Collaborative ring trial for the detection of cow, sheep and goat milk in dairy samples by real-time PCR targeting mitochondrial DNA

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1 Production of dairy samples

Dairy samples with different proportions (mass fractions) of cow (*Bos taurus*), sheep (*Ovis aries*) and goat (*Capra hircus*) milk were produced in the research dairy of the Max Rubner-Institut in Kiel.

1.1 Raw milk and milk processing

Three batches of each milk were obtained and delivered daily either from the research station Schädtbek (Max Rubner-Institut, Dobersdorf, Germany) in the case of cow milk or from two local organic dairy farms in case of sheep and goat milk, which were guaranteed free of admixtures. At the research dairy, all milk was pasteurised (72 °C; < 30 s) per single batch and kept in cold storage (6 °C) for a maximum of 24 h. Fat and protein contents were not further adjusted to avoid contamination. All dairy equipment was alkaline cleaned and rinsed thoroughly after each run. Different types of cheese or yoghurt were made consecutively from the same milk. Raw milk to be further processed to milk for direct consumption (drinking milk) or yoghurt was homogenised (250/50 bar) at 60 °C. Drinking milk was additionally pasteurised again. Somatic cell counts in all raw milk batches were determined using flow cytometry with a Fossomatic FC instrument (Foss Electric, Hillerød, Denmark).

1.2 Yoghurt manufacture

Homogenised and pasteurised milk was heated to 90 - 95 °C for 7 min, immediately cooled to 15 °C, and stored (6 °C) for another 1 - 2 days. One litre of milk was heated to 42.0 °C, inoculated with 20 g starter culture/100 L (Yo-Mix 215; Danisco Deutschland GmbH, Niebüll, Germany), split and sealed in 150 mL-retail containers, which were fermented in an incubator (C+10/350; CTS GmbH, Hechingen, Germany) at 42 °C. Temperature and pH were recorded (DAQ Factory Express v19.1; AzeoTech Inc., Ashland, USA) using a pre-calibrated pH sensor (SE555X/2-NMSN; Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany) in one of the containers. The fermentation was stopped at pH 4.60 (after approx. 6 h) by transferring them into a cold-storage room (< 10 °C). All yoghurt samples were stored at 10 °C for 6 days before further analysis.

1.3 Cheese manufacture

From sheep and goat milk, two different cheese types were produced: Feta-type cheese contain about 45 % dry matter, 17 % protein, and Picodon cheese contains about 50 % dry matter, 30 % protein and pH 5.8 (McSweeney et al. 2017), respectively. All cheeses were produced on a pilot plant scale involving four main process steps:

(1) Fermentation and coagulation: 6 L of pasteurised milk was brought to 36.0 °C (Feta) or 25.0 °C (Picodon) in a cheese vat to which 20 g (Feta) or 10 g $CaCl_2$/100 L (Picodon) were added. Each cheese milk was inoculated with its specific starter culture: Feta required 0.8 g/100 L of type Choozit FT 001 and Picodon 2.0 g/100 L of type Choozit Probat 222 plus 0.2 g/100 L of type Choozit Geo 17. All Choozit starter and ripening cultures were purchased from Danisco Deutschland GmbH (Niebüll, Germany). After 60 min (Feta) or 3 h (Picodon), 12 mL (Feta) or 15 mL (Picodon) of fermentation-produced chymosin/100 L (CHY-MAX M 1000; Chr. Hansen GmbH, Nienburg, Germany) was added for renneting.

(2) Cutting, draining, and molding: After 45 min (Feta) or 18 h (Picodon) of coagulation, the gel was cut into 1.0 cm grains. During the whey drainage, molding, and pressing, all whey was collected where possible. Relative to 100 L of initial cheese milk, a total mass of approx. 1.15 kg (Feta) and 0.7 kg (Picodon) yielded 170 g and 80 g, respectively, per individual cheese loaf.

(3) Salting: Feta-type cheeses were then immersed in a freshly prepared brine solution (6.0 % NaCl (*w/v*), 0.5 % $CaCl_2$ (*w/v*)) for 7 days, resulting in a salt content of approx. 3.5 g NaCl/100 g ripened cheese. For Picodon-type cheese, the required quantity of NaCl was pre-calculated to achieve a salt content of about 1.0 - 1.5% (*w/w*) in the raw cheese. The loaves (Picodon) were homogeneously coated until all the dry salt had been taken up, resulting in a salt content of approx. 2.0 g NaCl/100 g ripened cheese.

(4) Ripening and storage: Pre-dried Feta-type cheese loaves were stored in shrink bags (IP Ingredients GmbH, Süderlügum, Germany) and ripened at 7 °C for 14 days prior to analysis. Picodon-type cheese loaves were stored at 25 °C and > 65 % relative humidity and smeared (Choozit PC 22) for 2 - 3 days unless the cheese mold was clearly visible. These cheeses were then ripened at 15 °C and > 95 % relative humidity for 21 days.

2 DNA extraction

Total DNA of the dairy samples was extracted with the NucleoSpin Food kit (Macherey-Nagel, Düren, Germany). As the spin columns of the Food kit were only available in mini format, the spin columns of the NucleoSpin Blood kit (Macherey-Nagel) available in XL format were used for all DNA extractions.

In order to generate sufficient amounts of DNA, all milk and whey samples were centrifuged for enrichment of somatic cells. In particular, from all raw and yoghurt milk batches and the cheese milk batches that were not meant as DNA standards, two aliquots of 1 mL each were centrifuged for 1 h at 17 000 x g in an Avanti J-26S XPI ultracentrifuge (Beckman Coulter, Brea, CA, USA). Fat and supernatants were discarded, and one pellet from each sample was resuspended in 550 µL lysis buffer and transferred to the other tube of the sample for resuspending the second pellet. After addition of 10 µL Proteinase K, the samples were incubated at 65 °C for 1 - 2 h. The Picodon and Feta cheeses were finely chopped with a knife and 10 g to 18 g and 300 to 500 mg were sampled for the ring trial samples and for the samples for single-laboratory validation, respectively. From the yoghurts, 20 g and 500 mg were used as starting material for the ring trial samples and the samples for single-laboratory validation, respectively. The samples were then mixed with 10 mL lysis buffer and 100 µL Proteinase K (ring trial samples) or 550 µL lysis buffer and 10 µL Proteinase K (samples for single-laboratory validation). From the drinking milk, the whey samples and the cheese milks meant as DNA standards, 45 mL were centrifuged for 1 h at 12 0 00 x g in a microcentrifuge (Micro Star 17, VWR, Radnor, PA, USA). Fat and supernatants were discarded and another 45 mL were added to the pellets and centrifuged again. This procedure was repeated another 2 - 4 times resulting in pellets from 180 mL to 270 mL starting volumes. The pellets were resuspended in 10 mL lysis buffer (drinking milk and cheese milk) or 5 mL lysis buffer (whey) and 100 µL Proteinase K were added. DNA extractions of all samples were resumed according to manufacturer's instructions. All ring trial as well as the whey and cheese milk samples were processed with XL columns and finally eluted in 1 mL elution buffer whereas mini columns and 100 µL elution buffer were applied for all other samples. For each DNA extraction batch, an extraction blank control was processed together with the samples.

The DNA concentrations of all samples were measured fluorometrically with the DeNovix dsDNA High Sensitivity Kit (DeNovix, Wilmington, DE, USA) on a DS-11FX µL volume spectrophoto/fluorometer (DeNovix, Wilmington, DE, USA). The DNA of the cheese milks to be used as DNA standards were adjusted to 10 ng/µL whereas the DNA of the samples were tested with the real-time PCR methods in different dilutions to determine a concentration suitable for the real-time experiments with all species showing quantification cycle (Cq) values within the ranges of the standards and to rule out the activity of inhibiting substances.