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Aggregability and digestibility study of fruit juice fortified camel milk powder proteins

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ABSTRACT

In this work, we observed the effect of grape juice (% concentrated juice/% concentrated camel milk: GJ20/80, GJ50/50) and pomegranate juice (PJ20/80, PJ40/60) fortification on camel milk (CM) protein solubility and digestibility. Proteins were dissolved in sodium phosphate buffer to 50 mg/ml and defatted prior Bradford assay of protein concentration, then analyzed by Size Exclusion-Ultra High-Performance Liquid chromatography (SE-UHPLC). The CM protein aggregation and their stability were further monitored at different pH 2.0, 4.0, and 7.5 via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Freeze dried CM (FDCM) was the reference sample and our results showed that GJ50/50 and PJ40/60 with the highest fruit juice ratio had the lowest protein content in the supernatant, hence the decreased solubility. SE-UHPLC of supernatants showed a slight decrease in retention times of 11 kDa and 62 kDa proteins for GJ50/50 and PJ40/60 suggesting a possibility of adduct formation due to fortification leading to higher molecular weight. The simulated static *in vitro* gastrointestinal digestion of samples revealed that most soluble proteins were readily digested by pepsin, trypsin and chymotrypsin enzymes leading to small peptides. However, the SDS PAGE of pellets showed the partial resistance of casein and α-lactalbumin against peptic digestion.

1. Introduction

In many countries, camel milk (CM) is popular due to its perceived health-promoting properties. It is reported that CM contains high amounts of the immune-active proteins lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, as well as vitamin C and insulin, all of which play important roles in disease defense mechanisms (Habtegebriel, Edward, Wawire, Sila, & Seifu, 2018).

CM is highly perishable, especially problematic in its abundant regions with hot climates. Despite having been considered highly nutritious with higher vitamins, minerals, and immunoglobulins content, additional functional and medicinal properties compared to bovine milk (Laleye, Jobe, & Wasesa, 2008; Maqsood et al., 2019), CM is not consumed as much also owing to its less desirable sensorial properties. To overcome such hurdles, commercially flavored milks are brought in the market, but high amount of sugar is added. Therefore, an acidified, fortified, dried CM powder including the beneficial aspects without the addition of further added sugars is absolute solution. The ruminant's milk has been extensively used as a vehicle for fortification with nutrients, initially with Vitamin D to prevent rickets in children and osteoporosis in adults, iron to prevent anemia (FAO, 1996). The research suggested CM proteins itself could be potential functional food ingredient (Jafar, Kamal, Mudgil, Hassan, & Maqsood, 2018). The CM powder blended with the optimized ratio of fruit juice is commercially apt idea as it is further enriched with the micronutrients from fruit juice along with the heightened sensorial properties: the color developed due to the fruit juice, the mouthfeel, the flavor retention and so on. The product is naturally appealing to all ages of the consumers. The whole CM is

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usually dehydrated into powder for longer shelf life and instant availability upon reconstitution or as intermediate product for further processing into infant and special milk products (Belitz, Grosch, & Schieberle, 2009). Previously few researchers and technologists developed fruit juice fortified milk products: grape/peach fruit juice and cow's milk (Afifi, AbuShelaibi, Laleye, & Ismail, 2009), kiwifruit juice and skim milk (Sun-Waterhouse & Waterhouse, 2015) and studied on the changes on its thermophysical properties owing to the juice milk composition and ratio. However, the assessment of the impact of milk fortification component on milk's macronutrients' (proteins, carbohydrates, and fat) bioaccessibility is far less common. The overall quality of these readily available fortified milk products is reflected by their shelf life, re-dispersibility (cold and warm), sensorial properties like taste, aroma and flavor, microbiological features, and preservation of essential nutrients (proteins, vitamins) during and after production (Belitz et al., 2009). Therefore, amongst many techno-functional properties likely to alter owing to the fruit juice fortification, milk protein solubility is one of the important functional properties. Nevertheless, there has not been any work on fortified CM protein solubility although very limited research on solubility of camel whey protein (Laleye et al., 2008; Momen et al., 2018) were carried out in the past suggesting heat treatment decreased its solubility due to colloidal instability.

Also, the probable alteration on physiological response to fruit juice fortified CM might influence protein digestibility. Therefore, it is crucial to follow the complex digestive processes throughout the human digestive tract. CM protein comprises mostly of casein; β-, α- and κ-CN of which constitutes approximately 65, 21 and 3.47 percentage respectively (Kappeler, Farah, & Puhan, 2003; Salami et al., 2011). CM shows similarity to human milk as it contains a high amount of β-CN that could reflect differently on its digestibility in human GI tract and hypoallergenic to infants/adults, as β-CN is proved to be more sensitive to peptic hydrolysis than α-CN (El-Agamy, Nawar, Shamsia, Awad, & Haenlein, 2009). There has been numerous researches involving pepsin, trypsin and chymotrypsin enzyme hydrolysis of CM to study various parameters: effect on caseins and antioxidant activity (Jrad et al., 2014; Kumar, Chatli, Singh, Mehta, & Kumar, 2016), Angiotensin-converting enzyme inhibitory properties (Salami et al., 2011), anti-hypertensive and anti-hemolytic properties (Jafar et al., 2018), techno functional properties in*in vitro* and in food model systems (Al-Shamsi, Mudgil, Hassan, & Maqsood, 2018). Despite increasing production of fortified CM, there is a distinct lack of the digestibility studies post milk fortification. In this work, we primarily intend to study the impact of fruit juice fortification and their ratio on CM protein solubility. Secondarily, we focus on CM protein digestibility under standardized INFOGEST 2.0 static simulated *in vitro* digestion protocol adapted and modified from (Brodkorb et al., 2019) wherein we prepared samples mimicking real life conditions so as to observe the effect of real food matrix.

2. Materials and methods

All chemicals and enzymes were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared in ultra-pure water (Millipore, France) and filtered through 0.20 μm filter (Millipore) where applicable.

2.1. CM samples preparation

For the detailed CM powder and fruit juice fortified CM products, refer to the patent application publication number US 2019/0239527A1 (Ghnimi, Abdulhalim, & Al Kaabi, 2019). In short, fresh raw CM was purchased from a local farm in Al-Ain (UAE) and stored at 4 °C. CM was concentrated then dried using a pilot model spray dryer (Model FT80, Armfield Ltd, UK) under conditions described in the Supplementary information. This spray dried CM (SDCM) powder and/or Freeze-dried CM powder (FDCM) were used as control. Fortified CM powder were prepared at varying concentrated fruit juice to concentrated CM ratio. The fresh raw CM and fruit juice (commercial pasteurized juices purchased from local supermarket, free of added sugar, artificial colors and preservatives) were concentrated to 28 percentage and 45 percentage of total solids respectively (rising film evaporator). Grape juice (GJ) mixed with CM at 20 to 80 (v/v) ratio was labelled as GJ20/80. Similarly, 50/50 (v/v) GJ fortified with CM was labelled as GJ50/50. Pomegranate juice (PJ) was mixed with CM at 20 to 80 and 40 to 60 (v/v) ratios denoted as PJ20/80 and PJ40/60 respectively. For details, refer to the Supplementary information 2.1.1.

2.2. Protein determination

Five CM powder samples: FDCM, GJ20/80, GJ50/50, PJ20/80, and PJ40/60, each was dissolved in sodium phosphate buffer (20 mmol/L, pH 6.8) to a final concentration 50 mg/ml with gentle rocking at room temperature for 1 h. Samples were centrifuged at 12,300×*g* for 10 min. Supernatants were defatted by dichloromethane extraction (1:1 v/v). Afterwards, their protein concentration was determined by Bradford Protein Assay (BPA) (Bradford, 1976) in triplicates using bovine serum albumin (BSA) as standard. The CM protein aggregation and their stability in presence of fruit juice powders were further monitored at different pH values 2.0, 4.0, and 7.5 via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for both soluble (supernatant) and insoluble fraction (pellet) of the reconstituted CM sample solutions. For details, refer to the Supplementary information 2.2.1.

2.3. Size exclusion-ultra-high performance liquid chromatography (SE-UHPLC)

50 mg of each powdered CM sample was dissolved in 1 ml of 100 mmol/L phosphate buffer pH 6.8 which was also used as mobile phase. Samples were vortexed (15 s) and left 1 h with gentle rocking at room temperature. After centrifugation (12,300×*g*, 10 min) supernatants were defatted by dichloromethane extraction (1:1 v/v). Prior to SE-UHPLC analysis protein concentration in the samples was determined by the Bradford assay and samples were diluted to 0.35 mg/ml.

SE-UHPLC analyses were performed using ACQUITY UPLC Protein BEH SEC 125 Å column (4.6 × 150 mm I.D., 1.7 μm, Waters, Milford, MA, USA) connected to ultra-high-performance liquid chromatography (UHPLC) workstation Nexera XR (Shimadzu Corporation, Kyoto, Japan). Mobile phase flow was 0.3 ml/min and column temperature was set at 30°C. UV detection was done at 220 nm wavelength. The BEH125 SEC Protein Standard Mix (Waters, Milford, MA, USA), four component protein mixture (Thyroglobulin, Ovalbumin, Ribonuclease A and Uracil) were used for column calibration. Calibration curve for column was generated after three consecutive runs. The method run time was 60 min. Injection volumes were 4 μl for BEH SEC 125s Å column. The chromatographic control, data acquisition and analysis were performed using LabSolutions CS Analysis Data System (Shimadzu Corporation, Kyoto, Japan) version 5.73.

2.4. Standardized static in-vitro simulation of gastrointestinal digestion

In this adopted and slightly modified protocol by COST INFOGEST network revised by Brodkorb et al., 2019, we mimicked the successive oral, gastric and intestinal human digestive phases keeping in mind the parameters as the electrolytes in Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF), enzymes and their activity, bile and its concentration, pH and time of digestion are in accordance with the available physiological data based on an international consensus developed by the COST INFOGEST network (Brodkorb et al., 2019; Minekus et al., 2014). The concentrations of electrolytes in stock solutions of SSF, SGF and SIF along with their final reaction mixtures concentration are shown in Table S1. The exact calculated volumes of sample, fluid, enzymes, milliQ water and stopping reagent are explained in text under same title in Supplementary

document. For details and additional information, refer to the Supplementary information.

The powdered samples were reconstituted with milliQ water before proceeding to the oral phase digestion. The samples reconstitution was done in 2 ways: i) mimic real life consumption product composition i.e. 12.5 percentage fortified milk powder in milliQ water, ii) standardized protein content in each sample for comparative study wherein exactly 12.5 percentage CM protein was reconstituted with milliQ water (Assuming negligible or none amount of protein is in fruit juice powder). The detailed flow chart of reconstituted fruit juice fortified CM powder oral phase digestion to intestinal phase is also shown in Fig S1 in Supplementary information.

Additional to the reference sample; Reconstituted CM powder in milliQ water, control/stability sample tubes were also prepared to evaluate CM stability during exposure to simulated digestive fluids without enzymes, simultaneously with the oral, gastric and intestinal phases. The enzyme-blank tubes, i.e., the digestion tube containing quartz sand instead of CM sample with ①pepsin at 120′ time and ②Trypsin and Chymotrypsin at 120' time with bile were prepared. This proved to be the essential step in identifying enzyme and degradation products, bile salts during gel analysis of the oral phase (OP), gastric phase (GP) and intestinal phase (IP) digesta.

After the digestion, the proteins in the digesta supernatant at both GP endpoint and IP endpoint were separated and characterized via SDS-PAGE following the procedure as described in *2.5.1* under Materials and Methods section below. The pellets containing insoluble proteins, short digestion resistant peptides (SDRPs) were treated directly with the SDS sample buffer under reducing and non-reducing conditions. The equal volume of the pellets samples was loaded onto the gel for gel electrophoresis.

2.5. Protein profiling by gel electrophoresis

2.5.1. Native electrophoresis and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Freeze dried CM (FDCM) and fortified samples: GJ20/80, GJ50/50, PJ20/80, and PJ40/60 were characterized by native electrophoresis (See supplementary information for details) and SDS-PAGE under both reducing conditions and non-reducing conditions using 4–20 % gradient precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories). Protein concentration was determined by Bicinchoninic acid Assay (BCA) (Smith et al., 1985) in triplicates using Bovine serum albumin (BSA) as standard then the dilutions were made to maintain uniform protein concentration of 1 μg/μl among all the samples. Prior to sample loading, each sample was mixed well with the respective sample buffer, β-mercaptoethanol was used as a reducing agent while preparing sample under reducing conditions. The samples were prepared in the sample buffer without β-mercaptoethanol under native conditions and loaded onto the precast gel. The samples were heated at 95 °C for 5 min at 400 rpm on thermoshaker (Thermo Scientific), cooled to room temperature then 20 μg protein was loaded unto the gel well. The gels were run on Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories) following standard protocol: constant voltage of 200 V for 30–40 min (depending on the time taken by protein biomarker to reach the black reference line towards the anode of the gel). Precision Plus Protein Dual Xtra standard (2–250 KDa) was used for the quantification and molecular weight determination of the protein bands. After running the gel, they were stained with Coomassie Blue staining solution followed by destaining to visualize the gel. Gel visualization, image export and protein bands quantification were completed using Gel documentation unit: Chemidoc™ XRS+ and Image lab software version 6.0 (Bio-Rad Laboratories). Further, the protein bands were identified based on the determined molecular weight in parallel with the relevant published literatures.

3. Results and discussion

3.1. Soluble proteins profiling of the fruit juice fortified CM proteins

From Fig. 1, it is observed that with the increasing juice powder ratio to CM powder, overall protein concentration decreases. This is straightforward as the ratio of CM powder decreases in the fortified product, the protein quantity and thus concentration is bound to decrease. Besides, the milk and juice concentration, blending by agitation and drying processing were all optimized carefully keeping in mind the heat treatment could trigger the protein denaturation and aggregation. Since, it was discovered that spray drying enhances CM protein aggregation (Habtegebriel et al., 2018), the fruit juice milk blend were spray dried at 40–50 °C using rising film evaporation under vacuum to avoid damaging vitamins, pigments, or other thermally sensitive desirable substances (Ghnimi et al., 2019). It is established that freeze drying has no advantages over the spray drying treatment, instead it is more expensive and only of interest for special products.

However, depending on the type and ratio of fruit juice used for fortification, the protein concentration in the samples decreases with increasing fruit juice ratio (Fig. 1), suggesting that solubility of the proteins may be affected due to crosslinking of proteins or intensified protein aggregation in presence of sugars and added polyphenols since these compounds alter the surface hygroscopic properties that directly relates to the solubility of the CM samples (Al-Shamsi et al., 2018; Sun-Waterhouse & Waterhouse, 2015).

Irreversible protein aggregation is not only problematic for *in vitro* protein research and industry applications but is commonly associated with a large spectrum of human disease. The literatures suggested the undesirability of the milk protein aggregation, especially αs- and β-caseins in regards to the undesirable functional properties led by the aggregation (Chi, Krishnan, Randolph, & Carpenter, 2003; Yong & Foegeding, 2010). CM proteins lack β-Lg (lactoglobulin) and is mainly composed of ALA (Alpha-lactalbumin), a protein without free –SH group (Momen, Salami, Alavi, Emam-Djomeh, & Moosavi-Movahedi, 2019). Under native conditions, denaturing reducing and non-reducing conditions, CM powder and fortified CM powder evidently further shows the formation and varied nature of CM protein aggregates over the course of biphasic *in vitro* digestion, gastric and intestinal digestion (Refer to Fig. 5 A) and Fig. 5 B)). Previous research on bovine whey protein emulsions revealed the formation of some aggregates via covalent bonds and these aggregates with disulfide nature intensified upon increasing protein concentration and temperature (Momen et al., 2019). It was proven that the CM whey proteins are more heat stable over bovine proteins.

3.2. Aggregability study of the soluble CM proteins by size exclusion chromatography (SE-UHPLC)

Fig. 2SE-UHPLC chromatogram accompanied with the bar diagram illustrates the effect of fruit juice ratio on the elution profiles of defatted fortified CM samples. It must be considered that only the soluble fractions (supernatant) were analyzed by SE-UHPLC. The soluble non-covalent high MW aggregates with mass of about 200 kDa (Retention time 3 min) were present in all samples. Slightly increased MW for samples with higher ratio of juice powder; GJ50/50 (from 61.4 to 63.0 kDa) and PJ40/60 (from 61.4 to 62.8 kDa) can be observed for peaks around 61.40 kDa (Retention time, Rt = 3.8) referring to camel serum albumin (CSA) and 10.50 kDa (Rt = 5.36) to 11.48 kDa (Rt = 5.30) and 11.10 kDa (Rt = 5.33) respectively. This denotes the protein aggregation formation which could be probably because of increased glycosylation of proteins, as seen in overlaid chromatogram accompanied with the graphs with MW for details in Fig. 2 A) and 2 B). With decreased solubility, the higher MW protein aggregates formation that could probably occur due to sugars and polyphenols from juices, are insoluble. Nevertheless, this slight alteration in MW of proteins in case of fortified CM samples indicates that the protein aggregates must have formed owing

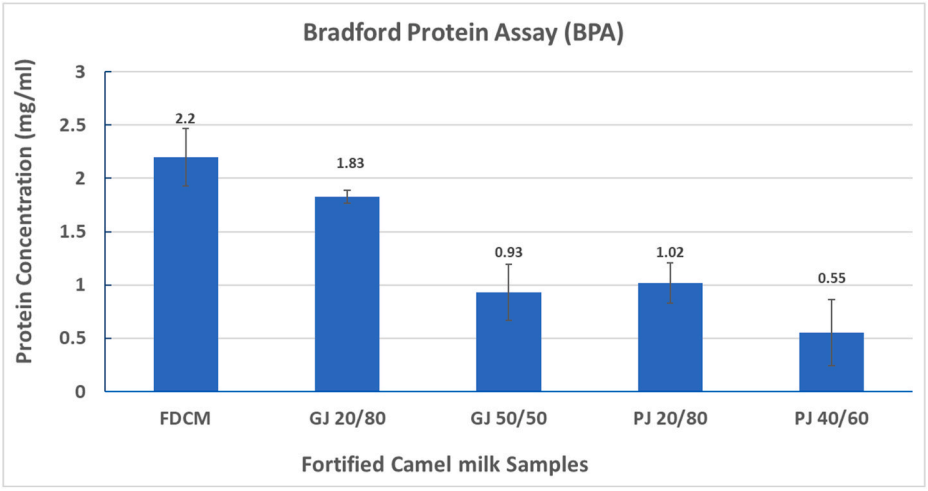

Fig. 1. Protein solubility in camel milk samples based on Bradford Protein Assay (BPA). [The protein concentrations were determined in triplicates] FDCM: Freeze dried Camel milk as control, GJ20/80: Grape Juice fortified CM at 20:80 ratio, GJ50/50: Grape Juice fortified CM at 50:50 ratio, PJ20/80: Pomegranate Juice fortified CM at 20:80 ratio, PJ40/60: Pomegranate Juice fortified CM at 40:60 ratio.

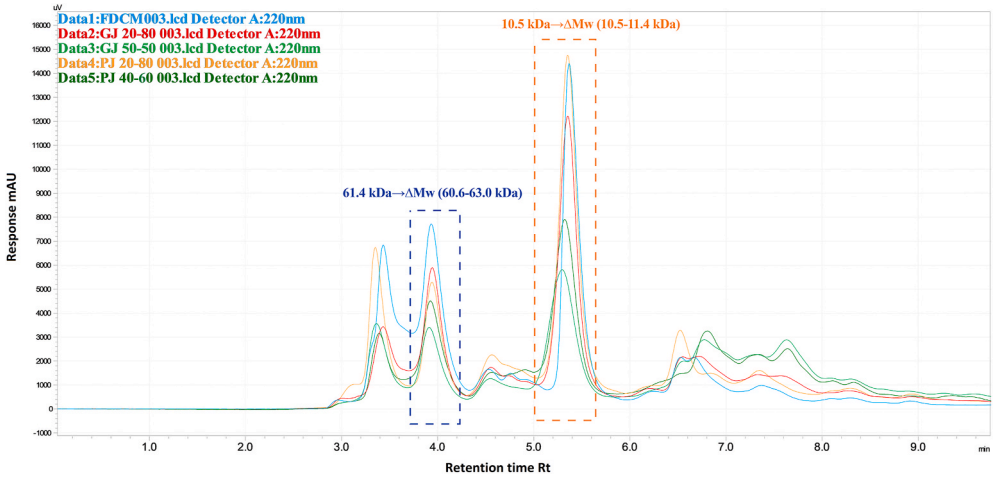

Fig. 2A. Ultra High-Performance size exclusion chromatography (SE-UHPLC) chromatograms of fortified camel milk powder samples in the order from FDCM as control (top), GJ20/80, GJ50/50, PJ20/80, PJ40/60 (bottom).

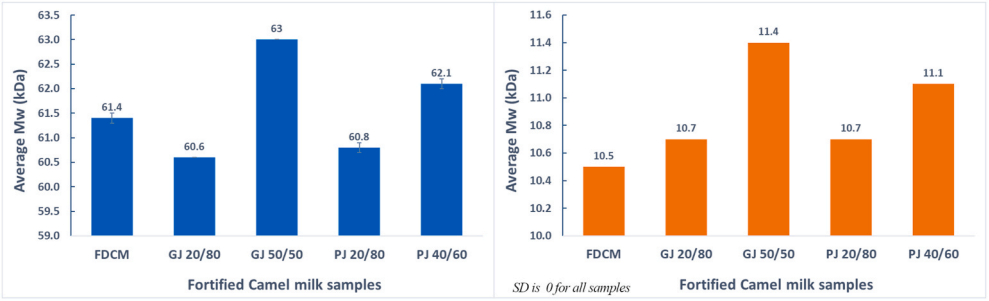

Fig. 2B. Bar diagram showing the shift in the MW of proteins around 60 kDa and 11 kDa in the samples. [Error bars indicate the standard deviation of the duplicate measurements].

to fruit juice fortification since the other parameters such as heat, temperature, enzyme or any chemical stimuli were maintained as they were. To further dig into the protein behavior, we proceeded along with the native electrophoresis and SDS-PAGE.

The elution peak within 5–6min retention time represents large protein aggregates, and its peak area increased slightly with increasing CM to fruit juice ratio which showed that aggregates with larger size were formed due to fortification. Although, the fortified CM was concentrated using a vacuum rising film evaporator at low temperature (40 °C to 50 °C), the blended mixture was dried using pilot spray dryer at higher temperature of 130 °C to 150 °C (for few seconds). While it was optimally designed production, the temperature may still adversely

affect the CM protein solubility as demonstrated in previous research wherein at higher spray drying temperature than 140 °C, the CM protein solubility was adversely affected (Habtegebriel et al., 2018). This phenomenon may also have slight effect on the protein aggregate formation. However, the protein aggregability is enhanced with higher fruit juice ratio.

3.3. Gel electrophoresis of fortified CM protein

The most abundant proteins found in CM are αs1-casein (~25–28 kDa), β-casein (~24 kDa) and k-casein (~19–22.4 kDa) with electrophoretic mobility corresponding to 19–28 kDa, as camel caseins are known for their lower electrophoretic mobility, in comparison to their bovine counterparts, depending on their phosphorylation pattern. As for whey proteins, the dominant protein bands were of camel serum albumin (CSA, ~66 kDa), α-Lactalbumin (ALA, ~14 kDa), and lactoferrin (~75–87 kDa) (Ereifej, Alu'datt, AlKhalidy, Alli, & ; Maqsood et al., 2019; Perusko et al., 2021; Salmen, Abu-Tarboush, Al-Saleh, & Metwalli, 2012).

Primarily, native electrophoresis and SDS-PAGE were carried out to compare and observe the proteins under native conditions and denaturing conditions. Native PAGE was performed to avoid protein denaturation or disruption of macromolecular complexes during electrophoresis to obtain native protein profile. Native electrophoretic profile of the proteins among the fortified samples shows the distinguished amount of protein retention on the gel well suggesting the formation of larger crosslinks of proteins those will not even migrate through the gel. It is possible that the protein aggregates might have occurred due to the concentration in the stacking gel, as common as the occurrence might be, we monitored in each gel under SDS-PAGE and noticed that the phenomenon shifts with the varying parameters as pH and sample buffer. It reinforces that the retention must not be due to stacking gel concentration. Comparatively, the lanes with higher fruit juice ratio; GJ50/50 and PJ40/60 in native electropherogram has fewer and fainter protein bands (mostly acidic or slightly basic proteins with pI range of about 3–8 in the native electropherogram), the protein retention on gel well of lower fruit juice ratio; GJ20/80 and PJ20/80 is more profound than in other samples as well as control. This might indicate aggregates formation in these two samples and shows that increased juice content can have protective effect on aggregation as less aggregates can be observed in samples with higher juice content. Additionally, the casein proteins; αs1-and β-caseins and whey proteins; lactoferrin, CSA, and ALA can be clearly observed under denaturing electrophoresis as labelled in Fig. 3 albeit there are not major differences between the samples' protein profile. These results are followed by additional observation under SDS PAGE of both supernatant (soluble proteins) and the pellets (insoluble proteins).

Secondarily, SDS-PAGE was performed to gain better understanding via visual observation of protein aggregates formation/crosslinks both in sample supernatant (Fig. 4) and pellet (Fig S4 in supplementary material). Since the structure or the size of protein aggregates could be effected by ionic strength, pH (Lajnaf et al., 2018; Sobhaninia, Nasirpour, Shahedi, Golkar, & Desobry, 2018), protein composition and other factors such as dielectric constant, ionic strength, temperature, we also executed SDS PAGE of these supernatant and pellets at different major pH: 2, 4 and 7.5. The protein profile of supernatant at this three different pH are shown in Fig. 4 (under reducing and nonreducing conditions), the protein profile of the pellets at different pH under reducing and nonreducing conditions are shown in Fig S4 in supplementary section.

From Fig. 4, we can observe that the casein protein bands (β-caseiñ24 kDa, α-caseiñ25–28 kDa) at around 19–28 kDa (Maqsood et al., 2019; Perusko et al., 2021; Salmen et al., 2012) are absent in the supernatant samples with more sugar; higher fruit juice ratio GJ50/50 and PJ40/60 at pH2 under both reducing and non-reducing conditions. In case of the pellet portion of samples at pH2, higher MW protein including Lactoferrin (around 75.3 kDa) (Kappeler, Ackermann, Farah, & Puhan, 1999) crosslinks can be observed under non-reducing conditions rather than the smaller proteins and they are less profound under reducing conditions (Fig S4 supplementary section). Significant amount of heavier protein has retained in the gel well itself regardless of pH conditions under non-reducing SDS PAGE. The protein retention is more pronounced in case of the fortified samples compared to the FDCM reference sample, more so in PJ fortified sample lane. This can be explained as the sugar and polyphenol content in pomegranate juice is unique and complex than in grape juice itself (Rozenberg, Howell, & Aviram, 2006), probably leading to bigger protein crosslinks that they cannot migrate through gels. Similarly, at pH4, in the supernatants gel lanes, there are no caseins in any of the samples under both reducing and non-reducing conditions. It is expected phenomenon because of their isoelectric point (pI) being within the range of 4.10–4.66, caseins (α, β, k-caseins~19–25 kDa) precipitate owing to acid coagulation as there is no electrostatic repulsion between molecules (Kappeler, Farah, & Puhan, 1998). Consequently, in the pellets gel at pH4, casein protein bands are observed dominantly under reducing conditions. The heavier protein bands including camel serum albumin (CSA) and Lactoferrin (75–150 kDa) are clearly observed in the pellet portion at all pH

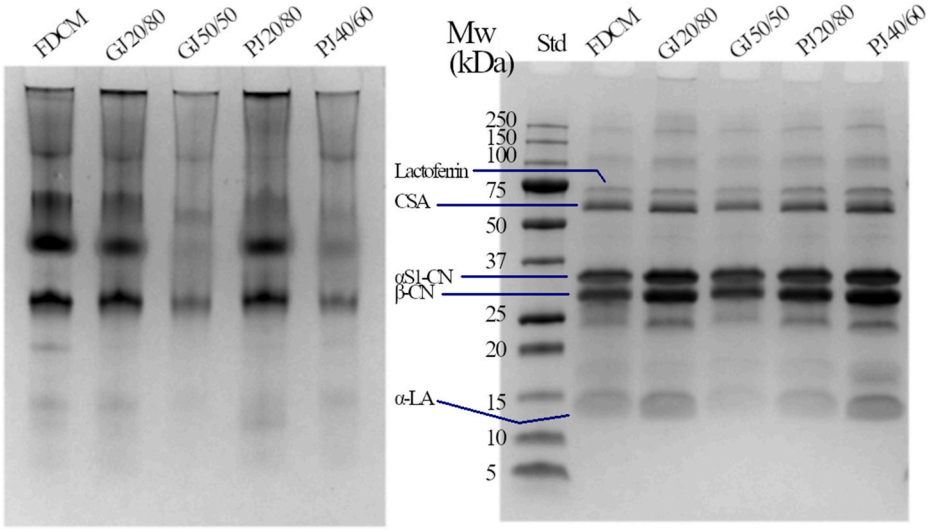

Fig. 3. Native electropherogram of the soluble supernatants of fortified CM powder samples (left) alongside the comparative SDS-PAGE gel of samples dissolved in the sample buffer on any kDa precast gel (Bio-Rad, California, USA) (right).

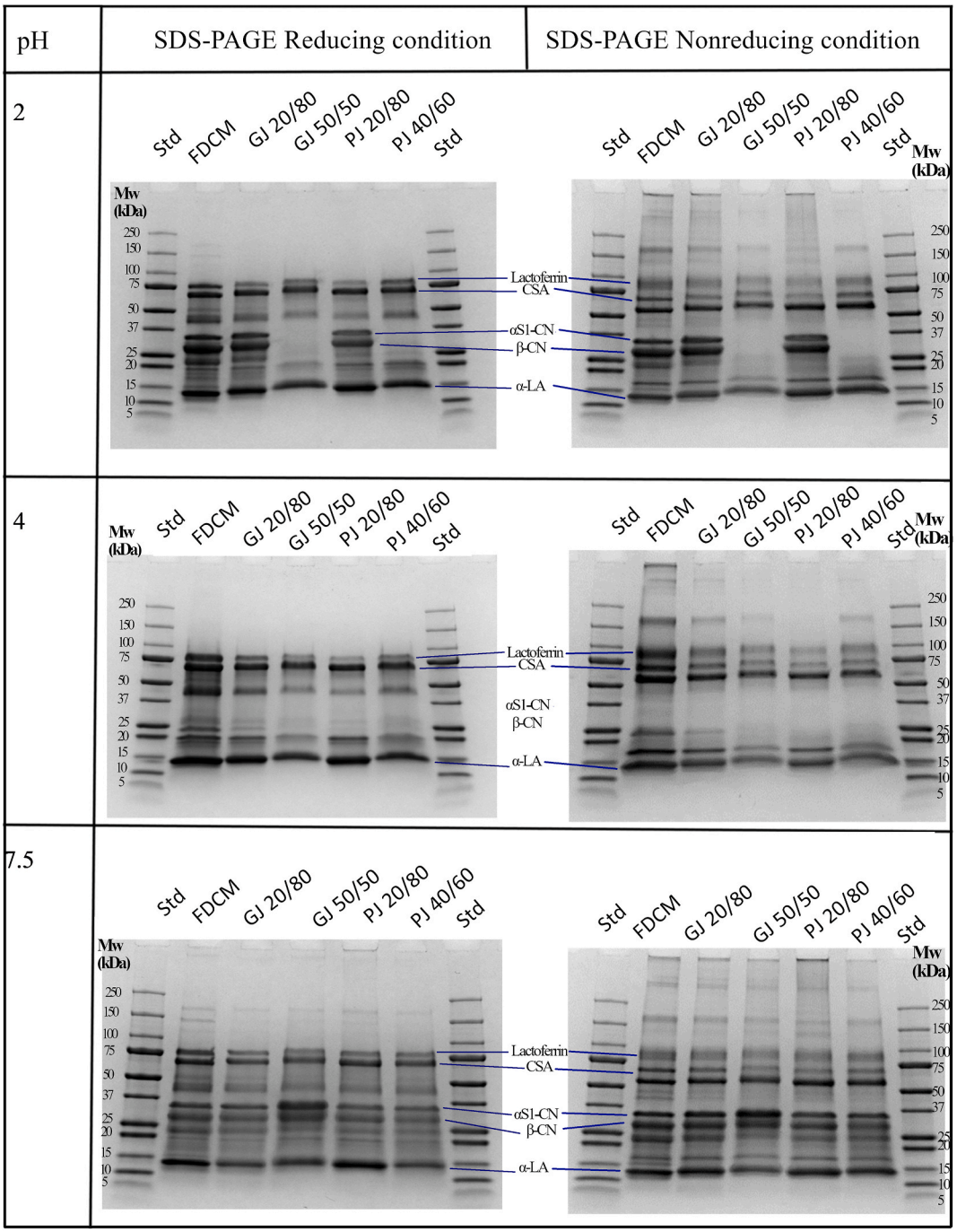

Fig. 4. SDS-PAGE electropherogram of supernatants (soluble protein) of fortified CM powder samples under reducing and nonreducing conditions at varying pH. FDCM: Freeze dried Camel milk as control, GJ20/80: Grape Juice fortified CM at 20:80 ratio, GJ50/50: Grape Juice fortified CM at 50:50 ratio, PJ20/80: Pomegranate Juice fortified CM at 20:80 ratio, PJ40/60: Pomegranate Juice fortified CM at 40:60 ratio, Std: Biorad precision plus protein standard.

conditions confirming that these major proteins are just partially soluble and significant amount retains in the pellet as well. On the other hand, these bands are not distinct at all in the pellets gel under non-reducing conditions denoting the large protein crosslinks formation. Additionally, the distinguished amount of protein retention on the gel well suggests the larger crosslinks of proteins those won't even migrate through the gel in all samples but profoundly under non-reducing conditions suggesting that those are *S*–S covalent aggregates. In the supernatants, however, the protein retention on the well is distinct under non-reducing conditions but majorly at pH 7.5 and pH 2.0. At pH7.5, there is no significant difference between samples as the protein solubility is at the maximum at neutral pH. As a result, the protein bands in the pellet gel at pH7.5 are not as intense as in the pellet gels at lower pH 4.0 and pH 2.0.

3.4. Digestibility of fortified CM powder

3.4.1. 1-D gel electrophoresis for fortified CM protein digestibility study in supernatant (soluble fraction)

Regardless of the fruit juice powder, either grape juice powder or the pomegranate juice powder, and their fortification ratio, 20:80, 40:60 or 50:50, the CM proteins were completely digested within 2 h of gastric digestion (pepsin) followed by additional 2 h of simulated intestinal digestion (trypsin and chymotrypsin) as shown in Fig. 5 A). The

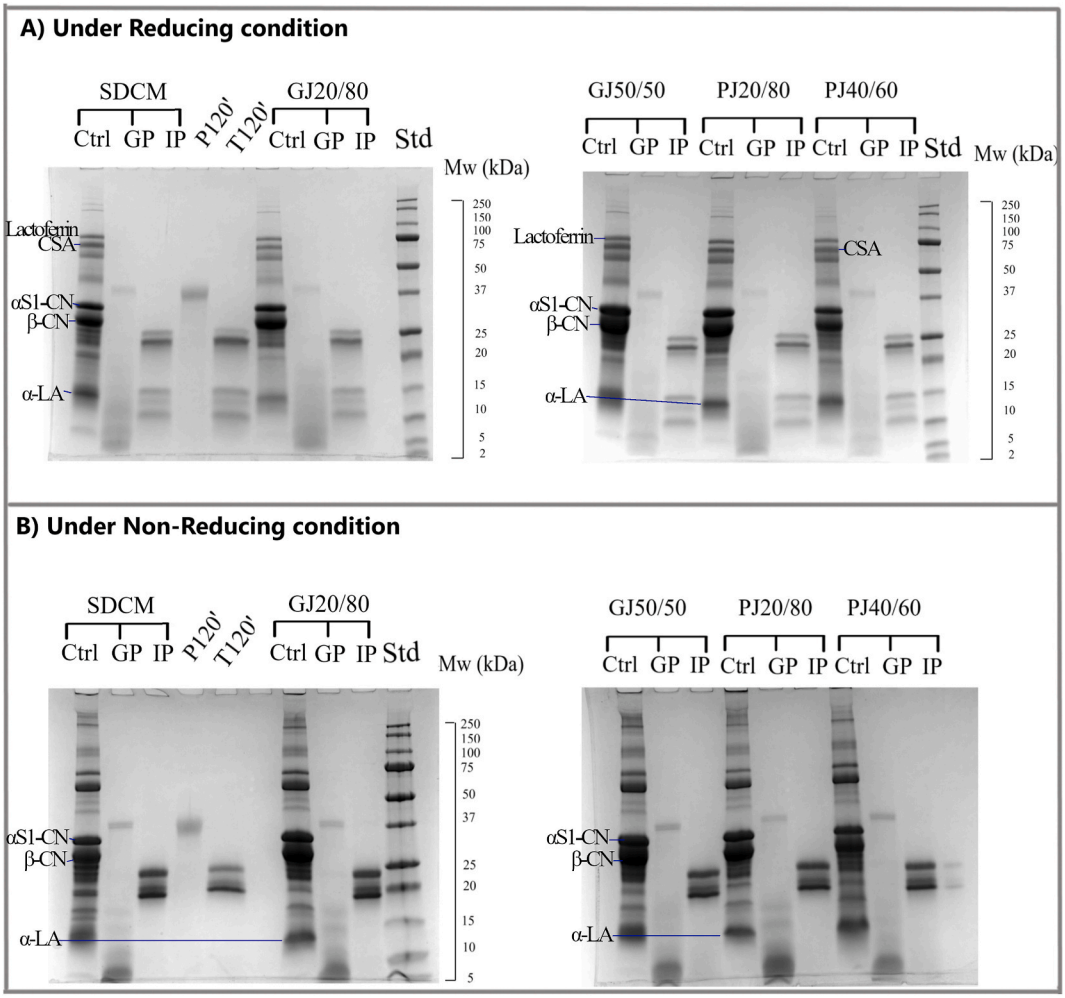

Fig. 5A. SDS-PAGE electropherogram of digested supernatant of fortified CM powder samples under reducing and non-reducing conditions along with the Control (Ctrl) at gastric phase (GP) digestion endpoint and upper intestinal phase (IP) digestion endpoint. SDCM: Spray dried Camel milk as control, GJ20/80: Grape Juice fortified CM at 20:80 ratio, GJ50/50: Grape Juice fortified CM at 50:50 ratio, PJ20/80: Pomegranate Juice fortified CM at 20:80 ratio, PJ40/60: Pomegranate Juice fortified CM at 40:60 ratio, P120': Pepsin blank at 120 min, T120': Trypsin and chymotrypsin blank at 120 min.

concentration of the reconstituted CM samples estimated by BCA are shown in Fig S3 in the supplementary information. As can be seen in all the gels Fig. 5, enzyme blanks conveniently highlighted the presence of additional enzyme-derived peptides due to the proteolytic enzyme autolysis, especially in case of trypsin and chymotrypsin.

Simply Reconstituted CM powder compared with the standardized protein samples showed no significant differences suggesting that the fruit juice powder regardless of the ratio with CM powder whatsoever neither intervenes nor improves the digestibility of CM proteins. It simply does not have major impact except for enhancing the sensorial qualities, retaining the bio accessibility of the vitamin C and phenolic compounds, eliminating the need of adding sugar to enhance the taste. Although in some of the research works, the large aggregates and CSA were susceptible to pepsin hydrolysis but the ALA band intensity however lowered, were pepsin resistant. They were digested with trypsin (Momen et al., 2018). In our results, the CM proteins including ALA were not pepsin resistant.

3.4.2. 1-D gel electrophoresis for fortified CM protein digestibility study in pellets (insoluble protein fraction)

From Fig. 5 B), it is obvious that majority of CM proteins are retained in the pellets meaning that significant portion of the total proteins remain insoluble in the digestive fluids inside the human GI tract. Compared to the CM protein profile from the supernatant control samples, the casein proteins bands are intensely characterized in terms of both volume and intensity in the pellets proving that the caseins must be less soluble in human intestine. Some of the previous works proved that the camel casein solubility is the highest at pH2 and pH8 (Ibrahim & Ateteallah, 2019; Post, Arnold, Weiss, & Hinrichs, 2012). In this research work, the gastric digesta and intestinal digesta protein profiles of fortified CM samples are significant. There are interesting findings regarding pepsin, trypsin and chymotrypsin digestion of these proteins with regards to the fruit juice fortification ratio. In pellets gastric digesta, the results show primarily that in addition to insolubility, the proteins do not only denature but also seem to form aggregates, especially lower Mw whey proteins. This is in direct comparison to supernatant gastric digesta, where we can observe that protein simply got denatured without any visible bands hence the absence of protein aggregates formation. Secondarily, the pellets casein protein bands did not completely fade off showing the partial resistance to pepsin compared to the supernatant where caseins are fully digested. Another interesting finding was during intestinal digestion in pellets. The whole protein in supernatant digested completely on trypsin and chymotrypsin digestion, however, in case of pellets, the lower Mw whey protein ALA seem to be less susceptible to enzymatic hydrolysis and probably modified as well. Additionally, a new unrecognized protein band appeared, relatively faint and in all samples under reducing conditions above 250 kDa region. This band disappeared but another faint band within 40–50 kDa

range was observed under non-reducing condition accompanied with the intensified protein band overlapping casein protein band at 24.9 kDa. When comparing the IP lane of the samples under SDS reducing and nonreducing PAGE conditions, it can be claimed that β-CN aggregates might have formed (see Fig. 5 B)) since this band around 24.9 kDa is visible under nonreducing condition of the samples due to the absence of reducing agent. In some research works, whey proteins, ALA and immunoglobulins G, were observed to be more resistant to the digestive proteolytic enzymes than other CM proteins (Jrad et al., 2014). The only explanation for this phenomenon could be the modification of whey protein followed by the denoted casein proteins in pellets because of protein aggregation. This explanation could also be supported with the observation of smearing of αs1-and β-caseins towards high Mw under non-reducing condition, suggesting their covalent modifications. Thus, electrophoretic analysis evidenced indiscrete gradual increase in protein molecular weights and acidification, indicating covalent modification of CM proteins.

4. Conclusions

The results conclude that in addition to the enhanced sensorial olfactory properties of CM powder upon fortification with grape juice and pomegranate juice, the protein digestibility remained dynamic making them bioaccessible. When the fruit juice powder ratio is increased with the CM powder then the protein aggregation augmented leading to lower CM protein solubility. Regardless of the fruit juice powder, either grape juice powder or the pomegranate juice powder, and their fortification ratio, 20, 40 or 50, the CM proteins in the soluble fraction of CM were completely digested with pepsin, trypsin and chymotrypsin. However, in the insoluble protein fractions in the pellet, the protein aggregation occurred. Both whey proteins and casein proteins seemed to be affected, casein and ALA were less susceptible to the enzymatic hydrolysis when digested under simulated static *in vitro* conditions. However, fruit juice fortification and the ratio does not necessarily affect the digestibility and hence the nutrients and protein's bio accessibility inside the human gastro-intestinal tract remain intact. Thus, this study showed the digestibility and technological viability of CM protein for the fabrication of fruit juice fortified CM powder.

CRediT author contribution statement

Urmila Khulal: Methodology, Formal analysis, and interpretation, Visualization, Writing – original draft, revising critically and editing. **Sami Ghnimi:** Methodology, Resources, Writing, Reviewing. **Nikola Stevanovic:** Formal analysis, Writing and Data Visualization. **Andreja Rajkovic:** Supervision, Writing, Reviewing. **Tanja Cirkovic Velickovic:** Methodology, Conceptualization, Design, Writing, revisions and editing, Project administration.

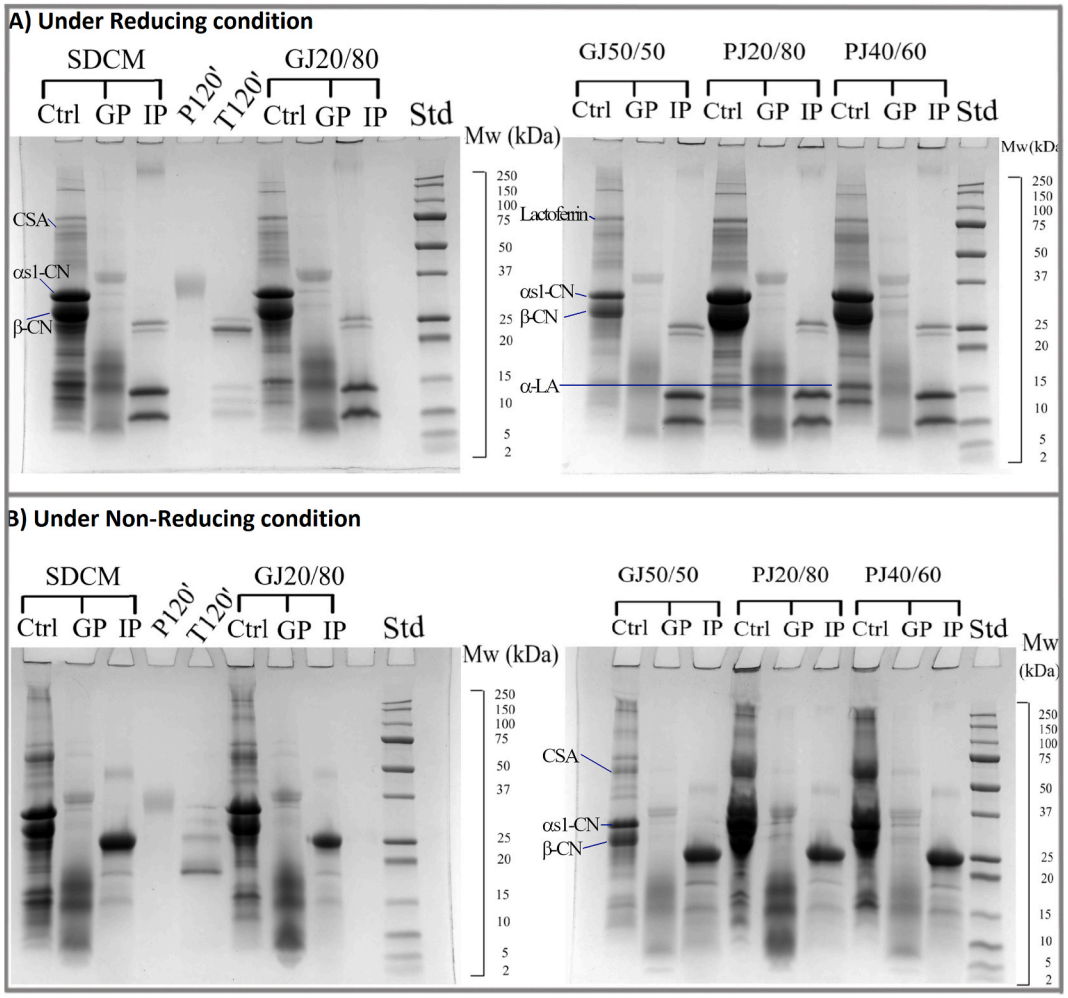

Fig. 5B. SDS-PAGE electropherogram of digested pellets of fortified CM powder samples under reducing and non-reducing conditions at gastric phase (GP) digestion endpoint and upper intestinal phase (IP) digestion endpoint. SDCM: Spray dried Camel milk as control, GJ20/80: Grape Juice fortified CM at 20:80 ratio, GJ50/50: Grape Juice fortified CM at 50:50 ratio, PJ20/80: Pomegranate Juice fortified CM at 20:80 ratio, PJ40/60: Pomegranate Juice fortified CM at 40:60 ratio, P120': Pepsin blank at 120 min, T120': Trypsin and chymotrypsin blank at 120 min, Std: Biorad precision plus dual Xtra protein standard.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lwt.2021.112250.

References

Afifi, H. S., Abu Shelaibi, A. A., Laleye, L. C., & Ismail, I. A. (2009). Measurement of thermal characteristics of spray-dried milk and juice blend. *International Journal of Food Sciences & Nutrition, 60*, 89–98. https://doi.org/10.1080/09637480802595526

Al-Shamsi, K. A., Mudgil, P., Hassan, H. M., & Maqsood, S. (2018). Camel milk protein hydrolysates with improved technofunctional properties and enhanced antioxidant potential in in vitro and in food model systems. *Journal of Dairy Science, 101*(1), 47–60. https://doi.org/10.3168/jds.2017-13194

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Food chemistry. In *le-tex publishing services oHG* (4th ed.). Leipzig: Springer-Verlag Berlin Heidelberg.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Chi, E. Y., Krishnan, S., Randolph, T. W., & Carpenter, J. F. (2003). Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation. [journal article]. *Pharmaceutical Research, 20*(9), 1325–1336. https://doi.org/10.1023/a:1025771421906

El-Agamy, E. I., Nawar, M., Shamsia, S. M., Awad, S., & Haenlein, G. F. W. (2009). Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Ruminant Research, 82*(1), 1–6. https://doi.org/10.1016/j.smallrumres.2008.12.016

Ereifej, K. I., Alu'datt, M. H., AlKhalidy, H. A., Alli, I., & Rababah, T. (2011). Comparison and characterisation of fat and protein composition for camel milk from eight Jordanian locations. *Food Chemistry, 127*(1), 282–289. https://doi.org/10.1016/j.foodchem.2010.12.112

FAO. (1996). *Food fortification: Technology and quality control. (FAO food and nutrition paper - 60). [Technical paper]. Report of an FAO Technical meeting* http://www.fao.org/3/W2840E/W2840E00.htm. Retrieved on 2020.03.24.

Ghnimi, S., Abdulhalim, I., & Al Kaabi, K. S. S. (2019). *Method of producing a fortified dairy product, 303* p. 303). Google Patents. *patent number US 2019/0239527A1* (15/891.

Habtegebriel, H., Edward, D., Wawire, M., Sila, D., & Seifu, E. (2018). Effect of operating parameters on the surface and physico-chemical properties of spray-dried camel milk powders. *Food and Bioproducts Processing, 112*, 137–149. https://doi.org/10.1016/j.fbp.2018.09.010

Ibrahim, F., & Ateteallah, H. (2019). Assessment some function properties of acid casein in different types of milk. *Journal of Food and Dairy Sciences, 10*(6), 171–173.

Jafar, S., Kamal, H., Mudgil, P., Hassan, H. M., & Maqsood, S. (2018). Camel whey protein hydrolysates displayed enhanced cholesteryl esterase and lipase inhibitory, anti-hypertensive and anti-haemolytic properties. *LWT-Food Science and Technology, 98*, 212–218. https://doi.org/10.1016/j.lwt.2018.08.024

Jrad, Z., El Hatmi, H., Adt, I., Girardet, J.-M., Cakir-Kiefer, C., Jardin, J., … Oulahal, N. (2014). Effect of digestive enzymes on antimicrobial, radical scavenging and angiotensin I-converting enzyme inhibitory activities of camel colostrum and milk proteins. *Dairy Science & Technology, 94*(3), 205–224.

Kappeler, S., Ackermann, M., Farah, Z., & Puhan, Z. (1999). Sequence analysis of camel (*Camelus dromedarius*) lactoferrin. *International Dairy Journal, 9*(7), 481–486. https://doi.org/10.1016/S0958-6946(99)00117-X

Kappeler, S., Farah, Z., & Puhan, Z. (1998). Sequence analysis of *Camelus dromedarius* milk caseins. *Journal of Dairy Research, 65*(2), 209–222. https://doi.org/10.1017/s0022029997002847

Kappeler, S., Farah, Z., & Puhan, Z. (2003). 5′-Flanking regions of camel milk genes are highly similar to homologue regions of other species and can be divided into two distinct groups. *Journal of Dairy Science, 86*(2), 498–508. https://doi.org/10.3168/jds.s0022-0302(03)73628-5

Kumar, D., Chatli, M. K., Singh, R., Mehta, N., & Kumar, P. (2016). Enzymatic hydrolysis of camel milk casein and its antioxidant properties. *Dairy Science & Technology, 96*(3), 391–404. https://doi.org/10.1007/s13594-015-0275-9

Lajnaf, R., Picart-Palmade, L., Cases, E., Attia, H., Marchesseau, S., & Ayadi, M. (2018). The foaming properties of camel and bovine whey: The impact of pH and heat treatment. *Food Chemistry, 240*, 295–303.

Laleye, L. C., Jobe, B., & Wasesa, A. A. H. (2008). Comparative study on heat stability and functionality of camel and bovine milk whey proteins. *Journal of Dairy Science, 91*(12), 4527–4534. https://doi.org/10.3168/jds.2008-1446

Maqsood, S., Al-Dowaila, A., Mudgil, P., Kamal, H., Jobe, B., & Hassan, H. M. (2019). Comparative characterization of protein and lipid fractions from camel and cow milk, their functionality, antioxidant and antihypertensive properties upon simulated gastro-intestinal digestion. *Food Chemistry, 279*, 328–338. https://doi.org/10.1016/j.foodchem.2018.12.011

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Momen, S., Salami, M., alavi, F., Emam-Djomeh, Z., Hosseini, E., Sheibani, N., et al. (2018). Effect of dry heating on physico-chemical, functional properties and digestibility of camel whey protein. *International Dairy Journal, 86*, 9–20. https://doi.org/10.1016/j.idairyj.2018.06.015

Momen, S., Salami, M., Alavi, F., Emam-Djomeh, Z., & Moosavi-Movahedi, A. A. (2019). The techno-functional properties of camel whey protein compared to bovine whey protein for fabrication a model high protein emulsion. *LWT-Food Science and Technology, 101*, 543–550. https://doi.org/10.1016/j.lwt.2018.11.063

Perusko, M., Ghnimi, S., Simovic, A., Stevanovic, N., Radomirovic, M., Gharsallaoui, A., et al. (2021). Maillard reaction products formation and antioxidative power of spray dried camel milk powders increases with the inlet temperature of drying. *LWT-Food Science and Technology, 143*, 111091. https://doi.org/10.1016/j.lwt.2021.111091

Post, A., Arnold, B., Weiss, J., & Hinrichs, J. (2012). Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of αS-and β-casein. *Journal of Dairy Science, 95*(4), 1603–1616.

Rozenberg, O., Howell, A., & Aviram, M. (2006). Pomegranate juice sugar fraction reduces macrophage oxidative state, whereas white grape juice sugar fraction increases it. *Atherosclerosis, 188*(1), 68–76. https://doi.org/10.1016/j.atherosclerosis.2005.10.027

Salami, M., Moosavi-Movahedi, A. A., Moosavi-Movahedi, F., Ehsani, M. R., Yousefi, R., Farhadi, M., et al. (2011). Biological activity of camel milk casein following enzymatic digestion. *Journal of Dairy Research, 78*(4), 471–478. https://doi.org/10.1017/S0022029911000628

Salmen, S. H., Abu-Tarboush, H. M., Al-Saleh, A. A., & Metwalli, A. A. (2012). Amino acids content and electrophoretic profile of camel milk casein from different camel breeds in Saudi Arabia. *Saudi Journal of Biological Sciences, 19*(2), 177–183. https://doi.org/10.1016/j.sjbs.2011.12.002

Smith, P. K., Krohn, R. I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., … Klenk, D. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*(1), 76–85.

Sobhaninia, M., Nasirpour, A., Shahedi, M., Golkar, A., & Desobry, S. (2018). Fabrication of whey proteins aggregates by controlled heat treatment and pH: Factors affecting aggregate size. *International Journal of Biological Macromolecules, 112*, 74–82.

Sun-Waterhouse, D., & Waterhouse, G. I. N. (2015). Spray-drying of green or gold kiwifruit juice–milk mixtures; novel formulations and processes to retain natural fruit colour and antioxidants. [journal article]. *Food and Bioprocess Technology, 8*(1), 191–207. https://doi.org/10.1007/s11947-014-1397-4

Yong, Y. H., & Foegeding, E. A. (2010). Caseins: Utilizing molecular chaperone properties to control protein aggregation in foods. *Journal of Agricultural and Food Chemistry, 58*(2), 685–693. https://doi.org/10.1021/jf903072g