Eye formation in semi-hard cheese: X-ray computed tomography as a non-invasive tool for assessing the influence of adjunct lactic acid bacteria

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ORIGINAL PAPER

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Abstract Eye formation is an important feature for various cheese varieties. This study firstly aimed to evaluate the potential of X-ray computed tomography (CT) and image analysis software as a non-invasive method to quantify cheese eye volume. The quantification of the eye volume by CT was validated with 12 eyeless hard cheeses made with the inclusion of 0–100 hollow PP balls (Ø=10 or 20 mm). The results obtained for the total volume of the 'artificial eyes' showed a good correlation with the volume of the added balls (R^2>0.998). In a second part of the study, the developed CT method was applied for the non-invasive investigation of the eye formation in semi-hard Tilsit-type cheese with adjuncts of *Lactobacillus casei* or *Lactobacillus plantarum*. Both adjuncts completely metabolized citrate in cheese, thus enhancing gas production. Total eye number, relative eye volume and the size distribution of eyes were determined in order to reveal the influence of the two adjunct cultures on eye formation. In comparison to the control, the addition of *L. casei* resulted in a significantly higher eye number and eye volume. In contrast, the addition of *L. plantarum* did not influence the eye formation in a significant way, though it yielded cheeses with more succinate and aspartate and less serine at significant levels. The results imply that, apart from CO_2 production, other factors, such as the presence of eye nuclei and the dynamics of CO_2 diffusion, interfere in an important way in the process of eye formation in cheese.

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1 Introduction

The demand for non-destructive monitoring of eye formation in cheese during and/or after ripening is increasing. The size, number, structure and distribution of eyes in cheese are important quality parameters for various varieties such as Emmental, Comté, Jarlsberg, Gouda, and Tilsit. The eye formation in cheese is primarily dependent on microorganisms and their metabolic pathways. The gas formation and the basic metabolic pathways during cheese ripening were described over 100 years ago. However, the development of new eye-forming cultures and the linkage with technological factors influencing the eye formation are still under investigation. The biochemistry of gas production by different microorganisms in cheese was reviewed by Polychroniadou (2001). Propionibacteria and heterofermentative lactic acid bacteria are the sources for the CO_2 formed during the ripening process of Swiss-type cheeses (Fröhlich-Wyder and Bachmann 2004; Thierry et al. 2010). Saturation of the cheese matrix with CO_2 gas is a prerequisite for eye formation and can only be realized by a high rate of CO_2 production and a relatively low diffusion out of the cheese (Van den Berg et al. 2004). The formation of CO_2 by heterofermentative lactic acid bacteria usually takes place after lactic acid fermentation by the starter bacteria, and CO_2 formation by propionibacteria usually occurs during the period of warm room storage and can be delayed by cooling the cheese to low temperatures (Fröhlich-Wyder and Bachmann 2004; Thierry et al. 2010).

The presence of slits, cracks and irregular eyes usually leads to a downgrading of the cheese. Quality control is traditionally performed by listening to the sound while tapping on the cheese surface, by visual inspection of a small cylinder of cheese using a cheese trier or by viewing a section of a cheese cut into halves. These traditional methods are dependent on an individual's skills, often imprecise and not quantifiable. Nowadays, different methods of image acquisition, using digital cameras and image analysis (Caccamo et al. 2004), ultrasound (Albrecht et al. 1998; Conde et al. 2007; Eskelinen et al. 2007), magnetic resonance imaging (MRI) (Rosenberg et al. 1992), X-rays (Kraggerud et al. 2009; Akkerman et al. 1989) and computed tomography (CT), are applied to the investigation of eye formation in cheese, in particular, with regard to the distribution of cheese eyes. CT technology is widely used in medicine; however, more recently it is increasingly used in industrial applications, such as in structural mechanics and failure analysis, and in archaeology. The CT method (Greco et al. 2009; Lee et al. 2012) allows materials of different densities to be distinguished; thus, cheese eye volumes can be quantified without destruction of the cheeses. Furthermore, the time required for a CT scan is much shorter than that for MRI (approximately 10 s and 30 min, respectively).

The objective of the present study was to evaluate and validate our image analysis method to measure and quantify the total eye volume of cheese by means of CT technology and to compare the influence of adjuncts of *Lactobacillus casei* and *Lactobacillus plantarum* on eye formation in a Tilsit-type semi-hard cheese without propionic acid fermentation.

2 Material and methods

2.1 Production of experimental hard cheeses for CT validation study

Two series of six cheeses were produced at the Agroscope Liebefeld-Posieux (ALP) pilot plant from a large cheese vat (each cheese from 90 l standardized raw milk; 37 $g \cdot kg^{-1}$ fat). Twelve 30-cm diameter, 8-kg eyeless cheeses were pressed individually after the inclusion of two different types of hollow polypropylene (PP) balls (diameter: 10 and 20 mm; Semadeni, Ostermundigen, Switzerland), as per Table 1, and ripened for 30 days in a plastic film at 5 °C. The PP balls were included as 'artificial eyes' because of their known shapes and volumes. The cavity volume of the PP balls was measured by randomly choosing five balls from each diameter and injecting water with a syringe into the balls and weighing the balls. This method was chosen, as in the CT images the skin of the PP balls (approx. density of 0.9 $g \cdot cm^{-3}$) and the cheese matrix of ripened cheese (density of >1.0 $g \cdot cm^{-3}$, depending on composition) are indistinguishable due to the similarity of the densities and atomic numbers of the materials involved.

2.2 Production of experimental Tilsit-type cheeses with two different adjuncts

Six semi-hard cheeses (30 cm in diameter, 5 kg) were produced on 2 different days at the ALP pilot plant from 70 l standardized and pasteurized (72 °C, 15 s) milk (37 $g \cdot kg^{-1}$ fat), by adding a starter culture consisting of *Lactobacillus delbrueckii* subsp. *lactis*, *Streptococcus salivarius* subsp. *thermophilus* and *Lactococcus lactis* subsp. *lactis* (cultures MK 401 and RMK 150; ALP Research Station, Switzerland). The ripening time was 4 months. In order to study the influence of facultative

Table 1 Experimental design of cheese production for CT validation study with increasing numbers of plastic balls ('artificial eyes') and hollow volumes

Cheese identification	Diameter of balls (mm)	Number of balls (−)	Expected hollow volume (mL)	Total volume of the cheeses (mL)	Relative hollow volume (%)
1	10	0	0.0	6454	0.03
2	10	20	6.7	8456	0.08
3	10	40	13.3	9311	0.14
4	10	60	20.0	8651	0.21
5	10	80	26.7	8687	0.30
6	10	100	33.3	8448	0.39
7	20	0	0.0	7699	0.05
8	20	20	61.8	8918	0.75
9	20	40	123.6	9079	1.48
10	20	60	185.5	8327	2.39
11	20	80	247.3	9319	2.62
12	20	100	309.1	9163	3.46

The total volume of the cheeses and the relative hollow volumes were calculated by means of CT and subsequent image analysis

heterofermentative cultures on eye formation, two cheeses were manufactured with adjuncts of either *L. casei* (strain FAM 8099) or *Lactobacillus plantarum* (strain FAM 20827).

2.3 X-ray measurement

X-ray radiographs were taken using a mobile Practix 21 system (Philips, Zürich, Switzerland) with the following parameters: 65 kV, 20 mA, 0.8 s. The total eye number in the experimental Tilsit-type cheeses was counted from the X-ray radiographs.

2.4 CT measurement

CT (Greco et al. 2009) of all the cheeses was performed at the end of the ripening time, using a CT scanner (Philips CT Brilliance 16P; Philips) at a nearby veterinary medicine facility with the following scan parameters: 120 kV, 150 mA and 0.5 mm slice thickness (Fig. 1). The pixel size in each slice was adjusted for each cheese individually, ranging from 0.423 to 0.508 mm. The individual pixel spacing was automatically calculated, as determined by the field of view. The cheeses were directly transported from the cold room (5 °C) to the CT scanner, still packed in a plastic bag, but not vacuumed. The CT scan time for each cheese was about 10 s. After scanning, the cheeses were returned to the cold room.

2.5 Image analysis

The software products used for the eye volume analysis were Dicom2Tiff Ver. 0.9.9., ImageMask Ver. 2.1.2 and Disect Ver. 3.1 (www.disectsystems.com; Disect Systems, Ipswich, England).

Each raw dicom file stack (one stack per cheese) was converted into a tiff file stack to enable segmentation by using the ImageMask software. To reduce the computational demand and effect of grey level variations at the cheese rind, some images at

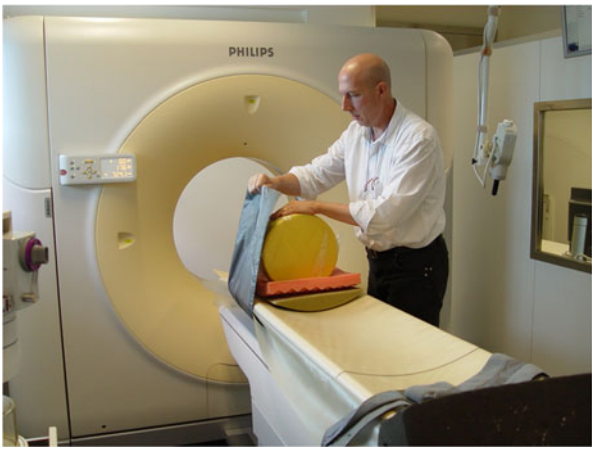

Fig. 1 CT scanner (Philips CT Brilliance 16P with cheese and operator)

the cheese rind were excluded after visual inspection. Parameters like slice thickness (0.5 mm), filter and rotation time were constant for all images. Segmentation was carried out in two steps as follows.

Step 1. For the segmentation of the space around the cheese, a luminance mask was used (upper threshold=255, lower threshold=56, Internal flood fill, Region=all, Invert Mask). A few speckles in the mask were removed with the dilation function, using a size of 1 pixel (software parameter); then, the new data set was saved.

Step 2. For the segmentation of cheese matrix around the cheese eyes, the grey level was set to 230, and a luminance mask was used (upper threshold=226, lower threshold=66, Region=All). A sequence of dilation functions with software parameters of 2, −3 and 2 pixels was applied to remove any unwanted black pixels; then, the new data set was saved.

The final image stack from either Step 1 or Step 2 was imported to Disect, and the total cheese volume (the total cheese volume was defined as the volume of the cheese matrix and the cheese eyes) and the absolute eye volume were calculated by integration of the remaining volumetric pixels (voxels). In order to calculate the quantitative cheese volume (after Step 1) and the absolute cheese eye volume (after Step 2), a grey level range of 0 to 65,534 was used, according to the instructions of Disect Systems. The ratio of eye volume to cheese volume was calculated for each cheese and expressed as a percentage of the total cheese volume. Figure 2 illustrates the image processing with a sample cheese (100 balls, 20 mm diameter).

The mean diameter d in (mm) of the eyes was calculated using the following equations:

$$\text{Mean eye volume} = \frac{\text{absolute eye volume}}{\text{total eye number}}, \mathrm{d} = 2 * r \tag{1}$$

$$= 2 * \sqrt[3]{\frac{3 * \text{ mean eye volume}}{4 * \pi}}$$

Absolute eye volume = the total eye volume (in mm^3; r=mean radius [in mm]). The total eye volume was calculated using Disect software with Step 2, whereas the total eye numbers were manually counted from the X-ray images. The manual counting of the cheese eyes for the calculation of the mean diameter according to Eq. 1 was necessary since the automatic counting of the eyes after Step 2 occasionally failed with the applied software (Matlab, Version 7.10.0 R2010a; see next the section) due to the presence of multiple conjoined eyes that were recognized as a single coherent area.

To calculate the volumes and the corresponding diameters of the individual eyes from the CT data, the images from Step 2 were additionally analysed with Matlab, Version 7.10.0 R2010a (MathWorks Inc., Natick, MA, USA). The image stacks obtained after Step 2 were loaded into Matlab, and a grey level threshold of 10,000 (the results from the DISECT software were saved in 16-bit format) was applied to select the cheese eyes. The pixels corresponding to the interior of the individual cheese eyes were compiled by using the connected component analysis methods of Matlab. The corresponding diameter of the individual eyes was calculated according

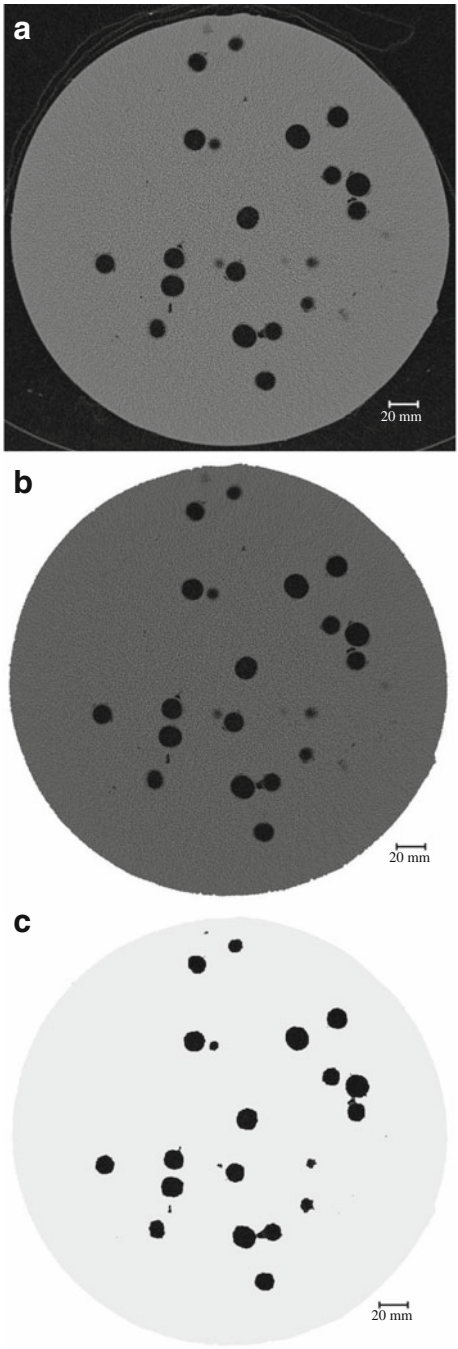

Fig. 2 The processing steps of the CT images are illustrated in the following three images of an experimental cheese (diameter= 30 cm) containing 100 plastic balls 'artificial eyes' (diameter= 20 mm). (**a**) Raw CT data as converted to tiff file; (**b**) ImageMask eliminated all data around the cheese (calculation of cheese volume inclusive of eye volume); (**c**) ImageMask eliminated all data of the cheese except the cheese eyes (calculation of eye volume)

to Eq. 1. The histograms of the size distribution were calculated using gawk (Free Software Foundation, Boston, MA, USA) and plotted with gnuplot (http://www.gnuplot.info/, accessed October 2012).

2.6 Determination of pH and analysis of cheese composition

pH was determined with a Metrohm pH-meter 605 (Metrohm AG, Herisau, Switzerland). The fat content of the cheese was determined by the Gerber-van Gulik method (ISO 3433 2008). Total nitrogen (TN) was determined by the Kjeldahl method (IDF (International Dairy Federation) 1993). Non-protein nitrogen (NPN) was determined according to IDF (2001). Water was determined by means of the dry matter-method (IDF 2003) as the mass difference of the cheese after drying at 102 °C for 4 h.

Total lactic acid, citrate and succinate were analyzed enzymatically according to the instruction protocol of the kit manufacturer (R-Biopharm, Darmstadt, Germany). Free amino acids were determined as described by Bütikofer and Ardö (1999) and biogenic amines as described by Bütikofer et al. (1990). Facultative heterofermentative lactobacilli were enumerated on FH-agar, as described by Isolini et al. (1990).

Short-chain fatty acids (C1–C4) were analyzed by gas chromatography and flame ionization detector with headspace technology after esterification with ethanol, as described by Badertscher et al. (1993). All measurements were performed in duplicate.

2.7 Sensory analysis

Sensory analysis of the experimental Tilsit-type cheeses was performed at the ALP pilot plant. The sensory quality of the cheeses was judged individually by a panel of six experts according to the ALP standard protocol. The following characteristics were judged: aroma intensity, aroma quality, number and size of eyes, eye quality, body elasticity and hardness, and texture quality. The score given was from one for lowest to five for highest intensity of the assessed characteristic. For each cheese, the average of the scores was calculated and used for the following statistical evaluation.

2.8 Statistical analyses

For the second part of the study, a simple experimental design with one control and two adjunct cultures (with either addition of *L. casei* or *L. plantarum*) was applied. The experiment was replicated on a second day.

Statistical analysis on instrumental data was carried out using analysis of variance (ANOVA) with the general linear model (GLM) using SYSTAT 12 (SPSS Inc., Chicago, IL, USA). Differences between the various factors are considered statistically significant at $P \leq 0.05$. The *Lactobacillus* strain and repetition factors were treated as categorical variables. The differences between the means of the experimental Tilsit-type cheeses were determined according to Fisher's least significant difference (LSD) test.

3 Results and discussion

3.1 CT validation study with cheeses with 'artificial eyes'

Figure 3 shows the expected and calculated hollow volumes of the 12 experimental cheeses from the validation study with 'artificial eyes'. The 'artificial eyes' volumes,

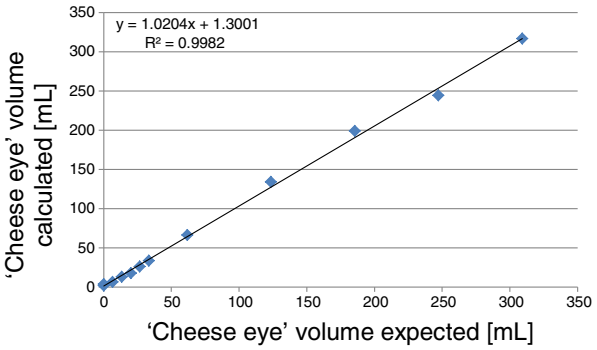

Fig. 3 Correlation between the expected (*x*-axis) and the calculated (*y*-axis) volumes of cheese eyes. The smaller volumes represent the run with the small plastic balls (diameter=10 mm) and the larger volumes represent the run with the large plastic balls (diameter=20 mm)

calculated by the CT method, showed a good correlation (R^2>0.998) in comparison with the given hollow volumes of the added PP balls. Table 1 shows the relative eye volume in percentages. The percentage values (>1%) of the cheeses with the larger PP balls (7–12) were representative for the range found in Swiss-type cheeses (Fig. 2c), whereas the cheeses with the smaller PP balls (1–6) were representative for the range (<1%) found in some Swiss semi-hard cheese varieties, such as Tilsit, Appenzeller or Raclette. Similar values from <1% up to 10% (relative gas volume) were reported in a study with six different Gouda-type cheeses exposed to different ripening conditions together with butyric acid bacteria (Lee et al. 2012). As postulated by Lee et al. (2012), the abnormally large gas hole volumes (>4%) were due to the active fermentation of butyric acid bacteria and the high temperature during the ripening time. An analysis of the distribution of the eye diameters with Matlab, as extracted from the CT images, indicated that the extracted volumes (Fig. 4) correspond to the average cavity volumes of the PP balls (0.33 mL for cheese 6 and 3.09 mL for cheese 12; Table 1). In a few cases, multiple conjoined eyes were recognized as a single eye due to the missing cheese matrix between the PP balls. Therefore, not only peaks at the value of the expected cavity volume, but also peaks at integer multiples, are present in Fig. 4. Counting the eyes with *n* times the volume of the cavity of a single PP ball as *n* eyes, the calculated eye number corresponds to the exact expected number of eyes.

3.2 Tilsit-type cheeses with adjunct lactobacilli

The composition of the experimental cheeses was analyzed, and no significant difference among the experimental cheeses was found. Fat, protein, water and pH values (30%, 25%, 39% and pH=5.6, respectively) were in the standard range for Tilsit-type cheese. Slightly more lactic acid was found in the control cheese and the cheese with *L. casei* compared to the *L. plantarum* cheese (Table 2). The content of the short-chain fatty acids (C1–C4) is also shown in Table 2. The differences for formic, propionic and butyric acid were not statistically significant. However, a remarkable stepwise increase of the acetic acid content was observed: the control cheeses showed the lowest content and the experimental cheeses with *L. plantarum* the highest. This indicates a strong (but differing) metabolic activity by the two tested

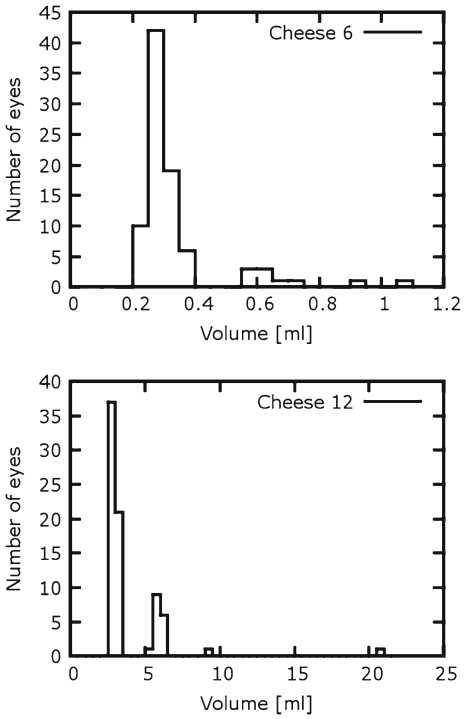

Fig. 4 Histograms of the eyes volumes from cheeses with 'artificial eyes' used for the validation study. One hundred plastic balls with a diameter of 10 mm were added to cheese 6; 100 balls with a diameter of 20 mm were added to cheese 12). In both plots, volumes <50 μL were attributed to noise in the images and, therefore, excluded

strains releasing acetic acid. By means of the analysis of covariance ('acetic acid' being the dependent variable), the production of acetic acid could be traced back to the metabolism of citrate and serine (Squared Multiple R=0.977 with P=0.004). Table 2 shows the contents of citrate and of serine measured in the cheeses ripened for 3 months. The *L. casei* and *L. plantarum* adjuncts metabolized the citrate completely. The level of serine in the *L. plantarum* experimental cheeses was ten times lower than in the control and in the *L. casei* cheeses. According to Liu et al. (2003), *L. plantarum* is capable of metabolizing serine, releasing, among other products, acetic acid. Thus, besides citrate, *L. plantarum* also metabolized serine, leading to the highest content of acetic acid found in the experiment.

In Table 2, the contents of succinic acid, some other amino acids (aspartic acid, leucine), the total amino acids and the counts of facultative heterofermentative lactobacilli are presented. In the *L. plantarum* experimental cheeses, the succinic acid and aspartic acid contents were increased compared to the control cheese. Succinic acid and the extra aspartic acid are probably derived from citrate metabolism by a reductive tricarboxylic acid pathway. This metabolic pathway was proposed for various *L. plantarum* strains (Dudley and Steele 2005). Accumulation of both compounds was also observed in washed curd, brine-salted Dutch-type cheese to which *L. plantarum* INF15D was added as adjunct culture (Skeie et al. 2008).

The cheese with the *L. casei* adjunct did not show increased levels for succinic acid and only slightly increased levels for aspartic acid. Therefore, it was concluded

Table 2 Contents of free fatty acids and organic acids in 3-month-aged experimental Tilsit cheeses, produced with adjuncts of *L. casei* and *L. plantarum*

Run		1	2	3	GLM-ANOVA	
					Factor: L. strain	Factor: day/ repetition
L. strain	(−)	control	*L. casei* (FAM 8099)	*L. plantarum* (FAM 20827)		
Formic acid	(mmol·kg^{-1})	0.78	1.75	3.06	n. s.	n. s.
Acetic acid	(mmol·kg^{-1})	7.20 A	11.84 B	17.58 C	***	*
Propionic acid	(mmol·kg^{-1})	1.05	0.76	0.68	n. s.	n. s.
Butyric acid	(mmol·kg^{-1})	1.95	1.53	1.46	n. s.	n. s.
Lactic acid	(mmol·kg^{-1})	97.0	93.0	89.5	n. s.	n. s.
Succinic acid	(mmol·kg^{-1})	1.4 AB	1.2 A	2.9 B	*	n. s.
Citrate	(mmol·kg^{-1})	6.2 A	0.0 B	0.0 B	*	n. s.
Total amino acids	(g·kg^{-1})	20.4	21.3	23.3	n. s.	n. s.
Aspartic acid	(mg·kg^{-1})	342 A	402 B	544 C	**	*
Leucine	(mg·kg^{-1})	2817 A	2916 A	3159 B	**	n. s.
Serine	(mg·kg^{-1})	434 A	407 A	37 B	**	n. s.
FHL 1d	(CFU·g^{-1})	3.2E+2 A	4.5E+8 B	1.3E+7 C	**	n. s.
FHL 120 d	(CFU·g^{-1})	1.4E+3 A	3.5E+7 B	2.0E+6 AB	n. s.	n. s.

Plate counts of facultative heterofermentative lactobacilli (FHL) were determined on day 1 and day 120. Values are averages of two independent experiments (N=2)

*P≤0.05, **P≤0.01, ***P≤0.001, n. s.: not significant; differences between means with the same letter are not significant (Fisher's least significant difference test, P≤0.05)

that *L. casei* metabolized citrate mostly to pyruvate and CO_2. Consequently, more eyes and an increased total eye volume were expected in the *L. casei* experimental cheeses compared to the *L. plantarum* experimental cheeses.

The amount of facultative heterofermentative lactobacilli (Table 2) was significantly increased in the cheeses with adjuncts for day 1 after the cheese production, but also after the ripening time of 3 months. It was observed that the added lactobacilli decreased during ripening time by around 1 order of magnitude and that the counts for *L. plantarum* were lower than for *L. casei*, which may also contribute to fewer eyes and less eye volume.

Biogenic amines are generated from decarboxylation of the corresponding amino acids. Since amino acids must first be available, the formation of biogenic amines is usually encountered during an advanced ripening of the cheese (Linares et al. 2011). The experimental cheeses with *L. casei* or *L. plantarum* did not show increased concentrations of cadaverine, histamine or tyramine when compared to the control cheese. The total amount of biogenic amines was in a low range (40–50 mg·kg^{-1}). Therefore, additional CO_2 from amino acid decarboxylation was not expected.

Figure 5 shows the X-ray radiographs taken from the experimental cheeses. *L. casei* seemed to generate the most eyes. The control cheeses had the fewest eyes,

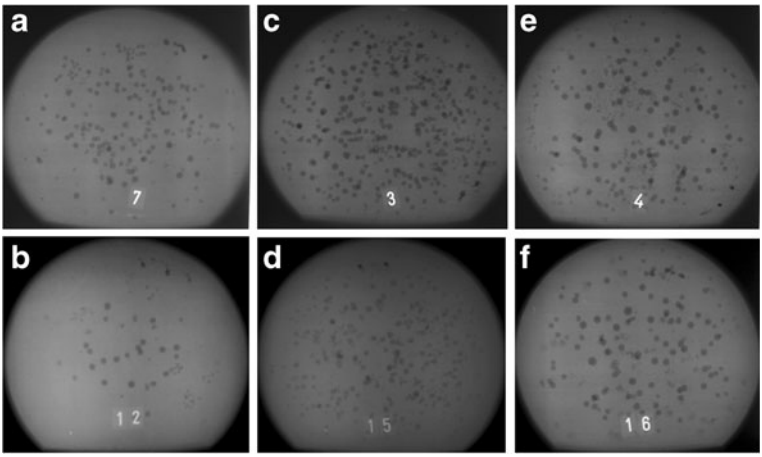

Fig. 5 Radiographs of experimental Tilsit-type cheeses (diameter=30 cm) at the end of the ripening time (4 months). From these pictures, the number of eyes was estimated for each individual cheese: the control at **a** day 1 and **b** day 2; the variant with *L. casei* (FAM 8099) supplement at **c** day 1 and **d** day 2; the variant with *L. plantarum* (FAM 20827) supplement at **e** day 1 and **f** day 2

whereas *L. plantarum* cheeses exhibited fewer eyes than *L. casei* cheeses did. From these radiographs, the total eye number was calculated manually to prove the visual impression (Table 3). The mean diameter of the eyes for *L. plantarum* seemed to be larger than for *L. casei*. This statement was also confirmed by the results of the CT analysis. The mean diameter of the eyes in the *L. plantarum* experimental cheeses (6.3 mm) was larger than that of the eyes in the *L. casei* experimental cheeses (5.9 mm) and the control cheeses (5.2 mm). In fact, it is a common, practical

Table 3 Quantitative analysis of eye formation in experimental cheeses with adjuncts of *L. casei* and *L. plantarum* by means of CT, X-ray radiographs and sensory evaluation

Run	L. strain	Relative eye volume[1]	Total eye number[2]	Mean of eye diameter[3]	Number of eyes[4]
	(−)	(%)	(−)	(mm)	(1–5)
1	control	0.27 A	142 A	5.22	2.0
2	*L. casei* (FAM 8099)	0.67 B	324 B	5.85	3.5
3	*L. plantarum* (FAM 20827)	0.48 AB	190 A	6.28	2.8
GLM-ANOVA					
Factor: L. strain		n. s.	*	n. s.	n. s.
Factor: day/repetition		n. s.	*	*	n. s.

Values are averages of two independent experiments (N=2)

*P≤0.05, **P≤0.01, ***P≤0.001, n. s.: not significant; differences between means with the same letter are not significant (Fisher's least significant difference test, P≤0.05)

[1] Relative eye volume calculated by CT technology (proportion of total eye volume to total cheese volume)

[2] Total eye number counted from X-ray radiographs

[3] Mean of eye diameter

[4] Number of eyes from sensory evaluation, sensory score: 1 for lowest intensity and 5 for highest intensity

observation that cheeses with a smaller number of eyes tend to have eyes with a larger diameter.

A major advantage of the CT data in comparison to the radiographies is that these data also provide reliable information on the size distribution of the eye diameters (Fig. 6) and the 3-D spatial distribution of the eyes. The main peaks in the size distribution of eye diameters obtained with the Matlab software fully matched the values of the mean diameters d in Table 3 that were calculated from Eq. 1. Remarkably, the size distribution of the eye diameters showed a second population of eyes with small diameters (<2 mm). These small eyes resulted from gray scale variations in the CT images that occurred predominantly in the cheese rind. Such artifacts could be significantly lowered in number when only eyes with a diameter of more than 2 mm (~4 voxels, corresponding to eyes with a total volume of about 64 voxels) were taken into account. In contrast, the diameters of the 'artificial eyes' in the cheeses used in the validation study (ball diameters of 10 and 20 mm were used) and the mean diameters of the eyes in the investigated Tilsit-style cheeses (4–6 mm) were significantly larger than this intrinsic resolution limit of the CT data. Thus, it can be concluded that the resolution of the CT methodology is suitable for the examination of eye formation in cheese varieties with eyes bigger than 4 mm.

The *L. casei* and *L. plantarum* experimental cheeses both showed a trend to greater eye volume than the control cheeses (Table 3). Furthermore, the microbial and biochemical data supported a higher relative eye volume and eye number in *L. casei* cheeses in comparison to the *L. plantarum* cheeses (e.g., 10-fold-higher counts of facultative heterofermentative lactobacilli and reduced production of CO_2 due to the reductive tricarboxylic acid pathway used for the conversion of citrate by *L. plantarum*). However, the pairwise comparisons of the experimental cheeses with each other and with the control revealed that only *L. casei* cheeses exhibited a significantly higher relative eye volume in comparison to the control cheeses. It has to be taken into consideration that an increase in the production of CO_2 is only one of the critical factors for eye formation. Another important factor, which was not influenced by the

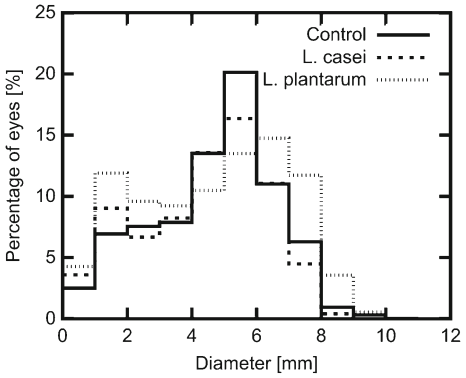

Fig. 6 Histogram of extracted diameters for the eyes in the experimental cheeses (Control, *L. casei* (FAM 8099) and *L. plantarum* (FAM 20827)), as extracted with Matlab. For each eye, the diameter of a sphere with volume equal to that of the extracted eye is calculated. From this information, the mean diameter of the cheese eyes calculated by Eq. 1 could be verified. Some images showed grey level fluctuations close to the cheese rind due to the slant mounting of the loaf in the scanner. These 'black pixels' were considered artifacts and, therefore, excluded in the verification of the mean eye diameter

study design, is the natural presence of particles that act as eye nuclei. Differences in the cheese milk regarding the presence or absence of eye nuclei have an important impact on the eye formation in cheese. For example, the almost complete absence of eye nuclei in microfiltered or bactofuged cheese milk usually yields almost eyeless cheeses, despite a sufficient production of CO_2. In such situations, the lack of eye formation is usually compensated by higher rates of CO_2 diffusion out of the cheese matrix, bigger eyes or even the formation of opening defects such as slits and cracks.

The sensory results for the cheese texture and flavor were not significantly different among the cheeses with adjuncts cultures and the control cheeses (data not shown). The number of eyes approximated by judging the sectional view of a cheese loaf cut in half (Table 3) showed a clear trend to more eyes in the *L. casei* and *L. plantarum* experimental cheeses. The relative eye volume (calculated from CT analyses) correlated most closely with the sensory property 'number of eyes'. Figure 7 represents the relationship between the sensorial attribute of 'number of eyes' and the CT eye volume (R=0.873). Although visual inspection of sectional views is not an accurate method, it enables a preliminary estimation of eye formation in cheese in practice.

4 Conclusions

The results of the present study showed that the validated CT methodology might be an interesting tool to study the eye formation in cheese. The quantification of the total volumes of 'artificial eyes' in cheeses by means of CT showed a very good correlation with the hollow volumes of the added PP balls (R^2>0.998). In combination with chemical, biochemical, microbial and sensorial analysis, the use of the CT methodology also allowed in depth investigation of the influence of two adjunct cultures on eye formation. Although the adjuncts of *L. casei* and *L. plantarum* both completely reduced citrate and tended to increase the eye volume in Tilsit-type semi-hard cheeses in comparison to the control cheeses, a significant impact was only obtained for adjuncts of *L. casei*. The results imply that the complex process of eye

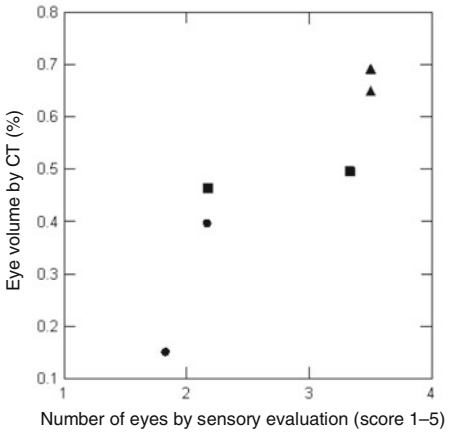

Fig. 7 Relationship between the number of eyes as assessed by sensory evaluation and the total eye volume, as calculated by CT (R=0.873 with $p \leq 0.05$). *filled circle*, control; *filled triangle*, *L. casei* (FAM 8099); *filled square*, *L. plantarum* (FAM 20827)

formation in the experimental cheeses was not only influenced by the addition of heterofermentative lactic acid bacteria, but also other factors, such as the presence of eye nuclei and the dynamics of CO_2 diffusion. More experiments are necessary to fully understand and control the eye formation in cheese. The use of CT data will contribute to elucidate the impact of individual factors on the eye formation in cheese.

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