Journal of Animal and Feed Sciences, 35, 2, 2026, e16, https://doi.org/10.22358/jafs/212108/2026
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

ORIGINAL PAPER

Influence of breed and feeding season on peptide levels in cow's milk

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KEY WORDS: biologically active peptides, breed, cow milk, feeding season, feeding system

Received: 17 July 2025
Revised: 15 September 2025
Accepted: 13 October 2025

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ABSTRACT. The aim of the study was to assess the influence of cow breed and feeding season on the concentration of health-promoting, bioactive peptides in cows' milk. Milk from Holstein-Friesian cows (HF) contained more bioactive peptides than milk from Simmental cows (SIM), regardless of season, including peptides with specific biological functions. HF milk showed higher concentrations of colostrinin (HF: 413.65 ng/ml; SIM: 371.14 ng/ml), casokinin (HF: 90.48 ng/ml; SIM: 68.89 ng/ml), casomorphin (HF: 87.73 µmol/ml; SIM: 76.17 µmol/ml), and ceruloplasmin (HF: 2141.63 ng/ml; SIM: 1547.94 ng/ml). These results indicate stronger neuroprotective, anticancer, antioxidant, immunomodulatory, and anti-obesity properties of HF milk. Milk collected in summer, particularly from cows fed silage and maize silage, showed more favourable characteristics. It contained higher concentrations of health-related components, including copeptin (summer: 467.84 ng/ml; winter: 406.26 ng/ml). Increased levels of bioactive substances in summer milk are likely attributable to a greater share of forage, especially silage, and a reduced proportion of legume feed. However, it was also observed that increasing the percentage of starch-rich feed, such as cereals and maize silage, negatively affected the biological value of milk and reduced the levels of biologically active peptides.

Introduction

Cow's milk is a source of biologically active peptides (BAP), which include fractions and products of casein hydrolysis, whey proteins, and proteins of the milk fat globule membrane (Cervantes-Garcia et al., 2020). In addition to their nutritional value, BAP retain many biological properties and can exert positive effects on physiological processes and overall health. Their activity depends mainly on amino-acid composition and sequence, as well as the chemical bonds within the molecule. Antioxidant properties are associated with hydrophobic and sulphhydryl groups, sulphur-containing amino acids, and a high proportion of proline residues (Cervantes-Garcia et al., 2020). Antimicrobial properties of BAP are linked to short chain length and a cationic structure. Antihypertensive properties are due to the presence of the proline-valine (Pro-Val) sequence, while immunomodulatory activity is linked to the glycine-histidine (Gly-His) sequence (Cervantes-Garcia et al., 2020). Opioid activity depends on the presence of aromatic aminoacids, while branched-chain amino acids,

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particularly abundant in the whey fraction, are important for muscle repair and development (Cervantes-Garcia et al., 2020). BAP can be released from precursor proteins, and most of its biological activity is encoded in the native protein sequence. They are believed to promote a variety of beneficial effects, including hypocholesterolaemic activity (by reducing cholesterol solubility and limiting its intestinal absorption), glycaemic control, antihypertensive activity, anticoagulant effects (inhibition of platelet aggregation), and immunomodulatory activity. They may also act as regulatory agents, with hormone-like functions (Cervantes-Garcia et al., 2020; Zaky et al., 2022). BAP also function as an antioxidant by inhibiting non-enzymatic peroxidation of essential fatty acids, and shows anti-inflammatory, anti-coagulant, and antibacterial effects. They can regulate gastric mucosal function, support ion transport, exert neuroprotective effects, and reduce anxiety. Some BAP also show opioid activity by binding to opioid receptors located in the central nervous system and gastrointestinal tract, and play a key role in responses to pain and stress (Nguyen et al., 2015).

The basic protein fractions of milk, also in the context of bioactivity, are casein and whey. Casein proteins form aggregates in the milk matrix, which give milk its white colour. The biological activity of peptides derived from casein depends on amino acid composition, the quantity of individual amino acids, and their capacity to transport minerals for passive absorption. Whey is a water fraction containing a significant number of high-quality proteins and peptides. Its amino acid profile includes many essential amino acids with high bioavailability and bioactivity (Mills et al., 2011). Of the 9 amino acids essential for humans found in whey, methionine, leucine, lysine, and tryptophan contribute most to its bioactive properties. The main peptides with distinct biological functions in this fraction are αlactalbumin and lactoferrin (Layman et al., 2018; Li et al., 2019). Milk fat globules and their membranes contain globular membrane proteins, phospholipids, glycoproteins (cerebrosides), lipoproteins, cholesterol, and monoglycerides (Kosmerl et al., 2024). This membrane acts as a natural emulsifier, covering the surface of the fat globule. Mucin is the principal bioactive peptide associated with this component. The type and concentration of BAP in milk likely depend on breed and feeding system. These factors influence the overall protein content, the amino acid composition of proteins and peptides, proteolytic enzyme activities, and microbiota community structure. Breed has a marked effect on the profile of endogenous peptides produced during protein hydrolysis in milk. The feeding system further modifies the composition and type of individual peptides with health-related properties for humans (Bhattacharya et al. 2019). Reklewska et al. (2003) also identified feeding system and season as dominant factors determining the content of biologically active milk components.

It is well-established that milk from cows with a high protein percentage contains many longer-chain peptides, such as casein and β-lactoglobulin. However, available literature lacks data on short-chain peptides, which possess unique health-promoting properties for humans.

Given this context, the research hypothesis was formulated that the content of biologically active peptides in milk depends on cow breed and feeding system. The objective was to determine whether these factors influence the variability of BAP levels in cows' milk with relevance to human health.

Material and methods

The research material consisted of bulk milk collected from cows from 9 conventional farms in eastern and south-eastern Poland. Samples were collected during two feeding periods: the summer season (June–September 2024) and the winter season (October 2024–January 2025). A total of 162 bulk milk samples were obtained in each feeding season. Feed on each farm was prepared individually, according to available raw materials. Farms 1, 3, 5, 8, and 9 kept Holstein-Friesian cows (HF, 367 cows), while farms 2, 4, 6, and 7 kept Simmental cows (SIM, 384 cows) (Table 1). Forage feed

Table 1. Characteristics of farms, cow breeds, type of farming and type of feed used

Farm No.	Cow breed	Type of cow housing system	Type of feed – summer season	Type of feed – winter season
1	HF	T	TMR (MS+S)	TMR (MS+S)
2	SIM	FS	TMR (MS+S)	TMR(MS+S)
3	HF	FS	TMR (MS+S)	TMR (MS+S)
4	SIM	T	TMR (MS+S)	TMR (MS+S)
5	HF	FS	TMR (S+BP)	TMR (S+BP)
6	SIM	FS	TMR (S+MS)	TMR (S+MS)
7	SIM	FS	TMR (S+BP)	TMR (S+BP)
8	HF	FS	TMR (S+MS)	TMR (S+MS)
9	HF	T	TMR (S+MS)	TMR (S+MS)

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, T – tethered housing system, FS – free-stall housing system, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage comprised approximately 75% of the mixtures. In the feeds used on farms 1–4, the forage (total mixed ration (TMR)) was maize silage (approximately 50% of the feed mixture) with the addition of silage TMR (MS+S). On farms 5 and 7 the feed was TMR (S+BP) – silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp. On farms 6, 8, and 9 it was TMR (S+MS) – silage (approximately 50% of the feed mixture) with the addition of maize silage (Table 1). Milk was collected in the mid-lactation period of the cows. Milk from cows of each breed (HF or SIM), fed TMR (S+BP), TMR (MS+S), or TMR (S+MS), was pooled. From the pooled six milk samples (HF, TMR (S+BP); HF, TMR (MS+S); HF, TMR (S+MS); SIM, TMR (S+BP); SIM, TMR (MS+S); SIM, TMR (S+MS), six independent milk samples were collected for biochemical analysis (36 samples).

Determination of bioactive peptides in bovine milk

All measurements were determined in raw cow's milk. Each sample was analysed in 6 replicates. ELISA kits were used to quantify casokinin, colostrinin, copeptin caseinomacropeptide, lysozyme, mucin, ceruloplasmin, casein-α, casein-β, casein-κ, lactoferrin, serum albumin, immunoglobulin, lactokinin, and β-casomorphin. All tests were produced by Qayee Bio-Technology Co. (Shanghai, China).

Statistical analysis

This experiment used a completely randomised 3 × 2 factorial design. Data are presented as means and were analysed using 2-way ANOVA to assess the effects of two cow breeds (HF and SIM) and three types of feed mixture: TMR (MS+S), TMR (S+BP), and TMR (S+MS). Homogeneity of variance and normality were verified using the Levene and Shapiro–Wilk tests. When a significant interaction effect was found, treatment means were compared using Tukey's post-hoc test. Differences were considered statistically significant at $P \leq 0.05$. Statistical analyses were conducted using STATISTICA, version 12.0 (StatSoft Corp., Krakow, Poland).

Results

Influence of breed, feeding system, and season on selected BAP levels in cow's milk

During summer, both feed type and breed significantly affected the level of bioactive peptides in milk (Table 2). A significant breed-by-feeding interaction ($P < 0.001$) was observed for the concentrations of β-casomorphin and colostrinin. This reflected higher concentrations in milk from cows receiving TMR (S+MS), irrespective of breed, while this pattern did not occur under TMR (MS+S) feeding or TMR (S+BP). Casokinin levels were higher ($P < 0.001$) in the milk of HF cows fed a TMR (MS+S), which increased ($P = 0.002$) the concentration of this component more than the other diets (Table 2).

Table 2. Level of β-casomorphin, casokinin and colostrinin in cow's milk - summer feeding season

Indices	β-casomorphin, µmol/ml	Casokinin, ng/ml	colostrinin, ng/ml
Breed effect (B)			
HF	95.00	104.21^a	393.66
SIM	89.39	81.34^b	357.26
Feeding effect (F)			
TMR (MS+S)	66.26^c	103.83^a	334.52^c
TMR (S+BP)	99.79^b	84.71^b	381.93^b
TMR (S+MS)	110.54^a	89.79^b	409.93^a
P -value			
B	0.042	<0.001	0.06
F	<0.001	0.002	<0.001
B x F	<0.001	0.66	0.011

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+s) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{abc} – means with different superscripts are significantly different at $P \leq 0.05$

Lysozyme levels were higher ($P = 0.002$) in milk from cows fed the TMR (MS+S) system and those receiving TMR (S+MS), compared with cows fed TMR (S+BP) (Table 3). In SIM cows, the level of mucin was higher ($P = 0.001$) than in HF cows. Feeding TMR (S+BP) or TMR (S+MS) also increased ($P = 0.011$) mucin levels in milk. A breed × feeding interaction ($P = 0.003$) was also observed for lactokinin (Table 3). TMR (S+MS) increased the level of this peptide in milk of SIM cows.

Compared to SIM cows, milk from HF cows had a higher ($P = 0.044$) ceruloplasmin concentration (Table 4). Feeding TMR (S+MS) increased ($P = 0.002$) the level of this peptide in milk. A significant breed and feeding interaction was noted for copeptin ($P = 0.036$). Feeding TMR (S+BP) or TMR (S+MS) increased its content in milk regardless of breed (Table 5). A breed × feeding interaction was also observed for α-casein ($P = 0.011$). Feeding TMR (S+MS) increased this protein level in the milk irrespective of breed (Table 4).

The use of the TMR (MS+S) significantly reduced albumin levels in cow's milk ($P < 0.001$).

Table 3. Level of α-lactoferrin, lysozyme, mucin and lactokinin in cow's milk - summer feeding season

Indices	α-lactoferrin, μg/ml]	Lysozyme, ng/ml	Mucin, ng/ml	Lactokinin, μg/ml
Breed effect (B)				
HF	275.92	94.58	234.16^{b}	1306.86
SIM	253.48	99.97	330.21^{a}	1416.23
Feeding (F)				
TMR (MS+S)	272.73	146.10^{a}	224.46^{b}	1334.37
TMR (S+BP)	256.61	41.36^{b}	313.50^{a}	1316.48
TMR (S+MS)	264.75	104.42^{a}	308.59^{a}	1409.58
P -value				
B	0.005	0.772	0.001	0.070
F	0.168	0.002	0.011	0.325
B x F	<0.001	0.156	0.056	

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{ab} – means with different superscripts are significantly different at $P \leq 0.05$

Table 5. Level of serum albumin, immunoglobulins, caseinomacropeptide, and copeptin in cow's milk – summer feeding season

Indices	Serum, albumin μg/ml	Immunoglobulins, μg/ml	Caseinomacropeptide, ng/ml	Copeptin, ng/ml
Breed effect (B)				
HF	950.72	2346.975^{b}	245.96^{b}	376.29
SIM	1171.80	3060.058^{a}	334.46^{a}	496.72
Feeding effect (F)				
TMR (MS+S)	933.85^{b}	1758.656^{c}	304.08	290.53^{b}
TMR (S+BP)	1145.25^{a}	3625.525^{a}	275.45	551.14^{a}
TMR (S+MS)	1104.70^{a}	2726.368^{b}	291.08	467.84^{a}
P -value				
B	0.08	<0.001	<0.001	0.062
F	0.001	<0.001	0.091	0.001
B x F	<0.001	0.562	<0.001	0.036

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{abc} – means with different superscripts are significantly different at $P \leq 0.05$

Immunoglobulin content was highest in milk from SIM cows ($P < 0.001$). TMR (S+BP) increased immunoglobulin levels ($P < 0.001$), whereas TMR (MS+S) reduced them ($P < 0.001$). Milk from SIM cows also contained more caseinomacropeptide than milk from HF cows ($P < 0.001$) (Table 5).

In the winter season, as in summer, both the feeding system and breed significantly affected several peptide levels. Regardless of feed type, casokinin levels were higher ($P < 0.001$) in milk from HF cows than in milk from SIM cows (Table 6).

Regardless of breed, feeding with TMR (MS+S) or TMR (S+MS) increased colostrinin levels in milk ($P < 0.001$) (Table 6). A higher content of lactoferrin ($P = 0.001$) and lysozyme ($P < 0.001$) was also detected in SIM cows' milk compared to HF cows. Breed did not influence mucin levels, whereas TMR (MS+S) increased this peptide concentration ($P = 0.018$) (Table 7).

Milk from SIM cows contained more lactokinin than milk from HF cows ($P = 0.021$) (Table 7). Milk from cows fed TMR (S+BP) showed a higher plasma κ-casein concentration ($P = 0.003$) than milk from cows fed TMR (MS+S) or TMR (S+MS) (Table 8). An interaction between breed and diet was recorded for milk albumin ($P < 0.001$) (Table 9). Milk from HF cows had a higher ceruloplasmin content than milk from SIM cows ($P = 0.032$), while TMR (MS+S) feeding increased ceruloplasmin levels to the greatest extent ($P = 0.049$) (Table 8).

The level of copeptin in milk was clearly modified by diet (Table 9), and TMR (S+MS) exerted the strongest effect ($P = 0.027$). Breed affected β-casein levels, which were higher in SIM cows ($P = 0.042$) (Table 8). Immunoglobulin levels were higher ($P < 0.001$) in HF cows, particularly when fed TMR (S+BP) ($P < 0.001$). For caseinomacropeptide, a breed × feeding interaction was identified ($P < 0.003$); cows receiving TMR (S+MS) or TMR (S+BP) showed higher concentrations than those

Table 4. Level of ceruloplasmin, α-casein, β-casein, and κ-casein in cow's milk – summer feeding season

Indices	Ceruloplasmin, ng/ml	α-casein, μg/ml	β-casein, μg/ml	κ-casein, g/ml
Breed effect (B)				
HF	1951.46^{a}	1089.63	544.15	135.09
SIM	1265.06^{b}	1066.39	633.62	148.95
Feeding effect (F)				
TMR (MS+S)	804.63^{c}	1010.33^{c}	674.14	142.08^{ab}
TMR (S+BP)	1461.82^{b}	1026.59^{b}	518.18	171.84^{a}
TMR (S+MS)	2558.31^{a}	1179.97^{a}	574.33	112.15^{b}
P -value				
B	0.044	0.226	0.085	0.335
F	0.002	0.018	0.058	0.014
B x F	0.079	0.011	0.022	0.027

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{abc} – means with different superscripts are significantly different at $P \leq 0.05$

Table 6. Level of β-casomorphin, casokinin, colostrinin and amyloid A in cow's milk - winter feeding season

Indices	β-casomorphin, μmol/ml	Casokinin, ng/ml	Colostrinin, ng/ml
Breed effect (B)			
HF	70.45	76.75^{a}	433.64
SIM	62.94	56.44^{b}	385.02
Feeding effect (F)			
TMR (MS+S)	74.94	65.71	466.49^{a}
TMR (S+BP)	56.94	63.20	293.92^{b}
TMR (S+MS)	68.21	70.87	467.58^{a}
P -value			
B	0.185	<0.001	0.092
F	0.051	0.220	<0.001
B x F	0.009	0.005	0.069

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{ab} – means with different superscripts are significantly different at $P \leq 0.05$

Table 7. Level of α-lactoferrin. lysozyme. mucin and lactokinin in cow's milk - winter feeding season

Indices	α-lactoferrin, μg/ml	Lysozyme, ng/ml	Mucin, ng/ml	Lactokinin, μg/ml
Breed effect (B)				
HF	412.29^{b}	111.76^{b}	379.14	349.13^{b}
SIM	508.54^{a}	160.72^{a}	346.12	401.78^{a}
Feeding effect (F)				
TMR (MS+S)	468.35^{ab}	125.90^{b}	521.94^{a}	403.13^{a}
TMR (S+BP)	539.16^{a}	150.08^{a}	341.51^{b}	224.82^{b}
TMR (S+MS)	373.75^{b}	132.74^{ab}	269.47^{b}	430.82^{a}
P -value				
B	0.010	<0.001	0.975	0.021
F	0.004	0.039	0.018	0.010
B x F	0.413	0.223	0.991	0.065

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{ab} – means with different superscripts are significantly different at $P \leq 0.05$

Table 8. Level of ceruloplasmin. α-casein. β-casein and κ-casein in cow's milk – winter feeding season

Indices	ceruloplasmin, ng/ml	α-casein, μg/ml]	β-casein, μg/ml	κ-casein, μg/ml
Breed effect (B)				
HF	2331.79^{a}	884.97	619.66^{b}	176.19
SIM	1830.81^{b}	847.35	700.96^{a}	164.76
Feeding effect (F)				
TMR (MS+S)	3047.68^{a}	654.88^{c}	896.55^{a}	155.14^{b}
TMR (S+BP)	1598.10^{b}	973.45^{b}	625.00^{ab}	188.43^{a}
TMR (S+MS)	1598.12^{b}	1082.61^{a}	459.39^{b}	167.86^{b}
P -value				
B	0.032	0.259	0.062	0.085
F	0.049	<0.001	0.012	0.003
B x F	0.801	0.119	0.072	0.079

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{abc} – means with different superscripts are significantly different at $P \leq 0.05$

Table 9. Level of serum albumin. immunoglobulins. caseinomacropeptide and copeptin in cow's milk – winter feeding season

Indices	Serum albumin, μg/ml	Immunoglobulins, μg/ml	Caseino-macropeptide, ng/ml	Copeptin, ng/ml
Breed effect (B)				
HF	1292.961	4226.83^{a}	380.25	306.18
SIM	1261.517	3274.71^{b}	321.27	370.61
Feeding effect (F)				
TMR (MS+S)	1161.37^{b}	3027.14^{b}	277.35^{b}	304.97^{b}
TMR (S+BP)	1191.30^{b}	4789.02^{a}	360.87^{a}	303.97^{b}
TMR (S+MS)	1479.05^{a}	3436.15^{b}	414.06^{a}	406.25^{a}
P -value				
B	0.414	<0.001	0.018	0.274
F	<0.001	<0.001	0.001	0.027
B x F	<0.001	0.473	0.003	0.694

HF – Holstein-Friesian cow breed, SIM – Simmental cow breed, TMR (MS+S) – forage was maize silage (approximately 50% of the feed mixture) with the addition of silage; TMR (S+BP) – forage was silage (approximately 50% of the feed mixture) with the addition of ensiled beet pulp, TMR (S+MS) – forage was silage (approximately 50% of the feed mixture) with the addition of maize silage; ^{ab} – means with different superscripts are significantly different at $P \leq 0.05$

fed TMR (MS+S), irrespective of breed (Table 9). Feeding TMR (MS+S) or TMR (S+MS) increased lactokinin levels compared with TMR (S+BP) ($P = 0.010$) (Table 7).

Seasonal analysis revealed that milk collected in the summer season contained a significantly higher concentration of β-casokinin than milk from the winter season ($P < 0.001$). Summer milk contained more copeptin ($P < 0.001$) than winter milk. Winter milk contained more colostrinin and caseinomacropeptide than summer milk ($P < 0.001$, Figure 1). Milk from the winter season had significantly higher concentrations of lysozyme, mucin, and ceruloplasmin ($P < 0.001$ for all) compared to milk from the summer season (Figure 2). For casein (Figure 3), summer milk contained more of α-casein fraction than winter milk ($P < 0.001$).

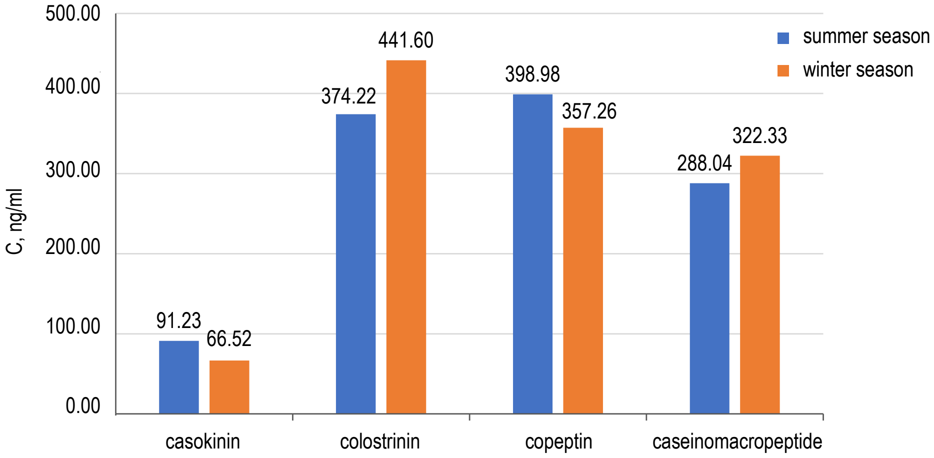

Figure 1. Concentration of casokinin, colostrinin, copeptin and caseinomacropeptide in bovine milk depending on feeding season

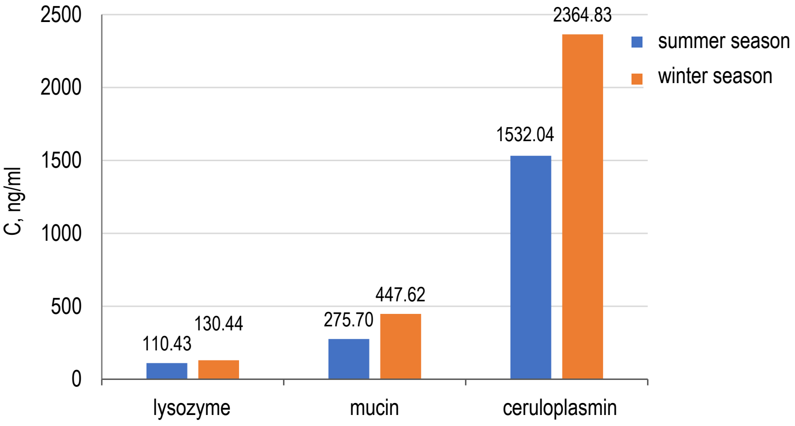

Figure 2. Concentration of lysozyme, mucin and ceruloplasmin in bovine milk depending on feeding season

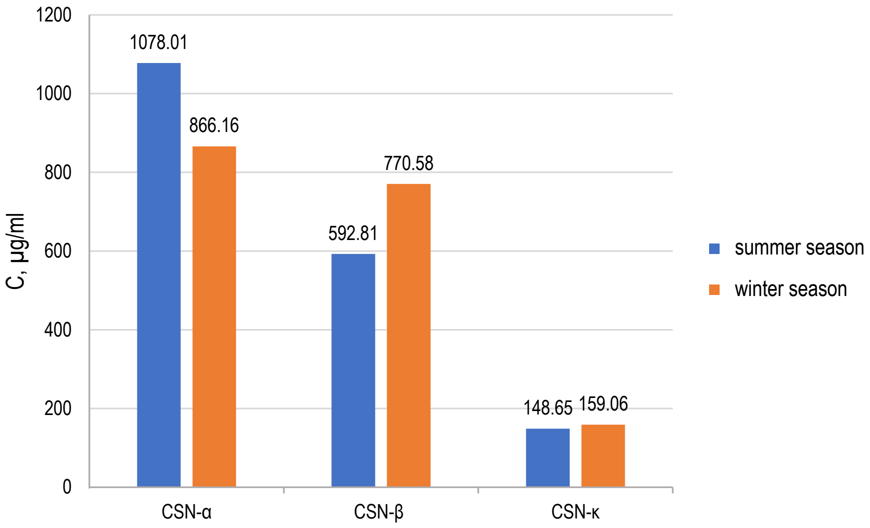

Figure 3. Concentration of CSN-α, CSN-β and CSN-κ in bovine milk depending on feeding season; CSN-α – casein-α, CSN-β – casein-β, CSN-κ – casein-κ

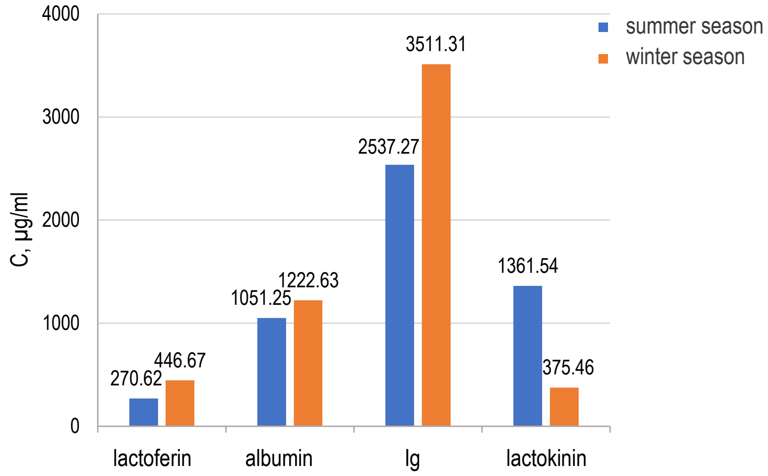

Figure 4. Concentration of lactoferrin, albumin, Ig and lactokinin in bovine milk depending on feeding season; Ig – total immunoglobulin

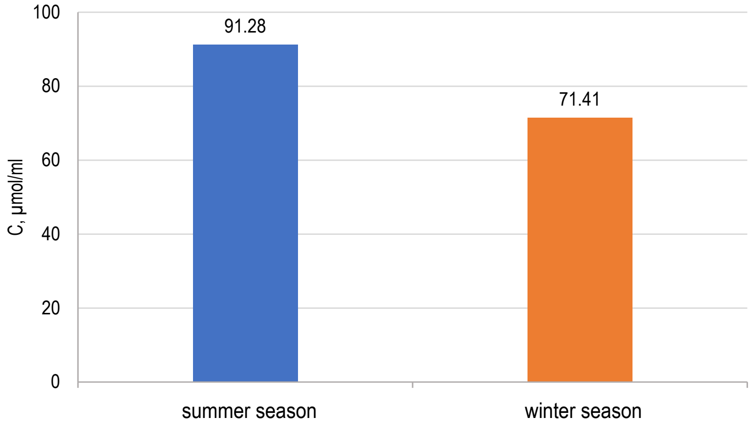

Figure 5. Concentration of β – casomorphin in bovine milk depending on feeding season

In contrast, β- and κ-casein levels were higher in winter milk ($P < 0.001$).

Summer milk contained more lactokinin than winter milk ($P < 0.001$). Winter milk contained more serum albumins and immunoglobulins ($P < 0.001$). Winter milk also contained more αlactoferrin than summer milk ($P < 0.001$; Figure 4). A significantly higher concentration of β-casomorphin was found in milk collected during the summer season compared to the winter period ($P < 0.001$; Figure 5).

Discussion

Our findings indicate that both breed and feeding season significantly modified the composition of milk peptides. Moreover, the type of feed or feeding system also influenced peptide levels. When comparing breeds in summer, HF milk contained more lactoferrin, while in winter, SIM milk had a higher concentration of this compound. Litwińczuk et al. (2011) also showed a seasonal effect on lactoferrin content, although their values were lower than in the present work. In the latter study, a TMR (corn silage, silage, and feed concentrate) feeding system was used in both winter and summer. In HF milk, lactoferrin reached 72 µg/ml in summer and 79.07 µg/ml in winter. In SIM milk, lactoferrin levels were 110.91 µg/ml in summer and 101.31 µg/ml in winter. Król et al. (2008) obtained lactoferrin concentration of 116.22 µg/ml in milk from cows fed mixtures with a predominance of silage and maize silage, while in TMR-fed cows, it was 88.34 µg/ml. Sobczuk-Szul et al. (2014) reported lower lactoferrin in HF milk from free-stall barns using TMR, at 198.84 µg/ml. Linehan et al. (2023) reported that lactoferrin levels in cows' milk ranged from 20 to 750 ng/ml, while Brodziak et al. (2021) recorded values between 123.8 to 125.9 µg/ml. Higher lactoferrin in winter milk indicates stronger antimicrobial properties than in summer milk. Lactoferrin is a glycoprotein that binds free iron, an essential component for bacterial growth and metabolism. By limiting iron availability restricts bacterial development in the digestive tract and reduces proliferation. Consequently, the consumption of lactoferrin in milk helps relieve intestinal infections and lowers their incidence. In addition, lactoferrin also shows cytotoxic activity (Nielsen et al., 2023) and supports the growth of commensal bacteria (Bhattacharya et al., 2019). According to Boldogh et al. (2008), lactoferrin is the most biologically active whey peptide due to its bacteriostatic and antioxidant properties, and its ability to limit β-amyloid formation in individuals with Alzheimer's disease.

In summer, milk of HF cows contained more β-casomorphin than SIM milk. Nguyen et al. (2015), using Elisa assays on HF milk collected in winter, obtained β-casomorphin levels of 76.72–85.41 µmol/ml. Meanwhile, Bartley et al. (2010) have argued that casomorphin present in cow's milk may increase mucin secretion by acting on opioid receptors on goblet cells.

In the present study, breed did not affect casein content in milk. Findings reported by Park and Haenlein (2021) were similar, as α-casein concentration was 1360 µg/ml, β-casein 980 µg/ml, and κ-casein 280 µg/ml. Amalfitano et al. (2020) obtained comparable results, with α-casein levels of 1158 µg/ml in HF milk and 1569 µg/ml in SIM milk; β-casein 1069 µg/ml and 1125 µg/ml; and κ-casein 549 µg/ml and 654 µg/ml, respectively; higher α-casein content in SIM milk was consistent with our data. A two-year study by Li et al. (2019) showed that seasonality did not influence casein micelle size. Caseins (α-, β-, and κ-casein) have immunomodulatory functions that, in individuals without casein allergy, are regarded as beneficial (Garg and Kumar, 2021) and help reduce oxidative stress. Additionally, the α- and β-casein fractions also show cytotoxic activity (Nielsen et al., 2023). Peptides derived from casein may exert a hypocholesterolaemic effect by reducing cholesterol absorption in the digestive tract.

Casokinin is a bioactive peptide originating from the α-casein fraction. In the present study, casokinin concentrations were higher in HF milk in both seasons. Comparison with other authors is difficult, as similar data are limited. However, it should be noted that casokinin shows antihypertensive, antithrombotic, antimicrobial, and immunostimulatory properties and acts as a mineral carrier. It supports the transport and uptake of certain minerals and induces cytotoxicity in malignant cells. It also acts as an opioid antagonist (Garg and Kumar, 2021), which allows it to modulate intestinal peristalsis and stimulate mucin secretion. Casokinin may also stimulate intestinal immune responses (Kayihura, 2023).

Another peptide, colostrinin, prevents DNA damage by neutralising intracellular free radicals and enhancing natural DNA repair mechanisms (Boldogh et al., 2008). This compound also has a neuroprotective effect by inhibiting β-amyloid-induced apoptosis (Boldogh and Kruzel, 2008). According to Boldogh et al. (2008), colostrinin consumption does not increase IgE/IgG1 levels nor does it induce skin hypersensitivity reactions or respiratory tract inflammation. It acts as an interferon inducer and is effective in preventing allergic reactions to external and internal allergens (Kosmerl et al., 2024). These findings indicate the potential use of colostrinin in alleviating allergic inflammation in humans (Boldogh et al., 2008).

Comparison between breeds shows that milk from SIM cows was richer in lysozyme than that from HF cows. Reported lysozyme concentrations in cow's milk range from 70 to 600 ng/ml, consistent with values reported by Linehan et al. (2023). In contrast, Litwińczuk et al. (2011) measured much lower lysozyme levels, as in summer, HF milk contained 7.94 ng/ml, and SIM had 10.22 ng/ml. In the winter season, these values were 8.18 ng/ml and 10.13 ng/ml, respectively. Brodziak et al. (2021) reported lysozyme levels of 11.14 µg/ml. Lysozyme is a valuable peptide with antibacterial properties, as it destroys bacterial cell walls by hydrolysing peptidoglycans. Lysozyme, similarly to lactoferrin, can act prebiotically and promote the growth of commensal bacteria (Bhattacharya et al., 2019).

In summer, milk of SIM cows contained higher levels of mucin. Its deficiency causes dysfunction of the intestinal mucosa barrier, which may reduce nutrient absorption and increase susceptibility to inflammatory processes and food allergies.

Copeptin levels were also higher in SIM milk during summer. In winter, no differences in the level of this component were observed between breeds. These results can be considered novel, as no previous studies were found in the available literature assessing the level of this peptide in cows' milk.

In the summer season, milk from SIM cows was richer in albumin, which was in line with results obtained by Lieske et al. (2005) using ELISA. Litwińczuk et al. (2011) reported slightly higher average albumin levels in milk collected during the summer and winter feeding seasons, ranging from 640 to 680 µg/ml. For the SIM breed, they recorded concentrations of 490 µg/ml in summer and 530 µg/ml in winter. According to Brodziak et al.

(2021), serum albumin level in milk is approximately 440 μg/ml. Bovine serum albumin is the third most abundant whey protein in milk after α-lactalbumin and β-lactoglobulin. In the present study, SIM cows' milk was richer in immunoglobulins in summer, whereas in winter, HF milk was more abundant in this component. Linehan et al. (2023) reported that the level of this indicator in cows' milk varied between 340 and 480 ng/ml.

With respect to caseinomacropeptide in our study, HF milk in the winter season contained lower levels of this peptide, whereas in summer, the results were opposite. During κ-casein hydrolysis, cleavage of the bond between phenylalanine and methionine produces a hydrophilic, phosphorylated, and glycosylated C-terminal fragment known as caseinomacropeptide. Lenardon et al. (2017) observed concentrations as high as 2520 ng/ml. This compound, derived from casein, is distinguished by its lack of aromatic residues and a high content of branched-chain amino acids, which may benefit diets aimed at supporting liver function. These branched-chain amino acids provide a carbon source that facilitates gluconeogenesis, reducing metabolic stress on the liver. Caseinomacropeptide also contributes to shaping the intestinal microbiota by promoting *Bifidobacterium* growth and showing strong bactericidal and toxin-binding activity. Caseinomacropeptide may assist in obesity management by stimulating cholecystokinin release, increasing satiety, and helping regulate food intake (Thomä-Worringer et al., 2006; Nielsen et al., 2023). Its antibacterial, immunomodulatory, and prebiotic properties are due to post-translational glycosylation of κcasein (Cordova-Davalos et al., 2019; Wilson et al., 2019). Similarly to lactoferrin and lysozyme, caseinomacropeptide can support digestive health and inhibit lipid oxidation, neutralise free radicals, and chelate metal ions such as calcium, iron, and zinc.

Ceruloplasmin content was consistently higher in HF milk during both seasons in the current trial. Research by Szczubiał et al. (2012) reported ceruloplasmin levels of 1352 ng/ml in HF milk from Lublin region herds using ELISA. This glycoprotein, synthesised by the mammary gland during lactation, is the main extracellular source of copper.

Considering the rising incidence of diet-dependent diseases, the role of bioactive peptides in milk deserves special attention. Through appropriate feeding and housing conditions for dairy cows, the concentration of these peptides can be optimised to utilise their significant health-promoting potential, particularly anti-atherosclerotic and anti-cancer properties.

Conclusions

The results do not allow a definitive conclusion regarding which breed produces milk richer in bioactive peptides beneficial to humans. However, milk from Holstein-Fresian cows contains higher levels of peptides such as colostrinin, casokinin, casomorphin, and immunoglobulins, conferring stronger neuro-protective and anticancer effects, improving the antioxidant status, strengthening the immune system, and supporting the fight against obesity. Regarding the feeding season, milk collected during winter shows better properties, especially from cows fed silage with maize silage. These feeds are rich in enzymes, bioactive bacterial metabolites, and organic acids produced during fermentation, which stimulate milk protein hydrolysis. Additionally, they contribute to favourable modifications in the milk protein profile and activate the microbiome in the digestive tract of dairy cows, increasing the content of bioactive peptides with health-promoting properties in milk.

Funding

The project 'Research network of natural science universities for the development of the Polish dairy sector' is funded through a special-purpose subsidy from the Ministry of Science and Higher Education (MEiN/2023/DPI/2862).

Conflicts of interest

The Authors declare that there is no conflict of interest.

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