Hypoallergenic properties of donkey's milk: a preliminary study

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Summary
Cow's milk protein allergy (CMPA) is an abnormal immunological response to cow milk proteins, which results in IgE-mediated reactions. The therapeutic strategy to respond to CMPA envisages the total elimination of milk or the administration of cow's milk substitutes. For this reason the use of milk from other mammalian species was tested. Among them, donkey's milk proved to be the best alternative in feeding infants affected by CMPA, since its chemical composition is comparable to human milk. In this work an *in vitro* study was performed in order to analyze the IgE reactivity to milk protein allergens from cow, donkey and goat. In particular, immunoblotting experiments using sera from milk-allergic and non-allergic adult volunteers were conducted with the aim of verifying the hypoallergenic property of donkey's milk. This study provided a preliminary evidence of the hypoallergenicity of donkey's milk when compared to bovine and goat milk. Considering the obtained results, it would be possible to develop a sensitive diagnostic method for CMPA detection, based on chromatographic and immunoblotting analysis.

Studio preliminare sulle proprietà ipoallergeniche del latte di asina

Parole chiave
APLV,
Cromatografia a fase inversa,
Cromatografia a scambio ionico,
Immunoblotting,
Ipoallergenicità,
Latte di asina,
Latte di capra,
Latte vaccino.

Riassunto
L'allergia alle proteine del latte vaccino (APLV) è una reazione immunologica IgE-mediata. La strategia terapeutica dell'APLV è basata sull'eliminazione totale del latte vaccino e sulla somministrazione di alcuni tipi di latte sostitutivi a quello vaccino. A tal proposito, il latte d'asina ha dimostrato di essere, per la sua composizione chimica molto simile a quella del latte umano, l'alternativa migliore nell'alimentazione di neonati affetti da APLV. In questo studio preliminare è stata analizzata la reattività delle proteine del latte di vacca, asina e capra con esperimenti di immunoblotting. Sono stati utilizzati sieri di soggetti adulti volontari allergici e non allergici (controlli) al latte vaccino. I risultati hanno permesso di evidenziare le proprietà ipoallergeniche del latte di asina rispetto al latte di capra ma, soprattutto, rispetto a quello vaccino. Lo studio può contribuire allo sviluppo di un metodo diagnostico veloce e sensibile per la rilevazione dell'APLV.

Introduction

Food allergy refers to an abnormal immunologic response to a food protein that occurs in a susceptible subject. This reaction occurs each time the food is ingested and it is often not dose dependent (Cianferoni and Spergel 2009). Food allergy affects around 11-26 million of the European population and, in general, is more frequent in the pediatric, rather than in the adult population (Fiocchi *et al.* 2010). The allergens responsible for more than 85% of food allergy are proteins contained in milk, egg, peanut, tree nuts, shellfish, wheat, sesame, seed and soy (Waserman and Watson 2011). The allergenic segments or 'epitopes' of these proteins tend to be small (from 10 to 70 kDa in size) water-soluble glycoproteins, which are generally resistant to denaturation by heat or acid and therefore can remain intact even after processing, storage, cooking and digestion (Waserman and Watson 2011). Examples of these glycoproteins include caseins in milk, vicilins in peanut, and ovomucoid in eggs. Based on the immunological mechanism involved, food allergies may be further classified in: IgE-mediated or mediated by IgE antibodies; cell-mediated, when the cell component of the immune system is responsible of the food allergy and mostly involves the gastrointestinal tract; mixed IgE mediated-cell mediate (Cianferoni and Seprgel 2009).

Cow milk protein allergy (CMPA) is clinically an abnormal immunological reaction to cow milk proteins, which may be due to the interaction between one or more milk proteins and one or more immune mechanisms, and results in immediate IgE-mediated reactions. The clinical manifestations of CMPA include gastrointestinal, respiratory, cutaneous as well as systemic anaphylactic symptoms (Bahna and Gandhi 1983). Cow milk is one of the most common food allergies in children, occurring in between 0.3 and 7.5% of the infant worldwide population (Hill *et al.* 1986), although this allergy is considered transient, with a remission rate of 85% after 3 years of age, there are still some children who exhibit it at the age of 10 and in the adult age (Giner *et al.* 2012). The main allergens in cow's milk are caseins (αs1- and β-caseins) followed by β-lactoglobulin and α-lactalbumin, although the latter occurs to a minor extent (Docena *et al.* 1996, Jarviner *et al.* 2001). The therapeutic strategy for CMPA envisages the total elimination of cow's milk and all its derivatives. In the first 2 years of life, however, milk represents an important source of nutrients and as such it cannot be eliminated from the everyday diet, making therefore necessary to use substitutive milks. In addition, oral desensitization cannot be performed before 2 years of age (Meglio *et al.* 2004). Soy milk or hydrolysed formulas may be considered good substitutes to human milk and allow to the children to grow up well, nevertheless it has been shown that 17-47% of milk allergic infants can have adverse reactions to soy. In order to find a good substitute to cow's milk, the use of milk from other mammalian species like goat, sheep, mare and donkey was considered in all the cases in which breast feeding was not possible and when it is not possible to use soy milk or hydrolyzed formulas (Iacono *et al.* 1992, Dean *et al.* 1993, Carroccio *et al.* 2000, Muraro *et al.* 2002, Restani *et al.* 2002). In particular, donkey's milk proved to be a good alternative for CMPA infants because of its similarity to human milk with regard to its composition in lipids, with particular regards to the triacylglycerol fraction and fatty acid profile (Chiofalo *et al.* 2011), protein fraction, mineral and lactose content (Monti *et al.* 2007, Vincenzetti *et al.* 2008). Furthermore, the high content of lactose confer to this milk a good palatability and optimize the intestinal absorption of calcium, essential for bone mineralization in infants.

The low allergenicity of donkey's milk is mainly due to the low casein content (Vincenzetti *et al.* 2007), which is very close to the casein content determined in human milk. In particular αs1- and β-caseins in different phosphorylated forms has been shown to be present in large amount in donkey's milk, κ-casein and αs2-casein are also present although in very small amounts (Vincenzetti *et al.* 2008, Criscione *et al.* 2009, Bertino *et al.* 2010) differently from cow's milk (Creamer 2003).

In this work, an *in vitro* study was performed in order to analyze the IgE reactivity to milk protein allergens from cow, donkey and goat by immunoblotting experiments using sera from milk-allergic and non-allergic adult volunteers. The cross-reactivity between sera of children with CMPA and milk proteins from some mammalian species such as sheep, goat and buffalo has been addressed in several studies (Carroccio *et al.* 1999, Restani *et al.* 2002, Monti *et al.* 2007), the primary aim of this work was to verify the renowned hypoallergenic property of this milk. To achieve such a goal, a purification and characterization of the caseins and whey proteins present in donkey, goat and cow milk were performed and each separated protein was used as antigen in the immunoblotting experiments. However, due to the restricted number of sera available, the present work must be intended as preliminary study that could be considered as a starting point for further analysis on the allergenicity of different types of milk.

Materials and methods

Milk samples

Bulk milk in midstage of lactation from cow, goat and donkey was obtained from local farms (Umbria region, May 2013). Skimmed milk was

obtained from 25 ml fresh milk by centrifugation at 3000g for 30 minutes at 15°C. Whole caseins were obtained from skimmed milk by adjusting the pH to 4.6 with 10% (v/v) acetic acid and centrifuged at 3000g for 30 minutes in order to obtain a supernatant of whey proteins and the isoelectrically precipitated caseins which were subsequently resuspended in 25 ml of buffer A (50 mM ammonium acetate, 8 M urea, pH 5.5). The protein concentration of both whey and casein fractions was determined by the Bradford protein assay method (Bradford 1976).

Casein and whey protein purification

A reversed-phase (RP-HPLC) and an ion exchange chromatography (Vincenzetti *et al.* 2008) were performed in order to purify and characterize the whey protein and the casein fractions in cow, goat and donkey milk. The HPLC system used was an Äkta Purifier (GE-Healthcare, Uppsala, Sweden).

One aliquot (500 µl) of whole caseins resuspended in buffer A was subjected to the cationic exchange chromatography on HPLC through a MONO S HR 5/5 column, (1.0 ml bed volume, GE Healthcare), equilibrated in buffer A (flow rate of 0.5 ml/min) and eluted by a linear gradient between buffer A and buffer B (1 M ammonium acetate, 8 M urea, pH 5.5). The gradient used was: %B = 0, time = 10 min; %B = 100, time = 100 min; %B = 100, time = 110 min. Whey proteins from bovine, donkey and goat were separated by a RP-HPLC using a C4 Prosphere (300 Å, 5 µm, 4.6 mm I.D., 150 mm., Alltech); 500 µL of whey proteins were added to 500 µl of CL buffer (0.1 M bis-tris, pH 8.0 containing 8 M urea, 1.3% trisodium citrate, 0.3% DTT) and 1 ml was loaded into the reversed phase column equilibrated in trifluoroacetic acid (TFA)/H_2O 1:1000 v/v (buffer A). Elution was achieved by the following step gradient with TFA/H_2O/acetonitrile 1:100:900 v/v (buffer B): %B = 0, time = 10 min; %B = 20, time = 10 min; %B = 40, time = 0.1 min; %B = 60, time = 40 min. The flow rate was 1 ml/min. In all cases, the proteins eluted from the columns were detected at 280 nm by a UV 900 Monitor included in the HPLC system. Each chromatographic peak eluted from each chromatographic course was collected and, subsequently, analyzed by SDS-PAGE. After the chromatographic courses, each purified peak casein and whey protein was dialyzed against 50 mM ammonium acetate buffer (pH 5.5), and 20 mM Tris/HCl buffer, respectively. After dialysis the concentration of proteins was determined by the Bradford protein assay method (Bradford 1976).

Immunoblotting analysis

Blood samples were obtained from a total of 6 volunteers: 3 milk-allergic and 3 non-allergic subjects (controls). Sera were obtained by centrifugation at 3000 g for 10 minutes at 4°C. Allergic volunteers were selected according to a positive case history (i.e. gastrointestinal symptoms upon controlled ingestion of milk products), positive skin-prick reactions and determination of specific IgE to cow's milk proteins. For the immunoblotting analysis, each purified casein and whey protein from cow, goat and donkey was firstly separated by SDS-PAGE and then transferred to a nitrocellulose membrane by a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories, Inc. Hercules, CA).

The nitrocellulose sheets were washed in 50 mM Tris; 150 mM NaCl; 0.05% Tween 20, pH 7.6 (TBST) and soaked in blocking solution (TBST, 1% BSA) and incubated for 16 hours.

After incubation with blocking solution, the nitrocellulose membranes were directly incubated with each whole serum diluted 1:500 in TBST for at least 3 hours, to allow IgE antibodies eventually present in serum to cross-react with a specific milk proteins. After a washing in TBST, the nitrocellulose sheets were incubated with mouse anti-human Ig-G secondary antibody conjugated with Alkaline Phosphatase (AP) diluted 1:20000 in TBST for another hour. The AP reaction was visualized by the Alkaline Phosphatase Conjugate chromogen/substrate kit (Bio-Rad) according to the manufacturer's instructions.

Allergenicity evaluation of milk proteins

Caseins and whey proteins separated from cow, goat and donkey milk by chromatography and electrophoresis were individually blotted onto a nitrocellulose membrane and incubated with each serum of subject affected by CMPA and controls.

The volunteer subjects involved in this preliminary screening were:

1A: subject affected by CMPA, 22 years old, female;

2A: subject affected by CMPA, 25 years old, female;

3A: subject with suspect CMPA, 25 years old, female;

C1: control, 48 years old, male;

C2: control, 46 years old, female;

C3: control, 76 years old, male.

Results and discussion

In this study, casein and whey protein fractions from cow, goat and donkey milk were separated using the different chromatographic procedures described in the Materials and methods section. The total whey proteins content was 1.7 mg/ml for bovine milk, 1.91 mg/ml for donkey milk and 1.77 mg/ml for goat

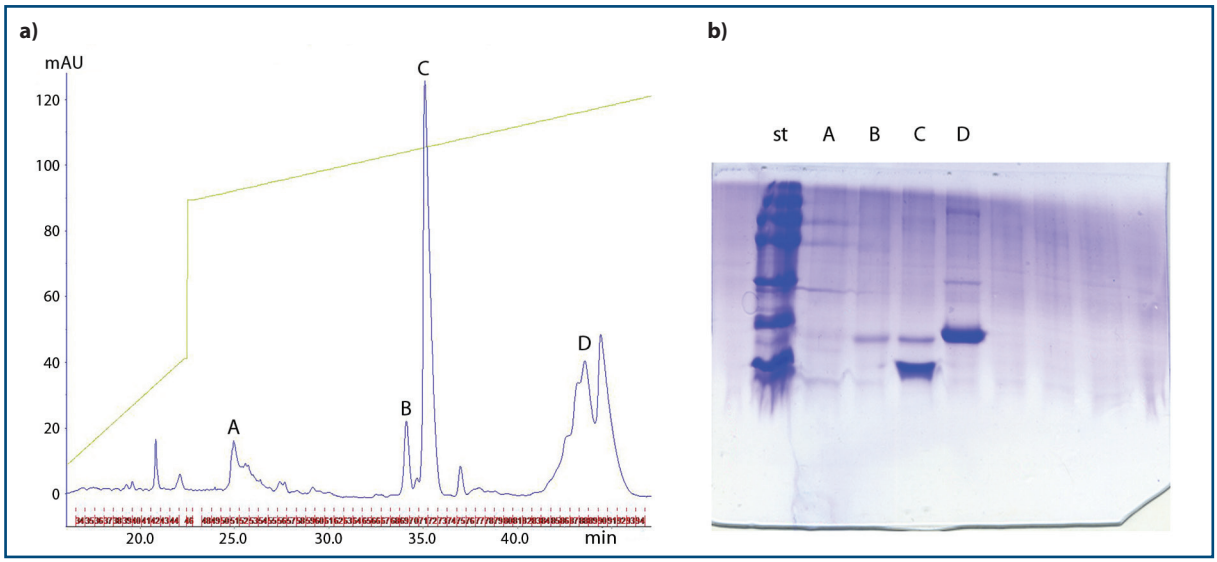

Figure 1. *(a) RP-HPLC of whey proteins from bovine milk. (b) 15% SDS-PAGE of peaks A, B, C and D eluted from RP-HPLC. St: Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa ovalbumin; 31.0 kDa carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).*

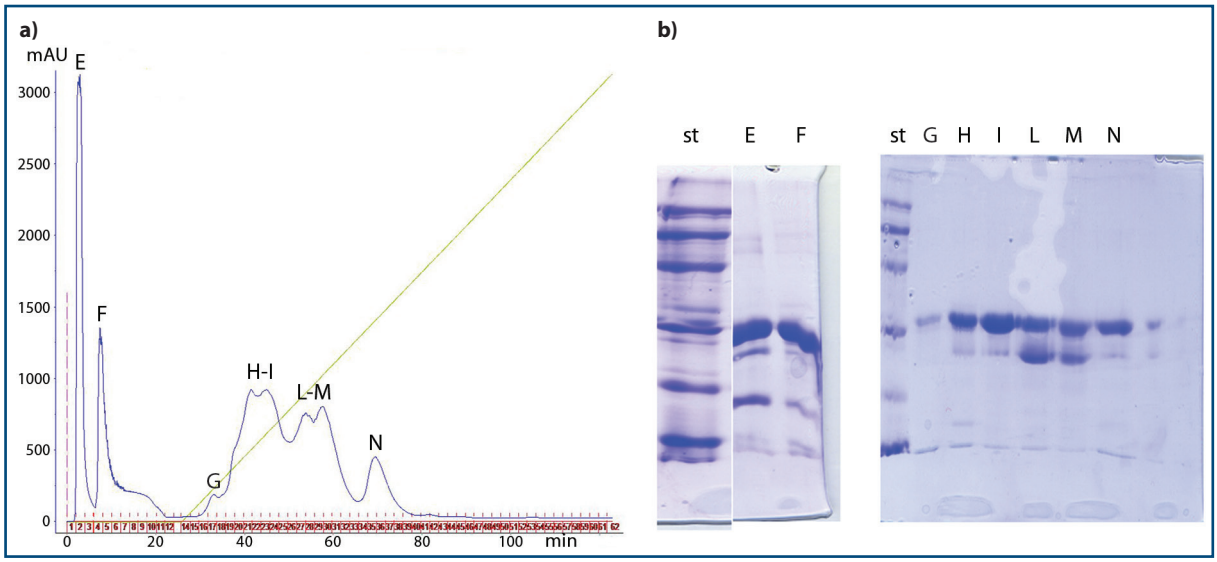

Figure 2. *(a) Cationic-exchange chromatography on HPLC on casein from bovine milk. (b) 13% SDSPAGE of the peaks eluted from the MONO S HR 5/5 column. St: Bio-Rad low molecular weight standard.*

milk, whereas the total casein protein content for cow, donkey and goat milk was 5.08, 2.42 and 5.37 mg/ml, respectively.

By RP-HPLC whey proteins from bovine milk were separated into 4 peaks, A, B, C and D as shown in Figure 1a, 15% SDS-PAGE analysis revealed that the peak C corresponds to α-lactalbumin with a molecular weight of 13.1 kDa and peak D correspond to β-lactoglobulin having a molecular weight of 18.97 kDa. For peak A and B, no proteins were identified. In according to data reported in literature, we did not find the presence of lysozyme in bovine milk (Figure 1b).

By a cationic-exchange chromatography (MONO S HR 5/5) caseins from bovine milk were separated into 8 peaks named E, F, G, H, I, L, M, N as shown in Figure 2a.

Each peak subjected to 13% SDS-PAGE showed a pattern similar to the one reported in the literature for bovine milk (Rasmussen 1994). By comparing protein migration patterns with those previously published for bovine milk, the chromatographic peaks were identified as follows: peaks E, F were assigned to the β-casein with a molecular weight of 32.56 kDa, the peaks H-I to a mixture of αs1 with a molecular weight of 33.52 kDa and κ-casein with a molecular weight of 26.55 kDa. The peaks L-M

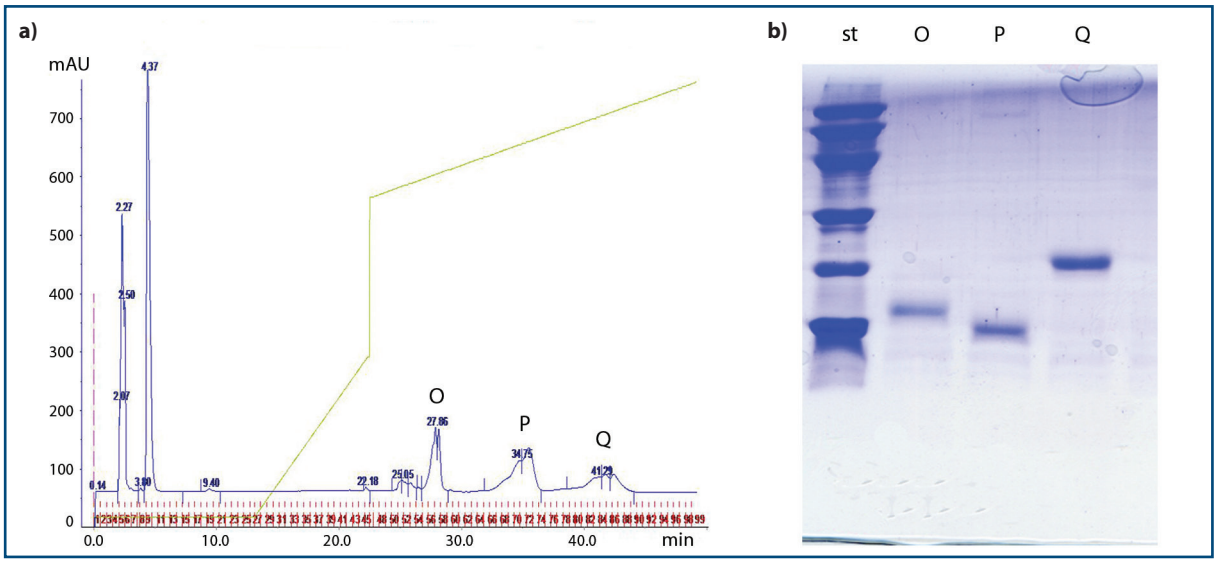

Figure 3. *(a) RP-HPLC of whey proteins from donkey milk. (b) 15% SDS-PAGE of peaks O, P and Q eluted from the reversed phase. St: Bio-Rad low molecular weight standard.*

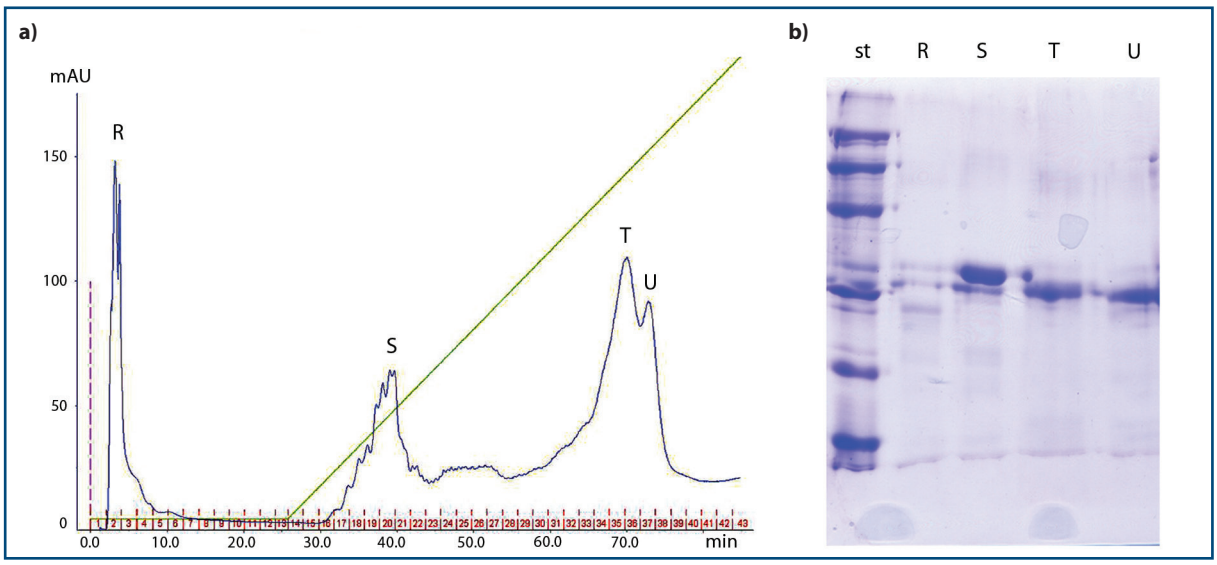

Figure 4. *(a) Cationic-exchange chromatography of casein from donkey's milk. (b) 13% SDSPAGE of the peaks eluted from the MONO S HR 5/5. St: Bio-Rad low molecular weight standard.*

were assigned to a mixture of αs1-, αs2- (molecular weight 32.03 kDa) and κ-casein whereas the peak N to the αs2 casein.

By RP-HPLC, whey proteins from donkey milk were separated into 3 peaks named O, P and Q as shown in Figure 3a, 15% SDS-PAGE analysis (Figure 3b) revealed that the peak O corresponds to lysozyme with a molecular weight of 14.6 kDa, peak P corresponds to α-lactalbumin with a molecular weight of 14.1 kDa and peak Q corresponds to β-lactoglobulin with a molecular weight of 22.4 kDa, as previously reported (Vincenzetti *et al.* 2008).

Caseins from donkey's milk were separated into 4 peaks, named R, S, T and U, by cationic-exchange chromatography (MONO S HR 5/5) as shown in Figure 4a. 13% SDS-PAGE analysis (Figure 4b) identifies mainly αs1 (32.3 kDa) and β-caseins (36.0 kDa): peak S was identified as β-casein, whereas peaks T and U were identified as αs1 casein. Peak R presents a weak band of 29.7 kDa presumably corresponding to an isoforms of αs1 casein (Vincenzetti *et al.* 2008). It was not possible to determine, in this experimental conditions, the presence of other types of caseins, such as αs2-, κ- and γ- , however other authors detected them in donkey's milk but in very small amount (Chianese *et al.* 2010).

Whey proteins from goat's milk were separated into 3 peaks named V, W and X by reversed-phase

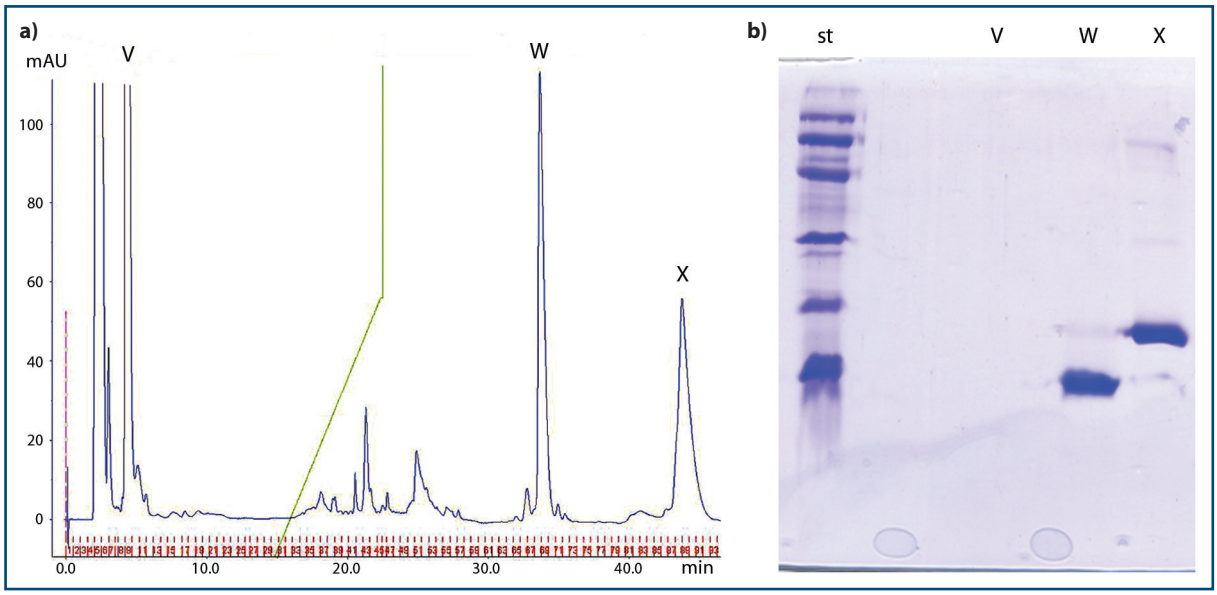

Figure 5. *(a) RP-HPLC of whey proteins from goat's milk. (b) 15% SDS-PAGE of peaks V, W and X.*

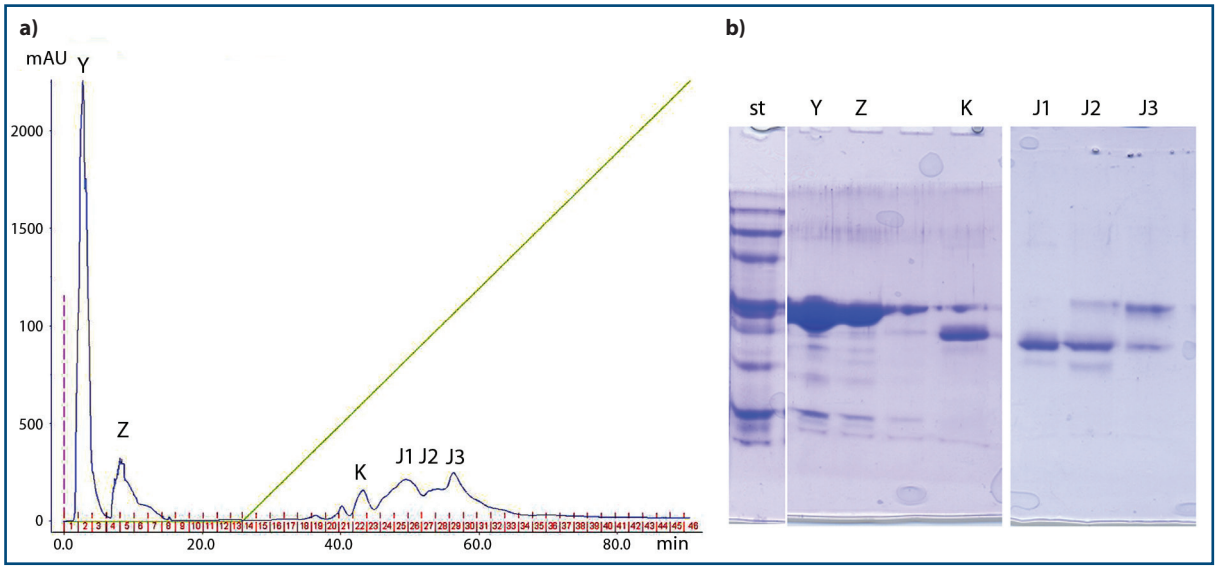

Figure 6. *(a) Cationic-exchange chromatography analysis of casein from goat's milk. (b) 13% SDSPAGE of the eluted peaks. St: Bio-Rad low molecular weight standard.*

chromatography (Figure 5a), 15% SDS-PAGE analysis (Figure 5b) revealed that the peak W corresponds to α-lactalbumin with a molecular weight of 12.7 kDa, peak X corresponds to β-lactoglobulin with a molecular weight of 18.27 kDa. Similarly to bovine milk, lysozyme was not present in goat's milk.

The goat milk's whole casein was separated into 6 peaks, named Y, Z, K, J1, J2 and J3 by cationic-exchange chromatography (MONO S HR 5/5), as shown in Figure 6a, 13% SDS-PAGE analysis on the peaks eluted from the cationic-exchange chromatography, showed a pattern similar to that reported in the literature for goat's milk (Greppi *et al.* 2008). By comparing protein migration patterns with those previously published for goat's milk, the chromatographic peaks were identified as follows (Figure 6b): the peaks Y-Z were assigned to the β-casein (molecular weight of 29.70 kDa), the peaks K-J1 correspond to the κ-casein (molecular weight of 26.42 kDa), the peak J2 resulted to be a mixture of αs2-casein (molecular weight of 32.66 kDa), κ-casein (predominant) and αs1-casein (molecular weight of 23.19 kDa).

The serum of the allergic subject 1A showed a weak cross-reactivity with bovine β-lactoglobulin (Figure 7, lane D), but a strong cross-reactivity was obtained towards the bovine αs1- and αs2- caseins (Figure 8a, lanes H-I, L and M), no cross-reactivity

in correspondence to the β-casein was observed (Figure 8a, lane G). In addition, the 1A subject showed cross-reactivity with goat αs1- and αs2- casein (Figure 8b), as well. No positivity was observed in correspondence of the donkey's milk proteins (Figure 7, lanes O-P-Q and Figure 8c).

The serum of allergic subject 2A did not show cross reactivity with any of the blotted whey proteins, indicating that its allergy is probably only due to casein fraction (data not shown). This subject showed in fact a very strong cross-reactivity towards all bovine casein fractions (Figure 9a), cross-reacted also with the αs2-casein from goat's milk (Figure 9b) but did not show cross-reactivity with the donkey's milk proteins (Figure 9c).

The serum of subject 3A with suspected CMPA did not show any cross-reactivity with any of the blotted whey proteins (data not shown), while a weak cross-reactivity towards the bovine casein fractions was observed, but not towards the goat and the donkey's milk proteins (Figure 10).

Control subjects C1, C2 and C3 did not show cross-reactivity with any of the blotted whey proteins and caseins from bovine, goat, donkey's milk.

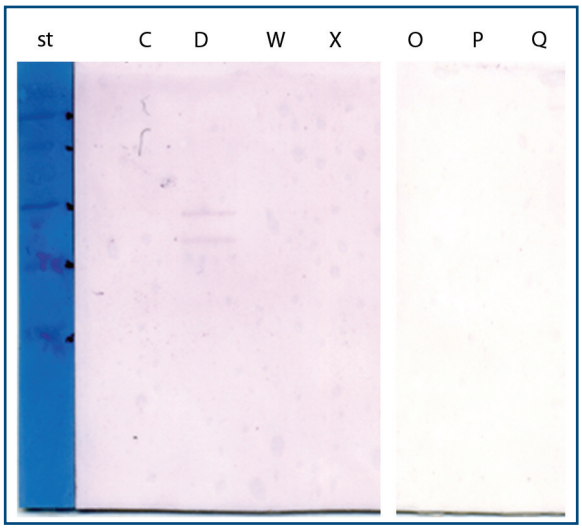

Figure 7. *Immunoblotting analysis of the serum of the subject 1A affected by CMPA towards: α-lactalbumin from bovine (C), goat (W), donkey (P); β-lactoglobulin from bovine (D), goat (X), donkey (Q) and lysozyme from donkey milk (O).*

Conclusions

The primary aim of this work was to verify the renowned hypoallergenic property of donkey's milk, given the common knowledge and the recent evidences of the importance of this milk as substitute of human milk in CMPA infants. .

The immunoblotting was performed with the serum of 6 volunteers, 2 of them (1A and 2A) were affected by CMPA, 1 (3A) was suspected to be affected by CMPA and 3 were healthy controls (C1, C2, C3).

The serum of subject 1A showed a strong cross-reactivity with the bovine caseins, a weak cross-reactivity with the goat caseins, but no reactivity with the donkey caseins. Moreover, a very weak cross-reactivity with 2 isoforms of bovine β-lactoglobulin was determined.

The serum of subject 2A showed a very strong cross-reactivity with the bovine casein fractions, a cross-reactivity (but lesser than bovine) with the

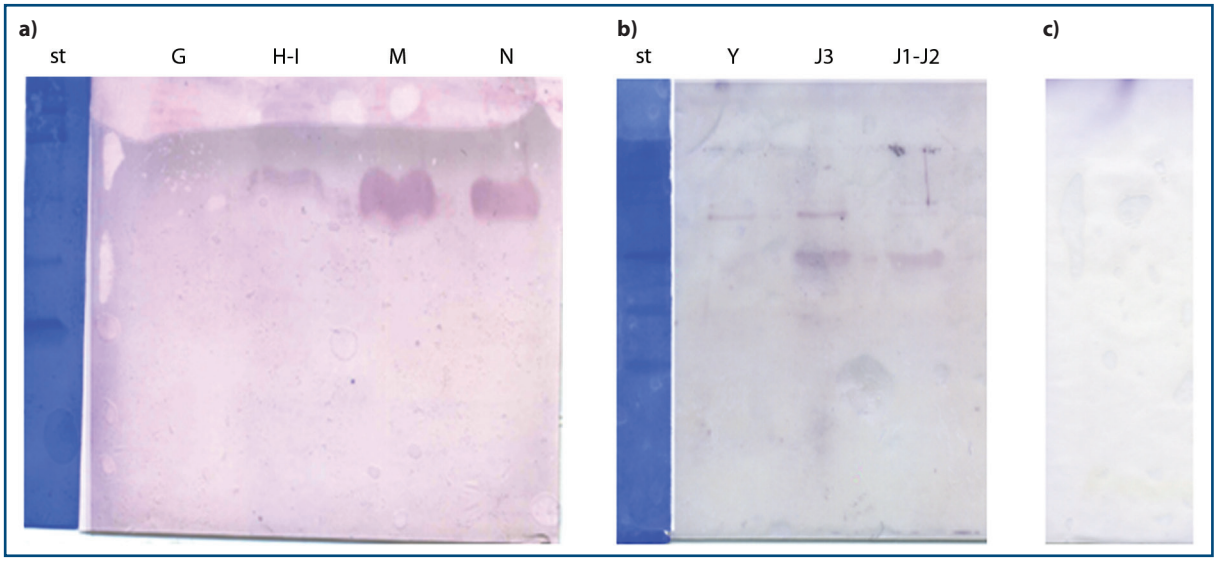

Figure 8. *Immunoblotting analysis of the serum of the subject 1A affected by CMPA towards bovine (a), goat (b) and donkey whole caseins (c).*
Lane G, bovine β-casein; lane H-I, bovine αs1- and κ-casein; lane L-M, bovine αs1 and αs2-caseins; lane Y, goat β-casein; lane J1-2-3, goat αs1 and αs2-caseins.

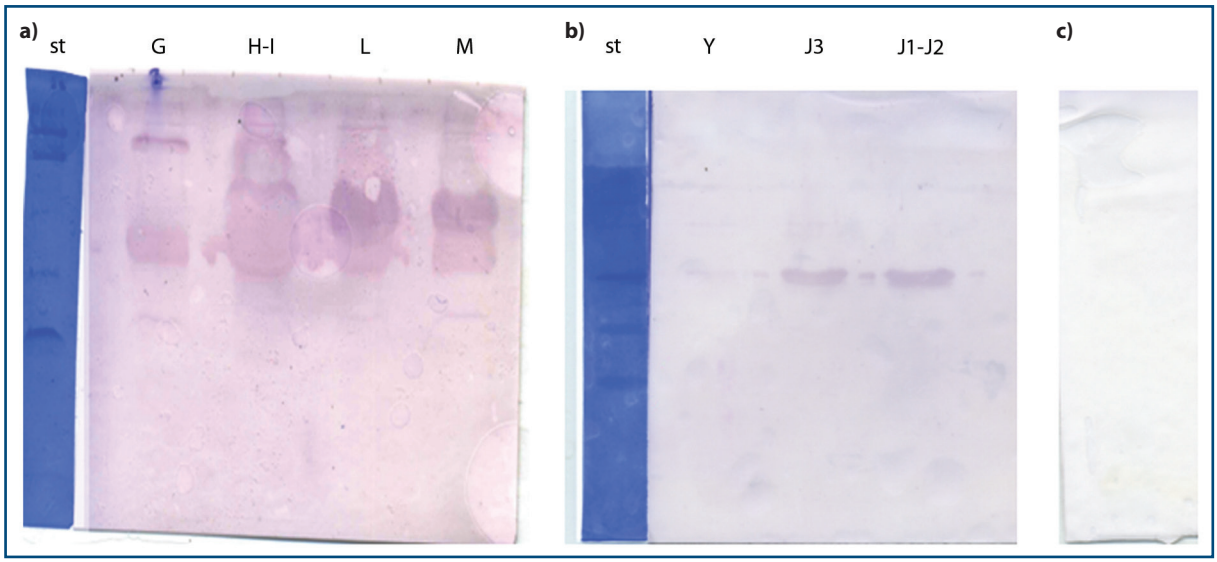

Figure 9. *Immunoblotting analysis of the serum of the subject 2A affected by CMPA towards bovine (a), goat (b) and donkey whole caseins (c).* Lane G, bovine β-casein; lane H-I, bovine αs1- and κ-casein; lane L-M, bovine αs1 and αs2-caseins; lane Y, goat β-casein; lane J1-2-3, goat αs1 and αs2-caseins.

Figure 10. *Immunoblotting analysis of the serum of the subject 3A with suspect CMPA towards bovine, goat and donkey whole caseins.*

goat caseins, and no reactivity with donkey caseins. The serum of A2 did not show cross-reactivity with any of the blotted whey proteins.

The weaker serum cross-reactivity with goat milk proteins and the absence of serum cross-reactivity with donkey milk proteins in 2 of the 3 allergic volunteers included in this study confirm the lower allergenic power of goat and donkey milk proteins compared to those of bovine milk.

The less allergenicity of goat's milk has been ascribed to its lower α-casein content. For this reason goat milk is one of the most frequently suggested alternative to cow milk, although evidence of its tolerability is reported only by few clinical studies (Fiocchi *et al.* 2010). Some studies (Carroccio *et al.* 2000, Vita *et al.* 2007) indicated donkey milk as a valid substitute to cow milk; it has been demonstrated to be more tolerated than cow and goat milk and also than hydrolyzed milks in CMPA patients. In this preliminary study donkey's milk proteins did not show any cross-reactivity with allergic sera, giving an important in vitro proof of its hypoallergenicity if compared to bovine and goat milk. However, due to the limited number of cases examined, further studies are needed to confirm these data.

Considering the results obtained in this study, it would be possible to develop a sensitive diagnostic method for CMPA detection, based on chromatographic and immunoblotting analysis.

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