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The effect of NaCl and metabolic profile of propionibacteria on eye formation in experimental Swiss-type cheese

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ABSTRACT

The influence of three brining times (0, 1, and 3 d) and two propionibacteria (PAB) cultures (Prop A and Prop B) on eye formation was investigated in experimental Swiss-type cheeses by comparing PAB counts, biochemical parameters, eye numbers, diameters and volumes. Prop A was strongly inhibited with increasing NaCl content. As a result, eye volume decreased considerably, as well as eye diameter especially towards the cheese surface. Prop B exclusively converted L-lactate and was much less affected by increasing NaCl content, hence, eye formation changed less. The salt sensitivity of the cultures also influenced the spatial distribution of the eyes. The width of the eyeless border zone was mainly affected by three factors: CO_2 diffusion to the outside, propionic acid fermentation, and body firmness. The study clearly showed that strain-specific salt sensitivity of PAB is an important feature that strongly influences eye formation in Swiss-type cheeses with different salt content.

1. Introduction

The term ‘Swiss-type cheeses’ is a collective term used for cheeses that have undergone propionic acid fermentation and therefore exhibit characteristic round eyes (or ‘holes’) and have a slightly sweet and fruity flavour (Fröhlich-Wyder et al., 2017). Out of the four dairy propionibacteria (PAB) species that are often part of the natural flora of raw milk (*Propionibacterium freudenreichii, Acidipropionibacterium thoenii, Acidipropionibacterium jensenii* and *Acidipropionibacterium acidipropionici*) it is usually *P. freudenreichii*, a species characterised by a relatively high tolerance to heat stress, that is used for the manufacture of Swiss-type cheeses. Apart from heat tolerance, individual strains may differ in other ways, such as salt resistance, growth at cold temperatures, amino acid catabolism, aspartase activity, lipolytic activity, the use of lactate isomers and interactions with lactic acid bacteria (Crow, 1986; Dherbécourt et al., 2010; Fröhlich-Wyder, Bachmann, & Casey, 2002; Jaros, Ginzinger, Tschager, Mayer, & Rohm, 1997; Thierry, Maillard, Hervé, Richoux, & Lortal, 2004; Turgay et al., 2011; Turgay, Ryser, Fröhlich-Wyder, Wechsler, & Lüdin, 2018; White, Broadbent, Oberg, & McMahon, 2003). This turns the control of the propionic acid fermentation into a challenging task. In particular, the sensitivity of PAB to NaCl is of high technological interest as the NaCl content of Swiss-type cheeses varies over a wide range from 0.3 to 1.7% (Fröhlich-Wyder et al., 2017).

Salt is an important element in the flavour, texture and overall acceptability of cheese. It controls survival of starter bacteria, growth of non-starter lactic acid bacteria, enzyme activity and contributes directly to flavour (Fox, Guinee, Cogan, & McSweeney, 2017; IDF, 2014). The effect of the salt, or more precisely the salt content in the aqueous phase, on the growth and metabolism of propionibacteria has been the subject of various studies, some of which were carried out in culture media and others in cheese. The sensitivity of propionibacteria to sodium chloride had already been emphasised by Orla-Jensen (1921), who noticed that the eye formation in cheese could be influenced by the salt content. Peltola (1940) found that CO_2 formation is delayed at salt concentrations above 4% (w/w). Rüegg, Glättli, and Blanc (1976) studied the effect of water activity on the growth rate of a PAB culture used for the manufacture of Swiss Emmentaler cheese in a whey-based culture medium by adding varying amounts of NaCl. The highest growth rate was observed at $a_w = 0.99$ (no NaCl added). At $a_w = 0.955$, corresponding to 6% NaCl, growth and acid production were completely inhibited. Similarly, Boyaval, Deborde, Corre, Blanco, and Bégué (1999) showed that growth of *P. freudenreichii* strain

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CIP 103027 was progressively inhibited when the NaCl concentration (0–4%) in the culture medium was increased. Carcano, Todesco, Lodi, and Brasca (1995) investigated the inhibitory effect of sodium chloride (0.5–3.0% NaCl) on 34 PAB strains isolated from Italian hard cheeses in culture broth (15 °C, 22 °C, 30 °C) and found that 36% and 5% of the *P. freudenreichii* strains were partially or strongly inhibited, respectively, at 1.5% NaCl under warm room conditions (22 °C). Interestingly, about one third of salt-insensitive PAB isolates grew faster at a NaCl content of 0.5% than in the absence of salt. Similarly, a study by Richoux, Faivre, and Kerjean (1998) in Swiss-type cheese concluded that salt tolerance of *P. freudenreichii* was strongly strain-dependent.

Acerbi, Guillard, Aliani, Guillaume, and Gontard (2016a) studied the effect of salt and ripening conditions on CO_2 formation in foil-packed cheese blocks. They found that the CO_2 formation rate during ripening followed a bell-like curve with a maximum achieved earlier and higher with decreasing salt concentration. Hollywood and Doelle (1984) investigated the spatial distribution of propionibacteria and chemical parameters such as NaCl and propionic acid in Swiss-type cheese at different stages of ripening. They observed only poor growth in the 1–5 cm zone from the edge and an increasing gradient of both bacterial counts and propionic acid towards the cheese centre. The authors assumed that this gradient was the result of factors such as salt, moisture and diffused oxygen.

Eye formation in cheese is a complex process influenced by a number of factors such as gas formation, gas diffusion, the presence of eye initiating nuclei, pH, elasticity of the cheese body and technological parameters (Fröhlich-Wyder et al., 2013). Recently, computed tomography was used to study the spatial distribution and the volume of eyes in cheese (Schuetz et al., 2013). Using magnetic resonance imaging (MRI), Huc, Challois, Monziols, Michon, and Mariette (2014a) showed that eyes in the centre of a cheese are bigger and more numerous than those in the peripheral zones. In a further study, Huc et al. (2014c) compared eye formation, CO_2 production and lactate metabolism of salted and unsalted semi-hard Swiss-type cheese. The study clearly confirmed that NaCl has an important impact on both the bacterial development and CO_2 production in Swiss-type cheese. Guggisberg et al. (2015) showed that the addition of small doses of tiny hay particles to micro-filtered vat milk allowed the control of the eye number in cheese in a dose-dependent manner. The addition of eye nuclei (tiny hay particles) to micro-filtered milk is an efficient measure to standardise eye formation preconditions in cheese and thus enables a detailed investigation of factors influencing eye formation in cheese. The objective of the present study was to quantitatively investigate the influence of three different brining times (0 d, 1 d, 3 d) and of two different PAB cultures (Prop A and Prop B) under standardised conditions on the eye formation in experimental Swiss-type cheeses.

2. Material and methods

2.1. Cultures

The two propionibacteria cultures that were used contained one strain of *Propionibacterium freudenreichii* (Prop B, FAM 23904) and two strains of *Propionibacterium freudenreichii* (Prop A, FAM 14176 & FAM 14177), respectively. All three strains are deposited in the Agroscope strain collection (Bern, Switzerland).

2.2. Cheese making and ripening

Twelve experimental hard Swiss-type cheeses, 30 cm in diameter, were manufactured from 90 L of milk each in the Agroscope pilot plant (Bern, Switzerland) in twelve stainless steel vats. The milk after skimming was micro-filtered and the fat content adjusted to 35 g kg^{-1}. The number of eye nuclei was standardised by the subsequent addition of powdered food grade hay to the milk. A suspension containing 200 mg of powdered hay (<100 μm; Agroscope, Bern, Switzerland) in 100 mL tap water was prepared in a flask with a screw cap. The powdered hay was kept in suspension by continuous shaking, and 10 mL of the suspension were added into the individual vat milks at the beginning of cheese making. Eight litres of water containing 9 mL aqueous copper sulphate solution (39.3 g L^{-1} of copper (II) sulphate pentahydrate) were added to the vat milk (90 L) at a level to ensure concentrations of Cu in the final cheese close to that of traditional Emmentaler Switzerland AOP (appellation d'origine protégée – protected designation of origin). Copper is known to regulate microbial activities (Rodriguez, Ritvanen, Joutsjoki, Rekonen, & Alatossava, 2011). After adding 200 mL of a bulk starter containing multiple strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *lactis* (RMK 101, Agroscope, Bern, Switzerland), the milk was pre-ripened at 31–32 °C for 30 min. Thereafter, the milk was inoculated with a culture of *P. freudenreichii* (either Prop A or Prop B, according to Table 1) at a level of approximately 10^3 cfu mL^{-1}. The manufacturing and ripening processes have been previously described in detail by Guggisberg et al. (2015); only the brine treatment varied according to Table 1. In total, six different variants were simultaneously produced on two days, the second day being a replicate of the first day (n = 2 for all variants). To maintain the round shape of the cheeses at a diameter of 30 cm, they were stabilised at the hoop side by a low-density polyethylene (LDPE) net with a mesh size of 6–7 mm (Bichsel Kasereibedarf, Grosshöchstetten, Switzerland).

2.3. Cheese sampling

Cheeses were sampled after 4 months of ripening. No sampling occurred at 24 h to keep the cheeses intact for the X-ray computer tomography (CT) measurements (section 2.7). After the CT measurements, the 4-months aged cheeses were cut in half. One half was used for the rheological analysis (section 2.6) and the chemical and biochemical analysis (section 2.4), and one quarter was used for microbiological analysis (section 2.5). The last quarter served as a retention sample and was deep-frozen. For all the analysis, the rind of the hoop side and of the two cheese faces was discarded with a thickness of 0.5 cm. For the analysis of the eyeless border zone the next 2.5 cm of the hoop side was used. For the analysis of the cheese core, another 2 cm of the hoop side was discarded and the remaining core used.

2.4. Chemical and biochemical analysis

For the chemical and biochemical analysis, the cheese was grated and mixed. Succinate and L- and D-Lactate were measured

Table 1
Experimental design of the Swiss-type model cheese manufacturing with two different propionibacteria cultures and three different brining conditions.

Cheese numbers	Production day (run)	PAB culture	Brining conditions
2 and 9	1 and 2	Prop A	no brining
3 and 10	1 and 2	Prop A	1 day (control)
4 and 11	1 and 2	Prop A	3 days
5 and 12	1 and 2	Prop B	no brining
1 and 8	1 and 2	Prop B	1 day (control)
6 and 7	1 and 2	Prop B	3 days

enzymatically according to the manufacturer's protocol of the kits (R-Biopharm, Darmstadt, Germany). Water content was determined with the dry matter method (IDF, 2004), and fat content according to ISO Standard 3433 (ISO, 1975). Volatile carboxylic acids (C1–C6) were analysed by gas chromatography with a flame ionisation detector and headspace technology after esterification with ethanol, as described by Fröhlich-Wyder et al. (2013). Sodium chloride content was determined using the potentiometric titration method (IDF, 2006). Total nitrogen (TN), nitrogen soluble at pH 4.6 (SN) and 12% trichloroacetic acid soluble nitrogen (TCA-SN) were measured using the Kjeldahl method (Collomb, Spahni, & Steiger, 1990). The specific activity of aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) of the two PAB cultures was determined in three culture replicates as previously described by Turgay et al. (2011).

2.5. Microbiological analysis

For the enumeration of propionibacteria in cheese, samples (10 g) were homogenised in 90 mL of peptone water (Merck, Buchs, Switzerland) for 3 min in a stomacher. Diluted suspensions were plated onto sodium lactate agar as described by Turgay et al. (2011). The plates were incubated under anaerobic conditions at 30 °C for 9–10 days.

2.6. Physicochemical analysis

The pH-value of the cheeses was determined with a pH measuring instrument (Metrohm 605, Metrohm AG, Herisau, Switzerland). A uniaxial compression test (ISO 17996:2006/IDF 4:2004; ISO, 2006) was carried out at 15 ± 1 °C using a Zwick universal machine (Zwick GmbH & Co., Ulm, Germany). Eyeless cylinders of cheese (height 15 mm, diameter 12.5 mm) were cut vertically out of the core cheese sample. The method has recently been described in Guggisberg et al. (2017).

2.7. Computed tomography of cheese and image analysis

X-ray CT measurements of all the cheeses were carried out after a ripening period of 120 days using a CT scanner (Somatom Volume Zoom, Siemens, Zürich, Switzerland). The following scan parameters were applied: 120 kV, 60 mA s and 0.5 mm slice thickness. The resolution for the CT cross-section was set constant by choosing a constant field of view for all cheeses. Before the scan procedure, the cheeses were packed in plastic bags (but not vacuumed) and placed on a Styrofoam-Plexiglas support for optimal positioning. The procedure and the image analysis with VG Studio Max, Version 2.2 (Volume Graphics, Heidelberg, Germany), were carried out as previously described by Guggisberg et al. (2015).

This software determines the volume for each eye and defines its centre using a three-dimensional coordinate system. The x and y coordinates of the cheese centre were determined by fitting a circle to the two-dimensional plot of the cheese wheel. Using the x and y coordinates of the centre of each eye, the radial distances (s_i) of the eyes to the central z-axis were calculated according to the following equation:

$$s_i = \sqrt{(x_i - x_c)^2 + (y_i - y_c)^2} \quad (1)$$

where x_i and y_i are the horizontal coordinates of the eye and x_c and y_c are the horizontal coordinates of the central axis of the cheese wheel.

Assuming a perfectly spherical eye shape, the eye diameters (d_i) were calculated with the following equation:

$$d_i = 2 \times \sqrt[3]{\frac{3V_i}{4\pi}} \quad (2)$$

where V_i is the volume of the eye.

In a final step, the eyes were sorted by their vertical position (z-axis). The diameter and radial position of the eyes within the 4 cm thick central layer of the cheeses were plotted in an x-y diagram as shown in Fig. 5. Eight cheeses were evaluated (Prop A and Prop B cheeses with a brining time of 0 d and 3 d, n = 2). The diagrams shown in Fig. 5 represent the results obtained from cheeses produced in run 1. Run 2 cheeses yielded very similar results (data not shown).

The eyeless border zone was visually analysed with the following procedure using 2 D plots. As a first step, the diameter (D_{plot}) of each 2 D plot was determined. Thereafter, the 10 outermost cheese eyes were taken and the distances from their centres to the border (x_{1-10}) were measured and added up. The mean value of the distances was converted to the original size of the cheese (D_{cheese} = 30 cm) using the following equation:

$$\bar{x} = \frac{\sum x_{1-10} \times D_{cheese}}{D_{plot}} \Big/ 10 \quad (3)$$

The average value $\bar{x}$ was taken as a measure for the eyeless border zone (Fig. 6).

2.8. Calculation of CO_2 formation

Formation of CO_2 during cheese ripening was estimated on the basis of the molar concentrations of propionic acid, butyric acid and succinate. The ratio to the CO_2 formed was 2 for propionic acid, 2 for succinate and 0.5 for butyric acid, resulting in the following equation:

$$c\,(CO_2) = \frac{c\,(propionic\ acid)}{2} + \frac{c\,(succinic\ acid)}{2} + 2 \times c\,(butyric\ acid) \quad (4)$$

The calculated amounts of CO_2 (mmol kg^{-1} cheese) were converted into mL kg^{-1} cheese (normal conditions; T = 273.15 K and P = 101.3 kPa). At these conditions the molar volume of an ideal gas is 22.4 L.

2.9. Statistical analysis

A factorial design with two factors (culture and brine duration, treated as categorical variables) on two levels (cultures Prop A and Prop B) and three levels (brine duration of 0, 1 and 3 days), respectively, was applied (Table 1). The experiment was replicated on a second day (N = 2 runs, treated as a categorical variable). Statistical analysis of the instrumental data was carried out using the method of analysis of variance with the general linear model (GLM). The software used was SYSTAT 13 (Systat Software, Inc., Chicago, IL, USA). Significant differences between the various levels within a factor were accepted at $P \leq 0.05$.

The linear dependence between two variables was described with the Pearson correlation coefficient r.

3. Results and discussion

3.1. Effect of brine salting on composition and growth of PAB

Salting in brine and the subsequent diffusion of salt into the cheese temporarily leads to elevated salt contents in the peripheral zone of the cheese. This elevation influences the zonal composition and the development of the cheese microbiome and thus prevents a uniform ripening process in larger cheese loafs (Gomes, Vieira, & Malcata, 1998; Guinee & Fox, 2017). Various parameters were analysed in both the core and in the eyeless border zone of the experimental Swiss-type cheeses at the end of ripening (120 days) to study zonal differences (Table 2). Depending on brining time, significant differences in the water content were found. As expected, water content was lower in the border zone than in the core. During brining, water diffuses out of the cheese and NaCl into the cheese due to osmotic pressure differences. As intended, increasing brining time resulted in a higher NaCl content in the aqueous phase in both the core and the border zone ($P < 0.001$). Equilibrium through salt diffusion from the border zone to the core was not reached after 120 days of cheese ripening. On average, the NaCl content was 0.4% higher in the border zone than in the core of the cheeses ($P < 0.001$), due to the method of brine salting itself and because of dry ripening with a natural rind. This decreased the water content in the border zone and consequently the speed of salt diffusion. Salt diffusion in the experimental cheeses was rather slow compared with previous findings by Goy et al. (2012), who found equilibrium of salt after 90 days in Gruyere AOP cheese

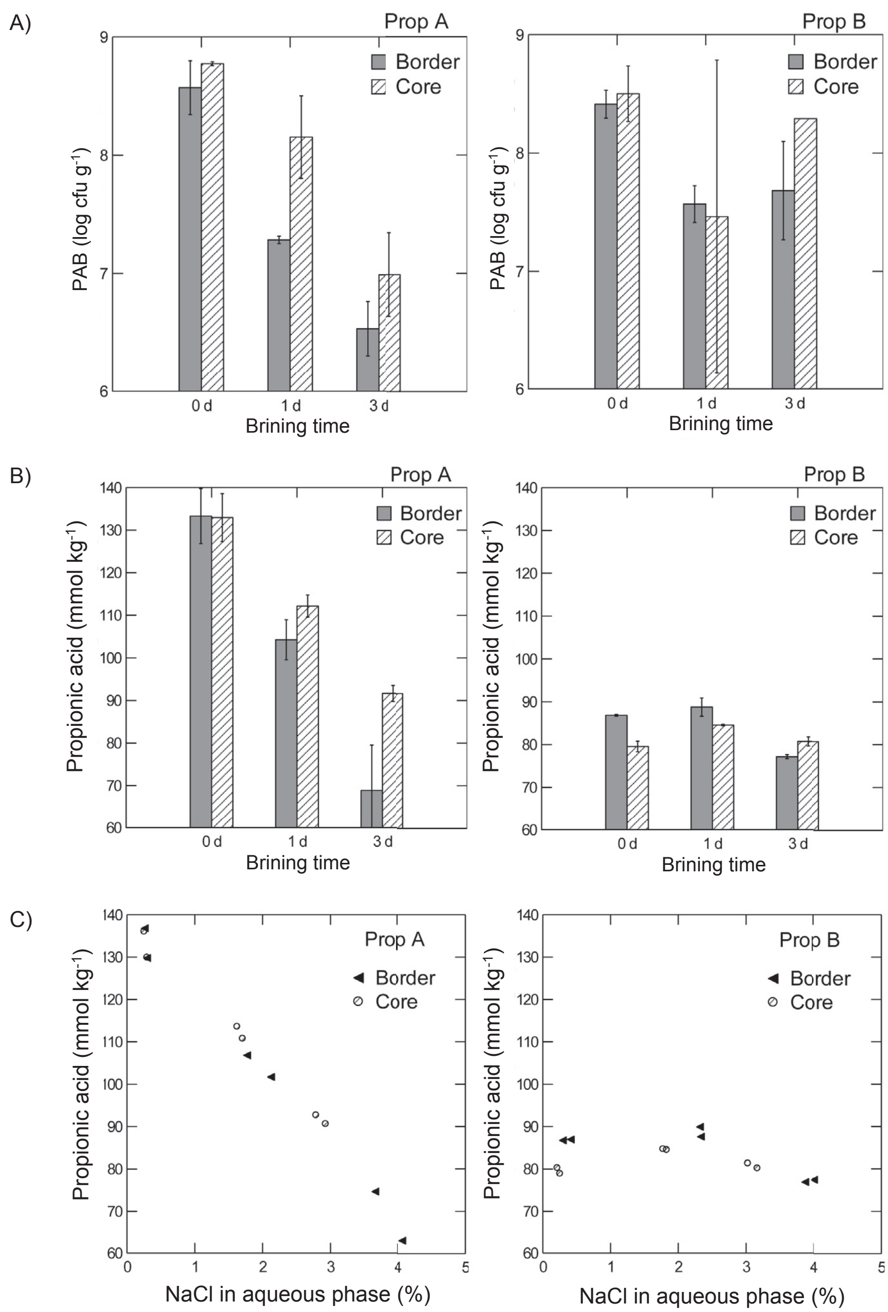

Fig. 1. Determination of propionic acid bacteria (PAB) and propionic acid in experimental Swiss-type cheeses (120 days) manufactured with three different brining conditions (0 d, 1 d, 3 d) and two different PAB cultures (Prop A and Prop B). Panel A: effect of brining time on PAB counts in the core (▨) and the border zone (■) of the cheeses made with Prop A (left) and Prop B (right). Panel B: effect of brining time on propionic acid formation by Prop A (left) and Prop B (right) in the core (▨) and the border zone (■) of the cheeses. Panel C: influence of the NaCl content in the aqueous phase from the core (○) and the border zone (◄) on propionic acid formation by Prop A (left) and Prop B (right). Error bars indicate standard error (N = 2 for each variant, except for PAB count in cheese core Prop B, 3 d).

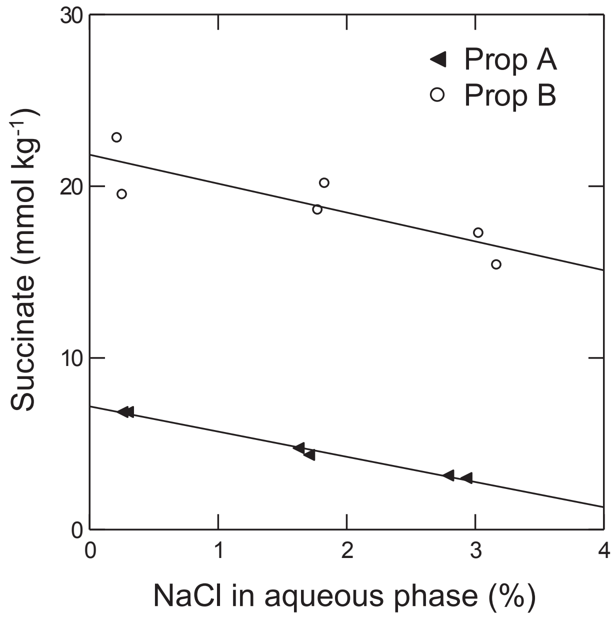

Fig. 2. Formation of succinate in experimental Swiss-type cheeses (120 days) manufactured with three different brining conditions (0 d, 1 d, 3 d) and two different propionic acid bacteria (PAB) cultures (Prop A, ◄, and Prop B, ◯). The NaCl concentrations in the aqueous phase of the experimental cheeses were strongly dependent on brining conditions ($P < 0.001$) and significantly affected succinate formation of the two PAB cultures ($P < 0.001$).

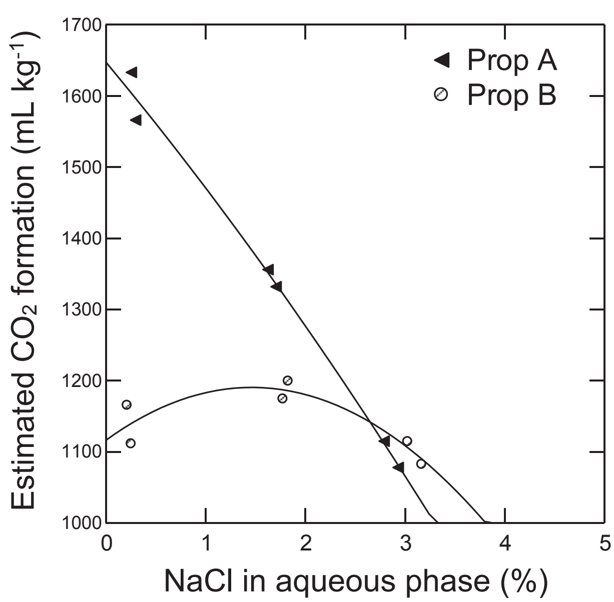

Fig. 3. Estimation of CO_2 formation in 120 days aged experimental Swiss-type cheeses manufactured with three different brining times (0 d, 1 d, 3 d) and two different propionic acid bacteria cultures (Prop A, ◄, and Prop B, ◯). In cheeses made with the salt-sensitive Prop A culture, CO_2 formation progressively decreased with increasing NaCl concentration. In cheeses made with Prop B the maximum of CO_2 formation was at medium salt content as indicated by the quadratic smoother.

having a humid smear surface, a diameter of 0.55–0.65 m and a height of 0.095–0.12 m. Guinee (2004) and Geurts, Walstra, and Mulder (1974) indicated a NaCl diffusion coefficient in cheese moisture of typically 0.2 $cm^2\ d^{-1}$ and a period of 83 days until equilibrium for salt in a hard Romano-type cheese.

The applied brining times (0 d, 1 d or 3 d) significantly influenced growth of PAB ($P < 0.001$; Table 2, Fig. 1A). The highest PAB counts were obtained in the unsalted cheeses (3.7×10^8 cfu g^{-1}), whereas brining times of 1 d and 3 d progressively inhibited the growth of PAB (4.1×10^7 cfu g^{-1} and 1.7×10^7 cfu g^{-1}, respectively). These findings are in good agreement with those of Boyaval et al. (1999) and Carcano et al. (1995), who both showed a progressive inhibitory effect of salt on the growth of PAB in a concentration range of 0–4% salt in moisture. Huc et al. (2014c) also found a lower PAB count of 8.4 log cfu g^{-1} in salted semi-hard Swiss-type cheese with 2.3% salt in moisture compared with 9.1 log cfu g^{-1} in the unsalted sample. Furthermore, a significant difference was found for the PAB counts in the cheeses with Prop A between the border and the core zone with lower counts in the border zone ($P = 0.004$, separate statistical analysis for cheeses with Prop A), confirming results of Reinbold, Hussong, and Stine (1958). In contrast, no significant zonal difference was found for the PAB counts in the cheeses with Prop B.

The applied brining times (0 d, 1 d or 3 d) not only affected NaCl content in the aqueous phase and PAB counts, but also caused highly significant differences in the content of propionic acid ($P < 0.001$). The average propionic acid content in cheeses with Prop B was about 23% lower compared with cheeses with Prop A, which can be explained by the different metabolic properties of the applied cultures: Prop A metabolised both D- and L-lactate, whereas Prop B only metabolised L-lactate (see section 3.2). In accordance with the results of the PAB counts, propionic acid concentration was lower in the eyeless border zone than in the core of the brine salted cheeses (1 d, 3 d) produced with Prop A culture ($P = 0.039$) whereas in the cheeses with Prop B the zonal differences were not significant (Fig. 1B). An interesting observation was made for cheeses with Prop B: cheeses salted in brine for 1 day showed slightly higher propionic acid contents in the core and the border than the unsalted cheeses and the ones brine salted for 3 days (Fig. 1B). Similarly, Carcano et al. (1995) and Todesco, Carcano, Lodi, and Crepaldi (2000) observed that salt tolerant PAB isolates sometimes grew even faster at a NaCl content of 0.5% than in the absence of salt.

In summary, the obtained results clearly showed that the two PAB cultures differ considerably in their salt sensitivity. In the cheeses with Prop A the formation of propionic acid strongly decreased with increasing salt content in the aqueous phase. In contrast, Prop B culture proved to be rather salt tolerant (Fig. 1C).

3.2. Metabolic differences of the applied PAB cultures

In Swiss-type cheeses, propionic acid fermentation constitutes the key metabolism for the formation of CO_2. The overall formation of CO_2 by PAB is mainly related to the growth of PAB (viable counts) and to strain-specific/culture-specific metabolic characteristics such as the ability to metabolise D- and/or L-lactate and to co-ferment aspartate during propionic acid fermentation (Fröhlich-Wyder et al., 2017). To investigate culture-specific metabolic properties, D- and L-lactate as well as succinate were analysed in the experimental cheeses after a ripening period of 120 days (Table 2). All experimental cheeses had been produced with the same commercial thermophilic starter (RMK 101, Agroscope, Bern, Switzerland), therefore, it can be assumed that the contents of D- and L-lactate were comparable in all the cheeses after the completion of lactic acid fermentation (24 h). From a previous study, it can be assumed that the lactic acid content was between 125 and 130 mmol kg^{-1}, of which approx. 60% was present as L-lactate (Fröhlich-Wyder et al., 2002). However, after ripening for 120 days, the contents of D- and L-lactate differed significantly between the cheeses made with Prop A and Prop B. Prop A used both

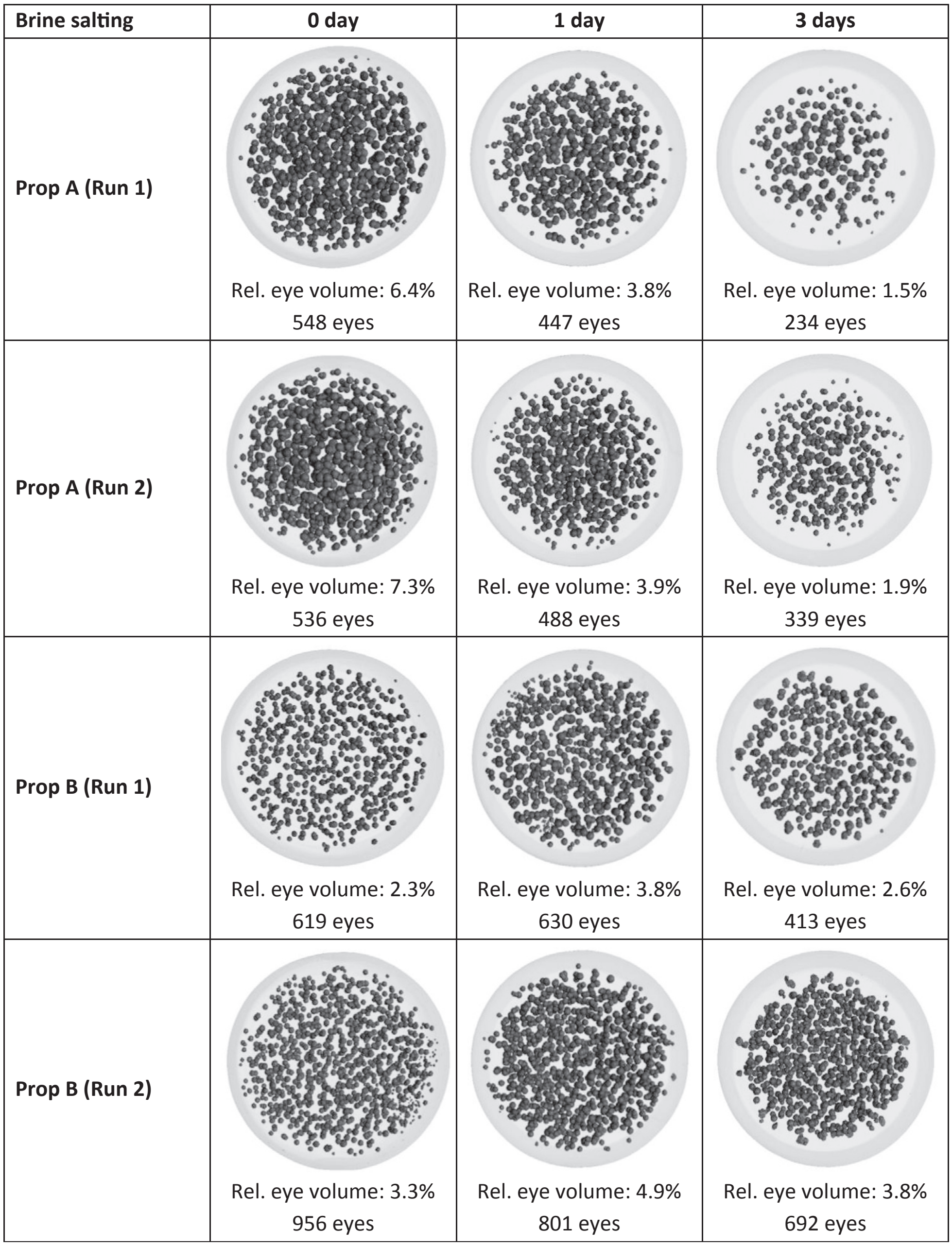

Fig. 4. Visualisation of the eye formation by X-ray computed tomography data in 120 days aged experimental Swiss-type cheeses manufactured with three different brining times (0 d, 1 d, 3 d) and two different propionic acid bacteria cultures (Prop A and Prop B). The relative eye volume indicates the total eye volume as a percentage of the total cheese volume.

isomers, in contrast to Prop B that only metabolised L-lactate. In the ripened cheeses with Prop B, L-lactate was depleted whereas the content of D-lactate seemed to remain at the original level. The obtained results strongly indicate that L-lactate was a limiting factor for the growth, the metabolic activity of Prop B, and hence, the production of PA and CO_2 (see section 3.3).

In the presence of free aspartate, the metabolism of lactate can be coupled with the metabolism of aspartate, which is deaminated to fumarate and further reduced to succinate (Crow & Turner, 1986). The determination of the specific aspartase activity is a reliable indicator to assess the potential for aspartate metabolism of individual PAB strains or PAB cultures. For Prop A and Prop B, a specific aspartase activity of 3.7 ± 1.1 and 140.4 ± 6.6 nmol min^{-1} mg^{-1} protein, respectively, was determined. Additionally, the determination of succinate allowed the estimation of the amount of co-fermented aspartate and the resulting amount of CO_2 (Table 2, Fig. 3). The aspartate metabolism releases one mole of CO_2 per mole of lactate, whereas the classic Fitz-pathway uses three moles of lactate to form one mole of CO_2 (Fröhlich-Wyder et al., 2017). Fig. 2 shows the succinate formation as a function of the NaCl content in the aqueous phase. The two lines are almost parallel and show greater aspartate metabolism in cheeses made with Prop B.

The propionic acid fermentation solely via the Fitz-pathway usually leads to ratio of propionic to acetic acid (PA/AA) of about two. This is the case for the Prop A culture (Table 2), in contrast to Prop B, where this ratio is significantly lower ($P < 0.001$). These findings confirm the presence of a much stronger aspartate metabolism for Prop B. Interestingly, the inhibitory effect of increasing

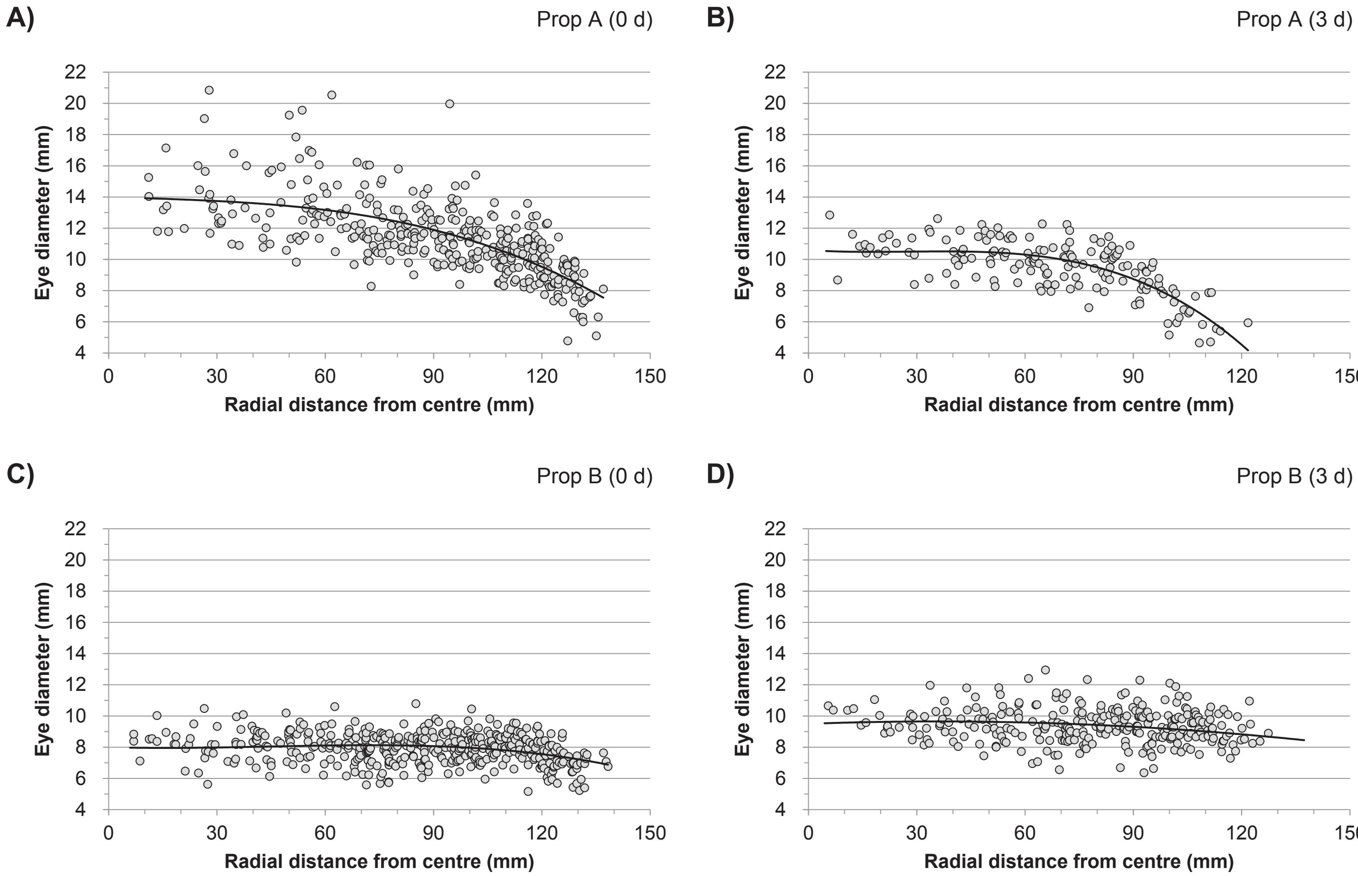

Fig. 5. Zonal variation of the eye diameters in 120 days aged experimental Swiss-type cheeses manufactured with propionic acid bacteria cultures Prop A (panels A and B) and Prop B (panels C and D) and brining times of 0 d (panels A and C; unsalted) and 3 d (panels B and D). The data of the individual eyes were extracted from X-ray computed tomography analysis.

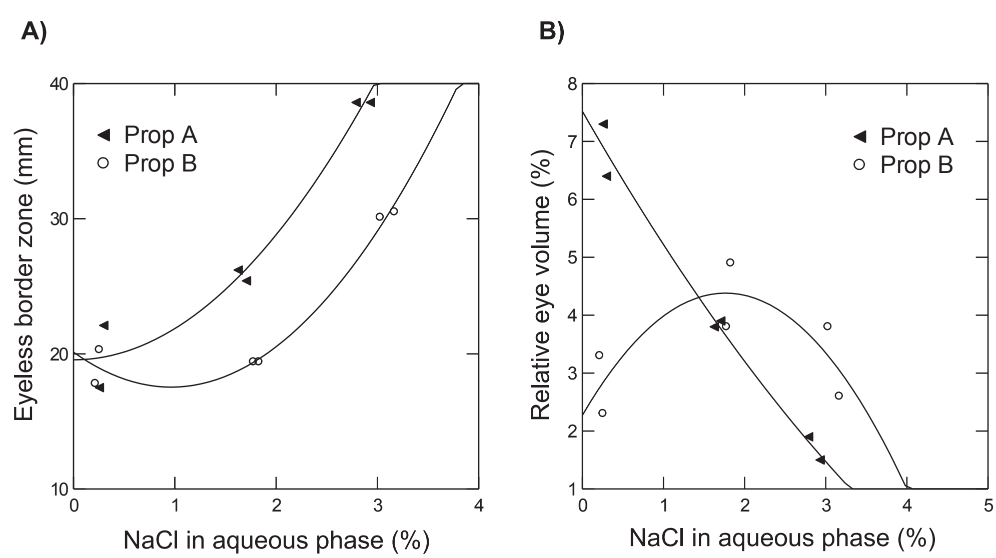

Fig. 6. Effect of the salt concentration in the aqueous phase on the width of the eyeless border zone, and on the relative eye volume (total eye volume expressed as a percentage of the total cheese volume) in 120 days aged experimental Swiss-type cheeses manufactured with three different brining times (0 d, 1 d, 3 d) and two different PAB cultures (Prop A, ◄, and Prop B, ◯). Quadratic smoothers are shown.

salt concentrations on succinate formation was similar for both PAB cultures, as shown by their parallel development in Fig. 2. This is in opposition to the salt-sensitivity of PAB growth and propionic acid formation (Fig. 1), as previously discussed.

The observed differences in the metabolism of the two PAB cultures underline the importance of selecting a suitable PAB culture to obtain the desired properties of the individual Swiss-type cheese varieties. For example, the application of Prop B culture is

Table 2
Mean values of moisture, fat, NaCl in the aqueous phase (NaCl aq.), counts of propionibacteria (PAB), propionic acid (PA), the ratio of propionic to acetic acid (AA), D- and L-lactate, succinate and force at 33% deformation in the experimental Swiss-type cheeses (120 days) manufactured with two different PAB cultures (Prop A and Prop B) and three different brining conditions (0 d, 1 d, 3 d).

Factor	Level	N	Moisture	Fat	NaCl aq.	PAB[a]	PA	PA/AA	D-lactate	L-lactate	Succinate	Force at 33% deformation
			($g\ kg^{-1}$)	($g\ kg^{-1}$)	(%, w/w)	($cfu\ g^{-1}$)	($mmol\ kg^{-1}$)	(–)	($mmol\ kg^{-1}$)	($mmol\ kg^{-1}$)	($mmol\ kg^{-1}$)	(N)
Culture	Prop A	6	347.6	323.0	1.8	5.2E+07	107.2	2.06	13.2	20.6	4.8	20.1
	Prop B	6	346.6	322.9	2.0	9.1E+07	82.9	1.82	73.7	0.0	19.0	25.6
Brining	0 day	4	354.1	320.8	0.3	3.7E+08	108.1	1.86	37.5	0.0	14.0	13.9
	1 day	4	347.9	320.6	1.9	4.1E+07	97.4	1.95	42.3	12.7	12.0	21.5
	3 days	4	339.4	327.5	3.4	1.7E+07	79.6	2.01	50.4	18.2	9.7	33.0
Zone	Core	6	350.6	323.0	1.7	1.0E+08	96.9	1.95	43.4	10.3	11.9	22.8
	Border	6	343.6	nd	2.1	4.7E+07	93.2	1.94	nd	nd	nd	nd
GLM	Culture		ns	ns	*	ns	***	***	***	***	***	***
(P- value)	Brining		***	ns	***	***	***	***	***	***	**	***
	Zone		***	–	***	0.051	ns	ns	–	–	–	–
	Run		ns	*	ns	ns	ns	*	ns	ns	ns	**
Zone × brining			0.057	–	***	ns	*	ns	–	–	–	–
Culture × brining			–	–	–	**	***	*	***	***	ns	*

[a] One sample was missing (Prop B, core, 3 d); nd not determined; -, not analysed. Statistical significance indicated by:*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns not significant.

recommended for the manufacture of fast-ripened Swiss-type cheeses with an elevated salt content. However, the use of cultures with a high aspartase activity usually yields cheeses that are prone to late fermentation and thus show poor ripening stability. In such cheeses, propionic acid fermentation cannot be slowed down by cold room storage (12 °C); because of the ongoing and uncontrolled CO_2 formation, cracks are formed (Turgay et al., 2011). However, none of the cheeses with Prop B showed symptoms of late fermentation. The sole metabolism of L-lactate is an important feature of Prop B, which is of great interest in terms of ripening stability. Lactate is also necessary as a substrate for the co-fermentation of aspartate. The inability to metabolise D-lactate limited not only propionic acid fermentation but also aspartate metabolism and thus contributed in different ways to limit the formation of CO_2 and the risk of late fermentation in the cheeses with Prop B. The cheeses produced with Prop A also showed a good ripening stability which can be explained by the low specific aspartase activity and the high salt sensitivity.

3.3. Estimation of the overall CO_2 formation

CO_2 formation by bacterial metabolism was estimated based on propionic acid, succinate and butyrate (traces, data not shown), according to equation (4) (section 2.8). By far the majority of CO_2 was formed by the classic Fitz pathway (about 95% in cheeses with Prop A and 80% in cheeses with Prop B). Co-fermentation of lactate and aspartate resulting in succinate contributed to about 4% and 19% of CO_2 for Prop A and Prop B, respectively, and butyric acid fermentation to as little as 1% for both cultures. The average CO_2 formation in cheeses with Prop A was significantly higher than in cheeses with Prop B ($P < 0.001$; Fig. 3). This again is reflecting the inability of Prop B to utilise D-lactate and is in agreement with the higher amounts of propionic acid formed by Prop A (Table 2). Nevertheless, as discussed above, salt sensitivity and the metabolic properties of the two PAB cultures were very different. As to be expected, the amounts of CO_2 formed by the two PAB cultures strongly depended on the salt concentration in the aqueous phase ($P < 0.001$ for interaction for culture × brining; Fig. 3). For Prop A, the volume dropped from 1600 to 1100 mL kg^{-1} for 0 and 3 days brining, respectively. For Prop B, it remained in a range of 1200 to 1100 mL kg^{-1}. A similar average value of 1027 mL kg^{-1} was reported by Guggisberg et al. (2015) in a previous study with experimental Emmental cheese. The calculated CO_2 volumes are up to forty times higher than in six months matured Gruyère, a cheese variety without propionic acid fermentation that typically lacks eye formation (Wenzel et al., 2018). For salt-less short-ripened semi-hard Swiss-type cheese, Acerbi, Guillard, Aliani, Guillaume, and Gontard (2015) reported a CO_2 formation of 3.4–6 mmol kg^{-1} d^{-1} at 19 °C and 20–22 mmol kg^{-1} d^{-1} at 25 °C at 15–27 days after renneting, which corresponds to 76–500 mL kg^{-1} d^{-1} at normal conditions (0 °C, 101.3 kPa). These comparisons illustrate the importance of the temporary warm-room ripening of Swiss-type cheeses, which strongly supports the formation of large amounts of CO_2 in a short time and thus enables the onset of eye formation. However, the results of the present study show that, in addition to the ripening temperature, the metabolic properties and salt sensitivity of PAB cultures strongly influence CO_2 formation.

3.4. Eye formation

The large variation of the salt content (0.3–1.7%) in Swiss-type cheeses is a challenge for the selection of PAB cultures and the control of eye formation. X-ray CT measurements at the end of ripening (120 days) assisted in studying the impact of the brining time and of the two PAB cultures on eye formation in the experimental cheeses. To quantify the eye formation, the number of eyes and the relative eye volume (total eye volume expressed as a percentage of the total cheese volume) were determined in the individual cheeses (Fig. 4). Furthermore, the variation of the eye diameters was studied in relation to the radial distance from the central axis of the cheese wheel (Fig. 5). The visualisation of the eye formation in the 12 experimental cheeses shows that the brining time and the choice of PAB culture strongly affected eye formation (Fig. 4).

Based on the visual inspection, three major effects were observed. Independently of the choice of culture, the highest eye numbers were generally recorded in the unsalted cheeses, whereas the lowest eye numbers were obtained in the three-day brine salted cheeses. Moreover, brine salting for 3 days considerably increased the width of the eyeless border zone (Figs. 4 and 6A). In addition to these general differences, some culture-specific differences were visible. In the cheeses made with the salt-sensitive Prop A culture, the relative eye volume, eye number and eye diameter strongly decreased with increasing brining time (Figs. 4 and 5). The larger eye diameter in the cheeses made with Prop A can be explained mainly by its capacity to utilise both lactate isomers. This contributed to a higher CO_2 concentration and consequently to the formation of larger eyes especially in the cheese core. In the unsalted cheese with Prop A, numerous outliers were observed in the upper eye diameter range, which can be explained by coalesced eyes (Fig. 5A). Salting for 3 days caused a general decrease in eye size in the cheese with the salt-sensitive culture Prop A (Fig. 5B); this effect was particularly pronounced towards the border zone. In contrast, in the cheeses with the salt-tolerant and exclusively L-lactate fermenting Prop B culture, zonal variation of eye size was generally smaller and less affected by the different brining conditions (Fig. 5C and D).

Surprisingly, for each brining condition, eye numbers were clearly higher with Prop B than with Prop A, despite the fact that Prop B utilised L-lactate only and formed less CO_2 at 0 d and 1 day brining. This may indicate that CO_2 formation by Prop B happened earlier than with Prop A, a phenomenon that needs further investigation. Only the size of the eyeless border zone increased as well, albeit at a lesser extent. Brine salting for 1 day yielded cheeses with rather similar eye patterns and relative eye volumes for both cultures (Figs. 4 and 6). The highly significant interaction between culture and brining confirmed the visual observations of the stronger influence of salt on eye formation for Prop A compared with Prop B. The separate statistical evaluation of the cheeses made with salt-sensitive Prop A showed that brining time had a significant effect on both the relative eye volume ($P < 0.01$) and eye numbers ($P < 0.05$). Both parameters progressively decreased with increasing salt content (Figs. 4 and 6B). In the cheeses with salt tolerant Prop B, the highest relative eye volume was obtained in the 1 day salted cheeses (P < 0.01). Similar culture dependent differences were observed for propionic acid (Fig. 1B). Both observations indicate that moderate salt concentrations in the cheese core slightly supported the metabolic activity of Prop B. In the core zone of unsalted and salted (2.3% salt in moisture) semi-hard Swiss-type cheese, Huc et al. (2014c) found no significant difference in the eye number between the two samples but a six times higher mean eye volume in the unsalted sample. This is in accordance with the present study where eye volume was much more influenced by brining conditions and culture than the eye number. The addition of eye nuclei in our study helped to reduce variation in eye numbers.

In the unsalted cheeses, the eyeless border zone had an average width of 19 mm. In this zone, the diffusion of CO_2 to the outside of the cheeses prevented the formation of eyes (Acerbi, Guillard, Guillaume, Saubanere, & Gontard, 2016b; Pauchard, Flückiger, Bosset, & Blanc, 1980). Salting of cheeses in brine for 1 and 3 days significantly increased the width of the eyeless border zone (23 mm and 35 mm, respectively, $P < 0.001$). It can be assumed that the initially high salt content in the border zone of the brined cheeses limited (at least temporarily) the growth of both PAB cultures. In cheeses made with the salt sensitive Prop A culture, the width of the eyeless border zone strongly correlated with propionic acid ($r = -0.973$, $P = 0.001$). These results allow the conclusion that a continuous increase in eye number towards the core resulted mainly from the decreasing salt content towards the core during propionic acid fermentation. In contrast, in cheeses made with the salt tolerant Prop B culture, the distribution of the eyes was more balanced (Fig. 4) and there was no correlation between propionic acid and the eyeless border zone ($r = -0.284$, $P = 0.586$). Eyes in the centre of a cheese are usually more numerous than those in peripheral zones (Huc et al., 2014b). However, our study using standardised conditions regarding the number of eye nuclei shows that the extent of this phenomenon depends on the salt sensitivity of the PAB culture.

The brining time also influenced the texture of the experimental cheeses. In Table 2 the forces at 33% deformation in the core of the experimental cheese are listed. As expected, the increase in brining time significantly increased the firmness and thus the deformation resistance of the cheeses ($P < 0.001$). Moreover, dry ripening contributed to a firmer texture in the border zone by the loss of water and thereby affected eye formation.

4. Conclusions

Considerable progress was achieved in recent years in the understanding of eye formation in cheese. The improved standardisation of the conditions for eye formation using micro-filtered milk and adding eye nuclei, as well as the use of X-ray computed tomography combined with advanced image analysis, have immensely spurred on the investigation of the influence of individual factors on eye formation in a quantitative manner. These advances make it possible to develop tailor-made PAB cultures and to reliably solve cheese quality problems related to eye formation defects.

In the present study we investigated the effect of salt on the technological properties of PAB with different salt sensitivity and different lactate metabolism. The results clearly demonstrate that salt sensitivity and lactate metabolism of PAB are some of the key factors for eye formation in Swiss-type cheeses. Despite the standardisation of the number of eye nuclei, zonal distribution and number of eyes varied considerably depending on the salt sensitivity of the applied cultures. The influence of salt on propionic acid fermentation and eye formation was most pronounced in the border zone. The initially high salt content and the associated inhibition of PAB, the firmer texture and the CO_2 diffusion out of the cheese, drastically reduced eye formation in the border zone. The use of a salt tolerant PAB culture and the ripening of cheeses in plastic films with a specific CO_2-permeability are technological measures to reduce this effect. The present study confirms that eye formation in cheese is a complex dynamic process which is strongly influenced by the properties of the applied PAB culture (salt sensitivity, metabolic properties), diffusion processes (CO_2, NaCl) and a number of technological parameters (salting and ripening conditions).

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References

Acerbi, F., Guillard, V., Aliani, M., Guillaume, C., & Gontard, N. (2015). Novel methodology for the in situ assessment of CO_2 production rate and its application to anaerobic ripened cheese. *Food Research International, 78*, 295–301.

Acerbi, F., Guillard, V., Aliani, M., Guillaume, C., & Gontard, N. (2016a). Impact of salt concentration, ripening temperature and ripening time on CO_2 production of semi-hard cheese with propionic acid fermentation. *Journal of Food Engineering, 177*, 72–79.

Acerbi, F., Guillard, V., Guillaume, C., Saubanere, M., & Gontard, N. (2016b). An appraisal of the impact of compositional and ripening parameters on CO_2 diffusivity in semi-hard cheese. *Food Chemistry, 194*, 1172–1179.

Boyaval, P., Deborde, C., Corre, C., Blanco, C., & Bégué, E. (1999). Stress and osmoprotection in propionibacteria. *Lait, 79*, 59–69.

Carcano, M., Todesco, R., Lodi, R., & Brasca, M. (1995). Propionibacteria in Italian hard cheese. *Lait, 75*, 415–426.

Collomb, M., Spahni, M., & Steiger, G. (1990). Estimation of nitrogen according to Kjeldahl in milk products and some of their nitrogen-containing fractions with an automatic system. *Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene, 81*, 499–509.

Crow, V. L. (1986). Utilization of lactate isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: Regulatory role for intracellular pyruvate. *Applied and Environmental Microbiology, 52*, 352–358.

Crow, V. L., & Turner, K. W. (1986). The effect of succinate production on other fermentation products in Swiss-type cheese. *New Zealand Journal of Dairy Science & Technology, 21*, 217–227.

Dherbécourt, J., Bourlieu, C., Maillard, M.-B., Aubert-Frogerais, L., Richoux, R., & Thierry, A. (2010). Time course and specificity of lipolysis in Swiss cheese. *Journal of Agricultural and Food Chemistry, 58*, 11732–11739.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017). *Fundamentals of cheese science. Chapter 9: Salting of cheese curd*. New York, NY, USA: Springer.

Fröhlich-Wyder, M. T., Bachmann, H. P., & Casey, M. G. (2002). Interaction between propionibacteria and starter/non-starter lactic acid bacteria in Swiss-type cheeses. *Lait, 82*, 1–15.

Fröhlich-Wyder, M. T., Bisig, W., Guggisberg, D., Jakob, E., Turgay, M., & Wechsler, D. (2017). Cheeses with propionic acid fermentation. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese: Chemistry, physics and microbiology. Vol 2. Cheese technology and major cheese groups* (4th ed., pp. 889–910). Amsterdam: The Netherlands Elsevier: Academic Press.

Fröhlich-Wyder, M. T., Guggisberg, D., Badertscher, R., Wechsler, D., Wittwer, A., & Irmler, S. (2013). The effect of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* on the eye formation of semi-hard cheese. *International Dairy Journal, 33*, 120–128.

Geurts, T., Walstra, P., & Mulder, H. (1974). Transport of salt and water during salting of cheese. 1. Analysis of the processes involved. *Netherlands Milk and Dairy Journal, 28*, 102–129.

Gomes, A. M. P., Vieira, M. M., & Malcata, F. X. (1998). Survival of probiotic microbial strains in a cheese matrix during ripening: Simulation of rates of salt diffusion and microorganism survival. *Journal of Food Engineering, 36*, 281–301.

Goy, D., Häni, J. P., Piccinali, P., Wehrmüller, K., Jakob, E., Fröhlich-Wyder, M. T., et al. (2012). *Salt and its significance in cheese making. ALP forum no. 59*. Bern, Switzerland: Agroscope.

Guggisberg, D., Schuetz, P., Winkler, H., Amrein, R., Jakob, E., Fröhlich-Wyder, M. T., et al. (2015). Mechanism and control of the eye formation in cheese. *International Dairy Journal, 47*, 118–127.

Guggisberg, D., Winkler, H., Bütikofer, U., Fröhlich-Wyder, M. T., Egger, L., Badertscher, R., et al. (2017). Influence of chemical and biochemical characteristics on the texture of Appenzeller® cheese. *International Dairy Journal, 75*, 111–119.

Guinee, T. P. (2004). Salting and the role of salt in cheese. *International Journal of Dairy Technology, 57*, 99–109.

Guinee, T. P., & Fox, P. F. (2017). Salt in cheese: Physical, chemical and biological aspects. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese: Chemistry, physics and microbiology, vol 1: General aspects* (4th ed., pp. 317–378). Amsterdam, The Netherlands: Elsevier, Academic Press.

Hollywood, N. W., & Doelle, H. W. (1984). The effect of sampling position and age on levels of propionibacteria and flavour-related compounds in Swiss-type cheese. *Australian Journal of Dairy Technology, 39*, 81–82.

Huc, D., Challois, S., Monziols, M., Michon, C., & Mariette, F. (2014a). Spatial characterisation of eye-growing kinetics in semi-hard cheeses with propionic acid fermentation. *International Dairy Journal, 39*, 259–269.

Huc, D., Mariette, F., Challois, S., Barreau, J., Moulin, G., & Michon, C. (2014b). Multiscale investigation of eyes in semi-hard cheese. *Innovative Food Science & Emerging Technologies, 24*, 106–112.

Huc, D., Roland, N., Grenier, D., Challois, S., Michon, C., & Mariette, F. (2014c). Influence of salt content on eye growth in semi-hard cheeses studied using magnetic resonance imaging and CO_2 production measurements. *International Dairy Journal, 35*, 157–165.

IDF. (2004). *Cheese and processed cheese – determination of the total solids content (reference method). IDF Standard 4*. Brussels, Belgium: International Dairy Federation.

IDF. (2006). *Cheese and processed cheese products – determination of chloride content – potentiometric titration method. IDF Standard 88*. Brussels, Belgium: International Dairy Federation.

IDF. (2014). *The importance of salt in the manufacture and ripening of cheese. IDF S1 Special Issue 1401.* Brussels, Belgium: International Dairy Federation.

ISO. (1975). *Cheese – determination of fat content – van gulik method. ISO standard 3433*. Geneva, Switzerland: International Organisation for Standardisation.

Jaros, D., Ginzinger, W., Tschager, E., Mayer, H. K., & Rohm, H. (1997). Effects of water addition on composition and fracture properties of Emmental cheese. *Lait, 77*, 467–477.

Orla-Jensen, S. (1921). *Dairy bacteriology*. London, UK: J. & A. Churchill.

Pauchard, J. P., Flückiger, E., Bosset, J. O., & Blanc, B. (1980). CO_2-Löslichkeit, -Konzentration bei Entstehung der Löcher und -Verteilung im Emmentalerkäse. *Schweizerische Milchwirtschaftliche Forschung, 9*, 69–73.

Peltola, E. (1940). The effect of salt on bacteria of importance in Emmental cheesemaking. *Meijeritieteellinen-Aikakauskija, 2*, 11–21.

Reinbold, G. W., Hussong, B. V., & Stine, J. B. (1958). Distribution of propionibacteria in Swiss cheese. *Journal of Dairy Science, 41*, 606–610.

Richoux, R., Faivre, E., & Kerjean, J.-R. (1998). Effect de la teneur en NaCl sur la fermentation due lactate par *Propionibacterium freudenreichii* dans des mini-fromages à pâte cuite. *Lait, 78*, 319–331.

Rodriguez, L. M., Ritvanen, T., Joutsjoki, V., Rekonen, J., & Alatossava, T. (2011). The role of copper in the manufacture of Finnish Swiss-type cheese. *Journal of Dairy Science, 94*, 4831–4842.

Rüegg, M., Glättli, H., & Blanc, B. (1976). Einfluss der Wasseraktivität auf Vermehrung und Stoffwechsel von Propionsäurebakterien. *Schweizerische Milchwirtschaftliche Forschung, 5*, 119–122.

Schuetz, P., Guggisberg, D., Jerjen, I., Fröhlich-Wyder, M. T., Hofmann, J., Wechsler, D., et al. (2013). Quantitative comparison of the eye formation in cheese using radiography and computed tomography data. *International Dairy Journal, 31*, 150–155.

Thierry, A., Maillard, M.-B., Hervé, C., Richoux, R., & Lortal, S. (2004). Varied volatile compounds are produced by *Propionibacterium freudenreichii* in Swiss-type cheese. *Food Chemistry, 87*, 439–446.

Todesco, R., Carcano, M., Lodi, R., & Crepaldi, P. (2000). Indirect conductimetry in the study of propionibacteria inhibition. *Lait, 80*, 337–346.

Turgay, M., Irmler, S., Isolini, D., Amrein, R., Fröhlich-Wyder, M. T., Berthoud, H., et al. (2011). Biodiversity, dynamics, and characteristics of *Propionibacterium freudenreichii* in Swiss Emmentaler PDO cheese. *Dairy Science & Technology, 91*, 471–489.

Turgay, M., Ryser, L., Fröhlich-Wyder, M. T., Wechsler, D., & Lüdin, P. (2018). *Relation between the lack of the d-lactate dehydrogenase gene and the metabolism of d-lactate by Propionibacterium freudenreichii. Poster presented at 10th Cheese Symposium. 04.04.2018, Rennes, France*.

Wenzel, C., Irmler, S., Bisig, W., Guggisberg, D., Roetschi, A., Portmann, R., et al. (2018). The effect of starters with a functional arginine deiminase pathway on cheese ripening and quality. *International Dairy Journal, 85*, 191–200.

White, S. R., Broadbent, J. R., Oberg, C. J., & McMahon, D. J. (2003). Effect of *Lactobacillus helveticus* and *Propionibacterium freudenrichii* ssp. *shermanii* combinations on propensity for split defect in Swiss cheese. *Journal of Dairy Science, 86*, 719–727.