

Article

Effect of *In Vitro* Digestion on the Antioxidant and Angiotensin Converting Enzyme (ACE)-Inhibitory Potential of Buffalo Milk Processed Cheddar Cheese

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Abstract: The purpose of this study was to develop an *in-vitro* digestion protocol to evaluate the antioxidant potential of the peptides found in processed cheddar cheese using digestion enzymes. We studied first antioxidant and angiotensin converting enzyme (ACE) inhibition and antioxidant activities of processed cheddar cheese with the addition of spices e.g. cumin, clove and black pepper made from buffalo milk and ripened for 9 months. Then we conducted an *in vitro* digestion of processed cheddar cheese by gastric and duodenal enzymes. Freeze dried water (WSE) and ethanol soluble fractions (ESE) of processed cheddar cheese were also monitored for their ACE inhibition activity and antioxidant activities. In our preliminary experiments, different levels of spices (cumin, clove and black pepper) were tested into cheese matrix and only one level 0.2g/100g (0.2%) on the basis of cheese weight was considered good concerning sensory evaluation. Significant increase in ACE-inhibition (%) of processed Cheddar cheese as well as its WSE and ESE was obtained. Lower IC₅₀ values were found after duodenal phase digestion compared to oral phase digestion.

Keywords: ACE-inhibition; antioxidant potential; processed cheddar cheese; water soluble extract; ethanol soluble extract

1. Introduction

Bioactive compounds are “extra nutritional” constituents occurring in foods in small quantities but having health impacts [1]. In milk, bioactive peptides are produced during fermentation with starter cultures (proteolytic), proteolytic enzymatic hydrolysis, and gastrointestinal digestion. Hence, they confer to processed dairy products such as cheese nutritional and health benefits. Cheese could provide antihypertensive peptides devoid of any premeditated functional part [2]. Several studies, concurring with this claim, report associations of dairy products intake with a decrease in blood pressure through specific biological pathways [3]. Rennin-angiotensin-aldosterone system is a biological pathway involved in blood pressure regulation in the human body and affected by bioactive peptides in cheese [4]. The possible health benefits and associations with cheese consumption of these bioactive peptides, resulting from proteolytic digestion of the parent milk proteins, can be monitored. This is possible when considering the natural concentrations of

these compounds within food products for example ripened cheese [5]. Antioxidant activity is another important property of bioactive compounds. Superfluous free radicals and oxygen species with high reactivity result in disastrous cellular possessions like apoptosis by cellular proteins (oxidized), enzymes, DNA, and cell membrane lipids. Antioxidants that occur candidly in foods could safeguard the humanoid form by delaying the progress of many chronic diseases [6,7,8].

The functional role of spices used as cheese additives have been reported in many studies. The bark of cinnamon contains “Cinnamaldehyde” as a foremost component with 2% of essential oil. The extract of methanol comprises anthraquinones, tannins, terpenoids, flavonoids, coumarins and glycosides [9]. Cinnamon contains polyphenolic polymers which were found effective in controlling diabetes and glucose intolerance and also act as antioxidants [10]. The clove oil contains ‘Eugenol’ as a principal component which exhibit significant anticancer, antioxidant, cardiovascular and anti-inflammatory properties in addition to these, triterpenes, galloyltannins, flavonoids, phenolic acids were also found in clove [11, 12]. Compounds e.g. Tannins, phenolics and flavonoids were found in cumin roots, leaves and flowers [13]. These pharmacological properties of *Nigella sativa* oil and seed were due to the occurrence of some essential elements e.g. alpha-hederin, thymoquinone, nigellidine, thymohydroquinone, nigellidine, dithymoquinone, nigellimine- N -oxide, thymol and carvacrol [14]. The “king of spices” was the name given to black pepper as it was one of the world’s ancient and best-well-notorious spices. Piperine is major element present in black pepper because of its capability to escalate the digestive capacity by motivating the digestive enzymes of pancreas which considerably decreases the transit time of food in gastro-intestinal tract. Black pepper’s essential oil was found to have anti-microbial property and was able to exhibit inhibitory properties against 25 diverse bacterial categories [15].

A few studies have been conducted on the supplementation of spices in cheese. Ahmed *et al.* [16] made spicy Mudaffara cheese using clove (*Syzygium aromaticum*), black cumin and black pepper and monitored their antioxidant activity. In another study, it was reported that manufacturing of paneer with turmeric powder (turmeric addition at 0.6 % proportion by weight of estimated paneer production) enhances its shelf stability fit for 15 days [17]. Josipovic *et al.* [18] manufactured the novel cottage cheese having spices and found that using pepper into the cheese matrix; it will have good antimicrobial and antioxidant activity. In this study, influence of stimulated *in vitro* digestion of processed Cheddar cheese (made with addition of clove, cinnamon, and black pepper) by gastric and duodenal enzymes on the release of bioactive peptides related to antioxidant activity and an antihypertensive activity during ripening was monitored.

2. Materials and Methods

2.1. Procurement of raw material for Cheddar cheese manufacturing

Buffalo milk was used for Cheddar cheese manufacturing. Milk (15 L) was collected from the local farm at Sargodha, Pakistan and standardized at 3.5 % fat for cheese manufacturing. All the apparatus was properly cleaned and manufacturing was done under hygiene condition in the laboratory. All the glassware used were properly washed and sterilized. Chemicals for analysis were purchased from Sigma Aldrich (Seelze, Germany) and Lab-Scan (Dublin, Ireland) available at local market in Sargodha, Pakistan.

2.2. Manufacturing of Processed Cheddar cheese

The procedure described by Lawrence *et al.* [19] with some modifications, was adapted to make processed cheddar cheese. After standardization of milk (15 L), pasteurization was done at 63 °C for 30 min. The active culture was added into the pasteurized milk at 32 °C (slow stirring), and ripening of milk was done for 3 h until the milk pH lowered to 6.4 (before addition of rennet, pH should be below 6.4 for better rennet activity). To speed up the coagulation, CaCl₂ (0.05% w/w) was added into the milk. Then rennet (3.5 mL/15 L) was added into the milk and stirring was stopped. After setting of the coagulum (30 min), it was cut to the size of peas. The whey was drained off by pouring in the

muslin cloth and after pressing for 15 min, cheddaring was done for 30 min. Then salt (1%) was added into the cheese matrix after milling and mellowing was done for 30 min. The cheese matrix was pressed (2 bar pressure) with hydraulic press for 60 min. The cheeses were packed and placed at the controlled atmosphere (4 °C) for ripening period of 9 months.

After melting of 9 months ripened cheeses made within kettle, three spices namely, cumin, cardamom and black pepper were added into each cheese. The inclusion levels of the spices were determined in a preliminary experiment where cheeses were rated by their sensory properties and the inclusion level of 0.2% (based on cheese weight) was adopted. During melting process, some emulsifying salts (phosphates 3%) were also added in order to avoid fat separation. The mixing of ingredients was done for about 10 minutes at a temperature of 80-85 °C with steam. The processed cheese was poured into moulds and stored at 5±1 °C until further analysis.

2.3. Preparation of freeze-dried water soluble and ethanol soluble extracts (pH 4.6 soluble fraction) of processed cheddar cheese

Water soluble extracts (pH 4.6 soluble fractions) of all the processed cheddar cheese were prepared by following method of Pripp *et al.* [20]. The first step was to mix the 15 g of grated cheese in 50 mL water and this mixture was placed in a water bath and heated at 40 °C for 5 minutes. The second step was homogenization of the above mixture for 2 min using an Omni-Mixer homogenizer (Omni International, Waterburg, CT). Then 2 M hydrochloric acid was used to adjust the pH at 4.6 and distilled water was added until the sample weight (above grated cheese sample mixture) was equal to 100 g. Again, the samples were placed in a water bath for 1 hour at temperature of 40 °C to soften or melt all the fat in the cheese. Then centrifugation of the samples was done at 4500 rpm (3000 g) for 30 min at 40 °C. After centrifugation, filtration was done using Whatman filter paper No. 1. The round bottom flask was used to collect water soluble fraction or extracts and then were freeze dried for further analysis. After freeze drying, powdered samples were weighed and stored at 20 °C in plastic tubes. The remaining 30 mL of supernatant was mixed with 70 mL (v/v %) of ethanol and retained for 1 hour at room temperature. Centrifugation for 10 minutes was again done to remove precipitates at 4500 rpm (3000 g) at a temperature of 20 °C. Supernatant labeled as the ethanol soluble fraction (ESF) 70% was filtered through Whatman No.1 filter paper. About 60 mL supernatant was collected in round bottle flask and place separately. Rotator evaporator was used for ethanol removal at 30 °C. Freeze-dried samples were stored at -20 °C until further use.

2.4. In Vitro Enzymatic Digestion of processed cheddar cheese

To simulate human digestion in the stomach and the duodenum, an *in vitro* digestion was performed according to Minekus *et al.* [21], with some modifications. For the preparation of cheese samples approximately 1.3 mL simulated salivary fluid (SSF) electrolyte stock solution + 5.0 µL of 0.3 M CaCl₂ was added to shredded cheese samples and mixed properly to get paste-like uniformity. The suggested time of interaction of sample with the enzyme was 2-3 minutes done at 37 °C, which require pre-warming of all components to 37 °C. The final volume of oral phase digestion was 2 mL. In gastric phase, 1.6 mL of stimulated gastric fluid (SGF) stock electrolyte solution was added in the oral bolus or liquid cheese samples to acquire an absolute ratio of samples to SGF 50:50 (v/v) after addition of water and other recipients. The pepsin solution was prepared by adding 3.71 mg porcine pepsin (EC 3.4.23.1) in 1 mL of distilled water. In the final digestion mixture, porcine pepsin (EC 3.4.23.1) was added to attain 2000 U mL⁻¹, followed by 1.0 µL of 0.3 M CaCl₂ addition to achieve 0.075 mM in the final digestion mixture. Nearly 250 µL of 1 M HCl was required for decreasing the pH of the solution up to 3.0. Lastly, for dilution of the stock solution of SGF, the essential volume of water was added. The measured amount of water was 851 µL to make the final volume of gastric phase up to 4 mL. The gastric digestion was done for 60 minutes at 37 °C. After the gastric phase the final volume of liquid sample was 4 mL. This 1.6 mL SIF electrolyte solution (stock electrolyte) containing porcine pancreatin was added in gastric samples-chyme. The total of

pancreatin added was calculated on the basis of activity of trypsin (100 U mL^{-1}) in the final mixture. The porcine bile in 0.5 mL distilled water. $8.0 \mu\text{L CaCl}_2$ is added to the mixture. To neutralize the mixture to pH 7.0, 100 μL NaOH was added to the digestion mixture. The intestinal digestion was done for 120 minutes at 37°C . The intestinal digestion was stopped by adding 96 μL PEFA block. The digested cheese samples were stored in plastic bags at 4°C for further analysis.

2.5. ACE-Inhibition Assay

The ACE-inhibition assay was performed using reversed-phase HPLC according to Qureshi *et al.* [22], following the method described by Hyun and Shin (2000), with some modifications. The liberated hippuric acid as a result of the reaction between HHL (hippuryl-histidyle-leucine) (substrate) and ACE (enzyme) in the presence or absence (control) of sample were quantified. The percent ACE inhibition was calculated from the following equation:

$$\text{ACE inhibition (\%)} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100$$

Where hippuric acid concentration was given as HA (control) and hippuric acid was liberated after reaction between substrate (without sample) and enzyme, while HA (sample) stands for hippuric acid released after enzyme and substrate (presence of sample) reaction.

2.6. Determination of Total Phenolic content of processed cheddar cheese

The total Phenolics contents were determined by using Folin-Ciocalteu reagent method with some modifications [23]. The freeze-dried samples of cheese samples were dissolved in ethanol and then filtered through $0.45 \mu\text{m}$ filters. The 0.5 mL sample was mixed with 1 mL of Folin-Ciocalteu reagent (10%). Then 2 mL of sodium carbonate (20%) solution was added into the above mixture after 6 min. The absorbance was taken at 760 nm using a spectrophotometer after 60 min incubation at 30°C .

2.7. Determination of total antioxidant capacity (TAC) of processed cheddar cheese

For the determination of total antioxidant capacity (TAC), the WSE and ESE of cheese samples were analyzed using the method described by Prieto *et al.* [24]. The freeze-dried cheese samples were dissolved in ethanol and then were filtered through $0.45 \mu\text{m}$ filters. 0.4 mL of sample was mixed with 4 mL of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) solution. After incubating the mixture for 95 min at 90°C , the absorbance was measured at 695 nm using a spectrophotometer. Spectrophotometer was calibrated with blank (methanol) solution prepared in the same manner.

2.8. Determination of DPPH radical scavenging activity of processed cheddar cheese

Using the method prescribed by Yi *et al.* [25] with some modifications, the capability of WSE and ESE of all the cheeses to scavenge 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) was measured. The ethanol was used to dissolve the freeze-dried samples of cheese and then filtration ($0.45 \mu\text{m}$ filters) was carried out before running the DPPH assay. 1 mL of water-soluble extract (WSE) and ethanol soluble extract (ESE) were taken and then added 2 mL of DPPH (60 μM in ethanol) solution into each separately. Then incubation was done for 30 minutes in the dark. Spectrophotometer was used to measure the absorbance at 517 nm. The preparation of control (ethanol) was also done using the same method.

2.9. Reducing power ability of processed cheddar cheese

The method described by Hegazy and Ibrahim [26] was adopted with some modifications in order to determine the reducing power of WSE and ESE of cheese samples. The freeze-dried samples of cheese were dissolved in ethanol and then filtered through $0.45 \mu\text{m}$ filters. 0.5 mL sample was mixed with 0.5 mL each sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$). After incubating the

mixture for 20 min (50 °C), 0.5 mL of trichloroacetic acid (%) was added into it. After 15 min, 0.2 mL of ferric chloride (0.1%) was added. The absorbance of the sample mixture was measured using spectrophotometer at 700 nm.

2.10. Sensory Evaluation

The score for overall acceptability, appearance, flavor, taste, texture and color for cheese samples was evaluated using 9 point hedonic scale. The sensory evaluation of cheese samples was done by panel of 15 judges including faculty members and students at Institute of Food Science and Nutrition, University of Sargodha, Sargodha.

2.11. Statistical analysis

Statistical analysis was performed using Minitab 16 software. 3-factors factorial design was executed with repeat batches (random variable), age (static variable; with the supposition that the individual cheeses were measured independently from the same batch) and milk type (fixed variable) of Cheddar cheese. Similarly, 3-factors factorial design was carried out with replicate block (random variable), digestion steps (fixed variable; including undigested samples) and milk type (fixed variable) of Cheddar cheese. Tukey test for pair wise comparison was used to test the differences between means. For all comparisons, the level of significance was set to $P < 0.05$.

3. Results

3.1. Total antioxidant activity (TAA) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC)

3.1.1. Total antioxidant activity (TAA) of freeze-dried water soluble and ethanol soluble extract of processed Cheddar cheese

The processed cheddar cheese with black pepper (6122.8 ± 205.4 $\mu\text{g/g}$ trolox equivalent) and clove (5897.5 ± 23.29 $\mu\text{g/g}$ trolox equivalent) powder shows the maximum values for TAA after *in vitro* digestion of ripened (PDC), while the minimum TAA was observed in case of cumin processed Cheddar cheese (4739 ± 153.02 $\mu\text{g/g}$ trolox equivalent) (Figure 1).

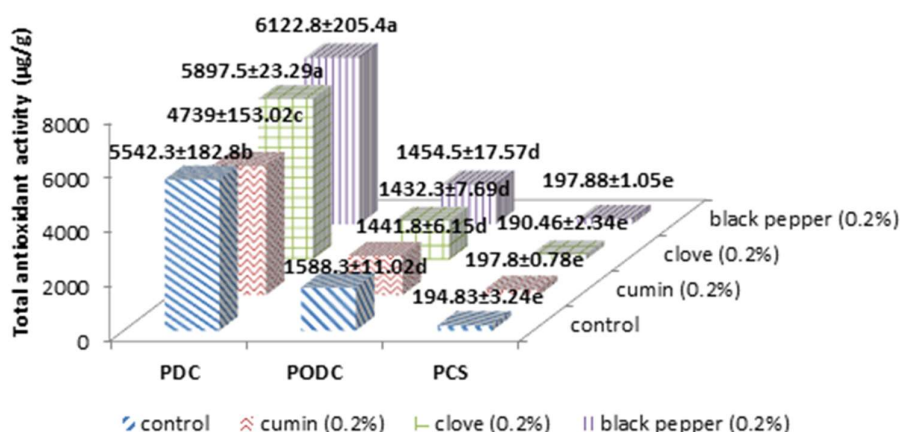


Figure 1. Mean values for Total antioxidant activity (TAA) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC) made from buffalo milk after ripening.

Freeze dried ethanol extract of processed Cheddar cheese showed increased total antioxidative potential with the addition of spices such as cumin, clove and black pepper powder with 0.2 % concentration as compared to water soluble extract. This may be because peptides which are responsible for antioxidative activity were more soluble in

ethanol as compared to water. Among the spices, the higher total antioxidant capacity (432.27 ± 2.28 $\mu\text{g/g}$ trolox equivalent) was observed in ethanol extract of cumin processed Cheddar cheese as compared to water soluble extract (Figure 2). The processed Cheddar cheese extracts (WSE and ESE) from cow and buffalo milk have greater potential for exhibiting total antioxidant potential as compared to control Cheddar cheese extracts (WSE and ESE). Thus, this may be due the antioxidative potential of individual spices as reported by different studies.

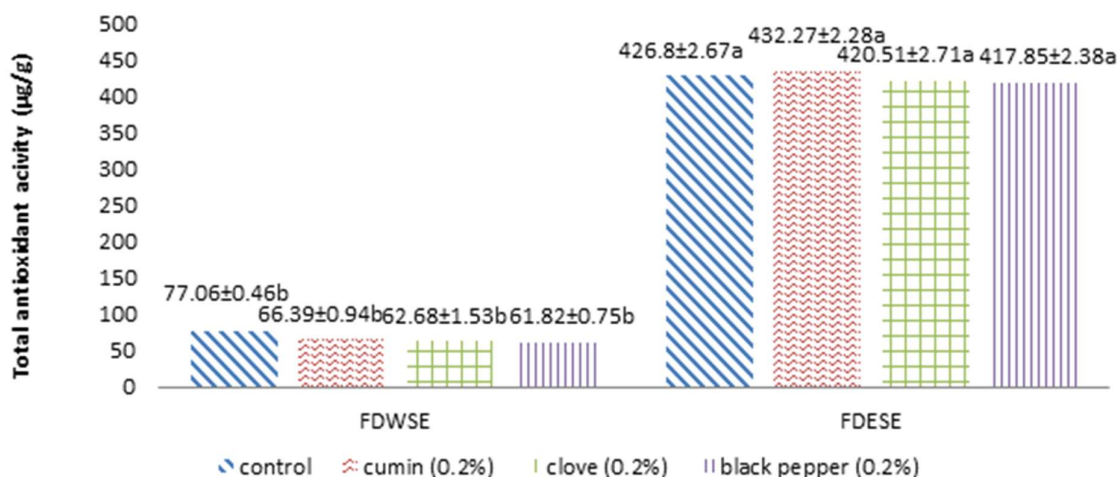


Figure 2. Mean values for Total antioxidant activity (TAA) of freeze-dried water soluble (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese made from buffalo milk after ripening.

3.2. Total phenolic content (TPC) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC)

The TPC of processed Cheddar cheese increased after oral and duodenal stimulated *in vitro* digestion, although no considerable effect of individual spices was observed on TPC of cheese after digestion. After *in vitro* digestion at 9 months of ripening, clove PODC (2982.1 ± 4.74 $\mu\text{g/g}$ gallic acid equivalent) and cumin PDC (15269 ± 1.16 $\mu\text{g/g}$ gallic acid equivalent) showed the maximum mean values (Figure 3). No significant increase in phenolic content of ripened processed Cheddar cheese was observed with the addition of spices before and after digestion.

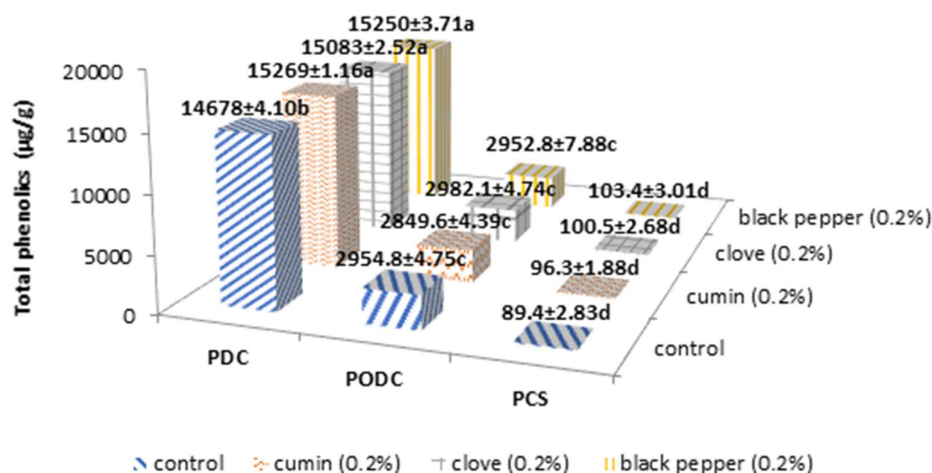


Figure 3. Mean values for Total phenolic content (TPC) (µg/g gallic acid equivalent) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC) made from buffalo milk after ripening.

3.2.1. Total phenolic content (TPC) of freeze-dried water soluble and ethanol soluble extract of processed Cheddar cheese

Phenolic content of WSE and ESE of processed cheese was not affected by the addition of spices. Higher phenolic content values were observed in water soluble extracts as compared to ethanol soluble extract (Figure 4). Spices were not found to contain any phenolic compounds. More studies need to be done to find their effect on antioxidant potential of cheese.

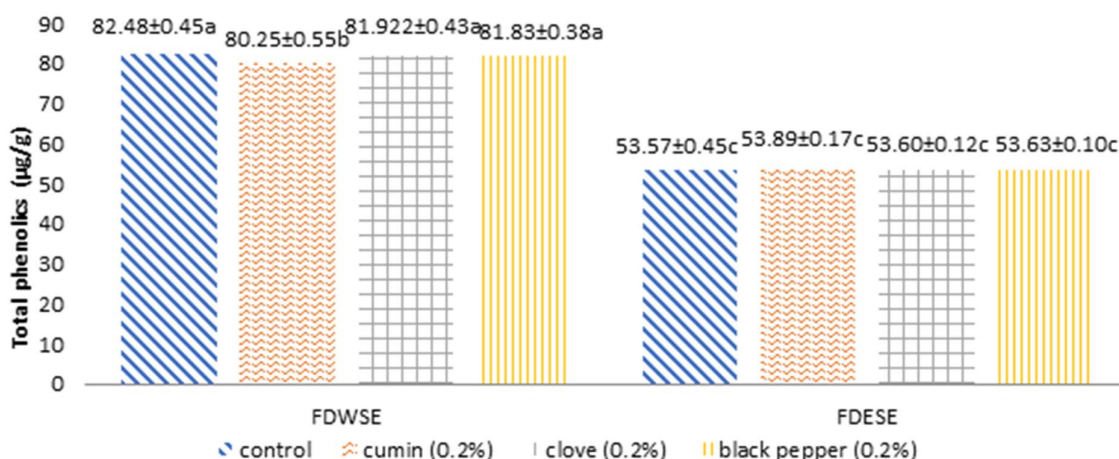


Figure 4. Mean values for Total phenolic content (TPC) (µg/g gallic acid equivalent) of freeze-dried water soluble (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese made from buffalo milk after ripening.

3.3. Reducing power (RP) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC)

Significant increase in reducing power ability of ripened processed Cheddar cheese was observed with the addition of spices before and after digestion. The RP of processed Cheddar cheese increased after oral and duodenal stimulated *in vitro* digestion, although effect of individual spices was observed on RP of cheese after digestion might be due to

release of compounds from cheese and spices which have redox potential. After *in vitro* oral and duodenal digestion at 9 months of ripening cumin PODC (943 ± 6.34 $\mu\text{g/g}$ trolox equivalent) and clove PDC (3772.7 ± 8.82 $\mu\text{g/g}$ trolox equivalent) showed the maximum mean values respectively (Figure 5).

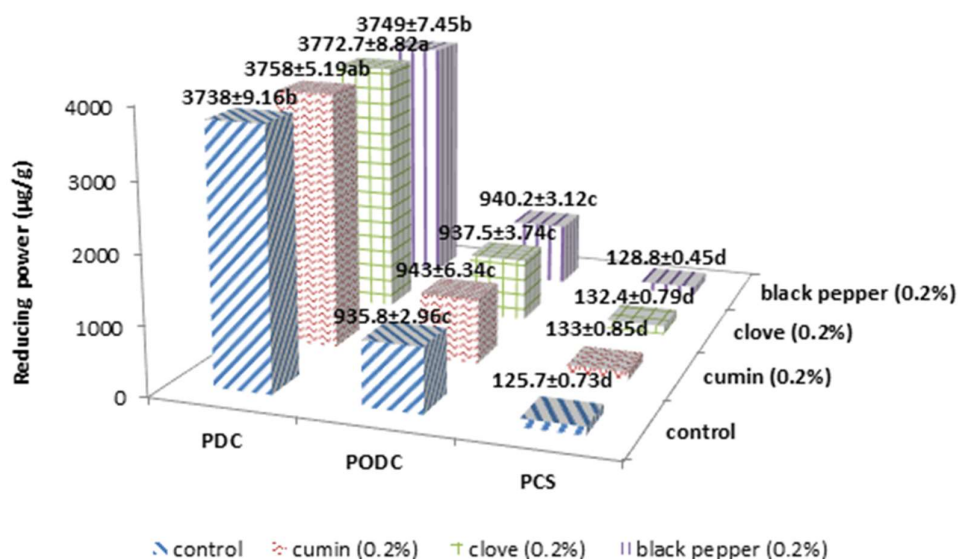


Figure 5. Mean values for Reducing power (RP) ($\mu\text{g/g}$ trolox equivalent) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC) made from buffalo milk after ripening.

3.3.1. Reducing power (RP) of freeze-dried water soluble (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese

Spices were found to contain limited compounds which exhibit reducing power, however processing and storage conditions might be responsible for reduced RP in WSE and ESE of processed Cheddar cheese. The highest reducing power was observed in black pepper cheese WSE (38.12 ± 0.61 $\mu\text{g/g}$ trolox equivalent) and black pepper cheese ESE (39.71 ± 0.11 $\mu\text{g/g}$ trolox equivalent) after ripening in buffalo processed Cheddar cheese (Figure 6).

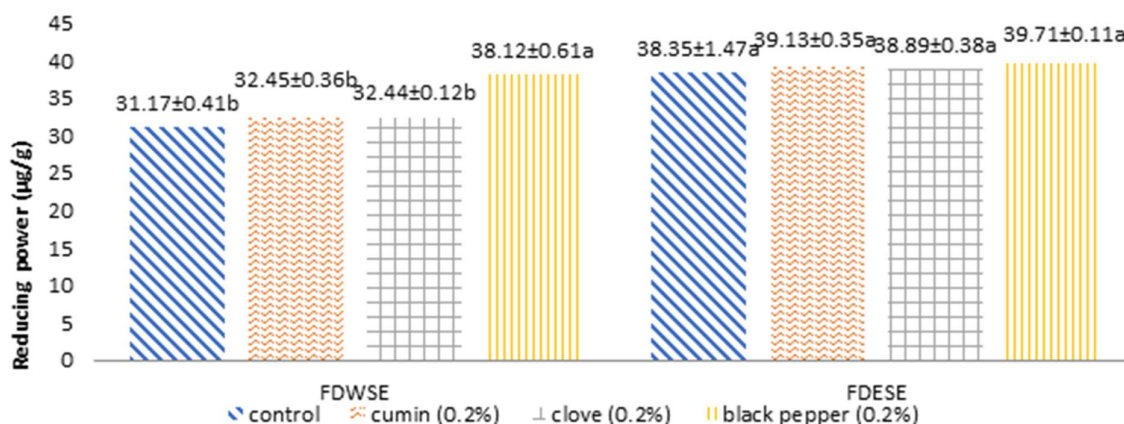


Figure 6. Mean values for Reducing power (RP) ($\mu\text{g/g}$ trolox equivalent) of freeze-dried water soluble and ethanol soluble extract of processed Cheddar cheese made from buffalo milk after ripening.

3.4. DPPH (Radical Scavenging Activity) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC)

Substantial increase in radical scavenging activity (DPPH) of ripened processed Cheddar cheese was observed with the addition of spices before and after digestion. The DPPH of processed Cheddar cheese increased after oral and duodenal stimulated *in vitro* digestion, although effect of individual spices was also observed on DPPH of cheese after digestion might be due to release of compounds from cheese and spices which have peptides with radical scavenging ability. Among processed Cheddar cheese samples, radical scavenging activity of cheese was affected by addition of spices (cumin, clove and black pepper). After *in vitro* digestion at 9 months of ripening, cumin PODC (5621.8 ± 5.21 $\mu\text{mol/ml}$ trolox equivalent) and cumin PDC (22533 ± 3.86 $\mu\text{mol/ml}$ trolox equivalent) showed the maximum mean values (Figure 7).

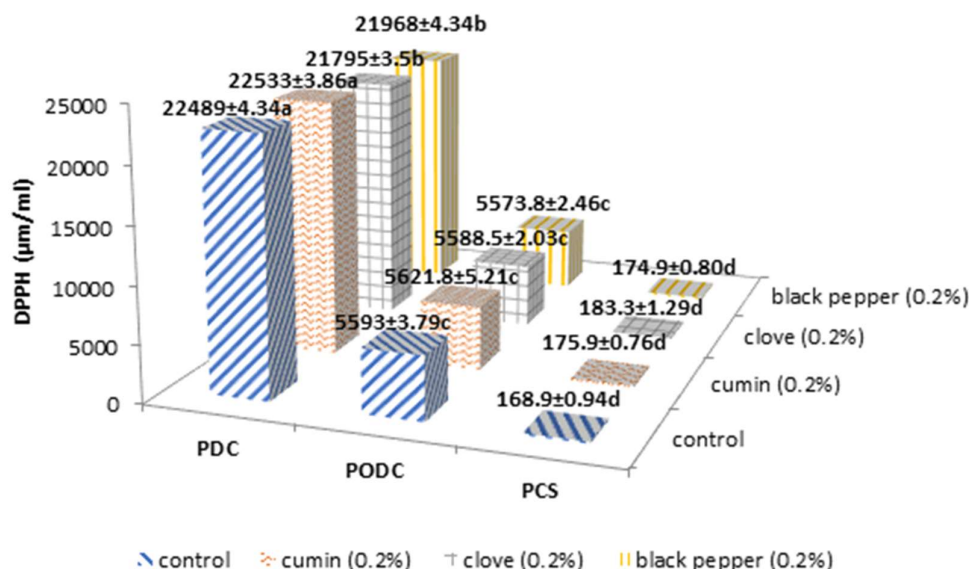


Figure 7. Mean values for DPPH (Radical Scavenging Activity) ($\mu\text{mol/ml}$ trolox equivalent) of processed Cheddar cheese (PCS), orally digested (PODC) and duodenal digested Cheddar cheese (PDC) made from buffalo milk after ripening.

3.4.1. DPPH (Radical Scavenging Activity) of freeze-dried water soluble (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese

The highest DPPH value was observed in cumin cheese WSE (1525.8 ± 1.43 $\mu\text{mol/ml}$ trolox equivalent). In case of ethanol soluble extract, black pepper cheese (1587.8 ± 0.55 $\mu\text{mol/ml}$ trolox equivalent) and clove (1586.1 ± 1.44 $\mu\text{mol/ml}$ trolox equivalent) showed maximum values (Figure 8).

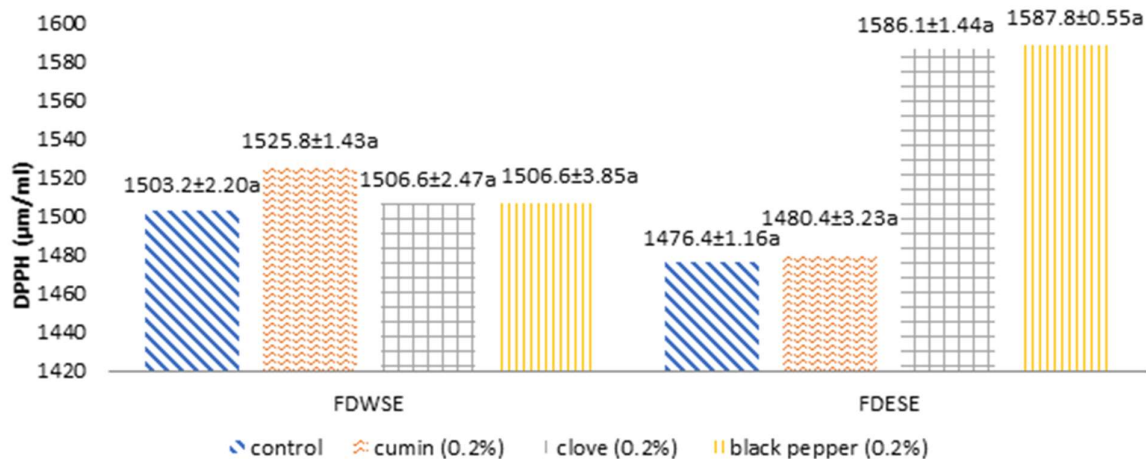


Figure 8. Mean values for DPPH (Radical Scavenging Activity) (µmol/ml trolox equivalent) of freeze-dried water soluble and ethanol soluble extract of processed Cheddar cheese made from buffalo milk after ripening.

3.5. Determination of ACE-Inhibition (%) and IC50 values (mg/ml) of processed Cheddar cheese

The inhibitory activity of angiotensin 1-converting enzyme (ACE) was estimated after *in vitro* oral and duodenal enzymatic digestion in buffalo milk processed Cheddar cheese (addition of spices e.g. cumin, clove and black pepper at 0.2g/100g concentration) after ripening. The inhibitory activity of ACE was also monitored in water soluble extract (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese. ACE inhibitory activity is expressed as IC₅₀ value (as the amount of protein content needed to inhibit 50% of the ACE inhibitors). In 9 months ripened processed Cheddar cheese (PODC) with the addition of spices e.g. cumin (CPODC), clove (LPODC), black pepper (BPPODC) after oral digestion the maximum value for ACE-inhibition % was observed in BPPODC 69.1±0.55% (IC₅₀ value 0.76±0.01mg/ml) (Table 1). The duodenal digestion significantly increases the percent inhibitory activity of ACE in Cheddar cheese with ripening which may be resulted from the production of more ACE inhibitory peptides after degradation of proteins in *in vitro* digestion. After duodenal digestion the maximum value for ACE-inhibition % was observed in black pepper BPPDC 91.2±0.75% (IC₅₀ value 0.56±0.01mg/ml) (Table 1).

Table 1. ACE inhibitory activity is expressed as IC₅₀ value (as the amount of protein content needed to inhibit 50% of the ACE inhibitors). Each observation is mean of three replicates of each batch. Results are expressed as mean of scores ± standard error of mean.

Cheddar cheese samples	Treatments	ACE-Inhibition activity	
		ACE-Inhibition (%)	IC ₅₀ Values mg/ml
Orally Digested processed cheddar cheese samples	CPODC	66.2 ^c ±1.00	0.95 ^a ±0.01
	LPODC	67.5 ^b ±0.47	0.86 ^b ±0.01
	BPPODC	69.1 ^a ±0.55	0.76 ^c ±0.01
Duodenal digested processed cheddar cheese samples	CPDC	89.5 ^b ±0.55	0.75 ^a ±0.03
	LPDC	90.3 ^{ab} ±0.33	0.67 ^b ±0.01
	BPPDC	91.2 ^a ±0.75	0.56 ^c ±0.01

CPODC: Orally digested processed Cheddar cheese sample (addition of cumin powder 0.2% after ripening)
LPODC: Orally digested processed Cheddar cheese sample (addition of clove powder 0.2% after ripening)
BPPODC: Orally digested processed Cheddar cheese sample (addition of black pepper powder 0.2% after ripening)
CPDC: Digested processed Cheddar cheese sample (addition of cumin powder 0.2% after ripening)

LPDC: Digested processed Cheddar cheese sample (addition of clove powder 0.2% after ripening)
BPPDC: Digested processed Cheddar cheese sample (addition of black pepper powder 0.2% after ripening)

3.5.1.Determination of ACE-Inhibition % and IC50 (mg/ml) values of freeze-dried water (WSE) and ethanol soluble extract (ESE) of processed Cheddar cheese

Lower IC50 values were obtained for freeze dried ESE as compared to WSE. More ACE inhibitory peptides may be present in ESE with more potent ACE inhibition activity as compared to WSE. The highest ACE inhibition percentage was found in BPFDESE 90.3±0.49% (IC50 value 0.19±0.02 mg/ml) while in freeze-dried water-soluble extract more percentage was found in BPFDWSE 88.4±0.32% (IC50 value 0.21±0.01mg/ml) (Table 2). Lower IC50 values were calculated in freeze dried ESE of black pepper processed Cheddar cheese as compared to WSE after ripening period of 9 months (Table 2).

Table 2. ACE inhibitory activity is expressed as IC50 value (as the amount of protein content needed to inhibit 50% of the ACE inhibitors). Each observation is mean of three replicates of each batch. Results are expressed as mean of scores ± standard error of mean.

	Cheddar cheese samples (Buffalo milk)	ACE-Inhibition activity	
		ACE-Inhibition (%)	IC ₅₀ Values mg/ml
Water Soluble Extract of processed cheddar cheese samples	CFDWSE	82.3 ^e ±0.47	0.26 ^a ±0.01
	LFDWSE	85.5 ^{cd} ±0.43	0.24 ^b ±0.02
	BPFDWSE	88.4 ^b ±0.32	0.21 ^e ±0.01
Ethanol Soluble extract of processed cheddar cheese	CFDESE	84.1 ^{de} ±0.43	0.23 ^c ±0.01
	LFDESE	87.2 ^{bc} ±0.33	0.22 ^d ±0.03
	BPFDESE	90.3 ^a ±0.49	0.19 ^f ±0.02

CFDWSE: Freeze dried water soluble extract cumin cheese sample (Cumin 0.2 %)
LFDWSE: Freeze dried water soluble extract clove cheese sample (Clove 0.2%)
BPFDWSE: Freeze dried water soluble extract BP cheese samples (Black pepper 0.2%)
CFDESE: Freeze dried ethanol soluble extract cumin cheese sample (Cumin 0.2 %)
LFDESE: Freeze dried ethanol soluble extract clove cheese sample (Clove 0.2%)
BPFDESE: Freeze dried ethanol soluble extract BP cheese samples (Black pepper 0.2%)

3.6. Sensory score of processed Cheddar cheese

All the cheese samples of processed Cheddar cheese (after ripening) were subjected to sensory evaluation with a panel of fifteen judges and evaluated for different sensory attributes like flavor, taste, color, texture and overall acceptability, following the 9 Point Hedonic Scale Performa presented to the panelists for recording scores. Highly significant (p<0.01) effect of spices was observed on sensory score of color, flavor, taste, texture and overall acceptability of processed Cheddar cheese (buffalo milk) after ripening. The best score for color (7.85±0.44), flavor (7.92±0.42), taste (7.96±0.45) and texture (7.95±0.42), overall acceptability (7.92±0.42) were observed for cumin processed cheddar cheese after ripening (Figure 9). The development of flavoring compounds was improved with the

progress of ripening. It means ripening positively affected the taste, texture, color and overall acceptability of processed Cheddar cheese made from buffalo milk.

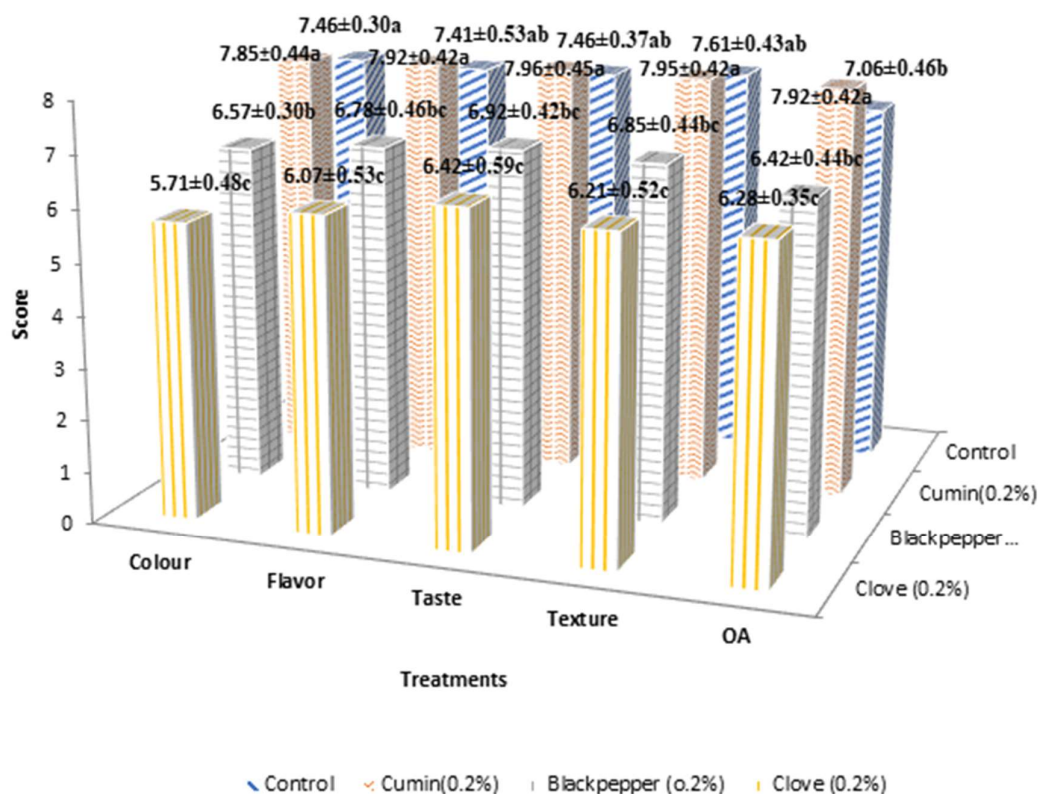


Figure 9. Mean values for sensory score of processed Cheddar cheese made from buffalo milk after storage.

4. Discussion

The results obtained regarding ACE-inhibitory activity after digestion of processed cheese using intestinal enzymes in the present study were concurrent to the studies conducted by Qureshi *et al.* [22] Srinivas *et al.* [27]. In addition, Srinivas *et al.* [27] and Barac *et al.* [28] also found increased antioxidant activity after digestion of bovine milk proteins and Serbian white-brined cheese respectively, using enzymes which is consistent with our results of the present study. No doubt, many bioactive peptides from milk proteins are also released during gastric phase [29, 22] but in the present study, we conducted oral phase and intestinal phase digestion experiments because most of the bioactive peptides are liberated in duodenal phase compared to gastric phase [22].

The peptides in the intact condition of original proteins are usually inactive within the sequence but can be liberated through the action of different enzymes from stomach (especially pepsin), intestine (chymotrypsin and others) in *in vitro* models [30, 31]. The liberated peptides through gastrointestinal digestion are active and have been shown different bioactivities like for instance, ACE-inhibitory and antioxidant activities, in different types of cheeses [32,33,34,35]. The increased bioactivities after simulated gastrointestinal digestion might be due to liberation of more and more low molecular weight peptides in the extracts.

Scarce data is available on the ACE-inhibitory and antioxidant activities of cheese with spices, to compare with our studies but Ahmed *et al.* [16] investigated antioxidant potential of plaited pickled cheese (Mudaffara) cheese with the addition of black pepper, black cumin. Both ACE-inhibitory and antioxidant activities of different types of cheeses

increased with the progress of ripening period [36,37,38,39] which corroborate the results obtained in the present study. In some previous studies, it was found that most of the bioactive peptides are dissolved in ethanol soluble fractions of cheeses and these fractions showed potent bioactivities compared to water soluble fraction [20]. Therefore, in the present study, both WSE and ESE were studied.

The results of TAA of processed cheddar cheese after in-vitro digestion results were similar to those by Lee *et al.* [40] who made soft goat cheese (unfrozen and 3 months frozen) with the addition of tocopherol. Ahmed *et al.* [16] determined the antioxidant activity of individual spices such as black cumin (*Nigella sativa*), clove (*Syzygium aromaticum*), and black pepper (*Piper nigrum*) using (DPPH free radical scavenging assay). The Mudaffara cheese was prepared using the spices and its sensory evaluation and antioxidative potential was measured [16]. According to research, highest antioxidative potential of ethanol and water-soluble extracts of black pepper were determined and confirmed [41,42,43]. As well as highest antioxidation potential was estimated and observed in clove. The extract obtained from clove was reported to have strongest antioxidative capacity which may be due to its grater metal chelating ability and hydrogen bonding capacity and additionally its efficiency was due to presence of free radicals, superoxide and may act as scavenger of hydrogen peroxide [44]. Increase values for TPC were obtained in digested cheese samples (fortified with increased amount of catechins) while the value for TPC was measured double as compared to the value of control in case of cheese fortified with 500 mg/kg catechin [45]. Various varieties of cheeses were investigated for their total phenolic content [46] e.g. smoked cheeses, goat milk cheese (where animals feed on different feeding systems) and production of cheeses from milk using different quantities of plant extracts [47,48]. According to research done by Levkov *et al.* [49], no survey was performed regarding the phenolics content of conventionally manufactured cheeses and the potential of these compounds to hinder oxidation process.

The results of RP were in accordance with the studies done by Liu *et al.* [50]. The results were reported to significantly increased ($P < 0.05$) for antioxidant activity after simulated gastrointestinal digestion. The reducing power values for Cheddar cheese samples B-1 (Cheddar cheese with starter culture) and B-2 (Cheddar cheese with starter culture and *Lactobacillus rhamnosus*) were 0.503 and 0.696 respectively which signifies an increase of 13.03 (%) and 17.57 (%) individually [50]. The results were in accordance with the finding by Abadía-garcía *et al.* [51]. According to research, extension in ripening period of Cheddar cheese might resulted in enhanced antioxidation potential due to the production of peptides which exhibit antioxidant potential [51]. The contribution ability of samples for protons and electrons was dependent on relation of peptide cleavages and reducing power [52]. The finding of DPPH was supported by studies of Ahmed *et al.* [16]. It was confirmed by the studies that addition of 0.5% black pepper (grounded form) during manufacturing significantly ($P \geq 0.05$) raised the antioxidant potential of Mudaffara cheese (65.3%) as compared to fresh cheese (48.4%) as well as the amount of tested ethanol (extract) increases from 50 to 200 μg . The concentrations of ethanol extract have considerable positive impact on antioxidative potential of cheese with ripening period of 4 or 8 weeks at 30 ± 2 or $7 \pm 2^\circ\text{C}$ temperature respectively. These results were confirmed by Burits and Bucar [53]. According to research, it was confirmed that peptides were present in WSE which were easily reachable to DPPH as well as Hydroxyl radicals [54]. The higher mean values for DPPH peptides were observed after 60 days of ripening but a decreasing trend was found after 120 days of ripening. According to another research, antioxidative potential of Cheddar cheese was observed higher in first ripening period while decreased in 2nd period of ripening [55]. Seed (*Piper nigrum* Linn.) of black pepper along with its WSE and ESE were reported to exhibit antioxidant as well as (DPPH) radical scavenging activities [15,56,57].

The results of ACE inhibition after in-vitro digestion were in closed agreement with Qureshi *et al.* [58]. According to research, it was reported that higher values for ACE inhibition was obtained in Gamalost (Norwegian cheese) which confirmed the production of more peptides (with potent ACE inhibitory potential) after gastrointestinal digestion.

More peptides were generated after digestion with human gastric juice and duodenal gastric juice which possesses additional activity as equated to undigested peptides, this resulted in lower IC_{50} values. The peptides which release after gastrointestinal digestion exhibited greater ACE inhibitory potential and possesses molecular mass less than 3kDa in Norvegia cheese and soluble fraction of Gamalost (pH 4.6); as the noticeable decrease in IC_{50} values was obtained after gastrointestinal digestion [58]. The results of ACE inhibition potential of water-soluble and ethanol-soluble fractions were similar to the study conducted by Bara' *et al.* [28]. It was reported that water soluble fractions of traditional cheeses exhibited different potential for ACE inhibition. The water-soluble fractions vary with variety and their IC_{50} values were from 2.26 to 4.61 (mg/ml). Generally, lower ACE inhibition activity was observed in sheep milk cheeses as compared to cow cheeses. The good ACE inhibitory potential was measured in WSF of cow cheese (Zlata) while WSF of sheep cheese (Sjenica) possesses lowest ACE inhibition potential.

The results regarding the change in color of cheese are similar to the findings of Mamo [59]. A significant change ($P < 0.05$) in color of cheese was obtained within ripening period. It was observed that color changes positively affect the sensory score of cheese as color gets better with ripening. The mean values for color score increased from 7.15 to 7.95 during ripening. Milk constituents, manufacturing approaches and ripening state may prejudice the color of cheese, while the cheese color vary with the variety of cheese and it have no adverse effect on cheese flavor [60]. The results of flavor score are like the findings of Mamo [59]. It was reported that ripening significantly ($P < 0.05$) affected the flavor of cheese. The flavor of cheese improved with ripening and the mean values for flavor ranged from 8.0 (ripened) to 4.60 (pre-ripened). Many compounds were responsible for flavor characterization of cheese e.g. sequence of microbial, metabolic, and biochemical alterations during cheese ripening as well as enzymes (milk and rennet), starter bacteria, lipases and microflora (secondary) may contribute to flavor development in cheese. Mamo *et al.* [59] observed significant difference for taste score of cheese samples with ripening. Ripened cheese tasted much well with mean value for taste score 8.15 as compared to pre-ripened cheese samples with mean values for taste score 5.80 [59]. It was identified that sodium chloride was added during cheese preparation was responsible for salty taste of Cheddar cheese due to its organic ions. Content of FAA and peptides stimulated the umami, bitter and sweet taste in Cheddar cheese [61]. Mamo *et al.* [59] reported positive increase in texture score of cheese with ripening. According to Murtaza *et al.* [62], different biochemical and microbial alterations occurred throughout ripening were responsible for texture changes and development. Mamo *et al.* [59] observed that the overall acceptability score was higher in ripened cheese (8.05) as compared to pre-ripened cheese (6.15). According to Ahmed *et al.* [16], spices affected the overall acceptability score of cheese (Mudaffara) during storage (room and refrigeration temperature). The black pepper cheese (19.0) and black cumin cheese (18.4) samples at 7 ± 2 °C temperature were found with more overall acceptability score during ripening (after 4th week) whereas, overall acceptability score for clove cheese was higher after 2nd week of ripening.

5. Conclusion

This study showed that the ACE-inhibitory potential was highest in processed cheese (with addition of 0.2% cumin, clove and black pepper) made from buffalo milk. Higher ACE-Inhibition percentage was observed in ethanol soluble extract as compared to water soluble extract of processed cheddar cheese. Among the spices, higher ACE-Inhibition percentage was found in black pepper processed cheddar cheese after ripening as compared to cumin and clove. The antioxidant activity was increased after *in vitro* digestion in processed cheddar cheese. Addition of spices also considerably increases the antioxidant potential of ripened processed cheddar cheese before and after *in vitro* digestion.

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investigation, A.S. and M.N.; resources, M.S.; data curation, A.S. and M.N.; writing—original draft preparation, A.S.; writing—review and editing, M.N.; M.S.; R.R.S.; O.K. and R.K. visualization, M.S.; R.R.S.; O.K. and R.K.; supervision, M.N.; project administration, M.S.; R.R.S.; O.K. and M.N. funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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