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Dietary deprivation of fermented foods causes a fall in innate immune response. Lactic acid bacteria can counteract the immunological effect of this deprivation

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Extrinsic factors such as maternal microbiota, bacterial load of the environment, diet and medication modulate the intestinal microbiota. Maturation and function of the immune system is influenced by established gut microbiota. In this work we describe the immunological effects of the dietary deprivation of fermented foods of healthy volunteers. Significant decreases in faecal lactobacillus and total aerobes counts and concentration of short chain fatty acids were observed following deprivation of fermented food of the normal diet. Moreover, a decrease in phagocytic activity in leukocytes was observed after two weeks of restricted diet. Therefore, the dietary deprivation of fermented foods could induce a decrease in innate immune response that might affect the capacity to respond against infections. The ingestion of a probiotic product containing the strains *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711 or a standard yogurt containing a conventional starter *Lactobacillus delbrueckii* sp. *bulgaricus* counteracted the fall in the immune response, although the probiotic product was more effective than the standard yogurt.

Keywords: Fermented food, probiotics, immune response.

The microbiota of the human intestine influences health and well-being. The list of beneficial functions attributed to intestinal bacteria is large and includes defence against pathogen infections, nutrient processing, the fine tuning and maturation of immune responses, etc. (Erickson & Hubbard, 2000; Isolauri et al. 2001; Fanaro et al. 2003; Grönlund et al. 2000). The establishment of the gut microbiota begins after birth and is a complex process influenced by microbial – host interactions, and by external and internal factors. Human milk is a major factor in the initiation and development of neonatal gut microbiota because it constitutes a continuous source of microorganisms to the infant gut during the first weeks after birth (Martín et al. 2003). Moreover, it also contains prebiotic substances, which selectively stimulate the growth of bacteria (Drasar & Roberts, 1990; Dai & Walker, 1999). After weaning, a bacterial community resembling the adult flora become established (at 2 years of age). Essential extrinsic modulating factors of microbiota include the bacterial load of the environment, the composition of the maternal microbiota, diet and medication (Fanaro et al. 2003).

Fermentation of foods has been in use for thousands of years for the preservation and improvement of a range of foods, unfortunately the consumption of fermented food is decreasing in the western diet. However, although this kind of food constitutes an important bacterial source, no studies have been carried out in order to investigate the effect of this lack in western diet. In recent years, the use of probiotic strains (particularly lactobacilli and bifidobacteria) has been encouraged as a mean to balance the gut microbiota and, in fact, their potential preventive and therapeutic effects have received a renewed research and industrial interest (Salminen et al. 1998; Ouwehand et al. 1999; Saavedra, 2001).

In this article we analysed the effect on microbiota and immune system during fermented food deprivation of healthy volunteers and the ability of a fermented product containing two probiotic strains, *Lactobacillus gasseri* CECT5714 isolated from breast milk (Martín et al. 2005a) and *Lactobacillus coryniformis* CECT5711 isolated from an

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artisan goat-cheese (Martín et al. 2005b) to counteract the effects of the restricted diet.

Material and Methods

Design of the trial

A total of 30 healthy adult human volunteers (15 females and 15 males) with an age ranging from 23 to 43 years were included in the study. Volunteers included in the study were regular consumers of fermented foods, consuming fermented milk products (yogurt and/or cheese) at least in an amount of 5 portions per week and other kinds of fermented food at least 3 portions per week. Exclusion criteria were lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders or metabolic diseases. The study was carried out according to the Helsinki declaration and the protocol was approved by the ethical committee of Puleva Biotech SA and informed written consent was obtained from all the subjects. The study consisted of two phases: a restricted diet period (2 weeks from day –14 to day 0) followed by a period of restricted diet supplemented with a specific fermented product (2 weeks from day 0 to day +14). The volunteers were asked to exclude from their diet any kind of fermented food or drink, such as fermented milk and dairy products including cheese, fermented meat, and fermented beverages like wine, beer or vinegar, and also any other kind of fermented food product such as cured olives. The only exception was bread. Volunteers were allowed to consume only the specific fermented products supplied as the object of this study and then only during the period corresponding to the second phase, while maintaining all the other diet restrictions. Volunteer's diet was controlled by daily surveys.

After two weeks of restricted diet volunteers were randomly distributed into two groups. Those belonging to the 'yogurt' group used as a control received, on a daily basis, 200 ml of a yogurt elaborated with a standard yogurt starter provided by Puleva Food (Granada, Spain) containing 10^8 cfu *Streptococcus thermophilus* and 4×10^9 cfu *Lactobacillus delbruekii* sp. *bulgaricus*. Those of the 'probiotic' group ingested, with the same frequency, 200 ml of a similar dairy product containing the same concentration of the starter *Str. thermophilus* but in which the *Lb. bulgaricus* starter strain had been replaced by approximately 2×10^9 cfu of each of the probiotic strains *Lb. coryniformis* CECT5711 and *Lb. gasseri* CECT5714.

Collection of faecal samples and counts of fecal bacterial groups

Once a week, fresh faecal samples were collected and individually homogenized in a peptone-saline solution (100 mg/ml). To estimate the concentration of bacterial groups, appropriate dilutions were spread in quadruplicate onto plates of MRS agar for lactic acid bacteria, MRS agar supplemented with 0·5 mg dicloxacilin/l, 1 g LiCl/l and 0·5 g L-cysteine hydrochloride/l for bifidobacterium and Reinforced Clostridial Agar containing 20 µg polymixin/ml for Clostridium. All media were obtained from Oxoid (Basingstoke, UK) whereas antibiotics and other supplements were obtained from Sigma Chemical Co. (St Louis, MO). Culture plates were incubated in absence of oxygen at 37 °C for 24 to 48 h. Similarly, 1 ml of suitable dilution was spread onto specific Count Plates Petrifilm (3 M St Paul, MN) for total aerobes and for Enterobacteriaceae. Plates were incubated at 37 °C for 24 h. After the incubation, the specific colonies grown on the selective culture media were counted and the number of viable microorganism per gram of faeces (cfu/g) was calculated. The mean and standard error per group were calculated from the log values of the cfu/g.

Quantification of short chain fatty acids in the faecal samples

The concentration of short chain fatty acids (SCFA) in the faecal samples was quantified similarly to the method described by Rodríguez-Cabezas et al. (2002). Faecal samples were homogenized with 150 mM-$NaHCO_3$ (pH 7·8) (1 : 5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37 °C and stored at –80 °C until extraction. To extract the SCFA, 50 µl 100 mM-2-methylvaleric acid (internal standard), 10 µl sulphuric acid and 0·3 ml ethyl acetate were added to 1 ml of the homogenate. The mix was centrifuged at 10 000 × ***g*** for 5 min at 4 °C. The supernatants were dehydrated with sodium sulphate (anhydrous) and re-centrifuged. Then, the sample (0·5 ml) was injected (splitless) into a gas chromatograph (CP-3800, Varian, Lake Forest, CA) equipped with an ID (CPWAX 52CB 60 m × 0·25 mm), and connected to a FID detector (Varian). Helium was used as the carrier and the make-up gas, with a flow rate of 1·5 ml/min. The injection temperature was 250 °C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of the resulting peaks using the Star Chromatography WorkStation program (version 5.5), which was connected on-line to the FID detector.

Collection of blood samples

After an overnight fast lasting at least 10 h, blood samples were taken from the volunteers at the beginning of the trial (Day –14); before probiotic supplementation (Day 0) and at the end of the trial (Day +14) just using EDTA-containing vacutainers (S-Monovette, Sarstedt, Gemany). Samples were individually analysed. An automatic cell counter system, SYMEX F-800 (Symex, Nashua, NH), was used to analyse neutrophils proportion. Major leukocyte subset phenotypes were counted in EDTA-treated whole-blood samples *via* flow cytometry on a FACScalibur (Becton Dickinson, Oxford, United Kingdom) by using

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Table 1. Effect of deprivation on bacterial groups in faeces. Log numbers of bacteria per gram of faeces are expressed

Values are means ± SEM for $n=30$

	Day −14	Day 0
Lactobacilli	8·13 ± 0·18	6·89 ± 0·14*
Bifidobacterium	8·99 ± 0·12	8·70 ± 0·13
Enterobacteriaceae	6·90 ± 0·18	6·96 ± 0·18
Clostridium	9·22 ± 0·15	9·20 ± 0·11
Total aerobes	7·41 ± 0·16	6·00 ± 0·19*

* Statistically significant difference ($P<0·01$) respect to Day −14

the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-CD3+, -CD19+, -CD4+, -CD8+, -CD45RO+, -CD56+, -CD25+, -CD14+. The results were expressed as the percentage of mononuclear cells that stained positively.

Phagocytic activity

In vitro phagocytic activity was determined by flow cytometry in whole-blood samples after the uptake of fluoresceinated *Escherichia coli* (Gill et al. 2000). Basically 100 µl heparin treated whole-blood were incubated for 10 min at 37 °C with 10 ml (10^8 cfu) fluoresceinated bacteria. Erythrocytes were lysed with 100 µl 40 g formaldehyde/l and 1 ml cool water. Samples were centrifuged to 2200 × ***g*** for 5 min and suspended in 0·5 ml 40 g formaldehyde/l PBS. Samples were analysed by flow cytometry and the results were expressed as the percentage of monocytes and granulocytes showing phagocytic activity.

Total immunoglobulin and cytokines measurements

Total IgA, IgG and IgE concentrations in sera and total IgA concentration in faeces were measured by ELISA quantitation kits (BETHYL, Montgomery, TX). Cytokines concentrations in sera were measured by ELISA quantitation kits (CytoSets, BIOSOURCE, Camarillo, CA) following manufacturer's recommendations in both cases.

Statistical analysis

The data were analysed using SPSS software (version 12.0, Chicago, USA). Data are expressed as means ± SEM. P values $<0·05$ were considered significant.

For Gaussian variables, the longitudinal effect of each yogurt within each group at the different time points of the study was analysed by one-way repeated measures ANOVA followed by paired t test (within-group comparison). Two-way repeated measured ANOVA was used to analyse statistical differences produced by the consumption of each fermented product followed by independent t test to asses in which time-points the groups differed.

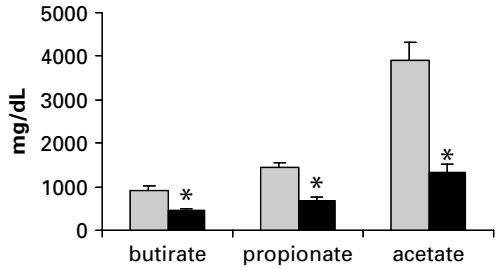

Fig. 1. SCFA concentrations (butyrate, propionate and acetate) after 24 h fermentation of the faecal samples are represented as means (± SEM) of the g/l at the beginning of the study (grey bars) and after two weeks of restricted diet (black bars). * Significant difference, $P<0·01$, between the two time points.

Results

Clinical status during the study

Throughout the study, none of the volunteers reported any adverse effect associated with the diet or the consumption of the dairy fermented products. Clinical examination by the medical staff of Puleva Biotech revealed that the health condition of all the participants was perfect during the study.

Effects of the fermented foods deprivation in diet – phase 1

Bacterial groups and quantification of SCFA in faecal samples. After two weeks of restricted diet a significant decrease in the number of faecal lactic acid bacteria was detected (Table 1). Changes in other bacterial groups such as bifidobacterium, enterobacteriaceae and clostridium could not be detected and only a significant decrease was observed in aerobes counts. With regards to SCFA a significant decrease was observed in butyric acid, propionic acid and acetic acid at the end of the deprivation phase (Fig. 1).

Effects on immune response. The phagocytic activity of granulocytes decreased significantly after the two week deprivation phase (Fig. 2). The proportion of neutrophiles was measured but no changes were observed (Table 2). In the case of monocytes the flow cytometric analysis showed a significant increase of CD14+ cells proportions (Table 2) but the phagocytic activity of these cells tended to decrease ($P=0·08$; Fig. 2).

Flow cytometric analysis of lymphocyte subsets was also performed. The proportion of cells staining positive for CD3+ (T lymphocytes), CD8+ (cytotoxic T lymphocytes), CD4+ (T helper lymphocytes), CD19+ (B lymphocytes), CD3+CD45RO+ (memory T lymphocytes), CD4+CD25+ (suppressor T lymphocytes), and CD56+ (Natural Killer cells) were in the ranges for haematologically normal Caucasian adults (Table 2). A significant increase was detected in T and B lymphocytes proportions. The T helper

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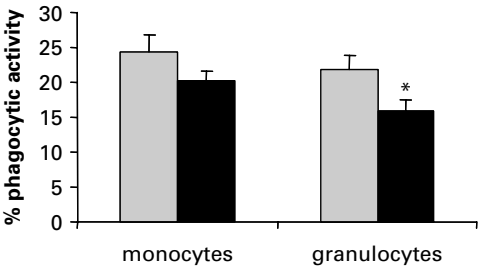

Fig. 2. Phagocytic activity of monocytes and granulocytes at the beginning of the study (grey bars) and after two weeks of restricted diet (black bars) is expressed as the mean (± SEM) of the percentage of cells containing fluoresceinated *Esch. coli* after in vitro incubation of the bacteria with fresh blood. * Statistically significant difference between the two time points $P<0{\cdot}01$.

lymphocytes increased during this period and, in contrast, a significant reduction in T memory lymphocytes was detected.

No significant changes were detected in cytokines and immunoglobulins concentrations during this period (Table 3). In faeces, however, a decrease was observed of approximately 20% in IgA concentration but differences were not statistically significant due to the variability of the samples.

Effects of the introduction of lactobacilli on diet-modified parameters – phase 2

Bacterial groups and quantification of SCFA in faecal samples. The consumption of the probiotic product caused a significant increase in faecal lactobacilli counts although these did not reach the initial values (Table 4). In contrast, the lactobacilli strains included in standard yogurt were not able to compensate for the lack of lactobacilli consumption during the period of restricted diet. The counts continued to fall during the period of supplemented diet. In the case of total aerobes, the initial values were recuperated after supplemented with both fermented products (Table 4). Regarding SCFA, the consumption of the standard yogurt did not arrest the decrease in concentrations that continued significantly during the period of supplementation. However, the probiotic product maintained the SCFA concentrations in faecal samples at the level reached at the end of phase 1 (Table 4).

Effects in immune response. With respect to immunological parameters, after consumption of both fermented products, a significant increase in phagocytic activity was observed that even overtook the values before the restricted diet began. The proportion of Natural Killer cells was not significantly modified during the two weeks of the restricted diet period, nor during the following two weeks of yoghurt consumption. However,

Table 2. Effects of the deprivation on % of white blood cells subsets and % of lymphocytes subsets

Results are expressed as mean ± SEM for $n=30$

	Day −14	Day 0
Monocytes	6·11 ± 0·35	7·10 ± 0·43*
Neutrophiles	51·97 ± 1·06	50·73 ± 1·30
Natural killer	13·61 ± 0·90	12·58 ± 0·87
T lymphocytes	59·15 ± 1·94	65·21 ± 1·66**
T helper	34·61 ± 1·54	38·98 ± 1·23*
T cytotoxic	23·55 ± 1·31	24·29 ± 1·07
T suppressor	17·82 ± 0·93	18·08 ± 0·94
T memory	43·01 ± 2·18	37·19 ± 1·57**
B lymphocytes	8·62 ± 0·84	10·40 ± 0·89**

Statistically significant difference respect to Day −14: *($P<0{\cdot}05$), **($P<0{\cdot}01$)

Table 3. Cytokines and immunoglobulins in serum or faeces

Results are expressed as mean ± SEM for $n=30$

	Day −14	Day 0
TNF (pg/ml)	38·49 ± 8·88	39·82 ± 7·70
IL-10 (pg/ml)	70·99 ± 24·2	62·70 ± 21·4
IL-12 (pg/ml)	24·87 ± 4·93	21·42 ± 3·75
IL-4 (pg/ml)	35·92 ± 7·64	30·52 ± 6·97
IgA (mg/g faeces)	237·53 ± 38·84	190·73 ± 26·19
IgA (mg/dL)	153·13 ± 15·37	143·17 ± 12·45
IgG (mg/dL)	1146·20 ± 121·6	1054·50 ± 128·3
IgE (mg/dL)	202·41 ± 40·52	191·53 ± 30·71

the consumption of the probiotic product increased the values above those obtained at the beginning of the clinical trial (Table 5). Regarding the proportions of lymphocytes subsets after the division into two groups of supplemented diet (yogurt and probiotic), the statistically significant differences were absent in some cases but in general the initial values were recovered after consumption of fermented products (Table 5).

Discussion

The effect of the diet on gut microbiota has been broadly demonstrated (Spanhaak et al. 1998; de Champs et al. 2003; Schultz et al. 2004; Valeur et al. 2004). In this work we have induced a significant decrease in lactobacillus counts by depriving fermented products from the normal diet. Intestinal colonization by lactobacilli is suggested to be a prerequisite to normal mucosa immune functions. In fact an inadequate level of lactobacilli may be involved in allergic diseases (Kalliomaki & Isolari, 2003; Savino et al. 2005). Thus, changes in lactobacillus populations in the gut could have consequences for the immune system. Microbial succession is an ongoing process influenced by numerous external and internal host-related factors. Some bacterial populations do not colonize the gastrointestinal tract permanently and need a periodic reintroduction of

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Table 4. Fecal parameters. Results are expressed as mean ± SEM for $n=29$

	Yogurt group			Probiotic group		
	Day −14	Day 0	Day +14	Day −14	Day 0	Day +14
Lactobacilli (logCFU/g)	7·99 ± 0·27	6·82 ± 0·23*	6·70 ± 0·27*	8·33 ± 0·20	6·97 ± 0·15*	7·50 ± 0·17*#‡
Aerobes (logCFU/g)	7·41 ± 0·23	6·02 ± 0·29*	8·05 ± 0·13#	7·42 ± 0·19	5·98 ± 0·20*	7·80 ± 0·23#
Propionate (g/dl)	1·235 ± 0·15	0·632 ± 0·07*	0·351 ± 0·05*#	1·616 ± 0·18	0·740 ± 0·09*	0·681 ± 0·08*#‡
Butyrate (g/dl)	0·747 ± 0·10	0·463 ± 0·08*	0·221 ± 0·04*#	1·036 ± 0·01	0·431 ± 0·05*	0·505 ± 0·08*#‡
Acetate (g/dl)	3·525 ± 0·37	1·368 ± 0·23*	0·620 ± 0·10*#	4·267 ± 0·73	1·322 ± 0·22*	1·284 ± 0·20*#‡

* Statistically significant differences with respect to day −14 ($P<0·05$)
# Statistically significant differences with respect to day 0 ($P<0·05$)
‡ Statistically significant differences between groups ($P<0·05$)

Table 5. Blood parameters. Results are expressed as mean ± SEM for $n=29$

	Yogurt group			Probiotic group		
	Day −14	Day 0	Day +14	Day −14	Day 0	Day +14
Monocytes (%)	6·24 ± 0·49	7·29 ± 0·58*	9·03 ± 1·26	5·48 ± 0·44	7·02 ± 0·53*	11·00 ± 1·40*#
Neutrophils (%)	51·44 ± 1·23	50·55 ± 1·75	57·48 ± 2·81*#	52·46 ± 1·73	50·90 ± 1·98	57·34 ± 2·37*#
Phagocytosis (%)						
monocytes	22·78 ± 2·47	19·57 ± 1·31*	33·71 ± 4·87*#	26·08 ± 4·06	22·44 ± 2·32*	37·39 ± 4·12*#
granulocytes	21·56 ± 3·06	18·19 ± 2·20*	27·39 ± 5·71*	22·24 ± 2·90	14·37 ± 2·03*	33·52 ± 4·71*#
Natural killer (%)	13·21 ± 1·10	12·22 ± 0·93	12·73 ± 1·07	14·29 ± 1·37	12·91 ± 1·41	17·29 ± 1·69*‡
T lymphocytes (%)	60·78 ± 3·04	67·65 ± 2·33	64·95 ± 2·09	57·63 ± 2·49	62·90 ± 2·36*	56·48 ± 2·68
T helper (%)	34·00 ± 2·03	37·56 ± 1·80	33·14 ± 1·89	35·21 ± 2·32	39·25 ± 1·43	36·75 ± 1·12
T cytotoxic (%)	24·70 ± 2·10	25·21 ± 1·73	25·45 ± 2·02	22·39 ± 1·53	23·74 ± 1·08	22·24 ± 1·40
T suppressor (%)	17·79 ± 1·17	17·52 ± 1·44	19·41 ± 2·09	17·85 ± 1·48	18·42 ± 1·36	18·54 ± 1·81
T memory (%)	45·26 ± 2·72	39·32 ± 2·33*	39·28 ± 4·10*	40·92 ± 3·36	35·10 ± 2·01	37·31 ± 2·69
B lymphocytes (%)	8·93 ± 1·55	11·38 ± 1·62*	11·01 ± 1·42*	8·32 ± 0·78	9·48 ± 0·70	8·74 ± 0·84

* Statistically significant differences with respect to day −14 ($P<0·05$)
# Statistically significant differences with respect to day 0 ($P<0·05$)
‡ Statistically significant differences between groups ($P<0·05$)

bacteria by repeated oral doses or by other means. Thus, the dietary deprivation of fermented food caused the decrease observed in this bacterial population. Moreover, a decrease in SCFA faecal concentration was detected suggesting the modification in the metabolism of intestinal microbiota. SCFA, mainly butyrate, affect differentiation, maturation and function of immune cells influencing the immune response (Millard et al. 2002; Rinne et al. 2005; van Nuenen et al. 2005). We have studied how the lack in fermented foods affects the immune response. We observed that, although a slight increase was detected in monocytes proportions, the phagocytic activity of these cells was lower. Normal flora is the main bacterial stimulus of the immune system thus, the absence of stimulus by lactobacillus groups seems to induce a loss in response capabilities (Doyle et al. 2004). Toll Like Receptor (TLR) activation by bacterial components triggers the rapid differentiation of monocytes into macrophages and dendritic cells and mediates the apoptosis mechanism (Kirschnek et al. 2005; Krutzik et al. 2005). The lack of this activation could explain the observed decrease in phagocytic activity, although the proportion of monocytes increased. Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response against microbial antigens which precedes the development of the acquired response (Karlsson et al. 2002). Natural Killer cells were also slightly affected by the absence of lactobacillus stimulus. Natural Killer cells play an important role in recognizing and killing of virus-infected cells and tumour cells. Therefore, the deprivation of fermented foods could induce a decrease in innate immune response that might affect the capability of the organism in the defence against infections or cell transformations.

Finally probiotic, but not standard yogurt, was able to increase the faecal lactobacilli counts and counteract the decrease in SCFA concentration, however the values did not reach the initial values. The capacity for SCFA production depends on the fermentative properties of each strain. Since the probiotic supplementation did not totally recover the pre-trial SCFA concentrations, it seems that other commercial starter strains and also wild-type lactic acid bacteria, which originate from the raw material or the environment, contained in other fermented products such as cheese or fermented meat contribute in an important

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way to the fermentative metabolism in gut. The lack of effect on the decreased SCFA in the yogurt group can be explained because this product was not able to compensate for the decrease in faecal lactobacilli. While the strains contained in the probiotic product have been demonstrated to survive the gastro-intestinal tract conditions (Olivares et al. 2006), the reported data about the survival of *Lb. delbruekki* sp. *bulgaricus* contained in standard yogurt are contradictory (del Campo et al. 2005; Mater et al. 2005).

Regarding the immune parameters, it was observed that the consumption of both fermented products induced the recovery of the phagocytic activity, even to a level that improved on the values at the beginning of the study. The fact that both fermented products induced similar results suggest that the viability of the lactobacillus strains is not essential to induce this activation probably because the interaction of the leukocytes with membrane components, which are also present in dead bacteria. However, in the case of Natural Killer cells, the yogurt strain did not modify their numbers, while the lactobacillus strains of the probiotic product boosted the proportion of these cells above those observed at the beginning of the study. These differences in the activation of Natural Killer cells proliferation could be dependent on the viability of the bacteria or of the intrinsic bacteria properties.

Dietary effects on acquired immune response were less evident. The dietary deprivation of fermented foods caused a slight increase in certain lymphocyte subsets, although in general, the initial levels of lymphocytes subsets proportions were recovered after consumption of both fermented products. In this sense it is known that probiotic bacteria can mediate suppression of lymphocyte proliferation (Isolauri et al. 2001). Thus the lack of suppressor effect by the decrease in lactobacillus load could lead to the increase in lymphocyte proliferation which is compensated by the administration of lactobacillus strains.

In conclusion, the dietary deprivation of fermented foods modified the gut microbiota and caused a decrease in the immune response mainly affecting the innate response that could affect the defence capacities of the immune system. These results are evidence of the importance of sustained oral ingestion of fermented products for health maintenance. Since the increasing lack of fermented foods in western diet, the capabilities of Lactobacillus strains to counteract the effect of deprivation of fermented food demonstrate the role that probiotic enriched products could play in the improving of this diet.

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