

Effects of two different domestic boiling practices on the allergenicity of cow's milk proteins

Cristina Lamberti,^{a*} Cristina Baro,^a Marzia Giribaldi,^{a,b} Lorenzo Napolitano,^a Laura Cavallarini^a and Maria Gabriella Giuffrida^a

Abstract

BACKGROUND: The sale of raw drinking milk through automatic dispensers is permitted in some EU member states, but consumers are usually advised to boil the milk before consumption. The present study has been conducted to evaluate the effects of two common domestic boiling techniques on the proteins of raw milk and, in particular, on their potential allergenicity.

RESULTS: Native one-dimensional electrophoresis, N-terminal amino acid sequencing and immunoblotting have been used to characterize the protein pattern and to evaluate the possible changes in the allergenic properties of the processed milk. The main result of this investigation is that heating induces the aggregation of β -lactoglobulin in higher-molecular-weight products, while caseins seem to be more resistant to the treatments. β -Lactoglobulin aggregates have been found to be non-immunoreactive with the sera of subjects suffering from cow's milk protein allergy.

CONCLUSION: Domestic boiling modifies the milk protein profile, causing a minor reduction in milk allergenicity.

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Keywords: raw milk; domestic boiling; allergenicity; β -lactoglobulin; caseins

INTRODUCTION

Cow's milk is considered to be an important source of lipids, proteins, amino acids, vitamins and minerals. Moreover, it contains anti-infectious components, hormones, growth factors, cytokines, nucleotides, peptides, polyamines and enzymes.¹ In particular, the protein fraction of cow's milk is rich in bioactive proteins and peptides, some of which show important antimicrobial activity (immunoglobulins, lysozyme, lactoperoxidase and lactoferrin).^{2,3} Whey proteins play a relevant nutritional role, as they are rich in essential amino acids. However, cow's milk is one of the most important sources of food allergens.⁴ The incidence of cow's milk protein allergy (CMPA) varies with age, showing a prevalence in early childhood (2–6% incidence) and decreasing in adulthood (0.1–0.5% incidence).⁵ For some years, the direct commercialization of raw cow's milk by producers through automatic dispensers has been authorized in some EU member states, including Italy. The European Food Safety Agency (EFSA) has recently released a Scientific Opinion publication in which the main microbial risks associated with the consumption of raw drinking milk have been identified.⁶ Since 2008, Italian farmers who sell raw milk have had to clearly indicate, on the automatic dispensers and on the bottles, that this milk has to be boiled before its consumption.⁷ Much is known about the industrial thermal processing of food, while there is a lack of information about the impact of domestic heat treatments (boiling) on the biochemical quality of milk. It has been demonstrated that industrial pasteurization may cause marked modifications of milk, such as enzyme inactivation, protein denaturation and/or aggregation, causing epitope destruction,

modification, masking or unmasking,^{8,9} crosslinking between proteins and other food components, such as lactosylation, lipid oxidation products and the generation of Maillard reaction products.¹⁰ Food processing is also known to modulate protein digestive stability, which is the main condition for a protein to become an allergen,¹¹ thereby influencing the antigenic structures presented to intestinal immune cells.¹² Both a decrease in the allergenicity of cow's milk and the generation of new allergens due to thermal treatments of milk have been reported.¹³ Milk allergenicity may also be affected by interactions with other food ingredients, thus influencing the allergen availability for the intestinal immune cells as well as its own digestibility. This feature may partially explain why some people can tolerate unprocessed food or a single ingredient but not the corresponding processed counterpart, or vice versa.¹⁴

In this context, the present study was aimed at evaluating whether two different common domestic treatments (boiling on

* Correspondence to: C Lamberti, Institute of Sciences of Food Production, National Research Council, Grugliasco, Italy
E-mail: cristina.lamberti@ispa.cnr.it

^a Institute of Sciences of Food Production, National Research Council, Grugliasco, Italy

^b Food Technology Research Unit, Council for Agricultural Research and Economics, Torino, Italy

a hotplate or in a microwave oven) could influence the protein fraction of milk from an allergenic point of view.

In our study, changes in the protein profile were investigated by means of the electrophoresis technique in native experimental conditions, in order to highlight any possible protein–protein aggregation, polymerization or co-migration. Furthermore, the allergenicity of raw milk was compared with that of milk boiled on a hotplate and in a microwave oven respectively by testing the sera of 20 subjects affected by CMPA. Moreover, some conventional milk nutrition parameters were evaluated in different experimental conditions.

EXPERIMENTAL

Chemicals

Boric acid, tris(hydroxymethyl)aminomethane (Tris), bromophenol blue, glycerol, glycine, Ponceau red, NaCl, Tween-20, gelatin, β -lactoglobulin and α_{s1} -casein were from Sigma-Aldrich (St Louis, MO, USA); TGX Precast Gel, Blue Coomassie Colloidal, nitrocellulose membranes, AP Conjugate substrate kit and *o*-phthaldialdehyde (OPA) were from Bio-Rad (Hercules, CA, USA); Immuno-CAP system was from Phadia (Thermo Fisher Scientific, Waltham, MA, USA); 2-D Quant kit was from GE Healthcare (Little Chalfont, UK); alkaline phosphatase-conjugated goat anti-human-IgE antibody (ϵ -chain specific) was from KPL (SeraCare Life Sciences, Milford, MA, USA); FOODLAB peroxidase assay kit and ϵ -fructosyl lysine FOODLAB kit were from CDR Mediated (Ginestra Fiorentina, Italy).

Serum samples

Serum samples from subjects sensitized to cow's milk were collected from Koelliker Hospital (Turin, Italy). The content of specific cow's milk immunoglobulin E (IgE) antibodies was measured using an Immuno-CAP system according to the manufacturer's instructions. Twenty subjects showing cow's milk IgE levels ranging from 0.57 to 82.3 kU_A L⁻¹ were enrolled in the study.

Sample preparation

Raw cow's milk (Rm) was collected in two subsequent samplings from 11 farms located in the north of Italy (Lombardy) that furnish automatic dispensers on a daily basis. After collection, the samples were stored at -40°C . After thawing, equal amounts of each sample were mixed to create a starting pool, which was divided into three aliquots. One aliquot (20 mL) was used untreated as a reference (Rm), while the others underwent two common domestic boiling procedures, one on a hotplate (16 min, $\sim 98^{\circ}\text{C}$) (HPm) and one in a microwave oven (1300 W, 60 s, $\sim 98^{\circ}\text{C}$) (MWm).

The total protein content of the three samples (Rm, MWm, HPm) was quantified using a 2-D Quant kit according to the manufacturer's instructions. Each of the three samples was then split into two aliquots prior to the analyses. One aliquot was skimmed by means of centrifugation ($2000 \times g$, 30 min, 6°C) in order to perform native polyacrylamide gel electrophoresis (native-PAGE) and immunoblotting analyses, while the other one was used, without further handling, to determine the available lysine, ϵ -fructosyl lysine and peroxidase activity.

Native-PAGE and electroblotting on nitrocellulose membrane

Native-PAGE was performed as already detailed by Monti *et al.*¹⁵ Briefly, a protein mixture of skimmed Rm, MWm and HPm was separated on 7.5 g kg⁻¹ Mini-PROTEAN ten-well TGX Precast Gel

using a Mini-PROTEAN Electrophoresis Tetra System cell (Bio-Rad). A 5 μg aliquot of total proteins was taken from each sample and added to 10 μL of boric acid/borax buffer (0.2 mol L^{-1} , pH 8.4), and this solution was further diluted 1:2 with native sample buffer (0.25 mol L^{-1} Tris–HCl, pH 6.8, 2.5 g L⁻¹ bromophenol blue, 150 mL L⁻¹ glycerol). Bovine β -lactoglobulin (7 μg) was used as native molecular weight standard, and Towbin buffer (192 mmol L^{-1} glycine, 25 mmol L⁻¹ Tris base, pH 8.3) was used as native running buffer. Protein separation was carried out at a constant voltage of 200 V for 40 min. The gels were stained with fresh home-made Blue Coomassie Colloidal stain¹⁶ and digitized using a GS-800 Densitometer (Bio-Rad).

A 2.5 μg aliquot of total proteins was loaded onto each well for the immunoblotting analyses. The gels were then transferred onto 2 μm nitrocellulose membranes using Towbin buffer at a constant voltage of 100 V for 1 h. The blotted membranes were reversibly stained with Ponceau red stain for 20 min and then cut into 4 mm wide slices, corresponding to the gel wells.

N-terminal amino acid sequencing of proteins

The bands of interest were excised from the native-PAGE, passively eluted and then microsequenced as previously described.¹⁷

Immunostaining

Immunolabelling was performed on blotted nitrocellulose membranes using the sera from 20 selected subjects affected by CMPA. Each membrane had previously been washed in Tris-buffered saline (TBS: 50 mmol L⁻¹ Tris, 150 mmol L⁻¹ NaCl, pH 7.4) in order to eliminate the Ponceau red stain and then blocked twice for 15 min using TBS solution containing 3 mL L⁻¹ Tween-20. The washed membranes were incubated overnight in the dark with the subject's serum diluted 1:5 in incubation solution (TBS, 0.5 mL L⁻¹ Tween-20, 0.5 g L⁻¹ gelatin). After incubation, the membranes were rinsed with washing solution (TBS, 0.5 mL L⁻¹ Tween-20) and incubated again for 1 h with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human-IgE antibody (ϵ -chain specific) in incubation solution. After three washing steps (10 min each), immunoblots were developed with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (AP Conjugate substrate kit), and the membranes were then digitized using a GS-800 Densitometer (Bio-Rad).

Immunoblot inhibition analysis

The sera of the selected subjects were pooled, diluted 1:5 in incubation solution and divided into three aliquots. Before immunodetection, each aliquot was incubated with 200 μg of β -lactoglobulin, α_{s1} -casein or their 1:1 mixture for 1 h at room temperature. Incubation and immunoreaction were performed on blotted Rm samples and β -lactoglobulin as described above.

Available lysine determination

The total available lysine content was measured on 20 μL of each unskimmed sample by means of the modified OPA method already described by Baro *et al.*¹⁸ Five technical replicates were performed.

Lactoperoxidase activity and ϵ -fructosyl lysine determination

The lactoperoxidase activity was measured using a FOODLAB peroxidase assay kit, while the amount of ϵ -fructosyl lysine was measured by means of an ϵ -fructosyl lysine FOODLAB kit in five technical replicates according to the manufacturer's instructions.



Statistical analysis

Densitometric analysis of native-PAGE (Rm, MWm, HPm) was conducted using QuantityOne software (Bio-Rad). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA). The bands with statistically significant differences ($P \leq 0.05$) were compared by Tukey's *post hoc* test using SPSS Version 15.0 software (SPSS, Chicago, IL, USA). In a similar way, for the lactoperoxidase activity and ϵ -fructosyl lysine content, the differences among samples were assayed using one-way ANOVA associated to a probability of less than 5%, followed by Tukey's *post hoc* test, whereas the total available lysine content was analyzed by means of one-way ANOVA associated to a probability of less than 5%. Statistical analysis was performed using KyPlot Version 2 Beta 15 software (KyensLab Inc.).

RESULTS AND DISCUSSION

As far as proteins are concerned, it is known that thermal treatment may affect their conformation as a function of temperature and time, thus leading to protein denaturation and/or aggregation and to potentially modified allergenicity.¹³ To date, only a limited amount of information is available on the effect of common domestic boiling procedures on the milk protein content and structure and, in particular, on its allergenicity.¹⁹

Native-PAGE

Native-PAGE analyses of the Rm, MWm and HPm samples were performed in order to detect the modifications induced by domestic boiling on the milk protein pattern (Fig. 1). All detectable bands were quantified by QuantityOne software (Table 1), except for band 1 which cannot be quantified because it is too close to the edge of the gel analyzed. Each band was excised and identified by means of N-terminal amino acid sequencing (Table 2); several bands were found to contain more than one protein in both the raw and processed milk. The choice to separate the cow's milk proteins in native conditions, without treating the sample with linearizing agents such as sodium dodecyl sulfate (SDS) or dithiothreitol (DTT), allowed us to obtain a more detailed characterization of the heating effects regarding denaturation, protein polymerization and protein-protein association. SDS is an anionic detergent, while DTT is a reducing agent; avoiding the use of a sample preparation protocol using DTT, SDS and other heating steps allowed us to discriminate the formation of aggregates or protein unfolding caused exclusively by the boiling procedures.

Subjecting the milk to a boiling treatment induces a cascade of protein modification events, as a result of the temperature changes, which include loss of secondary structure (55–70 °C), cleavage of disulfide bonds (70–80 °C), formation of new intra- and intermolecular interaction rearrangements of disulfide bonds (80–90 °C) and formation of aggregates (90–100 °C). These modifications reflect a progressive transition to disorganized structures, which passes through protein denaturation and unfolding and finally stabilizes into a random coil conformation.²⁰ In the present experiments, after both heat treatments, the whey protein bands became weaker (α -lactalbumin (α -LA) in band 6 showed a variation compared with Rm of –14.0% for MWm and –28.6% for HPm) or even non-detectable (β -lactoglobulin (β -LG) in band 12).

Wijayanti *et al.*²¹ have demonstrated that a heat treatment at 85 °C induces a progressive aggregation of β -LG into dimers and, afterwards, into larger polymers (trimers, tetramers, etc.). In the present experiment, β -LG disappeared from the bands in the lower

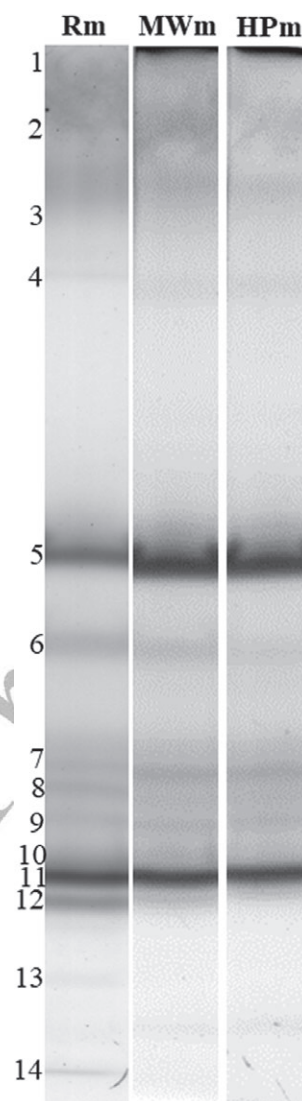


Figure 1. 1-D native-PAGE protein profile of raw (Rm), microwaved (MWm) and hotplate boiled (HPm) cow's milk. Numbers indicate band codes.

part of the gel (band 12 for both heat treatments and band 10 only for the hotplate treatment) and was detected in the band at the gel well level (band 1). This result is also in agreement with the results of Bloom *et al.*,²² who have recently demonstrated the formation of β -LG and α -LA high-molecular-weight aggregates after the boiling of cow's milk for more than 20 min. In the native conformation, β -LG contains two disulfide bonds, from residue Cys-106 to Cys-119 and from Cys-66 to Cys-160, with a free thiol group at Cys-121.⁸ According to the literature, two different polymerization mechanisms have been described for β -LG. As shown by Carrotta *et al.*,²³ heating β -LG above 65 °C causes the unfolding of the main helix with the exposure of the free sulfhydryl group that was previously hidden in the native protein, and this leads to protein-protein co-polymerization, which in turn results in the formation of dimers or/and higher-molecular-mass products. On the other hand, Mills *et al.*⁸ demonstrated that the unfolding of β -LG by heating reveals the buried Cys-121, which is then able to catalyze the disulfide interchange to form a non-native monomer in which Cys-119 is exposed, thus allowing the protein to become linked to other food proteins such as caseins. Differently, caseins

Table 1. Densitometric data of native-PAGE of raw (Rm), microwaved (MWm) and hotplate boiled (HPm) cow's milk from image analysis by QuantityOne software (Bio-Rad)

Band number	Sample	Relative quantity	SD	Variation from Rm (%)
1	Rm	Not quantified	–	–
	MWm	Not quantified	–	–
	HPm	Not quantified	–	–
2	Rm	4.435	0.72	
	MWm	3.443	0.35	–22.4
	HPm	3.100	0.70	–30.1
3	Rm	9.383	1.08	
	MWm	6.996	0.51	–25.4
	HPm	5.359	0.49	–42.9
4	Rm	1.535	0.10	
	MWm	1.964	0.49	+27.9
	HPm	2.100	0.39	+36.8
5	Rm	11.039	1.95	
	MWm	18.581	0.44	+68.3
	HPm	21.028	0.68	+90.5
6	Rm	5.033	0.29	
	MWm	4.327	0.53	–14.0
	HPm	3.593	0.31	–28.6
7	Rm	3.032	0.62	
	MWm	3.946	0.31	+30.1
	HPm	4.295	0.39	+41.6
8	Rm	3.718	0.56	
	MWm	1.826	0.53	–50.9
	HPm	1.753	0.48	–52.9
9	Rm	3.390	0.40	
	MWm	3.155	0.33	–6.9
	HPm	3.737	0.27	+10.2
10	Rm	1.280	0.07	
	MWm	1.100	0.05	–14.1
	HPm	1.090	0.06	–14.8
11	Rm	11.385	0.60	
	MWm	10.541	0.26	–7.4
	HPm	10.741	1.12	–5.7
12	Rm	4.378	0.31	
	MWm	2.483	0.03	–43.3
	HPm	2.050	0.31	–53.2
13	Rm	0.754	0.02	
	MWm	0.444	0.03	–41.1
	HPm	0.255	0.10	–66.2
14	Rm	1.670	0.03	
	MWm	0.350	0.02	–79.0
	HPm	0.310	0.04	–81.4

SD, standard deviation.

seem to be more heat-stable than whey proteins because they do not have secondary, tertiary or quaternary structures that can be disrupted by heating.²⁴ This is confirmed by the stable profile of α_{s1} -casein (band 11) before and after heat treatment. On the other hand, the behavior of β -casein seems to be in disagreement with the previous findings, since a band exclusively containing β -casein in the lower part of the gel (band 14) showed a decreased intensity after both heating treatments (–79.0% for MWm and –81.4% for HPm), which apparently corresponded to an increased protein abundance in band 5 halfway through the gel (+68.3% for MWm and +90.5% for HPm), thus suggesting its aggregation in a polymerized form.

Immunoblotting

In order to investigate the effect of the domestic heat treatments on the allergenic potential of the cow's milk, the reactivity pattern of the raw and heat-treated milk proteins against the sera of 20 subjects suffering from CMPA was investigated (Fig. 2). The subject sera preferentially recognized caseins (especially α_{s1} -casein and β -casein) in both the raw and boiled milk (as already demonstrated²⁵), while the β -LG aggregates identified in band 1 were found to be non-immunoreactive. The IgE reactivity did not change to any great extent before or after the milk was boiled; however, some of the bands showed some slight differences in the percentage of reacting sera among the treatments.

Table 2. Protein identification by means of N-terminal amino acid sequencing of gel bands excised from native-PAGE of raw (Rm), microwaved (MWm) and hotplate boiled (HPm) cow's milk

Band number	Sample	Protein	N-terminal sequence	UniProt entry	
1	Rm	No band	-	-	
	MWm	β -Lactoglobulin	LIVTQTMKGLD	P02754	
	HPm	β -Lactoglobulin	LIVTQTMKGLD	P02754	
2	Rm	α_{s1} -Casein	RPKHPIKHQG	P02662	
	MWm	Not identified	-	-	
	HPm	Not identified	-	-	
3	Rm	α -Lactalbumin	EQLTKXEVFRE	P00711	
	MWm	α -Lactalbumin	EQLTKXEVFRE	P00711	
	HPm	α -Lactalbumin	EQLTKXEVFRE	P00711	
4	Rm	Not identified	-	-	
	MWm	α -Lactalbumin	EQLTKXEVFRE	P00711	
	HPm	β -Casein	RELEXLNPGE	P02666	
5	Rm	Not identified	-	-	
	MWm	β -Casein	XELEXLNPGE	P02666	
	HPm	β -Casein	XELEXLNPGE	P02666	
6	Rm	α -Lactalbumin	EQLTKXEVFRE	P00711	
	MWm	α -Lactalbumin	EQLTKXEVFRE	P00711	
	HPm	α -Lactalbumin	EQLTKXEVFRE	P00711	
7	Rm	β -Casein	XELEXLNPGE	P02666	
		α_{s1} -Casein	RPKHPIKHQG	P02662	
		α_{s1} -Casein	RPKHPIKHQG	P02662	
	MWm	α_{s2} -Casein	KNTMEHVSSEE	P02663	
		α -Lactalbumin ^a	EQLTKXEVFRE	P00711	
		HPm	α_{s1} -Casein	RPKHPIKHQG	P02662
8	Rm	α_{s2} -Casein	KNTMEHVS	P02663	
		α -Lactalbumin ^a	EQLTKXEVFR	P00711	
		Not identified	-	-	
	MWm	Not identified	-	-	
		HPm	α_{s1} -Casein	RPKHPIKHQG	P02662
		α_{s2} -Casein	KNTMEHVSSEE	P02663	
9	Rm	α -Lactalbumin ^a	EQLTKXEVFRE	P00711	
		α_{s1} -Casein	RPKHPIKHQG	P02662	
		α_{s2} -Casein	KNTMEHVSSEE	P02663	
	MWm	α_{s1} -Casein	RPKHPIKHQG	P02662	
		HPm	α_{s1} -Casein	RPKHPIKHQG	P02662
		α_{s2} -Casein	KNTMEHVSSEE	P02663	
10	Rm	α_{s1} -Casein	RPKHPIKHQG	P02662	
		β -Lactoglobulin	LIVTQTMKGLD	P02754	
		α_{s1} -Casein	RPKHPIKHQG	P02662	
	MWm	β -Lactoglobulin	LIVTQTMKGLD	P02754	
		HPm	α_{s1} -Casein	RPKHPIKHQG	P02662
		α_{s2} -Casein	KNTMEHVSSEE	P02663	
11	Rm	α_{s1} -Casein	RPKHPIKHQG	P02662	
		β -Lactoglobulin ^a	LIVTQTMKGLD	P02754	
		HPm	α_{s1} -Casein	RPKHPIKHQG	P02662
	MWm	β -Lactoglobulin ^a	LIVTQTMKGLD	P02754	
		Rm	α_{s1} -Casein	RPKHPIKHQG	P02662
		β -Lactoglobulin	LIVTQTMKGLD	P02754	
MWm	α_{s1} -Casein	RPKHPIKHQG	P02662		
	HPm	α_{s1} -Casein	RPKHPIKHQG	P02662	
	Rm	Not identified	-	-	
14	HPm	Not identified	-	-	
	Rm	β -Casein	XELEXLNPGE	P02666	
	MWm	Not identified	-	-	
HPm	Not identified	-	-		

^a Proteins present in band in traces.

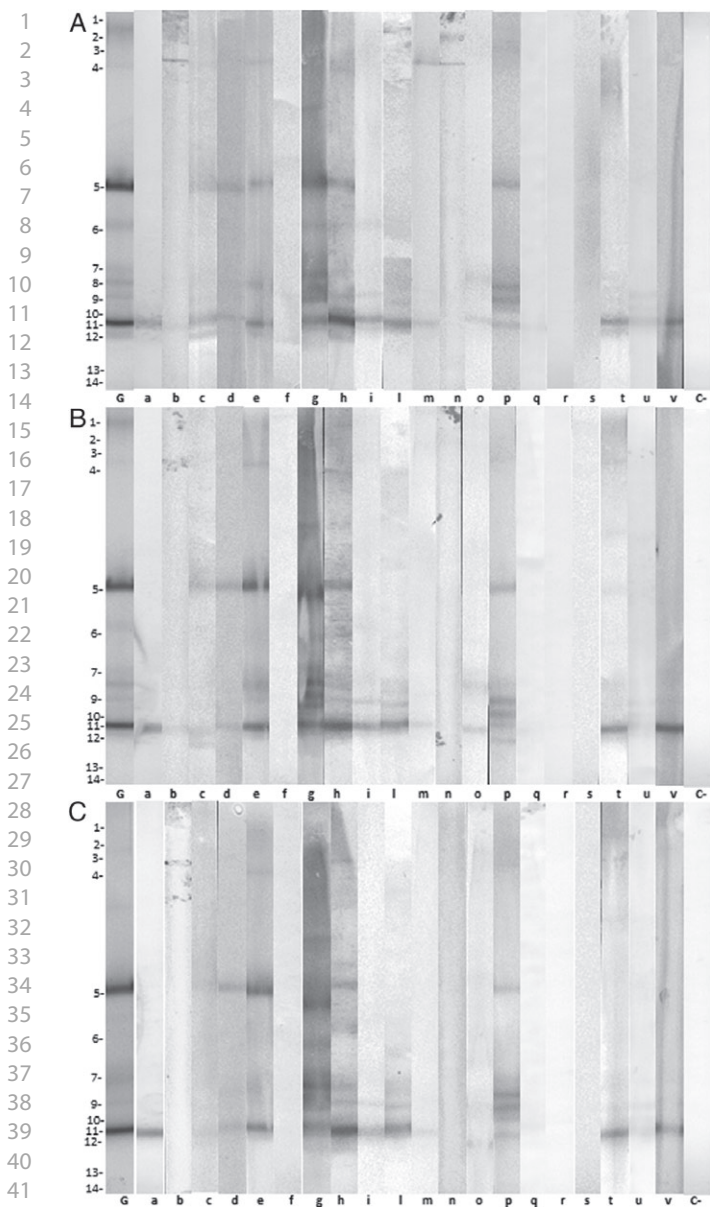


Figure 2. 1-D native-PAGE immunoblotting screening of sera from 20 subjects affected by CMPA: immunoblotting of (A) raw milk proteins, (B) milk proteins boiled in microwave oven and (C) milk proteins boiled on hotplate. In each panel: G, gel; C⁻, negative control; numbers indicate band codes; letters indicate codes of CMPA-affected subjects.

The proteins in band 11 showed that 75% of the subjects were reactive in all tested conditions. The proteins in band 12 showed 45% reactivity in Rm, 40% in MWm and 35% in HPm. The proteins in band 5 had 30% reactivity in all tested conditions. The proteins in band 7 showed that 30% of the subjects were reactive to Rm, 35% to MWm and 25% to HPm, while the proteins in band 9 had 20% of the subjects reactive to Rm, 30% to MWm and 25% to HPm. Finally, the new band at the gel well level, which was only detected after both of the boiling procedures (band 1), did not show any immune reactivity; this is probably due to the masking of the IgE-reactive epitopes, which was caused by protein–protein polymerization. The present results are in agreement with those of Ehn *et al.*²⁶ and Taheri-Kafrani *et al.*,²⁷ in which β -LG, isolated or present in whole milk, showed a slight, although significant, decrease in IgE

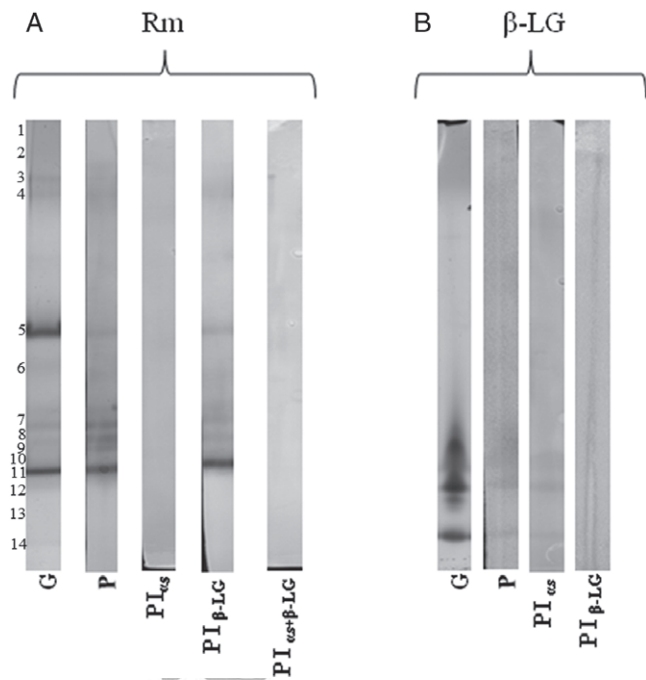


Figure 3. Inhibition assay on 1-D native-PAGE immunoblotting of (A) Rm sample and (B) pure β -LG: G, Blue Coomassie Colloidal-stained gel; P, incubation with pooled sera from 20 subjects affected by CMPA; PI_{α_1} , incubation with pooled sera depleted of α_{s1} -casein reactive IgEs; $PI_{\beta-LG}$, incubation with pooled sera depleted of β -LG reactive IgEs; $PI_{\alpha_1+\beta-LG}$, incubation with pooled sera depleted of both α_{s1} -casein and β -LG reactive IgEs.

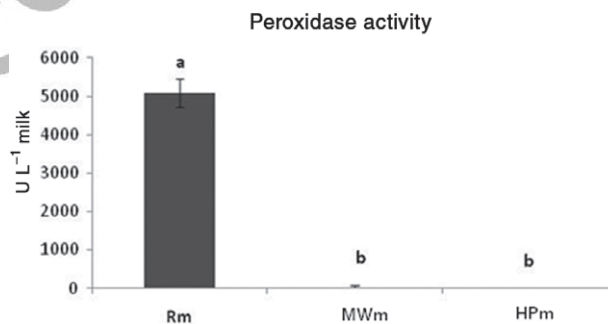


Figure 4. Peroxidase activity of raw (Rm), microwaved (MWm) and hotplate boiled (HPm) milk. Bars represent standard deviations ($n = 5$) and letters indicate Tukey test classes.

binding when heated above 74 °C, while a more pronounced decrease was found between 85 and 95 °C. Chen *et al.*²⁸ also showed that β -LG protein–protein aggregation can be detected by a specific monoclonal antibody and postulated that β -LG could be used as a specific marker to discriminate differences in milk processing procedures.

The results pertaining to the β -LG aggregate behavior should also be evaluated carefully *in vivo*. In fact, it has recently been demonstrated that heat treatment reduced the allergenicity of β -LG by at least two distinct mechanisms: by directly causing inter- and intramolecular conformational changes that disrupt the conformational epitopes and by increasing the susceptibility of β -LG to digestion, thus causing the loss of the structural integrity of the allergen and its capacity to elicit an IgE response.²⁹

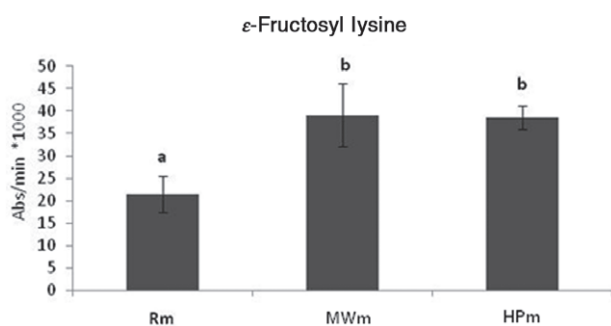


Figure 5. ϵ -Fructosyl lysine of raw (Rm), microwaved (MWM) and hotplate boiled (HPM) milk. Bars represent standard deviations ($n = 5$) and letters indicate Tukey test classes.

Inhibition assay

In order to understand which protein was responsible for the immunoreactivity of band 12, which contained co-migrating proteins (α_{s1} -casein and β -LG), an inhibition assay was performed. Figure 3 shows the results of the inhibition assay on the one-dimensional (1-D) native immunoblotting of the Rm sample. The blotted Rm proteins were incubated with (i) pooled sera from the selected subjects (Fig. 3A, lane P), (ii) pooled sera after depletion of the α_{s1} -casein reactive IgEs (Fig. 3A, lane $PI_{\alpha s1}$), (iii) pooled sera after depletion of the β -LG reactive IgEs (Fig. 3A, lane $PI_{\beta-LG}$) and (iv) pooled sera after depletion of both the α_{s1} -casein and β -LG reactive IgEs (Fig. 3A, lane $PI_{\alpha s1 + \beta-LG}$). No reactivity was detectable for the sera depleted of α_{s1} -casein reactive IgEs or of α_{s1} -casein and β -LG reactive IgEs, while a reaction was still detectable when the pooled sera were inhibited with β -LG reactive IgEs.

The same experiment was also performed on the 1-D native immunoblotting of pure β -LG (980 g kg^{-1}). In this case, the pooled sera showed a weak reactivity against β -LG, even when depleted of the α_{s1} -casein reactive IgEs (Fig. 3B, lane $PI_{\alpha s1}$). This result suggests that the immunoreaction was exclusively due to α_{s1} -casein, probably because the β -LG reactive epitopes were masked by α_{s1} -casein steric hindrance when the two proteins co-migrated in native conditions. This finding has been confirmed by the stable immunoreaction of band 12 (Fig. 2) which contains α_{s1} -casein and β -LG in the raw and heated milk, despite the disappearance of β -LG in the heated samples, as demonstrated by means of the protein identification analysis.

Process/nutritional parameters

Some control markers that are commonly used by the dairy industry to evaluate the pasteurization process of milk, such as ϵ -fructosyl lysine determination and peroxidase activity assay, were also considered. Domestic boiling, using both a hotplate and a microwave oven, caused a complete inactivation of peroxidase (Fig. 4), as expected for UHT milk and for over-pasteurized milk, thus suggesting that the domestic boiling of cow's milk is more severe than the common industrial pasteurization process. The ϵ -fructosyl lysine content (Fig. 5), which is considered as an index of severe heat treatment and/or of the addition of milk powder and UHT milk to raw and pasteurized milk, revealed that, although significantly different from raw and boiled milk samples, domestic boiling was only slightly responsible for the formation of early Maillard reaction products. This result is consistent with the steady amount of available lysine found in the three samples.

CONCLUSIONS

In the present study, milk whey proteins have appeared to be more heat-labile than caseins when subjected to domestic boiling procedures. Minor differences in allergenicity have been observed between raw milk and boiled milk, mainly due to the modifications in the protein profile following the thermal treatments. In boiled milk, β -lactoglobulin, in the aggregated form, did not elicit the immunorecognition of the sera of CMPA patients. The complete inactivation of peroxidase in the heated milk suggests that domestic boiling is a more severe treatment than common industrial pasteurization processes.

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