



# Influence of the inoculum level of *Lactobacillus parabuchneri* in vat milk and of the cheese-making conditions on histamine formation during ripening

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## ABSTRACT

Histamine formation by four histaminogenic *Lactobacillus parabuchneri* strains in experimental cheeses was investigated. Firstly, Raclette cheeses were manufactured from pasteurised milk inoculated with different levels of selected *L. parabuchneri* strains ranging from  $10^1$  to  $10^4$  cfu mL<sup>-1</sup>. Secondly, cheeses were produced using four different curd cooking conditions (20 min at temperatures of 44–56  C) to study survival of *L. parabuchneri* strains. The growth of *L. parabuchneri* during cheese ripening and the formation of biogenic amines was monitored using a species-specific qPCR assay and HPLC, respectively. The spoilage threshold in cheese milk was very low, at about  $10^1$ – $10^2$  cfu mL<sup>-1</sup>. Up to 992 mg of histamine kg<sup>-1</sup> was accumulated in the cheeses within 180 days. *L. parabuchneri* proved to be a rather heat-tolerant species; however, heating at 56  C for 20 min proved to be sufficient to inactivate *L. parabuchneri* and thus to prevent histamine formation.

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## 1. Introduction

Among the biogenic amines present in food, histamine has the highest toxicological impact. Its intake often leads to health problems such as headaches, diarrhoea, redness or even more serious symptoms. Histamine-sensitive or histamine-intolerant individuals are particularly affected (Benkerroum, 2016;  anlı;  enel, 2014). Cheeses with a very high histamine content are rare but represent a health risk, especially for children and histamine-sensitive and histamine-intolerant consumers. In the European Rapid Alert System for Food and Feed (RASFF), only one case is documented in which cheese with histamine led to an alert and a market withdrawal of the product (alert 2012.0391). In this incident, registered in 2012, Cheddar cheese containing 1227 mg histamine kg<sup>-1</sup> caused health problems in a group of children.

In cheese and other fermented foods, the main histamine producers are lactic acid bacteria (LAB) (Ascone et al., 2017; M oller, Ucok, & Rattray, 2020). Although facultatively heterofermentative *Lactobacillus* species usually predominate in the microbial

community in matured cheeses, obligately heterofermentative lactobacilli (OHL), such as *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus buchneri*, and *Lactobacillus parabuchneri*, may be found (Broadbent, Budinich, & Steele, 2011; Coton, Berthier, & Coton, 2008).

The species *L. buchneri* and *L. parabuchneri* are phylogenetically closely related. *L. parabuchneri* was first described by Farrow, Phillips, and Collins (1988) and has been found in a variety of habitats, such as human saliva, brewery yeasts, ropy beer, cheese, and silage (Beneduce et al., 2010; Sakamoto & Konings, 2003; Wang & Nishino, 2010; Wittwer, 2011). *L. parabuchneri* grows at 15  C but not at 45  C (Hammes & Hertel, 2006). The occurrence of a histamine-forming strain of *L. buchneri* in cheese was first mentioned in 1985, five years after a small outbreak of histamine poisoning associated with the consumption of Swiss-type cheese aged 18 months in New Hampshire, USA (Sumner, Speckhard, Somers, & Taylor, 1985). In a subsequent study, Sumner, Roche, and Taylor (1990) produced Swiss-type cheese from milk inoculated with *L. buchneri* strain St2A and showed that the presence of this strain during cheese ripening caused strong histamine formation. The strain St2A was later re-identified as *L. parabuchneri* and is available from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG 11773).

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The presence of the histidine decarboxylase (HDC, EC 4.1.1.22) is a strain-specific characteristic of *L. parabuchneri*. Wüthrich et al. (2017) showed that the HDC gene cluster is located on a genomic island and found strong evidence that this cluster was introduced to the genome of *L. parabuchneri* strains by horizontal gene transfer. Based on genome data of *L. parabuchneri* FAM 21731, genetic target sequences were identified, which enabled the development of a quantitative real-time polymerase chain reaction (PCR) and a simple and reliable PCR-based strain typing method (Berthoud et al., 2017).

A recent field study carried out by Ascone et al. (2017) showed that 19.1% of the raw milk samples originating from 67 farms were contaminated with histamine-forming bacteria. *L. parabuchneri* was detected in 97.4% of these histamine-positive raw milk samples, which emphasises the high importance of this species. The population density of *L. parabuchneri* in contaminated raw milk samples was typically  $< 10^2$  gene equivalents (GE) mL<sup>-1</sup>. Systematic testing of milking systems allowed the identification and elimination of the persistent contamination sources of *L. parabuchneri* at the farm level.

In the manufacture of traditional raw milk cheeses, raw milk quality and hygienic processing conditions are key factors in preventing the formation of biogenic amines. However, the accumulation of biogenic amines in cheese is also influenced by other factors, such as the type of coagulant and the ripening time (Tofalo et al., 2019). The most striking feature of histamine-contaminated cheeses is the burning taste caused by the inflammatory effect of histamine on the oral mucosa, easily recognisable above a concentration of approx. 200 mg kg<sup>-1</sup> (Ascone et al., 2017). In addition to histamine, considerable amounts of CO<sub>2</sub> are formed, causing split defects or other undesired eye formation (Bütikofer & Fuchs, 1997; Fröhlich-Wyder et al., 2013). An interesting approach to reduce the accumulation of biogenic amines during cheese ripening could be the use of biogenic amine-degrading adjunct cultures (Tittarelli, Perpetuini, Di Gianvito, & Tofalo, 2019).

Ladero, Sánchez-Llana, Fernández, and Alvarez (2011) investigated the thermal resistance of biogenic amine-producing strains of various LAB species inoculated in skimmed milk. *L. buchneri* B301 showed a reduction in viable number of about 2 log after a low-temperature-long-time pasteurisation (30 min at 63 °C). *L. brevis* (CECT 3810) did not survive this treatment, while some cells of *Lactobacillus curvatus* (VI6) survived even a 30 min treatment at 78 °C. Still, little is known about the heat resistance and survival of *L. parabuchneri* during cheese-making. The present study investigated how different inoculum levels in the cheese milk, and how curd cooking conditions during cheese production affect histamine formation during cheese ripening by *L. parabuchneri*. Four histaminogenic *L. parabuchneri* strains (FAM 21731, FAM 21823, FAM 21836, and FAM 23097) were applied, all of which had been isolated from samples of different types of commercial cheeses produced in geographically separate cheese dairies, and which showed high histamine levels.

## 2. Material and methods

### 2.1. *L. parabuchneri* strains

The four histaminogenic *L. parabuchneri* strains used in this study were obtained from the strain collection of Agroscope (Bern, Switzerland; former name "Forschungsanstalt für Milchwirtschaft", FAM). The strains were isolated from commercial cheese samples containing high amounts of histamine (Table 1) and identified as previously described in Berthoud et al. (2017). The

**Table 1**

Origin of the histaminogenic strains of *Lactobacillus parabuchneri* used in the present study.<sup>a</sup>

Strain	Origin	Age (months)	Histamine (mg kg <sup>-1</sup> )
FAM 21731	Emmentaler PDO (hard cheese)	12	1364
FAM 21823	Mont Soleil (semi-hard cheese)	13	277
FAM 21836	Raclette (semi-hard cheese)	10	945
FAM 23097	Tête de Moine PDO (semi-hard cheese)	3	~445

<sup>a</sup> Value for FAM 23097 is the average of nine analysed batches from the same cheese dairy with histamine contents of 282–682 mg kg<sup>-1</sup>.

presence of the *hdcA* gene was confirmed using the PCR assay described by Coton and Coton (2005). The capability to produce histamine was determined as described previously (Ascone et al., 2017). All strains were stored at -80 °C in sterile reconstituted 10% (w/v) skim milk until use.

### 2.2. Strain preparation and inoculation of vat milks

The frozen *L. parabuchneri* strains were reactivated in MRS broth (de Man, Rogosa, & Sharpe, 1960) and grown overnight at 30 °C. New MRS broth was then inoculated individually with 0.1% (v/v) of the overnight culture and incubated at 30 °C for 20 h. Serial dilutions were plated on MRS agar plates and incubated anaerobically at 37 °C for two days. At these conditions, cell counts in the range of 10<sup>8</sup> cfu mL<sup>-1</sup> were achieved. This value was used to calculate the volume needed for the inoculation of the vat milks.

### 2.3. Manufacture of experimental Raclette-type cheeses from milk inoculated with different population densities of *L. parabuchneri*

A total of 16 semi-hard cheeses (Raclette-type, diameter 30 cm, weight 5.2 kg) were produced from 50 L of pasteurised cow milk (fat content 33 g kg<sup>-1</sup>) with the addition of 10.0 mL CaCl<sub>2</sub> 35% (w/w, aqueous solution) (Dr. Grogg Chemie, Bern, Switzerland) according to the flow sheet in Fig. 1. A batch pasteurisation with a heating rate of +3 °C min<sup>-1</sup> to a maximal temperature of 70 °C during 15 s was applied; the cooling rate was -3 °C min<sup>-1</sup> accordingly. Four different strains of *L. parabuchneri* (FAM 21731, FAM 21823, FAM 21836, or FAM 23097) were used to inoculate vat milks at levels of 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> cfu mL<sup>-1</sup> each as outlined in Table 2.

After the addition of the starter (350 mL RSW 901; Liebefeld Kulturen AG, Bern, Switzerland), which consists of strains of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis* biovar *diacetylactis*, the milk was pre-ripened at 30 °C for 40 min. For coagulation, 12 mL of rennet (Winkler GR orange) was diluted in 1 L of water and added to the milk, which was then incubated at 32 °C for 30 min. According to the manufacturer's instructions, the strength of the rennet was as follows: 1 part of rennet clots 9000 parts of non-heated full-fat cow milk (pH 6.65 at 32 °C) within 30 min, equivalent to 194 IMCU mL<sup>-1</sup>. The coagulum was cut into cubes of about 4–8 mm using a cheese harp with vertical wires. Thereafter, 22 L of water was added to the curd grains/whey mixture, which was heated to 38 °C within 10 min, followed by a final stirring (38 °C, 30 min). For whey removal, the mixture was transferred into perforated moulds (Ø 30 cm) and pressed for 0.75 h at 30 °C. Thereafter, the cheeses were pressed and drained at 10,000 Pa, 25 °C, for about 5–6 h until they reached a pH of 5.2. Immersion in brine solution 20% (w/w) for 14 h at 11–13 °C and ripening at 10–11 °C and 90–96% relative humidity

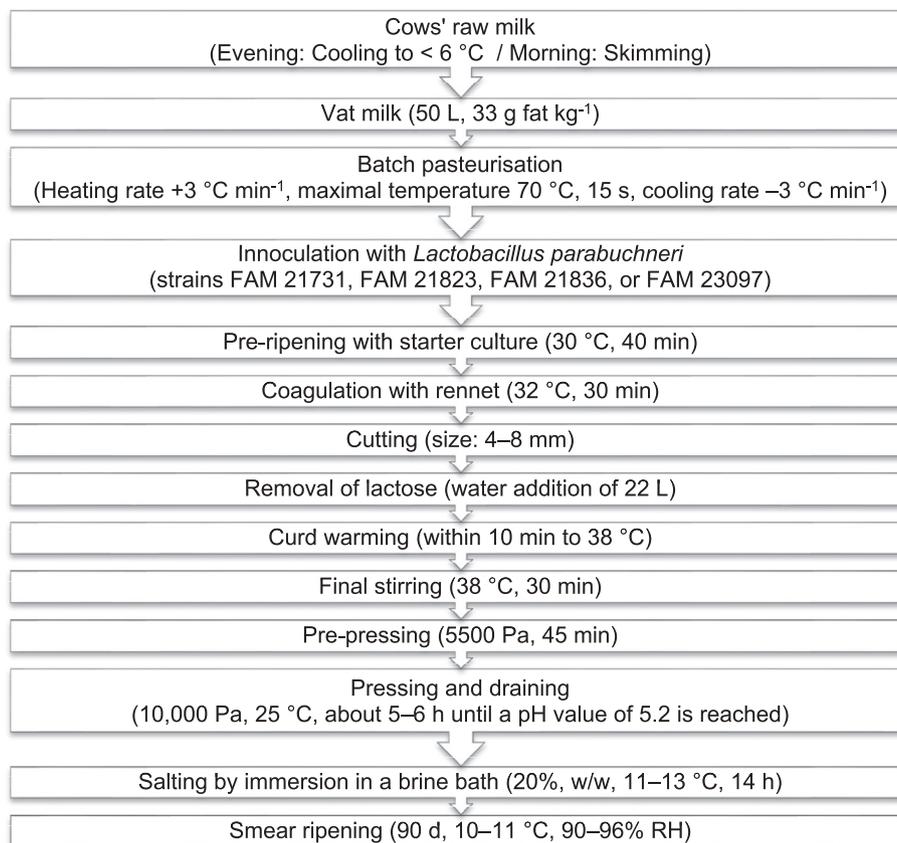


Fig. 1. Flow diagram for the manufacture of Raclette model cheese.

Table 2

Experimental design for the manufacture of Raclette-type cheese using vat milk with inoculums of histaminogenic strains of *Lactobacillus parabuchneri*.<sup>a</sup>

Cheese no.	<i>L. parabuchneri</i> strain	Inoculation level (cfu mL <sup>-1</sup> )
1	FAM 21731	10 <sup>1</sup>
2		10 <sup>2</sup>
3		10 <sup>3</sup>
4		10 <sup>4</sup>
5	FAM 21823	10 <sup>1</sup>
6		10 <sup>2</sup>
7		10 <sup>3</sup>
8		10 <sup>4</sup>
9	FAM 21836	10 <sup>1</sup>
10		10 <sup>2</sup>
11		10 <sup>3</sup>
12		10 <sup>4</sup>
13	FAM 23097	10 <sup>1</sup>
14		10 <sup>2</sup>
15		10 <sup>3</sup>
16		10 <sup>4</sup>

<sup>a</sup> In all cases the starter was RSW 901; inoculation level was the intended level in the vat milk.

for 90 d followed. During the first 10 d of ripening, the cheeses were smeared daily with brine solution (6%, w/v, NaCl) that had previously been inoculated with a mixture of *Brevibacterium linens*, *Arthrobacter* ssp., and *Debaryomyces hansenii* (OMK 702; Liebefeld Kulturen AG, Bern, Switzerland); afterwards, the brine solution was applied twice a week.

#### 2.4. Manufacture of semi-hard and hard cheeses from inoculated milk with different heat treatments

Two series of eight experimental cheeses were manufactured in the Agroscope pilot plant (Bern, Switzerland) from pasteurised milk with the addition of 10.0 mL CaCl<sub>2</sub> 35% (w/w, aqueous solution). The cheese milks were inoculated with one out of four different strains of *L. parabuchneri* each, at a level of 10<sup>3</sup> cfu mL<sup>-1</sup>, as outlined in Table 3. For the manufacture of the semi-hard cheeses, the milk (31 °C) was inoculated with 2‰ (v/v) of the bulk starters MK 401 (*Lc. lactis* subsp. *lactis*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *lactis*) and RMK 150 (*Str. thermophilus* and *L. delbrueckii* subsp. *lactis*; Liebefeld Kulturen AG, Bern, Switzerland). In contrast, two thermophilic bulk starters, RMK 101 and RMK 124 (*Str. thermophilus* and *L. delbrueckii* subsp. *lactis*; Liebefeld Kulturen AG), were used for the manufacture of the experimental hard cheeses.

After pre-ripening (32 °C, 30 min), the milk was coagulated in approximately 35 min, and the coagulum was cut into grains (semi-hard cheeses 4–8 mm, hard cheeses 3–6 mm). After the addition of water (20 L; solely for semi-hard cheeses), the mixture of curd grains and whey was warmed, as indicated in Table 3. Four different temperatures (44 °C, 48 °C, 52 °C, or 56 °C) were applied to study the impact of cheese-making on the survival of *L. parabuchneri* and its histamine formation during cheese ripening. After cooking (20 min), the mixture of curd grains and whey was filled into moulds and pressed (24 h). The cheeses (30 cm in diameter, about 6 kg) were immersed in a 20% (w/w) brine solution at 12 °C for 16 h

**Table 3**

Experimental design for the manufacture of different model cheeses made from vat milk inoculated with histaminogenic strains of *Lactobacillus parabuchneri* at a level of approximately  $10^3$  cfu mL<sup>-1</sup>.

Cheese no.	Starter	<i>L. parabuchneri</i> strains	Heat treatment		Cheese type
			Heating	Final stirring	
1	MK 401, RMK 150	FAM 21731	44 °C, 15 min	44 °C, 15 min	Semi-hard, similar to Appenzeller®
2		FAM 21823			
9		FAM 21836			
10		FAM 23097			
3	MK 401, RMK 150	FAM 21731	48 °C, 15 min	48 °C, 15 min	Semi-hard, similar to Tête de Moine PDO
4		FAM 21823			
11		FAM 21836			
12		FAM 23097			
5	RMK 101, RMK 124	FAM 21731	52 °C, 30 min	52 °C, 20 min	Hard, similar to Emmentaler PDO
6		FAM 21823			
13		FAM 21836			
14		FAM 23097			
7	101, RMK 124	FAM 21731	56 °C, 30 min	56 °C, 20 min	Hard, similar to Gruyère PDO
8		FAM 21823			
15		FAM 21836			
16		FAM 23097			

and 24 h for semi-hard cheeses and hard cheeses, respectively. Smear ripening for 180 d and 360 d for semi-hard and hard cheeses, respectively, was as described above for Raclette-type cheese.

### 2.5. Cheese sampling

Samples of the Raclette-type and the experimental semi-hard and hard cheeses were collected after 1 day as well after 15, 45, 90, and 180 days of ripening. Samples of hard cheeses were collected additionally after 360 days. At each sampling, a vertical cylinder of 2 cm diameter was cut from the loaf at a distance of half of the radius. The collected samples were analysed for chemical and microbial parameters, as described below.

### 2.6. Chemical analysis of cheeses

Fat content of the cheeses was determined using the Gerber van Gulik method (ISO 3433:2008/IDF 222:2008; ISO, 2008). Water content was determined with the dry loss method (ISO 5534:2004/IDF 4:2004; ISO, 2004) by measuring the weight difference of the cheese sample before and after drying at 102 °C for 4 h. Moisture content in the fat-free cheese matrix (MFFB) was calculated using the following formula:

$$\text{MFFB (g kg}^{-1}\text{)} = \text{Water content} \times 1000 / (1000 - \text{Fat content})$$

Total nitrogen (TN) was determined by the Kjeldahl method (ISO 8968-3:2007/IDF 20-3:2007; ISO, 2007). Protein content was calculated from  $\text{TN} \times 6.38$ .

Total lactate (D- and L-lactate) was determined enzymatically according to the instruction protocol of the kit manufacturer (Boehringer, Mannheim, Germany) using an automated spectrophotometric analyser (Gallery, Thermo, Switzerland).

Free amino acids were determined using high-performance liquid chromatography (HPLC) as described previously (Wenzel et al., 2018).

Volatile carboxylic acids (C1–C6, formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, caproic acid, and isocaproic acid) were measured using gas chromatography and flame ionisation detection (GC-FID) with headspace technology after esterification with ethanol, as described by Fröhlich-Wyder et al. (2013).

Propane-1,2-diol was determined after esterification with phenylboronic acid using gas chromatography–mass spectrometry

(GC–MS), as described by Badertscher, Freiburghaus, Wechsler, and Irmeler (2017).

Biogenic amines (cadaverine, histamine, isopentylamine, β-phenylethylamine, putrescine, tryptamine, tyramine, spermidine, spermine) were derivatised with dansyl chloride prior to ultra performance liquid chromatography (UPLC) separation, as previously described in detail by Ascone et al. (2017).

### 2.7. Molecular biological and microbiological analyses

OHL were determined as described by Isolini, Grand, and Glättli (1990). The presence of *L. parabuchneri* in broth, raw milk, and cheese was determined using the quantitative real-time PCR (qPCR) described by Berthoud et al. (2017).

## 3. Results and discussion

### 3.1. Histamine formation by *L. parabuchneri* in Raclette-type cheeses made from inoculated milk

*L. parabuchneri* has been identified as a nonstarter lactic acid bacteria (NSLAB) species that is often responsible for high histamine contents in ripened cheeses (Diaz et al., 2016). In the first cheese experiment, 16 Raclette-type cheeses made from pasteurised vat milk with different inoculum levels of *L. parabuchneri* were analysed at different steps of ripening. Pasteurisation of the cheese milk largely suppresses influences of the raw milk flora and improves the safety of dairy products derived thereof. In contrast to pathogens, which are inactivated by pasteurisation, low counts of aminogenic NSLAB still may be found in pasteurised milk (Ladero et al., 2011). However, cheeses made from pasteurised milk usually contain lower biogenic amine concentrations than cheeses made from raw milk (Novella-Rodríguez, Veciana-Nogués, Roig-Sagués, Trujillo-Mesa, & Vidal-Carou, 2004; Schneller, Good, & Jenny, 1997; Stratton, Hutkins, & Taylor, 1991).

In a recent study (unpublished results) we produced Raclette-type control cheeses (without inoculations of aminogenic bacteria into the pasteurised milk) using exactly the same manufacturing conditions as described in the present manuscript. The overall formation of biogenic amines in the four control cheeses was very low ( $<26$  mg kg<sup>-1</sup>;  $n = 4$ ) and only traces of cadaverine and tyramine were detected. All starters used in the experiments were free from biogenic amine-producing strains. Moreover, genomic analyses (data not shown) and the findings of current literature

(Benkerroum, 2016) indicate that the *L. parabuchneri* strains used in this study do not produce biogenic amines other than histamine.

The results of the first cheese experiment are summarised in Fig. 2, Table 4 and Supplementary material Table S1. A total of nine biogenic amines were determined with the UPLC-System. In the 180-day-matured Raclette-type cheeses (n = 16), histamine on average represented more than 99% of the total amount of biogenic amines. Apart from histamine, only traces of cadaverine were detected (0–26 mg kg<sup>-1</sup>, average 3 mg kg<sup>-1</sup>). These results indicate that biogenic amine-producing bacteria, such as those present in the raw milk, were largely inactivated by the batch pasteurisation applied to the vat milk, and that post-pasteurisation contamination with aminogenic bacteria was almost completely avoided. Therefore, it is unlikely that other bacteria than the added *L. parabuchneri* contributed to the histamine formation.

The four *L. parabuchneri* strains tested showed very similar behaviour regarding growth and histamine formation. For this reason, a strain-specific presentation of the results was omitted. The inoculum level in the vat milk only had a short-term influence on the population density of *L. parabuchneri* in the cheeses studied (Fig. 2A).

After only 45 days, the concentration of *L. parabuchneri* was similar in all the cheeses, independent of the initial concentration in the milk. These results show that histaminogenic strains of *L. parabuchneri* grow very rapidly in cheese, in contradiction of the findings of Sumner et al. (1990) who reported that the number of *L. parabuchneri* St2A in experimental Swiss-type cheeses was strongly dependent on the inoculum level and remained fairly constant during cheese ripening. In our 16 investigated cheeses, the GE of *L. parabuchneri* at 45 d ranged between  $1.8 \times 10^6$  and  $7.9 \times 10^7$  g<sup>-1</sup> (mean  $7.1 \pm 0.5$  log GE g<sup>-1</sup>), and the histamine contents ranged between 8 and 51 mg kg<sup>-1</sup> (Fig. 2B). At 90 days, the histamine content had roughly doubled (82–121 mg kg<sup>-1</sup>), and at the end of the ripening period (180 days), the cheeses clearly showed elevated histamine contents in the range of 270–394 mg kg<sup>-1</sup>, although no further growth of *L. parabuchneri* could be observed. It is worth noting that the inoculum level of *L. parabuchneri* in the vat milk had no influence on the histamine concentration in the ripened cheeses. In contrast, Sumner et al. (1990) found that histamine levels in Swiss-type cheeses aged 90 days (about 150–800 mg kg<sup>-1</sup>) were strongly dependent on the inoculum levels ( $10^2$ – $10^5$  cfu mL<sup>-1</sup>) of

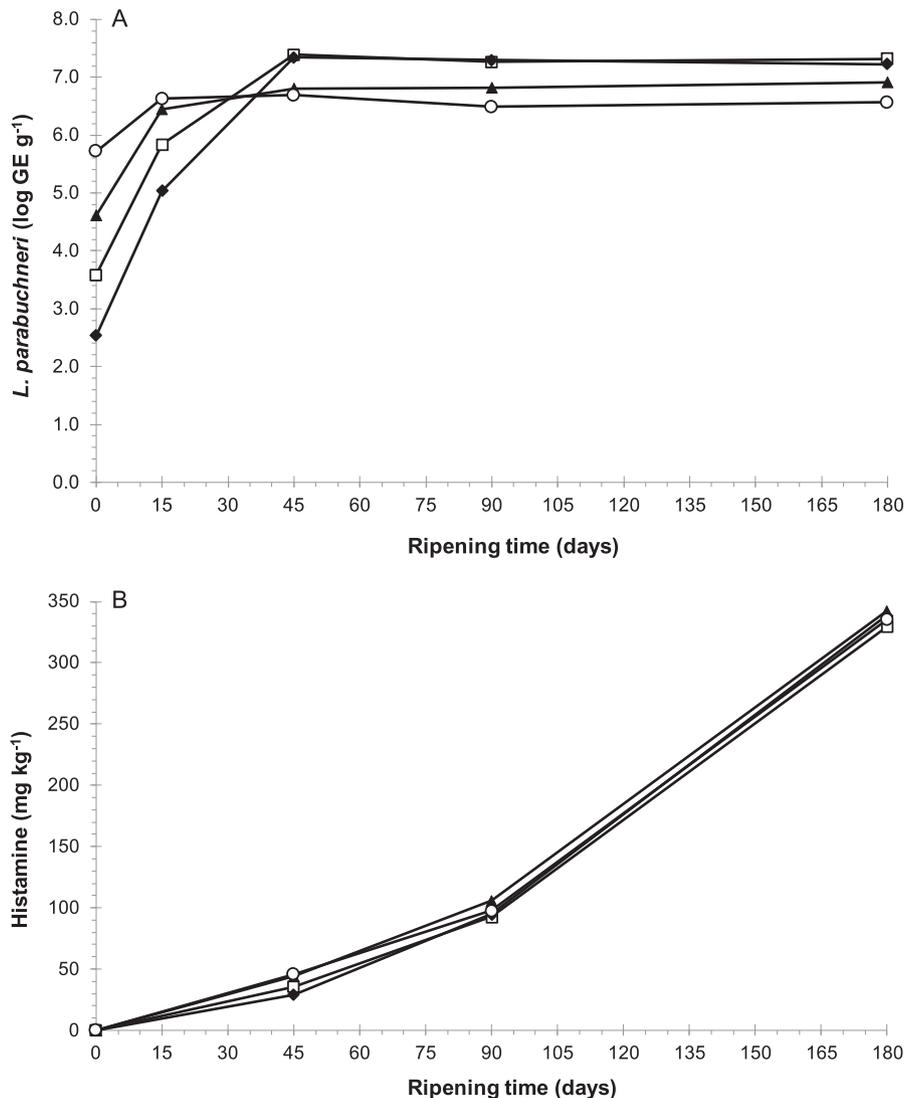


Fig. 2. Growth of *Lactobacillus parabuchneri* (A) and histamine formation (B) in experimental Raclette-type cheeses made from vat milks inoculated with *L. parabuchneri* strains at levels of about  $10^1$  cfu mL<sup>-1</sup> (◆),  $10^2$  cfu mL<sup>-1</sup> (□),  $10^3$  cfu mL<sup>-1</sup> (▲), and  $10^4$  cfu mL<sup>-1</sup> (○). The results represent the mean values of four cheeses, each produced from milk individually inoculated with one of the four *L. parabuchneri* strains FAM 21731, FAM 21823, FAM 21836, and FAM 23097. GE: gene equivalents.

**Table 4**

Chemical analysis of experimental Raclette-type cheeses manufactured from vat milk inoculated with different levels of *Lactobacillus parabuchneri* of about  $10^1$ ,  $10^2$ ,  $10^3$  and  $10^4$  cfu mL<sup>-1</sup>.<sup>a</sup>

Component	Day	<i>Lactobacillus parabuchneri</i> inoculum level			
		$10^1$ cfu mL <sup>-1</sup>	$10^2$ cfu mL <sup>-1</sup>	$10^3$ cfu mL <sup>-1</sup>	$10^4$ cfu mL <sup>-1</sup>
Total lactic acid (mmol kg <sup>-1</sup> )	1	161 ± 5	158 ± 3	159 ± 9	157 ± 10
Portion of L-lactate (%)	1	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
MFFB (g kg <sup>-1</sup> )	180	552 ± 9	548 ± 16	543 ± 18	547 ± 23
pH	180	5.87 ± 0.06	5.82 ± 0.11	5.88 ± 0.08	5.94 ± 0.10
Histamine (mg kg <sup>-1</sup> )	180	339 ± 25	330 ± 51	343 ± 54	335 ± 35
BAs other than histamine (mg kg <sup>-1</sup> )	180	0 ± 0	2 ± 3	7 ± 14	5 ± 9
Propane-1,2-diol (mg kg <sup>-1</sup> )	45	4.3 ± 2.0	5.8 ± 2.1	6.5 ± 1.8	9.3 ± 2.7
	90	7.3 ± 4.4	16.1 ± 7.2	17.9 ± 13.8	23.5 ± 14.7
	180	12.0 ± 8.4	24.6 ± 8.2	24.5 ± 14.8	24.5 ± 15.4
Histidine (free; mg kg <sup>-1</sup> )	180	143 ± 16	135 ± 6	147 ± 15	152 ± 18
Sum of FAAs (mg kg <sup>-1</sup> )	180	19,153 ± 778	18,457 ± 1682	17,702 ± 1872	17,437 ± 1478

<sup>a</sup> Abbreviations are: MFFB, moisture on a fat-free basis; BAs, biogenic amines; FAAs, free amino acids. Values are means and standard deviations of four cheeses each, which were individually inoculated with one of the *L. parabuchneri* strains FAM 21731, FAM 21823, FAM 21836, and FAM 23097.

*L. parabuchneri* St2A. The results obtained in that study should be critically reviewed, since not only the inoculum level but also the heat treatment during cheese production affect the survival of *L. parabuchneri* and histamine formation.

Berthoud et al. (2017) analysed eight commercial cheeses of different varieties with elevated histamine contents in the range of 270–1012 mg kg<sup>-1</sup> using the same species-specific qPCR assay. The results showed that *L. parabuchneri* was present in the corresponding cheeses in concentrations ranging from  $1.1 \times 10^7$  to  $8.5 \times 10^7$  GE g<sup>-1</sup>, which is consistent with our results obtained in the analysed Raclette-type cheeses. In summary, the data show that histaminogenic strains of *L. parabuchneri* typically reach a population density of about  $10^6$ – $10^7$  GE g<sup>-1</sup> in matured cheeses.

Similar to facultatively heterofermentative lactobacilli (FHL), OHL usually occur in very low numbers in the cheese milk, presumably originating from the milking or cheese-making environment. Depending on their metabolic properties and the availability of growth substrates, they can reach counts in the order of  $10^7$  cfu g<sup>-1</sup> during cheese ripening. In traditional cheese varieties such as Raclette, Appenzeller®, Tête de Moine PDO, Emmentaler PDO, and Gruyère PDO, lactose and galactose are usually completely metabolised by the starter cultures within 24 h. Other available energy sources for NSLAB are citrate, lactate, free amino acids and peptides, glycerol released by lipolysis, and microbial cell lysis products such as ribose or deoxyribose released from starter DNA. Oude Elferink et al. (2001) showed that *L. buchneri* and *L. parabuchneri* are capable of converting lactic acid into equimolar amounts of propane-1,2-diol and acetic acid and small amounts of ethanol under anoxic conditions. As propane-1,2-diol seems to be a specific metabolic product of these two *Lactobacillus* species, its detection can serve as an indicator for their growth (Badertscher et al., 2017).

To better understand the relevance of this metabolism for the growth of *L. parabuchneri* in cheese, the concentration of propane-1,2-diol was measured at different ripening times. The results showed that propane-1,2-diol accumulates during ripening, and that the four *L. parabuchneri* strains differed significantly in its level formed. The highest individual concentration of propane-1,2-diol was found to be 46.8 mg kg<sup>-1</sup> (0.6 mmol kg<sup>-1</sup>) in the cheeses made with the addition of *L. parabuchneri* strain FAM 21823. The concentration of this compound was in all cheeses generally low despite the high availability of lactic acid (Table 4). According to Oude Elferink et al. (2001), the anaerobic degradation of lactic acid to propane-1,2-diol does not support cell growth and is pH-dependent. In resting-cell suspensions, this metabolism was found to be active at pH values of 3.8 and 4.3 but, at pH values above

5.8, hardly any lactic acid degradation was observed. The results of our study indicate that this metabolism is less important in cheese than in silage, which can be explained by the fact that the pH in cheese is typically above 5.0 and rises continuously towards 5.8 or higher during ripening. In a previous study, it was also found that *L. parabuchneri* FAM 21731 could not metabolise citrate (Fröhlich-Wyder et al., 2015). In summary, it can be concluded that, apart from the arginine deiminase metabolism, the formation of histamine is the key factor enabling the growth of HDC-positive strains of *L. parabuchneri* in cheese.

### 3.2. Effect of curd cooking conditions on the growth of *L. parabuchneri*

In a second cheese experiment, the four histaminogenic *L. parabuchneri* strains FAM 21731, FAM 21823, FAM 21836, or FAM 23097 were added individually to milk to obtain a population density of approximately  $10^3$  cfu mL<sup>-1</sup>. This corresponds to the upper range of *L. parabuchneri* levels found in raw milk on farms (Ascone et al., 2017; Berthoud et al., 2017). The heat tolerance of the added strains was investigated using curd warming temperatures in the range of 44–56 °C. The applied curd warming temperatures were comparable with those used in the commercial production of Appenzeller® (43–44 °C), Tête de Moine PDO (48–52 °C), Emmentaler PDO (52–54 °C), and Gruyère PDO (56–57 °C) cheese (Table 3). To study the effects of the various heat treatments, the growth of *L. parabuchneri* and histamine formation were monitored during cheese ripening. The results obtained are summarised in Table 5, Supplementary material Table S2, and Fig. 3.

The curd grains/whey mixtures warmed at temperatures of 44 °C and 48 °C yielded semi-hard cheeses with an average MFFB of  $563 \pm 6$  for 44 °C and of  $553 \pm 5$  g kg<sup>-1</sup> for 48 °C (Table 5). In contrast, the experimental hard cheeses manufactured with curd warming temperatures of 52 °C and 56 °C showed, as expected, a lower average MFFB of  $534 \pm 3$  and  $512 \pm 4$  g kg<sup>-1</sup>, respectively. As a result of curd washing, the initial lactic acid contents of semi-hard cheeses (range 128–137 mmol kg<sup>-1</sup>) were about 10 mmol lower on average than those of the hard cheeses (range 133–150 mmol kg<sup>-1</sup>), and their pH-values were distinctively higher than those of the hard cheeses at the end of ripening (pH  $6.04 \pm 0.04$  versus  $5.82 \pm 0.04$ ).

Regarding the concentrations of propane-1,2-diol, strain-specific differences, as well as distinct influences of the warming conditions, were noted. In the cheeses manufactured with a warming temperature of 56 °C, only low concentrations of propane-1,2-diol (<2.1 mg kg<sup>-1</sup>) were detected at the end of ripening. In contrast, the cheeses manufactured with warming

**Table 5**

Chemical analysis of experimental semi-hard and hard cheeses made from vat milk inoculated with approximately  $10^3$  cfu mL<sup>-1</sup> of different strains of *Lactobacillus parabuchneri* using curd cooking conditions of 20 min at 44 °C, 48 °C, 52 °C and 56 °C.<sup>a</sup>

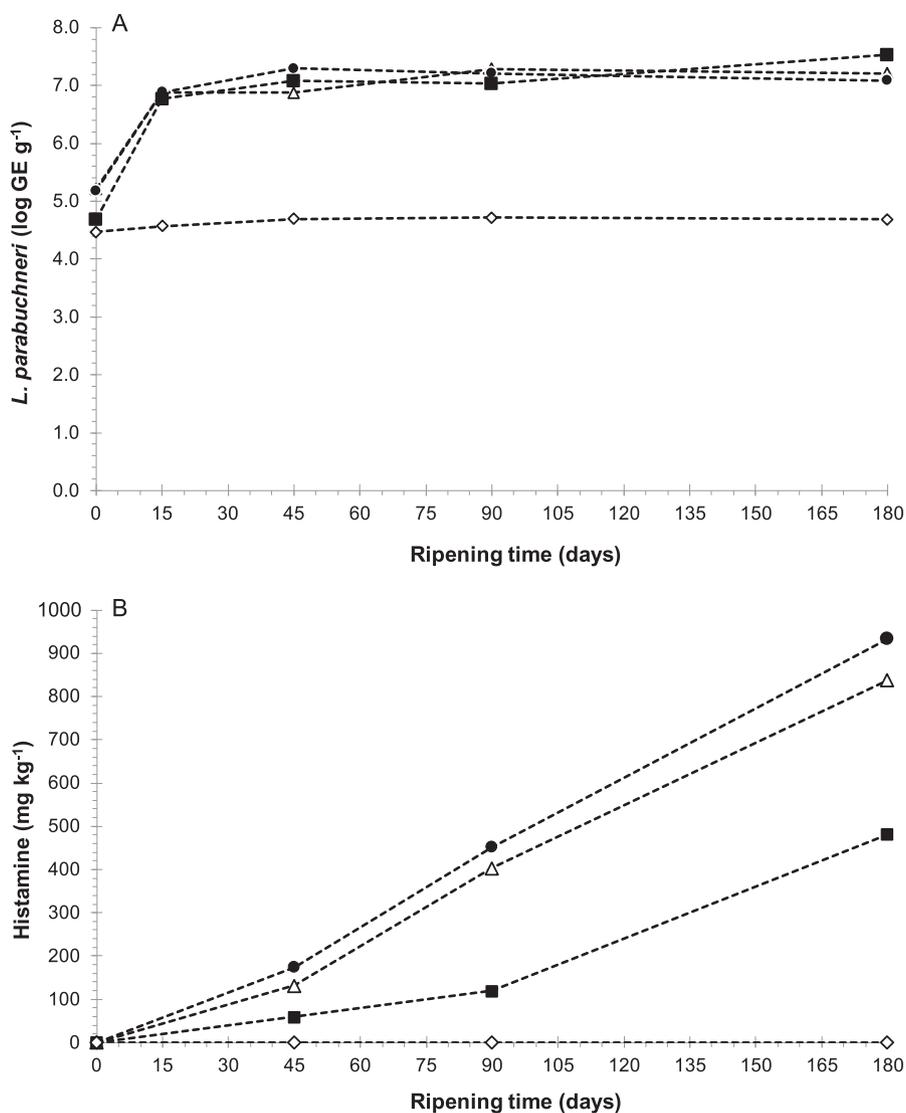
Parameter	Day	<i>Lactobacillus parabuchneri</i> strain			
		FAM 21731	FAM 21823	FAM 21836	FAM 23097
<b>44 °C</b>					
Total lactic acid (mmol kg <sup>-1</sup> )	1	134	135	137	131
MFFB (g kg <sup>-1</sup> )	180	562	571	556	560
pH	180	6.11	6.10	6.03	6.04
Histamine (mg kg <sup>-1</sup> )	180	962	992	910	865
BAs other than histamine (mg kg <sup>-1</sup> )	180	26	28	25	20
Propane-1,2-diol (mg kg <sup>-1</sup> )	45	10.3	14.5	3.9	8.2
	90	19.6	27.9	7.1	14.2
	180	19.6	39.5	10.0	15.6
Histidine (free; mg kg <sup>-1</sup> )	180	125	119	132	143
Sum of FAAs (mg kg <sup>-1</sup> )	180	36,084	31,418	34,266	32,869
<b>48 °C</b>					
Total lactic acid (mmol kg <sup>-1</sup> )	1	129	133	128	130
MFFB (g kg <sup>-1</sup> )	180	552	560	549	551
pH	180	5.98	6.04	6.02	6.02
Histamine (mg kg <sup>-1</sup> )	180	685	838	888	941
BAs other than histamine (mg kg <sup>-1</sup> )	180	18	22	18	23
Propane-1,2-diol (mg kg <sup>-1</sup> )	45	6.3	10.7	3.0	6.3
	90	9.7	31.9	3.6	10.5
	180	14.3	42.1	4.4	14.3
Histidine (free; mg kg <sup>-1</sup> )	180	125	117	131	130
Sum of FAAs (mg kg <sup>-1</sup> )	180	26,788	27,826	30,976	31,709
<b>52 °C</b>					
Total lactic acid (mmol kg <sup>-1</sup> )	1	143	139	145	148
MFFB (g kg <sup>-1</sup> )	360	534	531	532	538
pH	360	5.83	5.85	5.79	5.91
Histamine (mg kg <sup>-1</sup> )	360	851	844	12	790
BAs other than histamine (mg kg <sup>-1</sup> )	360	38	62	52	34
Propane-1,2-diol (mg kg <sup>-1</sup> )	45	3.9	4.2	0.9	5.3
	90	6.5	12.1	0.9	8.1
	180	8.1	27.2	0.4	18.7
Histidine (free; mg kg <sup>-1</sup> )	360	14.8	39.6	1.1	22.3
Sum of FAAs (mg kg <sup>-1</sup> )	360	28	33	732	30
Total lactic acid (mmol kg <sup>-1</sup> )	360	27,806	28,150	25,795	25,512
<b>56 °C</b>					
Total lactic acid (mmol kg <sup>-1</sup> )	1	143	133	142	150
Portion of L-lactate (%)	1	49.8	47.5	48.8	49.5
MFFB (g kg <sup>-1</sup> )	360	507	516	513	510
pH	360	5.81	5.80	5.81	5.82
Histamine (mg kg <sup>-1</sup> )	360	<2	<2	<2	<2
BAs other than histamine (mg kg <sup>-1</sup> )	360	29	48	66	102
Propane-1,2-diol (mg kg <sup>-1</sup> )	45	1.2	1.1	0.9	0.9
	90	0.8	0.8	0.8	0.7
	180	0.6	0.7	0.6	0.5
Histidine (free; mg kg <sup>-1</sup> )	360	1.3	1.3	2.1	1.1
Sum of FAAs (mg kg <sup>-1</sup> )	360	1468	1456	1349	1438
Total lactic acid (mmol kg <sup>-1</sup> )	360	44,037	44,021	40,360	42,905

<sup>a</sup> Abbreviations are: MFFB, moisture on a fat-free basis; BAs, biogenic amines; FAAs, free amino acids.

temperatures of 44, 48, and 52 °C contained up to 42.1 mg kg<sup>-1</sup> propane-1,2-diol, depending on the applied strain. Similarly, an accumulation of histidine but no formation of histamine was found in the hard cheeses warmed at a temperature of 56 °C (Table 5, Supplementary material Table S2 and Fig. 3B). The results of the species-specific qPCR assay show that there was no growth of *L. parabuchneri* in the cheeses warmed at this temperature. This most probably explains why hard-cooked raw-milk cheeses, such as Gruyère PDO and Sbrinz PDO, only contain very low histamine contents. For example, in 12 commercial Gruyère PDO cheeses, histamine was not detectable after a ripening period of 12 months (Wechsler, Walther, Jakob, & Winkler, 2009). In contrast, *L. parabuchneri* proved to be a highly potent histamine producer in all semi-hard experimental cheeses during ripening. By the end of the 180-day ripening period, histamine had accumulated in the semi-hard cheeses at concentrations ranging from 433 to 992 mg kg<sup>-1</sup>. Histamine was

the most abundant biogenic amine, with a share of more than 93% of the total, followed by tyramine, which was present in insignificant concentrations.

Emmentaler PDO curd is typically warmed to 52–54 °C. *L. parabuchneri* strain FAM 21836 showed a low heat resistance; neither growth nor histamine formation was observed in the cheese warmed to 52 °C. In contrast, the three other *L. parabuchneri* strains tested survived this curd warming treatment and even showed growth curves similar to those in the cheeses with curd warming temperatures of 44 °C and 48 °C (Fig. 3A) though a markedly slower formation of histamine was observed (Fig. 3B). The reason for the initially delayed histamine formation in these three cheeses remains unclear; possibly the thermophilic starter also had an influence. However, at the end of the 360-days ripening period, the histamine content in these three cheeses warmed to 52 °C also reached values between 790 and 851 mg kg<sup>-1</sup> (Table 5). These results confirm that even hard-cooked Swiss-type cheeses,



**Fig. 3.** Growth of *Lactobacillus parabuchneri* (A) and histamine formation (B) in experimental cheeses made from vat milks inoculated with *L. parabuchneri* strains at a level of approximately  $10^3$  cfu mL<sup>-1</sup> and heated at maximal temperatures of 44 °C (●; n = 4), 48 °C (△; n = 4), 52 °C (■; n = 3), and 56 °C (◇; n = 4). With one exception, the values represent the mean values of four cheeses, each produced from milk contaminated with one of the four *L. parabuchneri* strains FAM 21731, FAM 21823, FAM 21836, or FAM 23097. Strain FAM 21836 was inactivated at a maximum curd warming temperature of 52 °C and was therefore not included for the mean value. GE: gene equivalents.

such as Emmentaler PDO, may contain elevated histamine levels when contaminated with heat-tolerant *L. parabuchneri* strains.

In the study of Sumner et al. (1990), the heat stability of the histamine-producing *L. parabuchneri* strain St2A was evaluated in rehydrated skim milk. Treatments at temperatures of 49, 60, 65, and 80 °C for 0.5, 1, 2, 5, or 10 min were applied. Strain St2A was stable to heating for 10 min at 49 °C; in contrast, heating to 60 °C and 65 °C decreased the number of surviving bacteria by 1 log and 3 log, respectively. However, it was found that, even after 10 min at 80 °C, approximately  $10^3$  cfu mL<sup>-1</sup> survived. The exceptionally high heat-tolerance observed for the *L. parabuchneri* strain St2A could not be confirmed for the strains used in this study. All four strains tested were completely inactivated by a 20 min heat treatment at 56 °C, and strain FAM 21836 was even completely inactivated by a 20 min heat treatment at 52 °C. These findings show that results obtained from heat resistance tests in skimmed milk are only partially transferable to cheese making and underline the importance of cheese-making experiments. The distinctly lower heat

resistance observed for all strains of this study is probably due to the fact that the harsh environmental conditions in cheese made it more difficult for thermally stressed cells to resume growth.

Compared with the Raclette-type semi-hard cheeses of the first experiment, the semi-hard cheeses of the second experiment showed significantly higher histamine contents at the end of the 180-days ripening. The detected levels of free amino acids reveal that proteolysis was less advanced in the Raclette-type cheeses (Tables 4 and 5). The weaker proteolysis probably limited the histamine formation in the Raclette-type cheeses. This can be explained by the mesophilic starter containing *L. lactis*; the strains of this species tend to be less proteolytic than thermophilic lactobacilli, such as *L. delbrueckii* (Johnson, 2013). Moreover, it has been shown that other factors, such as pH value, influence the metabolic activity of *L. parabuchneri* during cheese ripening (Fröhlich-Wyder et al., 2015).

In addition to the qPCR analyses, the population densities of OHL were determined in the experimental cheeses. The results of these

analyses are in good agreement with the results of the qPCR analyses. However, a direct comparison of colony-forming units (cfu g<sup>-1</sup>) and gene equivalents (GE g<sup>-1</sup>) is difficult due to various methodological differences, such as the inclusion of non-viable cells and the morphology of LAB that occur singly, in pairs, or as short chains (Turgay et al., 2018).

#### 4. Conclusions

The occurrence of increased levels of histamine and other biogenic amines in cheese made from unpasteurised milk is a quality and food safety issue. So far, there has been only one study investigating the influence of the contamination level of *L. parabuchneri* in raw milk and cheese manufacturing conditions on the extent of histamine formation during cheese ripening. The experimental results presented in this study show that even a minimal inoculation level of *L. parabuchneri* in raw milk in the range of about 100 GE mL<sup>-1</sup> causes high levels of histamine formation in raw milk cheese. Furthermore, it could be shown that this rather heat-tolerant species survives the manufacturing conditions of most hard-cooked cheeses. An exception was found for cheeses such as Gruyère PDO, where the curd grains/whey mixture is heated for 20–40 min at a temperature of 56–57 °C. The results of this study indicate that *L. parabuchneri* is inactivated under such conditions, which is why histamine-sensitive individuals can consume such raw milk cheese varieties without health problems. Moreover, the study showed that an initial population density of *L. parabuchneri* in freshly produced cheese of about 100 GE g<sup>-1</sup> is sufficient to cause histamine accumulation of up to 992 mg kg<sup>-1</sup> within six months.

Despite the use of raw milk of good overall microbial quality with a total bacterial count of <10,000 cfu mL<sup>-1</sup>, specific contamination with *L. parabuchneri* may lead to an excessive accumulation of histamine during cheese ripening. Statements generalising that cheeses made from raw milk contain higher levels of biogenic amines are proven to be wrong. In raw milk cheeses with a curd cooking temperature of ≥56 °C, only low histamine values are to be expected. Good milking and processing hygiene is particularly important for raw milk cheeses and makes it possible to reduce the risk of contamination with *L. parabuchneri* and accumulation of histamine even in cheeses with lower cooking temperatures. Based on this study, the monitoring of *L. parabuchneri* in raw milk and cheese can be considered a crucial measure for producers of raw milk cheese to obtain high quality and safe raw milk cheeses.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.idairyj.2020.104883>.

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