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RESEARCH ARTICLE

ACE- inhibitory and antioxidant activities in probiotic Cheddar cheese incorporated with Inulin and Whey Protein Concentrate

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Abstract: The aim of this study was to compare angiotensin-converting enzyme (ACE) - inhibitory and antioxidant activities of Cheddar and probiotic Cheddar cheese using *Lactiplantibacillus plantarum* DSM 20174 with inulin and whey protein concentrate (WPC). Water soluble extracts (WSE) of probiotic Cheddar cheese exhibited higher ACE-inhibitory activity than their Cheddar cheese counterparts. A similar trend was observed for antioxidant activity. The highest antioxidant activity (2183.55 µM of Trolox) was obtained when cheese supplemented with WPC and probiotic (PCW) after 6 months of ripening among all the samples followed by probiotic cheese with inulin (PCI). Similarly, PCW had highest ACE- inhibitory activity (88.51%) after 6 months of ripening. The electrophoretic and RP-HPLC profiles indicated that the rate of degradation of proteins resulted in formation of smaller peptides were highest in PCW followed by PCI among all the samples. The results suggest the potential use of WPC in presence of *L. plantarum* DSM 20174 for production of Cheddar cheese enhanced ACE-inhibitory and antioxidant properties.

Keywords:ACE- inhibition, Antioxidant, Probiotic Cheddar Cheese, Inulin, WPC

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Introduction

Many chemical, biochemical changes occur during cheese ripening and these improve the taste, flavour and nutrimental properties of the cheese. Among the milk constituents, milk proteins and its hydrolysis/ hydrolysate received a renewed interest related to their physiological role in humans and are the main source of biologically active peptides (Pihlanto 2006). Most of these peptides are encrypted within the primary structure of the native protein and released from milk protein sequences by digestive proteases, microbial or plant enzymes or by fermentation using dairy starter cultures with proteolytic activities. Such peptides posses various biological properties including antimicrobial (Meira et al. 2012 ; Mohanty et al. 2014), cholesterol-lowering (Hartmann & Meisel 2007), mineral-binding (Vegarud,et al. 2000), immunomodulatory, opioid, antioxidative and antihypertensive (Korhonen 2009; Moslehishad et al. 2013; Perna et al. 2015).

Angiotensin-I-converting enzyme (ACE) increases blood pressure by converting angiotensin- I to angiotensin-II, a potent vasoconstrictor. ACE also inactivates the vasodilating peptide bradykinin (nonapeptide) and endogenous opioid peptide Met-enkephalin. ACE inhibition results in an antihypertensive effect and may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity. These peptides have already been isolated from a variety of fermented dairy products including cheese (Hartmann and Meisel 2007; Lu et al. 2016), yoghurt (Donkor 2007) and fermented bovine milk (Qian et al. 2011).

The role of free radicals and active oxygen species in various diseases, including aging, cancer, inflammation, neurodegenerative disorder, cardiovascular disease, cataract and the toxicity of numerous compounds, has been well documented. The body has its own dependent system against reactive oxygen species (ROS) on the basis of antioxidant enzymes (i.e. superoxide dismutase, catalase and glutathione peroxidase) and endogenous antioxidant (i.e. glutathione). Oxidative stress occurs when ROS overload the body's antioxidant defense or when the antioxidant defenses system loses its capacity for response (e.g. elderly people) and can lead to damage of vital cellular components. To

enhance antioxidant defense, the body must be provided with a constant supply of antioxidants through proper diet (Herna´ndez-Ledesma et al. 2005). The search for natural antioxidants as alternatives to synthetic ones is a subject of great interest nowadays. In recent years, some of the milk protein-derived bioactive peptides have been considered as a novel class of antioxidants (Pihlanto 2006). Health-Promoting role of *Lactobacillus plantarum* recently denominated as *Lactiplantibacillus* (Lpb.) *plantarum* isolated from fermented foods has been reviewed by Garcia-Gonzalez et al. (2021). Food-associated *Lpb. plantarum* showed a good adaptation and adhesion ability in the gastro-intestinal tract and the potential to affect host health through various beneficial activities, e.g., antimicrobial, antioxidative, antigen toxic, anti-inflammatory and immunomodulatory, in several in vitro and in vivo studies.

The benefits of the fermented dairy products in the diet are well accepted. The central role of microorganisms in fermentation, especially Lactic Acid Bacteria (LAB) is now widely acknowledged, and it is accepted that these microorganisms can exert beneficial effects through two mechanisms: direct effects or indirect effects during fermentation where these microbes act as cell factories for the generation of secondary metabolites with health promoting properties. Among the latter the most important components in fermented milk are bioactive peptides released from milk proteins by members of the genera *Lactobacillus*, *Bifidobacterium* and other LAB. There are few reports on the bioactive peptides release in cheeses during ripening.

Consumers are becoming more aware that foods directly contribute to their health improvement. Therefore, they are in search of a food which would facilitate to improve their health, i.e. functional food which contributes to reduce the risk of disease occurrence and helps in promoting health apart from satisfying hunger. Probiotic and prebiotic are combined to form a synbiotic food. *Lpb. plantarum* is versatile probiotic organism with various health benefits and it is very compatible with cheese matrix (Milesi et al. 2008). Enzymes produced by this microorganism hydrolyze cheese proteins and generate large and intermediate sized peptides which may be further hydrolyzed into smaller peptides and free fatty acids (Lynch et al. 2014) .More proteolytic activity of *Lactobacillus plantarum* (new *Lpb. plantarum*) stains in cheddar cheese as adjunct cultures has been observed by Duan et al. (2019). Inulin is a widely used prebiotic. It is a soluble dietary fibre forming a subset of nutraceutical ingredients that are increasingly used in food products. WPC is considered as functional ingredient. It serves as a source of nitrogen and amino acids. Incorporation of probiotic enhances the proteolysis and accelerates cheese ripening which improves functional properties. It is presumed that inulin & WPC act as growth promoters for probiotic bacteria, they may enhance functional properties and helps to reduce ripening time.

In this study, it is hypothesized that development of Cheddar cheese incorporated with functional ingredients (inulin & WPC) in presence of probiotic (*Lactobacillus plantarum*) would increase the rate of proteolysis and lipolysis. Higher proteolysis means formation of more number of low molecular weight bioactive peptides. These peptides have been proved to have beneficial health effects like being antihypertensive (with ACE-inhibitory property), opioid, antimicrobial, immunomodulatory and mineral binding activities. Thus, this study was aimed to enrich probiotic cheddar cheese with inulin & WPC and to assess their impact on rate of proteolysis and functional properties like antihypertensive & antioxidant activities.

Materials and Methods

The pooled cow milk, used for the manufacture of cheddar cheese, was obtained from Experimental Dairy Plant of National Dairy Research Institute, Bengaluru. Mesophilic mixed starter consisting of *Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis* was obtained from dairy bacteriology unit of the Institute. The probiotic culture of *Lpb.* DSM 20174 was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Two additives were used to prepare different types of Cheddar cheese: Inulin as prebiotic from M/s. DKSH India Pvt. Ltd. and Whey protein concentrates (WPC 70 % protein) as growth promoter from M/s Mahan proteins India Ltd. were procured for this investigation.

Cheddar cheese manufacture

Cheeses were manufactured according to the standard procedures (Kosikowski 1977). Whole milk was standardized with skim milk to adjust casein to fat ratio of 0.68- 0.70 and then pasteurized at 63 ºC for 30 min. The milk was then cooled to 30-31 ºC before inoculation of the culture. Cheddar cheese starter culture was added at 0.5% (v/v) to prepare control Cheddar cheese (CC), Cheddar cheese with inulin (CI) and Cheddar cheese with WPC (CW). Additional 1% probiotic bacteria was added along with 0.5% cheese starter culture for control probiotic Cheddar cheese (PCC), probiotic Cheddar cheese with inulin (PCI) and probiotic Cheddar cheese with WPC (PCW). The milk was kept for ripening for 30 min before the addition of rennet @ 3 g per100 L of milk. The milk coagulated after 45 min and the resulted curd was cut with cheese knives. The curd was cooked to 38 -39 ºC with slow agitation. Whey was drained and curd was cheddared at 39 ºC and turned every 15 min until the acidity increased to 0.45-0.50 % lactic acid. The cheddared curd was milled. Milled curd of 5 kg lots was mixed with 2% additive and 1% salt on the curd basis individually. The curd was hooped in cheese moulds and pressed overnight. The cheese blocks were turned next day and pressed further for 24 h. After pressing, cheese blocks were removed from press and kept in ripening room for 2 days for surface drying and then paraffined. The paraffined blocks of cheese were stored

at 7± 2 ºC for ripening. Three replicates of experimental (CI, CW, PCI & PCW) and control cheddar cheese (CC & PCC) were made and analysed for the ACE- inhibitory and antioxidant activity at zero day, 6 months and 10 months.

Preparation of Water soluble extract

Water soluble extracts of all Cheddar cheese were prepared using the method developed by Kuchroo and Fox (1982), with some modification. Grated Cheddar cheese of 20 g was mixed with 60 mL of distilled water in small mixer. The mixtures were centrifuged at 4250 g for 30 min at 4 ºC (cooling centrifuge, REMI, Mumbai). The fat layers were removed and the water extracts were filtered through Whatman No.1. The pH of the extracts was adjusted to 4.6 using 1N HCl. The precipitated proteins were removed by filtering through Whatman No.1. To further remove any impurities, the water soluble extracts were filtered through 0.22 µm pore size filter (Millipore, Billerica, MA, USA). The water soluble extract was kept in deep freeze and used for HPLC, ACE and antioxidant activity.

Analysis by Urea-PAGE

The Cheddar cheese samples were analysed by alkaline urea-polyacrylamide gel electrophoresis using the method of Creamer (1991). Samples were prepared by taking 0.3 g of each cheese into 15 mL of sample buffer (0.092 g EDTA, 1.08 g Tris base, 0.55 g boric acid, 36 g urea made up in 100 mL and adjusted to pH 8.4). Each sample was centrifuged at 3000 rpm for 30 min. 1 mL of middle portion was taken and were mixed with 3% each of 0.1% (w/v) bromophenol blue solution and mercaptoethanol. The resolving gel solution was made using 8 mL of a 30% solution of " acrylamide/ BIS 37.55:1.0" and 11.9 mL of resolving gel buffer(9.2g Tris, 54 g urea, 100 mL water, adjusted to pH 8.8 and made to 200 mL). The gel solution was mixed with 10 µL of TEMED and 100 µL of 10% (w/v) of freshly made ammonium persulfate solution. The gel solution was poured into the Biorad mini-protean apparatus and overlaid with about 0.5 mL of water. After the gel had set, the water was removed. The stacking gel as made from 10 mL of a gelling solution (1.08g Tris, 36 g urea, 0.55 g boric acid, 0.092g EDTA and 3.0 g "acrylamide/ BIS 37.55:1.0 made up to 100 mL and adjusted the pH to 8.4 with HCl) was mixed with 10 µL of TEMED and 50 µL of ammonium persulfate. The solution was poured and the comb was inserted. After gel formation, comb was removed and 30 µL of 2% cheese solutions was carefully put into each well. Stock chamber buffer (0.925g, EDTA, 10.79 g Tris and 5.5 g boric acid made upto 1L and adjusted to pH 8.4). was diluted 1:4 with water and then put into the apparatus. The electrophoresis was started at a 60 V till the sample crossed the stacking gel. Thereafter, the voltage was increased to 90V and it was run for about 3 h till the dye front was close to the bottom of the gel slab. The gel was stained with staining solution (1 g Comassie brilliant blue R, 500 mL isopropanol and 200 mL glacial acetic acid made up to 2L) for 1- 2 h and destained for 1-2 h with several changes of destaining solution (200 mL isopropanol and 200 mL glacial acetic acid made up to 1L) until a transparent gel background was obtained. The gel was then scanned for record.

RP-HPLC

RP-HPLC of water soluble extract of cheese samples were carried as per the procedure of Prithard et al. (2010). Extracts of the cheese samples were separated by a Sun Fire ™ C18 Column using RP-HPLC (Waters, Milliford, MA, USA). Solvent A was 0.1% Trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. For each sample, 50 µL was injected and run with a linear gradient 0.2–60% of solvent B (0.1% TFA in acetonitrile) to 50 min followed by 0.2% of solvent B to 55 min at a flow rate of 1 mL/min at room temperature. The separated peptides were monitored at 215 nm using UV detector.

ACE- inhibitory activity

Angiotensin converting enzyme (ACE) inhibitory activity was measured using the method Herna´ndez-Ledesma et al. (2003). The method is based on the liberation of hippuric acid from Hippuryl-l-histidyl-l-leucine (HHL) catalyzed by ACE. To 110 µl of substrate (5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) 20 µL of sample was added. The reaction was initiated by the addition of 20 µL (4 mU) of ACE solution and incubated at 37^0 C for 30 min. The reaction was terminated by addition of 250 µL of 1 N HCl. Subsequently, the hippuric acid formed in the enzymatic process was extracted with 1.5 mL ethyl acetate by centrifugation at 3000 g for 10 min. An aliquot of 1 mL of the upper organic layer was collected and dried out completely by heating at 95 ^{0}C for 20 min. The dried material was re dissolved in 1 mL of double distilled water and the absorbance was measured at 228 nm. The positive control of the reaction was carried out by adding only substrate, ACE and water (no sample). Results are the mean values of three triplicates.

The extent of inhibition was calculated as follows:% Inhibition of ACE (% ACE) = $\frac{[(A-B)-(C-D)] \times 100}{(A-B)}$

where,

A = the absorbance in the presence of ACE (substrate + ACE)

B = the absorbance of the reaction blank (substrate alone)

C = the absorbance in the presence of ACE and the sample/ inhibitor

(substrate + ACE + sample)

D = the absorbance of the sample blank (substrate + sample)

The inhibitory activity of the sample was expressed as percentage of ACE inhibition.

Antioxidant activity

Antioxidant activity was measured using the method of Herna´ndez-Ledesma et al. (2005). The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7mM ABTS stock solution with 2.45mM potassium persulfate (final concentration in 10 mL of water) and keeping the mixture in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted in 5mM phosphate-buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734nm in a 1cm cuvette at 30°C. After addition of 1mL of diluted $ABTS^{\bullet+}$ solution to 10 µL of samples or Trolox standard (0 to 2500 µM) in PBS, the absorbance was recorded every min for 10 min at 30 °C. Appropriate solvent blanks were run in each assay. Results are the mean values of three triplicates. The percent inhibition of absorbance at 734nm was calculated as

$$\% \text{ inhibition} = ((A734\ nm_{blank} - A734\ nm_{sample}) / A734\ nm_{blank}) \times 100$$

To calculate the Trolox equivalent antioxidant capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance versus sample concentration was divided by the gradient of the plot for Trolox. This gives the TEAC at the specific time.

Statistical Analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA, 2007).

Results and Discussion

Urea-PAGE

The alkaline Urea- PAGE of all Cheddar and probiotic Cheddar cheeses was performed to see the extent of proteolysis with ripening time and electrophoretic patterns are presented in Fig. 1-3. Protein bands were identified by comparison with standards of Sodium caseinate where, β- Casein (β-CN) and α- Casein (α-CN) and peptide positions are shown (plate1a). Cheese samples of different ages were analysed to compare the degradation pattern of these two caseins during ripening. αs_1-CN and αs_2-CN could not be separated on 12% acrylamide gel because the molecular weights of these two fractions are very similar. As the ripening progressed, the concentrations of α-CN & β-CN decreased and the breakdown products were formed. These breakdown products which appeared below α-CN, are low molecular weight peptides. The intensity of these peptides gradually increased till 6 months of ripening thereafter started decreasing till 10 months for all the cheeses. These peptides formation are due to hydrolysis of α-CN & β-CN by the action of microbial proteinase and peptidase leading to further formation of smaller peptides and amino acids. The degradation of β-casein by plasmin in all the cheeses **(Fig.1-3)** is clearly indicated by the decrease in the intensity of β-casein. However, during ripening β-CN did not undergo as much degradation as α-CN (Brandsma et al. 1994; Mistry and Kasperson 1998). This shows that the bacterial peptidases hydrolyse α-CN more readily than β–CN. This may be due to lower activity of natural enzyme of plasmin in cheddar cheese as it is neither cooked at high temperature nor any plasminogen activator was added (Fox et al. 2000).

The differences in the degradation and development of peptides at 6 months ripening of the Cheddar & probiotic Cheddar cheeses are shown in Fig. 4a &1b. The α-CN degradation was more than β -CN in all the cheese samples as the intensity of α-CN tended to be lower than β -CN among the six samples. Cheese made with Inulin and WPC showed higher hydrolysis of both the caseins as compared to control indicating a faster proteolysis in these cheeses.

Comparison between the electrophoretic pattern of 0 day and 10 months ripened cheese is shown in Fig. 2 & 3. There was not much peptides formation in fresh samples. Therefore, there was not much difference in the intensity of the peptides observed. At 10 months of ripening, hydrolysis of α-CN was higher in probiotic cheese (Fig. 3) as compared to Cheddar cheese (Fig. 2) irrespective incorporation of additives as visible by disappearance of α-CN band. All factors, which have influenced the rate of hydrolysis of both caseins, were kept constant during production & ripening, except probiotic addition. This faster α- CN degradation in probiotic was due to the action of probiotic peptidases (Ong et al. 2007) and more proteolytic activity of *Lpb plantarum* (Duan, 2019). In Cheddar cheese, WPC added cheese showed highest proteolysis among the five samples (Fig. 2, lane 7**)**. Similarly, WPC added cheese showed highest proteolysis among the five probiotic Cheddar cheese samples followed by inulin added cheese (Fig. 3, lane7). The increased rates of proteolysis in these two cheeses were mainly due to higher growth and survival of bacteria in presence Inulin and WPC, which might have resulted in high bacterial peptidase activity and may be due to higher growth and more proteolytic activity of *Lpb planatrum* (Duan et al. 2019).The results were also reflected in the amount of soluble amino nitrogen content (proteolysis) in probiotic cheeses.

RP-HPLC

The RP- HPLC chromatogram of standard α- lactalbumin (12 kDa) was run and eluted out at 35.806 min (Fig. 4). It was inferred that all peptides which eluted before 35 min are less than 12 kDa. Assuming this phenomenon, water soluble extracts (WSE) of all cheddar cheeses were analysed for RP-HPLC chromatogram restricting to 35 min run. Comparison between the RP-HPLC chromatograms of 6 months old, Cheddar cheese (data not shown) and probiotic cheddar cheese, with additives showed that total

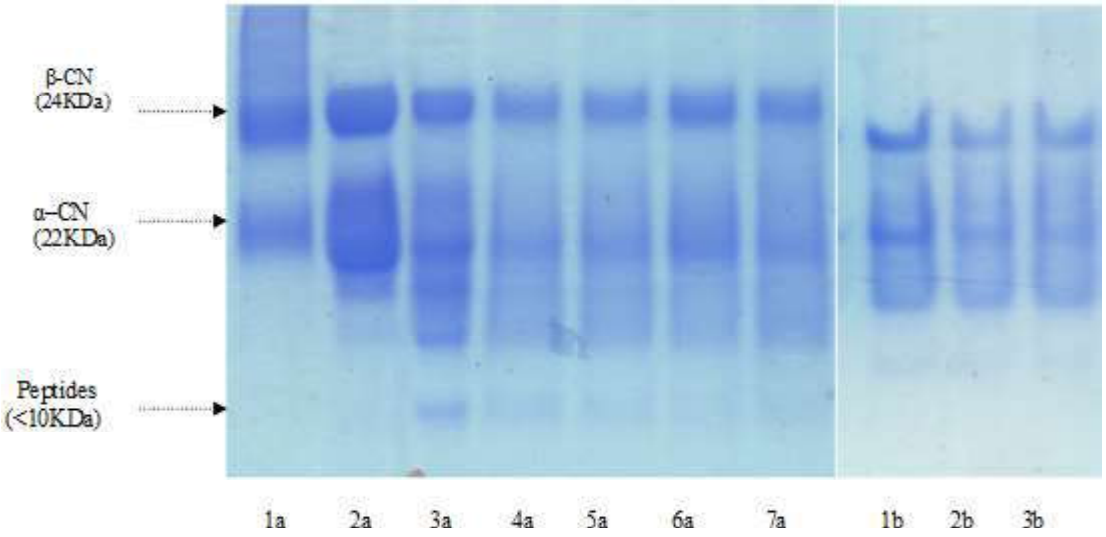


Fig. 1 Electrophoretic pattern of Urea-PAGE of Cheddar and Probiotic Cheddar cheese with additives at 6 months. Lane 1a- Standard (Na Casienate); lane 2a – 0 day; lane 3a- 2 months old; lane 4a- 6 months old Cheddar cheese (CC); lane 5a- 6 months old Cheddar cheese with inulin (CI); lane 6a- 6 months old Cheddar cheese with inulin -lactose; lane 7a- 6 months old Cheddar cheese with WPC (CW); lane 1b- 6 months old probiotic Cheddar cheese (PCC); lane 2b- 6 months old probiotic Cheddar cheese with inulin (PCI); lane 3b- 6 months old probiotic Cheddar cheese with WPC (PCW).

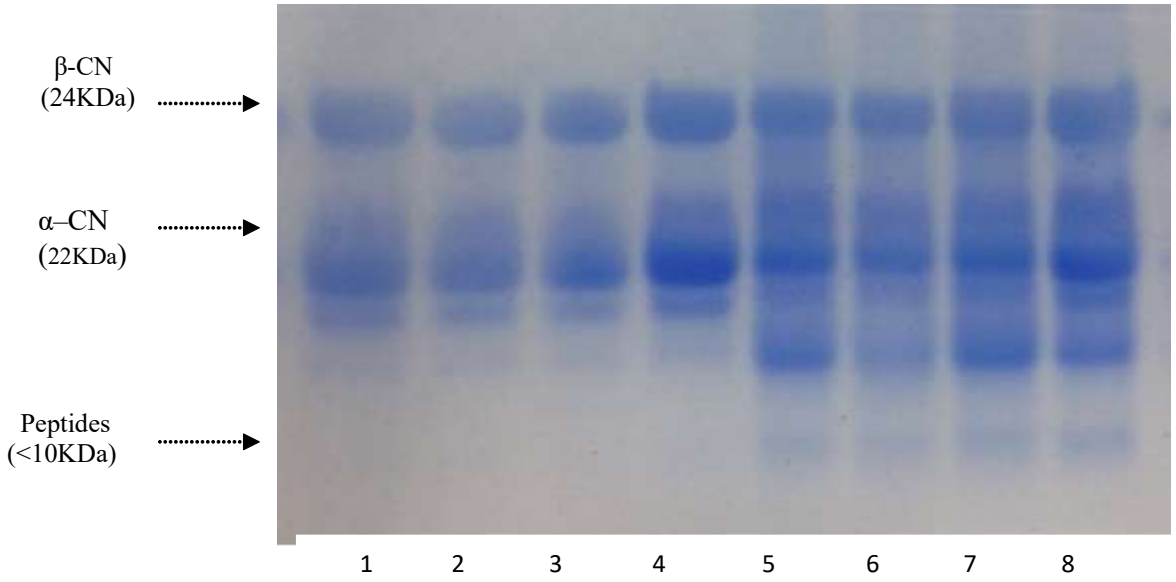


Fig. 2 Electrophoretic pattern of Urea-PAGE of Cheddar cheese with additives at 0 day & 10 months. Lane 1- 0 day ; lane 2 – 0 day with inulin; lane 3- 0 day with WPC; lane 4- 0 day with lactose; lane 5- 10 months old; lane 6- 10 months old with inulin; lane 7- 10 months old with WPC; lane 8- 10 months old with lactose.

number of peaks and their intensity were more in probiotic cheddar cheese as compared to corresponding Cheddar cheese. It was observed that more number of main peaks (15peaks) in probiotic cheddar cheese (PCC) than the control cheese (13 peaks). When peaks and intensity of CW & PCW were compared, it was found that PCW had 18 main peaks whereas CW had 15 main peaks and peak intensity was also higher in PCW. Since probiotic Cheddar cheese samples had higher number of peaks and higher intensity as compared with their corresponding Cheddar cheese samples, it may be inferred that probiotic addition would enhance the rate of proteolysis. The soluble amino nitrogen and electrophorograms also indicated that the rate of proteolysis was more in inulin and WPC added cheese.

A comparative study of peptide profile between PCC, PCW and PCI was done for 0 day, 6 and 10 months (Fig. 5). It can be inferred that as the age of cheese increased, new peaks appeared while existing peaks at the initial stages of ripening either increased or

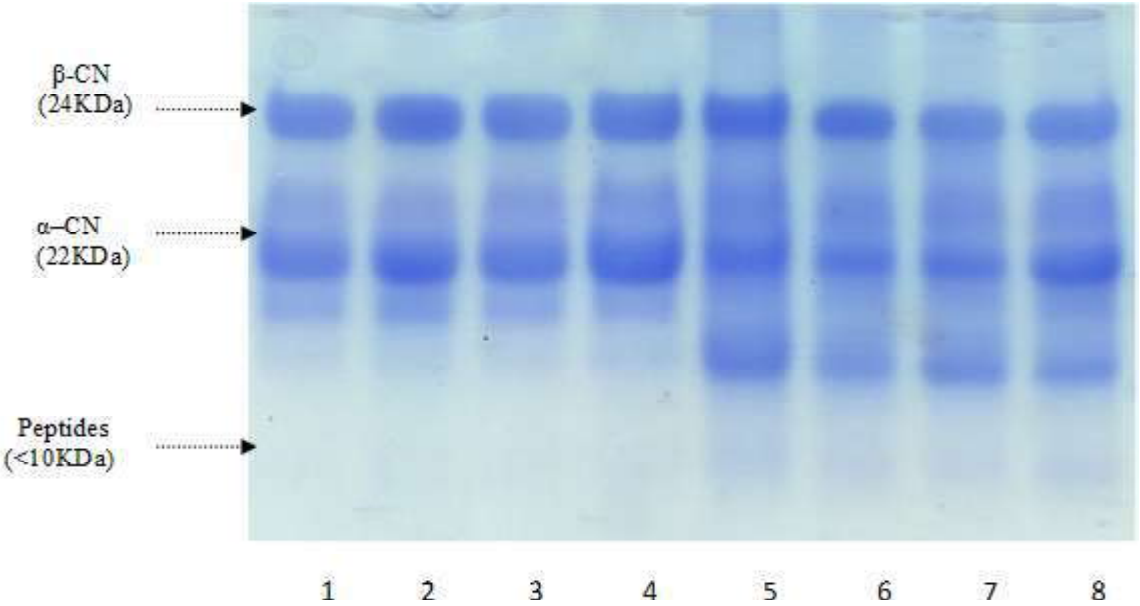


Fig. 3 Electrophoretic pattern of Urea-PAGE of probiotic Cheddar cheese with additives at 0 day & 10 months. Lane 1- 0 day ; lane 2 – 0 day with inulin; lane 3- 0 day with WPC; lane 4- 0 day with lactose; lane 5- 10 months old; lane 6- 10 months old with inulin; lane 7- 10 months old with WPC; lane 8- 10 months old with lactose.

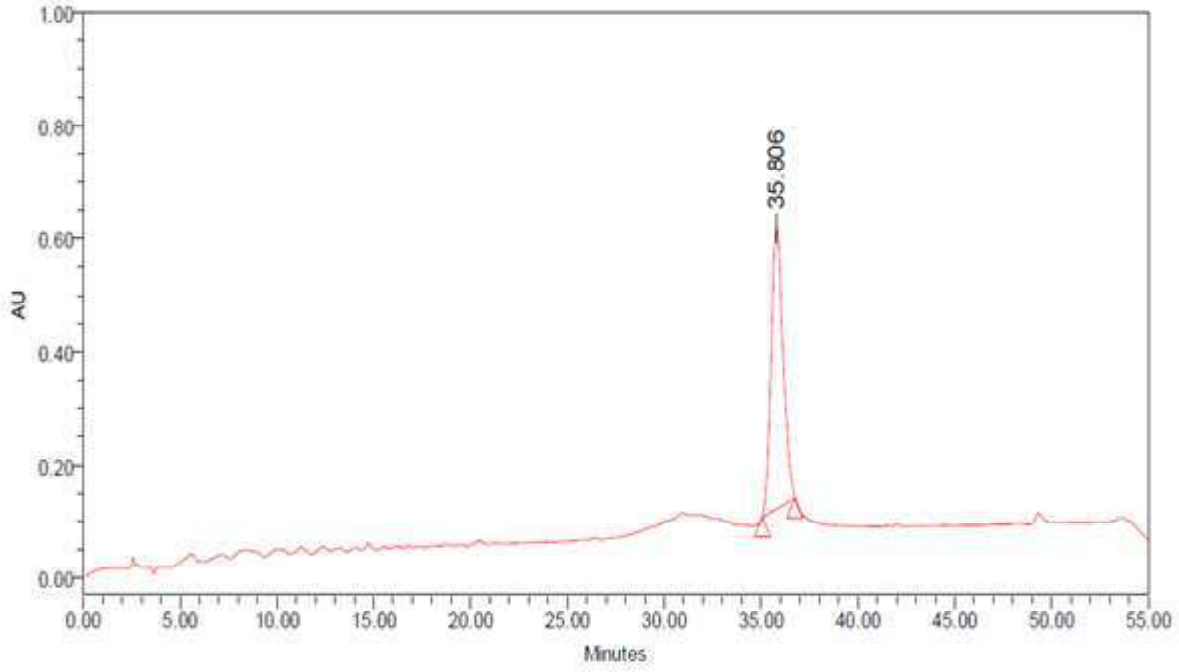
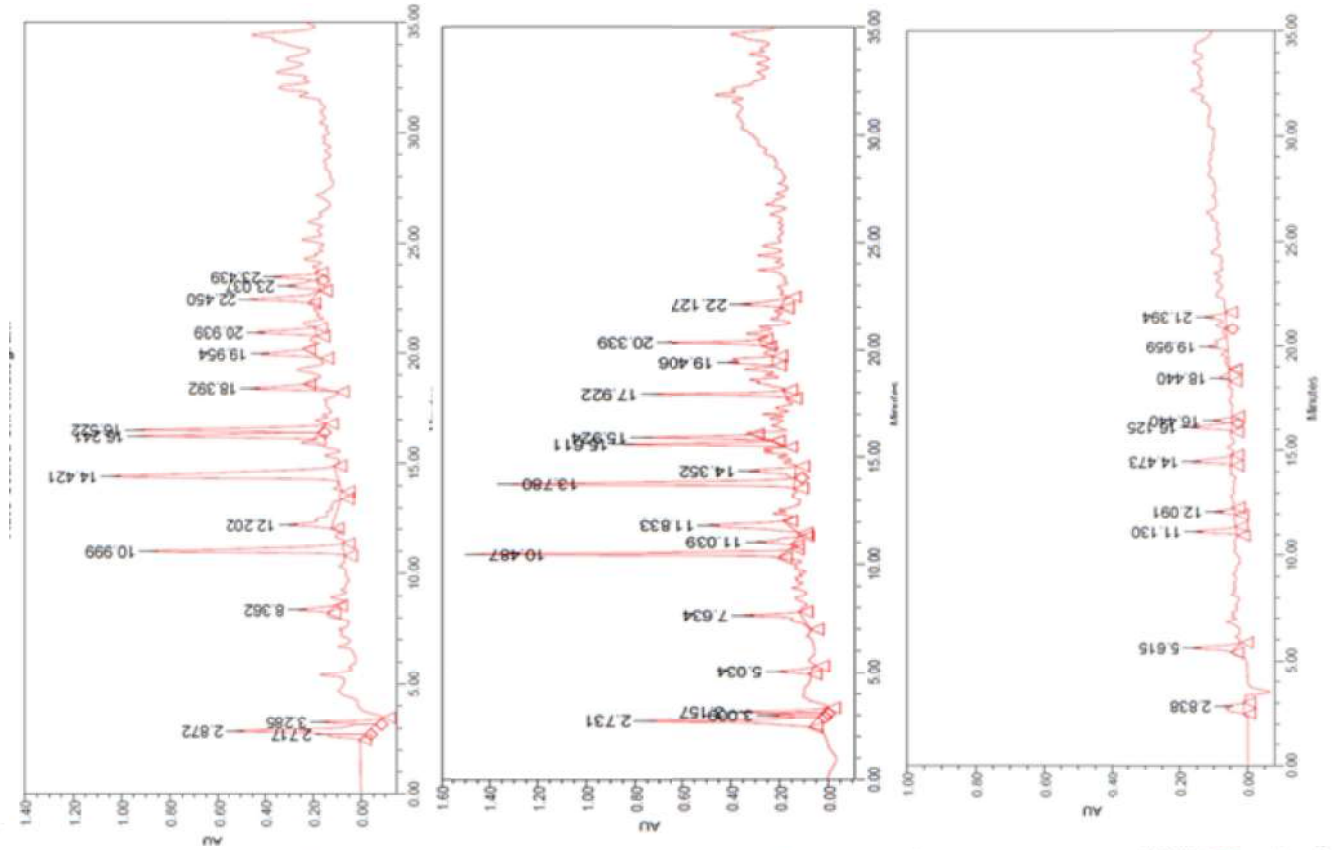
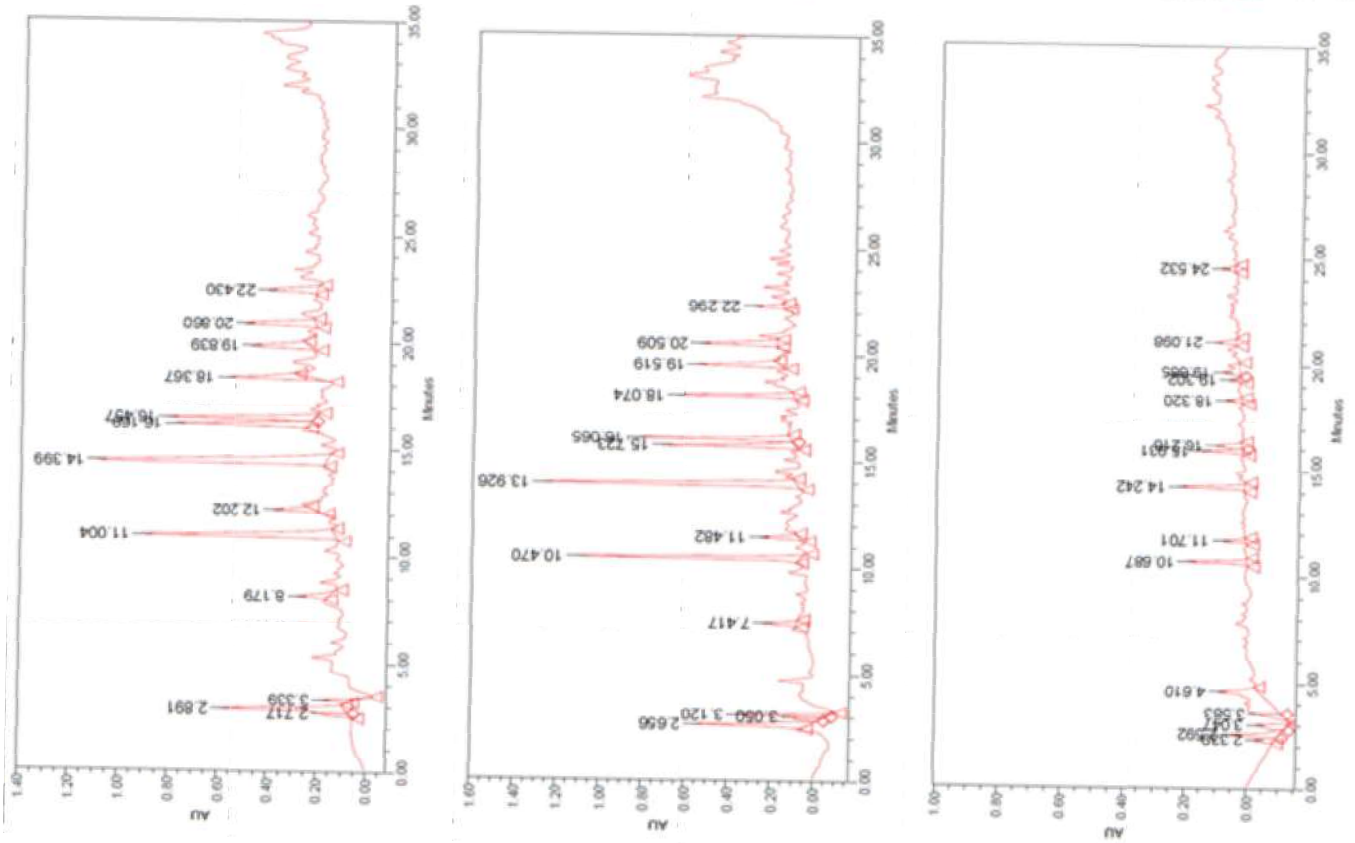


Fig. 4 RP-HPLC chromatograms of α lactalbumin standard

decreased. Peptide profile of cheeses at zero day showed that the number of peaks and their intensity were very less; however it did not differ among the samples. As the ripening increased the rate of formation of peptides in the cheeses made with WPC and inulin were higher as compared to control cheese. PCW had 18 main peaks, whereas 16 main peaks were observed in PCI and 13 main peaks in PCC. It was noted that the elution times of peptides with higher retention times of peptides were often affected by the average hydrophobicity on the reverse phase column (Champion and Starley 1982). Higher absorbance values of peaks indicate higher concentration of peptide contents in WSEs of Cheddar cheeses. With the increase in the ripening time from 6 to 10 months the electrophoretic pattern and elution profile changed. The number of peaks and their intensity was higher in cheese with WPC even after 10 months of ripening. However, there was decrease in number of peaks in RP- HPLC elution profile. This



5 b) PCI



5 a) PCC

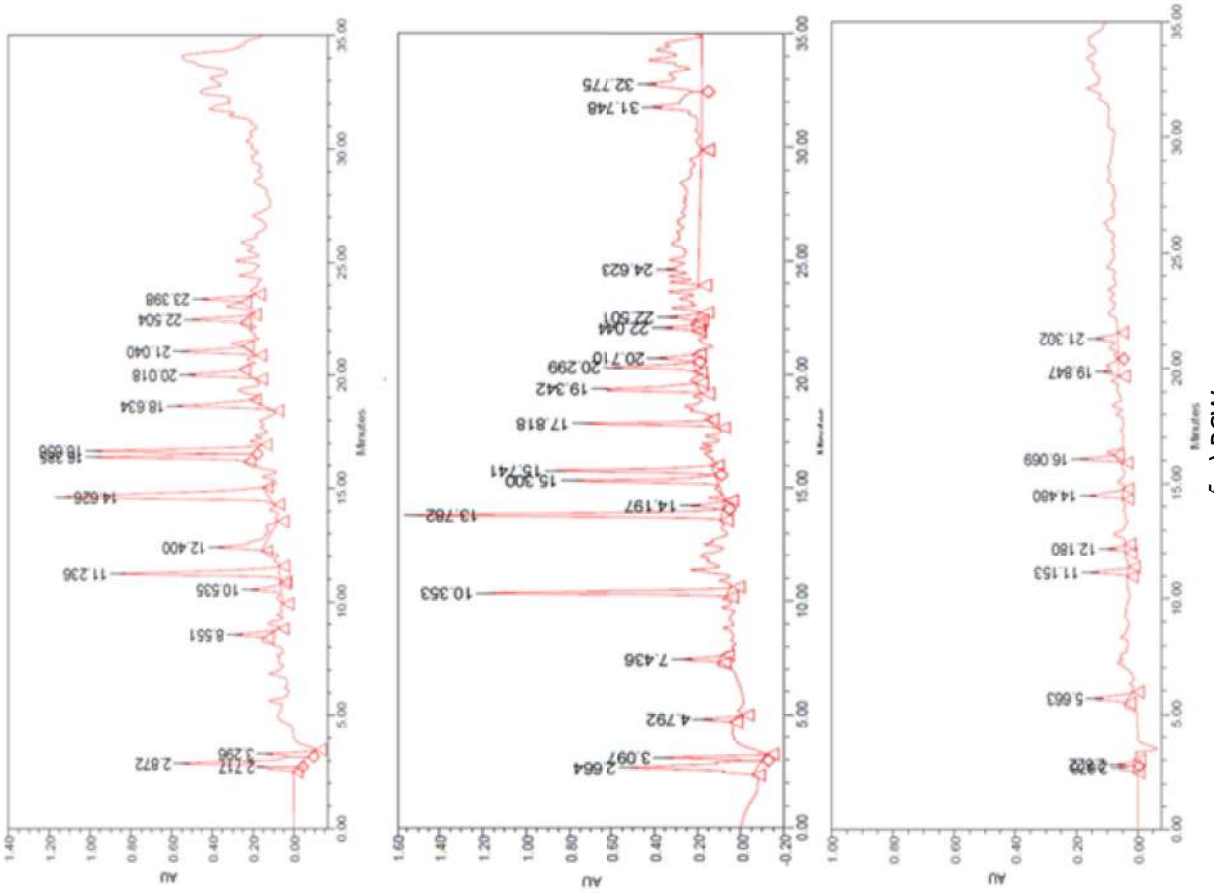


Fig. 5 Peptide profile of water-soluble extract (WSE) of (a) control probiotic Cheddar cheese (PCC), (b) probiotic Cheddar cheeses with inulin (PCI) and (c) probiotic Cheddar cheese with WPC (PCW) at 0 day, 6 mon & 10 mon. Eluent A was 0.01% TFA in water . Eluent B was 0.01% TFA in acetonitrile. Gradient: 0 to 50min, 0.2 to 60% eluent B; 50 to 55 min, 60 to 0.02% eluent B.. Detection was at 215 nm

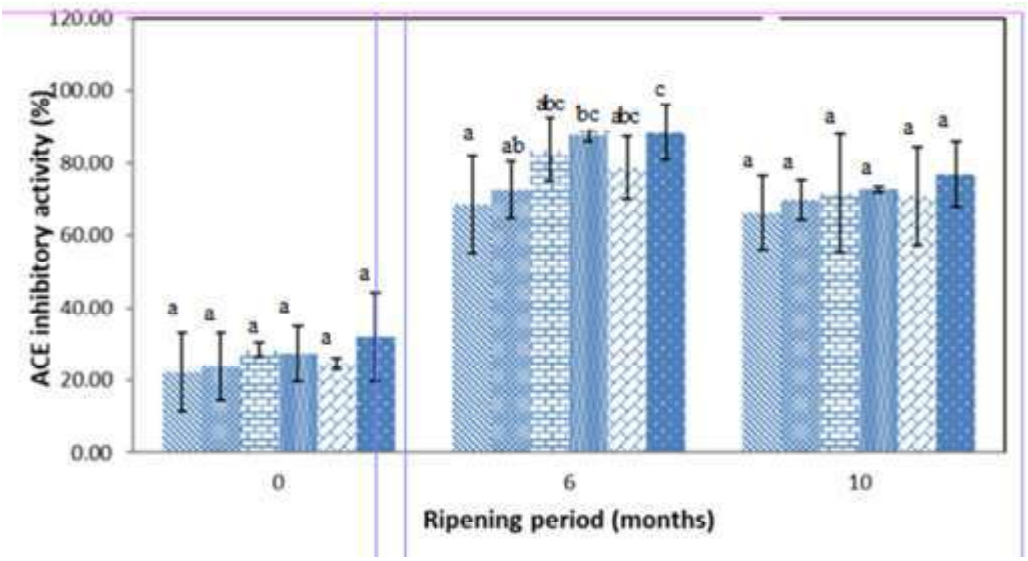


Fig. 6 ACE- inhibitory activity of WSE of CC ( ), PCC ( ), CI ( ), PCI( ), CW( ) and PCW( ). The data measured as Trolox equivalent antioxidant capacity (µM Trolox). Error bars show standard error of the mean of duplicate measurements of three independent experiments (batches).

may be due to further breakdown of peptides into smaller peptides and amino acids. As the ripening increased, more peptides were released into the cheeses and chromatogram became more complex which are similar to the observations for Cheddar cheese as reported earlier (Duan et al. 2019, Gupta et al. 2013, Ong et al. 2007 ; De Wit et al. 2005). It can be concluded that

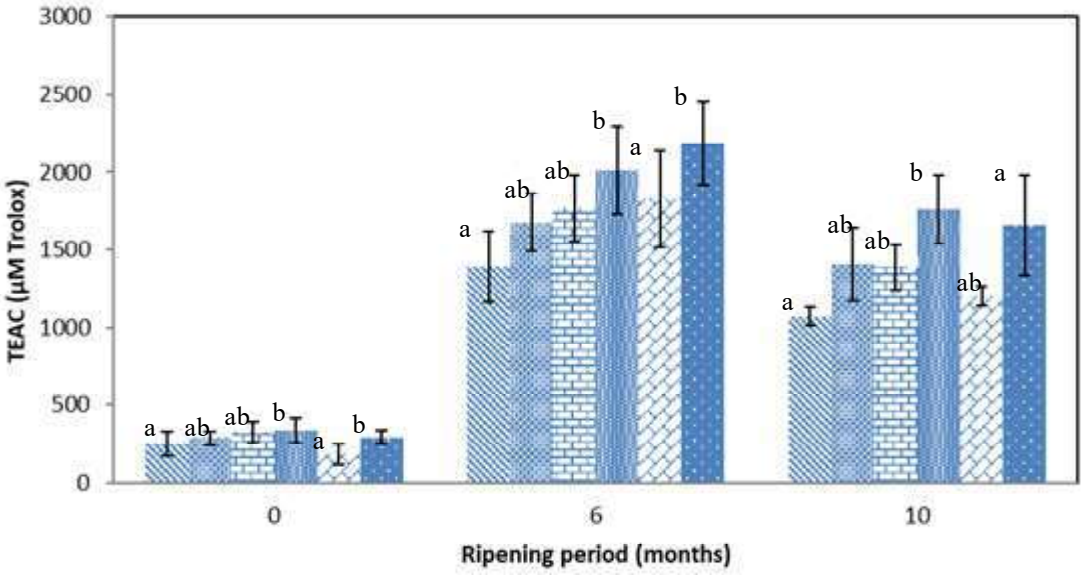


Fig. 7 Antioxidant activity of WSE of CC( ), PCC ( ), CI ( ), PCI( ), CW( ) and PCW( ). The data measured as Trolox equivalent antioxidant capacity (µM Trolox). Error bars show standard error of the mean of duplicate measurements of three independent experiments (batches). Mean values with different small letters for the same ripening period are significantly different ($P < 0.05$).

addition of WPC in presence of probiotic resulted in highest proteolysis.

ACE- inhibitory activity

The ACE- inhibitory activity of WSE of cheese samples collected at 0 day, 6 months and 10 months was compared by determining % ACE- inhibitory activity. Initial ACE- inhibitory activity of six cheeses with additives ranged between 22.21 to 32.00% (Fig. 6). The ACE- inhibitory activity among the cheeses at zero day of ripening did not show any significant difference ($p>0.05$).

All six cheese samples showed a significant increase in ACE-inhibitory activity after 6 months of ripening. ACE- inhibitory activity of PCW (88.51%) was highest among all the samples. It was significantly higher ($p<0.05$) than both of CC (68.68%) and PCC (72.50%) after 6 months followed by PCI (87.82%), CI (83.62%) & CW (78.80%). ACE- inhibitory activities of probiotic cheeses were more than that of Cheddar cheeses irrespective of additives.

ACE- inhibitory activity did not show any significant ($p>0.05$) difference among the samples after 10 months of ripening. ACE-inhibitory activity increases till 6 months and showed decease after 10 months of ripening. Our study suggests that ACE-inhibitory activity did not increase continuously till 10 months ripening unlike proteolysis but reached to a maximum value within 6 months and thereafter started decreasing. This may be due to some peptides produced at the early or intermediate stage of hydrolysis, getting degraded and subsequently forming new peptides which had less inhibition activity or may be further degraded and formed amino acid upon longer period of ripening.

Higher ACE inhibitory activity of probiotic Cheddar cheese compared to Cheddar cheese suggests that ACE- inhibitory activity is related to the bacterial strains used in the cheese ripening process. Similar trends were previously reported by Gupta et al. (2013) and Ong et al. (2007). ACE- inhibitory activity of probiotic cheese with WPC was highest among all the samples, may be due to extensive proteolysis in presence of WPC. Proteolysis was also highest for this sample as observed earlier. Many investigations showed that ACE- inhibitory activity is dependent on the degree of proteolysis and peptide formation. The activity increases until it reached maximum due some peptide formation, decreasing thereafter as a result of extensive proteolytic degradation of ACE- inhibitory peptides (Ong et al. 2007). (In contrast, Moslehishad et al. (2013) found that the ACE- inhibition did not follow any specific trend during 21 days of storage at 5 ºC in fermented milk.

Antioxidant activity

Antioxidant activities of all cheeses were evaluated by scavenging activity of 2, 2'- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical and expressed in Trolox equivalent antioxidant activity (TEAC) values. A comparative analysis of antioxidant activity between cheddar and probiotic cheddar cheeses with two additives (Inulin and WPC) including their control were studied at 0, 6 & 10 months of ripening (Fig. 7).

The TEAC values of Cheddar cheese with additives initially were low for all six samples and ranged from 183.09 to 334.19 µM of Trolox. The ABTS scavenging activities of both PCI & PCW samples were significantly higher than control samples at 0 day of ripening. There was a significant increase in ABTS scavenging activity till 6 months and reached to maximum value irrespective of the treatments. Highest TEAC value (1828.20 µM of Trolox) in Cheddar cheese was observed in the cheese with WPC followed by the cheese of CI and CC after 6 months of ripening. Similarly, in probiotic Cheddar cheese, ABTS scavenging activity was maximum in cheese with WPC (2183.55 µM of Trolox). When the antioxidant activity assays of all six samples was compared, it was observed that radical scavenging activity of cheeses at 6 months of ripening was significantly highest ($p<0.05$) in PCW as compared to CC but not significantly ($p>0.05$) different from PCC, followed by PCI. However, overall ABTS scavenging activity of the probiotic Cheddar cheeses was higher than Cheddar cheeses. The ABTS scavenging activity decreased after 6 months till 10 months of ripening irrespective of the treatment. TEAC value of PCI was highest among the six samples after 10 months of ripening. However, TEAC value (1385.87 µM of Trolox) was more in cheese with inulin as compared to the cheese with WPC (1200.26 µM of Trolox) after 10 months of ripening. Simliar trend was observed in probiotic Cheddar cheese.

ABTS scavenging activity was maximum at 6 months of ripening which may be due to hydrolysis of protein to smaller peptides having antioxidant activity. Reduction in ABTS scavenging activity after 6 months may be due to further hydrolysis of these peptides to smaller peptides and amino acids which may not have antioxidant activity as reported by Moslehishad et al. (2013). According to them, the 5-10 kDa peptide fractions exhibited the highest radical scavenging activities compared with lower molecular masses of peptides (3-5 kDa) in fermented milk.

ABTS scavenging activity of the probiotic Cheddar cheeses was higher than Cheddar cheeses may be due to the more hydrolysis of both α and β-CN by proteolytic/ peptidolytic enzymes of *Lpb.* In electrophoretic study (plate 1), more hydrolysis of α and β – CN was observed in probiotic cheeses. Gjorgievski et al. (2014) reported that the antioxidative capacity of fermented milk product (yogurt) was strain dependent. The lowest value of 45.17% indicates that milk was fermented with symbiosis of *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophiles* in the product. A few studies related to the production of antioxidant peptides in fermented milk with lactic acid bacteria indicated that the development of radical scavenging activity was a strain-specific characteristic and radical scavenging was related to proteolysis (Hernandez et al. 2005; Virtanen et al. 2007).

When the extent of proteolysis and peptide formation was compared with antioxidant activity, it was observed that changes in antioxidant activity were very similar to the change in the rate of formation of soluble peptides (proteolysis) in all the cheese samples till 6 months of ripening. As shown earlier, the soluble amino nitrogen was reported to be more in cheese made with WPC than in control cheese. Therefore, ABTS radical scavenging activity of probiotic cheese with WPC was highest. Perna et al. (2015) investigated antioxidant activity in Caciocavolla cheese by ABTS method and reported antioxidant activity varied depending on the rate of formation of soluble peptides during ripening. In Cheddar cheese and probiotic cheddar cheese, ABTS scavenging activity was highest in WPC added cheese after 6 months of ripening.

Conclusions

From the results, it can be concluded that addition of WPC increased the proteolysis more than inulin in cheddar cheese. Our results further indicate that proteolytic enzymes of probiotic culture, play an important role in increasing the production of peptides (detected via urea PAGE) which contributed towards ACE– inhibitory and antioxidant activity. Lower activity observed after 10 ripening may be due to further degradation and formation of amino acid upon longer period of ripening and also due to higher activity of *Lpb. plantarum* which was enhanced in the presence of WPC and thereby shown more ACE- inhibitory and antioxidant activity after 6 months of ripening.

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