



ANTIOXIDANT POTENTIAL OF CHEDDAR CHEESE PREPARED FROM COW AND BUFFALO MILK DURING RIPENING

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ABSTRACT

A study on antioxidant potential of cheddar cheese utilizing milk from cow and buffalo sources during ripening was conducted in 2019-20 at Institute of Food science and Nutrition, University of Sargodha, Pakistan. The cheese samples were placed at the controlled atmosphere (4°C) for ripening and were ripened for 3, 6 and 9 months. This research was conducted to evaluate antioxidant ability of cheddar cheese during ripening. Freeze dried water (WSE) and ethanol soluble fractions (ESE) of cheddar (cow and buffalo) were prepared and monitored for their antioxidant activities during ripening. Total antioxidant activity (TAA) of soluble fractions (WSE and ESE) was determined. Higher TAA was found in WSE (59.900 ± 1.59 µg/g trolox equivalent) and higher reducing power was observed in ESE (27.734 ± 5.76 µg/g) of buffalo milk cheddar cheese after 9 months of ripening. Increased values were found for total phenolic content (TPC) in freeze dried WSE (69.572 ± 5.88 µg/g gallic acid equivalent) and for DPPH in freeze-dried ESE (1436.1 ± 4.95 µmol/ml trolox equivalent) of cow milk cheddar cheese after 9 months of ripening. In short, it (cheddar cheese) can be utilized in manufacturing of functional foods due to its increased antioxidant potential.

KEYWORDS: Antioxidant activity; cheddar cheese; ethanol soluble extract; water soluble extract; Pakistan

INTRODUCTION

Fermentation is a process used to preserve milk into different products such as various cheese varieties. During ripening, a diverse proteolytic system was observed in cheddar cheese through the action of indigenous milk enzymes (peptidases and proteinases), coagulants, starter and non-starter lactic acid bacteria and adjuncts (McSweeney, 2004). Water-soluble and water insoluble peptides were produced as a result of proteolysis. These peptides are further hydrolysed to produce amino acids and smaller peptides. Crude extract of water-soluble peptides (WSPs) may synergistically act to perform several physiological functions as antihypertensive, anti-oxidative, anti-inflammatory, anticancer, antibacterial, antithrombotic, and cholesterol lowering properties (Ong *et al.*, 2007). There is a series of biochemical changes during the cheese ripening period, in which the degradation of protein is the major one. These peptides not only contribute to the formation of flavor and texture of

ripened cheese, but also attain bioactivities in certain time of ripening (Saito *et al.*, 2000). Importantly, bioactive compounds exhibit antioxidant activity. Superfluous free radicals and oxygen species with high reactivity resulted in disastrous cellular possessions such as apoptosis carried out by cell membrane lipids, DNA, oxidized cellular protein and enzymes. Naturally existing antioxidants in different foods could safeguard the human being by delaying the progress of many chronic diseases (Aliakbarian *et al.*, 2009; Aloğlu *et al.*, 2011; Liu *et al.*, 2005). Antioxidant activity of ripened cheeses is also considered important. Antioxidant activity of Cottage cheese increased with increased storage time. This might be due to proteolytic activity of microorganisms (Abadía-garcía *et al.*, 2013). Gupta *et al.* (2009) considered and described rise in antioxidant properties of cheddar cheese during ripening up to 4 months after which it decreased and remained persistent on or after 7 to 9 months of maturing. The goal behind this study was to categorize the antioxidant

ability of freeze-dried ethanol soluble extract (ESE) and water-soluble extract (WSE) of Cheddar cheese prepared using milk of cow and buffalo during ripening.

MATERIALS AND METHODS

This research was conducted to determine the effect of ripening on the development of bioactive components in cheddar cheese during ripening related to antioxidant activity made from cow and buffalo milk. The cheese samples were prepared and stored at Acha Dairy Industries, Lahore, Pakistan in year 2019. The initial research work was conducted at Food Analysis Laboratory and Microbiology Laboratory at the Institute of Food Science and Nutrition, University of Sargodha, Pakistan. The remaining research work was conducted at Food Science and Biochemistry Laboratory at Department of Chemistry, Biotechnology and Food Science (IKBM), Norwegian University of Life Sciences, as, Norway in year 2019-20.

Manufacturing of cheddar cheese

Cow and buffalo milk was collected to prepare Cheddar cheese. Both types of milk were standardized as per data in Table 1. All the apparatus was properly cleaned and manufacturing was done under hygiene conditions. The process followed by Lawrence *et al.* (2004) with few amendments, was selected to make cheddar cheese. The cheese production was carried out on three different days (batches) and the cheeses from each batch were randomly selected for ripening (3, 6 and 9 months). One cheese from each batch was also kept as fresh. Chemicals for analysis were purchased from Sigma Aldrich (Seelze, Germany) and Lab-Scan (Dublin, Ireland) available at local market in Sargodha, Pakistan.

Grating of the cheddar cheese

Conferring to the International Dairy Federation (IDF) standard 50C, all the cheeses (ca. 250 g) were cut identically to get four equally sampled pieces (IDF, 1995). The cheese samples were stored in glass containers in cold storage after grating with a grinder (manual) until used for further prescribed analysis.

Packaging and Storage of cheddar cheese

Modified atmosphere packaging was used for sustaining quality of cheese for the period of storage in order to prevent water loss by dehydration and to preclude the development and occurrence of

detrimental microorganisms, specifically pathogens. The packaging of cheese was done under the supervision of expertise in packaging section at Achha Dairies Industries, Lahore, by means of wrapping films with minimum water vapor diffusion: semi-barrier (low density polyethylene or polypropylene). The storage of cheddar cheese was done in cheese cellar at 4°C for 3, 6 and 9 months at Achha Dairies Industries-Pvt-Ltd Lahore.

Preparation of water-soluble (WSE) and ethanol-soluble extracts cheddar cheese

Extracts of all the cheeses were prepared by following method of Pripp *et al.* (2006) (Fig. 1). Cheese (15.001 g) was firstly grated and then blended with water (50 mL). The mixture was heated for 5 minutes at 40°C using water bath. Homogenization of above mixture was carried out for 2 minutes using an Omni-Mixer homogenizer (Omni International, Waterburg, CT). pH 4.6 was adjusted and the weight of mixture was made up to 100 g. The samples were put again in water bath for 1 hour at a temperature of 40°C to soften or melt all the fat in the cheese. Then the samples were centrifuged at 4500 rpm (3000 g) for 30 min while maintaining a temperature of 40°C. Then Whatman filter (No.1) was used to filter and remove the middle soluble layer and this fraction was designated as WSE which was freeze dried for further analysis. Samples after freeze drying were kept at -21°C.

Total Phenolic content of WSE and ESE of cheese

Folin-Ciocalteu reagent technique was used to estimate the total phenolic contents (Singleton *et al.*, 1999). The freeze-dried samples of all the cheeses were dissolved in ethanol and then 0.45 µm thick filters were used for filtration. A mixture of 0.5 mL sample with 1.0 mL of Folin-Ciocalteu reagent (10%) was prepared. Then, after 6 minutes, twenty percent solution of sodium carbonate approximately 2.0 mL was poured into the above blend. The UV- visible light spectrophotometer (Shimadzu UV 1800) was used to take the readings of absorbance at 760 nm after 60 min incubation at 30°C. All the experiments were done twice and readings were taken thrice to reduce any ambiguity.

Total antioxidant capacity (TAC) of WSE and ESE of cheese

For calculating the TAC, the WSE and ESE of all the cheeses were tested using the method adopted by

Table 1. Basic composition of milk used for manufacturing of cheddar cheese

Milk sample	Total solids %	Moisture %	Fat %	Protein %	Lactose %	Minerals %
Cow milk	13.73±0.03	84.66±0.05	3.53±0.01	3.56±0.02	4.82±0.02	0.69±0.02
Buffalo milk	13.87±0.01	84.69±0.18	3.59±0.04	3.69±0.04	4.96±0.02	0.71±0.01



Fig. 1. Preparation of water and ethanol soluble extract using various approaches

Prieto *et al.* (1999). The filtration of freeze-dried samples of different types of cheese was done using 45 μm filters after dissolving in ethanol. 0.4 mL of sample was mixed with 4.0 mL of reagent (4mM $(\text{NH}_4)_2\text{MoO}_4$, 0.6 M H_2SO_4 and 28 mM Na_2PO_4) solution. After incubation of mixture at 90°C for 95 minutes, the transmission density was calculated at 695 nm using a UV- visible light spectrophotometer (CECIL_CE 7200). All the experiments were done twice and readings were taken thrice to reduce any ambiguity.

DPPH radical scavenging activity of WSE and ESE of cheese

Using the technique adopted by Yi *et al.* (2008) with some alterations, the capability of WSE and ESE of all the cheeses to scavenge DPPH was recorded. Ethanol was used to dissolve the freeze-dried samples of all the cheeses and then filtration (0.45 μm filters) was carried out before running the DPPH assay. 1.0 mL of water-soluble extract (WSE) and ethanol soluble extract (ESE) were taken and then added 2.0 mL of DPPH (60 μm in ethanol) solution into each separately. Then incubation was done for 30 minutes in the dark. UV- visible light spectrophotometer (CECIL_CE 7200) was used to measure the absorbance at 517 nm. The preparation of control (ethanol) was also done using the same method. All the experiments were done twice and readings were taken thrice to reduce any ambiguity.

Reducing power ability of WSE and ESE of cheese

The method described by Hegazy and Ibrahim (2012) was chosen with some changes in order to determine the reducing power of WSE and ESE of all the cheeses. The freeze-dried samples of all the cheeses were dissolved in ethanol and then filtered through 0.45 μm filters. A mixture of 0.5 mL sample with 0.5

milliliter (0.2 M, pH 6.6) each Na_2PO_4 buffer and $\text{K}_3\text{Fe}(\text{CN})_6$ was prepared. The incubation of the mixture was done for 19 min (51degree Celsius), afterwards, 0.5 mL (%) of TCA (trichloroacetic acid) was added into it. After 15 min, 0.2 mL of FeCl_3 (0.1%) was mixed in the solution. The UV- visible light spectrophotometer (CECIL_CE 7200) was utilized to record absorbance of sample mixture at 700 nm. All the experiments were done twice and readings were taken thrice to reduce any ambiguity. Data were analyzed by using the statistical procedure described Steel *et al.* (1997).

RESULTS AND DISCUSSION

Total antioxidant activity of freeze-dried water-soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese

Total antioxidant activity (TAA) of freeze-dried water and ethanol soluble fractions of cheddar cheese from the milk of cow and buffalo was determined with the ripening period of 9 months. In cow milk cheddar cheese, the mean value of TAA of freeze dried WSE ($38.322 \pm 1.11 \mu\text{g/g}$) was found higher as compared to ESE ($27.911 \pm 2.19 \mu\text{g/g}$ trolox equivalent) throughout the ripening period and the maximum antioxidant potential of freeze dried WSE and ESE was observed after 9 months of ripening period. The mean value increased from 13.513 ± 0.68 to $47.460 \pm 5.22 (\mu\text{g/g}$ trolox equivalent) after 9 months of ripening period (Fig. 2). Positive correlation exists between TAA and ripening because during ripening intensive proteolysis occur and the concentration of amino acid and peptides increased. Similar results were found in freeze dried WSE and ESE of buffalo milk Cheddar cheese during storage. The higher mean value for TAA was observed in WSE ($59.900 \pm 1.59 \mu\text{g/g}$ trolox equivalent) as compared to ESE ($28.005 \pm 1.58 \mu\text{g/g}$ trolox equivalent)

in the Cheddar cheese prepared from buffalo milk. The mean value increased from 28.166 ± 1.15 to 64.873 ± 1.26 ($\mu\text{g/g}$ trolox equivalent) after 9 months of ripening period (Fig. 3). The antioxidant activity of Cheddar cheese produced from utilizing milk of cow and buffalo increases all through the accelerated and conventional ripening process of 120 days. In case of conventional ripening (120 days), TAA of Cheddar cheese using milk of buffalo and cow was 72.91% and 52.42% respectively. In case of accelerated ripening (120 days), total antioxidant potential of cow cheese was 77.76% and buffalo cheese was 88.30% (Batool *et al.*, 2018).

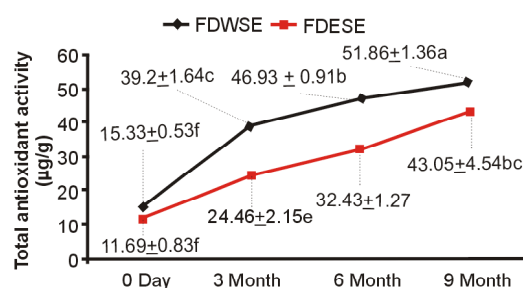


Fig. 2. Total antioxidant activity (TAA) ($\mu\text{g/g}$ trolox equivalent) of freeze-dried water-soluble extract (FDWSE) and ethanol soluble extract (FDESE) of cheddar cheese made from cow milk

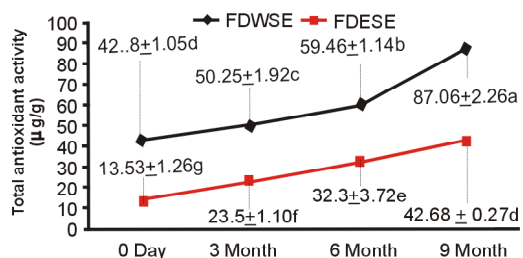


Fig. 3. Total antioxidant activity (TAA) ($\mu\text{g/g}$ trolox equivalent) of freeze-dried water-soluble extract (FDWSE) and ethanol soluble extract (FDESE) of cheddar cheese made using milk from buffalo

Total phenolic content (TPC) of freeze-dried water-soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese

TPC of freeze-dried water (WSE) and ethanol soluble fractions (ESE) of Cheddar cheese using milk from buffalo and cow was estimated with ripening period from 0 day to 9 months. In cow milk cheddar cheese, the mean value for TPC of freeze dried WSE ($69.572 \pm 5.88 \mu\text{g/g}$ gallic acid equivalent) was found higher as compared to ESE ($52.113 \pm 0.77 \mu\text{g/g}$ gallic acid equivalent) throughout the ripening period. The mean value for TPC increased from 53.460 ± 2.99 to 67.820

$\pm 8.81 \mu\text{g/g}$ gallic acid equivalent after 9 months of ripening period in cow milk cheddar cheese (Fig. 4). Similar results were found for freeze dried WSE and ESE of buffalo milk Cheddar cheese during storage. The higher means value for TPC was observed in WSE ($65.430 \pm 6.98 \mu\text{g/g}$ gallic acid equivalent) as compared to ESE ($51.635 \pm 0.97 \mu\text{g/g}$ gallic acid equivalent) of buffalo milk Cheddar cheese. The mean value increased from 50.515 ± 1.24 to 68.220 ± 8.81 ($\mu\text{g/g}$ gallic acid equivalent) after 9 months of ripening period (Fig. 5).

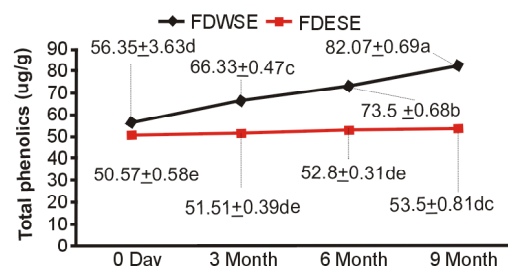


Fig. 4. Total phenolic content ($\mu\text{g/g}$ gallic acid equivalent) of freeze-dried water-soluble extract (FDWSE) and ethanol soluble extract (FDESE) of cheddar cheese made from cow milk

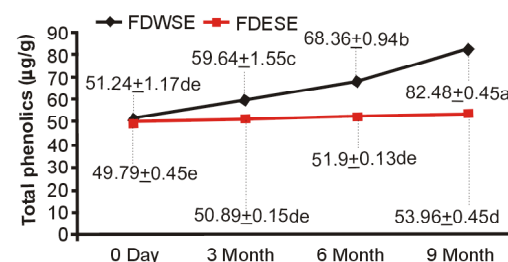


Fig. 5. Total phenolic content ($\mu\text{g/g}$ gallic acid equivalent) of freeze-dried water-soluble extract (FDWSE) and ethanol soluble extract (FDESE) of cheddar cheese made from buffalo milk

As limited data was available on total phenolic content of water-soluble and ethanol-soluble fractions of Cheddar cheese using milk from cow and buffalo sources. Although, different researches were done on determination of phenolic compounds from different cheese varieties with fortification or addition of plant extracts in order to determine the role of phenolic compounds. According to research, the polyphenolic content of cheese and milk was dependent on the animal feed ($P < 0.0001$) as well as milk processing and heat treatment ($P < 0.0008$). Heat treatment such as pasteurization process causes the destruction of phenolic compounds and catabolism of amino acids thus adversely affects concentration of polyphenolic compound (De-Feo *et al.*, 2006), and increased

concentration of polyphenols was found in cheese samples made from grazing goat milk e.g., GR (raw goat milk utilized for cheese manufacturing) and GP (Pasteurized goat milk used for cheese production) (O'Connell and Fox, 2001; Lawrence *et al.*, 2004).

Reducing power (RP) of water-soluble extract (WSE) and ethanol soluble extract (ESE) of freeze-dried cheddar cheese

Reducing power of water-soluble (WSE) and ethanol-soluble fractions (ESE) of freeze-dried Cheddar cheese using milk from cow and buffalo was investigated with the ripening period from 0 day to 9 months. Fig. 6 shows the mean values for RP of freeze dried WSE and ESE of cow milk Cheddar cheese during storage. The mean value for RP of freeze-dried ESE ($26.32 \pm 6.02 \mu\text{g}$) was found higher as compared to WSE ($21.27 \pm 6.43 \mu\text{g/g}$). The mean value for RP increased from 11.139 ± 0.51 to $37.177 \pm 3.40 \mu\text{g/g}$ trolox equivalent after 9 months of ripening period in cow milk cheddar cheese (Fig. 6).

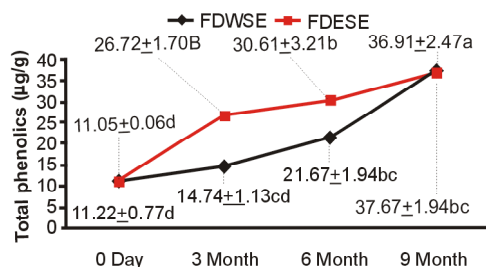


Fig.6. Reducing power (RP) ($\mu\text{g/g}$ trolox equivalent) of freeze - dried water - soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese made from cow milk

Similar results were found for RP of freeze dried WSE and ESE of buffalo milk Cheddar cheese during storage. The higher mean value for RP was observed in ESE ($27.734 \pm 5.76 \mu\text{g/g}$) as compared to WSE ($20.972 \pm 4.49 \mu\text{g/g}$) of buffalo milk Cheddar cheese. The mean value increased from 12.728 ± 0.43 to $34.765 \pm 2.43 (\mu\text{g/g trolox equivalent})$ after 9 months of ripening period (Fig. 7).

Meira *et al.* (2012) studied the water-soluble bioactive peptides in different ovine cheeses. The RP of most cheeses were increased with the ripening days as their values ranged between 0.2 to 0.3 units at 700 nm absorbance. It was reported that these cheeses contain protons and electrons for maintaining specific redox potential at specified concentration (mg/mL). The higher value for RP was estimated in roquefort (0.428) while feta (0.131) exhibited lower values. In addition to this, Roquefort-type cheese was considered merely the single sample to quench DPPH and

possesses increased reducing power ability owing to the occurrence of more residues of hydrophobic amino acids subsequently for these dual characteristics lower polarity was crucial (Hernández-ledesma *et al.*, 2005).

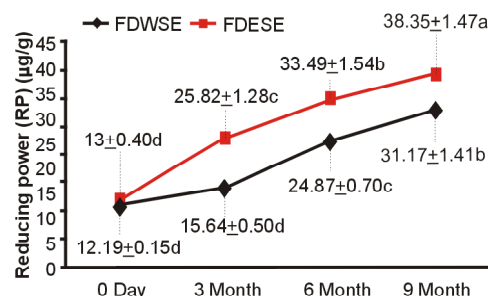


Fig. 7. Reducing power (RP) ($\mu\text{g/g}$ trolox equivalent) of freeze - dried water - soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese made from buffalo milk

DPPH (radical scavenging activity) of freeze-dried water-soluble extract (FDWSE) and ethanol-soluble extract (FDESE) of Cheddar cheese

The mean value for DPPH of freeze-dried ESE ($1436.1 \pm 4.95 \mu\text{mol/ml trolox equivalent}$) was found higher as compared to WSE ($1110.6 \pm 3.65 \mu\text{mol/ml trolox equivalent}$) (Fig. 8). The higher mean value for DPPH was observed in ESE ($1435.3 \pm 2.74 \mu\text{mol/ml trolox equivalent}$) as compared to WSE ($1121.8 \pm 2.58 \mu\text{mol/ml trolox equivalent}$) of buffalo milk Cheddar cheese (Fig. 9). The DPPH activity of WSE and ESE were found higher. The ethanol soluble fractions give more values as compared to water soluble fractions after ripening of 9 months. This may show the presence of more free radicals originated from peptides after ripening due to extensive proteolysis in ethanol soluble fractions which have ability to show radical scavenging activity (DPPH).

These findings were similar to Gupta *et al.* (2009) who studied that quantity of DPPH was reduced at specific absorbance (with characteristic wavelength) on interacting with proton radical scavengers. The DPPH activity of Cheddar cheeses WSE was studied. The values of WSE of most cheeses (4 month ripened) for DPPH increases but reduces after 5 and 6 months. After 4 months of ripening period the DPPH and SOSA (superoxide scavenging activity) increased noticeably as confirmed by antioxidant activity assays. Additional, significant difference in anti-oxidative potential was obtained in 5 month ripened cheeses (with different starter cultures) but production of soluble peptides remained same. As a result, it was concluded that rate of proteolysis and type of starter cultures utilized for cheese manufacturing influenced antioxidant activity (TEAC, SOSA and RSA) but up to some extent. In

antioxidant potential in fermented milk (lactic acid bacteria used for fermentation) reported that particular strain was associated with DPPH activity production with radical scavengers correlated with proteolysis (Hernández-ledesma *et al.* 2005; Kudoh *et al.*, 2001).

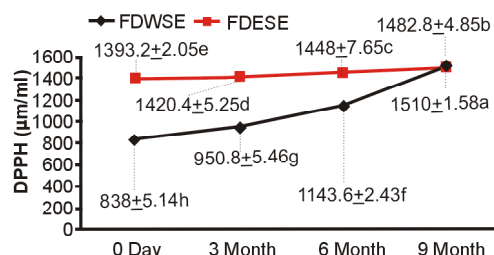


Fig. 8. DPPH (Radical scavenging activity)(μmol/ml equivalent of freeze - dried water - soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese made using milk from cow

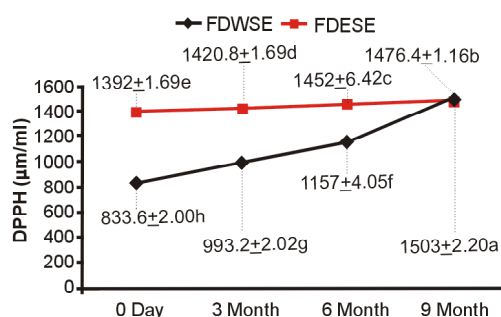


Fig. 9. DPPH (Radical scavenging activity)(μmol/ml equivalent of freeze - dried water - soluble extract (WSE) and ethanol soluble extract (ESE) of cheddar cheese made using milk from buffalo

CONCLUSION

Antioxidant potential of water soluble (WSE) and ethanol soluble extracts (ESE) of freeze-dried cheddar cheese made using milk from buffalo and cow was investigated as the ripening proceed from 0 day to 9 months. The purpose of this research was to take account of antioxidant ability of ethanol-soluble and water-soluble fractions prepared from cow and buffalo cheddar cheese during ripening. Ethanol soluble extracts of cheddar cheese from both kind of milk shows higher DPPH and RP ability as compared to water soluble fractions up to 6 months ripening. After 9 months of ripening increasing trend in water soluble fractions was observed for DPPH and reducing power ability. Water-soluble extracts (WSE) of cheddar cheese (freeze dried) shows higher total antioxidant activity and total phenolic activity as compared to ethanol soluble extracts during ripening. Form the study it can be concluded that the Cheddar cheese develops the bioactivity (anti-oxidant activity) during the ripening. Inference of my experimentation is that it

may be suggested that cheddar cheese can be utilized in the manufacturing of functional foods due to its increased antioxidant potential

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CONTRIBUTION OF AUTHORS

Sr. No.	Author's name	Contribution	Signature
1.	Amal Shaukat	Conducted the research work and prepared the initial draft of manuscript	
2.	Muhammad Nadeem	Helped in research work	
3.	Tahir Mahmood Qureshi	Analysed the data	
4.	Mian Anjum Murtaza	Helped in lab analysis	
5.	Muhammad Luqman	Proof read the manuscript	
6.	Lala Rukh	Performed analysis	
7.	Muhammad Bilal Shahid	Helped in data analysis and proof read the manuscript	