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Cheese Matrix Microstructure Studied by Advanced Microscopic Techniques

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The functional properties of cheese and cheese ingredients are dictated by the structure; therefore knowledge of how cheese structure is produced and develops during ripening is of great importance to the cheese manufacturer [1]. To determine the influence varied manufacture process parameters (e.g. altered cook temperature and salting methods) have on cheese matrix structure during manufacture and ripening, we need to clarify the influence of these parameters on protein, fat and bacterial structures within the matrix both individually and as an integrated structure. A greater understanding of these influences will enable the development of treatment strategies to produce cheeses of greater consistency and also to develop novel cheese-type. Protein, fat and calcium salts (particularly Calcium phosphate but also Calcium lactate) are key to cheese matrix microstructure and also strongly influence cheese texture, sensory and quality attributes.

Cheese trials were conducted in triplicate consisting of 4 vats of 454 kg of mid lactation milk. Novel dry or brine salted cheeses were manufactured using thermophilic cultures and varying maximum cook temperatures (40 or 50 °C) as described in [2] and outlined in Table 1. Cheese fat and proteins were stained by Nile Red and Nile Blue as described in [3] and live/dead bacteria were differentiated using LIVE/DEAD BacLight viability stain according to [4].

For cryo-SEM a thin piece of cheese was quickly frozen in liquid nitrogen, moved into a vacuum chamber (ACE600, Leica Microsystems) where it was freeze-fractured and shortly sublimated at -95°C. Next the sample was moved at high vacuum using a shuttle (VCT100, Leica Microsystems) into the SEM (Magellan, FEI) equipped with a cold stage and the fractured structure was observed in 1 keV e^-beam at -140°C without any metal coating.

The confocal laser scanning microscope Leica TCS SP8 AOBS MP system equipped with wide range of visible lasers, spectral tunable and NDD detectors was used for optical data acquisition. Ar laser line 488 nm was used for one photon fluorescence excitation. The Second Harmonic Generation (SHG) acquisition based on non-centrosymmetric crystalline structure in the sample was excited by Spectra Physics InSight DeepSee femtosecond laser tunable in wide range of 680-1300 nm. The output laser wavelength of the femtosecond laser was set to 860 nm and frequency doubled signal observed through 420-440 nm bandpass filter in transmission geometry by TLD-NDD detector.

Four different cheese types were examined as described in Table 1. The cheese matrix surface at high resolution was visualized by the cryo-SEM that enables observation of the calcium phosphate / lactate salt crystals, fat globules and bacteria. The cheese matrix in 3D was acquired by confocal laser scanning microscope with labeling of the fat/protein compartments and live/dead bacteria at 3 days, 7 days and 3 weeks old. This approach enables us to compare changing structures of fat and protein compartments

and changing ratios of live/dead bacteria. Images captured by CLSM (Confocal Laser Scanning Microscopy) in fluorescence and SHG mode were utilized to create three-dimensional (3D) visualizations (see Fig. 1). The potential applications of SHG imaging and cryo-SEM were explored during our experiment. We looked both for overview and detailed images of large areas of cheese, including crystalline inclusions.

The SHG imaging data are the first of its kind obtained to show calcium phosphate/lactate salts crystals in cheese, *in situ* and combined with fat and protein distributions as shown in Fig. 1.

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	Vat 1	Vat 2	Vat 3	Vat 4
Starter Culture	S. *thermophilus* L. *helveticus*	S. *thermophilus* L. *helveticus*	S. *thermophilus* L. *helveticus*	S. *thermophilus* L. *helveticus*
Max Scald	50°C	50°C	40°C	40°C
Salting Method	Brine Salted	Dry Salted	Brine Salted	Dry Salted
Mould	Wheel	Block	Wheel	Block

Table 1.) Manufacture parameters used in various vats for trials 1-4.

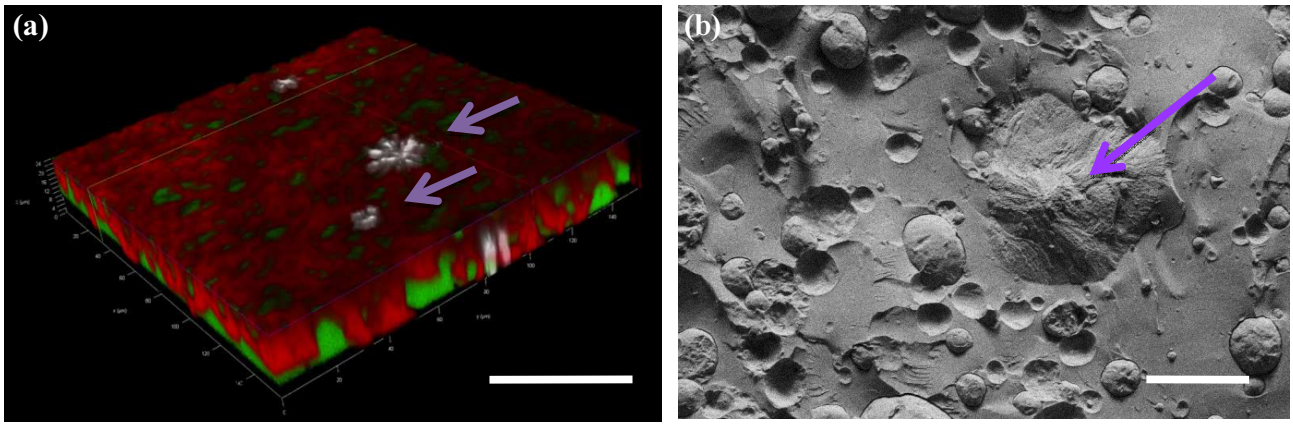

Figure 1. (a) 3D reconstruction of Z-stack acquired by CLSM – combination of one photon excitation fluorescence showing fat (green, Nile Red) and protein (red, Nile Blue) and SHG imaging (gray, calcium phosphate/lactate salt crystals) in cheese matrix, scale bar = 50 µm, **(b)** cryo-SEM image showing crystalline inclusion (arrow), scale bar = 10 µm.

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