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Ectoine as a natural component of food: detection in red smear cheeses

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Ectoine is a compatible solute accumulated in halophilic bacteria in response to high salt concentrations and offers protection from osmotic stress. The occurrence of compatible solutes is widespread among bacteria, yet ectoine has never been detected in foods. The use of an ectoine producing microorganism (*Brevibacterium linens*) in the surface ripening of red smear cheeses led to the question whether ectoine can be found in cheese. Therefore we examined samples from a variety of cheese manufacturers and different types of red smear cheeses for the presence of ectoine using HPLC and HPLC/MS analysis. Ectoine solely appears in the rind and was detected up to 178 mg/200 g red smear cheese, depending on several factors like ripening status and conditions throughout the cheese production process (e.g. salt concentrations of used brine baths).

Keywords: Ectoine, *Brevibacterium linens*, red smear cheese.

Ectoine belongs to a group of natural compounds, termed compatible solutes, which can be defined as organic osmolytes that are accumulated by the cell in high concentrations (up to several moles per liter) without disturbing vital cellular functions and the correct folding of proteins (Brown, 1976, reviewed by Roberts, 2005). Compatible solutes maintain the cell turgor under conditions of low water activity by counteracting the efflux of water from the cell. In addition they have a stabilizing influence on the native structure of proteins and other cellular structures (Lippert & Galinski, 1992). Ectoine shows a high solubility in water and is non-ionic at physiological pH (Galinski et al. 1985). Currently the main use of ectoine is as a functional ingredient in skin care products, where it is used as a moisturizer and cell protectant (Bünger, 1998, 1999; Bünger et al. 2001; Bünger & Driller, 2004). Osmoprotective compounds like ectoine also gained increasing interest for biotechnological applications and as highly effective protectants of proteins and cells (reviewed in Margesin & Schinner, 2001; Roberts, 2005; Lentzen & Schwarz, 2006).

The organic osmolyte ectoine occurs only in eubacteria and is widespread among halophilic and halotolerant eubacteria (Sauer & Galinski, 1998). The surface of smear-ripened cheeses harbours a highly diverse bacterial flora of aerobic halophilic bacteria, e.g. *Corynebacterium casei* (Monnet et al. 2006), *Staphylococcus saprophyticus* (Mounier et al. 2006), *Arthrobacter spec.* (Mounier et al. 2005) and *Brevibacterium linens* (Rattray & Fox, 1999).

Brevi. linens is strictly aerobic, with a rod-coccus growth style and a temperature growth optimum of 20 to 30 °C. It is a halotolerant microorganism with optimum growth at pH 6·5 to 8·5 (Rattray & Fox, 1999) and produces ectoine as an osmoprotectant (Jebbar et al. 1998). The main reasons for the application of *Brevi. linens* in the cheese production process are development of flavour (Bockelmann et al. 2001), colour development (Loessner, 2000, Bockelmann et al. 1996) and protection against growth of mildews (Jäger et al. 2000).

The conditions throughout the cheese production process, especially the salinity of brine baths used and the occurrence of the ectoine producing microorganism *Brevi. linens* led to the question whether ectoine can be found in surface-ripened cheeses. Therefore the aim of this study was to examine different types of red smear cheeses for the presence of ectoine.

Materials and Methods

Collection of cheese samples

Samples of red smear cheeses from a variety of cheese manufacturers were purchased from local supermarkets (Table 1). Harzer cheese used for ripening experiments was provided directly from the cheese plant by

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Table 1. List of cheese samples and analysis results

All cheese samples are produced by German manufacturers, except of manufacturer I (France) and J (UK) and were sampled from local retailers. The age of the respective cheese is displayed by the time span before the expiry date and the ectoine content was measured as described in Materials & Methods

Cheese	Manufacturer	Ectoine content mg/200 g cheese	Age of cheese (weeks before expiry date)
Harzer	A	73	1
Limburger	B	44	3
Romadur	B	34	3
Limburger	C	22	2
Chaumes	I	18	3
Harzer	D	n.d.	3
Harzer	E	n.d.	3
Feta	F	n.d.	10
Camembert	G	n.d.	2
Gouda	H	n.d.	4
Cheddar	J	n.d.	4

n.d. = not detected. The limit of detection was approx. 0·28 mg/200 g cheese

Sachsenmilch AG, Leppersdorf, Germany (manufacturer A in Table 1). Other types of cheeses (e.g. Feta and Gouda) were analysed for control purposes.

Preparation, extraction and analysis of cheese samples

Extraction method used for cheese samples. A modification of the extraction method of Bligh & Dyer (1959) was used for cheese preparation prior to HPLC and HPLC/MS analysis. All cheese samples were extracted and analysed as follows: The rind of a whole package of cheese was cut off to the depth of approximately 2–4 mm and separated from the inside mass. Rind and inside mass were mixed separately with a kitchen blender until the sample was homogeneous. To 5 g of each sample, 30 ml of a mixture of methanol, chloroform and water (10 : 5 : 4) was added and mixed thoroughly in a falcon tube on a shaker (Janke & Kunkel, IKA-Labortechnik, KS 250) for 60 min. Then 7·8 ml chloroform and 7·8 ml water were added and mixed for another 4 h followed by centrifugation for 10 min at 3940 ***g*** (Heraeus Labofuge 400R). The aqueous phase was removed, its volume measured and 200 µl analysed by HPLC. Experiments were conducted in duplicate.

To get accurate results for HPLC/MS analysis it was necessary to process a larger amount of cheese. For the preparation of a Limburger extract (manufacturer B), a modified extraction method was used: the rind of 2·2 kg Limburger cheese was cut off and extracted (as described above) with 2 l solvent. The aqueous phase was reduced under vacuum at 70 °C to a volume of 500 ml. The liquid was desalinated by electrodialysis (Berghof, BEL – 500). The diluate was reduced under vacuum at 70 °C to a volume of 135 ml followed by ion exchange chromatography (column material: Dowex Marathon C, column height: 47 cm, column diameter: 2 cm, column volume 147·6 ml, pump system: Watson Marlow 101 U). The fractions, where ectoine was detected (HPLC analysis), were pooled and desalinated again, followed by reduction under vacuum at 70 °C to complete dryness. A solid matter of 3·26 g remained.

Ripening experiments. Ripening experiments were performed with cheese batches of Harzer cheese from manufacturer A. Cheese samples were incubated at 4 °C and extracts prepared and analysed at the indicated dates.

Additionally, *Brevi. linens* population was measured by the method of Toolens & Koning-Theune (1970). Harzer cheese samples were analysed at different ripening stages (4 times; from 1 week before, to 2 weeks after, the expiry date) for the population of *Brevi. linens* (cfu/g). *Brevi. linens* strain DSM 20425 was used as a positive control.

Experiment of alkaline hydrolysis. In this experiment we used the feature that under alkaline conditions and room temperature ectoine slowly hydrolyzes to α-N-Acetyl-2, 4-diaminobutric acid and γ-N-Acetyl-2,4-diaminobutric acid (1 : 3). A Limburger cheese extract was prepared as described above. Limburger extract (100 mg) was dissolved in 5 ml H_2O. To 1 ml of this solvent approximately 5 µl 10 M-NaOH were added to adjust the pH to 12·8 and then mixed for 24 h (Eppendorf Thermomixer comfort). The ectoine content was measured at the beginning, after 5 and 24 h by HPLC. Additionally, an ectoine sample (7 g ectoine/l) was analysed identically for control purposes. We used external standards (α-N-Acetyl-2,4-diaminobutric acid and γ-N-Acetyl-2,4-diaminobutric acid, bitop AG) for the identification of the hydrolysis products.

HPLC analysis. Ectoine detection and quantification was performed by HPLC according to the method of Severin et al. 1992. The HPLC instrumentation included a pump system (422 Biotek – Kontron), detector (Kontron 430), column (CC 125/4 Macherey+Nagel), stationary phase (nucleosil 100–5 NH_2), pre column (CC 8/4 Macherey+ Nagel) and eluent (acetonitril/phosphate buffer 70 : 30).

HPLC/MS analysis. Identity of the corresponding HPLC peak with ectoine was confirmed by HPLC/MS analysis. The aim of the HPLC/MS analysis was to identify ectoine according to the mol peak of 142 g/mol. The HPLC/MS instrumentation included a liquid chromatography processor (spectra system Finnigan Mat), a pump system (P4000/AS 3000) and a mass spectrometer TSQ (Thermoquest Finnigan).

Results and Discussion

In the microflora of bacterial smear surface-ripened cheeses such as Limburger, Harzer and Chaumes,

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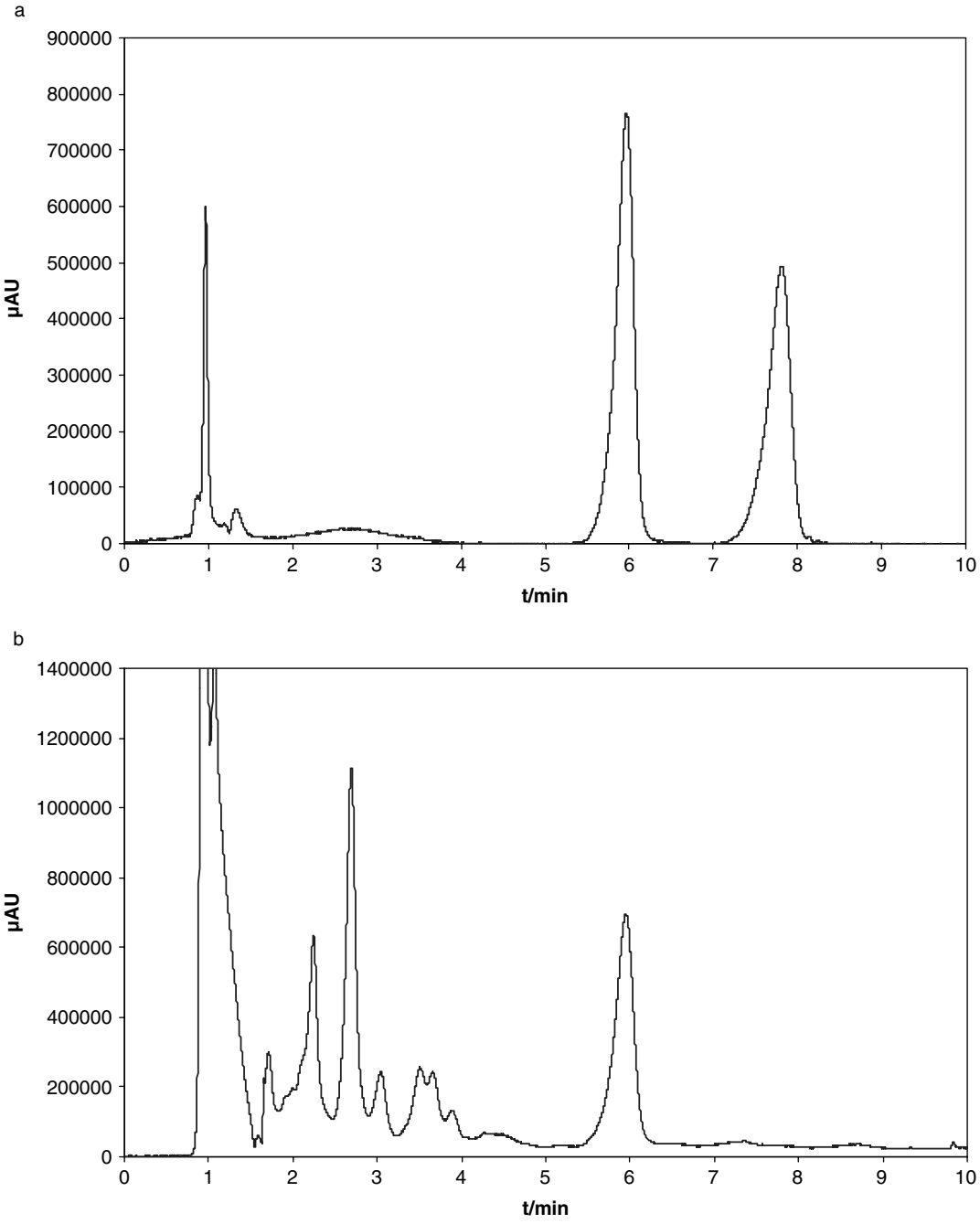

Fig. 1. (a): HPLC reference chromatogram of ectoine and hydroxyectoine measured from the pure substances. Retention time ectoine: 5·97 min. Retention time hydroxyectoine: 7·81 min. **(b):** HPLC chromatogram of Limburger rind. At a retention time of 5·95 min a single peak is visible which corresponds well with the retention time of ectoine shown in figure 1 a. No peak is visible at the retention time of hydroxyectoine.

microorganisms such as *Brevibacterium* spp., *Arthrobacter* spp., *Micrococcus* spp., *Corynebacterium* spp. are found as the dominant bacteria. Some manufacturers use the ectoine producing organism *Brevi. linens* as a pure starter culture, others use it in a mixture with different microorganisms. To test whether ectoine occurs in red smear cheeses which use *Brevibacterium* as a starter culture, we tested two red smear cheeses, Harzer cheese (manufacturer A) and Limburger cheese (manufacturer B), for the presence of ectoine. Extracts of the rind and the inside mass of the cheese were prepared and analysed by HPLC. A HPLC chromatogram of a Limburger cheese extract is shown in Fig. 1. A component of the extract elutes at 5·95 min, corresponding well with the elution time of

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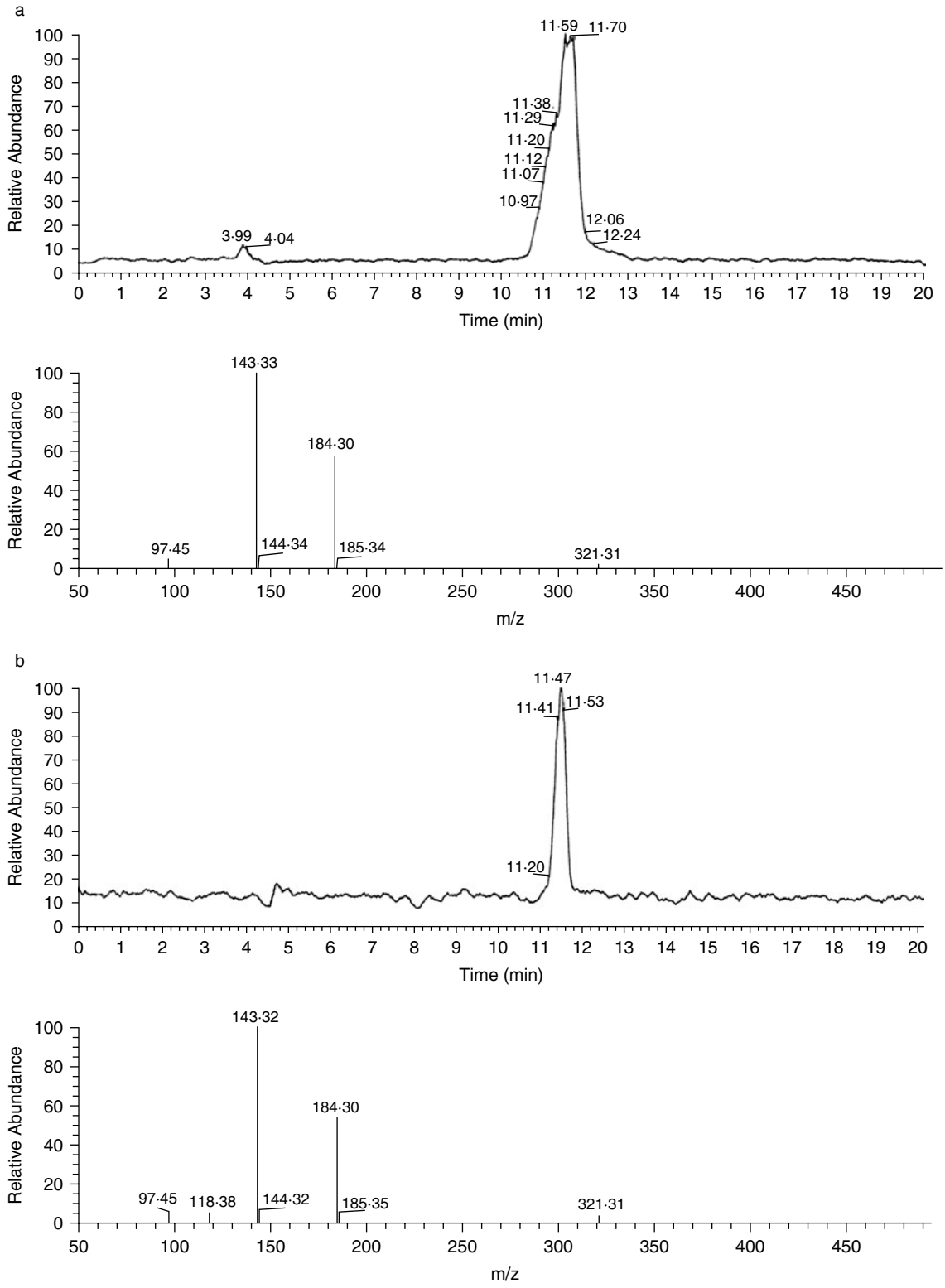

Fig. 2. (a): LC/MS reference chromatogram of ectoine measured from the pure substances. **(b):** LC/MS chromatogram of Limburger extract. In the mass spectrum referring to the UV-peak, all mass peaks corresponding to ectoine can be identified.

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ectoine in a control run (5·97 min). A Harzer cheese sample was also analysed by HPLC and also showed a peak at the retention time of ectoine (data not shown).

Ectoine hydrolyzes under alkaline conditions into α-N-Acetyl-2,4-diaminobutric acid and γ-N-Acetyl-2, 4-diaminobutric acid. This feature was used to confirm that the observed HPLC peak corresponds to ectoine. A sample of pure ectoine (7 g/l) was hydrolyzed under alkaline conditions and analysed by HPLC. A reduction of the peak corresponding to ectoine and the appearance of two new peaks at 7·18 min and 11·8 min, corresponding to α-N-Acetyl-2,4-diaminobutric acid and γ-N-Acetyl-2, 4-diaminobutric acid, respectively was observed. Alkaline treatment of a Limburger extract led to very similar results: the area under the peak at 5·95 min is reduced by 90% after 24 h of alkaline treatment. The hydrolysis products, which appear in the HPLC chromatograms of the ectoine sample and Limburger extract, elute exactly at the same retention time as the used reference (α-N-Acetyl-2, 4-diaminobutric acid and γ-N-Acetyl-2,4-diaminobutric acid; data not shown). This hydrolysis behaviour gave a strong indication that the HPLC peak from Limburger extract corresponds to ectoine.

To finally confirm the identity of the compound, we used HPLC/MS analysis. A sample of the Limburger extract was analysed with HPLC/MS. Here, ectoine elutes in the retention time area of 11·55 min to 11·80 min (Fig. 2). In the mass spectrum referring to the UV-peak, we found different mass peaks corresponding to ectoine. The mass m/z=143·3 refers to the protonized form of ectoine, m/z=184·30 refers to acetonitril plus ectoine, m/Z=97·45 refers to ectoine minus one CO_2-molecule, m/z=185·34 refers to ectoine plus acetonitril and plus one proton, m/z=144·34 refers to ectoine plus two protons. Furthermore, a mass of m/z=321·31 (composition unknown) was found in the ectoine sample.

The HPLC/MS spectrum of the limburger extract shows a peak in the chromatogram of the UV-spectrum within the retention time area of 11·35 min to 11·58 min which can be assigned to ectoine. All of the masses which were assigned to different connections of molecules in the spectrum of the ectoine control sample can be retrieved in the mass spectrum referring to the UV-peak (m/z=143·3; 184·30; 97·45; 144·32; 185·34; 321·31). The mass spectrometry results confirm that the substance detected by HPLC analysis of cheese extracts is ectoine.

To further test the occurrence of ectoine in cheese, samples of red smear cheeses from different manufacturers, purchased from local supermarkets were extracted and analysed by HPLC. Other types of cheeses like Feta, Gouda and Camembert were analysed for control purposes.

Table 1 shows that, in addition to Harzer and Limburger cheese, ectoine can also be found in Romadur and Chaumes. When comparing Harzer cheese from different manufacturers, we found that only the cheese from supplier A (Sachsenmilch AG, Leppersdorf, Germany) contained ectoine. Here *Brevi. linens* is used as a starter culture for ripening (supplier A, personal communication). The lack of ectoine in the Harzer cheeses from manufacturers D and E may be due to differences in the production process, e.g. other starter cultures or lower salt concentrations. Also, the composition of the cheese microflora changes during the ripening process and starter culture organisms may disappear during this process (Bockelmann et al. 1997; Feurer et al. 2004). Samples from other types of cheeses (Feta, Gouda, Cheddar and Camembert) did not contain ectoine. This may be due to the lack of ectoine-producing, surface-ripening bacteria like *Brevi. linens* in general (Feta and Gouda cheese) or in the specific ripening process (e.g. salt concentration) employed by the manufacturer (Camembert and Cheddar samples). It has also been shown that the resident microflora specific for a manufacturing site can be more important for the composition of the surface microflora than the starter culture (Mounier et al. 2005).

Table 2. Ripening experiment with Harzer cheese

The ectoine content of Harzer cheese samples from manufacturer A was analysed at different ripening stages. Experiments were conducted in different independent trials and data show the typical progression of the ectoine concentration in one experiment

Ripening status	Ectoine content in mg/200 g cheese
18 days before end of shelf life	0
8 days before end of shelf life	13·2
End of shelf life	21
6 days after end of shelf life	111
12 days after end of shelf life	178·7

Quantitation of the HPLC peaks showed an ectoine content varying between 18 mg in 200 g (common package size) of Chaumes and 73 mg in a Harzer cheese (200 g). Ectoine was only found in the rind and not in the inside mass of the cheese. This corresponds well with the production of ectoine being linked to the occurrence of aerobic ectoine-producing bacteria like *Brevi. linens*.

The microflora of red smear cheeses is complex and changes significantly during ripening (Bockelmann et al. 1997). At the end of the ripening process bacteria (*Brevibacterium* spp., *Arthrobacter* spp., *Micrococcus* spp., *Corynebacterium* spp.) dominate the microflora (Corsetti et al. 2001). We performed HPLC analysis of cheese extracts at different ripening stages and found that the ectoine content increased with time (Table 2).

Using Harzer cheese from manufacturer A, we tested the assumption that *Brevi. linens* population, and hence ectoine content, increases with ripening time, by plating out dilutions of homogenized cheese rind on plates selective for *Brevibacterium* (Toolens & Koning-Theune, 1970). The lowest *Brevibacterium* content was measured one week before the expiry date ($1·3 \times 10^8$ cfu/g). The population increased continuously with ripening time. The sample which was analysed on the expiry date had a population of $7·5 \times 10^8$ cfu/g, one week later a content of

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$1{\cdot}95 \times 10^9$ cfu/g was measured and the cheese which was analysed two weeks after the expiry date showed the highest population (1×10^{10} cfu/g). The correlation between the increase in ectoine content and *Brevibacterium* population indicates that *Brevibacterium* is responsible for the occurrence of ectoine in cheese. However, the possibility cannot be excluded that also other ectoine-producing bacteria like *Halomonas* sp., which have recently been reported to occur in the surface of red-smear cheeses (Maoz et al. 2003; Mounier et al. 2005) contribute to the build-up of ectoine in the cheese rind.

In this report we demonstrated for the first time the occurrence of ectoine in food by analysing cheese products in which ectoine-producing bacteria are used in the production process. Ectoine may also occur in other foodstuff that involves the fermentation of halotolerant or halophilic bacteria under high-salt conditions like soy sauces, fermented fish sauces and cured meat.

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