Characterization of ACE inhibitory and antioxidant peptides in yak and cow milk hard chhurpi cheese of the Sikkim Himalayan region

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ABSTRACT
In this study, simulated in vitro GI digestion of the Himalayan hard chhurpi cheese resulted in the increase of hydrolyzed protein content, antioxidant and ACE-inhibitory activities. LC-MS/MS-based peptidomics revealed a total of 1473 peptides in the samples originating from different milk proteins, including α-S1-casein, α-S2-casein, β-casein, κ-casein, γ-casein, α-lactalbumin, and β-lactoglobulin, out of which 60 peptides have been reported for different functional properties. A total of 101 peptides were predicted to be antihypertensive using the bioactivity prediction web servers, AHTpin and mAHTPred. In silico molecular docking studies predicted 20 antihypertensive peptides, exhibiting non-bond interactions between hard chhurpi peptides and ACE catalytic residues. A peptide, SLVYPFPGPI, identified in GI digested cow hard chhurpi and undigested, and GI digested samples of yak hard chhurpi, showed a stronger binding affinity towards ACE. Identifying antioxidant and ACE inhibitory peptides in hard cheese products adds value to them as functional foods of the Himalayan region.

Introduction
Hypertension is one of the leading causes of cardiovascular illness. It is linked to a variety of health consequences, including myocardial infarction, heart failure, stroke, renal disease, and kidney dysfunction (Rai, Sanjukta, & Jeyaram, 2017). Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase belonging to the zinc protease class that plays a crucial physiological role in blood pressure regulation. It is critical in maintaining the renin-angiotensin (RAS) and kallikrein-kinin (KK) systems. ACE cleaves two amino acids from angiotensin-I to form a vasconstrictor angiotensin-II and also inactivates the potent vasodilator peptide, bradykinin, resulting in high blood pressure (Mudgil et al., 2019). ACE inhibitors, such as captopril, enalapril, and lisinopril, are synthetic antihypertensive drugs that are therapeutically available to reduce the effects of hypertension by blocking the conversion to angiotensin-II and relaxing the blood vessels (Mudgil et al., 2019). Due to some of the adverse symptoms such as headache, chronic dry cough, angioneurotic edema, and impaired taste perception, researchers are focusing on nutraceutical alternatives as the preventive measure to control hypertension (Lin et al., 2020). During the last two decades, many studies have shown that peptides derived from dietary proteins exhibit ACE inhibitory properties in vitro and antihypertensive affects in vivo (Lin et al., 2020; Rai, Sanjukta, & Jeyaram, 2017).

Yaks (Bos grunniens or Poephagus grunniens) are long-haired bovid mammals found in Russia, Mongolia, the Tibetan Plateau, and the Himalayan region, with over 5% of India’s total yak population present in the Sikkim Himalayan region (Pereoze, Ray, Singh, & Singh, 2019; Jiang et al., 2020). Yak milk is rich in nutritional components such as proteins, fats, minerals, lactose and essential amino acids, apart from functional and bioactive compounds (Lin et al., 2018). One of the significant characteristics of yak milk is higher protein content than bovine milk. However, both milk types have a similar amino acid composition in protein. For years, yak milk has been used to produce ghee, butter, cheese, yoghurt and other fermented products, which have been a significant source of nutrients for individuals living, especially in high altitudes (Agyare & Liang, 2021). Milk proteins have been reported to be a
good source of bioactive peptides, which exert numerous health beneficial effects, such as antimicrobial, antiviral, antioxidant, antithrombotic, opioid, immunoregulatory, and antihypertensive activities (Chourasia et al., 2020, 2021). These peptides are released from milk proteins by the proteolytic activity of microorganisms during milk fermentation (Chourasia et al., 2021). Curd, yoghurt and cheese production results in enhanced functional property of milk due to the release of bioactive peptides (Chourasia et al., 2021).

Chhurpi is a type of traditional cheese product famous in the Sikkim Himalayan region. It is consumed in both the forms of soft and hard cheese types (Rai, Kumari, Sanjukta, & Sahoo, 2016). Hard chhurpi is generally prepared from both yak and cow milk. By the process of milk churning, a product rich in fat content, ghee/mar (crude butter), is obtained. Chhurpi, rich in casein soft product, is called soft chhurpi variety. It is generally consumed as soup/curry and relish along with meals. Soft chhurpi is dehydrated to produce the hard type of chhurpi, called chhur.Kumari, Singh, Sahoo, and Rai (2022) and Sanjukta, Rai, Muhammed, Jeyaram, and Talukdar (2015), with minor modifications. The powders obtained from the hard chhurpi samples were dissolved in distilled water in the ratio 1:10 (w/v), and pH was adjusted to 2.0 using 1 M HCl. After the addition of pepticin (4% w/w of the protein), the mixture was incubated under shaking conditions for 2 h at 37 °C. Then, the pH of the mixture was adjusted to 7.5 using 1 M NaOH. Further, pancreatin (4% w/w of the protein) was added for digestion and incubated under shaking conditions for 4 h at 37 °C. The mixture was boiled for 15 min to terminate the enzymatic activity and then cooled down to room temperature and centrifuged at 10,000 g for 30 min. The supernatant was collected and stored at −20 °C for further analysis.

**Estimation of hydrolyzed protein content**

Hydrolyzed protein content was determined using the method explained by Rai, Sanjukta, Chourasia et al. (2017), with certain modifications. An equal volume of 10% TCA (w/v) was added to extracts and GI digested protein hydrolysate. The mixture was then incubated at room temperature overnight, followed by centrifugation at 8000 g for 15 min. The supernatant was collected, and protein estimation was done by using Lowry’s method (Lowry, Rosebrough, Farr, & Randall, 1951). TCA soluble protein was expressed as mg tyrosine equivalent (TE) per g of sample.

**ACE-inhibitory assay**

The ACE-inhibitory activity was estimated using the method described by Cashman and Cheung (1971). ACE solution (25 µL of 250 mU/mL) was added to an equal volume of extract (10 mg/mL) and incubated at 37 °C for 10 min. Then, 75 µL of hippuryl-l-histidyl-l-leucine (HHL) solution (8.3 mM HHL in 50 mM HEPES buffer containing 0.5 M NaCl, pH 8.3) was added to the mixture and incubated at 37 °C for 45 min. The enzymatic reaction was terminated by the addition of 125 µL of 1 M HCl. Hippuric acid (HA) was extracted by the addition of 1 mL ethyl acetate. The mixture was vortexed vigorously and then centrifuged for 5 min at 5000 g. The supernatant was collected, and 700 µL of this was transferred to a test tube for evaporation of ethyl acetate at 80 °C. HA obtained was then dissolved in 1 mL Milli Q water, and the absorbance was measured at 228 nm using a spectrophotometer. ACE-inhibitor captopril (10 ng/mL) was used as a positive control. The percentage of ACE-inhibition was calculated using the formula mentioned below.

\[
ACE - inhibition(\%) = \left( \frac{(A - B) - (C - D)}{A - B} \right) \times 100
\]

where A is the absorbance in the presence of enzyme and absence of extract, B is the absorbance in the absence of both enzyme and extract, C is the absorbance in the presence of both enzyme and extract, whereas D is the absorbance in the absence of enzyme and presence of extract.

**Antioxidant activities**

The undigested and simulated in vitro GI digested aqueous extracts of cow, and yak hard chhurpi cheese was analyzed for DPPH radical scavenging activity, superoxide radical scavenging activity, reducing power potential activity, and total antioxidant activity. All the experiments were performed in triplicates.

**DPPH radical scavenging activity**

DPPH radical scavenging activity was determined using the method described by Rai et al. (2011). It was estimated by adding 2 mL of 0.16 mM DPPH solution to 200 µL of extracts and incubating in the dark at room temperature for 30 min. After incubation, the absorbance was measured using Shimadzu UV-1800 spectrophotometer at 517 nm. The scavenging effect was calculated using the formula mentioned below:

\[
\text{Scavenging effect}(\%) = \left( \frac{C_{abs} - B_{abs}}{S_{abs}} \right) \times 100
\]

where \(S_{abs}\) is the absorbance of samples, \(B_{abs}\) is the absorbance of sample blank containing 200 µL sample and 2 mL methanol, and \(C_{abs}\) is the absorbance of control containing 200 µL methanol and 2 mL DPPH.
solution. The DPPH radical scavenging activity was expressed as mg ascorbic acid equivalent (AAE) per g of the hard *chhurpi* sample.

**Superoxide radical scavenging activity**

Superoxide radical scavenging activity was determined using the method described by Rai et al. (2011). In 200 µL of extract 1.8 mL of phosphate buffer (50 mM, pH 8.2) was added, followed by the addition of freshly prepared 3 mM pyrogallol dissolved in 10 mM HCl. At times 0 min and 10 min, the absorbance of superoxide radical scavenging activity was measured at 325 nm using a spectrophotometer. The ability to scavenge the superoxide radical was estimated by using the formula below:

\[
\text{Scavenging effect} (%) = \left[1 - \frac{(S_{10} - S_0)}{(C_{10} - C_0)}\right] \times 100
\]

where \(S_0\) and \(S_{10}\) are the absorbances of the sample at time 0 min and 10 min, whereas \(C_0\) and \(C_{10}\) are the absorbances of control (200 µL distilled water and 2 mL 50 mM phosphate buffer with pH 8.2) at time 0 min and 10 min, respectively. The superoxide radical scavenging activity was expressed as mg ascorbic acid equivalent (AAE) per g of the hard *chhurpi* sample.

**Reducing power potential**

To determine the reducing power potential activity, the method described by Rai et al. (2011) was followed. It was estimated by adding 900 µl of 0.2 M phosphate buffer with pH 6.6, and 900 µl of freshly prepared 1% potassium ferric cyanide to 100 µl of extract. After mixing it thoroughly, the solution was incubated for 20 min at 50 °C, after mixing it thoroughly. After incubation, 900 µl of 10% TCA was added, mixed and centrifuged for 10 min at 8000g. The supernatant was (900 µl) was mixed with an equal volume of distilled water and freshly prepared 0.1% FeCl₃ solution. The absorbance of reducing activity was measured at 700 nm using a spectrophotometer. The reducing power potential activity was expressed as mg ascorbic acid equivalent (AAE) per g of the hard *chhurpi* sample.

**Total antioxidant activity**

The method described by Rai et al. (2009) was followed to determine the total antioxidant activity. Initially, 3 mL reagent solution (0.6 M sulphuric acid: 28 mM sodium phosphate: 4 mM ammonium molybdate – 1:1:1) was added to 200 µL extract. The reaction mixture was incubated in a water bath for 90 min at 95 °C, and then cooled down to room temperature. The absorbance of total antioxidant activity was measured at 695 nm using a spectrophotometer. The total antioxidant activity was expressed as mg ascorbic acid equivalent (AAE) per g of the hard *chhurpi* sample.

**Peptidomics analysis**

The aqueous extracts of undigested and GI digested hard *chhurpi* samples were freeze-dried (Labconco, Kansas, USA) prior to LC–MS/MS analysis. The lyophilized powder was dissolved in 7 M urea, followed by sonication for 15 min. A molecular filter with 3 kDa cut off was used to filter 200 µL of the sample, and the filtrate was centrifuged using an Amicon® Ultra-0.5 centrifugal filter unit (UFC200324) at 14, 000 g for 30 min. EASY nLC 1200 and an Orbitrap Fusion MS, coupled with a Nano-flow liquid chromatographic system (Thermo Fisher Scientific), were used in a mass scan range of 375–1500 (m/z) for the LC-MS/MS analysis. About 10 µL sample, with 0.1% formic acid in water (solvent A), and 80% acetonitrile and 0.1% formic acid in water (solvent B), was injected into an EASY SPRAY PEPPMAP RSLC C18 (3 µm; 50 cm × 75 µm; 100 Å) analytical column, and run at a constant flow rate of 300 nL/min for 60 min. Analysis was done with a gradual increase in solvent B from 2% to 20% in 2 min, to 55% in 37 min, to 95% in 50 min, and finally, the flow was held at 2% solvent B for an additional 10 min. The most abundant ions, along with ten times charged precursor ions from the survey scan, were chosen for MS data. The RAW files were analyzed using Mascot daemon v 2.6.2 (Matrix Science, UK) against the reference proteome database UNIPROT Bos taurus, with the ions score cut off of 43. The peptide mass and fragment mass limitations were set to 10 ppm and 0.6 Da for the database search, respectively (Albenzio et al., 2015).

**Identification of bioactive peptides**

Peptide sequences obtained from the LC-MS/MS analysis of the selected samples were subjected to screening for the identification of bioactive peptides. The peptide sequences were searched using web server MBPDB (Nielsen, Beverly, Qu, & Dallas, 2017) and BIOPEP-UWM (Minkiewicz, Iwania, & Darewicz, 2019). These databases contain all the potentially bioactive peptide sequences, which have been reported in various food sources.

**In silico prediction of antihypertensive peptides**

Different bioactivity prediction servers, such as mAHTPred (Manavalan, Basith, Shin, Wei, & Lee, 2019) and AHTpin (Kumar et al., 2015), were used to predict the antihypertensive peptide sequences obtained from the LC-MS/MS analysis of the selected samples. Their amino acid composition and physicochemical properties were used for prediction. The peptides with optimal probability (≥0.99) of being antihypertensive were chosen for further study. The web server ProtParam (Gasteiger et al., 2005) was used to calculate physicochemical properties, grand average of hydropathy, molecular weight, and isoelectric point of the predicted antihypertensive peptides.

**Molecular docking**

In *silico* molecular docking of the predicted peptides was done with ACE. The non-bond interactions between the peptideyl residues and the catalytic amino acids of ACE were screened. The PEPstrMOD webserver was used for the construction of 3D structures of the predicted peptides (Singh et al., 2015). The X-ray crystallographic 3D structure of human ACE was retrieved from the protein data bank (PDB ID: 1O8A). The structure file of the candidate peptides and the ACE enzyme was imported to Discovery Studio Visualizer (Dassault Systèmes, France). The peptide and protein structures were prepared by manually deleting all water molecules, hydrogen atoms, and heteroatoms and typed with the CHARMM forcefield. Molecular docking was performed using ZDOCK 3.0.2, a rigid-body protein–protein docking algorithm based on the use of fast Fourier transforms (FFTs) (Pierce et al., 2014). ZDOCK 3.0.2 uses interface atomic contact energy (IFACE) to obtain scoring functions that include electrostatics, shape complementarity, and a pairwise atomic statistical potential. It is developed using the contact propensities of transient protein complexes that result in a highly improved predictive ability (Pierce, Hourai, Weng, & Keskin, 2011). The top 10 docked conformation predictions according to the default ZDOCK scores were retained for further assessment. Non-bond interactions including hydrogen bonds, hydrophobic interactions and binding affinity of the docked complexes were examined using Discovery Studio Visualizer.

**Statistical analysis**

All the experiments were performed in triplicates (n = 3), and results were expressed as mean ± standard deviation. Tukey’s test for one-way analysis of variance (ANOVA) was performed with a confidence of 95% (P < 0.05) using the Minitab 19 statistical software (State College-PA, USA).
Results and discussion

Hydrolysed protein content and ACE-inhibitory activity of hard chhurpi cheese

Yak milk is gaining popularity among consumers due to its high-quality nutrition and functional properties, including anti-hypertension, anti-diabetes, and anticancer properties (Wang et al., 2020). Recent studies have demonstrated that peptides from yak milk casein have anti-inflammatory, free radicals scavenging, zinc-binding, and angiotensin I-converting enzyme (ACE) inhibitory properties (Lin et al., 2018). Natural fermentation of milk during cheese production involves the hydrolysis of milk proteins by a wide variety of proteolytic enzymes. The proteolysis results in the liberation of small peptides with diverse functional properties (Chourasia et al., 2021). A higher hydrolyzed protein content of 0.299 ± 0.052 mg TE/g sample was observed for yak hard chhurpi than cow hard chhurpi (0.246 ± 0.074 mg TE/g sample) (Table 1).

The health beneficial effects of milk-based fermented foods are truly realized after the digestion of the food product. Upon consumption, proteolytic enzymes of the gastrointestinal (GI) tract, including pepsin in the stomach, and trypsin and chymotrypsin (pancreatic digestive enzymes) in the small intestine, further hydrolyze the food proteins and peptides into smaller fragments, which may result in the increase or decrease in the health-beneficial effect of the consumed food (Chourasia et al., 2021). Thus, it is necessary to evaluate the effect of GI digestion on the health-beneficial function of the peptide enriched fermented foods (Chourasia et al., 2022). Simulated GI digestion by using pepsin and pancreatin, increased hydrolyzed protein content of both yak and cow hard chhurpi cheese. A significant increase in hydrolyzed protein content was observed after pepsin digestion of yak hard chhurpi (11.378 ± 0.070 mg TE/g sample), as compared to cow hard chhurpi (8.004 ± 0.241 mg TE/g sample). Further digestion by pancreatin resulted in a slight increase in protein hydrolysis with similar hydrolyzed protein content in both yak and cow hard chhurpi (Table 1). After consumption, the bioactivity of a functional food can vary significantly due to the hydrolysis of proteins and peptides catalyzed by proteolytic enzymes of the GI tract (Xue, Yin, Howell, & Zhang, 2021). Generation of new bioactive peptides by further proteolysis during GI digestion and sustenance of bioactive peptides resistant to GI enzymes result in enhanced functionality of the food after consumption (Sanjukta et al., 2015). However, hydrolysis of bioactive peptides into inactive fragments during digestion can lead to a decreased health beneficial effect of the functional food (Chourasia et al., 2021).

Captopril is a chemically synthesized 1-proline derivative and is widely used in the therapy of hypertension (Memarpoor-Yazdi, Zare-Zardini, Mogharrab, & Navapour, 2020). About 73.65% ACE-inhibition was observed for the positive control captopril at the concentration of 10 ng/ml. Fermented milk products have been reported to contain a diverse range of bioactive peptides that exert several functional properties (Chourasia et al., 2020). ACE-inhibitory peptides are highly desirable in fermented foods to limit hypertension, which is strongly linked to coronary heart disease, stroke, and diabetes (Rai et al., 2017). In the present study, a significantly higher (P < 0.05) ACE-inhibitory activity was observed for undigested yak hard chhurpi (10.610 ± 0.328 %), as compared to undigested cow hard chhurpi (8.771 ± 0.447 %), demonstrating a superior hypotensive property of undigested yak hard chhurpi. ACE-inhibition by both yak and cow hard chhurpi was significantly lower (P < 0.05) than the activity expressed by captopril; however, the lack of side effects of bioactive peptides promotes ACE-inhibitory peptide enriched foods as hypertension preventive functional food products. Traditional Tibetan fermented yak milk products, Chula (acid curd cheese) and Kurut (fermented yak milk) have previously been reported to exert antihypertensive effects due to the release of ACE-inhibitory peptides (Jiang, Chen, Ren, Luo, & Zeng, 2007). Simulated GI digestion increased the ACE-inhibitory activity of both yak and cow hard chhurpi, indicating further release of ACE-inhibitory peptides upon proteolysis by GI enzymes (Table 1). The ACE-inhibitory activity of the hard chhurpi samples during in vitro GI digestion was closely related to an increase in hydrolyzed protein content at different digestion stages. After digestion by pepsin, the hydrolyzed protein content of yak hard chhurpi increased to 11.378 ± 0.070 mgTE/g from 0.299 ± 0.052 mgTE/g, while the hydrolyzed protein content of cow hard chhurpi increased from 0.246 to 8.004 mgTE/g. This was complemented by higher ACE-inhibitory activity of yak hard chhurpi pepsin digest (42.677 ± 1.558 %), as compared to cow hard chhurpi pepsin digest (40.916 ± 1.683 %) (Table 1). However, an increase in proteolysis was observed for cow hard chhurpi upon further digestion by pancreatin. This resulted in a substantial increase in ACE-inhibitory activity of cow hard chhurpi, as compared to yak hard chhurpi (Table 1). After digestion with pepsin and pancreatin, the highest ACE-inhibitory activity of 60.281 ± 2.486 % was observed for cow hard chhurpi. A similar increase in the ACE-inhibitory activity of cheese upon proteolysis by GI enzymes has been reported by recent studies (Jiang et al., 2020). In vitro ACE-inhibitory activity assays are effectively used for the determination of potential antihypertensive properties of functional foods. However, potential in vivo physiological changes of peptides prevent the establishment of a proper relation between ACE-inhibition in vitro and the antihypertensive effect of the food in vivo (Xue et al., 2021). Due to the higher protein content of yak milk as compared to cow milk and the identification of ACE-inhibitory peptides in yak milk, yak milk-based fermented foods offer a higher potential of functional foods that could prevent hypertension. Besides, novel ACE-inhibitory peptides identified from fermented yak milk foods can be used in the development of nutraceuticals against hypertension and cardiovascular diseases.

| Table 1 | Hydrolysed protein content and ACE-inhibitory activity of undigested and GI digested yak and cow hard chhurpi cheese. |
|---------|----------------------------------------------------------------------------------------------------------|
| Hard Chhurpi types | In vitro GI digestion | Protein hydrolysis (mg TE/g sample) | ACE-inhibitory activity (%) |
|---------|----------------------------------------------------------------------------------------------------------|
| Yak hard chhurpi | Undigested | 0.299 ± 0.052 | 10.610 ± 0.328 |
|         | Pepsin digest | 11.378 ± 0.070 | 42.677 ± 1.558 |
|         | Pancreatin digest | 11.752 ± 0.123 | 49.861 ± 1.11 |
| Cow hard chhurpi | Undigested | 0.246 ± 0.074 | 8.771 ± 0.447 |
|         | Pepsin digest | 8.004 ± 0.241 | 40.916 ± 1.683 |
|         | Pancreatin digest | 8.104 ± 0.101 | 60.281 ± 2.486 |

Superscript letters mean values for hard chhurpi at the same digestion stage for the same activity without common letters are significantly different (P < 0.05) (n = 3). GI = Gastrointestinal; TE = tyrosine equivalent; ACE = Angiotensin-I converting enzyme.

Antioxidant effect of undigested and GI digested yak and cow hard chhurpi cheese

Antioxidant activity of foods has received significant attention in functional food research due to its association with other health-beneficial properties, e.g. antihypertensive and anticancer activities (Tadesse & Emire, 2020). Antioxidant peptides mitigate oxidative stress caused by free radicals generated during oxidation reactions by inactivating reactive oxygen species, hydroperoxide reduction, chelating oxidative metals, and scavenging free radicals (Sanjukta et al., 2015). Higher DPH radical scavenging (0.162 ± 0.012 mg AAE/g), superoxide radical scavenging activity (0.330 ± 0.012 mg AAE/g), reducing power potential (0.272 ± 0.016 mg AAE/g), and total antioxidant activity (0.666 ± 0.029 mg AAE/g) were recorded for cow hard chhurpi as compared to yak hard chhurpi (Table 2). Simulated GI digestion by pepsin and pancreatin increased antioxidant activity of both cow and yak hard chhurpi, suggesting the further release of antioxidant peptides.
upon proteolysis by GI enzymes (Table 2). A recent study has reported a similar increase in antioxidant activity after simulated in vitro GI digestion of soft chhurpi cheese by controlled fermentation (Chourasia et al., 2022). The increase in antioxidant activity of both soft chhurpi and hard chhurpi, after simulated in vitro GI digestion, suggests that the chhurpi cheese product is a potential functional food containing bioactive peptides and their precursors that can help in the prevention of several diseases upon consumption. Upon pepsin treatment of an undigested sample of cow hard chhurpi, the DPPH radical scavenging activity was increased from 0.162 ± 0.012 mg AAE/ g to 0.462 ± 0.019 mg AAE/ g. Its subsequent digestion by pancreatin further increased the DPPH radical scavenging activity to 0.479 ± 0.015 mg AAE/ g. Similarly, DPPH radical scavenging activity of undigested samples of yak hard chhurpi was increased from 0.084 ± 0.013 mg AAE/ g to 0.463 ± 0.015 mg AAE/ g, after treatment by pepsin and pancreatin. A similar increase in superoxide radical scavenging activity, reducing power potential and total antioxidant activity, has been observed in GI digests of cow and yak hard chhurpi (Table 2). The antioxidant peptides released during hard chhurpi production and its simulated GI digestion could potentially exert other bioactive properties, thereby increasing the health beneficial effects of yak and cow hard chhurpi upon consumption.

### Table 2

Antioxidant activity of undigested and GI digested yak and cow hard chhurpi cheese.

| Hard Chhurpi types | In vitro GI digestion | DPPH radical scavenging activity (mg AAE/ g sample) | Superoxide radical scavenging activity (mg AAE/ g sample) | Reducing power potential (mg AAE/ g sample) | Total antioxidant activity (mg AAE/ g sample) |
|-------------------|-----------------------|------------------------------------------------------|----------------------------------------------------------|--------------------------------------------|--------------------------------------------|
| Yak hard chhurpi   | Undigested            | 0.084 ± 0.013<sup>b</sup>                           | 0.175 ± 0.004<sup>b</sup>                               | 0.225 ± 0.025<sup>b</sup>                  | 0.662 ± 0.028<sup>b</sup>                 |
|                   | Pepsin digest         | 0.382 ± 0.026<sup>b</sup>                           | 1.030 ± 0.008<sup>b</sup>                               | 0.875 ± 0.031<sup>b</sup>                  | 2.845 ± 0.071<sup>b</sup>                |
|                   | Pancreatin digest     | 0.463 ± 0.015<sup>b</sup>                           | 1.300 ± 0.030<sup>b</sup>                               | 2.075 ± 0.037<sup>b</sup>                  | 3.345 ± 0.075<sup>b</sup>                |
| Cow hard chhurpi   | Undigested            | 0.162 ± 0.012<sup>a</sup>                           | 0.330 ± 0.012<sup>a</sup>                               | 0.272 ± 0.016<sup>a</sup>                  | 0.666 ± 0.029<sup>a</sup>                |
|                   | Pepsin digest         | 0.462 ± 0.019<sup>a</sup>                           | 0.750 ± 0.010<sup>a</sup>                               | 0.857 ± 0.046<sup>a</sup>                  | 2.991 ± 0.047<sup>a</sup>                |
|                   | Pancreatin digest     | 0.479 ± 0.015<sup>a</sup>                           | 1.000 ± 0.005<sup>a</sup>                               | 1.186 ± 0.041<sup>a</sup>                  | 3.578 ± 0.030<sup>a</sup>                |

Superscript letters mean values for hard chhurpi at the same digestion stage for the same activity without common letters are significantly different (P < 0.05) (n = 3).

GI = Gastrointestinal; DPPH = 2,2-diphenyl-1-picrylhydrazyl; AAE = ascorbic acid equivalent.

Peptidomics of undigested and simulated GI digested yak and cow hard chhurpi

LC-MS/MS-based peptidomics of the undigested and GI digested cow, and yak hard chhurpi samples were performed for the identification of bioactive peptides (Supplementary Fig. S2). A total of 499, 603, 767, and 568 non-redundant peptides were identified in undigested cow hard chhurpi, GI digested cow hard chhurpi, undigested yak hard chhurpi, and GI digested yak hard chhurpi, respectively. β-Casein was the primary source protein for the peptides identified in all the samples, followed by α-S1-casein, and the whey protein, β-lactoglobulin (Fig. 1A). Several peptides were commonly identified in undigested and digested hard chhurpi.

![Fig. 1. Distribution and Venn diagram of peptides identified from undigested and GI digested yak and cow hard chhurpi. CN = casein; LA = lactalbumin; LG = lactoglobulin; BSA = bovine serum albumin; CUNH = Undigested cow chhurpi; CH = GI digested cow chhurpi; YUNH = Undigested yak chhurpi; YH = GI digested yak chhurpi. A: Distribution of total and bioactive peptides based on source protein; Venn diagram, B: Total peptides; C: Bioactive peptides; and D: ACE-inhibitory peptides.](image-url)
The presence of such common peptides in chhurpi produced using different milk types indicates that specific protein sites, targeted by proteolytic enzymes, are shared by different microorganisms during milk fermentation. Conversely, unique peptides were also identified specific to the GI digest stage of the hard yak hard chhurpi, and GI digested yak hard chhurpi, respectively. The presence of unique peptides in different digestion stages of hard chhurpi products, prepared by using different milk types, suggests that the production of certain bioactive and precursor peptides is based on specific protein substrates, specific milk proteases, and specific proteolytic mechanisms of the fermenting starter strains (Nandan & Nampoothiri, 2020). Raw milk contains several protease enzymes, including plasmin, plasminogen, and aminopeptidases, which upon activation, release fragments equipped with bioactive properties (Nielson et al., 2017). These bioactive peptides can be consistent in raw milk and raw milk cheese products. However, the chhurpi cheese production process includes the milk boiling stage that considerably inactivates the major milk proteases, plasmin and plasminogen (Leite et al., 2021; Panda et al., 2016). This ensures that the major concentration of bioactive peptides identified in the chhurpi cheese product is the result of milk proteolysis by starter microorganisms.

Bioactivity search in selected databases and available literature revealed the presence of a total of 60 bioactive peptides in the 4 chhurpi samples. Among these, 38, 33, 45, and 30 bioactive peptides were identified in undigested cow hard chhurpi, GI digested cow hard chhurpi, undigested yak hard chhurpi, and GI digested yak hard chhurpi, respectively. Proteolytic hydrolysis of bioactive peptides by GI enzymes during digestion can result in extensive peptide transformations, leading to changes in peptide bioavailability and ultimately the bioactivity of the functional food (Xue et al., 2021). However, the identification of bioactive peptides in GI digested cow, and yak hard chhurpi indicates the sustenance of functionality of the chhurpi products. The bioactivities exerted by the selected biopeptides include ACE-inhibitory, antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, immunomodulatory, and anxiolytic activities (Table 3). The source protein distribution of the identified bioactive peptides in all the samples was comparable to that of the total identified peptides derived from β-casein, followed by α-S1-casein and β-lactoglobulin (Fig. 1A). Among the identified bioactive peptides, a total of 17 peptides were common to undigested and digested samples of both cow and yak hard chhurpi (Fig. 1C). A total of 22 peptides were common to undigested, and GI digested cow hard chhurpi, and 23 peptides were common to undigested, and GI digested yak hard chhurpi. These peptides had escaped GI digestion and are of significance for the development of nutraceuticals. Unique bioactive peptides,

| Peptide Sequence | Source Protein | m/z | Bioactivity | Protein accession | Samples |
|------------------|----------------|-----|-------------|------------------|---------|
| SDPNPGESENKEK    | α-S1-casein    | 743.8549 | Antimicrobial | P02662 | CUNH, CH, YUNH, YH |
| TPEVDDEALEK      | β-lactoglobulin | 623.296 | DPP-IV inhibitory | P02754 |
| PVPVFFPLQFR     | β-casein       | 611.3476 | Antimicrobial | P02666 |
| YYVEELKPEQGDEILQK| β-lactoglobulin | 1157.133 | Hypcholesterolemic | P02754 |
| LLYQHPLQFR     | β-casein       | 692.4035 | ACE-inhibitory | P02666 |
| VLYQVQLQFQ     | α-S1-casein    | 634.5654 | Antioxidant | P02662 |
| YKVQLEVPVPAEER   | α-S1-casein    | 936.4971 | Promote calcium uptake | P02662 |
| DMINPQAFLQYPFHP   | β-casein       | 729.3944 | Anti-inflammatory | P02662 |
| FVAPPEFVFK      | α-S1-casein    | 692.8686 | ACE-inhibitory | P02662 |
| YVFFPapeh      | PEP inhibitor  | 513.7742 | Antiamnestic | J9U164 |
| ALNEINEQFQ     | α-S2-casein    | 684.3513 | ACE-inhibitory | P02663 |
| FOSEEQQETDELODK | β-casein       | 991.4345 | Promote calcium uptake | P02666 |
| DIGSESDQAMEDIK  | α-S1-casein    | 884.3829 | Promote calcium uptake | P02662 |
| LHLPLPL        | β-casein       | 401.7629 | ACE-inhibitory | P02666 | CUNH, CH, YH |
| AYDSILLDQAQPRL | β-casein       | 814.4356 | Antimicrobial | P02754 |
| FVVPFFEPVG   | α-S1-casein    | 555.2871 | ACE-inhibitory | P02662 | CUNH, YUNH, YH |
| LVYPFPFP     | β-casein       | 501.7868 | ACE-inhibitory | P02666 |
| LLYQPHPLVPFPVRPFP | β-casein     | 1054.1187 | ACE-inhibitory | P02666 |
| YQEPVLPVPFPPF  | β-casein       | 834.5953 | Antimicrobial | P02666 |
| NLIPLPLL       | β-casein       | 515.3266 | ACE-inhibitory | P02666 |
| YLEQLLR       | α-S1-casein    | 467.7714 | Antimicrobial | P02666 |
| SWMIHQHLQPFPDPT | β-casein       | 778.3777 | Antioxidant | P02666 |
| PFPVAPFVG      | α-S1-casein    | 660.4776 | ACE-inhibitory | P02662 |
| IVLNPQDQVK     | β-casein       | 606.3425 | Antimicrobial | P02663 |
| RDMPQAF       | β-casein       | 489.2475 | ACE-inhibitory | P02666 |
| VIESSPERNFVQ  | α-casein       | 663.5351 | Antioxidant | P02668 |
| VLNELRUR    | α-S1-casein    | 485.7876 | Antimicrobial | P02662 |
| VLPVQKAVYPPQOR | β-casein       | 531.3154 | Antimicrobial | P02666 |
| SLAMASDSSL     | β-lactoglobulin | 596.3183 | Antimicrobial | O77777 | CH, YUNH |
| FSQSEEOQETDELODK | β-casein     | 826.0476 | Promote calcium uptake | P02666 | CH, YH |
| TDELODKHIP     | β-casein       | 491.2404 | Antimicrobial | P02668 |
| YVEELRTFQEDGL | β-casein       | 745.375 | Antioxidant | BSBD04 |
| NMAINSK       | α-S2-casein    | 437.7262 | ACE-inhibitory | P02663 |
| YLYEIRAR       | Serum albumin  | 464.2501 | ACE-inhibitory | AA1407897 | CUNH |
| LYPVEVPLVGRPPFP | β-casein       | 997.5774 | Immunomodulatory | P02666 |
| KLVVPDKQ      | β-casein       | 454.8 | Antioxidant | P02662 |
| KHQLQPEVLNELL | α-S1-casein    | 577.9842 | Antioxidant | P02662 |
| APSSFDNPPGENSE  | α-S1-casein    | 880.9027 | Antioxidant | P02662 |
| VRGPPFIP      | β-casein       | 499.3134 | ACE-inhibitory | P02666 |
| LQPNPPLT      | β-casein       | 496.7925 | DPP-IV inhibitory | P02666 |
| LVVFPPFIP      | β-casein       | 550.3133 | ACE-inhibitory | P02666 |
| LIVYYTMK      | β-lactoglobulin | 467.2756 | Cytotoxic | BSBD04 |

CUNH - Undigested cow chhurpi; CH- GI digested cow chhurpi; YUNH - Undigested yak chhurpi; YH- GI digested yak chhurpi, m/z = peptide mass by charge ratio; ACE = angiotensin-I converting enzyme.
specific to a particular sample, included 3, 3, 8, and 1 peptide in undigested cow hard chhurpi, GI digested cow hard chhurpi, undigested yak hard chhurpi, and GI digested yak hard chhurpi, respectively.

The most common bioactive peptides identified in fermented milk products are the ACE-inhibitory peptides (Rai, Sanjukta, & Jeyaram, 2017). Yak milk casein has been considered as a functional ingredient, with studies reporting the release of ACE-inhibitory peptides upon hydrolysis of yak milk by fermentation or commercial proteases (Jiang et al., 2020). The presence of aromatic (His, Phe, Trp, and Tyr) and hydrophobic (Ala, Val, Leu, Ile, and Gln) amino acids are associated with the expression of antioxidant and ACE-inhibitory properties of bioactive peptides (Sanjukta et al., 2021). Peptide conformations and the presence of specific amino acids can render a multifunctional peptide. Multifunctional peptides are preferred over single-activity peptides as these peptides can simulate or inhibit multiple physiological pathways simultaneously, thereby aiding in the prevention of several related diseases (Aguilar-Toalá et al., 2017). A total of 18 multifunctional peptides were identified in undigested and GI digested cow and yak hard chhurpi cheese (Table 4). Six multifunctional peptides were common to undigested and digested samples of both cow and yak hard chhurpi. Combined ACE-inhibitory, antidiabetic and antioxidant activities were reported to be exerted by the majority of the identified multifunctional bioactive peptides (Table 4). The highly potent β-casein-derived peptide, YQEPVLGPVRGPFPIIV, previously reported to express ACE-inhibitory, antioxidant, immunomodulatory, antithrombotic, and anti-inflammatory activity (Sowmya, Bhat, Bajaj, Kapila, & Kapila, 2019), was identified in undigested and GI digested samples of both cow and yak hard chhurpi.

In silico prediction of antihypertensive property and molecular docking of identified peptides

Two web-based softwares were used for the prediction of antihypertensive peptides identified in undigested and GI digested yak and cow hard chhurpi cheese.

| Peptide Sequence | Source Protein | m/z | Bioactivity | Protein Accession | Samples |
|------------------|----------------|-----|-------------|-------------------|---------|
| YQEPVLGPVRGPFPIIV | β-casein | 532.2949 | Anti-inflammatory / ACE-inhibitory | P02754 | CH, YH |
| YQEPVLGPVR | β-casein | 626.3583 | Opioid / C3a Receptors agonist | P02668 | CH, YH |
| FALPQYLK | α-S2-casein | 490.2845 | ACE-inhibitory / Antioxidant | P02663 | |
| YQEPVLGPVR | β-casein | 617.6506 | ACE-inhibitory / Antioxidant | P02666 | |
| YQEPVLGPVR | β-casein | 579.3195 | ACE-inhibitory / Immunomodulatory, Antithrombotic / Antioxidant / Anti-inflammatory | P02666 | |
| LYQEPVLGPVR | β-casein | 635.8615 | ACE-inhibitory / Antioxidant | P02666 | |
| IDALNENK | β-lactoglobulin | 458.7404 | Stimulates proliferation / Antimicrobial | P02754 | CH, YUNH, YH |
| VGINYWLAHK | β-lactoglobulin | 600.83 | ACE-inhibitory / DPP-IV inhibitory | P00711 | CH, YUNH, YH |
| VLPVPPK | β-casein | 390.7525 | ACE-inhibitory / DPP-IV inhibitory | P02666 | CH, YUNH, YH |
| RELEELNVGVEISLSSSEESIR | β-casein | 934.8048 | Caseinophosphopeptide / Immunomodulatory / Promotes calcium uptake | P02666 | |
| VENLHPFLFL | β-casein | 629.3825 | ACE-inhibitory / Anticancer | P02666 | CH, YH |
| IPIQGPTVP | β-casein | 483.287 | ACE-inhibitory / DPP-IV inhibitory | P02666 | CH |
| VFPPGFI | β-casein | 445.2448 | PEP-inhibitory / ACE-inhibitory | P02666 | CH |
| WMHQHIPPLPTV | β-casein | 734.8614 | ACE-inhibitory / ACE-inhibitory | P02666 | |
| FVPPGOFN | β-casein | 550.7923 | ACE-inhibitory / ACE-inhibitory | P02666 | CH |
| AVPPQOR | β-casein | 415.7297 | ACE-inhibitory / ACE-inhibitory | P02666 | YUNH |
| YQEPVLGPVRGPFPIIV | β-casein | 941.0368 | ACE-inhibitory / ACE-inhibitory | P02666 | |
| LYYWPVWTR | Hemorphin-9 | 581.3244 | ACE-inhibitory / ACE-inhibitory | E1B976 | YH |

CUNH - Undigested cow chhurpi; CH - GI digested cow chhurpi; YUNH - Undigested yak chhurpi; YH - GI digested yak chhurpi; m/z = peptide mass by charge ratio; ACE = angiotensin-I converting enzyme.
ACE-inhibitory peptides identified in the present study. The β-casein derived multifunctional peptide LYQEPVLGPR, identified in undigested and digested samples of both cow and yak hard chhurpi, demonstrated strong non-bond interactions with hydrophobic residues of ACE. Conventional hydrogen bonds with Glu411 and Tyr523, alkyl hydrophobic interactions with His353, His383, and His513 were observed for residues of LYQEPVLGPR (Fig. 2A, 2B), supporting the in vitro ACE-inhibition observed for the hard chhurpi samples.

A total of 20 antihypertensive predicted peptides demonstrated non-bond interactions with catalytic amino acids of ACE (Supplementary Table S2). All 20 peptides were predicted as non-toxic by the web-based tool ToxinPred (Gupta et al., 2013). Among the predicted peptides demonstrating interactions with the catalytic residues of ACE, three peptides, FFVAPFPEVFG, NQFLPYPY, and SLVYPFPGPIPN, were identified in undigested and GI digested samples of both cow and yak hard chhurpi. Similarly, the β-casein derived peptide, SLVYPFP GPIPN, interacted strongly with active site residues of ACE with a ZDOCK score of 1412.254. A 10-residue long derivative, SLVYPFP GPI, identified in GI digested cow hard chhurpi, and undigested and GI digested samples of yak hard chhurpi, showed a stronger binding affinity for ACE than SLVYPFP GPIPN, with a ZDOCK score of 1546.843 (Table S2). Non-bond interactions were observed for SLVYPFP GPI with ACE catalytic residues, including Gln281, His353, Ala354, His387, and His513 (Fig. 2C, D). Molecular docking study of two novel peptides, VLPVPQ and VAPFPE, released by the action of proteases on bovine milk, demonstrated strong non-bond interactions with S1 and S2 pockets of ACE, suggesting the use of such peptides for the development of antihypertensive nutraceuticals and functional foods (Chen, Shangguan, Bao, Shu, & Chen, 2020). Studies have reported that ACE-inhibitory activity of peptides is closely related to the presence of hydrophobic amino acids such as proline, isoleucine, leucine, and tryptophan (Sanjukta et al., 2021). Further, the presence of proline at the C-terminal end highly enhances the ACE-inhibitory potential of a peptide by increasing the hydrophobic interaction at the ACE catalytic sites (Chourasia et al., 2021). Strong conventional hydrogen bonds were observed between SLVYPFP GPI residues and catalytic S2 active site residues of ACE, including Gln281, His353, and His513 (Fig. 2C, D). Similar interactions with ACE S2 catalytic residues have been reported for captopril, suggesting the high potential of SLVYPFP GPI as an ACE-inhibitory peptide. Binding of drug candidates and peptides at the catalytic pockets of ACE through non-bond interactions results in competitive inhibition of ACE activity. The non-bond interaction between ACE and inhibitor peptides prevents the binding of angiotensin I to ACE and the substrate hydrolysis to the vasoconstrictor angiotensin II (Chourasia et al., 2020). The peptides such as SLVYPFP GPI that escape hydrolysis by GI enzymes can maintain the antihypertensive property of the food, even after digestion, thus exerting health beneficial effects upon consumption (Rai, Sanjukta, & Jeyaram, 2017). However, further extensive studies including, in vivo antihypertensive analyses are necessary to validate further the functionality of these peptides and the hard chhurpi containing such peptides. ACE-inhibitory peptides resistant to GI digestion can be used for the development of nutraceuticals with functional properties.

**Conclusions**

Yak and cow milk-based naturally fermented hard chhurpi cheese are less explored traditional fermented food of the Sikkim Himalayan region. Research on the functional properties of the hard chhurpi cheese products can help in exploring them for the development of nutraceuticals and functional food products. Yak milk has attracted several food processors due to the higher content of proteins and overall nutritious value as compared to cow milk. Identifying novel peptides with enhanced ACE-inhibitory activity from yak milk-based fermented foods can bring us closer to developing natural nutraceuticals enriched with highly active ACE-inhibitory peptides. Virtual screening techniques such as molecular docking studies have made it convenient to screen...

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**Fig. 2.** Non-bond interactions between catalytic residues of ACE and peptides identified in yak and cow hard chhurpi. (A): Illustration showing molecular docking of the multifunctional bioactive peptide LYQEPVLGPR within catalytic cavity of ACE. (B): 2D diagram of LYQEPVLGPR-ACE interactions including hydrogen bonds and hydrophobic interactions. (C): Illustration showing molecular docking of the predicted peptide SLVYPFP GPI within catalytic cavity of ACE. (D): 2D diagram of SLVYPFP GPI-ACE interactions including hydrogen bonds and hydrophobic interactions.
potential bioactive peptides. The peptides selected in the present study can be studied in detail on synthesis and validation of ACE-inhibitory activity by in vitro and in vivo methods. Furthermore, the development of bioprocesses for the controlled milk fermentation using defined proteolytic starter strains is necessary to produce bioactive peptide enriched functional yak and cow hard chhupri cheese.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi.org/10.1016/j.fodchem.2022.100231.

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