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Comparative study on antioxidant, antimicrobial, emulsifying and physico-chemical properties of purified bovine and camel β-casein

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ABSTRACT

Emulsifying properties and *in vitro* antioxidant and antimicrobial activities of the isolated camel and bovine β-casein (β-CN) were investigated and compared. Antioxidant assay showed that both β-CN had significant reducing power, iron chelating and antiradical activities especially for camel β-CN. Camel β-CN also exhibited strong antifungal activities against *Aspergillus tamarii* and *Aspergillus sclerotiorum.* The maximum emulsion activity was achieved by both β-CN samples at pH 7.0 and 9.0 than 5.0, with higher values for the camel β-CN. This behavior was linked to the relative high electronegative charge and interfacial properties of proteins under conditions away from their isoelectric-point as confirmed by the ζ-potential and interfacial tension measurements. Further, the stability of emulsions decreased at pH 5.0 because of the β-CN precipitation and aggregation despite its high surface hydrophobicity. This study concluded that the camel β-CN has antimicrobial, antioxidant, and techno-functional properties in agricultural and food industries.

1. Introduction

Camel milk is an essential component of the human nutrition for populations in many hot and arid regions of the world. This milk provides to consumer all the essential nutrients which are already found in bovine milk (El-Agamy, Abou-Shloue, & Abdel-Kader, 1998). Recent studies have reported that camel milk is even richer in iron, vitamin C and lactoferrin. It can also be consumed as a potential therapeutic food due to its anti-diabetic, anti-hypertensive, carcinogenic properties (Al haj & Al Kanhal, 2010).

Camel milk proteins are classified according to their solubility into two main components: caseins and whey proteins. Casein is the main protein fraction in camel milk representing between 61.8% and 88.5% of the total camel milk proteins with an average value of 75.5% comparing to 80% of bovine milk (Ereifej, Alu'datt, Alkhalidy, Alli, & Rababah, 2011).

Camel milk is similar to human milk in its high β-casein (β-CN) content which reflected its higher digestibility rate and its lower allergy incidence on the consumer (El-Agamy, Nawar, Shamsia, Awad, & Haenlein, 2009). Thus, the β-CN represents the main protein in camel milk with an average concentration of 12.78 g/L representing 44% of total milk proteins which is significantly higher than that of bovine milk (11.66 g/L representing 37.4% of total bovine milk proteins) (Lajnaf, Zouari, Trigui, Attia, & Ayadi, 2020b; Omar, Harbourne, & Oruna-Concha, 2016).

Bovine β-CN is an acidic protein with an isoelectric point (pI) ranging between 4.8 and 5.1. It consists of 209 amino-acids with 12 different genetic variants (A1, A2, A3, B, C, D, E, F, G, H1, H2, I) based on gene polymorphisms and protein sequences. The two variants A1 and A2 of β-CN are most frequently found and well characterized (Atamer, Post, Schubert, Holder, & Marcel, 2016).

Camel β-CN presents some structural differences as compared with the bovine one; it's slightly bigger with a molecular mass of 24.65 kDa containing 217 amino-acid residues and with a pI of 5.7 (Esmaili et al., 2011; Kappeler, 1998). Hence, sequence alignment of bovine and camel β-CN shows that the sequence similarity and identity between these two caseins are 84.5% and 67.2%, respectively (Barzegar et al., 2008).

Physical, chemical, and microbiological factors are essential for food additives. For many years, manufacturers and consumers have been using synthetic additives in food industries but their consumption can lead to some allergic effects, cancer, intoxications and other degenerative diseases (Bhavaniramya, Vishnupriya, Al-Aboody, Vijayakumar, & Baskaran, 2019). Thus, aiming at the reduction of the use of chemical additives in the food industry, there has been growing interest recently

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in the use of natural food additives with antimicrobial and antioxidant activities that do not have any negative effects on human health (Alves-Silva et al., 2013).

Emulsification is a common operation in food industry and is encountered with mayonnaise soft drinks, soups, sauces, cream, salad dressings, butter and margarine (Guzey & McClements, 2006). Overall, oil-in-water emulsions are produced by the homogenizing of oil and aqueous phases in the presence of one or more emulsifiers. During homogenization, emulsifiers are adsorbed onto the surfaces of freshly formed oil-droplets leading to the reduction of the interfacial tension, which facilitates droplet disruption. The most common emulsifiers used in the food industry are caseins such as β-CN which is the most surface-active dairy proteins and can be considered as an effective emulsifier in formulated emulsion systems (Dickinson, 1998).

Previous works have shown that camel β-CN showed significant Angiotensin Converting Enzyme (ACE)-inhibitory activities after hydrolysis with pepsin as well as a highest antioxidant activity after hydrolysis with chymotrypsin when compared to bovine β-CN (Salami et al., 2008). However, only few works exist on the biological and techno-functional properties of camel β-CN. Most importantly, this paper is dedicated to discuss the differences between camel and bovine β-CN in comparative antioxidant, antimicrobial, emulsifying and physico-chemical studies. Thus, the examination of biological and techno-functional properties of camel β-CN might provide useful information to guide the application of this protein in food industry.

2. Material and methods

2.1. Materials

Camel milk samples (35 samples) (*Camelus dormedarius*) were purchased from a modern farm of camels of Gabes region of Tunisia. Fresh raw camel milk was collected from 20 different healthy Dromedary camel females ranging between 2 and 12 months in lactation in local cattle located in the south of Tunisia (region of Gabes). Fresh bovine (*Bos taurus*) milk samples were collected from 20 different Holstein cows. Cow milk was supplied by a local farmer in the region of Sfax in Tunisia.

Once purchased, milk samples were immediately cooled to 4 °C. For both milks, fat was removed by centrifugation at 3000g during 20 min at 4 °C (Felfoul, Lopez, Gaucheron, Attia, & Ayadi, 2015) and skim milk was then stored at −18 °C for further experimental analysis.

2.2. Purification of camel and bovine β-CN

Camel and bovine β-CN was purified from cold renneted caseins according to the modified methods of Huppertz, Hennebel, Considine, Kelly, and Fox (2006) as previously described by Lajnaf, Picart-Palmade, Attia, Marchesseau, and Ayadi (2016) and Lajnaf, Gharsallah, Jridi, Attia, and Ayadi (2020a).

First, camel and bovine skimmed milk samples were warmed to 37 °C. Casein fraction was separated from the whey by rennet coagulation using rennet addition (0.46 mL and 1.4 mL per liter of bovine and camel skimmed milk, respectively) (Rennet enzyme reference: M. *miehei*, strength = 1:10,000, Laboratories Arrazi, Parachimic, Sfax Tunisia) followed by a centrifugation at 5000 g for 20 min at 20 °C. A volume of heated demineralized water at 80 °C equal to that of the discarded whey was added to the curd and the mixture was kept at the temperature of the water (80 °C) during 5 min to enable the action of rennet enzyme, and then centrifuged for 5000 g for 15 min at 20 °C. After discarding the supernatant, the curd was kept, mancreated and suspended in demineralized water (5 °C) at a volume equal to that of the previously discarded whey. Finally, the protein suspension was kept at 5 °C for up to 24 h for the cold-extraction of the β-CN and the mixture was centrifuged at 5000 g for 15 min at 5 °C.

Once isolated, both camel and bovine β-CN samples were lyophilized (Bioblock Scientific Christ ALPHA 1–2) for further experiments.

The purity of β-CN was checked using RP-HPLC (Agilent 1260 Infinity quaternary LC, Germany) through a separation column C18 (Zorbax Eclipse Plus, 250 mm length x 4.6 mm, particle size 5 μm) (Yüksel & Erdem, 2010).

Briefly, 500 μL of the isolated camel and bovine β-CN (at a protein concentration of 5 g/L) were added to 3.7 mL of the solvents A and B mixture in 70:30 ratio (v/v) as follows: solvent A (acetonitrile, water, and trifluoroacetic acid in the ratio 100:900:1, (v/v/v)) and solvent B (acetonitrile, water, and trifluoroacetic acid in the ratio 900:100 (v/v/v)).

Afterwards, 20 μL of the filtered mixture (through 0.45 μm nylon filter) were injected into the RP-HPLC column. A gradient was then generated by increasing linearly the proportion of solvent B as function of time from 20% to 46% in 40 min at a column temperature of 25 °C and a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 220 nm.

Purchased standard (bovine β-CN from Sigma Aldrich) was dissolved in deionized water (from Milli-Q system, Millipore, USA) and then diluted in solvents A and B mixture (70:30) as purified samples separately.

2.3. Preparation of β-CN solutions

Camel and bovine β-CN were dissolved in 20 mmol/L Tris-HCl buffer (pH 7.0) at a protein concentration of 1 g/L (Lajnaf et al., 2016) and the solution was passed through filter membrane to avoid creation of large protein aggregates, according to Esmaili et al. (2011).

The pH values of the camel and bovine β-CN solutions were adjusted from the initial to 5.0, 7.0 or 9.0, using 1 mol/L HCl and 1 mol/L NaOH followed by mechanically stirring as described by Lam and Nickerson (2015b). pH values of this work were chosen according to previous works (Dan et al., 2014; Wüstneck et al., 2012).

The purpose of β-CN solution preparation is to work under the same conditions (protein concentration and pH level) for both camel and bovine β-CN in order to obtain a significant comparison for both camel and bovine β-CN samples.

2.4. Antioxidant activities

2.4.1. DPPH assay

The determination of radical-scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed according to Bersuder, Hole, and Smith (1998) at different protein concentrations of 0.1, 1 and 5 g/L (Bamdad, Shin, Suh, Nimalaratne, & Sunwoo, 2017). Briefly, 500 μL of each β-CN sample was added to 125 μL of the DPPH reagent (0.02% DPPH dissolved in 95% ethanol w/v)) 375 μL of ethanol (99%) in glass test tubes. The samples were shaken vigorously and then incubated in the dark at room temperature for 1 h. The absorbance of the incubated samples was measured at λ = 517 nm.

The DPPH radical-scavenging activity was expressed as the percentage of DPPH free radicals inhibition by the isolated β-CN proteins using the following equation Eq. (1):

$$\text{DPPH radical} - \text{scavenging activity} = \frac{\boldsymbol{C} + \boldsymbol{B} - \boldsymbol{S}}{\boldsymbol{C}} \times 100 \quad (1)$$

where C (Control) is the absorbance of 500 μL of water, 125 μL of DPPH-ethanol solution and 375 μL of ethanol at 517 nm; B (Blank) is the absorbance of 500 μL sample extract and 500 μL ethanol at 517 nm and finally S (Sample) is the absorbance of 500 μL β-CN sample added to 375 μL of ethanol and 125 μL of DPPH-ethanol solution at 517 nm.

2.4.2. Ferric-reducing antioxidant power (FRAP)

The FRAP method was performed according to the procedure described by previous works (Benzie & Strain, 1999). Results were expressed as μmol of Trolox-equivalents (μmol TE/g) of β-CN (0.1, 1 and

5 g/L in agreement with Bamdad et al. (2017)) based on a standard curve of Trolox (0–50 μmol/L).

2.4.3. Ferrous iron-chelating activity

The chelating activities of the isolated β-CN samples towards Fe^{2+} were measured by the methods of Boyer and McCleary (1987). The ferrous iron-chelating activity exhibited by the β-CN samples at concentrations of 0.1, 1 and 5 g/L was calculated and expressed as a percentage inhibition of ferrozine-Fe^{2+} complex formation (Bamdad et al., 2017). Indeed, 4.7 mL of each β-CN sample were added and mixed with 0.1 mL of 2 mmol/L $FeCl_2$ and 0.2 mL of 5 mmol/L ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine). The reaction mixtures were then incubated during 20 min at room temperature and the absorbance was read at 562 nm. The ferrous iron-chelating activity (FCA) exhibited by the β-CN sample was calculated using the following equation Eq. (2):

$$\text{FCA}\ (\%) = \left[1 - \frac{\boldsymbol{A\ sample}}{\boldsymbol{A\ blank}}\right] \times 100 \quad (2)$$

where A_{sample} and A_{blank} are the absorbance of the β-CN sample and control reaction respectively at 562 nm. Indeed, the blank was prepared as described previously, except that sample was replaced by distilled water.

2.5. Detection of antimicrobial activities

2.5.1. Antibacterial activity

Two Gram-positive bacteria (*Enterococcus faecalis* ATCC 25912 and *Staphylococcus aureus* ATCC 25923) and two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) were used as indicator strains in this work. Bacterial pathogens were provided by the laboratory of microbiology at the Habib Bourguiba University Hospital, Sfax, Tunisia.

100 μL of an overnight culture (10^7 CFU/mL) were inoculated in the LB agar plates. The β-CN solutions (concentration of 5 g/L) were delivered into wells (5 mm diameter) which are cut into the agar plates (Håkansson et al., 2000). The measurement of the antibacterial activities of the purified camel and bovine β-CN were realized for the diluted camel and bovine β-CN at a 5 g/L proteins concentration using deionized water which is reported to be the optimum concentration of the antimicrobial activities of the purified α_{S2}-CN from goat milk (Triprisila, Suharjono, Christianto, & Fatchiyah, 2016).

Diffusion step was carried out at 4 °C for 2 h. The plates were incubated during 24 h at 37 °C and the inhibition zones were measured. The antibacterial activity of the β-CN samples was evaluated by measuring the diameter of inhibition zones around the wells (in millimeters). Negative controls were prepared using sterile water.

2.5.2. Antifungal activity

Antifungal activity tests of the β-CN protein were conducted using the following phytopathogenic fungi provided by Tunisian Olive Institute: *Aspergillus protuberus* (MH137674.1), *Aspergillus sclerotiorum* (MG204869.1), *Aspergillus tamarii* (KY828882.1) and *Penicillium bilaiae* (MF681615.1).

A volume of 100 μL of each fungal suspension (containing approximately 10^6 conidia per mL) was spread on the PDA surface. Wells (5 mm diameter) were punched and filled with the β-CN solutions. Plates were finally incubated at 25 °C during 5 days. The antifungal activity of the β-CN was evaluated by measuring the diameter of inhibition zones around the wells. Sterile water was used as a negative control.

The purified camel and bovine β-CN were tested at a concentration of 5 g/L (Triprisila et al., 2016).

2.6. Emulsifying properties

20 mL (85%, v/v) of the β-CN samples (1 g/L, pH 5.0, 7.0 and 9.0) were homogenized with 3.5 mL (15%, v/v) of corn oil at 21,500 rpm during 30 s using the Ultra-Turrax T25 high-speed mixer (IKA-LAbortechnik, Staufen Germany) (Lajnaf, Trigui, Samet-Bali, Attia, & Ayadi, 2019). 100 μL of each freshly created emulsion were dispersed into 900 μL of 0.1% SDS (sodium dodecyl-sulfate) solution (w/v). The SDS-emulsion mixture was vortexed for 10 s and the absorbance was read at 500 nm using a UV mini-1240 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

For the optical microscopy, 100 μL were pipetted from the SDS diluted emulsion and placed between lame and lamella to be an optical light microscope (Nikon Eclipse E400, Kanagawa, Japan) with a 40× objective magnification and connected to digital camera (Laleye, Jobe, & Wasesa, 2008).

After 10 min, another volume of 100 μL was taken from emulsion mixture and dispersed into 900 μL of 0.1% (w/v) SDS solution. The absorbance was also measured at 500 nm as described above. Emulsifying activity index (EAI, m^2/g) and emulsion stability index (ESI, %) of β-CN solutions were determined using Eq. (3) and Eq. (4) suggested by Pearce and Kinsella (1978):

$$\text{EAI}\ (m^2/g) = [2 \times 2.303 \times A_{500} \times \text{dilution}] / [C \times (1\text{-}\Phi) \times 10^4] \quad (3)$$

$$\text{ESI}\ (\%) = [A_{10}/A_0] \times 100 \quad (4)$$

where, A_{500} represents the absorbance of diluted emulsion at 500 nm, C is the β-CN concentration (g/mL), Φ is the volume of the oil fraction (Φ = 0.15), 100 is the dilution, A_0 and A_{10} represent respectively the absorbance values of the diluted emulsion at 500 nm at time zero and after 10 min.

2.7. Interfacial properties

The interfacial tension values at oil/water (O/W) interface for each β-CN solution (concentration of 1 g/L) was measured using a TSD-971 Tensiometry System Digital (Gibertini Elettronica, Italia) equipped with the "Du Noüy methodology" as previously mentioned in the works of Lajnaf, Gharsallah, et al. (2020a) and Lam and Nickerson (2015b).

2.8. Determination of the hydrophobicity

The surface hydrophobicity of the purified β-CN (concentration of 1 g/L) was determined using the method of Al-Shamsi, Mudgil, Hassan, and Maqsood (2018) and expressed as micrograms of BPB-bound amount (bromophenol-blue) per gram of protein. In this assay, a higher amount of bound-BPB reflects a higher proteins' hydrophobicity.

2.9. Electrical charge (ζ-potential measurements)

The ζ-potential (mV) of the isolated β-CN solutions was determined at 25 °C using a Zeta-sizer Nano-ZS90 (Malvern Instruments, Westborough, MA) as previously described (Lam & Nickerson, 2015b). Experiments were performed at a concentration of 1 g/L.

2.10. Statistical analysis

The significance of the main effects of protein (camel and bovine β-CN) along with their associated interactions on emulsifying properties (EAI and ESI), physico-chemical characteristics (ζ-potential, surface hydropohobicity and interfacial tension measurements), antioxidant and antimicrobial activities was tested by three-way analysis of variance (ANOVA) followed by post hoc Duncan test to compare the means ($p < 0.05$).

IBM-SPSS software (Version 19) was used to perform statistical analyses. All experiments were performed at least in triplicate and results

were reported as the mean ± one standard deviation.

3. Results and discussion

3.1. Purification of camel and bovine β-CN

The results of purification of β-CN from bovine and camel milk according to Huppertz et al. (2006) showed that the isolation of this protein can be achieved by an easy two step purification procedure using rennet coagulation to separate caseins from whey in milk followed by a cold storage of caseins curd in water to solubilize the β-CN.

Since β-CN is the most hydrophobic among caseins (α- and κ-CN) (Cheng et al., 2020) and it is loosely bound to the other caseins through hydrophobic interactions, it tends to dissociate when these hydrophobic bonds are weaker by lowering the temperature to 4 °C (Pierre & Brule, 1981). The cold solubilzation allow the recovery of a mixture of β-CN variants which are A1, A2 without preference for a genetic variant in agreement with previous works (Petrat-Melin, 2014; Petrat-Melin et al., 2015).

The RP-HPLC chromatograms of supernatant containing the isolated β-CN from camel and bovine milk are shown in Fig. 1. Chromatograms showed that after cold storage, the isolated bovine camel β-CN proteins were found in the supernatant obtained after centrifugation, whereas α-CN and κ-CN were kept in the discarded curd. The bovine β-CN percentage rose from 37.4% in camel milk to 72.4% after purification. On the other hand, the purity of camel β-CN increased from 44% in camel milk to 81.5% after cold-extraction. Solubilization of β-CN on cold storage is related to the weakening of hydrophobic bonds as well as increased solubility of β-CN at low temperature (Huppertz et al., 2006; Pierre & Brule, 1981; Sullivan et al., 1955). Thus, higher camel β-CN purity when compared to bovine β-CN can be attributed to the higher β-CN content in camel milk when compared to cow milk as reported by Kappeler, Farah, and Puhan (2003) or the higher solubility of camel β-CN at low temperature.

3.2. Antioxidant properties

Antioxidant capacity of the purified samples of camel and bovine β-CN was determined via DPPH radical-scavenging activity, FRAP and FCA methods (Table 1). First, as expected, Table 1 shows that the antioxidant activities increased with the protein concentration (0.1, 1 and 5 g/L) regardless of the used method and the β-CN origin in agreement with previous studies (Bamdad et al., 2017).

Furthermore, camel β-CN presented significantly higher antioxidant capacities as compared to its bovine counterpart regardless of the protein concentration in both DPPH radical-scavenging and ferrous-chelating activities. Indeed, the DPPH rates (in %) at protein concentration of 5 g/L were 49.86 ± 7.11% and 32.07 ± 4.0 3% for the camel

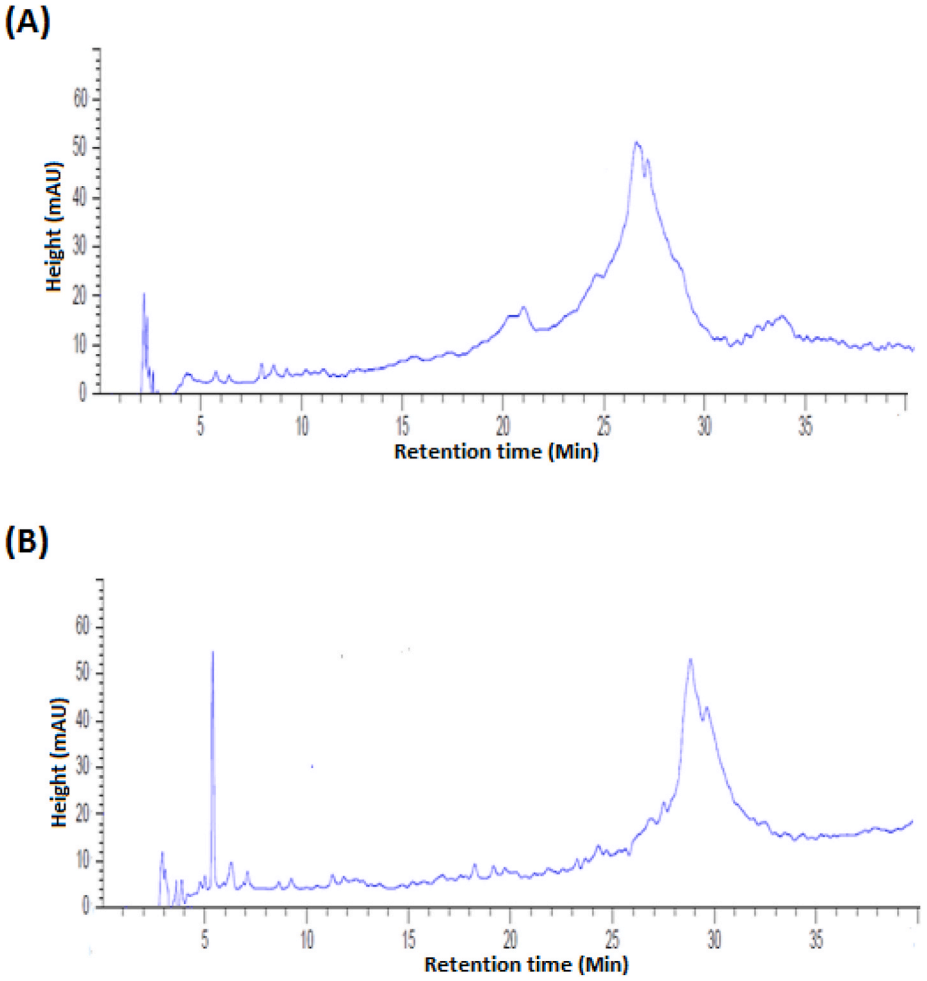

Fig. 1. HPLC chromatograms recorded at 220 nm for purified bovine and camel β-CN by extracting rennet curd, heated at 80 °C for 5 min, with demineralized water at 5 °C for 24 h (chromatograms A and B, respectively).

Table 1
Antioxidant properties (DPPH, FRAP and FCA) of camel and bovine β-CN at different concentrations (0.1, 1 and 5 g/L). a-e Different letters in the same column indicate significant differences ($p < 0.05$) between protein fractions.

Protein	Protein concentration	DPPH (%)	FRAP (μmol TE/g of protein)	FCA (%)
Bovine β-CN	**0.1 g/L**	10.39 ± 3.42^{d}	12.25 ± 1.23^{c}	18.15 ± 8.38^{e}
	1 g/L	19.37 ± 1.82^{c}	25.91 ± 1.93^{b}	44.03 ± 4.60^{d}
	5 g/L	32.07 ± 4.03^{b}	60.93 ± 4.36^{a}	62.67 ± 1.43^{b}
Camel β-CN	**0.1 g/L**	16.69 ± 1.99^{c}	13.22 ± 2.55^{c}	16.11 ± 2.80^{e}
	1 g/L	28.34 ± 1.12^{b}	25.35 ± 2.21^{b}	51.89 ± 1.51^{c}
	5 g/L	49.86 ± 7.11^{a}	59.97 ± 3.64^{a}	73.58 ± 2.07^{a}

and bovine β-CN, respectively, while FCA values of 73.58 ± 2.07% and 62.67 ± 1.43% were obtained for camel and bovine β-CN, respectively. Thus, camel β-CN exhibited significantly greater antioxidant activity when compared to its bovine counterpart. Salami et al. (2011) reported that camel β-CN carried higher antioxidant activity when compared to camel caseins (α_S-, β- and κ-CN). These authors attributed the interesting antioxidant properties of camel β-CN to its highest hydrophobicity index and to the primary sequence of this protein which plays an important role in free radical-scavenging. Indeed, camel β-CN has greater antioxidant amino-acids content as Tyr, Met, Ile, Leu and Pro as compared to bovine β-CN (Esmaili et al., 2011). Not only the amino-acid residues composition of proteins is important in antioxidant capacities but also their positioning and their accessibility (Elias, McClements, & Decker, 2005; Salami et al., 2011).

No significant differences of FRAP values were observed between camel and bovine β-CN (~60 μmol TE/g of proteins at 5 g/L). The FRAP values of the camel β-CN obtained in the present study are consistent with Al-Shamsi et al. (2018) who reported that FRAP of camel caseins solution achieved a value of 58 μmol TE/g. Thus, these findings showed that both caseins exhibited the same reducing activity towards ferric ions, and suppress their pro-oxidant effect. This behavior could be explained by the high sequence similarity between these two proteins (~84.5%) suggesting a similar exposure of the electron-rich side chains.

Several studies have been reported on the antioxidant activities of milk proteins and especially after enzymatic hydrolysis in order to improve their antioxidant behavior (Al-Shamsi et al., 2018; Kumar, Chatli, Singh, Mehta, & Kumar, 2016; Lajnaf, Gharsallah, et al., 2020a; Petrat-Melin et al., 2015; Salami et al., 2011).

Lajnaf, Gharsallah, et al. (2020a) reported that the purified camel α-lactalbumin is more effective in scavenging the DPPH radicals when compared to its bovine counterpart in its both calcium-loaded (holo) and calcium depleted (apo) states. These authors found also that apo camel α-lactalbumin displayed higher FRAP and iron chelating activities compared to its bovine counterpart due to the different molecular structure of both proteins and the different amino-acid composition. Petrat-Melin et al. (2015) found that cold-extracted β-CN, when subjected to enzymatic hydrolysis, resulted in increased antioxidant capacity toward the ABTS radicals. Similarly, Kumar et al. (2016) and Al-Shamsi et al. (2018) noted an increase in the DPPH radical-scavenging activity and in the metal-chelating activity of the camel caseins after hydrolysis using different enzymes due to breakdown of proteins leading to generate active peptides with antioxidant potential and due to the exposure amino-acid residues with carboxyl groups. Kamau and Lu (2011) also reported that the DPPH radical-scavenging activity of hydrolyzed whey protein concentrate (WPC) was dependent on the used enzyme as well as the hydrolysis conditions (pH and temperature). Nevertheless, Al-Shamsi et al. (2018) and Luo, Pan, and Zhong (2014) found that the unhydrolyzed camel and bovine caseins carried higher FRAP values when compared to the hydrolysates because of the decreased length of peptide chains after hydrolysis.

3.3. Antimicrobial activities

Antimicrobial activity results of camel and bovine β-CN are presented in Table 2. The bovine and camel β-CN had no bactericidal activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Furthermore, bovine β-CN had no antifungal activity against *Penicillium bilaiae*, *Aspergillus tamarii*, *Aspergillus sclerotiorum* and *Aspergillus protuberus* even at a concentration of 5 g/L.

The same trends were reported for bovine caseins by Pellegrini et al. (2003) who noted that native caseins exhibited no antimicrobial activity, they just release a number of bioactive peptides after digestion. These peptides have both antibacterial and immunostimulating properties. Indeed, milk protein-derived bioactive are inactive within the sequence of milk proteins, whereas, they can be released *in vivo* by digestive proteases or *in vitro* by enzymatic hydrolysis. Once bioactive peptides are released, they act as regulatory compounds in the host organism with specific activities such as antioxidant, and antimicrobial activities (López-Expósito, Quirós, Amigo, & Recio, 2007; Salami et al., 2011).

Table 2 shows that the analyzed camel β-CN at a concentration of 5 g/L exerted strong antifungal activities towards *Aspergillus tamarii* and *Aspergillus sclerotiorum* with inhibition zones of 20 ± 0.9 and 30 ± 1.5 mm, respectively. This behavior can be explained by the ability of camel β-CN to inhibit the synthesis of the fungal cell wall or perturb fungal membrane structure, leading to fungal cell lysis (Selitrennikoff, 2001). However, camel β-CN had non-inhibitory effect on *Aspergillus protuberus* and *Penicillium bilaiae* at a concentration of 5 g/L.

At the same protein concentration (5 g/L), the α_{S2}-CN from goat milk was reported to have antimicrobial effects against various Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Bacillus cereus* and *Shigella flexneri*. Thus, caprine α_{S2}-CN protein was considered as an important antimicrobial agent (Triprisila et al., 2016). Among all milk proteins, lactoferrin exhibits significant antifungal activities against *Candida albicans* at protein concentrations ranged from 0.2 to 6.4 g/L and against *Candida glabrata* at protein concentrations of 6 g/L (Fernandes & Carter, 2017).

On the other hand, higher protein concentrations of camel caseins (in the range of 20–40 g/L) are needed to inhibit some Gram-positive and Gram-negative bacteria as *Escherichia coli* and *Listeria innocua* (Jrad et al., 2015). Otherwise, in the presence of 5 g/L of purified camel β-CN, the growth of *Escherichia coli* strains was not affected. This behavior was explained not only by the difference in the protein concentration (20 g/L in the work of Jrad et al. (2015)) but also by the difference in the protein composition and the presence of the other caseins (α- and κ-CN) which could exhibit antibacterial activities. Thus, camel and bovine β-CN doesn't exhibit antibacterial activities against the studied Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*).

Table 2
Antimicrobial activities: antifungal activities (zone of inhibition in mm) of camel β-CN (at a concentration of 5 g/L). Bovine β-CN exhibited no antimicrobial activity.

Protein	Antimicrobial activities: Antifungal activities			
–	***Aspergillus protuberus***	***Penicillium bilaiae***	***Aspergillus tamarii***	***Aspergillus sclerotiorum***
Bovine β-CN	–	–	–	–
Camel β-CN	–	–	20 ± 0.9 mm	30 ± 1.5 mm

3.4. Emulsifying properties

EAI and ESI values of the purified β-CN solutions as a function of pH value (5.0, 7.0 and 9.0) and milk origin (camel and bovine milk) at a protein concentration of 1 g/L are shown in Fig. 2A and B. The electronic microscopy images of the β-CN created emulsions are shown in Fig. 3.

As expected, β-CN coated the oil-droplets better at pH 7.0 and 9.0 than those at pH 5.0 with higher EAI values of at pH 9.0 (EAI ~ 29.4 and 36.5 m^2/g for bovine and camel β-CN, respectively) (Fig. 2A). Fig. 3 showed also that the oil-droplet diameter of the β-CN emulsion visually decreased with the increase of pH level during emulsion preparation. On the other hand, better emulsification activity values were obtained with the camel β-CN when compared to its bovine counterpart regardless of pH value.

These results are in consistence with those of Augusta et al. (2007) and Leman, Kinsella, and Kilara (1989) carried out with bovine caseins. These authors reported a similar decrease in the emulsifying properties in acidic conditions caused by changes in the proteins charge and solubility. Indeed, a substantial reduction in surface charge of coated droplets by milk proteins near the pI (pH~ 5) leads to a loss of electrostatic stabilization (Dickinson, 1997).

ESI values of both β-CN samples increased when the pH values rose from 5.0 to 9.0 regardless of the protein origin (Fig. 2B) in agreement with previous studies of bovine caseins (Augusta Rolim Biasutti, Vieira, Capobiango, Silva, & Silvestre, 2007; Marinova et al., 2009; Mellema & Isenbart, 2004). Bovine and camel β-CN gave the highest stability of emulsions at pH 9.0 reaching 52%. On the other hand, at pH 7.0, ESI values of bovine β-CN were found to be significantly higher as compared to its camel counterpart (ESI~ 26.4 and 21.7% for bovine and camel β-CN, respectively). This behavior could be explained by the difference in the structure between both β-CN. Indeed, Lajnaf, Gharsallah, et al. (2020a) found that bovine β-CN carried a different secondary structure as compared to camel β-CN, especially in the β-sheet structure. This different structure explained the high foam stability of bovine milk as the β-CN plays the key role in stabilizing milk foams.

Furthermore, emulsions made camel β-CN at pH 5.0 were found to be unstable (ESI < 2%). Similarly, it has been previously reported that emulsion stability of caseins is higher at pH values far from their pI. Indeed, the higher production of charged molecules during the creation of the emulsions would increase the repulsion forces between protein films and improve the ESI of the emulsions (Biasutti et al., 2007). On the contrary, the acidification usually leads to the decrease in emulsion stability because of precipitation and aggregation which violates the amphiphilic nature of milk proteins (Mellema & Isenbart, 2004).

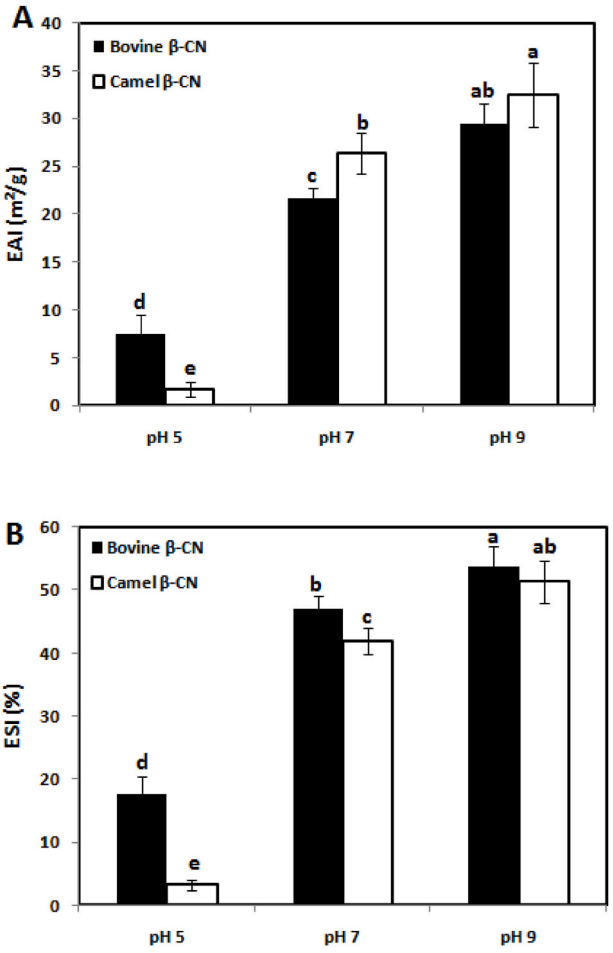

Fig. 2. Emulsifying Activity Index (EAI) (A) and Emulsion Stability Index (ESI) (B) of camel and bovine β-CN, at a protein concentration of 1 g/L and as a function of pH value (5.0, 7.0 and 9.0).
[a-e] Samples represented with different letters are significantly different from each other ($p < 0.05$). Error bars show the standard deviations of mean values of EAI and ESI.

3.5. Surface characteristics

3.5.1. Surface hydrophobicity

The surface hydrophobicity (BPB-bound amount) of the purified β-CN samples at a protein concentration of 1 g/L as a function of pH value (pH 7.0, 5.0 and 9.0) is shown in Table 3. Overall, BPB-bound amounts of camel β-CN were significantly higher than those of bovine β-CN.

Indeed, at pH 7, the BPB-bound amounts were 9.3 ± 1.5 μg/mL and 14.1 ± 2.0 μg/mL for bovine and camel β-CN, respectively. At pH 5.0, both β-CN samples carried higher surface hydrophobicity values when compared to those at pH 7.0 and 9.0. The maximum values were achieved with camel β-CN under these conditions: BPB-bound amounts reached 24.9 and 29.5 μg/mL of the protein solution, respectively.

In support of these results, Lajnaf, Gharsallah, et al. (2020a) reported that camel β-CN shows different structural characteristics and greater surface hydrophobicity as compared to bovine β-CN due to its higher content of Ile in primary sequence. Previous studies (Cases, Rampini, & Cayot, 2005; Mellema & Isenbart, 2004) noted that at low pH (~4.6) milk proteins presented higher surface hydrophobicity due to the greater exposure of hydrophobic residues in caseins, from which β-CN, αs-CN and κ-CN, have been dissociated.

Lam and Nickerson (2015a) confirmed that, at neutral pH milk proteins molecules were strongly negatively charged which may repel BPB from binding to the hydrophobic part of proteins. On the contrary, the reduction of the electrostatic repulsion near protein's pI could enhance protein–BPB interactions (Lajnaf et al., 2019).

Finally, despite the highest surface hydrophobicity of β-CN samples in acidic conditions, lower emulsifying properties were found regardless of the protein origin. These findings suggest that surface hydrophobicity of proteins is not a major factor determining the emulsifying and interfacial properties of proteins in agreement with Leman et al. (1989).

3.5.2. Determination of ζ-potential

Table 3 shows the surface charge values (ζ-potential) of bovine and camel β-CN at three pH values: 5.0, 7.0 and 9.0. The ζ-potential values of bovine β-CN were about ~-32.9 ± 1.1 mV and −20.9 ± 1.9 mV at pH 7.0 and 5.0, respectively. These results are in agreement with previous works carried with bovine β-CN (McCarthy, Kelly, O'Mahony, & Fenelon, 2014). Indeed, the ζ-potential of emulsions prepared with bovine β-CN was highly negative since it is away from its pI value (~35 mV at pH > 6), and increased with decreasing pH to ~-20 mV at pH 5 (McCarthy et al., 2014). The ζ-potential of camel β-CN was approximately ~-28.8 ± 1.5 mV, ~-24.8 ± 1.0 mV and ~-18.1 ± 0.5 mV at pH 9.0, 7.0 and 5.0, respectively. Hence, camel β-CN was found to carry a

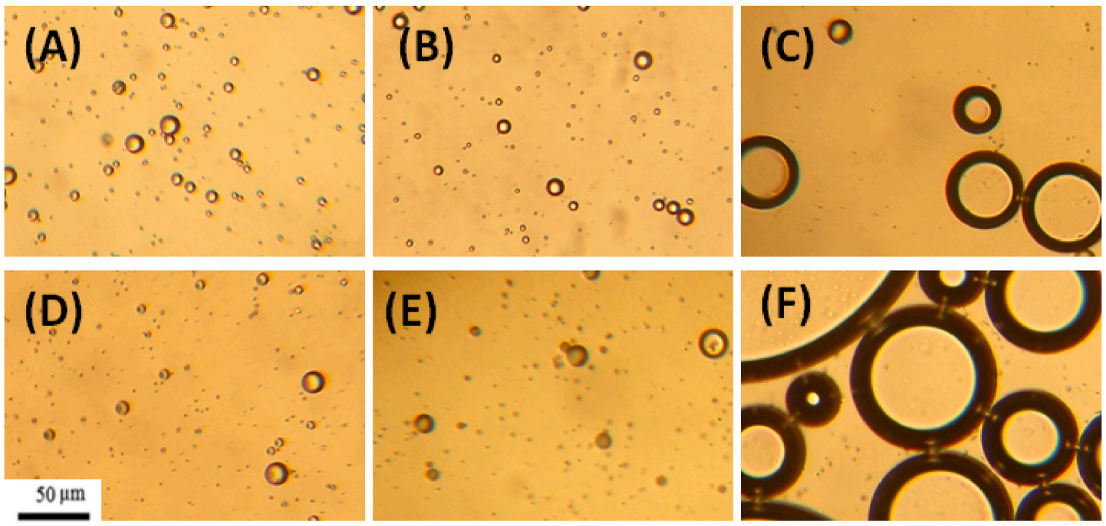

Fig. 3. Microscopy images of oil-in-water emulsions stabilized by bovine and camel β-CN: A: bovine β-CN pH 9.0; B: bovine β-CN pH 7.0; C: bovine β-CN pH 5.0; D: camel β-CN pH 9.0; E: camel β-CN pH 7.0; F: camel β-CN pH 5.0. The experiments were performed in 20 mmol/L Tris-HCl buffer at 25 °C and at a protein concentration of 1 g/L. Bar = 50 µm.

Table 3
Surface properties: ζ-potential measurements (mV), Surface hydrophobicity (µg of BPB-bound) and interfacial tension at the O/W interface (mN/m) of camel and bovine β-CN as a function of pH (5.0, 7.0 and 9.0). The experiments were performed in 20 mmol/L Tris-HCl buffer, pH 7.0, at 25 °C at a protein concentration of 1 g/L.

Protein	pH value	ζ-potential (mV)	Surface hydrophobicity (µg of BPB-bound)	interfacial tension at O/W interface (mN/m)
Bovine β-CN	pH **5.0**	-20.9 ± 1.9^{b}	24.9 ± 1.1^{b}	26.1 ± 0.5^{a}
	pH **7.0**	-30.0 ± 1.5^{d}	9.3 ± 1.5^{d}	19.9 ± 0.7^{b}
	pH **9.0**	-32.9 ± 1.1^{e}	6.9 ± 2.5^{de}	16.7 ± 0.1^{cb}
Camel β-CN	pH **5.0**	-18.1 ± 0.5^{a}	29.5 ± 2.6^{a}	25.9 ± 1.1^{a}
	pH **7.0**	-24.8 ± 1.0^{c}	14.1 ± 2.0^{c}	17.6 ± 0.6^{c}
	pH **9.0**	-28.8 ± 1.5^{d}	4.6 ± 1.5^{e}	14.1 ± 0.6^{e}

a-e Different letters in the same measurement indicate significant differences ($p < 0.05$) between protein fractions.

significantly lower negative charge as compared to its bovine counterpart regardless of pH value ($p < 0.05$) in agreement with the results of Hinz, O'Connor, Huppertz, Ross, and Kelly (2012) who reported that camel caseins have a lowest net-negative charge when compared to bovine, caprine and buffalo caseins.

3.5.3. *Interfacial properties*

The interfacial tension values at O/W interface of camel and bovine β-CN in response to pH values (5.0, 7.0 and 9.0) are shown in Table 3. Overall, all β-CN samples were found to significantly reduce the interfacial tension value from 29.1 mN/m ($p < 0.05$), which is estimated to be close to the surface tension values of pure water at the O/W interface.

Table 3 shows that the order of effectiveness to reduce the surface tension at the O/W interface was: camel β-CN, pH 9.0 ($\gamma\sim$ 14.1 mN/m) > camel β-CN pH 7.0 and bovine β-CN pH 9.0 ($\gamma \sim$ 17 mN/m) > bovine β-CN pH 7.0 ($\gamma\sim$ 20 mN/m) > camel and bovine β-CN pH 5.0 ($\gamma\sim$ 26 mN/m). These results are in agreement with the highest EAI values of camel and bovine β-CN at pH 7.0 and 9.0 (section 3.3) and with Cases et al. (2005) who reported that surface tension at O/W interface of bovine β-CN solution (at a concentration of 11 mg/L and pH 6.7) achieved a value of 13.9 mN/m within 1000 s. Mellema and Isenbart (2004) reported that acidification of caseins solutions to pH$\sim$ 4.6 leads to caseins precipitation and aggregation. The resulting aggregates are less surface active, with a lower efficiency to reduce the interfacial tension at the O/W interface than that at pH 6.7.

The more important efficiency of camel β-CN in reducing the interfacial tension at O/W interface as compared to bovine β-CN can be explained by the difference in amino-acid residues composition and the highest surface hydrophobicity of camel β-CN as compared to bovine β-CN (section 3.4.1). Indeed, camel β-CN is suggested to have a more disordered structure when compared to bovine β-CN due to its higher amount of Pro and Gln residues whose percentages are significantly higher than those of bovine β-CN (Barzegar et al., 2008; Faizullin, Konnova, Haertlé, & Zuev, 2013).

4. Conclusion

The emulsifying properties and *in vitro* antioxidant and antimicrobial activities of isolated camel and bovine β-CN were investigated and compared in this work. Findings indicated that camel β-CN samples exhibited important antioxidant activities when compared to bovine β-CN especially with respect to iron chelating and antiradical activities which could be explained by the higher content of antioxidant amino-acid residues in camel β-CN and the different conformational features between bovine and camel β-CN. On the other hand, no significant differences were observed for the reducing power of both proteins.

Furthermore, camel β-CN showed strong antifungal activities towards *Aspergillus tamarii* and *Aspergillus sclerotiorum* with inhibition zones of 20 ± 0.9 and 30 ± 1.5 mm, respectively, due to its ability to inhibit the synthesis of the fungal cell wall or perturb fungal membrane structure, leading to fungal cell lysis.

Emulsifying properties of β-CN solutions depended on pH level (pH 9.0, 7.0 and 5.0) and milk origin of proteins (camel and bovine milk). A higher surface coverage of the oil-droplets (EAI) was obtained at pH 7.0 and pH 9.0 especially with camel β-CN which carried the highest ability to reduce the surface tension at O/W interface. On the other hand, stability of the created emulsions (ESI) seemed greatest at pH 9.0 and pH 7.0 due to the presence of electrostatic repulsive forces between β-CN molecules as confirmed by the ζ-potential measurements. Changes in pH (pH 5.0) affected the physico-chemical properties of bovine and camel β-CN by increasing the surface hydrophobicity and also decreasing the negative charge and the efficiency to reduce the interfacial tension at O/W interface. These findings confirmed the caseins precipitation, in agreement with the decrease in emulsifying and interfacial behavior of the purified β-CN samples in acidic conditions. For instance, the acidified milk proteins as the β-CN could be used as an interesting agent in

food industry. Indeed, acidified caseins become unstable and precipitate which would be useful in production of acidified milk products with different physical and rheological properties as fermented dairy products and soft white cheese. Furthermore, it may help to solve some difficulties encountered in food industry as the production of low-fat and non-fat yogurts which requires poor texture and whey proteins separation.

Finally, these results confirmed the strong potential of camel β-CN for potential applications in food, pharmaceutical and cosmetic industries.

CRediT authorship contribution statement

Roua Lajnaf: Conceptualization, Methodology, Software, Visualization, Investigation, Writing - original draft, preparation, reviewing and editing. **Houda Gharsallah:** Conceptualization, Visualization, Investigation, Software. **Hamadi Attia:** Supervision. **M.A. Ayadi:** Conceptualization, Supervision, Reviewing and Editing.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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