Top-down proteomics investigation of age gelation mechanisms in genetically selected milks

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Introduction

Ultra-high temperature (UHT) treatment (135-150 °C for 2-6 s) is widely used to sterilize milk and extend its shelf-life up to 4-6 months at room temperature. The demand for UHT milk is steadily increasing every year as urbanisation continues and refrigerated storage becomes economically and environmentally unviable. UHT milk processing, whilst producing sterile products, induces major physicochemical changes to the milk components, particularly the heat sensitive whey proteins. These physiochemical changes can lead to a suite of negative sensory attributes such as browning, bitterness, particle formation and gelation that consumers reject, dictating the shelf life of UHT milk rather than shelf life restriction from microbial infection.

Age gelation is the most prominent defect affecting UHT milk and can be described as a two stage process. Firstly, during the UHT of milk, beta-lactoglobulin (bLG), the major whey protein in milk, denatures as a result of the applied heat and complexes with kappa-casein (kCN) forming a bLG-kCN complex on the surface of the casein micelles, which comprise alpha-casein (aCN), beta-casein (bCN) and kCN. The second stage results in a three dimensional gel network. The protein variants of these major milk proteins are known to strongly influence milk functionality. The most common variants in dairy cows of these three proteins are kCN A and B, bCN A1 and A2, and bLG A and B with each containing a slightly altered amino acid sequence and protein functionality.

We set out to explore the relationship between the commonly found protein variants of bCN, kCN and bLG and their influence on the age gelation in UHT milk over 9 months to ascertain whether there is an optimal genetic profile to produce a more stable UHT milk and to probe the mechanism of age gelation.

Materials and Methods

12 milk samples with defined combinations of the genetic variants of k- and bCN and bLG were obtained from the DEDJTR research herd maintained at Ellinbank (VIC).

Viscosity was measured using a Paar-Physica 302 fitted with a cup and bob geometry.

Microbes were isolated based on Murphy *et al*. (2002). Variable region 4 from the prokaryote 16S rRNA gene was amplified by PCR. Illumina MiSeq system was used to generate DNA sequence data from the PCR fragments.

Group	kCN	bCN	bLG
G01	AA	A1A1	AB
G02	AA	A1A1	BB
G03	AA	A1A2	AB
G04	AA	A1A2	BB
G05	AA	A2A2	AB
G06	AA	A2A2	BB
G07	AB	A1A1	AB
G08	AB	A1A1	BB
G09	AB	A1A2	AB
G10	AB	A1A2	BB
G11	AB	A2A2	AB
G12	AB	A2A2	BB

Table 1: genetic variants of the 12 milk groups.

For intact protein LC-MS analyses, milk samples were processed as described in Vincent *et al*. (2016). MS/MS experiments were performed on the Bruker maXis Qq-ToF (60,000 resolution) using CID fragmentation mode.

Results

Milk viscosity in groups 9 and 11 increases from 6 months onwards (Fig 1A). They are the only groups displaying age gelation. These groups also show the least microbial diversity (Fig. 1B).

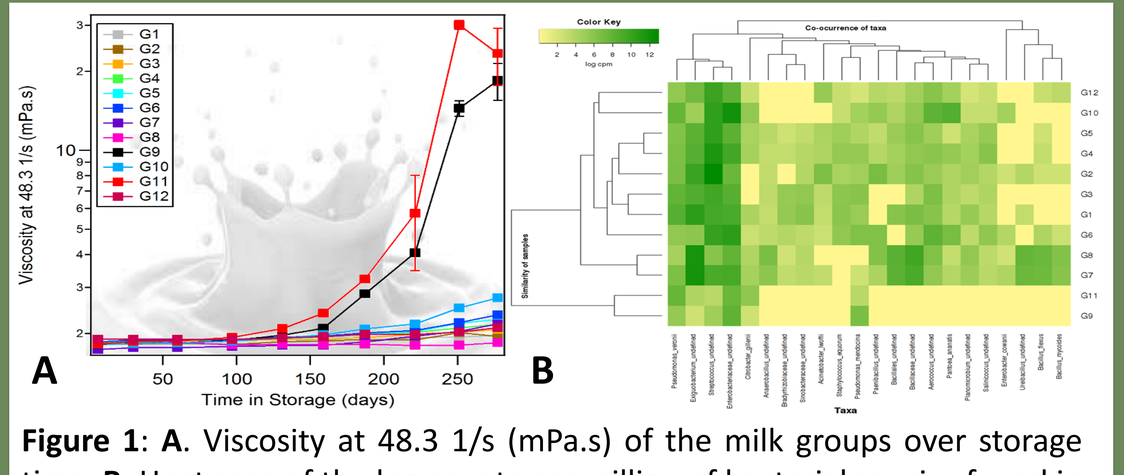

Figure 1: **A**. Viscosity at 48.3 1/s (mPa.s) of the milk groups over storage time. **B**. Heat map of the log counts per million of bacterial species found in the milk samples before UHT treatment.

Top-down proteomics analysis highlights 3 general trends (Fig. 2 & 3):

- Decreases in the abundance of all of the native intact milk proteins.
- Accumulation of peptides arising from intact proteins.
- Accumulation of the lactose-modified proteoforms post-UHT treatment.

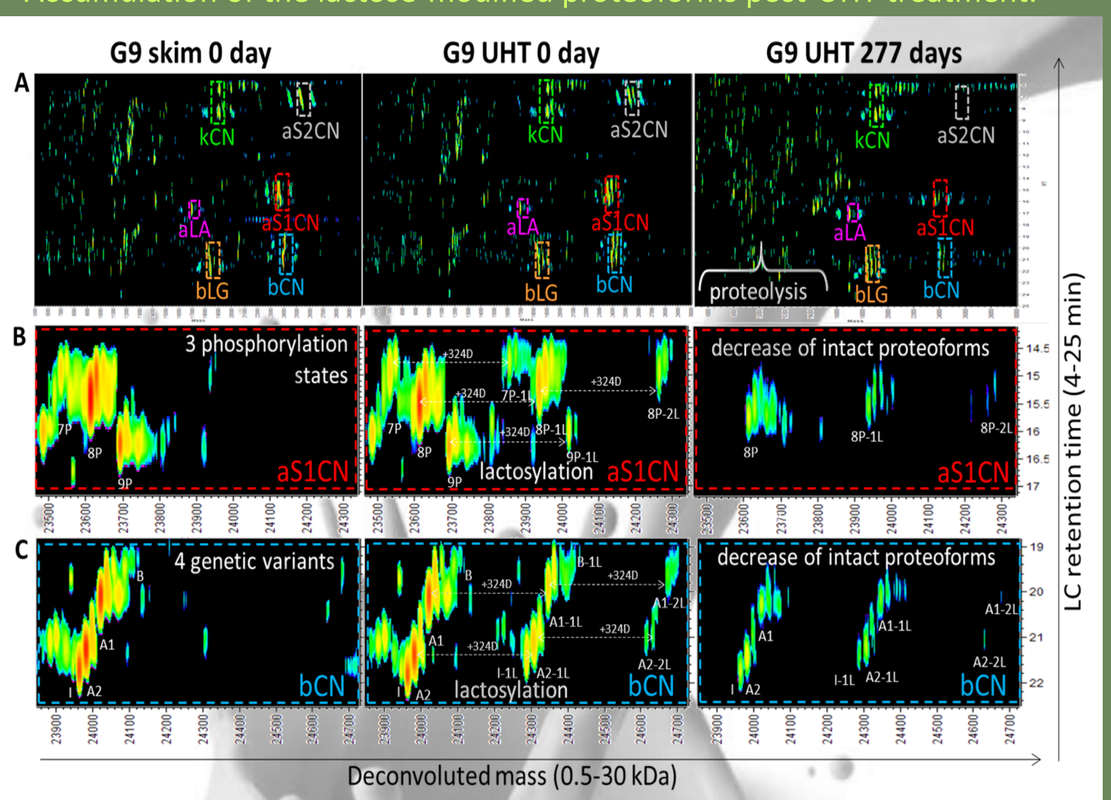

Figure 2: Proteomic 2-D map of compounds contained in Group 09 (G9) according to their retention time and accurate deconvoluted mass displayed in Genedata Expressionist. **A**. Full scale. **B**. Zoom in on aS1CN proteoforms. **C**. Zoom in on bCN proteoforms.

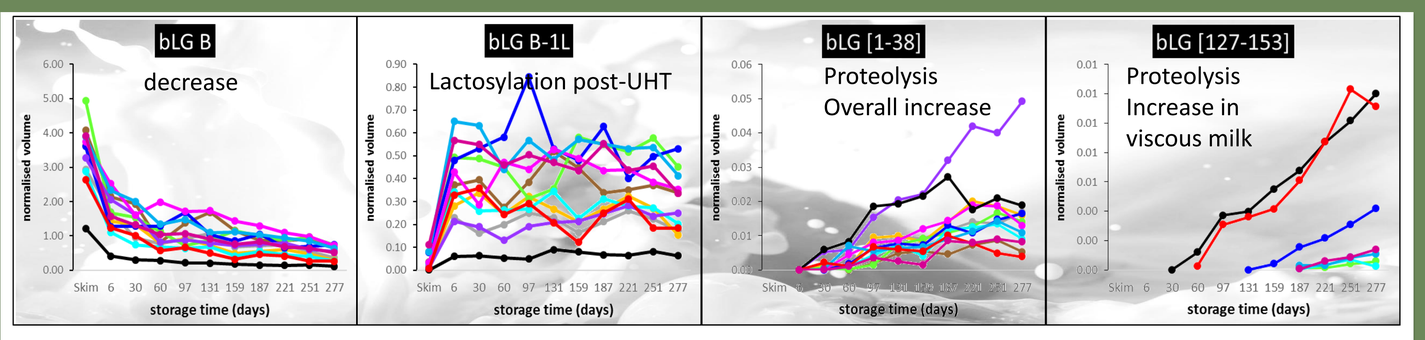

Figure 3: Accumulation patterns of bLG variant B over storage time.

A total of 58 intact proteoforms and 151 peptides including PTMs were annotated (Fig. 4).

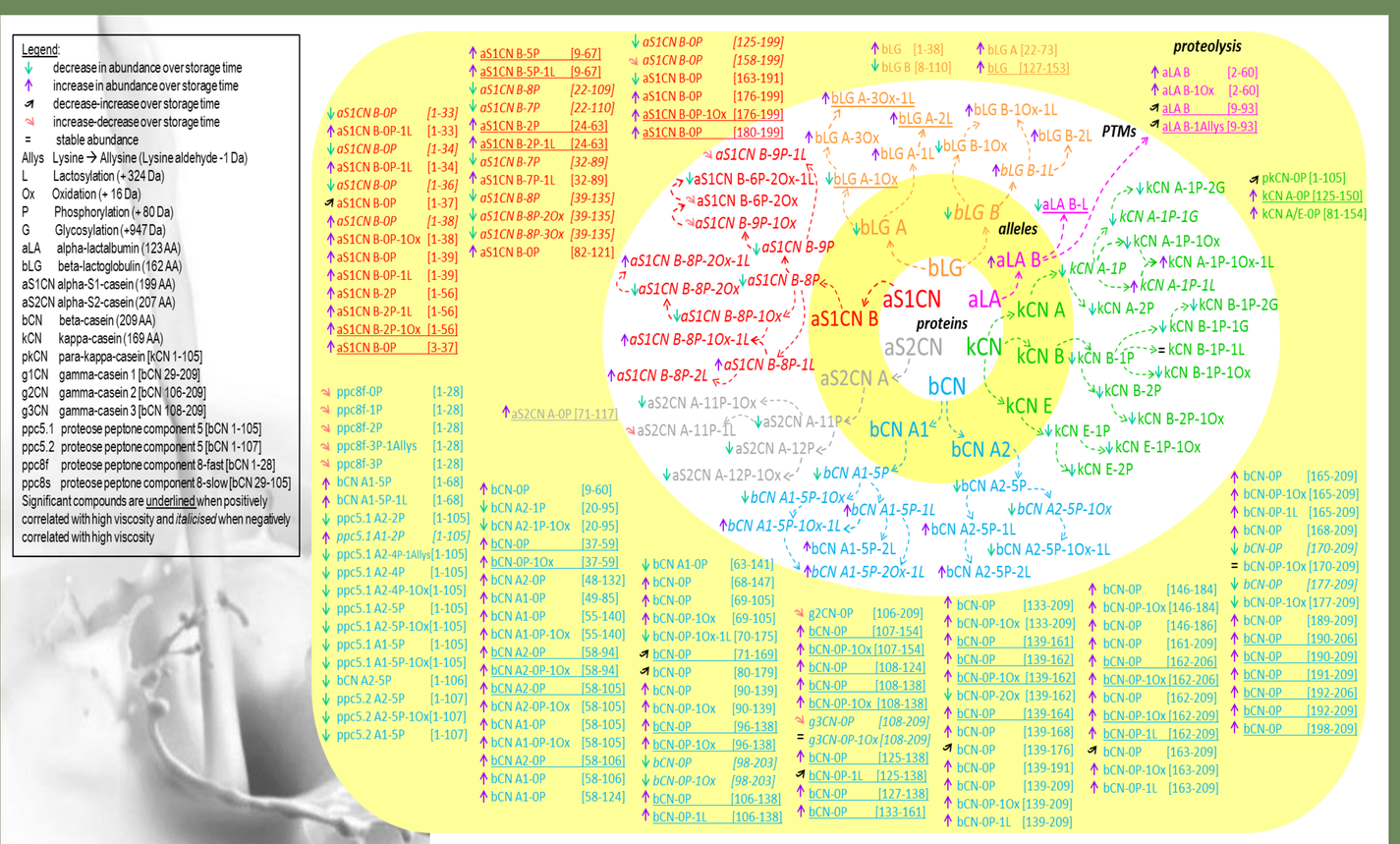

Figure 4: Overview of proteins and peptides found in all the milk groups by LC-MS over storage time. The different proteins are in the innermost circle, their allelic variants in the 2nd ring, their PTMs in the 3rd ring, and their proteolysed products around the outside.

Conclusions

Proteolysis is a leading mechanism for the instability of stored UHT milk. Peptides that were associated with viscous milk were predominantly from bCN. Most have previously been shown to be cleavage sites from ArpX protease, implying that heat stable bacterial proteases could be preventing age gelation by inhibiting ArpX. Where such bacteria are absent, ArpX is active.

Citations:

-Murphy, M. A., Shariflou, M. R., & Moran, C. (2002). High quality genomic DNA extraction from large milk samples. The Journal of Dairy Research, 69(4), 645–9.

-Vincent, D., Elkins, A., Condina, M. R., Ezernieks, V., & Rochfort, S. (2016). Quantitation and Identification of Intact Major Milk Proteins for High-Throughput LC-ESI-Q-TOF MS Analyses. PLOS ONE, 11(10), e0163471.

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