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Differences in protein fraction from goat and cow milk and their role on cytokine production in children with cow's milk protein allergy

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ABSTRACT

Garganica goat milk was characterized for protein composition and the impact of milk protein mixture, casein and β -lactoglobulin fraction on cytokine production by cultured peripheral blood mononuclear cells from infants with cow's milk protein allergy. Bulk milk samples were collected in a dairy goat farm located in the Gargano area (Southern Italy), cow milk was used as control. Ten patients with cow's milk protein allergy (6 males; mean age 8.4 ± 6.4 months) and ten non-allergic control subjects (6 males; mean age 7.4 ± 3.1 months) were consecutively included in the study. Although the amount of total casein was comparable in cow and goat milk (56.08% vs. 55.33%, respectively) the distribution of principal casein fractions was different according to species. α -CN on total casein was more than 50% both in raw and pasteurized cow milk whereas it was always lower than 40% in goat milk and an opposite trend was observed for β -CN. Production of tumor necrosis factor- α after exposure to goat milk casein and β -lactoglobulin was lower than after exposure to the same fractions from cow milk. Goat milk induced higher levels of regulatory interleukin-10 by peripheral blood mononuclear cells than cow milk. Results on tumor necrosis factor- α evidenced that it is important to test the immune reactivity against each protein fraction before considering goat milk as a safe substitute for feeding infant with cow's milk protein allergy.

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1. Introduction

The Garganica goat is an autochthonous breed of economical weight for Gargano promontory and the neighboring areas of the Southern Italy. Milk protein characteristics, casein genotype and milk casein expression of

the Garganica goat breed were studied by [Albenzio et al. \(2006, 2009\)](#).

The role of goat milk in feeding children with cow's milk protein allergy (CMPA) is controversial: a study suggested that goat milk of particular α_{s1} -CN genotypes can be used in the preparation of modified formulas for selected groups of allergic patients ([Ballabio et al., 2011](#)); other studies showed that goat milk should be as allergic as cow milk ([Restani et al., 2002](#)). Genetic polymorphisms of goat milk protein influence the presence and level of synthesis of each protein fraction in milk playing thus important role in eliciting different degrees of allergic reaction ([Park, 1994](#)). Low levels of α_{s1} -CN in goat milk means that its casein

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profile is closer to human milk than that of cow milk (Clark and Sherbon, 2000) as human milk lacks α_{s1} -CN (Silanikove et al., 2010). Indeed, milk from goats having mild alleles can be employed to produce milk for allergic subjects (Roncada et al., 2002). Due to high frequency of weak and null alleles for α_{s1} -CN locus, and to high frequency of A0 genotype at α_{s2} -CN locus in Garganica breed population, Albenzio et al. (2009) suggested that such milk could be exploited for feeding CMPA subjects.

In this study Garganica goat milk was tested for (i) the effect of heat treatment on protein composition and (ii) the impact of milk, casein and β -lactoglobulin (β -Lg) on cytokine production by cultured peripheral blood mononuclear cells (PBMCs) from infants with cow's milk protein allergy. In this study bovine milk was used as control.

2. Materials and methods

Bulk milk samples were collected from 200 lactating Garganica goats in a dairy goat farm located in the Gargano area (Southern Italy) three times over one week. Bulk milk from Friesian cow, reared in a dairy farm located in province of Foggia (Italy), was used in this study as control milk. Bulk milk samples from cow and goat were analyzed for chemical composition using an infra-red Spectrophotometer (Milko Scan 133B; Foss Electric, Hillerød, Denmark). Mean values ($\pm$ SEM) of protein and casein were 3.35 ± 0.05 and 2.61 ± 0.04 , 3.86 ± 0.06 and 2.95 ± 0.04 , in cow and goat milk, respectively. Defatted milk was pasteurized (63°C , 30 min) and then precipitation with sodium acetate and acetic acid (10%, w/v) was performed to obtain casein and whey protein fractions. β -Lg fraction was obtained according to the method proposed by Aschaffenburg and Drewry (1957). Raw and pasteurized cow and goat milk were analyzed by SDS-PAGE (Laemmli, 1970) using a Protean II xi vertical slab gel unit (Bio-Rad, Watford, UK). The destained gels were acquired by means of a Gel Doc EQ™ system using a white light conversion screen and analyzed with the Quantity One software (Bio-Rad, Watford, UK) to determine the signal intensity (Optical Density) of the defined bands. The bands detected by densitometry were visually identified as previously determined by Park and Jin (1998), Park (2000) and Santillo et al. (2009). Lactoferrin, serum albumin, immunoglobulin were grouped to minor whey proteins, and peptides deriving from α - and β -CNs hydrolysis were grouped as casein degradation products. Given 100% the sum of the intensity of the defined bands in a lane, the relative quantity of each band was determined as the percentage of the signal intensity of the defined band in a lane.

Ten patients with CMPA (6 males; mean age 8.4 ± 6.4 months) and ten non-allergic control subjects (6 males; mean age 7.4 ± 3.1 months) recruited at the University Clinic of Pediatrics and Pediatric Research Center of Foggia were consecutively included in the study. All CMPA subjects had various clinical manifestations suggestive of CMPA, including gastrointestinal, skin, and respiratory symptoms (Vandenplas et al., 2007). The diagnosis of CMPA was based on a successful cow's milk avoidance diet, with clinical relapse after re-exposure to cow's milk proteins (Berni Canani et al., 2008). Metabolic disorders, anatomical abnormalities, coeliac disease and other enteropathies, pancreatic insufficiency, non immunological adverse reactions to food, allergic reactions to other food allergens or other substances were excluded.

Informed consent for inclusion in the study was obtained from parents. The study followed the requirements of the International Conference on Harmonisation Good Clinical Practice, respected the principles outlined by the Declaration of Helsinki on the Ethical Principles for Medical Research Involving Human Subjects, and it was approved by the Ethical Committee for Pediatric Studies of the Medical School of the University of Foggia.

Production of inflammatory and regulatory cytokines tumor necrosis factor- α (TNF- α), interleukin-10 (IL-10) and interleukin-12p70 (IL-12p70) has been studied in cow's and goat's milk proteins stimulated PBMCs from CMPA patients and controls, due to their recognized major role as key regulators of allergic diseases. PBMCs were obtained from children with CMPA and from controls. PBMCs were isolated from heparinized blood (3–5 ml) by gradient centrifugation in Ficoll-Hypopaque (Sigma). PBMC were suspended in culture medium consisting in RPMI 1640 supplemented with glutamine, sodium pyruvate, nonessential amino acids,

Hepes buffer, penicillin/streptomycin, and 10% FCS (Sigma). PBMCs were distributed at a concentration of 1.5×10^5 cells per well in 0.2 ml of calcium medium into flat-bottomed 96-well microtiter plates, cultivated for 5 days at 37°C in a 5% CO_2 incubator in presence of the following milk allergenic proteins: milk protein mixture (casein: whey protein, 80:20, w/w), casein fractions mixture, and β -Lg from cow and goat milk. Concentration of the tested proteins used for stimulation was 100 $\mu\text{g/ml}$. Phytohemagglutinin (10 $\mu\text{g/ml}$) was used as positive control stimuli to ensure normal immune reactivity to control stimuli in the subjects. Medium RPMI was used as negative control. At the end of the incubation period, triplicate culture supernatants were harvested and stored at -20°C until used in cytokine assays. IL-10, IL-12 p70 and TNF- α levels in the culture supernatants were determined in duplicate using commercial ELISA kits (R&D Systems, Minneapolis, MN); results were expressed in pg/ml. The intra and inter assay coefficients of variation were 3.5% and 4.2%, respectively. As expected cytokines level in control patients did not evidence differences both for milk species and protein fractions; mean values of cow and goat milk were 391 ± 80 , 132 ± 20 , 45 ± 5 for TNF- α , IL-10, and IL-12p70, respectively.

All the variables were tested for normal distribution using the Shapiro–Wilk test (Shapiro and Wilk, 1965). Data on milk chemical composition, protein profile and cytokine were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2011). The effects of milk source, milk treatment and their interaction were tested for protein profile; the effects of species, milk protein fraction and their interaction were tested for cytokine levels. When significant effects were found (at $P < 0.05$), the Student *t*-test was used to locate significant differences between means.

3. Results and discussion

Previous research on Garganica goat's milk evidenced a major complexity for protein profile which was associated with high genetic polymorphism (Albenzio et al., 2009). Such characteristics could be involved in hypoallergenic features of Garganica goat's milk thus it is worth testing milk protein fractions in clinical studies with CMPA infants.

Relative percentage of milk protein fractions in raw and pasteurized milk from cow and Garganica goat was reported in Table 1. Species affected the percentage of α - and β -CNs whereas pasteurization affected caseins and minor whey proteins and casein degradation products. Although the amount of total casein was comparable in cow and goat milk (56.08% vs. 55.33%, respectively) the distribution of principal casein fractions was different according to species. α -CN on total casein was about 50% both in raw and pasteurized cow milk whereas it was always lower than 40% in goat milk; an opposite trend was observed for β -casein on total casein.

In this study milk protein fractions exhibited a different distribution after heat treatment suggesting a possible modification in the chemical structure and interactions among protein fractions. Pasteurization reduced the relative percentage of minor whey proteins probably as a consequence of heating denaturation due to coagulation induced by heating. Indeed, proteins differ markedly in their resistance to heat treatment, bovine serum albumin (BSA) being the most labile (Bahana and Gandhi, 1983). Moreover minor whey protein fraction interacts with caseins in particular with β -casein fraction as reported by Creamer and Sawyer (2003). This partly explains the increase of the intensity of the band ascribed to β -casein both in pasteurized cow and goat milk; also percentage of casein degradation products was changed by heat treatment.

Table 1Relative percentages ($\pm$ SEM) of milk protein fractions in raw and pasteurized milk from cow's and goat's.

Parameters	Treatment	Species		SEM	Effects, <i>P</i>		
		Cow	Goat		Species	Treatment	Interaction
Minor whey protein	Raw	14.26	13.13	0.6	NS	**	NS
	Pasteurized	10.44	10.05				
α -CNs	Raw	26.38	21.18	0.5	***	**	**
	Pasteurized	26.53	19.18				
β -CNs	Raw	19.56	23.97	1.3	**	**	**
	Pasteurized	26.85	30.25				
κ -CNs	Raw	10.14	10.18	0.25	NS	NS	NS
	Pasteurized	5.55	9.85				
Caseins degradation products	Raw	5.95	6.42	0.35	NS	**	NS
	Pasteurized	4.2	4.76				
β -Lactoglobulin	Raw	21.65	21.63	2.01	NS	NS	NS
	Pasteurized	19.44	17.37				
α -Lactalbumin	Raw	6.08	4.09	0.8	NS	NS	NS
	Pasteurized	6.34	5.26				

NS, not significant.

** $P < 0.01$.*** $P < 0.001$.**Table 2**

Cytokine levels (pg/ml) in cultured supernatants of lymphocytes of children stimulated against protein fractions of cow's and goat's milk.

	Species	Milk protein fraction			SEM	Effects, <i>P</i>		
		Protein mixture ^a	Casein	β -Lg		Species	Protein fraction	Interaction
TNF- α	Cow	833.67A	1362.38bA	2401.81bB	220	*	*	NS
	Goat	651.02	380.37a	366.45a				
IL-10	Cow	236.82aA	482.17B	242.49A	70	*	***	**
	Goat	460.94bB	448.81AB	441.94AB				
IL-12	Cow	164.66	106.64	210.21	52	NS	NS	NS
	Goat	130.61	74.05	173.3				

Mean followed by different letters differ for $P < 0.05$: a and b for species effect; A and B for milk fraction effect. NS, not significant.^a Protein mixture = casein: whey protein (80:20 w/w).* $P < 0.05$.** $P < 0.01$.*** $P < 0.001$.

Cytokine levels in cultured supernatants of lymphocytes of children stimulated against milk protein mixture, casein fraction, and β -Lg fraction of cow and goat pasteurized milk were reported in Table 2. PBMC produced significantly higher TNF- α levels after exposure to cow milk casein ($P < 0.05$) and β -Lg ($P < 0.001$) than after exposure to the same fractions from goat milk. TNF- α is one of the mediators involved in adverse reactions to cow milk proteins as gastrointestinal manifestations, and respiratory and cutaneous symptoms (Motrich et al., 2003). Although total casein percentage was not different among species higher level of TNF- α induced by cow milk casein fraction may be ascribed to different relative distribution of α - and β -CNs fraction. Indeed, caseins and especially α -CN are among the most important milk allergens (Restani et al., 2009; Ballabio et al., 2011). Secretion of IL-10 after PBMC stimulation was influenced by milk protein source being higher in goat's than cow's milk. Within cow milk protein fractions, casein induced higher levels of regulatory cytokine than β -Lg and milk protein mixture. IL-10 is one of the major cytokines produced by regulatory T-cells and

exerts inhibitory actions on monocytes and T-cells, partly suppressing the formation of pro-inflammatory cytokines like TNF- α in T-cells and monocytes (Reuss et al., 2002). Tiemessen et al. (2003) investigated the role of IL-10 in T-cells reactivity of children with CMPA suggesting that activated allergen-specific T-cells might contribute to an active form of immune suppression *in vivo* through the production of IL-10 and thereby prevent aberrant reactions towards antigens such as cow's milk. The production of IL-12 is of particular interest for its role in shaping balanced T_H1 – T_H2 responses therefore playing a critical role in the development of food allergy (Temblay et al., 2007). No differences between species and milk protein fractions were detected in levels of IL-12p70 cytokine.

In conclusion, cow and goat milk exhibited a different protein composition in particular as regard casein fractions with α -CN showing lower levels in goat's than in cow's milk. Different casein profile was associated with different TNF- α levels produced by PBMC; this cytokine displayed a lower level after stimulation with casein fractions isolated from goat than from cow milk in children with CMPA.

Results on TNF- α evidenced that it is important to test the immune reactivity against each protein fraction before considering goat's milk as a safe substitute for feeding infant with CMPA. More studies are needed to better evaluate the role of goat's milk in the therapeutic approach of children with CMPA.

References

- Albenzio, M., Caroprese, M., Marino, R., Muscio, A., Santillo, A., Sevi, A., 2006. Characteristics of Garganica goat milk and Caciocotta cheese. *Small Rum. Res.* 64, 35–44.
- Albenzio, M., Santillo, A., d'Angelo, F., Sevi, A., 2009. Focusing on casein gene cluster and protein profile in Garganica goat milk. *J. Dairy Res.* 76, 83–89.
- Aschaffenburg, R., Drewry, J., 1957. Improved method for the preparation of crystalline β -lactoglobulin and α -lactalbumin from cow's milk. *Biochem. J.* 65, 273–277.
- Bahana, S.L., Gandhi, M.D., 1983. Milk hypersensitivity II. Practical aspects of diagnosis, treatment and prevention. *Ann. Allergy* 50, 295–301.
- Ballabio, C., Chessa, S., Rignanese, D., Gigliotti, C., Pagnacco, G., Terracciano, L., Fiocchi, A., Restani, P., Caroli, A., 2011. Goat milk allergenicity as a function of α s1-casein polymorphism. *J. Dairy Sci.* 2, 998–1004.
- Berni Canani, R., Ruotolo, S., Discepolo, V., Troncone, R., 2008. The diagnosis of food allergy in children. *Curr. Opin. Pediatr.* 20, 584–589.
- Clark, S., Sherbon, J.W., 2000. Alpha s1-casein, milk composition and coagulation properties of goat milk. *Small Rum. Res.* 38, 123–134.
- Creamer, L.K., Sawyer, L., 2003. Beta-lactoglobulin. In: Roginski, H., Fuquay, J.W., Fox, P.F. (Eds.), *Encyclopedia of Dairy Sciences*, 3. MPG Books Ltd., Bodmin, Cornwall, UK, pp. 1932–1939.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 15 (227), 680–685.
- Motrich, R.D., Gottero, C., Rezzonico, C., Rezzonico, C., Riera, C.M., Rivero, V., 2003. Cow's milk stimulated lymphocyte proliferation and TNF alpha secretion in hypersensitivity to cow's milk protein. *Clin. Immunol.* 109, 203–211.
- Park, Y.W., 1994. Hypo-allergenic and therapeutic significance of goat milk. *Small Rum. Res.* 14, 151–159.
- Park, Y.W., 2000. Proteolysis and lipolysis of goat milk cheese. *J. Dairy Sci.* 84E (Suppl.), E84–E92.
- Park, Y.W., Jin, Y.K., 1998. Proteolytic patterns of Caciotta and Monterey Jack hard goat milk cheeses as evaluated by SDS- PAGE and densitometric analyses. *Small Rum. Res.* 28, 263–272.
- Restani, P., Ballabio, C., Di Lorenzo, C., Tripodi, S., Fiocchi, A., 2009. Molecular aspects of milk allergens and their role in clinical events. *Anal. Bioanal. Chem.* 395, 47–56.
- Restani, P., Beretta, A., Fiocchi, C., Ballabio, C., Galli, L., 2002. Cross-reactivity between mammalian proteins. *Ann. Allergy Asthma Immunol.* 89, 11–15.
- Reuss, E., Fimmers, R., Kruger, A., Becker, C., Rittner, C., Höhler, T., 2002. Differential regulation of interleukin-10 production by genetic and environmental factors—a twin study. *Genes Immun.* 3, 407–413.
- Roncada, P., Gaviraghi, A., Liberatori, S., Canas, B., Bini, L., Greppi, G.F., 2002. Identification of caseins in goat milk. *Proteomics* 2, 723–726.
- Santillo, A., Kelly, A.L., Palermo, C., Sevi, A., Albenzio, M., 2009. Role of indigenous enzymes in proteolysis of casein in caprine milk. *Int. Dairy J.* 19, 655–660.
- S.A.S Institute, 2011. *SAS User's Guide: Statistics*. Version 9.2 ed. SAS Inst. Inc., Cary, NC.
- Shapiro, S.S., Wilk, M., 1965. An analysis of variance test for normality. *Biometrika* 52, 591–601.
- Silanikove, N., Leitner, G., Merin, U., Prosser, C.G., 2010. Recent advances in exploiting goat's milk: quality, safety and production aspects. *Small Rum. Res.* 89, 110–124.
- Temblay, J.N., Bertelli, E., Arques, J.L., Regoli, M., Nicoletti, C., 2007. Production of IL-12 by Peyer patch-dendritic cells is critical for the resistance to food allergy. *J. Allergy Clin. Immunol.* 120, 659–665.
- Tiemessen, M.M., van Ieperen-Van Dijk, A.G., Bruijnzeel-Koomen, C.A.F.M., Garssen, J., Knol, E.F.K., Van Hoffen, E.V., 2003. Cow's milk-specific T-cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. *J. Allergy Immun.* 5, 932–939.
- Vandenplas, Y., Brueton, M., Dupont, C., Hill, D., Isolauri, E., Koletzko, S., Oranje, A.P., Staiano, A., 2007. Guidelines for the diagnosis and management of cow's milk protein allergy in infants. *Arch. Dis. Child.* 92, 902–908.