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ORIGINAL CONTRIBUTION

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Effects of a nutritional intervention with yogurt on lymphocyte subsets and cytokine production capacity in anorexia nervosa patients

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Summary *Background* The benefits of probiotic therapy in immunocompromised subjects still need strong scientific evidences. *Aim of the study* To assess the effects of yogurt on certain immunological parameters in anorexia nervosa (AN) patients during refeeding. *Methods* A parallel 10-week nutritional intervention with yogurt was conducted on a group of patients with AN and on a group of healthy adolescents (HA). In total, 16 AN patients and 16 HA consumed 375 g/d of yogurt containing *L. bulgaricus* and *S. thermophilus* (groups AN-y and HA-y, respectively). The control groups for AN patients (n = 14) and healthy subjects (n = 19) consumed 400 ml/d of semi-skimmed milk (groups AN-c and HA-c, respectively). Blood lymphocyte subsets were assessed by flow cytometry and the *in vitro* production of IL-2, IFN-γ, IL-1, IL-6 and TNF-α by PHA-stimulated PBMC was measured by ELISA. *Results* A significant combined effect of time and nutritional intervention was found for the CD8+ subset and IFN-γ production, both in HA and AN patients. The CD8+ subset showed a significant increase after 10 weeks in HA-c and AN-c. As a consequence, the CD4+/CD8+ ratio was significantly lower in AN-c than in AN-y after treatment. A significant increase in IFN-γ production was found after yogurt intake in AN-y, while it decreased significantly in AN-c. *Conclusion* The findings suggest that the inclusion of yogurt in the refeeding therapy of AN patients may exert positive effects on the immunological markers related to the nutritional status of these patients, such as the CD4+/CD8+ ratio and the production of IFN-γ by lymphocytes.

Key words yogurt – immune system – anorexia nervosa patients – adolescents

Introduction

Adolescence is a critical age when a number of important changes take place at the physical and psychological levels of the individual. During this stage in life, erroneous eating habits are very frequent, including skipping meals, abusing fast food, high calorie foods, carbonated drinks and even alcohol consumption. A different eating pattern is exhibited by adolescent patients with anorexia nervosa (AN), who drastically restrict their diet to an extent that puts their life at risk. From previous studies our group has described an immunocompromised status in patients with AN that share some similar characteristics with typical malnutrition situations. These include leucopoenia and an impaired cell mediated immunity as reflected by a reduced DHT (delayed hypersensitivity test) response, depleted T cell subset counts in com-

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parison with a control group and an altered cytokine production in response to a stimulus [1–5].

Yogurt is fermented milk containing live lactic-acid bacteria (LAB) with a number of proved beneficial effects on health beyond nutrient supply, such as a balancing effect on the intestinal microflora, improvement of lactose tolerance, shortening of diarrhoea episodes, etc., [6]. In addition, the results of human and animal studies support the notion that LAB can also exert immunomodulatory effects. Different animal studies have shown that those LAB contained in yogurt may enhance natural and acquired immunity [7, 8]; however, human studies published in this field are lacking [9]. Moreover, despite the fact that the yogurt fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* exclusively is the most consumed in the world, relatively few studies have looked into the effects of this conventional yogurt, without any additional *Lactobacillus* or Bifidobacteria strains, on the immune system in humans. Two of these studies have found a decrease in allergy symptoms in young persons and in adults after 1 year of conventional yogurt intake [10, 11]. Other studies have described that the LAB contained in conventional yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) significantly enhance cytokine production from peripheral blood mononuclear cells (PBMC) of healthy humans when added into culture as viable bacteria [12–15]. Theoretically, an enhanced capacity to produce IFN-γ could lead to a better response against pathogens during infection processes, especially in malnourished and immunocompromised subjects. However, dietary intervention studies on human volunteers, which are fed conventional yogurt and their PBMC functionality tested "*ex vivo*", are very scarce. This type of immune system modulation could be of special interest in the case of AN patients if yogurt can be shown to ameliorate their immunocompromised status.

The present work aims to explore the influence of a nutritional intervention with yogurt, on the immunocompetent cell numbers and function in AN patients, measuring the capacity of their isolated PBMC to produce a panel of cytokines including IFN-γ, IL-2, IL-1β, IL-6 and TNF-α. The same protocol and parameters were tested in a healthy adolescent population in order to check the coincidence or disparity of the outcomes linked to yogurt intake in a physiological situation as opposed to the pathological condition of the AN patients.

Materials and methods

Subjects

A total of 35 healthy adolescents and 30 patients with AN were included in this study. The healthy adolescents were female students recruited from a secondary education school. They had no history of milk allergy or intolerance, and no other atopic symptoms. They were also free from any other organic or mental illness and were not receiving pharmacological treatment at the time of the study. AN patients were recruited upon hospital admission for refeeding and multidisciplinary treatment in the Niño Jesus University Children Hospital of Madrid (Spain), all of them meeting DSM IV diagnostic criteria [16]. In order to increase the homogeneity of the study group only female patients within the age range 13–19 years were included. Treatment was provided in an eating-disorders unit, through combined cognitive-behavioural treatment and psychopharmacological medication (Selective serotonin uptake inhibitors: 59% of patients; ansiolytic drugs [benzodiazepine derivatives]: 25%; tricyclic antidepressants: 24%; antipsychotic drugs: 31%; antiparkinson agents: 20%; antiepileptic agents: 4%; cyanosides: 4%, estrogens: 7%; calcium: 4% and several pharmaceuticals for different gastrointestinal symptoms: 22%).

Study design

The study was prospective, randomised, controlled and parallel. Both AN patients and healthy adolescents participating in the study were randomly divided into two groups consuming either yogurt in the intervention group or milk in the control group, as follows: 16 AN patients and 16 healthy adolescents consumed 375 g/d of yogurt for 10 weeks (AN-y and HA-y groups, respectively) and 14 AN patients and 19 healthy adolescents consumed 400 ml/d of semi-skimmed milk (groups AN-c and HA-c, respectively). The amount of milk was selected in order to approximately match the energy and nutrients supplied by 375 g of yogurt (400 ml of semi-skimmed milk: 172 kcal, 11.8 g protein, 6.2 g fat and 17.6 g carbohydrates; 375 g of yogurt: 225 kcal, 12 g protein, 12 g fat and 14.25 g carbohydrates). The yogurt used in this study was conventional natural yogurt, which contains the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* at a concentration of 10^7–10^8 cfu/ml, each. All measurements and analysis were performed on three occasions during the study according to the following time points: (*T*0) baseline, which in the case of the AN patients coincided with hospital admission, (*T*6) after 6 weeks of the dairy product consumption, (*T*10) after 10 weeks of the dairy product consumption. The AN patients received supervised feeding during their in-hospital stay including either two glasses of semi-skimmed milk or three yogurts a day as part of their dietary prescription, which accounted for 400 ml of

milk or 375 g of yogurt, respectively. According to the hospital's refeeding programme for AN patients, initial calorie intake was around 1,500 kcal/d for the first few days in hospital, and was increased by 500 kcal in 3–4 days and again by another 500 kcal in around 7–10 days to ensure an acceptable rate of weight gain (0.5–1.5 kg/wk). Dietary management was supported by psychological therapy in order to correct abnormal eating behaviour. After discharge, generally occurring 4–6 weeks after admission, a normocaloric diet was prescribed together with psychological ambulatory therapy in order to maintain healthy food habits, nutrient intakes and anthropometric parameters, as well as to normalise psychosexual maturation and body image perception. At this time, AN patients received instructions from the physician in charge to follow a dairy intake in accordance with the intervention protocol. In the milk-consuming groups, subjects were not allowed to eat yogurt and inversely, no milk was allowed in the HA-y and AN-y groups. A home delivery system ensuring cold transport conditions was used for free yogurt supply to all outpatients and healthy subjects. The HA groups received the same oral instructions as the AN patient groups for their respective dairy product consumption, and in order to keep a record they had to write down in a chart every dose of the product consumed per day. With the exception of the dairy products intake, they were advised to continue their habitual diets. A simple questionnaire about previous habits regarding dairy product consumption was carried out in order to assess basal consumption and to avoid the inclusion of subjects with manifest dislike or intolerance towards yogurt or milk. The HA groups had a regular consumption of milk and yogurt (7 and 4 day/week, respectively), while AN patients consumed skimmed milk every day and occasionally low-fat yogurt. In the HA groups, the consumption of two glasses of milk per day was not much higher than their regular milk intake.

Prior to start this study, the protocol was approved by the Ethic's committee of the Niño Jesus University Children Hospital. All the students and patients, or their parents in the case of minors, provided written informed consent. The study was conducted in accordance with the ethical rules of the Helsinki Declaration as revised in 1983 and the EEC Good Clinical Practice guidelines (document 111/3976/88 of July 1990).

A total of 20 patients were first included in the AN-c but 1 committed suicide and 5 were excluded due to failure to meet the dairy product intake prescribed. In the AN-y group this occurred with 2 patients out of 18 recruited. In both groups, patients belonged to the restricting-type, except for 2 patients in each of them who belonged to the binge/purging type of AN. The mean age of the subjects in the four different subgroups was similar (HA-c: 15.5 ± 1.5; HA-y: 15.3 ± 1.5; AN-c: 16.5 ± 2.2; AN-y: 15.8 ± 1.5). On admission the mean evolution period of the illness was 14.9 ± 10.8 months in AN-c and 16.3 ± 11.3 months in AN-y, and it was their first admission to hospital except for 4 patients in the AN-c group and for 6 patients in the AN-y group.

Measurements and analysis

At every time point basic anthropometric data and blood samples were obtained by the standard procedure after an overnight fast. Blood was collected in the early morning by puncture of the cubital vein. Routine blood haematology using whole blood collected into EDTA tubes was assessed using an automated analyser (Technicon H1, Bayer, Tarrytown, NY).

Lymphocyte subsets were measured by flow cytometry (FACScan, BD, Sunnyvale, CA). Whole blood collected into EDTA tubes was incubated with monoclonal antibodies (BD Biosciences): CD2 [total T cells + natural killer (NK) cells], CD3 (mature T cells), CD4 (helper cells), CD8 (cytotoxic/suppressor cells) and CD19 (B cells), for 45 min at room temperature, and processed using the Immunoprep system (Q-prep, Coulter Corporation, Hialeah, Florida). Lymphocytes were gated by forward and side scatter and pan-leucocyte marker expression (CD45, BD Biosciences).

Mononuclear cells were isolated from heparinised peripheral blood on Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway). Separated PBMC were washed twice with RPMI-1640 medium (BioWhittaker, Verviers, Belgium) and suspended in the same medium, supplemented with 10% fetal bovine serum (BioWhittaker) after decomplementation and containing 1% penicillin/streptomycin (5,000 UI/ml: 5,000 μg/ml, BioWhittaker). A volume of 1 ml culture medium containing 10^6 PBMC was placed into each of 4 wells of a 24-well plate (Becton Dickinson, Sunnyvale, CA). The mitogen phytohemagglutinin (PHA) (Gibco BRL, Paisley, UK) was used as a stimulus at a final concentration of 7 μg/ml, which probed to induce maximum proliferation. Cultures were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, culture media from the four wells were collected and pooled and cells removed by centrifugation. The supernatants were kept at −20 °C until assayed. Cytokine concentrations in the supernatants were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) utilising commercially prepared kits (Bender MedSystems, Vienna, Austria). The detection limits of the IFN-γ, IL-2, TNF-α, IL-6

and IL-1β assays were 1.5, 15, 5, 1.4 and 1 pg/ml, respectively.

Statistical analysis

All data are expressed as means and standard deviations. Data were analysed by a one-way ANOVA with repeated measures to assess the effect of time (T), the effect of the dietary intervention with yogurt (D) and the combined effect of both factors ($T \times D$) within each group (HA and AN). Repeated-measures ANOVA was also performed on each subgroup, HA-c, HA-y, AN-c and AN-y. When the analysis indicated a significant effect of time ($P < 0.05$), the Bonferroni method, which encompasses a downward adjustment for the number of statistical tests conducted, was used for a pairwise comparison between the time points and for calculation of 95% confidence limits for the differences between the time points. Values of $P < 0.05$ were considered significant. Previous to the statistical analysis, a logarithmic transformation of the variables measuring cytokine production was calculated in order to obtain normality in the distribution of the data. To further test the effect of the dairy product consumption, comparisons between the diets (control versus yogurt) were performed by Student's t-tests within each time point and for each state (HA and AN), separately. Values of $P < 0.05$ were considered significant. All statistical analyses were performed using SPSS 12.0 software.

Results

Anthropometrical measurements are presented in Table 1. Weight and BMI at baseline were significantly lower in the AN patients than in the healthy adolescents. These anthropometrical parameters did not change along the study in the healthy subjects but an increase was observed in the AN patients in $T6$, that was maintained in $T10$; however, they did not reach the levels of the HA groups. No differences in weight or BMI values were found between subjects consuming milk and those consuming yogurt (AN-c versus AN-y and HA-c versus HA-y)

Leukocyte and lymphocyte counts as well as lymphocyte subsets are presented in Tables 2 and 3. Leukocyte and lymphocyte counts were lower in the AN patients than in the healthy adolescents during the entire study (Table 2). A significant combined effect of time and type of dairy product consumed was observed on the CD8+ subset in the HA and the AN patients (Table 3), and further analysis found a significant increase of CD8+ cells in the HA-c and AN-c groups after 10 weeks of milk intake. This outcome is also reflected on the CD4+/CD8+ ratio, which decreased significantly after 10-week milk consumption in the AN patients, reaching at $T10$ significantly lower values than in the AN-y group. Regarding this ratio, significant combined effects of time and nutritional intervention with the dairy products were observed both in the HA group and in the AN group. No other $T \times D$ (time × dairy product) effect was found; therefore, the rest of the changes observed are not influenced by the dairy products intake, such as the significant increase in leukocytes in the HA-c group in $T10$ with parallel increases in CD2+, CD3+ and CD4+ subsets and the significant reduction in CD19+ cells found in $T6$ and $T10$ in the AN-y group, which, although not significant, was very similar in the AN-c group too.

Cytokine production by PHA-stimulated PBMC is presented in Table 4. The capacity to produce IFN-γ

Table 1 Anthropometrical measurements in healthy female adolescents and AN patients with yogurt consumption

	T0	*T6*	*T10*	
Weight (kg)				
HA-c	53.90 ± 9.38	54.48 ± 9.42	54.26 ± 8.75	
HA-y	55.03 ± 9.16	55.31 ± 9.08	54.37 ± 8.94	
AN-c†	40.93 ± 7.00[a]	48.16 ± 4.77[b]	48.17 ± 5.16[b]	*T*
AN-y†	38.27 ± 4.14[a]	45.63 ± 3.70[b]	46.29 ± 4.70[b]	
BMI (kg/m^2)				
HA-c	20.20 ± 2.62	20.36 ± 2.63	20.21 ± 2.38	*T*
HA-y	21.03 ± 2.81	21.05 ± 2.77	20.73 ± 2.81	
AN-c†	15.29 ± 1.98[a]	18.06 ± 1.14[b]	18.04 ± 1.19[b]	*T*
AN-y†	14.94 ± 1.33[a]	17.80 ± 0.94[b]	18.02 ± 1.15[b]	

Values are mean ± SD
HA-c and AN-c: received 400 ml semi-skimmed milk a day during 10 weeks; HA-y and AN-y: received 375 g natural yogurt a day during 10 weeks
T: One-way ANOVA, repeated measures ($P \leq 0.05$). Significant effect of time
†: One-way ANOVA, repeated measures ($P \leq 0.05$), for differences among time points within each group [ab]: Different superscripts mean significant differences between time points. Bonferroni test ($P \leq 0.05$)

Table 2 Leukocyte and lymphocyte numbers in healthy female adolescents and AN patients with yogurt consumption

	T0	*T6*	*T10*	
Leucocytes (cel./μl)				
HA-c†	6,767 ± 1,200[a]	7,258 ± 1,660[ab]	7,318 ± 1,541[b]	
HA-y	7,563 ± 1,861	7,483 ± 1,717	7,808 ± 1,378	
AN-c	4,838 ± 1,135	5,220 ± 1,282	5,067 ± 1,395	
AN-y	5,702 ± 1,356	5,837 ± 1,410	5,971 ± 1,695	
Lymphocytes (cel./μl)				
HA-c	2,481 ± 509	2,438 ± 668	2,709 ± 710	*T*
HA-y	2,734 ± 543	2,552 ± 733	2,789 ± 560	
AN-c	2,039 ± 592	1,976 ± 459	2,111 ± 549	
AN-y	2,123 ± 566	2,035 ± 458	2,256 ± 685	

Values are mean ± SD
HA-c and AN-c: received 400 ml semi-skimmed milk a day during 10 weeks; HA-y and AN-y: received 375 g natural yogurt a day during 10 weeks
T: One-way ANOVA, repeated measures ($P \leq 0.05$). Significant effect of time
† : One-way ANOVA, repeated measures ($P \leq 0.05$), for differences among time points within each group
[ab]: Different superscripts mean significant differences between time points. Bonferroni test ($P \leq 0.05$)

was significantly modified by the combination of time and the nutritional intervention with the dairy products, both in the AN patients and the HA. The results of the ANOVA within the AN groups with Bonferroni post hoc tests showed that 10-week milk intake decreased significantly IFN-γ production while 10-week

Table 3 Lymphocyte subsets in healthy female adolescents and AN patients with yogurt consumption

	T0	*T6*	*T10*	
CD2, Cel./μl				
HA-c†	1,975 ± 396[a]	1,974 ± 609[ab]	2,072 ± 562[b]	
HA-y	2,142 ± 504	2,057 ± 616	2,086 ± 497	
AN-c	1,674 ± 498	1,646 ± 423	1,672 ± 506	
AN-y	1,568 ± 416	1,536 ± 430	1,718 ± 549	
CD3, Cel./μl				
HA-c†	1,626 ± 347[a]	1,643 ± 540[ab]	1,802 ± 487[b]	*T*
HA-y	1,770 ± 355	1,653 ± 555	1,890 ± 452	
AN-c	1,476 ± 533	1,438 ± 347	1,519 ± 460	
AN-y†	1,367 ± 373[a]	1,282 ± 430[ab]	1,582 ± 558[b]	
CD4, Cel./μl				
HA-c†	928 ± 262[ab]	889 ± 300[a]	999 ± 257[b]	*T*
HA-y	1,055 ± 236	939 ± 308	1,120 ± 357	
AN-c	863 ± 364	683 ± 289	808 ± 330	*T*
AN-y†	726 ± 167[ab]	693 ± 227[a]	854 ± 292[b]	
CD8, Cel./μl				
HA-c†	450 ± 177[a]	520 ± 243[a]	586 ± 213[b]	*T*, *T*×*D*
HA-y	575 ± 188	595 ± 284	541 ± 131	
AN-c†	541 ± 147[a]	571 ± 239[ab]	707 ± 191[b]	*T*, *T*×*D*
AN-y†	491 ± 232[ab]	427 ± 166[a]	560 ± 223[b]	
CD19, Cel./μl				
HA-c	259 ± 81	273 ± 93	288 ± 112	
HA-y	256 ± 91	294 ± 133	283 ± 132	
AN-c	274 ± 168	162 ± 113	170 ± 87	*T*
AN-y†	286 ± 147[a]	175 ± 75[b]	175 ± 47[b]	
CD4:CD8, Cel./μl				
HA-c	2.27 ± 0.85	1.82 ± 0.48	1.79 ± 0.31	*T*, *T*×*D*
HA-y†	1.94 ± 0.51[ab]	1.72 ± 0.51[a]	2.13 ± 0.80[b]	
AN-c†	1.71 ± 0.69[a]	1.34 ± 0.60[ab]	1.19 ± 0.54[b]	*T*, *T*×*D*
AN-y	1.69 ± 0.60	1.72 ± 0.56	1.61 ± 0.42*	

Values are mean ± SD
HA-c and AN-c: received 400ml semi-skimmed milk a day during 10 weeks; HA-y and AN-y: received 375g natural yogurt a day during 10 weeks
T; *T* × *D*: One-way ANOVA, repeated measures ($P \leq 0.05$). *T*: significant effect of time; *T* × *D*: significant combined effect of time and dairy product consumption
†: One-way ANOVA, repeated measures ($P \leq 0.05$), for differences among time points within each group.
[ab]: Different superscripts mean significant differences between time points. Bonferroni test ($P \leq 0.05$)
* Student's *t*-tests ($P \leq 0.05$) HA-y versus HA-c; AN-y versus AN-c

Table 4 Cytokine production by PHA-stimulated PBMC from healthy female adolescents and AN patients with yogurt consumption

	*T*0	*T*6	*T*10	
IL-2, pg/ml				
HA-c†	189 ± 155[a]	83 ± 57[b]	157 ± 127[ab]	*T*
HA-y	178 ± 222	181 ± 314	88 ± 68	
AN-c	261 ± 274	149 ± 168	230 ± 329	*T*
AN-y†	126 ± 78[a]	90 ± 146[b]	151 ± 125[a]	
IFN-γ, pg/ml				
HA-c	467 ± 445	366 ± 232	260 ± 354	*T*×*D*
HA-y	435 ± 419	672 ± 508	721 ± 634**	
AN-c†	713 ± 395[a]	342 ± 323[a]	72 ± 59[b]	*T*; *T*×*D*
AN-y†	390 ± 377[a]	381 ± 430[a]	831 ± 808[b,**]	
IL-1, pg/ml				
HA-c†	37 ± 31[a]	88 ± 76[b]	99 ± 103[ab]	*T*
HA-y	90 ± 80**	165 ± 209	149 ± 204	
AN-c	398 ± 240	454 ± 307	347 ± 195	
AN-y	379 ± 325	663 ± 431	460 ± 246	
IL-6, pg/ml				
HA-c	2,273 ± 1,854	2,394 ± 2,091	3,172 ± 3,665	
HA-y	3,707 ± 2,295	4,483 ± 7,237	3,742 ± 2,002	
AN-c	10,997 ± 5,892	15,273 ± 5,838	10,240 ± 6,520	*T*
AN-y	10,843 ± 8,876	14,344 ± 6,984	15,538 ± 8,711	
TNF-α, pg/ml				
HA-c	150 ± 123	236 ± 159	120 ± 100	*T*
HA-y	194 ± 190	302 ± 277	138 ± 90	
AN-c	99 ± 110	52 ± 66	51 ± 23	*T*
AN-y†	50 ± 82[ab]	46 ± 94[a]	66 ± 34[b]	

Values are mean ± SD
HA-c and AN-c: received 400 ml semi-skimmed milk a day during 10 weeks; HA-y and AN-y: received 375 g natural yogurt a day during 10 weeks
T; *T* × *D*: One-way ANOVA, repeated measures ($P \leq 0.05$). *T*: significant effect of time; *T* × *D*: significant combined effect of time and the dairy product consumption
†: One-way ANOVA, repeated measures ($P \leq 0.05$), for differences among time points within each group. [ab]: Different superscripts mean significant differences between time points. Bonferroni test ($P \leq 0.05$)
*,** Student's *t*-test (*$P \leq 0.05$; **: $P \leq 0.01$). HA-y versus HA-c; AN-y versus AN-c

yogurt intake significantly increased this production. At the study end-point (*T*10) significantly higher IFN-γ values were observed in the AN-y group than in the AN-c group. This outcome was also found when comparing HA-y and HA-c in *T*10. In fact, in the control subjects, although not significant, milk also showed a trend to decrease IFN-γ production capacity and yogurt to increase it. The production of other cytokines measured in this study was not modified by the dairy products used in the intervention and did not show significant differences between the basal and end points in the study in any of the four groups.

Discussion

The anthropometrical characteristics of the AN patients in this study are those typical of AN patients in the acute stage of the illness. They were extremely thin and showed BMI values close to 15 on admission, which defines the emaciation threshold [17] (43% of AN patients showed BMI values below 15 in *T*0 while they all were above 15 in *T*6 and *T*10). Despite showing a significant weight gain in *T*6, as expected from patients receiving refeeding therapy, they did not gain further weight during the 4 weeks that followed until *T*10, when they were receiving out-patient treatment. This limited period as out-patients was obviously too short to observe a more substantial physical recovery.

This study explored the effect of yogurt intake on the immunocompetence of AN patients and healthy adolescents. Agreement between both populations was found regarding the effect of milk and yogurt on the CD8+ subset. The CD8+ subset change, led to the decrease of the CD4+/CD8+ ratio in the AN-c group. This ratio is accepted as an indicator of nutritional status since it has been found to be decreased in malnourished children with kwashiorkor [18]. In addition, in the studies performed in the past by our group on the nutritional status of AN patients we found that in those patients with eating disorders whose illness was diagnosed in an advanced state, this CD4+/CD8+ ratio showed lower values than in the control group [1, 19]. Therefore, yogurt seems to be a good alternative to prevent the decrease observed in the CD4+/CD8+ ratio in AN patients, which means that yogurt could be a healthy food choice to include in the refeeding therapy of these patients. Some studies have found similar relationships between milk

and yogurt intakes and the CD8+ subset of peripheral lymphocytes to those described in our subjects. Fermented milk containing *L. casei* has been shown to downregulate allergic skin inflammation by reducing the size of the hapten-specific $CD8^+$ effector T cell pool, an effect possibly mediated by bacterial wall cell components [20]. On the other hand, CD8 subset and the CD4+/CD8+ ratio have been respectively shown to be higher and lower in those cow's milk allergic infants fed a cow's milk based formula in comparison with those fed breast milk [21]. These changes in the composition of blood mononuclear leukocyte populations are relevant for immunocompetence. In humans, a CD4/CD8 ratio lower than 1.5 has been associated with immunosuppression [22], which might be the case for the subjects in the AN-c group showing a CD4+/CD8+ ratio of 1.19 in *T*10. However, no infection episodes and no fever were observed during this study in any of the subjects in the four subgroups, irrespective of their status and/or the dairy product consumed. Regarding the control subjects, we believe that the increase in CD8+ in the healthy girls is probably due to a combination of factors, which might include a seasonal variation as well as perhaps the fact that they were obliged to refrain from any fermented milk intake. Thus, after evaluating the results it seems that the study would have benefit from the inclusion of another HA control group in which no dietary intervention was performed (*ad libitum* dairies consumption), hence providing an independent evaluation of any time effects. On the other hand, an appropriate run-in period would have required no milk or yogurt intake in a previous period to the start of the study. However, this is ethically not admissible in AN patients admitted for refeeding treatment. Despite this, the prior overall consumption of yogurt was very low in the patients. Although in the healthy adolescents the "no dairies"-period could have been performed more easily, three yogurts a day are, however, a significantly higher amount of yogurt compared to their previous intake. On the other hand, two glasses of milk a day are close to their habitual intake, and therefore, are used as a control diet. Finally, we chose an intervention period of 10 weeks because in such a long period the physiological effects of yogurt can be shown.

The mean leukocyte values in the AN patients, although in the normal range, were significantly lower than in the healthy adolescents at all time points. Mild leukopoenia (4,000–4,800 cell/μl) was observed in 23% of the AN patients at baseline (*T*0) and severe leukopoenia (<4,000 cell/μl) in 13% of them, which remained unmodified in *T*10. However, there is a trend towards an increase in the T lymphocyte subsets in the AN patients, that is more marked in the AN-y group. On the other hand, the patients' therapy for the first 10 weeks of treatment led to a decrease in B lymphocyte numbers in AN patients. It is generally admitted that in malnourished AN patients cell-mediated immunity is impaired, while B cells and humoral immunity are better preserved [3, 23–26]. The decrease in B cells found in this study in the AN patients could be an attempt to normalise the high percentage of CD19+ cells exhibited by the patients in *T*0 (higher than in the healthy adolescents; data not shown). This effect, however, is not affected by the type of dairy product consumed. There are no published studies quantifying the effect of conventional yogurt containing *L. bulgaricus* and *S. thermophilus*, on lymphocyte numbers and subset distribution in humans. However, the effect of fermented milk containing *L. acidophilus* has been assessed in a limited number of studies. Wheeler et al. [27] have not found significant changes in peripheral cell counts in patients with moderate asthma consuming yogurt containing *L. acidophilus* for 1 month, and no differences either were noted by Schiffrin et al. [28] in lymphocyte subsets in healthy adults consuming a similar product during a 3-week period. Although these studies used different LAB strains and the duration of the intervention is shorter than in the current one, there is a coincidence in the lack of effect on the lymphocyte subsets as a consequence of LAB intake.

Regarding cytokine production, the only effects that were due to the nutritional intervention with the dairy products were those observed on IFN-γ. Yogurt enhanced IFN-γ production while milk inhibited this production. The rest of the cytokines were not affected by the consumption of these dairy products. In agreement with our findings, previous results in the literature have found that yogurt bacteria induce IFN-γ production from human blood mononuclear cells *in vitro* [12, 29]. However, a concurrent stimulation of IL-1β and TNF-α was also observed [29]. Other authors have also pointed out the capacity of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to increase the production of other cytokines, such as IL-6 and TNF-α, by a macrophage cell line [14]. However, it is worth noting that we present *ex vivo* results, while in the studies referred above, the immune cells were exposed *in vitro* to the LAB in a co-culture system. On the other hand, there is a previous *ex vivo* study that has found an increase in IFN-γ production in healthy adults after yogurt consumption for 15 days, as reflected by an increase in 2′–5′ A synthetase activity, an enzyme induced by IFN, in blood mononuclear cells [30]. In the current study, the effect of yogurt consumption on IFN-γ production was more significant in the AN patients than in the healthy adolescents. This could be explained by the capacity of the LAB to exert immune modulation depending on the cell status. In this

sense, the release of TNF-α by inflamed Crohn's disease mucosa has been shown to be significantly reduced by co-culture with *L. casei* or *L. bulgaricus*, while no changes are induced in non-inflamed mucosa [31]. Although it is difficult to establish how this cytokine modulation by yogurt may influence the immune system of the AN patients, some benefits could be presumed from this enhanced IFN-γ production since previous results by Schattner et al. [32] and Polack et al. [33] pointed out an inhibition of PHA-induced IFN-γ production by PBMC from AN patients in relation to that of age-matched controls.

In conclusion, this study showed that yogurt intake for 10 weeks can increase PHA-stimulated IFN-γ production in malnourished patients with AN. Moreover, the evolution of the lymphocyte subsets when yogurt is used in the refeeding therapy is different from that observed when milk is used. In this sense, the CD4+/CD8+ ratio, which is considered a good marker of the nutritional status, showed a non desirable decrease after 10-week milk intake in the AN patients that was not observed during the intervention with yogurt. Thus, the inclusion of yogurt in the nutritional therapy of AN patients may exert positive effects on their immunological markers of nutritional status.

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