

**EFFECTS OF TWO DIFFERENT DOMESTIC BOILING PRACTICES ON THE
ALLERGENICITY OF COW MILK PROTEINS**

RUNNING TITLE: DOMESTIC BOILING AND ALLERGENICITY

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ABSTRACT

BACKGROUND: the sale of raw drinking milk through automatic dispensers is permitted in some European 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

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techniques on the proteins of raw milk, and, in particular, on their potential allergenicity. RESULTS: Native 1-D 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 result 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 minor reduction in milk allergenicity.

KEYWORDS LIST

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 remarkable modifications of milk, such as enzyme inactivation, protein denaturation and/or aggregation causing epitope destruction, modification, masking or unmasking [8,9], cross-linking 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 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 cow milk protein allergy (CMPA). Moreover, some conventional milk nutrition parameters were evaluated in different experimental conditions.

EXPERIMENTAL

Chemicals

Boric acid, TRIS, Bromophenol blue, Glycerol, Glycine, Ponceau Red, NaCl, Tween-20, Gelatine, β -lactoglobulin and α_{s1} -casein were from Sigma-Aldrich (Saint Louis, MI); TGX Precast Gel, Blue Coomassie Colloidal, Nitrocellulose membranes, AP Conjugate substrate kit and o-phthaldialdehyde were from Bio-Rad (Hercules, CA); Immuno-CAP system was from Phadia (Thermo Fisher Scientific, Waltham, MA); 2DE Quant kit was from GE Healthcare (Little Chalfont, Buckinghamshire, UK); alkaline phosphatase-conjugated goat anti-human-IgE antibody (ϵ -chain specific) was from KPL (SeraCare Life Sciences, Milford, MA); FOODLAB peroxidase assay kit and ϵ -fructosil lysine FOODLAB kit were from CDR Mediated (Ginestra Fiorentina, Italy).

Serum samples

Serum samples from subjects sensitized to cow's milk were collected from the Koelliker Hospital (Turin, Italy). The content of specific cow's milk IgE antibodies was measured by using the

Immuno-CAP system, according to the manufacturer's instructions. Twenty subjects showing cow milk IgEs levels ranging from 0.57 to 82.3 kU_A/L were enrolled in the study.

Sample preparation

Raw cow's milk (Rm) was collected in two subsequent samplings, from eleven 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 3 aliquots. One aliquot (20 ml) was used untreated as a reference (Rm), while the others underwent to two common domestic boiling procedures, one on a hotplate (16 min. and ~98°C) (HPm) and one in a microwave oven (power: 1300 W, 60 sec. and ~98°C) (MWm), respectively.

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

Native-PAGE and electroblotting on nitrocellulose membrane

Native-PAGE was performed, as already detailed by Monti and co-workers [15]. Briefly, a protein mixture of skimmed Rm, MWm and HPm was separated on a 7.5% Mini-PROTEAN 10-well TGX Precast Gel, using the Mini-PROTEAN Electrophoresis Tetra System cell (Bio-Rad). Five µg of total proteins was taken from each sample and added to 10 µL of boric acid-borax buffer (0.2M, pH 8.4) and this solution was further diluted 1:2 with native sample buffer (0.25M Tris-HCl pH 6.8, 0.25% (w/v) Bromophenol blue and 15% (v/v) glycerol). Seven µg of bovine β-lactoglobulin was used as native molecular weight standard, and the Towbin buffer (192 mM Glycine, 25 mM 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 a fresh home-made Blue Coomassie Colloidal stain [16] and digitized using a GS-800 Densitometer (Bio-Rad).

An aliquot of 2.5 µg 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 1h. 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 the 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 cow milk protein allergy (CMPA). Each membrane had previously been washed in Tris Buffered Saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4) in order to eliminate the Ponceau Red stain, and then blocked twice for 15 min using a TBS solution containing 0.3% v/v Tween-20. The washed membranes were incubated overnight in the dark with the subject's serum diluted 1:5 in an incubation solution (TBS, 0.05% v/v Tween-20, 0.05% w/v gelatin). After incubation, the membranes were rinsed with a washing solution (TBS, 0.05% v/v Tween-20), and incubated again for 1h with a 1:1000 dilution of an alkaline phosphatase-conjugated goat anti-human-IgE antibody (ε-chain specific) in an incubation solution. After three washing steps (10 minutes each), immunoblots were developed with a BCIP/NBT alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (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 was pooled, diluted 1:5 in an incubation solution and divided into 3 aliquots. Before immunodetection, each aliquot was incubated alternatively with 200 µg of β -lactoglobulin, α_{s1} -casein or with a mixture (1:1) of them for 1h, at room temperature. Incubation and immunoreaction were performed on blotted Rm samples and on the 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 (o-phthaldialdehyde) already described by Baro and co-workers [18]. Five technical replicates were performed.

Lactoperoxidase activity and ϵ -fructosil lysine determination

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

Statistical analysis

Densitometric analysis of Native-PAGE (Rm, MWm, HPm) was obtained using QuantityOne software (Bio-Rad). Statistical analysis of the data was performed by One-Way ANOVA. The bands with statistically significant differences ($P \leq 0.05$) were compared by post-hoc with Tukey's test, using SPSS software version 15.0 (SPSS, Chicago, Illinois). In a similar way as for the lactoperoxidase activity and ϵ -fructosil lysine content, the differences among the 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%. A statistical analysis was performed using the Kyplot software package, ver.2 beta 15 (KyensLab Inc.).

RESULTS AND DISCUSSION

As far as proteins are concerned, it is known that the 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

The 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 the detectable bands were quantified by QuantityOne software (Tab. 1), only band 1 cannot be quantified because too close to the edge of the gel analyzed. Each band were excised and identified by means of N-terminal amino acid sequencing (Tab. 2); several bands were found to contain more than one protein in both the raw and processed milk. The choice to separate the cow milk proteins in native conditions, without treating the sample with linearizing agents, such as SDS or DTT, allowed us to obtain a more detailed characterization of the heating effects regarding denaturation, protein polymerization and the protein-protein association. SDS is an anionic detergent, while DTT is a reducing agent: by 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 a loss of secondary structure (55–70°C), cleavage of disulphide bonds (70–80°C), formation of new intra-/intermolecular interactions rearrangements

of the disulphide 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 to R_m of -14% for MWm and -28,6% for HPm) or even non-detectable (β -lactoglobulin (β -LG) in bands 12).

Wijayanti and co-workers [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 part of the gel (bands 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 and co-workers [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 literature, two different polymerization mechanisms have been described for β -LG. As shown by Carrota and co-workers [23], β -LG heating above 65°C causes the unfolding of the main helix with the exposure of the free sulphhydryl group that was previously hidden in the native protein, and this leads to protein-protein copolymerization, which in turn results in the formation of dimers or/and higher molecular mass products. On the other hand, Mills and co-workers [8] demonstrated that the unfolding of β -LG by heating reveals the buried Cys-121, which is then able to catalyze the disulphide 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 resulted 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 the α s1-casein (band 11) before and after the 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 of the heating treatments (-79% 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 demonstrated [25]), 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. The protein in band 11 showed that 75% of the subjects were reactive in all the tested conditions. The proteins in band 12 showed 45% reactivity in Rm, 40% in MWm and 35% in HPm. The protein in band 5 had 30% reactivity in all the 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 IgEs reactive epitopes, which was caused by protein-protein polymerization. The present results are in agreement with those of Ehn and co-workers [26] and Taheri-Kafrani [27], in which β -LG, isolated or present in whole milk, showed a slight, although significant, decrease in IgE binding when heated above 74°C, while a more pronounced decrease was found between 85° and 95°C. Chen and co-workers [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 behaviour should also be evaluated carefully “in vivo”. In fact, it has recently been demonstrated that the heat treatment reduced the allergenicity of β -LG by at least two distinct mechanisms: by directly causing intermolecular 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].

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. Fig. 3 shows the results of the inhibition assay on the 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 $PI_{\alpha s}$), 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 the β -LG reactive IgEs (Fig. 3A, lane $PI_{(\alpha s+\beta-LG)}$). 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 was inhibited with β -LG reactive IgEs.

The same experiment was also performed on the 1-D native immunoblotting of pure β -LG (98% w/w). 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 $PI_{\alpha s}$). 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 -fructosil 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 milk is more severe than the common industrial pasteurization process. The ϵ -fructosil 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 results to be a more severe treatment than common industrial pasteurization processes.

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Figure 1. 1-D Native-PAGE protein profile of raw (Rm), microwave (MWm) and hotplate (HPm) boiled cow's milk. The numbers indicate the band codes.

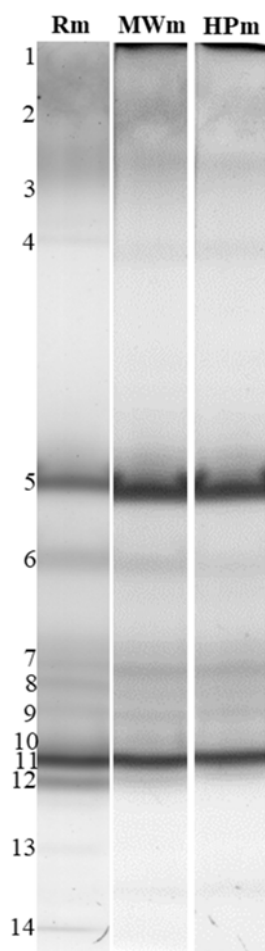


Figure 2. 1-D Native-PAGE immunoblotting screening of sera from 20 subjects affected by CMPA. A: immunoblotting of the raw milk proteins; B: immunoblotting of the milk proteins boiled in the microwave oven; C: immunoblotting of the milk proteins boiled using a hotplate. In each panel: G = gels; C⁻ = negative control; the numbers indicate the band codes; the letters indicate the codes of the CMPA affected subjects.

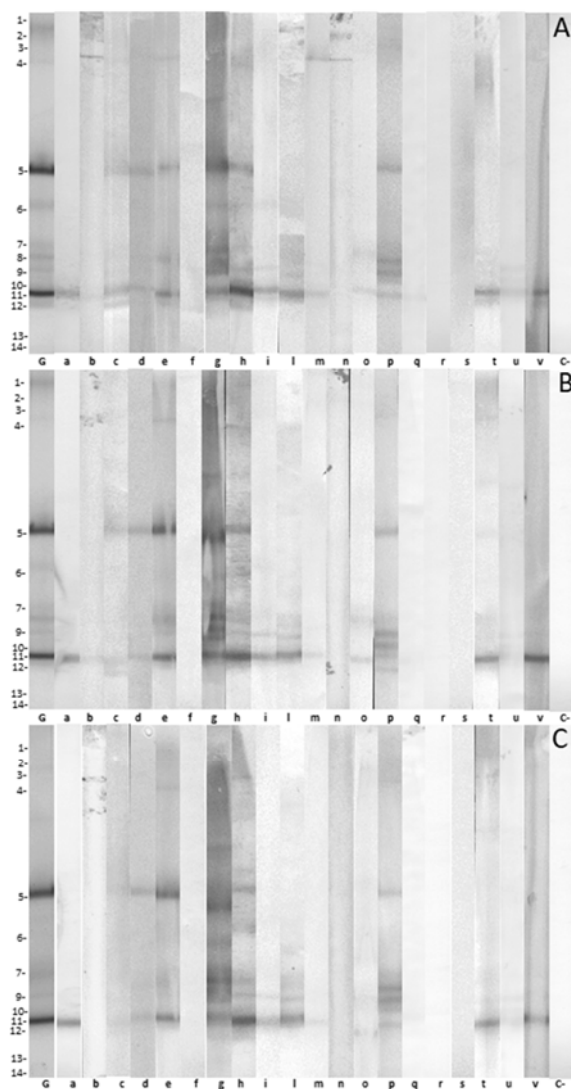


Figure 3. Inhibition assay on 1-D Native-PAGE immunoblotting of the Rm sample (A) and pure β -LG (B). G: Blue Commassie stained gel; P: incubation with pooled sera from 20 subjects affected by CMPA; $PI_{\alpha s}$: incubation with pooled sera depleted of $\alpha s1$ -casein reactive IgEs; $PI_{\beta-LG}$: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

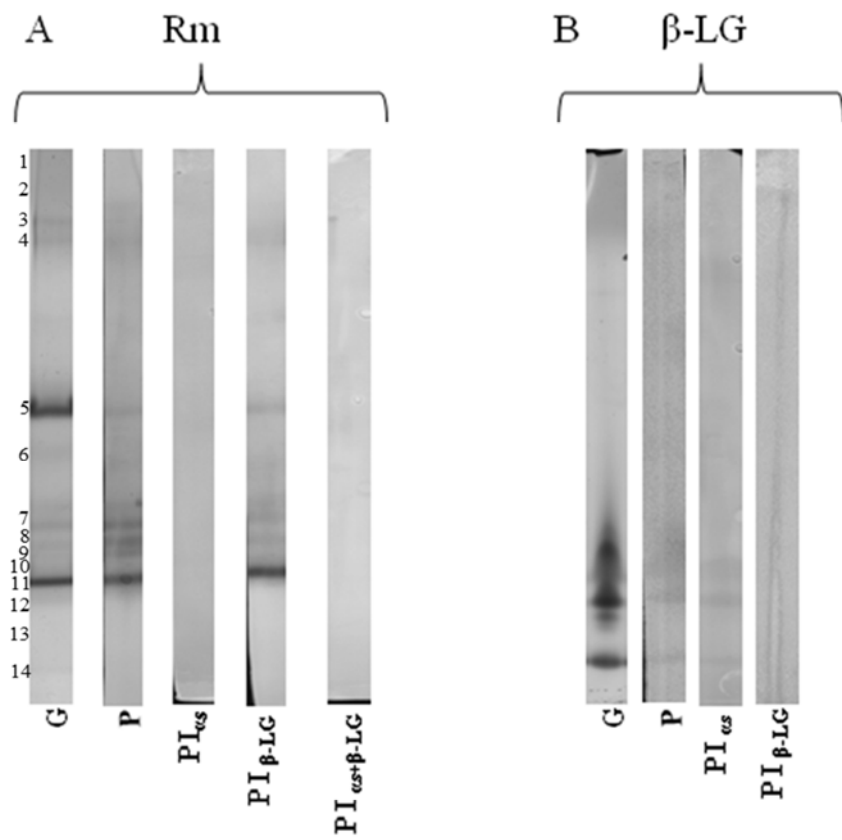
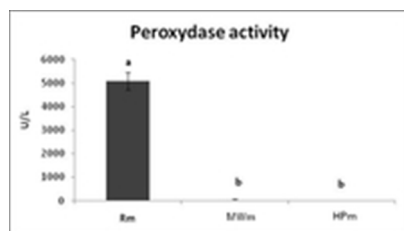
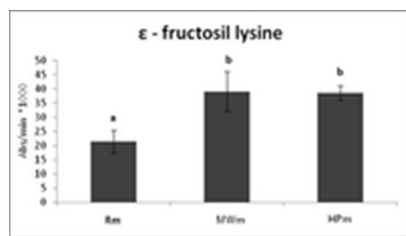


Figure 4. Peroxidase activity (U/L of milk) of raw milk (Rm), microwaved milk (MWm), and hotplate boiled milk (HPm). The bars represent Standard Deviations (n=5) and the letters indicate the Tukey test classes.

Figure 5. ϵ -fructosil lysine (Abs/min*1000) of raw milk (Rm), microwaved milk (MWm), and hotplate boiled milk (HPm). The bars represent Standard Deviations (n=5) and the letters indicate the Tukey test classes.



Peroxidase activity (U/L of milk) of raw milk (Rm), microwaved milk (MWm), and hotplate boiled milk (HPm). The bars represent Standard Deviations (n=5) and the letters indicate the Tukey test classes



ϵ -fructosil lysine (Abs/min*1000) of raw milk (Rm), microwaved milk (MWm), and hotplate boiled milk (HPm). The bars represent Standard Deviations (n=5) and the letters indicate the Tukey test classes.

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Table 1. Densitometric data of Native-PAGE of Rm, MWm, HPm, from image analysis by QuantityOne software (Bio-Rad).

Band n.	sample	relative quantity	SD	% variation to Rm
1	Rm	<i>not quantified</i>	-	-
	MWm	<i>not quantified</i>	-	-
	HPm	<i>not quantified</i>	-	-
2	Rm	4,435	0,72	
	MWm	3,443	0,35	-22,4
	HPm	3,100	0,700	-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	1,67	0,03	
	MWm	0,350	0,02	-79,0
	HPm	0,310	0,04	-81,4

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

Band n°	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 β-casein	EQLTKXEVFRE RELEXLNVPGE	P00711 P02666		
	HPm	Not identified	—	—		
5	Rm	β-casein	XELEXLNVPGE	P02666		
	MWm	β-casein	XELEXLNVPGE	P02666		
	HPm	β-casein	XELEXLNVPGE	P02666		
6	Rm	α-lactalbumin	EQLTKXEVFRE	P00711		
	MWm	α-lactalbumin	EQLTKXEVFRE	P00711		
	HPm	α-lactalbumin	EQLTKXEVFRE	P00711		
7	Rm	β-casein α _{s1} -casein	XELEXLNVPGE RPKHPIKHQG	P02666 P02662		
	MWm	α _{s1} -casein α _{s2} -casein α-lactalbumin*	RPKHPIKHQG KNTMEHVSSEE EQLTKXEVFRE	P02662 P02663 P00711		
		HPm	α _{s1} -casein α _{s2} -casein β-casein α-lactalbumin*	RPKHPIKHQG KNTMEHVSSEE XELEXLNVPGE EQLTKXEVFRE	P02662 P02663 P02666 P00711	
			8	Rm	α _{s1} -casein α _{s2} -casein	RPKHPIKHQG KNTMEHVS
	MWm			α-lactalbumin*	EQLTKXEVFR	P00711
		HPm		— Not identified	— —	— —
9	Rm	α _{s1} -casein α _{s2} -casein α-lactalbumin*	RPKHPIKHQG KNTMEHVSSEE EQLTKXEVFRE	P02662 P02663 P00711		
		MWm	α _{s1} -casein α _{s2} -casein	RPKHPIKHQG KNTMEHVSSEE	P02662 P02663	
			HPm	α _{s1} -casein α _{s2} -casein	RPKHPIKHQG KNTMEHVSSEE	P02662 P02663
	10	Rm		α _{s1} -casein β-lactoglobulin	RPKHPIKHQG LIVTQTMKGLD	P02662 P02754
		MWm		α _{s1} -casein β-lactoglobulin	RPKHPIKHQG LIVTQTMKGLD	P02662 P02754
			HPm	α _{s1} -casein α _{s2} -casein	RPKHPIKHQG KNTMEHVSSEE	P02662 P02663

11	Rm	α_{s1} -casein	RPKHPIKHQG	P02662
	MWm	α_{s1} -casein β -lactoglobulin*	RPKHPIKHQG LIVTQTMKGLD	P02662 P02754
	HPm	α_{s1} -casein β -lactoglobulin*	RPKHPIKHQG LIVTQTMKGLD	P02662 P02754
12	Rm	α_{s1} -casein β -lactoglobulin	RPKHPIKHQG LIVTQTMKGLD	P02662 P02754
	MWm	α_{s1} -casein	RPKHPIKHQG	P02662
	HPm	α_{s1} -casein	RPKHPIKHQG	P02662
13	Rm	α_{s1} -casein	RPKHPIKHQG	P02662
	MWm	<i>Not identified</i>	—	—
	HPm	<i>Not identified</i>	—	—
14	Rm	β -casein	XELEXLNVPGE	P02666
	MWm	<i>Not identified</i>	—	—
	HPm	<i>Not identified</i>	—	—

*proteins present in the band in traces