Production of Low-Fat Camel Milk Functional Ice creams Fortified with Camel Milk Casein and its Antioxidant Hydrolysates

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Abstract

Background and objective: The objective of this study was to produce functional low-fat camel milk ice creams enriched with native camel milk casein or its antioxidant hydrolysates produced by chymotrypsin.

Material and methods: Native or hydrolyzed camel milk caseins (0, 2 and 4%) were added to camel milk low-fat ice creams. Hydrolysates were characterized for molecular weights and antioxidant activities. Physical (hardness, overrun and melting resistance) and sensorial attributes of the final products were assessed.

Results and conclusion: Results showed that the chymotrypsin-mediated hydrolysis significantly (P<0.05) increased 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity of the native camel milk casein. Apparent viscosity and consistency coefficient of the ice creams were increased by addition of proteins and hydrolysates due to their water holding capacity. Protein/hydrolysates-fortified samples showed higher melting resistances but lower overruns and softer textures, compared to control ice creams with no added native or hydrolyzed camel milk casein. Sensory analysis showed that only samples enriched with 2% of casein hydrolysate included sensory properties similar to those of control camel milk low-fat ice creams and other samples received lower sensory scores. Generally, this study has suggested that camel milk can be used to produce low-fat ice creams. Properties of these ice creams can be modified by adding various concentrations of native and hydrolyzed camel milk caseins.

Conflict of interest: The authors declare no conflict of interest.

1. Introduction

Ice cream is a complex colloidal system which contains high concentrations (typically 10-16%) of fats, which limit ice cream consumption [1]. Regarding relationships between the high intake of fats and obesity, metabolic and cardiovascular diseases, studies have been carried out to develop low-fat ice creams [2]. However, reduction or removal of fat contents results in ice creams with poor sensory and textural qualities [3]. To overcome undesirable properties caused by the reduction of fat contents, various types of fat replacers such as octenyl succinylated starch [4] and inulin [5] were used in low fat ice creams. Food proteins such as whey proteins [2] and milk protein concentrates (MPC) [6] have been used as fat replacers to produce low-fat ice creams with acceptable quality due to ability of these replacers to interact with water, proteins and flavor compounds.

In recent years, supplementation of food products with biologically active or bioactive peptides has greatly been popular due to important roles of these peptides in In vivo bio functionalities of dietary proteins [7]. Such peptides are multi-functional biologically active fragments with positive effects on body functions, which are normally included in food proteins and can be liberated through various ways such as gastrointestinal digestion, microbial fermentation and enzymatic hydrolysis. Of these various approaches, enzymatic hydrolysis is the most commonly used method to release biologically active peptides from native proteins [8,9]. Camel milk, as a common product in arid regions, is
one of the sources of bioactive peptides, which is extensively interested over the last years [10]. Several nutritional and therapeutic properties such as low cholesterol levels and antidiabetic, antihypertensive, antioxidant and anti-tuberculosis effects have been reported experimentally for camel milk [11,12]. Zero or low allergic effects have also been reported for camel milk attributing to its higher digestibility and lack of β-lactoglobulin [10]. Superior biological properties, especially antioxidant activities, have been shown for camel milk proteins and bioactive peptides generated by limited proteolysis [13] and microbial fermentation [14], compared to bovine milk peptides. Better techno-functional properties such as higher viscosity have been demonstrated for camel milk, compared to those demonstrated for cow milk [15].

Casein and whey proteins of the camel milk have been used to produce biologically active peptides and hydrolysates. Camel milk casein was reported to include high susceptibilities to enzymatic hydrolysis as well as high abilities to generate bioactive peptides [11]. Accordingly, camel milk casein and its derived peptides were introduced as novel natural anti-hypertensive and antioxidant agents by Salami et al. [16]. Good antimicrobial activities against food spoilage and pathogenic microorganisms were reported for the camel milk casein enzymatic hydrolysates and fractions [12]. Camel milk casein hydrolysates were used as potential food ingredients. Kumar et al. [17] incorporated camel milk casein enzymatic hydrolysates into goat meat emulsions and reported lower lipid oxidation and microbiological counts, compared to hydrolysate-free samples. Generally, camel milk casein-originated bioactive peptides can be used as valuable ingredients in formulation of functional foods with health-promoting properties due to their therapeutic and preventive activities. No reports have been published on use of camel milk with camel milk casein and its enzymatic hydrolysates in production of low-fat ice creams. In the current study, camel milk low-fat (2.6%) ice creams containing various concentrations of native and hydrolyzed camel milk casein were produced and their physicochemical, textural, rheological and sensorial attributes were characterized.

2. Materials and methods

2.1. Materials

Camel milk samples were collected from Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran (Tehran, Iran) and stored at 4°C during transportation to the laboratory. Ingredients used in ice creams were purchased from local markets in Tehran, Iran. Chymotrypsin (EC 3.4.21.1, activity of 45 U mg⁻¹ protein), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2’-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Munich, Germany). Other chemicals with analytical grades (Sigma-Aldrich, Munich, Germany) were used without further purifications.

2.2. Preparation of the camel milk casein and its enzymatic hydrolysates

Whole camel milk casein was isolated using method of Salami et al. [16]. Camel milk was defatted using centrifugation at 6000 ×g for 15 min at 37°C. The pH of skim milk was adjusted to 4.6 using 1 N HCl at 37°C for 30 min. Solution was centrifuged (8000 ×g, 60 min, 4°C) to separate caseins from whey proteins and then washed three times with distilled water (D.W.). Caseins were neutralized using NaOH and then were freeze-dried and stored at -20°C until use. Hydrolysis of the camel milk casein was carried out using chymotrypsin at pH 7.8 for up to 1.5 h at 37°C under slow stirring. The enzyme-to-protein substrate ratio was 1:250 (w w⁻¹). Enzyme was inactivated by heat-treating at 85°C for 10 min. Hydrolysates were freeze-dried and stored at -20°C until use.

2.3. Characterization of the hydrolysates

Briefly, 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with reducing condition was used to show changes in molecular weight of the camel milk casein hydrolyzed for various time intervals (0, 10, 30, 60 and 90 min) according to Salami et al. [18]. Trolox equivalent antioxidant capacity reported by Salami et al. [13] was used to assess antioxidant activity of the native camel milk casein and its enzymatic hydrolysates. The ABTS radical cation (ABTS⁺) was produced by oxidizing 7 mM ABTS stock solution in water through treatment with 2.45 mM of potassium persulphate at a molar ratio of 1:0.5 for 12-16 h at room temperature in dark. Solution was diluted in 5 mM phosphate buffer with pH 7.4 to achieve 0.70 U ±0.2 at 734 nm using UV-visible spectrophotometer (Cecil CE2502, Cecil, Cambridge, UK). Then, 10 μl of the sample was mixed with 1 ml of ABTS radical solution and incubated at 25°C for 5 min. Scavenging of the ABTS⁺ was monitored spectrophotometrically by an absorbance decrease at 734 nm. A blank solvent was used in each assay. A standard curve of Trolox, as a water-soluble analogue of vitamin E, was used to calculate Trolox equivalent values.

2.4. Formulation and production process of the ice creams

In the current study, five camel milk low-fat ice cream formulations containing various concentrations of camel milk casein and its enzymatic hydrolysates were developed. These included control ice creams with no proteins or hydrolysates, samples with 2% of native casein (C-2%), samples with 4% of native casein (C-4%), samples with 2% of casein hydrolysates (CH-2%) and samples with 4% of casein hydrolysates (CH-4%). Other ingredients of the formulations were as follows: salep (0.6%), corn syrup DE
48 (5%), k-carrageenan (0.1%), vanilla (0.15%), fat (2.6%) and milk solid non-fat (7%). Fat and milk solid non-fat contents were associated to the camel milk. Ice creams were prepared according to Akalin et al. [1] with minor modifications. Raw camel milk, containing 2.6% of fats, was weighed, heated and then mixed with dry ingredients. When temperature reached 30-40°C, corn syrup was added to the mixture followed by pasteurizing at 75°C for 30 min. This rapidly cooled to 4°C using ice bath. The cool mixture was stored at 4°C for 24 h to guarantee complete hydration of all compounds. Then, vanilla was added to the mixture and the mixture was transferred to a laboratory-scale ice cream maker (Clatronic, Germany) for 20 min. Ice creams were packed and stored at 18°C until further assessments.

2.5. Rheological properties of the ice cream mixes

Rheological properties of unfrozen ice cream mixes were assessed at 4°C using Brookfield Viscometer (LVDV-II Pro, Brookfield Engineering, USA) equipped with a cylindrical LV spindle (No. 4). Totally, 25 ml of each sample were transferred to the measuring cylinder and subjected to a shear rate linearly increasing from 10 to 140 s⁻¹ with 5 s intervals. Resulting data were fitted to Power Law Model using Rheocalc Software v.3.2 to characterize rheological attributes of the samples using the following formula:

\[ \tau = K \cdot \gamma^n \]  

Where, \( \tau \), \( K \), \( \gamma \) and \( n \) were shear stress (Pa), consistency coefficient (Pa sⁿ), shear rate (s⁻¹) and flow behavior index (dimensionless), respectively.

2.6. Analysis of the ice creams

In this study, penetration test was carried out to assess hardness of the ice creams at ambient temperature (20°C ±1) using universal texture analyzer apparatus (M350-10CT, Testometric, Lancashire, UK) equipped with a stainless steel cylindrical probe. Ice creams in plastic cups were tempered at room temperature and penetrated using probe to a depth of 20 mm at a constant speed of 60 mm min⁻¹. Hardness, as the maximum force (N) needed to penetrate a depth of 20 mm at a constant speed of 60 mm min⁻¹, was measured by comparing weight of a certain volume of unfrozen ice cream mix with ice creams using the following formula:

\[ \text{Overrun} \% = \left( \frac{\text{weight of ice cream mix} - \text{weight of ice cream}}{\text{weight of ice cream}} \right) \times 100 \]  

Method of Innocente et al. [19] with some modifications was used to assess melting properties of various camel milk low-fat ice creams with or without camel milk casein and its enzymatic hydrolysates. Briefly, 50 g of the ice creams were transferred to a 20-wire plate and set to melt at room temperature (21°C ±0.5) for 60 min followed by recording weight of the melted ice creams. Percentage of the melted ice creams was calculated and reported. Sensory characteristics of the ice creams, including texture, flavor, odor and overall acceptance, were judged by a panel of 15 untrained assessors based on a 5-point Hedonic scale ranging from 1 (very bad) to 5 (very good). Various ice creams were packed and coded using 3-digit random numbers and served randomly by the panelists. Panelists were asked to drink water between the testing rounds to clear their mouth.

2.7. Statistical analysis

Data were analyzed using SPSS Software v.16 (IBM software, NY, USA) and one-way ANOVA. Duncan’s test at 0.05 level of \( p \) was used to show differences between the mean values.

3. Results and discussion

3.1. Characterization of the hydrolysates

Figure 1 presents reducing-mode sodium dodecyl sulfate polyacrylamide gel electrophoresis profiles of the samples. Two major bands were seen for the casein isolated from camel milk, corresponding to \( \alpha \)-casein and \( \beta \)-casein. Intensities of these bands decreased by enzymatic hydrolysis with chymotrypsin, indicating that caseins were converted to low molecular weight peptides and almost degraded after 10 min of hydrolysis. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that camel milk casein included high susceptibility to chymotrypsin. Salami et al. [18] reported a great chymotrypsinolyis for camel milk casein due to a great number of potential chymotrypsin-specific cleavage sites in its primary structure. An ABTS radical cation assay was used to assess antioxidant capacity of the camel milk casein during enzymatic hydrolysis. Results are shown as Trolox equivalent antioxidant capacity (μM) in Figure 2. As expected, hydrolysis significantly (\( P<0.05 \)) increased ABTS radical scavenging activity of the native camel milk casein and reached its maximum after 90 min. Salami et al. [16] and Kumar et al. [12] similarly reported higher antioxidant activities for camel milk casein hydrolysates prepared by enzymatic hydrolysis, compared to those for whole native camel milk casein. Augmented antioxidant activity of the protein hydrolysates was attributed to exposure of certain amino acids with effective radical scavenging activity (e.g. His, Trp, Tyr, Phe, Pro, Met and Cys) in native conformation of the proteins as well as increased availability of the hydrogen ions due to enzymatic hydrolysis [8]. Moreover, hydrolysis of the camel milk casein by chymotrypsin resulted in generation of peptides (mostly with aromatic and hydrophobic AAs) with high antioxidant activities in their C-terminals [16].
Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis profiles of the camel milk casein and its enzymatic hydrolysates

Figure 2. Antioxidant capacity of the camel milk casein hydrolysates at various hydrolysis times. Data are expressed as Trolox equivalent antioxidant capacity (μM). Means followed by different letters are significantly different (P≤0.05). TEAC = Trolox equivalent antioxidant capacity

3.2. Rheological properties of the unfrozen ice creams

Apparent viscosity of various unfrozen ice creams (as a function of shear rate) is represented in Figure 3 and their rheological parameters are shown in Table 1. Addition of camel milk casein or its enzymatic hydrolysates increased apparent viscosity of the camel milk low-fat ice creams. Consistency coefficient (K) of the ice creams, indicating their relative thickness, increased with increasing proteins and hydrolysates. In the present study, the most improvement in viscosity and consistency coefficient were observed in ice creams supplemented with 4% of camel milk casein hydrolysates using chymotrypsin followed by those supplemented with 4% of camel milk casein. Danesh et al. [2] and Akalin et al. [1] reported that addition of proteins into low-fat ice creams increased their viscosity and relative thickness mainly due to the high ability of proteins to hold water. In a study by Yilsay et al. [20], addition of whey protein fat replacers improved viscosity of low-fat vanilla ice creams attributing to the liquid binding/water holding capacity of proteins. Gani et al. [21] showed that water absorption capacity of the casein drastically increased using enzymatic hydrolysis due to the dissociation of proteins into smaller subunits with further water binding sites. This can explain the higher viscosity and K value of the ice creams with added camel milk casein hydrolysates, compared to those with native camel milk casein. There was also no statistically significant difference between the flow behavior index (n) of different ice cream mixes and all of the samples showed a pseudoplastic behavior (n<1) where the viscosity decreased with the increase of shear rate. Other studies also reported that the ice cream mixes with increased protein content [22] as well as the reduced-fat ice cream mixes containing various types of fat replacer such as whey protein isolate [2] and inulin [1] were shear-thinning.

Figure 3. Viscosity as a function of shear rate for various unfrozen ice creams supplemented with various concentrations of camel milk casein (C) or its hydrolysates (CH)
Table 1. Power law parameters of unfrozen ice creams containing various concentrations of camel milk native casein (C) or its enzymatic hydrolysates (CH)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K$ (Pa s$^n$)</th>
<th>$n$</th>
<th>Confidence of fit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.94 ± 0.05$^a$</td>
<td>0.35 ± 0.02$^a$</td>
<td>99.78 ± 0.11</td>
</tr>
<tr>
<td>C-2%</td>
<td>1.32 ± 0.10$^d$</td>
<td>0.36 ± 0.01$^a$</td>
<td>99.52 ± 0.34</td>
</tr>
<tr>
<td>C-4%</td>
<td>2.11 ± 0.08$^b$</td>
<td>0.34 ± 0.01$^a$</td>
<td>99.56 ± 0.07</td>
</tr>
<tr>
<td>CH-2%</td>
<td>1.64 ± 0.11$^c$</td>
<td>0.35 ± 0.01$^a$</td>
<td>99.60 ± 0.19</td>
</tr>
<tr>
<td>CH-4%</td>
<td>2.71 ± 0.09$^e$</td>
<td>0.34 ± 0.02$^a$</td>
<td>99.25 ± 0.77</td>
</tr>
</tbody>
</table>

Means with different superscripts within a column are different significantly (P<0.05).

3.3. Properties of the ice creams

In this study, hardness of various ice creams was assessed (Figure 4). Control ice creams were significantly harder than those supplemented with camel milk casein proteins or peptides (P<0.05). Danesh et al. [2] used milk proteins (whey proteins) as fat replacers to produce low-fat ice creams and reported a softer texture of the ice creams with added proteins; possibly because of decreased ice crystallization due to the formation of protein networks in ice creams. However, hardness of the ice creams increased with increasing protein or hydrolysate concentrations in the present study as samples containing 4% of the camel milk casein enzymatic hydrolysates needed the highest force to penetrate, in contrast to control ice creams. Firmer structures of the ice creams in the presence of casein hydrolysates in comparison with the native casein could occur due to their lower ability to inhibit ice crystallization. Damodaran [23] studied inhibition of ice crystal growth in ice creams using gelatin hydrolysates prepared by papain and reported that protein hydrolysates greater than 7000 Da did not inhibit growth of the ice crystals.

![Figure 4. Hardness of various ice cream samples including control camel milk low-fat ice creams and samples with 2% of camel milk casein (C-2%), 4% of camel milk casein (C-4%), 2% of casein hydrolysates (CH-2%) and 4% of casein hydrolysates (CH-4%). Different letters (a-e) represent significant differences between the treatments (P<0.05).](image)

Overrun, as an important factor affecting quality of ice creams, is an indicator of incorporated air into ice creams and is usually defined as the volume of ice cream achieved in excess of the volume of mixtures (Figure 5) [24]. In the current study, the highest overrun was achieved in control ice creams whereas the lowest overrun was reported in ice creams containing 2% of the native camel milk casein. The overrun generally decreased with the addition of native and hydrolyzed camel milk caseins, which could be linked to increased viscosity. Increased viscosity of ice creams resulted in decreased incorporation of air [25]. Previous studies demonstrated that overrun of ice creams decreased when viscosity of the ice creams increased [2,20,26]. Interestingly, ice creams with 2% of the camel milk casein hydrolysates showed a greater overrun than ice creams with 2% of the native casein, despite a higher viscosity (Figure 3). This could occur due to a higher interfacial activity of the hydrolysates, compared to native caseins. Furthermore, a lower overrun of the ice creams with 4% of the casein hydrolysates in comparison with ice creams with 2% of the casein hydrolysates could be due to a higher viscosity of the former ice creams that limited the incorporation of air. van der Ven et al. [27] and Gani et al. [21] reported that foam-forming ability of the casein was improved using enzymatic hydrolysis due to the generations of amphiphilic peptides and increases in polypeptide contents, which allowed further incorporation of air.

![Figure 5. Overrun of various ice cream samples including control camel milk low-fat ice creams and samples with 2% of camel milk casein (C-2%), 4% of camel milk casein (C-4%), 2% of casein hydrolysates (CH-2%) and 4% of casein hydrolysates (CH-4%). Different letters (a-d) represent significant differences between the treatments (P<0.05).](image)

After 60 min of incubation at room temperature (21°C ±0.5), 45.54% of the control camel milk sample and 5.46% of the ice cream sample supplemented with 2% of the native casein were melted. In contrast, ice creams containing 4% of the native camel milk casein and various concentrations of the casein hydrolysates (2 and 4%) completely resisted melting at the highlighted temperature. Generally, addition of camel milk casein and its enzymatic hydrolysates improved melting resistance of the ice creams. Innocente et al. [19] and Daw and Hartel [22] showed a higher melting resistance for the ice creams enriched with proteose-peptone (a heat-stable protein fraction of milk) and MPC, respectively. Melting resistance is an important property...
used to show the physical stability of ice creams and is highly associated with various parameters such as viscosity, overrun and presence of fat clusters and agglomerates which can decrease melting rates by decreasing the thermal diffusivity [5,26]. It is well-known that melting resistance of ice creams increases by increasing in viscosity [24]. In this study, a higher resistance of ice creams supplemented with native and hydrolyzed camel milk caseins could be linked to their higher viscosity and consistency. Danesh et al. [2] showed a lower melting resistance in ice creams with decreased fats than that in ice creams treated with whey proteins and transglutaminase due to their lowest viscosity rates.

In the present study, sensory attributes of the ice creams were assessed because of their importance for the consumers (Figure 6) [28]. In general, most of the sensory characteristics were affected by adding various concentrations of the native and hydrolyzed camel milk caseins. Danesh et al. [2] and Yilsay et al. [20] similarly stated that the addition of proteins significantly affected sensory properties of decreased fat ice creams. In the current study, sensory panelists stated that control ice creams and those containing 2% of the camel milk casein hydrolysates included better flavor and odor, compared to ice creams containing native casein and higher concentrations of the hydrolysates. Despite a softer texture, ice creams supplemented with native camel milk casein received a lower score for the texture than that control ice creams and ice creams supplemented with 2 or 4% of the casein hydrolysates did. Danesh et al. [2] reported a lower texture score for low-fat ice creams with a lower hardness attributing to the fact that the overall perception of ice cream texture was dictated by viscosity rather than the hardness. The overall acceptability scores showed that control ice creams and ice creams supplemented with 2% of the casein hydrolysates were further accepted by the panelists. Based on the sensory scores, adding appropriate quantities of the camel milk casein hydrolysates does not affect the sensory quality of ice cream adversely.

4. Conclusion

In the present study, camel milk low-fat ice creams were produced and supplemented with various concentrations of native camel milk casein or antioxidant casein hydrolysates. Addition of proteins and hydrolysates increased viscosity and consistency of the ice creams and decreased hardness and overrun of the samples. Enrichment with native and hydrolyzed camel milk caseins drastically improved melting resistance of the products. From the panelists’ views, only the ice cream samples containing 2% of camel milk casein hydrolysates included sensory properties similar to sensory properties of control ice creams. Generally, it can be concluded that the camel milk can successfully be used to produce low-fat ice creams with adjustable properties by adding various concentrations of native and hydrolyzed camel milk caseins. Results encourage use of camel milk proteins and their derived antioxidant hydrolysates as functional ingredients in food formulations with health-promoting effects. Further studies are necessary to assess techno-functional attributes of camel milk proteins and hydrolysates and their effects on quality attributes of the food products.

Figure 6. Sensory attributes of various ice cream samples including control camel milk low-fat ice creams and samples with 2% of camel milk casein (C-2%), 4% of camel milk casein (C-4%), 2% of casein hydrolysates (CH-2%) and 4% of casein hydrolysates (CH-4%)

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflict of interest.

References


تولید بستنی فراستتودمند کم‌چرب بر پایه شیر شتر گنی‌شده با کازئین شیر شتر و هیدرولیزهای آنتی‌اکسیدان‌ان

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چکیده
سابقه و هدف: هدف این مطالعه تولید بستنی کم‌چرب فراستتودمند از شیر شتر است که هیچ افزودن کازئین دست‌نخورده را نیاز ندارد (کازئین‌های تولیدی مورد بررسی قرار گرفت). ویگی‌های فیزیکی ( أصبح جسم، حجم و مقاومت به ذوب) و حسی محصولات نهایی نیز مورد ارزیابی قرار گرفتند.

مواد و روش‌ها: کازئین شیر شتر‌های فراستتودماند 200، 400 و 600 درصد به بستنی کم‌چرب شیر شتر اضافه گردید و سپس با افزودن هیدرولیزهای آنتی‌اکسیدان‌ان هزینه شد. وزن مولیولی و فعالیت آنتی‌اکسیدان‌ان بر روی هیدرولیزات در مقایسه با بستنی‌های راه‌نمای شامل کازئین دست‌نخورده و بدون افزودن هیدرولیزات، بررسی شد. هیچ نوع این افزودن کازئین دست‌نخورده یا هیدرولیزات به تولید بستنی‌های کم‌چرب کم‌سیاهی نیاز نداشت.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که هیدرولیزات آنتی‌اکسیدان‌ان به طور معنی‌داری (P<0/05) بهبودی در خصوصیات فیزیکی و حسی بستنی‌های کم‌چرب شیر شتر باعث شد و افزودن کازئین دست‌نخورده باعث افزایش مقاومت به ذوب شیر شتر و رطوبت شیر شتر شد. افزودن هیدرولیزات، خصوصیات ویژه و صنایع غذایی برای بازیابی کیفیت محصولات نهایی نیز حیاتی بود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.