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Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during Gouda cheese ripening

Renata G.K. Leuschner*, Rima Kurihara, Walter P. Hammes

Institut für Allgemeine Lebensmitteltechnologie und -mikrobiologie der Universität Hohenheim, Garbenstr. 25, 70599 Stuttgart, Germany

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Abstract

The effect of enhanced proteolysis on amine formation by amino acid decarboxylase positive *Lactobacillus* sp. during Gouda cheese ripening was examined. A commercial proteolytic enzyme preparation was added to pasteurized milk prior to cheese preparation. The effect of this manipulation on the formation of putrescine, histamine and tyramine was investigated in the presence and absence of amino acid decarboxylase-positive strains during a 12-week ripening period. Four batches were supplemented with a proteolytic enzyme. Batch I contained the proteolytic enzyme only, whereas batches II–IV were additionally supplemented with *Lactobacillus delbrueckii* LTH 1260 (batch II), *Lactobacillus buchneri* LTH 1388 (batch III) or *Lactobacillus brevis* LTH 2560 (batch IV). In batch I putrescine was detected with 4 mg/kg, in batch II, 42 mg/kg putrescine, 238 mg/kg histamine and 636 mg/kg tyramine were found. Batch III contained 13 mg/kg putrescine and 418 mg/kg histamine, whereas in batch IV, 26 mg/kg putrescine and 776 mg/kg tyramine were present. Batch V was supplemented with all three lactobacilli but did not contain the proteolytic enzyme. In this experiment, 4 mg/kg putrescine, 179 mg/kg histamine and 337 mg/kg tyramine were detected. A control cheese batch (VI) without addition of amine forming lactobacilli or a proteolytic enzyme was produced and only 4 mg/kg putrescine were detected. An increase in amine concentration during cheese ripening under conditions of enhanced proteolysis in the presence of starter and spoilage lactobacilli was evident from the experiments.

Keywords: Biogenic amines; Cheese; Proteolysis; Lactobacilli

1. Introduction

Biogenic amines may be of endogenous origin at low concentrations in non-fermented food such as fruits, vegetables, meat, milk and fish. High concentrations have been found in fermented foods as a result of a contaminating microflora exhibiting amino acid decarboxylase activity (Silla Santos, 1996). Since biogenic amines, in particular histamine and tyramine, have been reported to affect the well-being of susceptible consumers, their occurrence in food is not desired (Anderson et al., 1993).

Technological measures taken to accelerate cheese

*Corresponding author. Present address and address for correspondence: Unilever Research Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK 44 1LQ, UK. Tel.: +44 1234 222 682; fax: +44 1234 222 599; e-mail: renata.leuschner@unilever.com

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ripening rest on an increase of proteolysis e.g. by addition of proteolytic enzymes or proteolytic starter cultures, often in combination with peptidases to ensure the breakdown of bitter peptides (El Soda, 1986; Hayashi et al., 1990; Tan et al., 1993; Folkertsma et al., 1996). The degradation products of proteolysis increase the concentration of peptides and amino acids (Sood and Kosikowski, 1979) which both serve as precursors for amine formation in the presence of amino acid decarboxylase-positive microorganisms (Straub et al., 1994). The effectiveness of this sequence of events in cheese was shown by Joosten and Northolt (1989). These authors have added a suspension of heat-treated starter cells to accelerate proteolysis in combination with an inoculum of a histidine-decarboxylase positive strain of *Lactobacillus buchneri* and observed a significant increase of histamine concentration in the end product. However, *L. buchneri* is a spoilage organism and its presence in fermented food is not desired. Amino acid decarboxylases were shown to be present as well in lactic acid bacteria which are used for food fermentation purposes (Straub et al., 1995).

In the present study the effect of amino acid decarboxylase containing lactic acid bacteria on putrescine, tyramine and histamine formation under the conditions of enhanced proteolysis during cheese ripening was investigated.

2. Material and methods

2.1. Strains and culture conditions

Strains used in this investigation as shown in Table 1 were of food origin and were mainly taken from the culture collection of the Institute of Food Technology (Hohenheim University, Germany). *L. delbrueckii* ssp. *lactis* LTH 1266 was kindly provided by Prof. Busse (Technische Universität München-Weihenstephan, Germany).

Lactobacilli were grown in MRS broth (Merck, Darmstadt, Germany) at 30°C and enterococci were cultivated in M17 broth (Biokar, Beauvais, France). Viable counts were determined on agar plates composed of the medium described above and solidified with 15 g/l agar No. 1 (Oxoid, Wesel, Germany).

2.2. Determination of amine formation by microorganisms

Biogenic amine formation by resting cells was investigated in milk supplemented with 0.01 g/l lysine, ornithine, histidine and tyrosine (Serva, Heidelberg, Germany). Samples were taken after 6 days. The incubation temperature was 30°C and the cell concentrations were 10^8–10^9 cfu/ml. Preparation of samples for HPLC analysis was carried out according to Straub et al. (1995).

2.3. Determination of viable counts in cheese

The determination of microorganisms in cheese samples was carried out as follows: 10 g cheese was homogenized in 100 ml Ringer solution (Merck) using an Ultra-turrax (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany). For plate count determination the homogenate was diluted and of each dilution 0.1 ml were transfered to agar plates. Identification of amino acid decarboxylase-positive microorganisms in cheese was carried out on a decarboxylase assay medium composed of (g/l):

Table 1
Experimental design for the study of biogenic amine formation in cheese

Batch	Supplements to cheese milk	
	Microorganism species	Proteolytic enzyme
I	None	added
II	*Lactobacillus delbrueckii* ssp. *lactis* LTH 1266	added
III	*Lactobacillus buchneri* LTH 1388	added
IV	*Lactobacillus brevis* ssp *brevis* LTH 2560	added
V	*Lactobacillus delbrueckii* ssp. *lactis* LTH 1266, *Lactobacillus buchneri* LTH 1388, *Lactobacillus brevis* ssp. *brevis* LTH 2560	not added
VI	None (control cheese)	not added

Tryptone (Merck) 5, yeast extract (Merck) 5, NaCl (Merck) 5, glucose (Merck) 0.5, bromcresole purple (Sigma) 0.06, agar (Oxoid) 20, histidine or tyrosine (Serva) 10 and a pH of 5.5. Parallel plate counts were performed on MRS agar plates.

2.4. Analyses

Biogenic amines were analysed using reverse phase HPLC and two eluents composed of 1-hexanesulphonic acid (Merck) and acetonitrile (Merck) as described by Straub et al. (1993). Extraction of biogenic amines from cheese samples was carried out as follows: 10 g cheese were added to 40 ml of 0.6 M perchloric acid (Merck) and homogenized with an Ultra-turrax. The supernatant was filtered through a 0.2 μm membrane filter (Schleicher and Schuell, Dassel, Germany) and the filtrate was subjected to HPLC analysis.

The dry matter of the cheese samples was determined after 2 weeks, prior to coating of the cheese with paraffin wax (Tilsiter Super Käsewachs, Rehm, Rinteln, Germany), using an electronic moisture determinator (MA 30, Sartorius, Göttingen, Germany) which operated at a temperature of 110°C.

Fat determination of cheese samples was carried out according to the standard method of van Gulik (Kotterer and Münch, 1978) using calibrated specialized butyrometers.

Sensory evaluation was carried out using a triangle test. The tests were performed in duplicate by five test persons and were repeated on an independent occasion. The selected persons were familiar with cheese quality parameters.

2.5. Production of Hohenheimer Gouda cheese

Cheese was produced according to the following protocol: A bulk starter (BS) culture for cheese preparation was obtained by heating skim milk for 20 min at 90°C. After cooling a freeze dried culture (Probat 505, Wiesby, Niebüll, Germany) was added and the mixture was incubated for 20 h at 20°C. For cheesemaking lots of 20 l pasteurized milk (fat 3.5%, protein 3.2%, pH 6.7) were inoculated with 3 ml/l of the BS culture and incubated for 16 h at 13°C. Thereafter, the milk was supplemented with 10 ml/l BS starter and 0.1 g/l $CaCl_2$ and matured for 1 h at 32°C. This maturation was terminated when the pH reached 6.5. Prior to the addition of 32 ml/100 l pure calf rennet (activity: 1/10000, Hauser original) the proteolytic enzyme and decarboxylase-positive microorganisms (contaminants) were added to selected cheese batches (Table 1). The inoculum of model contaminants was 10^7 colony forming units (cfu)/ml milk and the proteolytic enzyme (PROMOD™215, Biocatalysts, Pontypridd, England) was added as 0.15% of the end product weight. After incubation for 50 min the curd was cut, stirred and heated to 40°C prior to transfer into cheese forms in which the cheese was pressed (300–350 kPa) for 4 h. At the next day the cheese was brined (150 g/l NaCl, pH 5.5) for 8 h. The cheeses were ripened for 12 weeks at 14 ºC and 90% rel. humidity. After a ripening period of two weeks the cheese was covered with a paraffin wax coating (Rehm).

3. Results

3.1. Amino acid decarboxylase activity

The potential of *L. delbrueckii*, *L. buchneri* and *L. brevis* to form biogenic amines was tested in milk supplemented with the precursor amino acids lysine, ornithine, phenylalanine, tyrosine and histidine. Amine formation in milk was quantified (Table 2). None of the strains was able to decarboxylate lysine. *L. delbrueckii* formed putrescine, histamine and tyramine. *L. buchneri* decarboxylated phenylalanine and more efficiently histidine, whereas *L. brevis* displayed the potential to produce phenylethylamine and tyramine.

3.2. Growth of amine forming microorganisms in cheese

One of three lactobacilli was added to cheese batches II–IV. Batch V contained all three strains. To the batches II–IV further commercial proteolytic enzyme was added. The viable counts of the amine forming lactobacilli were investigated in the cheese batches II–V. All lactobacilli were inoculated with 10^7 cfu/ml and increased their viable counts by a factor of ten in the batches II–IV (data not shown). In batch V no significant growth was observed but the initial viable counts were 10^8 cfu/ml and a

Table 2
Formation of biogenic amines in milk supplemented with 0.01% of ornithine, histidine, tyrosine, phenylalanine and lysine

Microorganism	Strain/Source	Biogenic amine[a]				
species	LTH	Put	His	Tyr	Phe	Cad
Lactobacillus						
delbrueckii	1260 apple mash	+	+	+	−	−
buchneri	1388 cheese	−	+ +	−	+	−
brevis	2560	−	−	+	+	−

[a] Putrescine (Put), histamine (His), tyramine (Tyr), phenylethylamine (Phe), cadaverine (Cad).
Biogenic amine concentration: (−) ≤5mg/ml, (+) ≥5mg/ml, (+ +) ≥50mg/ml.

comparison of amine formation in the batches II–V was therefore independent from the cell concentration. The viable counts of all microorganisms remained stable within the 12 weeks of ripening.

The dry matter of the cheese was determined in each cheese batch and revealed an average value of 59.59% (s.d. 2.18) and the fat content was found to be 55.1% (s.d. 1.67).

3.3. The combined effects of amine producing lactobacilli and enhanced proteolysis on cheese quality

The formation of biogenic amines was examined in all cheese batches (I–VI). Under conditions of a conventional Gouda cheese production (batch VI) only low concentrations of putrescine were detected (after 10 weeks 1.54 ppm and after 12 weeks 3.87 ppm). The supplementation of a conventional Gouda cheese production with a proteolytic enzyme (batch I) resulted in no higher amine concentrations and after 12 weeks 4 mg/kg putrescine were detected.

The amine concentrations in the batches containing the proteolytic enzyme and one amine producing *Lactobacillus* strain (batches II–IV) are given in Table 3. The amine concentrations found in batch V where no proteolytic enzyme was added but all three lactobacilli are shown in Table 4. An increased proteolytic activity in cheese resulted as expected in higher concentrations of putrescine, histamine and tyramine in the final product.

The texture of the cheese produced with a proteolytic enzyme (batch I–IV) was found to be softer when compared with cheese not containing the proteolytic enzyme (batches V and VI). The batches (I–IV) were further characterised by a bitter off flavour and an atypical odour. The consistence of the cheese batches V and VI displayed no differences due to the added lactobacilli. These results indicated that the proteolytic enzyme applied in the cheese batches

Table 3
Putrescine (Put), histamine (His) and tyramine (Tyr) concentrations in the cheese batches II–IV supplemented with a proteolytic enzyme in combination with one amine producing *Lactobacillus* strain. Batch II contained *Lactobacillus delbrueckii* LTH 1260; batch III *Lactobacillus buchneri* LTH 1388 and batch IV *Lactobacillus brevis* LTH 2560

Time (week)	Amine concentration (mg/kg)						
	Batch II			Batch III*		Batch IV[a]	
	Put	His	Tyr	Put	His	Put	Tyr
Start	nd	nd	nd	nd	nd	nd	nd
2	nd	nd	85.21	nd	37.86	nd	105.73
4	5.41	nd	168.32	nd	71.1	nd	263.69
6	6	nd	250.89	2.15	122.77	3.14	416.82
8	16.71	104.45	322.52	5.06	168.75	4.33	504
10	29.74	183.41	468.74	8.83	289.36	9.32	621.78
12	41.57	237.94	636.19	12.68	418.32	25.67	776.28

nd: not detectable, * tyramine formation not detected; [a] histamine formation not observed.

Table 4
Putrescine, histamine and tyramine concentrations in cheese batch V supplemented with *Lactobacillus delbrueckii* LTH 1260, *L. buchneri* LTH 1388 and *L. brevis* LTH 2560

Ripening time (week)	Amine concentration (mg/kg)		
	Putrescine	Histamine	Tyramine
Start	nd	nd	nd
2	nd	nd	64.92
4	nd	nd	102.23
6	nd	40.85	90.08
8	1.84	84.07	217.05
10	1.72	121.99	272.7
12	3.95	178.9	337.32

nd: not detectable.

was affecting the quality properties of the cheese to an undesired extent.

4. Discussion

The concentrations of tyramine, putrescine and histamine found in the cheese batch supplemented with *L. delbrueckii*, *L. buchneri* and *L. brevis* are within a range which is considered to affect human well-being (Edwards and Sandine, 1981). It was previously shown by Joosten (1988) that the tyramine formation in cheese by a tyrosine decarboxylase-positive strain of *L. brevis* was dependent on tyrosine and the degree of proteolysis. Joosten and Van Boekel (1988) described furthermore that the concentration of histamine was related to the concentration of the precursor histidine and the presence of a histidine-decarboxylase positive *Lactobacillus* sp. In our investigation we increased the degree of proteolysis by addition of a proteolytic enzyme.

Our results were significantly lower when compared with the observations of Joosten and Northolt (1989). The authors found a dramatic increase in histamine concentration in cheese under conditions of accelerated ripening when a histidine-decarboxylating strain of *L. buchneri* at a low inoculum of 0.2 cfu/ml was used and the histamine concentration increased to 1060 ppm when the inoculum was increased by a factor of 25. In their investigation *L. buchneri* achieved viable counts of 1×10^8 cfu/g and remained at this level during a six month ripening period. Similarly, in our investigation the lactobacilli remained at levels of 1×10^8 cfu/g but the initial inocculum was already 10^7 cfu/ml and our final amine concentrations did not reach the high levels observed in the work presented by Joosten and Northolt (1989). An explanation for the findings might be the three month longer ripening period of their cheeses.

Beside the amine formation by spoilage lactobacilli the potential of *L. delbrueckii*, a commonly used starter culture to form amines was addressed and indicates the need for a selection of amino acid decarboxylase-negative cultures.

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