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The Antithrombotic Potential of Bioactive Peptides Induced by Buffalo Milk Probiotic Cheddar Cheese

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ABSTRACT

Cheddar cheese undergoes significant changes resulting in numerous microbiological and biochemical processes called glycolysis, lipolysis, and proteolysis, accountable for a unique texture, aroma, appearance, and taste. Specific bioactive peptides developed during these biochemical reactions impart health benefits. Addition of probiotics boots the development of bioactive peptides in foods. **Objective:** The current research investigated the therapeutic potential of water-soluble peptides (WSPs) extracts from buffalo milk probiotic Cheddar cheese regarding anti-thrombic facets. **Methods:** The appropriateness of Buffalo milk for Cheddar manufacturing was assessed by analysing its pH, acidity, fat, protein, and total solids content. Two batches of Cheddar cheese were produced, one having a mixture of Probiotic microorganisms and commercially available mesophilic cheese starter and the second containing only commercially available cheese starters. Both of the cheese batches were analysed for their physicochemical properties. Water-soluble extract of Cheddar cheese samples was analysed for anti-thrombic effects after two-month intervals during ripening. **Results:** Three concentrations of WSE of buffalo milk cheddar cheese were used to assess the antithrombotic effect during 60, 120, and 180 days of ripening at 4°C. Antithrombotic activity increased with the ripening period for both control and probiotic cheddar cheese samples **Conclusion:** A significantly increased effect of antithrombotic activity was observed by Probiotic adjunct on control cheddar cheese.

INTRODUCTION

Due to growing public awareness of diet and health in recent years, there has been a tremendous rise in the market for functional foods. Probiotic strains are used to prepare various foods, including yogurt, cheese, ice cream, dried yogurt (frozen), and fruit juices. Foods containing live probiotic bacteria are linked to several health advantages, such as the treatment of lactose intolerance, diarrhoea, cancer, high blood pressure, and immune system diseases [1]. At the time of intake, the probiotics must be viable within the recommended range in the food product. During processing and storage conditions of particular food items, probiotic strains' viability must be considered. The number of viable probiotic strains for better health benefits must not be less than 10^9 CFU/100 gram or ml. In Japan, probiotic meals are suggested to include a minimum of 10^7 cells per 100g or ml [2]. In dairy products, the growth and survival of probiotic strains are supported. Yogurt and fermented milk are the best options for probiotic delivery during processing and storage conditions. The viability of probiotics in probiotic yogurt may be affected by factors such as low pH, the need for aerobic processing and packaging, hydrogen peroxide, and inhibiting compounds.

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The factors affecting the viability of probiotic strains could be overcome by selecting suitable probiotic strains [3]. Cheese has a higher pH, solid consistency, and fat than freshly fermented dairy foods like yogurt. Cheese is the best dairy food for effective probiotic delivery [4]. It provides a protective environment to probiotic bacteria during their passage through the gastrointestinal tract. The buffering capacity of cheese is higher as compared to that of yogurt. Compared to cow milk, essential and no essential fatty acids, casein proteins, vitamins, peptides, and other bioactive substances are abundant in buffalo milk. Buffalo milk is characterized by more conjugated linoleic acid, medium-chain fatty acids, total protein, retinol, and tocopherol. Certain kinds of gangliosides are only found in the milk of buffaloes [5]. The Buffalo milk differs from cows, goats, camels, and humans in that it has higher fat levels, total solids, proteins, lactose, and ash. It has been established that buffalo's milk is the best raw material for making dairy goods. Due to the composition of buffalo milk, yogurt and cheese are inherently thick. When making cheese or yogurt, no additional milk protein or other gelling agents are needed for buffalo milk. For this reason, buffalo milk is preferred by milk processors [6]. Buffalo cheese is recognized for its distinctive flavor, characteristic texture, and juicy consistency. Buffalo milk mozzarella cheese is treated as a premium product. Buffalo milk is exceptional for creating various dairy products because of its improved churning ability and increased heat stability [7]. There are numerous ways for encrypted bioactive peptides to emerge from precursor proteins like proteolysis, during the processing of milk and enzymatic hydrolysis by digestive enzymes, or a combination of two or more conditions. Milk protein release bioactive peptides in the digestive tract by digestive enzymes such as pepsin and pancreatic enzymes like trypsin, chymotrypsin, carboxyl, and aminopeptidases [8]. Bioactive peptides are released as a result of a number of structural and chemical changes that take place during food processing. By creating more inter- or intra-molecular connections, alkalizing and heating food can prevent it from hydrolyzing. Proteolytic starter cultures are utilized in the dairy sector to produce bioactive peptides [9]. The sequencing and makeup of the amino acids in a peptide determine its action. Opioid, antithrombotic, antihypertensive, immunomodulatory, anti-oxidative, antibacterial, anti-cancer, mineral reserve, and growth-inducing properties are among milk-derived bioactive peptides [10]. Bioactive peptides produced from milk may manifest their activity after absorption in the gastrointestinal system or throughout the body. The bioavailability of bioactive peptides should always be taken into account in vivo. The antithrombotic efficacy of buffalo milk probiotic cheese was investigated in this study after ripening for 0, 60, 120, and 180 days at 4°C.

METHODS

Probiotic Cheddar cheese manufacturing

Buffalo milk was purchased from the local dairy farm. Milk was analysed for pH, fat, acidity, SNF, and protein. Control and probiotic cheddar cheese were manufactured using Murtaza *et al.*, [11] with some modifications. Typical mesophilic starter culture was used to prepare the control sample; *lactobacillus acidophilus* and distinct mesophilic starter cultures were used as probiotic strains while manufacturing probiotic cheddar cheese. The cheese samples were hermetically packed and stored for ripening at 4°C for 180 days.

Analysis of probiotic cheddar cheese

The control and probiotic cheddar cheese samples were analysed for pH, fat, protein, and acidity after 60, 120, and 180 days of ripening[12].

Extraction of bioactive peptides from control and probiotic cheddar cheese

Cheddar cheese samples were mixed with distilled water and homogenized to prepare the slurry. The pH of the cheese slurry was adjusted to 4.6 by adding 0.1 M HCl. The samples were heated in the water bath at 40°C for 60 minutes, followed by centrifugation at 4000 rpm for 30 minutes. The temperature during centrifugation was adjusted to 4°C. After centrifugation, the supernatant was filtered out using Whatman filter no 40. The supernatant was further used for antithrombic activities -[13].

Antithrombotic Activity Assay

Prasad *et al.* studied the anti-thrombolytic action of peptides [14]. He selected twenty healthy volunteers for the said study. Selected subjects had their venous blood extracted. The blood was incubated at 37°C for 45 minutes in pre-weighed sterile microcentrifuge tubes. Clots occur as a result of incubation. Serum was extracted. Each tube's clot weight was estimated by weighing it again. (wt. of clot = clot weight including tube weight – empty tube weight). Clot-containing tubes were labelled correctly. The tubes were filled with Streptokinase (100 µl) and manifold dilutions in sterile distilled water. Distilled water was added as a negative thrombolytic control in one of the clot-containing tubes. At 90 minutes of incubation at 37°C, clot lysis was seen. After incubation, the tubes were weighed again by draining the acquired fluid. Clot lysis % was defined as the difference in weight before and after clot lysis.

Statistical analysis

The resulting data were statistically analysed using *ANOVA* in Minitab, and *Tukey's test* was utilized for multiple comparisons (a = 0.05) between means. The findings were presented as mean values with standard error(SE)

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RESULTS

Milk composition

Milk samples were analysed for compositional assays to assure their suitability for Cheddar cheese production. The physicochemical composition of milk for Cheddar cheese production is described in table 1. Buffalo milk is concluded to be the best milk source for the preparation of various dairy products, including cheese, by several findings.

Component	Composition (%)
Fat	5.54
Protein	3.92
pH	6.78
Acidity	0.13
SNF	38.54
Moisture	85.83

Table 1: Physicochemical composition of milk for Cheddar cheese

Cheddar Cheese composition:

Produced Control and probiotic cheddar cheese samples were analysed for their fat, protein, pH, and acidity during 60, 120, and 180 days of ripening at 4°C (Table 2). Overall a non-significant relation was observed between fat and protein assays of control and probiotic cheddar cheese samples. The pH of control and probiotic cheddar cheese samples decreased with an increase in ripening time. The lowest pH was observed in control and probiotic cheddar cheese during 180 days of ripening. The results indicate that the addition of probiotic adjunct did not significantly affect the composition of cheddar cheese.

Component (%)	Control			Probiotic Cheddar cheese		
Ripening (days)	60	120	180	60	120	180
Fat	30.52±0.66	30.72±0.81	30.98±0.54	30.82±0.46	31.72±0.52	32.12±0.32
Protein	26.88±0.22	26.92±0.34	27.12±0.22	27.12±0.28	27.82±0.29	28.18±0.26
pH	5.15±0.02	5.01±0.12	4.91±0.06	5.14±0.02	5.00±0.12	4.89±0.06
Acidity	0.90±0.01	0.93±0.01	0.96±0.01	0.91±0.01	0.92±0.01	0.95±0.01
Moisture	38.74±0.14	37.43±0.12	36.22±0.16	37.88±0.11	37.12±0.13	36.11±0.13

Table 2: Composition percentage of control and probiotic cheddar cheese

Antithrombotic activity of control and probiotic cheddar cheese samples:

Thrombosis situations emerge in the human body due to an imbalance in hemostatic systems that results in the formation of a clot (thrombus) in arteries, veins, or the heart chamber. Aside from platelet attachment, dissemination and aggressiveness on the extracellular matrix contribute to thrombus formation. For control sample anti-thrombic activity was recorded as 4.8 ± 0.34 (%), 16.0 ±0.31(%) and 38.2 ± 0.96(%) at 60, 120 and 180 days of ripening respectively (Table 3). A highly significant increase in anti-thrombic activity was observed with an increase in the ripening period for both control and probiotic Cheddar cheese adjuncts. Bioactivities of control and probiotic Cheddar cheese for anti-thrombic activity were estimated using three different concentration levels (250 µg/mL, 500 µg/mL, and 750 µg/mL) of water-soluble extracts. The maximum anti-oxidant activity was observed at 750 µg/mL concentration of WSE of peptides. Recorded values for anti-thrombic activity at concentration level 750 µg/mL of WEPs extract is 8.1±0.19 (%), 18.5±0.27 (%) and 42.3±0.66 (%) respectively. The table describes the interaction of WSPs extract and storage level. In current exploration, control and probiotic Cheddar cheese displayed a steady increase in anti-thrombic activity as the ripening proceeds.

Samples	Level	Storage (days)			Means
		60	120	180	
Control	250 µg/mL	3.6±0.11	15.3±0.74	36.7±0.94	18.5±4.86
	500 µg/mL	5.0±0.20	16.3±0.38	38.0±2.15	19.8±4.88
	750 µg/mL	5.9±0.17	16.4±0.27	39.9±1.73	20.8±5.05
La	250 µg/mL	5.7±0.16	17.8±0.64	40.2±2.69	21.2±5.11
	500 µg/mL	7.1±0.25	18.2±0.27	40.1±0.58	21.8±4.85
	750 µg/mL	7.9±0.12	18.4±0.48	42.1±2.01	22.8±5.10

Table 3: Effect of cheese samples, storage and concentration level on anti-thrombic activity (%) of WSE of probiotic cheddar cheese

DISCUSSION

Some variations in calcium contents of milk were reported in this assay. These changes are associated with feed, season, and lactation stages. Murtaza *et al.* [11] observed the variations in calcium contents and other minerals in the milk of Mediterranean buffaloes throughout the year due to these factors. The most critical factor during dairy product manufacturing is milk pH. Protein conformation, enzymatic activity, and milk acid dissociation are associated with milk pH. Various research works support milk pH in this study. The results of this study for moisture analysis of control and probiotic cheddar cheese during 180 days of ripening are in line with Moller *et al.*[15]. The present results show no direct effect on cheese moisture contents by adding probiotic adjuncts, which confirms the findings of Gardiner *et al.[16].* The results of this study for fat analysis of control and probiotic cheddar cheese during 180 days of ripening are close to the effects of Ong *et al. [17].* Fat is retained within the cheese matrix in buffalo milk. Milk fat undergoes enzymatic hydrolysis during ripening by lipase and esterase (lipolytic) and oxidative changes. During ripening, fat is hydrolyzed into free fatty acids, mono and diglycerides, and glycerol [18]. In cheese, probiotic bacteria contain proteolytic systems, which contribute to the release of small peptides and free amino acids. The addition of probiotic bacteria Lb has observed an increased rate of proteolysis. *paracasei,* or *Lb. Acidophilus* in Cheddar cheese, especially in forming low molecular mass peptides and free amino acids [19]. But the net quantity of nitrogenous components within the cheese matrix remains almost the same. However, a minute change in fat might be observed by reduced moisture contents[20]. The

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results of this study for pH analysis of control and probiotic cheddar cheese during 180 days of ripening are compatible with the findings of Papetti and Carelli [21] that the pH of cheese varies within limits of 5.12 to 5.58 depending upon various factors. Ong *et al.*, in their study on probiotic Cheddar cheese, found pH in the range of 5.1 to 5.4 for different probiotic adjuncts. He also concluded that the nature of starter cultures (either probiotic or non-probiotic) does not significantly affect the pH of the product. These results are in line with the present study. Most lactose contents (about 98%) are drained off, and the whey. The rest of the lactose contents (0.8 to 1.8 %) remain present till the end of cheese manufacturing. In the early ripening stage, cheese's pH is decreased due to the residual lactose contents. Salting is another factor for the drop in pH. Starter lactic acid bacteria also participate in a decrease in pH. Reduced pH may affect the mineral content and growth of non-starter lactic acid bacteria in Cheddar cheese[22]. Furthermore, defects in platelet function may add to the complications of thrombotic events, ultimately connected to cardiovascular disease [23]. In the present study, a highly significant *(p<0.01)* effect on % age of anti-thrombic activity of bioactive peptides was observed in the ripening stage, the concentration of WSE (a water-soluble extract) of bioactive peptide and cheese samples (control and probiotic adjuncts). A significant *(p<0.01)* trend was observed in probiotic species' interactive effect with ripening days. The rapid evolving gene and post-translational modifications are also related to considerable variations in the primary sequences of α and β caseins. The water-soluble crude extracts of cheeses that contain major peptides formed during ripening may act synergistically to exert several physiological roles, including anti-thrombic activity -[24]. It is an exciting fact that functional similarities exist between milk (κ casein) clotting and blood clotting (fibrinogen)[25]. The peptides casoplatelins derived from κ casein fractions showed anti-thrombic activity by inhibiting fibrinogen binding platelets. The caseino glycopeptidase (CGP) from bovine, caprine, and ovine sources has been demonstrated to inhibit thrombic formation[26]. Like the present findings, Qian *et al.* [5] found the anti-thrombic effect of sheep κ casein fraction in a dose-dependent manner. The present study is also supported by Sharma *et al. [27],* who revealed the dose-dependent anti-thrombic effect of bovine GMP..

CONCLUSION

The acidity of control and probiotic cheddar cheese increased with an increase in ripening. A significant difference in anti-thrombic activities was observed for probiotic Cheddar cheese and control Cheddar cheese. The percentage of clot lysis increased with the concentration of water-soluble peptides extracted from both control and probiotic Cheddar cheese. The maximum anti-thrombic activity was observed in water-soluble peptides extracts at 180 days of ripening.

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