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# Processing affects beta-casomorphin peptide formation during simulated gastrointestinal digestion in both A1 and A2 milk

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ABSTRACT

Variation between bovine A1-or A2-type β-caseins are suggested to affect beta-casomorphin-7 (BCM-7) formation, which may affect health. Studies assessing BCM-7 formation often use raw milk, but processing of milk is known to affect digestion. Using in vitro digestion and stable-isotope assisted peptide quantification, we reveal that BCM-7 is formed under intestinal conditions from both A1-and A2-type milk. Moreover, formation of BCM-7 was affected by industrial relevant heating in both A1 and A2 milk. Further studies with a large number of single A1-and A2-type cows are needed to elaborate if amounts of BCM-7 formed are different between A1 and A2 milk. Both longer and shorter BCM peptide sequences that may display similar activities should be taking into account as a longer BCM-7 containing peptide (BCM-7 +2) could also be detected in A2 milk intestinal digesta.


## 1. Introduction

Different genetic variants of β-casein in bovine milk have been postulated to affect health outcomes differentially (Bell, Grochoski, & Clarke, 2006; Brooke-Taylor, Dwyer, Woodford, & Kost, 2017; Küllenberg de Gaudry et al., 2019). In this respect, a single amino acid variation at position 67 of the bovine β-casein protein sequence distinguishes the A1 ($His^{67}$) or A2 ($Pro^{67}$) variants of β-casein (Pal, Woodford, Kukuljan, & Ho, 2015) although, in fact, nomenclature and amino acid difference for bovine β-casein variants are more elaborate (Farrell et al., 2004). A total of 12 different bovine β-casein variants have been described (Farrell et al., 2004). The single amino acid difference at β-casein position 67 is thought to affect the formation of the adjacent bioactive sequence beta-casomorphin-7 (BCM-7; YPFPGPI) during gastrointestinal digestion. Since the 1970s, an opioid-like activity has been assigned to milk, particularly to a casein digest fraction (Teschemacher, Brantl, & Haarmann, 1978). Both in vitro and in vivo studies localised this activity to the N-terminal region of β-casein containing the BCM-7 sequence from which several bioactive sequences with different activities can be formed (Teschemacher et al., 1978; Thiruvengadam, Venkidasamy, Thirupathi, Chung, & Subramanian, 2021).

Several studies with diverse outcomes have investigated the formation of BCM-7 from β-casein A1 and A2 in milk (Table 1). Milk from single cows of multiple breeds, bulk milk and model products have been tested using different digestion procedures and analytical methods to detect BCM peptide formation (Asledottir et al., 2017, 2018; Cieślińska, Kaminski, Kostyyra, & Sienkiewicz-Szłapka, 2007; Cieślińska et al., 2012; Duarte-Vázquez, García-Ugalde, Villegas-Gutiérrez, García-Almendárez, & Rosado, 2017; Jinsmaa & Yoshikawa, 1999; Nguyen, Busetti, Smolenski, Johnson, & Solah, 2021; De Noni, 2008; Ul Haq, Kapila, & Kapila, 2015). However, considering today's standard for simulating gastrointestinal digestion and analytical methods, physiological relevance varies among the studies listed in Table 1. In addition, most of these studies were performed with isolated casein fractions. However, considering conclusions for milk as a whole or products thereof, protein purifications before digestion should ideally be limited as separation of milk protein fractions will affect outcomes as, e.g., endogenous casein peptides (of which some have been reported to contain the BCM sequence (Guerrero et al., 2015)) are lost in the whey fraction when separating intact casein and whey proteins. Moreover, the majority of these studies have assessed raw milk or (β-)casein isolated from raw milk, whereas practically all milk consumed is processed which may affect outcomes as a result of protein denaturation and other processing-induced changes.

As virtually all of the discussed studies employed different digestion and analytical procedures this study was performed

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adapting the international standard for in vitro digestion (INFOGEST) and sensitive stable-isotope assisted quantification of BCM peptide sequences during the digestion of A1 and A2 milk samples from 6 A1-type and 6 A2-type Holstein Friesian cows from a single herd. Moreover, to further elucidate the effect of milk processing on the formation of a range of BCM peptides, A1 and A2 milk was processed adapting industrial relevant processing procedures at pilot scale and BCM peptide formation was compared with raw milk. In line with previous findings of milk processing and effects on digestion (e.g., reviewed in van Lieshout, Lambers, Bragt, & Hettinga, 2020), we hypothesised that heating may affect digestion and peptide formation from β-casein in A1-and A2-type milk.

## 2. Materials and methods

### 2.1. Milk collection and processing

Milk samples were collected from a local dairy farm where all cows were previously genotyped. In short, for assigning β-casein variants, relevant SNP according to Caroli, Chessa, and Erhardt (2009) were genotyped with the EuroGenomics genotyping beadchip, using the Infinium assay technology (Illumina Inc., San Diego, CA, USA). Six A1-type cows and 6 A2-type cows were selected for milk collection by evaluation of β-casein amino acid position 67. Milk was collected in the morning and equal volumes of the A1-type (n = 6) or A2-type (n = 6) milk from each cow were mixed to form representative A1 and A2 milk samples, respectively. Milk was transported cooled to the lab for processing (Fig. 1). Skimming was performed by centrifugation (4000× *g* for 30 min at 5 °C) and subsequent filtration through glass wool. Homogenisation (200/30 bar at 50 °C) was performed on a GEA NIRO Soavi type NS1001LK2 homogeniser. Heat treatment at either 85 °C for 30 s or 140 °C for 5 s was done by continuous flow indirect heating. Accordingly, heated full fat (homogenised and unhomogenised) and heated skimmed milk samples were produced of both A1 and A2 milk. All samples, including a full fat and skimmed raw milk control, were stored at 4 °C until the next day when samples were subjected to in vitro digestion.

### 2.2. Milk protein characterisation

Milk protein composition was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection at 220 nm as described previously (Visser, Slangen, & Rollema, 1991). Degree of whey protein denaturation was determined on the same setup and was based on calculation of total and pH 4.6 soluble α-lactalbumin and β-lactoglobulin (Gazi & Huppertz, 2015). Data analyses were done with Chromeleon software (ThermoFisher Scientific, Waltham, MA, USA).

Genetic variants of β-casein were quantified using an Agilent 6530 LC-QTOF system (Agilent, Santa Clara, CA, USA) where samples were injected on a wide-pore C18 analytical column maintained at 40 °C. Proteins were eluted with a gradient of trifluoroacetic acid, water and acetonitrile. Data analyses was done with the Mass Hunter software (Agilent). As a control a representative milk sample of the average Dutch bovine milk was used that was a weekly pooled milk sample collected from fourteen Dutch dairy factories by the Dutch Milk Control Institute (QLIP, Zutphen, the Netherlands).

### 2.3. In vitro digestion

Raw and processed milk samples were subjected to simulated gastric and intestinal digestion adopting the international consensus semi-dynamic INFOGEST model as described previously (Mulet-Cabero et al., 2020). In short, all samples were brought to pH 6.0 and 37 °C before addition of simulated gastric fluid (1:1 ratio) containing pepsin from porcine gastric mucosa (Sigma−Aldrich, St. Louis, MO, USA). Subsequently acidification towards pH 2.0 within 120 min was initiated by following a predefined acidification curve. Upon completion of simulated gastric digestion, simulated intestinal fluid was added (1:1 ratio) containing pancreatin from porcine pancreas and bovine bile acids (both from Sigma−Aldrich) and pH was set at 7.0 and incubated for another 120 min. Samples for peptide quantification were taken upon completion of the gastric phase by applying Pepstatin A (Sigma−Aldrich) to stop the enzymatic reactions and after completing simulated intestinal digestion by applying Pefabloc (Sigma−Aldrich) to stop the enzymatic reactions.

### 2.4. Stable-isotope assisted peptide quantification

Stable isotope labelled peptides including BCM-7, BMC-5 and longer peptides from the BCM-sequences from β-casein A1 and A2 were obtained from ThermoFischer (Table 2). Peptides and samples were diluted in 5% (v/v) acetonitrile with 1 mL $L^{-1}$ formic acid and cleaned using C18 micro columns as described previously (Lu et al., 2011). A 6-point calibration curve of all labelled peptides in both a gastric and intestinal digest samples was prepared to determine linearity. Based on this calibration and the signal of the endogenous peptides, a single concentration of all labelled sequences was selected and used for quantification in all samples. Samples were loaded directly onto a 0.10 × 250 mm ReproSil-Pur 120 C18-AQ 1.9 μm beads analytical column (prepared in-house) with a maximum pressure of 825 bar, generally giving a flow rate of 700 nL $min^{-1}$ using formic acid in water. Peptides were eluted at a flow of 0.5 μL $min^{-1}$ with a linear gradient from 9% to 34% acetonitrile with 1 mL $L^{-1}$ formic acid using a Thermo EASY nanoLC 1000 (Thermo Fisher Scientific). An electrospray potential of 3.5 kV was applied directly to the eluent via a stainless-steel needle fitted into the waste line of the micro cross that was connected between the nano-LC and the analytical column. A nano-Blow was installed to limit contaminants as described previously (Humphrey, Crossett, & Parker, 2019). Full scan positive mode FTMS spectra were measured between *m/z* 380 and 1400 on a Q-Exactive HFX (Thermo electron, San Jose, CA, USA) in the Orbitrap at resolution 60,000. MS and MSMS AGC targets were set to $3 \times 10^6$ or 50,000, respectively, or maximum ion injection times of 50 ms (MS) and 25 ms (MSMS) were used. HCD fragmented (Isolation width 1.2 *m/z*, 24% normalised collision energy) MSMS scans (resolution of 15,000) of the 25 most abundant 1−5+ charged peaks in the MS scan were recorded in data dependent mode (Threshold 1.2e5, 15 s exclusion duration for the selected *m/z* ± 10 ppm). QualBrowser of Thermo Xcalibur 4.1.50 was used to create chromatograms (3 points boxcar smoothing enabled) and determine peak areas of calculated *m/z* values of light and heavy peptides with a mass tolerance of 10 ppm.

## 3. Results and discussion

### 3.1. Milk protein characterisation

Milk from 6 previously genotyped β-casein A1-type and from 6 β-casein A2-type Holstein-Friesian cows collected from a single herd was blended together to form representative A1-type and A2-type milk samples, respectively, and to control for possible cow-to-cow difference as observed in previous studies assessing the formation of BCM-7 from single cows (Cieślińska et al., 2007; Nguyen et al., 2021). Milk protein composition was determined to quantify individual milk proteins (Supplementary material Table S1).

**Table 1**
Overview of previous β-casein A1 and A2 digestion studies.[a]

| Milk source | Milk processing | Digestion procedure | Analytical method | Outcome | Ref. |
|---|---|---|---|---|---|
| Single Holstein (unspecified genotype), isolated β-CN, synthesised peptides from the BCM region | No | Combinations of pepsin, pancreatin, trypsin, chymotrypsin, elastase & leucine amino peptidase | RP-HPLC | BCM-7 could only be formed when a His was present at position 67 | Jinsmaa and Yoshikawa (1999) |
| Homozygous A1/A1 (n = 5) and A2/A2 (n = 5) Polish Holstein Friesian, isolated β-CN | No | Pepsin | RP-HPLC | BCM-7 could be detected in both A1/A1 and A2/A2 although levels in A1 were ~4-fold higher | Cieślińska et al. (2007) |
| Bulk milk & homozygous A1/A1 (n = 3), A2/A2 (n = 3), B/B (n = 3) from Jersey and Holstein, isolated β-CN | No | Pepsin followed by further hydrolyses with Corolase PP™ | LC-MS/MS | BCM-7 could be detected in bulk and A1/A1 and B/B variants only, BCM-5 could not be detected | De Noni (2008) |
| Holstein Friesian, A1/A1 (n = 3), A1/A2 (n = 3) and A2/A2 milk (n = 3) | No | Pepsin followed by trypsin, followed by elastase | BCM-7 ELISA | ELISA signal could be detected in all samples, highest signal in A1/A1 milk | Cieślińska et al. (2012) |
| Single Karan Fries (n = 1), A1/A1, A1/A2 and A2/A2, isolated β-CN; synthesised peptide from BCM-7 region | No | Combinations of pepsin followed by trypsin and chymotrypsin or pepsin followed by pancreatin | HPLC<br>LC-MS/MS<br>ELISA | BCM-7 formation did not occur from A2/A2 | Ul Haq et al. (2015) |
| Milk and model infant formula of A2/A2 jersey from a single herd or non-selected Friesian Holstein, whole casein isolates | Model formula; Yes Milk; No | Pepsin followed by trypsin, chymotrypsin, elastase and carboxypeptidase | LC-MS/MS | BCM-7 could be detected in all infant formula casein digests although the amount in A2 formula was 2–3 fold lower.<br>BCM-7 was about 3 fold lower in raw milk casein digests from A2/A2 | Duarte-Vázquez et al. (2017) |
| Single homozygous (n = 1) A1, A2 & I Danish Holstein, isolated β-CN | No | INFOGEST recommendations for gastric and intestinal digestion but using human gastrointestinal aspirates | LC-MS/MS | BCM-7 was detected in all intestinal digesta although somewhat higher levels in A2 | Asledottir et al. (2017) |
| Single homozygous A1, A2 & I Danish Holstein and a homozygous F native cow breed | Skimming only | INFOGEST recommendations for gastric and intestinal digestion but using human gastrointestinal aspirates | LC-MS/MS | BCM-7 was detected in all intestinal digesta although somewhat higher levels in A2 and F | Asledottir et al. (2018) |
| Single A1/A1 (n = 3), A1/I (n = 2) and A2/A2 (n = 3) Holstein Friesian or Holstein Friesian-Lai Zebu | Skimming, heat treatment | Pepsin followed by pancreatin | LC-MS/MS (stable isotope assisted) | BCM-7 could be detected in A1/A1 (2 out of 3 cows), A1/I (1/2) but not in A2/A2, Heating 121 °C for 12 min increased formation of BCM-7 | Nguyen et al. (2021) |

[a] The majority of the studies employed raw milk or (β-)casein isolated from raw milk; in addition, digestive and analytical procedures varied among the studies.

Although small differences were observed, predominantly related to the concentration of κ-casein, concentrations of all major milk proteins were comparable between the A1 and A2 milk. Although we cannot exclude that differences in κ-casein levels will affect the digestion of β-casein, this is unlikely to be a major factor because this interaction has not been demonstrated in previous digestion studies and differences are only small. In line with expectations, analyses of whey protein denaturation (Supplementary material Table S1) showed that heating increased whey protein denaturation, predominantly β-lactoglobulin, in both full fat and skimmed milk. Important for the interpretation of the digestive results, whey protein denaturation in A1 and A2 milk was comparable.

The Holstein-Friesian dominant β-casein variants A1, A2, B and I were quantified by LC-MS (data not shown) to confirm levels of A1 and A2-type β-caseins at the protein level in our mixed milk samples. A representative Dutch bulk milk sample was analysed as control of which LC-MS results were in agreement with previous genetic data that demonstrate a characteristic distribution of 70% for A2-type and 30% A1-type β-casein variants in Dutch milk (Heck et al., 2009; Visker et al., 2011). The combined A1 milk sample

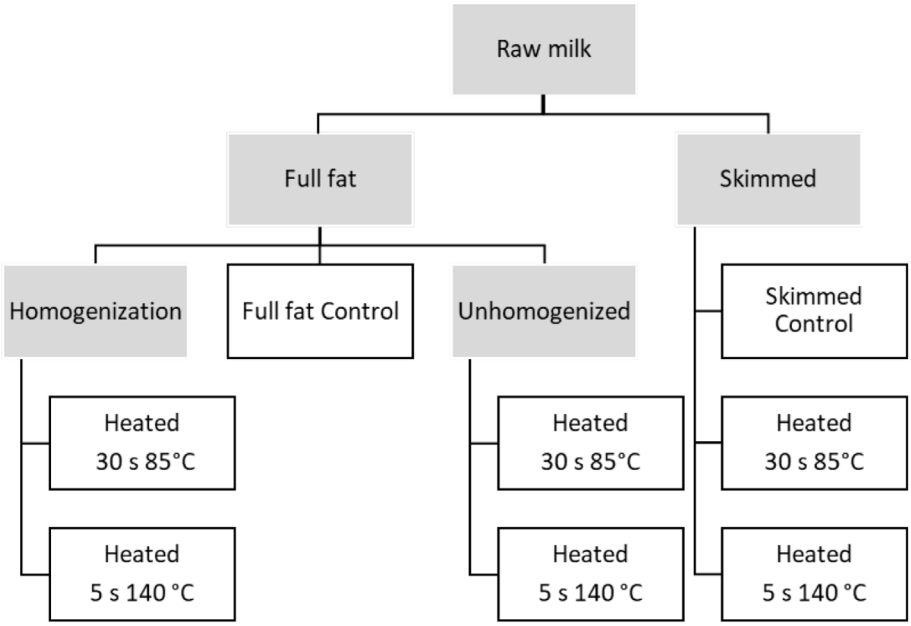


**Fig. 1.** Overview of processing variants used in this study.

**Table 2**
Peptides selected for quantification.[a]

| Sequence | Stable isotope-labelled peptide | Name | A1 or A2 |
|---|---|---|---|
| YPFPGPI | YPFPGP[I] (+7 Da) | BCM-7 | Both |
| YPFPGP | YP[F]PGP (+10 Da) | BCM-6 | Both |
| YPFPG | YP[F]PG (+10 Da) | BCM-5 | Both |
| YPFPGPIP | YPFPGP[I]P (+7 Da) | BCM-7 + 1 | A2 |
| YPFPGPIH | YPFPGP[I]H (+7 Da) | BCM-7 + 1 | A1 |
| YPFPGPIPN | YPFPGP[I]PN (+7 Da) | BCM-7 + 2 | A2 |
| YPFPGPIHN | YPFPGP[I]HN (+7 Da) | BCM-7 + 2 | A1 |

[a] Selected BCM-7 and BCM-7-related sequences; stable isotope amino acids indicated added either 7 or 10 Da to total peptide mass.

contained a small contamination of A2-type β-casein although A1-type β-caseins were still highly dominant (>90% of total β-casein). LC-MS analyses further revealed that the combined A2 milk sample contained exclusively A2-type β-caseins, which allowed us to further investigate formation of BCM-7 in A2 milk and assess the effect of processing thereon.

### 3.2. Formation of BCM peptides during simulated gastrointestinal digestion

Processed milk samples were subjected to in vitro digestion and BCM peptides were quantified by LC-MS/MS using stable-isotope assisted peptide quantification. Besides the previously quantified BCM-5 and BCM-7, C-terminal BCM-7 +1 and + 2 peptides were also selected for quantification (Table 2), as in previous peptidomic analyses of β-casein digests the A2 derived sequence YPFPGPIPN (BCM-7 +2) was identified (Asledottir et al., 2017). In addition, another truncated form of BCM-7 (BCM-6) was selected. Importantly, depending on the exact functional readout, both shorter and longer forms of BCM-7 have previously been

**Table 3**
Concentrations of BCM7 +2 (YPFPGPIPN) and BCM7 (YPFPGPI) present after in vitro gastro-intestinal digestion.[a]

| Milk | Fat | Homogenisation | Heating | YPFPGPIPN | | YPFPGPI | |
|---|---|---|---|---|---|---|---|
| | | | | μM | mg $g^{-1}$ β-casein | μM | mg $g^{-1}$ β-casein |
| A1 | Full fat | No | No | 0.10 | 0.04 | 2.69 | 0.83 |
| | Full fat | No | 30 s, 85 °C | 0.10 | 0.04 | 1.86 | 0.57 |
| | Full fat | No | 5 s, 140 °C | 0.07 | 0.03 | 1.71 | 0.51 |
| | Full fat | Yes | 30 s, 85 °C | 0.07 | 0.03 | 1.98 | 0.61 |
| | Full fat | Yes | 5 s, 140 °C | 0.09 | 0.03 | 1.73 | 0.53 |
| | Skimmed | No | No | 0.15 | 0.06 | 2.92 | 0.87 |
| | Skimmed | No | 30 s, 85 °C | 0.12 | 0.04 | 1.94 | 0.57 |
| | Skimmed | No | 5 s, 140 °C | 0.07 | 0.03 | 1.54 | 0.44 |
| A2 | Full fat | No | No | 0.61 | 0.24 | 1.72 | 0.54 |
| | Full fat | No | 30 s, 85 °C | 0.53 | 0.21 | 1.57 | 0.50 |
| | Full fat | No | 5 s, 140 °C | 0.43 | 0.17 | 1.31 | 0.42 |
| | Full fat | Yes | 30 s, 85 °C | 0.45 | 0.18 | 1.38 | 0.43 |
| | Full fat | Yes | 5 s, 140 °C | 0.38 | 0.15 | 1.46 | 0.45 |
| | Skimmed | No | No | 0.69 | 0.27 | 1.87 | 0.58 |
| | Skimmed | No | 30 s, 85 °C | 0.65 | 0.25 | 1.32 | 0.41 |
| | Skimmed | No | 5 s, 140 °C | 0.42 | 0.16 | 1.02 | 0.31 |

[a] Processed milk samples were subjected to in vitro digestion and peptides from the BCM-region were quantified at the end of the simulated intestinal digestion step by LC-MS/MS using stable-isotope assisted peptide quantification. Based on results from the milk protein quantification and taking into account the dilution during simulated gastrointestinal digestion, the amount of peptide per gram of casein was calculated.

demonstrated to display similar activities as BCM-7 (Eisele, Stressler, Kranz, & Fischer, 2013; Kondrashina, Brodkorb, & Giblin, 2020; Meisel, 1986; Meisel, Frister, & Schlimme, 1989; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). These also include shorter sequences (e.g., YPFP), which are often neglected during assessment by untargeted LC-MS because peptides smaller than 5 amino acids are typically not identified in automated LC-MS identifications. Thus, from the perspective of possible BCM-driven differences among A1 and A2 milk, both longer and shorter peptide sequences from the BCM-7 region should be taken into account.

In the gastric digests, none of the selected BCM sequences could be identified, regardless of milk type and processing conditions applied (data not shown). This is in contrast to previous studies reporting the formation of BCM-5 and BCM-7 in pepsin digests of purified β-casein variants and milk from homozygous A1 and A2 cows (Cieślińska et al., 2007, 2012), but in line with other, more recent, studies adopting a similar design and more physiological relevant digestive conditions (Asledottir et al., 2017; De Noni, 2008). Thus, most likely these discrepancies are a result of different digestive conditions and analytical procedures to detect BCM-5 and BCM-7.

In the intestinal digests of both A1-type and A2-type milk, 2 of the selected peptides were detected including BCM-7 and the BCM-7 + 2 (YPFPGPIPN) form of the A2 variant (Table 3). In line with the milk LC-MS data demonstrating the presence of a A2-type β-casein contamination in the combined A1 milk, a small amount of the BCM-7 + 2 sequence was found in A1 milk as well. Taking into account the quantified amounts of the peptides, the total amount of β-casein in the milk (Supplementary material Table S1) and the dilution within the digestion model, the percentage of peptide per amount of casein was calculated for each individual sample (Table 3). In all A1 and A2 milk samples, the amount of BCM-7 formed was only a fraction of the total amount of that could be formed from the β-casein present, although the amount of BCM-7 appeared to be slightly higher in the A1 samples compared with the A2 samples, but differences did not exceed a factor of 1.5 when comparing identically processed or control milk samples. The amounts of BCM-7 detected in the intestinal digestions, as well as the slightly higher levels from A1 milk, are in line with previous observations (Asledottir et al., 2017, 2018), although the differences between A1 and A2 BCM-7 formation appeared to be somewhat smaller in the current study, employing a milk sample from multiple cows. A larger data set of multiple single homozygous cows (preferably from a single herd to accommodate potential effects of farm management/feed) may be required to further quantitatively elucidate possible differences in the amount of BCM-7 formed in A1 and A2 milk.

Heat treatment of the milk at 85 °C for 30 s or 140 °C for 5 s decreased the formation of BCM-7 in both full-fat (both unhomogenised and homogenised) and skimmed milk samples both in A1 and A2 milk (Table 3). These effects of heat load are somewhat different than observed previously in a study where the authors concluded that relative to pasteurisation temperatures, UHT treatment increased the formation of BCM-7 in A1/A1 milk although this effect was not observed in A2/I milk, and BCM-7 formation in raw milk was not analysed (Nguyen et al., 2021). Overall, in this study processing thus appears to hinder the formation of BCM-7 independent of the genetic origin of the milk. Most likely this is the result of heating induced protein denaturation which has previously been demonstrated to affect the digestion kinetics of milk proteins (e.g., reviewed in van Lieshout et al., 2020). However, it is currently unclear if the effects of reduced BCM-7 formation in this study are caused by a reduced hydrolyses of β-casein or a result of increased hydrolyses of β-casein and the BCM-7 region into smaller fragments.

Although, relative to BCM-7, about 2-fold lower, the A2-variant of BCM-7 +2 was also detected in intestinal digest samples of A2 milk in line with previous studies (Asledottir et al., 2017). As discussed, in functional assays longer BCM-sequences (including BCM-7 +2) were identified to display similar activities as BCM-7. Interestingly, when taking the identified amount of this sequence into account differences between A1 and A2 milk get even smaller. This further supports that, from the perspective of possible functional BCM-driven differences between A1 and A2 milk, both longer and shorter peptide sequences of the β-casein BCM region should be taken into account. In this respect, also the whey fraction of milk contains β-casein fragments in the form of proteose peptones which are formed as a result of the activity of the endogenous milk protease plasmin on β-casein (Bastian & Brown, 1996; Deeth & Bansal, 2018). In addition, elaborate milk peptidomics has revealed the presence of milk endogenous β-casein derived peptides containing the BCM-7 sequence (Guerrero et al., 2015). Thus, from a food manufacturing perspective it should be noted that whey protein ingredients used for manufacturing of food products are a potential source for BCM-7 containing β-casein fragments.

## 4. Conclusion

Simulated in vitro digestion of milk and direct stable isotope-label assisted peptide quantification reveals that BCM-7 can be formed from both A1 and A2 milk. Milk processing at pilot scale revealed that the formation of BCM-7 is inhibited by typical pasteurisation and UHT-treatments, with and without homogenisation, in both A1 and A2 milk in a similar fashion. Further studies with a large range of single homozygous A1-and A2-type cows are needed to elaborate if absolute amounts of BCM-7 formed are different between A1 and A2 milk. From a functional perspective both longer and shorter BCM sequences should be taking into account as a longer BCM-7 containing peptide (BCM-7 +2) could also be detected in A2 milk.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2021.105099.

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