



A feasibility study on the pilot scale manufacture of fresh cheese from skim milk retentates without acid whey production: Effect of calcium content on bitterness and texture

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ABSTRACT

The sensory and structural properties of fresh cheeses (8.0%, w/w, protein) made from fermented skim milk retentates [concentrated-fermented (CoF) fresh cheese] with different calcium-protein ratios (Ca:Prot; 15.9–36.6 mg g⁻¹) by means of pilot plant scale microfiltration (MF) and MF in the diafiltration (DF) mode without acid whey (AW) production were characterised. Calcium reduction reduced the bitterness level significantly (by 53.4%) and the bitter peptide content (by ≈ 33%) of the CoF fresh cheese after ≤ 1 week of storage; however, storage for ≥ 2 weeks resulted in an increase of both bitterness and bitter peptide content, and quantitatively changed the peptide pattern, possibly due to lack of thermisation. Fresh cheese with a lower bitterness level can be successfully manufactured, while avoiding AW production, if the Ca:Prot of the milk retentate is decreased to ≤ 15.9 mg g⁻¹ (calcium content = 1240 mg kg⁻¹ at 7.81%, w/w, protein) prior to fermentation.

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1. Introduction

A total of 1.6 billion litres of acid whey (AW) are generated per year during the manufacture of fresh cheese and other protein-enriched fermented dairy products such as strained (“Greek-style”) yoghurt (Chandrapala et al., 2015). The manufacture of fermented-concentrated (FCo) fresh cheese currently involves two main steps. The first is the gelation of milk *via* acidification by means of starter cultures, optionally supported by the addition of small amounts of chymosin. Secondly, the stirred curd-whey mixture is separated by centrifugation or ultrafiltration (UF) into a highly concentrated microgel suspension such as fresh cheese, with AW/acid permeate as the coproduct (Hahn, Sramek, Nöbel, & Hinrichs, 2012a; Schulz-Collins & Senge, 2004).

To concentrate AW, for example by means of UF, the flux is impaired by increased fouling caused by the precipitation of calcium phosphates and the high acid content, resulting in a low pH, which causes insolubility of some whey protein fractions (Konrad, Kleinschmidt, & Faber, 2012). It has been noted that high levels of lactic acid promote agglomeration of powder particles and sticky deposits on spray dryer surfaces (Nishanthi, Chandrapala, & Vasiljevic, 2017). Consequently, AW is mostly wasted today and is hence an undesired coproduct (Chandrapala et al., 2015; Shon & Haque, 2007); thus, minimising or avoiding the generation of AW at the point of manufacture is highly recommended. Strategies to reduce the amount of AW by ≈ 40 to ≈ 78% during manufacture of fresh cheese and protein-enriched yoghurt currently involves the following steps: (i) a pre-concentration step of the milk by means of microfiltration (MF; 0.05–0.2 μm pore size), UF or nanofiltration (NF) to ≤ 8.3% (w/w) protein; (ii) a retentate fermentation step; and (iii) a post-concentration step for the fermented retentate by means of centrifugation, UF or cloth bag filtration to the final protein content (Hülens, 2002; Schkoda & Kessler, 1996; Skrzypek & Burger, 2010;

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Uduwerella, Chandrapala, & Vasiljevic, 2018). According to the aforementioned authors, this hybrid-processing of concentration-fermentation-concentration (CoFCo) steps generally leads to fresh cheese and yoghurt products without sensory or structural defects.

However, an economically interesting and environmentally friendly approach is to completely avoid production of AW during fresh cheese manufacture and to generate a valuable sweet whey/MF permeate (Schäfer et al., 2018b; Sebald, Dunkel, Schäfer, Hinrichs, & Hofmann, 2018). Focusing on this idea, pH native (\approx pH 6.7) skim milk can be fractionated into a permeate and retentate by means of MF. According to Alsaed et al. (2013) and Jelen (2011), cheese whey of pH \geq 6.0 is classified as sweet whey and can be used for further applications. Due to an absence of any classification for MF permeates obtained by microfiltering pasteurised skim milk, Schäfer et al. (2019) proposed the use of the same pH limit to differentiate between AW and MF permeate that can be processed similarly to sweet whey. However, due to the absence of cheese fines, fat, salt, rennet, starter culture, colour and glycomacropptide, the MF permeate represents a “virgin whey protein stream” that can be used for the manufacture of whey protein concentrates and isolates or whey-based beverages/desserts (Ardisson-Korat & Rizvi, 2004; Hülsen, 2002). The MF milk retentate is adjusted to the final composition, e.g., by adding fat, and then acidified/fermented to obtain concentrated-fermented (CoF) fresh cheese.

During prior investigations of fresh cheese and protein-enriched yoghurt manufactured from UF skim milk retentates, several sensory defects concerning texture and taste were observed (Table 1), especially that CoF fresh cheeses and yoghurt had a bitter off-taste. Those authors, cited in Table 1, postulate that an elevated mineral content, especially calcium, but also protein degradation products such as (bitter) peptides are responsible for the bitterness. Wolfschoon-Pombo (personal communication) recommended a (maximum) calcium threshold of 1800 mg kg⁻¹ to avoid calcium-triggered bitterness as observed in cream cheese.

Nevertheless, systematic investigations of (i) overall appearance, (ii) texture and (iii) sensory properties, especially bitter taste, of CoF fresh cheese manufactured with different calcium contents and avoiding AW production, are absent. Data regarding the bitterness level of CoF fresh cheese obtained by a professional sensory panel as well as the sequences and content of bitter peptides as a function of storage time have not yet been published to our knowledge. Based on the above-mentioned considerations, our hypothesis was that the bitterness level of CoF fresh cheese (\approx 8.5% (w/w) protein) and the amount of bitter peptides generated during fermentation would be reduced by decreasing the calcium content.

2. Material and methods

2.1. Manufacture of fresh cheeses

All fresh cheeses were processed at pilot scale in the Dairy for Research and Training (D-BW 100 EG) of the University of

Hohenheim (Stuttgart, Germany). FCo fresh cheese with a target protein content of 8.5% (w/w) and a calcium-protein ratio (Ca:Prot), which is the quotient of the calcium and protein content, of \leq 17.0 mg g⁻¹ was manufactured as standard (Fig. 1, flowchart 1). CoF fresh cheeses with 8.5% (w/w) protein and calcium contents of 2850 and 1360 mg kg⁻¹ (Ca:Prot are 33.5 and 16.0 mg g⁻¹, respectively) were manufactured according to Fig. 1 (flowcharts 2 and 3). To obtain FCo and CoF fresh cheeses of the same age, manufacture was synchronised so that the fermentation of all samples was stopped within one day.

As raw material for all fresh cheeses, pasteurised skim milk was prepared according to the method of Schäfer et al. (2018a). Fresh raw bovine milk, provided by the agricultural experimental station Meiereihof (University of Hohenheim, Stuttgart, Germany), was skimmed (\leq 0.1% (w/w) fat) via a separator (SA 10-T, Frautech S.r.L., Thiene, Italy) at a temperature of 60 °C, and subsequently pasteurised (72 °C for 32 s) by means of a plate heat exchanger (KS8FS1545, ATS-Südmo GmbH, Feldkirch, Germany). To provide comparable feed fluid conditions during the concentration processes via MF, the pasteurised skim milk was standardised to a protein content of 3.4% (w/w) according to the procedure of Körzendörfer, Nöbel, & Hinrichs, 2017. This paper represents a feasibility study and, due to the scale of the manufacturing process, the analytical results are generated from one individual experimental run.

2.1.1. Manufacture of fermented-concentrated fresh cheese (standard)

The pasteurised skim milk was heated to 95 °C for 256 s to denature the whey proteins (>90%) by means of a tubular pilot plant heat exchanger (150 L h⁻¹; Asepto-Therm, Asepto GmbH, Dinkelscherben, Germany) (Fig. 1, flowchart 1). Approximately 130 g of frozen starter (Choozit 230, Danisco Deutschland GmbH, Niebüll, Germany) was added to 870 g of skim milk to ensure a homogenous distribution of the two species *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. This stock solution (13%, w/w) was prepared at ambient temperature with occasional stirring until thawed. The high heat-treated skim milk was inoculated with 0.01% (w/w) of the starter culture stock solution and 0.001% (w/w) (i.e., 1.0 g 100 kg⁻¹) of chymosin (minimum activity: 190 IMCU mL⁻¹, Chy-Max Plus, Chr. Hansen, Nienburg/Weser, Germany). Fermentation was performed in a 200 L sterile tank (custom made, Pentair Südmo GmbH, Riesbürg, Germany and Thrun Maschinen-und Behälterbau GmbH, Nesselwang, Germany) until pH 4.6–4.7 was reached. After in-situ stirring for \approx 5 min, the fermented milk (145 kg) was pre-heated to 38 °C and concentrated via cross-flow MF at pilot plant scale (TFF unit, Pall GmbH, Dreieich, Germany) equipped with multi-channel gradient of permeability ceramic membranes (0.1 μ m cut-off, 1.69 m² total filtration area, 4 mm channel diameter, 7P19-40 GP Membralox[®] Module, 99.7% α -alumina, Pall Exekia, Bazet, France) at 38 °C and transmembrane pressure (TMP) of 0.1 MPa until a mass reduction ratio (MRR, the

Table 1

Sensory defects of concentrated-fermented fresh cheeses and protein-enriched yoghurt made from skim milk retentates obtained via ultrafiltration as found in the literature.^a

Product	Dry matter (%)	Protein (%)	Sensory defect	Postulated reason for sensory defect	Reference
Fresh cheese	17.8	12.2	Shiny, gluey and smeary appearance, cardboard flavour	High mineral content, especially calcium	Flüeler and Puhán (1977)
	\geq 16.0	n.d.	Bitter taste after some days of storage	High calcium content	Friis (1981)
	17.0–18.0	n.d.	Bitter taste	High calcium content	Bäurle, Walenta, and Kessler (1984)
	18.6	12.5	Gluey and smeary appearance, bitter taste, lacking in acid taste	High calcium content	Patel et al. (1986)
Protein-enriched yoghurt	n.d.	11.8	Rubbery texture, bitter taste	Presumably caused by an increased level of peptides	Biliaderis, Khan, and Blank (1992)

^a Abbreviation: n.d., not determined.

ratio of initial feed mass to retentate mass at the end of filtration) of 2.65 was reached. The FCo fresh cheese was filled into sterilised 400 mL polyethylene containers under a laminar flow cabinet (Ehret Biosafe 2, Dipl.-Ing. W. Ehret GmbH, Emmendingen, Germany), immediately cooled and stored at $\approx 6^\circ\text{C}$ until analyses were performed.

2.1.2. Fermented-concentrated fresh cheese (reference)

A commercially available FCo fresh cheese (Cremiger Quark mit frischem Joghurt, Berchtesgadener Land Chiemgau eG (D-BY 110 EG), Piding, Germany) was used as a commercial reference. In Germany, all unripened cheeses with a dry matter of $\geq 18\%$ (w/w) are labelled as speisequark, otherwise they have to be labelled as fresh cheese in general (Käseverordnung, 1986). This fresh cheese product was chosen based on results of an in-house sensorial evaluation by a consumer panel, which indicated that the bitterness level was ≤ 2 (slightly bitter) during 5 weeks of storage at $\approx 6^\circ\text{C}$ (data not shown). For the present study, reference FCo fresh cheese of the same batch number was kindly provided by the manufacturer.

2.1.3. Manufacture of concentrated-fermented fresh cheeses with different calcium contents

For manufacture of the CoF fresh cheese with target protein content of 8.5% (w/w), calcium content of 2850 mg kg^{-1} , and a Ca:Prot of 33.5 mg g^{-1} , a feed mass of 145 kg skim milk was concentrated to a MRR of 2.83 (Fig. 1, flowchart 2). The concentration process utilised cross-flow MF at 50°C at a TMP of 0.1 MPa. The MF retentate was cooled to $<10^\circ\text{C}$, pasteurised ($\approx 75^\circ\text{C}$ for $\approx 15\text{ s}$) using a batch heater (Pasteurisierer C600/45, Kälterudi GmbH & Co. KG, Keltern-Dietlingen, Germany) and subsequently cooled in-situ to the fermentation temperature of 22.5°C .

The pasteurised MF retentate was inoculated with 0.01% (w/w) of the starter culture stock solution (preparation according to section 2.1.1) and 0.00038% (w/w) (i.e., $0.38\text{ g }100\text{ kg}^{-1}$) of chymosin (minimum activity: 190 IMCU mL^{-1} , Chy-Max Plus, Chr. Hansen). It should be mentioned here that during MF processing of the fermented milk for the FCo fresh cheese, a fraction of the chymosin is removed along with acid (whey) permeate, leading to $\approx 0.00038\%$ chymosin in the FCo fresh cheese. To have equal amounts of chymosin in both FCo and CoF fresh cheeses, 0.00038% (instead of 0.001%) chymosin was added to the MF retentates during the CoF process. Finally, the inoculated MF retentate was filled into sterilised 400 mL polyethylene containers under a laminar flow cabinet. Fermentation at 22.5°C was performed in a climate chamber (PR-2KP, Spec Corp., Osaka, Japan) to pH 4.6–4.7. To stop the fermentation, containers were immediately cooled and stored at $\approx 6^\circ\text{C}$ until the analyses were performed.

For manufacture of calcium-reduced CoF fresh cheese (target protein content = 8.5%, w/w), featuring a calcium content of 1360 mg kg^{-1} and a Ca:Prot of 16.0 mg g^{-1} , calcium-reduced MF retentate was prepared according to Schäfer et al. (2019) (Fig. 1, flowchart 3). This calcium content is well below the threshold of 1800 mg kg^{-1} to avoid calcium-triggered bitterness, as recommended by Wolfschoon-Pombo (personal communication). However, to partially solubilise the micellar calcium and increase the amount of soluble calcium, the skim milk (160 kg) was slowly acidified in a stainless steel tank (200 L, custom made, Alfred Bolz Apparatebau GmbH, Wangen, Germany) at a temperature below 10°C until pH 6.2. A pH of 6.2 was used because the resulting MF permeate has a pH ≥ 6.0 and thus the fresh cheese manufacturing process can be classified as free of AW (Schäfer et al., 2019). Acidification was performed by adding citric acid (1 M, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) dropwise under the liquid level using a syringe (50 mL, Omnifix, B. Braun Melsungen AG,

Melsungen, Germany) equipped with a long cannula ($0.9 \times 70\text{ mm}$, Sterican, B. Braun Melsungen AG, Melsungen, Germany) with continuous mixing. The pH was monitored using a pH meter (WTW pH 330i, WTW GmbH, Weilheim, Germany) with a glass electrode (Sentix 81, WTW GmbH, Weilheim, Germany). To ensure steady-state pH, the skim milk was stirred for an additional 30 min and, if necessary, citric acid was added again. The acidified skim milk was heated in situ with a temperature gradient of approximately 1°C min^{-1} to 50°C .

Concentration processing of 145 kg of acidified skim milk was carried out by cross-flow MF under the same conditions as mentioned for processing of CoF fresh cheese (see section 2.1.1). The acidified MF retentate was cooled to $<10^\circ\text{C}$ and slowly acidified in-situ to pH 5.6 (according to the same procedure used to acidify skim milk to pH 6.2). Finally, the MF retentate was heated in-situ to 50°C and six repeated diafiltration (DF) stages using demineralised water at a dilution ratio of 1:1.54 (DF medium: feed medium) were performed to elute soluble calcium. During MF in DF mode (MF-DF), lactose was also eluted from the MF retentate. Consequently, the calcium-reduced MF retentate was standardised by adding lactose monohydrate (RAB G, Molkerei Meggle Wasserburg GmbH & Co. KG, Wasserburg, Germany) to 4.46% (w/w) lactose. Further processing was performed similarly to the manufacture of the CoF fresh cheese, as described previously.

2.2. Chemical analysis

2.2.1. Composition

The nitrogen content of all fresh cheese samples was determined by the method of Dumas according to IDF-method 185:2002 (IDF, 2002) using a nitrogen analyser (Dumatherm DT, Gerhardt Analytical Systems, Königswinter, Germany). Total protein content was calculated from the nitrogen content via a conversion factor of 6.38. The fat, dry matter and calcium contents were determined according to the method of Gerber (C 15.3.2; VDLUFA, 2003), the sea sand method (C 35.3; VDLUFA, 2003) and a complexometric method (C 10.6.8; VDLUFA, 2003), respectively. The latter method is based on the complexometric titration of calcium ions by means of ethylenediaminetetraacetic acid (EDTA) using calconcarboxylic acid as indicator. Lactose content was determined by an accredited (DIN EN ISO/EC 17025:2005) lab (Landwirtschaftliches Zentrum Baden-Württemberg (LAZBW), Wangen, Germany) using an enzymatic method. All analyses were performed in triplicate.

2.2.2. Quantitative analysis of selected bitter peptides

To quantify selected previously reported bitter peptides in different manufacturing processes, water-soluble extracts of the standard FCo, CoF and calcium-reduced CoF fresh cheeses at 0 and 2 weeks of storage were prepared and analysed using LC-MS/MS in MRM-mode according to Sebald et al. (2018). Measurements were performed in triplicate.

2.3. Microbiological analysis

To ensure food safety, all fresh cheese samples manufactured within this study were tested and cleared prior to sensorial evaluations for enterobacteria, yeasts and moulds according to EN ISO 21528-2:2017 (ISO, 2017) and ISO 21527-1:2008-07 (ISO, 2008), respectively.

2.4. Sensorial evaluation of the bitterness level

The level of bitterness of all fresh cheese samples was evaluated by means of a professional sensory panel for fresh cheese and yoghurt after 1 and 3 weeks of storage. The panel consisted of 7–8

members (male/female/diverse; age: ≈ 25 –50 years) and were trained according to DIN EN ISO 8586:2014-05 (DIN, 2014) and DIN ISO 3972:2013-12 (DIN, 2013). Most fresh cheese varieties consumed in Europe have a paste- or spread-like (but not gel-like) consistency (Schulz-Collins & Senge, 2004). To compare the CoF and FCo fresh cheeses, they should have a similar appearance and consistency; thus, prior to the sensorial evaluation, both CoF fresh cheeses were mechanically treated as follows: (i) breaking up the intact gel of two containers (800 mL) of each CoF fresh cheese by means of a food processor equipped with a whisk (KM260 series, Kenwood, Neu-Isenburg, Germany) at level three for $t = 90$ s, (ii) shearing the broken gel by means of a hand blender (Zauberstab M 180, Unold AG, Hockenheim, Germany) at level two for $t = 90$ s until a visibly smooth mass with a paste-like consistency, i.e., a microgel suspension, was obtained.

Samples (≈ 50 –60 g) of each CoF and FCo fresh cheese having a temperature of ≈ 10 °C were served in plastic containers coded with random 3-digit numbers. To neutralise any carry-over taste during the sensorial evaluation, tap water and matzo bread (Pico Food GmbH, Tamm, Germany) were also served. Panellists evaluated the bitterness level using a non-structured linear scale (0, not bitter; 1, very slightly bitter; 2, slightly bitter; 3, bitter; 4, very bitter; 5, extremely bitter) by means of FIZZ software (version: 2.51 A, Sarl Biosystemes, Couternon, France). The evaluation was performed in two independent sessions, held ≈ 4 h apart, giving a total of ≥ 14 measurement repetitions of each fresh cheese sample.

2.5. Physical analysis

2.5.1. Visual appearance

The visual appearance of all fresh cheese samples was evaluated after 1 and 3 weeks of storage. Before analyses were started, all fresh cheese samples were stored for at least 12 h at 10 °C. The CoF fresh cheese gels were carefully removed from the container and placed in polystyrene petri dishes (diameter = 145 mm). A portion (≈ 75 g) of each mechanically treated CoF fresh cheese (sample preparation described in section 2.4) and both FCo fresh cheeses were put into petri dishes. Before and after four hours of aging at 10 °C, photos of the CoF fresh cheese (before and after mechanical treatment) as well as the FCo fresh cheeses were taken using a camera (Canon Power Shot G16, Canon, Tokyo, Japan).

2.5.2. Textural properties

Colour determination. The colour of all fresh cheese samples (CoF fresh cheese: mechanically treated samples only, sample preparation described in section 2.4) was determined with a colorimeter (CR-400, Konica Minolta Sensing Europe B.V., München, Germany) after 1 week of storage at a sample temperature of 10 °C by putting the measurement port onto a glass jar filled with ≈ 100 g of each sample. The lightness is given via the L^* value (0: black; 100: white) and the chromatic coordinates via a^* (-a: red; +a: green) and b^* values (-b: blue; +b: yellow). Measurements were performed in triplicate.

Confocal laser scanning microscopy. The microstructure of the CoF fresh cheese gels was visualised using confocal laser scanning microscopy (CLSM) after 1 and 3 weeks of storage. The protein phase was labelled with 35 μ L of rhodamine B protein dye ($M = 479$ g mol⁻¹, Art.-No. 107599, CAS 81-88-9, Merck KGaA, Darmstadt, Germany) solubilised in distilled water at 0.016 g 100 g⁻¹ (Heilig, Göggerle, & Hinrichs, 2009). Three specimens of each CoF fresh cheese gel were prepared according to Heilig et al. (2009). Images were taken using a microscope (Eclipse-C1, Nikon GmbH, Düsseldorf, Germany) equipped with a 63 \times oil immersion

objective and controlled via NIS-Elements Confocal software (Nikon GmbH, Düsseldorf, Germany). Four images were taken at 10 °C of each specimen in clockwise rotation, giving a total of 12 images of each CoF fresh cheese sample. The CLSM images shown are those most representative of the gels investigated.

Syneresis. The syneresis, i.e., whey separation, of all fresh cheese samples (CoF fresh cheese: mechanically treated samples only, sample preparation described in section 2.4) was determined by a centrifugal method according to Hahn et al. (2012b) after 1 and 3 weeks of storage. Before analyses were started, all fresh cheese samples were stored for at least 12 h at 10 °C. Fresh cheese samples were gently stirred and ≈ 45 g was weighed into each centrifuge tube (50 mL). The centrifuge (Heraeus, BioFuge Stratos, Thermo Electron Corporation, Waltham, MA, USA) was tempered (without the samples) to 10 °C at 20 \times g for 20 min, then the fresh samples were centrifuged at 5000 \times g for 15 min at 10 °C (Harwalkar & Kalab, 1983; Kneifel, Paquin, Abert, & Richard, 1991). The supernatant (expelled whey) was removed and sediment (including the centrifuge tube) was weighed and syneresis was calculated as the mass ratio of expelled whey to initial sample weight (≈ 45 g). Measurements were performed in triplicate.

Gel firmness. The gel firmness of the CoF fresh cheese gels was determined by means of a penetration test after 1 and 3 weeks of storage. Before analyses were started, all fresh cheese samples were stored for at least 12 h at 10 °C. The penetration tests were done by means of a universal testing machine (load cell: 50 N, 5944, Instron, Norwood, MA, USA) with Bluehill 3 software (Instron). A cylindrical probe (diameter = 10 mm) penetrated the gels at a sample temperature of 10 °C with a test speed of 0.5 mm s⁻¹ for 60 s and force at the first local maximum was determined. The gels of 4 containers were penetrated once, giving a total of four measuring repetitions of each sample.

2.6. Statistical analysis

Significant differences in syneresis, gel firmness and bitterness level of the fresh cheeses were identified by conducting an analysis of variance (ANOVA). If significant, a subsequent Fisher's least significant difference (LSD) test was applied to discriminate affected samples using XLSTAT 2015 software (version 2015.4.01.20575; Addinsoft, New York, NY, USA). All significance levels were set at $\alpha = 0.05$ ($P < 0.05$).

3. Results and discussion

3.1. Chemical composition

During the general manufacture of FCo fresh cheese, skim milk is commonly fermented at ≈ 20 –30 °C to pH 4.6–4.8 causing solubilisation of calcium to an extent of ≈ 98 to 96% (Koutina, Knudsen, Andersen, & Skibsted, 2014). Solubilised calcium was removed via the acid (whey) permeate, leading to calcium contents of 1330 ± 30 and 1320 ± 20 mg kg⁻¹ at protein contents of 8.19 ± 0.06 and $9.16 \pm 0.15\%$ (w/w), and resulting in calcium-protein ratios of 16.2 and 14.4 mg g⁻¹ for the standard and reference FCo fresh cheese, respectively (Table 2).

The effect of CoF and FCo technology on the calcium content of the fresh cheeses is depicted in Table 2. Since $\approx 71\%$ of milk calcium is micellar-bound at native pH (≈ 6.6) and 20 °C (Koutina et al., 2014), the calcium content increases during MF concentration of skim milk at native pH along with protein content in a linear relationship. Consequently, CoF fresh cheese had a calcium content

Table 2
Chemical composition of the fresh cheeses.^a

Fresh cheese sample	Protein (% w/w)	Calcium (mg kg ⁻¹)	Calcium–protein ratio (mg g ⁻¹)	Fat (% w/w)	Lactose (% w/w)	Dry matter (% w/w)
FCo (standard)	8.19 ± 0.06	1330 ± 30	16.2	0.15 ± 0.01	3.81	13.67 ± 0.03
FCo (reference)	9.16 ± 0.15	1320 ± 20	14.4	0.15 ± 0.01	3.60	14.27 ± 0.02
CoF	8.26 ± 0.09	3020 ± 160	36.6	0.15 ± 0.01	3.11	13.89 ± 0.05
CoF (Ca ²⁺ red.)	7.81 ± 0.36	1240 ± 40	15.9	0.18 ± 0.01	3.28	12.26 ± 0.10

^a Abbreviations are: FCo, fermented-concentrated; CoF, concentrated-fermented; Ca²⁺ red., calcium-reduced. Values represent the means and standard deviations (i = 1, n = 3) except for analyses of lactose content that were performed in triplicate by an accredited laboratory; no standard deviations were provided. The calcium–protein ratio is the quotient of the calcium and protein content.

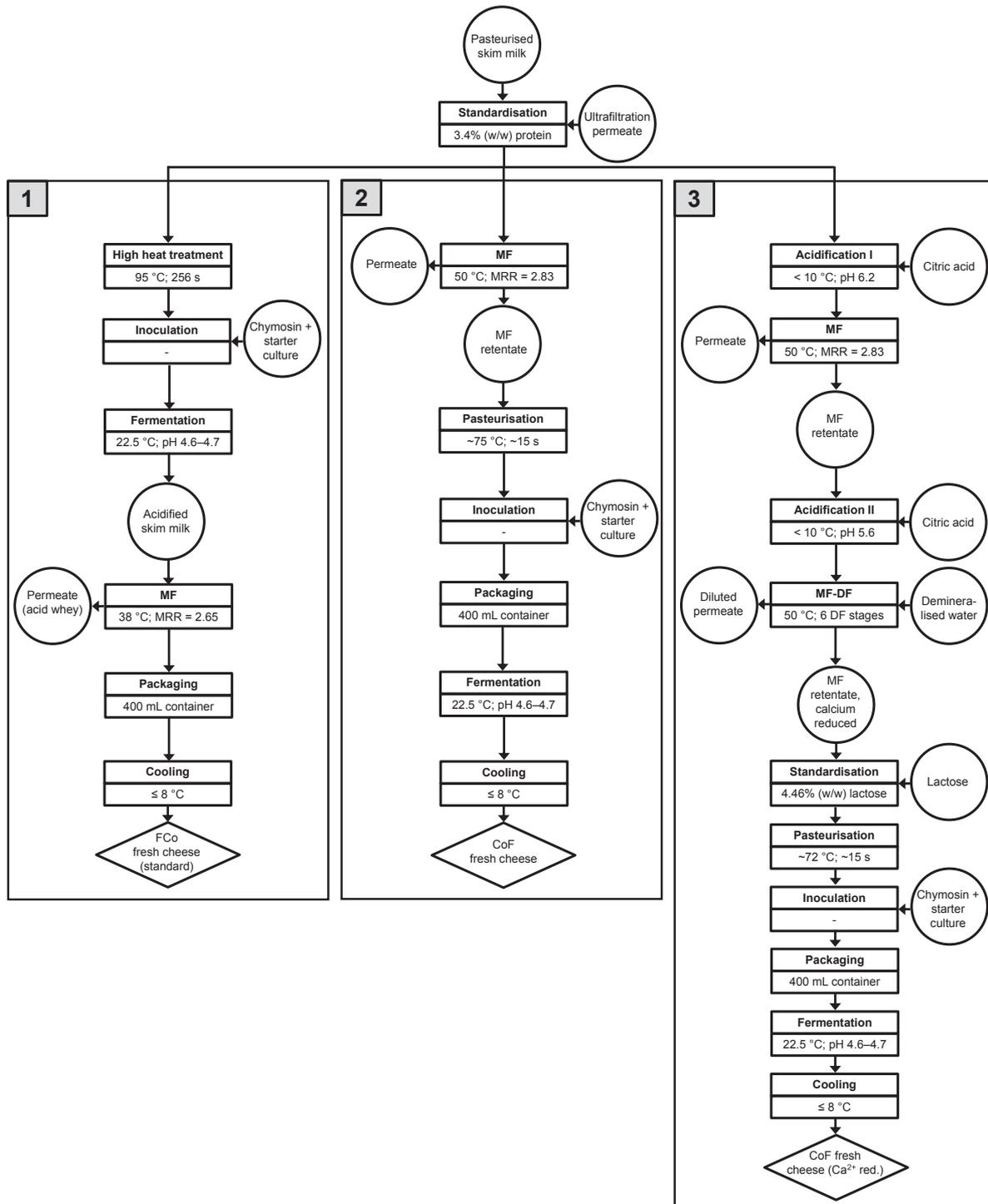


Fig. 1. Flowcharts for the manufacture of standard fermented-concentrated (FCo) fresh cheese (1), concentrated-fermented (CoF) fresh cheese (2), and calcium-reduced (Ca²⁺ red.) concentrated-fermented (CoF) fresh cheese (3) with a target protein content of 8.5% (w/w). Abbreviations are: MF, microfiltration; MF-DF, microfiltration in diafiltration mode; MRR, mass reduction ratio.

of $3020 \pm 160 \text{ mg kg}^{-1}$ at a protein content of $8.26 \pm 0.09\%$ (w/w) and thus a Ca:Prot of 36.6 mg g^{-1} . Resulting from the two-stage acidification procedure and MF-DF, the calcium content of calcium-reduced CoF fresh cheese was successfully lowered to $1240 \pm 40 \text{ mg kg}^{-1}$, which is in the range of skim milk ($\approx 1050\text{--}1300 \text{ mg kg}^{-1}$, Hurt, Adams, & Barbano, 2015; Walstra, Wouters, & Geurts, 2006), but at a higher protein content ($7.81 \pm 0.36\%$, w/w), resulting in a Ca:Prot of 15.9 mg g^{-1} .

3.2. Sensory properties

Microbiological analysis showed that standard FCo and CoF fresh cheese(s) were free of enterobacteria and that yeast and mould counts were below the typical reference values of 1.0×10^4 and $1.0 \times 10^3 \text{ cfu g}^{-1}$, respectively, as given by Klein and Schütze (2015). It can also be stated that both MF retentates (Fig. 1, flowcharts 2 and 3) could be successfully batch pasteurised before fermentation, despite their higher viscosity compared to skim milk.

Beginning with standard FCo fresh cheese, it was observed that the bitterness level was 2.41 ± 1.37 after 1 week of storage and the bitterness increased slightly to 2.81 ± 1.32 at 3 weeks of storage, but this was not significant (Fig. 2). The reference FCo fresh cheeses were also slightly bitter (1.76 ± 0.92) after 1 week of storage, but no significant increase was detected during 3 weeks of storage in accordance with observations of Sohal, Roehl, and Jelen (1988). It is assumed that, during the manufacture of reference FCo fresh cheese, which consists predominantly of speisequarg (Table 2), the milk coagulum was low heat-treated (known as thermisation), which is typically performed at $\approx 60^\circ \text{C}$ for 3 min, as the majority of speisequarg is produced in Germany by means of this processing technique (Schulz-Collins & Senge, 2004). Sachdeva, Reuter, Prokopek, and Klobes (1993) found that thermisation of acidified, coagulated milk resulted in speisequarg without stale, bitter or metallic flavours during 5 weeks of storage; in contrast without a thermisation step prior to separation the speisequarg was kept well for approx. 2 weeks only. Thermisation reduces the starter culture

and enzyme activity, consequently reducing proteolysis arising from various bacterial proteinases during storage of fresh cheese (Zakrzewski, Stepaniak, Abrahamson, & Sørhaug, 1991).

By decreasing the Ca:Prot of the CoF fresh cheese, by 56.6% from 36.6 to 15.9 mg g^{-1} , bitterness level was significantly decreased by 53.4% from 3.48 ± 1.31 to 1.61 ± 1.53 (Fig. 2, storage time = 1 week). Patel, Reuter, and Prokopek (1986), who treated UF skim milk retentate at native pH with UF in the DF (UF-DF) mode using water and fermented it, also stated that the resulting fresh cheese was less bitter. After 1 week of storage, no significant differences in bitterness level were observed between the calcium-reduced CoF fresh cheese and both FCo fresh cheeses. However, at 3 weeks of storage, the bitterness level of calcium-reduced CoF fresh cheese increased significantly from 1.62 ± 1.53 to 2.86 ± 1.61 , whereas CoF fresh cheese showed little change in bitterness level (3.48 ± 1.31 (1 week) and 3.91 ± 1.45 (3 weeks)). The absence of a thermisation step, which was already discussed as a possible explanation for the increased bitterness level of standard FCo fresh cheese after 3 weeks storage, is also assumed to cause the increased bitterness level of both CoF fresh cheeses after storage (3 weeks).

3.3. Quantification of selected bitter peptides

Identification of the investigated bitter peptides, as well as their quantification using LC-MS/MS-MRM, was previously described by Sebald et al. (2018). During fermentation, the peptides were released by degradation of α_{S1} -, β -, and κ -caseins and the results of bitter peptide quantification are summarised in a heatmap (Fig. 3), in which data in the respective columns are scaled to visualise relative differences between the analysed samples, independent of their absolute content. Several trends regarding sample type and storage were observed; firstly, the non-stored (0 weeks) FCo fresh cheese sample showed generally lower concentrations

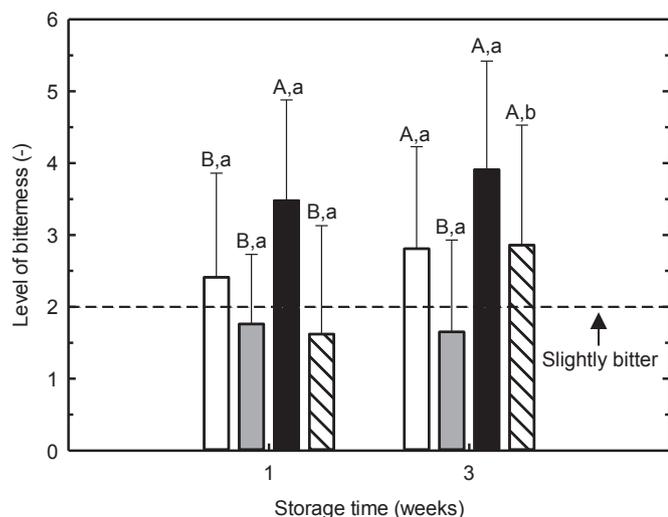


Fig. 2. Level of bitterness of the fermented-concentrated (FCo) (white bars, standard FCo fresh cheese; grey bars, reference FCo fresh cheese) and concentrated-fermented (CoF) fresh cheeses (black bars, CoF fresh cheese; hatched bars, calcium-reduced CoF fresh cheese) as a function of storage time. Scores are: 0, not bitter; 1, very slightly bitter; 2, slightly bitter; 3, bitter; 4, very bitter; 5, extremely bitter; bars with different uppercase letters are significantly different related to the type of fresh cheese, bars with different lowercase letters are significantly different related to the storage time of each individual fresh cheese. Values represent the means and standard deviations ($i = 1, n \geq 14$).

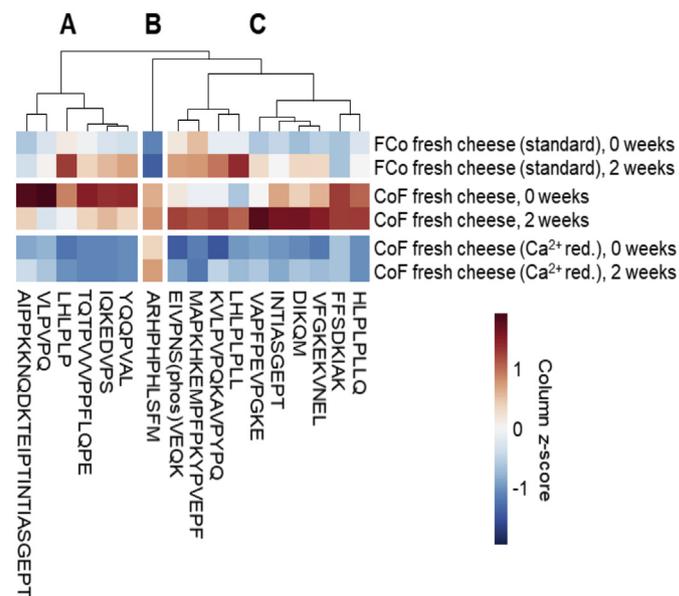


Fig. 3. Quantitative mapping of the formation of selected bitter peptides (identified in the former study by Sebald et al. (2018)) in the standard fermented-concentrated (FCo) fresh cheese, concentrated-fermented (CoF) fresh cheese, and calcium-reduced (Ca²⁺ red.) concentrated-fermented (CoF) fresh cheese as a function of storage time (0 and 2 weeks). The letters A, B, C represent different clusters of peptides. Negative column z-scores (in blue) represents lower, and positive column z-scores (in red) represents higher peptide concentration as compared to the average. Values represent the means ($i = 1, n = 3$).

($\Sigma = 40 \mu\text{mol kg}^{-1}$) of the analysed peptide compounds compared with CoF cheese ($\Sigma = 69 \mu\text{mol kg}^{-1}$), with the exception of the peptides LHLPLLL and MAPKHKEMFPKYPVEPF. Furthermore, the amount of analysed bitter peptides was lower in calcium-reduced CoF (fermentation of $6 \times \text{DF}$ retentate) fresh cheese ($\Sigma = 23 \mu\text{mol kg}^{-1}$) than in the CoF fresh cheese sample ($\Sigma = 69 \mu\text{mol kg}^{-1}$). This effect may be due to a lower calcium content, meaning that the formation of bitter peptides during fermentation is reduced in calcium-reduced milk retentates. Due to these quantitative results, there seems to be a correlation between the sensory and quantitative data, as calcium-reduced CoF fresh cheese had a lower level of bitter peptides than the bitter-tasting CoF fresh cheese.

Furthermore, during 2 weeks of storage, the sum of measured peptides increased from 40 to $59 \mu\text{mol kg}^{-1}$ in FCo fresh cheese, from 69 to $85 \mu\text{mol kg}^{-1}$ in CoF fresh cheese and from 23 to $36 \mu\text{mol kg}^{-1}$ in calcium-reduced CoF fresh cheese. In all cases, an increased sum of bitter peptides observed was possibly due to the fact that the fermented dairy products were not thermised, resulting in greater starter bacterial and enzymatic activity during storage.

The panellists described the calcium-reduced CoF fresh cheese as being more bitter after ≥ 2 weeks of storage, as compared with the 1st week of storage, which is in accordance to the quantitative data. Regarding changes during storage of the CoF fresh cheese sample, the investigated peptides could be divided into two clusters. The concentrations of the peptides in cluster A (Fig. 3), such as AIPPKKNQDKTEIPTINTIASGEPT or VLPVPQ, were decreasing during storage, whereas the concentration of peptides in cluster C, such as EIVPNS(phos)VEQK to VFGKEKVNEL, increased during 2 weeks of storage in CoF fresh cheese.

In summary, higher concentrations of bitter peptides were found in CoF fresh cheese versus FCo fresh cheese. However, in calcium-reduced CoF fresh cheese, the total amount of peptides was decreased by MF-DF treatment.

3.4. Structural properties

All mechanically treated fresh cheeses showed a typical milky white to faintly yellowish colour, smooth body and comparable overall appearance, independent of their storage and aging time (Fig. 4). Nevertheless, two samples showed abnormalities; firstly, the calcium-reduced CoF fresh cheese had a brighter colour than the other samples, which correlates well to the lowest a^* and b^* values of -2.1 ± 0.1 and 2.9 ± 0.1 , respectively, while, for all other fresh cheeses, a^* values of -3.2 to -3.8 and b^* values of 6.0 – 9.3 were measured. Consequently, calcium-reduced CoF fresh cheese had the lowest green and yellowish pigment content, due to the permeation of riboflavin, which is responsible for the yellowish-greenish colour of whey (Belitz, Grosch, & Schieberle, 2012), during MF-DF. Secondly, the reference FCo fresh cheese appeared to be considerably yellowish, which correlates well with the highest b^* value of 9.3 ± 0.1 . This could be explained by the fact that this fresh cheese was a blend of speisequarg and 9% of skim milk yoghurt, colouring the mixture yellowish, because during yoghurt manufacture there is usually no whey drainage.

The volume of macroscopic visible expelled whey after 4 h of aging at 10°C was the highest for calcium-reduced CoF fresh cheese and lowest for CoF fresh cheese, whereas the FCo cheeses showed intermediate volumes (Fig. 4) (mechanically treated samples, storage time = 1 and 3 weeks). The amounts of syneresis, as determined by means of the centrifugal method after 1 week of storage, were 61.17 ± 0.11 and $36.16 \pm 0.05\%$ for calcium-reduced CoF and CoF fresh cheese, respectively, as well as 45.47 ± 0.04 and $51.62 \pm 0.96\%$ for the standard and reference FCo fresh cheese, respectively (Table 3). The significant differences in amount of syneresis reflect the same trends as stated for the amounts of macroscopic visible expelled whey shown in Fig. 4 (Table 3). Three weeks of storage had no significant impact on syneresis behaviour, with the exception of the standard FCo fresh cheese, which showed a slight increase in the amount of syneresis, from 45.47 ± 0.04 to $46.86 \pm 0.43\%$.

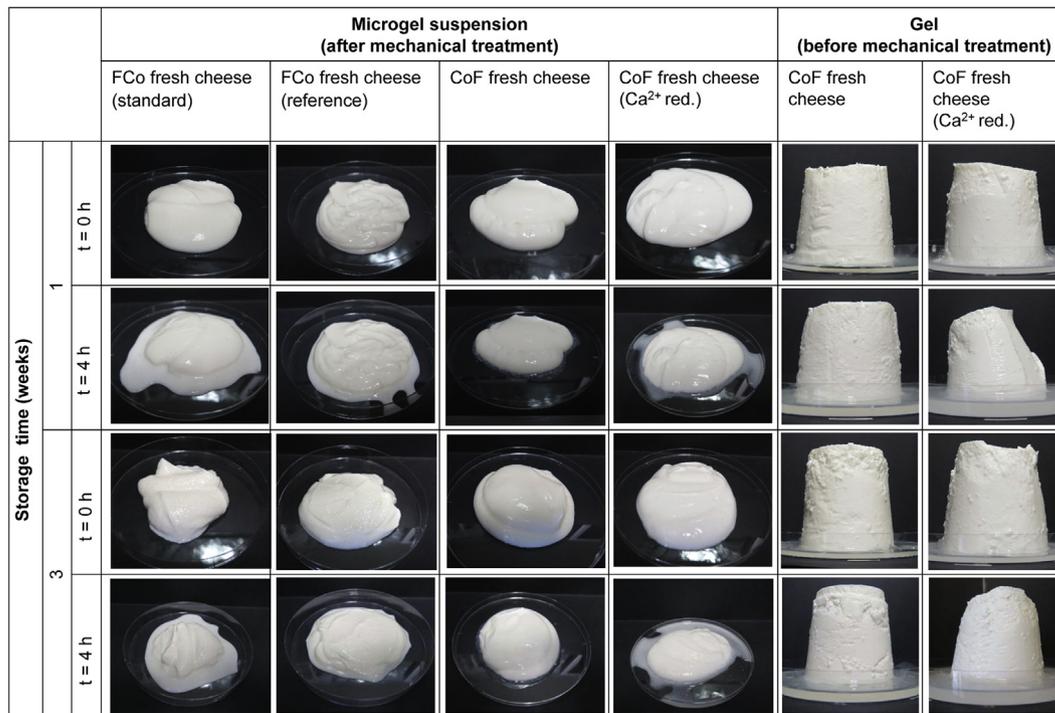


Fig. 4. Photos of the fermented-concentrated (FCo) and concentrated-fermented (CoF) fresh cheeses before and after a mechanical treatment of their gels (sample preparation described in section 2.4) at different aging times ($t = 0$ h, $t = 4$ h at 10°C) as a function of storage time. The FCo fresh cheeses were mechanically treated previously during manufacturing. Ca²⁺ red., calcium-reduced.

Table 3
Syneresis and the maximum force representing the gel firmness of fresh cheeses.^a

Storage time (weeks)	Microgel suspension, syneresis (%)				Gel, maximum force (mN)	
	FCo (standard)	FCo (reference)	CoF	CoF (Ca ²⁺ red.)	CoF	CoF (Ca ²⁺ red.)
1	45.47 ± 0.04 ^{A,a}	51.62 ± 0.96 ^{B,a}	36.16 ± 0.05 ^{C,a}	61.17 ± 0.11 ^{D,a}	1601.0 ± 87.4 ^{A,a}	2293.0 ± 125.5 ^{B,a}
3	46.86 ± 0.43 ^{A,b}	50.60 ± 0.55 ^{B,a}	36.71 ± 0.45 ^{C,a}	60.90 ± 0.30 ^{D,a}	1623.2 ± 85.1 ^{A,a}	2492.1 ± 98.2 ^{B,b}

^a Abbreviations are: FCo, fermented-concentrated; CoF, concentrated-fermented; Ca²⁺ red., calcium-reduced. Values represent the means and standard deviations (i = 1, n = 3 for syneresis and n = 4 maximum force data); values with different superscript uppercase letters in a row are significantly different concerning the type of fresh cheese; values with different superscript lowercase letters in a column are significantly different concerning the storage time.

During sample preparation prior to taking CLSM images of the intact CoF fresh cheese gels, it was observed that the calcium-reduced CoF fresh cheese showed a coarser (micro)structure, which was also observed by sensory evaluation of CoF fresh cheese made from calcium-reduced skim milk retentate obtained by UF-DF according to Patel et al. (1986). As indicated by the CLSM images (Fig. 5), the calcium reduction led to gels of a coarser microstructure with larger pores compared with those gels with native calcium content, with no impact of storage time on gel microstructure. In case of the calcium-reduced CoF fresh cheese, there seemed to be a correlation between a coarser gel microstructure with larger pores and high syneresis. This is consistent with the results of Aichinger et al. (2003), who found that gels made from fermented skim milk concentrates with different levels of chymosin showed increased syneresis if they had an increased number of large pores. As described by Meletharayil, Patel, and Huppertz (2015), gels with reduced calcium content showed also a low water holding capacity (i.e., high syneresis and a high porosity), but also low gel firmness. Surprisingly, the gels of calcium-reduced CoF fresh cheese revealed significantly much higher gel firmness (maximum force = 2293.0 ± 125.5 mN) as compared with the CoF fresh cheese gels with native calcium content (maximum force = 1601.0 ± 87.4 mN) (Table 3, storage time = 1 week). At 3 weeks of storage, there was a slight increase in the maximum force for both gels from 2293.0 ± 125.5 to 2492.1 ± 98.2 mN (significant increase; calcium-reduced CoF fresh

cheese) and 1601.0 ± 87.4 to 1623.2 ± 85.1 mN (no significant increase; CoF fresh cheese).

4. Conclusion

The present investigations indicated that calcium reduction prior to fermentation resulted in CoF fresh cheese with significantly reduced bitterness and a lower bitter peptide content. During storage (≥ 2 weeks) both bitterness level and bitter peptide content increased and the peptide pattern changed quantitatively, possibly due to lack of thermisation. However, to master the challenge of debittering CoF fresh cheese, it is recommended to stop fermentation by means of a thermisation step of the microgel suspension to reduce the activity of endo- and exogenous milk enzymes during storage. Poulsen and Mogensen (1981) acidified UF retentate with glucono delta lactone and perceived no bitterness and thus it is clear that the milk calcium itself cannot be held solely responsible for the bitter taste, but it may play an indirect role in relationship with the starter culture. The findings of this study contribute to the body of knowledge towards development of a manufacturing process for non-bitter fresh cheese avoiding AW production, but generating desirable MF permeate which can be processed similarly to sweet whey. Furthermore, during MF-DF a large volume of dilute permeate is accumulated which represents a well-standardised by-product for, e.g., biogas production or lactose fractionation. Whether this entire manufacturing process is economically interesting and/or environmentally friendly should be investigated by means of a life-cycle assessment in the future. Further research is needed to investigate the interactions of calcium and starter culture in protein-enriched fermented dairy products, such as CoF fresh cheese, to understand the formation of bitter off-tastes which is currently being investigated by the authors.

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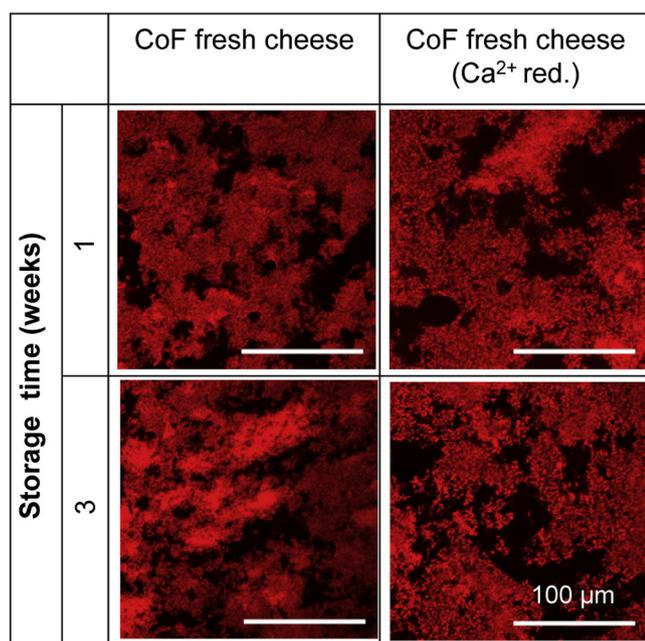


Fig. 5. Microstructure of concentrated-fermented (CoF) fresh cheese gels (before mechanical treatment) as a function of storage time. Samples were visualised by confocal laser scanning microscopy with Rhodamine B as protein dye (63 × magnification) at 10 °C. Ca²⁺ red., calcium-reduced.

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