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Physicochemical Properties of Cheddar Cheese made from *Citrus reticulata* Blanco Crude Flowers Extract

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ARTICLE INFO	ABSTRACT
	Citrus reticulata Blanco crude flowers extracts (CFE) at four different concentrations (1, 2, 3 and 4%, v/v) were used as natural milk coagulant instead of rennet to apply for Cheddar cheese making from buffalo milk. The physicochemical properties of Cheddar cheeses were compared with cheese made with 0.002% (v/v) rennet (control cheese). Physicochemical properties of Cheddar cheese showed that cheese made with 1% and 2% of CFE had a crumbly and slightly softer texture/appearance. While cheeses containing 3 and 4% crude flowers extracts had semi-hard texture/appearance of curd similar to rennet added cheese. Protein analysis shows that crude flowers extracts made cheese had significantly higher protein content than control. The Cheddar cheese with 3% and 4% CFE were preferred by panelists instead of 1% and 2% CFE for their taste, texture/appearance and overall acceptability. Conclusively, crude flowers extracts coagulated Cheddar cheese fulfills the compositional requirement with acceptable organoleptic characteristics and at the same time provides nutritional health benefits.

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Introduction

Cheese as one of the major fermented dairy product is an excellent source of high quality protein and milk fat needed for human health. It is a rich source of fat soluble vitamins and also an important source of minerals (calcium and phosphorous) and concentrated source of energy. Cheese is valued for its long life and differs from country to country in colors, flavor, and texture (Fox et al., 2000; Donnelly et al. 2010).

Cheese industry in Pakistan is in growing stage and needs new innovation and technologies for its development and sustainability. The basic demand of food industry is to have consistent and reproducible characteristics for quality and processing of food products in food industry (Murtaza et al., 2008).

Traditionally, rennet is abomasum source of slaughtered young calves. In Pakistan, there is now decrease in slaughtering of young calves according to Pakistan Food Authority rules, as nowadays calves are grown for their meat production and which ultimately leads to less availability and shortage of natural rennet. So to fill this gap alternatives of rennet are used in cheese production. In countries with majority of vegetarian population, there is less availability of rennet from animals for cheese production. Therefore, alternatives in milk coagulation process will be economical for cheese production in developed countries (Fox et al., 2000; Andren, 2011).

Several alternative proteases obtained from different vegetable and fruit sources (e.g., kiwi, melon and papaya), roots (e.g., ginger), latex (e.g., papaya fruit and sodom apple) and flowers (e.g., *Citrus aurantium* sp., *Cynara cardunculus* and *Centaurea calcitrapa*) have been recommended as milk-clotting agents in cheese processing (Adetunji and Salawu, 2008; Domsalla and Melzig, 2008; Fernandez-Salguero et al. 2002). Additionally, these plant extracts have unique functional properties in flavor and texture, and will lead to innovative applications in new product development (Harboe et al., 2010).

Pakistan is in the sixth position in production of kinnow and oranges in the world with 1.9 million tons production yearly (Sharif et al., 2014). Proteins and enzymes present in kinnow are nutritional compounds which are also beneficial for human health. Kinnow (*Citrus reticulata* Blanco) known as bitter orange grows in most of subtropical climate and most of its fruits and flowers are discarded. Therefore, it is important to make more proficient use of agricultural and industrial products like seeds, leaves, flowers and other organic materials to have benefits in the economical, ecological and technological points of view (Silvestre et al., 2012).

Kinnow has proteolytic enzyme which can be obtained from generative organs of flowering plants. Plant tissues present in kinnow contain a wide range of proteins varying in functional properties. Protein content of prepared crude flowers extract (CFE) was in the range of 20 to 35 mg/mL and soluble protein in the citrus flower increased up to 85% of the total protein content at final floral development (Radlowski, 2005).

Rennet production is time consuming and costly. CFE has a typical aroma and flavor and it contains citrase with the activity to coagulate the milk (Mazorra-Manzano et al., 2013). Furthermore, Pakistan is a Muslim country and there is a controversy in using rennet for the production of cheese. Therefore, the objectives of this study is to provide economical, easily available and new milk coagulant, *Citrus reticulata* Blanco CFE to use in Cheddar cheese production technology as a source of milk-clotting enzyme in cheese production technology. The physicochemical characteristics and sensory evaluation of Cheddar cheese made with CFE were compared to rennet coagulated Cheddar cheese.

Materials and Methods

Materials

Flowers of *Citrus reticulata* Blanco were collected during blossom periods (mid of March to end of April and mid of September to end of October, 2015) from Sargodha District nurseries, Faisalabad Road, Sargodha. Flowers were collected and stored in freezer in food grade bags at -20°C to keep them fresh. Five liters of buffalo milk were used for each treatment of Cheddar cheese production. Buffalo milk was procured from the Dairy Animals Training and Research Center, UVAS, Ravi Campus, Pattoki.

Preparation of Citrus reticulata Blanco CFE

CFE was prepared by blending citrus flowers with 5 parts of cold buffer (20 mmol/L of Tris-HCl) having pH 7.20 in an electrical blender for 3 intermittent periods of 15 seconds. The blended sample was filtered by using a cheese cloth and then centrifuged at 850 g, 10°C for 30 minutes (Hemle Bench Top Centrifuge, Wehingen, Germany). Centrifugation of extract with Tris–HCL buffer leads to the activation of citrase. Then separate the aqueous phase of CFE from settled pallet by gradually pouring in the plastic test tubes. Then aqueous extract was kept in a refrigerator at 4°C until analysis (Mazorra-Manzano et al., 2013).

Manufacturing of Cheddar Cheese

Prior to the cheese making, five liters of buffalo milk for each treatment were pasteurized at 65°C for 30 minutes and then cooled at 33°C. Then, the milk was inoculated with 2% of starter cultures (v/v) (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, availed from Danisco Dairy Cultures, Paris, France) (Walstra et al., 2006). A control sample (T_0) was coagulated with 0.002% rennet (Rennet strength is 20 International Milk-Clotting Units, IMCU/mL) at 33°C for 45 minutes and it was obtained from Danisco Dairy Cultures (Paris, France) while the other four samples were coagulated using *Citrus reticulata* Blanco CFE at different concentrations of 1% (T_1), 2% (T_2), 3% (T_3) and 4% (T_4) (v/v) at 33°C for 45 minutes. After the curd was firm enough, it was then cut and stirred to drain whey. Then, curd was cooked and milled at 33°C for 15 minutes. After that blocks were turned up and down to separate more whey from the curd of cheese (Andren, 2011). This block of cheese was kept warm and then cheddaring of block led to acid development at final pH of 5.52 to 5.54. Salting was done at the rate of 2.5% (w/w) and whey was removed by pressing the cheese blocks with cheese presser. Storage and ripening of cheese was done at 10°C for 63 days (Walstra et al., 2006). Waxing of Cheddar cheese was done by pure paraffin wax (obtained from BW Chemicals, Lahore, Pakistan) to preserve it from molds and other contaminants (Figure 1).

Physicochemical Analysis

Moisture, fat, protein, and salt contents and pH values of Cheddar cheeses were done according to the methods prescribed by AOAC (2000) at storage (during 0 and 63 days).

Sensory Evaluation

Samples of Cheddar cheeses were coded with three digit random numbers and randomly served to semi-trained panels for sensory evaluation at 63 days of storage. The sensorial attributes were carried out on basis of texture/appearance, taste, color, odor and overall acceptability on a 9-point hedonic scale (9 = dislike very much; 1 = like very much) (Meilgaard et al., 2007). The panelists (comprised of 50 semi-trained panelists) were provided with respective cheese samples at room temperature. Panelists were given deionized water to cleanse their palates after evaluating each sample.
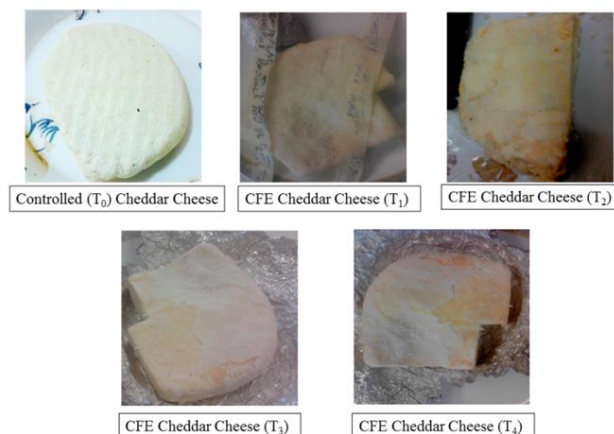

Figure 1 Control (T_0) as rennet coagulated and treatments (T_1, T_2, T_3 and T_4) as crude flowers extracts (CFE) coagulated cheeses after 63 days of storage period

Statistical Design

All data were analyzed using two-way Analysis of Variance (ANOVA) technique under Completely Randomized Design (Steel et al., 1997). Duncan's Multiple Range Test at P<0.05 was used for comparison of significant differences (SAS 9.1 Statistical Software).

Results and Discussion

Physicochemical Analysis

The Cheddar cheeses prepared using 1% and 2% of CFE showed higher fat contents from 0 to 63 days of ripening while cheese prepared with 3% and 4% of CFE have lower fat contents than control one (Table 1). Fat contents lead to a fatty appearance, specific aroma and flavor development in Cheddar cheeses (Singh et al., 2003). High protein contents were observed in Cheddar cheeses with 4% CFE after 63 days of storage because CFE contains protein which ranges from 20 to 35 mg/mL. Rennet coagulated Cheddar cheese showed protein content of 26.53% at 0 days of storage which decreased to 26.22% after 63 days of storage. This protein content is similar to standard protein contents for Cheddar cheese which range from 24% to 27% protein content (Chang, 2010). *Citrus reticulata* Blanco CFE provides enhancement of protein level in Cheddar cheese. Proteolysis of proteins increases at storage temperature of 10°C which leads to development of flavor and aroma as described by Wang et al. (2011). There was a decreasing trend in moisture content after 63 days of storage at 10°C because of the environment and storage variations. Cheddar cheeses made from 1% and 2% of crude flower extract showed slightly higher moisture contents while Cheddar cheeses prepared from 3% and 4% of CFE showed 33% moisture content which is almost similar to the rennet coagulated Cheddar cheese with 32% moisture content as reported by Zheng et al. (2011). The pH of cheese samples ranged from 5.54 to 5.52 throughout the storage (Table 1). CFE Cheddar cheese prepared with 1, 2 and 3% showed pH of 5.53 at 0 days and decreased to 5.52 which is standard pH of the Cheddar cheese as described by Pastorino et al. (2003). The decrease in pH of Cheddar cheese was due to breakdown of lactose into lactic

acid during ripening stage of 10°C (Onga et al., 2007). Cheeses with a normal (1.7%) and high (2.3%) salt content suppress bitterness and un-natural flavor of cheese (Moller et al., 2013). Salt concentration of Cheddar cheeses decreases in ripening stage as salt come out with water after pressing of cheese blocks or during storage at 10°C (O' Mahony et al., 2005).

Sensory Evaluation

Results showed that cheeses made with 1% and 2% of CFE had a crumbly and slightly softer texture/appearance. Cheeses contained 3% and 4% CFE had semi-hard texture/appearance which is similar to rennet added cheese as reported by Prinsloo (2007). Cheddar cheese coagulated with 1% CFE showed light yellow color while 4% CFE was yellow in color during all the ripening period due to the higher amount of the CFE. The color observed by using 4% CFE was quite resembled to standard Cheddar cheese (Young et al., 2004). Control Cheddar cheese prepared with rennet resulted "salty", "fatty' and "slight sour" taste while 3% CFE resulted with "low sour", "sweet", 'buttery" and "high salty" taste. Cheddar cheese with 1% and 4% CFE showed "bitter "and "umami" tastes (Caspia et al. 2006). Sources of bitterness were excessive accumulation of small hydrophobic peptides and a lack of masking compounds (primarily salt) in curd of cheese (Singh et al., 2003). Cheddar cheese with 3% and 4% CFE showed higher aroma intensity while 1% and 2% CFE showed less intense aroma as compared to standard Cheddar cheese which have typical aroma. The better sensory score was taken by 3% and 4% of CFE coagulated Cheddar cheeses as compared to 1% and 2% CFE at 63 days of storage. Cheddar cheese with higher CFE developed bitter flavor. The most liked Cheddar cheese sample was rennet coagulated Cheddar which showed smooth/creamy, hard, compact, smooth surface, rubbery, fatty, smeary, sticky and shiny appearance. Texture of Cheddar cheese changes as ripening accelerates after 12 months' period of storage. Significant differences observed in firmness and crumbliness after analyzing which showed that there are texture/appearance differences in CFE coagulated Cheddar cheeses with comparison to control one (Table 2).

Table 1 Effect of Different Treatments of Crude Flowers Extracts (CFE) on Physico-Chemical Properties of Control (Rennet Coagulated) and CFE Coagulated Treatments

Treatments	Storage Days	Physicochemical Analysis of CFE Cheddar cheese				
		Fat (%)	Protein (%)	Moisture (%)	pH	Salt (%)
T0	Day 0	33.37±0.17[a]	26.53±0.01[ab]	37.09±0.020[d]	5.54±0.000[d]	2.50±0.01[a]
	Day 63	32.28±0.34[a]	26.22±0.003[a]	35.94±0.010[c]	5.52±0.003[a]	2.47±0.01[b]
T1	Day 0	32.95±0.17[a]	27.34±0.10[b]	36.78±0.030[c]	5.53±0.003[bc]	2.50±0.01[a]
	Day 63	32.57±0.44[ab]	29.05±0.03[d]	34.31±0.010[b]	5.52±0.003[a]	2.41±0.01[b]
T2	Day 0	33.90±0.33[b]	28.66±0.08[a]	35.30±0.020[a]	5.53±0.003[cd]	2.5±0.01[a]
	Day 63	32.04±0.17[bc]	30.45±0.01[e]	34.22±0.006[a]	5.52±0.006[a]	2.42±0.01[b]
T3	Day 0	33.13±0.17[a]	27.47±0.02[ab]	36.28±0.010[c]	5.53±0.003[b]	2.49±0.02[b]
	Day 63	31.25±0.17[c]	30.53±0.01[c]	33.12±0.004[d]	5.52±0.003[a]	2.43±0.07[a]
T4	Day 0	34.03±0.17[b]	28.21±0.24[b]	35.62±0.020[b]	5.52±0.003[a]	2.50±0.01[c]
	Day 63	31.20±0.44[ab]	32.38±0.01[b]	32.95±0.006[d]	5.53±0.003[a]	2.42±0.01[c]

*All values are triplicate means of each treatment, **Different lower case alphabets showing significant differences among different treatments (P<0.05), T_0= Controlled Cheddar cheese with rennet, T_1= Cheddar cheese prepared with 1% *Citrus aurantium* CFE, T_2= Cheddar cheese prepared with 2% *Citrus aurantium* CFE, T_3= Cheddar cheese prepared with 3% *Citrus aurantium* CFE, T_4= Cheddar cheese prepared with 4% *Citrus aurantium* CFE

Table 2 Effect of Crude Flowers Extracts (CFE) on Overall Acceptability of Cheddar cheese

Treatments	Sensory Parameters					
	Color	Taste	Aroma	Acidity	Texture/Appe arance	Overall Acceptability
T_0	2.00 ± 0.27^{ab}	2.50 ± 0.56^{a}	2.50 ± 0.33^{ab}	2.75 ± 0.36^{ab}	2.25 ± 0.31^{ab}	2.00 ± 0.46^{c}
T_1	2.12 ± 0.29^{ab}	3.50 ± 0.50^{a}	3.00 ± 0.38^{ab}	2.88 ± 0.55^{ab}	2.88 ± 0.44^{a}	4.37 ± 0.37^{a}
T_2	2.75 ± 0.25^{a}	3.25 ± 0.25^{a}	3.50 ± 0.38^{a}	3.75 ± 0.25^{a}	2.50 ± 0.33^{ab}	5.12 ± 0.22^{a}
T_3	1.50 ± 0.19^{b}	2.37 ± 0.26^{a}	2.00 ± 0.27^{b}	2.25 ± 0.16^{b}	1.62 ± 0.26^{b}	1.75 ± 0.25^{c}
T_4	1.62 ± 0.26^{b}	2.87 ± 0.29^{a}	2.50 ± 0.38^{ab}	2.25 ± 0.16^{b}	2.62 ± 0.37^{ab}	3.00 ± 0.33^{b}

*All values are triplicate means of each treatment, **Different lower case alphabets showing significant differences among different treatments ($P<0.05$).

Overall acceptability is based on panelists' likeness/dis-likeness, quality, appearance, taste, flavor or color preferences. Overall acceptability decreased by 1% and 2% CFE treatments because both showed lower bitterness in taste while 3% CFE was more bitter and accepted by panelists (Table 2). Cheddar cheese acceptance varies distinctively among consumers and related to consumer preference for distinct flavor profile of Cheddar cheese (Young et al., 2004). Cheddar cheese with 3% and 4% CFE were preferred by panelists instead of 1% and 2% for their better taste, texture/appearance and overall acceptability but they were less preferred over standard Cheddar cheese (control) due to the presence of bitter and intense aroma compounds of *Citrus reticulata* Blanco flowers.

Thus, additional research must be carried out to find ways to remove the bitterness in CFE coagulated Cheddar cheese. Amino acids profiling is needed to better understand the taste development and to have pleasant aroma.

Conclusions

This research project was designed to implement and introduce the use of *Citrus reticulata* Blanco CFE as an economical, easily available rennet substitute in Cheddar cheese production. The CFE was evaluated for its first time usage as coagulant in Cheddar cheese production and compared with rennet for its physico-chemical characteristics and sensory evaluation. The Cheddar cheeses made with 1% and 2% of CFE had a longer and slightly softer texture/appearance. While, cheeses contained 3% and 4% CFE had semi-hard appearance of curd similar to rennet added cheese which is similar to the standard Cheddar cheese made with rennet. The Cheddar cheese with 3% and 4% were preferred by panelists instead of 1% and 2% for their better taste, appearance and overall acceptability. In future, this research will provide benefits to the cheese industry by introducing a new economical, nutritional and easily available rennet substitute source of milk coagulation. Pakistan is producing largest amount of *Citrus reticulata* Blanco, so export of CFE to other countries for usage as an alternate of rennet and to provide economic benefits to Pakistan. Furthermore, this research will open new ways for researchers to find out the characterization of extract and enzyme of CFE. However, efficiency and characterization of citrase is still unknown so only its activity is mentioned.

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