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Casein protein from Gir and Jersey cow milk: biochemical profiling, MALDI-TOF/MS peptide fingerprinting, and cytotoxic effects on HT-29 cells

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Abstract

Dietary chemoprevention offers a low-toxicity strategy to complement traditional cancer therapies. Bovine casein, the primary milk protein fraction, can produce bioactive peptides with reported anticancer properties. However, the influence of cattle genetic lineage on casein composition and activity remains insufficiently understood. This study compared the characteristics of casein-derived native and hybrid cows, evaluated their cytotoxic and apoptotic effects on human colorectal cancer HT-29 cells, and assessed their biocompatibility with Vero cells. Gir milk showed higher protein (10.7 vs. 1.6 mg/mL) and carbohydrate (2.4 vs. 1.6 mg/mL) contents than Jersey milk, while fat levels differed (4.34 vs. 5.44%), with total solids being similar (~16.6–16.8%). Native-PAGE revealed a major casein band along with minor bands in Gir, whereas SDS-PAGE identified two prominent bands (~35–25 kDa for αs1- and β-casein) and additional minor bands. Chromatographic analysis and MALDI-TOF/MS displayed a single prominent peak in Gir and three in Jersey, with Gir exhibiting higher m/z features (up to ~2020.985) and Jersey exhibiting lower m/z features (~719.638). Both chromatographic and mass spectrometry analyses indicated peptide profiles, with Gir having higher-mass features and Jersey having lower-mass features. Gir casein demonstrated poor biocompatibility, as evidenced by a dose-dependent decrease in Vero cell viability and an approximately 93.8% loss of viability in Jersey cells. Both caseins showed moderate, dose-dependent cytotoxicity toward HT-29 cells (67.50% for Gir and 75.80% for Jersey at 50 µg/mL; 44.80% and 50.09% at 350 µg/mL), with morphological apoptosis confirmed by PI staining. Variations in casein and peptide composition among breeds influence their safety and efficacy profiles, highlighting their potential as natural supplements for colorectal cancer; further research is needed to clarify their molecular mechanisms and in vivo effects.

Keywords SDS-PAGE, DEAE, Native, Hybrid, DNA fragmentation

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Introduction

Cancer is one of the most common and deadly health issues due to its heterogeneity and deadly global health issues due to its heterogeneity and evolutionary adaptability (Bray et al., 2021). Cancer develops due to inherited mutations, hormones, and immune conditions, and environmental/acquired factors (Anand et al., 2008). Cancer kills 16.8% of people worldwide and 22.8% of people with noncommunicable diseases (NCD). The cancer causes 30.3% of premature NCD deaths in 30-69-year-olds worldwide and is among the top three causes of death in the age group in 177 of 183 countries (Bray et al., 2021). Research suggests that 30–40% of all cancers might be preventable through dietary modifications (Donaldson et al., 2004). Bovine milk, a complex biological fluid, provides essential nutrients and bioactive proteins that contribute to human health; it contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes, and other bioactive peptides (Haug et al., 2007). These proteins are primarily classified as caseins, which represent 80% of the nitrogen content in bovine milk and 40% in human milk (Huppertz et al., 2018). The levels of casein in milk vary with animal breed and lactation stage, typically ranging from 24 to 29 g/L (Amalfitano et al., 2020).

Casein micelles consist of four major subtypes, αs1- (40%), αs2- (10%), β- (35%), and κ-caseins (15%), in relative proportions. Protein-protein interactions and the presence of calcium and phosphate are also associated with casein molecules (Broyard et al. 2015). Caseins and derivatives have demonstrated promising anti-cancer effects, exhibiting antitumor and cytotoxic properties against cells from various tumor types while sparing normal cells (Romero-Trejo et al. 2024). Specifically, β- and κ-caseins have shown anti-tumor activity against a range of cancers, including breast cancer (Nekipelaya et al. 2008; Leischner et al. 2021), melanoma (Azevedo et al. 2012), and ovarian cancer (Wang et al. 2013).

The genetic background of cattle significantly influences milk protein composition. Genetic variants of milk proteins have been a topic of interest and controversy for many decades, especially about the A1 and A2 variants of β-CN. Some studies have aimed to link A1 β-CN with heart disease, type 2 diabetes, and autism; however, no definitive link has been established (Kelly et al., 2025). This genetic distinction is highly relevant when comparing Native (Gir) and Hybrid (Jersey) breeds, as these variations may alter the structural and functional properties of the milk proteins (Khan et al., 2023; Kamiński et al., 2025). Casein has been observed to exhibit anticarcinogenic activity, potentially by inhibiting enzymes that convert procarcinogens or by enhancing immune functions. However, the comparative efficacy of caseins derived from different genetic lineages remains an area for investigation. This study aims to provide a comparative biochemical characterization of casein proteins isolated from the milk of Native (Gir) and Hybrid (Jersey) cows. Furthermore, the research evaluates their respective cytotoxic and apoptotic effects on the human colorectal cancer (HT-29) cell line to establish a scientific rationale for their potential application as natural therapeutic agents.

Materials and methods

Collection of milk samples

Fresh milk samples from native (Gir) and hybrid (Jersey) cows were collected during evening milking at the Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai. Samples were transferred in an ice box and stored at -4 °C upon arrival at the laboratory.

Biochemical analysis

Isolation of casein protein

Casein was isolated using isoelectric precipitation. Raw milk was centrifuged at 5000 rpm for 20 min to separate the lipids. The upper fat layer was removed, and the pH of the remaining aqueous phase was adjusted to 4.6 using 1 N HCl to precipitate the casein. The precipitate was filtered through double cheesecloth, washed with double-distilled water and ethanol, and further treated with diethyl ether to extract residual fats. The isolated casein was air-dried at room temperature (Marika Yamauchi et al., 2018).

Qualitative estimation

Protein concentration was quantified using the Bradford method (1976). Carbohydrate content was determined by the Roe (1955) method, fat content by the Rose-Gottlieb method (1961), and total solids were measured using the Clark et al. method (1989).

Electrophoretic profiling

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed according to the method of Maurer (1970). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted following the Laemmli (1970) method to determine molecular weights. Samples were run on 12% and 16% polyacrylamide gels in a Tris-Glycine buffer (pH 8.3) and visualized with Coomassie Brilliant Blue R-250, and molecular weights were estimated using a standard protein marker (BioLit, SRL; 14–220 kDa).

Purification of casein protein

Casein was purified by DEAE-cellulose ion-exchange chromatography (Sigma-Aldrich). A 5 g matrix was equilibrated in 100 nm sodium phosphate buffer (pH 7.6) and packed into a Bio-Red column (1.7 × 15 cm). Crude

casein (2 mL) was applied at a flow rate of 1 mL/min, and proteins were eluted using a linear sodium chloride gradient (0–1 M NaCl). 2 mL fractions were collected and monitored at 280 nm (Saliha et al., 2013).

Characterization by MALDI-TOF/MS

To ensure reliable peak assignment, the analysis was performed on purified intact proteins. Characterization was conducted using a Q-TOF hybrid mass spectrophotometer (MicroMass, Manchester, UK) equipped with a nano-spray source. Dried samples were analyzed with an accelerating potential of 20 kV in reflector mode. Precursor ions were accelerated at 8 kV and fragment ions at 19 kV. Protein annotation was confirmed via MASCOT database searches against the NCBI protein database.

Anti-cancer activity of crude and purified casein protein

Cancer cell line and chemicals

HT-29 (Human colorectal adenocarcinoma) and Vero (African green monkey kidney) cell lines were obtained from the National Center for Cell Science, Pune, India. DMEM, FBS, MTT, and antibiotics were sourced from Tarsons Products Pvt., Kolkata.

Toxicity assessment of crude casein protein on the Vero cell line

Cytotoxicity in the Vero cell line was evaluated using the MTT assay, as described by Sheth (2022). Vero cells at 5×10^3 cells per milliliter were seeded in 96-well plates containing DMEM supplemented with 10% FBS. Plates were incubated for 24 h in a humidified atmosphere at 37 °C with 5% CO_2. After incubation, the medium was removed, and the cells were washed with PBS. Fresh medium was added, and the mixture was incubated for 1 h under the same conditions. Control wells received fresh medium (DMEM, 50 µg/mL), while treatment wells were exposed to casein concentrations of 50, 100, 150, 250, and 350 µg/mL. Plates were reincubated under the same conditions. After 24 h, 10 µL of MTT solution was added to each well, and plates were incubated for an additional 3 h. DMSO (100 µL) was added to each well to dissolve the formazan crystals, then the plates were gently pipetted. Absorbance was measured at 570 nm with a microplate reader.

Cytotoxicity on the HT-29 cell line

Cell viability was assessed using the MTT assay, as described by Abedi et al. (2023) and Dolati et al. (2021). The antiproliferative effects of purified caseins were evaluated in HT-29 cells seeded at 5 × 10^3 cells/well in 96-well plates. After a 24-hour stabilization period, cells were washed with PBS and treated with the crude extract at concentrations of 50, 100, 150, 250, and 350 µg/mL for 24 h. After treatment, 10 µL of MTT was added to each well, and the wells were incubated for 3 h. The resulting crystals were then solubilized in 100 µL of DMSO. In the control treatment, DMEM, 50 µg/mL was used. Absorbance was measured at 570 nm, and the percentage of growth inhibition was calculated using the following formula:

$$Growth\ Inhibition\ \% = \frac{Absorbance\ control - \ Absorbance\ treated)}{Absorbance\ control} \times 100$$

Morphological observation

To observe phenotypic changes, HT-29 cells (5 × 10^4 cells/mL) were seeded in 6-well plates and incubated for 24 h. Cells were then treated with native and hybrid caseins at their determined low concentration (50 µg/mL for purified casein) and up to 350 µg/mL. In the control treatment, DMEM, 50 µg/mL was used. Following the incubation period, morphological changes such as cell shrinkage, rounding, and loss of adhesion were examined and documented using an inverted light microscope (Radical) at 40x magnification.

Propidium iodide (PI) staining

Staining of apoptotic nuclei was performed following the protocol outlined by Riccardi et al., (2006). Apoptotic nuclear changes were assessed by PI staining. HT-29 cells were seeded at 5 × 10^4 cells/mL in 6-well plates and incubated for 8 h. The concentrations of native and hybrid casein range from 50 µg/mL (minimum) to 350 µg/mL (maximum). After incubation, cells were washed with PBS, fixed in methanol: acetic acid (3:1, v/v) for 10 min, and stained with PI (50 µg/mL) for 20 min. In the control treatment, DMEM, 50 µg/mL was used. Nuclear morphology, specifically the presence of nuclear condensation and apoptotic bodies, was visualized immediately using a fluorescence microscope (Radical) at 40x magnification.

Statistical analysis

All experiments were performed in triplicate ($n = 3$), and results are presented as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 12.5.

Results

Biochemical analysis

Native and hybrid cow milks were successfully separated by isoelectric precipitation to isolate casein protein. The isolated samples were lyophilized and stored at -20 °C for further analyses. Reproducibility and reliability of the data were assessed by triplicate testing ($n = 3$) of all biochemical parameters, including protein, carbohydrate, fat, and total solids. The results are presented as mean ± SD in Table 1. Biochemical analyses showed

Table 1 Comparative biochemical composition of native and hybrid cow's milk

Biochemical components	Native milk sample	Hybrid milk sample
Protein (mg/mL)	10.7 ± 0.40	07.6 ± 0.30
Carbohydrate (mg/mL)	02.4 ± 0.20	01.6 ± 0.40
Fat (%)	4.34 ± 0.15	5.44 ± 0.21
Total solids (%)	16.61 ± 0.82	16.78 ± 0.75

Data represents the mean ± SD of triplicate measurements ($n = 3$)

significant differences in the composition of the milk of the two breeds. The protein concentration of native milk was significantly higher than that of hybrid milk (10.7 vs. 7.6 mg/mL). This is especially important for the study objectives, since caseins are the main precursors of bioactive peptides that may have anti-cancer properties, and the native breed has a higher protein content. On the other hand, the native milk sample had a lower fat content (5.44%) than the hybrid sample (4.34%), with total solids fairly similar in both (16.61% vs. 16.78%). The variation in protein and carbohydrate levels between the Gir and Jersey breeds justifies further comparative investigation of their respective therapeutic potentials.

Analysis of protein profile

Native–PAGE and SDS-PAGE

First, crude casein protein was extracted from the native and hybrid cow milk and electrophoretically profiled using native polyacrylamide gel electrophoresis (Native-PAGE) to visualize the proteins in their native state. Then, 4 mg of crude casein was prepared in 1 mL of sodium phosphate buffer (pH 7.6), and 20 µL of this solution was loaded per well. The resulting profiles showed one distinct, large band in both samples, with identical mobility, suggesting homology between the two breeds. However, two additional minor bands were observed in native casein but not in the hybrid sample. The presence of minor protein variations suggests that the casein fraction of the native breed is more structurally complex than that of other breeds shown in Fig. 1a.

Denaturing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to determine molecular weights and further characterize protein fractions. Analysis identified two major bands: α-S1-casein and β-casein in both native and hybrid samples. The major fractions were estimated to be 25–35 kDa. The high-resolution gel revealed 7–8 minor bands in each sample, confirming that casein fractions in milk comprise several protein components beyond the

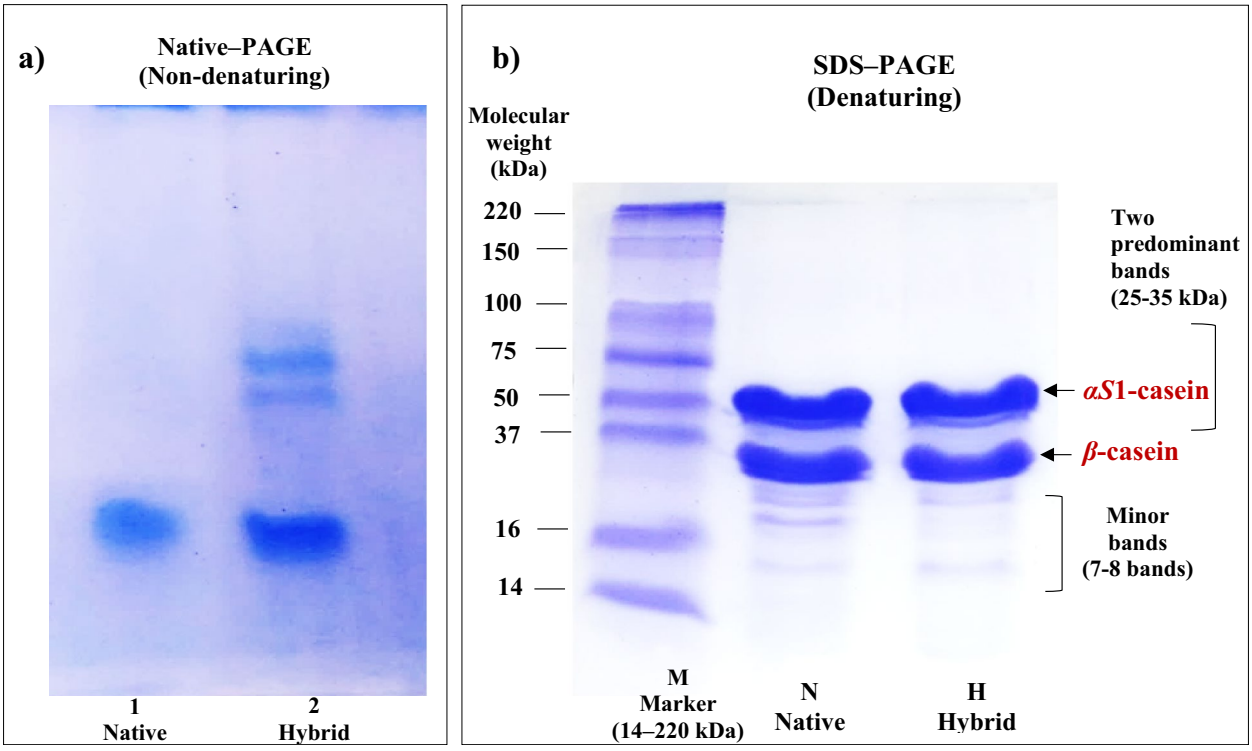


Fig. 1 Electrophoretic analysis of native and hybrid milk casein protein. **a** Native PAGE analysis under non-denaturing conditions. Lane 1: Native milk casein protein; Lane 2: hybrid milk casein protein. **b** SDS-PAGE analysis under denaturing conditions. Lane M: Molecular weight marker (14-H-220 kDa); Lane N: Native milk casein protein; Lane H: Hybrid milk casein protein. Two predominant bands corresponding to αS1-casein and β-casein (25–35 kDa) were observed in both samples

major milk casein subtypes. A standard protein marker (BioLit, SRL) with a range of 14–220 kDa was used to estimate molecular weight. The near-identical molecular weights of α-S1-casein (23,614 Da) and β-casein (23,974 Da) are thought to account for their similar migration patterns (Fig. 1b).

Purification of casein protein by DEAE–cellulose anion exchange chromatography

The crude caseins from native and hybrid milks were purified by DEAE-cellulose anion-exchange chromatography. The sample was injected into a column (1.7 cm × 15 cm) equilibrated with 100 nM sodium phosphate buffer (pH 7.6). The unbound proteins were eluted with equilibration buffer, and the bound proteins were eluted with a linear gradient of sodium chloride (0–1 M NaCl) at a flow rate of 1 mL/min. 40 fractions were collected and monitored at 280 nm. The chromatographic profiles showed that the two breeds had different elution profiles (Fig. 2). The native casein profile showed a single main peak, indicating that the casein fraction had been enriched to a single peak.

The hybrid casein profile, however, showed three peaks, indicating a more complex elution profile or the presence of multiple protein isoforms and complexes in the hybrid sample. Native-PAGE (10%) and SDS-PAGE (12%) were used to confirm the purity of peak fractions. For both, a single clear band was observed on PAGE of native milk, indicating successful separation of casein proteins from other milk components. These purified fractions were separated into two bands under denaturing conditions (SDS-PAGE), revealing the two major casein subtypes (αS1- and β-casein). Because SDS-PAGE is a denaturing method, the oligomeric state (dimer, trimer, etc.) of the casein species cannot be determined from the purification profiles. However, there are clear differences in the elution characteristics of native and hybrid caseins. There appear to be differences in protein association or composition between the breeds, as indicated by differences in peak numbers.

Characterization of purified protein by MALDI–TOF/MS

To develop a molecular fingerprint for each milk type, the purified casein fractions from native and hybrid milk were characterized by MALDI-TOF mass spectrometry. The intact molecular weights of α S1-casein and β-casein are approximately 23,614 Da and 23,974 Da, respectively. Therefore, the mass spectra obtained in the low m/z region (350–2020) do not represent the intact casein proteins but rather fragments of the protein or low-molecular-weight bioactive components. The resulting spectra were highly reproducible and showed a clear peptide profile for each sample. Major peaks were observed at m/z values of approximately 909.721, 1037.7779, 1165.874, 1233.976, 1465.159, 1593.290, 1661.492, and 1822.894, suggesting the presence of distinct casein-derived peptides or isoforms. The most intense peak was observed at m/z ~ 2020.985, corresponding to the major molecular species or a highly abundant peptide fragment in the purified sample (Fig. 3).

The MALDI-TOF mass spectrum of the purified hybrid casein showed a clear peptide distribution in the m/z range of 200–1200, indicating a relatively low molecular mass distribution compared with native casein. The spectrum revealed several well-defined peaks, with the most intense signal at m/z ~ 719.638, suggesting that this is the dominant peptide or fragment in the hybrid casein sample (Fig. 4). Other significant peaks appeared at m/z values around 795.619, 902.690, 946.739, and 984.729, likely corresponding to several peptide components or modified casein fragments. The moderate-intensity peaks at m/z ~ 1082.750, 1129.915, and 1173.943 further confirm

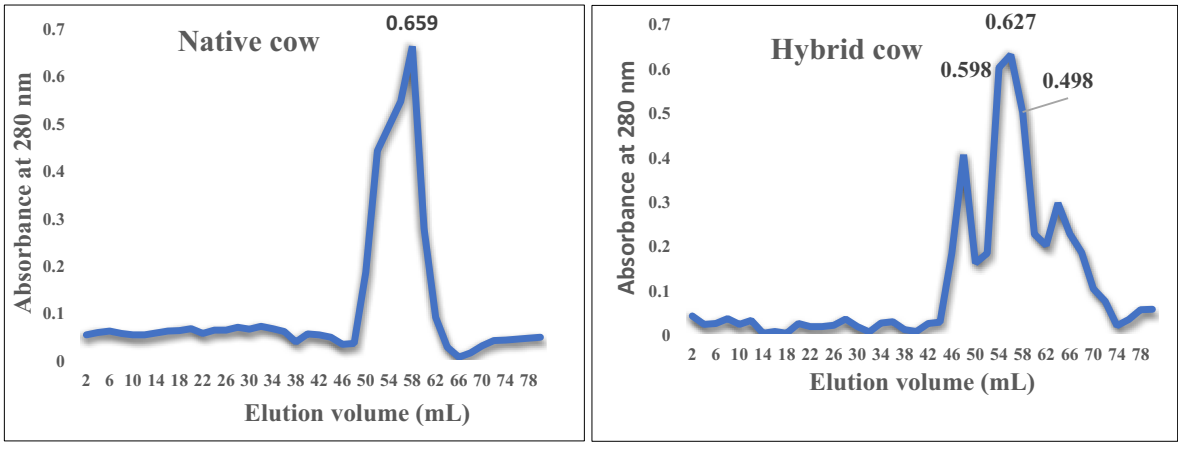
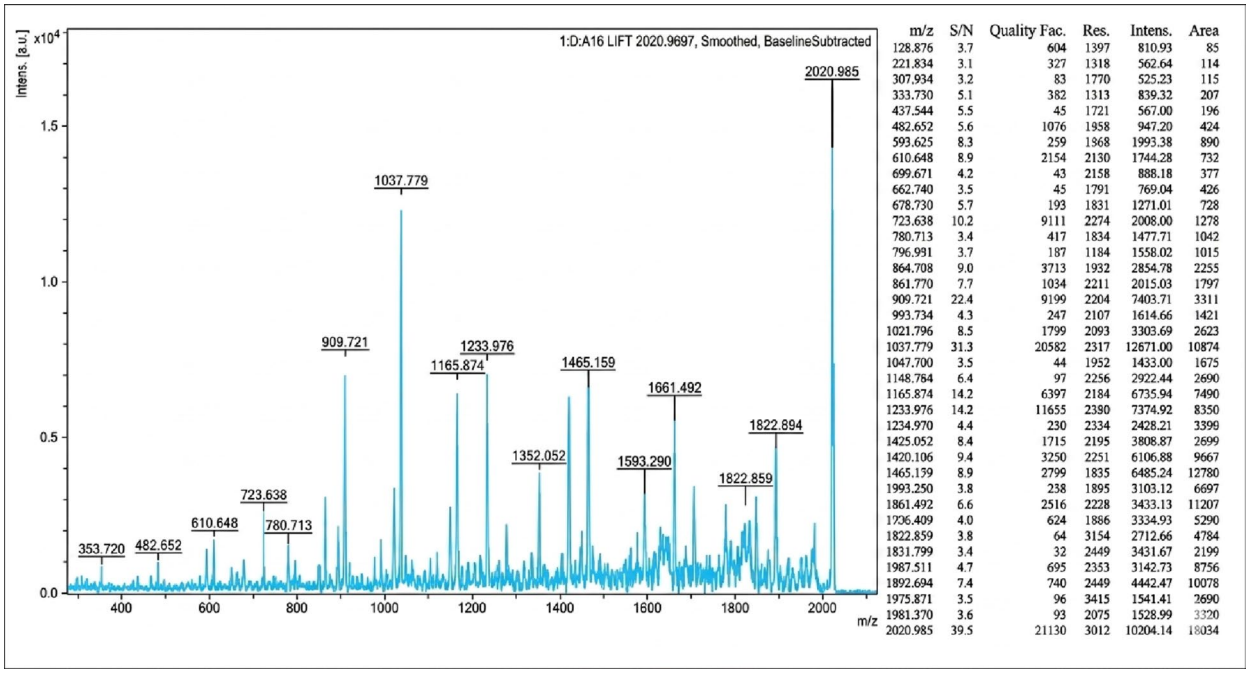
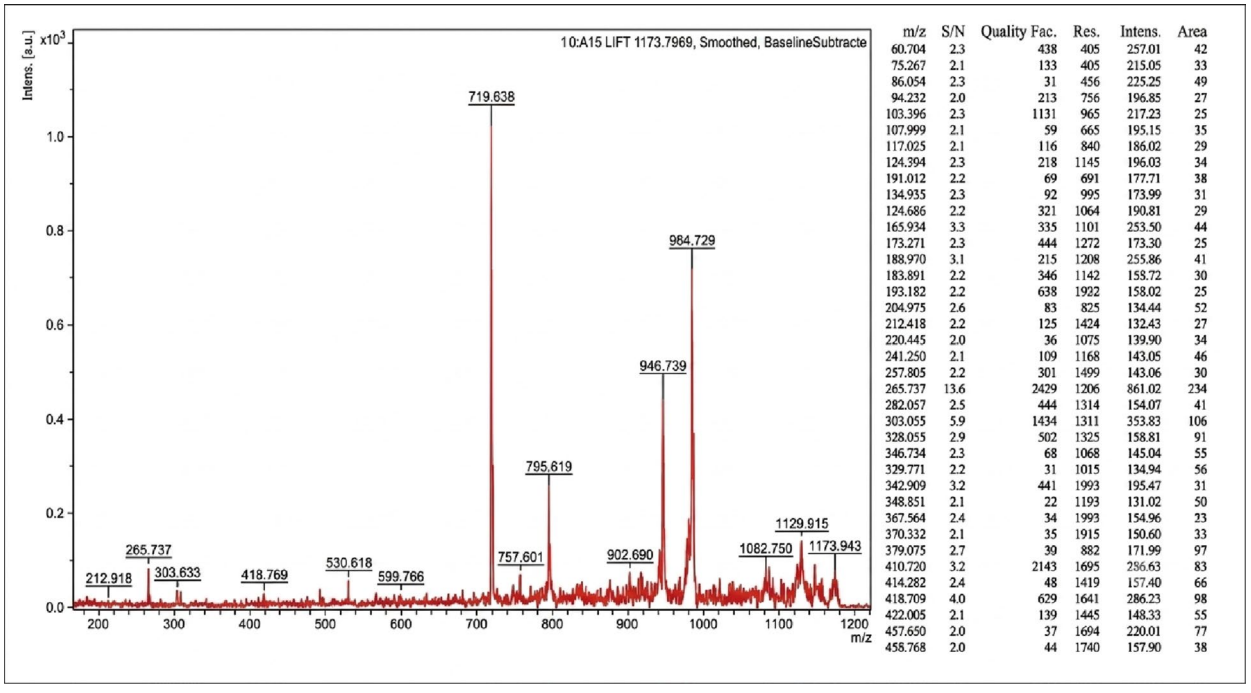


Fig. 2 DEAE-cellulose anion-exchange chromatographic profiles of casein proteins isolated from (**a**) native and (**b**) hybrid cow's milk. Proteins were separated on a DEAE-cellulose column equilibrated with 100 mM sodium phosphate buffer (pH 7.6) and eluted with a linear NaCl gradient (0–1 M) at a flow rate of mL min^{-1}. Protein-containing fractions were collected and monitored at 280 nm. Fraction numbers and the NaCl gradient profile are indicated



m/z	S/N	Quality Fac.	Res.	Intens.	Area
128.876	3.7	604	1397	810.93	85
221.834	3.1	327	1318	562.64	114
307.934	3.2	83	1770	525.23	115
333.730	5.1	382	1313	839.32	207
437.544	5.5	45	1721	567.00	196
482.652	5.6	1076	1958	947.20	424
593.625	8.3	259	1868	1993.38	890
610.648	8.9	2154	2130	1744.28	732
699.671	4.2	43	2158	888.18	377
662.740	3.5	45	1791	769.04	426
678.730	5.7	193	1831	1271.01	728
723.638	10.2	9111	2274	2008.00	1278
780.713	3.4	417	1834	1477.71	1042
796.991	3.7	187	1184	1558.02	1015
864.708	9.0	3713	1932	2854.78	2255
861.770	7.7	1034	2211	2015.03	1797
909.721	22.4	9199	2204	7403.71	3311
993.734	4.3	247	2107	1614.66	1421
1021.796	8.5	1799	2093	3303.69	2623
1037.779	31.3	20582	2317	12671.00	10874
1047.700	3.5	44	1952	1433.00	1675
1148.784	6.4	97	2256	2922.44	2690
1165.874	14.2	6397	2184	6735.94	7490
1233.976	14.2	11655	2380	7374.92	8350
1234.970	4.4	230	2334	2428.21	3399
1425.052	8.4	1715	2195	3808.87	2699
1420.106	9.4	3250	2251	6106.88	9667
1465.159	8.9	2799	1835	6485.24	12780
1993.250	3.8	238	1895	3103.12	6697
1861.492	6.6	2516	2228	3433.13	11207
1706.409	4.0	624	1886	3334.93	5290
1822.859	3.8	64	3154	2712.66	4784
1831.799	3.4	32	2449	3431.67	2199
1987.511	4.7	695	2353	3142.73	8756
1892.694	7.4	740	2449	4442.47	10078
1975.871	3.5	96	3415	1541.41	2690
1981.370	3.6	93	2075	1528.99	3320
2020.985	39.5	21130	3012	10204.14	18034

Fig. 3 MALDI-TOF/MS peptide profile of purified native (Gir) casein



m/z	S/N	Quality Fac.	Res.	Intens.	Area
60.704	2.3	438	405	257.01	42
75.267	2.1	133	405	215.05	33
86.054	2.3	31	456	225.25	49
94.232	2.0	213	756	196.85	27
103.396	2.3	1131	965	217.23	25
107.999	2.1	59	665	195.15	35
117.025	2.1	116	840	186.02	29
124.394	2.3	218	1145	196.03	34
191.012	2.2	69	691	177.71	38
134.935	2.3	92	995	173.99	31
124.686	2.2	321	1064	190.81	29
165.934	3.3	335	1101	253.50	44
173.271	2.3	444	1272	173.30	25
188.970	3.1	215	1208	255.86	41
183.891	2.2	346	1142	158.72	30
193.182	2.2	638	1922	158.02	25
204.975	2.6	83	825	134.44	52
212.418	2.2	125	1424	132.43	27
220.445	2.0	36	1075	139.90	34
241.250	2.1	109	1168	143.05	46
257.805	2.2	301	1499	143.06	30
265.737	13.6	2429	1206	861.02	234
282.057	2.5	444	1314	154.07	41
303.055	5.9	1434	1311	353.83	106
328.055	2.9	502	1325	158.81	91
346.734	2.3	68	1068	145.04	55
329.771	2.2	31	1015	134.94	56
342.909	3.2	441	1993	195.47	31
348.851	2.1	22	1193	131.02	50
367.564	2.4	34	1993	154.96	23
370.332	2.1	35	1915	150.60	33
379.075	2.7	39	882	171.99	97
410.720	3.2	2143	1695	286.63	83
414.282	2.4	48	1419	157.40	66
418.709	4.0	629	1641	286.23	98
422.005	2.1	139	1445	148.33	55
457.650	2.0	37	1694	220.01	77
458.768	2.0	44	1740	157.90	38

Fig. 4 MALDI – TOF/MS peptide profile of purified Hybrid (Jersey) casein

the complexity of the hybrid protein structure. There is also a decrease in the m/z range and in the number of high-mass peaks compared to native casein, suggesting possible structural modifications, fragmentation, or processing effects in the hybrid form.

The results showed that the genetic origins of the major proteins are the same, but the fragmentation patterns or peptide profiles associated with each protein differ between the two breeds. The selected mass spots were subjected to MS/MS analysis and a MASCOT database search, followed by BLAST alignment with NCBI's protein database to confirm protein identity. These results indicate the potential of MALDI-TOF/MS for determining peptide fingerprints suitable for breed discrimination,

but the spectra observed are the result of the peptide complexity of milk and do not represent the actual amino acid differences between the milk parent proteins (such as Proline to Histidine).

Toxicity assessment of native and hybrid purified casein protein on the Vero cell line

After 24 h of exposure, the cytotoxicity assay showed that cell viability decreased in a concentration-dependent manner in both native and hybrid casein-treated Vero cells. Cell viability decreased significantly from 84.50% at 50 µg/mL to 45.20% at 350 µg/mL in native casein, indicating that higher concentrations were more cytotoxic. Hybrid casein, on the other hand, showed relatively stable cell viability across all concentrations tested, ranging from 93.84% (at 50 µg/mL) to 93.86% (at 350 µg/mL). Hybrid casein also demonstrated significantly lower cytotoxicity (89.77, 84.44, and 78.41%, respectively) at intermediate concentrations when compared to native casein (73.21, 65.12, and 56.70%, respectively) (Fig. 5). The results indicate that hybrid casein is less toxic to Vero cells and more biocompatible than native casein. Overall, the data suggest that structural changes in hybrid casein may help reduce cytotoxic effects and enhance cell compatibility.

Anticancer activity against the HT-29 human colon cancer cell line

The anticancer activity of native and hybrid cow milk casein was tested against the human colorectal cancer cell line (HT-29), as shown in Fig. 6. Cell viability decreased in a concentration-dependent manner for both casein samples, indicating cytotoxic activity against HT-29 cells. Native casein at the lowest concentration (50 µg/mL) reduced cell viability by 67.50%, whereas hybrid casein showed a relatively weak cytotoxic effect, with 75.80% cell viability. At 100 µg/mL, cell viability decreased to 62.20% and 63.70% for native and hybrid casein, respectively. At 150 and 250 µg/mL, further reductions were observed: native casein at 58.20% and 53.30%, and hybrid casein at 57.30% and 55.00%, respectively. Casein samples showed increased anticancer activity at higher concentrations. At 350 µg/mL, cell viability decreased to 44.80% and 50.09% for native and hybrid casein, respectively. Overall, both native and hybrid cow milk casein exhibited moderate cytotoxic activity against HT-29 cells, with native casein showing a slightly greater growth-inhibitory effect at high concentrations. These results indicate that casein proteins have anticancer properties and can dose-dependently inhibit the proliferation of colorectal cancer cells.

Cytomorphological observations

The effects of crude native and hybrid casein on the cytomorphology of HT-29 human colorectal cancer cells are shown in Fig. 7. Control cells exhibited normal morphology, with a confluent monolayer, well-structured cell architecture, and a typical polygonal shape, indicating healthy growth and attachment to the culture surface. However, cells exposed to crude native and hybrid casein showed concentration-dependent morphological changes. Compared with the control, HT-29 cells

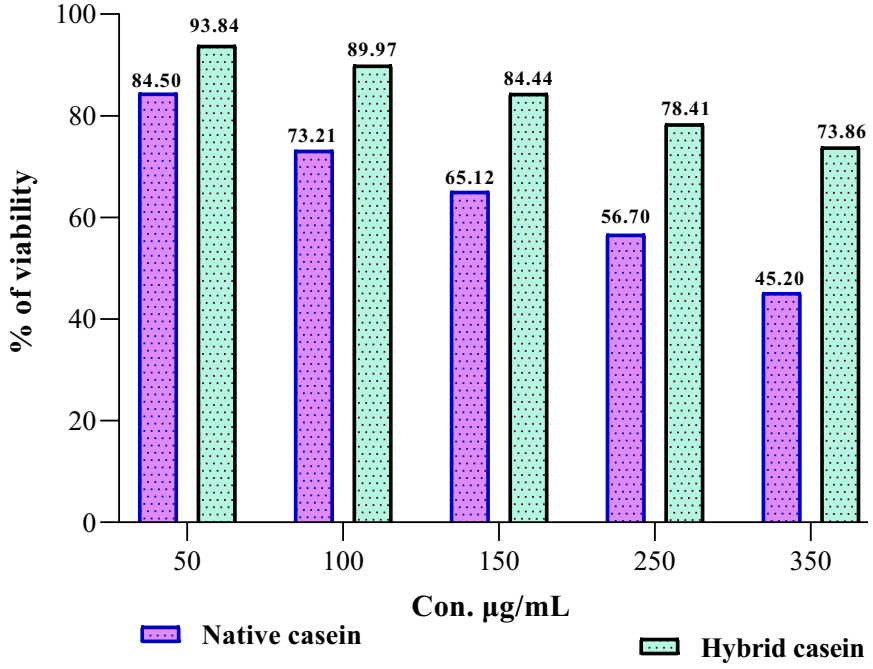


Fig. 5 Comparative dose-dependent cytotoxicity of native and hybrid casein in Vero cells after 24 h exposure

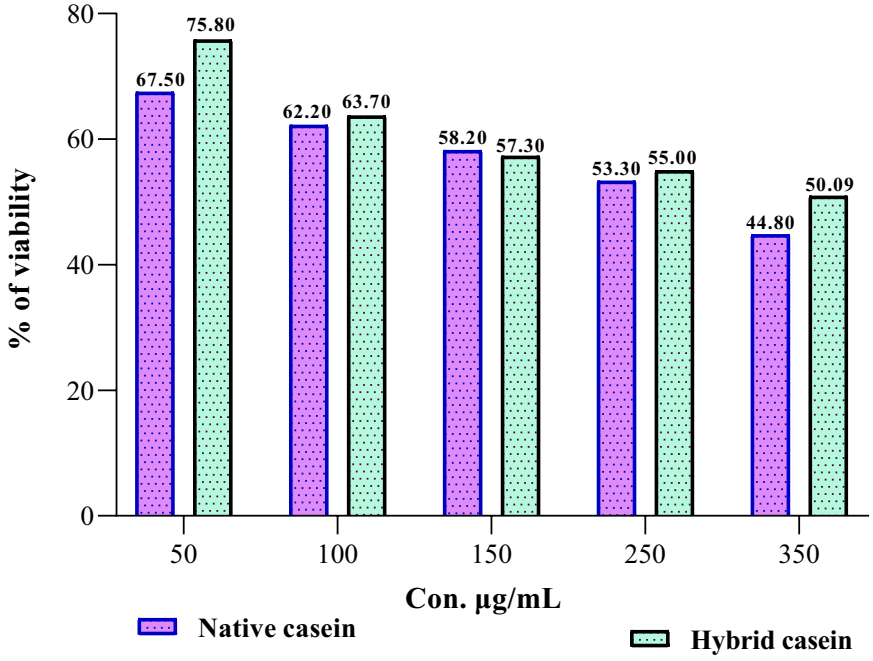


Fig. 6 Effect of purified casein from native and hybrid cow's milk on the viability of HT-29 human colon cancer cells at different concentrations

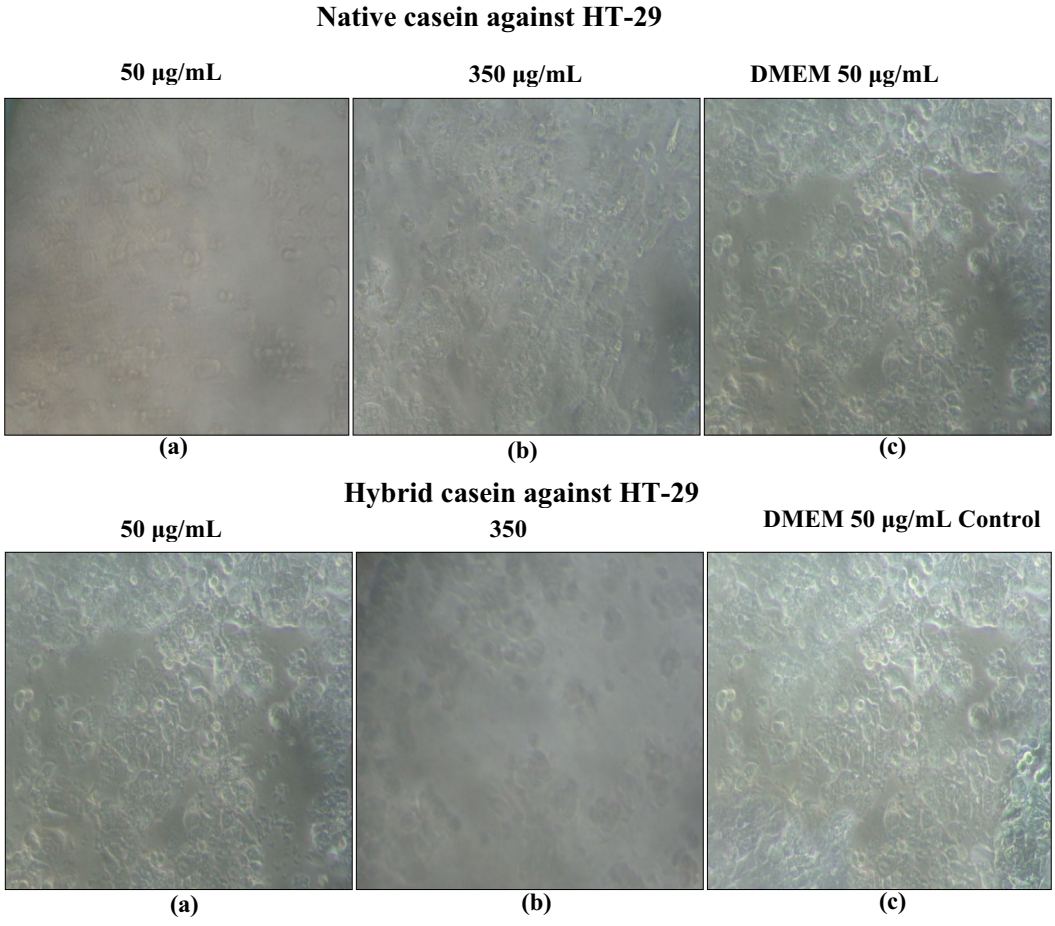


Fig. 7 Cytomorphological observation of cells treated with crude native & hybrid casein on HT-29 cells. **a** - Minimum concentration (50 µg/mL); **b** - Maximum concentration (350 µg/mL); **C** – Control

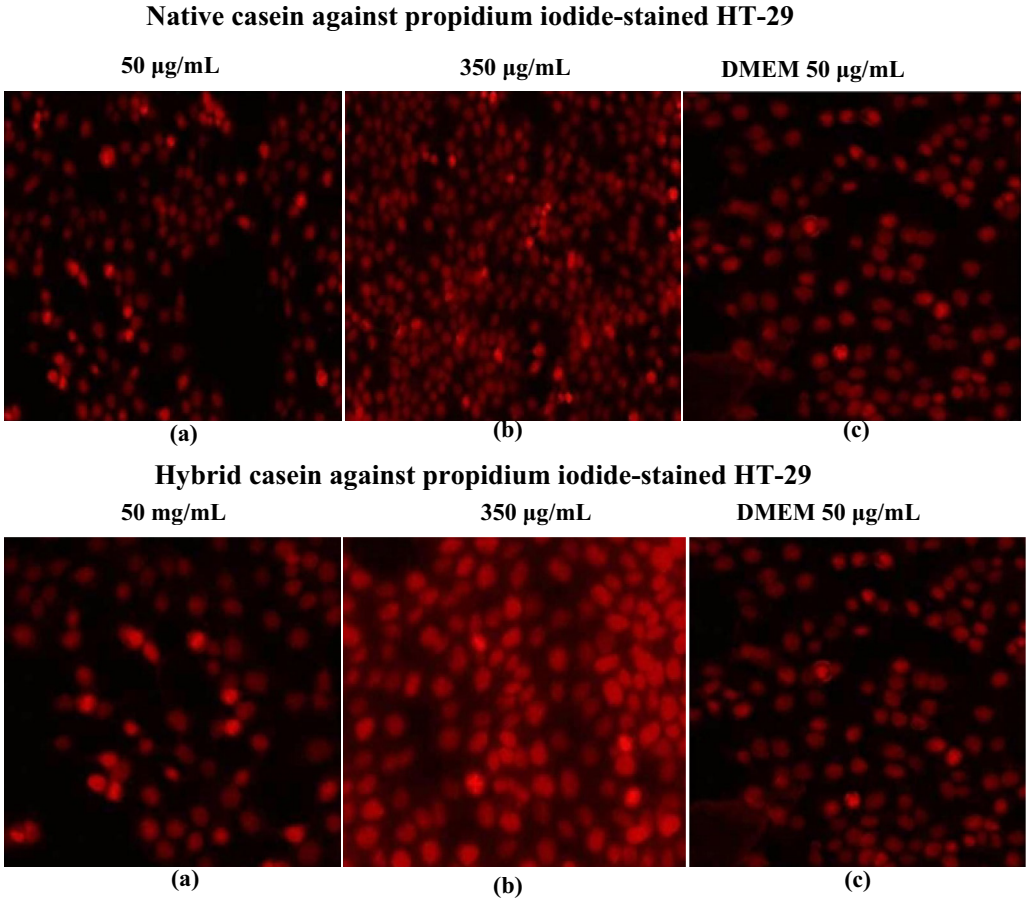


Fig. 8 Photomicrographs showing the effect of purified native and hybrid casein protein on propidium iodide-stained HT 29 cells (40X Magnification). **A** – minimum concentration (50 µg/ml); **B** - maximum concentration (350 µg/mL); **C** – Control

exhibited reduced cell density, reduced cell-to-cell contact, cell shrinkage, and rounding at concentrations of 50 and 350 µg/mL for native casein and hybrid casein. These observations indicated that cytotoxic effects and inhibition of cell growth had begun.

Propidium iodide (PI) staining

To confirm apoptosis, HT-29 cells were stained with propidium iodide after treatment with both crude and purified casein proteins. Treated cells showed a very bright red stain, indicating nuclear condensation and the formation of apoptotic bodies, whereas control cells had a dim red stain and intact nuclear morphology. The effect of purified native and hybrid caseins on PI uptake in HT-29 cells is shown in Fig. 8. A time-dependent increase in the number of PI-positive cells was detected after 24 h of treatment. Staining was moderate at the lower concentration (50 µg/mL), and the higher concentration (350 µg/mL) showed a marked increase in fluorescence intensity. This progressive increase in the number of PI-positive cells demonstrates that casein treatment induces apoptosis in HT-29 cells.

Discussion

Cancer is the second most common cause of death worldwide and accounts for approximately 10 million deaths annually. Globally, about 1 in 6 deaths is due to cancer (https://www.who.int/news-room/facts-in-pictures/detail/cancer). Radiotherapy, chemotherapy, and surgery are considered first-line treatments (Amjad et al., 2023). Despite some success in curbing tumor growth, these therapies have not cured malignancies and have left patients with a host of serious side effects, such as medication sensitivity, nausea and vomiting, and hair loss. Radiation therapy is usually used to treat systemic spread of esophageal cancer, lung cancer, nasopharyngeal cancer, and skin cancer, but once cancer has spread to the tissue, radiation is not effective (Evans et al., 2018). This underscores the urgent need to identify natural anticancer agents that are both safe and effective. Dietary compounds with chemopreventive properties have attracted interest as promising, low-toxicity alternatives to conventional drugs for cancer prevention and treatment (Langner et al., 2012). Nutrition-based approaches to chemoprevention offer an innovative and practical strategy for controlling cancer progression and improving

overall health. Among natural foods, milk and its proteins stand out as potential sources of bioactive peptides with anticancer activity (Phung et al., 2026).

Bovine milk primarily contains two major protein families: caseins and whey proteins (De Azeredo et al., 2014). Milk-derived peptides exhibit a broad spectrum of biological activities, including antimicrobial, immunomodulatory, antioxidant, antihypertensive, anticancer, and mineral-binding effects (Phung et al., 2026). Milk is nutritionally dense, providing essential nutrients such as fats, proteins, lactose, minerals, and non-fat solids, making it an important global food (Malik et al., 2024). In our study, the native milk sample had a higher fat content (5.44%) than the hybrid sample (4.34%), with total solids fairly similar in both (16.61% vs. 16.78%). Numerous biochemical analyses have been reported previously. During early lactation (phase I), Gir cow milk contained 4.611, 8.629, 3.152, 4.764, and 0.702% fat, solids-not-fat, protein, lactose, and salt, respectively. In contrast, during mid-lactation (phase II), the values were 5.142%, 8.586%, 3.131%, 4.743%, and 0.699%, respectively (Chaudhari et al., 2025). Singhai et al. (2013) reported mean fat, solids-not-fat, and protein content of Gir cow milk as 4.54, 8.53, and 3.85%, respectively. Gajbhiye et al. (2019) reported the contents of milk fat, solids-not-fat, protein, and lactose as 4.15%, 8.85%, 3.28%, and 4.83%, respectively.

Electrophoretic analysis (NATIVE-PAGE) of crude casein from both samples revealed one major and two minor protein bands. Under denaturing conditions (SDS-PAGE), two main and several minor bands were observed—seven in native casein and eight in hybrid casein. The two predominant bands corresponded to αs1-CN and β-CN, with molecular weights of 25–35 kDa. These results align with previous research (Iukalo et al., 2014), which reported similar banding patterns. The close molecular weights of αs1-CN (23,614 Da) and β-CN (23,974 Da) account for their similar migration behavior, complicating their separation. MALDI–TOF analysis indicated that the purified native casein exhibited 53% similarity to a hypothetical protein from *Streptomyces daghestanicus* (7,541 Da, pI 11.48), whereas hybrid casein showed 31% similarity to a hypothetical protein from *Pteropus alecto* (11,831 Da, pI 6.8). Both proteins exhibited comparable mass and score values, indicating similar structural properties.

Bonuccelli et al. (2012) discovered that three genes encoding the protein casein (α-, β-, and κ-casein) can significantly inhibit the migration of mouse and human breast cancer cells, with α-casein being the most effective. Expression of α-casein in tumor cells significantly inhibits tumor growth and lung metastasis in mice by decreasing the 'stemness" of tumor cells and promoting a more differentiated, apoptosis-prone phenotype via the STAT1 pathway. The casein nanodispersion and p-naphthoquinone were subjected to a cytotoxicity assay, yielding an IC_{50} of 46.35 µg for the drug nanodispersion, suggesting anticancer properties (Duraikannu et al., 2026). Casein is known to have strong antimutagenic activity, as demonstrated in mutagen models (Parodi et al., 2007; van Boekel et al., 1993). In several animal experiments, casein also showed an inhibitory effect on mutagens (McIntosh et al., 1995). In mice fed 20% casein diets, the incidence of colon cancer was significantly lower than in the control group when stimulated with 1,2-dimethylhydrazine (Papenburg et al., 1990). Casein may be protective against the development of colon and breast cancer (Bonuccelli et al., 2012). The proliferation of prostate cancer cells treated with α-casein (228%) and casein (166%) was higher than that of untreated cells (Park et al., 2014).

Recent studies have shown that milk protein is an important factor in the development and modulation of prostate cancer cell proliferation (Tate et al., 2011; Pettersson et al., 2012). Tate et al. (2011) found that prostate cancer cells (LNCaP) grew nearly as well in the presence of cow's milk as in digested whole milk. There was no increase in breast cancer cell growth (MCF-7) in either casein or digested milk, consistent with our results. Casein was found to inhibit the growth rate of breast cancer cells, but not that of prostate cancer cells. Moreover, Bonuccelli et al. (2012) demonstrated that α-casein was effective in appreciably reducing the proliferation and metastatic ability of one murine mammary tumor cell line (Met-1 cells) and two human breast cancer cell lines (MCF10A-H-Ras and MDA-MB-231 cells). Moreover, α-casein exerted its tumor-suppressor activity via STAT1 signaling. They hypothesized that α-casein might be able to provide a 'differentiation' therapy for breast cancer.

Conclusion

The results of this study show that casein proteins in native and hybrid cow milk differ in biochemical composition, structural profiles, and biological activities. The protein content of the native milk was higher, and the casein profile was more complex than that of the hybrid milk. Native casein exhibited slightly higher anticancer activity than other casein types in the human colorectal cancer cell line HT-29, with dose-dependent cytotoxic and apoptotic effects. The hybrid casein appears less cytotoxic to normal Vero cells, indicating better biocompatibility. MALDI-TOF/MS analysis showed that peptide fragmentation patterns differed between breeds, suggesting structural differences that could underlie functional differences. In general, this study confirms the promising potential of casein proteins, especially from native breeds, as natural anticancer agents, which should be further explored for their mechanisms of action and applications in cancer prevention and treatment.

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Generative AI statement
The author(s) declared that generative AI was used in the creation of this manuscript. During manuscript preparation, the authors used Grammarly Premium to correct language errors. This AI-assisted tool checked spelling, grammar, punctuation, clarity, and plagiarism, suggesting corrections where needed. This collaboration enhanced the manuscript's quality by combining human expertise with AI efficiency, and Turnitin was used to detect plagiarism and AI-generated content.

Author contributions
CRediT: SS: Funding acquisition, investigation, methodology, project administration, validation, writing– original draft. MA: Conceptualization, data curation, formal analysis. AS: Conceptualization. VR: Conceptualization, data curation. RM: Formal analysis, investigation, project administration, software, writing– original draft, writing– review & editing.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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