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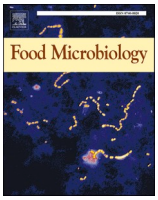

Histamine-forming ability of *Lentilactobacillus parabuchneri* in reduced salt Cheddar cheese

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ABSTRACT

Lentilactobacillus parabuchneri, a member of the non-starter microbiota in cheese, was recently associated with fast and effective histamine-formation ability, a safety issue. The present study was performed to investigate *Lentilactobacillus parabuchneri* KUH8, a histamine-producer (HP) in reduced-salt Cheddar cheese. Four cheeses were manufactured: 1) normal-salt (NS); 2) reduced-salt (RS); 3) normal-salt with HP (NS+HP); 4) reduced-salt with HP (RS+HP). Two replicates were produced with milk from the same batch, and the cheeses ripened at 10 and 15 °C. Cheeses were sampled immediately after manufacture and after 1, 3 and 6 months of ripening. Ultra-high-performance-liquid chromatography indicated that with the HP, histamine reached higher levels in reduced-salt cheeses (3.5–3.7% S/M) at 15 °C (86, 1112, 2149 and 3149 mg kg^{-1}), compared to normal-salt cheeses (5.4–6.3% S/M) at 10 °C (78, 584, 593 and 1389 mg kg^{-1}), at each respective cheese-sampling point. Higher salt-content reduced the growth rate of non-starter microbiota, but after six months the levels in all cheeses were similar, according to the ripening temperature: at 10 °C (8.05–8.30 log_{10} cfu g^{-1}), and at 15 °C (6.00–6.94 log_{10} cfu g^{-1}). A correlation between increased histamine levels, non-starter-cell development and pH was found. This study highlights the importance of normal-salt content and low-ripening temperature as measures to control histamine-formation and to improve safety in cheese.

1. Introduction

Histamine formation through the microbial decarboxylation of histidine is a safety concern, especially in long-time ripened cheeses such as Cheddar, Emmental, Gouda, Manchego and Swiss type cheeses (Ascone et al., 2017; Daly et al., 2010; Diaz et al., 2016; Švarc-Gajić and Stojanović, 2011). Most of the lactic acid bacteria able to use histidine as an energy source, are members of the non-starter lactic acid bacteria (NSLAB) and derived from the milk or the processing environment (Ascone et al., 2017; Moniente et al., 2021). Development of NSLAB is an important parameter for flavour formation in cheese (Gobbetti et al., 2015), but considering the safety issue related to the presence histamine-producers, there is a need for excluding histamine-formation ability as a parameter for starter and ripening culture selection (Møller et al., 2020; Moniente et al., 2021).

The major safety concern is related to the ingestion of high levels of histamine. High ingestion may cause urticarial, rashes, headache and hypertensive crisis. The symptoms can be more severe in individuals that are not able to metabolize and eliminate histamine efficiently (Pino-Ángeles et al., 2012). There is no established histamine limit for cheese, but for fish the limit is 100 mg kg^{-1} (Commission of the European Communities, 2007). In 2011, EFSA reported a level of 1870 mg kg^{-1} in a cheese implicated in an incident of histamine intoxication.

Many lactic acid bacteria have been generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA), and they have been placed on the qualified presumption of safety (QPS) list by the European Food Safety Authority (EFSA), due to their long history of use in fermented food products (Hill et al., 2018). To be included in these categories, the lactic acid bacterium needs to have its safety assessed according to the requirements for entering the food chain (EFSA, 2017). This is important because some of the lactic acid bacteria members may be of safety concern due their histamine-production ability (Møller et al., 2020). The main lactic acid bacteria associated with histamine production in cheese are *Lentilactobacillus parabuchneri* and *Streptococcus thermophilus* (Diaz et al., 2016). In fact it has been reported that *L. parabuchneri* is usually, if not exclusively, responsible for histamine formation in cheese (Ascone et al., 2017; Fröhlich-Wyder et al., 2017). In this sense, histamine-forming *L. parabuchneri* strains are undesirable

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in dairy industry (Diaz et al., 2016).

The use of high salt-in-moisture (4.7–5.7%) and low temperatures of ripening have been applied to suppress growth of undesirable micro-organisms, reducing safety risks and ensuring high quality products (Guinee and Sutherland, 2011; Khan and Anand, 2016). However, a challenge for the food industry is the demand from consumers for healthier products such as cheeses with reduced salt content, and in this context, histamine-producing bacteria may present a risk to the dairy industry. An additional problem with producing reduced salt cheese is that it lacks flavour intensity which may be addressed by addition of a ripening or adjunct culture (Gobbetti et al., 2015). This in itself may also be problematic as such cultures are often highly proteolytic (Ascone et al., 2017; Choi et al., 2020; Di Cagno et al., 2006; Sadat-Mekmene et al., 2013) resulting in the release of high levels of free amino acids, the precursors for biogenic amine formation.

Recently, Møller et al. (2020) reported a fast and effective histamine-producing *L. parabuchneri* strain (KUH8), isolated from cheese. This lactic acid bacterium is an important species of the NSLAB microbiota of cheese and it may have the histidine decarboxylase operon present (Ascone et al., 2017; Cruz Martín et al., 2005). Therefore, the histamine-forming ability of this cheese isolate needs to be further investigated, especially considering the new trends for fast production of healthier cheeses, such as the case of use of proteolytic adjunct culture, salt reduction and increased temperature of ripening.

In this study, the ability of *L. parabuchneri* KUH8 was investigated during ripening of Cheddar cheese. Besides the addition of a proteolytic adjunct culture and the use of a reduced salt content, an increased temperature of ripening was also examined.

This approach is expected to support the dairy industry, and to highlight the importance of regulating, monitoring and controlling the histamine-forming ability of lactic acid bacteria in cheese.

2. Material and methods

2.1. Confirming the histamine-forming ability and the identity of the strain

L. parabuchneri KUH8, recently isolated and identified (16S rRNA gene sequencing) by Møller et al. (2020) as a histamine-producer strain, was investigated to confirm the histamine-forming ability and the identity of the strain.

2.1.1. Histamine-forming ability

After thawing and mixing, 100 μL of the stock culture (kept in 20% glycerol at −81 °C) were transferred to 10 mL restricted broth without glucose and with 1.0% histidine and a pH indicator added (Møller et al., 2020). The culture was incubated at 30 °C for 48 h, a loop was streaked on MRS agar (Difco™, Le Pont le Claix, France) and incubated anaerobically at 30 °C for 5 d. Twenty colonies were then selected and tested individually in 5 mL of restricted broth without glucose and with 1.0% histidine and a pH indicator added. After 5 d incubation at 30 °C, 100 μL were spread on restricted agar plates (1.7% agar in the restrict broth without glucose and with 1.0% histidine and a pH indicator added) and incubated anaerobically at 30 °C for 5 d. Colour change due to raise of pH in the restricted media was considered as an indication of histamine production.

2.1.2. Identity of the strain

2.1.2.1. DNA preparation. Three of the isolates tested for colour change in restricted broth were randomly selected. A loop of each of the three isolates, as well as a loop of the original frozen stock, were grown individually in 5 mL MRS broth for a week at 30 °C. Each grown culture was then centrifuged (4,400 g for 10 min), the supernatant discarded, the pellets resuspended in 2.0 mL of 0.9% saline, and transferred to two Eppendorf tubes. After centrifugation (10,000 g for 5 min), the supernatant was discarded and the DNA from the pellet was extracted with Gravity A&A extraction kit (A&A Biotechnology, Gdansk, Poland), according to the manufacture's instructions. DNA concentration was measured with Qubit™ 4 Fluorometer (ThermoFisher Scientific Inc., Hvidovre, Denmark), following the manufacture's instructions.

2.1.2.2. Whole genome sequencing. The DNA library was prepared following RAPID barcoding protocol (SQK-RBK004, Version: RBK_9054_v2_rewQ_14Aug2019, https://nanoporetech.com), loaded on R9.4.1 flow cell and sequenced using GridION Mk1 (Oxford Nanopore Technologies, Oxford UK).

2.1.2.3. Analysis of sequencing data. The raw data (fast5) was base-called and demultiplexed using Guppy (v4.4.2, Oxford Nanopore Technologies, Oxford UK). Fastq files were quality-controlled (≥14 average base quality: ≥98% identity and a minimum size of 5 kb) using NanoFilt (De Coster et al., 2018). High-quality reads were then subjected to *de-novo* assembly-only using wtdbg2 (Ruan and Li, 2020). For every isolate, a single genome-fragment was retrieved and their taxonomic annotation was determined as follows: ORF calling and gene predictions were performed with Prodigal (Hyatt et al., 2010), the predicted proteins were blasted (blastp – DIAMOND) (Buchfink et al., 2014) against NCBI NR bacterial and archaeal protein database. Using Basic Sequence Taxonomy Annotation tool (BASTA) (Kahlke and Ralph, 2019), the Lowest Common Ancestor (LCA) for every genome was estimated based on percentage of hits of LCA of 85, minimum identity of 0.85, minimum alignment of 0.85 and a minimum number of hits for LCA of 100. Average nucleotide identity (ANI) of the assemble genomes was calculated with fastANI (Jain et al., 2018), and compared against NCBI RefSeq (January 2021). The assembled genomes were screened for the presence of histamine decarboxylase operon of *Lentilactobacillus buchneri* (Cruz Martín et al., 2005).

2.2. Preparation of the histamine producing strain for the cheese trial

A stock of the isolate *L. parabuchneri* KUH8 (kept in 20% glycerol at −81 °C) was thawed, mixed and 100 μL transferred to 10 mL MRS broth, incubated for 24 h at 30 °C and centrifuged (6000×*g* for 10 min at 4 °C). To ensure that no histamine residue would be transferred to the cheese, the cell pellet was washed twice with 0.9% NaCl solution followed by centrifugation. The washed cells were then used to inoculate the cheese milk at a level of 5.0 log cfu mL^{-1}. Plate counting experiments established the appropriate dilution in order to achieve this target level in the cheese milk.

2.3. Culture preparation and cheese recipes

The starter culture used was a mesophilic homofermentative culture, type O, containing strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Frozen-Direct Vat Set (F-DVS) R-604, Chr. Hansen A/S, Hørsholm, Denmark). A thermophilic *Lb. helveticus* adjunct culture (F-DVS LH-32, Chr. Hansen A/S, Hørsholm, Denmark) was also included to promote proteolysis and to ensure high levels of histidine in the cheese matrix.

In all four recipes, the cheeses were produced with (w/w): 0.012% starter culture and 0.002% adjunct culture. The four recipes were: 1) **NS**, cheese with normal salt-in-moisture (target 5.5% w/w); 2) **RS**, cheese with reduced salt-in-moisture (target 3.5% w/w); 3) **NS+HP**, cheese with normal salt-in-moisture (target 5.5% w/w) and *L. parabuchneri* KUH8; 4) **RS+HP**, cheese with reduced salt-in-moisture (target 3.5% w/w) and *L. parabuchneri* KUH8.

Cheeses were manufactured according to these four recipes in four different 200 L vats, and produced on the same day. The same batch of milk was also used to perform the whole operation again on the next

day, generating the second replicate.

2.4. Cheese trial

In each vat, 100 L of 3.5% fat, non-homogenized and pasteurized cow milk (Arla Foods, Slagelse, Denmark) was heated to 32 °C. All cultures and the histamine producing strain were added simultaneously to the vat, according to the designed recipe. After 45 min, the coagulant (CHY-MAX® Plus, 200 IMCU mL^{-1}, Chr. Hansen A/S) was added at a level of (w/w) 0.018%, held for 40 min, and then cut into 10 mm cubes. The curd grains were heated to 38 °C over 40 min and then cooked for a further 20 min at 38 °C. The whey was drained, the curd was cut into blocks and cheddaring took place until the pH of 5.2 was reached. The curd was milled, salted and extensively mixed over 30 min to ensure even salt distribution. After salting, the cheeses were initially pressed at 2 bar for 30 min, and then at 5 bar for 16 h. Each of the cheeses was then cut into 12 blocks (weighing approximately 800 g) and vacuum packed. Half of the blocks of each cheese was stored at 10 °C, while the other half was stored at 15 °C. The cheese manufacture took place at the dairy pilot plant of the University of Copenhagen.

2.5. Analytical design

2.5.1. Chemical analysis in milk

Besides measurement of pH, the composition of the milk was analysed using an automatic milk analyser. About 50 mL of milk at 32 °C was collected from each of the vats, injected in duplicate to a calibrated milk analyser (Milko-Scan FT2 analyzer, Foss Electric, Hillerød, Denmark) and levels of fat, protein, casein and lactose were measured.

2.5.2. Chemical analysis in cheese

NaCl (ISO 5943 IDF88, 2006), moisture (ISO 5534: IDF4, 2004) and pH (Ardö and Polychroniadou, 1999) were determined seven days after the cheeses' manufacture. Determination of protein with the Kjeldahl method (Ardö and Polychroniadou, 1999) and fat content with the Gerber method (ISO 488 IDF105, 2008) were also performed.

2.5.3. Microbiological analysis

Milk samples from each of the eight vats and cheese samples from all treatments and time points were analysed by spreading 100 μL of appropriated diluted milk or cheese extract (10 g cheese and 90 g of 2.0% sterile tri-sodium citrate mixed in a Stomacher® blender for 2 min at high speed) in 0.9% saline. Subsequently serial dilution on agar plates was performed using conditions for starter lactic acid bacteria (SLAB) (M17 agar at 25 °C for 4 days, aerobic) and *Lentilactobacillus parabuchneri* and other non-starter lactic acid bacteria (NSLAB) (MRS agar pH 5.4 at 30 °C for 5 days, anaerobic). Pre-experiments confirmed no growth of the ripening culture, *L. helveticus* on these media and incubation conditions. Media were obtained from OXOID (Hampshire, UK). Milk sampling took place just before adding the cultures to the vats. Cheeses were sampled immediately after manufacturing and after 1, 3 and 6 months ripening at 10 and 15 °C.

2.5.4. Analysis of amino compounds

Amino compounds were extracted in triplicate from the cheeses in plastic bags (1.0 g cheese and 9.0 g of 0.1 M HCl with 0.2% 3,3′-thiodipropionic acid) mixed in Stomacher® for 1 min at high speed. For deproteinization the mixture was held at 30 °C for 30 min in an ultrasound bath. The bags were then mixed in a Stomacher® for 1 min at high speed and 1.2 mL sample was transferred to an Eppendorf tube. The sample was centrifuged at 9600×*g* for 30 min at 4 °C, and then 1 mL of the supernatant was filtered through a 0.45 μm cellulose acetate membrane. Finally, for removal of peptides, 500 μL of the filtrate was transferred to a 3000 Da (3 kDa) cut-off Amicon ultra-0.5 centrifugal filter device (Sigma-Aldrich, Copenhagen, Denmark) and centrifugated at 9600 x for 30 min at 4 °C. Derivatization of 100 μL filtrate (non-diluted for samples before ripening and 10-fold dituted in 0.1 M HCl for samples during ripening time) was performed as described by Redruello et al. (2013). The conditions for measuring the amino compounds in the ultra performance liquid chromatography (UPLC) were the same as those of Møller et al. (2020) and the gradient of Redruello et al. (2013), but with a reduced molarity of the ammonium acetate buffer (10 mM). Reagents were HPLC grade and obtained from Sigma-Aldrich (Copenhagen, Denmark), except for the standard with 18 amino compounds, which was obtained from Thermo Fischer Scientific (Roskilde, Denmark).

2.6. Data analysis

Analysis of variance (ANOVA) with post-hoc Tukey HSD were applied to evaluate the cheese composition and to identify if differences in values of salt, moisture, protein, fat and pH were significant at the level of 0.05%, using R studio software (Version 3.6.1). Furthermore, Principal Component Analysis (PCA) was carried out using the MixOmics (6.14.0) (Rohart et al., 2017) and ggplot2 (3.3.3) (Wickham, 2016) packages on the R platform version 4.0.3.

3. Results and discussion

A *Lentilactobacillus parabuchneri* strain, isolated from a Gouda cheese, was recently associated with fast and effective histamine-formation ability (Møller et al., 2020). Whole genome sequencing was performed, the identity of the strain was confirmed and the genes for histamine production were identified (Table 1). This strain of *L. parabuchneri* KUH8 was used in the present study at two different target salt-in-moisture (S/M) contents (5.5 and 3.5% S/M) and two ripening temperatures (10 and 15 °C) in cheeses manufactured in duplicate. The investigated cheeses at 10 °C or 15 °C were: 1) cheese with normal salt-in-moisture ($NS_{10°C}$ or $NS_{15°C}$); 2) cheese with reduced salt-in-moisture ($RS_{10°C}$ or $RS_{15°C}$); 3) cheese with normal salt-in-moisture and *L. parabuchneri* KUH8 ($NS+HP_{10°C}$ or $NS+HP_{15°C}$); 4) cheese with reduced salt-in-moisture and *L. parabuchneri* KUH8 ($RS+HP_{10°C}$ or $RS+HP_{15°C}$). The same batch of milk was used to produce the cheeses in replicates 1 (Day 1) and replicates 2 (Day 2), which minimized variation in the composition of the milk tested in each of the eight vats. Composition of the milk was: fat (3.54 ± 0.01%), protein (3.25 ± 0.01%), casein (2.47 ± 0.02%), and lactose (4.56 ± 0.01%). Values of pH (6.7 ± 0.04) were also measured. LAB able to grow on M17 agar at 25 °C in aerobic conditions (mainly *Lactococcus* species) were found (3.41 ± 0.10 $\log_{10}$ cfu mL^{-1}) as well as LAB able to grow on MRS (ph 5.4) at 30 °C in anaerobic conditions (mainly *Lactobacillus* species) in the level of 1.17 ± 0.20 $\log_{10}$ cfu mL^{-1}. These results indicate that the

Table 1
Average Identity Nucleotide (ANI) comparison between isolates and references genomes, as well as the presence of histidine decarboxylase operon.

Isolate	Replicate	Colour change[a] assay	Taxonomy	size (Mb)	NCBI RefSeq	ANI	Target-Operon (TO)	% ID TO	% CoverQuery TO
KUH8	1	+	*L. parabuchneri*	2.58	NZ_CP018796.1	97.78	ENA|AJ749838|AJ749838.1	99.74	100
KUH8	2	+	*L. parabuchneri*	2.63	NZ_CP018796.1	97.83	ENA|AJ749838|AJ749838.1	99.91	100
KUH8	3	+	*L. parabuchneri*	2.62	NZ_CP018796.1	97.77	ENA|AJ749838|AJ749838.1	99.90	100
KUH8	4	+	*L. parabuchneri*	2.65	NZ_CP018796.1	97.80	ENA|AJ749838|AJ749838.1	99.95	100

[a] Positive (+) results in the colour change assay (Møller et al., 2020) is related to a raise in pH, which may be an indication of histidine decarboxylation.

milk characteristics were inside the expected range for all tested parameters. As indicated in Table 2, the composition of the eight manufactured cheeses was also within the range expected for Cheddar cheese. As anticipated, the differences were mainly significant at the level of 0.05% between the group of cheeses with distinct target salt content (NS = 5.5 and RS = 3.5% S/M). The level of S/M of the NS cheeses ranged between 5.35 and 6.25%, which is acceptable considering that the range for high quality Cheddar cheese with normal salt content is 5.4–6.4% S/M (Ong et al., 2017). A high standard deviation for histidine and histamine was found for NS+$HP_{10\,°C}$ (Fig. 2 C). This is explained by a difference in the S/M values of the replicates; S/M values for NS+$HP_{10\,°C}$ in replicate 1 (Fig. 2C-Rep1) and in replicate 2 (Fig. 2C-Rep2) were 5.37 and 6.25% (Fig. 2C-Rep2), respectively. This difference in S/M was primarily due to the combined effect of a higher salt content and low moisture content in replicate 2 (Table 2). It is believed that the lower S/M (5.37) in replicate 1 was due to insufficient mixing of the salt and curd chips during the salting step. While the magnitude of the S/M difference was relatively small (0.88) it had a large effect on the histamine development in the cheeses. In replicate 1 histamine levels reached 2408 mg kg^{-1}, after 6 months at 10 °C (Fig. 2C-Rep1), while in replicate 2 the level of histamine reached only 371 mg kg^{-1} after 6 months at 10 °C (Fig. 2C-Rep2). These results clearly show that histidine decarboxylaton by *L. parabuchneri* in cheese is effectively controlled by S/M values at or greater than 6.25 %. The RS cheeses ranged between 3.29 and 3.93% S/M. Salt content is very important in influencing the microbiota composition of cheese. The effect of salt reduction on Cheddar cheese parameters resulting in increased moisture, reduced S/M, and decreased pH have been reported (McCarthy et al., 2016). It also impacts directly the cheese composition and flavour development, which is a result of biochemical changes such as proteolysis and free amino acid levels during the ripening (Guinee, 2004). When producing high quality Cheddar cheeses, less variation of the parameters is required, which may be achieved by managing manufacture conditions in reduced salt cheeses, such as milk pre-acidification for less impact on pH, and changes in gel cutting and cooking temperatures to reduce moisture (McCarthy et al., 2016). The focus of this study was mainly to investigate the behaviour of *L. parabuchneri* in a reduced salt cheese. Changes in manufacture conditions such as increased cooking temperatures could affect the initial microbiota within the cheeses (Gatti et al., 2014; Sheehan et al., 2007). To avoid variation on the initial microbiota due other parameters rather than salt content, no changes in manufacture conditions were made. By reducing salt content, moisture increased but protein and fat were present at reduced levels (Table 2). Yet, NaCl content explained a low percentage of variance (Fig. 1A) on counts (M17 & MRS), total AA, histamine and histidine (Fig. 1F), as compared to the presence of histamine-producer (Fig. 1C), temperature (Fig. 1B) and time (Fig. 1D) of ripening.

As expected, the cheeses with *L. parabuchneri* KUH8 added (closed symbols in Fig. 1C) showed increased levels of histamine and counts in MRS (Fig. 1F), while the cheeses without the histamine-producer added (open symbols in Fig. 1C) corresponded with increased levels of histidine. High counts in M17 (Fig. 1F) were related to early stages of ripening, while throughout the ripening period (Fig. 1D) an increase in pH (Fig. 1E) and total free amino acids was observed.

3.1. Evaluation of histamine production

As shown in Fig. 1C and F, only cheeses with *L. parabuchneri* KUH8 added had a large impact on histamine formation. For cheeses ripened at 10 °C no histamine was found in the normal salt ($NS_{10°C}$) and reduced salt ($RS_{10°C}$) cheeses until the sixth month of ripening (Fig. 2A and B), when histamine started to be produced at very low levels (111 ± 0 mg kg^{-1}), in both cheeses. These levels are below the values of histamine found in raw milk semi-hard cheeses at earlier stage of ripening (2.5 months), which averaged between 210 and 360 mg kg^{-1} depending on

Table 2
Chemical composition of Cheddar cheese with normal salt (NS) or reduced salt (RS) content, made with or without the histamine producer (HP) *L. parabuchneri* KUH8.

Measurement	unit	Parameters applied to the manufactured Cheddar cheeses							
		Normal Salt (NS)		Reduced Salt (RS)		*L. parabuchneri* KUH8 in Normal Salt (NS+HP)		*L. parabuchneri* KUH8 in Reduced Salt (RS+HP)	
		Rep 1[β]	Rep 2[β]	Rep 1[β]	Rep 2[β]	Rep 1[β]	Rep 2[β]	Rep 1[β]	Rep 2[β]
Salt[α]	(%)	1.98 (±0.05)[a]	1.97 (±0.02)[a]	1.54 (±0.12)[b]	1.30 ± (0.06)[b]	1.99 (±0.02)[a]	2.25 (±0.07)[a]	1.38 (±0.03)[b]	1.43 (±0.21)[b]
moisture[α]	(%)	36.98 (±0.48)[a]	36.41 (±0.33)[a]	39.11 (±0.12)[b]	39.46 ± (0.25)[b]	37.06 (±0.34)[a]	35.97 (±0.29)[a]	38.91 (±0.31)[b]	38.96 (±0.19)[b]
Protein[α]	(%)	27.38 (±0.18)[a]	27.07 (±0.36)[a]	26.28 (±0.37)[b]	26.70 ± (0.37)[b]	26.90 (±0.37)[a,c]	27.04 (±0.24)[a,c]	26.78 (±0.02)[b,c]	26.70 (±0.15)[b,c]
Fat[α]	(%)	31.75 (±0.25)[a]	31.25 ± (0.25)[a]	29.50 (±0.50)[b]	29.00 ± (0.50)[b]	31.50 (±0.25)[a]	31.50 (±0.25)[a]	30.25 (±0.25)[c]	30.00 (±0.25)[c]
pH[α]		5.1 (±0.05)[a]	5.1 ± (0.09)[a]	4.9 (±0.05)[a]	5.0 ± (0.02)[a]	5.1 (±0.11)[a]	5.1 (±0.09)[a]	5.0 (±0.03)[a]	5.0 (±0.03)[a]
S/M[γ]	(%)	5.35	5.41	3.93	3.29	5.37	6.25	3.54	3.68
Cheese after pressing	(kg)	9.78	9.81	9.81	9.99	9.75	9.53	10.05	10.11

β Cheddar cheeses were manufactured in day 1 (Rep 1) and day 2 (Rep 2) with milk from same batch.
ϰ Determination of salt, moisture, protein, fat and pH were performed in triplicate. Averaged values (standard deviation between brackets) for each replicate in the respective tested cheese. Analysis of variance (ANOVA) with post-hoc Tukey HSD were applied for comparing these multiple measurements. Different superscript letters in the same row indicate statistical significant difference of the measurements at the level of 0.05%.
γ S/M (salt-in-moisture) was calculated using the averaged values of salt and moisture indicated for each replicate in the respective tested cheeses.

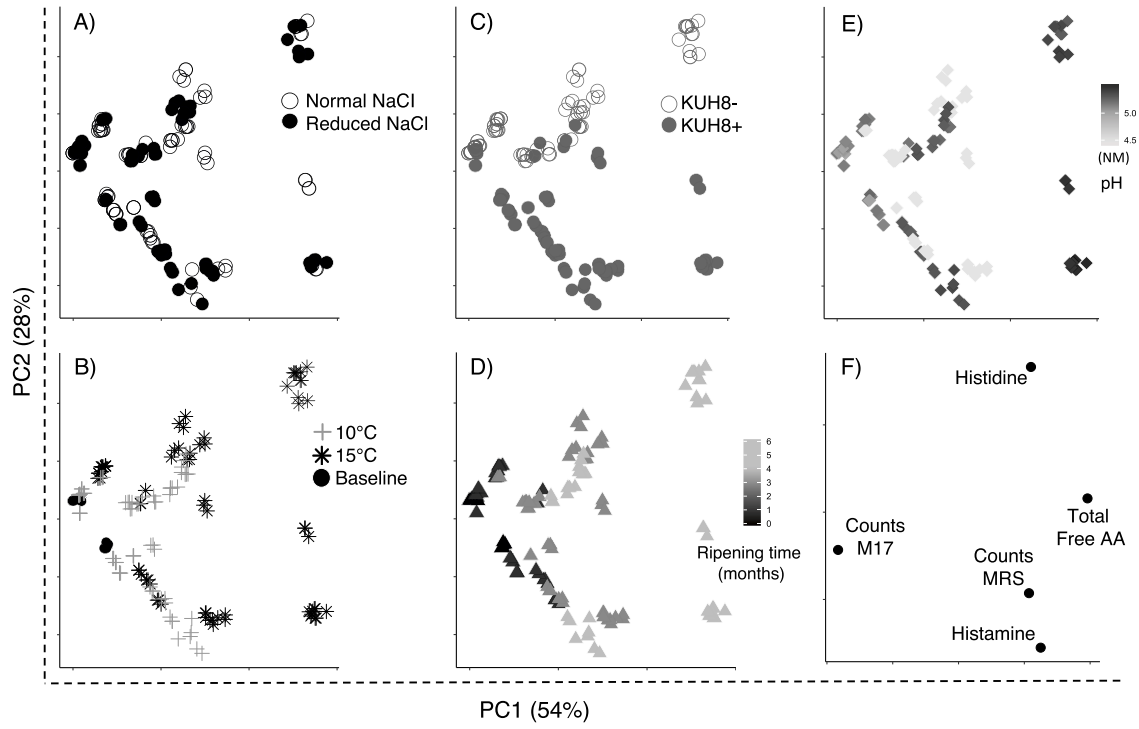

Fig. 1. Principle Component Analysis (PCA) of different measurements obtained from Cheddar cheeses during ripening: A) Content of NaCl (Normal = target 5.5% w/w; Reduced = target 3.5% w/w); B) Before ripening (baseline) and temperature applied during ripening (10 and 15 °C); C) presence (+) and absence (−) of histamine-producer *L. parabuchneri* KUH8; D) before ripening (0) and during ripening time (after 1, 3 and 6 months); E) pH levels before ripening (pH values in Table 2), and after 1 (mean value of 5.1 in all cheeses) and 6 (mean pH value = 5.2 in normal salt without KUH8 and 5.3 for the other cheeses at 10 °C, and mean pH value = 5.3 in normal salt without KUH8 and 5.4 for the other cheeses at 15 °C) months of ripening; F) loadings of the measurements used to built the PCA. The four obtained cheeses were produced in day 1 (Replicate 1) and in day 2 (Replicate 2) with milk from the same batch.

the origin of the milk (Ascone et al., 2017), indicating a possible effect of pasteurization on microbial quality of the milk, when comparing to our results from cheeses made with pasteurized milk. The levels of histidine after 6 months ripening for $NS_{10°C}$ and $RS_{10°C}$ were 1629 ± 78 mg kg^{-1} and 1810 ± 377 mg kg^{-1}, respectively. Guinee et al. (2008) found that levels of histidine in vintage Cheddar cheeses from retail varied between 1142 and 2210 mg kg^{-1}, which is an indication that the values found in the present study are within the range expected for Cheddar cheese, especially considering the proteolytic *Lb. helveticus* added as an adjunct culture. It seems that this was also the explaining factor for the high levels of total free amino acids (TFAA) in cheeses $NS_{10°C}$ (3.10 ± 0.38%, w/w) and $RS_{10°C}$ (3.45 ± 0.28%, w/w), after six months of ripening. Nevertheless, these levels were still within the range of TFAA reported by Guinee et al. (2008) in vintage Cheddar cheeses (2.43–3.56%, w/w). After six months of ripening, histamine formation at high levels was seen in the cheeses inoculated with *L. parabuchneri* KUH8. The level of histamine produced in the reduced salt cheeses ($RS+HP_{10°C}$) was 2204 ± 393 mg kg^{-1} (Fig. 2D), while in the normal salt cheeses ($NS+HP_{10°C}$) the level of histamine (1389 ± 1044 mg kg^{-1}) was much lower (Fig. 2C), when ripened at 10 °C. The level of histidine was nearly depleted 155 ± 0 mg kg^{-1} in the $RS+HP_{10°C}$ cheese and present at the level of 1060 ± 924 mg kg^{-1} in the $NS+HP_{10°C}$ cheese. For the cheeses ripened at 15 °C, no histamine was found in the normal salt ($NS_{15°C}$) and reduced salt ($RS_{15°C}$) cheeses until six months of ripening (Fig. 3A and B), were it started to rise to 167 ± 56 mg kg^{-1} and 296 ± 189 mg kg^{-1}, respectively. The levels of histidine at this time point were similar, 3077 ± 107 and 3103 ± 237 mg kg^{-1} in the normal salt ($NS_{15°C}$) and reduced salt ($RS_{15°C}$) cheeses, respectively. The level of histamine in the reduced salt cheese $RS+HP_{15°C}$ was 3149 ± 52 mg kg^{-1} (Fig. 3D) and in the normal salt cheese $NS+HP_{15°C}$ the level of histamine was 2631 ± 532 mg kg^{-1} (Fig. 3C), indicating the combined effect of reduced salt content and higher temperature of ripening on histamine formation during ripening. The effect of increased temperature on accelerating histamine formation in Cheddar cheese has been described by Madejska et al. (2018), who reported that in commercially available hard cheeses stored at 22 °C, histamine reached levels double as high as when compared to storage at 4 °C. The effect of reduced salt content on histamine production in Cheddar cheese has been reported previously (Stratton et al., 1991). However, levels of histamine after 6 months ripening were reported to be very low (less than 50 mg kg^{-1}) probably a reflection of very low proteolysis due to the fact that no adjunct or ripening culture was added. Adding an adjunct culture to the milk as a measure to accelerate cheese ripening and to obtain a fast release of amino acids in cheese has been described (Gobbetti et al., 2015; Saiki et al., 2018). In the present study, *Lb. helveticus* was added to the cheeses due its effectiveness to increase the level of histidine during cheese ripening (Ardö et al., 2009; Hickey et al., 2018). The effect on *Lb. helveticus* on enhancing proteolysis in cheese has been reported (Griffiths and Tellez, 2013; Sadat-Mekmene et al., 2013; Slattery et al., 2010). Nateghi (2012) reported that when *Lb. helveticus* was added as an adjunct culture to Cheddar cheese making, the level of histidine after 75 days at 12 °C reached 1.40 nmol/g cheese, while it remained undetectable when no *Lb. helveticus* was added. In addition, the level of total free amino acids was more than four times higher (34.86 nmol/g cheese) when *Lb. helveticus* was added, in relation to the control cheese without *Lb. helveticus* added (8.10 nmol/g cheese). As expected, the level of histidine available in the cheeses was high, indicating the efficiency of the added *Lb. helveticus* in accelerating proteolysis and releasing this amino acid. In the cheeses RS+HP, regardless of ripening temperature, the level of histidine was always found to be much lower (Figs. 2D and 3D) than NS+HP (Figs. 2C and 3C). This low level of histidine in reduced salt cheeses occurred even at earlier stages, and already after one month of ripening the levels of histamine formed in these cheeses were high, particularly in the reduced salt cheese ($RS+HP_{15°C}$) ripened at 15 °C (Fig. 3D) with histamine at the level of

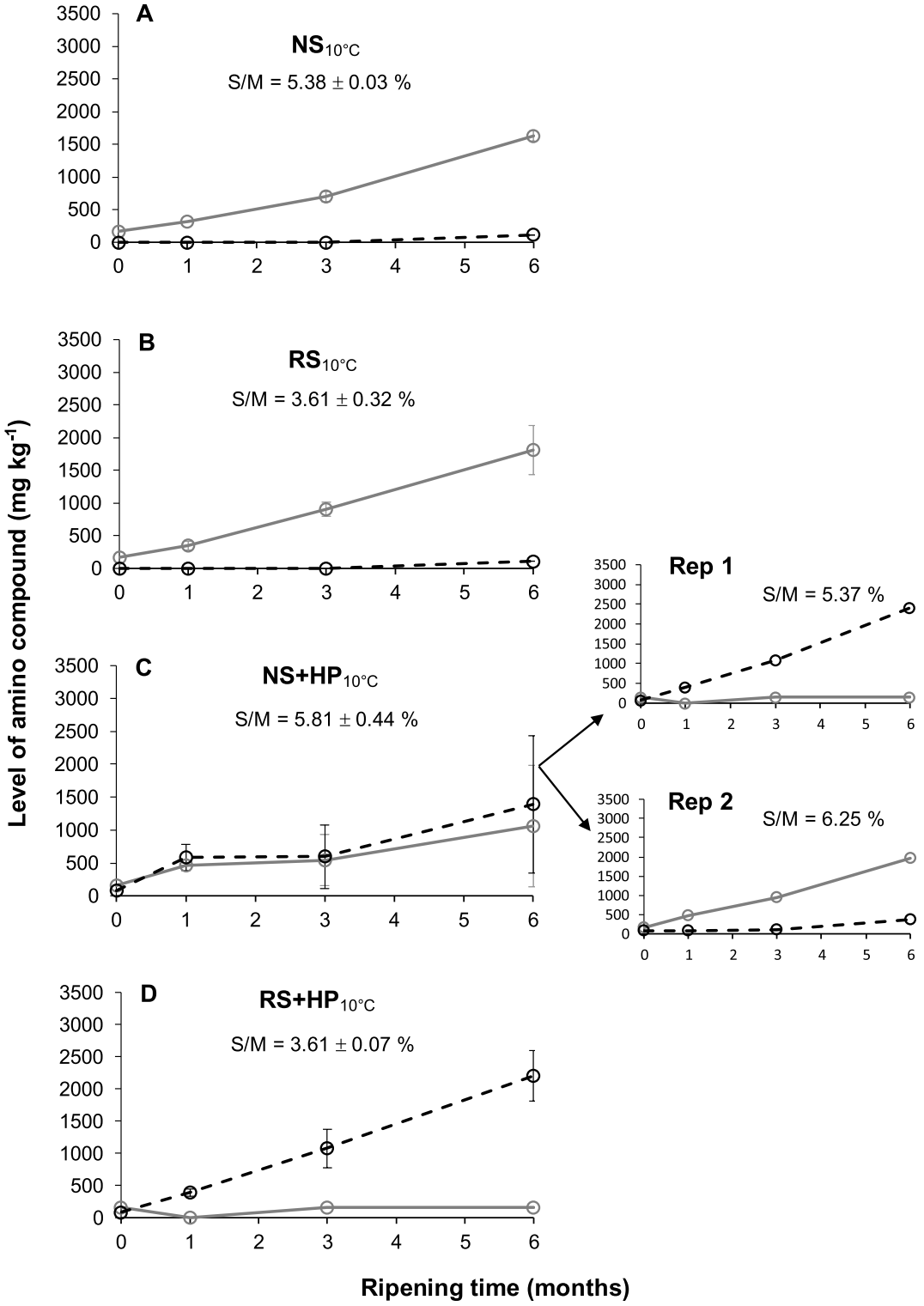

Fig. 2. Changes in levels of histidine (continuous grey lines) and histamine (dashed black lines) in ripening at 10 °C of Cheddar cheeses: (A) **NS** (normal salt-in-moisture, target 5.5% w/w); (B) **RS** (reduced salt-in-moisture, target 3.5% w/w); (C) **NS+HP** (normal salt-in-moisture, target 5.5% w/w, and *Lentilactobacillus parabuchneri* KUH8); and (D) **RS+HP** (reduced salt-in-moisture, target 3.5% w/w, and *L. parabuchneri* KUH8) were sampled immediately after manufacture and after 1, 3 and 6 months of ripening. Amino compounds were measured in triplicate with UPLC in each of the two replicates (Rep 1 and Rep 2) manufactured in day 1 and day 2 with the same batch of milk. Averaged values ± standard deviation (error bars) are shown. Values for salt-in-moisture (S/M) are indicated for each cheese as average (followed by ± standard deviation) or as absolute values for Rep 1 and Rep 2 (for NS+HP cheese).

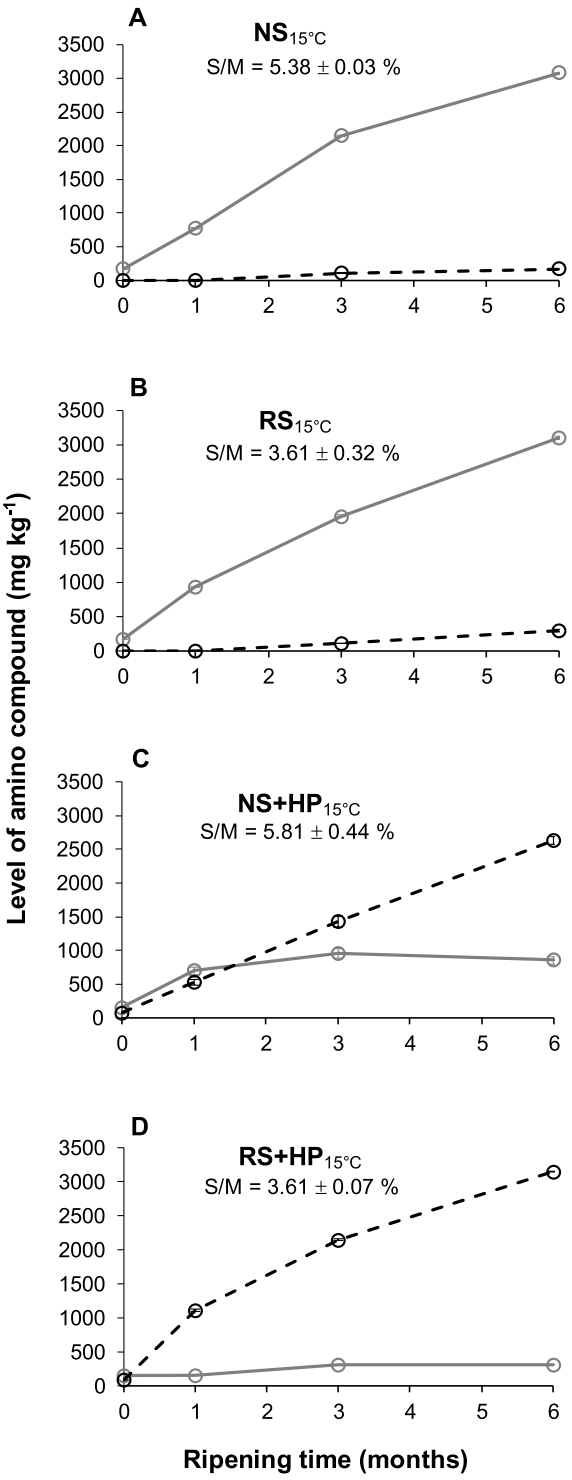

Fig. 3. Changes in levels of histidine (continuous grey lines) and histamine (dashed black lines) in ripening at 15 °C of Cheddar cheeses: (A) **NS** (normal salt-in-moisture, target 5.5% w/w); (B) **RS** (reduced salt-in-moisture, target 3.5% w/w); (C) **NS+HP** (normal salt-in-moisture, target 5.5% w/w, and *Lentilactobacillus parabuchneri* KUH8); and (D) **RS+HP** (reduced salt-in-moisture, target 3.5% w/w, and *L. parabuchneri* KUH8) were sampled immediately after manufacture and after 1, 3 and 6 months of ripening. Amino compounds were measured in triplicate with UPLC in each of the two replicates (Rep 1 and Rep 2) manufactured in day 1 and day 2 with the same batch of milk. Averaged values ± standard deviation (error bars) are shown.

1093 ± 175 mg kg^{-1}. Estrada et al. (2019) reported that in raw sheep milk cheese the level of proteolysis increased in cheese with higher salt content (3.0%) when compared to cheese with lower salt content (2.2%). However, Dugat-Bony et al. (2019) highlighted that the effect of salt on proteolysis is highly dependent on the cheese type. When the histamine-producer was present, the levels of histamine were higher at nearly all time points for the RS+HP cheese, regardless of ripening temperature. These results show that when present in the cheese matrix, the histamine-producer converts histidine to histamine at a faster rate in reduced-salt content. This clearly indicates the need to monitor the presence of histamine-producers in the milk and to apply strategies to avoid transfer of these microorganisms to the cheese during manufacture. This is of particular relevance to reduce risks related to histamine and to improve safety of long time ripened cheeses (Møller et al., 2020). In the cheese with higher level of S/M (Fig. 2C-Rep2), the levels of histamine were always low during the whole ripening time, and only after six months of ripening it raised to 371 (±52) mg kg^{-1}. Even before ripening, the counts in MRS were higher in all cheeses with the HP added (Fig. 4B and D), and after three months of ripening the level of NSLAB in these cheeses were close to the maximum density, indicating that despite the presence of the HP strain added, the high salt content inhibited production of histamine at 10 °C (Fig. 2C-Rep2). Joosten and Northolt (1989) tested the histamine-producer *L. parabuchneri* at levels of about 1 log_{10} cfu mL^{-1} milk and after 3 months of ripening the histamine produced in the Gouda cheese reached a level of 1060 mg kg^{-1}, which is closely related to the level found after 3 months at 10 °C in this study (Fig. 2D) for RS+$HP_{10°C}$ cheese (1149 ± 228 mg kg^{-1}) and almost the double of the level found for NS+$HP_{10°C}$ (Fig. 2C) cheese (593 ± 483 mg kg^{-1}). As expected, before ripening the counts in MRS for the cheeses without the HP were low (below the detection limit of 2.0 log cfu g^{-1}), both for NS cheese (Fig. 4B and D) and RS cheese (Fig. 4B and D). As also expected, the initial counts in MRS were detected at a higher level in the cheeses with HP added, varying from 4.61 ± 2.61 log cfu g^{-1} in the NS+HP cheese (Fig. 4B and D) to 7.15 ± 0.08 log cfu g^{-1} in the RS+HP cheese (Fig. 4B and D). The same trend between these two cheeses regarding histamine formation at a higher level in the RS+HP cheese, was also seen in the in MRS counts, which was faster in the RS+HP cheese (Fig. 4B and D). In the cheese NS+$HP_{10°C}$ (Fig. 4B) the same level of development took three months to occur as in RS+$HP_{10°C}$ (Fig. 4D). The increase in the level of LAB counts in MRS in the cheese NS+HP was also shown to occur when the cheese was ripened at 15 °C (Fig. 4D), but at a faster rate since after only at 1 month of ripening the level of LAB in the cheese NS+$HP_{15°C}$ (Fig. 4D) was already about the same as in RS+$HP_{15°C}$ (Fig. 4D). Levels of LAB counts in MRS at six months of ripening were about the same in all cheeses, ripened at the same temperature. Despite the fact that pasteurization reduces the level of LAB counts in MRS from the milk to about or under 2.0 log_{10} cfu g^{-1}, unknown species of histamine-producers still remain present as members of this microbiota, which may explain the fact of cheeses made with pasteurized milk been implicated in histamine intoxication (Barbieri et al., 2019; EFSA, 2011; Gobbetti et al., 2015). Values of pH also indicates the effect of salt content, especially after six months of ripening when lower values of pH (5.2–5.3) were measured in the NS cheeses, and values of pH (5.3–5.4) in reduced salt cheeses. Effect of salt reduction on increase of pH in Cheddar cheeses has been reported, especially in reduced fat content (McCarthy et al., 2015). Increased values of pH is an indication that compounds able to raise the pH were formed, as it is the case of histamine. The increase in pH due to histamine formation is also likely to accelerate the rate of proteolysis in the cheeses due to increased activities of plasmin and intracellular LAB peptidases at pH values closer to their optima (around neutral pH) (McSweeney, 2017). After six months of ripening the cheeses with the higher pH also had the higher levels of TFAA: NS+$HP_{10°C}$ (3.53 ± 0.61%, w/w) and RS+$HP_{10°C}$ (3.74 ± 0.61%, w/w) were at pH 5.3, while NS+$HP_{15°C}$ (10.55 ± 0.12%, w/w) and RS+$HP_{15°C}$ (11.13 ± 0.43%, w/w) at pH 5.4. The increase in values of pH was higher in

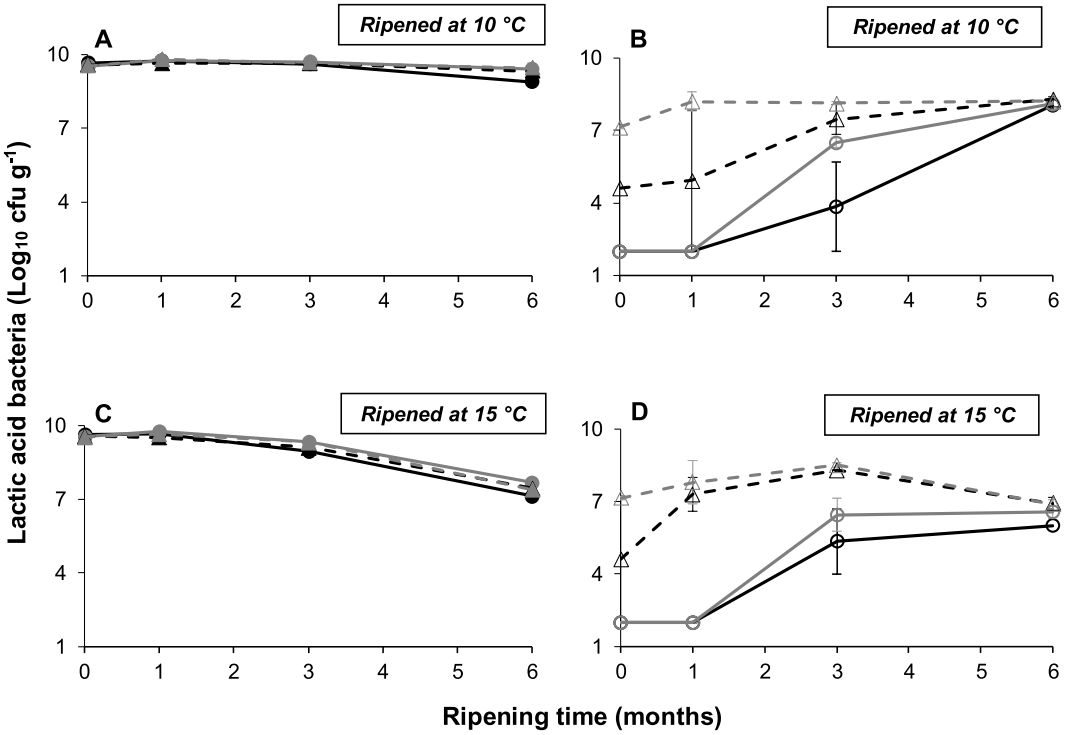

Fig. 4. Changes in levels of starter on M17 agar (closed symbols, to the left in A and C) and non-starter on MRS agar (open symbols, to the right in B and D) lactic acid bacteria in Cheddar cheeses. Averaged values ± standard deviation (error bars) of the two cheeses (manufactured in day 1 and day 2 with the same batch of milk) for each treatment are shown. The manufactured cheeses: **NS**) normal salt-in-moisture, target 5.5% w/w (continuous black lines), **RS**) reduced salt-in-moisture, target 3.5% w/w (continuous grey lines), **NS+HP**) normal salt-in-moisture, target 5.5% w/w, and *L. parabuchneri* KUH8 (dashed black lines) and **RS+HP**) reduced salt-in-moisture, target 3.5% w/w, and *L. parabuchneri* KUH8 (dashed grey lines) were ripened at 10 °C (A and B) and 15 °C (C and D).

cheeses at 15 °C (5.4), which supports the histamine levels measured. Effect of higher temperatures of ripening on increasing the level of proteolysis have also been reported in Cheddar cheese (Ong and Shah, 2009). Therefore, the pH monitoring during cheese ripening is an important indicator of quality and safety. A correlation between increased level of histamine produced, accelerated stage of LAB development in MRS, and high value of pH was shown in the cheeses during ripening.

4. Conclusions

To meet the demands faced by the dairy industry for healthier products, reduced salt Cheddar cheeses, with and without the addition of a histamine producer, were produced and ripened at different temperatures. Changes in the levels of histamine and LAB in MRS were monitored in the cheeses, over 6 months of ripening. Normal salt content limits the histamine formation at low temperature of ripening. High levels of histamine were formed in reduced salt cheeses and it was particularly problematic at high ripening temperatures, where levels as high as 3100 mg kg^{-1} were observed. Besides UPLC, the monitoring of pH is a good strategy to follow formation of compounds with the ability to raise the pH, such as the case of histamine. This study highlights the importance of applying the best combination of parameters to promote the safety and quality in cheese ripening. Besides monitoring of flavour development and traditional attributes of quality and safety, the monitoring of histamine formation is suggested to support the dairy industry in the challenges of new product development.

Declaration of competing interest

No conflicts of interest.

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