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^-1. 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^-1. Up to 992 mg of histamine kg^-1 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; Şanli; Şenel, 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^-1 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; Møller, 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, rropy 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, Specklhall, Somers, & Taylor, 1985). In a subsequent study, Sumner, Roche, and Taylor (1990) produced Swiss-type cheese from milk inoculated with *L. buchneri* strain S2A and showed that the presence of this strain during cheese ripening caused strong histamine formation. The strain S2A was later re-identified as *L. parabuchneri* and is available from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG 11773).
The presence of the histidine decarboxylase (HDC, EC 4.1.1.22) is a strain-specific characteristic of \textit{L. parabuchneri}. \textcite{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 \textit{L. parabuchneri} strains by horizontal gene transfer. Based on genome data of \textit{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 (\textcite{Berthoud et al., 2017}).

A recent field study carried out by \textcite{Ascone et al. (2017)} showed that 19.1\% of the raw milk samples originating from 67 farms were contaminated with histamine-forming bacteria. \textit{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 \textit{L. parabuchneri} in contaminated raw milk samples was typically < 10^2 gene equivalents (GE) mL^-1. Systematic testing of milking systems allowed the identification and elimination of the persistent contamination sources of \textit{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 (\textcite{Tofalo et al., 2019}). The most striking feature of histamine-contaminated cheeses is the burning taste caused by the concentration of approx. 200 mg kg^{-1} histamine. The catalysis and elimination of the persistent contamination sources of \textit{L. parabuchneri} at the farm level.

The frozen \textit{L. parabuchneri} strains were reactivated in MRS broth (\textcite{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 10^8 cfu mL^{-1} were achieved. This value was used to calculate the volume needed for the inoculation of the vat milks.

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

The frozen \textit{L. parabuchneri} strains were reactivated in MRS broth (\textcite{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 10^8 cfu mL^{-1} 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 \textit{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^{-1}) with the addition of 10.0 mL CaCl\textsubscript{2} 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^{-1} to a maximal temperature of 70 °C during 15 s was applied; the cooling rate was ~3 °C min^{-1} accordingly.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Age (months)</th>
<th>Histamine (mg kg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM 21731</td>
<td>Emmentaler PDO (hard cheese)</td>
<td>12</td>
<td>1364</td>
</tr>
<tr>
<td>FAM 21823</td>
<td>Mont Soleil (semi-hard cheese)</td>
<td>13</td>
<td>277</td>
</tr>
<tr>
<td>FAM 21836</td>
<td>Raclette (semi-hard cheese)</td>
<td>10</td>
<td>945</td>
</tr>
<tr>
<td>FAM 23097</td>
<td>Tête de Moine PDO (semi-hard cheese)</td>
<td>3</td>
<td>–445</td>
</tr>
</tbody>
</table>

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

The frozen \textit{L. parabuchneri} strains were reactivated in MRS broth (\textcite{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 10^8 cfu mL^{-1} were achieved. This value was used to calculate the volume needed for the inoculation of the vat milks.

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After the addition of the starter (350 mL RSW 901; Liebefeld Kulturen AG, Bern, Switzerland), which consists of strains of \textit{Lactococcus lactis} subsp. \textit{lactis}, \textit{L. lactis} subsp. \textit{cremeris}, and \textit{L. lactis} subsp. \textit{lactis} biovar \textit{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) in 30 min). For whey removal, the curd grains/whhey 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.
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$_2$ 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$^3$ cfu mL$^{-1}$, as outlined in Table 3. For the manufacture of the semi-hard cheeses, the milk (31 ± 4°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 ± 4°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 ± 4°C, 48 ± 4°C, 52 ± 4°C, or 56 ± 4°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.
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:

\[
\text{MFFB} = \frac{\text{Water content} \times 1000}{1000 - \text{Fat content}}
\]

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 (\( \text{L}- \)and \( \text{L}- \)lactate) was determined enzymatically according to the instruction protocol of the kit manufacturer (Boehringer, Manheim, Germany) using an automated spectrophotometric analyser (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 Irmler (2017).

Biogenic amines (cadaverine, histamine, isopentylamine, \( \beta \)-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-Sagüés, 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\(^{-1}\); \( n = 4 \)) 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 \textit{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\(^{-1}\), average 3 mg kg\(^{-1}\)). 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 \textit{L. parabuchneri} contributed to the histamine formation.

The four \textit{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 \textit{L. parabuchneri} in the cheeses studied (Fig. 2A).

After only 45 days, the concentration of \textit{L. parabuchneri} was similar in all the cheeses, independent of the initial concentration in the milk. These results show that histaminogenic strains of \textit{L. parabuchneri} grow very rapidly in cheese, in contradiction of the findings of Sumner et al. (1990) who reported that the number of \textit{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 \textit{L. parabuchneri} at 45 d ranged between \(1.8 \times 10^6\) and \(7.9 \times 10^7\) g\(^{-1}\) (mean \(7.1 \pm 0.5\) log GE g\(^{-1}\)), and the histamine contents ranged between 8 and 51 mg kg\(^{-1}\) (Fig. 2B). At 90 days, the histamine content had roughly doubled (82–121 mg kg\(^{-1}\)), 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\(^{-1}\), although no further growth of \textit{L. parabuchneri} could be observed. It is worth noting that the inoculum level of \textit{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\(^{-1}\)) were strongly dependent on the inoculum levels (\(10^2–10^5\) cfu mL\(^{-1}\)) of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2}
\caption{Growth of \textit{Lactobacillus parabuchneri} (A) and histamine formation (B) in experimental Raclette-type cheeses made from vat milks inoculated with \textit{L. parabuchneri} strains at levels of about 10\(^3\) cfu mL\(^{-1}\) (●), 10\(^2\) cfu mL\(^{-1}\) (□), 10\(^3\) cfu mL\(^{-1}\) (▲), and 10\(^4\) cfu mL\(^{-1}\) (○). The results represent the mean values of four cheeses, each produced from milk individually inoculated with one of the four \textit{L. parabuchneri} strains FAM 21731, FAM 21823, FAM 21836, and FAM 23097. GE: gene equivalents.}
\end{figure}
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⁻¹ 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 × 10⁷ to 8.5 × 10⁸ GE g⁻¹, 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 10⁸–10⁹ GE g⁻¹ 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 10⁷ cfu g⁻¹ 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⁻¹ (0.66 mmol kg⁻¹) 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 10³ cfu mL⁻¹. 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 ± 6 for 44 °C and of 553 ± 5 g kg⁻¹ 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 ± 3 and 512 ± 4 g kg⁻¹, respectively. As a result of curd washing, the initial lactic acid contents of semi-hard cheeses (range 128–137 mmol kg⁻¹) were about 10 mmol lower on average than those of the hard cheeses (range 133–150 mmol kg⁻¹), and their pH-values were distinctly higher than those of the hard cheeses at the end of ripening (pH 6.04 ± 0.04 versus 5.82 ± 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⁻¹) were detected at the end of ripening. In contrast, the cheeses manufactured with warming
In contrast, ripening period of 12 months (Wechsler, Walther, Jakob, Gruy

very low histamine contents. For example, in 12 commercial

concentrations ranging from 433 to 992 mg kg⁻¹. Histamine was

accumulation of histidine but no formation of histamine was

emmentaler PDO curd is typically warmed to 52°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⁻¹ (Table 5). These results confirm that even hard-cooked Swiss-type cheeses,

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⁻¹ (Table 5). These results confirm that even hard-cooked Swiss-type cheeses,
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^-1^ 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⁻¹) and gene equivalents (GE g⁻¹) 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⁻¹ 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/wheway 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⁻¹ is sufficient to cause histamine accumulation of up to 992 mg kg⁻¹ within six months.

Despite the use of raw milk of good overall microbial quality with a total bacterial count of <10,000 cfu mL⁻¹, 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

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References


