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Effect of Heat Treatment and Fermentation on Bioactive Behavior in Yoghurt Made from Camel Milk

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Abstract In this study, the possibility to release bioactive peptides from camel milk using heat treatments and fermentation of camel milk was investigated. Camel milk was heated at 80℃ for 30, 60, 90 and 120min. Samples of yoghurts after their fermentation and during storage were determined for proximate physicochemical and bioactive activities. Results showed that heat treatments and fermentation process, decreased significantly the pH (P<0.01) and increased the total solids and protein (P<0.01) of yoghurt during storage. The ash content was almost unchanged, when the time of heat treatments of camel milk increased. Also, fermentation increased the antioxidant activities in yoghurt. SDS–PAGE electrophoretic patterns of camel milk showed that after heating camel milk at 80℃ for 60min; α-S2caseins (α-S2-CN) was not detected, while after fermentation α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were not significantly detected. The peptide fractions from yoghurt showed good potential to inhibit growth of *Bacillus cereus* (ATCC 9639), *Escherichia coli* (ACCT 8739) and *Staphylococcus aureus* (ATCC 6538). For all the above, the non-standard heat treatment of camel's milk and fermentation are necessary for a future application in dairy industry to produce a bioactive peptides.

Keywords: *heat treatment, bioactive antioxidant, physicochemical, and antimicrobial attributes, camel milk and yoghurt*

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1. Introduction

Bioactive peptides derived from milk and vary from 2-20 amino acid residues [1]. There are several ways to release the bioactive peptides from milk protein. Among them: during fermentation of milk by starter; gastrointestinal digestion like pepsin; proteolytic enzymes from microorganisms [2]. The activity of peptides is depending on their composition and amino acid sequence [2]. The therapeutic benefits effects of bioactive peptides on human health have been documented by several approaches [2,3,4]. Bioactive peptides show a broad range of biological activities such as anti-oxidant, anti-microbial (as bactericidal), anti-hypertension, anti-carcinogenic activities and anti-angiotensin [5,6]. Also, bioactive peptides can lower the blood pressure [7]. Addition to, the bioactive peptides are used as health-promoting food supplements or as food grade bio-preservatives in the food industry. The common source of bioactive peptides is egg proteins [8], muscle protein, plant proteins, fish proteins [9], casein [10] and whey proteins [8].

Camel milk has therapeutic and nutritional properties which are widely exploited for human health in several countries [11]. It contains high percentage of vitamin C, higher amounts of essential fatty acids compared to other species' milk, and antimicrobial agents due to the presence of lactoferrin, lysozyme, immunoglobulin, lactoperoxydase and bacteriocins [12]. Currently, a few approaches have investigated the effect of heat and fermentation treatment on camel milk [13,14].

Heat treatment of milk is a traditional technique to deactivate enzymes and prolong its shelf life by either complete sterilization of milk or partial destruction of microorganisms [15,16]. Also, Heat treatment of milk affect functional properties of milk proteins; such as hydrolysis of proteins and lipids, degradation of lactose, destruction of some vitamins and enzymes and denaturation of whey proteins [12,13,17,18]. After heating of milk above 70℃, k-casein interacted with sulfhydryl-disulfide bond [19]. The production of soluble and micelle-bound thermal co-aggregates affected further acid egelation properties of casein micelles and gelled network structuration. In camel milk at 80℃ for 60 min, camel peptidoglycan recognition protein (PGRP) and α-lactalbumin and were not detected while camel serum albumin was significantly diminished [14].

Lactic acid bacteria (e.g. *Streptococcus thermophilus and Lactobacillus helveticus*) can release bioactive peptides during fermentation by using tripeptidases, endo-peptidases, amino-peptidases and di-peptidases that derived from such microorganisms [20].

Bioactive peptides have attracted the interest of researchers as a health promoting functional food, so the goals of this research were to study the effects of heat

treatment, fermentation and storage on bioactive activities (Antibacterial and antioxidant activities) and physicochemical in yoghurt made from camel milk.

2. Materials and Methods

2.1. Milk Samples

Camel milk (12 L) was collected from reared camels using an ice box within 4 hrs., once reaching the laboratory at 4℃, pH (744-pH meter, Model HI 8424; HANNA instrument, Porto, Portugal**)** was determined.

2.2. Heat Treatment Experiments

The camel milks (2.25% fat, 9.40% TS and pH 6.62) were divided in four parts: each part was to 3000 mL and the four parts were heated at 80℃ for 30, 60, 90 and 120min; respectively. Heat treatments consisted in heating over a hot plate without agitation [21]. The experiments were reproduced at least 3 times.

2.3. Processing of Yoghurt

Prior to manufacturing of yoghurt, all equipment used was sterilized after wash cleaning in an autoclave for 30 min; while the plastic equipment were placed in boiling water for 30 min. The four parts of heat milk were cooled quickly to 45^{o}C and inoculated with yoghurt starter culture (YO-MIX495 LYO 250 DCU, DANISCO, and Denmark). The inoculated was poured into plastic cups (100 ml). The yoghurt cups (100 ml) were incubated at 42℃ until coagulation, and then stored at 5℃, the physicochemical attributes, antioxidant and antimicrobial evaluations of yoghurt samples was determined at zero time(after one day) and after 7, 15, and 21days intervals. The yoghurt making experiment was repeated three times.

2.3.1. Physicochemical Analysis

Proximate composition of yoghurt samples was analyzed: About 100 g of prepared yogurt was blended and the pH was measured by a digital pH meter, ash content was measured by dry ash method [22], total solids of the yogurt was determined by drying at102 ± 2℃ until constant mass., the protein content was determined by Kjeldahl method and the fat content was determined by Gerber method [22,23].

2.3.2. Bioactive Activity Analysis

2.3.2.1. Preparation of Samples

Samples of yoghurt were prepared by centrifugation at 6000 g, 4℃ for 15 min; and the supernatant was used to determine the bioactive activities.

2.3.2.2. Antioxidant Activity

DPPH (2,2-Diphenyl-1-picrylhydrazil) assay was determined by the method of Rangkadilok (2007) [24], while the Reducing power assay was measured according to Zhu, *et al*., (2008) [25].

2.3.2.3. Antibacterial Activity Assay

Bacillus cereus (ATCC 9639), *Escherichia coli* (ACCT 8739), *Salmonella typhimurium* (ACCT 25566), and *Staphylococcus aureus* (ATCC 6538) were used in assay protocol [26].

2.3.2.4. (SDS–PAGE) of Fractions

The supernatant (water soluble peptide extract) were separated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis according [27]. Runs were carried out at 75V in stacking gel then increased to 125V until the end of electrophoresis. After electrophoresis, proteins were localized in gels using Coomassie blue 0.1%. Electrophoresis experiments were carried out using a Bio-Rad apparatus (Mini-Protean II cell (Bio-Rad), Belgium) of gels in vertical slabs.

2.3.2.5. Determination of Protein Molecular Weight

Molecular weight (kDa) of protein fractions were estimated according to the method of Weber and Osborn (1969) [28] after fractionation on SDS-PAGE and using standard protein markers.

2.4. Statistical Analysis Method

Data were analysed using the Statistical Analysis System software package [29]. Analyses of variance were performed using ANOVA procedures. Significant differences between mean were determined using Duncan's multiple range test. Principal Component Analysis (PCA) was applied to the average values of sensory evaluation data [30].

3. Results and Discussion

3.1. Physicochemical Composition of Yoghurt Samples

The results as Table 1 illustrated the changes in pH of the yogurt made from heated camel milk at 80℃/30 or 60 or 90 or 120 min. The values of pH were 4.55, 4.42, 4.41 and 4.42 after 7 days, respectively. Decreasing in pH values is the result of lactose fermentation by the associative growth of the two thermophilic, homo-fermentative lactic acid bacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* [31,32,33,34]. The bacterial enzyme β-galactosidase is not completely stopped by the cold storage of yogurt [35]. The storage of yogurts significantly $P<0.01$ affected the decreasing of the pH value. A slower acidification in camel milk yoghurt has been ascribed to the presence of the antimicrobial agents in the camel milk. It was clear that a gradual decrease in pH value was observed on increasing the time of cold storage. This may be due to the presence of antioxidant of camel milk, which displayed a significant scavenging ability on the peroxyl radicals such as H_2O_2 thus retard the growth [36].

The chemical composition of yoghurt samples was (Table 1) within the following ranges: total solids (T.S)

content (9.97–11.66%), fat (2.15–2.52%), protein (2.77–3.24%) and ash (1.00–1.23%). The content of total solids, fat and protein significantly increased (P<0.01) as time of cold storage increased. These results were similar to that estimated by Salih and Ahmed (2013) [37] and Price Weston (2008) [37]. Also, the lower total solids content of yoghurt may be due to the high water content of camel milk [39] and that could explain the watery texture of yoghurt made with camel milk [40]. Stahl *et al*., (2006) [41] reported that camel milk contains smaller amounts of short chain fatty acids and small size of the camel fat globules. The lower protein content in yoghurt may be due to the increase of proteolytic activity during fermentation of milk [42].

To show the relationship among total solids (TS), fat (F), protein (Pr) and ash (A), Performance Analytics was performed (Figure 1). As shown in the chart correlation, there are a positive correlation among total solids, fat and protein, i.e. the correlation factor between total solids and protein was r=+1.00, while it was r=+0.99 between protein and fat.

Table 1. Changes of pH, total solids (%), fat (%) protein (%) and ash contents in *yoghurt* made with camel milk during cold storage

Temperature	Time(min.)	Storage period(days)	pH value	T.S (%)	Fat (%)	Protein (%)	Ash (%)
80℃	30	0	4.60*^{a}	9.97^{h}	2.15^{h}	2.77^{g}	1.00^{ab}
		7	4.55^{a}	10.01^{h}	2.16^{gh}	2.78^{g}	1.00^{ab}
		15	4.44^{bc}	10.10^{h}	2.18^{gh}	2.81^{fg}	1.02^{ab}
		21	4.38^{cde}	10.35^{g}	2.22^{fgh}	2.86^{efg}	1.03^{ab}
80℃	60	0	4.45^{b}	10.46^{fg}	2.26^{efgh}	2.90^{defg}	1.04^{ab}
		7	4.42^{bcd}	10.55^{ef}	2.27^{efg}	2.94^{cdefg}	1.05^{ab}
		15	4.36^{def}	10.68^{e}	2.30^{def}	2.97^{bcdefg}	1.07^{ab}
		21	4.32^{efg}	10.87^{d}	2.35^{cde}	3.02^{abcdef}	1.08^{ab}
80℃	90	0	4.45^{b}	10.90^{d}	2.35^{cde}	3.03^{abcdef}	1.09^{ab}
		7	4.41^{bcd}	10.91^{d}	2.36^{cde}	3.05^{abcde}	1.10^{ab}
		15	4.37^{def}	10.96^{d}	2.37^{bcde}	3.06^{abcde}	1.10^{ab}
		21	4.31^{fg}	11.19^{c}	2.41^{abcd}	3.11^{abcd}	1.12^{ab}
80℃	120	0	4.45^{b}	11.34^{bc}	2.45^{abc}	3.15^{abc}	1.16^{ab}
		7	4.42^{bcd}	11.37^{b}	2.45^{abc}	3.15^{abc}	1.18^{ab}
		15	4.38^{cde}	11.45^{b}	2.48^{ab}	3.18^{ab}	1.20^{ab}
		21	4.27^{g}	11.66^{a}	2.52^{a}	3.24^{a}	1.23^{a}

*Means of triplicates. Means followed by the same superscript are not significantly different, P<0.01.

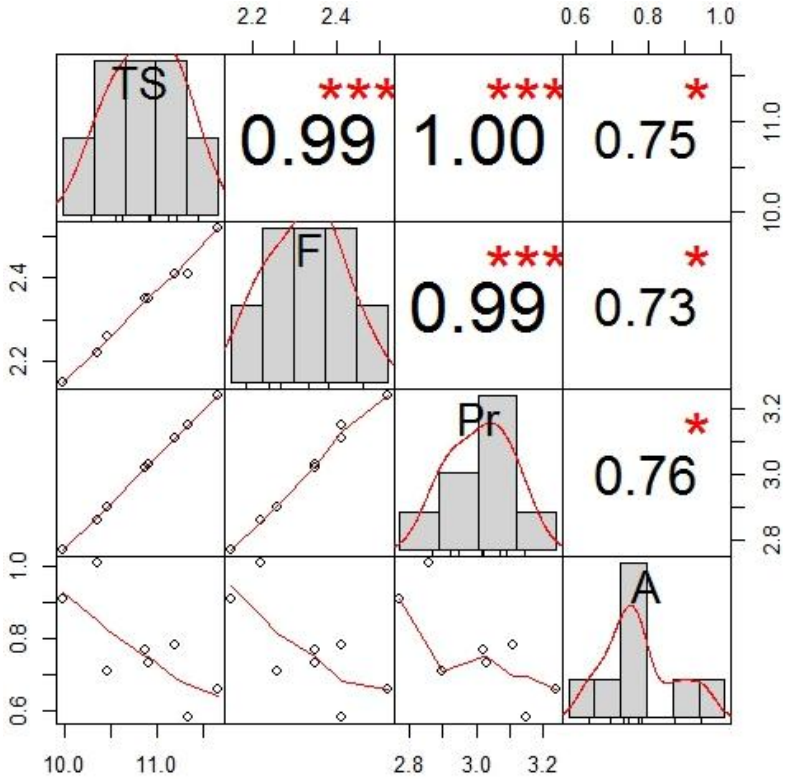

Figure 1. Chart correlation of total solids (TS), fat (F), protein (Pr) and ash (A), of yoghurt samples made after heating camel milk 80℃/30 or 60 or 90 or 120 min

3.2. Antibacterial Activity Assay

The inhibition zone assay (wells diffusion method) is a common method used to test the antimicrobial activity of commonly used food antimicrobials (Table 2). The bioactive peptides shows Antimicrobial activity against of *Bacillus cereus* (ATCC 9639), *Escherichia coli* (ACCT 8739), *Salmonella typhimurium* (ACCT 25566), and *Staphylococcus aureus* (ATCC 6538) with inhibition zones of 4, 3 and 3mm after 15 days of storage at 5℃, respectively. The diameter of inhibition zones values for all tested bacteria strains were in the range of 0-5mm. The diameter of inhibition zones was the maximum value for *Bacillus cereus* (ATCC 9639) in the case heat treatments at 80° C for 30 and 90 min, followed by *Escherichia coli* (ACCT 8739) and *Staphylococcus aureus* (ATCC 6538).However, no difference in diameter of inhibition zones values was found among *Escherichia coli* (ACCT 8739) and *Staphylococcus aureus* (ATCC 6538) at the same heat treatment after 15 days. Mohanty *et al*., (2014) [43] reported that *Escherichia coli* MTCC82, *Salmonella typhi* MTCC3216, *Bacillus cereus* ATCC10702, *Salmonella typhimurium* SB300, S. *enteritidis* 125109, *Staphylococcus aureus* MTCC 96 were inhibited with bioactive peptides derived from milk. Samaržija, (2015) [44] and Guzel-Seydim *et al*., (2011) [45] found that the bacteria in kefir grains hydrolyze proteins into bioactive peptides from milk during the fermentation process. Low molecular mass peptides released during the fermentation exhibit the highest inhibitory effect [46]. Galia *et al*., (2009) [47] reported that *Streptococcus thermophilus* strains were able to liberate peptides from the casein depending on their proteolytic activity and some of that had been classified as antimicrobial peptides [48]. Similarly, when β-casein and αs1-casein were hydrolyzed with *Lactobacillus delbrueckii* subsp. *lactis* CRL 581, a great number of peptides were released from both caseins. Lactoferrampin, isolated as a fragment of lactoferrin displays inhibitory activity against *Streptococcus mutans*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* [49]. This may be due to the bioactive peptides, which play a regulatory role in oxidative metabolism. Also, when an excess of free radicals is released, they oxidize membrane lipid, cellular protein, enzymes and DNA that cause shutting down of cellular respiration and oxidative DNA-damage [50,51,52].

3.3. Antioxidant Activity

As shown in Table 3, the reducing power assay in samples of yogurt, which made after heating camel milk 80° C/30 or 60 or 90 or 120 min., were 16.93, 17.50, 18.10 and 19.21% at zero time (after 1 day) of storage. This resulted may be due to heat treatment of milk, which increased antioxidant activity because the protein unfolding and exposure of thiol groups that can act as hydrogen donor. Under severe heating, pro-oxidant molecules may be consumed in the Maillard reaction pathway, generating melanoidin with strong antioxidant activity [1,53]. Free radicals released from casein peptides may influence scavenging activity [54,55]. It has been reported that many antioxidant peptides contain the hydrophobic amino acid residues Leu or Val at the N-terminus of the peptides and histidine, tyrosine or proline in the sequence [56,57,58]. During 21 days of cold storage the reducing activity of all samples significantly (P<0.01) decreased up to the end of storage period.

Table 3 shows the antioxidant activities of DPPH. Generally, the DPPH of yogurt significantly increased with increase the time of heat treatments of camel milk followed by a slight decrease after 21 days of cold storage. The percentages of DPPH were 22.7, 24.5, 25.6and 28.7% of the yogurt made from heated camel milk at 80° C/30 or 60 or 90 or 120 min., respectively. This may be due to the pro-oxidant effects of bioactive peptides produced after fermentation of camel milk and presence of functional groups such as hydroxyl groups on phenolic compounds [59]. Also, demonstrated that a number of food-derived peptides were capable of interacting and quenching DPPH radicals [10,60].

Table 2. Antibacterial activity (zone of inhibition: mm) of yoghurt made from camel milk by the well diffusion method

strain	Period of heat treatment at 80℃(min.)	Inhibition zone (mm)						
		control	After heat	Zero time	After 3 days	After 7 days	After 15 days	After 21 days
Bacillus cereus (ATCC 9639)	30	_	_	1	2	3	4	6
	60	_	_	_	_	2	_	_
	90	_	_	_	2	2	4	5
	120	_	_	2	2	_	3	3
Escherichia coli (ACCT 8739)	30	_	_	_	_	2	3	4
	60	_	_	_	_	_	_	_
	90	_	_	_	_	_	3	2
	120	_	_	_	_	_	_	_
Salmonella typhimurium (ACCT 25566)	30	_	_	_	_	_	_	_
	60	_	_	_	_	_	_	_
	90	_	_	_	_	_	_	_
	120	_	_	_	_	_	_	_
Staphylococcus aureus (ATCC 6538)	30	_	_	_	2	_	3	2
	60	_	_	_	_	2	_	_
	90	_	_	_	_	_	3	2
	120	_	_	_	_	_	4	3

*Means of triplicates. Means followed by the same superscript are not significantly different, P<0.01.

Table 3. Changes of reducing power assay (%inhibition) and DPPH (%inhibition) contents in *yoghurt* made with camel milk during cold storage

Temperature	Time(min.)	Storage period(days)	reducing power assay (%inhibition)	DPPH (%inhibition)
80℃	30	0	16.93^{defg}	22.7^{fgh}
		7	16.52^{efg}	21.9^{ghi}
		15	16.0^{gh}	21.3^{hi}
		21	15.32^{h}	19.9^{hi}
80℃	60	0	17.50^{bcde}	24.5^{cdef}
		7	17.01^{cdefg}	23.8^{efg}
		15	16.37^{fgh}	23.12^{fgh}
		21	15.91^{gh}	22.7^{fgh}
80℃	90	0	18.10^{abc}	25.6^{cde}
		7	17.57^{bcde}	24.4^{cdefg}
		15	17.31^{cdef}	24.1^{defg}
		21	16.73^{fgh}	23.6^{efg}
80℃	120	0	19.21^{a}	28.7^{a}
		7	18.51^{ab}	27.9^{ab}
		15	17.93^{bcd}	26.5^{abc}
		21	17.12^{cdef}	26.3^{bcd}

*Means of triplicates. Means followed by the same superscript are not significantly different, $P<0.01$.

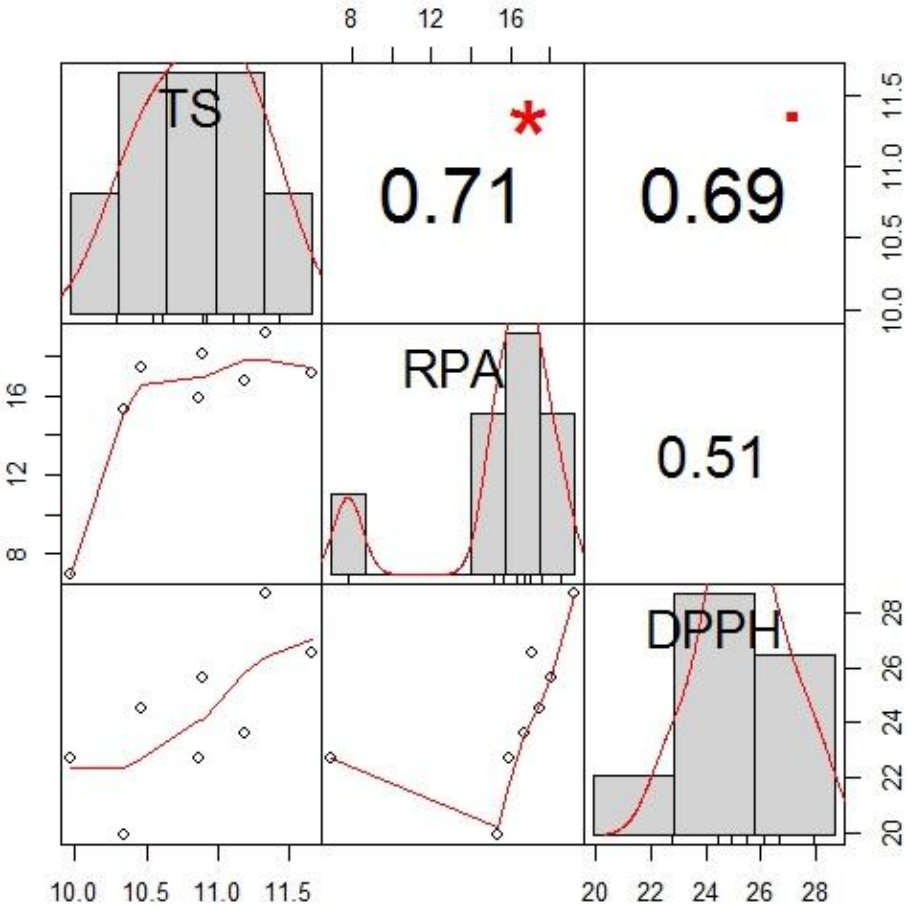

Figure 2. Chart correlation of total solids (TS), reducing power assay (RPA) and 2,2-Diphenyl-1-picrylhydrazil (DPPH), of yoghurt samples made after heating camel milk 80℃/30 or 60 or 90 or 120 min

To show the relationship among total solids (TS), reducing power assay (RPA) and 2,2-Diphenyl-1-picrylhydrazil (DPPH), Performance Analytics was performed (Figure 2). As shown in the chart correlation, there are a positive correlation among total solids, reducing power and 2,2-Diphenyl-1-picrylhydrazil, i.e. the correlation factor between total solids and reducing power was r=+0.71, while it was r=+0.69 between total solids and 2,2-Diphenyl-1-picrylhydrazil.

3.4. SDS–PAGE Analysis

The SDS–PAGE patterns of camel milk, the yoghurt supernatant are shown in Figure 3. After heating cow milk at 80℃ for 60, 60 and 120min, no band was observed on the gel patterns comparison with the protein molecular weight standard. Also, the milk proteins were separated into three major zones, namely caseins (14–21 kDa), and the three major proteins, α-S2caseins (α-S2-CN) (21 kDa),

β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa). α-S2caseins (α-S2-CN) was not detected, while after fermentation α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were not significantly detected [61].

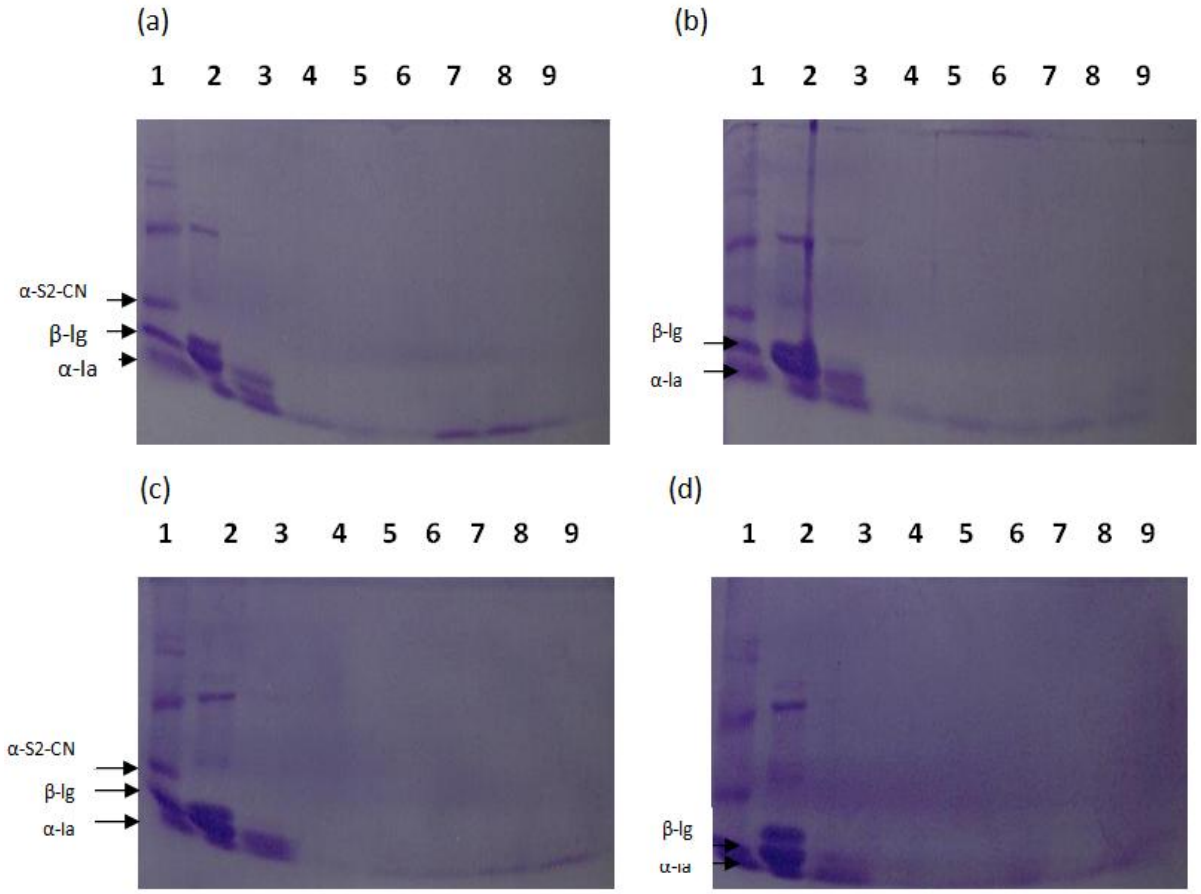

Figure 3. SDS-PAGE separation of camel milk proteins (a): heated samples at 80℃ for 30min,(b): heated samples at 80℃ for 60min (c): heated samples at 80℃ 90, (d): heated samples at 80℃ 120 min.;α-S2-CN, α-S2caseins; α-la, a-lactalbumin; β-lg, β-lactoglobulin. Panel A shows: lane 1, standard protein marker; lane 2, camel milk before heat treatment; lane 3, camel milk after heat treatment; lanes 4, yoghurt at zero time; lane 5, yoghurt after 3 days; lane 6, yoghurt after 7 days; lane 7, yoghurt after 15 days; lane 8, yoghurt after 21 days and lane 9, sample buffer. The electropherograms shown are representative of three independent experiments

4. Conclusion

From this study, it can be concluded that treatment of camel milk with heating and fermentation had significant impact on yoghurt composition and bioactive peptides derived from milk. Camel yoghurts had strong antioxidant activities in all assays and their activities significantly increased during their fermentation. The obtained results showed that camel α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were significantly affected by heat treatment and fermentation. It is possible to produce camel's milk yogurt that will have bioactive peptides and good quality over the storage period of 21 days by using non-standard heat treatment of camel's milk at a temperature of 80℃/30 min; and fermentation by starter.

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