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Effect of digestive enzymes on antimicrobial, radical scavenging and angiotensin I-converting enzyme inhibitory activities of camel colostrum and milk proteins

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Abstract Camel milk and colostrum are known to be a rich source of bioactive proteins. Camel milk, colostrum and colostral whey proteins were successively hydrolysed by pepsin and pancreatin using an in vitro protocol mimicking gastro-intestinal digestion. The degradation of proteins was characterised by electrophoresis and reversed-phase ultra-high performance liquid chromatography. Two whey proteins, α-lactalbumin and immunoglobulins G, were more resistant to the digestive proteolytic enzymes than other camel milk and colostrum proteins. Undigested and digested samples were assayed for their antioxidant, angiotensin I-converting enzyme inhibitory and antimicrobial

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properties. Camel colostrum, colostral whey and milk proteins had unveiled angiotensin I-converting enzyme (ACE) inhibitory activity following in vitro enzymatic digestion and a higher free radical scavenging activity than before their digestion. Moreover, *Escherichia coli* XL1 blue and *Listeria innocua* LRGIA01 cells growth were both inhibited by undigested and digested samples, suggesting that antimicrobial proteins resisted to the action of digestive enzymes or that antimicrobial fragments of camel milk and colostrum proteins were released or both. After pepsin and pancreatin hydrolysis, camel milk and colostrum proteins digests still had an antibacterial activity and their antioxidative and ACE-inhibitory activity even increased, suggesting that bioactive fragments of camel milk and colostrum proteins such as antioxidative and ACE-inhibitory peptides were released. Among 181 peptides identified by tandem mass spectrometry, 25 were homologous to known bioactive peptides, particularly with ACE inhibitors and free radical scavengers.

Keywords Angiotensin I-converting enzyme inhibition · Antibacterial activity · Antioxidant activity · Camel colostrum proteins · Camel milk proteins · Digestive enzymes

1 Introduction

Besides providing essential amino acids, colostrum and to a lesser extent milk proteins can exhibit different biological activities (Korhonen and Pihlanto 2007). For instance, lactoferrin (Lf) present in the milk or colostrum of various species of mammals exerts antimicrobial activities (Farnaud and Evans 2003). Camel milk and colostrum protein composition and structure have been reported to differ from those of milk and colostrum proteins of the other mammals (Farah 1993; El Hatmi 2007). These differences mainly concern whey proteins: for example β-lactoglobulin (β-LG), the main bovine whey protein, is absent from camel whey, while cow's milk is deficient in some camel milk proteins such as peptidoglycan recognition protein (PGRP; Kappeler et al. 2004) and camel whey basic protein (CWBP; Ochirkhuyag et al. 1998). Moreover, according to Abd El-Gawad et al. (1996), the concentration of Lf in camel milk (0.34 g.L^{-1}) and colostrum (5.10 g.L^{-1} at the second day after parturition) has been reported to be higher than in its bovine counterparts (0.06 g.L^{-1} in milk and 0.50 g.L^{-1} in colostrum at the second day after parturition). Camel milk is a rich source of proteins with potential antimicrobial and antioxidative activities (Salami et al. 2010). As it is the main diet of some desert nomads and as the latter use it in traditional medicine for the treatment of gastric problems, the biological activities of the proteins deserve further study. Some prerequisites have to be considered: (1) a bioactive protein might have an effect on the health of its consumers once ingested and its biological activity should not be impaired following its hydrolysis by digestive proteolytic enzymes, or (2) a protein might possess biologically active regions encrypted in its sequence that should be liberated by the digestive enzymes.

Numerous authors have thus reported that peptides inhibiting the angiotensin I-converting enzyme (ACE; EC 3.4.15.1) in vitro can be released from the milk proteins by digestive proteolytic enzymes (Hernandez-Ledesma et al. 2004; Jäkälä and Vapaalo 2010; Ricci et al. 2010; Rousseau-Ralliard et al. 2011; Qureshi et al. 2013). Interestingly, as ACE is a peptidyl carboxydipeptidase which catalyses the production

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of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin, molecules significantly inhibiting its activity can exert an antihypertensive activity in vivo, although it is clear that a significant inhibition of ACE in vitro does not warrant any antihypertensive effect in vivo (Hernández-Ledesma et al. 2011). Recently, Salami et al. (2011) reported that ACE-inhibitory peptides were released following the successive actions of pepsin and trypsin, or chymotrypsin on camel whole casein (CN). Salami et al. (2010) also reported that the antioxidative and the antimicrobial activities of camel milk whey proteins can be increased after a limited proteolysis by trypsin or chymotrypsin.

However, to our knowledge, biological activities such as radical scavenging and ACE-inhibitory activities of camel colostrum proteins have never been studied neither before, nor after enzymatic digestion. Therefore, the effect of the action of pepsin and then pancreatin (to mimic the gastric and intestinal phases of digestion, respectively) on camel (*Camelus dromedarius*) colostrum (CP), colostral whey (CWP) and milk proteins (MP) on their antioxidative, ACE-inhibitory and antibacterial activities were investigated in the present study. Identification of peptides released by the action of the digestive enzymes was performed by tandem mass spectrometry, and some peptides with putative biological activity were found by sequence alignment with known bioactive peptides.

2 Materials and methods

2.1 Samples collection

Colostrum and milk were obtained from five healthy dromedaries (*C. dromedarius*) all belonging to the experimental herd of the Livestock and Wildlife Laboratory (Institute of Arid Land, Medenine, Tunisia). Colostrum samples were collected from 1 h to 144 h post-partum and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) to determine which sample contained the highest content of Lf, the main antimicrobial protein. Camel milks were collected between the second and fourth week post-partum and mixed. All samples, collected manually in sterile bottles once per day (usually in the morning), were divided into three aliquots and were immediately stored at −20 °C until used.

2.2 Whey protein and casein separation

Colostral whey proteins (CWP) were prepared as described in a previous work for milk whey protein preparation (El Hatmi et al. 2007) with some modifications. Camel colostrum was skimmed by centrifugation (5,000×*g*, 30 min, 4 °C) and warmed at 30 °C (the camel milk was also skimmed with the same experimental conditions). Caseins were separated from whey proteins following pH adjustment to 4.2 with 1 mol.L^{-1} HCl to enhance their precipitation. After a centrifugation at 5,000×*g* for 30 min at 20 °C, CWP (supernatant) were neutralised by 1 mol·L^{-1} NaOH before dialysis (cut-off of dialysis membranes = 100–500 Da, SpectraPor; Spectrum Labs Inc., Rancho Dominguez, CA, USA) against 200 vol of distilled water for 72 h and freeze-dried before use.

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2.3 In vitro digestion

All samples were in vitro digested by the simplified procedure described by Parrot et al. (2003). For the gastric digestion step, 20 mg of freeze-dried samples, i.e. MP, CWP and CP, were dissolved in 1 mL of ultra-pure (Milli-Q) water (Millipore, Bedford, MA, USA). The pH was adjusted to 2.0 with 1 mol.L^{-1} HCl. Pepsin (EC 3.4.23.1) at 1 g.L^{-1} in 0.01 mol.L^{-1} HCl [from porcine stomach mucosa; specific activity of 3260 units (U).mg^{-1} protein; Sigma-Aldrich Co., St. Louis, MO, USA] was added at an enzyme-to-substrate mass ratio of 1:200. The digestion was performed at 37 °C for 30 min. To mimic the intestinal digestion step, the pH was increased to 7.5 with 30 % w/w NaOH added dropwise to simulate the pH in the duodenum. Twenty-five microliters of pancreatin at 2 g.L^{-1} in water (from bovine pancreas, activity equivalent to 8 U.S.P. specifications according to the supplier Sigma-Aldrich) was added to 975 μL of peptic digest to get an enzyme-to-substrate mass ratio of 1:400. Simulated intestinal digestion of samples was carried out at 37 °C for 4 h. Then, the protein hydrolysis reaction was stopped by heating at 85 °C for 5 min. Digested samples were directly stored at −20 °C until analysed, while samples for antimicrobial activity assays were dialysed (cut-off of dialysis membranes = 100−500 g.mol^{-1}) for 48 h against 200 vol of Milli-Q water and freeze-dried prior to storage at −20 °C.

2.4 SDS–PAGE analysis

The protein composition of the different fractions was determined by SDS–PAGE according to Laemmli and Favre (1973). SDS–PAGE was running in 4.9 % w/v polyacrylamide stacking gel at pH 6.8 and in 15.4 % w/v polyacrylamide resolving gel at pH 8.8. Samples were dissolved in 0.125 mol.L^{-1} tris(hydroxymethyl)aminomethane (Tris)–HCl buffer, pH 6.8, in the presence of 0.1 % w/v SDS, 5 % v/v 2–mercaptoethanol, 10 % v/v glycerol, and 0.01 % w/v bromophenol blue. Volumes of 30 μL of samples (2 g.L^{-1}) were loaded on the gel and the protein bands were stained by Coomassie Brilliant Blue R-250. Precision Plus Protein Prestained All Blue Standards (Bio-Rad, Hercules, CA, USA) were used as molecular mass standards.

2.5 Protein and peptide quantification

The protein concentration was determined in triplicates by the Bradford method using bovine serum albumin (BSA) as protein standard (Ku et al. 2013) and the data are expressed as milligrams per liter equivalent (eq.) to BSA.

The peptide concentration in different hydrolysates samples was estimated by using the o-phthaldialdehyde (OPA) method of Frister et al. (1988). The OPA solution was prepared as follows: 100 mmol.L^{-1} sodium tetrahydroborate buffer, pH 9.3, containing 1 % w/v SDS, 6 mmol.L^{-1} OPA (dissolved in an aliquot of pure methanol), and 14 mmol.L^{-1} of 2-mercaptoethanol. A quantity of 3 mg of freeze-dried powder was dissolved in 1 mL of water and aliquots of 20 μL were mixed to 200 μL of OPA reagent. The solutions were mixed briefly and their absorbance at 340 nm was measured after a 3-min reaction time at room temperature using an MRX® microplate reader (ThermoLabsystems, Chantilly, VA, USA). Calibration curve was established with leucine. The concentration was expressed in micromoles per liter eq. NH$_2$. All the assays were carried out in triplicates.

2.6 Reversed phase ultra-high performance liquid chromatography (RP-UHPLC)

The protein fractions and their corresponding hydrolysates (1 mg.mL^{-1} of freeze-dried sample) were analysed by RP-UHPLC. Volumes of 10 μL were loaded onto a XB-C18 analytical column (150-mm length, internal diameter of 2.1 mm, 2.6-μm particle size, 10-nm porosity; Phenomenex, Le Pecq, France) connected to a UHPLC system (Shimadzu, France) equipped with a photodiode array detector SPD M20A and a gradient pump LC 30AD. Peptides were then eluted for 90 min by a linear gradient from 5 to 50 % v/v acetonitrile in water and in the presence of 0.1 % v/v trifluoroacetic acid (TFA) at 27 °C. The flow rate was 0.5 mL.min^{-1}, detection was carried out at 215 nm and the separation was analysed with the LabSolutions version 4.2 software (Shimadzu).

2.7 Nano-electrospray ionisation tandem mass spectrometry coupled with liquid chromatography (LC/nESI–MS/MS)

Peptide hydrolysate was analysed by on-line LC/nESI–MS/MS on a nanoLC Dionex RSLC system coupled to a ThermoScientific Q Exactive Quadrupole-Orbitrap mass spectrometer (Dionex/ThermoScientific, Villebon-sur-Yvette, France). A quantity of 0.3 μg (10 μL) was loaded onto a RP-HPLC C18 Acclaim PepMap100 column (150-mm length, internal diameter of 75 μm, 3-μm particle size, 10-nm porosity; Dionex/ThermoScientific). Chromatographic separation was performed with a linear gradient of 4 to 60 % v/v acetonitrile in water in the presence of 0.08 % v/v formic acid and 0.1 % v/v TFA for 50 min at a flow rate of 0.3 μL.min^{-1}. Ionization of peptides by a Proxeon ESI source was performed in positive mode at a voltage of 2 kV. Each MS spectrum was acquired over the range of m/z values from 300 to 2,000 and MS/MS fragmentation was performed on the 10 most intense ions.

Peptides were identified from MS/MS spectra using X!Tandem pipeline software (Plateforme d'Analyse Protéomique de Paris Sud-Ouest, INRA, Jouy-en-Josas, France, http://pappso.inra.fr). The peptide identification database was composed of the *Camelus* taxonomy (n°9836) downloaded from www.uniprot.org on the 13th of August 2013. Database search parameters were specified as follows: an unspecific enzyme cleavage was selected; a 0.05-Da mass error was allowed on fragment ions while 10 ppm mass error was allowed for parent ions; phosphorylation of serine was selected as a variable modification; and for each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered to be a prerequisite for valid peptide identification.

Sequence alignments with known peptides displaying any biological activity were performed with the PepBank database available on http://pepbank.mgh.harvard.edu/search/blast.

2.8 Radical scavenging activity measurements

The free radical scavenging activity of gallic acid and Trolox as well as proteins and peptides was carried out according to the method of Re et al. (1999) adapted to small reaction volumes by Sadat et al. (2011). Briefly, the ABTS$^{+•}$ (ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation was produced by

dissolving 7 mmol.L^{-1} of cation ABTS$^+$ in 2.45 mmol.L^{-1} potassium persulfate and keeping the mixture in the dark for 15 h at room temperature. The ABTS$^{+\bullet}$ radical cation reagent was then diluted with 5 mmol.L^{-1} sodium phosphate buffer, pH 7.4 to reach an absorbance of ca. 0.7 at 740 nm. The radical was stable for at least 1 h at 22 °C. Gallic acid, Trolox, and the protein and peptide fractions were dissolved in 150 μL of a solution of 20 % v/v acetonitrile in water and added to 150 μL of the ABTS$^{+\bullet}$ radical reagent, and incubated for 10 min at 30 °C, then absorbance was measured at 740 nm with an MRX® microplate reader as a function of concentration. All the experimental points of the curves of the radical scavenging activity versus the proteins or peptides concentration were mean values of five measurements±SD (standard deviation). The antioxidant activity was calculated as follows:

$$\text{Activity}(\%) = [1 - (A_r - A_b)/(A_i - A_b)] \times 100 \tag{1}$$

where A_i is the absorbance of the initial ABTS$^{+\bullet}$ radical cation, A_r is the absorbance of the remaining radical and A_b is the absorbance of the blank (in the case of phosphate buffer, $A_b = 0.09$).

The IC$_{50}$ value is defined as the concentration of sample able to transform 50 % of ABTS$^{+\bullet}$ to ABTS$^+$, i.e. when the absorbance of the remaining radical ($A_r - A_b$) was the same than that of the scavenged radical ($A_i - A_r$). Thus, log (IC$_{50}$) corresponds to the x-intercept of the curve:

$$\log(A_r - A_b)/(A_i - A_r) \, \text{vs.} \log[\text{sample}] \tag{2}$$

where the concentrations of samples were expressed in micromolar for gallic acid and Trolox, micromolar eq. NH$_2$ for the peptide fractions and milligrams per liter eq. BSA for the protein fractions.

The Trolox-equivalent antioxidant capacity (TEAC) measures the free radical scavenging capacity of a given substance, as compared to the standard, Trolox. The TEAC is the ratio of the slope of the plot of activity versus concentration of the given substance over the slope of the plot of Trolox (Re et al. 1999). The TEAC is expressed as micromoles Trolox equivalent (TE) per milligram protein in the case of CP, CWP and MP, or micromoles TE per micromole eq. NH$_2$ in the case of the hydrolysates. Student's t tests were also performed on data to assess differences between the TEAC values of undigested samples CP and CWP compared with MP and those of digested ones with MP hydrolysate, and differences were considered significant when $p < 0.05$.

2.9 Determination of the ACE-inhibitory activity

The ACE-inhibitory activity was determined according to Tauzin et al. (2002) with minor modifications. A volume of 20 μL of ACE from rabbit lung (3.33 U.L^{-1}; Sigma-Aldrich) was added to 100 μL of 4.5 mmol.L^{-1} hippuryl-L-histidyl-L-leucine (HHL; Sigma-Aldrich) and 30 μL of 50 mmol.L^{-1} 2-(cyclohexylamino)ethanesulfonic acid buffer containing 300 mmol.L^{-1} NaCl and 5 % v/v absolute ethanol (pH 8.3) or 30 μL of samples at final concentrations of 150 μmol.L^{-1} eq. NH$_2$ for peptide fractions or

150 mg.L^{-1} for protein fractions. The mixture was incubated at 37 °C for 1 h. Then, the enzyme reaction was stopped by adding captopril (32 μmol.L^{-1}), trisodic ethylenediaminetetraacetate at 10 μmol.L^{-1} and TFA at 0.13 mol.L^{-1}. After that, the released hippuric acid was quantified by RP-HPLC onto a Symmetry C$_{18}$ column (150×2 mm, 5-μm particle size, 10-nm porosity; Waters Corp., Milford, MA, USA) connected to an Alliance HPLC unit 2690 (Waters) and elution was performed for 7 min by a linear gradient of acetonitrile in water from 13 % to 50 % v/v in the presence of 0.1 % v/v TFA. The flow rate was 0.25 mL.min^{-1} and detection was carried out at 228 nm with a Waters 996 photodiode array detector. All the assays were carried out in triplicates and the percentages of inhibition, compared to the maximum activity of ACE without inhibition, were expressed as mean values±SD. Data were analysed by t test and level of significance was considered when p values were <0.05.

2.10 Bacterial growth inhibition assay

The two tested bacterial strains (*Escherichia coli* XL1 blue and *Listeria innocua* LRGIA01 strains) were cultured in brain heart infusion (BHI) broth at 30 °C as described by Kouadio et al. (2011). These two harmless strains were chosen for their similarity to foodborne pathogenic bacteria (enterohaemorrhagic *E. coli* and *L. monocytogenes* strains, respectively). Freeze-dried undigested and digested samples were dissolved in BHI broth and sterilised by filtration onto 0.2-μm-pore-size membranes (Millipore Corp., Billerica, MA, USA). Antimicrobial tests were performed in sterile 100 wells microplates, which were incubated at 30 °C for 24 h. Optical density at 600 nm (OD$_{600\ nm}$) was recorded every 15 min using a Bioscreen® plate reader (Thermo Fisher Scientific Inc., Illkirch, France). Volumes of 30 μL of overnight cultures containing 1.2 10^7 and 1.1 10^7 colony-forming units (cfu).mL^{-1} of *L. innocua* and *E. coli*, respectively, were used to inoculate each well containing 270 μL of BHI broth (positive controls) or 270 μL of BHI broth supplemented with digested or undigested camel milk or colostrum fractions in order to obtain 10, 20 and 40 g.L^{-1} final concentrations. Triplicates were run for each concentration. In addition to the positive controls, negative controls were also included. The negative controls contained 270 μL of BHI broth supplemented with undigested or digested samples in all the above mentioned concentrations and 30 μL of sterile BHI broth.

The growth curves of *E. coli* XL1 blue and *L. innocua* LRGIA01 were obtained by plotting the OD$_{600\ nm}$ as a function of time. The percentage of inhibition of bacterial growth after 15 h of incubation at 30 °C was estimated by comparing OD$_{600\ nm}$ values measured with the mean values of the positive controls and was calculated as follows:

$$\% \ of\ inhibition = \left[\left(OD_{600\ nm\ control} - OD_{600\ nm\ sample}\right)/OD_{600\ nm\ control}\right] \times 100 \quad (3)$$

As all cultures were performed in triplicates, OD$_{600\ nm}$ values were given as mean value±SD. For the antimicrobial activity assays, a t test (two-samples, assuming unequal variances) was run to compare bacterial growth curves in the presence or absence of CP, CWP or MP, or their hydrolysates at different concentrations. The differences were considered significant when p <0.05.

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3 Results

3.1 Characterisation of MP, CWP and CP and their respective hydrolysates

The protein and peptide contents of native camel milk, colostral whey or colostrum proteins and of their hydrolysates were analysed by SDS–PAGE and RP-UHPLC, respectively.

As Lf is a well-known antimicrobial protein that is abundant in colostrum of mammals, it was used as a reference for our study in order to choose the right moment of collection of colostrum rich in Lf. Thus, the colostrum for which Lf displayed the highest band relative intensity (determined by densitometry at 633 nm from SDS–PAGE profiles of 11 samples collected from 1 to 144 h post-partum; Fig. 1) corresponded to the sample collected 48 h post-partum.

Fractions CP (collected at 48 h post-partum), CWP (prepared from 48 h post-partum colostrum) and MP were thus prepared. Their corresponding hydrolysates (named CPH, CWPH and MPH, respectively) were obtained by successive action of pepsin and pancreatin. Overall, the SDS–PAGE profiles (Fig. 2a) showed the absence of β-LG, the main whey protein in bovine milk (Beg et al. 1985). Little amounts of CN were observed in CWP, as they precipitated very slowly when the pH was lowered to 4.2, especially in colostrum, whereas very high relative amounts of immunoglobulins G (IgGs) were found in CWP. The CP fraction contained Lf, α-lactalbumin (α-LA) and camel serum albumin (CSA).

After hydrolysis by pepsin followed by hydrolysis by pancreatin, at least two of the initial bands remained visible on the electrophoretic profiles although they were far less intense than before the action of enzymes (Fig. 2b). They corresponded to α-LA and to IgGs in MPH, CWPH and CPH, respectively.
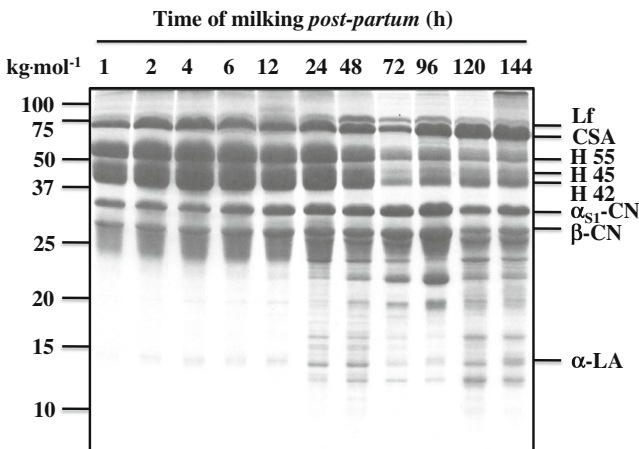
Fig. 1 SDS–PAGE profiles of total proteins from camel colostrums collected from 1 to 144 h post-partum. *Lf* lactoferrin, *CSA* camel serum albumin, *H* heavy chains of immunoglobulins G, *CN* casein, *α-LA* α-lactalbumin. The H42, H45 and H55 heavy chains were identified on the electropherogram according to Lauwereys et al. (1998) and the other proteins according to the proteomic analysis of Hinz et al. (2012)
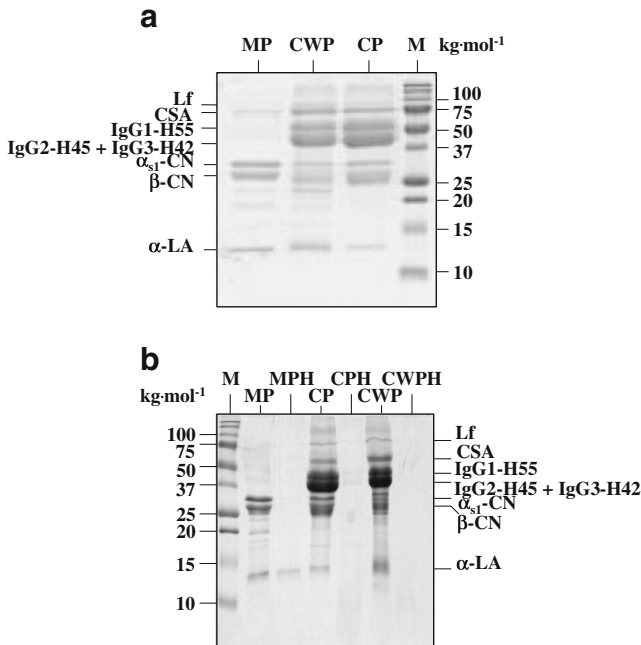
Fig. 2 a SDS–PAGE profiles of camel milk proteins (*MP*), colostral whey proteins (*CWP*) and colostrum proteins (*CP*) (CWP and CP were prepared from colostrum collected 48 h post-partum). **b** SDS–PAGE profiles of the corresponding hydrolysates MPH, CPH and CWPH. *M* molecular mass standards, *Lf* lactoferrin, *CSA* camel serum albumin, *H* heavy chains of immunoglobulins G (IgG), *CN* casein, *α-LA* α-lactalbumin. The H42, H45 and H55 heavy chains were identified on the electropherogram according to Lauwereys et al. (1998) and the other proteins according to the proteomic analysis of Hinz et al. (2012)

As most of camel milk and colostrum proteins were degraded into smaller fragments that were not detectable by SDS–PAGE analysis after the action of digestive enzymes, each protein fraction as well as their corresponding hydrolysates were characterised by RP-UHPLC analysis (Fig. 3). Three major fractions were observed after retention time of 60 min on the MP chromatogram, but were not present on that of MPH. The first peak corresponded to the elution of α_{s1}-CN, the second to α-LA, the main soluble protein of milk, and the third peak corresponded to the elution of β-CN, as shown by SDS–PAGE control (Fig. 3). The chromatogram was markedly modified after hydrolysis, as a number of peaks appeared between 5 and 60 min showing that the majority of proteins were hydrolysed into small peptides. The same conclusion can be drawn when comparing the chromatograms of intact colostral and colostral whey proteins with those of their respective hydrolysates.

The major peaks on chromatograms of CWP and CP were also eluted after 60 min. However, these peaks were smaller, likely because these fractions did contain little amounts of CN and α-LA comparatively to the high amounts of IgGs, the latter being poorly resolved by RP-UHPLC analysis. Particularly, the CWP profile was more complex probably due to a number of soluble proteins concentrated in whey after the acidification treatment of the colostrum. Before digestion of CP and CWP, almost no
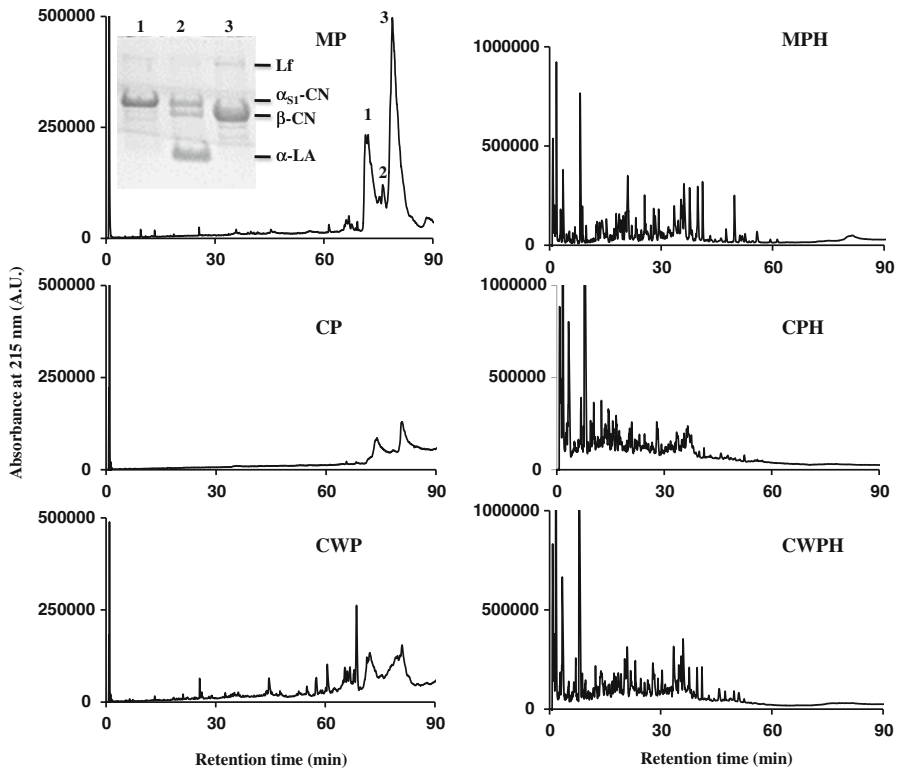
Fig. 3 RP-UHPLC chromatograms of camel milk proteins (*MP*), colostral whey proteins (*CWP*) and colostrum proteins (*CP*), and their respective hydrolysates MPH, CWPH and CPH. Separation conditions were detailed in the "Materials and methods" section. In the case of MP, fractions 1–3 were collected and analysed by SDS–PAGE. *Lf* lactoferrin, *CN* casein, *α-LA* α-lactalbumin, *A.U.* absorbance unit

peak was observed before 60 min. This indicated that only very little amounts of peptides are present in camel milk, colostral whey or colostrum.

3.2 Effect of enzymatic digestion

As camel milk, colostrum and colostral whey contain bioactive proteins (antimicrobial proteins) or proteins with bioactive regions encrypted in their sequence that might be liberated by gastro-intestinal digestion (antioxidant or ACE-inhibitory peptides), the effect of an in vitro hydrolysis by digestive proteolytic enzymes on biological activities of MP, CP and CWP was investigated. Three biological activities that can be ascribed to proteins or their fragments were assayed in vitro: the free radical scavenging activity, the ACE-inhibitory activity and the antibacterial activity. The fact that a bioactive protein can resist to the action of proteolytic enzymes, be hydrolysed into inactive fragments or even into more active fragments than the parent protein justified the interest of such a study.

3.2.1 Radical scavenging activity

Two potent antioxidant molecules, gallic acid and Trolox, a soluble analogue of vitamin E, were used as positive controls (Fig. 4). The TEAC value of gallic acid was
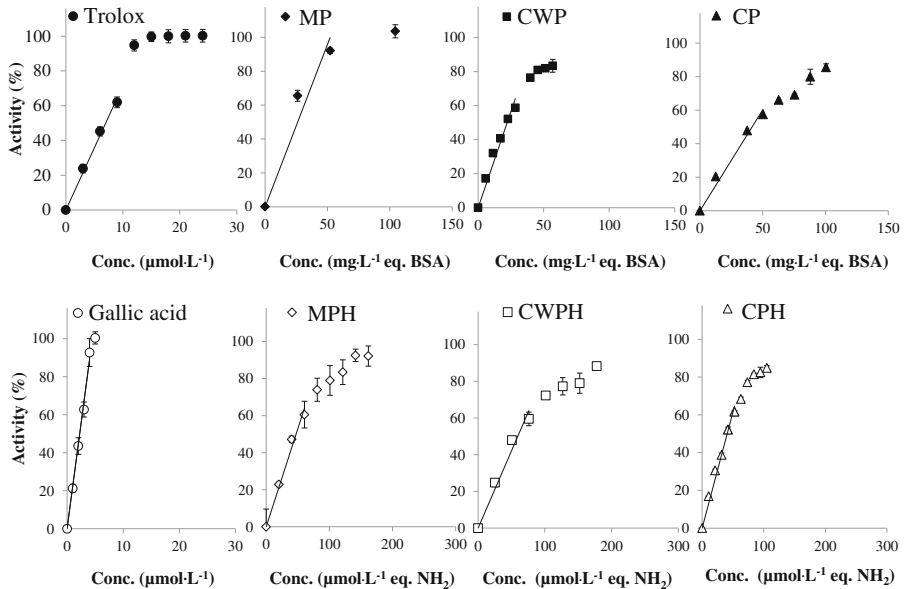
Fig. 4 ABTS radical scavenging activity of Trolox, gallic acid, camel milk proteins (*MP*), colostral whey proteins (*CWP*), colostrum proteins (*CP*) and their respective hydrolysates MPH, CWPH and CPH

3 µmol TE.µmol^{-1}, showing that gallic acid was a greater free radical scavenger than Trolox with an antioxidant power 3-fold higher, in accordance to our previous work (Sadat et al. 2011). The IC$_{50}$ value of gallic acid (2.0 µmol.L^{-1}) was also close to the value IC$_{50}$ (2.5 µmol.L^{-1}) reported by Sadat et al. (2011) and lower than the IC$_{50}$ of Trolox (6.5 µmol.L^{-1} in the present study and 5.6 µmol.L^{-1} in the study of Sadat et al. 2011).

The ABTS-scavenging capacities of MP, CWP and CP as well as their respective hydrolysates are reported in Fig. 4 and the IC$_{50}$ and TEAC values in Table 1. Among the three protein fractions, CWP exhibited a higher activity with TEAC ca. 2-fold higher than that of CP and IC$_{50}$ ca. 2-fold lower than the IC$_{50}$ of CP. The antiradical scavenging power of MP was intermediate between those of CP and CWP. After digestion by pepsin and pancreatin, CPH that was generated from the lowest active fraction, CP, displayed similar IC$_{50}$ and similar TEAC to MPH and CWPH, showing that enzymatic hydrolysis generated peptides with radical scavenging properties.

Table 1 Radical scavenging activity (TEAC and IC$_{50}$) of camel milk proteins (MP), colostral whey proteins (CWP), colostrum proteins (CP) and their respective hydrolysates

Sample	TEAC	*p* value	IC$_{50}$
	µmol TE.mg^{-1} protein		mg.L^{-1} eq. BSA
MP	0.26±0.03	–	24.7
CWP	0.31±0.08	0.012	19.4
CP	0.16±0.04	0.025	36.1
	µmol TE.µmol^{-1} eq. NH$_2$		µmol.L^{-1} eq. NH$_2$
MPH	0.15±0.03	–	43.4
CWPH	0.11±0.02	0.870	54.3
CPH	0.17±0.05	0.320	35.6

Nevertheless, the radical scavenging activities of the camel milk and colostrum hydrolysates remained low comparatively to that of Trolox (Table 1).

3.2.2 ACE-inhibitory activity

The in vitro ACE-inhibitory activity of MP, CWP, and CP and their respective hydrolysates was measured and the percentages of inhibition of ACE are given in Table 2. ACE is namely inhibited by different source of peptides. As expected, MP, CWP and CP which have low peptides content (as observed by RP-UHPLC analysis) did not significantly inhibit ACE activity. However, the ACE-inhibitory activity of these protein fractions was enhanced after their enzymatic digestion, suggesting that ACE-inhibitory peptides corresponding to camel milk and colostrum protein fragments have been released.

3.2.3 Antimicrobial activity

The CP, CWP and MP fractions and their respective enzymatic hydrolysates were screened for antimicrobial activities against E. coli XL1 blue and L. innocua LRGIA 01. Characteristic L. innocua growth curves in the presence of CP and CPH are presented in Fig. 5. The respective percentages of inhibition of the growth of the two bacterial strains are presented in Table 3. The results obtained indicate that CP exhibits a higher antimicrobial activity than CWP and MP. After enzymatic hydrolysis, CWPH still exhibited an antimicrobial activity indicating that either antimicrobial proteins resisted to the action of proteolytic enzymes or that new antimicrobial fragments of camel colostrum whey proteins might have been released by their action. Both CP and CPH significantly inhibited E. coli XL1 blue strain growth. Interestingly, even at a low concentration (i.e. 10 g.L^{-1}), CPH significantly inhibited E. coli growth. As reported in Table 3, CPH also significantly inhibited L. innocua LRGIA 01 growth.

3.2.4 Peptides identification

Peptides present in CPH, the hydrolysate displaying the highest bioactivities among the fractions tested, were identified by LC/nESI–MS/MS. Among the 181 peptides identified in the present work (data not shown), 25 peptides presented sequence homology with casein fragments reported to possess various biological activities including ACE-inhibitory and free radical scavenging activities (Table 4).

Table 2 ACE-inhibitory activity of camel milk proteins (MP), colostral whey proteins (CWP), colostrum proteins (CP) tested at 150 mg.L^{-1} and their respective hydrolysates tested at 150 μmol.L^{-1} eq. NH$_2$

Undigested/digested sample	ACE inhibition (%)		p value
	Undigested samples	Digested samples	
MP/MPH	0	17±1	<0.001
CWP/CWPH	0	9±3	0.220
CP/CPH	2±1	18±4	0.009
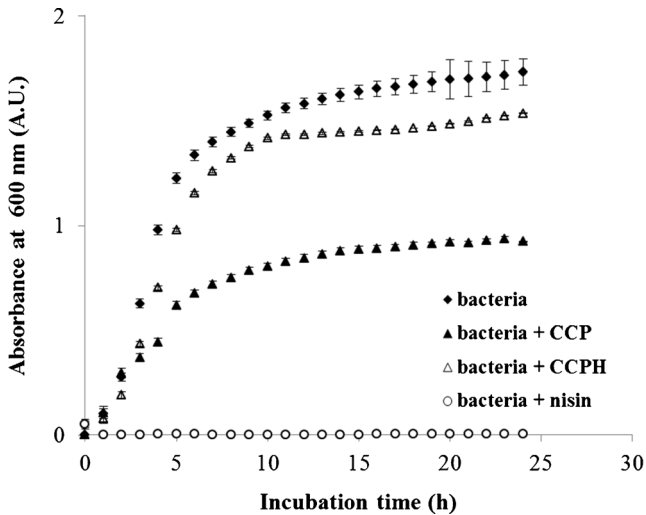
Fig. 5 Growth curves at 30 °C of *L. innocua* LRGIA01 in BHI broth in the presence of 40 g.L^{-1} of CP or 10 g.L^{-1} of CPH. *Listeria* growth was totally inhibited by nisin (2,400 IU.mL^{-1}) used as positive control

4 Discussion

4.1 Effect of hydrolysis by pepsin and pancreatin on camel colostral, colostral whey and milk proteins

Electropherograms and RP-UHPLC chromatograms of camel milk and colostrum proteins were consistent with previous studies regarding their respective compositions: colostrum contained far less CNs than milk and a high amount of IgGs, especially IgG2 and IgG3 (ca. 58 g.L^{-1} 1 h post-partum, but not less than ca. 12 g.L^{-1} 48 h post-partum; El Hatmi et al. 2007). The other soluble proteins detected by SDS–PAGE in colostrum of the camels from our experimental herd had been assayed by immunological method in a previous study: at first milking, it contained ca. 2.2 g.L^{-1} of α-LA, ca. 1.2 g.L^{-1} of Lf and ca. 8.5 g.L^{-1} of CSA (El Hatmi et al. 2006). Before 60-min elution time, no peaks and only a few peaks were present on RP-UHPLC chromatograms of camel

Table 3 Inhibition (%) of *E. coli* XL1 blue and *L. innocua* LRGIA 01 growth by camel milk proteins (MP), colostral whey proteins (CWP), colostrum proteins (CP), colostral whey proteins hydrolysates (CWPH) and colostrum proteins hydrolysates (CPH) after incubation for 15 h at 30 °C in BHI broth

Sample	Conc. (g.L^{-1})	*E. coli*	*p* value	*L. innocua*	*p* value
MP	20	4.00±0.53	0.007	7.90±0.84	0.006
	40	9.80±0.42	<0.001	12.90±1.11	<0.001
CWP	40	22.00±1.53	<0.001	16.00±1.04	0.001
CP	20	62.50±5.75	<0.001	26.70±1.2	<0.001
	40	62.00±3.92	<0.001	48.30±3.03	<0.001
CWPH	10	9.00±0.45	0.05	11.10±0.52	0.873
CPH	10	29.00±3.28	<0.001	15.40±1.28	0.007

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Table 4 Tandem mass spectrometry identification of peptides in colostrum protein hydrolysate (CPH) displaying at least seven identical residues (bold character) with known bioactive peptides

Camel protein source	Camel peptide	Homologous bioactive peptide (origin)	Putative bioactive activity	Reference
β-Casein	D^{130}**LENLHLPLPL**^{140} L^{128}T**DLENLHLPLPL**^{140} T^{129}**DLENLHLPLP**^{139} T^{129}**DLENLHLPLPL**^{140} T^{127}LT**DLENLHLPLPL**^{140}	N^{130}**LHLPLPLL**^{138} (bovine β-casein)	ACE inhibition	Robert et al. (2004)
	S^{165}LSQ**FKVLPVPQQ**MVPYPQRAMPV^{188}	S^{166}**QSKVLPVPQ**^{175} (bovine β-casein)		Hayes et al. (2007)
	F^{34}K**IEEQQQTEDEQQDK**^{49} K^{35}**IEEQQQTEDEQQDK**^{49} K^{35}**IEEQQQTEDEQQD**^{48} K^{35}**IEEQQQTEDEQ**^{46} Q^{40}**QTEDEQQDKIYT**^{51} Q^{40}**QTEDEQQDKI**^{49}	F^{33}Qp**SEEQQQTEDELQDK**^{48} (bovine β-casein)	Free radical scavenging	Sohn et al. (2009)
	S^{62}**HTEPIPYPI**LPQN^{75} S^{62}**HTEPIPYPI**LPQ^{74} S^{62}**HTEPIPYPI**^{71} V^{60}YS**HTEPIPYPI**LPQN^{75} V^{60}YS**HTEPIPYPI**LPQ^{74} Y^{61}S**HTEPIPYPI**^{71}	GW**THLEPGPYPI** (human VIP; vasoactive intestinal peptide)	Neuropeptide	Tan et al. (2003)
α$_{s1}$-Casein	L^{47}**KDTRNEPTEDH**^{58}	DRSNT**LHLKDYRNEENERH** (*Plasmodium falciparum* EBA-175 protein)	Red blood cell binding	Rodriguez et al. (2000)

Table 4 (continued)

Camel protein source	Camel peptide	Homologous bioactive peptide (origin)	Putative bioactive activity	Reference
α_{s2}-Casein	I^{83}**VMNPWDQ**GKTRAYPFIPT^{101} I^{83}**VMNPWDQ**GKT^{93} I^{83}**VMNPWDQ**G^{91} Q^{80}GQIV**MNPWDQ**GKT^{93}	N^{107}**PWDQ**^{111} (bovine α_{s2}-casein)	Food allergy prevention	Tanabe et al. (2006)
κ-Casein	I^{98}**AIPPKK**TQDKTVNPA^{113}	M^{106}**AIPPKKNQDK**^{116} (bovine κ-casein)	Casoplatelin	Jolles et al. (1986)
	V^{31}QS**RYPSYGIN**^{41}	Y^{35}**PSYGLN**^{41} (bovine κ-casein)	Opioid antagonist	Chiba et al. (1989)

colostrum and milk proteins, respectively. This was consistent with the fact that non-protein nitrogen (NPN), including peptides and free aminoacids, in camel colostrum and milk is far lower than protein nitrogen: the respective NPN/TN (total nitrogen) ratios of camel colostrum and milk reported in the literature were 27 g.kg^{-1} at 2 h post-partum (Zhang et al. 2005) or 37 g.kg^{-1} at first milking (El Hatmi et al. 2007) and 111 g.kg^{-1} (Shamsia, 2009), respectively.

After hydrolysis by pepsin and pancreatin, only α-LA and IgGs bands were still visible on SDS–PAGE electropherograms. Other proteins were hydrolysed into fragments which were detected by RP-UHPLC but not by SDS–PAGE, suggesting thus that most of these fragments were low molecular mass fragments. Since α-LA and IgG electrophoretic bands were less intense than before the action of digestive enzymes, this suggests that they are also hydrolysed into low molecular mass fragments but likely more slowly than other milk proteins. These soluble camel milk and colostrum proteins have already been reported to be more resistant toward proteolysis (Holt et al. 2003; Salami et al. 2008, 2009).

4.2 Effect of digestive enzymes on camel colostral, colostral whey and milk bioactivities

The radical scavenging, the ACE-inhibitory and antibacterial activities of camel milk, colostrum and colostral whey proteins were assayed in vitro before and after hydrolysis by digestive enzymes.

Although α-LAs of bovine milk (IC$_{50}$ of 40 µmol.L^{-1} according to Sadat et al. 2011) and camel milk (TEAC of 30 µmol.L^{-1} according to Salami et al. 2009) possess strong radical scavenging activities, comparable to that of Trolox, the remarkable antioxidant properties of the camel α-LA were not observed in the present work, probably because IgG concentrations in CP as well as in CWP were far higher than corresponding α-LA concentrations: IgGs would have limited their antioxidant activity. MP contained the highest relative amount of caseins (CNs) comparatively to CWP and CP (Fig. 2). Particularly in bovine milk, the CNs have been extensively investigated, as they are a source of antioxidant peptides obtained either by enzymatic hydrolysis or by fermentation. Indeed, radical scavenging peptides corresponding to β-CN (Kansci et al. 2004) and α$_{s1}$-CN (Suetsuna et al. 2000) fragments have been identified. The increase of radical scavenging activity of camel milk and colostral proteins after their hydrolysis by digestive proteolytic enzymes is consistent with the observation of Salami et al. (2010) showing that the capability of camel milk whey proteins to scavenge free radicals was increased after hydrolysis by proteinase K. In the present work, the increase of radical scavenging activity of camel colostrum proteins after the action of digestive enzymes was unveiled. This was in agreement with the presence of peptides displaying a strong identity with the bovine 33–48 β-caseinopeptide known to form stable radical (Table 4).

To our knowledge, the only studies reporting the ACE-inhibitory activity of peptides generated from camel milk proteins concerned caseins. In a former study, one ACE-inhibitory peptide, κ-CN(107–115), has been identified from fermented camel milk prepared with *Lactobacillus helveticus* 130B4 (Quan et al. 2008). Recently, Salami et al. (2011) have shown that a peptic hydrolysate of purified camel β-CN displayed a high ACE-inhibitory activity; this is consistent with the increase of camel milk proteins ACE-inhibitory activity after hydrolysis by pepsin followed by pancreatin observed in

the present study. In this study, we have shown that the digestive proteolytic enzymes also generated ACE-inhibitory fragments from camel colostrum proteins. Some peptides generated by pepsin and pancreatin action on colostrum proteins presented strong sequence similarity with known ACE inhibitors and therefore might have an ACE inhibitory action, putatively (Table 4).

The antibacterial activity of camel milk and colostrum proteins was tested with a Gram-positive (*L. innocua*) and a Gram-negative (*E. coli*) bacterial strain. Colostral proteins had a higher antibacterial activity against both bacteria than colostral whey proteins and milk proteins. This ranking was consistent with the observations of Benkerroum et al. (2004) and El-Agamy et al. (1992) who also noted an antimicrobial effect against Gram-positive and Gram-negative bacteria, including *E. coli*. Camel CPH still significantly inhibited the growth of *E. coli* and to a lesser extent *L. innocua*. This suggests that fragments of proteins with a higher antimicrobial activity than that of antimicrobial proteins present in camel colostrum could be released following the hydrolysis of camel colostral proteins by digestive enzymes. Salami et al. (2010) recently reported that a limited hydrolysis by proteinase K of whole camel milk whey proteins significantly enhanced their inhibition of *E. coli* growth in Luria-Bertani broth. However, a limited hydrolysis by either trypsin or chymotrypsin did not significantly enhance their antimicrobial activity. Since camel colostrum is rich in Lf, the possibility that proteolytic enzymes could release fragments of camel Lf with a higher antimicrobial activity than intact Lf should now be investigated. As reported by Tomita et al. (2009), a limited hydrolysis of bovine Lf by pepsin significantly increases the inhibition of *E. coli* growth, namely because Lf fragments with a higher antimicrobial activity than intact Lf, such as lactoferricin, are released.

5 Conclusions

Following hydrolysis by pepsin and pancreatin to mimic digestion of camel milk, colostral whey and colostrum proteins, most of the present proteins were hydrolysed into peptides. Interestingly, the radical scavenging and the ACE-inhibitory activities of the hydrolysates increased following digestion, showing that bioactive peptides were released by digestion of camel milk and colostrum proteins.

Regarding antimicrobial activity, it is of interest to observe that camel milk, colostral whey and colostrum proteins still inhibited *L. innocua* LRGIA01 and *E. coli* XL1 blue growth after the action of digestive enzymes. These antimicrobial activities are namely due to proteins such as Lf. In the case of colostrum, the *E. coli* growth inhibitory activity did not decrease following enzymatic digestion: this strongly suggested that antimicrobial fragments of colostrum proteins having a higher activity than native Lf were released. As a consequence, ongoing work concerns the identification of fragments of camel Lf, which could exhibit a higher antimicrobial activity than intact camel Lf. Although these preliminary in vitro assay results were promising, the biological activities have also to be studied by other approaches, as they cannot be extrapolated in vivo. For instance, in vitro digestion experiments should be performed with more complex digestion protocols better mimicking human digestion by either adults or newborn infants and/or mimicking different physiological situations.

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