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In Silico Determination of Physicochemical Properties of Lactoferrin Peptides Isolated from Equine Milk

Meiramkul Narmuratova,[1,*] Zhanar Narmuratova,[1, 2,*] Shattyk Kanayat,[1] Aliya Meldebekova[1] and Yus Aniza Yusof[3, 4]

Abstract

Whey protein contains low-molecular-weight bioactive peptides with various medicinal properties, such as anti-inflammatory, antioxidant, and antimicrobial effects. The presence of these low-molecular-weight peptide molecules plays a significant role in promoting health through food. For peptides to function as effective food ingredients, they must be digested and absorbed. Current research includes information on 56 peptides from the most active fractions of lactoferrin (LF) isolated from equine milk hydrolysate. The potential bioactivity, including allergenicity, toxicity, and physicochemical properties, as well as the applicability of these peptides, was determined using the Peptide Ranker online database (http://distilldeep.ucd.ie/PeptideRanker/). The studied peptides were classified as cationic (13), anionic (23), and neutral (20). The findings revealed that only the cationic and neutral peptides demonstrated significant biological activity (>0.75). Furthermore, peptide bioactivity was positively correlated with phenylalanine content. These research findings can significantly contribute to the MS-based proteomics of equine milk LF and shed light on the composition of its bioactive peptides. Further research is required to comprehensively investigate the biochemical nature and pathways of bioactive peptides responsible for the antimicrobial and antioxidant properties of LF from equine milk.

Keywords: Equine's milk; Lactoferrin; Peptides composition; Bioactivity; Amino acid composition.
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1. Introduction

Equine milk is a significant source of nutrition for nomadic people in Central Asian countries such as Kazakhstan, Kyrgyzstan, Tajikistan, and Mongolia. Today, residents of these regions still consume both fresh (saumal) and fermented (koumiss) types of equine milk.[1] It remains an important component of the Kazakh diet, with populations relying on horses as a primary nutrient source for many centuries. Equine milk has a unique biochemical composition. Although its protein content is lower than that of cow's milk, it contains a higher percentage of whey proteins-around 40%, compared to 20% in cow's milk-classifying it as albumin-type milk.[2] Its whey proteins include β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin, and lysozyme. Lactoferrin (LF), a globular protein present in bodily fluids like milk, saliva, and tears, is found in higher concentrations in equine milk (0.2–2 g L^{-1})[3,4] compared to cow's milk (0.03–0.1 g L^{-1})[5] but is lower than in breast milk (1–7 g L^{-1}).[6]

Numerous studies suggest that equine milk closely resembles breast milk in its physicochemical composition and nutritional and therapeutic values.[7] It is rich in vitamin C and lactose, making it easily digestible and health-promoting. According to Miraglia *et al.*,[8] equine milk is used in both the food and non-food sectors, including cosmetics. It is particularly beneficial for sensitive consumers, such as immunocompromised individuals and children allergic to cow's milk protein.[8] Additionally, equine milk is used to treat tuberculosis, skin disorders, chronic hepatitis, and cow milk allergies.[9]

However, the specific bioactive compounds in equine milk responsible for its antimicrobial,[10] antioxidant,[11,12] anti-

[1] *Department of Biotechnology, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, Al-Farabi Almaty, 71, 050040, Kazakhstan.*
[2] *Department of Chemical and Biochemical Engineering, Satbayev University, Satbayev 22a, Almaty 050013, Kazakhstan.*
[3] *Laboratory of Halal Science Research, Halal Products Research Institute, University Putra Malaysia, Putra Infoport, Serdang, Selangor 43400, Malaysia.*
[4] *Department of Process and Food Engineering, Faculty of Engineering, University Putra Malaysia, Serdang, Selangor 43400, Malaysia.*
**Email:* m.narmuratova@gmail.com (Z. Narmuratova)

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diabetic,[13] and anti-inflammatory[4,14] properties are not well understood. This study hypothesized that whey protein LF isolated from equine milk and its peptides might be more effective in protecting human health compared to other whey proteins. The biological properties of LF-such as antioxidant, antimicrobial, and metal ion-binding activities-can enhance its digestibility through the peptides formed during enzymatic hydrolysis in the gastrointestinal tract.

This study aims to identify bioactive peptides derived from equine LF which are responsible for the physicochemical properties of equine milk. To determine LF bioactivity, the LF protein was isolated from equine milk and analyzed using mass spectrometry (MS) to identify the peptides amino acid residues. The bioactivity of these peptides was then assessed using the Peptide Ranker online database.

2. Materials and methods

2.1 Whey proteins isolation

To isolate whey proteins from equine milk, fresh milk was obtained from a local farm. The fresh milk was cooled at 4 °C for 30 min before the isolation process. The cooled milk was then poured into 50 mL tubes and centrifuged at 5000 rpm for 30 min. The fat layer that formed on the surface was removed. To separate the whey, the pH of the milk was adjusted to 4.2 using 1 M HCl, followed by another centrifugation at 5000 rpm for 30 min. The pH of the resulting supernatant, which contained the whey proteins, was then returned to the initial level of 6.8 using 1 M NaOH. The supernatant was subsequently dialyzed in distilled water (dH_2O) using a dialysis membrane bag with a molecular weight (M_r) cutoff of 6-8000 Da (SpectraPor; Spectrum Labs Inc., Rancho Dominguez, California, USA) at 4 °C for 72 h to remove salts.

2.2 Equine LF purification

Equine whey proteins (1.0 g) were suspended in 10 mL of 20 mM Tris-HCl buffer at pH of 8.0 (100 mg mL^{-1}) and then fractionated by cation-exchange fast protein liquid chromatography (FPLC) using a HiTrap SP Fast Flow column. This strong cation exchange chromatography column, connected to an AKTA-pure device (GE Healthcare, Uppsala, Sweden), was used for high-resolution, small-scale protein purification according to El Hatmi *et al.*[15] Briefly, a 2 mL sample was loaded onto the column, which had been equilibrated in 30 mM Tris-HCl buffer at pH of 8.0. A 0-0.5 M NaCl linear gradient in the same buffer was applied for elution. The flow rate was 2 mL min^{-1}, and detection was monitored at 280 nm. Several runs were carried out, and the collected fractions were pooled, dialyzed against dH_2O at 4 °C for 3 days, and freeze-dried. The protein composition of each fraction was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli and Favre.[16]

2.3 Protein extraction

Each gel band was cut into 1 mm^2 pieces. The gel pieces were incubated with a mixture containing equal amounts of 100 mM ammonium bicarbonate and 100% acetonitrile. Reduction was performed by adding 5 mM dithiothreitol (DTT) and incubating at 60 °C for 10 min. Subsequently, alkylation was carried out by adding 100 mM iodoacetamide and incubating for 10 min at 37 °C. Afterward, the supernatant was removed, and the sample was washed three times with a solution containing 50 mM ammonium bicarbonate and 100% acetonitrile, with 5-min incubations at 37 °C for each wash. Following reduction and alkylation, the samples were digested overnight at 37 °C with 20 ng μL^{-1} trypsin (Thermo, USA). The resulting peptide mixtures were purified and concentrated using ZipTip-C18 (Millipore, Ireland). The eluted peptides were then dried using a centrifugal evaporator (Eppendorf, Germany), re-suspended in 10 μL of 0.1% trifluoroacetic acid, and stored at -20 °C until liquid chromatography tandem mass spectrometry (LC-MS/MS).

2.4 Mass spectrometry analysis

Peptide mixtures were analyzed using online nano-flow reversed-phase C_{18} liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatography was performed with a trapping column (Acclaim PepMap 100 C_{18} pre-column) and a Dionex nanoHPLC pump. Peptides were separated on an Acclaim Pep-Map RSLC column (Thermo, USA) using a 75-min multistep acetonitrile gradient at a flow rate of 0.3 mL min^{-1}. An unmodified captive spray ion source (capillary 1300 V, dry gas 3.0 L min^{-1}, dry temperature 150 °C) was used to interface the LC system with the Impact II ESI-QUAD-TOF mass spectrometer (Bruker Daltonics, Germany). Full-scan MS spectra were acquired at a spectral rate of 2.0 Hz, followed by the acquisition of one MS/MS spectrum.

The MS/MS peak list data were analyzed using DataAnalysis 3.4 software (Bruker Daltonics, Germany) and saved in Mascot generic format (*.mgf). The MS/MS peak lists in Mascot generic format were searched on a local server using Mascot 2.6.1 software (Matrix Science, UK) against the Swiss-Prot protein database (release 2024_02, 571,282 sequences; 206,678,396 residues) taxonomically restricted to "Other Mammalia," containing 13,494 sequences. The search parameters included methionine oxidation as a variable modification and carbamidomethylation of cysteine residues as a fixed modification. Mass error windows of 100 ppm for MS and 0.05 Da for MS/MS were allowed.[17]

2.5 Peptide bioactivity and applicability in silico determination

Amino acid composition, molecular weight (*Mr*), and isoelectric point of peptides were determined using the Expasy ProtParam online platform (https://web.expasy.org/protparam/).[18] The potential bioactivity was predicted using the Peptide Ranker online database (http://distilldeep.ucd.ie/PeptideRanker/).[19,20] The prediction focused on amino acid residues, as certain classes of bioactive peptides have specific structural features and

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amino acid sequences.[21] Peptides were scored from 0 to 1, with higher values indicating a higher probability of being bioactive.

The potential peptide allergenicity was predicted using the AllerTOP v. 2.0 online database (http://www.ddg-pharmfac.net/AllerTOP/index.html).[22,23] Peptides were classified using the *k*-nearest neighbor algorithm based on a training set containing 2,427 known allergens and 2,427 non-allergens from different species.

The water solubility of the obtained peptides was estimated using the Peptide Properties Calculator available at https://pepcalc.com/peptide-solubility-calculator.php, and the sequence of active water-soluble peptides was determined.

Peptide toxicity and physicochemical properties (i.e., hydrophobicity, amphipathicity, steric hindrance, and M_r) were studied using the ToxinPred online database (http://crdd.osdd.net/raghava/toxinpred/).[24,25] Peptide toxicity was predicted based on amino acid composition and position, using models developed through machine learning techniques and quantitative matrices involving more than 1,805 toxic peptides.

2.6 Statistical analysis

Pearson correlation and principal component analysis (PCA) were performed using RStudio software (version 2023.06.0, build 421, RStudio PBC, 2023).

3 Results & discussion

Equine milk whey proteins were fractionated by cation-exchange FPLC. The main proteins with acidic isoelectric points—α-lactalbumin (α-LA), β-lactoglobulin (β-LG), and serum albumin—were not retained on the column and were recovered in the flow-through. In contrast, lysozyme and equine lactoferrin (eLF) (isoelectric point: 8.32) were eluted at retention times of 14-15 and 20-25 min, respectively, with high purity (Fig. 1). The purity of eLF and the electrophoretic profiles of equine whey proteins were assessed using Bio-Rad's SDS-PAGE System, following the method described by Laemmli[16] with slight modifications, under reducing conditions on a 12% separation gel.

To determine the purity and homogeneity of each individual protein fraction from equine milk whey, Bio-Rad's SDS-PAGE was carried out. In the whey protein fractions shown in Fig. 1, protein bands with an apparent M_r of around 75 kDa were observed, identified as lactoferrin. According to ProtParam analysis, the theoretical M_r of eLF protein is 75-76 kDa. Additionally, goat milk lactoferrin (81 kDa),[26] human lactoferrin (77 kDa),[27] and bovine lactoferrin (85 kDa)[26,28] have similar molecular weights. The electrophoresis results confirmed the correct isolation of lactoferrin from equine milk. The isoelectric point (pI) of eLF was found to be 8.30, consistent with literature data reporting that the pI of equine milk varies between 8.0 and 9.7.[29,30]

The amino acid sequence of eLF was determined by MALDI-TOF MS analysis. The eLF molecule consists of a single polypeptide chain with 695 amino acid residues (Fig. 2). Peptide chains consist of amino acid residues linked by peptide bonds. Equine milk lactoferrin was treated with trypsin enzyme, resulting in the identification of 56 short-chain and long-chain peptides. Among these, 13 were cationic

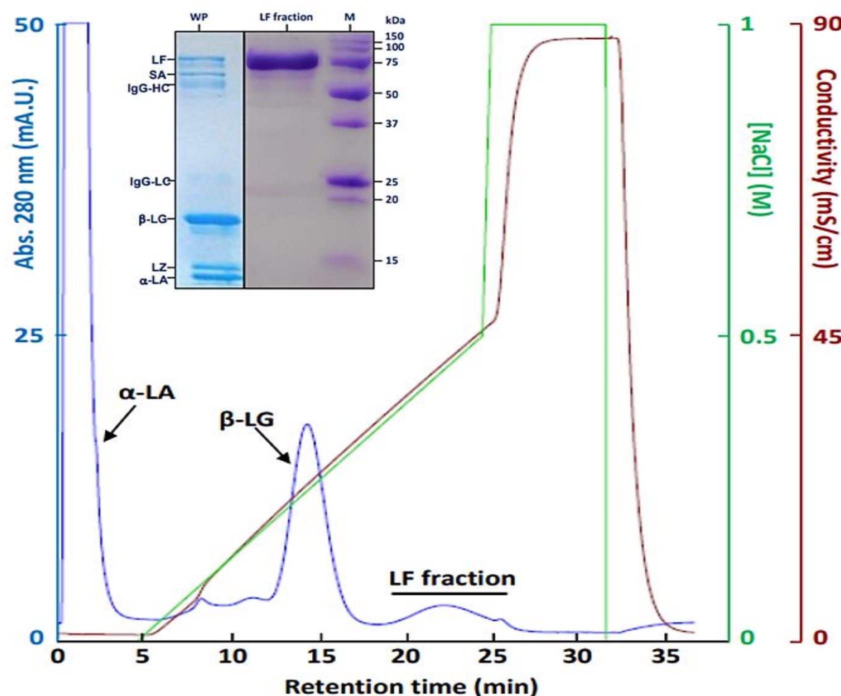

Fig. 1 Cation-exchange Fast Protein Liquid Chromatography of equine whey proteins and SDS-PAGE analysis of the lactoferrin fractions collected. Modified from Narmuratova *et al*.[4] Notes: A.U. - absorbance unit; M – marker.

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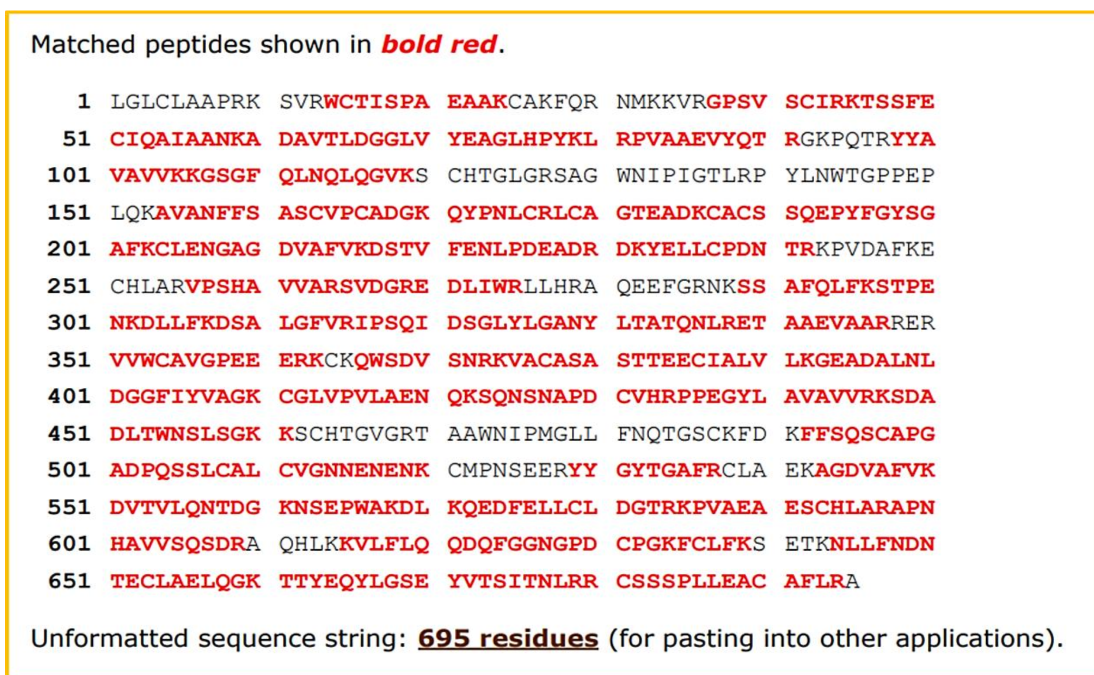

Fig. 2 Amino acid sequence of equine lactoferrin.

peptides, 23 were anionic peptides, and 20 were neutral peptides. All the studied peptides are nontoxic, as they are derived from milk lactoferrin protein.

The composition of lactoferrin peptides includes short-chain peptides with 5 amino acids (*e.g.*, 635-639 FCLFK) and long-chain peptides with 36 amino acids (*e.g.*, 375-410 VACASASTTEECIALVLKGEADALNLDGGFIYVAGK). The amino acid sequences of these peptides are shown in Table 1.

The theoretical M_r and pI values of these peptides were determined using the ProtParam software. Accordingly, the peptide with the lowest theoretical M_r of 656.84 Da was FCLFK, while the peptide with the highest M_r of 3601.10 Da was VACASASTTEECIALVLKGEADALNLDGGFIYVAGK. Additionally, the M_r differences of 28 lactoferrin peptides were determined both theoretically and through MS analysis.

Table 1. Peptides of equine lactoferrin.

№	Position	Amino acid sequence	Bioactivity score	No. of amino acids	M_r		pI	Formula	Net charge at pH of 7	Water solubility	Ion exchange type
					Theoretical	MS calc.					
1	635-639	FCLFK	0.946878	5	656.84	713.348	8,22	$C_{33}H_{48}N_6O_6S_1$	1	Poor	cationic
2	681-694	CSSSPLLEACAFLR	0.906249	14	1496.76	1609.77	5,99	$C_{64}H_{105}N_{17}O_{20}S_2$	-0.1	Poor	neutral
3	680-694	RCSSSPLLEACAFLR	0.855151	15	1652.95	1765.86	8,07	$C_{70}H_{117}N_{21}O_{21}S_2$	1	Good	cationic
4	289-296	SSAFQLFK	0.853383	8	927.07	926.48	8,47	$C_{44}H_{66}N_{10}O_{12}$	1	Poor	cationic
5	187-203	CACSSQEPYFGYSGAFK	0.76135	17	1845.03	1957.80	5,99	$C_{82}H_{113}N_{19}O_{26}S_2$	-0.1	Poor	neutral
6	171-177	QYPNLCR	0.749108	7	893.03	949.44	8,22	$C_{38}H_{60}N_{12}O_{11}S_1$	1	Poor	cationic
7	154-170	AVANFFSASCVPCADGK	0.692434	17	1686.92	1799.80	5,86	$C_{73}H_{111}N_{19}O_{23}S_2$	-0.1	Poor	neutral
8	316-338	IPSQIDSGLYLGANYLTATQNLR	0.687433	23	2508.81	2507.32	5,83	$C_{111}H_{178}N_{30}O_{36}$	0	Poor	neutral
9	303-315	DLLFKDSALGFVR	0.65786	13	1480.73	1479.80	5,96	$C_{69}H_{109}N_{17}O_{19}$	0	Good	neutral

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№	Position	Amino acid sequence	Bioactivity score	No. of amino acids	M_r		pI	Formula	Net charge at pH of 7	Water solubility	Ion exchange type
					Theoretical	MS calc.					
10	529-537	YYGYTGAF R	0.624263	9	1097.20	1096.50	8,5	$C_{53}H_{68}N_{12}O_{14}$	1	Poor	cationic
11	308-315	DSALGFVR	0.623991	8	863.97	863.45	5,84	$C_{38}H_{61}N_{11}O_{12}$	0	Good	neutral
12	37-44	GPSVSCIR	0.60367	8	817.96	874.43	8,25	$C_{33}H_{59}N_{11}O_{11}S_1$	1	Good	cationic
13	447-461	KSDADLTW NSLSGKK	0.590291	15	1649.82	1648.85	8,5	$C_{71}H_{116}N_{20}O_{25}$	1	Good	cationic
14	217-242	DSTVFENLP DEADRDKY ELLCPDNT R	0.576631	26	3056.26	3111.42	4,03	$C_{129}H_{199}N_{35}O_{49}S_1$	-5.1	Good	anionic
15	178-203	LCAGTEAD KCACSSQE PYFGYSGA FK	0.547725	26	2734.02	2903.23	4,68	$C_{118}H_{173}N_{29}O_{40}S_3$	-1.2	Good	anionic
16	270-275	EDLIWR	0.538623	6	830.94	830.43	4,37	$C_{38}H_{58}N_{10}O_{11}$	-1	Good	anionic
17	107-119	GSGFQLNQ LQGVK	0.531161	13	1375.55	1374.73	10,1 2	$C_{60}H_{98}N_{18}O_{19}$	1	Poor	cationic
18	615-634	KVLFLQQD QFGGNGPD CPGK	0.52697	20	2148.42	2204.08	5,95	$C_{95}H_{146}N_{26}O_{29}S_1$	-0.1	Good	neutral
19	60-79	ADAVTLDG GLVYEAGL HPYK	0.521757	20	2089.33	2088.05	4,54	$C_{95}H_{145}N_{23}O_{30}$	-1.9	Poor	anionic
20	616-634	VLFLQQDQ FGGNGPDC PGK	0.520063	19	2020.25	2075.99	4,21	$C_{89}H_{134}N_{24}O_{28}S_1$	-1.1	Good	anionic
21	572-584	QEDFELLCL DGTR	0.518295	13	1538.69	1594.736 4	3,92	$C_{65}H_{103}N_{17}O_{24}S_1$	-3.1	Good	anionic
22	393-410	GEADALNL DGGFIYVA GK	0.51662	18	1809.99	1808.900 3	4,03	$C_{81}H_{124}N_{20}O_{27}$	-2	Good	anionic
23	569-584	DLKQEDFE LLCLDGTR	0.507307	16	1895.11	1950.943 5	4,11	$C_{81}H_{131}N_{21}O_{29}S_1$	-3.1	Good	anionic
24	492-520	FFSQSCAPG ADPQSSLC ALCVGNNE NENK	0.502186	29	3031.29	3200.37	4,14	$C_{125}H_{192}N_{36}O_{46}S_3$	-2.2	Good	anionic
25	447-460	KSDADLTW NSLSGK	0.498055	14	1521.65	1520.76	5,96	$C_{65}H_{104}N_{18}O_{24}$	0	Good	neutral
26	448-460	SDADLTWN SLSGK	0.496031	13	1393.47	1392.66	4,21	$C_{59}H_{92}N_{16}O_{23}$	-1	Good	anionic
27	106-119	KGSGFQLN QLQGVK	0.472252	14	1503.72	1502.82	10	$C_{66}H_{110}N_{20}O_{20}$	2	Good	cationic
28	562-568	NSEPWAK	0.471141	7	830.90	830.39	6,00	$C_{37}H_{54}N_{10}O_{12}$	0	Good	neutral
29	297-307	STPENKDLL FK	0.416373	11	1291.47	1290.68	5,79	$C_{58}H_{94}N_{14}O_{19}$	0	Good	neutral
30	585-597	KPVAEAES CHLAR	0.410762	13	1410.61	1466.74	6,75	$C_{59}H_{99}N_{19}O_{19}S_1$	0	Good	neutral
31	366-373	QWSDVSNR	0.408838	8	991.03	990.45	5,84	$C_{41}H_{62}N_{14}O_{15}$	0	Good	neutral

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№	Position	Amino acid sequence	Bioactivity score	No. of amino acids	M_r		pI	Formula	Net charge at pH of 7	Water solubility	Ion exchange type
					Theoretical	MS calc.					
32	543-550	AGDVAFVK	0.384077	8	805.93	805.43	5,88	$C_{37}H_{59}N_9O_{11}$	0	Good	neutral
33	204-216	CLENGAGD VAFVK	0.374738	13	1322.50	1378.66	4,37	$C_{57}H_{91}N_{15}O_{19}S_1$	-1.1	Good	anionic
34	265-275	SVDGREDLI WR	0.36692	11	1345.48	1344.69	4,56	$C_{58}H_{92}N_{18}O_{19}$	-1	Good	anionic
35	14-24	WCTISPAEA AK	0.296886	11	1176.35	1232.59	5,99	$C_{52}H_{81}N_{13}O_{16}S_1$	-0.1	Good	neutral
36	411-422	CGLVPVLA ENQK	0.286924	12	1270.51	1326.69	5,99	$C_{55}H_{95}N_{15}O_{17}S_1$	-0.1	Poor	neutral
37	375-392	VACASAST TEECIALVL K	0.254547	18	1809.12	1921.962 0	4,53	$C_{76}H_{133}N_{19}O_{27}S_2$	-1.1	Poor	anionic
38	351-363	VVWCAVGP EEERK	0.245985	13	1501.72	1557.77	4,79	$C_{66}H_{104}N_{18}O_{20}S_1$	-1.1	Good	anionic
39	45-59	KTSSFECIQ AIAANK	0.243536	15	1610.85	1666.84	8,2	$C_{69}H_{115}N_{19}O_{23}S_1$	1	Good	cationic
40	256-264	VPSHAVVA R	0.240973	9	935.09	934.53	9,73	$C_{41}H_{70}N_{14}O_{11}$	+ 1.25	Poor	cationic
41	423-446	SQNSNAPD CVHRPPEG YLAVAVVR	0.235445	24	2579.87	2635.30	6,47	$C_{110}H_{175}N_{35}O_{35}S_1$	0	Good	neutral
42	46-59	TSSFECIQAI AANK	0.232932	14	1482.67	1538.74	5,66	$C_{63}H_{103}N_{17}O_{22}S_1$	-0.1	Poor	neutral
43	644-660	NLLFNDNT ECLAELQG K	0.228142	17	1922.14	1977.95	4,14	$C_{82}H_{132}N_{22}O_{29}S_1$	-2.1	Good	anionic
44	374-392	KVACASAS TTEECIALV LK	0.227817	19	1937.30	2050.05	6,13	$C_{82}H_{145}N_{21}O_{28}S_2$	-0.1	Good	neutral
45	351-362	VVWCAVGP EEER	0.201303	12	1373.55	1429.67	4,25	$C_{60}H_{92}N_{16}O_{19}S_1$	-2.1	Good	anionic
46	233-242	YELLCPDN TR	0.200803	10	1223.37	1279.60	4,37	$C_{52}H_{82}N_{14}O_{18}S_1$	-1.1	Good	anionic
47	80-91	LRPVAAEV YQTR	0.126952	12	1402.62	1401.77	8,75	$C_{62}H_{103}N_{19}O_{18}$	1	Good	cationic
48	598-609	APNHAVVS QSDR	0.123789	12	1280.36	1279.63	6,79	$C_{52}H_{85}N_{19}O_{19}$	0.1	Good	neutral
49	375-410	VACASAST TEECIALVL KGEADALN LDGGFIYVA GK	0.119972	36	3601.10	3712.864 1	4,18	$C_{157}H_{255}N_{39}O_{53}S_2$	-3.1	Poor	anionic
50	217-232	DSTVFENLP DEADRDK	0.10765	16	1850.91	1849.84	3,96	$C_{77}H_{119}N_{21}O_{32}$	-4	Good	anionic
51	217-230	DSTVFENLP DEADR	0.0986029	14	1607.65	1606.72	3,77	$C_{67}H_{102}N_{18}O_{28}$	-4	Good	anionic
52	98-105	YYAVAVVK	0.0912264	8	912.10	911.51	8,5	$C_{45}H_{69}N_9O_{11}$	1	Poor	cationic
53	551-561	DVTVLQNT DGK	0.0757897	11	1189.29	1188.60	4,21	$C_{49}H_{84}N_{14}O_{20}$	-1	Good	anionic

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					Theoretical	MS calc.					
54	661-679	TTYEQYLGSEYVTSITNLR	0.069327	19	2238.44	2237.10	4,53	$C_{99}H_{152}N_{24}O_{35}$	-1	Poor	anionic
55	661-680	TTYEQYLGSEYVTSITNLRR	0.0680152	20	2394.62	2393.20	5,81	$C_{105}H_{164}N_{28}O_{36}$	0	Good	neutral
56	339-347	ETAAEVAAR	0.0597562	9	916.99	916.46	4,53	$C_{37}H_{64}N_{12}O_{15}$	-1	Good	anionic

The difference between the M_r of the peptides corresponded to the atomic mass of the iron (Fe) ion (~56 Da), indicating a potential close connection between the lactoferrin protein and its special property of binding, transporting, and regulating Fe levels.

As a result of MS analysis, the M_r difference corresponding to 1 Fe ion was identified in 19 peptides, the M_r difference corresponding to 2 Fe ions – in 7 peptides, and the M_r difference corresponding to 3 Fe ions – in 2 peptides. For example, the FCLFK (635-639) peptide binds 1 Fe molecule. The RCSSSPLLEACAFLR (680-694) peptide binds 2 Fe molecules. The FFSQSCAPGADPQSSLCALCVGNNENENK (492-520) peptide is notable for binding 2 Fe molecules; this peptide was cited in the research of Gallina *et al.*.[31]

In comparison with donkey milk peptides, the FCLFK peptide is located between amino acid positions 629-633 in lactoferrin, and the long-chain peptide FFSQSCAPGADPQSSLCALCVGNNENENK is located between amino acid residues 486-514.

The physicochemical characteristics of amino acids depend on their side chains, which determine the interaction between ligand-binding residues and metal ions.[31] For example, cysteine (Cys) has a thiol group that binds Fe effectively, playing a crucial role in proteins by binding to various metal ions. This binding capability is also significant in biologically active peptides.

Amino acids can form stable five-membered chelates with metal ions through their amino and carboxylate groups (N, O-chelation). Additionally, some amino acids have extra metal-binding sites in their side chains, allowing them to form metal complexes with different structures. Important metal-binding sites in proteins include the imidazole ring of histidine (His), the phenol ring of tyrosine (Tyr), the thiol group of Cys, and the β- and γ-carboxylate groups of aspartate (Asp) and glutamate (Glu), respectively. The thioether moiety of methionine (Met) is also often involved in metal binding.[32-35] According to Trejos *et al.*,[36] polar amino acids such as arginine (Arg), lysine (Lys), phenylalanine (Phe), and Cys are particularly significant due to their capacity to interact with and disrupt lipid layers, a common component of antimicrobial activity. The presence of polar and small amino acids in certain peptides may indicate the need for specific conformations to ensure water solubility and effective interaction with targets.[34,35]

The biological function of proteins and peptides relies on the interaction of amino acid residues with metal ions, which bind the ligand. The molecular mechanism of peptide activity involves the binding of metal ions to specific residues in proteins.[37,38]

Food-derived bioactive peptides are important as functional food supplements to improve health and prevent chronic diseases due to their high bioactivity, low toxicity, and metabolic rejuvenating properties. Identifying bioactive peptides using traditional methods is time-consuming and hampers detailed studies. However, bioinformatics methods can rapidly address these issues.[22,23]

In the current study, bioinformatics software was used to characterize the peptides' bioactivity and water solubility. Peptides with bioactivity indicators higher than 0.50 are shown in Table 1. The higher the bioactivity index, the more likely the peptide is active.[24] According to Kęska *et al.*,[39] peptides consisting of 7 to 21 amino acids with a PeptideRanker score of 0.50 to 0.97 were considered biologically active. Twenty-four (24) out of fifty-six (56) peptides had a PeptideRanker threshold greater than 0.5, indicating their potential as bioactive peptides.[37-39]

Water solubility was also assessed as a parameter of bioactivity. Thirty-nine (39) peptides were identified as sufficiently soluble, while 17 – poorly soluble. Fourteen (14) of the twenty-four (24) bioactive peptides also exhibited good water solubility.

Descriptive statistical analysis, clustering analysis, correlation analysis, and principal component analysis (PCA) were conducted on the 56 peptides. Pearson correlation was applied to a data set consisting of 32 variables, including amino acid composition, ion exchange, water solubility, M_r, biological activity, pI, and allergenicity, to select the most influential variables.

Figure 3 presents the correlation heat map showing only significant coefficients. The target parameters of correlation were peptide bioactivity, allergenicity, water solubility, and pI. The results indicate that peptide bioactivity has a slight positive correlation with ion exchange type (IE; 0.34), pI

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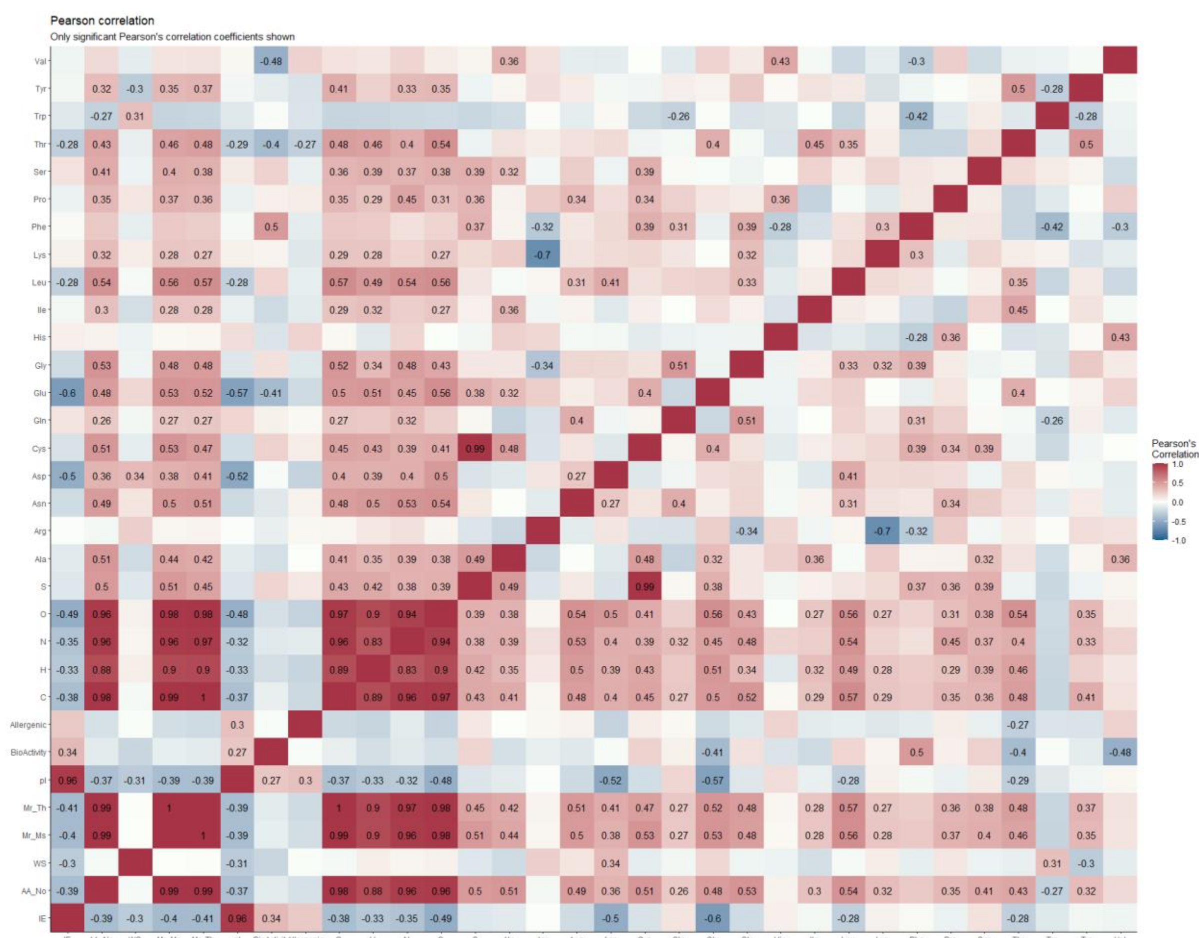

Fig. 3 Pearson correlation heat map.

(0.27), and Phe (0.50). Conversely, bioactivity negatively correlates with glutamine (Glu; -0.41), threonine (Thr; -0.40), and valine (Val; -0.48).

Peptide allergenicity shows a slight negative correlation with Thr (-0.27) and a positive correlation with pI (0.30). Peptide water solubility (WS) correlates negatively with ion exchange type, pI, and Tyr, and positively with asparagine (Asp) and tryptophan (Trp), with correlation coefficients ranging from -0.31 to 0.34.

The PCA scree plot shows that Principal Component 1 (PC1) accounts for 26.2% of the dataset variability, while PC2 accounts for 24.0% (Fig. 4a). Based on the PCA results and correlation analysis, the 9 most influential variables were selected from the initial 32. The first two PCs, accounting for approximately 50% of the dataset variability, were used to develop a biplot.

PC1 was primarily influenced by the M_r of peptides obtained from mass spectrometry (Mr_MS), as well as by Cys, Phe, Gly, and Glu (Fig. 4b). PC2 was mainly influenced by peptide bioactivity, Glu, Phe, and Val (Fig. 4c).

To create a biplot, the investigated peptides were grouped based on bioactivity levels: <0.5 (not bioactive), 0.5 < x < 0.75 (moderately bioactive), and >0.75 (highly bioactive) (Fig. 5). Additionally, peptides were grouped by ion exchange type to determine whether IE can influence bioactivity, given the slight positive correlation shown in the heat map (Figs. 3 and 5).

Five (5) highly (2 neutral and 3 cationic) and 19 moderately (9 anionic, 5 cationic, and 5 neutral) bioactive peptides were identified. This suggests that peptide bioactivity can be linked to the ion exchange type, with higher bioactivity expected in cationic or neutral peptides (Fig. 5). Both groups of bioactive peptides (highly and moderately) are separated from the non-bioactive ones by PC1 and PC2.

Considering PC1, which is greatly influenced by Cys and Gly, it can be concluded that peptide bioactivity tends to be higher if Cys and/or Gly are present in its composition. For PC2, mainly influenced by Val, Glu, Arg, and Phe, bioactivity is expected to be greater if Phe is present in its composition, whereas the presence of Arg, Val, and Glu may potentially contribute to peptide inactivity (Fig. 5).

It should be noted that the studied peptides did not contain the amino acids methionine (Met), pyrrolysine (Pyl), or selenocysteine (Sec). Among the 56 studied peptides, only cationic and neutral peptides demonstrated excellent biological activity (>0.75). These peptides exhibited superior biological activity compared to others due to the following reasons.[40-45]

1. Cationic peptides, being positively charged, can attach to negatively charged components of biological membranes,

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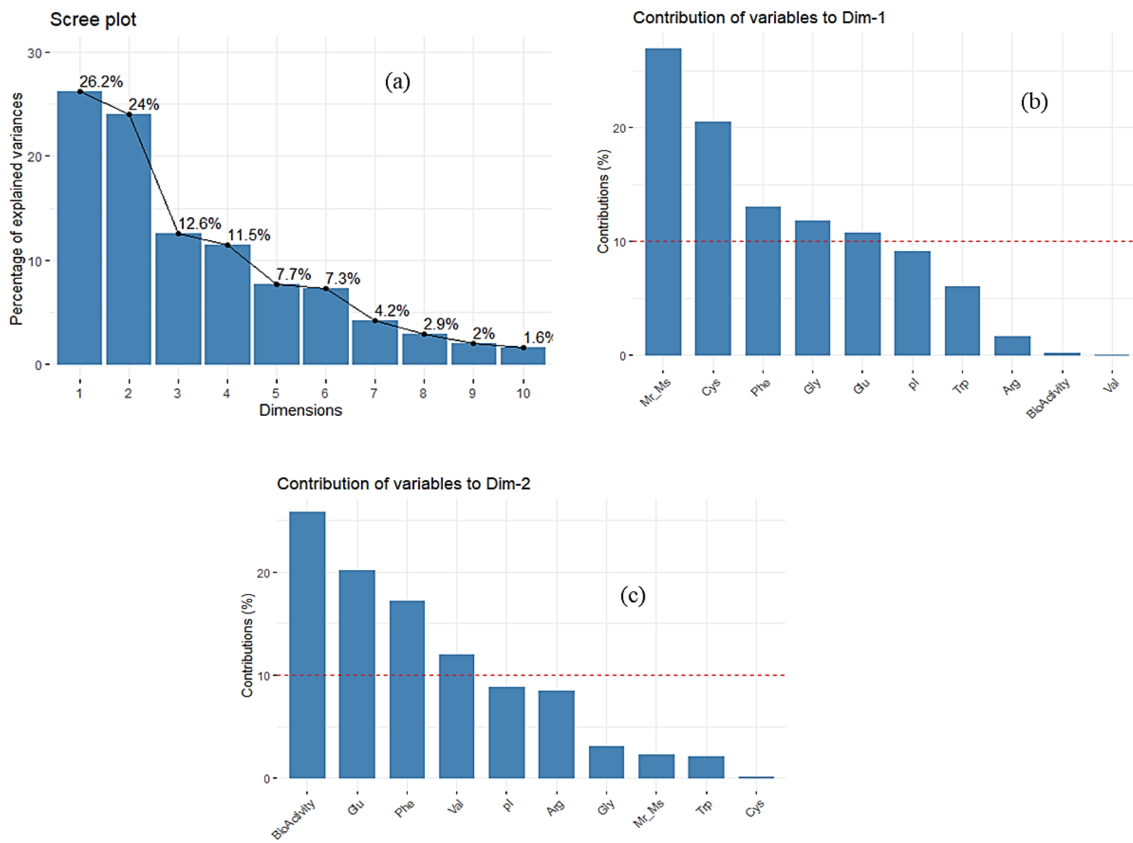

Fig. 4 PCA results. a) Scree plot; b) variables contribution to PC1; c) variables contribution to PC2.

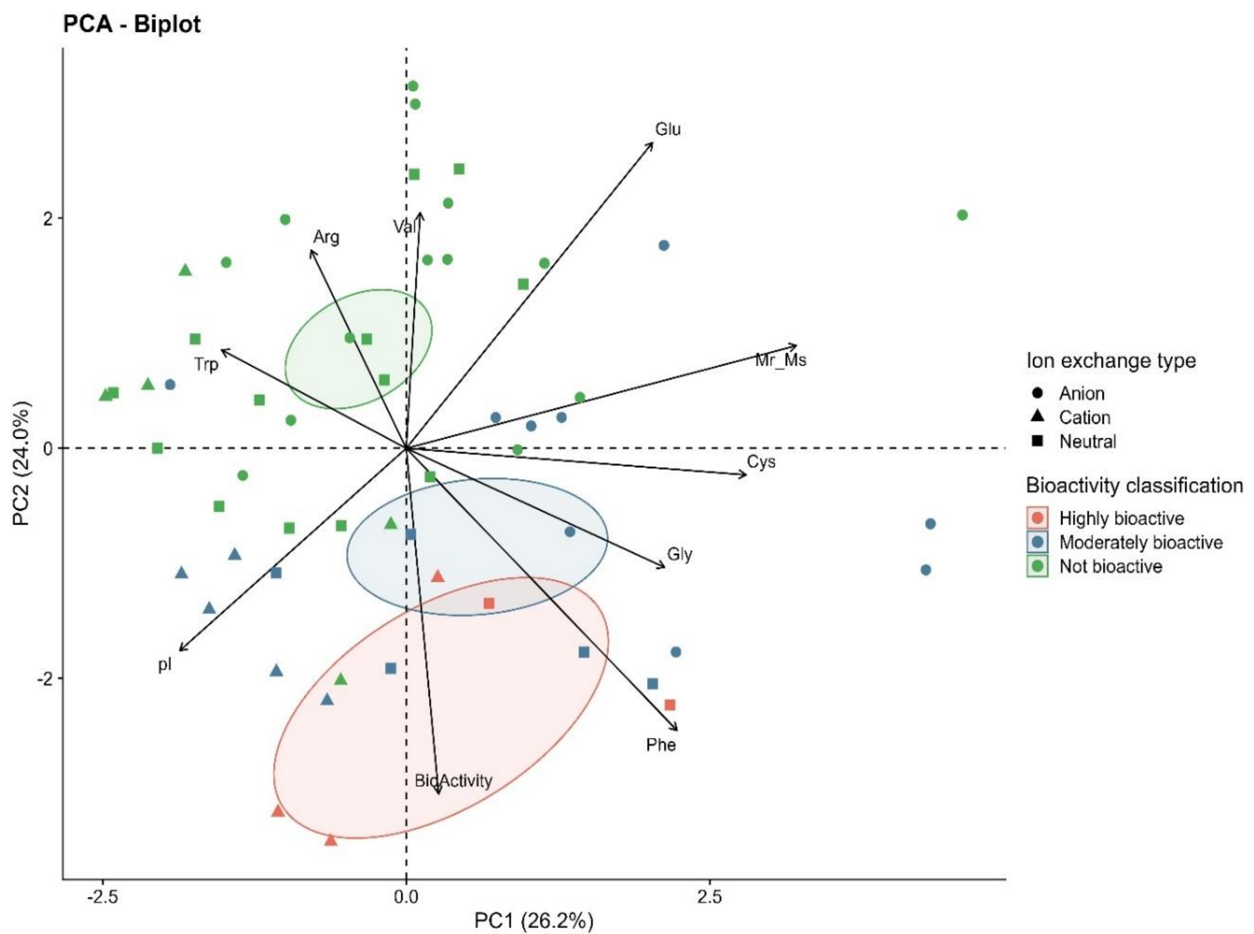

Fig. 5 Biplot of PC1 & PC2.

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such as proteins and lipids. This interaction allows the peptide to penetrate the cell membrane, enhancing biological activity.
2. Cationic and neutral peptides are generally more stable than anionic peptides. Their stable environment protects against proteases, allowing the peptides to remain active for longer periods.
3. Cationic and neutral peptides typically have a higher binding affinity for their target molecules, such as receptors and enzymes, compared to anionic peptides. The stronger binding interaction contributes to increased biological activity. This enhanced interaction, stability, and binding affinity explain the superior biological activity of cationic and neutral peptide.

Thus, it can be concluded that peptide bioactivity is positively correlated with Phe and negatively with Val. It is important to note that the correlations observed in this study may not universally apply to all peptides, but the following explanations can be offered: a) phenylalanine is an aromatic amino acid that plays a crucial role in the structure and function of proteins, contributing to peptide stability and binding affinity, thereby enhancing interaction with target molecules and increasing bioactivity; b) valine is a nonpolar amino acid typically found in the hydrophobic regions of proteins.[41-50] While valine itself is not the cause of negative bioactivity, its properties at specific peptide sequence sites may limit interaction with target molecules, reducing bioactivity.

4. Conclusion

The study findings offer a new perspective on equine milk proteins and their bioactive peptides as health-promoting products. Observation made that peptides containing phenylalanine are more likely to be bioactive suggests that this amino acid may play a crucial role in conferring bioactivity to peptides. Among the 56 obtained peptides, five with high biological activity (>0.75) were identified. These include the cationic peptides FCLFK, RCSSSPLLEACAFLR, and SSAFQLFK, as well as the neutral peptides CSSSPLLEACAFLR and CACSSQEPYFGYSGAFK. These peptides exhibit various biological activities, such as antimicrobial, anti-inflammatory, antioxidant, wound healing, and immunomodulatory properties, making them highly beneficial for health. Thus, further investigations are required to provide substantial evidence on the mechanisms by which these bioactive peptides exert antimicrobial and antioxidant effects of equine lactoferrin.

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Not applicable.

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