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ROBERTO KAPPES

EFFECT OF β-CASEIN A1 OR A2 MILK ON BODY COMPOSITION, PERFORMANCE, AND SERUM β-CASOMORPHIN-7 IN HOLSTEIN, SIMMENTAL, AND CROSSBRED DAIRY CALVES

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RESUMO

KAPPES, Roberto. **Efeito do leite β-caseína A1 ou A2 na composição corporal, desempenho e β-casomorfina-7 sérica em bezerros leiteiros Holandês, Simental e mestiços.** 2024. 98p. Tese (Doutorado em Ciência Animal -Area: Produção Animal) – Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência Animal, Lages, 2024.

O objetivo deste estudo é comparar os efeitos da alimentação com leite β-CN A1 ou A2 homozigoto em bezerros Holandês Alemão, Simental Alemão e mestiços leiteiros de ambos os sexos, portadores do genótipo β-CN A1A1, A1A2 e A2A2 na composição corporal, consumo de leite, desempenho de crescimento, escore fecal médio, dias com diarreia e níveis séricos de β-casomorfina-7 (BCM-7), durante as duas primeiras semanas de vida. Foram avaliados 104 bezerros (n = 54 fêmeas – f e n = 50 machos – m) dos grupos raciais HA (n = 23), SA (n = 61) e mestiços HA x SA (M; n = 20). Os bezerros foram pesados após o nascimento e receberam colostro ad libitum. No segundo dia, foi coletada amostra de sangue para determinação da proteína sérica total e genotipagem para β-caseína. No segundo dia, os bezerros foram alojados alternadamente aos pares em sistemas de iglu duplo de acordo com a ordem aleatória de nascimento e receberam leite A1 (n = 52; 27 f/25 m) ou leite A2 (n = 52; 27 f/25 m). Foram fornecidos 7,5 litros/dia e registrado o consumo total individual de leite. O consumo diário de leite corrigido para energia também foi calculado com base na composição do leite (gordura e proteína). O escore fecal foi registrado diariamente. No 15° dia, os bezerros foram pesados e o volume do tecido adiposo visceral (TAV) foi avaliado por ressonância magnética (RM) e absorciometria de raio-X de dupla energia (DXA). Além disso, a massa gorda e magra (g), bem como o conteúdo mineral ósseo (g) e a densidade mineral óssea (g/cm²), foram determinados por DXA. Uma amostra de sangue foi coletada para avaliar a concentração sérica de BCM-7. A composição corporal, a ingestão de leite e o crescimento foram semelhantes entre os dois tipos de leite nas duas primeiras semanas de vida. As bezerras fêmeas tinham mais TAV e massa gorda, e menos massa magra que os bezerros machos. Os bezerros HA e M tiveram mais TAV e menos massa magra que os bezerros SA. Os bezerros machos foram mais pesados que as fêmeas após o nascimento e no dia 15. A média de dias com diarreia e ocorrência de diarreia foi semelhante entre bezerros alimentados com leite A1 e A2 e entre ambos os grupos sexuais. Os bezerros SA apresentaram um pouco mais de dias com diarreia e maiores chances de ter diarreia em

comparação aos bezerros HA, não diferindo dos bezerros M. Os níveis séricos de BCM-7 foram semelhantes entre os dois grupos de alimentação, genótipo, sexo e raça, e não teve associação com probabilidade de dias com diarreia. Os bezerros com o genótipo β-CN A2A2 tiveram maior risco de ter mais dias com diarreia em comparação com os bezerros do genótipo β-CN A1A1 ou A1A2. Bezerros com genótipo A1A2 β-CN apresentaram menor média de escore fecal e dias com diarreia do que bezerros A2A2 nas primeiras duas semanas de vida, não diferindo dos bezerros A1A1, o que refletiu em um ganho médio diário ligeiramente melhor para bezerros A1A2 e A1A1. A composição corporal foi similar entre os três genótipos β-CN. Não houve interação entre tipo de leite e genótipo.

Palavras-chave: absortometria de raio-X de dupla energia; consumo diário de leite; escorre fecal; ressonância magnética

ABSTRACT

KAPPES, Roberto. **Effect of β-casein A1 or A2 milk on body composition, performance, and serum β-casomorphin-7 in Holstein, Simmental, and crossbred dairy calves.** 2024. 98p. Thesis (Doctorate in Animal Science – Area: Animal Production) – Santa Catarina State University. Post-Graduation Program in Animal Science, Lages, 2024.

The objective of this study is to compare the effects of feeding homozygous β-CN A1 or A2 milk in German Holstein, German Simmental, and crossbred dairy calves of both sex, carrying the β-CN genotype A1A1, A1A2, and A2A2 on body composition, milk intake, growth performance, mean fecal score, days with diarrhea, and serum levels of β-casomorphin-7 (BCM-7), during the first two weeks of life. A total of 104 calves (n = 54 female – f and n = 50 male – m) from the breed groups GH (n = 23), GS (n = 61), and crossbred GH x GS (CR; n = 20) were evaluated. Calves were weighed after birth and received colostrum *ad libitum*. On the second day, a blood sample was collected to determine the total serum protein and the β-casein genotype. On the second day, calves were alternately housed in pairs in double-igloo systems according to their random birth order and received either A1 milk (n = 52; 27 f / 25 m) or A2 milk (n = 52; 27 f / 25 m). They were offered 7.5 liters/day, and the individual actual total milk intake (TMI) was recorded. Daily energy-corrected milk intake was also calculated based on the milk composition (fat and protein). Fecal scores were recorded daily. On day 15, the calves were weighed and visceral adipose tissue (VAT) volume was assessed by open magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA). In addition, fat, and lean mass (g), as well as bone mineral content (g) and bone mineral density (g/cm^2), were determined by DXA. A blood sample was collected to evaluate the serum concentration of BCM-7. The body composition, milk intake, and growth were similar between the two types of milk in the first two weeks of life. Female calves had more VAT and fat mass, but less lean mass than male calves. GH and CR calves had more VAT and less lean mass than GS calves. Male calves were heavier than female calves after birth and on day 15. The average days with diarrhea and diarrhea occurrence were similar between calves fed A1 and A2 milk and between both sex groups. GS calves presented slightly more days with diarrhea and increased odds of having diarrhea compared to GH calves, not differing from CR. The serum levels of BCM-7 were similar between the two feeding groups, genotype, sex, and breed, and had no association with odds of days with diarrhea. Calves with the A2A2 β-CN genotype had increased odds of having more days

with diarrhea in comparison with A1A1 or A1A2 β-CN genotype calves. Calves with A1A2 β-CN genotype had lower mean fecal score and days with diarrhea than A2A2 calves in the first two weeks of life, not differing form A1A1 calves, which reflected in a slightly better average daily gain for A1A2 and A1A1 calves. The body composition was similar among the three β-CN genotypes. There was no interaction between milk type and genotype.

Keywords: daily milk intake; dual energy X-ray absorptiometry; fecal score; magnetic resonance imaging

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Figure 1. Results of the Logistic regression analysis of the daily Diarrhea records (Group 1: 0-1 day with diarrhea; Group 2: 2-4 days with diarrhea; Group 3: >4 days with diarrhea) for the classes milk type (A1A1 vs A2A2), sex (f=female, m =male), breed (CBREED with CR=crossbred, GS = German Simmental, GH = German Holstein), β-CN calf genotype (GT_CALF A1A1, A1A2, A2A2), combined with the covariates birth body weight (BBW), and concentration of β-casomorphin-7 (CNC_BCM7). The model was chosen due to the lowest AIC. 95

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ABBREVIATIONS LIST

ADG – average daily gain

Arg - arginine

Asn – asparagine

BBW – birth body weight

BCM - β-casomorphin

BCM-5 - β-casomorphin-5

BCM-7 - β-casomorphin-7

BCM-9 - β-casomorphin-9

BCS – body condition score

BFT – backfat thickness

BHBA - β-hydroxybutyrate

BMC – bone mineral content

BMD – bone mineral density

CN – casein

CR / CBREED – crossbred

CT – computed tomography

DMI – dry matter intake / daily milk intake

DMI_ECM - daily energy-corrected milk intake

DPP-IV - dipeptidyl peptidase-IV

DXA - dual X-ray absorptiometry

EBV- estimated breeding values

EBW – end body weight

F – female / fat

F1 - first crossbreeding generation

FS – fecal score

GH – German Holstein

GL – gestation length

Gln - glutamine

Glu - glutamic acid

Gly – glycine

GS – German Simmental

His – histidine

H – Holstein

Ig - immunoglobulin

Ile – isoleucine

J - Jersey

Leu – leucine

LDL-C – low density lipoprotein

Lys – lysine

M – Montbeliarde / male

Met – methionine

MRI – magnetic resonance imaging

NEFA - non-esterified fat acids

OR – odds ratio

Phe – Phenylalanine

Pro – proline

PTA - predicted transmitting abilities

R1 - first backcross generation

S – Simmental

SCC – somatic cell count

SCS – somatic cell score

Ser – serine

STP – serum total protein

TECMI – total energy-corrected milk intake

TMI – total milk intake

US – ultrasound / United States

VAT – visceral adipose tissue

VR – Vicking Red

SUMMARY

INTRODUCTION

Milk and dairy products present high nutritional value, providing essential micro- and macronutrients and indispensable amino acids for human nutrition (Smith et al., 2022). In an assessment of global nutrient availability for the human population, it was determined that milk contains 28 out of the 29 essential nutrients for human nutrition. Additionally, it emerged as one of the top five contributors to 23 out of the 29 essential nutrients (Smith et al., 2022). One of the most important nutrients is protein, which releases a variety of essential amino acids and bioactive peptides (Smith et al., 2022; Stelwagen, 2022; de Vasconcelos et al., 2023). Caseins (CN) are the main milk proteins, corresponding to more than 80% of milk protein, divided into four main fractions: α-s1, α-s2, β, and k-casein (Chitra, 2022; Stelwagen, 2022). β-casein (β-CN) is one of the most important fractions, accounting for almost one-third of the total milk protein content (Stelwagen, 2022). β-CN can be found in 15 different variants (Gallinat et al., 2013; Sebastiani et al., 2020), although the most common are A1 and A2 (Bisutti et al., 2022; Scott et al., 2023). However, nowadays a discussion about the effect of A1 β-CN is taking place as an important cause of non-communicable diseases in humans, mainly because of a peptide released during the enzymatic digestion of the A1 β-CN known as β-casomorphin-7 (BCM-7) (Kullenberg de Guadry et al., 2019; de Vasconcelos et al., 2023).

The effect of A1 and A2 milk in calves was assessed by only one study (Hohmann et al., 2021). Differing from their hypothesis, calves fed A1 milk had a higher daily milk intake (7.28 vs. 6.96 l) and feed efficiency (9.2 vs. 10.5 l/kg) and a tendency for higher average daily gain (750 vs. 640 g) compared with A2, respectively. The authors also found a lower mean fecal score (1.97 vs. 2.56) and diarrhea occurrence (6 vs. 10%) in the first 21 days in calves fed A1 milk compared to calves fed A2 milk, respectively, even though the levels of BCM-7 were five times higher (Hohmann et al., 2021). This is in contrast to the studies on humans, which report an increase in stool consistency and frequency, an increase in plasma concentration of inflammation-related biomarkers, flatulence, bloating, and abdominal pain with the intake of A1 β-CN variant, mainly associated with BCM-7 (Ho et al., 2014; Jianqin et al., 2016; He et al., 2017).

It is expected that the higher milk intake and feed efficiency observed in calves fed A1 milk by Hohmann et al. (2021) had a higher amount of body fat compared to calves fed A2 milk. A high plane of nutrition and high milk replacer intake in early life increases the growth rates and contributes to the development of adipose depots and improves carcass composition in heifers at 21 weeks of life (Keogh et al., 2021). Additional adipose tissue reserves in young calves (less than 3 weeks) is a desirable trait, as when they get sick, the milk intake decreases, and the extra body fat may assist them in recovering from disease (Law, 2022). Furthermore, variations in body composition can be expected among different breeds under the same dietary conditions. This can also be correlated by the different average daily gain (ADG) among breeds, as observed by Hohmann et al. (2021) where German Holstein calves had a higher ADG (710 g) than German Simmental and crossbred calves (650 g). Scholz et al. (2003) reported lower body fat mass and a higher percentage of lean mass in German Holstein calves compared to German Simmental and crossbred calves. Hampe et al. (2005) found significantly higher body fat and lower lean percentages for German Simmental compared with German Holstein between 6 to 50 days of age. The four different crossbred combinations had medium body fat and lean percentages in comparison to the two purebred lines. It can be inferred that different breeds can exhibit variations in fat storage patterns, also associated with the purpose of the breed (milk, meat, or dual purpose).

The effects on calf performance and diarrhea occurrence of carrying A1A1 or A2A2 genotypes are also scarce. In cows, some studies have evaluated the productive and reproductive performance and survival. Heck et al. (2009) and Comin et al. (2008) found lower milk and protein yield in A1A1 cows compared to A2A2. Lu et al. (2020) reported similar values for milk, fat, and protein yield and similar values for reproductive traits between A1 and A2 genotypes. No interaction between CSN2 gene and predicted transmitting abilities (PTA) values for milk, fat, protein, and daughter pregnancy rate was reported by Ardicli et al. (2023). Scott et al. (2023) reported that A1A2 and A2A2 animals had superior estimated breeding values (EBVs) for production traits and inferior EBVs for fertility, health, and survival compared to A1A1 animals, mainly associated with the increased selection and inbreeding in A2 cows. Arens et al. (2023) found similar values for 305d milk, fat, protein yield, and fertility traits between A1A1, A1A2, and A2A2. However, the survival rate to the first and second lactation was higher for A1A2 and lower for A1A1, not differing from A2A2.

The selection for animals producing A2A2 milk has increased in the past years, as the A2 milk market is prominent (Juan and Trujillo, 2022; Ardicli et al., 2023; Arens et al., 2023). To meet this demand, more milk producers will be pushed to produce A2A2 milk (Arens et al., 2023). This intensive selection for A2A2 animals can lead to an increase in inbreeding (Scott et al., 2023), with adverse effects considering productive and reproductive aspects (Arens et al., 2023; Scott et al., 2023) that should be monitored along the years (Ardicli et al., 2023). It is worth noting that A2A2 milk is an important product to meet a specific market of A1 β-CN milk-intolerant consumers and can be selected for specific applications during processing the milk (Gai et al., 2021). For that, the industry should offer incentive payments for farmers to produce only A2A2 milk to meet this specific market (Arens et al., 2023). However, the β-CN variant should not be the only characteristic to be considered for animal breeding.

This study assessed the effects of feeding either homozygous A1A1 or A2A2 β-casein milk and the impacts of carrying the A1A1, A1A2, and A2A2 β-CN genotype on the performance, body composition, diarrhea occurrence, and levels of β-casomorphin-7 during the first two weeks of life in German Holstein, German Simmental, and crossbred dairy calves of both sexes.

CHAPTER I

LITERATURE REVIEW

β -casein and β-casomorphin-7

Bovine milk is composed of 3.2 to 3.5% of total protein. The most abundant protein are caseins, representing 82.2 %, β-Lactoglobulin (9.6 %), and α-Lactalbumin (3.8 %). Caseins are subdivided into four main groups, α-s1, α-s2, β, and k-casein. α-s1 and β are the mainly caseins, representing 31.3 and 29.3 %, respectively; and k and α-s2-caseins represent 10.5 and 8.4 %, respectively, of the total milk protein. α-s1, β, k, and α-s2-caseins represent 38.1, 35.7, 12.8, and 10.2% of total caseins, respectively (Stilwagen, 2022). β-CN is encoded by the CSN2 gene on chromosome 6 and possesses 209 amino acids on its chain (Bisutti et al., 2022). The β-CN alleles are co-dominant, meaning that a cow carrying one copy of each of the A1 and A2 alleles produces A1 and A2 β-CN in equal amounts and is commonly termed an 'A1A2' cow (Arens et al., 2023; Woodford, 2021). Up to now, 15 different β-CN variants were identified in dairy herd, being: A1, A2, A3, A4, B, C, D, E, F, H1, H2, I, G, J, K, and L (Gallinat et al., 2013; Sebastiani et al., 2020), with the most common A2, A1, and B.

Heck et al. (2009) investigated the allele frequency for β-CN in 1,912 first lactation Dutch Holstein Frisian cows from 398 commercial herds in the Netherlands. The authors found an allele frequency of 49.8 %, 46.2 %, and 2.6 % for A2, A1, and B variants, respectively. Sebastiani et al. (2020) investigated the allele frequency of 1,629 Italian Holstein Friesian cows in 17 farms located in central Italy and found 60.6 %, 30.3 %, and 5.6 % for A2, A1, and B variants, respectively. The genotype frequency was 36.9 % for A2A2, 35.7 % A1A2, and 9.8 % A1A1. In north of Italy, Bisutti et al. (2022) investigated 1,133 Holstein cows in 5 different herds and reported an allele frequency of 56 %, 36 %, and 2 % for A2, A1, and B. The genotype frequency was 14 %, 43 %, and 33 % for A1A1, A1A2, and A2A2. Hohmann et al. (2021) investigated 111 German Holstein, Simmental, and crossbred cows in one farm in Germany. They found an allele frequency of 51.6%, 46.5%, and 1.9% for A2, A1, and B variants. The genotype frequency was 11.4 % for A1A1, 68 % for A1A2, and 16.8 % for A2A2. Scott et al. (2023) found a mean genotype frequency of 45.62% with A2A2, 10.51% with A1A1, and

43.87% with A1A2 in Holstein, Jersey, and crossbred cows from Australia. The authors also found that between the years 2000 to 2017, the frequency of the A2A2 genotype increased by 20% in Holstein cows (from 32% to 52%). Arens et al. (2023) found an allele frequency of 65% for A2 in US organic dairy farms, suggesting an increase of the A2 allele through selection across the last 10 to 15 years. Oliveira et al. (2021) evaluated 421 Holstein x Gir crossbred cows in Brazil and found an allele frequency of 28.27% for the A1 allele and 71.73% for the A2 allele. The genotype frequencies were 52.96% (223/421) for A2A2; 37.53% (158/421) for the A1A2 genotype; and 9.50% (40/421) for the A1A1 genotype. Hortolani et al. (2023) found an allele frequency of 0.11 for A1 and 0.89 for the A2 allele in Gir catlle in Brazil. The genotype frequencies were 0.01, 0.19 and 0.80 for A1A1, A1A2 and A2A2, respectively.

The A2 variation is supposed to be the original β-CN, from which the variations A1, A3, E, H1, H2, and I variants originated through a mutation in European herds a few thousand years ago. The variations B, C, D, F and G arose due a mutation in A1 variation, the first one to be discovered (Pal et al., 2015; Brooke-Taylor et al., 2017; Chitra, 2022). Each variant differs from the other variants in terms of an amino acid substitution at a fixed position (Chitra, 2022). The change in the amino acid sequence of β-CN variants is presented in Table 1; adapted from Sebastiani et al. (2020).

Table 1. Change in the amino acid sequence of β-CN variants; adapted from Sebastiani et al., (2020).

β-casein	**Amino Acid Position**								
variant	**36**	**37**	**67**	**72**	**88**	**93**	**106**	**122**	**138**
A2	Glu	Glu	Pro	Gln	Leu	Met	His	Ser	Pro
A1	Glu	Glu	His	Gln	Leu	Met	His	Ser	Pro
A3	Glu	Glu	Pro	Gln	Leu	Met	Gln	Ser	Pro
B	Glu	Glu	His	Gln	Leu	Met	His	Arg	Pro
C	Glu	Lys	His	Gln	Leu	Met	His	Ser	Pro
E	Lys	Glu	Pro	Gln	Leu	Met	His	Ser	Pro
I	Glu	Glu	Pro	Gln	Leu	Leu	His	Ser	Pro
D	Glu	Glu	Pro	Gln	Leu	Met	His	Ser	Pro
F	Glu	Glu	His	Gln	Leu	Met	His	Ser	Leu
G	Glu	Glu	His	Gln	Leu	Met	His	Leu	Pro
H1	Glu	Glu	Pro	Gln	Ile	Met	His	Ser	Pro

H2	Glu	Glu	Pro	Glu	Leu	Leu	His	Ser	Glu

Arg: arginine; Gln: glutamine; Glu: glutamic acid; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Pro: proline; Ser: serine.

The A1 and A2 variants are distinguished solely by an amino acid difference at the position 67. The A2 variant has a Proline and A1 has Histidine (Brooke-Taylor et al., 2017; Chitra, 2022). During digestion in the small intestine, the enzymatic actions of pepsin and leucine aminopeptidase release the tyrosine residue in the amino terminal, by cleaving the Val59-Try60 peptide, and pancreatin cleaves Ile66-His67 in A1 β-CN milk (de Vasconcelos et al., 2023). This short chain of 7 amino acids (Tyr60 -Pro61 -Phe62 - Pro63 - Gly64 - Pro65 - Ile66) released during this process is known as β-casomorphin-7 (BCM-7; Figure 1), a bioactive peptide, with opioid characteristics similar to morphine (Kullenberg de Guadry et al., 2019; Chitra, 2022; de Vasconcelos et al., 2023). However, the release of BCM-7 during digestion is not exclusively from the A1 β-CN milk. Recently, Asledottir et al. (2017, 2018) found BCM-7 after 65 and 180 minutes of ex-vivo digestion of A2 milk, although in very low amount compared to A1, F, and I variants. Hohmann et al. (2021) also reported the presence of serum BCM-7 in dairy calves after 2-3 hours after A2 milk intake, although, the values were almost 5 times lower than A1 milk. The peptide bond between Ile66-Pro67 in the A2 variant is more resistant to proteases during digestion, however the cleavage occurs between Asn68- Ser69 (Figure 1), releasing a nine amino acid long peptide known as β-Casomorphin-9 (BCM-9; Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66-Pro67-Asn68) (de Vasconcelos et al., 2023). BCM-9 is also an opioid, however, it is considered as a potential beneficial bioactive compound with antihypertensive and antioxidant properties (Woodford, 2021).

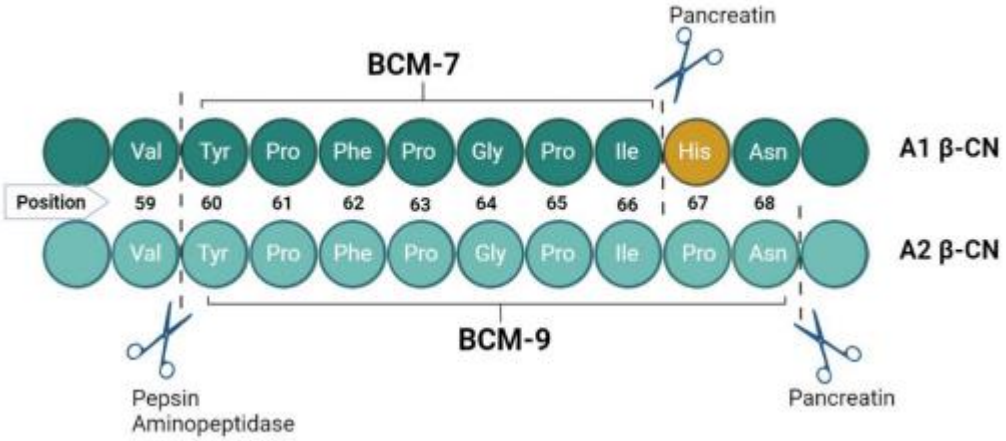

Figure 1. Amino acid sequence of A1 and A2 β-CN, cleavage sites, and release of BCM-7 and BCM-9 (de Vasconcelos et al., 2023).

The BCM-7 is normally broken down from the N-terminus, with the dipeptidyl peptidase-IV (DPP-IV) enzyme removing the dipeptide that contains proline (Pro61) in N-terminal peptides (Woodford, 2021; de Vasconcelos et al., 2023). As the C-terminus is hardly broken down (Gly64-Pro65) because of the resistance to enzymatic degradation, shorter-chain casomorphins like BCM-5 are of limited practical importance, although it has a higher opioid activity (Woodford, 2021). The high levels of BCM-7 or BCM-5 in the plasma of allergic individuals indicate that the DPP-IV activity is lower (Thiruvengadam et al., 2021). When not broken down, the BCM-7 peptide activates μ-opioid receptors that are expressed mainly throughout the central nervous system, gastrointestinal tract, bladder, and the cells of immune and endocrine system (de Vasconcelos et al., 2023) leading to different effects that include analgesia, sedation, reduction of blood pressure, nausea, decreased respiration, decreased bowel motility by reducing the frequency and amplitude of intestinal contractions, mucus and hormone production, and others (Pal et al., 2015; Kullenberg de Guadry et al., 2019; Chitra, 2022).

Kullenberg de Guadry et al. (2019) summarize in their review several studies presenting the effect of A1 milk and the occurrence of health alterations. Four of 8 studies reported a significant correlation between A1 milk intake and the incidence of type-1 diabetes. Three of seven studies did not observe effects of A1 or A2 milk on the evaluated cardiovascular parameters. One study showed higher plasma LDL-C concentrations and three studies correlated with a higher mortality due to cardiovascular disease through increasing A1 milk consumption compared to A2. Neurological alterations were reported in two studies. One study reported higher urinary levels of BCMs after A1 milk vs A2

milk consumption in autistic children, while age-matched neurotypical children had almost no BCM in their urine. In the same study a positive significant correlation between A1 β-CN exposure and mortality due to neurological disorders was reported. The other study reported slower response times and higher error rates of information processing in 45 healthy adults after 2 weeks of A1 milk intake compared with A2 milk intake. de Vasconcelos et al. (2023) also summarized 3 different studies evaluating BCM-7 in autistic children compared to neurotypical children. Two of the three studies reported higher BCM-7 in autistic children compared to neurotypical children and one study showed the presence of a gene associated to the BCM-7. However, the three studies did not make any dietary intervention. Kullenberg de Guadry et al. (2019) also observed that two of three studies presented some gastrointestinal alterations in participants consuming A1 milk compared to A2. Ho et al. (2014) evaluating participants consuming A1 or A2 milk for 2 weeks, reported an increase in stool consistency (Bristol stool scale) for A1 participants (3.87) compared to A2 (3.56), with no stool frequency difference. In participants with self-reported milk intolerance, higher rates of flatus, bloating, abdominal pain, and voiding difficulty was reported by participants consuming A1 milk compared with A2 milk. Jianqin et al. (2016) found a stool consistency of 4.42 and 4.35 in participants consuming A1A2 milk in their first and second trial period, respectively, after 2 weeks intake. For participants consuming A2A2 milk, the values were lower; 4.05 and 4.08 in their first and second trial period, respectively, after 2 weeks intake. The mean stool frequency in participants consuming A1A2 milk was 10.6 times a week, while in participants consuming A2A2 milk was 8 times a week. The whole gastrointestinal transit time after a 2-week intake of A1A2 milk increased by 6.3 hours in comparison with A2 milk. The authors also found an increase on plasma concentration of inflammation-related biomarkers as IL-4, IgG, IgE, and IgG1 in consumers of A1A2 milk. Higher BCM-7 levels were also reported, however, the results were not presented. He et al. (2017) evaluated 600 Chinese adults with self-reported lactose intolerance consuming milk containing A1A2 β-casein versus A2A2 β-casein. All patients consuming A2A2 milk reported lower occurrence of borborygmus, flatulence, bloating, abdominal pain, stool frequency, and stool consistency, compared to A1A2 after 1, 3, and 12 hours after intake. The symptoms were also lower in participants with confirmed lactose intolerance, when consuming A2A2 milk. The authors conclude that A2A2 milk attenuated acute gastrointestinal symptoms of milk intolerance, and A1A2 milk reduce lactase activity and

increased gastrointestinal symptoms. In many cases, the milk intolerance is due ingestion of A1 β-casein rather than lactose. Pal et al. (2015) describe some mechanisms relating to the interaction between lactose intolerance and the effects of BCM-7. First, the inflammation by BCM-7 may affect lactase production and activity, exacerbating the symptoms of lactose accumulation on the gut. Second, colon inflammation affects the processing of malabsorbed lactose, possibly via changes in the gut microbiota that occur with gut inflammation, and third; a delay of the gastrointestinal transit increases the lactose fermentation.

In 2009, the European Food Safety Authority published a review on the association between A1 milk intake or BCMs and various diseases, such as sudden infant death syndrome, autism, increased levels of LDL, heightened risk for atherosclerosis, cardiovascular disease, and type 1 diabetes. Several studies have reported or suggested these connections. The authors conclude that, for all the aforementioned diseases, the results are inconclusive or not convincing due to a lack of clear evidence indicating a relationship. The authors attribute this uncertainty to the multifactorial nature of these diseases, and some studies even present evidence supporting the opposite conclusion (Noni et al., 2009). Kullenberg de Guadry et al. (2019) conclude in their review, above mentioned, that A2 β-CN compared with A1 provides some benefits to digestive health, but low or very low certainty for other health benefits, so the results are still inconclusive. More recently, Daniloski et al. (2021), in their systematic review, evaluated 19 randomized controlled studies in human and animals reporting several health alterations such as gastrointestinal tract problems, neurological disorders, pulmonary inflammation, diabetes, and cardiovascular diseases associated to the A1 milk intake or serum BCM-7. The authors reported that the A2 milk can have some beneficial effects on the gastrointestinal tract, but it does not support the idea that A1 milk cause a negative effect in human health. In addition, the studies evaluated had inadequate clinical analysis, short intervention trials, and an insufficient number of participants, which hindered a detailed analysis of the findings.

Although some studies associate the consumption of A1 β-CN or the presence of BCM-7 with the occurrence of some diseases, other issues should be considered, as the most commercially available milk worldwide contains a mix of A1 and A2 β-CN (Juan and Trujillo, 2022). So, it seems that the adverse effects of A1 β-CN or BCM-7 are not correlated with the entire population, and individual health conditions may trigger

different physiological responses under the effect of BCM-7 (de Vasconcelos et al., 2023). The intestinal microflora and association with individual health conditions must be considered to understand the different responses to the A1 β-CN or BCM-7 exposure (de Vasconcelos et al., 2023). The animals health and performance, mainly in calves, must also be considered as they are dependent exclusively of milk on the first weeks of life.

Hohmann et al. (2021) is the only study evaluating the effect of A1 and A2 β-casein in dairy calves' performance, fecal score, and diarrhea occurrence. The authors reported higher daily milk intake (7.28 vs. 6.96 l) and a tendency for higher average daily gain (750 vs. 640 g; P = 0.07) for calves fed A1 milk compared with A2, respectively. The calves fed A1 milk presented better feed efficiency, consuming 1.3 l less of milk for every kg of body weight gain (10.5 vs 9.2 l for A2 and A1, respectively). The serum BCM-7 level was almost 5 times higher in calves fed A1 than A2 milk, however, the mean fecal score was 1.97 and 2.56, and the diarrhea occurrence was 6 and 10 %, respectively, on the first 21 days of life. Umbach et al. (1985) found plasma BCM-7 in 20 of 24 calves after the first milk intake, the authors suggest an important role of this peptide in gastrointestinal motility and to prevent a possible withdrawal syndrome. However, the β-casein milk offered was not reported. The lower fecal score and diarrhea occurrence in calves fed A1 could be possibly related to the antidiarrheal effect of BCMs, which stimulate electrolyte and water absorption and inhibition of intestinal motility (Noni et al., 2009; Fosset and Tomé, 2022). Hohmann et al. (2021) observed similar rectal body temperature (38.94 ± 0.06 and 38.96 ± 0.05 °C) between A1 and A2 groups, respectively. The respiratory frequency also did not differ between the two groups (P = 0.13), but it was slightly lower for A1 compared to A2 (46.60 ± 3.64 and 53.10 ± 3.50) as proposed by Pal et al. (2015) and Kullenberg de Guadry et al. (2019) in humans.

Although further investigations about health issues on A1 and A2 milk are still necessary (Noni et al., 2009; Kullenberg de Guadry et al., 2019; Daniloski et al., 2021), a growing trend towards the selection of only A2 homozygous animals is taking place, as the A2 milk market is prominent (Juan and Trujillo, 2022). However, all aspects about A1 and A2 milk must be considered before a targeted selection, and calves and cows' performance is an important aspect to be considered. Heck et al. (2009) reported lower milk protein yield in A1A1 cows compared to A2A2, with intermediate values for A1A2, resulting probably from a decrease in milk yield. Comin et al. (2008) also observed

favorable milk and protein yield with the presence of A2 variant instead of A1. The percentage of protein and fat is not affected by the β-CN variant (Ikonen et al., 2001; Heck et al., 2009). Juan and Trujillo (2022) found similar values for total solids and protein content between A2 and a mix of A1 and A2 milk. Lu et al. (2020) reported similar values for milk, fat, and protein yield and similar values for reproductive traits between A1 and A2 genotype. Arens et al. (2023) did not observe differences between A1A1, A1A2, and A2A2 for 305d milk, fat, and protein yield and somatic cell score for Holstein cows in US organic herds. The same was observed for fertility traits such as days open, first service conception rate, and number of times bred per pregnancy. However, the survival rates for the first and second lactation were higher for A1A2 and lower for A1A1, not differing from A2A2. Olenski et al. (2010) estimated the breeding value of milk performance traits of Polish Holstein– Friesian bulls for β-CN. The authors found, significantly ($P < 0.028$), an estimated EBV advantage of 47.65 (±20.480) kg for milk yield and of 2.08 (±0.547) kg for protein yield in favor of the A2 allele, while A1 had a genetic advantage of 0.031 (±0.014) in the fat percentage in comparison with A2. Scott et al. (2023) observed that animals carrying one or two copies of the A2 allele had superior estimated breeding values (EBVs) for production traits and inferior EBVs for health and fertility traits compared to A1A1 animals. A2A2 cows had an advantage of 0.2 genetic standard deviations for milk yield and 0.24 genetic standard deviations for protein yield compared to A1A1, while A1A1 were by 0.2 genetic standard deviations superior for fertility, 0.15 for somatic cell count, and 0.04 for survival compared to A2A2. The authors associated this with the increased selection and inbreeding depression in A2A2 cows. In Germany, Mugambe et al. (20240 estimated the inbreeding values in 24,489 Holstein German cows using different methods. The mean inbreeding ranged from −0.003 to 0.243. In this study the β-CN genotype was not considered in the analysis.

About the milk physicochemical properties, Jensen et al. (2012) reported that the A2 variant was associated with poorly coagulating characteristics, while the B variant was observed only in milk with good coagulation ability. A2 milk presented longer rennet coagulation time and lower curd firming rate compared to A1 milk, suggesting poor coagulation properties (Poulsen et al., 2013). Gustavsson et al. (2014) show that A1A2 and A2A2 β-CN variants are associated with poor gelation properties in Swedish Red cows' milk, and a selection towards A1A1 would have a favorable effect on the milk rennet ability. Marzilali and Ng-Kwai-Hang (1986) observed higher cheese yield with

A1A1 milk than A1A2. Vigolo et al. (2023) evaluated the cheese yield with different proportions of A1 and A2 milk. In the first 48 hour, the addition of ≥ 50% of A2 milk to the mix significantly worsened the cheese yield. The whey composition of the 100% A1 milk had lower protein and fat content, confirming more efficiency in the cheese yield. However, Comin et al. (2008) did not find a difference in coagulation time and curd firmness between A1 and A2, but the presence of at least one allele B of β-CN improves the coagulation properties of the milk. On the contrary, Juan and Trujillo (2022) found similar clotting time between A2A2 milk and A1A2, however, A2A2 milk had higher curd firmness, gel density, and potential cheese yield than A1A2 milk.

The coagulation traits are strongly associated with CSN3 gene (k-CN) whereas the CSN2 (β-CN) is more associated with milk and protein yields (Comin et al., 2008). Although A2 and A1 milk present some slight differences in some technological parameters, it is possible to create dairy products in a similar way with both variants (Juan and Trujillo, 2022). Although some studies reported lower coagulation traits, the A2 variant can be beneficial for specific markets due to its suitability for yogurt production or just whole milk (Gai et al., 2021), attending people with allergies to the A1 milk for example.

No studies – to the present knowledge - have evaluated A1 and A2 genotypes effects on calf performance.

Holstein, Simmental, and Crossbred

Crossbreeding between Holstein (H) and the dual-purpose breed Simmental (S) is a recent practice in Brazil. The first study reporting the crossbred system was published by Knob et al. (2016) who found a better calving interval (381 ± 8.7 vs 445 ± 5.7), calving to first service interval (65 ± 3.2 vs 89 ± 2.5), conception rate (37,3% vs 33.6%), and longevity for first crossbreeding generation (F1) H x S crossbred cows, compared to pure H, respectively. In another study from Knob et al. (2018), F1 H x S crossbred cows had higher milk yield (31.95 vs 30.55 kg/d) and better milk composition ($P < 0.05$), and lower somatic cell score (2.81 vs 4.46) than pure H, respectively. More recently, Knob et al. (2019) reported a lower calving interval for first backcross generation (R1) S and F1 compared with pure H, not differing from R1 H (386, 393.9, 438, and 420.6 days, respectively), same for calving to first service interval (79.8, 75.1, 87.9, and 80.2 days, respectively), and conception rate (44.7, 38.6, 33, and 38.9 %, respectively), with no

difference for energy corrected milk yield and somatic cell score. In another study, there was no difference for milk yield (44.5 vs 43.8 kg/d), composition (P > 0.1), dry matter intake (DMI; 24.51 vs 24.11 kg/d), and feed efficiency (1.82 vs 1.83 l of milk/kg of DMI) when comparing pure H and F1 H x S crossbred cows, respectively (Knob et al., 2023).

In Germany, or in Europe generally, the practice of crossing between Holstein and Simmental is slightly more common and has been performed for a long time, as these breeds originate there. Several studies performed there have been reporting satisfactory results in utilizing crossbred cows for milk yield, to improve milk composition, and fertility (Schichtl, 2007; Freyer et al., 2008; Brähmig, 2011; Nemes et al., 2012; Nolte, 2019; Knob et al., 2021a). In Germany, Schichtl (2007) reported that H x S cows did not differ from H cows for milk yield (7.93 vs. 8.19 kg per milking) and fat content (3.71 vs. 3.54 %), respectively, but showed a higher protein content (3.53 vs. 3.39 %, respectively). Knob et al. (2021a) did not observe differences in milk yield and fat content among pure H and S and their crosses. Brähmig (2011) reported lower SCC for H x S cows in comparison with pure H (104.000 x 250.000, respectively). In Serbia, Nemes et al. (2012) also did not observe differences in milk yield but observed higher fat content for crossbred H x S cows. Schichtl (2007) observed a lower calving interval (393 x 422 days) and a higher conception rate in the second calving 27.5 x 23.8 % for H x S cows than for pure H cows. de Haas et al. (2013) also reported a lower calving interval for H x S cows (392 days) compared to pure H cows (422 days).

Concerning the body conformation and energetic metabolites, Knob et al. (2016) did not observe a body weight difference between pure H (640.6 kg) and F1 H x S (651.7). However, F1 H x S had higher body condition scores (BCS) than H cows along the entire lactation (3.63 vs 2.94, respectively). After calving, F1 H x S cows showed an increase of the BCS until the end of the lactation, while H cows declined in BCS after calving (Knob et al., 2016). In a further study, evaluating the middle of lactation, Knob et al. (2023) reported higher body weight and BCS for F1 H x S than H cows, with no difference for β-hydroxybutyrate (BHBA). During the prepartum and postpartum periods, F1 H x S cows had higher BCS (4.13 and 3.64, respectively) than H cows (3.59 and 2.86, respectively), with no difference for BHBA (Knob et al., 2021b). During the first 150 days of lactation, S and R1 S cows had higher BCS than H, R1 H, and F1 H x S. However, the backfat thickness (BFT) was also higher for S and R1 S, not differing from H and F1; while R1 H had lower BFT but not differing from H and F1 cows. The BHBA and non-

esterified fat acids (NEFA) were lower for S cows compared with the other genetic groups (Knob et al., 2021a). In crossbreds with another dual-purpose breed (Brown Swiss), Blöttner et al. (2011) observed higher body weights for Holstein x Brown Swiss cows during the first and second lactation (620.8 ± 6.5 and 677.7 ± 7.0 kg, respectively) compared to H cows (593.9 ± 7.0 and 655.7 ± 7.5 kg, respectively). The BFT only differed during the first lactation, being higher for Holstein x Brown Swiss than H cows (18.2 ± 0.6 vs. 15.8 ± 0.7 mm, respectively). Phillips et al. (2017) conducted a comparative analysis of carcasses from Holstein, Holstein x Montbéliarde x Viking Red, and Normande x Jersey x Viking Red crossbred steers at approximately 490 days of age. No difference was found in fat percentage in the kidney, pelvic, and heart regions. Additionally, the marbling score and BFT were also found to be similar among the three genetic groups.

Calf performance

The studies evaluating the calf performance comparing the purebred Holstein with other milk-specialized breeds are still limited. However, Heins et al. (2010) compared purebred Holstein (H) calves with F1 Montbeliarde x Holstein (M x H) calves from multiparous H cows for gestation length (GL) and body weight (BBW) at birth traits. The GL of M x H calves was 4.8 days longer than H calves (278.4 ± 0.4 vs. 283.2 ± 0.5, respectively). The authors suggest that this longer GL influenced the BBW of M x H calves, which were 5 kg heavier than H calves (48.3 ± 0.5 vs. 43.3 ± 0.4 kg, respectively). Nevertheless, there were no differences for calving difficulty and stillbirth. The authors also evaluated the same traits by comparing pure H and 3-breed rotational crossbred M x (H x Jersey -J) calves from primiparous and second and third-calving cows. MHJ crossbred calves from primiparous cows had 2.6 days longer GL compared with purebred H (280.3 ± 0.7 vs. 277.7 ± 0.8 days, respectively), however, this longer GL did not affect the BBW. Indeed, MHJ calves were lighter than H calves (37.6 ± 0.6 vs. 38.9 ± 0.6 kg, respectively). Calves from dams with two or three calvings did not differ in BBW (42.5 ± 0.6 vs. 41.9 ± 0.6 kg for H and MHJ, respectively); but they differed for GL, being 3.6 days longer for MHJ. Pereira et al. (2022) also found a longer GL for a 3-breed rotational crossbred between Viking Red – VR x (M x H) in both primiparous and multiparous cows (280 ± 0.23 and 281 ± 0.19 days, respectively) compared to H (278 ± 0.24 and 279 ± 0.20 days, respectively). The crossbred calves from primiparous and multiparous cows were

0.9 and 1.1 kg heavier than pure H (40.3 ± 0.23 and 44.3 ± 0.25 kg vs. 39.4 ± 0.25 and 43.2 ± 0.25 kg, respectively). Although there are some differences in BBW between purebreds and crossbreds, Hazel et al. (2017) did not observe differences in age at first calving, being 23.9 months for H, 23.8 for M x H, and 23.7 months for VR x H, combined with similar GL.

In Brazil, Knob et al. (2018) compared the development of H and F1 (H x S) crossbred calves from birth up to an age of 24 months. The authors observed similar BBW (46.9 vs. 45.8 kg) and at 24 months of age (496.1 vs. 480.7 kg) for H and H x S, respectively. Hohmann et al. (2021) reported a BBW of 44.3 ± 3.05 kg for German Holstein (GH), 43.7 ± 6.51 kg for German Simmental (GS), and 41.1 ± 6.37 kg for the crosses between these two breeds (CR). At an age of 21 days, the calves weighed 59.7 ± ± 4.77, 57.8 ± 7.70, and 55.2 ± 3.77 kg, respectively. The authors observed an ADG of 710 ± 22 g for GH and 650 ± 16 g for GS and GH x GS. Scholz et al. (2003) evaluated GH, GS, and different crossbreeding types at an average age of 28 days. GH and R1 GH calves were lighter (50.3 ± 0.95 and 50.8 ± 1.20 kg, respectively) than R1 GS and F1 GS x GH (56.9 ± 2.13 and 54.9 ± 0.90 kg, respectively); GS and F1 GH x GS presented intermediate values (52.9 ± 0.96 and 52.7 ± 0.78 kg, respectively). Hampe et al. (2005) evaluated the same breeding type. The authors observed that F1 GS x GH, R1 GS, and GS were heavier (54.6 ± 0.7, 54.0 ± 0.9, and 53.2 ± 0.6 kg, respectively), than F1 GH x GS, R1 H, and GH (52.6 ± 0.6, 52.0 ± 0.8, and 50.4 ± 0.6 kg, respectively).

Body composition

Evaluating GH, GS, and CR calves in a dual X-ray absorptiometry equipment from 4 to 50 days of live, Scholz et al. (2003) reported that the GH calves showed a lower body fat content and higher lean content than CR calves, not differing from GS. GH calves also had a significantly lower bone mineral density (BMD- g/cm²) and bone mineral content (BMC- %) compared to the other lines, with the highest values for these traits found in offspring calves of GS bull and F1 dam. The authors did not observe differences for fat content (4.04 vs 4.05%), lean content (92.08 vs 92.06%), BMC (3.88 vs 3.88%), and BMD (0.96 vs 0.97 g/cm²) between male and female calves, respectively. Hampe et al. (2005) reported significantly higher body fat and lower lean percentages for GS (4.53 and 91.65%, respectively) compared with GH (4.17 and 92.18%, respectively) between 6 to 50 days of age. The four different crossbred combinations between GS and GH had

medium body fat and lean percentages in comparison to the two purebred lines. GH presented lower BMD and BMC compared with the other breed lines. These are the only two studies evaluating body composition comparing Simmental and Holstein calves, both using dual energy X-ray absorptiometry.

Imaging methods in animal production/breeding

The use of non-invasive procedures to measure body composition has gained more interest in recent years. Predicting *in vivo* body composition or carcass composition is important for selection in breeding programs or studies on growth performance, nutrition, housing, and behavior or farm animal welfare (Scholz et al., 2015). Traditionally, the body composition is assessed in slaughtered animals by manual dissection and chemical analysis. However, these procedures, are destructive, high-cost and time-consuming (Sanchez et al., 2022; Lobo et al., 2023). Although these methods are precise and considered a reference standard, it is not possible to monitor further growth (Chapman et al., 2017).

Currently, image-based techniques such as dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound imaging (US) are being applied for body and carcass evaluations (Scholz et al., 2015; Sanchez et al., 2022). These techniques use electromagnetic or mechanical energies, which are able to pass completely or partially through body tissues such as muscle, adipose tissue, and bone. The energy interacts with tissues at the atomic or molecular level, resulting in secondary or attenuated signals detected by the instruments and analyzed quantitatively to measure, for example, tissue depths, areas, volumes, or distributions of fat, muscle and partly bone or bone mineral (Scholz et al., 2015).

DXA - dual-energy X-ray absorptiometry

Body composition measured with DXA is divided into three compartments: mass in g or percentage of soft tissue (fat + lean soft tissue) and bone mineral tissue. Soft Lean tissue is composed of all non-fat tissues and all non-bone mineral tissues. The sum of bone mineral and soft tissues provides the total tissue mass or the live body weight for *in vivo* measurements (Figure 2) (Pietrobelli et al., 1996; Danesh Mesgaran et al., 2020). DXA measurements are based on the mass attenuation of X-ray photons passing through

a body (*in vivo*) or a carcass (*post mortem*). Every type of tissue or element in the body is characterized by specific mass attenuation coefficients, which also depend on the photon energy level applied for measurement. Two different X-ray photon energy levels are applied, high and low. High X-ray attenuation coefficients (R values) define bone (mineral), and lower ones define soft tissue (lean and fat tissue) (Scholz et al., 2015; Danesh Mesgaran et al., 2020). "The ratio (by using the natural logarithm = ln) of the attenuated (l) and the initial X-ray photon number (l_0) for the low (L) and the high (H) energy levels provides the so-called R value (X-ray attenuation coefficient). This R value is – depending on the energy levels used – a unique trait for a certain element or compound tissues, such as bone mineral, soft, lean or fat tissues" (Scholz et al., 2015). Usually, immediately after the scan is completed, the result of the whole-body analysis is available. A regional analysis can also be performed by manually defining the region(s) of interest (Danesh Mesgaran et al., 2020).

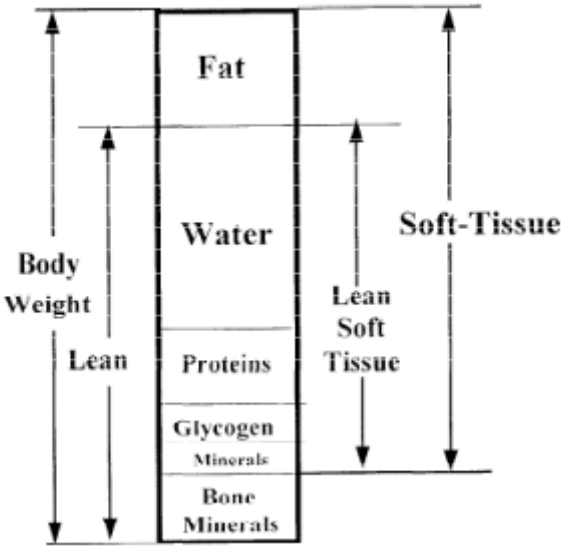

Figure 2. Model of body composition based on DXA evaluations. (Pietrobelli et al., 1996).

Several studies have reported the use of DXA to estimate live body composition in pigs (Mitchell and Scholz, 1998; Mitchell et al., 2001; Scholz et al., 2007; Rothammer et al., 2017; Bernau et al., 2020; Weigand et al., 2020), sheep/lamb carcasses (Scholz et al., 2012; Miller et al., 2018) and calves/calf carcasses (Scholz et al., 2003; Hampe et al., 2005; Lobo et al., 2023). Scholz et al. (2012) found a higher accuracy in pigs, followed by turkeys, lambs, and finally calves. "Whole body analysis in sheep and calves *in vivo* is

particularly strongly affected by the ruminant gastrointestinal tract leading to lower relationships between DXA body composition and reference measures" (Scholz et al., 2012). Lobo et al. (2023) found a lower correlation between DXA and chemical analyses for carcass fat in veal; it might be due to the animals' meat was almost fat-free. The relatively low absolute amount of fat leads to relatively large errors in percentage values for lean and fat tissues (Scholz et al., 2015). However, DXA can predict carcass and lean tissue weight, and mineral and protein content in veal (Lobo et al., 2023).

MRI – magnetic resonance imaging

Magnetic resonance imaging (MRI) is a non-invasive technique that maps internal body structures (Katti et al., 2011). It is "based on signal intensity differences of body tissues, such as fat, muscle or bone, dependent on the electro-magnetic proton-proton or proton-non-proton interaction, and differences of the proton densities, defined as volume (in mm^3) or area (mm^2) of the tissue or organ of interest" (Danesh Mesgaran et al., 2020).

"Generally, MRI measurements are based on the electro-magnetic properties of nuclei with an uneven number of protons and neutrons characterized by an 'electro-magnetic' spin of the nuclei under investigation. The defined nuclei (e.g. hydrogen = 1H = proton) precess with a defined frequency (Larmor frequency) in a static magnetic field with a field strength between, for example, 0.2 and 7.0 Tesla along the field lines in a parallel or anti-parallel direction. A defined radio (wave) signal matching the 'resonance' frequency of the nucleus or nuclei of interest provides an energy input and leads to tissue dependent signal intensities of the relaxing protons after the radio wave signal (sequence) stops delivering energy. Tissue-dependent signal intensities vary due to different (net magnetization) relaxation times (T1 or T2, T2*), affected by spin-spin (T2 = e.g. 1H−1H) or spin-lattice (T1 = e.g. 1H−13C or 1H−31P) interactions. A T1-weighted image [usually fat = bright signal and other tissues less bright (grey or dark) signals] may be acquired by a short time of (sequence) repetition (e.g. TR <700 ms) and an even shorter time between (spectral signal peak) echoes (e.g. TE <30 ms), which might be modulated by different flip angles (of the nuclei of interest). In addition to whole body analysis, MRI is most often used for a regional analysis requiring special (gradient) coils, such as body coil, wrist coil, head coil etc." (Danesh Mesgaran et al., 2020). These procedures "create a number of cross-sectional images with a 3D voxel definition for the x-, y- and z-axis direction. A Fourier transformation helps in recalculating the signal information from the

spectral domain into pixel (or voxel)-wise signal intensity values in a ‘gray scale domain’ visible on the ‘computer’ screen” (Scholz et al., 2015).

Before the scan starts, a localizer sequence imaging is required to define the position and directions of the 'slice' of the region of interest. After the scan, a quantitative image analysis is required in order to measure the volume of the regions of interest. For these measurements, various free or commercial software packages are available to segment muscle/lean meat, fat, and bone (Scholz et al., 2015; Danesh Mesgaran et al., 2020).

Some studies have been performed in farm animals using MRI. Kremer et al., (2013) compared the loin eye area (MRI-LA) and its above fat area (MRI-FA) in the region of the 14th vertebra of pigs. Weigand et al. (2020) evaluated the visceral adipose tissue from the origin of the last rib and the top of the iliac crest also in pigs. Bernau et al. (2018) reported that immunocastrated boars tend to have greater subcutaneous fat layers (belly, shoulder, and back fat) than entire boars, and entire boars have larger testis volumes and a higher amount of body fat (especially belly fat). These results were associated with a higher level of androstenone. The authors claim that a comparably greater testis volume and a thicker fat layer of entire boars might become co-predictors of boar taint in live pigs. Bernau et al. (2016, 2017, 2018a) used the MRI to evaluate the local tissue reaction to vaccination in piglets and sheep, respectively. Sanchez et al. (2022) reported the use of MRI for food quality and safety monitoring through the evaluation of body tissue composition and structures. No studies have been performed in calves *in vivo*. In general, the MRI is a good tool to evaluate carcass traits and body composition *in vivo* with a high accuracy (Kremer et al., 2013; Bernau et al., 2018b; Weigand et al., 2020). However, MRI has limited access; high investment costs for running, temperature control, and general maintenance; prolonged scan time, and prolonged time for measuring the tissues of interests (Weigand et al., 2020). So far, no study evaluated calves by using MRI *in vivo*. Only Danesh Mesgaran et al. (2020) describe the MRI procedures to perform the evaluations, tested on one calf.

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HYPOTHESIS

- Calves fed A1 β-CN variant present better growth performance and higher volume of visceral adipose tissue than calves fed A2 β-CN variant.
- Calves fed A1 β-CN variant present higher serum levels of BCM-7 and lower mean fecal scores and days with diarrhea compared to A2 β-CN variant.
- German Simmental calves present higher amount of visceral adipose tissue and lower lean tissue volumes or masses than German Holstein calves, and crossbred calves present intermediate values for both traits.
- Female and male calves present similar amounts of visceral adipose tissue and lean tissue.

OBJECTIVES

General

Compare the effects of feeding homozygous β-CN A1 or A2 milk in German Holstein, German Simmental, and crossbred dairy calves of both sexes, carrying the β-CN genotype A1A1, A1A2, and A2A2 on body composition, milk intake, growth performance, mean fecal score, days with diarrhea, and serum levels of β-casomorphin-7, during the first two weeks of life.

Specifics

- Compare the effects of feeding homozygous β-CN A1 or A2 milk on milk intake, growth performance, and days with diarrhea in dairy calves during the first two weeks of life.
- Compare the effects of feeding homozygous β-CN A1 or A2 milk on body composition in dairy calves at the 15th day of life.
- Compare the body composition, milk intake, growth performance, and days with diarrhea of German Holstein, German Simmental, and crossbred dairy calves during the first two weeks of life.
- Compare the body composition between male and female calves at the 15th day of life.
- Determine the serum levels of β-casomorphin-7 in calves fed homozygous β-CN A1 or A2 milk and its relationship with fecal score.
- Compare the milk intake, average daily gain, feed conversion, mean fecal score, and days with diarrhea in calves carrying the β-CN genotype A1A1, A1A2, and A2A2 during the first two weeks of life.
- Compare the body composition among the β-CN genotype A1A1, A1A2, and A2A2 at the 15th day of life.

CHAPTER II

Effect of β-casein A1 or A2 milk on body composition, milk intake, and growth in Holstein, Simmental, and crossbred dairy calves of both sexes

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Effect of β-casein A1 or A2 milk on body composition, milk intake, and growth in Holstein, Simmental, and crossbred dairy calves of both sexes

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ABSTRACT: The aim of this study was to compare the effects of feeding homozygous β-casein A1 or A2 milk, on the body composition, milk intake, and growth of German Holstein (GH), German Simmental (GS), and crossbred dairy calves of both sexes during the first two weeks of life. A total of 104 calves (n = 54 female - f and n = 50 male - m) from the breed groups GH (n = 23), GS (n = 61), and crossbred GH x GS (CR; n = 20) were evaluated. Calves were weighed after birth and received colostrum ad libitum. On the second day, calves were alternately housed in pairs in double-igloo systems according to their random birth order and received either A1 milk (n = 52; 27 f / 25 m) or A2 milk (n = 52; 27 f / 25 m). They were offered 7.5 liters/day, and the individual actual total milk intake (TMI) was recorded. Daily energy-corrected milk intake was also calculated based on the milk composition (fat and protein). Fecal scores were recorded daily. On day 15, visceral adipose tissue (VAT) volume was assessed by

open magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA). In addition, fat, and lean mass (g), as well as bone mineral content (g) and bone mineral density (g/cm²), were determined by DXA. The body composition, milk intake, and growth were similar between the two types of milk in the first two weeks of life. Female calves had more VAT and fat mass, but less lean mass than male calves. GH and CR calves had more VAT and less lean mass than GS calves. Male calves were heavier than female calves after birth and on day 15. The average days with diarrhea and diarrhea occurrence were similar between calves fed A1 and A2 milk and between both sex groups. GS calves presented slightly more days with diarrhea and increased odds of having diarrhea compared to GH calves, not differing from CR.

KEYWORDS: daily milk intake; dual energy X-ray absorptiometry; fecal score; magnetic resonance imaging

INTRODUCTION

β-casein (β-CN) constitutes approximately 33% of the total protein found in bovine milk (McMahon and Brown, 1984). Dairy cattle possess 12 variants of β-CN, with the most common being A1, A2, and B, exhibiting different genotype frequencies (Sebastiani et al., 2020; Hohmann et al., 2021). The A1 and A2 variants are distinguished solely by an amino acid difference at position 67 of the 209-amino acid protein, where A2 has Proline and A1 has Histidine due to a mutation from the A2 variant (Brooke-Taylor et al., 2017; Chitra, 2022). When the A1 variant undergoes enzymatic digestion, it releases a bioactive opioid peptide consisting of seven amino acids, known as β-casomorphin-7 (BCM-7) (Kullenberg de Guadry et al., 2019). This peptide is not digested in the human body, activating μ-opioid receptors and increasing the risk of diseases such as type-1 diabetes, cardiovascular diseases, higher plasma LDL-C concentrations, as well as neurological and mainly digestive disorders, including abdominal discomfort and diarrhea (Jianqin et al., 2016; He et al., 2017; Kullenberg de Guadry

et al., 2019; Woodford, 2021; Chitra, 2022). However, Ho et al. (2014) reported significantly higher stool consistency scores in individuals consuming A1 β-CN milk compared to A2 milk. Petrat-Melin et al. (2015) observed a higher in vitro digestion rate for A1 milk compared to A2 milk. In their review, Kullenberg de Guadry et al. (2019) concluded that the results from several studies evaluating the influence of A1 milk as a cause of different diseases were inconclusive, due to the significant variability in the results.

In addition to the health aspects associated with the intake of A1 and A2 milk in humans, the performance and health of animals must also be considered. Umbach et al. (1985) reported the presence of β-CM7 in the plasma of 20/24 newborn calves after the first milk intake. The authors suggest that β-CM7 may have important effects on gastrointestinal motility. Hohmann et al. (2021) found β-CM7 values five times higher in the plasma of dairy calves fed A1 milk. However, calves fed A1 milk exhibited lower fecal consistency scores and lower prevalence of diarrhea compared to those fed A2 milk. The authors also observed higher milk intake and average daily gain (ADG) in calves fed A1 milk. Increased milk intake can lead to an increase in body fat content and alterations in body composition (Kristensen et al., 2007; Keogh et al., 2021). The crude protein content in milk replacers and concentrates can also influence the amount of body fat and lean tissue (Blome et al., 2003; Stamey Lanier et al., 2021). Furthermore, variations in body composition can be expected among different breeds under the same conditions. Scholz et al. (2003) reported lower body fat mass and a higher percentage of lean mass in German Holstein (GH) calves compared to German Simmental (GS) and crossbred GH x GS calves.

To assess body composition, dissection and chemical analysis have traditionally been used; however, these procedures are destructive, expensive, time-consuming, and prone to inaccuracies due to variations in human evaluations (Sanchez et al., 2022). Consequently, they are not possible for in vivo evaluations. Non-invasive imaging methods serve as an alternative for predicting tissue depths, areas, volumes, or distributions of fat, muscle (water, protein), bone

mineral content, and density in both live animals and carcasses (Scholz et al., 2015). Currently, emerging image-based techniques such as dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound imaging (US) are being applied for body and carcass evaluations (Scholz et al., 2015; Sanchez et al., 2022). Several studies have reported the use of DXA to estimate live body composition in pigs, sheep, and cattle (Scholz et al., 2003; Hampe et al., 2005; Rothammer et al., 2017; Bernau et al., 2020; Weigand et al., 2020). MRI studies have also been conducted on live pigs and sheep (Kremer et al., 2013; Bernau et al., 2016, 2017, 2018; Weigand et al., 2020) but not on calves. Therefore, this study represents the first attempt to evaluate a large number of dairy calves using both DXA and MRI techniques to assess the body composition. The aim of this study was to compare the effects of feeding homozygous β-CN A1 or A2 milk, on the body composition, milk intake, and growth of German Holstein, German Simmental, and crossbred dairy calves of both sexes during the first two weeks of life.

MATERIAL AND METHODS

All procedures performed on animals in this study were approved by the Animal Ethics Committee of the Government of Upper Bavaria under protocol number ROB-55.2-2532.Vet_03-22-20. The experiment was conducted at the Livestock Center Oberschleissheim, Veterinary Faculty of the Ludwig-Maximilians-University of Munich, from September 2022 to June 2023. All calves born in this period were included in the study and no calves were excluded from the study due to illness or death.

Animals, feeding, and evaluations

A total of 104 calves (54 females and 50 males), from the breed groups German Holstein (GH), German Simmental (GS), and crossbred GH x GS (CR), were used in the experiment.

The calves were randomly assigned to one of two feeding groups according to their birth order, not balanced by sex and breed. After every two calves born, the feed group is changed: (1) an A1 milk diet with homozygous β-CN genotype A1A1 (n = 52; 27 females, 25 males; 7 GH, 31 GS, and 14 CR), or (2) an A2 milk diet with homozygous β-CN genotype A2A2 (n = 52; 27 females, 25 males; 16 GH, 30 GS, and 6 CR). After birth, the calves were weighed to determine their birth body weight (BBW), received umbilical cord and navel disinfection, and double set of ear tags with an identification number. The calves were individually housed in calf boxes to receive maternal colostrum ad libitum within the first 24 hours (at least 10% of the body weight, with IgG > 50 mg/ml measured in a colostrometer). The total colostrum intake was measured (offered - leftover). Blood samples were collected from the jugular vein using EDTA-coated tubes on the second day to determine the transfer of passive immunity. Serum was separated and placed on the measuring surface of the digital refractometer to estimate serum total protein (STP g/l). On the second day, the calves were housed alternately in pairs in double-igloo systems bedded with straw, according to their random birth order, with ad libitum access to water and hay but no access to calf starter. As the hay intake during their first two weeks of life is negligibly small, it does not have to be considered in the feeding ratio. The igloos were identified with the respective treatment. The calves received either A1 or A2 milk in an 8 liters nipple bucket three times daily: 06:00 (3 l), 11:30 (1.5 l), and 17:30 (3 l). The individual actual milk intake of the calves was recorded daily (DMI). The milk was collected separately from known homozygous A1 or homozygous A2 cows by using the Lely M4USE system. The composition of the milk offered was determined daily by the Lely A3 or A3 next robotic milking system (Lely Deutschland, Waldstetten, Germany). As we did not balance the nutrient content of the diet between the two treatments, we calculated the daily energy-corrected milk intake (DMI_ECM) using the following equation: (0.327 * DMI) + (12.95 * F * DMI/100) + (7.65 * P * DMI/100), where F = fat percentage and P = protein percentage. On the 15^{th} day, the calves were deprived of the first feeding and weighed to determine the end body weight (EBW) and

calculate average daily weight gain (ADG) and body weight gain throughout the study period. We determined the feed efficiency, based on body weight gain divided by total energy-corrected milk intake (TECMI).

Every day, during the second feeding time, we recorded the fecal score (FS), where 1 = normal, 2 = soft, 3 = runny, and 4 = watery/diarrhea. Scores of 3 and 4 were considered as indicators of diarrhea (Renaud et al., 2020). Calves with FS 4 and rectal body temperature > 39.5 °C were offered an electrolyte solution and received an extra treatment of Parofor 140 mg/ml oral solution for 3 days. The same person consistently measured and recorded all parameters in all calves each day.

Before the magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA) evaluations on day 15, the health status of each calf was assessed. For the imaging evaluations, the calves were lightly sedated with Xylazine 2% (0.4 mg/kg i.m.) for induction and Ketamine 10% (1-2 mg/kg i.v.) for sedation maintenance. The anesthetic volumes were adjusted based on vital parameters and eyelid reflex to prevent movements during the evaluations. The study ended at 15 days of age, as this was the day of dehorning, for which calves must be sedated in Germany. An experienced veterinarian performed all procedures.

MRI evaluations

To assess the amount of visceral adipose tissue (VAT), an open low-field MRI system (Siemens Magnetom C!) with a magnetic field strength of 0.35 Tesla was used for the scans, previously established for calves as described by Danesh Mesgaran et al. (2020) and for pigs as described by Weigand et al. (2020). The imaging examinations were performed by one and the same person according to appropriate previous training. Before each evaluation, an automated quality control was conducted by the Siemens Magnetom C! system. For the abdominal evaluation, the calves were positioned in a prone position with the forelimbs and hind limbs extended (Figure 1A). The space between the origin of the last rib and the sacral

tuberosity was used as an anatomical landmark for consistent positioning, ensuring reproducible and comparable images of the same body region (Figure 1B). Two sequences were employed to cover the entire region of interest. The first sequence, called "ViscFat sequence," encompassed most of the abdomen, while the second sequence, the "Ham sequence", included the remaining part of the abdomen and the pelvic region. The images were evaluated using the "Synedra" View Personal software (Version 16.0.0.3, Synedra information technologies GmbH, Innsbruck, Austria) and the FDA approved Able 3D-Doctor software (Release 4.0; Able Software Corp., Lexington, MA, USA). The "Synedra" software was utilized to identify the images covering the region of interest. Subsequently, the Able 3D-Doctor software was employed to manually define the fat depots (white mass – Figure 2B) beneath the abdominal wall, around the kidneys, and in the abdominal cavity for every single slice (Figure 2A). The volume of VAT was quantified in cm^3 from a 3D model, based on the slice number, slice thickness, and slice distance (Figure 2C).

DXA evaluations

Following the MRI scan, the body composition of the calves was measured using DXA. The GE Lunar *i*DXA scanner was utilized for the whole-body scan (Figure 3A). The technical procedures applied for DXA are described in detail in Danesh Mesgaran et al. (2020). In this study, the "standard" mode of the scanner was employed to obtain information regarding the quantity and percentage of fat and lean mass, bone mineral content, and bone mineral density. The duration of the whole-body scan in "standard" mode was 7.45 minutes, with a radiation dose of 3 µGy. During the DXA examination, the calves were positioned in a prone position with the forelimbs flexed and hind limbs extended. Prior to the examination, a quality control check of the *i*DXA system was performed. The results of body composition and bone mineral measurements were automatically generated after the scan. The android region (origin of the last rib and the sacral tuberosity) was adjusted individually to encompass the same region of

interest as in the MRI scans (Figure 3B), ensuring a meaningful comparison of VAT volume between the two methods. The obtained results included body weight (kg), bone mineral density (BMD, g/cm²), bone mineral content (BMC, g), fat and lean mass (g) of the whole body, and VAT volume (cm³) and mass (g) in the android region.

Statistical analyses

The GPower software (Faul et al. 2007, Rasch et al. 2014, Kang, 2021) served as tool for sample size calculation. A medium Cohen's effect size (Cohen, 1988) based on numerator degrees of freedom of n = 2 combined with a power (1-beta) of 0.8 and an alpha error of 0.05 led to a sample size of n = 100. We assumed that the special effect (milk type) would explain 10% of the error variance (100%) that resulted in Cohen's effect size of f = 0.316.

All traits were automatically tested for normal distribution by the software SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA) applying Kolmogorov-Smirnov Test. A variance analysis (ANOVA) was performed by using a mixed model procedure and restricted maximum likelihood (REML) estimation with SAS 9.4 (The MIXED Procedure, SAS Institute Inc. 2015. SAS/STAT® 14.1 User's Guide. Cary, NC, USA). No exclusion of data points was applied. Milk type, sex, milk type x sex, breed, and sex x breed were defined as fixed effects with end body weight as covariate and birth date as random effect for all MRI and DXA body composition traits. No covariate was applied for the records of milk intake, total protein in blood, and abdominal length. In contrast, birth body weight was used as covariate for feed efficiency and average daily gain. A modified model was used for the daily recorded energy corrected milk intake with milk type, sex, milk type x sex, breed, sex x breed, age, milk type x age as fixed effects, measurement date as random effect, and calf as repeated effect. The significance level was in all cases set to $P < 0.05$.

Since the daily recorded fecal score does not follow an ideal normal distribution, we added a logistic regression analysis using again SAS 9.4 (The LOGISTIC procedure, SAS

Institute Inc. 2013. SAS/STAT® 13.1 User's Guide. Cary, NC) for the daily recorded trait "diarrhea" defined as no diarrhea for fecal scores < 3 and diarrhea for fecal scores > 2. We calculated, the Odds Ratios for the binary logit model by applying the Fisher's scoring optimization technique containing the fixed effects milk type, sex, breed, combined with the covariates birth body weight (BBW), daily energy corrected milk intake (DMI_ECM), rectal temperature, and serum total protein. We applied a backward selection of the covariates and kept the model with the lowest AIC. In addition, we performed a logistic regression analysis between diarrhea (yes/no) and the daily energy corrected milk intake. Finally, we performed a Bland-Altman analysis to compare DXA VAT with MRI VAT by using a combination of SAS procedures as are described by Johnson and Waller (2018).

RESULTS AND DISCUSSION

No interaction between feeding groups and sex was observed, only between sex and breed for the variables DXA VAT, fat mass, and BMD (Table 1). The body composition was similar for the two feeding groups, possibly related to the similar milk intake and ADG. Some differences, nonetheless, were expected based on the results of Hohmann et al. (2021), who reported higher DMI and ADG for calves fed A1 milk. The short treatment period (15 days) may not have been sufficient to observe any differences in body composition between the feeding groups. Consequently, a more extended observation period would be necessary, but may be restricted by the limited spatial capacity of the MRI equipment. However, female calves had more MRI VAT (353 ± 16 cm^3 vs. 300 ± 18 cm^3, $P < 0.05$) and more DXA body fat (3246 ± 42 g vs. 2978 ± 49 g, $P < 0.001$) compared to male calves, respectively. In contrast, male calves had more DXA lean mass than female calves (47130 ± 59 g vs. 46825 ± 50 g, $P < 0.05$). No BMD and BMC differences were observed (Table 1). Scholz et al. (2003) found no differences between male and female calves in fat and lean tissue percentage, BMD, and BMC between days 6 and 50 of age; only the body weight differed significantly with an advantage

for male calves (54.68 ± 0.72 kg vs. 51.52 ± 0.66 kg). Irshad et al. (2012) claim that heifers and castrated males have fatter carcasses, while young bulls produce carcasses with less external and internal fat and more lean mass. These effects are related to the hormonal status of the animals, even in very young animals. Keogh et al. (2021) reported higher systemic concentrations of IGF-1 and insulin in Angus x Holstein heifers fed a high plane of nutrition than in heifers fed a moderate plane of nutrition from 3 to 21 weeks of life. The authors postulate that these two anabolic hormones (IGF-1, insulin) may contribute to VAT development by inducing adipogenesis in pre-adipocytes.

In both MRI and DXA evaluations, CR and GH calves had higher amounts of VAT compared to GS ($P < 0.05$), although there was no difference in the abdominal size (length: approximately 21 cm). CR and GH calves also had more DXA fat mass, but less DXA lean mass compared to GS ($P < 0.05$; Table 1). Due to differences in morphological traits, carcass condition, and finality, fat storage occurs in different locations and amounts in the body. Breeds specialized for milk production tend to store more fat tissue, while breeds specialized for beef production store more energy in form of muscle tissue (Pfuhl et al., 2007). Berry (2021) also claims that Holstein and dairy x beef crossbreds had more fat cover than beef breeds. Pfuhl et al. (2007) found that Holstein bulls had more visceral fat, higher marbling scores, and more intramuscular fat at 18 months of age, while Charolais bulls put on 3.4% more carcass meat. The same relationship is found in crosses between specialized dairy breeds and dual-purpose breeds. Scholz et al. (2003), however, reported that the GH calves showed a lower body fat content and higher lean content than CR calves, not differing from GS between 4 to 50 days, while Hampe et al. (2005) reported significantly higher body fat percentages for GS compared with GH between 6 to 50 days of age. The four different crossbred combinations between GS and GH had medium body fat and lean percentages in comparison to the two purebred lines GS or GH, respectively. Phillips et al. (2017) conducted a comparative analysis of carcasses from Holstein steers and Holstein x Montbéliarde x Viking Red crossbred steers at approximately

490 days of age. The study found that the fat percentage in the kidney, pelvic, and heart regions was similar between the two breeding lines. Additionally, marbling score and backfat thickness (BFT) were also found to be similar. Blöttner et al. (2011) observed higher BFT in Holstein x Brown Swiss cows compared to Holstein cows throughout the first lactation. In a more recent study, Knob et al. (2021) reported higher BFT in Simmental and R1 Simmental cows compared to Holstein, R1 Holstein, and F1 cows during the first 150 days of lactation. Based on these findings, it can be inferred that different sexes and breeds at different ages exhibit variations in fat storage patterns.

The gold standard for VAT evaluations is the dissection procedure compared to non-invasive techniques (Abate et al. 1994). However, these destructive procedures do not allow for future evaluations on the calves. In our study, we assumed MRI as the reference method for VAT assessment because it is possible to visualize and define the fat depots in all images. Additionally, MRI was considered as reference based on the results derived in pigs (Weigand et al. 2020) and on the findings of van der Kooy and Seidell (1993) and Abate et al. (1994) for the measurement of human visceral adipose tissue. Compared to MRI VAT, DXA VAT was overestimated by 46 cm^3 (standard error 17 cm^3, $P < 0.01$, result of a Bland-Altman method comparison using SAS 9.4 with a Student's t test). Weigand et al. (2020) also observed overestimated DXA values for VAT in pigs compared with MRI data. The accuracy of whole body analysis in vivo, however, is severely compromised by the gastrointestinal tract of ruminants, resulting in a reduced relationship between body composition from DXA measurements and reference measures (Scholz et al., 2015).

There was no difference in BBW and EBW between feeding groups and among breeding lines, what is expected as there was no difference in milk consumption. However, male calves were heavier than the female calves in both BBW (43.5 ± 0.8 kg vs. 39.6 ± 0.9 kg) and EBW (53.3 ± 0.8 kg vs. 49.6 ± 0.7 kg), respectively. Other studies revealed also higher BW for male calves due to distinct sexual dimorphism in cattle (Kertz et al., 1997; Dhakal et al., 2013;

Hohmann et al., 2021). Knob et al. (2018) found similar growth rates for calves and heifers comparing Holstein and F1 Holstein x Simmental. Contrary from our findings, Heins et al. (2010) reported that Holstein x Montbeliarde calves weighed 5 kg more than Holstein calves at birth. Hampe et al. (2005) and Scholz et al. (2003) also found higher BW for GS and CR calves with higher proportion of GS than GH and CR calves with higher proportion of GH. Hohmann et al. (2021) reported that GH calves were the heaviest, followed by GS and CR calves, in both birth and at 21 days of age.

DMI excluding milk energy was similar between sexes and breeds/crossbreds, but showed a tendency ($P = 0.07$) toward a higher value for A1 milk than A2 milk, (6.64 ± 0.0 l vs. 6.46 ± 0.0 l, respectively). Hohmann et al. (2021) observed a higher DMI for calves fed A1 than A2 (7.28 ± 0.12 vs. 6.96 ± 0.11, $P = 0.02$, respectively). The authors hypothesized that A2 milk contains a higher protein content, which could explain the lower milk intake. However, when we considered the milk energy (DMI_ECM), there was no difference between the feeding groups ($P = 0.58$). The same observation was made for TECMI, which was also similar among sexes and breeds, with an average of 103.6 liters during the experimental period. With similar milk intakes, we observed similar ADG among the feeding groups, sexes, and breeds/crossbreds. Although not significant ($P = 0.07$), Hohmann et al. (2021) observed a slightly better ADG for calves fed A1 milk with about 110 g/d than calves fed A2 milk during three weeks of evaluation. In our study, A1 gained 699 ± 27 g/d and A2 only slightly less with 670 ± 27 g/d. A1 and A2 calves gained 96 (± 3.2) and 94 (± 3.3) grams of liveweight, respectively, per liter of milk. This was expected considering the similar TECMI and weight gain. In contrast to our study, Hohmann et al. (2021) found better feed conversion for A1 milk compared to A2 milk, 9.2 l/kg gain and 10.5 l/kg gain, respectively. Male calves had better feed efficiency than female calves (100 ± 3.4 g/l vs. 90 ± 3.3 g/l, $P = 0.02$, respectively).

In our study, mean days with diarrhea were similar between feeding groups ($P = 0.22$, Table 1). There was no association of feeding treatment with likelihood of diarrhea (OR:

0.785; 95% CI: 0.536 – 1.15; P = 0.21, Figure 5). This is in agreement with Hohmann et al. (2021), who observed higher mean fecal scores (2.56 ± 0.17 vs. 1.97 ± 0.18) and diarrhea prevalence (10% vs. 6%) in calves fed A2 than A1 milk. Generally, the occurrence of diarrhea is negatively associated with the daily energy corrected milk intake. DMI_ECM decreased by 36.7 % if calves had diarrhea (Odds Ratio Estimate: 0.633; 95% Confidence Limits: 0.584 - 0.687, Pr > ChiSq: < 0.0001). A similar relationship was reported by de Paula et al. (2017), who observed lower milk replacer intake during the first three weeks, when calves had more frequent diarrhea. Although all calves had an adequate passive immune transfer (serum total protein > 60 g/l) on the second day (Tyler et al., 1996; Wilm et al., 2018), GS calves had slightly more mean days with diarrhea (Table 1) and slightly increased odds of having diarrhea (Figure 5) than GH calves, whereas CR calves did not differ from GH and GS calves (Table 1). 20 (of 20) CR, 21 (of 23) GH, and 57 (of 61) GS calves had at least one day with diarrhea. These differences could possibly be related to the different gastrointestinal microbiota (Paz et al., 2016), which can be influenced by the interaction between the host and microbiota, as well as external factors such as the maternal microbiota, calving, diet, and use of antibiotics (Du et al., 2023). Gastrointestinal microbiota has a significant effect on animal health and productivity as well as diseases (Klein-Jöbstl et al., 2014; Du et al., 2023). However, some groups of microorganisms such as *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* have been associated to a decrease in the incidence of diarrhea, increase in body weight, and better feed conversion rates (Du et al., 2023). Further studies are needed to compare the gastrointestinal microbiota between different breeds/crossbreds at an early age under different health conditions, especially diarrhea.

Currently, there is a tendency to select animals for A2 β-CN (Juan and Trujillo, 2022), mainly because the adverse effects associated with the A1 β-CN variant on human health (Kullenberg de Guadry et al., 2019; Woodford, 2021; Giribaldi et al., 2022, Gonzales-Malca et al., 2023), as well as the observed positive correlation of the A2 β-CN variant with higher milk

and protein yields compared to A1 β-CN (Heck et al., 2009; Gai et al., 2021). However, considering the coagulating capacity, Gai et al. (2021) and Giribaldi et al. (2022) reported a higher incidence of the A2 variant in non-coagulating milk, a longer coagulation time, looser curd formation, and a lower cheese yield. Nevertheless, the A2 variant can be beneficial for specific markets due to its suitability for yogurt production or just whole milk. Prior to the targeted selection of a specific milk type, it is crucial to consider various factors encompassing human and animal health and performance, as well as physicochemical, technological, and functional characteristics of the milk.

In future research endeavors, it is recommended to extend the evaluation period, encompassing the weaning and post-weaning stages. This long period will facilitate the observation of potential impacts of A1A1 and A2A2 milk on key parameters such as ADG, FS, and body composition assessed by DXA. It is noteworthy that DXA provides ample space for the assessment of calves weighing up to 150 kg, previously established by Danesh Mesgaran et al. (2020). The authors also set a benchmark of 150 kg for the use of MRI in assessing calves. However, our observations have revealed a significant limitation when employing MRI in a prone position for VAT assessment. Specifically, we have noted that calves weighing up to 70 kg do not fit within the coil. This constraint compromises the quality of imaging, and in some cases, renders image acquisition entirely unfeasible. This did not compromise the MRI images in our study, as the heaviest calf in our study weighed less than 69 kg.

CONCLUSION

This is the first study to use MRI analysis to provide insights into the body composition of dairy calves. However, feeding A1 or A2 milk to calves has no effect on body composition, milk intake and growth of calves during the first two weeks of life. Female calves had more visceral fat and total body fat, while male calves had more lean mass. CR and GH calves had a higher percentage of VAT and a lower amount of lean mass compared to GS calves. Milk β-CN type had no effect on health parameters such as days with diarrhea and diarrhea occurrence, but

GS had slightly more mean days with diarrhea and increased odds of having diarrhea than GH. Further studies are needed to investigate the effects of A1 and A2 milk on gastrointestinal microbiota considering different breeds/crossbreds.

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Table 1. Least squares means (± standard error), and significance levels (P value) for body composition, body weight, milk intake, average daily gain, feed efficiency, days with diarrhea, and serum total protein for feeding groups, sex (F- female and M- male), and breed (crossbred- CR, German Holstein- GH, and German Simmental- GS).

Variables	Feeding groups			Sex			Breed				P value
	A1A1	A2A2	P value	F	M	P value	CR	GH	GS	P value	sex x breed
MRI VAT, cm³[1]	319 ± 15.6	333 ± 15.2	0.46	353 ± 15.9	300 ± 18.3	0.02	362 ± 23.7 a	333 ± 21.3 a	284 ± 13.9 b	<0.001	0.51
DXA VAT, cm³	390 ± 19.6	377 ± 19.2	0.61	413± 19.7	354 ± 22.8	0.05	405 ± 30.2 a	421 ± 27.0 a	325 ± 17.1 b	0.003	0.05
DXA fat mass, g	3090 ± 42.6	3134 ± 42.0	0.43	3246 ± 42	2978 ± 49	<0.001	3157 ± 66 a	3220 ± 59 a	2959 ± 36 b	<0.001	0.01
DXA lean mass, g	46992 ± 51	46963 ± 50	0.66	46825 ± 50	47130 ± 59	<0.001	46913 ± 79 b	46869 ± 72 b	47152 ± 43 a	<0.001	0.29
DXA BMC, g[2]	1700 ± 17	1706 ± 17	0.79	1720 ± 17	1686 ± 20	0.20	1707 ± 26	1702 ± 24	1700 ± 15	0.97	0.08
DXA BMD, g/cm²[3]	0.819 ± 0.01	0.819 ± 0.01	0.99	0.829 ± 0.01	0.809 ± 0.01	0.13	0.824 ± 0.01	0.805 ± 0.01	0.827 ± 0.01	0.28	0.04
Abdominal length, cm	21.2 ± 0.1	21.0 ± 0.1	0.31	21.0 ± 0.1	21.2 ± 0.2	0.54	21.0 ± 0.2	21.2 ± 0.2	21.1 ± 0.1	0.88	0.35
Birth body weight, kg	41.5 ± 0.7	41.6 ± 0.7	0.93	39.6 ± 0.9	43.5 ± 0.8	<0.001	40.9 ± 1.1	41.1 ± 1.0	42.7 ± 0.6	0.24	0.81
End body weight, kg	51.5 ± 0.7	51.4 ± 0.7	0.86	49.6 ± 0.7	53.3 ± 0.8	<0.001	51.5 ± 1.1	50.5 ± 1.0	52.4 ± 0.6	0.24	0.54
Daily milk intake, l	6.64 ± 0.0	6.46 ± 0.0	0.07	6.59 ± 0.0	6.51 ± 0.0	0.45	6.67 ± 0.1	6.50 ± 0.1	6.47 ± 0.0	0.30	0.47
DMI_ECM, l[4]	7.17 ± 0.1	7.09 ± 0.1	0.58	7.19 ± 0.1	7.07 ± 0.1	0.44	7.24 ± 0.1	7.15 ± 0.1	7.00 ± 0.0	0.42	0.78
TECMI, l[5]	103.7 ± 1.8	103.7 ± 1.7	0.98	104.6 ± 1.7	102.7 ± 2.1	0.47	106.3 ± 2.8	103.2 ± 2.4	101.5 ± 1.6	0.31	0.87
Average daily gain, g/d [y]	699 ± 27	670 ± 28	0.40	660 ± 28	708 ± 29	0.20	715 ± 50	658 ± 33	680 ± 23	0.63	0.82
Feed efficiency, g/l [y][6]	96 ± 3.2	94 ± 3.3	0.58	90 ± 3.3	100 ± 3.4	0.02	98 ± 5.9	90 ± 3.9	97 ± 2.8	0.31	0.68
Days with diarrhea	1.90 ± 0.2	2.22 ± 0.2	0.22	2.02 ± 0.2	2.10 ± 0.2	0.79	2.10 ± 0.3 ab	1.64 ± 0.3 b	2.44 ± 0.2 a	0.07	0.88
Serum total protein, g/l	64.1 ± 1.2	66.7 ± 1.2	0.11	64.2 ± 1.2	66.6 ± 1.4	0.20	67.7 ± 1.8	64.7 ± 1.8	63.6 ± 1.2	0.17	0.06

[1]Visceral adipose tissue.
[2]Bone mineral content.
[3]Bone mineral density.
[4]Daily energy corrected milk intake.
[5]Total energy corrected milk intake.
[6]Feed efficiency based on total energy corrected milk intake: (0.327 * DMI) + (12.95 * F * DMI/100) + (7.65 * P * DMI/100).
[y]Model includes birth body weight as covariate.
* Different letters within lines describe significant differences with $P < 0.05$.

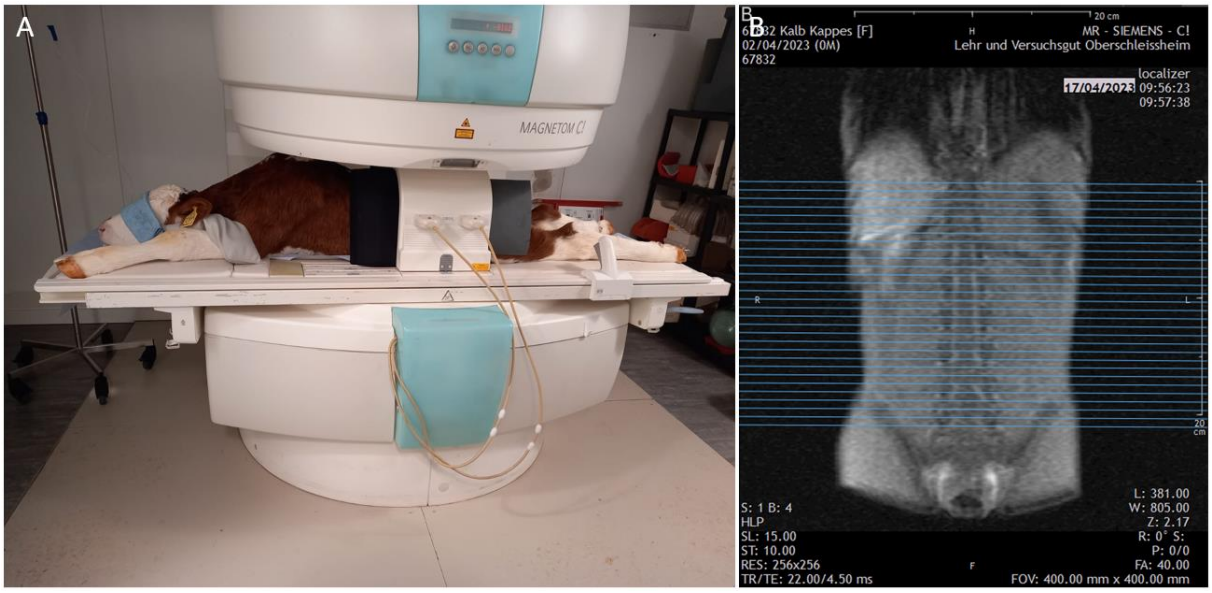

Figure 1. MRI examination of the abdomen. (A) Position of the calf for the examination of the abdomen by a Siemens Magnetom C! system. (B) Localizer of the "ViscFat-Sequence" with defined area encompassing visceral adipose tissue. Each line represents an axial sectional image of the abdomen starting at the origin of the last rib and the sacral tuberosity.

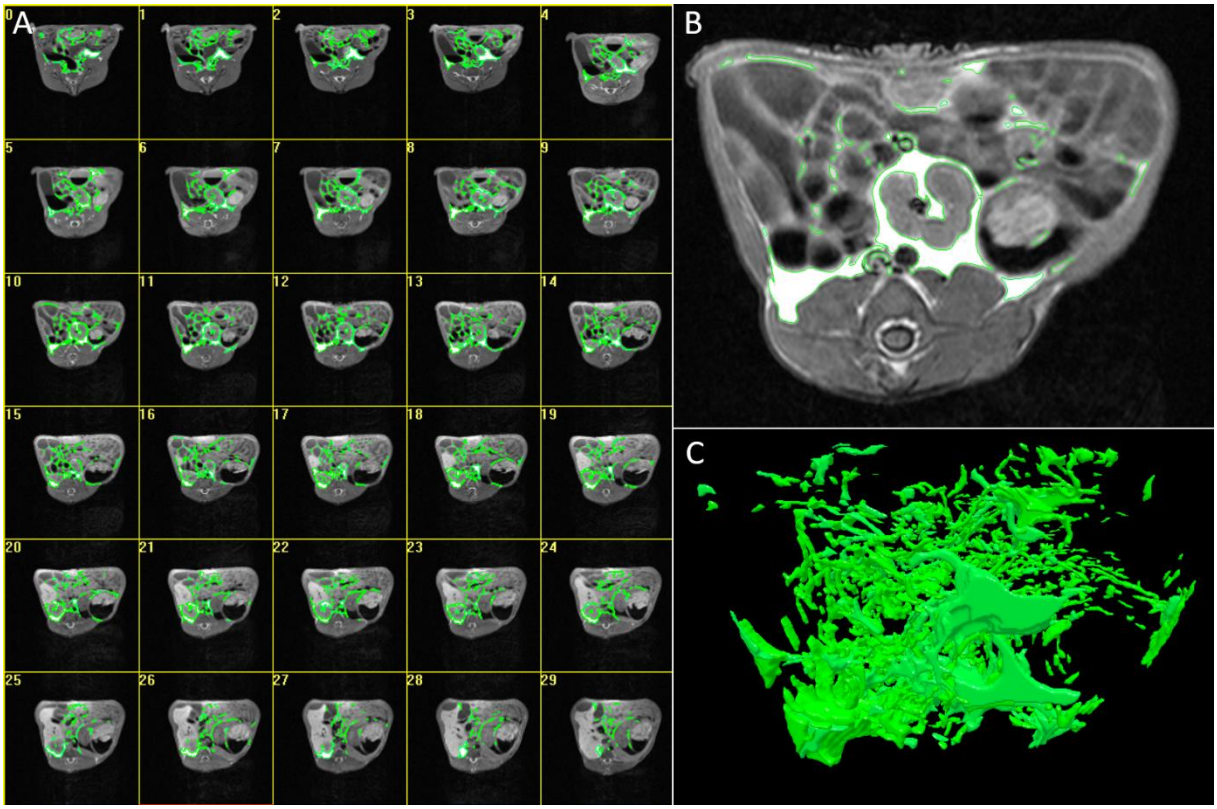

Figure 2. Evaluation of MR images using the Able 3D-Doctor software. (A) Analysis of all slices in the defined body region. (B) T1-weighted axial MR image with green boundaries, including the visceral adipose tissue. (C) Reconstruction of a 3D-model from the selected boundaries of VAT.

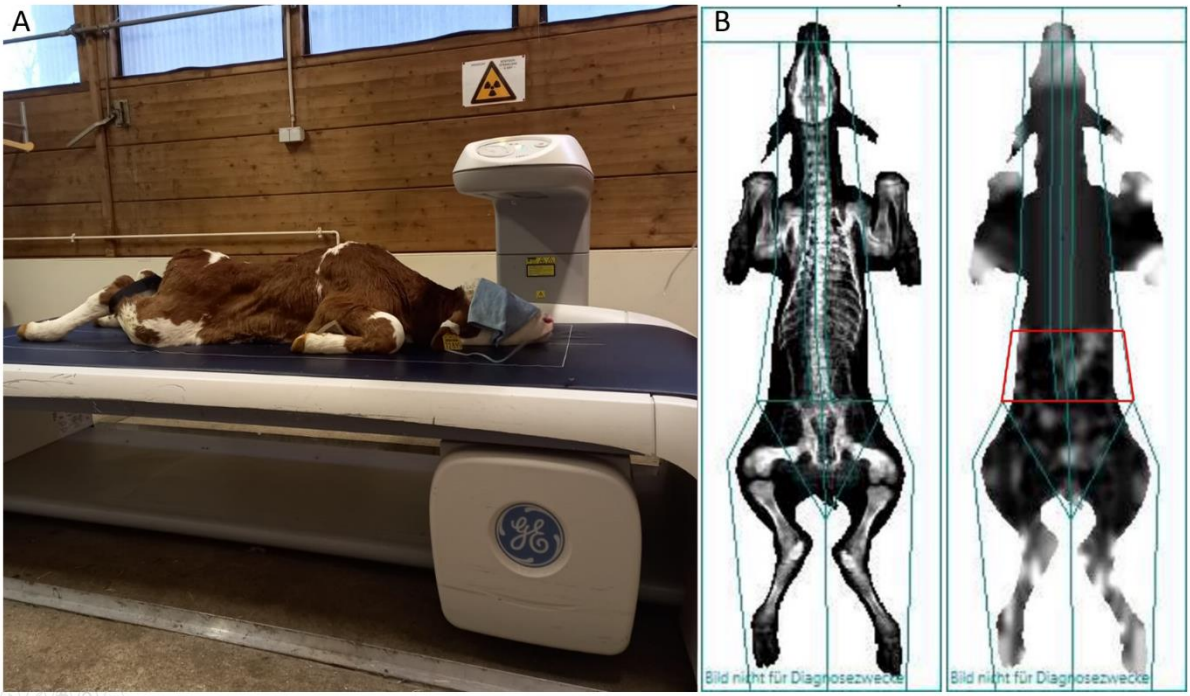

Figure 3. (A) DXA examination. (B) DXA evaluation with enCore software. Mineral tissue (left side), soft tissue (right side). The red lines define the android region (from the origin of the last rib to sacral tuberosity).

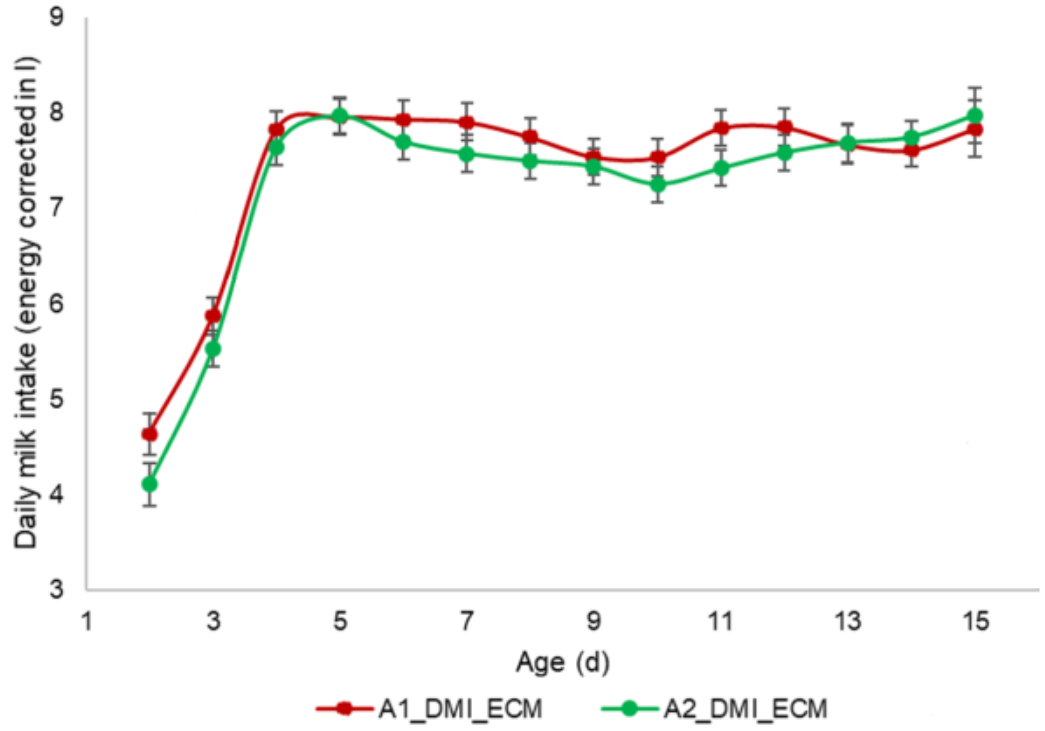

Figure 4. Least squares means for daily milk intake (energy corrected in l) of calves receiving A1 or A2-milk during the experimental period of 15 days. Black bars indicate the standard errors of estimation. There is no association of treatment with energy corrected milk intake (P = 0.1615).

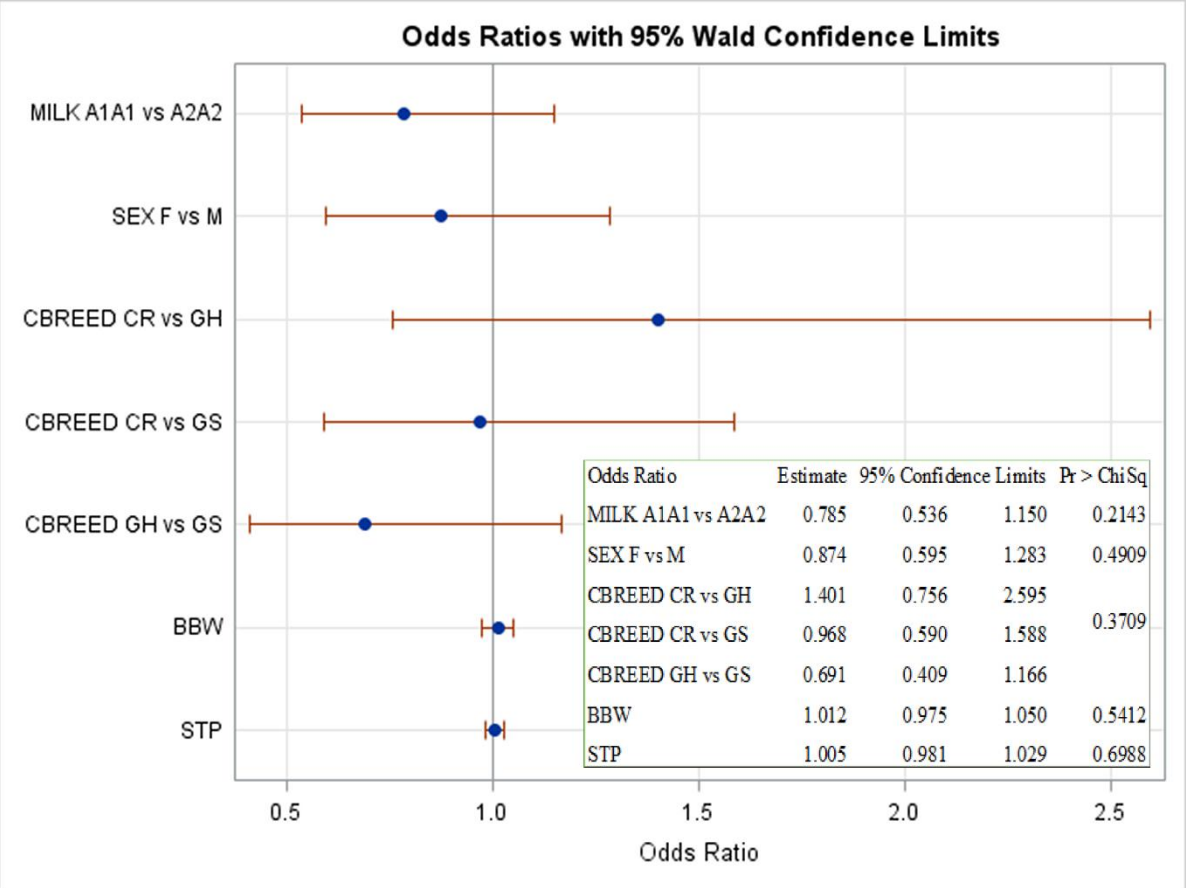

Figure 5. Results of the Logistic regression analysis of the daily Diarrhea records (0/1) for the classes milk type (A1A1 vs A2A2), sex (f=female, m =male), breed (CBREED with CR=crossbred, GS = German Simmental, GH = German Holstein), combined with the covariates birth body weight (BBW), and serum total protein (STP). The model was chosen due to the lowest AIC compared to models that include additional covariates such as daily energy-corrected milk intake and rectal temperature.

CHAPTER III

Effects of β-casein A1 and A2 milk and β-casein genotype on body composition milk intake, growth, β-Casomorphin-7 levels, and fecal score in dairy calves

*Paper written according to the Journal of Dairy Science

Effects of β-casein A1 and A2 milk and β-casein calf genotype on body composition, milk intake, growth, β-Casomorphin-7 levels, and fecal score in dairy calves

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ABSTRACT: The objective of this study was to evaluate the body composition, performance, mean fecal score, days with diarrhea, and serum levels of β-casomorphin-7 (BCM-7) in calves fed A1 and A2 β-CN milk carrying the A1A1, A1A2, or A2A2 β-CN genotype. A total of 104 calves (n = 54 female - f and n = 50 male - m) from the breed groups German Holstein (GH; n = 23), German Simmental (GS; n = 61), and crossbred GH x GS (CR; n = 20) were evaluated during the first fourteen days of life. After birth, calves were weighed and received colostrum ad libitum. On the second day, two blood samples were collected to determine the serum total protein and to genotype for β-CN. On the second day, calves were alternately housed in pairs in double-igloo systems according to their random birth order and received either A1 milk (n = 52; 27 f / 25 m) or A2 milk (n = 52; 27 f / 25 m). They were offered 7.5 liters/day, and the individual actual total milk intake was recorded. Total energy-corrected milk intake was also calculated based on the milk composition (fat and protein). Fecal scores were recorded daily. On

day 15, the calves were weighed and evaluated on an open magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA) for visceral adipose tissue (VAT) volume, fat, and lean mass (g), as well as bone mineral content (g) and bone mineral density (g/cm^2). A blood sample was collected from the jugular vein to determine the serum levels of BCM-7. The body composition, total milk intake, average daily gain, feed conversion, mean fecal score, and days with diarrhea were similar between the two feeding groups. The serum levels of BCM-7 were similar between the two feeding groups, genotype, sex, and breed, and had no association with odds of days with diarrhea. Calves with the A2A2 β-CN genotype had increased odds of having more days with diarrhea in comparison with A1A1 or A1A2 β-CN genotype calves. Calves with A1A2 β-CN genotype had lower mean fecal scores and less days with diarrhea than A2A2 calves in the first two weeks of life, not differing form A1A1 calves, what resulted in a slightly better average daily gain for A1A2 and A1A1 calves. The body composition was similar among the three β-CN genotypes.

KEYWORDS: Allele frequency, average daily gain, days with diarrhea, visceral adipose tissue.

INTRODUCTION

The growing consumer interest in A2 milk, driven by its purported significance in human health and commercial potential, has resulted in an increased selection of bulls and cows for homozygous A2 β-CN milk (Lu et al., 2020; Arens et al., 2023; Scott et al., 2023). However, an exploration of the effects of the A2 β-casein variant on milk production and composition, reproductive performance, health, and survival is essential before incorporating the A2 genotype as an additional criterion in bull selection (Lu et

al., 2020; Arens et al., 2023). Some studies have evaluated the productive and reproductive performance and survival in cows. Heck et al. (2009) and Comin et al. (2008) reported higher milk and protein yield in cows carrying the A2A2 genotype, while Lu et al. (2020), Ardicli et al. (2023), and Arens et al. (2023) found similar values for milk, fat, and protein yield, as well as reproductive traits between A1A1, A1A2, and A2A2 genotypes. Scott et al. (2023) reported superior estimated breeding values for production traits in A1A2 and A2A2 animals, while A1A1 were superior for fertility, health, and survival, mainly associated with lower inbreeding depression. Arens et al. (2023) found that the survival rate for the first and second lactation was higher for A1A2 and lower for A1A1, not differing from A2A2. No studies have assessed the performance and health of calves considering the β-CN genotype.

Hohmann et al. (2021) assessed the effect of feeding calves with milk containing A1 and A2 β-CN variants on performance and diarrhea occurrence during the first 21 days of life. Calves fed A1 β-CN milk exhibited higher daily milk intake, feed efficiency, and a tendency for a higher average daily gain compared to those fed A2 β-CN. Kappes et al. (2024) observed similar growth performance, body composition (visceral adipose tissue, fat and lean body mass), and days with diarrhea during their first two weeks of life. Hohmann et al. (2021) reported lower fecal scores and prevalence of diarrhea, along with higher levels of β-casomorphin-7 (BCM-7) in calves fed A1 compared to A2 β-CN milk. BCM-7 is an important bioactive peptide released from the digestion of A1 β-CN milk (Asledottir et al., 2017; Giribaldi et al., 2022; de Vasconcelos et al., 2023), associated with gastrointestinal alterations, including diarrhea in humans (Jianqin et al., 2016). However, an antidiarrheal effect is reported due to the stimulation of electrolyte and water absorption and the inhibition of intestinal motility (Noni et al., 2009; Fosset and Tomé, 2022).

Based on the study of Hohmann et al. (2021) and Kappes et al. (2024), we hypothesize is that calves fed the A1 β-CN variant will exhibit higher serum levels of BCM-7, with no adverse effects on mean fecal score and days with diarrhea when compared to those fed the A2 β-CN variant. Due to the absence of studies evaluating the performance and body composition of calves considering the β-CN genotype, no hypotheses were postulated. Therefore, the aim of this study is to assess the body composition, performance, mean fecal score, days with diarrhea, and serum levels of BCM-7 in calves fed A1 and A2 β-CN milk carrying the A1A1, A1A2, or A2A2 β-CN genotype.

MATERIAL, ANIMALS AND METHODS

The experiment was conducted at the Livestock Center Oberschleissheim, Veterinary Faculty of the Ludwig-Maximilians-University of Munich from September 2022 to July 2023. All procedures were approved by the Animal Ethics Committee of the Government of Upper Bavaria under protocol number ROB-55.2-2532.Vet_03-22-20.

The methodologies are described in detail in Kappes et al. (2024). Briefly, 104 German Holstein (GH), German Simmental (GS), and crossbred GH x GS (CR) calves were used in the experiment. The calves were randomly assigned to one of two feeding groups according to their birth order, not balanced by sex, breed, or β-CN genotype. The feeding groups were: (A1) an A1 milk diet with homozygous β-CN genotype A1A1 (n = 52; 27 females, 25 males), or (A2) an A2 milk diet with homozygous β-CN genotype A2A2 (n = 52; 27 females, 25 males).

Immediately after birth, the calves were weighed and received ad libitum colostrum (at least 10% of the body weight, with IgG > 50 mg/ml). On the second day, we collected blood samples from the jugular vein using EDTA-coated tubes to estimate

serum total protein (STP g/l) using a digital refractometer and to genotype the calves for β-CN. The calves were housed in pairs in double-igloo systems, according to their random birth order. The calves had ad libitum access to water and hay but no access to calf starter. They were offered 7.5 l of either A1 or A2 milk divided into three times daily: 06:00 (3 l), 11:30 (1.5 l), and 17:30 (3 l). Leftovers were measured to calculate daily milk intake (DMI) and daily energy-corrected milk intake (DMI_ECM).

The fecal score was recorded daily by the same person, after the second feeding time. The evaluations were based on the methodology of Renaud et al. (2020), where fecal score 1 = normal, 2 = soft, 3 = runny, and 4 = watery/diarrhea. Scores of 3 and 4 were considered as diarrhea. The fecal score was transformed as the mean fecal score during the evaluation period. Calves with FS 4 and rectal body temperature > 39.5 °C were offered an electrolyte solution and received an extra treatment of Parofor 140 mg/ml oral solution for 3 days.

On the 15th day, the calves were weighed to determine the end body weight (EBW), average daily gain (ADG), total milk intake (TMI), and feed efficiency. After that, the calves were lightly sedated with Xylazine 2% (0.4 mg/kg i.m.) for induction and Ketamine 10% (1–2 mg/kg i.v.) for sedation maintenance, for the imaging evaluations. The visceral adipose tissue (VAT) volume was assessed by an open magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DXA). The fat, and lean mass (g), as well as bone mineral content (g) and bone mineral density (g/cm^2) was assessed by DXA. All procedures for imaging evaluations and analysis are described in details by Kappes et al. (2024). A blood sample was collected from the jugular vein using EDTA-coated tubes to determine the concentration of β-casomorphine-7 (BCM-7). The blood sample was collected while the calves were sedated, which means approximately 13-14 hours after the last milk intake. The blood sample was centrifugated at 2000× g at 4 ◦C

for 25 min. Blood plasma was separated and stored in a −20 ◦C freezer until subsequent analyses. Serum levels of BCM-7 were measured according to Hohmann et al. (2021) using a commercial ELISA Kit (Creative Diagnostics, Shirley, NY, USA) and a Bio-Rad iMark 1.04.02 microplate reader (Bio-Rad, Neuberg, Germany), in accordance with the latest manufacturer's instructions (User's Manual, Bovine Beta-casomorphin-7, ELISA Kit, DEIA-XYZ57, Version 10-09/22, Creative Diagnostics).

The beta-casein genotypes of the calves were determined by using a 50k bovine oligonucleotide (SNP) chip analysis (Illumina BeadChip, Bovine Infinium® iSelect-96/XT) based on the genomic DNA extracted from the above described blood sampling at the facilities of GeneControl (Poing, Germany).

The statistical analysis was performed using the software SAS 9.4. The data were tested for normal distribution by applying Kolmogorov-Smirnov Test. A variance analysis (ANOVA) was performed by using the MIXED procedure. For all traits evaluated, the model included the fixed effects β-CN milk type, β-CN calf genotype, sex, breed, and β-CN milk type x β-CN calf genotype. The significance level was in all cases set to $P < 0.05$. As the data of BCM-7 had no normal distribution, we performed the non-parametric Wilcoxon and Kruskal-Wallis test using the NPAR1WAY Procedure. As the Wilcoxon test transforms the data into a mean score, we used the median value of the BCM-7 to present the data. The significance was set to Pr > Chi-Square < 0.05. We performed a logistic regression analysis using SAS 9.4 (The LOGISTIC procedure, SAS Institute Inc. 2013. SAS/STAT® 13.1 User's Guide. Cary, NC) for the daily recorded trait "diarrhea" defined as no diarrhea for fecal scores < 3 and diarrhea for fecal scores > 2. The calves were divided into three groups based on days with diarrhea Group 1: 0-1 day with diarrhea; Group 2: 2-4 days with diarrhea; Group 3: >4 days with diarrhea. We calculated, the Odds Ratios for the binary logit model by applying the Fisher's scoring

optimization technique containing the fixed effects milk type, sex, breed, β-CN calf genotype, combined with the covariates birth body weight (kg; BBW), and concentration of BCM-7 (ng/ml; CNC_BCM7). We applied a backward selection of additional covariates as were average daily gain, mean fecal score, total energy corrected milk intake and retained the model with the lowest AIC. The analysis includes n=103 calves, because one calf did not have a CNC_BCM7 value.

RESULTS AND DISCUSSION

No interaction was observed between β-CN milk type fed to the calves and β-CN genotype of the calves (Table 2), which means that the performance, mean fecal score, and days with diarrhea are influenced either by the genotype or the milk type feed. The effects of sex and breed have been previously presented and discussed by Kappes et al. (2024). Serum levels of BCM-7 were similar between calves fed A1 (0.728 mg/ml) and A2 (0.694 ng/ml) milk (Table 1; $P = 0.24$), which differs from the findings of Hohmann et al. (2021) who reported higher levels of BCM-7 in calves fed A1 milk (55.82 ng/ml) than A2 milk (12.73 ng/ml) on 21 days of life. The lack of difference may be related to the time of blood collection for BCM-7 analysis after the last feeding. In our study, blood samples were collected approximately 13-14 hours after the last feeding, whereas Hohmann et al. (2021) collected after 2-3 hours. Asledottir et al. (2018) also observed a higher concentration of BCM-7 in A1A1 milk after 1 and 2 hours of ex-vivo digestion. During the 13-14 hours period between milk feeding and blood sampling, the concentration of BCM-7 decreases due to metabolization by dipeptidyl-peptidase IV (DPP-IV) and excretion in urine (Sun et al., 2003; de Vasconcelos et al., 2023), although BCM-7 is released for 30 minutes to 6 hours after milk ingestion (de Vasconcelos et al., 2023). Hohmann et al. (2021) associated serum levels of BCM-7 with diarrhea

prevalence. Calves fed A1A1 milk had almost five times higher BCM-7 and a lower mean fecal score (1.97 vs. 2.56) and diarrhea prevalence (6% vs. 10%) in the first 21 days compared to calves fed A2A2 milk, respectively. Noni et al. (2009) and Fosset and Tomé (2022) reported an antidiarrheal effect of the BCMs, which stimulate electrolyte and water absorption and inhibit intestinal motility. In our study, the mean fecal score (1.4 vs. 1.5) and days with diarrhea (1.7 vs. 2.2 days) were similar between A1 and A2, respectively (Table 2), perhaps related to the similar BCM-7 levels. However, when evaluating the three β-CN calf genotypes, BCM-7 levels were similar among the three genotypes (P = 0.29), while A1A1 and A1A2 calves had a lower mean fecal score (P = 0.02) and less days with diarrhea (P = 0.01; Table 2). Similar results were obtained from a logistic regression analysis (Figure 1), in which there was no association between BCM-7 concentration and days with diarrhea (OR: 1.332; 95% CI: 0.745 – 2.383; P = 0.33). This suggests that the mean fecal score is influenced by factors other than the presence of BCMs. BCM-7 was not influenced by sex (P = 0.87) or breed/crossbred line (P = 0.23; Table 1).

The body composition, total milk intake, total energy-corrected milk intake, average daily gain, and feed conversion based on energy-corrected milk were similar between the two feeding groups (Table 2). These results have been previously presented and discussed by Kappes et al. (2024). Briefly, our results differ from Hohmann et al. (2021), who observed higher daily milk intake (7.28 vs. 6.96 l/day), average daily gain (0.75 vs. 0.64 kg/day), and feed conversion (9.2 vs. 10.5 l/kg gain) for calves fed A1 milk compared to A2 milk, respectively. The better performance is likely related to the lower mean fecal score and diarrhea prevalence in calves fed A1. In our study, the similar performance can also be attributed to the similar mean fecal score and days with diarrhea (de Paula et al., 2017; Feldmann et al., 2019).

Fifteen calves were A1A1 (14.4%), 44 calves were A1A2 (42.3%), and 45 calves were A2A2 (43.2%), corresponding to an allele frequency of 64.3% for A2. The genotype and allele frequencies were similar to those found by Arens et al. (2023) in Holstein dairy cows in organic farms in the United States. The present study was conducted on the same herd as the study by Hohmann et al. (2021), who reported an A2 allele frequency of 51.6%, more than 10% smaller than in our study, suggesting a selection toward the A2 allele. The same trend was reported by Scott et al. (2023), who observed a 20% increase in the A2A2 genotype frequency over 17 years in female Holstein herds in Australia, reaching 52% in 2017, while for bulls, the A2A2 genotype frequency was almost 60%. The increased selection towards the A2 allele may lead to an increase in inbreeding (Scott et al., 2023). This can negatively affect the cows and calf performance.

The similar body composition of the calves among the three β-CN genotypes can be related to the similar EBW. Only abdominal length differed among the three β-CN genotypes (P = 0.04; Table 2), being longer for A1A1 than A1A2, not differing from A2A2 (21.5 ± 0.2, 20.7 ± 0.9, and 21.1 ± 0.1 cm, respectively). No explanation was found for this difference, as the EBW was similar among them.

Calves with the A1A2 genotype had almost one day less with diarrhea (1.6 days) than A2A2 calves (2.5 days), with no significant difference from A1A1 calves (1.7 days; P = 0.01); the same trend was observed for mean fecal score (P = 0.02; Table 2). The similar mean fecal score and days with diarrhea for A1A1 and A2A2 can partly be explained by the lower number of A1A1 calves, which increases the standard error estimate. This is confirmed by the logistic regression, where calves with β-CN genotype A2A2 had increased odds of having more days with diarrhea in comparison with A1A1 or A1A2 β-CN genotype calves (P = 0.01; Figure 1). The lower mean fecal score and days with diarrhea affected the average daily gain, being slightly better for A1A2 and

A1A1 compared to A2A2 calves (P = 0.10; Table 2). de Paula et al. (2017) and Feldmann et al. (2019) also showed the negative influence of diarrhea on average daily gain (ADG). The occurrence of diarrhea is, only in part, determined by genetics. Mahmoud et al. (2017) observed a heritability of 0.06 for calf diarrhea in German Holstein calves, while Fuerst-Waltl et al. (2010) estimated a very low heritability of 0.027 for the liability to diarrhea in Austrian dual-purpose Fleckvieh (Simmental) heifer calves. Another factor that can contribute to diarrhea occurrence is the elevated inbreeding coefficient. Anderson et al. (2003) reported that inbred beef calves (inbreeding coefficient of 0.20) had significantly more often diarrhea (41 %) than outbred beef calves (28 %). Fuerst-Waltl and Fuerst (2012) observed that Austrian Brown Swiss calves with inbreeding coefficients of 0.10 had, on average, 1.8% higher mortality than non-inbred calves during 48 hours to 30 days of life.

Scott et al. (2023) found higher estimated breeding values (EBV) for somatic cell count (SCC) and survival in A1A1 cows, and higher EBV for SCC in A1A1 bulls compared to A2A2. The authors also associated these findings with higher inbreeding depression in A2A2 animals, which can negatively influence animal health. Sørensen et al. (2006) reported that a cow with 5% inbreeding had a higher SCC and a higher incidence of mastitis compared with a cow with 2% inbreeding. McParland et al. (2007) also reported that inbreeding increased somatic cell score (SCS). Arens et al. (2023) found similar SCS between the three genotypes (A1A1, A1A2, and A2A2). The survival rate to the first and second lactation was similar between the A1A1 and A2A2 genotypes, being lower than A1A2. The authors suggested that some farms may have used the β-CN genotype results to cull cows, thus inflating the number of A1A1 culled cows, although the survival was similar to A2A2. This shows that the survival rate is influenced by factors

253 other than health, and a selection towards one characteristic can influence culling
254 decisions.
255

256 **CONCLUSION**

257 The A1 and A2 β-CN milk did not affect body composition, total milk intake,
258 average daily gain, feed conversion, mean fecal score, and days with diarrhea. The serum
259 levels of BCM-7 were similar between the two feeding groups, genotypes, sexes, and
260 breeds, and had no association with the odds of days with diarrhea. Calves with the A2A2
261 β-CN genotype had increased odds of having more days with diarrhea in comparison with
262 A1A1 or A1A2 β-CN genotype calves. Calves with the A1A2 β-CN genotype had a lower
263 mean fecal score and fewer days with diarrhea than A2A2 calves in the first two weeks
264 of life, not differing from A1A1 calves, which resulted in a slightly better average daily
265 gain for A1A2 and A1A1 calves. The body composition was similar among the three β-
266 CN genotypes. The absence of an interaction between β-CN milk type and β-CN calf
267 genotype indicates that performance is influenced either by the genotype or the the
268 ingested milk type. Further studies are needed to investigate the effect of the β-CN calf
269 genotype on performance and health throughout the entire pre-weaning period and
270 productive life, involving a larger number of animals.
271

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Table 1. Median of the concentration of β-casomorphin-7 (BCM-7) expressed as ng/ml and the significance (Pr > Chi-Square) for the fixed effects of β-CN milk type, β-CN genotype, sex, and breed.

Fixed effects		Median of BCM-7 (ng/ml)	Pr > Chi-Square
β-CN milk type	A1	0.728	0.24
	A2	0.694	
β-CN calf genotype	A1A1	0.717	0.29
	A1A2	0.766	
	A2A2	0.59	
Sex	Female	0.702	0.87
	Male	0.718	
Breed	German Holstein	0.717	0.23
	German Simmental	0.796	
	Crossbred	0.539	

Table 2. Means (± standard error), and significance levels (P-value) for body composition, body weight, milk intake, average daily gain, feed conversion, mean fecal score, and days with diarrhea for the fixed effects β-CN milk type and β-CN genotype and interaction.

Variables	β-CN milk type		P-value	β-CN calf genotype			P-value	P-value interaction[1]
	A1	A2		A1A1	A1A2	A2A2		
MRI VAT cm[32]	316.5 ± 17.1	337.9 ± 17.7	0.38	352.0 ± 27.5	305.1 ± 17.5	324.5 ± 17.4	0.34	0.61
DXA VAT, cm[33]	385.1 ± 25.4	348.8 ± 26.2	0.31	371.4 ± 40.7	357.4 ± 25.9	372.0 ± 25.7	0.90	0.49
DXA fat mass, g	3129.2 ± 74	3139.7 ± 76	0.92	3244.2 ± 119	3066.1 ± 75	3093.0 ± 75	0.46	0.29
DXA lean mass, g	47258 ± 697	46536 ± 720	0.46	47697 ± 1118	46932 ± 712	46063 ± 707	0.40	0.28
DXA BMC, g[4]	1711.9 ± 35	1688.3 ± 36	0.63	1737.0 ± 56	1678.7 ± 35	16684.6 ± 35	0.68	0.54
DXA BMD, g/cm[25]	0.820 ± 0.01	0.816 ± 0.01	0.80	0.835 ± 0.01	0.808 ± 0.01	0.810 ± 0.01	0.50	0.90
Abdominal length, cm	21.1 ± 0.1	21.0 ± 0.1	0.73	21.5 ± 0.2 a	20.7 ± 0.9 b	21.1 ± 0.1 ab	0.04	0.37
Birth body weight, kg	41.9 ± 0.8	41.1 ± 0.8	0.52	41.7 ± 1.3	41.1 ± 0.8	41.6 ± 0.8	0.90	0.47
End body weight, kg	52.1 ± 0.7	51.0 ± 0.8	0.31	52.2 ± 1.2	51.6 ± 0.8	50.9 ± 0.8	0.62	0.38
Average daily gain, g	704 ± 29	678 ± 32	0.54	710 ± 49	723 ± 30	639 ± 31	0.10	0.75
Total milk intake, l	96.6 ± 1.3	94.9 ± 1.5	0.40	98.0 ± 2.3	95.2 ± 1.4	94.1 ± 1.4	0.38	0.44
TMI_ECM, l[6]	105.0 ± 1.8	104.7 ± 2.0	0.93	107.6 ± 3.1	103.1 ± 1.9	103.9 ± 2.0	0.50	0.18
Feed conversion, l/kg[7]	10.5 ± 0.3	10.7 ± 0.3	0.73	10.5 ± 0.6	10.3 ± 0.3	10.9 ± 0.3	0.40	0.56
Mean fecal score	1.4 ± 0.05	1.5 ± 0.05	0.20	1.4 ± 0.08 ab	1.3 ± 0.05 b	1.5 ± 0.05 a	0.02	0.82
Days with diarrhea	1.7 ± 0.2	2.2 ± 0.2	0.15	1.7 ± 0.3 ab	1.6 ± 0.2 b	2.5 ± 0.2 a	0.01	0.93

[1]Interaction between β-CN milk type* β-CN calf genotype.
[2]Visceral adipose tissue evaluated by magnetic resonance imaging
[3]Visceral adipose tissue evaluated by dual energy X-ray absorptiometry
[4]Bone mineral content
[5]Bone mineral density
[6]Total energy-corrected milk intake.
[7]Feed conversion based on the total energy-corrected milk intake per kg of body weight gain.
* Different letters within lines describe significant differences with $P < 0.05$.

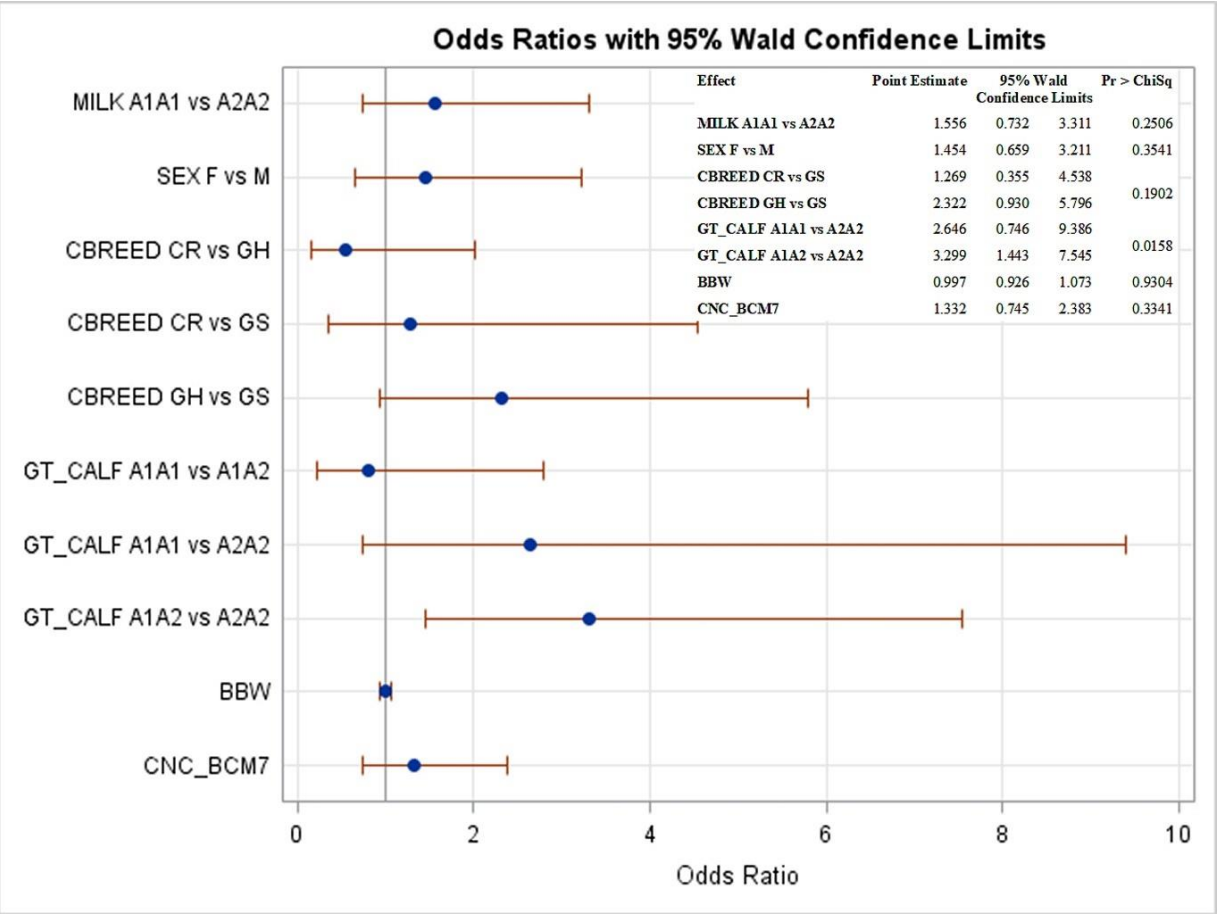

Figure 1. Results of the Logistic regression analysis of the daily Diarrhea records (Group 1: 0-1 day with diarrhea; Group 2: 2-4 days with diarrhea; Group 3: >4 days with diarrhea) for the classes milk type (A1A1 vs A2A2), sex (f=female, m =male), breed (CBREED with CR=crossbred, GS = German Simmental, GH = German Holstein), β-CN calf genotype (GT_CALF A1A1, A1A2, A2A2), combined with the covariates birth body weight (BBW), and concentration of β-casomorphin-7 (CNC_BCM7). The model was chosen due to the lowest AIC.

FINAL CONSIDERATIONS

Currently, the selection of bulls and cows for homozygous A2 β-CN milk is increasing in several countries due to the growing niche market for the so-called "A2 milk," considered as a "healthier" milk. With this trend, more farmers will be compelled to produce A2A2 milk to meet this new demand. However, this can be worrisome in the near future, as the increasing selection towards one characteristic may lead to an elevation in inbreeding depression, resulting in negative effects on animals' performance, including milk production and composition, fertility, health, and consequently, survival.

Although several studies report and suggest some adverse effects of consuming A1 β-CN milk on human health, this evidence is not clear and conclusive yet, and more studies in humans need to be performed with clear methodology and controlled trials. Besides that, calves' health must be considered, as the milk offered on farms is usually a mix of A1 and A2 β-CN. The effects of feeding A1 or A2 milk, or carrying the A1 or A2 β-CN alleles need to be investigated for longer periods, in a larger number of animals, and in other parameters such as respiratory disorders. Nevertheless, the A2 β-CN variant is important for specific markets to meet the needs of A1 β-CN allergic individuals. This creates an opportunity for farms and the industry to produce niche products such as "A2 milk, cheese, yogurt," and various other dairy products based on A2 milk, with added value.

Further studies need to be performed to evaluate the concentrations of BCM-7 in blood and urine at different times after A1 and A2 milk intake. We also suggest the evaluation of this peptide concentration under different volumes of milk offered and correlate these results with the daily fecal score and with plasma concentration of inflammation-related biomarkers such as IL-4, IgG, IgE, and IgG1.

Maybe the mutation on the β-CN loci is part of the evolution process to naturally improve the health of the calves. This idea can be suggested based on some evidence found in previous and in our study. However, to better understand the relationship between the β-CN genotype and the ingestion of A1 and A2 milk on performance and health, future studies need to be performed, considering the pre- and post-weaning period and productive life. Perhaps there is a residual effect through the productive life due to initial growth performance and the early alteration of the ruminal and gut microbiome.

Body composition is a trait explored by only a few studies. This was the first study evaluating visceral adipose tissue in dairy calves using magnetic resonance imaging (MRI) equipment. The limited space on the MRI equipment is a limiting factor for evaluations in older calves (weaning period). However, dual-energy X-ray absorptiometry (DXA) can be used in calves up to 150 kg and might be a better option. The measurement of the visceral adipose tissue (VAT) can be an important tool for determining calves' performance, mainly associated with body weight gain, as bone, lean, and fat tissue have different weights considering the same volume (e.g., cm^3).

As studies exploring body composition are scarce, the VAT, lean, and fat tissue volume measured by DXA and MRI during the pre-weaning period can be correlated with future performance (e.g., weaning weight, age at first service and calving, milk yield during the first lactation). It may become an important trait for indirect selection in early life if there is a correlation with performance in adult life (milk yield, reproductive performance, energetic balance during the transition period, etc.). The amount of fat and lean tissue explored in our study helps, in part, to understand the different energetic metabolism in Holstein, Simmental, and crossbred cows.

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Effect of β-casein A1 or A2 milk on body composition, milk intake, and growth in Holstein, Simmental, and crossbred dairy calves of both sexes

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ABSTRACT

The aim of this study was to compare the effects of feeding homozygous β-casein A1 or A2 milk, on the body composition, milk intake, and growth of German Holstein (GH), German Simmental (GS), and crossbred dairy calves of both sexes during the first 2 weeks of life. A total of 104 calves (n = 54 female - f and n = 50 male - m) from the breed groups GH (n = 23), GS (n = 61), and crossbred GH x GS (CR; n = 20) were evaluated. Calves were weighed after birth and received colostrum ad libitum. On the second day, calves were alternately housed in pairs in double-igloo systems according to their random birth order and received either A1 milk (n = 52; 27 f / 25 m) or A2 milk (n = 52; 27 f / 25 m). They were offered 7.5 L/day, and the individual actual total milk intake (TMI) was recorded. Daily energy-corrected milk intake was also calculated based on the milk composition (fat and protein). Fecal scores were recorded daily. On d 15, visceral adipose tissue (VAT) volume was assessed by open magnetic resonance imaging (MRI) and dual-energy x-ray absorptiometry (DXA). In addition, fat and lean mass (g), as well as bone mineral content (g) and bone mineral density (g/cm^2), were determined by DXA. The body composition, milk intake, and growth were similar between the 2 types of milk in the first 2 weeks of life. Female calves had more VAT and fat mass, but less lean mass than male calves. GH and CR calves had more VAT and less lean mass than GS calves. Male calves were heavier than female calves after birth and on d 15. The average days with diarrhea and diarrhea occurrence were similar between calves fed A1 and A2 milk and between both sex groups. GS calves presented slightly more days with diarrhea and increased odds of having diarrhea compared with GH calves, not differing from CR.

Key words: daily milk intake, dual energy X-ray absorptiometry, fecal score, magnetic resonance imaging

INTRODUCTION

β-casein (β-CN) constitutes approximately 33% of the total protein found in bovine milk (McMahon and Brown, 1984). Dairy cattle possess 12 variants of β-CN, with the most common being A1, A2, and B, exhibiting different genotype frequencies (Sebastiani et al., 2020; Hohmann et al., 2021). The A1 and A2 variants are distinguished solely by an amino acid difference at position 67 of the 209-amino acid protein, where A2 has Proline and A1 has Histidine due to a mutation from the A2 variant (Brooke-Taylor et al., 2017; Chitra, 2022). When the A1 variant undergoes enzymatic digestion, it releases a bioactive opioid peptide consisting of 7 amino acids, known as β-casomorphin-7 (BCM-7) (Kullenberg de Guadry et al., 2019). This peptide is not digested in the human body, activating μ-opioid receptors and increasing the risk of diseases such as type-1 diabetes, cardiovascular diseases, higher plasma LDL-C concentrations, as well as neurological and mainly digestive disorders, including abdominal discomfort and diarrhea (Jianqin et al., 2016; He et al., 2017; Kullenberg de Guadry et al., 2019; Woodford, 2021; Chitra, 2022). However, Ho et al. (2014) reported significantly higher stool consistency scores in individuals consuming A1 β-CN milk compared with A2 milk. Petrat-Melin et al. (2015) observed a higher in vitro digestion rate for A1 milk compared with A2 milk. In their review, Kullenberg de Guadry et al. (2019) concluded that the results from several studies evaluating the influence of A1 milk as a cause of different diseases were inconclusive, due to the significant variability in the results.

In addition to the health aspects associated with the intake of A1 and A2 milk in humans, the performance and health of animals must also be considered. Umbach et al. (1985) reported the presence of β-CM7 in the

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