probe (5′-(FAM)ATACCGATGAAGTAGCTTTCCAAATCATCCA(BHQ-1)-3′), and 6 μL of 2× qPCR MasterMix No ROX (Eurogentec, Seraing, Belgium). All reactions were performed under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The fluorescence of the reporter dye (FAM) was measured during amplification at 510 nm. Data were analysed using Rotor-Gene 1.7 software with a threshold of 0.03 and the 'Dynamic Tube Normalization' option. The standard curve was generated by plotting the threshold cycle (Cq) values as a function of the concentration of recombinant *pheS* standard gene copies μL^{-1}. All standard and sample reactions were run in triplicate.

2.5. Construction of the plasmid standard

A part of the *pheS* gene, including the target sequence for the qPCR assay, was amplified with the primers StdLhF (5′-CGTGATGTTGCCCCAGAAAA-3′) and StdLhR (5′-GGTGTGAGTGAG-TAGCATCG-3′) from the gDNA of *L. helveticus* FAM1450. The amplification was performed at a final volume of 50 μL, containing 500 nM of each primer, 2.5 U AmpliTaq Gold® Polymerase (Roche, Bale, Switzerland), 0.2 mM dNTPs, and 5 μL 10× PCR buffer (Roche). The reactions were run on a thermocycler at 95 °C for 10 min followed by 35 cycles of 95 °C for 20 s, 60 °C for 30 c, and 72 °C for 30 s, as well as a final extension for 5 min at 72 °C. The amplicon was purified using the QIAquick PCR Purification kit (Qiagen) and then inserted into the pGEM®-T Easy vector (Promega, Madison WI, USA). *Escherichia coli* MAX Efficiency® DH5α™ competent cells (Invitrogen, California, USA) were transformed with the recombinant plasmids. Clones carrying inserts in the plasmid were selected via blue/white screening on plates containing X-gal. Plasmid DNA (pGEM/*pheS*) was isolated from 1 mL of a bacterial overnight culture with the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol. The orientation and DNA sequence of the insert were determined via Sanger sequencing.

2.6. qPCR specificity, sensitivity, and efficiency

First, the specificity of the primer design was evaluated using the NCBI Primer-BLAST tool. Furthermore, the specificity of the primer/probe was assayed experimentally with gDNA isolated from various bacterial strains (Table 1).

The sensitivity of the qPCR assay was evaluated by preparing a serial tenfold dilution of the plasmid pGEM/*pheS*, which had been linearised via restriction with the *Pst*I restriction enzyme, in 10 mM Tris(hydroxymethyl)aminomethane (pH 8.0). The DNA concentration was determined using the Qubit dsDNA BR Assay kit (LubioScience, Lucerne, Switzerland), and the molar concentration of the plasmid was calculated using the OligCalc oligonucleotide properties calculator (Kibbe, 2007). Eleven independent dilution series were prepared by different operators to control for inter-operator variations. Three dilution series per operator were prepared by Operators A and B, and five dilution series were prepared by Operator C. The dilutions were tested by qPCR and the calculated DNA copy numbers were used to assess the limit of detection (LOD) and the linearity of the qPCR assay. The LOD was defined as the concentration at which 95% of runs gave a positive result. The standard curve was generated by plotting the Cq values of all dilutions as a function of the

Table 1
Bacterial strains used in this study to test the specificity of the quantitative polymerase chain reaction (qPCR) assay.[a]

Strain	Reference	Isolation source	qPCR result
Lactobacillus helveticus			
FAM1450	ACC	NA	+
FAM1476	ACC	NA	+
FAM21493	ACC	MSS	+
FAM22081	ACC	NA	+
FAM21339	ACC	MSS	+
FAM22076	ACC	NWC	+
FAM21456	ACC	MSS	+
FAM8104	ACC	Raw milk cheese (Tilsit)	+
FAM13019	ACC	NWC	+
FAM8627	ACC	NA	+
FAM22155	ACC	NWC	+
FAM2888	ACC	NA	+
FAM20575	ACC	NWC	+
FAM22330	ACC	MSS	+
FAM1213	ACC	NA	+
FAM22074	ACC	NWC	+
FAM1172	ACC	NA	+
FAM8105	ACC	Raw milk cheese (Tilsit)	+
FAM22079	ACC	NWC	+
FAM22472	ACC	NA	+
FAM13019	ACC	NWC	+
B02	Chr. Hansen Holding A/S, Denmark	Starter culture	+
LH32	Chr. Hansen Holding A/S, Denmark	Commercial strain	+
DSM 20075^T	DSMZ	Emmental cheese	+
Lactobacillus gallinarum			
DSM 10532^T	DSMZ	Chicken crop	−
LMG 14751	BCCM	Chicken faeces	−
LMG 14754	BCCM	Chicken faeces	−
LMG 14755	BCCM	Chicken faeces	−
LMG 18181	BCCM	Chicken intestine	−
LMG 22870	BCCM	Laying hen vagina	−
Lactobacillus kefiranofaciens subsp. *kefiranofaciens*			
DSM 5016^T	DSMZ	Kefir grains	−
Lactobacillus kefiranofaciens subsp. *kefirgranum*			
DSM 10550^T	DSMZ	Kefir grains	−
Lactobacillus crispatus			
DSM 20584^T	DSMZ	Eye	−
Lactobacillus acidophilus			
DSM 20079^T	DSMZ	Human	−
Lactobacillus johnsonii			
DSM 10533^T	DSMZ	Human blood	−
Lactobacillus delbrueckii subsp. *bulgaricus*			
DSM 20081^T	DSMZ	Yoghourt	−
Lactobacillus delbrueckii subsp. *lactis*			
DSM 20072^T	DSMZ	Emmental cheese	−
Lactobacillus fermentum			
DSM 20052^T	DSMZ	Fermented beets	−
Lactobacillus casei			
ATCC 334	ATCC, Virginia, USA	Emmental cheese	−
FAM18121	ACC	Gruyère PDO cheese	−
Lactobacillus casei subsp. *tolerans*			
DSM 20012	DSMZ	Pasteurised milk	−
DSM 20258^T	DSMZ	Pasteurised milk	−
Lactobacillus paracasei subsp. *paracasei*			
DSM 5622^T	DSMZ	Milk products	−
Lactobacillus rhamnosus			
CCUG 34291	CCUG	Human faeces	−
Enterococcus faecium			
FAM8492	ACC	MSS	−
Streptococcus thermophilus			
DSM 20617^T	DSMZ	Pasteurised milk	−
Lactobacillus amylovorus			
DSM 20531^T	DSMZ	Cattle waste corn fermentation	−

[a] Abbreviations are: ACC, Agroscope Culture Collection (Switzerland); DSMZ, German Collection of Microorganisms and Cell Cultures; BCCM, Belgian Co-Ordinated Collections of Micro-Organisms; CCUG, Culture Collection University of Göteborg (Sweden); NWC, natural whey culture; MSS, mixed strain starter; NA, information about isolation source not available. ^{T} Indicates type strain; + and − indicate presence (+) or absence (−) of a qPCR signal.

concentration and calculating the linear regression in R (version 3.1.3, R Core Team, 2015). The qPCR efficiency was calculated from the standard curves with an equation PCR efficiency of $(10^{-1/\text{slope}}) - 1$.

The efficiency of the sum of DNA extraction plus qPCR was determined by comparing the calculated copy numbers with the colony forming units (cfu). For this, a culture of *L. helveticus* FAM1450 consisting of 9.93×10^8 cfu mL^{-1} was serially diluted tenfold in raw milk. Two dilution series were prepared and DNA from each dilution sample was extracted as described above before subjecting it to qPCR. The concentration of cfu in the MRS broth was determined via plate counting using modified MRS agar plates that contained lactose (20 g L^{-1}) instead of glucose. The plates were incubated at 37 °C for 48 h under aerobic conditions.

To test for qPCR inhibitors, tenfold serial dilution series of DNA extracts from raw milk, cheese, and NWCs were measured in triplicate via qPCR. The Cq values were plotted as a function of the dilution steps, and the linear regression was calculated using R v3.1.3 (R Core Team, 2015).

2.7. *Enumeration of* L. helveticus *in dairy products*

The applicability of the qPCR assay was evaluated by analysing various dairy samples (Table 2). The estimated number of copies per µL reaction ($C_{Reaction}$) for each reaction tube was calculated using the following formula:

$$C_{Reaction} = 10^{\frac{Cq - b}{m}}$$

where b is the intercept of the regression line of the standard curve and m is the slope of the regression line of the standard curve. To account for the dilution steps and volume transformation during sample preparation, the $C_{Reaction}$ values were multiplied by 10,000, 100, 10 and 25 for the lysed-cell fraction, cheese, NWC and raw milk samples, respectively, to obtain the gene copy number g^{-1} or mL^{-1}, respectively (C_{Sample}). Only samples for which all experimental replicates gave a Cq value above the LOD were regarded as positive.

2.8. *Cheesemaking*

Model raclette-type cheeses were produced from 300 L of pasteurised milk, using the mesophilic starters CHOOZIT MD 88 and CHOOZIT MA 14 (Danisco, France), without and with a mixture of *L. helveticus* (FAM13019, FAM23236, and FAM23237) as an adjunct culture. Milk was pre-ripened at 28–32 °C for 40 min. Coagulation occurred for 30 min at 32 °C in the presence of rennet. Cutting and stirring were performed at 38 °C for 30 min. The whey-curd

Table 2
Dairy samples used for detection and quantification of *L. helveticus* by qPCR.[a]

Sample	log *pheS* copies g^{-1} or mL^{-1}
Long-ripened hard cheese (produced with NWC), Switzerland	7.16 ± 0.04
Tilsit (produced with NWC), Switzerland	8.72 ± 0.42
Goat cheese, Switzerland	6.32 ± 0.03
Cheese with high leucyl aminopeptidase activity (133 IU kg^{-1}), USA	6.98 ± 0.11
Mozzarella di Bufala Campana PDO, Italy	8.55 ± 0.01
Grana Padano PDO, Italy	7.34 ± 0.03
Parmigiano Reggiano PDO, Italy	6.02 ± 0.04
Provolone, Italy	8.75 ± 0.07
Tête de Moine PDO, Switzerland	No *L. helveticus* detected
Tilsit (no NWC used for the production), Switzerland	No *L. helveticus* detected
Emmental PDO, Switzerland	No *L. helveticus* detected
Gruyère PDO (ripened for 24 h), cheese factory 1, Switzerland	8.29 ± 0.01
Gruyère PDO (ripened for 6 months), cheese factory 1, Switzerland	6.92 ± 0.08
NWC (incubated at 38 °C for 20 h), cheese factory 1, Switzerland	6.82 ± 0.29
Gruyère PDO (ripened for 24 h), cheese factory 2, Switzerland	8.49 ± 0.01
Gruyère PDO (ripened for 6 months), cheese factory 2, Switzerland	7.11 ± 0.4
NWC (incubated at 32 °C for 20 h), cheese factory 2, Switzerland	7.23 ± 0.28
NWC (incubated at 38 °C for 20 h), cheese factory 2, Switzerland	7.48 ± 0.05
Gruyère PDO (ripened for 24 h), cheese factory 3, Switzerland	7.58 ± 0.06
Gruyère PDO (ripened for 6 months), cheese factory 3, Switzerland	7.06 ± 0.21
NWC (incubated at 32 °C for 20 h), cheese factory 3, Switzerland	7.46 ± 0.03
NWC (incubated at 38 °C for 20 h), cheese factory 3, Switzerland	8.38 ± 0.04
NWC (incubated at 38 °C for 10 h), cheese factory 3, Switzerland	7.41 ± 0.04
Raw cheesemaking milk (6th January 2015), cheese factory, Switzerland	No *L. helveticus* detected
Raw cheesemaking milk (13th January 2015), cheese factory, Switzerland	No *L. helveticus* detected
Raw cheesemaking milk (14th April 2015), cheese factory, Switzerland	2.36 ± 0.19
Raw cheesemaking milk (21st April 2015), cheese factory, Switzerland	3.59 ± 0.07

[a] Mean values for triplicate measurements (±standard deviation) are shown. PDO, Protected Designation of Origin; NWC, natural whey culture.

mixture was filled in moulds and pressed at 30 °C for 0.75 h. After brining (11–13 °C, 16 h), the cheeses were smear-ripened at 10–11 °C and 90–96% relative humidity. Samples were taken after 24 h, 80 days, and 120 days of ripening.

3. Results

3.1. Specificity of the pheS targeted primers

In silico analysis using the Primer-BLAST tool against the RefSeq database revealed that the primer aligned with 100% identity to the *pheS* gene of *L. helveticus* only (data not shown). To experimentally assess the specificity of the primer, qPCR experiments were performed with gDNA extracted from 24 *L. helveticus* strains and 23 strains belonging to other LAB species. The target region was amplified in all *L. helveticus* strains. No signal was detected in the 23 strains from the other LAB species tested (Table 1).

3.2. Copy number of pheS in L. helveticus genomes

Twenty-two *L. helveticus* genomes available in the GenBank database (for accession numbers, see Fig. S1) were searched for the *pheS* gene. All genomes were found to contain a single copy of the gene.

3.3. Sensitivity and linearity of the pheS-specific qPCR

The sensitivity and efficiency of the qPCR assay were evaluated with a plasmid containing a 490-bp region of the *pheS* gene and by preparing a dilution series of *L. helveticus* FAM1450 in raw milk.

The plasmid was restricted to obtain a linearised form. Eleven dilution series of the linear plasmid showed a linear relationship from 10 to 10^8 copies μL^{-1} (Fig. 1). The equation of the linear regression was $Cq = -3.46x + 37.59$ with a correlation coefficient (R^2) of 0.994. The efficiency was calculated as 95%. Below 10 copies, no signal was obtained in all triplicate measurements. Therefore, the limit of detection was set to 10 copies μL^{-1}.

L. helveticus FAM1450 was also serially diluted in milk. The relationship between the logarithm of bacterial cells per mL milk and the DNA extracted from the milk samples was linear from 9.9 to 9.9×10^8 cfu mL^{-1} (Fig. 2). The equation of the linear regression was $Cq = -3.408x + 38.357$, with a correlation coefficient (R^2) of 0.999. Triplicate measurements of lower dilutions did not produce a signal.

3.4. Applicability of the novel qPCR assay

First, the applicability of the qPCR assay was examined with model raclette-type cheeses that were produced under defined conditions. The cheeses were produced with and without an *L. helveticus* adjunct culture. *L. helveticus* was detected in all cheeses to which it had been added during cheesemaking, whereas no *L. helveticus* was detected in the control cheese samples. The population level of *L. helveticus*, represented by the copy number, was 2.52 (±0.59) × 10^8 copies g^{-1} cheese on average after 24 h of ripening dropping to 6.03 (±0.51) × 10^6 copies g^{-1} cheese after 80 days and 2.32 ± 1.1 × 10^6 after 120 days of ripening (Fig. 3). We applied a sterilising filtration to the cheese extracts, as described by Gatti et al. (2008), to quantify the number of lysed cells. After 24 h of ripening, no signal of the presence of *L. helveticus* was detected in the lysed-cell fraction. After ripening periods of 80 d and 120 d, the lysed-cell fraction contained more than 10^7 *pheS* copies g^{-1} cheese (Fig. 3).

The qPCR assay was also tested with NWCs, cheese, and milk samples derived from three Gruyère PDO cheese factories (Table 2). All of the NWCs tested showed an amplification signal

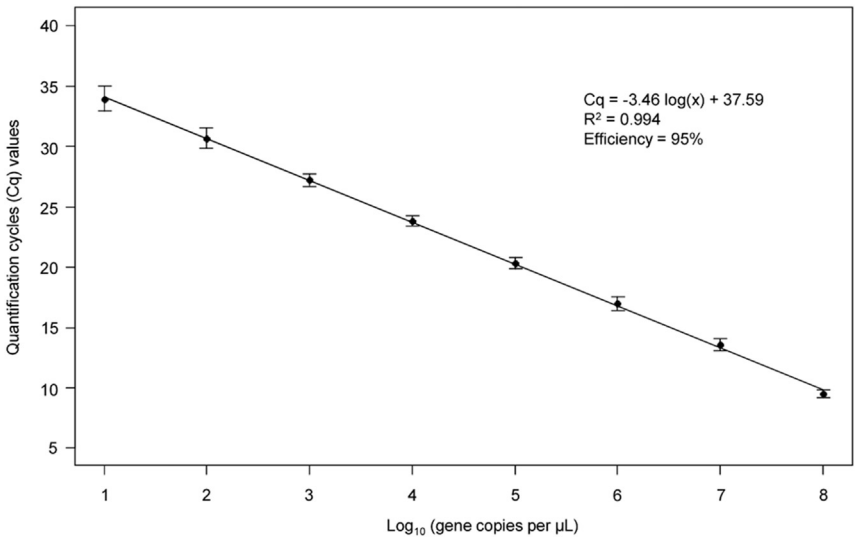

Fig. 1. Plasmid standard curve obtained by plotting the threshold cycle (Cq) values against the logarithm of the calculated gene copy numbers from serial tenfold dilutions of plasmid pGEM/pheS. Error bars indicate the standard deviations (n = 11).

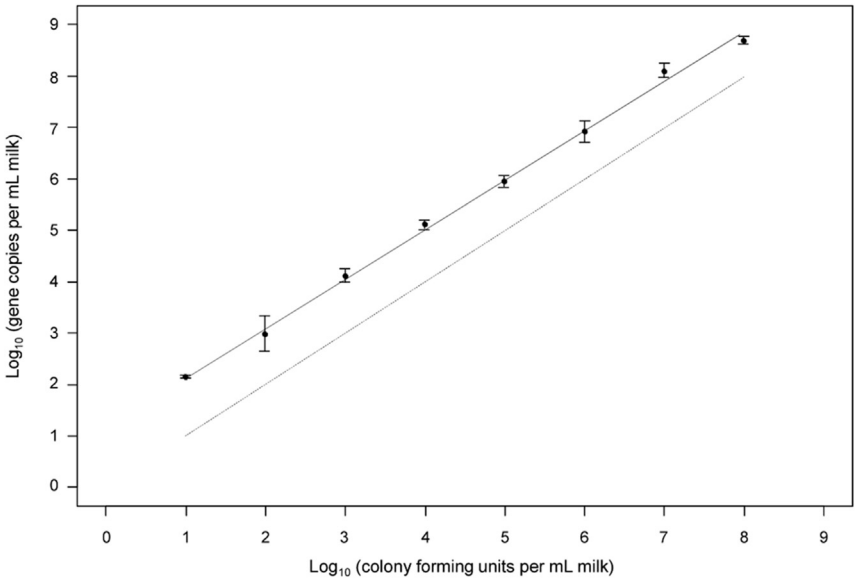

Fig. 2. Comparison of qPCR against plate counting of *L. helveticus* FAM1450 in milk. The solid line represents the regression curve determined experimentally; the dashed line illustrates the theoretical expected curve. The DNA extractions were performed twice. Error bars represent standard deviations of these two assays.

for the *pheS* gene within a range of 7.54×10^6 copies mL^{-1} and 2.43×10^8 copies mL^{-1}. All three Gruyère PDO cheeses tested positively for the presence of *L. helveticus* and contained a mean of 1.8×10^8 and 1.8×10^7 *pheS* copies g^{-1} after 24 h and 6 months, respectively.

We also analysed retail cheeses (Table 2). All cheeses that we knew to be produced with natural whey cultures tested positively for the presence of *L. helveticus*. Remarkably, a cheese sample from the USA that we found to exhibit high leucyl aminopeptidase activity (133 IU kg^{-1}), which is an indicator for the presence of *L. helveticus* (unpublished data), also tested positively for *L. helveticus*.

We detected *L. helveticus* in two cheesemaking milk samples collected from Gruyère PDO cheese factories (Table 2). The counts for the milk samples were low, but Sanger sequencing revealed that the sequence of both amplicons aligned with 100% identity to the *pheS* gene of *L. helveticus* DPC4571 (data not shown).

3.5. Absence of inhibitory compounds in the DNA extractions used for qPCR

DNA extracts from a milk, a NWC and a cheese were tested for PCR inhibitors by examining tenfold serial dilutions using qPCR. All samples showed a linear correlation between the Cq value and the dilution step, with correlation coefficients (R^2) of 0.998, 0.995, and 0.999 for the NWC, cheese, and milk, respectively (Supplementary data Fig. S2).

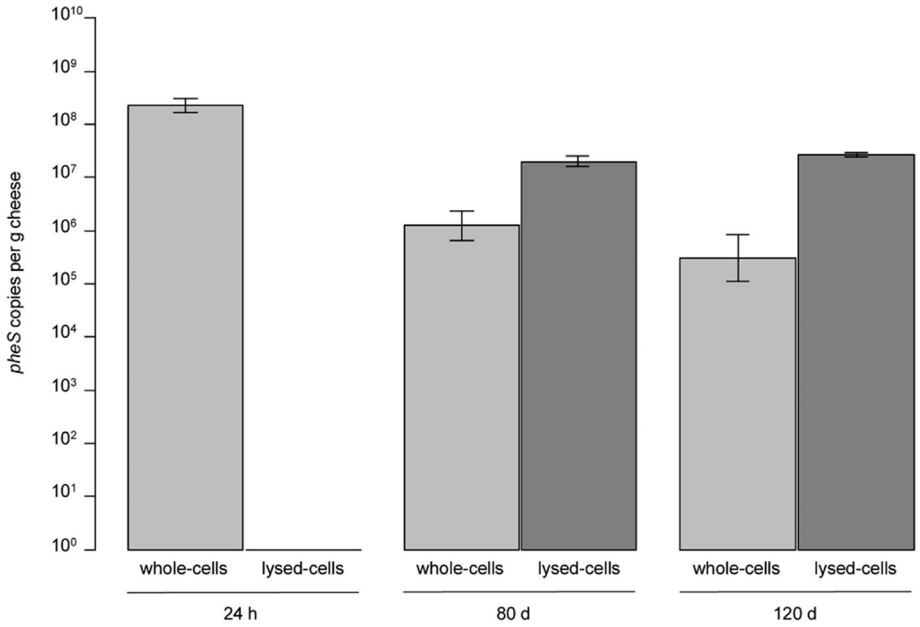

Fig. 3. Enumeration of *L. helveticus* in cheese; whole cells and lysed cells corresponding to *pheS* copy numbers were enumerated by qPCR in model-type cheeses. Samples were taken after 24 h, 80 days, and 120 days of ripening. Error bars represent the standard deviation of two cheese samples.

4. Discussion

DNA-based methods, such PCR and qPCR, are recognised as valuable alternatives to culture-dependent methods for the enumeration of bacteria (Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011). The former methods are sensitive, precise, and rapid and can be used to specifically detect and quantify bacteria with a variety of origins and in mixed populations. Some PCR-based methods for the identification of *L. helveticus* have been reported in the past. These techniques amplify parts of the characteristic genes encoding S-layer proteins, peptidases, or peptidoglycan hydrolases, or the 16S–23S rRNA spacer region (Fortina, Ricci, Mora, Parini, & Manachini, 2001; Jebava, Chuat, Lortal, & Valence, 2014; Tilsala-Timisjarvi & Alatossava, 1997; Ventura, Callegari, & Morelli, 2000).

Desfossés-Foucault et al. (2012) describe a qPCR that targets the *tuf* gene of *L. helveticus*, and the authors determined a limit of detection with 10^5 cfu per g cheese. Multiplex real-time PCR systems have also been described that amplify parts of the *pheS*, *hsp60*, *prtH*, or 16S rRNA genes of *L. helveticus* (Bottari, Agrimonti, Gatti, Neviani, & Marmiroli, 2013; Cremonesi et al., 2011; Herbel et al., 2013; Lu, Kong, Yang, & Kong, 2015). The purpose of these assays is the simultaneous detection and enumeration of *L. helveticus* in LAB mixtures. The detection limits described ranged from 10^4 cfu per mL (Herbel et al., 2013) to 183 copies (Bottari et al., 2013).

Remarkably, none of the mentioned qPCR systems was tested with gDNA from *Lactobacillus gallinarum*, which is the closest relative of *L. helveticus*. To our knowledge, only two PCR methods that can be used for the qualitative detection of *L. helveticus* have used *L. gallinarum* for specificity testing (Jebava et al., 2014; Ventura et al., 2000). Although *L. gallinarum* is typically found in the intestinal tracts of poultry, it has also been detected in cheese (Van Hoorde, Verstraete, Vandamme, & Huys, 2008). Therefore, we assume that differentiating between *L. gallinarum* and *L. helveticus* is an important requirement for a qPCR detection method in the dairy field.

In the present study, a simple and fast qPCR assay for the detection and quantification of *L. helveticus* in food was developed. Specific primers and a hydrolysis probe were designed to target a region of the phenylalanyl-tRNA synthase (*pheS*) gene, which has previously been proven to be a suitable target gene for the specific identification of LAB species (Naser et al., 2007). We tested our qPCR assay with 48 strains belonging to 17 LAB species (Table 1). We found that the assay was highly specific to *L. helveticus* because neither the closely related *L. gallinarum*, *Lactobacillus kefiranofaciens*, *Lactobacillus crispatus*, and *Lactobacillus acidophilus* nor any of the other LAB species were detected with the assay.

We used a plasmid standard for absolute quantification. Because previous studies have shown that the use of circular plasmid standards in qPCR leads to a significant overestimation of the expected concentration (Hou, Zhang, Miranda, & Lin, 2010; Lin, Chen, & Pan, 2011), we used linearised plasmids in our qPCR assays. The qPCR showed a linear quantification over a range of 8 logs, with a limit of detection of ten copies per reaction (Fig. 1). Given the assumption that the *pheS* gene is a single copy gene, ten bacterial cells can be detected. The method described herein is therefore more sensitive than the methods mentioned above.

In comparison with colonies identified via plate counting, we determined approximately one additional log of copies of the *pheS* gene. We assume that this difference is mainly explained by cell chains that are not separated during plating. In fact, upon microscopic inspection, we observed that the *L. helveticus* strains used in this study form cell chains (data not shown). A further explanation could be the presence of dead bacterial cells. Discrepancies between culture-dependent and culture-independent methods were also reported by other researchers (Postollec et al., 2011). Viability dyes, such as ethidium monoazide or propidiume monoazide, which covalently modify the DNA of bacteria with compromised membranes and consequently cannot be amplified further by PCR, could be used to estimate the number of dead cells (Elizaquivel, Aznar, & Sanchez, 2014).

To evaluate the applicability of this method, we assayed NWCs, cheese, and milk (Table 2). The NWCs were collected from three cheese factories producing Gruyère PDO. We found that all NWCs contained *L. helveticus* ranging from 10^6 to 10^8 cells (copies) mL^{-1}, indicating that *L. helveticus* is one of the predominant species present in these NWCs. *L. helveticus* was also found to be one of the predominant species present in the natural starter cultures used for

the production of Grana Padano and Parmigiano Reggiano (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014). It is noteworthy that the production parameters for Gruyère PDO differ significantly from those for Italian cooked, extra-hard cheeses and that the microbial composition of these NWCs has not yet been studied in detail. Further studies will reveal whether the microbial composition of the NWCs used for Gruyère PDO production are similar to or different from that of Italian natural whey starters.

The detection of *L. helveticus* at a low population density in raw milk implies that this ecosystem is also a habitat for *L. helveticus* strains. Coppola et al. (2006) also reported the presence of this species in raw milk that was used for the manufacturing of a pasta-filata cheese.

With regard to cheese, we were able to detect and enumerate *L. helveticus* in Gruyère PDO cheese and various other cheese types. The appearance of intracellular high-molecular-weight compounds, such as DNA, RNA, and bacterial enzymes, can be viewed as indicators of bacterial autolysis, which is associated with enhanced protein degradation during cheese ripening (Lortal & Chapot-Chartier, 2005). DNA, as long as it is not completely degraded, has the advantage of being a species-specific and sensitive indicator of autolysis in a mixed population. By applying a filter technique that removes whole cells, we observed, in a model-type cheese manufactured with controlled production parameters, that *L. helveticus* reached more than 10^8 copies g^{-1} cheese after 24 h. No free DNA of this species was detected at this time-point. However, more than 10^7 free *pheS* gene copies g^{-1} were detected after 80 and 120 days of ripening, indicating the leakage of DNA out of the cells into the cheese matrix. This free DNA was obviously not rapidly consumed by other bacteria. This is in agreement with Gatti et al. (2008) who also detected significant amounts of free DNA from *L. helveticus* in raw milk cheese that had ripened for 6 months. Furthermore, Treimo, Vegarud, Langsrud, and Rudi (2006) amplified regions of the 16S rDNA gene of lactococci and propionibacteria present in the supernatant of a liquid cheese model after five weeks of cultivation. Therefore, it can be assumed that bacterial free DNA, even if considerably fragmented, is stable enough to be amplified by PCR during the first months of cheese ripening. Compared with the more laborious immunoassays, which also detected species-specific intracellular constituents, a PCR-based method, combined with filter techniques, seems to be an easy-to-use alternative to investigate bacterial autolysis and its impact on a cheese ecosystem harbouring a mixed population.

5. Conclusions

The qPCR assay developed herein for the detection of *L. helveticus* proved to be highly specific; we were able to discriminate between *L. helveticus* and its closest relative, *L. gallinarum*. Its high specificity and sensitivity make this qPCR assay a suitable tool for the detection and quantification of *L. helveticus* in dairy products. The assay can be used for the rapid identification of colonies on agar plates; for the study of the spatial and temporal distributions of *L. helveticus* during cheese ripening; for the identification of autolytic strains indicated by the presence of “free” DNA in medium or food; and for the detection of habitats of *L. helveticus* in the process of milk production and transformation. Overall, the qPCR assay presented here is a useful tool for various applications in research and industry.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2016.12.007.

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