J. Dairy Sci. 107:6425–6436
https://doi.org/10.3168/jds.2024-24403

The A1/A2 β-casein genotype of cows, but not their horn status, influences peptide generation during simulated digestion of milk

A.-M. Reiche,[1,*†] M.C. Martín-Hernández,[2†] A. Spengler Neff,[3] B. Bapst,[4] C. Fleuti,[2] F. Dohme-Meier,[1] H.D. Hess,[1‡] L. Egger,[2§] and R. Portmann[2§]
[1]Ruminant Nutrition and Emissions, Agroscope, 1725 Posieux, Switzerland
[2]Method Development and Analytics/Biochemistry of Milk and Microorganisms, Agroscope, 3097 Liebefeld, Switzerland
[3]Department of Livestock Sciences, Research Institute of Organic Agriculture (FiBL), Ackerstrasse 113, CH-5070 Frick, Switzerland
[4]Qualitas AG, 6300 Zug, Switzerland

ABSTRACT

The effect of the horn status of cows on their milk composition and quality is a controversial research topic. In this study, 128 milk samples from 64 horned and 64 disbudded Brown Swiss and Original Braunvieh cows were collected from alpine farms where both horned and disbudded cows were grazing on mountain pastures. The samples were analyzed for their detailed composition and protein digestion in a simulated in vitro digestion (INFOGEST). To exclude probable influences on digestion, the β-CN genotype with its variants A1 and A2 was also included in the study. The effects of horn status and β-CN genotype were investigated in linear mixed models, which included additional influencing random factors such as Original Braunvieh blood proportion, stage of lactation, and farm. Horn status did not have any effect on milk composition or digestion. In contrast, milk from A1A1 cows showed a different protein digestion than milk of A1A2 and A2A2 cows in the gastric phase, including smaller amounts of β-casomorphin(BCM)21-associated peptides and larger amounts of BCM11-associated peptides. Abundances of BCM7 did not differ between β-CN genotypes. At the end of the intestinal phase, the digested milk of A1A1 and A2A2 β-CN genotypes did not differ.
Key words: beta-casomorphin, horns, in vitro digestion, milk quality and composition

INTRODUCTION

The objective of the present study was to investigate the effects of horn status on milk composition and digestion while considering the β-CN genotype, which has been shown to influence milk digestibility. Disbudding, the removal of the horn buds from young calves, is a routine practice in dairy farming for both economical and safety reasons. In the United States, Europe, and Switzerland, 94%, 61%, and 73% of dairy cows are hornless, respectively, mostly due to disbudding (USDA, 2010; KAGfreiland, 2014; Cozzi et al., 2015). In the short term, disbudding causes acute and chronic pain in calves (Mirra et al., 2018; Casoni et al., 2019); in the long term, horn status affects behavior, stress physiology, and meat quality, including the muscle proteome (Knierim et al., 2015; Reiche et al., 2019, 2020a,b). Very little is known about the potential long-term effects of disbudding on milk yield, composition, and digestion. At low-ambient temperatures (minimum temperatures ranging from −6°C to +2°C; Baars et al., 2019) and when comparing pooled milk samples from cows with versus without horns (Wohlers, 2011), horn status did not alter the main composition of milk (lactose, protein, fat, urea, cell count) but did alter its concentrations of a few individual proteins (for example, β-LG), free AA (for example, proline), and fatty acids. Based on the horn's possible role in thermoregulation, it was hypothesized (Baars et al., 2019) that the differences found would reflect a greater cold stress of horned cows due to heat loss via the horns. However, the role of the horn in thermoregulation was not confirmed in other works (Kiltie, 1985; Bro-Jørgensen, 2007; Wohlers and Stolz, 2022; Reiche et al., 2023). Taken together and with several limitations (small sample sizes, pooled samples and confounding factors, lack of evidence), these studies do not provide robust evidence for horn status-related effects on milk. However, it is frequently argued, especially in biodynamic circles,

Received November 7, 2023.
Accepted February 12, 2024.
*Corresponding author: anna-maria.reiche@agroscope.admin.ch
†These authors contributed equally to this work.
‡Current address: Canton of Luzern, Department of Building, Environment and Economy, Office of Agriculture and Forest, 6210 Sursee, Switzerland.
§These authors contributed equally to this work.

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

that milk of horned cows might be better digestible and healthier than that of disbudded cows.

The digestion of milk and milk proteins is strongly influenced by genetic factors. Natural genetic polymorphisms are known for all 6 major milk proteins (caseins as well as α-LA and β-LG), which lead to both a variety of variants for each (Caroli et al., 2009) and to different digestion products (peptides) due to differences in AA composition. In recent years, the β-CN polymorphism has gained attention due to its potential impact on human health through milk consumption (Caroli et al., 2016). The β-CN account for approximately one-third of milk proteins; 13 genetic variants have been identified to date, with A2 (the ancestral, oldest variant), A1, and B (both have a single AA mutation) being the most common (Farrell et al., 2004; Kamiński et al., 2007). The original variant, A2, has a proline at position 67, which is mutated to histidine in the A1 and B variants. The B variant has an additional mutation at position 122, with the original serine being replaced by arginine. The prevalence of A2 and A1 variants depends on the animal breed, with A2 being more common in Simmental and Brown Swiss than in Holstein cows (Kamiński et al., 2007). After milk consumption, the digestive process leads to the release of peptides of different lengths, variable sequence, and abundance, depending on the β-CN variant. Some of these peptides, the so-called β-casomorphins (**BCM**), have bioactive, namely opioid, properties that may be associated with undesirable gastrointestinal symptoms in humans (EFSA, 2009). For example, BCM have been associated with discomfort, delayed gastrointestinal transit time, and possibly even the onset of chronic diseases (EFSA, 2009; Jianqin et al., 2015; Brooke-Taylor et al., 2017; Daniloski et al., 2021). Similar negative effects have also been described for BCM-associated peptides (that is, peptides containing the sequences of BCM-4 [YPFP], BCM-5 [YPFPG], BCM-7 [YPPFGPI], BCM-8 [YPFPGPI P/H], BCM-13 [YPFPGPI P/H NSLPQ], and BCM-21 [YPFPGPI P/H NSLPQNIPPLTQT]; Jinsmaa and Yoshikawa, 1999; EFSA, 2009). The release of BCM-7, the peptide with the highest in vitro binding capacity to opioid receptors (Jinsmaa and Yoshikawa, 1999), depends on the cleavage efficiency of the precursor peptides BCM-8, -13, and -21, which is probably greater in the A1 than in the A2 β-CN variant (Jinsmaa and Yoshikawa, 1999; Asledottir et al., 2017).

Our aim was to precisely investigate the influence of horn status on milk composition and milk protein digestion with consideration of an animal-related factor likely to influence the protein digestion, namely the β-CN genotype. Based on the reported importance of position 67 of the β-CN in the formation of casomorphic peptides during digestion and the minor importance of other variants (Sebastiani et al., 2020), we distinguished only between variants with His^{67} (i.e., A1, B, and C, referred to as A1 throughout the article) and those with Pro^{67} (i.e., A2, A3, D, and E, referred to as A2; EFSA, 2009; Brooke-Taylor et al., 2017). Our working hypothesis was that both milk composition and digestion differ according to horn status and A1 or A2 β-CN genotype.

MATERIALS AND METHODS

All experiments were approved (GR 2019_09) by the cantonal veterinary offices of Grisons, Uri, Glarus, and Wallis, Switzerland.

Animals, Milk Sampling, and Genotyping

Milk samples were collected in July and August 2019 on 6 alpine summer pasture farms in the cantons of Uri, Grisons, Glarus, and Wallis in Switzerland between 1,350 and 2,450 m above sea level (Supplemental Table S1, see Notes). On each farm, both horned and disbudded lactating Brown Swiss cows were housed for summer grazing (herd sizes between 26 and 120 cows). The animals were registered in the Herdbook of Braunvieh Schweiz (Zug, Switzerland, https://homepage.braunvieh.ch/en/). Therefore, information was available on their genetic indices, genetic percentage of Original Braunvieh (**OB**), performance, lactation number, and DIM. An equal number of samples was selected for each group, randomly numbered, and all analyses were performed blinded.

Milk. Milk samples were obtained during an evening milking. In total, milk samples from 316 cows were collected. The maximum amount of milk sampled per cow was 150 mL. The milk was collected in 3 tubes of 50 mL each, which were immediately cooled on ice. The samples were then taken to the laboratory, where they were frozen at −20 °C no later than 6 h after sampling and kept frozen until further analysis.

Hair. A tail hair sample was collected from each cow to determine the β-CN genotype. Samples were analyzed using a commercial SNP array (Swiss Axiom Microarray, IFN Schönow GmbH, Bernau, Germany). Three β-CN genotypes were determined: homozygous A1A1 (including A1, B, and other A1-like genotypes), A2A2 (including A2 and A2-like genotypes), and heterozygous A1A2 (including the same genotypes).

Milk Analyses

Milk Composition. Milk protein, fat, and lactose concentrations were analyzed as described in Walther et al. (2022). In brief, the total nitrogen content was analyzed according to Kjeldahl (ISO 8968–3:2007–09 [IDF

20–3: 2007]; ISO, 2007) and multiplied by a factor of 6.38 to obtain the CP concentration. Milk fat content was analyzed gravimetrically with the method ISO 2446 (IDF 226:2008; ISO, 2008). For samples with volumes below 20 mL (n = 22), fat was analyzed with the ISO 1211:2010 (IDF 1:2010; ISO, 2010) method. Milk lactose and urea content were analyzed with an automated spectrophotometer (Gallery Analyzer, Thermo, Reinach, Switzerland), using adapted methods based on kits (lactose kit E8130 from R-Biopharm, Sugiez, Switzerland; urea kit, Megazymes, Wicklow, Ireland). Somatic cell counts in the milk were analyzed with the routine method ISO 13366–2:2006 (IDF 148–2:2006; ISO, 2006) on a Somacount-FC instrument (Bentley Instruments, Marœuil, France).

Main Milk Proteins. The 20 major milk proteins were quantified by MS as previously described (Bär et al., 2020). Briefly, the proteins were precipitated with ice-cold acetone. The pellet was solubilized in digestion buffer (10% acetonitrile, 25 mmol/L ammonium bicarbonate) and incubated with trypsin. A selected proteotypic peptide was synthesized for each of the proteins and used as an isotopically labeled internal standard. The digested peptide mixture was injected into a liquid chromatography–mass spectrometer along with a known concentration of the internal standard and quantified relative to it. The SDS-PAGE was performed according to Egger et al. (2016). Briefly, milk samples were diluted 10× in sample buffer (Tris–HCl 350 m*M*, pH 6.8, SDS 10%, dithiothreitol 100 mmol/L, glycerol 50%) before separation by SDS-PAGE (15% polyacrylamide). A molecular weight marker (Benchmark, Invitrogen) was included on each gel. Thereafter, the gels were stained with colloidal Coomassie Blue (Kang et al., 2002).

Amino Acids. Free AA were analyzed according to ISO/DIS 4214:2022 (ISO/DIS, 2022) with the following modifications: Instead of acid hydrolysis followed by neutralization of the samples, the milk proteins were precipitated with 30 mmol/L hydrochloric acid and 15 g/L trichloroacetic acid at 4 °C for 30 min. The supernatant was derivatized and analyzed analogously to ISO/DIS 4214:2022 (ISO/DIS, 2022).

Fatty Acid Composition. Fatty acids were analyzed as described in Bär et al. (2020). The internal standard (C9) was added to the fat, which was previously dissolved in hexane. After a transesterification, the 68 fatty acids were determined by high-resolution gas chromatography (Agilent 6890 Plus, Agilent Technologies, Basel, Switzerland) with flame ionization detection, and peaks were manually integrated. Peaks with an unknown position in the C-chain and *cis-trans*-configuration of the double bound are presented as "u," with u1 corresponding to the first unknown peak found, u2 to the second unknown peak found, and so on.

Metabolites. Milk metabolites were measured in an untargeted metabolomics approach. Milk proteins were precipitated with 75% acetonitrile (50 mL of sample and 150 mL of acetonitrile) on ice for 20 min. Supernatants were separated in ultra-performance liquid chromatography (Vanquish, Thermo Scientific, Reinach, Switzerland) over an Acquity HSS T3 column (2.1 × 150 mm^2, 1.8 um, Waters, Baden, Switzerland). A gradient from mobile phase A (H_2O/0.1% formic acid) to mobile phase B (acetonitrile/0.1% formic acid) was used. The mixture was left at 5% mobile phase A for 2 min and was then linearly increased to 95% mobile phase A within 13 min. The eluting metabolites were measured using a Q Exactive Plus (Thermo Scientific, Reinach, Switzerland) mass spectrometer with a mass range of 66 to 1,000 m/z at a resolution of 70,000. The spectra were subsequently aligned, normalized, and quantified using ProgenesisQI software (version 2.3.6198.24128, Waters, Baden, Switzerland). The feature selection criteria were as follows: signal intensity >3× the blank and variability of standard intensity <30%.

In Vitro Digestion

Raw milk (1 mL of each sample) was digested according to the INFOGEST in vitro method (Brodkorb et al., 2019) without salivary amylase and rabbit gastric extract. Enzymes were purchased from Merck (Zug, Switzerland), and activities were tested according to the protocol. Pepsin activity was 3,368 U/mg (Sigma-Aldrich, P7012, Lot SLBW 6530), pancreatin was 6.6 U/mg (Sigma-Aldrich, P7545, Lot SLCD7175), and bile extract had a bile acid concentration of 0.72 mmol/g (Sigma-Aldrich, SLBR0091V). Briefly, 1 mL of simulated salivary fluid was added to 1 mL of milk and incubated at 37 °C for 2 min. For the gastric phase, 2 mL of simulated gastric fluid containing pepsin (2000 U/mL of digesta) and 0.09 mL of HCl (1 mol/L) to reach pH 3 were added and incubated at 37 °C for 2 h. For the intestinal phase, pH was raised to 7 by addition of 0.09 mL of NaOH (1 mol/L). Pancreatin (100 U/mL of digesta trypsin activity) and bile salts (10 mmol/L) were added together with the simulated intestinal fluid and further incubated at 37 °C for 2 h, reaching a final volume of digesta of 8 mL. Digestion in gastric samples was stopped by raising the pH to 7 with NaOH, and intestinal digestion was stopped by the addition of 4-(2-aminoethyl)-benzenesulfonyl fluoride AEBSF (1 mmol/L, Roche, Switzerland). Samples were snap frozen in liquid nitrogen and kept at −20 °C until analysis.

Determination of Protein Hydrolysis Degree. The degree of protein hydrolysis was determined as previously described (Sousa et al., 2020), after quantification of primary amines using the o-phthaldialdehyde (**OPA**) method in supernatants after gastric and intestinal in vi-

tro digestion. Therefore, gastric samples were diluted 5× and intestinal samples were diluted 10× in perchloric acid (0.5 mol/L) for precipitation of undigested proteins and longer peptides. The primary amines were derivatized with OPA in the presence of 2-mercapto-ethansulfonic acid, and absorption of the 1-alkylthio-2-alcylisonindol compound was measured with a UV/visible spectroscopy photometer at 340 nm. The released primary amines were calculated based on a glutamic acid standard curve. Normalization between different sample sets was performed with a digest of skim milk powder that was digested in parallel with each set of the unknown milk samples. Samples were analyzed in duplicate.

Analysis of Peptide Patterns. The milk digesta were analyzed using mass spectrometry as previously described (Kopf-Bolanz et al., 2012; Kopf-Bolanz et al., 2014; Egger et al., 2016; Egger et al., 2017). In summary, after filtration of 0.5 mL of digesta over Microcon columns (Ultracel YM-30, Millipore, Schaffhausen, Switzerland), the samples were separated using a Rheos 2200 HPLC instrument (Flux Instruments, Reinach, Switzerland) equipped with an XTerra MS C18 column (3.5 µm, 1.0 × 150 mm^2, Waters, Baden, Switzerland). Peptides were analyzed using an ion trap mass spectrometer (LTQ, Thermo Scientific, Reinach, Switzerland) in a mass range between 100 and 1,300 *m*/*z* and measured in several overlapping mass windows. The MS/MS spectra of the overlapping mass windows were merged, and the peptides were identified in Mascot (Matrix Science, London, United Kingdom), using an in-house database containing all typical milk proteins from different species. Peptides of the 5 major milk proteins (β-CN, α_{s1}-CN, α_{s2}-CN, κ-CN, and β-LG) were monitored. To visualize peptide abundance, the individual AA within the identified peptides were summed up along the sequence and presented in a line graph, where the protein sequence is shown along the x-axis and the abundance of AA identifications along the y-axis.

Relative Quantification of Specific Peptides. The instrumental approach (equipment, column, gradient, etc.) for the quantification of peptides was analogous to that in the analysis of peptide patterns. However, specific mass transitions for the individual peptides were measured for quantification of specific peptides. The parent and daughter masses of the peptides (parent → daughter) were taken from the peptide pattern experiments in which these peptides were identified. For the gastric phase, the mass transitions 1,196.1 → 656.4 (VYPFPGPIPNSLPQNIPPLTQT), 811.4 → 656.4 (VYPFPGPIHNSLPQNIPPLTQT), 651.1 → 794.4 (VYPFPGPIPNSL), 671.2 → 834.4 (VYPFPGPIHNSL), 908.4 → 690.3 (PFTESQSL), 489.2 → 733.8 (PFTERQSL), 889.3 → 627.4 (VYPFPGPI), and 867.4 → 473.3 (PVVPPFL) were used. For the intestinal phase, the transitions 1,002.4 → 627.4 (LVYPFPGPI), 889.3 → 627.4 (VYPFPGPI), 790.2 → 383.2 (YPFPGPI), and 867.4 → 473.3 (PVVVPPFL) were used. For peptides YPFPGPI (BCM7), VYPFPGPIPNSL ([Val^0-Pro^8]-BCM11), VYPFPGPIHNSL ([Val^0-His^8]-BCM11), LVYPFPGPI (BCM9), and VYPFPGPI (BCM8), synthetic peptides were analyzed in equal amounts beforehand. The amounts found were normalized to BCM7 according to the response ratios to the synthetic peptides to also allow a relative comparison of the amounts found between the peptides.

Sample Selection and Statistical Analyses

Sample Selection. Of the 316 milk samples collected, 22 were excluded because the cows followed an antibiotic treatment. The remaining 294 samples included 10% A1A1, 40% A1A2, and 50% A2A2 samples. These proportions were consistent with previous studies (Kiddy et al., 1966; Van Eenennaam and Medrano, 1991). From the 294 samples, 128 samples from 64 horned and 64 disbudded cows were selected for further analysis by balancing subgroups as best as possible considering the alpine farm, lactation number, DIM, milk performance at the previous lactation, genetic proportion of Original Brown Swiss, and genetic indices (for SCC and yields of milk, fat, and protein). The sample selection was performed using the matchIt package of the R environment (R Core Team, 2023). Based on the limiting factor, namely, the low proportion of A1A1 genotype animals, matching groups of A1A2 and A2A2 animals similar to the limiting A1A1 group were compiled, and the resulting sample subset of 128 samples as presented in Table 1 was generated. Five samples (3 A1A2 and 2 A2A2) with SCC above the legal limit for milk delivery in Switzerland (SCC values >350 × 10^3 cells/mL, n = 20) were excluded, since they would not be delivered and therefore not be consumed by humans, and replaced by another milk sample of a cow with the corresponding β-CN genotype and horn status.

Statistical analyses. All statistical analyses were performed using the R environment (R Core Team, 2023). Linear mixed models were used to examine the effects of horn status and β-CN genotype (using the lme4 package). The models included the fixed effects of horn status, β-CN genotype, OB percentage, and DIM, as well as the random effects of alpine farm and lactation number. *P*-values of fatty acids were corrected for multiple testing according to Benjamini and Hochberg (1995). To detect significant effects due to influential data points, robust linear models were calculated on trimmed data that included only data within the 1.96-SD interval. Effects are presented only if they were significant in both statistical models and showed the same tendency in both genetic lines (OB and Brown Swiss). For the metabolite analyses,

Table 1. Number and characteristics of the animals used in the study by β-CN genotype and horn status

	β-CN genotype					
	A1A1		A1A2		A2A2	
Item	Mean	SE	Mean	SE	Mean	SE
Horned (H^+)						
Number of animals	16		24		24	
Age (yr)	5.5	0.7	5.2	0.4	5.6	0.4
DIM	242	10.6	241	13.1	247	13.4
Original Braunvieh percentage (%)	86	7.9	59	8.9	57	9.2
Genetic milk index[1]	−830	110	−288	84.2	−264	101
Disbudded (H^-)						
Number of animals	6		29		29	
Age (yr)	6.6	0.9	5.8	0.5	5.5	0.4
DIM	247	12.3	247	13.9	241	14.8
Original Braunvieh percentage (%)	21	15.9	38	7.9	23	6.8
Genetic milk index[1]	83	110	−309	85.7	13	99.6

[1]Herdbook of Braunvieh Schweiz (https://homepage.braunvieh.ch/en/).

only detected features with mean values more than twice those of blank samples were used. Multivariate analysis included principal component analysis (**PCA**) and sparse partial least-squares discriminant analysis (**sPLS-DA**, mixOmics package). Model fits of sPLS-DA analyses were evaluated using the explained variability (R^2) and predictive validity (Stone-Geisser's Q^2) of the model. Models with R^2 of >0.8 and Q^2 of >0.5 were considered to be of good quality.

RESULTS

Milk Composition, Major Proteins, AA, and Fatty Acids of Milk Samples

The β-CN genotype and horn status did not affect total protein, total fat, lactose, urea, or SCC in the milk (Table 2, all $P > 0.10$). The patterns of the major milk proteins, after separation on SDS-PAGE, were not visibly different (Figure 1). The quantification of 20 major milk proteins by mass spectrometry confirmed that the samples did not differ by β-CN genotype or horn status (Supplemental Table S2, see Notes), which is graphically represented in the PCA score plots by the lack of separation between groups (Figure 2). No differences by β-CN genotype or horn status were found in the 20 free AA (all $P > 0.10$, Figure 3). Milk from cows with the A1A2 genotype contained higher concentrations of C17, C18:1 t6–9, and C18:2 u3 and lower concentrations of C16:1 u2 than milk from A1A1 and A2A2 cows (all $P < 0.05$; Supplemental Table S3, see Notes). Despite these differences in individual fatty acids, the overall fatty acid profile did not differ by β-CN genotype or horn status, as graphically represented by the lack of separation between groups in the PCA score plots (Figure 4).

Differences During and After In Vitro Digestion

The hydrolysis degrees in both the gastric and intestinal phases increased with increasing DIM ($P < 0.05$) but not by horn status or genetic background ($P > 0.10$, data not shown). In the gastric phase, the abundance of 6 out of 8 identified β-CN peptides differed by the β-CN genotype (Supplemental Figure S1B, see Notes), but not by horn status (Supplemental Figure S1A). Milk from A1A1 cows had lower amounts of the peptide YPFPGPIPNSLPQNIPPLTQT ([Pro^8]-BCM21; $P < 0.05$) and higher amounts of PFTERQSL (arbitrary intensity

Table 2. Means and effects of milk composition by β-CN genotype and horn status[1]

	β-CN genotype			Horn status			*P*-value			
Item	A1A1	A1A2	A2A2	H^+	H^-	SEM	Genotype	Horn status	OB percentage	DIM
Protein (%)	3.50	3.48	3.44	3.48	3.46	0.030	0.833	0.698	0.93	<0.001
Fat (%)	4.50	4.31	4.62	4.43	4.51	0.084	0.321	0.604	0.99	0.13
Lactose (%)	4.55	4.58	4.53	4.59	4.52	0.019	0.330	0.380	0.95	0.002
Urea (mg 100 g^{-1} milk)	23.4	23.7	23.6	23.1	24.1	0.551	0.469	0.932	0.98	0.89
SCC (10^3 cells mL^{-1} milk)	122	132	131	116	145	7.613	0.750	0.291	0.96	0.050

[1]H^- = horned cows, H^+ = disbudded cows; OB = Original Braunvieh.

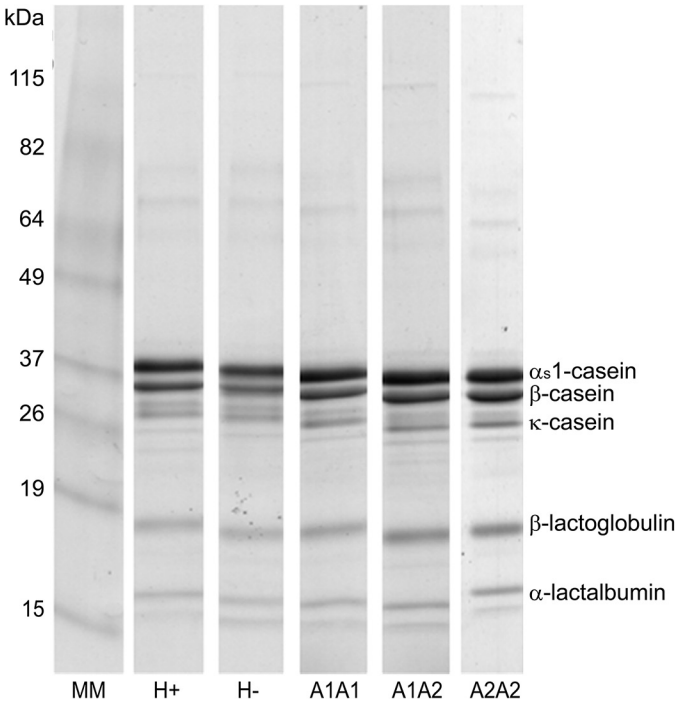

Figure 1. Gel electrophoresis of milk proteins from horned (H+) and disbudded (H-) cows with different β-CN variants A1A1, A1A2, and A2A2. A molecular size marker (MM) was run in the first lane. The bands are labeled with the corresponding identified proteins.

of 0.87, 0.20, and 0.01 for A1A1, A1A2, and A2A2, respectively; $P < 0.05$) than milk from A1A2 and A2A2 cows. Milk from A2A2 cows had lower amounts of YPFPGPIHNSLPQNIPPLTQT ([His^{8}]-BCM21) and greater amounts of VYPFPGPIPNSL ([[Val^{0}, Pro^{8}]-BCM11; all $P < 0.05$; Supplemental Figure S1B) than milk from A1A1 and A1A2 cows. Amounts of VYPFPGPIHNSL ([Val^{0}, His^{8}]-BCM11) were greatest in milk from A1A1 cows and lowest in milk from A2A2 cows, with intermediate values for milk from A1A2 cows ($P < 0.05$). The opposite trend was observed for PFTESQSL ($P < 0.05$). The amounts of the gastric peptides VYPFPGPI ([Val^{0}]-BCM7) and PVVVPPFL did not differ by genotype, horn status, OB percentage, or DIM (data not shown). In the PCA plot including gastric peptides, the groups of β-CN genotypes (A1A1, A1A2, and A2A2) were well separated (Figure 5B), while the horn status groups were overlapping (Figure 5A). The average abundance of each AA, identified within a peptide, was also altered by the β-CN genotype (Figure 6). In particular, the protein regions between the AA near position 67 of the A1/A2 mutation and at position 122 of the A1/B mutation differed by β-CN genotype. Differences were also observed in neighboring regions (especially AA positions 80 to 90 and 130 to 140). No differences along the whole protein sequence were observed in milk from horned versus disbudded cows (Figure 6). The gastric peptides of the other 4 most abundant milk proteins (a_{s1}-CN, a_{s2}-CN, k-CN, and β-LG) were not altered by β-CN genotype or horn status (data not shown).

In the intestinal phase, peptide abundances did not differ by β-CN genotype or horn status (Supplemental Figures S1C and S1D), except for the peptide LVYPFPGPI ([Leu^{-1}-Val^{0}]-BCM7), which was most abundant in milk from A1A2 cows and least abundant in milk from A2A2 cows ($P < 0.05$), whereas A1A1 milk had intermediate levels. The amounts of the intestinal peptides VYPFPGPI ([Val^{0}]-BCM7 and YPFPGPI (BCM7) were not affected

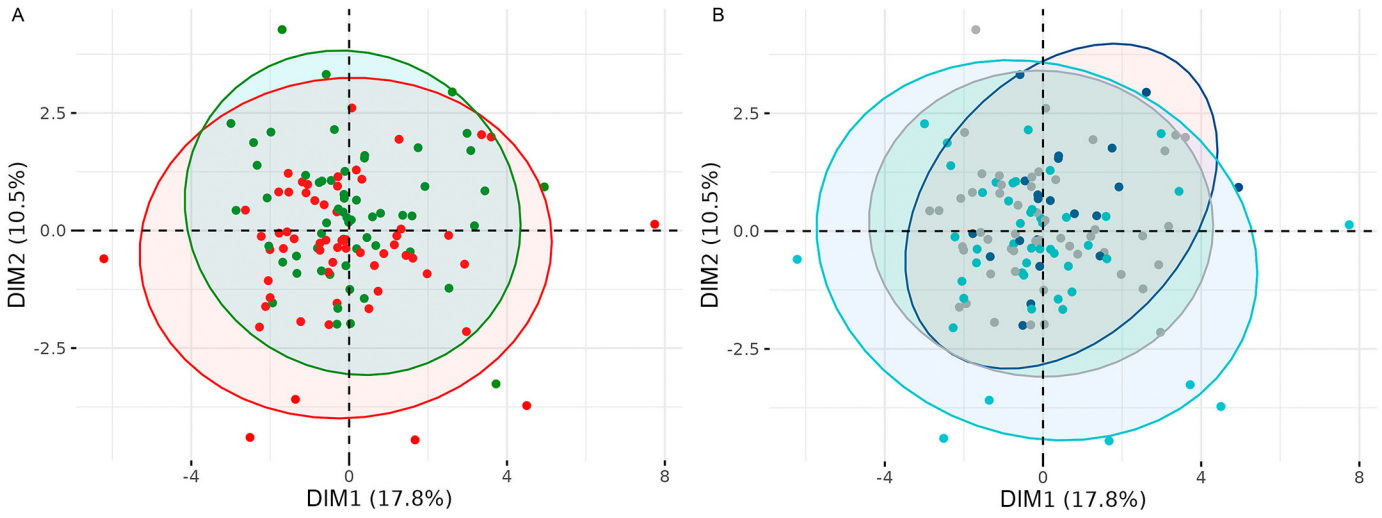

Figure 2. Principal component analysis score plots of milk main proteins. (A) Green and red dots represent samples of horned and disbudded cows, respectively. (B) Dark blue, gray, and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively. Ellipses represent 95% CI.

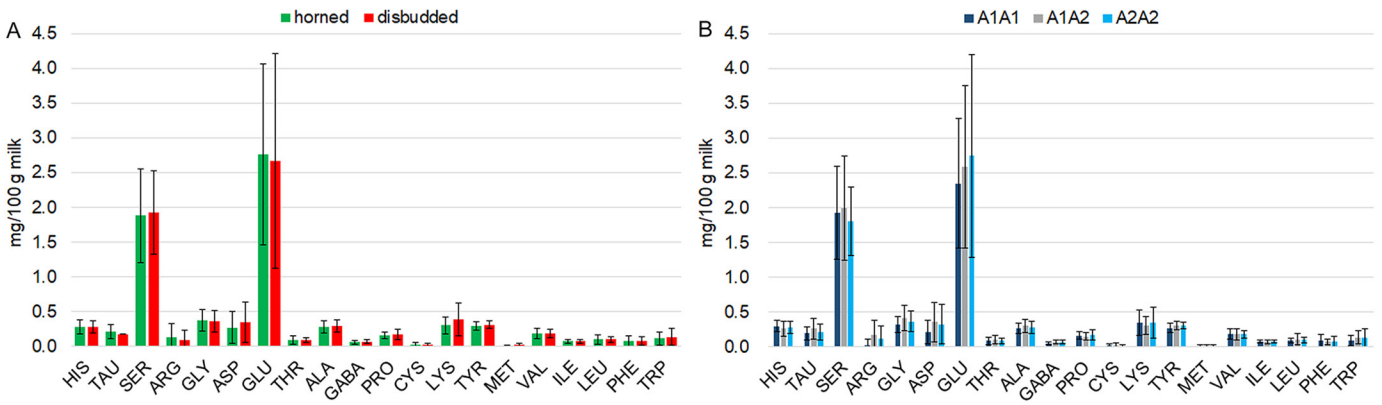

Figure 3. Bar plots of concentrations of free AA with error bars representing the standard deviation of the mean. (A) Green and red bars represent the means of samples of horned and disbudded cows, respectively. (B) Dark blue, gray, and turquoise bars represent the means of samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively.

by genotype, horn status, OB percentage, or DIM (all $P > 0.10$). The amount of PVVVPPFL increased with increasing number of DIM ($P < 0.05$).

Metabolites

Of ~23,000 detected features, 523 fulfilled the selection criteria. Sample separation by PLS-DA based on genotype (Supplemental Figure S2B, see Notes) and horn status (Supplemental Figure S2A) was of low predictive accuracy (R^2 and $Q^2 < 0.4$ for both genotype and horn status). A slightly better discrimination but with only moderate predictive accuracy was achieved by alpine farm (R^2: 0.6 and Q^2: 0.4; Supplemental Figure S2C). Milk samples from farms 1, 4, 5, and 6 were relatively similar, while samples from farms 2 and 3 formed distinct clusters. The most influential discriminating metabolites involved those with the highest values in farm 2 (6 on component 1 and 5 on component 2, colored in orange, Supplemental Figure S2D), followed by several metabolites that were elevated for farm 3 (2 on component 1 and 4 on component 2, colored in gray, Supplemental Figure S2D). Because the PLS-DA models were of low predictive accuracy and the best discrimination was observed between the different farms, no further statistical evaluations of the abundances of metabolites were undertaken.

DISCUSSION

We studied the influences of horn status and β-CN genotype on milk composition and digestion with 128 milk samples. To the best of our knowledge, this is the

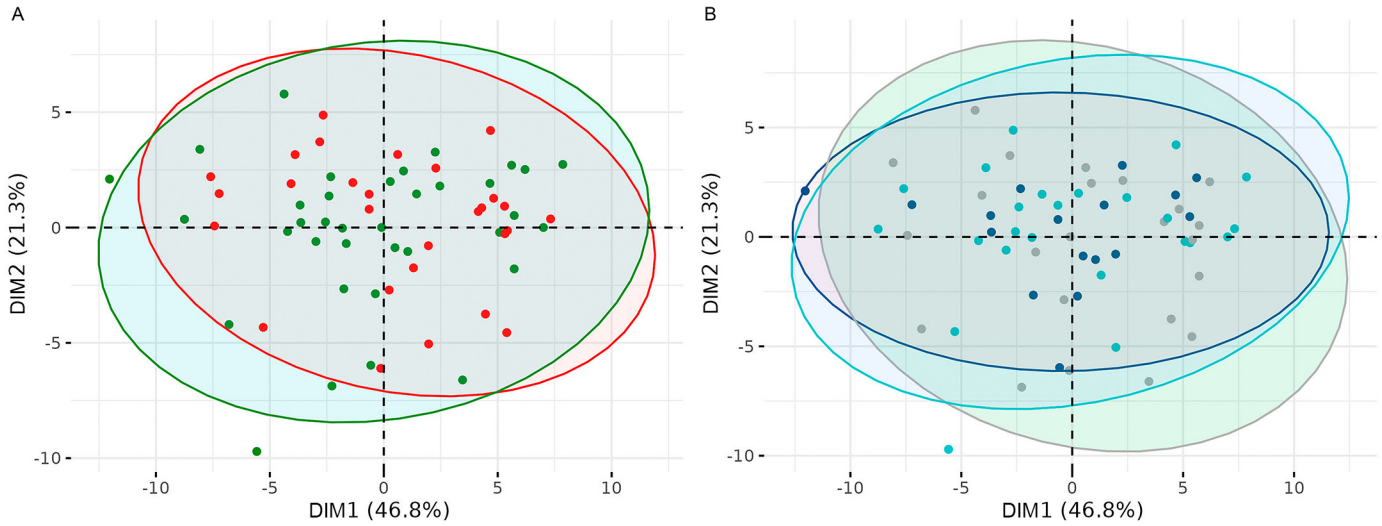

Figure 4. Principal component analysis score plots of milk fatty acids. (A) Green and red dots represent samples of horned and disbudded cows, respectively. (B) Dark blue, gray, and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively. Ellipses represent 95% CI.

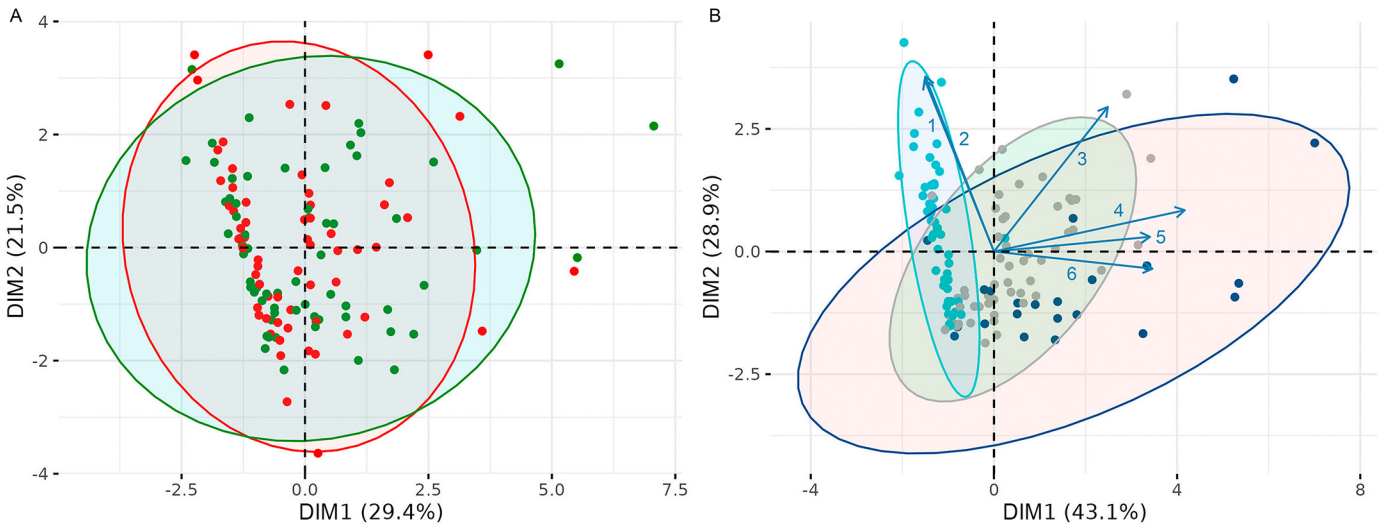

Figure 5. Principal component analysis plots of gastric peptides during in vitro digestion. (A) Score plot. Green and red dots represent samples of horned and disbudded cows, respectively. (B) Biplot. Dark blue, grey and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively. Ellipses represent 95% CI. Arrows represent the scaled loadings of the corresponding variables on the first 2two dimensions for the peptides (1) VYPFPGPIPNSL = [Pro^{8}]-BCM11, (2) PFTESQSL (β-CN; 133:140), (3) PVVVPPFL (β-CN; 96:103), (4) VYPFPGPIHNSL = [His^{8}]-BCM11, (5) YPFPGPIHNSLPQNIPPLTQT = [His^{8}]-BCM21, and (6) PFTERQSL (β-CN, 133:140).

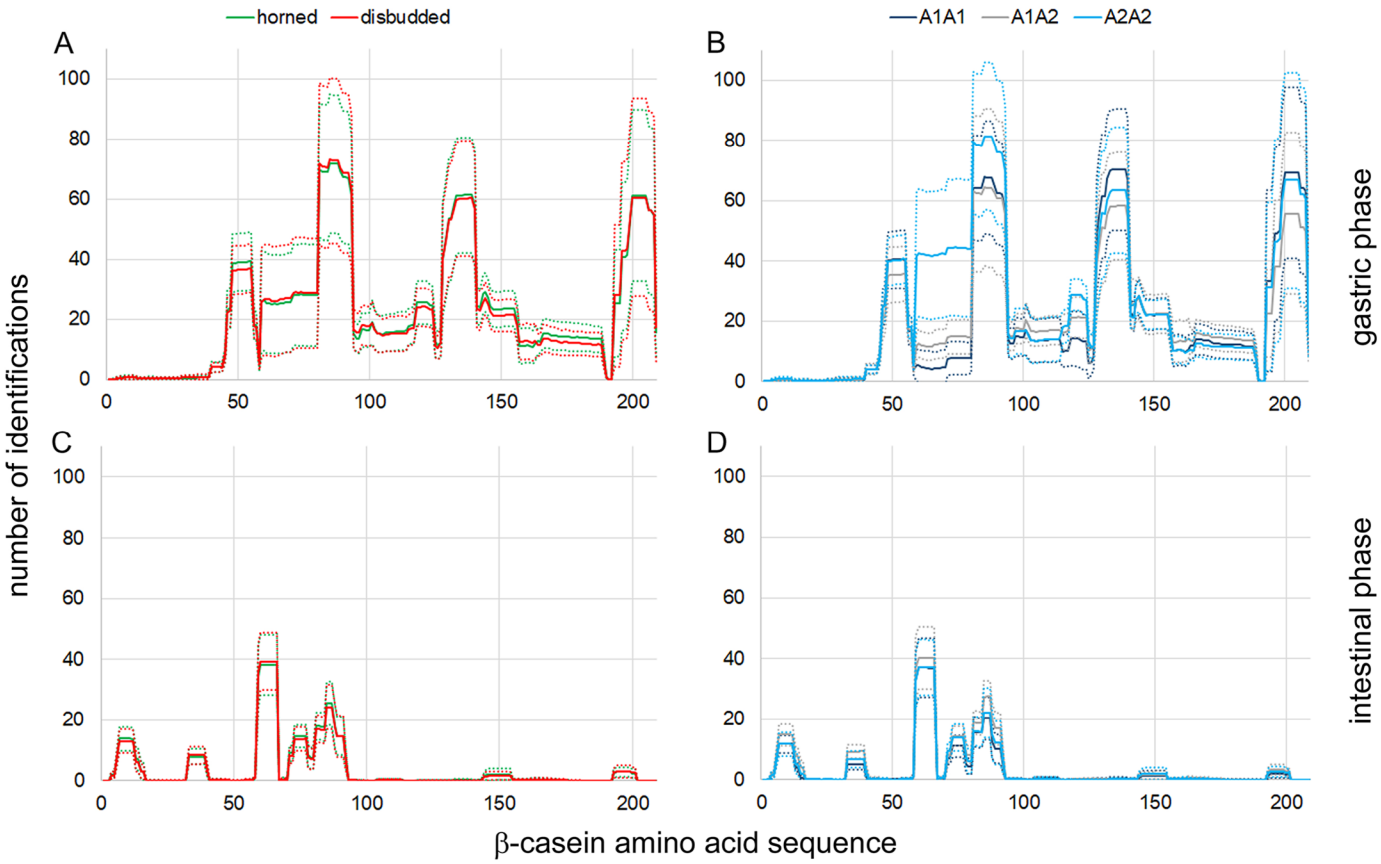

Figure 6. Frequency of peptides during in vitro digestion.

first study to investigate the influences of the 2 factors using a representative number of cows. While horn status had no measurable effect, the β-CN genotype altered the in vitro digestion of milk assessed using the harmonized INFOGEST IVD protocol, which was previously validated with in vivo data from humans and pigs (Egger et al., 2017; Sanchón et al., 2018). The differences found between A1 and A2 milks concerned casomorphic peptide abundances after the gastric phase; they were no longer present after the small intestine phase. The digestion of proteins other than β-CN was not influenced by the β-CN genotype, as represented by similar degrees of hydrolysis. Milk metabolite profiles did not differ by β-CN genotype but did differ by farm.

Horn Status Without Measurable Influence on Milk

Horn status had no effect on gross milk composition, which agrees with earlier studies (Wohlers, 2011; Baars et al., 2019; Reiche et al., 2023). In contrast to our study, these studies (using pooled milk, n = 56 and n = 10 samples, respectively) reported a general shift between horned and disbudded cows in milk fatty acid profiles and differences in specific AA and metabolites under cold weather conditions and in pooled milk samples. The present study (n = 128) did not reveal such differences. This might be related to the relatively large sample size and therefore lower sampling bias of the present study. Further, the influence of horn status on the simulated digestion of milk, which has not been investigated before, revealed that protein digestion after the gastric and intestinal steps in the INFOGEST in vitro digestion system did not differ in peptide patterns between milk from horned versus disbudded animals. We therefore conclude that the gross and detailed compositions of milk, including its digestion, do not differ by horn status.

β-CN Genetics Alter Peptide Patterns in the Gastric Phase but Not in the Intestinal Phase of In Vitro Digestion

As in previous studies (de Vitte et al., 2022), milk composition was not influenced by the β-CN genotype, but milk peptides from cows of different genotypes showed large differences in the gastric phase of simulated digestion. The different amounts of the peptides VYPFPGPIPNSL ([Val^0-Pro^8-]-BCM11), VYPFPGPIHNSL ([Val^0-His^8-]-BCM11), YPFPGPIPNSLPQNIPPLTQT ([Pro^8-]-BCM21), and YPFPGPIHNSLPQNIPPLTQT (([His^8-]-BCM21)), identified after gastric digestion, are explained by the inherent difference between the genetic variants A1 and A2, namely, the AA in position 67 (A1: His, A2: Pro). Obviously, the A1A1 and A2A2 milk contained almost uniquely the peptides with His and Pro at position 67, respectively, whereas the milk from heterozygous animals, possessing both alleles (A1 and A2), had intermediate amounts. When comparing the amounts of BCM irrespective of their AA prefix, the digestion of A1A1 milk released markedly greater amounts of BCM11 and markedly lower amounts of BCM21 than the digestion of A1A2 and A2A2 milk. The markedly greater amounts of the A2-derived casomorphic peptide ([Pro^8-]-BCM21) in A2A2 milk compared with that of the A1-derived casomorphic peptide [His^8-]-BCM21 in A1A1 milk agrees with earlier studies (Jinsmaa and Yoshikawa, 1999; Asledottir et al., 2017) and would confirm the hypothesis that the A2 variant is more resistant to pepsin cleavage. However, the present study could not confirm the cleavage resistance of A2 peptides for BCM11. This is in contrast to a study in which, in duodenal extracts of minipigs, only the A2-derived peptide [Val^0-Pro^8]-BCM11 was identified, while the A1-derived variant [Val^0-His^8]-BCM11 was not found (Meisel, 1986). It should be noted, however, that Meisel (1986) collected the samples in the duodenum and not after the gastric phase as in the present study.

The peptide [Val^0]-BCM7 was detected in both the gastric and intestinal phases; the peptides BCM7 and [Leu^{-1}-Val^0]-BCM7 were found in the intestinal phase only. All these peptides were present in equal amounts in the milk digesta of the 3 β-CN genotypes, except for [Leu^{-1}-Val^0]-BCM7, levels of which were increased in the intestinal phase for A1A2 cows. These findings are in contrast to earlier in vitro studies where the cleavage of the Pro (A2) at position 67 did not occur and thus led to lower amounts of BCM7 in A2 milk samples in both the gastric and intestinal phases (Jinsmaa and Yoshikawa, 1999; EFSA, 2009; Asledottir et al., 2017).

Other non-BCM-related peptides, namely PFTESQSL and PFTERQSL, also differed significantly in their amounts between A1A1 and A2A2 samples in the gastric phase. This difference is explained by another natural mutation at position 122 of the β-CN (serine instead of arginine), which is specific to variant B. Since the mutation leading to the B variant has a His at position 67, like the A1 variant, the variant B is counted as variant A1 in the genotype test used. Finally, the peptide abundances in the vicinity, but outside of the A2/A1 and A1/B mutations (especially AA positions 80 to 90 and 130 to 140), were also slightly different in the genetic groups.

Therefore, we conclude that the A1/A2 genotype affects the digestion of total β-CN in the gastric phase and thereby influences the gastric digestibility of the whole β-CN protein. According to the mutation at position 67, the mutated A1A1 genotype produces greater amounts of the casomorphins 11 and 21 with His at position 67, and the "wild" A2A2 genotype produces greater amounts of casomorphins with Pro at position 67. The A1A2 geno-

type generates intermediate peptide levels for the peptides PFTESQSL and BCM-11, as would be expected, since this genotype contains one A1 allele and one A2 allele. However, for BCM-21, the A1A2 genotype did not show intermediate levels. This might be explained by the variability between cows, i.e., cows with the A1A2 genotype may express their alleles differently, or possible interferences with other A1-like and A2-like β-CN variants that may influence the cleavage of peptides. However, the link between β-CN variant digestion and the amount and type of casomorphin released remains to be elucidated in view of the varying study results, as does the influence of other β-CN variants not considered but perhaps present in this (and in previous) work.

Possible Effects of Casomorphins on Human Digestion

According to the literature, BCM mainly act through m-type opioid receptors (EFSA, 2009), which are expressed in different peripheral tissues in the human body. As the gastrointestinal absorption of peptides larger than di- or tripeptides is limited, the action of BCM released during digestion is expected to occur mainly in the gastrointestinal tract. In the latter, m-type opioid receptor expression has been confirmed in the stomachs of rats and in the small and large intestines of humans and rats (Wittert et al., 1996; Sternini et al., 2004; DeHaven-Hudkins et al., 2008). In vitro, different BCM differ in their binding capacity to opioid receptors. BCM7 showsed the highest binding capacity compared with other BCM released during the digestion of bovine β-CN (Jinsmaa and Yoshikawa, 1999). BCM11 and BCM21 exhibited a distinct, albeit one lower than BCM7, affinity for opiate receptors of rat brain membrane (Meisel, 1986) and guinea pig ileum (Jinsmaa and Yoshikawa, 1999), respectively. Whether the casomorphic peptides [Val^0-Pro^8]-BCM11, [Val^0-His^8]-BCM11, [His^8-]-BCM21, and [Pro^8-]-BCM21 identified in this study also have distinct opioid receptor affinities needs to be tested. Despite contrary findings, the consumption of A1 milk has been associated with delayed intestinal transit and digestive symptoms, while the consumption of A2 milk was reported to result in improved digestive comfort (reviewed by Brooke-Taylor et al., 2017; Küllenberg de Gaudry et al., 2019; Daniloski et al., 2021). Although it is generally thought that the described negative effects on digestion are caused by the opioid activity of BCM, the mechanisms underlying these findings remain to be elucidated (Daniloski et al., 2021). In the present study, the differences in BCM amounts occurred at an early, namely the gastric, stage of digestion but were no longer measurable at the end of the intestinal phase. The effect of gastric BCM and BCM-associated peptides on gastric opioid receptors has not yet been studied. Therefore, it is not clear whether the differences observed in BCM-associated peptides in the present study could have physiological or pathological implications.

β-CN Genetics Influence Single Milk Fatty Acids

The milk of cows with the heterozygous genotype A1A2 showed the highest levels of the fatty acids C17, C18:1 t6–9, and C18:2 u3 and lowest levels of the fatty acid C16:1 u2 compared with milk from homozygous cows. Increased levels of fatty acids in the milk of A1A2 cows have been described (C11:0 and C18:2, Guantario et al., 2020); C14:1 n-5, C15:1 c10, C18:2 c9, and C22:6 n-3, de Vitte et al., 2022). The study-dependent variation of these fatty acids might be related to the type of cow used in the studies (present study: Brown Swiss, individual cows; previous studies: Simmental × Holstein crossbreeds, pooled milk). The genotype effect on fatty acids might relate to genetic differences in rumen microbiota and, subsequently, ruminal fermentation including fatty acid production. Despite the differences between the studies, we can conclude that the genotype has an influence on individual fatty acids, depending on the breed.

Milk Metabolites Mainly Influenced by Farm

Milk metabolite profiles differed by farm, irrespective of the cows' horn status and β-CN genotype. On all farms, the cows were housed on mountain pastures in full-time grazing systems. Although the milk samples were taken in the same season, it is probable that herbage offer, composition, and quality varied by farm, as the farms were located in different regions of Switzerland. As milk metabolite profiles are highly dependent on diet (reviewed by Rocchetti and O'Callaghan, 2021), the farm-dependent metabolite profiles may be explained by diet-related differences. The presence of a farm effect in the absence of an effect of horn status or β-CN genotype indicates that a factor such as nutrition has a visible influence on milk metabolites, while the influences of β-CN genotype and horn status are negligible.

CONCLUSIONS

We investigated whether the horn status of cows and their A1/A2 genotype influence the composition of milk and, ultimately, human digestion. Despite minimizing selection bias by balancing experimental groups for phenotypic and genetic traits and by avoiding or accounting for factors such as feeding, horn status had no influence on the type and quantity of components and metabolites in the milk, in contrast to our working hypothesis; neither did it affect digestion. The β-CN genotype also did

not affect gross and detailed milk composition, apart from the proportions of 4 (i.e., 1%) of the 68 quantified fatty acids. The β-CN genotype influenced the digestion pattern of β-CN in the stomach, especially the region around the BCM peptides. However, these differences disappeared after small intestine passage. Whether this genotype-dependent digestion affects human digestion and well-being should be investigated. Our methods were sensitive enough to detect differences of milk varying in one single AA of one milk protein—even at the level of human digestion. Despite the highly sensitive methods, no effects of horn status were detected. Therefore, we conclude that the horn status has no effect on milk quality, composition, or in vitro digestion.

NOTES

The authors gratefully acknowledge financial support from Foundation Sur-la-Croix (Basel, Switzerland). The authors thank the farmers and the persons in charge of the mountain farms Albin, Binneralp, Alpe Binntal, Falätscha, Wannelen, and Wichlen for their participation in the study, the milk inspectors of Braunvieh Schweiz, Giovanni Lazzari from the Ruminant Nutrition and Emissions group of Agroscope, and students (Daniel Wild, Lukas Ossberger, Aurélie Garnier from the University of Natural Resources and Life Sciences [BOKU] Vienna, Austria, and the Ecole Supérieure des Agricultures [ESA] Angers, France) for their help during the milk sampling. Braunvieh Schweiz is thanked for providing access to herdbook data. Supplemental material for this article is available at https://doi.org/10.5281/zenodo.11655184. All experiments were approved (GR 2019_09) by the cantonal veterinary offices of Grisons, Uri, Glarus, and Wallis, Switzerland. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: BCM = beta-casomorphin; MM = molecular size marker; OB = Original Braunvieh; OPA = o-phthaldialdehyde; PCA = principal component analysis; sPLS-DA = sparse partial least-squares discriminant analysis.

REFERENCES

Asledottir, T., T. T. Le, B. Petrat-Melin, T. G. Devold, L. B. Larsen, and G. E. Vegarud. 2017. Identification of bioactive peptides and quantification of β-casomorphin-7 from bovine β-casein A1, A2 and I after ex vivo gastrointestinal digestion. Int. Dairy J. 71:98–106. https://doi.org/10.1016/j.idairyj.2017.03.008.

Baars, T., G. Jahreis, S. Lorkowski, C. Rohrer, J. Vervoort, and K. Hettinga. 2019. Short communication: Changes under low ambient temperatures in the milk lipodome and metabolome of mid-lactation cows after dehorning as a calf. J. Dairy Sci. 102:2698–2702. https://doi.org/10.3168/jds.2018-15425.

Bär, C., M. Sutter, C. Kopp, P. Neuhaus, R. Portmann, L. Egger, B. Reidy, and W. Bisig. 2020. Impact of herbage proportion, animal breed, lactation stage and season on the fatty acid and protein composition of milk. Int. Dairy J. 109:104785. https://doi.org/10.1016/j.idairyj.2020.104785.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the False discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Bro-Jørgensen, J. 2007. The intensity of sexual selection predicts weapon size in male bovids. Evolution 61:1316–1326. https://doi.org/10.1111/j.1558-5646.2007.00111.x.

Brodkorb, A., L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies, and I. Recio. 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14:991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Brooke-Taylor, S., K. Dwyer, K. Woodford, and N. Kost. 2017. Systematic review of the gastrointestinal effects of A1 compared with A2 β-casein. Adv. Nutr. 8:739–748. https://doi.org/10.3945/an.116.013953.

Caroli, A. M., S. Savino, O. Bulgari, and E. Monti. 2016. Detecting β-casein variation in bovine milk. Molecules 21:141. https://doi.org/10.3390/molecules21020141.

Caroli, A. M., S. Chessa, and G. J. Erhardt. 2009. Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. J. Dairy Sci. 92:5335–5352. https://doi.org/10.3168/jds.2009-2461.

Casoni, D., A. Mirra, M. R. Suter, A. Gutzwiller, and C. Spadavecchia. 2019. Can disbudding of calves (one versus four weeks of age) induce chronic pain? Physiol. Behav. 199:47–55. https://doi.org/10.1016/j.physbeh.2018.11.010.

Cozzi, G., F. Gottardo, M. Brscic, B. Contiero, N. Irrgang, U. Knierim, O. Pentelescu, J. J. Windig, L. Mirabito, F. Kling Eveillard, A. C. Dockes, I. Veissier, A. Velarde, C. Fuentes, A. Dalmau, and C. Winckler. 2015. Dehorning of cattle in the EU Member States: A quantitative survey of the current practices. Livest. Sci. 179:4–11. https://doi.org/10.1016/j.livsci.2015.05.011.

Daniloski, D., N. M. D. Cunha, N. A. McCarthy, T. F. O'Callaghan, S. McParland, and T. Vasiljevic. 2021. Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials. Trends Food Sci. Technol. 111:233–248. https://doi.org/10.1016/j.tifs.2021.02.073.

de Vitte, K., S. Kerziene, J. Klementavičiūtė, M. de Vitte, R. Mišeikienė, I. Kudlinskienė, J. Čepaitė, V. Dilbiene, and R. Stankevičius. 2022. Relationship of β-casein genotypes (A1A1, A1A2 and A2A2) to the physicochemical composition and sensory characteristics of cows' milk. J. Appl. Anim. Res. 50:161–166. https://doi.org/10.1080/09712119.2022.2046005.

DeHaven-Hudkins, D. L., R. N. DeHaven, P. J. Little, and L. M. Techner. 2008. The involvement of the mu-opioid receptor in gastrointestinal pathophysiology: Therapeutic opportunities for antagonism at this receptor. Pharmacol. Ther. 117:162–187. https://doi.org/10.1016/j.pharmthera.2007.09.007.

EFSA. 2009. Scientific Report of EFSA prepared by a DATEX Working Group on the potential health impact of β-casomorphins and related peptides. EFSA Sci. Rep. 231:1–107.

Egger, L., O. Ménard, C. Delgado-Andrade, P. Alvito, R. Assunção, S. Balance, R. Barberá, A. Brodkorb, T. Cattenoz, A. Clemente, I. Comi, D. Dupont, G. Garcia-Llatas, M. J. Lagarda, S. Le Feunteun, L. JanssenDuijghuijsen, S. Karakaya, U. Lesmes, A. R. Mackie, C. Martins, A. Meynier, B. Miralles, B. S. Murray, A. Pihlanto, G. Picariello, C. N. Santos, S. Simsek, I. Recio, N. Rigby, L.-E. Rioux, H. Stoffers, A. Tavares, L. Tavares, S. Turgeon, E. K. Ulleberg, G. E. Vegarud, G. Vergères, and R. Portmann. 2016. The harmonized INFOGEST in vitro digestion method: From knowledge to action. Food Res. Int. 88:217–225. https://doi.org/10.1016/j.foodres.2015.12.006.

Egger, L., P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brügger, D. Dürr, P. Stoll, G. Vergères, and R. Portmann. 2017. Physi-

ological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. Food Res. Int. 102(Supplement C):567–574. https://doi.org/10.1016/j.foodres.2017.09.047.

Farrell, H. M. Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk–sixth revision. J. Dairy Sci. 87:1641–1674. https://doi.org/10.3168/jds.S0022-0302(04)73319-6.

Guantario, B., M. Giribaldi, C. Devirgiliis, A. Finamore, E. Colombino, M. T. Capucchio, R. Evangelista, V. Motta, P. Zinno, S. Cirrincione, S. Antoniazzi, L. Cavallarin, and M. Roselli. 2020. A comprehensive evaluation of the impact of bovine milk containing different beta-casein profiles on gut health of ageing mice. Nutrients 12:2147. https://doi.org/10.3390/nu12072147.

ISO (International Organization for Standardization). 2010. 1211:2010 (IDF 1:2010). Milk - Determination of fat content - Gravimetric method (Reference method).

ISO (International Organization for Standardization). 2006. 13366–2:2006 (IDF 148–2:2006). Milk - Enumeration of somatic cells - Part 2: Guidance on the operation of fluoro-opto-electronic counters.

ISO (International Organization for Standardization). 2008. 2446 (IDF 226:2008). ISO-2446 (IDF 226:2008) - Milk - Determination of fat content.

ISO (International Organization for Standardization). 2007. 8968–3:2007–09 (IDF 20–3: 2007). ISO 8968–3:2007–09 / IDF 20–3: 2007 Milk - Determination of nitrogen content - Part 3: Block-digestion method.

ISO/DIS (International Organization for Standardization/Draft International Standard). 2022. 4214:2022. Milk and Milk Products - Determination of amino acids in infant formula and other dairy products.

Jianqin, S., X. Leiming, X. Lu, G. W. Yelland, J. Ni, and A. J. Clarke. 2015. Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. Nutr. J. 15:35. https://doi.org/10.1186/s12937-016-0147-z.

Jinsmaa, Y., and M. Yoshikawa. 1999. Enzymatic release of neocasomorphin and beta-casomorphin from bovine beta-casein. Peptides 20:957–962. https://doi.org/10.1016/S0196-9781(99)00088-1.

KAGfreiland. 2014. Bericht Hörner-Umfrage.

Kamiński, S., A. Cieslińska, and E. Kostyra. 2007. Polymorphism of bovine beta-casein and its potential effect on human health. J. Appl. Genet. 48:189–198. https://doi.org/10.1007/BF03195213.

Kang, D., Y. Gho, M. Suh, and C. Kang. 2002. Highly sensitive and fast protein detection with Coomassie Brilliant Blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Communications to the editor. Bull. Korean Chem. Soc. 23:11.

Kiddy, C. A., R. F. Peterson, and F. G. Kopfler. 1966. Genetic control of variants of β-casein. J. Dairy Sci. 49(Suppl. 1):742.

Kiltie, R. A. 1985. Evolution and function of horns and hornlike organs in female ungulates. Biol. J. Linn. Soc. Lond. 24:299–320. https://doi.org/10.1111/j.1095-8312.1985.tb00377.x.

Knierim, U., N. Irrgang, and B. A. Roth. 2015. To be or not to be horned—Consequences in cattle. Livest. Sci. 179:29–37. https://doi.org/10.1016/j.livsci.2015.05.014.

Kopf-Bolanz, K. A., F. Schwander, M. Gijs, G. Vergeres, R. Portmann, and L. Egger. 2012. Validation of an in vitro digestive system for studying macronutrient decomposition in humans. J. Nutr. 142:245–250. https://doi.org/10.3945/jn.111.148635.

Kopf-Bolanz, K. A., F. Schwander, M. Gijs, G. Vergères, R. Portmann, and L. Egger. 2014. Impact of milk processing on the generation of peptides during digestion. Int. Dairy J. 35:130–138. https://doi.org/10.1016/j.idairyj.2013.10.012.

Küllenberg de Gaudry, D., S. Lohner, C. Schmucker, P. Kapp, E. Motschall, S. Hörrlein, C. Röger, and J. J. Meerpohl. 2019. Milk A1 β-casein and health-related outcomes in humans: A systematic review. Nutr. Rev. 77:278–306. https://doi.org/10.1093/nutrit/nuy063.

Meisel, H. 1986. Chemical characterization and opioid activity of an exorphin isolated from in vivo digests of casein. FEBS Lett. 196:223–227. https://doi.org/10.1016/0014-5793(86)80251-4.

Mirra, A., C. Spadavecchia, R. Bruckmaier, A. Gutzwiller, and D. Casoni. 2018. Acute pain and peripheral sensitization following cautery disbudding in 1-and 4-week-old calves. Physiol. Behav. 184:248–260. https://doi.org/10.1016/j.physbeh.2017.11.031.

R Core Team. 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Reiche, A. M., S. L. Amelchanka, B. Bapst, M. Terranova, M. Kreuzer, B. Kuhla, and F. Dohme-Meier. 2023. Influence of dietary fiber content and horn status on thermoregulatory responses of Brown Swiss dairy cows under thermoneutral and short-term heat stress conditions. J. Dairy Sci. 106:8033–8046. https://doi.org/10.3168/jds.2022-23071.

Reiche, A.-M., F. Dohme-Meier, and E. M. Claudia Terlouw. 2020a. Effects of horn status on behaviour in fattening cattle in the field and during reactivity tests. Appl. Anim. Behav. Sci. 231:105081. https://doi.org/10.1016/j.applanim.2020.105081.

Reiche, A.-M., A. K. Hankele, H. Hess, F. Dohme-Meier, and S. E. Ulbrich. 2020b. The ACTH challenge and its repeatability in fattening bulls—Influences of physiological state, challenge time standardization, and horn status. Domest. Anim. Endocrinol. 72:106360. https://doi.org/10.1016/j.domaniend.2019.03.001.

Reiche, A.-M., J.-L. Oberson, P. Silacci, J. Messadène-Chelali, H.-D. Hess, F. Dohme-Meier, P.-A. Dufey, and E. M. C. Terlouw. 2019. Pre-slaughter stress and horn status influence physiology and meat quality of young bulls. Meat Sci. 158:107892. https://doi.org/10.1016/j.meatsci.2019.107892.

Rocchetti, G., and T. F. O'Callaghan. 2021. Application of metabolomics to assess milk quality and traceability. Curr. Opin. Food Sci. 40:168–178. https://doi.org/10.1016/j.cofs.2021.04.005.

Sanchón, J., S. Fernández-Tomé, B. Miralles, B. Hernández-Ledesma, D. Tomé, C. Gaudichon, and I. Recio. 2018. Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. Food Chem. 239:486–494. https://doi.org/10.1016/j.foodchem.2017.06.134.

Sebastiani, C., C. Arcangeli, M. Ciullo, M. Torricelli, G. Cinti, S. Fisichella, and M. Biagetti. 2020. Frequencies evaluation of β-casein gene polymorphisms in dairy cows reared in central Italy. Animals (Basel) 10:252. https://doi.org/10.3390/ani10020252.

Sousa, R., R. Portmann, S. Dubois, I. Recio, and L. Egger. 2020. Protein digestion of different protein sources using the INFOGEST static digestion model. Food Res. Int. 130:108996. https://doi.org/10.1016/j.foodres.2020.108996.

Sternini, C., S. Patierno, I. S. Selmer, and A. Kirchgessner. 2004. The opioid system in the gastrointestinal tract. Neurogastroenterol. Motil. 16(Suppl 2):3–16. https://doi.org/10.1111/j.1743-3150.2004.00553.x.

USDA. 2010. Dairy 2007, Heifer calf health and management practices on U.S. dairy operations, 2007.

Van Eenennaam, A. L., and J. F. Medrano. 1991. Differences in allelic protein expression in the milk of heterozygous κ-casein cows. J. Dairy Sci. 74:1491–1496. https://doi.org/10.3168/jds.S0022-0302(91)78308-2.

Walther, B., D. Guggisberg, R. Badertscher, L. Egger, R. Portmann, S. Dubois, M. Haldimann, K. Kopf-Bolanz, P. Rhyn, O. Zoller, R. Veraguth, and S. Rezzi. 2022. Comparison of nutritional composition between plant-based drinks and cow's milk. Front. Nutr. 9:988707. https://doi.org/10.3389/fnut.2022.988707.

Wittert, G., P. Hope, and D. Pyle. 1996. Tissue distribution of opioid receptor gene expression in the rat. Biochem. Biophys. Res. Commun. 218:877–881. https://doi.org/10.1006/bbrc.1996.0156.

Wohlers, J. 2011. Ermittlung geeigneter Methoden zur Differenzierung und Qualitätsbeurteilung unterschiedlicher Milchqualitäten aus verschiedenen on-farm-Experimenten. PhD thesis. University of Kassel, Witzenhausen, Germany.

Wohlers, J., and P. Stolz. 2022. The importance of cow-individual effects and diet, ambient temperature, and horn status on delayed luminescence of milk from Brown Swiss dairy cows. Dairy 3:513–527. https://doi.org/10.3390/dairy3030037.