Food Chemistry: X 13 (2022) 100231

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x

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

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ARTICLE INFO

Keywords:
Sikkim Himalaya
Hard *chhurpi*
Proteomics
Bioactive peptides
Antihypertensive
Molecular docking

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, 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 (KKS) systems. ACE cleaves two amino acids from angiotensin-I to form a vasoconstrictor 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 (Feroze, 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

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https://doi.org/10.1016/j.fochx.2022.100231
Received 23 October 2021; Received in revised form 8 January 2022; Accepted 24 January 2022
Available online 27 January 2022

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, *gheu/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 *churkam.* Due to the sweetness and chewiness properties, it is used for mastication (Panda et al., 2016). Selected traditional cheese products around the globe, including cheddar cheese, Festivo cheese, Crescenza, Mozzarella, Gorgonzola, Manchego, etc., have been reported to contain ACE inhibitory peptides (Rai et al., 2017). The present investigation illustrates the comparative analysis of the yak and cow hard *chhurpi*, subjected to simulated *in vitro* GI digestion, exploring the functional properties and bioactive properties therein. Studies on the identification of bioactive peptides in hard cheese products will add value and make them popular as functional food products of the Himalayan region. This is the first study on bioactive peptides present in yak and cow hard *chhurpi* (cheese) from the Himalayan region.

Materials and methods

Chemicals and reagents

2-Diphenyl-1-picrylhydrazyl (DPPH), ACE-inhibitor captopril, acetronitrile, Amicon® Ultra-0.5 centrifugal filter unit (UFC200324), ammonium molybdate, copper sulfate (Cu_2SO_4), ethyl acetate, ferric chloride (FeCl3), Folin–Ciocalteu reagent (FCR), formic acid, hippuric acid, hippuryl-L-histidyl-L-leucine solution, hydrochloric acid (HCl), methanol (CH_3OH), pancreatin, pepsin, potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium sodium tartrate tetrahydrate, pyrogallol, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate, sulphuric acid (H_2SO_4), trichloroacetic acid (TCA), tyrosine, and urea were procured from Sigma-Aldrich (St. Louis, MO, USA).

Collection of yak and cow hard chhurpi

Hard *chhurpi* of yak and cow milk were collected from the local market of Gangtok, Sikkim in sterile bags and stored in refrigerated condition (Supplementary Fig. S1). Aqueous extract of the hard *chhurpi* samples was prepared by crushing the samples into a fine powder and dissolving each sample individually in sterile distilled water in the ratio 1:10 (w/v), followed by periodic shaking at 180 rpm for an hour and centrifugation for 30 min at 10,000*g*. The supernatant was gently pipetted to collect only the peptide and protein fraction of the extract. The supernatant was stored at −20 °C for further analysis.

Gastrointestinal digestion of hard chhurpi

In vitro GI enzymes (pepsin and pancreatin) effect was studied on hard *chhurpi* samples, following the method described by Chourasia, 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 pepsin (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 centrifugated 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 hydrolysates. 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 Cushman 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 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 for 10 min. 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.

$$\text{ACE} - \text{inhibition}(\%) = [((A - B) - (C - D))/(A - B)] \times 100$$

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:

$$\text{Scavenging effect}(\%) = [1 - (S_{abs} - B_{abs})/C_{abs}] \times 100$$

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:

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

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 8000*g*. The supernatant was (900 μL) was mixed with an equal volume of distilled water and freshly prepared 0.1% $FeCl_3$ 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, Kansan, 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 PEPMAP 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, Iwaniak, & 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 peptidyl 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 tACE 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

Hydrolyzed 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 L-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, Qula (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 5), 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[a]	10.610 ± 0.328[a]
	Pepsin digest	11.378 ± 0.070[a]	42.677 ± 1.558[a]
	Pepsin + Pancreatin digest	11.752 ± 0.123[a]	49.861 ± 1.111[b]
Cow hard *chhurpi*	Undigested	0.246 ± 0.074[a]	8.771 ± 0.447[b]
	Pepsin digest	8.004 ± 0.241[b]	40.916 ± 1.683[a]
	Pepsin + Pancreatin digest	11.953 ± 0.101[a]	60.281 ± 2.486[a]

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 DPPH 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

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^{b}	0.175 ± 0.004^{b}	0.225 ± 0.025^{b}	0.662 ± 0.028^{a}
	Pepsin digest	0.382 ± 0.026^{b}	1.030 ± 0.008^{a}	0.875 ± 0.031^{a}	2.845 ± 0.071^{b}
	Pepsin + Pancreatin digest	0.463 ± 0.015^{a}	1.300 ± 0.030^{a}	2.075 ± 0.037^{a}	3.345 ± 0.075^{b}
Cow hard *chhurpi*	Undigested	0.162 ± 0.012^{a}	0.330 ± 0.012^{a}	0.272 ± 0.016^{a}	0.666 ± 0.029^{a}
	Pepsin digest	0.462 ± 0.019^{a}	0.750 ± 0.010^{b}	0.857 ± 0.046^{a}	2.991 ± 0.047^{a}
	Pepsin + Pancreatin digest	0.479 ± 0.015^{a}	1.000 ± 0.005^{b}	1.186 ± 0.041^{b}	3.578 ± 0.030^{a}

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.

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.

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

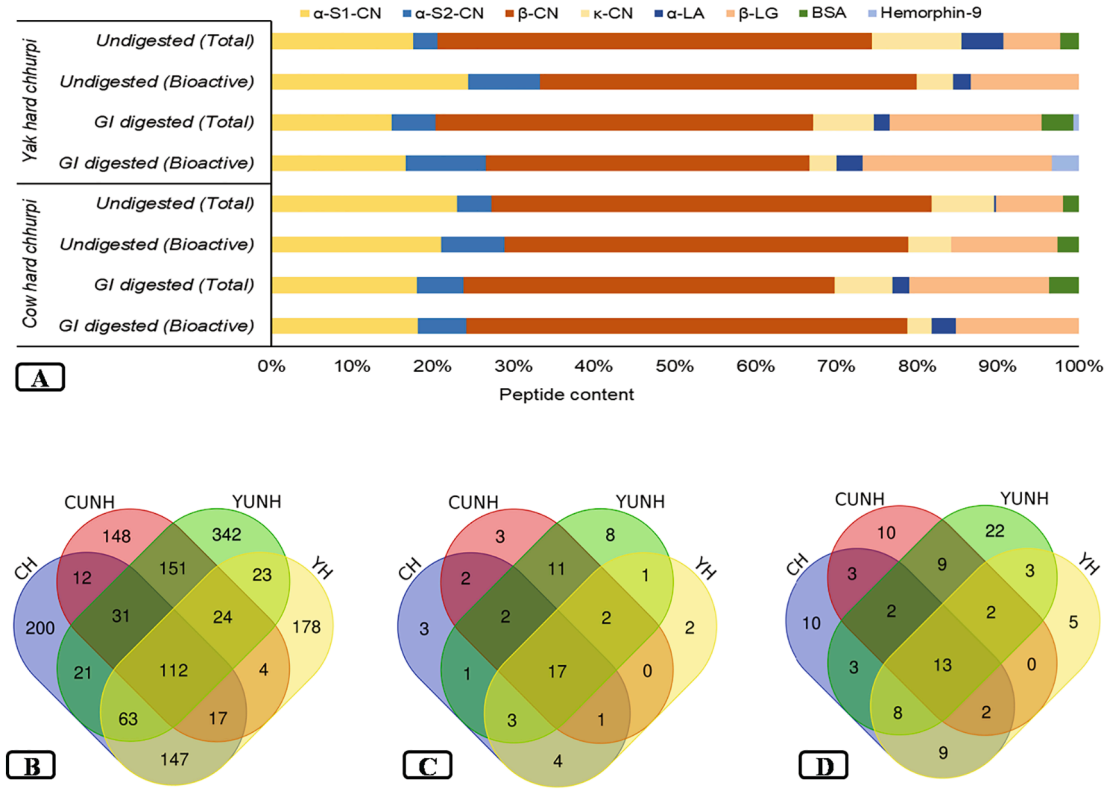

Fig. 1. Distribution and Venn diagram of peptides identified from undigested and GI digested yak and cow hard *chhurpi*. CN = casein; LA = lactalbumin; LG = lactoglobulin; BSA = bovine serum albumin; CUNH = Undigested cow *chhurpi*; CH = GI digested cow *chhurpi*; YUNH = Undigested yak *chhurpi;* YH = 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.

chhurpi samples, with 112 peptides commonly identified in undigested and digested samples of both cow and yak hard *chhurpi* (Fig. 1B). 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 (Chourasia et al., 2022). Conversely, unique peptides were also identified specific to the GI digest stage of the hard *chhurpi* samples. For example, 148, 200, 342, and 178 unique peptides were identified in undigested cow hard *chhurpi*, GI digested cow hard *chhurpi*, undigested 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 (Nielsen 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, antidiabetic, 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,

Table 3
Bioactive peptides identified in undigested and GI digested yak and cow hard *chhurpi* cheese.

Peptide Sequence	Source Protein	*m/z*	Bioactivity	Protein accession	Samples
SDIPNPIGSENSEK	α-S1-casein	743.8549	Antimicrbial	P02662	CUNH, CH, YUNH, YH
TPEVDDEALEK	β-lactoglobulin	623.296	DPP-IV inhibitory	P02754	
PVVVPPFLQPE	β-casein	611.3476	Antimicrobial	P02666	
VYVEELKPTPEGDLEILLQK	β-lactoglobulin	1157.133	Hypocholesterolemic	P02754	
LLYQEPVLGPVR	β-casein	692.4035	ACE-inhibitory	P02666	
YLGYLEQLLR	α-S1-casein	634.3564	Anxiolytic	P02662	
YKVPQLEIVPNSAEER	α-S1-casein	936.4971	Promote calcium uptake	P02662	
DMPIQAFLLYQEPVLGPVR	β-casein	729.3944	Anti-inflammatory	P02666	
FFVAPFPEVFGK	α-S1-casein	692.8686	ACE-inhibitory	P02662	
VYPFPGPIH	PEP inhibitor	513.7742	Antiamnestic	J9UHS4	
ALNEINQFYQK	α-S2-casein	684.3513	ACE-inhibitory	P02663	
FQSEEQQQTEDELQDK	β-casein	991.4345	Promote calcium uptake	P02666	CUNH, CH,YUNH
DIGSESTEDQAMEDIK	α-S1-casein	884.3829	Promote calcium uptake	P02662	
LHLPLPL	β-casein	401.7629	ACE-inhibitory	P02666	CUNH, CH, YH
AASDISLLDAQSAPLR	β-lactoglobulin	814.4356	Antimicrobial	P02754	CUNH, YUNH,YH
FVAPFPEVFG	α-S1-casein	555.2871	ACE-inhibitory	P02662	CH, YUNH, YH
LVYPFPGPI	β-casein	501.7868	ACE-inhibitory	P02666	
LLYQEPVLGPVRGPFPIIV	β-casein	1054.1187	ACE-inhibitory	P02666	CUNH, CH
YQEPVLGPVRGPFPI	β-casein	834.9593	Antimicrobial	P02666	
NLHLPLPLL	β-casein	515.3266	ACE-inhibitory	P02666	CUNH, YUNH
YLEQLLR	α-S1-casein	467.7714	Antimicrobial	P02662	
SWMHQPHQPLPPT	β-casein	778.3777	Antioxidant	P02666	
PFPEVFGK	α-S1-casein	460.7476	ACE-inhibitory	P02662	
IVLNPWDQVK	α-S2-casein	606.3425	Antimicrobial	P02663	
RDMPIQAF	β-casein	489.2475	ACE-inhibitory	P02666	
VIESPPEINTVQ	κ-casein	663.3515	Antioxidant	P02668	
VLNENLLR	α-S1-casein	485.7876	Antimicrobial	P02662	
VLPVPQKAVPYPQR	β-casein	531.3154	Antimicrobial	P02666	
SLAMAASDISLL	β-lactoglobulin	596.3183	Antimicrobial	O77777	CH, YUNH
FQSEEQQQTEDELQDKIHPF	β-casein	826.0476	Promote calcium uptake	P02666	CH, YH
TEDELQDKIHPF	β-casein	491.2404	Antimicrobial	P02666	
YVEELKPTPEGDL	β-lactoglobulin	745.375	Antioxidant	B5B0D4	
NMAINPSK	α-S2-casein	437.7262	ACE-inhibitory	P02663	YUNH, YH
YLYEIAR	Serum albumin	464.2501	ACE-inhibitory	A0A140T897	CUNH
LYQEPVLGPVRGPFPIIV	β-casein	997.5774	Immunomodulatory	P02666	
KVLPVPQK	β-casein	454.8	Antioxidant	P02666	YUNH
KHQGLPQEVLNENLL	α-S1-casein	577.9842	Antioxidant	P02662	
APSFSDIPNPIGSENSE	α-S1-casein	880.9027	Antioxidant	P02662	
VRGPFPIIV	β-casein	499.3134	ACE-inhibitory	P02666	
LPQNIPPLT	β-casein	496.7925	DPP-IV inhibitory	P02666	
LVYPFPGPIP	β-casein	550.3133	ACE-inhibitory	P02666	
LIVTQTMK	β-lactoglobulin	467.2756	Cytotoxic	B5B0D4	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.

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., 2007). Similar results were obtained in the present study, with ACE-inhibitory peptides being the most dominant bioactivity reported for peptides in all the undigested and GI digested hard *chhurpi* samples (Table 3). Other bioactivities reported for most of the identified peptides include antioxidant and antimicrobial properties. The peptide RELEEL identified in yak milk hydrolysate demonstrated superoxide anion and hydroxyl radical scavenging activity, indicating the presence of natural antioxidant peptides in hydrolyzed yak milk (Liu, Yang, Zhao, & Yang, 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, YQEPVLGPVR, 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.* AHTpin predicts the antihypertensive property of a peptide, based on the peptides' atomic and amino acid composition, as compared to previously reported antihypertensive peptides (Kumar et al., 2015). The sequence-based meta predictor, mAHTPred, uses several machine-learning algorithms, including support vector machine, random forest, gradient boosting, and extremely randomized tree for the prediction of antihypertensive peptides (Manavalan et al., 2019). In total, 101 peptides were predicted as antihypertensive by both the predictive softwares (Supplementary Table S1). Among these, 13 peptides were commonly identified in undigested and GI digested samples of both yak and cow hard *chhurpi* (Fig. 1D). The peptides predicted as antihypertensive were subjected to molecular docking study, determining interactions with ACE catalytic residues and potential inhibition of the enzyme. Compared with the traditional experimental approaches that are expensive and time-consuming, virtual screening methods, such as molecular docking, can simplify the drug discovery and receptor-ligand binding study with the help of *in silico* computational power (Chourasia et al., 2020). The molecular docking approach is used for studying interactions between ligands (small molecules and peptides) and target receptors at the atomic level, identifying potential drug candidates without the need of synthesizing each candidate and analyzing their activity (Chourasia et al., 2020).

ACE is a dipeptidyl carboxypeptidase that regulates the contraction of blood vessels, thereby maintaining blood pressure (Xue et al., 2021). The tACE is a 732 residue long polypeptide that contains catalytic residues identical to the catalytic C-domain of the somatic ACE (sACE), except a 36 amino acid stretch at its *N*-terminal end (Xue et al., 2021). The 3D structure of the tACE was used for observing non-bond interactions between the enzyme and predicted antihypertensive peptides. The catalytic site of ACE includes the zinc binding domain (His387, His383, and Glu411), and the three active site pockets, S1 (Ala354, Glu384, and Tyr523), S1′ (Glu162), and S2 (Gln281, His353, Lys511, His513, Tyr 520, and His523) that are not only critical for ACE activity but also the main binding targets for ACE inhibitory drugs (Xue et al., 2021). Captopril, the positive control used as ACE-inhibitor in this study, shows non-bond interaction with the ACE S2 residues, Gln281, His353, His513, Lys511, Tyr520, and Glu 384, of the S1 active site pocket (Memarpoor-Yazdi et al., 2020). Non-bond interactions with identical residues were observed for previously reported and predicted

Table 4
Multifunctional bioactive peptides identified in undigested and GI digested yak and cow hard *chhurpi* cheese.

Peptide Sequence	Source Protein	*m/z*	Bioactivity	Protein Accession	Samples
VLVLDTDYK	β-lactoglobulin	533.2949	Antibacterial / DPP-IV inhibitory	P02754	CUNH, CH,
YIPIQYVLSR	κ-casein	626.3583	Opioid / C3a Receptors agonist	P02668	YUNH, YH
FALPQYLK	α-S2-casein	490.2845	ACE-inhibitory / Antioxidant	P02663	
HQPHQPLPPTVMFPPQ	β-casein	617.6506	Anti-inflammatory / ACE-inhibitory	P02666	
YQEPVLGPVR	β-casein	579.3195	ACE-inhibitory / Immunomodulatory, Antithrombotic / Antioxidant / Anti-inflammatory	P02666	
LYQEPVLGPVR	β-casein	635.8615	Anti-inflammatory / ACE-inhibitory	P02666	
IDALNENK	β-lactoglobulin	458.7404	Stimulates proliferation / Antimicrobial	P02754	CUNH, YUNH, YH
VGINYWLAHK	α-lactalbumin	600.83	ACE-inhibitory / DPP-IV inhibitory	P00711	CH, YUNH, YH
VLPVPQK	β-casein	390.7525	Antioxidant / Antimicrobial / ACE-inhibitory / Wound healing / Osteoanabolic / Anti-apoptotic	P02666	CUNH, YUNH
RELEELNVPGEIVESLSSSEESITR	β-casein	934.8048	Caseinophosphopeptide / Immunomodulatory / Promotes calcium uptake	P02666	
VENLHLPLPLL	β-casein	629.3825	ACE-inhibitory / Anticancer	P02666	CH, YH
IPPLTQTPV	β-casein	483.287	DPP-IV inhibitory / ACE-inhibitory	P02666	CUNH
VYPFPGPI	β-casein	445.2448	PEP-inhibitory / ACE-inhibitory	P02666	CH
WMHQPHQPLPPT	β-casein	734.8614	Anti-inflammatory / ACE-inhibitory	P02666	
VYPFPGPIPN	β-casein	550.7923	Antioxidant / ACE-inhibitory	P02666	
AVPYPQR	β-casein	415.7297	Antioxidant / Antimicrobial / ACE-inhibitory	P02666	YUNH
YQEPVLGPVRGPFPIIV	β-casein	941.0368	Immunomodulatory / Anti-thrombin / Antimicrobial / ACE-inhibitory	P02666	
LVVYPWTQR	Hemorphin-9	581.3244	Opioid / 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 LYQEPVLGPVR, identified in undigested and GI digested samples of both cow and yak hard *chhurpi*, demonstrated strong non-bond interactions with key catalytic residues of ACE. Conventional hydrogen bonds with Glu411 and Tyr523, alkyl hydrophobic interactions with His353, His383, and His513 were observed for residues of LYQEPVLGPVR (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, SLVYPFPGPIPN, interacted strongly with active site residues of ACE with a ZDOCK score of 1412.254. A 10-residue long derivative, SLVYPFPGPI, 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 SLVYPFPGPIPN, with a ZDOCK score of 1546.843 (Table S2). Non-bond interactions were observed for SLVYPFPGPI with ACE catalytic residues, including GLN281, LYS511, 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 SLVYPFPGPI 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 SLVYPFPGPI 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 SLVYPFPGPI 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

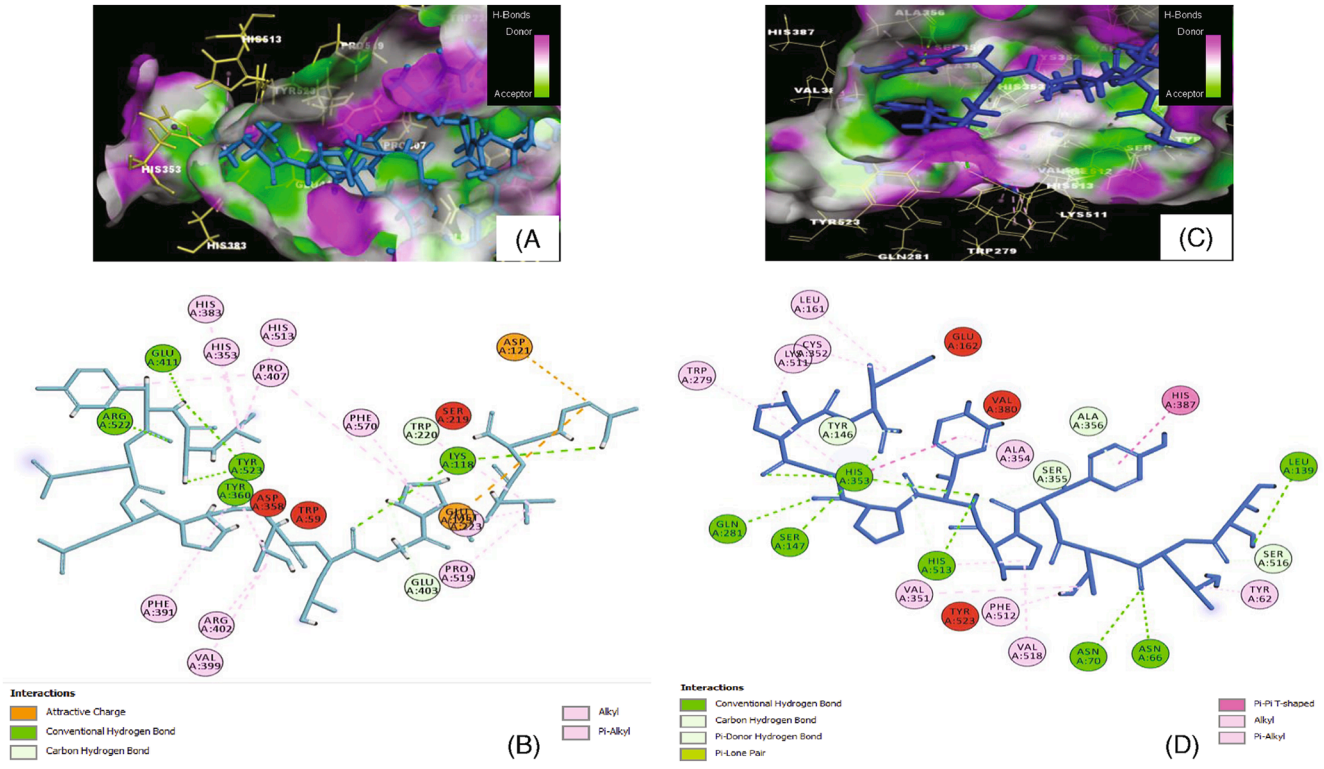

Fig. 2. Non-bond interactions between catalytic residues of ACE and peptides identified in yak and cow hard *chhurpi*. (A): Illustration showing molecular docking of the multifunctional bioactive peptide LYQEPVLGPVR within catalytic cavity of ACE. (B): 2D diagram of LYQEPVLGPVR-ACE interactions including hydrogen bonds and hydrophobic interactions. (C): Illustration showing molecular docking of the predicted peptide SLVYPFPGPI within catalytic cavity of ACE. (D): 2D diagram of SLVYPFPGPI -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 *chhurpi* cheese.

Declaration of Competing Interest

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

Acknowledgements

The authors would like to thank the Department of Biotechnology (DBT), Govt. of India. AKR and SPS acknowledge DBT for funding the colloborative research work on traditional fermented milk products (BT/PR31829/NDB/39/649/2019). We thank Director, IBSD, and CEO, CIAB, for the kind support and encouragement. We acknowledge Centre for Cellular And Molecular Platforms (C-CAMP) for the peptidomics analysis. The manuscript corresponds to IBSD manuscript number IBSD/2020/01/069.

Appendix A. Supplementary data

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

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