J. Dairy Sci. TBC
https://doi.org/10.3168/jds.2024-24403
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The A1/A2 β-casein genotype of cows, but not their horn status, influences peptide generation during simulated digestion of milk

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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-casein genotype with its variants A1 and A2 was also included in the study. The effects of horn status and β-casein 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-casein genotypes. At the end of the intestinal phase, the digested milk of A1A1 and A2A2 b-casein 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-casein 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 USA, 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 (Alessandro et al., 2018; Casoni et al., 2019); in the long term, horn status affects behavior, stress physiology, and meat quality, including the muscle proteome (Reiche et al. (2019); Reiche et al. (2020a); Reiche et al. (2020b); and reviewed by Knierim et al. (2015)). 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 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, b-lactoglobulin), free amino acids (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 (Bro-Jørgensen, 2007; Kiltie, 2008; 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, that milk of

Received November 7, 2023.
Accepted February 12, 2024.
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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-lactalbumin and b-lactoglobulin), which lead to both a variety of variants for each (Caroli et al., 2009) and to different digestion products (peptides) due to differences in amino acid composition. In recent years, the b-casein polymorphism has gained attention due to its potential impact on human health through milk consumption (Caroli et al., 2016). b-caseins 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 amino acid 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 b-casein variant. Some of these peptides, the so-called β-casomorphins (BCMs), have bioactive, namely opioid, properties that may be associated with undesirable gastrointestinal symptoms in humans (EFSA, 2009). For example, BCMs have been associated with discomfort, delayed gastrointestinal transit time, and possibly even the onset of chronic diseases (EFSA, 2009; Jianqin et al., 2016; 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), −5 (YPFPG), −7 (YPPFGPI), −8 (YPFPGPI P/H), −13 (YPFPGPI P/H NSLPQ), and −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 b-casein 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-casein genotype. Based on the reported importance of position 67 of the β-casein 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/A2 β-casein genotype.

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 1350 m and 2450 m above sea level (Table S1). 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). Therefore, information was available on their genetic indices, genetic percentage of Original Braunvieh (OB), performance, lactation number, and days in milk (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-casein genotype. Samples were analyzed using a commercial SNP array (Swiss Axiom® Microarray, IFN Schönow GmbH, Bernau, Germany). Three β-casein 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), 2007) and multiplied by a factor of 6.38 to obtain the crude protein concentration. Milk fat content was analyzed gravimetrically with the method ISO 2446 (IDF 226:2008) (2008). For samples with volumes below 20 mL (n = 22) fat was analyzed with the ISO 1211:2010 (IDF 1:2010) (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 (SCCs) in the milk were analyzed with the routine method ISO 13366–2:2006 (IDF 148–2:2006) (2006) on a Somacount-FC instrument (Bentley Instruments, Marœuil, France).

Main milk proteins. The 20 major milk proteins were quantified by mass spectrometry 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. SDS-PAGE was performed according to Egger et al. (2016). Briefly, milk samples were diluted 10 times in sample buffer (Tris–HCl 350 mM, pH 6.8, SDS 10%, DTT 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 amino acids were analyzed according to ISO/DIS 4214:2022 (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 (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 sample and 150 mL acetonitrile) on ice for 20 min. Supernatants were separated in UPLC (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 66–1000 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 times 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 3368 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 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

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previously described (Sousa et al., 2020), after quantification of primary amines using the o-phthaldialdehyde (OPA) method in supernatants after gastric and intestinal in vitro digestion. Therefore, gastric samples were diluted 5 times and intestinal samples were diluted 10 times 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/VIS 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 1300 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-casein, α_{s1}-casein, α_{s2}-casein, κ-casein, and β-lactoglobulin) were monitored. To visualize peptide abundance, the individual amino acids 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 amino acid 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 1196.1- > 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 1002.4- > 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 SCCs 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 b-casein genotype and horn status.

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 b-casein genotype (using the lme4 package). The models included the fixed effects of horn status, b-casein 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

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

	β-casein genotype					
	A1A1		A1A2		A2A2	
Horned (H$^+$)	mean	SE	mean	SE	mean	SE
Number of animals	16		24		24	
Age (years)	5.5	0.7	5.2	0.4	5.6	0.4
Days in milk	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 (years)	6.6	0.9	5.8	0.5	5.5	0.4
Days in milk	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.

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, 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, amino acids, and fatty acids of milk samples

The β-casein 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-casein genotype or horn status (Table S2), which is graphically represented in the PCA score plots by the lack of separation between groups (Figure 2). No differences by β-casein genotype or horn status were found in the 20 free amino acids (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 $P < 0.05$; Table S3). Despite these differences in individual fatty acids, the overall fatty acid profile did not differ by β-casein 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 b-casein peptides differed by the b-casein genotype (Fig. S1B), but not by horn status (Fig. 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 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 $P < 0.05$; Fig. 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 b-casein genotypes (A1A1, A1A2, and A2A2) were well separated (Figure 5B), while the horn status groups were overlapping (Figure 5A). The average abundance of each amino acid, identified within a peptide, was also

altered by the b-casein genotype (Figure 6). In particular, the protein regions between the amino acids near position 67 of the A1/A2 mutation and at position 122 of the A1/B mutation differed by b-casein genotype. Differences were also observed in neighboring regions (especially amino acid 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}-casein, a_{s2}-casein, k-casein, and b-lactoglobulin) were not altered by b-casein genotype or horn status (data not shown).

In the intestinal phase, peptide abundances did not differ by b-casein genotype or horn status (Fig. S1C, D), 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 by genotype, horn status, OB percentage, or DIM (all $P > 0.10$). The amount of PVVVPPFL increased with increasing number of DIM ($P < 0.05$).

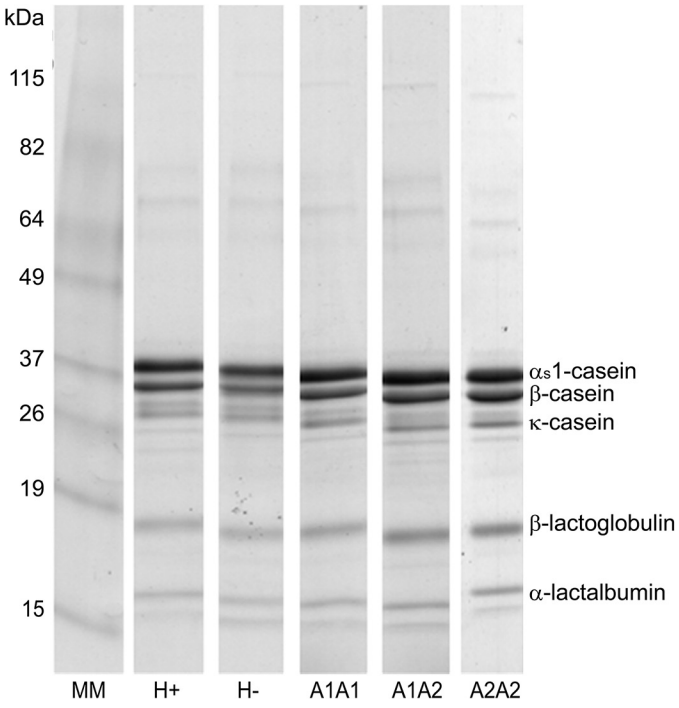

Figure 1. Gel electrophoresis of milk proteins from horned (H+) and disbudded (H-) cows with different β-casein variants A1A1, A1A2, and A2A2. A molecular size marker (MM) was run in the first lane. The bands are labelled with the corresponding identified proteins.

Table 2. Means and effects of milk composition by β-casein genotype and horn status

	β-casein genotype			Horn status			*P*-values			
	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
Somatic cell count (10^{3} cells mL^{-1} milk)	122	132	131	116	145	7.613	0.750	0.291	0.96	0.050

H-: horned cows, H^{+}: disbudded cows; OB: Original Braunvieh; DIM: days in milk.

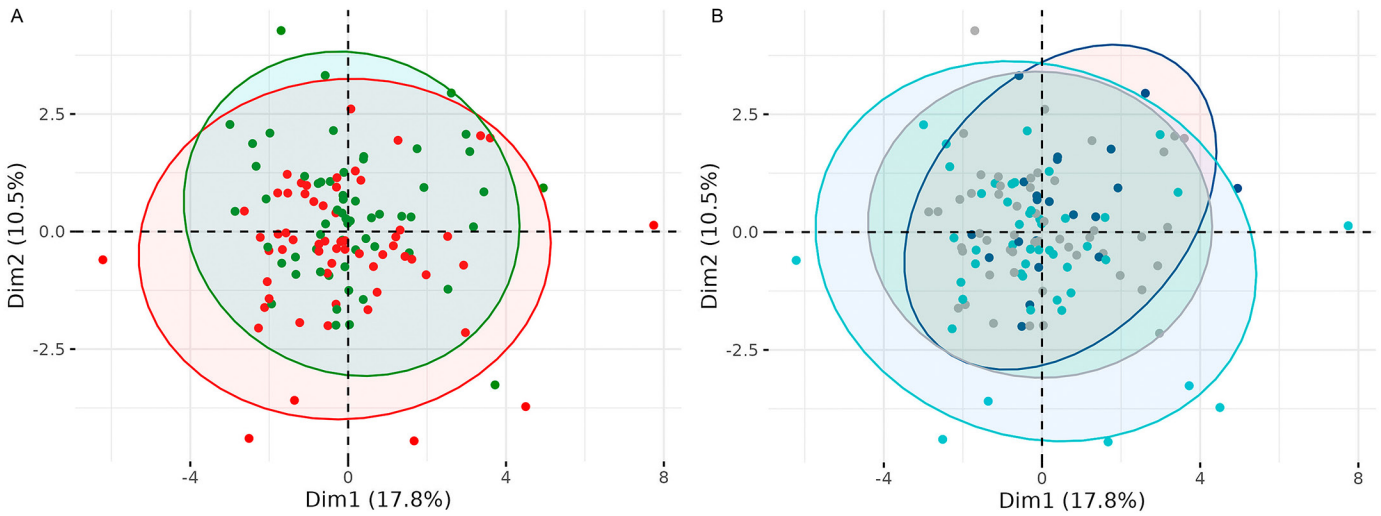

Figure 2. Principal component analysis score plots of milk main proteins. A) Green and red dots represent samples of horned and disbudded cows, respectively. B) Darkblue, grey and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively. Ellipses represent 95% confidence intervals.

Metabolites

Of approximately 23,000 detected features, 523 fulfilled the selection criteria. Sample separation by PLS-DA based on genotype (Fig. S2B) and horn status (Fig. 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; Fig. 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, Fig. S2D), followed by several metabolites that were elevated for farm 3 (2 on component 1 and 4 on component 2, colored in gray, Fig. 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 b-casein genotype on milk composition and digestion with 128 milk samples. To the best of our knowledge, this is the first study to investigate the influences of the 2 factors using a representative number of cows. While horn

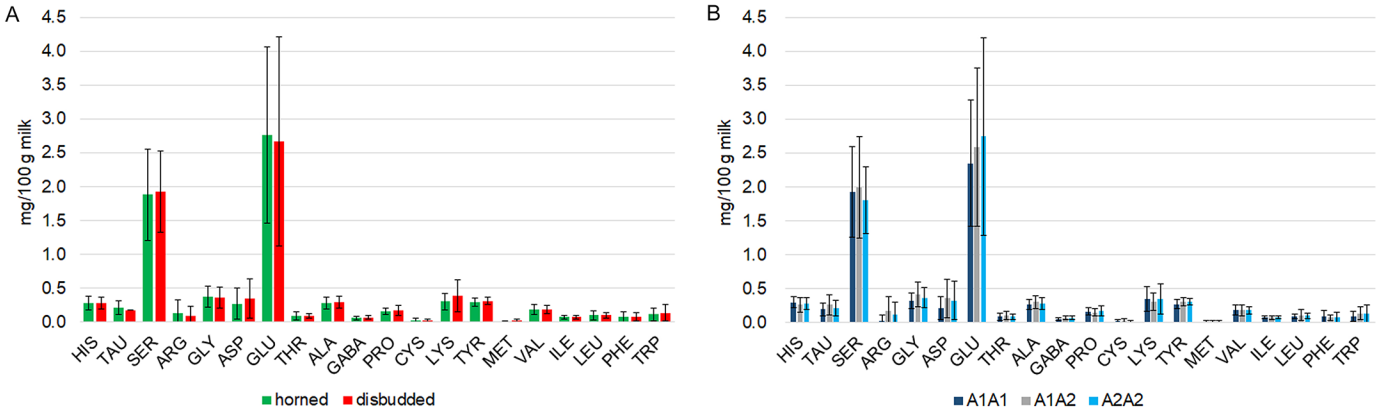

Figure 3. Bar plots of concentrations of free amino acids with error bars representing the standard deviation of the mean. A) Green and red bars represent the means of samples of horned and disbudded cows, respectively. B) Darkblue, grey and turquoise bars represent the means of samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively.

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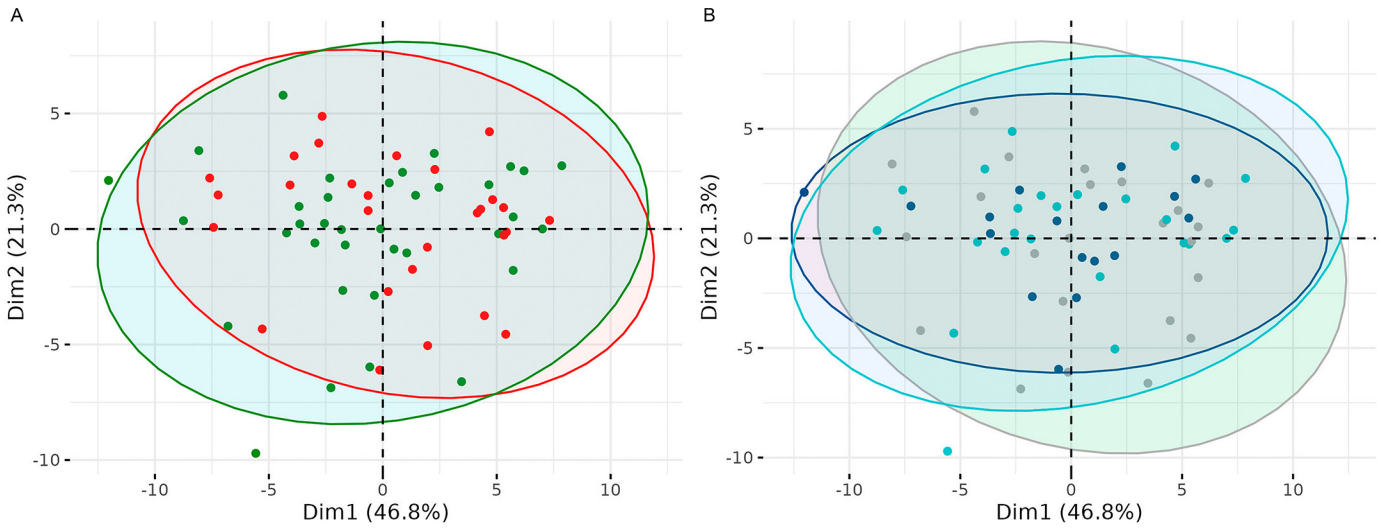

Figure 4. PCA score plots of milk fatty acids. A) Green and red dots represent samples of horned and disbudded cows, respectively. B) Darkblue, grey and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively. Ellipses represent 95% confidence intervals.

status had no measurable effect, the b-casein 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 b-casein was not influenced by the b-casein genotype, as represented by similar degrees of hydrolysis. Milk metabolite profiles did not differ by b-casein genotype but did differ by farm.

Horn status without measurable influence on milk

Horn status had no effect on gross milk composition, which is in agreement with earlier studies (Wohlers, 2011; Baars et al., 2019; Reiche et al., 2023). In contrast to our study, these studies (using pooled milk,

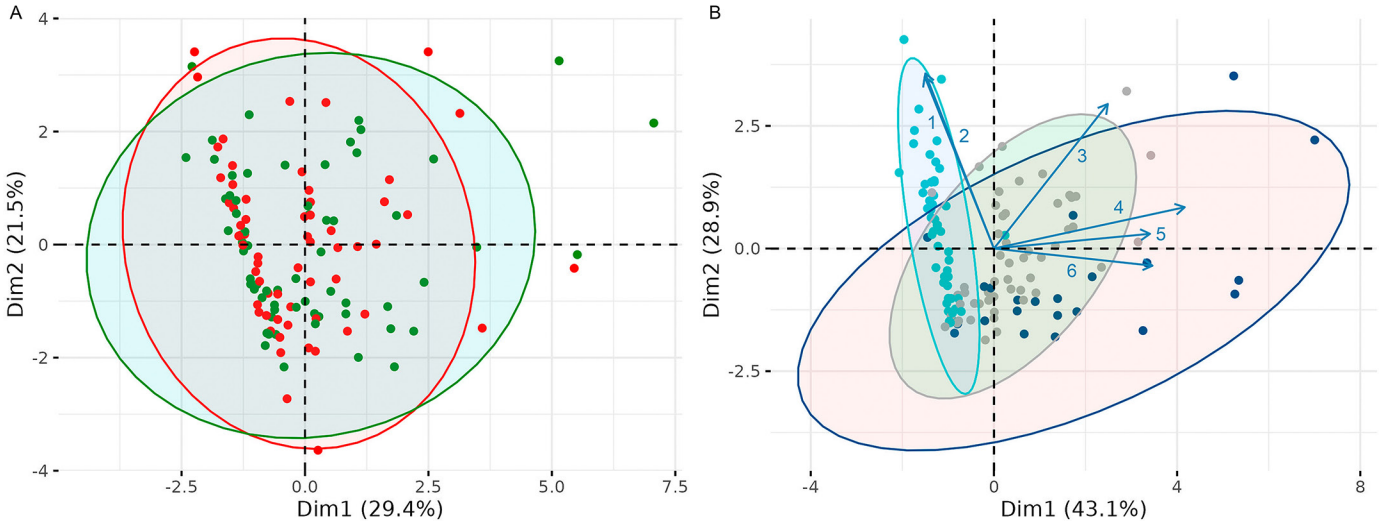

Figure 5. Principal component analysis plots of gastric peptides during in vitro digestion. A) Score plot. Green and red dots represent samples of horned and disbudded cows, respectively. B) Biplot. Darkblue, grey and turquoise dots represent samples of cows of the A1A1, A1A2, and A2A2 genotype, respectively.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

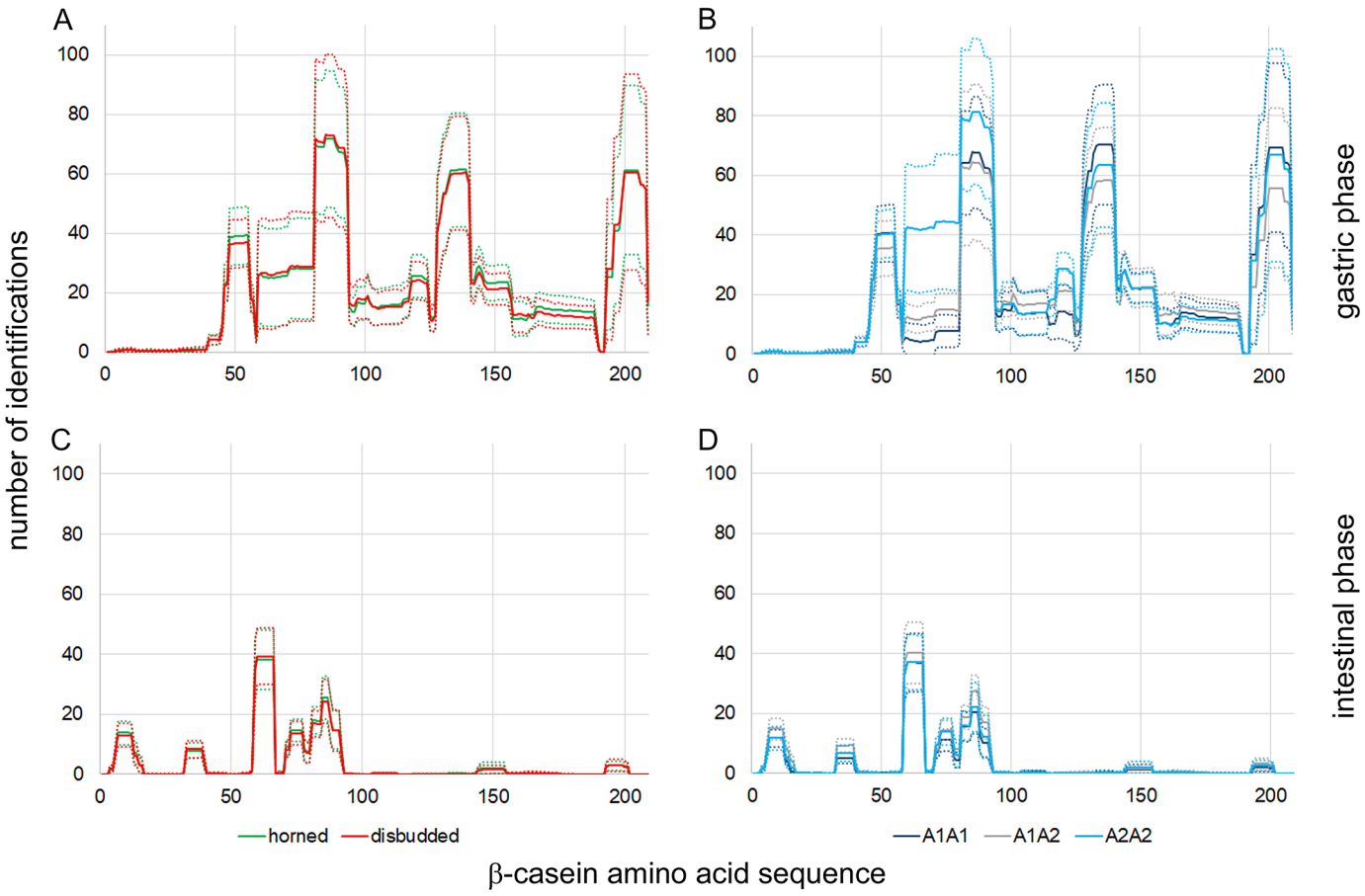

Figure 6. Frequency of peptides during in vitro digestion.

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 amino acids 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.

b-casein 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 b-casein 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 amino acid in position 67 (A1: histidine [His], A2: proline [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 amino acid prefix, the digestion

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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 is in agreement 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 b-casein 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 b-casein (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 amino acid 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 b-casein in the gastric phase and thereby influences the gastric digestibility of the whole b-casein 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 genotype 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-casein variants that may influence the cleavage of peptides. However, the link between b-casein 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-casein variants not considered but perhaps present in this (and in previous) work.

Possible effects of casomorphins on human digestion

According to the literature, BCMs 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 BCMs 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 BCMs differ in their binding capacity to opioid receptors. BCM7 showsed the highest binding capacity compared with other BCMs released during the digestion of bovine β-casein (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 BCMs, 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

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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-casein 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-casein 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-casein genotype indicates that a factor such as nutrition has a visible influence on milk metabolites, while the influences of β-casein genotype and horn status are negligible.

CONCLUSION

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-casein 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 b-casein genotype influenced the digestion pattern of β-casein 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 amino acid 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.

ACKNOWLEDGMENTS

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.

Funding: The authors gratefully acknowledge financial support from Foundation Sur-la-Croix, Basel, Switzerland.

Conflict of interest: The authors have not stated any conflicts of interest.

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