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Research Article

# Fermented Milks from Small Ruminant: Effect on Metabolism and Immune Status of Mice Fed Mild Caloric Restricted Diet

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**Abstract**

The purpose of this study was to evaluate the effect of the administration of Fermented Goat´S Milks (FGMs) in mice fed mild caloric restricted diet. The ability of these FGMs to ameliorate immune and metabolic parameters related to mild caloric restriction was studied. We also analyzed if the time (45 or 90 days) of caloric restriction have influence on leptin secretion, IgA+ cells number and phagocytic activity of peritoneal macrophages, and also test the correlation between these parameters. Mice were fed with a mild caloric restricted diet during 45 or 90 days. After these periods of caloric restriction, mice were refed with balanced conventional diet (BCD) plus goat´s milks or BCD plus goat´s milks fermented with *Lactobacillus rhamnosus* CRL1425 (FGM-Lr) or *Lactobacillus casei* CRL431 (FGM-Lc). All renutrition diets induced an increase of serum glucose, triglycerides, total proteins and leucocytes cells. A decrease in cholesterol levels was observed after the diets. FGM-Lr induced lower triglycerides values than others diets. All FGMs restore IgA+ cells in intestinal mucosa, and the FGM-Lc group had higher positive cells number than the *ad libitum* control. Furthermore, phagocytic activity of peritoneal macrophages only increased in mice fed with FGM-Lc. Mild caloric restriction induced a decrease on leptin circulating values at 90 days. The renutrition with FGM-Lr lead to lower leptin levels than mild caloric restriction controls, while FGM-Lc induced higher leptin levels. Positive correlation between serum leptin concentration and immune parameters was observed in all groups under study. We showed that leptin levels could positively predict the immune mucosal competence in mild caloric restricted mice. These results suggest that the FGMs are able to modulate, in different way, serum leptin levels, IgA+ cells number and phagocytic activity. Furthermore, FGM-Lc could be more effective for nutritional treatment in malnutrition status.

**Keywords:** Mild caloric restriction; Leptin renutrition; Goat´s milk; Probiotic

## Abbreviations

GM: Goat´s Milk; FGM: Fermented Goat´S Milk; FGM-Lr: Fermented Goat´S Milk with *Lactobacillus Rhamnosus* CRL1425; FGM-Lc: Fermented Goat´S Milk with *Lactobacillus casei* CRL431; BCD: Balanced Conventional Diet; AD: *Ad libitum* control group;

CR: Caloric Restricted group. Group of mice which after diet adaptation period (2days) received 75% of BCD; AD45: *Ad libitum* group of 45 days; AD90: *Ad libitum* group of 90 days; CR45: group of mice which after diet adaptation period (2days) received 75% of BCD during 45 days; CR90: group of mice which after diet adaptation period (2days) received 75% of BCD during 90 days; CR/Re: Caloric Restricted / Renourished group; CR45/Re-Lr: Caloric Restricted mice during 45 days were renourished with *ad libitum* BCD plus goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus FGM with *Lactobacillus rhamnosus* CRL1425; CR45/Re-Lr: Caloric Restricted mice during 45 days were renourished with *ad libitum* BCD plus goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus FGM with *Lactobacillus rhamnosus* CRL1425; CR90/Re+Lr: Caloric Restricted mice during 90 days were renourished with *ad libitum* BCD plus goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus FGM with *Lactobacillus rhamnosus* CRL1425; CR45/Re-Lc: Caloric Restricted mice during 45 days were renourished with *ad libitum* BCD plus goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus FGM with *Lactobacillus casei* CRL431; CR90/Re-Lc: Caloric Restricted mice during 90 days were renourished with *ad libitum* BCD plus goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus FGM with *Lactobacillus casei* CRL431; CR45/Re-GM: Caloric Restricted mice during 45 days were renourished with *ad libitum* BCD plus whole goat´s milk during 12 days; T: effect of time determinate by ANOVA test; D: effect of diet determinate by ANOVA test

## Introduction

Clinically, malnutrition is characterized by inadequate intake of protein, energy and micronutrients. Generally, it is associated with frequent infections and disorders. Currently, nearly 12% of the global population is estimated to be undernourished, and the vast majority of these live in developing countries [1], therefore, the management


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of undernourishment has continued being a public health priority.

According the WHO child growth standards, underweight is defined as weight-for-age below 2 SD the median [2]. Actually, there is a prevalence of stunting and underweight among children under-five years of age worldwide. This situation leads to millions of children still remain at risk [2]. Children with mild malnutrition have an increased risk of mortality. If some of these children do not receive adequate support, they may progress towards a severe acute malnutrition or a severe stunting.

The adaptation to caloric restriction diet is characterized by metabolic, endocrine, and immunologic changes. There is robust evidence that leptin could be a sensitive marker of nutritional status [3]. It is directly correlated with several biochemical and anthropometric parameters [3]. Leptin, the product of the obesity gene, is a 16-kDa circulating hormone that has been recognized to have a major influence on energy balance [4]. Leptin is a mediator of long-term regulation of energy balance via hypothalamic-mediated effects. This hormone suppresses food intake, by inhibiting orexigenic neuropeptides and stimulating anorexigenic ones, as well as increases energy expenditure [5,6]. The presence of leptin receptor in multiple biologic systems, as well as in various immune cells, suggest this hormone also intervene in the modulation of immune function [7]. During caloric restriciton the loss of body fat results in profound reductions in circulating levels of leptin, amongst other adipokines [8]. However, the decline in leptin in response to caloric restriction appears to be partially dependent on other factors, such as the composition of the diet [9,10]. Also, intensity and duration of caloric restriction seems to affect leptin drop. In a previous study we showed that a short–term (12 days) mild calorie-restricted diet (set at around a 25 % reduction in energy consumption) does not significantly modify serum leptin levels in mice [11].

It is known that nutritional deficiencies can adversely affect the number and activity of immune cells leading to a depression of the immune system [12,13]. Reduced body fat or nutritional deprivation, typically associated with hypoleptinemia, is a direct cause of secondary immunodeficiency and increased susceptibility to infection [4]. Matarese *et al.* suggested that leptin plays an important role in the regulation of the immune system in energy- or leptin-deficient states [14]. The malnutrition could be reversed with an appropriate renutrition [12,15,16]. In this regard, fermented dairy products could be useful in malnourished groups [17]. Probiotic dairy products are widely accepted as health products and included within functional foods [17]. Probiotics defined as live microorganisms that, when provided in adequate amounts, confer a health benefit on the host, are included as important components of the daily diet [17]. In this context, there is sufficient scientific evidence demonstrating that probiotic fermented milks are responsible for restoring gastrointestinal and immune functions in moderate malnutrition [12,15,16].

Milks from small ruminants, such as goat´s milk, are an important and cheap source of milk and especially in tropical countries, and could be an alternative for nutritional treatment [11,18,19]. The nutritive quality of milk is determined by its composition. These no conventional milks differ in lipid, protein, carbohydrate, vitamin and mineral contents, and thus in their nutritional properties, compared with cow´s milk [2,11]. Investigations have shown that small ruminant milks, and particularly goat´s milk, have functional benefits for the treatment of malnutrition [11,18]. However, little is known regarding the potential role of fermented goat´s milks on metabolism and immune status during mild malnutrition recovery. Therefore, in this study we evaluate the effect of the administration of Fermented Goat´S Milks (FGMs) in mice fed caloric restricted diet. The ability of these fermented milks to ameliorate immune and metabolic parameters related to mild caloric restriction was studied. We also analyzed if the time (45 or 90 days) of caloric restriction have influence on leptin secretion.

## Material and Methods

### Bacterial strains, media and culture conditions

Two lactic acid bacteria strains were selected as probiotic cultures for fermentation of goat´s milk due to their source or ability to stimulate the immune system. *Lactobacillus rhamnosus* CRL1425 and *Lactobacillus casei* CRL431 were obtained from Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina) culture collection. *L. rhamnosus* CRL1425 is a microorganism with proved technological properties which was isolated from goat´s cheese [20]. *L. casei* CRL431 was isolated from intestine of healthy children and has extensively showed immunomodulatory properties in malnourished hosts [12,15,16]. The cultures were stored at −70°C and activated in Man–Rogosa–Sharpe (MRS) broth (1% v/v inoculums). The cells were harvested by centrifugation at 3,000 g for 10 min and washed three times with sterile 0.01 mol/L Phosphate-Buffered Saline (PBS), pH 7.2 for development of fermented goat´s milks (FGMs).

### Preparation of goat´s milks fermented with Lactobacillus rhamnosus CRL1425 or Lactobacillus casei

**CRL431:** Fresh, non-fortified, whole goat´s milk was provided by INTA "Santa Cruz" (Catamarca, Argentina). The microbiological quality of the milk was assessed according Salva et al. [18]. Goat´s milk fat content, protein and non-fat solids were determined with a milk analyzer (Ekomilk M). Before use, the goat´s milk was pasteurized (85°C, 15 min) and cooled to 42°C. Goat´s milk was fermented with *Lactobacillus rhamnosus* CRL1425 (named as FGM-Lr) or *Lactobacillus casei* CRL431 (named as FGM-Lc). FGMs were obtained by the inoculation (1%) of a fresh (overnight 18 h) culture of these strains into goat´s milk followed by 8 h of incubation (37°C, aerobiosis). FGMs were microbiologically analyzed at the end of milk fermentation. The samples were diluted in sterile peptone water (0.1% v/v) and subsequently plated in duplicate onto the selective media. For total viable counts of bacteria, spread plating was performed in duplicate on MRS agar. The plates were incubated at 37°C for 48–72 h. The counts of *Lactobacillus casei* CRL431 and *L. rhamnosus* CRL1425 reached a maximum of 1x $10^9$cfu/ml and 7.21 x $10^8$, respectively, after fermentation process. These FGMs were prepared daily for the feeding trials.

### Animals and experimental design

The experimental protocol was approved by the Institutional of Laboratory Animals Care and Use of CERELA, and experimental procedures were carried out in accordance with the present laws of Argentina, according to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) requirements (Resolution D,Nº 1047/05) for laboratory animal research.

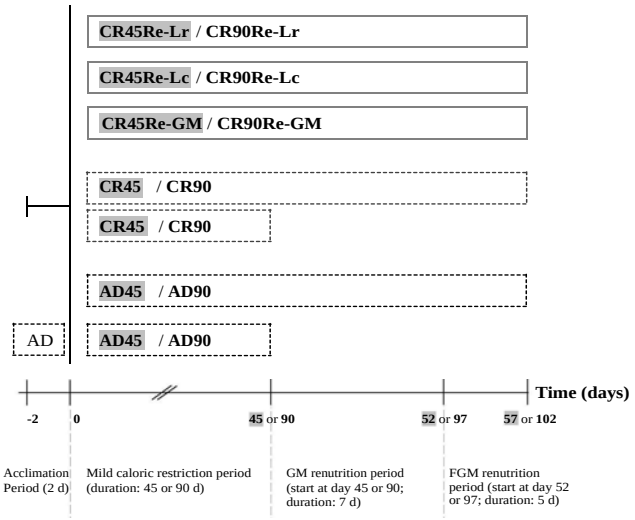


**Figure 1:** Flow chart of the study design. All mice received food *ad libitum* during the acclimation period (2 days). After this period, the mice were divided into three main groups:
1) *Ad libitum* main group (AD; ) (n = 6 for each AD groups): One group of mice was killed at day 0 (baseline values group). Two other groups of animals were killed, one at day 45 and the other at day 90 (in coincidence with the end of the period of caloric restriction). Two other groups of mice were killed after 12 days, in coincidence with the end of the period of renutrition (one group at day 57 and the other at day 102).
2) Caloric restricted group (CR; ) (n = 6 for each CR groups): animals were fed along 45 (CR45) or 90 (CR90) days with 75% of their normal diet intake. One group of mice was killed at day 45 and other at day 90 (end of CR45 and CR90 caloric restriction periods, respectively). Two other groups of mice were killed after 12 days, in coincidence with the end of the period of renutrition (one group at day 57 and the other at day 102).
3) Caloric Restricted / Renourished group (CR/Re; ) (n = 6 for each CR/Re groups): after both caloric restriction period, animals were renourished with *ad libitum* BCD plus goat´s milk (GM) during 7 days followed by 5 days in which animals were fed with BCD plus FGM. Two groups of mice, CR/Re45-Lr and CR/Re90-Lr, were fed with *ad libitum* BCD and FGM with *L. rhamnosus* CRL1425 until days 57 and 102, respectively. Two other groups of mice, CR/Re45-Lc and CR/Re90-Lc, were fed with *ad libitum* BCD and FGM with *L. casei* CRL 431 until days 57 and 102, respectively. Two other groups of animals, CR/Re45-GM and CR/Re90-GM, were kept under renutrition with *ad libitum* BCD and goat´s milk for the entire duration of the experiment (57 and 102 days, respectively). All mice of this group were killed at the end of the renutrition periods (57 or 102 days).

The overall experimental protocol is summarized in Figure 1. Weanling (21 d) male albine Swiss mice were supplied by CERELA Institute (Tucumán-Argentina). All animals were housed in individual metabolic cages and acclimated to 22°C with a 12 h light - 12 h dark cycle. Mice were fed with Balanced Conventional Diet (BCD) providing 21% calories as protein (casein), 66% as carbohydrates (corn starch) and 13% as fat (soy bean oil), supplemented with vitamin mixture (2.2%) (ICN, Biomedicals, Inc., Ohio, USA), and salt mixture (4%) (ICN, Biomedicals, Inc., Ohio, USA) and given water *ad libitum*. After 2 day diet adaptation period, mice were matched by weight and assigned to one of three main groups where the effects of mild caloric restriction (during 45 or 90 days) and renutrition diets were studied:

(1) *Ad libitum* control group (AD): after a period of adaptation to the diet (2 days), mice (n=30) were provided with *ad libitum* access to BCD for all assay period. Six mice were killed at day 0 (baseline values). Other 6 mice were killed at day 45 or 90 (in coincidence with the end of the period of caloric restriction). Other 6 mice were killed after 12 days (in coincidence with the end of the period of renutrition). All these mice constituted the AD45 and AD90 groups.

(2) Caloric restricted group (CR): after diet adaptation period (2 days), one animal group (n=24) received food in an amount equivalent to 75% of the *ad libitum* diet, to match 10-25% weight loss compared with the AD group (as was previously set) (11). Six (6) mice were killed at day 45 or 90 (end of the period of caloric restriction). Other 6 mice were killed after 12 days (in coincidence with the end of the period of renutrition). All these mice constituted the CR45 and CR90 groups.

(3) Caloric Restricted / Renourished group (CR/Re): after caloric restriction period, mice (n=36) were separated into three feeding groups for each period of caloric restriction. Animals of these test groups were refed with *ad libitum* BCD plus whole goat´s milk during 7 days followed by 5 days in which animals were fed with BCD plus (a) FGM-Lr or (b) FGM-Lc or (c) goat´s milk (goat´s milk control = GM). Previous studies showed that treatment with fermented milks for 5 days is the optimum time for a better recovery of metabolic and immune status (12,15,16). These mice constituted the CR45/Re-Lr or CR90/Re-Lr; CR45/Re-Lc or CR90/Re-Lc and CR45/Re-GM or CR90/Re-GM groups, respectively. Nutrient composition of FGMs is presented in Table 1. FGMs and GM were given twice daily (about 4 ml/mouse) during the renutrition period in replacement of drinking water. FGMs and GM were entirely consumed, ensuring the same energy consumption in all groups.

Throughout the trial, mice were sacrificed at beginning of treatment period (0 day), at the end of the two caloric restriction periods (45 or 90 days), and at the end of renutrition periods (after 12 days, that is to say at day 57 or 102, respectively) (Figure 1). Animals were fasted (12 hours) before killing. Blood samples were collected from cardiac puncture, stored at room temperature for 1 h and kept overnight at 4 °C. Then, it was centrifuged at 1,000 x*g* for 10 min to collect the serum and stored at −70 °C until analysis. The small intestines were removed for immunological studies. Macrophages were isolated from peritoneum to evaluate the phagocytic activity.

## Biochemical measurements

Serum glucose, cholesterol and triglycerides were determined by oxidase/peroxidase method according to Trinder [21] (GT Lab, Rosario, Argentina). Total protein concentration was determined using Bradford technique [22]. Haematocrit (HTO), and the number of leukocytes were determined by the haematocytometric methods. Haemoglobin concentration was determined by colorimetric assays (GT Lab, Rosario, Argentina). Serum leptin concentration was assayed using a sensitive commercially available ELISA kit according

**Table 1:** Composition of goat´s milk and fermented goat´s milks with *Lactobacillus rhamnosus* CRL1425 or *Lactobacillus casei* CRL431.

| Components | Goat´s milk | FGM-Lr | FGM-Lc |
|---|---|---|---|
| Proteins (%; w/v) | 3.28 | 3.4 | 3.33 |
| Total lipid (%; w/v) | 4.16 | 4.15 | 4.19 |
| Carbohydrate (%; w/v) | 4.45 | 4.5 | 4.43 |
| Calories (KJ/ml) | 286.21 | 288.34 | 285.88 |

FGM-Lr: Fermented goat´s milk with *L. rhamnosus* CRL1425 ; FGM-Lc: Fermented goat´s milk with *L. casei* CRL431.

**Table 2:** Effect of goat's milk and fermented goat's milks by *L. rhamnosus* CRL1425 (FGM-Lr) and *L. casei* CRL 431 (FGM-Lc) on metabolic and haematologic parameters in CR45 mice under feeding conditions.

| Experimental Groups | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variables** | **AD** | | **CR45** | | **CR/Re** | | **CR45/Re** | | **CR45/Re** | | ***P* (A_OVA)** | | |
| | | | | | **FGM-Lr** | | **FGM-Lc** | | **GM** | | | | |
| **Glucose (g/l)** | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | T | D | T*D |
| | 1.63[a] | 0.06 | 0.40[b] | 0.03 | 1.59[a] | 0.27 | 1.58[a] | 0.09 | 1.53[a] | 0.06 | NS | NS | NS |
| **Cholesterol (mg/dl)** | 127.50[a] | 8.94 | 137.00[a] | 6.32 | 104.00[b] | 3.79 | 101.00[b] | 10.12 | 115.00[c] | 8.05 | <0.001 | <0.001 | <0.001 |
| **Triglycerides (mg/dl)** | 109.00[a] | 1.26 | 95.00[b] | 1.26 | 102.00[d] | 8.94 | 115.50[d] | 4.43 | 117.5[d] | 13.28 | NS | <0.001 | NS |
| **Total Protein (g/l)** | 4.60[a] | 0.20 | 4.05[b] | 0.35 | 4.64[a] | 0.80 | 4.70[a] | 0.63 | 4.8[a] | 0.80 | NS | NS | NS |
| **Albumin (g/l)** | 3.12[a] | 0.48 | 2.88[a] | 0.40 | 2.99 | 0.63 | 3.09 | 0.27 | 3.15[a] | 0.41 | NS | NS | NS |
| **Haematocrit (%)** | 45.75[a] | 2.48 | 43.70[a] | 2.14 | 43.15[b] | 1.79 | 42.90 [b] | 1.07 | 43.30 [b] | 1.39 | NS | NS | NS |
| **Haemoglobin (g/dl)** | 14.02[a] | 2.15 | 14.80[a] | 1.88 | 13.8 [a] | 1.07 | 13.75[a] | 0.44 | 13.60[a] | 0.80 | NS | NS | NS |

*Animals were fed with a caloric restriction diet during 45 days (caloric restriction period), after which received *ad libitum* BCD plus goat´s milk (GM) during 7 days and then mice were fed with *ad libitum*
BCD plus fermented goat´s milk (FGM) with *L. rhammnosus* CRL1425 (CR/Re45-Lr) or *L. casei*
CRL431 (CR/Re45-Lc), or GM (CR/Re45-GM) during 5 days (renutrition period).
Values are means ± standard deviation (n = 6 for all groups). Means for each raw without a common superscript letter differ significantly ($P<0.05$, Student's t test). T: effect of time of caloric restriction; D: effect of different diet, T*D: interaction between time of caloric restriction and diet (ANOVA test). NS: no significant.

**Table 3:** Effect of goat´s milk (GM) and fermented goat´s milks by *L. rhamnosus* CRL1425 (FGM-Lr) and *L. casei* CRL 431 (FGM-Lc) on metabolic and haematologic parameters in CR90 mice under feeding conditions.

| Experimental groups | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variables** | **AD** | | **CR90** | | **CR/Re** | | **CR90/Re** | | **CR90/Re** | | ***P* (A_OVA)** | | |
| | | | | | **FGM-Lr** | | **FGM-Lc** | | **GM** | | | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | T | D | T*D |
| **Glucose (g/l)** | 1.64[a] | 0.10 | 0.48[b] | 0.04 | 1.52[a] | 0.27 | 1.55[a] | 0.13 | 1.56[a] | 0.07 | NS | NS | NS |
| **Cholesterol (mg/dl)** | 133.00[a] | 14.10 | 110.00[b] | 6.32 | 100.00[c] | 7.49 | 115.00[b] | 6.96 | 120.00[b] | 5.06 | <0.001 | <0.001 | <0.001 |
| **Triglycerides (mg/dl)** | 121.00[a] | 11.50 | 102.00[b] | 7.16 | 130.00[c] | 6.05 | 140.00[d] | 8.94 | 148.00[d] | 9.84 | NS | <0.001 | <0.001 |
| **Total Protein (g/l)** | 4.63 [a] | 0.89 | 4.09[b] | 0.54 | 4.80[a] | 0.36 | 4.70[a] | 0.63 | 4.95[a] | 0.49 | NS | NS | NS |
| **Albumin (g/l)** | 3.12[a] | 0.45 | 2.80[a] | 0.20 | 3.10[a] | 0.15 | 2.90[a] | 0.30 | 3.16[a] | 0.18 | NS | NS | NS |
| **Haematocrit (%)** | 45.75[a] | 2.77 | 42.00[a] | 2.58 | 44.24[a] | 2.29 | 41.35[a] | 3.47 | 43.68[a] | 2.24 | NS | NS | NS |
| **Haemoglobin (g/dl)** | 14.02[a] | 2.40 | 13.98[a] | 1.16 | 13.96[a] | 1.30 | 13.30[a] | 1.10 | 13.87[a] | 1.30 | NS | NS | NS |

* Animals were fed with a caloric restriction (CR) diet during 90 days (caloric restriction period), after which received *ad libitum* BCD plus goat´s milk (GM) during 7 days and then mice were fed with *ad libitum* BCD plus fermented goat´s milk (FGM) with *L. rhammnosus* CRL1425 (CR/Re90-Lr) or *L. casei*
CRL431 (CR/Re90-Lc), or GM (CR/Re90-GM) during 5 days (renutrition period).
Values are means ± standard deviation (n = 6 for all groups). Means for each raw without a common superscript letter differ significantly ($P<0.05$, Student's t test). T: effect of time of caloric restriction; D: effect of different diet, T*D: interaction between time of caloric restriction and diet (ANOVA test). NS: no significant.

to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

## Immunofluorescence assay for IgA secreting cells

The small intestines were removed after 12 days of renutrition period and washed with saline solution (NaCl 0.15 M). The tissues were prepared using the modified method described by Sainte-Marie [16]. Serial paraffin sections (4 μm) were made and used for direct immunofluorescence assays to determine the number of IgA+ cells in the lamina propria. After deparaffinization using xylene and rehydration in a decreasing gradient of ethanol, slides were incubated with α-chain monospecific antibody conjugated with fluorescein isothiocyanate (FITC, Sigma, St. Louis, USA) for IgA+ cells. The results are expressed as the number of positive cells per 10 fields of vision (magnification 100×) using a fluorescent light microscope.

## *Ex vivo* phagocytosis assay of peritoneal macrophages

After 12 days of renutrition period, peritoneal macrophages were obtained according to De Moreno de LeBlanc et al. [23] and the cell concentration were adjusted at $1 \times 10^6$ cells/ml. Phagocytosis assay was performed using a *Saccharomyces boulardii* suspension at a concentration of $10^7$ cells/ml. Opsonized yeast in mouse autologous serum (10%) were added to 0.2 ml of macrophage suspension. The mixture was incubated for 30 min at 37°C. The percentage of phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells counted in an optical microscope.

## Statistical analysis

Statistical analyses were carried out using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). The experimental data were expressed

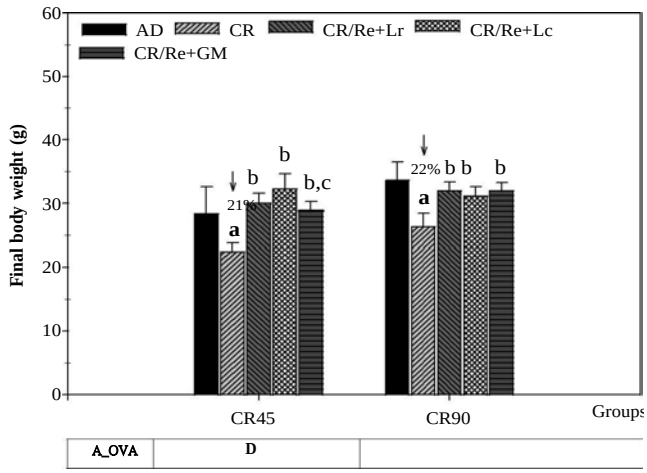


**Figure 2:** Effect of caloric restriction and renutrition diet on mice growth. Body weight was recorded daily. Animals were fed with a caloric restricted diet during 45 or 90 days (caloric restriction period), after which they were refed with *ad libitum* BCD plus whole goat´s milk (GM) during 7 days followed by 5 days in which animals were fed with BCD plus Fermented Goat´S Milk (FGM) with *L. rhammnosus.*

CRL1425 (CR/Re45-Lr, CR/Re90-Lr) or *L. casei* CRL431 (CR/Re45-Lc, CR/Re90-Lc), or GM (CR/Re45-GM, CR/Re90-GM) (renutrition period). Data are expressed as means ± SD (n = 6 for each group) and statistically significant differences are established at $P<0.05$. T: effect of time of caloric restriction; D: effect of different diet; TxD: interaction between time of caloric restriction and diet (two-way ANOVA). Significant differences: **a**: AD vs CR; **b**: CR vs CR/Re-Lr or CR/Re -Lc or CR/Re-GM; **c**: CR/Re-GM vs CR/Re-Lr or CR/Re -Lc (Student's t test). The percentages represent the decrease of body weight as a consequence of the caloric restriction diet.

as mean ± SD and were statistically evaluated by two-way ANOVA. Tukey's test (for pairwise comparisons of the means of the different groups) was used to test for differences between the groups. Single comparisons between groups were assessed by Student's t test. Differences were considered significant at $P < 0.05$. As an exploratory step, a principal component analysis was performed, including IgA cells number, leukocytes number, phagocytic activity and leptin concentration. Pearson correlation coefficients were used to detect correlations, where appropriate. *P* values of <0.05 were considered to be significant.

## Results

### Effects of mild caloric restriction/renutrition on body weight

Body weight was followed throughout the trials (Figure 2). As expected, mild caloric restriction diet resulted in lower body weight in mice of both CR groups (CR45 and CR90), with a decrease of approximately 21% in CR45 and 22% in CR90 group. At the end of renutrition period, all diets in both CR groups, caused a significant increase in body weights in comparison with the respective CR group of the same age. ANOVA test showed that there was no effect of Time (T) of caloric restriction diet on the body weight in both CR groups and there was a significant effect of diet (D) only in CR45 groups. Student's t test revealed that the FGM-Lc induced a higher increase in body weight than GM.

### Effects of mild caloric restriction/renutrition on serum biochemical variables

Table 2 and 3 shows serum glucose, cholesterol, triglycerides, total proteins, and haematological parameters (haematocrit, haemoglobin) values of CR animals. As it was expected, in both CR groups, the caloric restriction diet induced a significant decrease in serum glucose, triglycerides and total proteins (Table 2 and 3). All renutrition diets induced a significant increase of glucose, triglycerides and total proteins values in comparison with CR45 or CR90 controls (Table 2 and 3). In CR90/Re animals, an influence of diet (D) on the triglyceride levels were observed; FGM-Lr induced lower triglyceride values than FGM-Lc and GM administration (ANOVA test) (table 3). Cholesterol values not changed significantly at 45 days of caloric restriction diet. But, a significant fall was observed at 90 days of diet (ANOVA test) (Table 2 and 3). Both, FGM-Lr and FGM-Lc induced a hypocholesterolemic effect compared with CR45/Re-GM mice, while in CR90/Re group, only FGM-Lr administration was able to keep lower values of cholesterol (Table 2 and 3). Serum albumin modifications were not observed in any groups (Table 2 and 3). Also, the haematocrit percentage and haemoglobin concentration did not modify with any treatment in comparison with control groups (Table 2 and 3).

The results of serum leptin levels are shown in Figure 3. The 45 days of caloric restriction induced a slight increase of serum leptin, while 90 days of caloric restriction diet, produced a significant descent (ANOVA test). In the renutrition period, effect of time (T) of caloric restriction on leptin levels was not observed. However, influence of diet (D) was demonstrated (ANOVA test). Serum leptin values decreased after administration of FGM-Lr compared to CR and AD control, while FGM-Lc induced a significant increase on serum leptin when it was administered to both CR groups. The values observed with GM were similar to AD control mice.

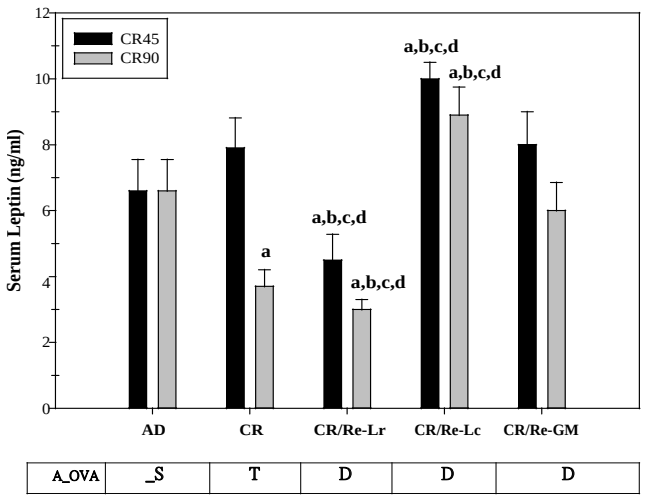


**Figure 3:** Serum leptin levels in caloric restricted and renourished mice. Animals were fed with a caloric restriction (CR) diet during 45 or 90 days (caloric restriction period), after which received *ad libitum.*

BCD plus whole goat´s milk (GM) during 7 days followed by 5 days in which animals were fed with BCD plus Fermented Goat´S Milk (FGM) with *L. rhammnosus* CRL1425 (CR/Re45-Lr, CR/Re90-Lr) or *L. casei* CRL431 (CR/Re45-Lc, CR/Re90-Lc), or GM (CR/Re45-GM, CR/Re90-GM) (renutrition period). Data are expressed as means ± SD (n = 6 for each group) and statistically significant differences are established at $P<0.05$. T: effect of time of caloric restriction; D: effect of different diet; TxD: interaction between time of caloric restriction and diet (ANOVA Test). Significant differences: **a**: AD vs CR; **b**: CR vs CR/Re-Lr or CR/Re-Lc or CR/Re-GM; **c**: CR/Re-GM vs CR/Re-Lr or CR/Re-Lc; **d**: CR/Re-Lr vs CR/Re-Lc (Student's t test).

### Impact of mild caloric restriction/renutrition on immune response. Effect on IgA secreting cells of the small intestine and phagocytic activity of macrophages isolated from peritoneum

Table 4 shows the number of leukocytes, the number of IgA secreting cells and the percentage of phagocytic activity of peritoneal macrophages in mice fed with caloric restriction diet and renourished with FGMs. Effect of time (T) of caloric restriction was observed in leukocytes number (ANOVA test). The administration of different renutrition diets (D) has influence on the studied immune parameters (ANOVA test). In both CR groups, leukocytes number diminished significantly. The administration of the FGMs during the renutrition period significantly improved the number of leukocytes, but without retrieving a value similar to the controls. The number of IgA+ cells significantly decreased in both CR group and FGMs feeding restore the count of these cells, and exceeding in the case of FGM-Lc administration, the AD control values. The percentage of phagocytic activity of peritoneal macrophages, markedly affected due to caloric restriction diet, only increased significantly after renutrition period with FGM-Lc in both CR/Re mice.

Figure 4 shows the principal component analysis (PCA) with focus on groupings of diets with respect to leptin and immune parameters levels. The analysis revealed that two components can be extracted, which together accounted for 82.6% of the variability in the grouping of diets. The first principal component discriminated better the animals according to their diets, specially AD animals and those renourished with FGM+Lc, from the other diets, and was more influenced by leukocytes number. The second principal component singled out AD animals and was more influenced by serum leptin levels. The loading plot showed that all the animals fed with the same diet formed a group well separated from other groups. Only CR groups were discriminated between animals fed during 45 days and those fed during 90 days, which demonstrates the influence of the time of caloric restriction with respect to immune parameters and leptin levels.

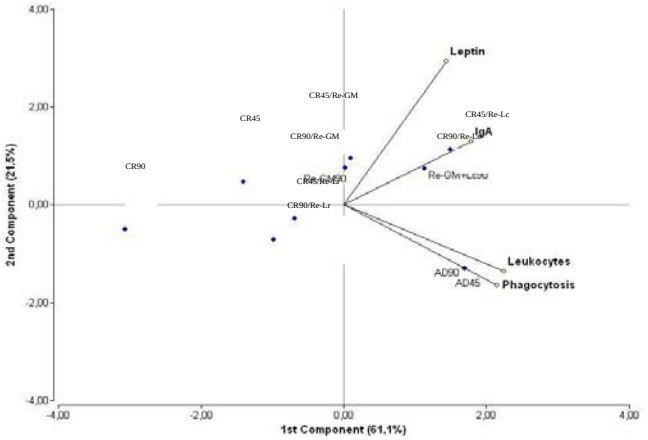


**Figure 4:** Biplot of the principal component analysis with focus on groupings of diets with respect to leptin and immune parameters level (IgA+ cells number; leukocytes number; peritoneal phagocytic activity) using the first and second principal components. Animals were fed with a caloric restriction (CR) diet during 45 or 90 days (caloric restriction period), after which received *ad libitum* BCD plus whole goat´s milk (GM) during 7 days followed by 5 days in which animals were fed with BCD plus fermented goat´s milk (FGM) with *L. rhammnosus* CRL1425 (CR/Re45-Lr, CR/Re90-Lr) or *L. casei* CRL431 (CR/Re45-Lc, CR/Re90-Lc), or GM (CR/Re45-GM, CR/Re90-GM) (renutrition period).

Correlations of serum leptin levels with IgA+ cells number, leukocytes number and peritoneal phagocytic activity are illustrated in Table 5. Significant and positive strong correlation between leptin concentration and immune parameters were found in all studied groups when they were analyzed individually. Thus, serum leptin

**Table 4:** Effect of goat´s milk and fermented goat´s milks by *L. rhamnosus* CRL1425 (FGM-Lr) and *L. casei* CRL 431 (FGM-Lc) on leukocytes number, IgA-secreting cells on the small intestine and phagocytic activity of peritoneal macrophages in CR45 or CR90 mice under feeding conditions*.

| Experimental Groups | | Leukocytes (cells/∝l) | RC45/Re IgA+ cells _umber (positive cells per 10 villi) | Peritoneal phagocytic activity (%) | Leukocytes (cells/∝l) | RC90/Re IgA+ cells _umber (positive cells per 10 villi) | Peritoneal phagocytic activity (%) |
|---|---|---|---|---|---|---|---|
| **Control** | ***AD*** | 6.83$^{a}$ ± 0.84 | 108.25$^{a}$ ± 7.89 | 46.45$^{a}$ ± 7.11 | 6.80$^{a}$ ± 0.66 | 108.25$^{a}$ ± 7.89 | 46.45$^{a}$ ± 7.11 |
| | ***CR*** | 3.75$^{b}$ ± 0.62 | 73.75$^{b}$ ± 7.54 | 24.95$^{b}$ ± 5.02 | 2.55$^{b}$ ± 0.66 | 66.50$^{b}$ ± 6.45 | 20.20$^{b}$ ± 4.10 |
| | **A_OVA** | **T** | | | **T** | | |
| **CR/Re** | **FGM-Lr** | 4.45$^{c}$ ± 0.76 | 118.75$^{a,c}$ ± 4.35 | 26.25$^{b}$ ± 4.86 | 4.47$^{c}$±0.86 | 117.00$^{a,c}$ ± 5.29 | 25.25$^{b}$ ± 2.87 |
| | **A_OVA** | | **D** | **D** | | **D** | **D** |
| | **FGM-Lc** | 5.00$^{c}$ ± 0.66 | 139.75$^{d}$ ± 5.62 | 37.40$^{c}$ ± 4.50 | 5.00$^{c}$±0.73 | 135.50$^{d}$ ± 4.93 | 36.00$^{c}$ ± 4.69 |
| | **A_OVA** | | **D** | **D** | | **D** | |
| | **GM** | 5.10$^{c}$ ± 0.84 | 113.25$^{a,c}$ ± 2.36 | 24.50$^{b}$ ± 4.65 | 4.99$^{c}$ ± 0.51 | 115.75$^{a,c}$ ± 4.35 | 25.25$^{b}$ ± 5.74 |
| | **A_OVA** | | **D** | **D** | | **D** | **D** |

* Animals were fed with a caloric restriction (CR) diet during 45 or 90 days (caloric restriction period) after which received *ad libitum* BCD plus goat´s milk (GM) during 7 days and then mice were fed with *ad libitum* BCD plus fermented goat´s milk (FGM) with *L. rhammnosus* CRL1425 (CR/Re45- Lr) or *L. casei* CRL431 (CR/Re45-Lc), or GM (CR/Re45-GM) during 5 days (renutrition period). The number of IgA+ cells was determined by direct immunofluorescence on the small intestine tissue slides from mice of different experimental groups. The results were expressed as the number of positive cells per ten fields of vision (1000X). Mice were sacrificed at the end of the study period to isolate the macrophages from peritoneum. The phagocytic activity of these cells was determined using *Saccharomyces boulardii*. The values are expressed as mean of percentage of phagocytosis expressed as the percentage of phagocytic macrophages in 200 cells counted. Values are means ± standard deviation (n = 6 for all groups). Means for each column without a common superscript letter differ significantly ($P$<0.05, Student's t test). T: effect of time of caloric restriction; D: effect of different diet, T*D: interaction between time of caloric restriction and diet (ANOVA test).

**Table 5:** Relationship between serum leptin levels and immune parameters (IgA-secreting cells on the small intestine, phagocytic activity of peritoneal macrophages and blood leukocytes number) in *Ad-libitum*, CR and CR/Re mice under feeding conditions*.

| Leptin | IgA+ cells _umber | Peritoneal phagocytic activity | Leukocytes _umber |
|---|---|---|---|
| | *r* | *r* | *r* |
| **AD** | 0.880 [a] | 0.885 [a] | 0.711 [a] |
| **CR45** | 0.870 [a] | 0.961 [a] | 0.753 [b] |
| **CR90** | 0.923 [a] | 0.944 [a] | 0.781 [b] |
| **FGM-Lr45** | 0.927 [a] | 0.951 [a] | 0.758 [b] |
| **FGM-Lr90** | 0.975 [a] | 0.944 [a] | 0.899 [a] |
| **FGM-Lc45** | 0.976 [a] | 0.882 [a] | 0.909 [a] |
| **FGM-Lc90** | 0.890 [a] | 0.902 [a] | 0.911 [a] |
| **GM45** | 0.822 [b] | 0.810 [b] | 0.865 [a] |
| **GM90** | 0.990 [a] | 0.912 [a] | 0.869 [a] |
| **Total#** | **0.405** [a] | **0.382** [a] | **0.389** [a] |

* Animals were fed with a caloric restriction (CR) diet during 45 or 90 days (caloric restriction period) after which received *ad libitum* BCD plus goat´s milk (GM) during 7 days and then mice were fed with *ad libitum* BCD plus fermented goat´s milk (FGM) with *L. rhammnosus* CRL1425 (FGM-Lr) or *L. casei.* CRL431 (FGM-Lc), or GM during 5 days (renutrition period).

***r***, Pearson correlation; [a] Correlation is significant at the 0.01 level; [b] Correlation is significant at the 0.05 level.

#, Relation between leptin and immune parameters regardless of the type of diet.

levels were positive predictors of the number of IgA+ cells, leukocytes and phagocytic activity, with Pearson correlation coefficients ranging from 0.711 and 0.990 ($P < 0.05$). Even when the data were analyzed regardless of the type of diet, leptin concentration still significantly and positively correlated with IgA+ cells number ($r = 0.405$; $P < 0.01$), leukocytes number ($r = 0.389$; $P< 0.01$) and peritoneal phagocytic activity ($r = 0.382$; $P < 0.01$).

## Discussion

The results obtained in this work showed some beneficial effects of mild caloric restriction on health that are comparable to previous reports, such as decline in serum triglycerides, cholesterol, glucose and leptin levels [9,24]. However, long term mild caloric restriction adversely affects other parameters and may compromised immune function.

Fermented dairy products are widely accepted as health products and actually, the development of new functional foods are required by specific benefits on health. The goat´s milk represented an alternative option for food industry for development of different functional food with important impact on malnutrition therapy. We selected goat´s milk for nutritional rehabilitation because in a previous study, and in agreement with other authors, we showed that it could be effectively used, as an alternative milk source, for gaining weight and for improving the altered metabolic parameters due to malnutrition [11]. In accordance with our previous results obtained in a mild caloric restriction model we also demonstrate here the efficiency of FGMs to restore (during refeeding period) metabolic parameters values, such as serum glucose and triglycerides. Lower levels of triglycerides obtained with FGM-Lr administration could be related to liporegulatory effects of leptin. Previous studies have already demonstrated a strain-dependent effect on cholesterol and triglycerides levels in rodents and also in humans [25,26].

It is well known that leptin could be used as a sensitive marker of nutritional status [27]. In a previous work we showed that the short–term (12 days) mild caloric restriction does not significantly modify serum leptin levels in mice [11]. In the current work, mice were maintained in this nutritional condition during 45 and 90 days. We observed that mice subject to mild caloric restriction diet for a period of 45 days showed a slight increase on serum leptin concentration. Those leptin values suggested that this hormone could play a role in a metabolic adaptive phenomenon necessary in the active growth period of weaned mice. Gat-Yablonsky *et al.* reported a significant stimulatory effect of leptin on longitudinal growth in a mouse model, even in the presence of low caloric intake [28,29]. The leptin levels decreased significantly only after implementation of 90 days of mild caloric restriction diet, probably related to a decrease in total white adipose tissue mass associated to the drop in the body weight of CR90 mice. Dagon *et al.* analyzed leptin functionality under different degrees of restriction diet in rats; and these authors observed that serum leptin concentrations decreased promptly after implementation of both, 40% or 60% of caloric restriction diets [30]. The discrepancies between our results (decrease of leptin concentration only after 90 days of caloric restriction diet) and those of Dagon *et al.*, could be attributed to the mild caloric restriction animal model used in this work, where animals only suffer 25% of caloric restriction.

Some strains of lactobacilli are known to modulate mucosal immune functions when were administered orally [13,15,16,31]. Several studies showed variable effects of *Lactobacillus* on the production of leptin. The main reason for the discrepancy is the route of administration of *Lactobacillus* strain and/or animal species or breed used. Bleau *et al.* [34] described *Lactobacillus*-induced decrease in leptin release by adipocyte derived from SJL mice, whereas the same *Lactobacillus* preparation led to an increase in leptin release when used to treat C57BL/6 adipocytes. In another report, probiotic capsules containing *L. acidophilus* had no effect on plasma leptin in a group of men even after 2 months of oral intake [33]. Oral administration of *L. plantarum* to smokers led to a decrease on leptin concentrations in plasma and other parameters which were attributed to their anti-inflammatory properties [35]. In other study, it was shown that direct administration of *Lactobacillus* supernatant into the brains of rats resulted in weight loss without a decrease in food

consumption and this was accompanied with an increase in leptin expression in neurons and peripheral adipose tissues as intestine [36]. This modulatory effect on leptin secretion was also observed with other probiotics, such as Bifidobacterium genus. In previous studies we demonstrated that administration of *B. pseudocatenulatum* CECT 7765 significantly reduced leptin levels in hight-fat-diet fed mice [37]. In the current work we proved that fermented milks impact on leptin circulating levels and this effect is strain dependent. Despite body weight recovery was similar after renutrition with both FGMs, we showed that FGM-*L. rhammnosus* ETC14 and FGM-*L. casei* CRL431 were able to induce changes in serum leptin levels differently. The leptin concentration was significantly decreased with FGM-*L. rhammnosus* ETC14 administration, while FGM-*L. casei* CRL431 displayed higher values than AD and CR controls.

Principal component analysis clearly discriminated between diets, which were grouped with respect to serum leptin concentration and immune parameters, regardless of the time of caloric restriction, except for CR controls. Specially, serum leptin levels and immune parameters positively influenced the differences between animals renourished with FGM-Lc and the others groups. However, PCA revealed a scarce impact of leptin and immune response in FGM-Lr groups.

According to previous studies, IgA+ cells number and phagocytic capacity of peritoneal macrophages can be used as immunologic parameters to evaluate probiotic properties of fermented milks [9,16,38]. In this work, we demonstrated that *L. casei* CRL431 was able to improve immune competence in mild calorie restricted mice [15]. This result is in concordance with other studies in malnourished and immunosuppressed mice [39–41]. Since the administration of FGM-Lc also increases serum leptin levels, it could be expected that this hormone intervene in the immunomodulatory mechanisms triggered by this microorganism. It is know that leptin is able to set up macrophages to be more responsive to lipopolysaccharide [42] and exerts direct effects on CD4+ T lymphocyte proliferation, macrophage phagocytosis, and secretion of inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF) α [31]. In line with this agreement, La Cava *et al.* showed that the restoration of leptin concentration in *ob/ob* mice might boost immune responses, via increase of thymic T cell output and cell mediated Th1 immune responses [4,44]. In addition, leptin-deficient mice have been reported to display reduced cellularity in spleen and thymus, in cell-mediated proinflammatory immune response, and in antibody production [13,45].

*L. rhammnosus* ETC14 is a microorganism with proved technological properties [20]. However, it showed low probiotic effects (up-regulation of immune response) because it produce a slight increase of IgA producing cells without change on phagocyte activity. The immune response triggered by *L. rhammnosus* ETC14 is accompanied by a decrease in serum leptin concentration. Acording to Bleau *et al.* the inhibition of leptin secretion may thus be a new way by which some lactobacilli strains may reduce inflammatory diseases [34]. Leptin is involved in several autoimmune conditions such as diabetes [46] or arthritis [47]. La Cava *et al.* suggested that modulation of circulating leptin levels may be considered as a newer possible strategy to intervene on some inflammatory and autoimmune conditions [44]. In this context, our results suggested that FGM-Lr could be used as dowregulator of leptin secretion, which could be helpful in inflammatory diseases. Studies of leptin (*OB*) mRNA expression levels in white adipose tissue should be performed to test this hypothesis.

Considering this background it could be assumed that leptin may contribute to improved macrophage phagocytosis and adaptative response (production of IgA-antibody-secreting cells) in malnourished individuals. As expected, leptin levels positively correlate with IgA+ cells number and peritoneal phagocytic activity, but also with leukocytes number, in all the groups studied. Furthermore, when the data were analyzed together, regardless of the type of diet, the correlation between leptin and the immune parameters remained positive. As was previously mentioned, numerous studies have shown a multifunctional role of leptin in modulating immune and inflammatory reactions, both in humans and experimental animals [42,48–51]. Since probiotic bacteria improve the function of immune system in malnourished humans and experimental animals, and as we demonstrated, these bacteria can regulate leptin levels in mice, it could be expected that probiotic bacteria produce the same response in humans and could be part of the mechanisms by which probiotics modulate the immune system.

## Conclusion

In conclusion, the regulation of serum leptin secretion during mild caloric restriction appears to depend on food deprivation period. Long time food restriction could lead to a decrease in leptin secretion. Whereas, during short-time moderate food restriction, leptin levels could be sustained as a way to regulate the energy homeostasis. On the other hand, not traditional fermented milk products could modify circulating leptin levels in a strain-dependent way, feature that could be exploited in hosts with different nutritional status or in inflammatory diseases. Thus, renutrition diets with both FGMs are able to modulate in different way, leptin secretion, IgA+ cells and phagocytic activity, and FGM-Lc could be useful in malnutrition status.

A developing area of research is the study of the complex link between leptin, nutrition, immune status and probiotics. We suggested that caloric restriction represents an ideal model to investigate how changes in the nutritional status influence neuroendocrine, reproductive, and immune functions.

Therefore, we aimed to gain further insight into the mechanisms that could underlie the link between nutritional and immune status. One way to take into account is performing a more extensive analysis of leptin levels and their relationship with other immune parameters in animals exposed to caloric restriction and renourished with probiotics.

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