International Dairy Journal 77 (2018) 80–88

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

A field study investigating the effectiveness of vat milk controls by qPCR for the prevention of undesired propionic acid fermentation in Sbrinz PDO cheese

Meral Turgay*, Walter Schaeren, Hans Ulrich Graber, Elvira Wagner, Rudolf Amrein, Ueli Bütikofer, Daniel Wechsler

Agroscope, CH-3003 Bern, Switzerland

ARTICLE INFO

Article history:
Received 17 March 2017
Received in revised form
9 June 2017
Accepted 29 June 2017
Available online 31 July 2017

ABSTRACT

The accurate monitoring of propionic acid bacteria (PAB) in cheese milk is of high importance since they can provoke undesired propionic acid fermentation during the ripening of unpasteurised milk cheeses. In the present study, we analysed 560 vat milk samples and 40 corresponding Sbrinz cheeses to evaluate the effectiveness of qPCR based controls of vat milks for the prevention of undesired propionic acid fermentation in Sbrinz PDO cheese. Two qPCR assays were used: one for the thermoduric species *Propionibacterium freudenreichii* and the other for the combined detection of the thermosensitive species *Propionibacterium thoenii*, *Propionibacterium jensenii*, and *Propionibacterium acidipropionici*. Thermoduric and thermosensitive PAB were detected in 35% and 19%, respectively, of the vat milks. However, in defective Sbrinz cheeses with propionic acid fermentation *P. freudenreichii* was the predominant species, indicating that low contaminations of thermosensitive PAB in vat milk do not present a risk in the manufacture of hard cooked cheeses.

1. Introduction

Sbrinz PDO cheese is a traditional, extra-hard, full-fat cheese made from raw cows' milk, and is produced in central Switzerland. The round-shaped loaves weigh 35–48 kg, with a diameter of 50–62 cm and a height of 14–17 cm. The association of Sbrinz PDO cheese consists of 27 small-scale cheese dairies, of which eight are alpine dairies with seasonal cheese production. The annual production is only approximately 1700 tons. Sbrinz PDO cheese has a fruity aroma, with the lightly roasted character of chicory, and a salty and slightly sweet taste. Raw milk is delivered from the dairy farms to the dairy twice a day, immediately after milking, and is processed once a day. After the milk has been warmed to 30–32 °C over a period of 15–60 min in a copper vessel, rennet and a starter culture, composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii*, are added. Following coagulation, the cheese curd is heated to 54–57 °C for 40–50 min, scalded at 53–55 °C for another 10–20 min, poured into moulds, pressed, and brine-salted (at 10–15 °C for at least 15 days). When ripening begins, the cheeses are stored at 12–18 °C for approximately 20 days and then ripened at 10–14 °C for up to a minimum of 18 months (Eugster-Meier, Fröhlich-Wyder, Jakob, & Wechsler, 2017). After 11 months of ripening, the quality of the cheese is assessed by an independent expert panel prior to commercialisation. Quality defects in Sbrinz PDO cheese frequently arise from the undesirable growth of propionic acid bacteria (PAB). When present in low numbers in raw milk, PAB may form visible brownish spots in the cheese body (Fessler, Casey, & Puhan, 1999a). If growth of PAB exceeds 10^6 cfu g^{-1} during cheese-ripening, an atypical sweet taste may become perceptible, and cracks may occur, due to the formation of CO_2. In Sbrinz PDO cheese of good quality, a maximal concentration of propionic acid of $\leq$2.0 mmol kg^{-1} is tolerable.

To detect and enumerate PAB in milk and cheeses a conventional plate count method (PCM) is usually performed (de Freitas et al., 2013; Thierry & Madec, 1995). However, these methods are very slow (7–10 days), and do not allow for species-specific detection of PAB. We recently described four species-specific qPCR methods for the determination of *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, and *Propionibacterium thoenii* in raw milk, and showed its usefulness in rapid

* Corresponding author. Tel.: +41 58 463 82 62.
E-mail address: meral.turgay@agroscope.admin.ch (M. Turgay).

http://dx.doi.org/10.1016/j.idairyj.2017.06.008
0958-6946/

and sensitive control of vat milk under practical conditions (Turgay, Schaeren, Wechsler, Bütikofer, & Graber, 2016). These four species are commonly referred to as "dairy PAB", because they constitute the typical species present in raw milk and cheese (Cummins & Johnson, 1986). It has been shown that other species, such as *Propionibacterium cyclohexanicum*, *Propionibacterium australiense*, *Propionibacterium acidifaciens*, *Propionibacterium microaerophilum*, *Propionibacterium olivae* and *Propionibacterium damnosum* are phylogenetically related to dairy PAB (Lucena-Padros, Gonzales, Caballero-Guerrero, Ruiz-Barba, & Maldonado-Barragan, 2014). However, these six species were isolated from other environments (Thierry et al., 2011).

Of the dairy PAB, *P. freudenreichii* is the most greatly feared contaminant in raw milk. In contrast with the other three dairy PAB species, it is characterised by a relatively high tolerance to heat stress. As a result of the heat stress adaptation, which leads to survival at temperatures of up to 55 °C, contamination can give rise to undesirable propionic acid fermentation in various cooked cheeses, such as Sbrinz PDO or Gruyère PDO (Chamba & Irlinger, 2004). Therefore, the distinction between *P. freudenreichii* and the other dairy PAB in supplier and vat milk is of high importance in the manufacture of traditional raw milk cheeses.

The main objective of the present study was to evaluate the effectiveness of qPCR based controls of vat milks for the prevention of undesired propionic acid fermentation in Sbrinz PDO cheese. Additionally, we wanted to investigate the survival of thermosensitive PAB species in the manufacture of Sbrinz PDO cheese to clarify whether they contribute to the development of undesired propionic acid fermentation during the ripening of this long-ripened cheese variety. Finally, we aimed to clarify whether minor contaminations of thermoduric and/or thermosensitive PAB in vat milks can be tolerated in the manufacture of this cheese variety without immediately running the risk of a faulty fermentation.

Accordingly, two primer systems were used to analyse vat milk samples and corresponding cheese samples from 20 Sbrinz cheese dairies: one system was used to determine the thermoduric species *P. freudenreichii* and the other was a newly designed primer system that was used for the combined detection of the three thermosensitive dairy PAB species *P. thoenii*, *P. jensenii*, and *P. acidipropionici* (3dPAB). This methodological improvement reduces the number of qPCR reactions from four to two, thus reducing the costs of the routine control of vat milk samples. In addition, propionic acid concentration was analysed in 40 selected Sbrinz cheeses after a ripening period of 11 months. The efficacy of monitoring PAB contamination in vat milk to minimise the risk of undesirable propionic acid fermentation in Sbrinz PDO cheeses was evaluated on the basis of the results obtained.

2. Materials and methods

2.1. Design of primers and hydrolysis probe for qPCR

Parizzi et al. (2012) located an ADP-forming acetyl-CoA synthetase gene (ADP-ACS) in the genome of *P. acidipropionici* ATCC 4875, and showed that this gene was absent from the genome of *P. freudenreichii*. However, screening (CLC main Workbench CLCbio) revealed the presence of this single copy gene in the genomes of the three strains *P. thoenii* FAM 22284, *P. jensenii* FAM 19038, and *P. acidipropionici* FAM 19036 (culture collection of the Agroscope Institute for Food Sciences, Bern, Switzerland). With the alignment of these four gene sequences, the primers and the hydrolysis probe were manually designed in regions with homology within the three 3dPAB species. The primers (forward: 5′-GGATCTGGCCGCTCATCAA-3′, reverse: 5′-GTTCATCTCGGTCTCGGTGA-3′) and the hydrolysis probe (5′-TCGGCCACCAGGGTCGAGAACCG-3′) were purchased from Microsynth, Balgach, Switzerland. The hydrolysis probe was labelled with FAM at the 5′ end and with BHQ-1 (Microsynth) at the 3′ end. This primer system, named 3dPAB-(ADP-ACS), was designed for the combined detection of the 3dPAB species *P. thoenii*, *P. jensenii*, and *P. acidipropionici*.

2.2. Real-time quantitative PCR

The qPCR assays for the individual determination of *P. freudenreichii*, *P. acidipropionici*, *P. jensenii*, and *P. thoenii* were conducted as described in Turgay et al. (2016).

The qPCR for the 3dPAB-(ADP-ACS) primer system was carried out in a reaction volume of 25 μL, which contained 12.5 μL qPCR™ MasterMix No ROX (Eurogentec, Seraing, Belgium), 300 nM forward and reverse primer, 100 nM hydrolysis probe, and 5 μL DNA extracted from the bacterial pellets obtained from the milk or cheese samples. The qPCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 60 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCRs were run on Rotor-Gene 6000 (Corbett, Qiagen). Rotor-Gene 6000 Software 1.7 was used for the analysis. The samples were run in duplicate, unless otherwise specified, and the qPCR was run with 2 ng genomic DNA to evaluate the analytical sensitivity and specificity of the primer system.

2.3. Construction of the qPCR-standard

The qPCR-standard for the 3dPAB-(ADP-ACS) primer system was constructed as follows: A fragment of the ADP-ACS gene from *P. acidipropionici* strain ATCC 4875, comprising the target sequence, was amplified (primers: 5′-GAGCTCATCCTCGGATCCA-3′, 5′-CCCCAGGTGCGAGTACTT-3′) at 95 °C for 10 min, followed by 35 cycles: denaturation at 95 °C for 30 s, annealing at 58.1 °C for 1 min, extension at 72 °C for 1 min, and was completed by a 10-min step at 72 °C (Biometra, T Gradient).

The amplicon (381 bp) was incorporated in a plasmid using pGEM®-T Easy Vector Systems (Promega Corporation, Madison, WI, USA) and then transformed in DH5-α cells, according to the manufacturer's instructions. Plasmid isolation was carried out using a QIAprep® Spin Miniprep Kit (QIAGEN). The plasmid was then linearised with the *Pst*I restriction enzyme. With the given size (bp) of the plasmid plus insert and the measured concentration (ng μL^{-1}), the numbers of copies μL^{-1} of the target sequences were calculated (see technical manual instructions of pGEM®-T Easy Vector Systems).

A serial dilution of the linearised plasmid in 10 ng μL^{-1} of 10 mM Tris–HCl, pH 8.0, DNA from herring sperm (Sigma–Aldrich, Dr. Grogg Chemie AG, Stettlen, Switzerland) was prepared for the standard curve, covering a range of 10^1–10^6 copies per reaction. The qPCR reactions were run in triplicate.

2.4. Verification of primer specificity by qPCR

The analytical sensitivity of the 3dPAB-(ADP-ACS) primer system was verified with type strains and various test strains of *P. acidipropionici* (20), *P. jensenii* (26) and *P. thoenii* (20). The specificity was tested with type strains and various test strains of non-target species of the genus *Propionibacterium*: *P. freudenreichii* (22), *P. acidifaciens* (1), *P. cyclohexanicum* (1), *P. australiense* (1), *P. microaerophilum* (1), *P. olivae* (1), and *P. damnosum* (1). Moreover, 36 strains of various other species (34) commonly found in raw milk were tested (Table 1). All type strains were obtained from the German Collection of Microorganism and Cell Cultures (Braunschweig, Germany), and the test strains were obtained from the culture collection of the Agroscope Institute for Food Sciences. Bacterial

Table 1
Quantification cycle values (Cq) obtained for the three dairy propionic acid bacteria target species (3dPAB), *P. acidipropionici*, *P. jensenii*, and *P. thoenii*, with the newly developed 3dPAB-(ADP-ACS) primer system designed for the combined detection of these species.[a]

Bacteria	Strains (*n*)	Cq ± SD	Bacteria	Strains (*n*)	Cq ± SD	Bacteria	Strains (*n*)	Cq ± SD
Dairy propionic acid bacteria species			Strains of other taxa cont ….			Strains of other taxa cont …		
P. acidipropionici	20	18.62 ± 0.05	*Lb. plantarum*		n.d.	*Enterococcus*	2	
P. jensenii	26	19.00 ± 0.62	*Lb. acidophilus*		n.d.	*E. faecium*		n.d.
P. thoenii	20	19.94 ± 0.69	*Streptococcus*	4		*E. faecalis*		n.d.
P. freudenreichii	22	n.d.	*S. salivarius* subsp. *thermophilus*		n.d.	*Luteococcus*	2	
Phylogenetically related propionic acid bacteria species			*S. agalactiae*		n.d.	*L. japonicus*		n.d.
P. cyclohexanicum^T	1	n.d.	*S. dysgalactiae*		n.d.	*L. sp.*		n.d.
P. olivae^T	1	n.d.	*S. uberis*		n.d.	*Clostridium*	4	
P. australiense^T	1	36.85	*Lactococcus*	2		*C. tyrobutyricum*		n.d.
P. acidifaciens^T	1	37.05	*Lc. lactis* subsp. *lactis*		n.d.	*C. beijerinckii*		n.d.
P. microaerophilum^T	1	19.63	*Lc. lactis* subsp. *cremoris*		n.d.	*C. sporogenes*		n.d.
P. damnosum^T	1	20.39	*Pediococcus*	2		*C. butyricum*		n.d.
Strains of other taxa			*Ped. pentosaceus*		n.d.	*Staphylococcus*	4	
Lactobacillus	9		*Ped. acidilactici*		n.d.	*Staph. chromogenes*		n.d.
Lb. delbrueckii subsp. *delbrueckii*		n.d.	*Leuconostoc*	2		*Staph. simulans*		n.d.
Lb. delbrueckii subsp. *bulgaricus*		n.d.	*L. lactis*		n.d.	*Staph. xylosus*		n.d.
Lb. delbrueckii subsp. *lactis*		n.d.	*L. mesenteroides* subsp. *mesenteroides*		n.d.	*Staph. aureus*		n.d.
Lb. helveticus		n.d.	*Brevibacterium*	1		*Bifidobacterium*	2	
Lb. rhamnosus		n.d.	*B. linens*		n.d.	*Bif. breve*		n.d.
Lb. brevis		n.d.				*Bif. animalis*		n.d.
Lb. fermentum		n.d.						
Lb. casei		n.d.						
Lb. buchneri		n.d.						

[a] The specificity of the primer system was tested with various type strains^T and test strains of non-target species of the genus *Propionibacterium* and various other species commonly found in raw milk. SD, standard deviation; n.d., not detectable.

DNA extraction, standardisation, and qPCR were conducted as described in Turgay et al. (2016).

2.5. Spiking of milk samples

Raw milk was collected by hand-milking, and microbiological analyses were used to test for the absence of PAB. Raw milk samples free of PAB were then spiked with liquid stock culture of *P. acidipropionici* (FAM 19036), *P. jensenii* (FAM 19038), or *P. thoenii* (FAM 22284). The three spiked raw milk samples were then serially diluted 10-fold in raw milk. The DNA extraction was carried out as described below, and the qPCR reactions were run in triplicate. Liquid stock cultures were prepared as described in Turgay et al. (2016).

2.6. Sampling of vat milk and cheese

Vat milk was collected from 20 Sbrinz cheese dairies during June 2014. A sample was taken from each dairy every morning immediately before the addition of rennet, and was deep frozen (−20 °C)

Table 2
Contamination by *P. freudenreichii* (PF) and the three other dairy propionic acid bacteria species (3dPAB), *P. acidipropionici*, *P. jensenii*, and *P. thoenii*, in vat milk collected from 20 Sbrinz cheese dairies, as determined by qPCR.[a]

Dairy	Number of analysed vat milk samples	Vat milk samples with quantifiable/detectable contamination of PF or 3dPAB		No. of contaminated samples containing both PF and 3dPAB	Total number of contaminated samples	No. of analysed cheeses made from vat milk with/without PAB contamination
		PF	3dPAB			
01	30	14/11	0/11	9	27	6/1
02	30	9/18	0/7	7	27	4/1
03	30	5/12	0/9	7	19	6/1
04	25	4/13	0/9	6	20	0/0
05	30	3/14	0/6	5	18	4/0
06	29	0/5	1/17	3	20	1/1
07	29	0/3	1/3	1	6	1/1
08	30	0/8	1/3	1	11	0/0
09	27	0/20	0/3	3	20	0/0
10	30	0/15	0/1	1	15	0/0
11	30	0/5	0/11	0	16	0/0
12	30	0/5	0/8	1	12	0/0
13	29	0/10	0/2	0	12	0/0
14	30	0/6	0/5	3	8	0/0
15	28	0/6	0/1	1	6	0/5
16	16	0/5	0/1	1	5	0/0
17	30	0/5	0/1	0	6	0/2
18	26	0/0	0/4	0	4	0/4
19	21	0/1	0/0	0	1	0/0
20	30	0/0	0/0	0	0	0/2
Total	560	35/162	3/102	49	253	22/18

[a] The lower limit of quantification for PF and 3dPAB was ≥50 copies mL^{-1}.

until analysis. A total of 560 samples were collected (Table 2). Due to production interruptions in some of the dairies, the number of samples taken from the individual cheese dairies varied between 16 and 30.

The sampling of Sbrinz cheeses was based on the results of the vat milk screening, as shown in Table 2. A total of 22 cheese samples were collected from cheeses made from vat milk contaminated with PAB. Another 18 cheese samples originated from cheeses made from vat milk without detectable PAB contamination. The 40 selected cheese loaves originated from 10 different cheese dairies and differed in their production date. The cheese samples were taken after a ripening period of 11 months.

2.7. Extraction of DNA from raw milk and cheese samples

The isolation of bacteria from raw milk and the extraction of DNA from bacterial pellets were conducted as previously described by Turgay et al. (2016). The isolation of bacteria from cheese was carried out as described by Berthoud et al. (2017). The pellets containing bacteria were processed in a similar manner to those obtained from the vat milk. Each cheese was analysed in duplicate.

2.8. Microbiological analyses

The enumeration of PAB from the liquid stock cultures and the 40 cheese samples was carried out using the plate count method (PCM) as described by Turgay et al. (2011). All samples were analysed in duplicate.

2.9. Assay controls

A positive extraction control (spiked raw milk) and a negative extraction control (PAB-free raw milk) were co-processed with the examined vat milk samples. An internal amplification control (IAC) was added to exclude negative results resulting from qPCR inhibition. To this end, 5×10^6 copies of a plasmid containing a partial sequence of tobacco mosaic virus (Tentcheva et al., 2004) was added to the sample during the DNA extraction process, following the enzymatic treatment step. The IAC was prepared as described in Turgay et al. (2016). Vat milk samples were considered to be contaminated if both reactions of the corresponding qPCR assay were positive, with a standard deviation of $\leq$1 Cq. The co-processed negative extraction control had to be negative.

2.10. Chemical analysis of cheeses

Propionic acid was analysed by gas chromatography and a flame ionisation detector with headspace technology after esterification with ethanol, as described by Fröhlich-Wyder et al. (2013).

3. Results

3.1. Real-time quantitative PCR assay performance

The qPCR with the 3dPAB-(ADP-ACS) primer system showed a linear relationship between the concentration of the qPCR-standard (10^1–10^6 copies per reaction) and the quantification cycle. The correlation coefficient (R) over this 6-log range was 0.999, and the amplification efficiency was 0.86 (results not shown). The limit of quantification was 10^1 copies per reaction.

3.2. Specificity of the 3dPAB-(ADP-ACS) primer system

It was shown that the target sequence of the ADP-ACS gene was present in all 66 tested strains of *P. thoenii*, *P. jensenii*, and *P. acidipropionici* (Table 1). The quantification cycle values (Cq), which ranged from 18.62 to 19.94, confirmed early amplification.

The primer system did not cross-react with either the 22 strains of the *P. freudenreichii* species or with the 36 strains of the other taxa. However, although major cross-reactions were observed with the phylogenetically related *P. microaerophilum* (Cq = 19.63) and *P. damnosum* (Cq = 20.39) species, the Cq values for the *P. cyclohexanicum* and *P. acidifaciens* (Cq > 36) species were considered as insignificant.

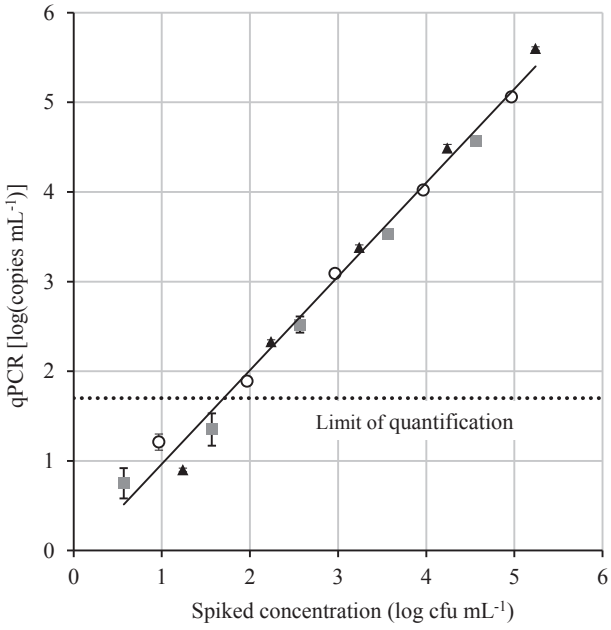

Fig. 1. Limits of detection and quantification of the real-time quantitative polymerase chain reaction (qPCR) with the 3dPAB-(ADP-ACS) primer system for the species *P. acidipropionici* (○), *P. jensenii* (▲), and *P. thoenii* (■) with 10-fold serially diluted spiked raw milk samples. The Cq values were transformed into copies mL^{-1} using the standard curve generated. Each data point represents the mean value and standard deviation of three qPCR replicates.

3.3. Determination of the limit of quantification in milk

To determine the limit of quantification, raw milk samples were individually spiked with liquid stock cultures of *P. acidipropionici*, *P. jensenii* or *P. thoenii*, and then serially diluted with raw milk. Since DNA was extracted from 4 mL raw milk and recovered in an elution volume of 100 µL, of which 5 µL were used for the qPCR assay, the 10 copies per reaction of the standard correspond to a theoretical limit of quantification of 50 copies mL^{-1} milk [(10 copies × 20)/4 mL]. Fig. 1 shows that the limit of quantification generated from standard curve is adequate for milk samples as well. The coefficient of correlation (R) over this 5-$\log_{10}$ range was 0.994 for all spiked samples. Concentrations below 50 copies mL^{-1} could still be detected, but they were not quantified since the obtained Cq-values were outside the linear range of the corresponding standard curve.

3.4. Monitoring PAB in vat milk samples

The results of the analysis of the 560 vat milk samples are shown in Table 2. In 197 samples (35%), contamination with *P. freudenreichii* was detectable or quantifiable, while contamination with other dairy PAB was detectable or quantifiable in 105 samples (19%). In total, 253 samples (45%) were contaminated with dairy PAB, of which 49 (9%) samples were contaminated with both

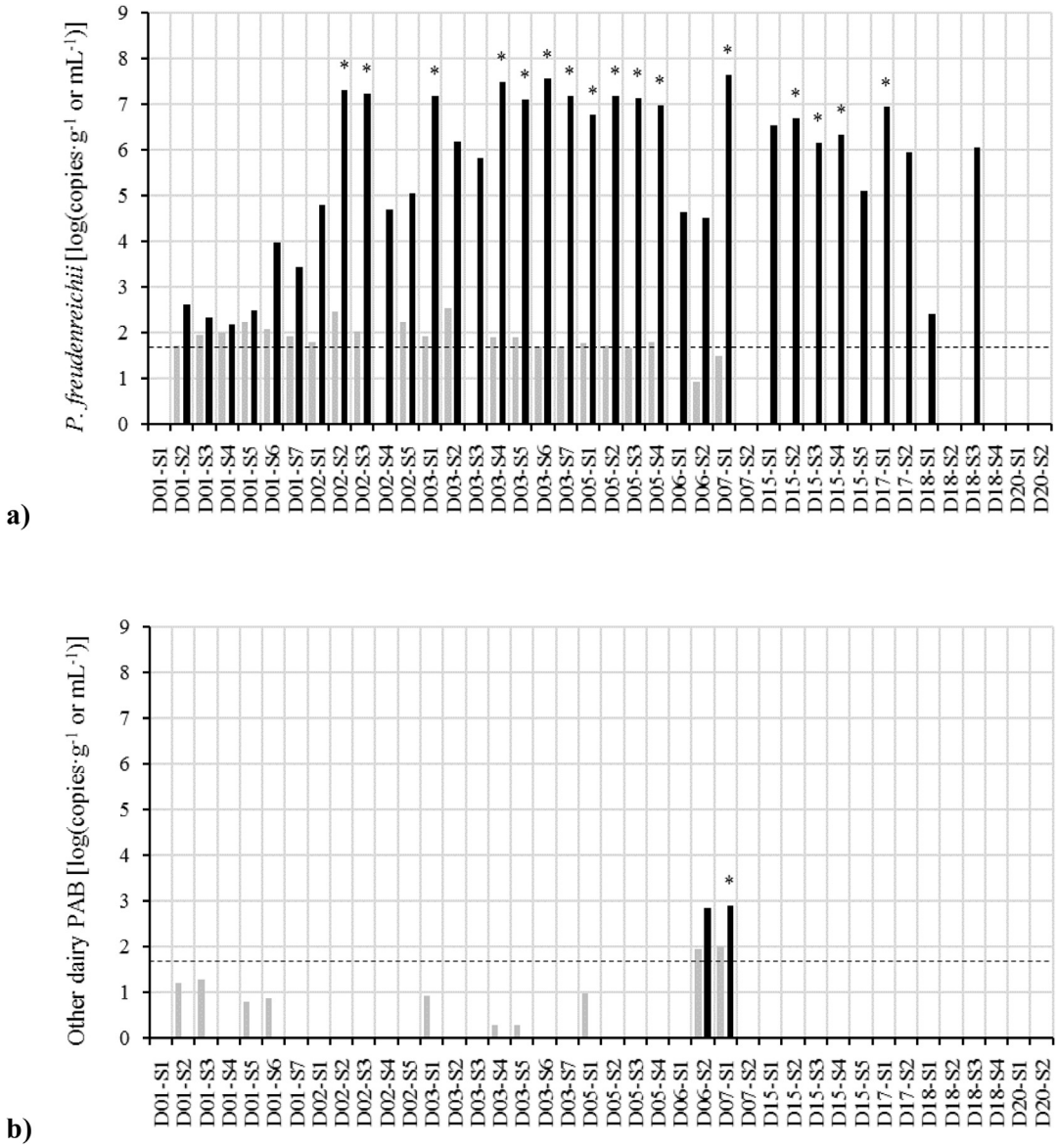

Fig. 2. Population density of *P. freudenreichii* (a) and the three other dairy propionic acid bacteria (PAB) species *P. acidipropionici*, *P. jensenii*, and *P. thoenii* (b) in 40 analysed Sbrinz cheeses (black bars) and corresponding vat milks (grey bars). A total of 22 cheeses were made from vat milks with quantifiable PAB contamination, and the remaining 18 cheeses were made from vat milk without detectable PAB contamination. The dashed line shows the lower limit of quantification of *P. freudenreichii* and other dairy PAB in vat milk samples. Sample pairs of cheeses and corresponding vat milks (S1, S2, …) were collected from 10 different dairies (D01, D02, …). Cheeses marked with an asterisk (*) were considered as defective, due to their propionic acid content >2.0 mmol·kg^{-1}.

P. freudenreichii and other dairy PAB. In the 35 samples of vat milk with quantifiable contamination with *P. freudenreichii*, the maximum population density was 347 copies mL^{-1}, and the geometric mean value was 94 copies mL^{-1}. In the three vat milk samples with quantifiable contamination with other dairy PAB, the maximum population density was 103 copies mL^{-1}, and the geometric mean value was 85 copies mL^{-1} (data not shown). Important differences regarding the prevalence of PAB contamination in the 20 cheese dairies involved were observed. Quantifiable contamination with PAB was found in the vat milk samples from eight cheese dairies only (dairy 01 to dairy 08), and five of these dairies (dairy 01 to dairy 05) had more than one sample with quantifiable PAB contamination. PAB contamination was also detected in the vat milk samples from 11 other cheese dairies (dairy 09 to dairy 19), but these were always below the limit of quantification. None of the 30 vat milk samples from dairy 20 contained detectable PAB contamination. In contrast, 90% of the analysed samples from each of the dairies, dairy 01 and dairy 02, were contaminated.

Overall, contamination with *P. freudenreichii* was approximately twice as frequent as contamination with other dairy PAB species. However, exceptions were noticed in the vat milk samples from five dairies (dairy 06, dairy 07, dairy 11, dairy 12, and dairy 18), whereby contamination with other dairy PAB occurred more frequently than contamination with *P. freudenreichii*.

3.5. Determination of PAB in ripened Sbrinz cheeses

Of the 40 sampled Sbrinz cheeses, 22 had been made from vat milk with quantifiable PAB contamination, while the remaining 18 had been made from vat milk with no detectable PAB contamination (Fig. 2). As expected, in all 22 Sbrinz cheeses that had been made from vat milk with quantifiable PAB contamination, PAB were

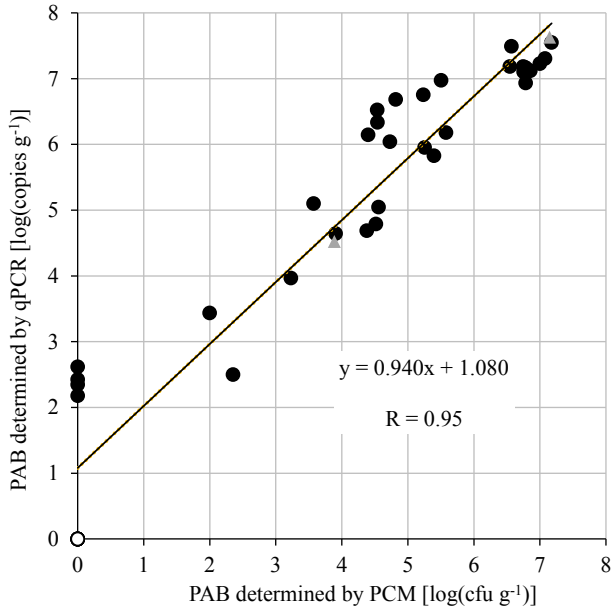

Fig. 3. Determination of propionic acid bacteria (PAB) in 40 Sbrinz cheeses via the conventional plate count method (PCM) and the real-time quantitative polymerase chain reaction (qPCR). The values determined with qPCR analysis represent the sum of the number of obtained copies of *P. freudenreichii* and the three other dairy PAB species (*P. acidipropionici*, *P. jensenii* and *P. thoenii*). With the exception of two samples (▲), the analysed cheeses contained only *P. freudenreichii* (●). In six cheese samples, no PAB were detected with either method (○).

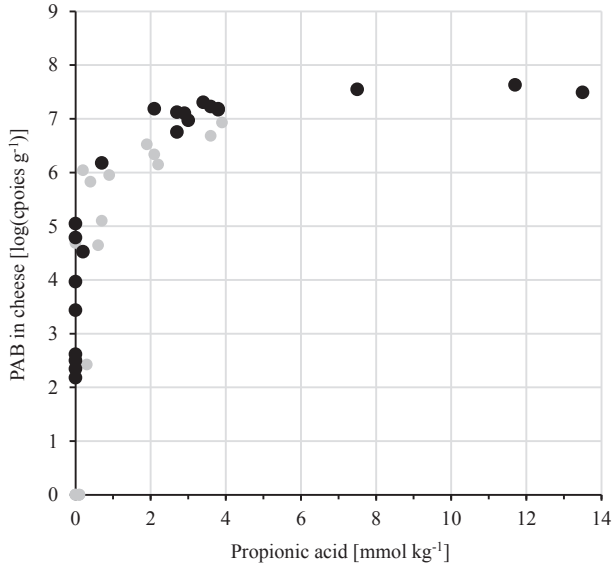

Fig. 4. Comparison of the concentrations of propionic acid bacteria (PAB) and propionic acid in 40 analysed Sbrinz cheeses, of which 22 were made from vat milk with quantifiable PAB contaminations (●). The remaining 18 cheeses were made from vat milk without detectable PAB contamination (●), in which no PAB were detectable by qPCR in six cheeses.

found at levels ranging from 1.5×10^2 to 4.3×10^7 copies g^{-1}. In all these cheeses, *P. freudenreichii* was the dominant PAB species (Fig. 2a). Positive qPCR signals were detected in only two Sbrinz cheeses (D06-S2 and D07-S1) with the primer system 3dPAB-(ADP-ACS) (Fig. 2b). With regard to cheese quality, this minor contamination with other dairy PAB was not relevant, since the same two cheeses also contained distinctly higher population densities of *P. freudenreichii*. Additional investigations with species-specific qPCR assays revealed only *P. thoenii* contamination in cheese D07-S1, whereas contamination with all three species, *P. acidipropionici*, *P. jensenii*, and *P. thoenii*, was found in cheese D06-S2. The analysis of the two corresponding vat milk samples with the species-specific qPCR systems allowed the presence of these species to be traced back to the vat milk (data not shown). Surprisingly, *P. freudenreichii* was present in a population density ranging from 2.7×10^2 to 8.5×10^6 copies g^{-1} in 12 cheeses that had been made from vat milk without detectable PAB contamination. In another six cheeses that had been made from vat milk without detectable PAB contamination, no PAB were found, as expected.

In addition to the determination of *P. freudenreichii* and other dairy PAB by qPCR, PAB counts were made in the same 40 cheeses, using conventional PCM. The comparison of the $\log_{10}$ transformed results is shown in Fig. 3. The correlation coefficient (R) was 0.955, indicating that the qPCR results correlated well with the viable PAB counts. The results obtained via qPCR were approximately 1 $\log_{10}$ higher than those obtained by PCM (regression curve equation: $y = 0.940x + 1.080$). In six of the 40 cheeses, no PAB were found with either method, and in four cheeses (D01-S2 to D01-S4 and D18-S1; Fig. 2) PAB was only detected via qPCR.

3.6. Determination of propionic acid in ripened cheeses

In Sbrinz cheeses, a propionic acid content of >2.0 mmol kg^{-1} is considered indicative of cheeses of low quality. Of the 40 cheeses analysed, 16 showed undesirable propionic acid fermentation, with propionic acid contents ranging from 2.1 to 13.5 mmol kg^{-1}. In all of these cheeses, *P. freudenreichii* was found in a population density ranging from 1.4×10^6 to 4.3×10^7 copies g^{-1} (Fig. 4). Of these 16 defective cheeses, four had been produced from vat milk without detectable PAB contamination, and three of these four had originated from the same dairy (Fig. 2a; cheeses D15-S2 to D15-S4). The results obtained for the IAC did not indicate an inhibition of the qPCR in the analysis of these vat milk samples. The Cq values were below the acceptable Cq delay ≥1, which was established as a cut-off inhibition by Postollec, Falentin, Pavan, Combrisson, and Sohier (2011).

The remaining 24 cheeses contained almost all <1 mmol kg^{-1} propionic acid, despite 10 of them having been made from milk with quantifiable PAB contamination.

4. Discussion

4.1. Sensitivity and specificity of the 3dPAB-(ADP-ACS) primer system

A new primer system was designed, enabling the combined detection of the three thermosensitive dairy PAB species *P. acidipropionici*, *P. jensenii* and *P. thoenii* in one single qPCR. The consequently achieved reduction of analytical costs is an important step in the implementation of systematic controls for the detection of PAB in vat milk in practice. The LOQ of 50 copies mL^{-1} of the newly developed qPCR assay with the 3dPAB-(ADP-ACS) primer system was equivalent to those of previously validated species-specific primer systems (Turgay et al., 2016) (Fig. 1). However, specificity was not limited to the three target species alone. The 3dPAB-(ADP-ACS) primer system also cross-reacted with type strains of *P. microaerophilum* and *P. damnosum* (Table 1). These two species were isolated from the olive processing environment (Koussémon et al., 2001; Lucena-Padros et al., 2014) and to our knowledge they were never detected in dairy products until now. Irrespective of these cross-reactions, it can therefore be expected that positive qPCR reactions with this primer system will most

likely result from contamination with the *P. acidipropionici*, *P. jensenii*, or *P. thoenii* species in the analysis of dairy products. PAB contamination detected with this primer system can be further analysed using species-specific primer systems, if required (Turgay et al., 2016).

4.2. Prevalence of dairy PAB in vat milk samples

In the present study, 560 vat milk samples were collected from 20 Sbrinz cheese dairies over the duration of a month, and analysed for contaminations with thermoduric and thermosensitive PAB (Table 2). The predominant species, *P. freudenreichii*, was found in 35% of the analysed vat milk samples, at a maximum level of 347 copies mL^{-1} (geometric mean 94 copies mL^{-1}). In 6% of the vat milk, *P. freudenreichii* was present at a population density of $\geq$50 copies mL^{-1}, which is too high for the manufacture of high-quality raw milk cheeses (Bachmann et al., 2011). Contaminations with other dairy PAB species were distinctly less frequent (19%), and only 0.5% of the samples contained quantifiable contamination (maximum level 103 copies mL^{-1}; geometric mean 94 copies mL^{-1}). These findings are similar to the results of our previous study, which was carried out using 51 vat milk samples that were collected from 50 cheese dairies (Turgay et al., 2016), and are in high accordance with other previous studies on the diversity of dairy PAB reporting that *P. freudenreichii* is the dominant PAB species in milk, followed by *P. jensenii* and *P. acidipropionici* (Fessler, Casey, & Puhan, 1999b; de Freitas et al., 2015). Carcano, Todesco, Lodi, and Brasca (1995) counted PAB with PCM in 306 milk samples that were directly obtained from Italian farms, and found that the PAB concentration was below 500 cfu mL^{-1} in 79% of the samples analysed. In contrast, Montel et al. (2014) indicated lower PAB counts for bulk milk, in the range of 10^1–10^2 cfu mL^{-1}, which is in line with the PAB population densities that were found in most of the vat milk samples in our study.

4.3. Presence of PAB in ripened Sbrinz cheeses

In the present study, 40 cheeses, produced by 10 different Sbrinz PDO cheese dairies, and samples of corresponding vat milk, were analysed for contamination with *P. freudenreichii* and other dairy PAB after a ripening period of 11 months, using qPCR. In addition, the concentration of propionic acid was analysed in the same cheeses to evaluate the efficacy of systematic control of vat milk for PAB with regard to preventing undesirable propionic acid fermentation during cheese ripening. As was observed in the vat milk, it was found that *P. freudenreichii* was also the predominant PAB species in the ripened Sbrinz cheeses (Fig. 2a). In 16 defective Sbrinz cheeses with propionic acid contents >2.0 mmol kg^{-1}, *P. freudenreichii* was detected at a population density ranging from 1.4×10^6 to 4.3×10^7 copies g^{-1}. Another 18 cheeses contained mostly lower, *P. freudenreichii* contamination (range 1.5×10^2 to 3.4×10^6 copies g^{-1}), and only six cheeses revealed an absence of *P. freudenreichii*. Dairy PAB other than *P. freudenreichii* were detected at low concentrations (7.2×10^2 copies g^{-1} to 8.0×10^2 copies g^{-1}) in only two cheeses from dairy 06 and dairy 07, despite 10 of the corresponding vat milk samples showing contamination with thermosensitive PAB species (Fig. 2b). These results strongly indicate that *P. acidipropionici*, *P. jensenii*, and *P. thoenii* are mostly inactivated in the manufacture of hard cooked raw milk cheeses. In contrast, *P. freudenreichii* mostly survived the harsh thermal treatment during manufacture, as a result of its higher heat resistance. Similar observations were made in a former study in Emmental PDO cheese (Turgay et al., 2011). However, the results obtained for the four sample pairs D01-S2 to D01-S5 (Fig. 2a) suggest that strains of *P. freudenreichii* were also occasionally inactivated. In three of these four samples (D01-S2 to D01-S4), enumeration of PAB with conventional PCM yielded negative results. The apparent weak growth of *P. freudenreichii* in these cheeses, as determined by qPCR, can most likely be explained by the fact that inactivated PAB entrapped in the curd were slightly concentrated during cheese-making, due to whey drainage. Similarly, cheese D18-S1 showed an absence of cultivable PAB, although 2.7×10^2 copies g^{-1} of *P. freudenreichii* were detected via qPCR. Overall, the qPCR results correlated well with the viable PAB counts in the ripened cheeses ($R = 0.955$) (Fig. 3). However, the qPCR values were approximately a power of 10 higher than the PCM values, which can probably be explained by the presence of non-cultivable cells, and the morphology of PAB that occur mostly in pairs.

4.4. Predictability of propionic acid fermentation in ripened Sbrinz cheeses

In the present field study a total of 45% of the analysed vat milks were contaminated with thermoduric and/or thermosensitive PAB. An important goal of our study was to examine whether contaminated vat milks must be strictly excluded from processing to avoid the risk of faulty propionic acid fermentation or whether minor contaminations in vat milks can be tolerated. The results obtained from the determination of PAB in ripened Sbrinz cheeses imply that minor contaminations (<10^2 copies mL^{-1}) of thermosensitive PAB (*P. thoenii*, *P. jensenii* and *P. acidipropionici*) in vat milk do not present a risk for the quality of hard cooked cheeses. Despite such contaminations were observed in 19% of the analysed vat milks, in none of the 16 Sbrinz cheeses showing a propionic acid fermentation (propionic acid content >2.0 mmol kg^{-1}) the defect was caused by thermosensitive PAB (Fig. 2).

For the thermoduric species *P. freudenreichii* it was difficult to establish a clear connection between contaminations in vat milk and faulty propionic acid fermentation in the ripened cheeses: Surprisingly, out of 22 Sbrinz cheeses that had been made from vat milk with quantifiable PAB contamination (containing on average 9.5×10^1 copies mL^{-1} of PAB), only 12 cheeses (54%) showed a propionic acid fermentation. In the remaining 10 cheeses, only low concentrations of propionic acid (<0.7 mmol kg^{-1}) were found (Fig. 4). With the exception of one cheese, the population density of PAB ranged between 1.5×10^2 and 1.1×10^5 copies g^{-1} in these 10 cheeses. The limited predictability of propionic acid fermentation in cheeses made from vat milk with quantifiable PAB contamination can be explained by strain-dependent differences in the heat resistance and metabolic characteristics of the PAB involved (Falentin et al., 2010; Turgay et al., 2011). Of the dairy PAB, *P. freudenreichii* is the most feared contaminant due to its higher tolerance to heat stress (Chamba & Irlinger, 2004). However, individual strains may vary considerably in their resistance to heat and their ability to metabolise aspartate (Blasco, Kahala, Tupasela, & Joutsjoki, 2011). Turgay et al. (2011) showed that the wild-type strains of *P. freudenreichii* growing in Emmental PDO cheese all exhibited high aspartase activity, indicating that this strain-specific property is important for a strong growth during cheese-ripening. Moreover, the study revealed considerable differences in the ability of *P. freudenreichii* strains to grow at a temperature of 11 °C. In addition, other factors, such as sensitivity to salt and low pH-values, as well as interactions with lactic acid bacteria complicate the prediction of growth and survival of individual strains during cheese-ripening (Fröhlich-Wyder & Bachmann, 2004). Thermosensitive PAB species were detected only in two cheeses at a very low level (Fig. 2b).

In 18 cheeses that had been made from vat milk with no detectable PAB contamination some unexpected results were found (Fig. 4). Surprisingly, only six of these 18 cheeses showed an

absence of PAB. In eight cheeses with propionic acid contents ≤2.0 mmol kg^{-1}, a considerable growth of *P. freudenreichii* was detected, and another four cheeses even had to be downgraded, due to propionic acid fermentation. Cheeses with unexpected PAB growth originated mainly from dairy 15 (5 cheeses), dairy 17 (2 cheeses), and dairy 18 (2 cheeses). As shown in Table 2, some of the analysed vat milk samples from these three cheese dairies were contaminated with *P. freudenreichii*. Therefore, a possible explanation could be that in some cases even *P. freudenreichii* contamination that were below the detection limit of the qPCR assay used resulted in undesirable growth of PAB during the ripening period. It is also possible that PAB contamination occurred after scalding during the filling of the cheese moulds with the curd in some of the cheese dairies. It is well known that contamination of PAB in raw milk is primarily due to persistent contamination in milking systems at the farm level (Maurer, Haldemann, Ascone, & Wechsler, 2016). Biofilms or deposits harbouring dairy PAB and other heat resistant bacteria can occasionally be found on the surfaces of insufficiently cleaned dairy equipment (e.g., tubes with large diameters in filling systems, seals, cheese moulds, and press plungers). Contamination with in-house microbiota is possible, as was shown by Calasso et al. (2016). Aburjaile et al. (2015) showed the long-term survival of *P. freudenreichii* in a context of nutrient shortage. Such persistent contamination sources usually affect cheese quality over extended periods of production, consistently yielding cheeses with the same sub-standard quality.

5. Conclusions

In the present study, we tested under practical conditions a newly developed qPCR assay for the combined detection of the thermosensitive PAB species *P. thoenii*, *P. jensenii*, and *P. acidipropionici*, and a recently developed qPCR assay for the determination of the thermoduric PAB species *P. freudenreichii* in vat milk and ripened cheeses. Contaminations with thermoduric and thermosensitive PAB were detected in 35% and 19%, respectively, of the 560 analysed vat milks, underlining the high importance of frequent and accurate stage-by-stage controls of the production process in cheese dairies producing unpasteurised milk cheeses. A main objective of the present study was to evaluate the predictability of undesirable propionic acid fermentation in Sbrinz cheese with regard to the contamination level of thermoduric and thermosensitive PAB in vat milk. In the long-ripened Sbrinz cheeses almost exclusively contaminations of *P. freudenreichii* were observed, indicating that contamination in vat milk with thermosensitive dairy PAB do not put a risk for the quality of Sbrinz cheese since this group of PAB obviously did not survive the severe scalding conditions. However, in the case of uncooked raw milk cheeses (e.g., Raclette du Valais PDO) contaminations with P. *thoenii*, *P. jensenii*, and *P. acidipropionici* are likely to be as detrimental to cheese quality as contaminations with *P. freudenreichii*. Therefore, the monitoring of thermosensitive PAB species contributes to reveal deficiencies in raw milk quality, and thus helps to implement corrective measures at an early stage.

Unfortunately, in the present study we failed to predict in a reliable way undesired propionic acid fermentation in Sbrinz cheese by monitoring *P. freudenreichii* in vat milk samples. There are three reasons for this: the extremely low damage threshold of *P. freudenreichii*, strain-dependent differences in the heat resistance, and the occurrence of faulty fermentations caused by PAB contaminations originating from the dairy environment/equipment. These factors made it finally impossible to establish a correlation between contaminations in vat milk and the growth of *P. freudenreichii* during cheese ripening. In view of the ambiguous study results the use of the two qPCR assays in practice seems to be questionable. To take a better advantage of the newly developed qPCR assays it would be necessary to analyse supplier milks instead of vat milks. In Sbrinz dairies, a typical vat milk represents a mixture of about 10–30 supplier milks. The pooling of the numerous supplier milks helps to reduce the number of samples but at the same time it leads to a dilution of the target being detected. The results of the present study clearly revealed that the cost-saving analysis of vat milks does not provide a reliable protection against undesired propionic acid fermentation.

In some of the investigated cheeses PAB contaminations originating from the dairy environment and equipment were likely to be the main cause for the observed cheese quality defects. Therefore, regular microbial controls of the dairy equipment should be envisaged as complementary measure. In comparison with the traditional plate count method for the determination of PAB the application of the newly developed qPCR assays offers for raw milk, cheese and environmental samples a faster and more specific control. At the moment the costs for qPCR analyses are still too high for a systematic monitoring of PAB contaminations in supplier milks and environmental samples. However, high throughput qPCR systems using microfluidics have been developed recently that allow a massive cost reduction. It can be expected that in the near future such systems will establish themselves successfully in routine analyses. Especially in the manufacture of unpasteurised milk cheeses this will offer new opportunities to intensify microbial controls at affordable costs and thus to improve the prevention against faulty fermentations during cheese ripening.

Acknowledgements

The authors would like to thank the cheese dairies involved, and the association of Sbrinz PDO cheese for their excellent collaboration and their support for this study.

References

Aburjaile, F. F., Madec, M. N., Parayre, S., Miyoshi, A., Azevedo, V., Le Loir, Y., et al. (2015). The long-term survival of *Propionibacterium freudenreichii* in a context of nutrient shortage. *Journal of Applied Microbiology, 120*, 432–440.

Bachmann, H. P., Fröhlich-Wyder, M. T., Jakob, E., Roth, E., Wechsler, D., Beuvier, E., et al. (2011). Cheese: Raw milk cheeses. In J. W. Fuquay, P. F. Fox, & P. L. H. Mcsweeney (Eds.), *Encyclopedia of dairy science* (2nd edn., pp. 652–660). London, UK: Elsevier Limited.

Berthoud, H., Wüthrich, D., Bruggmann, R., Wechsler, D., Fröhlich-Wyder, M., & Irmler, S. (2017). Development of new methods for the quantitative detection and typing of *Lactobacillus parabuchneri* in dairy products. *International Dairy Journal, 70*, 65–71.

Blasco, L., Kahala, M., Tupasela, T., & Joutsjoki, V. (2011). Determination of aspartase activity in dairy Propionibacterium strains. *FEMS Microbiology Letters, 321*, 10–13.

Calasso, M., Ercolini, D., Mancini, L., Stellato, G., Minervini, F., Di Cagno, R., et al. (2016). Relations among house, rind and core microbiotas during manufacture of traditional Italian cheeses at the same dairy plant. *Food Microbiology, 54*, 115–126.

Carcano, M., Todesco, R., Lodi, R., & Brasca, M. (1995). Propionibacteria in Italian hard cheeses. *Lait, 75*, 415–426.

Chamba, J. F., & Irlinger, F. (2004). Secondary and adjunct cultures. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), *Major cheese groups: Vol. 2. Cheese chemistry, physics and microbiology* (pp. 191–206). Amsterdam, The Netherlands: Elsevier Academic Press.

Cummins, C. S., & Johnson, J. L. (1986). Genus 1. Propionibacterium Orla-Jensen 1909, 337AL. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, & J. G. Holt (Eds.), *Bergey's manual of systematic bacteriology* (Vol. 2, pp. 1346–1353). Baltimore, USA: Williams & Wilkins.

Eugster-Meier, E., Fröhlich-Wyder, M. T., Jakob, E., & Wechsler, D. (2017). Cheese from Switzerland. In P. Papademas, & T. Bintsis (Eds.), *Global cheesemaking technology: Cheese quality and characteristics (Part II, section 15)*. Chichester, UK: John Wiley & Sons, Ltd.

Falentin, H., Deutsch, S. M., Jan, G., Loux, V., Thierry, A., Parayre, S., et al. (2010). The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1, a hardy actinobacterium with food and probiotic applications. *PLoS ONE, 5*, e11748.

Fessler, D., Casey, M. G., & Puhan, Z. (1999a). Identification of propionibacteria isolated from brown spots of Swiss hard and semi-hard cheeses. *Lait, 79*, 211–216.

Fessler, D., Casey, M. G., & Puhan, Z. (1999b). Propionibacteria flora in Swiss raw milk from lowlands and alps. *Lait, 79*, 201–209.

de Freitas, R., Chuat, V., Madec, M. N., Nero, L. A., Thierry, A., Valence, F., et al. (2015). Biodiversity of dairy Propionibacterium isolated from dairy farms in Minas Gerais, Brazil. *International Journal of Food Microbiology, 203*, 70–77.

de Freitas, R., Pinheiro Luiz, L. M., Pinheiro Alves, M., Valence-Bertel, F., Augusto Nero, L., & Fernandes de Carvalho, A. (2013). Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic Count plates added to Lithium Glycerol broth. *Journal of Dairy Research, 80*, 270–275.

Fröhlich-Wyder, M. T., & Bachmann, H. P. (2004). Cheeses with propionic acid fermentation. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.) (3rd edn.,*Major cheese groups: Vol. 2. Cheese chemistry, physics and microbiology* (pp. 141–156). Amsterdam, The Netherlands: Elsevier, Academic Press.

Fröhlich-Wyder, M. T., Guggisberg, D., Badertscher, R., Wechsler, D., Wittwer, A., & Irmler, S. (2013). The effect of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* on the eye formation of semi-hard cheese. *International Dairy Journal, 33*, 120–128.

Koussémon, M., Combet-Blanc, Y., Patel, B. K. C., Cayol, J., Thomas, P., Garcia, J., et al. (2001). *Propionibacterium microareolphilum* sp. nov., a microaerophilic bacterium isolated from olive mill wastewater. *International Journal of Systematic and Evolutionary Microbiology*, 1373–1382.

Lucena-Padros, H., Gonzales, J. M., Caballero-Guerrero, B., Ruiz-Barba, J. L., & Maldonado-Barragan, A. (2014). *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style green olives. *International Journal of Systematic and Evolutionary Microbiology, 64*, 2980–2985.

Maurer, J., Haldemann, J., Ascone, P., & Wechsler, D. (2016). *[Recommendations for producers of cheese milk to avoid contaminations of propionic acid bacteria and* Lactobacillus parabuchneri *in milking systems]*. Fiche Technique Agroscope 1/2016 at: https://www.agroscope.admin.ch/agroscope/fr/home/publications/recherche-publications/fiches-techniques/_jcr_content/par/externalcontent.external.exturl.pdf/aHR0cHM6Ly9pcmEuYWdyb3Njb3BlLmNoL2ZyLUNlL0VpbnplbH/B1Ymxpa2F0aW9uL0Rvd25sb2FkRXh0ZXJuP2VpbnplbHB1Ymxp/a2F0aW9uSWQ9MzY5Nzc=.pdf Accessed 12.01.17.

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., et al. (2014). Traditional cheeses rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology, 177*, 136–154.

Parizzi, L. P., Grassi, M. C., Llerena, L. A., Carazzolle, M. F., Queiroz, V. L., Lunardi, I., et al. (2012). The genome sequence of *Propionibacterium acidipropionici* provides insights into its biotechnological and industrial potential. *BMC Genomics, 13*. Article 562.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., & Sohier, D. (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiology, 28*, 848–861.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M. E., et al. (2004). Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and Varroa destructor mite populations in France. *Applied and Environmental Microbiology, 70*, 7185–7191.

Thierry, A., Deutsch, S. M., Falentin, H., Dalmasso, M., Cousin, F. J., & Jan, G. (2011). New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology, 149*, 19–27.

Thierry, A., & Madec, M. N. (1995). Enumeration of propionibacteria in raw milk using a new selective medium. *Lait, 75*, 315–323.

Turgay, M., Irmler, S., Isolini, D., Amrein, R., Fröhlich-Wyder, M. T., Berthoud, H., et al. (2011). Biodiversity, dynamics, and characteristics of *Propionibacterium freudenreichii* in Swiss Emmentaler PDO cheese. *Dairy Science and Technology, 91*, 471–489.

Turgay, M., Schaeren, W., Wechsler, D., Bütikofer, U., & Graber, H. U. (2016). Fast detection and quantification of four dairy propionic acid bacteria in milk samples using real-time quantitative polymerase chain reaction. *International Dairy Journal, 61*, 37–43.