

Donkey and Goat Milk Intake and Modulation of the Human Aged Immune Response

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Abstract: In a group of 14 healthy aged subjects, donkey and goat milk was administered respectively, for a period of one month. Cytokine profile [interleukin (IL)-12, IL-10, IL-1 β , IL-8, IL-6 and Tumor Necrosis Factor (TNF)- α] was assessed before and after milk intake by means of a cytometric bead array test.

Data demonstrated that IL-12 was undetectable, while IL-10, IL-1 β and TNF- α were released in very low amounts.

Quite interestingly, IL-8 was increased by donkey milk administration, while same cytokine was dramatically decreased following goat milk intake. Same pattern of response was noted with IL-6 even if levels of these cytokine were lower than those detectable in the case of IL-8.

Taken together, these findings indicate that administration of donkey milk in the aged host is able to upregulate the immune response, while goat milk seems to reduce the exaggerated acute phase response in elderly.

Keywords: Donkey milk, goat milk, cytokine, immunosenescence, elderly.

INTRODUCTION

Immunosenescence is characterized by a progressive decline of immune functions with age and both innate and adaptive immune responses are severely impaired [1-8]. This fact seems to account for the increased susceptibility of old people to infections, tumors and autoimmune diseases [7,8]. In particular, immune dysfunction of T cells in elderly seems to be more severe than that of other cells, and Henson and associates [9] have reported that receptors, such as CTLA-4 and PD-1, generate negative signals for T cell activation, thus leading to tumor survival and establishment of chronic infections in senescence.

Nutrition seems to be fundamental in the mechanism of immune recovery in the elderly and, for instance, Zinc (Zn) supplementation in Zn-deprived mice could normalize the otherwise subverted immune responsiveness as well as the thymic architecture [10]. In this respect, there is evidence that in aged people Zn-deficiency correlates with a poor immune response [10]. On the other hand, vitamin E administration to aged subjects led to an increase of interleukin (IL)-2 production with a reduction of prostaglandin E2 level and lipid peroxidation [11]. Furthermore, β -carotene supplementation could increase Natural Killer (NK) cell function in the elderly [11].

On the other hand, several clinical studies conducted in senescent individuals have clarified the beneficial effects of lactobacillus administration either in terms of immune recovery or less frequency of infectious episodes. In particular, the normalization of intestinal microflora by probiotics could counteract the main alterations of immunosenescence such as: 1. Shrinkage of the cell repertoire and accumulation of oligoclonal expansions of memory/effector cells directed toward ubiquitous infectious agents; 2. Involution of the thymus and the exhaustion of naïve T cells; 3. Chronic inflammatory status [12].

Quite interestingly, Guigor and associates [13] have pointed out that an altered host-microbiotic interaction at intestinal level is able

to maintain a low systemic inflammatory status in the elderly and therefore, prebiotics, probiotic or symbiotics might be useful as nutritional targets at the mucosal level.

In general terms, attempts have been made at defining immunological risk phenotypes in aged individuals and proinflammatory phenotypes seem to be predictive of an unsuccessful ageing [14]. Therefore, a nutritional regimen which arrests the inflammatory process in ageing should be pursued. On these bases, the immune protection conferred by milk to human being and, especially, to immunocompromised hosts should be taken into serious consideration.

It is well known that human milk possesses several components of immunological pertinence such as secretory s-IgA antibodies, Interferon- γ , Lactoferrin, Lysozyme, Fibronectin, and various Hormones and Growth Factors which exert defensive mechanisms [15].

In this respect, donkey milk represents the best substitute of human milk mostly for its content in lactose (equal to that of human milk) [16].

A series of reports has emphasized the beneficial effects of donkey milk in the immunocompromised host for the inhibition of bacterial activity and prevention of atherosclerosis because of its antioxidant properties [17,18].

In a recent report, we have evaluated the *in vitro* effects of colostrum and milk from donkeys on human healthy peripheral blood mononuclear cells [19]. Colostrum induced secretion of s-IgA, while milk prevalently promoted production of IgG. On the other hand, release of regulatory, pro-inflammatory and anti-inflammatory cytokines was confined to milk only. Quite interestingly, both milk and colostrum were able to induce generation of nitric oxide, more pronounced in the case of milk. Taken together, these data further support the use of donkey milk in the treatment of human diseases, even including prevention of atherosclerosis.

Here, 14 aged healthy subjects have been administered with donkey milk for one month and compared to another group administered with goat milk. The effects of both milks on the serum cytokine profile will be discussed.

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SUBJECTS AND METHODS

Aged Subjects

A total of 14 aged subjects (9 females and 5 males) ranging from 72 to 97 years were recruited from the Geriatric Center "Mamma Rosa", Turi (Italy). Before blood sampling, each subject was clinically evaluated in order to exclude concurrent diseases or drug assumption which might have affected the immune responsiveness. Therefore, on the basis of these criteria, only healthy aged people were included in this study, after obtaining an informed consent.

Normal Donors

Ten healthy blood donor sera as controls were collected and stored at -80°C.

Source of Milk

Donkey milk was obtained from Azienda Agricola "Di Masi", Alberobello (Italy). Goat milk was a commercial preparation.

Milk Administration Regimen

All subjects were administered with 1 milk cup (200 ml/day) for one month.

In a double blind trial, donkey milk was administered to 6 elderly (all females), while goat milk was administered to 8 elderly (5 males and 3 females).

Determination of Serum Cytokines

Blood for serum samples was collected in vacutainers and allowed to clot. The serum was separated by centrifugation, divided into aliquots, frozen and stored at -80°C until withdrawn for analysis.

Cytokine assay: Determination of cytokines was performed in serum of aged subjects, before (time T0) and after administration (T1) of milk, using innovative methods such as the multiplexing assay in cytofluorometry: Cytometric Bead Array (CBA) (Beckton Dickinson Biosciences, San Jose, CA, USA). We used the "CBA Human Inflammation kit" to assay IL-12, tumor necrosis factor alpha (TNF)- α , IL-10, IL-6, IL-1 β and IL-8.

CBA allows the determination of six different cytokines simultaneously in the same sample of serum. The principle of the method is the sandwich immunofluorescence (like ELISA). Therefore six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-1 β , IL-6, IL-10 and TNF- α proteins. The six bead populations were mixed together, and were resolved in a red channel (FL3) of a flow cytometer. The amount of cytokines linked to beads was determined later, through incubation with a mixture of antibodies conjugated with Phycoerythrin (PE) against the same cytokines but without competing with the bead's antibodies for the same site. So, every bead was separated on FL3, according to its specificity, and on FL2 according to the amount of cytokine and that was correlated to the concentration of cytokines in the sample.

Ten μ l/test of each bead suspension were mixed and washed by centrifugation. After, mixed beads were resuspended in appropriate buffer and incubated for 30 min at room temperature (RT) and protected from light. Fifty μ l of mixed beads were transferred to each assay tube. Lyophilized standards of Human Cytokine were reconstituted in appropriate diluent and incubated for 15 min and, then, diluted by serial dilutions using the appropriate diluent. Standard dilutions and samples were added to the appropriate sample tubes (50 μ l/tube).

The beads and the cytokines, in standard or in serum, were incubated for 1.5 h at RT protected from light. After, samples were washed with 1ml of wash buffer, centrifuged and added with PE-detection Antibodies (50 μ l/test).

So, the beads linked to cytokines, in standard or in serum and the PE-conjugated anti-human cytokines were incubated for 1.5 hour at RT protected from light and they formed a ternary complex (bead-Antibodies-Cytokine-Antibodies)

After, samples were washed with 1 ml of Wash Buffer, centrifuged and 300 μ l of Wash Buffer were added to each assay tube to analyze samples.

Flow cytometer "FACSCalibur" (Becton Dickinson), "CellQuest Software" program and "CBA Analysis Software" were used.

Statistical Evaluation

Statistical analyses were performed using the GraphPad Prism release 5.0 for Windows Vista. Paired t-test was used for comparison between the individuals before and after intake milk group. Statistical significance was set at $p < 0.05$.

RESULTS

Serum cytokine evaluation was performed in all 14 aged subjects, and no side effects were recorded.

In Table 1 normal values of cytokines in the younger counterpart are shown.

Table 1. Normal Values in Serum of 10 Healthy Young People

Cytokine	Range (pg/ml)
IL-8	1.5 – 6.0
IL-1 β	0.0 – 265.0
IL-6	0.0 – 9.5
IL-10	0.0 – 9.0
TNF- α	0.0 – 7.0
IL-12	0.0 – 28.0

Effects of Donkey Milk

As indicated in Fig. (1), serum levels of IL-12 were undetectable either before or after administration with donkey milk.

Conversely, basal levels of IL-10 were present within a normal range (2.2 pg/ml), but milk administration did not lead to a remarkable increase (2.3 pg/ml), as illustrated in Fig. (2).

A very dramatic increase of IL-8 (2081.7 pg/ml) was observed after donkey milk administration, as expressed in Fig. (3). Of note, baseline values of this cytokine (352.3 pg/ml) were *per se* comparable to the young counterpart.

Also in the case of IL-6 T0 values were moderately high (6.7 pg/ml) and increased (23.0 pg/ml) following milk administration Fig. (4).

Quite interestingly, both IL-1 β Fig. (5) and TNF- α Fig. (6) serum amounts were not detected at T0, while slightly increased after milk administration (IL-1 β = 5.0 pg/ml; TNF- α = 1.0 pg/ml), thus ranging within normal ranges.

Effects of Goat Milk

In aged individuals treated with goat milk the following findings were observed at the end of the trial.

IL-12 serum concentration slightly decreased at T1 (0 vs 0.23 pg/ml) Fig. (7).

On the other hand, IL-10 values were higher than those of IL-12 and no significant modifications were determined at T1 (2.4 vs 2.6 pg/ml) Fig. (8).

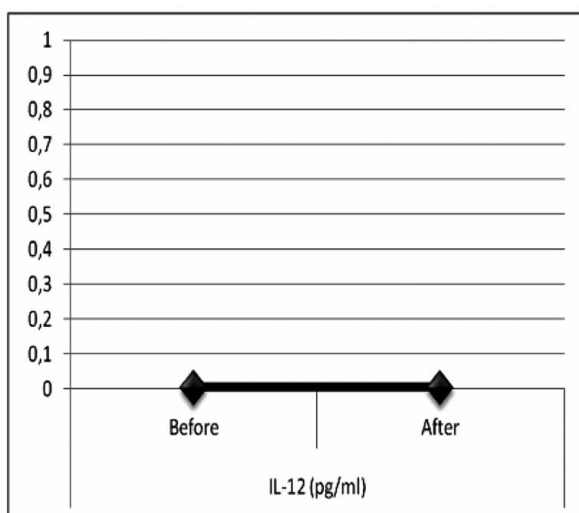


Fig. (1). Evaluation of serum IL-12 before and after donkey milk administration. Cytokine levels were evaluated by the Cytometric bead array (CBA) inflammation kit.

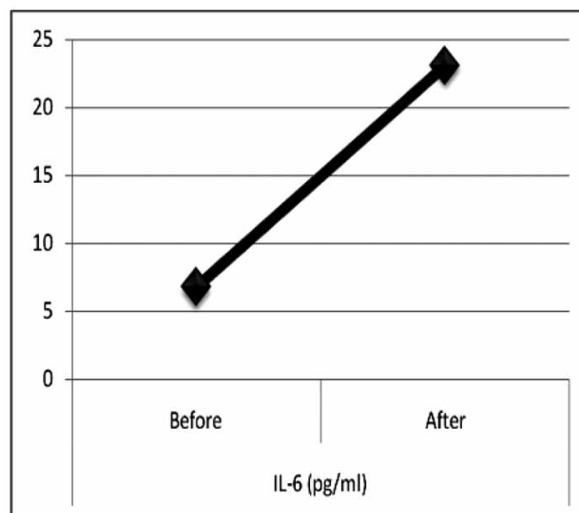


Fig. (4). Evaluation of serum IL-6 before and after donkey milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

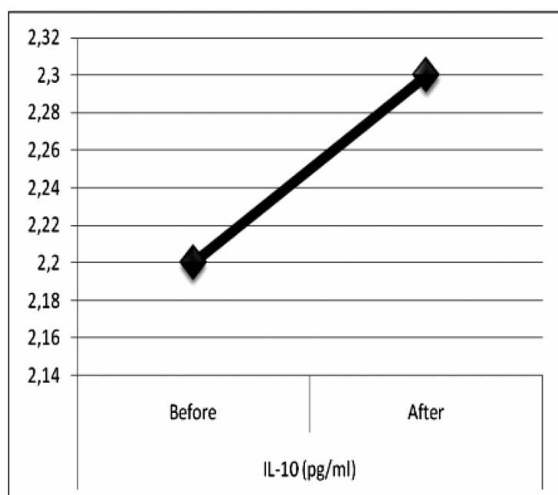


Fig. (2). Evaluation of serum IL-10 before and after donkey milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

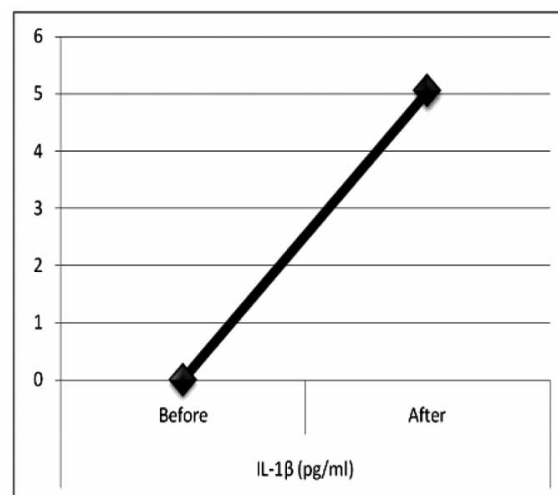


Fig. (5). Evaluation of serum IL-1β before and after donkey milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

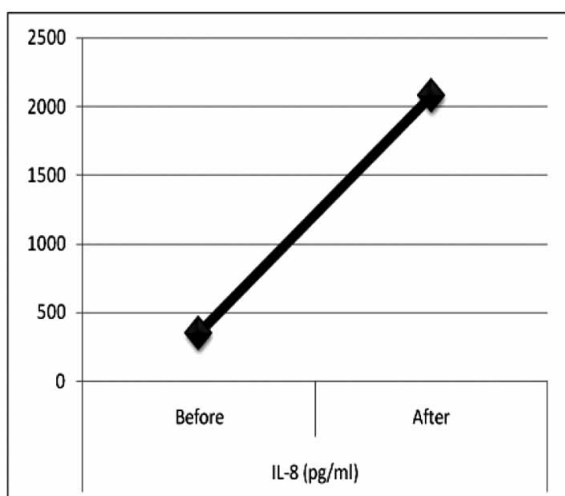


Fig. (3). Evaluation of serum IL-8 before and after donkey milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

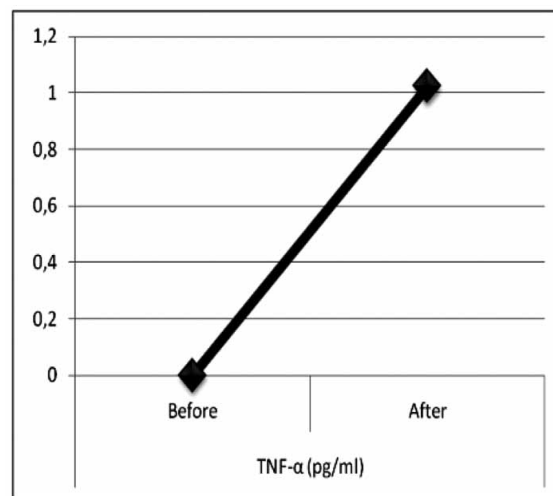


Fig. (6). Evaluation of serum TNF-α before and after donkey milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

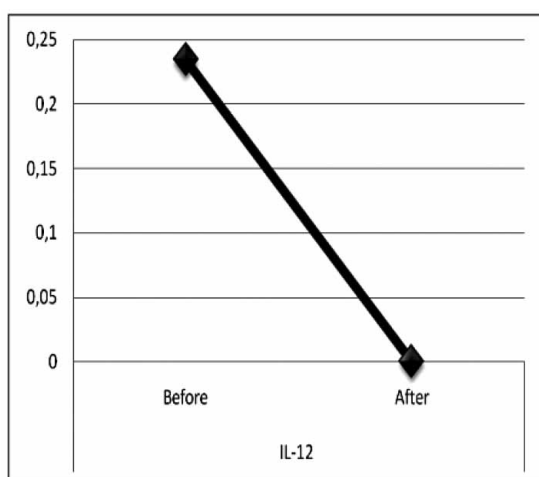


Fig. (7). Evaluation of serum IL-12 before and after goat milk administration. Cytokine levels were evaluated by the Cytometric bead array (CBA) inflammation kit

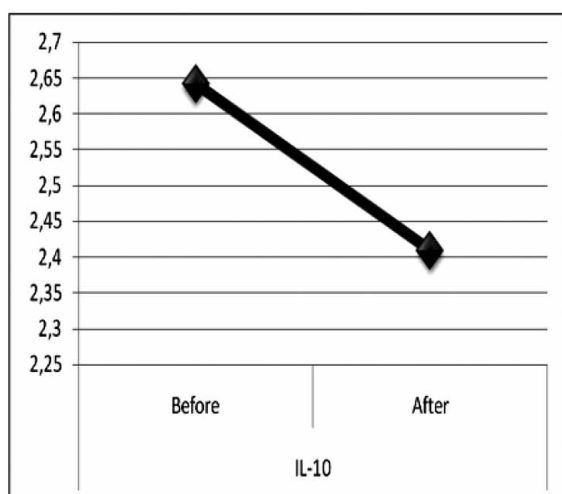


Fig. (8). Evaluation of serum IL-10 before and after goat milk administration. Cytokine levels were evaluated by the CBA inflammation kit

IL-8 concentration was higher than normal values at T0 (2991.3 pg/ml) and dramatically dropped at T1 (103.8 pg/ml) Fig. (9). The same was true in the case of IL-6 [22.4 pg/ml (T0) vs 9.5 pg/ml (T1)] even if values were falling within normal ranges Fig. (10).

A trend to a decrease was noted in the case of TNF- α Fig. (11): [0.32 pg/ml (T0); 0.23 pg/ml (T1)], being values in the low range of normality.

Same pattern of response was also evident in the case of IL-1 β Fig. (12): [3.5 pg/ml (T0) vs 0 pg/ml (T1)].

DISCUSSION

It is clear-cut that in our group of aged individuals, despite the fact that they are healthy, certain basic immune alterations are present. The first observation is that IL-12 serum concentration is very low or not detectable, thus indicating a consequential impairment of the Th1 response [5]. On the contrary, IL-10 levels are present even if in a relatively low amount, thus, indicating a potential of regulatory effect on the inflammatory arm of the immune response.

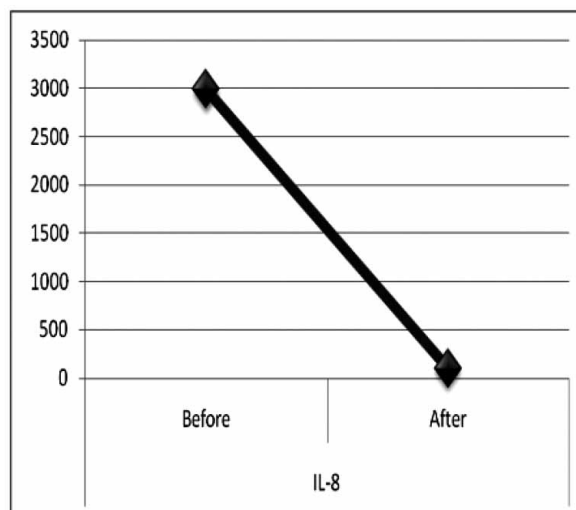


Fig. (9). Evaluation of serum IL-8 before and after goat milk administration. Cytokine levels were evaluated by the CBA inflammation kit.

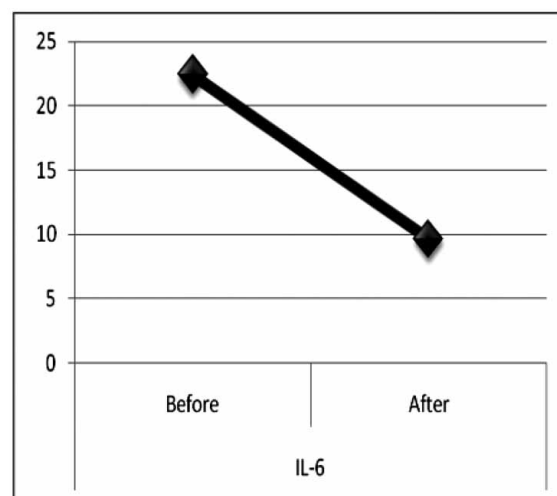


Fig. (10). Evaluation of serum IL-6 before and after goat milk administration. Cytokine levels were evaluated by the CBA inflammation kit

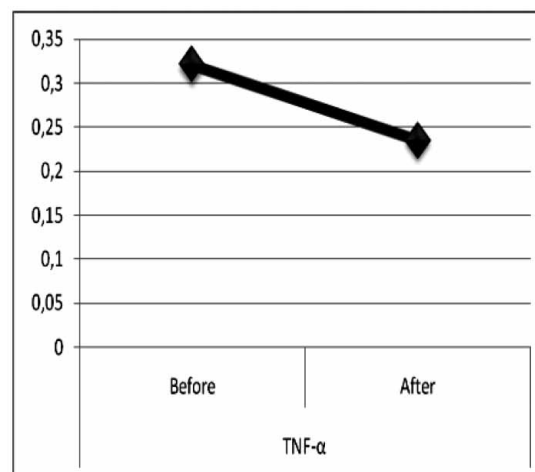


Fig. (11). Evaluation of serum TNF- α before and after goat milk administration. Cytokine levels were evaluated by the CBA inflammation kit

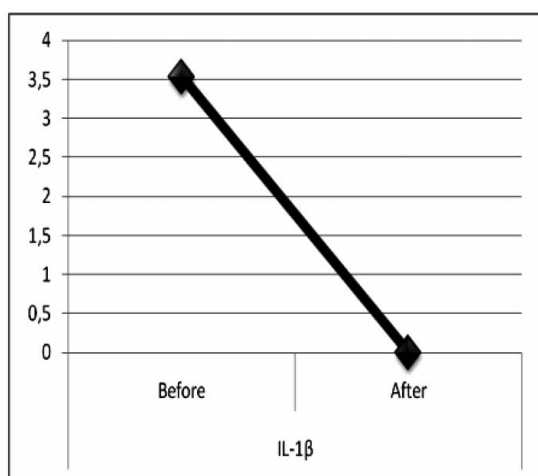


Fig. (12). Evaluation of serum IL-1 β before and after goat milk administration. Cytokine levels were evaluated by the CBA inflammation kit

Acute phase response in elderly seems to be preserved with higher levels of IL-8 than those observed in the case of IL-6. Finally, chronic pro inflammatory cytokines such as TNF- α or IL-1 β are always undetectable or present in a very low concentration.

In synthesis, these data suggest a good capacity of aged subjects to respond to an acute challenge with production of IL-8 [20], a neutrophil chemo attractant, and IL-6 [21], a protective acute phase protein. By contrast, no exaggerated production of both of TNF- α and IL-1 β is evident, thus suggesting a low proinflammatory profile in these group of healthy aged subjects.

According to our findings one can postulate the following hypothesis. Both milks do not affect levels of IL-12 and IL-10. Instead as far as IL-8 and IL-6 production is concerned they behave in an opposite manner. Donkey milk significantly augments IL-8 and IL-6 response in comparison to baseline values, while goat milk reduces the exaggerated basal secretion of both cytokines.

The effects of both milks on IL-1 β and TNF- α are less evident since these cytokines are scarcely secreted in sera. Nevertheless, donkey milk up-regulates, while goat milk down-regulates IL-1 β and TNF- α production, respectively.

A critical analysis of these data leads to the conclusion that donkey milk is an enhancer of acute phase response and, to a lesser extent, of proinflammatory cytokine response. Therefore, its daily use in the diet of immunocompromised aged patients should be recommended.

Goat milk is rather a down regulator of acute inflammation, thus preventing massive infiltration of neutrophils into the senescent lung and, consequentially further damage to vital organs. In this respect, in two recent reports [22,23] the ability of goat milk to attenuate experimental colitis in rats has been pointed out in virtue of its oligosaccharide content. In fact, oligosaccharides can function as a prebiotic or as a receptor for pathogens [22].

Again, basal low levels of TNF- α and IL-1 β limit our understanding on the full capability of goat milk to act as an anti inflammatory agent in the case of chronic clinical conditions.

The observed differences between donkey and goat milk in eliciting human cytokine productions IL-6 and IL-8 may reside on their different biochemical profiles. Donkey milk is quite similar to human milk in terms of ω -3 and ω -6 fatty acids, lactose and lysozyme content and total proteins [24]. Instead goat milk exhibits less content of short-chain fatty acids and cholesterol, being total proteins very close to the bovine milk.

In conclusion human trials with donkey and goat milk merit to be pursued for a better understanding of their immunomodulating capacities in elderly.

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ABBREVIATIONS

CBA	=	Cytometric Bead Array
IFN	=	Interferon
IL	=	Interleukin
TNF	=	Tumor Necrosis Factor
Zn	=	Zinc

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