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Development of new methods for the quantitative detection and typing of *Lactobacillus parabuchneri* in dairy products

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ABSTRACT

Thirty-one isolates of *Lactobacillus parabuchneri* were obtained from cheese containing histamine; of these, 26 were found to possess the *hdcA* gene encoding histidine decarboxylase. By analysing the genome data of 13 isolates, specific targets for the development of PCR-based detection and typing systems for *L. parabuchneri* were identified. The real-time PCR for detection showed a linear quantification over a range of 7 logs and a detection limit of 10 gene equivalents per reaction. The strain typing method utilised the amplification of repeat sequences and showed discrimination comparable with a phylogenetic tree, based on genome comparisons. The method was suitable for detecting and monitoring the development of *L. parabuchneri* in raw milk and cheese.

1. Introduction

In recent years, increasing attention has been paid to the presence of histamine in food, as the intake of this substance can result in health problems, such as headaches or diarrhoea. People with histamine intolerance are particularly affected. In cheese, histamine is mainly produced by bacteria that display histidine decarboxylase activity (Landete, De Las Rivas, Marcobal, & Munoz, 2008). Two classes of histidine decarboxylases can be distinguished: one is dependent on pyridoxal-phosphate and is mainly found in Gram-negative bacteria, and the other is dependent on pyruvoyl-phosphate and is typically present in Gram-positive bacteria. The latter is encoded by the *hdcA* gene, and the gene product has been well characterised in *Lactobacillus saerimneri* 30a (formerly known as *Lactobacillus* 30a).

The species *Lactobacillus parabuchneri* sp. nov. was first described by Farrow, Phillips, and Collins (1988). The type strain was originally isolated from human saliva, but other isolates have been obtained from cheese, brewery yeasts, ropy beer, and silage (Beneduce et al., 2010; Sakamoto & Konings, 2003; Wang & Nishino, 2010; Wittwer, 2011). Sumner, Speckhard, Somers, and Taylor (1985) reported that a histamine poisoning outbreak was related to the consumption of a type of Swiss cheese. The authors isolated the histamine-forming bacteria from the suspicious cheese and identified these biochemically and via a DNA–DNA hybridisation study as *Lactobacillus buchneri*. Later, Sumner, Roche, and Taylor (1990) manufactured cheese with one of the *L. buchneri* strains, namely strain St2A, and showed that the presence of this strain led to histamine formation during cheese ripening. The strain St2A has since been re-identified as *L. parabuchneri* and is available, for example, from the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG 11773).

L. parabuchneri is a Gram-positive, facultative anaerobic, and catalase negative rod that grows at 15 °C, but not at 45 °C. It can convert arginine into ornithine, CO_2, and ammonia using the arginine deiminase (ADI, EC 3.5.3.6) pathway (Manca De Nadra, Pesce de Ruiz Holgado, & Oliver, 1988). It is an obligate heterofermentative lactic acid bacterium that ferments arabinose, melezitose, and melibiose as well as ribose, galactose, glucose, fructose, maltose, and sucrose, but not cellobiose or trehalose (Hammes & Hertel, 2006). Although the species *L. buchneri* and *L. parabuchneri* are closely related phylogenetically, the latter is not able to metabolize xylose (Fröhlich-Wyder et al., 2013). Under anaerobic conditions, both species are capable of converting lactic acid to acetic acid, CO_2, and 1,2-propanediol (Oude Elferink et al., 2001). Histidine decarboxylase (HDC, EC 4.1.1.22) and glutamate

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decarboxylase (GAD, EC 4.1.1.15) activity are strain-specific characteristics of *L. parabuchneri* and *L. buchneri*, respectively (Cho, Chang, & Chang, 2007; Fröhlich-Wyder et al., 2013).

In cheese, the metabolic activity of *L. buchneri* and *L. parabuchneri* often leads to the accumulation of biogenic amines, the excessive formation of CO_2, and an increase in pH, since proteolysis continuously releases free amino acids (Fröhlich-Wyder et al., 2013). Owing to the many different metabolic activities, it is not surprising to find *L. buchneri* and *L. parabuchneri* in fermented products that have low oxygen but high lactic acid availability. At the end of the last century, *L. buchneri* was implemented in silage starters to increase aerobic stability against deterioration by yeasts and moulds (Holzer, Mayrhuber, Danner, & Braun, 2003). In contrast, the presence of *L. buchneri* is undesired in fermented foods, such as cheese, beer, or wine (Beneduce et al., 2010; Leuschner, Kurihara, & Hammes, 1998; Sakamoto & Konings, 2003).

In this paper, we report the isolation of *L. parabuchneri* from various cheeses containing histamine. By exploiting genome data of *L. parabuchneri* FAM21731, genetic target sequences were identified and used to develop a quantitative real-time PCR and a simple and reliable PCR-based strain typing method.

2. Materials and methods

2.1. Bacterial strains and media

The strains and isolates used in this study are listed in Tables 1 and 2. All strains/isolates were stored at −80 °C in sterile reconstituted skim milk powder (10%, w/v) and grown in MRS broth (de Man, Rogosa, & Sharpe, 1960) at 30 °C.

2.2. Isolation of L. parabuchneri from cheese

To isolate histaminogenic bacteria from cheese, the rind of each cheese was removed and 10 g of the remainder cheese sample was homogenised in 40 °C warm peptone water (10 g L^{-1} peptone from casein, 5 g L^{-1} sodium chloride, 20 g L^{-1} tri-sodium citrate dihydrate, pH 7.0) for 3 min in a stomacher (Masticator, IUL Instruments GmbH, Königswinter, Germany). Serial dilutions of the homogenate were then plated on modified decarboxylating agar containing histidine (MDA-H), which was prepared as described by Maijala (1993). In addition to the original protocol, 50 mg L^{-1} of pyridoxal-5-phosphate was added to the medium. Colonies were inoculated and purified in MRS broth. Isolates that grew in the MRS broth were identified by partial sequencing of the *tuf* gene (Chavagnat, Haueter, Jimeno, & Casey, 2002). The presence of the *hdcA* gene was tested as described by Coton and Coton (2005).

2.3. DNA extraction from bacteria

DNA was extracted from 1 mL of cultures with the EZ1 DNA Tissue Kit (Qiagen, Hombrechtikon, Switzerland) after pretreatment of the sample, as described previously (Chavagnat et al., 2002). Pretreatment consisted of incubation in 0.05 M sodium hydroxide for 15 min at room temperature, and then in TES buffer [100 mmol L^{-1} Tris(hydroxymethyl)-aminomethane, 10 mmol L^{-1} ethylenediaminetetraacetic acid, 25%, w/v, sucrose, pH 8.0] with 1 mg mL^{-1} lysozyme for 1 h at 37 °C. The DNA was eluted in 100 μL buffer EB provided in the kit (Qiagen).

Table 1
Cheeses used for the isolation of *L. parabuchneri* strains.

Cheese type	Inspected samples	Number of isolates[a]
Emmental	2	2 (2)
Tête de Moine	3	9 (9)
Mont Soleil	1	1 (1)
Tilsit	3	3 (3)
Alpine cheese	1	15 (10)
Raclette	1	1 (1)

[a] The number in brackets indicate how many of the isolated strains possessed the histidine decarboxylase gene *hdcA*.

2.4. DNA extraction from milk

A preparation of bacterial pellets from 4 mL of raw milk samples and DNA extraction was made as described by Turgay, Schaeren, Wechsler, Bütikofer, and Graber (2016). Following their description as well, 150 μL of an overnight culture of *Lactobacillus casei* FAM19404 grown in MRS broth was added to the milk as the carrier for the centrifugation steps.

2.5. DNA extraction from cheese

DNA was extracted from samples taken aseptically from the inner part of the cheese. The sample (10 g) was homogenised in 40 °C warm peptone water as described above. After adding 50 μL of 10% sodium dodecyl sulphate, the homogenate was centrifuged (4000 × *g*, 21 °C, 30 min). The pellet containing bacteria was treated with lysozyme and DNA was then extracted as described above.

2.6. Whole-genome sequencing

Twelve strains were selected for whole-genome sequencing with Ion Torrent technology (ThermoFisher Scientific, Zug, Switzerland). Library preparation, amplification, and sequencing were performed using an Ion Xpress Plus Fragment Library Kit, Ion PGM Template OT2 400 Kit, and Ion PGM Sequencing 400 Kit v2 (ThermoFisher Scientific) according to the manufacturer's instructions. Libraries were bar coded (Ion Xpress Barcode Adapters 1–16 Kit, ThermoFisher Scientific), and then pooled and sequenced on an Ion 318 chip (ThermoFisher Scientific). The PacBio sequencing and annotation of the genome from *L. parabuchneri* FAM21731 was described by Wüthrich et al. (unpublished data).

2.7. Core genome-based maximum likelihood tree construction

The Ion Torrent reads were assembled using SPAdes (version 3.1.0) using the options: –careful –mismatch-correction -k 21, 33, 55, 77, 99, 127 (Bankevich et al., 2012). Contigs with a lower mean read-depth than 10% of the mean read-depth of the whole genome and contigs shorter than 500 bp were excluded.

Prokka (version 1.8) (Seemann, 2014) was then used to perform the annotation of the genomes of the 12 *L. parabuchneri* strains.

Orthologous gene clusters (OGCs) were constructed using Ortho-MCL (version 2.0.9, default parameters) (Li, Stoeckert, & Roos, 2003). All proteins that were present as single orthologs in the 13 strains were used to construct the phylogenetic tree. The nucleotide sequences of these proteins were aligned separately for each OGC using Clustal Omega (version 1.1.0) (Sievers et al., 2011). The merged alignments of 1718 OGCs were used to construct a maximum-likelihood tree with RAxML (version 8.1.2, options: -m GAMMAWAG -# 1000) (Stamatakis, 2014).

2.8. Target mining and the design of the primers

To find unique nucleotide sequences for *L. parabuchneri*, the assembled genome data from *L. parabuchneri* FAM21731 was aligned against the genomes of the *L. buchneri* strains CD034 and NRRL B-30929 using BLASTN. Regions of the genome of FAM21371 homologous to any of the genomes from the two *L. buchneri* strains

Table 2
Strains used to assay the specificity of the quantitative PCR assay.[a]

Strain	Origin	Source
Lactobacillus parabuchneri		
DSM 5707[T]	Human saliva	DSMZ
DSM 5987	Cheese	DSMZ
LMG 11773	Cheese	BCCM
FAM21731, FAM21822, FAM21829	Emmental cheese	This study
FAM21809, FAM23163, FAM23164, FAM23165, FAM23166, FAM23167, FAM23168, FAM23097	Tête de Moine cheese	This study
FAM21823	Mont Soleil cheese	This study
FAM21831, FAM21832, FAM21834	Tilsit cheese	This study
FAM21836	Raclette cheese	This study
FAM21838 (hdc negative)	Alpine cheese	This study
Lactobacillus buchneri		
LMG 11439	Unknown	BCCM
LMG 11975	Marinated herring	BCCM
LMG 11985	Oral cavity	BCCM
DSM 20057[T]	Tomato pulp	DSMZ
Lactobacillus hilgardii		
LMG 11450	Sugar beet factory	BCCM
DSM 20176[T]	Wine	DSMZ
Lactobacillus malefermentans		
LMG 11416	Beer	BCCM
Lactobacillus casei		
FAM19280, FAM19404	Emmental cheese	ACC
Lactobacillus rhamnosus		
FAM21825	Emmental cheese	ACC
DSM 20021[T]	Unknown	DSMZ
FAM17803	Gruyère cheese	ACC
Lactobacillus delbrueckii subsp. *lactis*		
DSM 20072[T]	Emmental cheese	DSMZ
FAM19356	Emmental cheese	ACC
Lactobacillus plantarum		
FAM22729	Unknown	ACC
FAM20713	Tilsit cheese	ACC
Lactococcus lactis subsp. *lactis*		
FAM17953	Tilsit cheese	ACC
FAM17957	Gruyère cheese	ACC
FAM3984	NCFB 176	ACC
FAM18385, FAM1746	Unknown	ACC
Lactococcus lactis subsp. *cremoris*		
FAM22494	Whey	ACC
FAM17878	Milk	ACC
Leuconostoc lactis		
DSM 20192	Cheese	DSMZ
Pediococcus acidilactici		
FAM19149	Sbrinz cheese	ACC
FAM18988	Tête de Moine cheese	ACC
Pediococcus pentosaceus		
FAM19145	Sbrinz cheese	ACC
FAM19067	Tête de Moine cheese	ACC
Streptococcus thermophilus		
FAM20427	Gruyère cheese	ACC
FAM21633	Undefined starter culture	ACC
Enterococcus faecalis		
FAM20752, FAM20822	Tilsit cheese	ACC
FAM20853	Raclette cheese	ACC
Enterococcus faecium		
FAM20768	Tilsit cheese	ACC
FAM20342	Gruyère cheese	ACC
FAM20567	Raclette cheese	ACC
Enterococcus durans		
FAM22245	Whey	ACC
FAM20391	Emmental cheese	ACC
DSM 20622[T]	Dried milk	DSMZ

[a]Abbreviations are: DSMZ, German Collection of Microorganisms and Cell Cultures, BCCM, Belgian Coordinated Collections of Microorganisms; ACC, Agroscope Culture Collection; [T]designates the type strain of the species.

were removed for downstream analysis, as they are not unique for *L. parabuchneri*. Subsequently, Ion Torrent reads of 12 *L. parabuchneri* strains (Table 1) were mapped onto the genome of *L. parabuchneri* FAM21371 using Bowtie 2 (Langmead & Salzberg, 2012). We used the resulting alignments to perform SNP-calling using Samtools with the options: mpileup -d 10000 -L 1000 -Q 7 -h 50 -o 10 -e 17 -m 4 (Li et al., 2009) to find highly conserved regions on the nucleotide sequence level. A region that showed no variance was selected for the design of primer/probe sets (Table 3).

To identify targets for strain typing, repeated sequences were searched in the genome sequence of *L. parabuchneri* FAM21731 using the Tandem Repeats Finder tool (Benson, 1999) and CRISPR Finder (Grissa, Vergnaud, & Pourcel, 2007). Primers and probes were designed with Primer Express Software V2.0 (ThermoFisher Scientific) and synthesised by Microsynth (Balgach, Switzerland).

2.9. Real-time PCR conditions

The quantitative real-time PCR (qPCR) assays were performed in a reaction volume of 12 μL containing 6 μL of qPCR™ MasterMix No ROX (Eurogentec, Seraing, Belgium), 300 nM of forward and reverse primer, 100 nM of hydrolysis probe (Table 3), and 2 μL of DNA. The qPCR conditions were 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 1 min. All qPCR assays were run on Corbett Rotor-Gene 3000 (Qiagen), and a serial dilution of the plasmid pGEM-T/tmp was included in each run. The analysis was performed using Rotor-Gene 6000 Software 1.7 using a threshold of 0.05 for the quantification cycle (Cq) value determination. If an amplification was not detected in replicate measurements, a Cq value of 40 was assigned to the corresponding reaction and included in the calculation of the mean.

2.10. PCR specificity, sensitivity, and efficiency

Specificity was tested against strains of *L. parabuchneri* and other lactic acid bacteria from other genera (Tables 1 and 2). To evaluate the sensitivity, we analysed a plasmid carrying the target sequence and *L. parabuchneri* diluted in milk and cheese homogenate, respectively.

The plasmid was constructed by cloning a part of the *tmp* gene that had been amplified with the primer pair tmp_F/_R (Table 3) from the genomic DNA of *L. parabuchneri* FAM21731 into the pGEM-T Easy vector (Promega, Switzerland). The plasmid called pGEM-T/*tmp* was maintained in *Escherichia coli* DH5α and isolated with QIAprep Spin Miniprep Kit (Qiagen). The amount of plasmid was quantified in triplicate using NanoDrop (ThermoFisher Scientific), and the number of copies μL^{-1} was calculated using the constant value 660Da as the average weight of base pairs. Three independently-prepared dilution series were prepared in 10 mM Tris(hydroxymethyl)-aminomethane (pH 8.0) containing 10 ng μL^{-1} of herring sperm DNA (Sigma–Aldrich, Switzerland). One of the dilution series was measured in sextuplicate and the other two in duplicate.

Standard curves were constructed by plotting Cq against gene equivalents (GE) per reaction. PCR efficiencies were calculated taking the slope from the standard curves using the following equation:

$$\text{PCR efficiency} = 10^{(-1/\text{slope})} - 1 \quad (1)$$

2.11. Spiking of milk and cheese homogenates with L. parabuchneri

With regard to milk, raw milk obtained from one cow by hand-milking was used. Hand-milking was performed to avoid the risk of contaminating the collected milk with a high bacterial load. The absence of indigenous *L. parabuchneri* in the collected milk was confirmed using the qPCR assay. A culture of *L. parabuchneri* FAM21731, of which the population level had been determined by

Table 3
Primer, probes, and plasmids used in this study.

Primer	Sequence (5′ to 3′)	Used for
Lbpb_F/R	TGGAATTAACGGCTTGCTCTTAC CAACCCAATTAGGCCAAGGA	Primer pair for the specific detection of *L. parabuchneri*
Lbpb_FAM	CCGGTTCTGCTCATTGGCGCA	Hydrolysis probe for the specific detection of *L. parabuchneri*
tmp_F/_R	ATTTGGCGTTATCGGTCTTGC AATTTAAGCAGTAATGGACCTAAGAACC	Partial cloning of the *tmp* gene
pGEM-T/*tmp*	Plasmid pGEM-T containing a part of the *tmp* gene	Standard curve
MS1_F/R	GACGAAACCGTCCCTCCA GGGCTCCGGGGTTTCA	Primer pair for strain typing
MS2_F/R	ACCTAATCAGCCGAGTCAACCTAG TGACCTGGCTTAGTTGGCTTT	Primer pair for strain typing
MS3_F/R	ACACCAAAACGGTCACCGTTA GCTTTTACAACTCGTTTCTTTTTAGTCTTAGT	Primer pair for strain typing
MS4_F/R	TCGTTCAGTGACAAAGCCACTT GGTGATTATGAAGAACCGGCA	Primer pair for strain typing
MS5_F/R	GAATAGCCTGCTACCAGCTTATGAG ATTCKTTTATAGTAAACTGTTAGTAGGATCGTTAGA	Primer pair for strain typing
HDC3/HDC4	GATGGTATTGTTTCKTATGA CAAACACCAGCATCTTC	Coton and Coton (2005)

plate counting, was then tenfold serially diluted in this milk. DNA extracted from the spiked milks was measured by qPCR in duplicate. The spiking procedure was repeated two times. To assess the precision of the qPCR method at low levels of *L. parabuchneri*, milk samples containing less than 100 colony forming units (cfu) mL^{-1} of milk, based on the estimation by plate counting, were extracted five times.

With regard to cheese, a Gruyère cheese homogenate was prepared, and the absence of indigenous *L. parabuchneri* was confirmed using the qPCR assay. Then, a culture of *L. parabuchneri* FAM21731 was tenfold serially diluted in the cheese homogenate. The population level of the *L. parabuchneri* culture used for spiking was again determined by plate counting. DNA was extracted from each dilution step and tested using qPCR in duplicate. The procedure was repeated two times.

To determine the PCR efficiencies, Cq was plotted against the $\log_{10}$ cfu mL^{-1} and cfu g^{-1} for milk and cheese, respectively.

2.12. Determination of L. parabuchneri in milk and cheese

The gene equivalents (GEs) in milk and cheese were determined using the plasmid standard curves performed for each analysis run. The results from the milk and cheese samples were multiplied with dilution factors of 12.5 and 50, respectively.

2.13. Determination of histamine

The presence of histamine in culture supernatants was assayed as described by Fröhlich-Wyder et al. (2013). The level of histamine in cheese was determined by high performance liquid chromatography using pre-column derivatisation of biogenic amines with dansyl chloride (Fröhlich-Wyder et al., 2013).

2.14. Multiplex-PCR conditions for strain typing

Multiplex amplification was performed in a 25 μL reaction mixture containing 12.5 μL of 2× QIAGEN Multiplex PCR Master Mix, 2.5 μL of 10× primer mix (2 μM of each primer listed in Table 3), and 2 μL of DNA (with 1 ng μL^{-1}). After the initial heat activation at 95 °C or 10 min followed 35 cycles at 94 °C for 30 s, 58 °C for 90 s, 72 °C for 90 s, and the final extension by 72 °C for 10 min. The amplification products were separated using the DNF-910 dsDNA 910 Reagent Kit (separation range 15–1500 bp) on a Fragment Analyzer™ (Advanced Analytical Technologies, Ankeny, IA, USA) according to the manufacturer's instructions. The results were evaluated and compared with the PROSize software (Advanced Analytical Technologies).

3. Results

3.1. Isolation of L. parabuchneri from cheese

To isolate aminogenic bacteria, we used retail cheeses (Table 1) that had been found to contain histamine. When the cheese homogenates were plated on MDA-H agar, in all cases we observed the growth of purple colonies with an irregular, umbonate morphology. The formation of a purple colour is provoked by a pH shift caused by the production of alkaline metabolites such as histamine. To confirm that histamine in particular was produced, we transferred the colonies to MRS broth supplemented with 0.2% L-histidine. Following incubation for three days, we detected that all isolates formed histamine by thin-layer chromatography. Additionally, we confirmed the presence of the *hdcA* gene in all isolates using PCR. By analysing the sequence of the *tuf* gene, all isolates were identified as *L. parabuchneri* strains.

In one cheese, an Alpine cheese, we observed, in addition to the purple colonies, white colonies that exhibited the same morphology as the purple ones. Analysis based on a partial sequence of the *tuf* gene revealed that these colonies were *L. parabuchneri* strains. These isolates neither produced histamine nor possessed the *hdcA* gene.

3.2. Performance of qPCR assay

To establish a qPCR assay, we sequenced the genomic DNA of 13 *L. parabuchneri* isolates. By exploiting the genome data, a unique protein-coding sequence (CDS) that was present as a single copy gene and comprised 17,682 bp was identified. Meanwhile, the genome sequence of *L. parabuchneri* DSM 5707 was deposited in the GenBank database (NZ_AZGK00000000) and the unique CDS is accessible with the locus_tag FC51_RS03280. The function of the gene is unknown and an Interpro scan (https://www.ebi.ac.uk/interpro/) of the deduced protein sequence predicted a signal peptide and transmembrane domains. For clarity, we will use the gene name *tmp* for this locus in this report. The nucleotide sequence of the *tmp* gene was used to design a primer/probe set for a qPCR assay. To evaluate the specificity of the primer/probe set, we tested 31 isolated *L. parabuchneri* strains and three strains of

L. parabuchneri from other culture collections (Table 2). Additionally, the primer/probe set was tested against closely related lactobacilli, such as *L. buchneri*, *Lactobacillus hilgardii*, *Lactobacillus malefermentans*, and other lactic acid bacteria often present in cheese (Table 2). Only *L. parabuchneri* strains yielded a positive signal with the primer/probe set that was used. For all other species, no signal was obtained.

To evaluate the performance of the qPCR assay, first, tenfold serial dilutions of the plasmid pGEM-T/*tmp* were assayed. The plasmid dilution series showed linearity over 7 logs with a correlation coefficient (R^2) of 0.999 (Fig. 1A). A PCR efficiency of 1.02 was calculated. The lower limit of detection (LOD) was set at 10 GE per reaction since the sixfold determination of 10 copies of pGEM-T/*tmp* per reaction yielded positive results. The lower limit of quantification (LOQ) was set at 100 GE per reaction. At this concentration, the standard deviation of the six replicates was smaller than ±0.5 Cq.

Furthermore, bacterial cultures of *L. parabuchneri* FAM21731 containing between 4×10^8 and 8×10^8 cfu mL^{-1} were tenfold serially diluted in milk that had been tested negative for the presence of *L. parabuchneri*. The DNA extracts from spiked milks showed a linearity over 7 logs with a correlation coefficient (R^2) of 0.995 (Fig. 1B). The PCR efficiency was calculated to be 1.02. To assess the precision at low concentrations of *L. parabuchneri* in milk, five DNA extractions per dilution were carried out. Fig. 2 shows that above 100 GE per reaction, the standard deviation was smaller than ±0.5 Cq, allowing accurate quantification. Between 10 and 100 GEs per reaction, 8 of 10 reactions, 9 of 10 reactions, and 10 of 10 reactions obtained a positive signal (Cq below 40).

Finally, a Gruyère cheese homogenate was spiked with a culture of *L. parabuchneri* FAM21731 that contained 8×10^8 cfu mL^{-1}. The DNA extracted from tenfold dilution series showed linearity over 7 logs with a correlation coefficient (R^2) of 0.999 (Fig. 1C). The PCR efficiency was 0.99. At the lower limit of linearity, a mean Cq value of 31.9 with a standard deviation of ±0.4 Cq (N = 6) was obtained.

3.3. Applicability for milk from farms

To determine the naturally occurring population levels of *L. parabuchneri* in milk coming from farms, we analysed 29 raw milk samples from milk producers that delivered their milk to a cheese-maker whose cheese showed histamine formation during ripening. Three samples were within the range of quantification and showed 153, 168, and 192 GE per reaction, which corresponded to 1.9×10^3, 2.1×10^3, and 2.4×10^3 GE mL^{-1} milk, respectively. Nine samples were between 10 and 100 GE per reaction and considered positive. The remaining 17 samples were below the LOD.

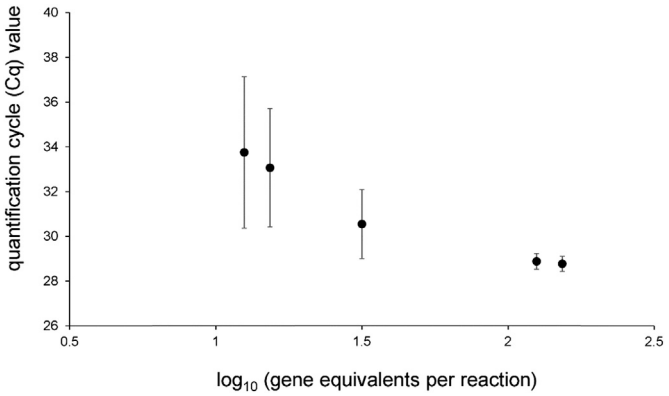

Fig. 2. Precision of the quantitative PCR assay at low population levels of *L. parabuchneri* FAM21731 in raw milk. Error bars represent the standard deviation from five DNA extractions measured in duplicate.

3.4. Applicability for commercial cheeses

Eight commercial cheeses with histamine contents ranging between 270 and 1012 mg per kg of cheese were analysed for the presence of *L. parabuchneri* (Table 4). The results of the qPCR analysis revealed that *L. parabuchneri* was present in all cheeses with population levels between 10^7 and 10^8 GE g^{-1} cheese.

3.5. Evaluation of repeats for the development of a typing method

In silico analysis revealed repeated sequences within the genome of *L. parabuchneri*. Thirty-three primer pairs predicted to amplify these repeated sequences were designed. Each primer pair was tested individually with a set of 10 *L. parabuchneri* strains from diverse origins (FAM21731, LMG 11773, FAM21822, FAM21823, FAM21829, FAM21834, FAM21835, FAM21836, FAM21838, and

Table 4
Gene equivalents (GE) of *Lactobacillus parabuchneri* determined in various commercial European cheese containing histamine.

Cheese[a]	Origin	Histamine (mg kg^{-1})	*L. parabuchneri* (GE g^{-1})
Farmhouse cheese	Netherlands	957	8.5×10^7
Emmentaler PDO	Switzerland	270	1.3×10^7
Tête de Moine PDO	Switzerland	330	6.0×10^7
Goat milk cheese	Switzerland	1012	2.6×10^7
Manchego PDO	Spain	749	1.1×10^7
Alpine cheese	Switzerland	774	2.3×10^7
Abondance de Savoie PDO	France	291	8.0×10^7
Queijo Sao Jorge PDO	Portugal	545	1.7×10^7

[a] PDO, Protected Designation of Origin.

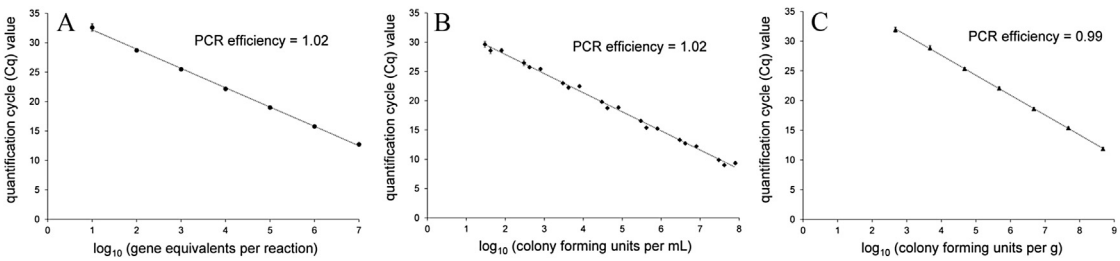

Fig. 1. Standard curves obtained by quantitative PCR analysis for pGEM-T/*tmp* (A), *L. parabuchneri* FAM21731 spiked into raw milk (B), and *L. parabuchneri* inoculated in cheese homogenate (C). Quantification cycle values were plotted against log gene equivalents per reaction for pGEM-T/*tmp*, $\log_{10}$ cfu mL^{-1} for milk, and $\log_{10}$ cfu g^{-1} for cheese. Error bars represent the standard deviation (n = 6 for plasmid; n = 2 for milk; n = 6 for cheese).

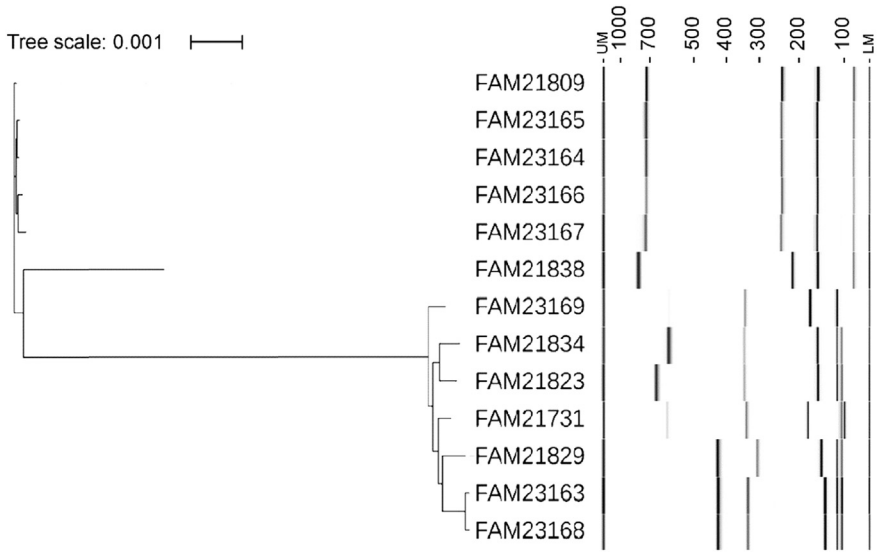

Fig. 3. The relationship of *L. parabuchneri* strains revealed by a phylogenetic tree based on core genome data (left-hand side) and by multiplex PCR-based typing (right-hand side). For the latter, the amplicons were separated with capillary electrophoresis and are presented in a gel-like view. Numbers above the right-handed side illustration represent the size of the DNA ladder in bp. UM, upper marker; LM, lower marker.

DSMZ 5707). Most of the primer pairs showed only two alleles, dividing the strains into two groups (data not shown). The first, group A, contained the strains FAM21731, FAM21823, FAM21829, FAM21834, LMG 11773, and DSM 5707, and the second, group B, contained the strains FAM21822, FAM21835, FAM21836, and FAM21838. Three primer pairs, namely MS2, MS3, and MS5 (Table 3), generated amplicons with the highest variability, and the amplification products ranged between 220 and 350 bp for MS2, 140 and 180 bp for MS3, and 430 and 800 bp for MS5, depending on the strain. The primer pair MS1 (Table 3) did not amplify a DNA fragment using genomic DNA from the group B strains. For the group A strains, the amplicon was 100 bp, except for DSM 5707, which showed an amplicon of 90 bp. The primer pair MS4 amplified a DNA fragment of approximately 120 bp and 70 bp for the strains from group A and group B, respectively, except for FAM21731, of which an amplicon of 100 bp was obtained. Based on these results, these five primer pairs were combined for the multiplex PCR reaction. The pattern obtained from these PCR reactions allowed the differentiation of the 10 strains mentioned above (data not shown).

In an additional step, the multiplex PCR was applied to the 13 genome-sequenced strains and the pattern was compared with the maximum-likelihood tree based on core genome data (1718 OGCs, Fig. 3). We found that strains with identical PCR patterns also clustered closely together in the core genome-based tree.

4. Discussion

We repeatedly isolated and detected *L. parabuchneri* in cheese containing histamine, indicating that this species plays an important role in histamine formation. The association of *L. parabuchneri* with histamine formation has been previously described by Fernandez, del Rio, Linares, Martin, and Alvarez (2006) and Schirone et al. (2013) as well. The authors used a qPCR system that targets the *hdcA* gene of *L. buchneri* B301 (actually a *L. parabuchneri* strain; M. A. Alvarez, personal communication) and showed that the presence of this gene in cheese was associated with histamine. In addition, cheese trials using *L. parabuchneri* as adjuncts demonstrated that the species in fact produces high amounts of histamine in cheese (Fröhlich-Wyder et al., 2013; Joosten & Northolt, 1989; Sumner et al., 1990).

In this study, a new qPCR assay was established that can be used to detect and enumerate *L. parabuchneri* in raw milk and cheese. By comparing genome data, we found the unique 17,682 bp locus *tmp* of which we did not find homologues in other species using BLAST (data not shown). The primer/probe set, which targets a 73-bp region of the *tmp* gene, only generated amplicons from genomic DNA of *L. parabuchneri* and was found to be suitable for quantitative analysis with an LOD and LOQ of 10 and 100 GE per reaction, respectively (corresponding to 125 and 1250 GE mL^{-1} milk and 500 and 5000 GE g^{-1} cheese). Since the PCR efficiencies for milk and cheese extracts were close to the PCR efficiency of the plasmid standard, the plasmid standard can be used for the relative quantification of dairy samples.

The results obtained from the analysis of milk samples used in a cheese factory to make cheese showed that approximately 40% of the milk producers contributed to *L. parabuchneri* contamination. Therefore, the qPCR assay is suitable for the rapid identification of milk samples containing *L. parabuchneri* and can be used to classify samples with low (below LOQ) and high (above LOQ) population levels.

Interestingly, in one case (Alpine cheese, Table 1) we isolated five *L. parabuchneri* strains that lack the *hdcA* gene and consequently did not produce histamine. Strain typing using the multiplex-PCR methods revealed no genetic differences between these isolates (data not shown), indicating that these all belong to the same strain. This case demonstrates that non-histamine forming *L. parabuchneri* strains can also be present in cheese. In such a case, the qPCR method can overestimate of the histamine-forming *L. parabuchneri* present in cheese. The use of a qPCR enumerating the *hdc* gene (Fernandez et al., 2006) and of the qPCR for *L. parabuchneri* is a possible strategy to study the occurrence and distribution of non-histamine forming *L. parabuchneri* in cheese.

It also needs to be considered that *L. parabuchneri* shows heterolactic fermentation, produces 1,2-propanediol, and possesses an active arginine deiminase pathway (Fröhlich-Wyder et al., 2013). These metabolic activities are accompanied with gas production, which influences cheese quality. Consequently, at the current state of knowledge, the presence of *L. parabuchneri* in cheese is undesirable and the qPCR method provides a valuable tool for the detection and tracking of this species in the food chain. Moreover,

the qPCR assay can also be used to study factors that limit the growth of *L. parabuchneri* in cheese, such as the use of food additives or of appropriate starter and adjunct cultures.

The study also presents an easy-to-use species-specific typing method for *L. parabuchneri*. The established multiplex PCR method used five primer pairs and was found to be an efficient tool for the discrimination of strains of *L. parabuchneri*. Recently, Ascone et al. (unpublished data) employed the herein described methods to trace strains from *L. parabuchneri* from farms to cheese factories. By this means, sources that contaminate milk with *L. parabuchneri* were detected and eliminated (Ascone et al., unpublished data). As a consequence, cheeses showed reduced formation of histamine during ripening.

5. Conclusions

The recurrent isolation and detection of *L. parabuchneri* in cheese with histamine indicates that this bacterium is a main cause of histamine accumulation in hard and semi-hard cheeses produced from raw milk. This study describes two methods: first, a qPCR assay to detect and enumerate of *L. parabuchneri* in dairy products and second, a multiplex PCR for differentiation and strain typing. The two methods can be combined to reveal contamination sources and to track bacterial strains.

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