British Journal of Nutrition (2002), **87**, 367–373
DOI: 10.1079/BJN2001527

Oral ingestion of lactic-acid bacteria by rats increases lymphocyte proliferation and interferon-γ production

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(Received 27 September 2001 – Accepted 3 December 2001)

The effect of feeding lactic-acid bacteria on indices of functions of lymphocytes obtained from Peyer's patches, peripheral blood and spleen from inbred Wistar-Furth rats were studied. Rats were fed on purified diets supplemented with 350 g milk or yoghurt/kg diet for 4 weeks. At the end of the feeding period, immune cells from the three sites were isolated and proliferation, interferon-γ production and lymphocyte subset composition were studied. Rats consuming yoghurt had a greater *in vitro* proliferative response to yoghurt bacteria in the three lymphoid compartments, a greater interferon-γ production in response to bacteria and concanavalin A in Peyer's patches and spleen, and a greater number of Peyer's patches B lymphocytes than milk-fed rats. Macrophage and T lymphocyte proportions and lymphocyte subset composition in the three sites were unaffected by yoghurt. These results indicate that feeding live bacteria contained in yoghurt may interact with the intestinal immune system, and influence the systemic immune system.

Lactic-acid bacteria: Intestinal and systemic immunity: Interferon: Rats

It is known that when an antigen enters the body through the oral route, the first response that normally occurs, through intra-epithelial lymphocytes of the small intestine, is tolerance. When tolerance is abrogated an immune response is produced. The antigen is transported into Peyer's patches (PP) and presented to T cells by dendritic cells or macrophages, and T cells then present the antigen to B cells. These activated lymphocytes pass to the mesenteric lymph nodes through the thoracic duct and circulate in the bloodstream from where they can be distributed to the intestinal lamina propria and to lymphoid organs such as the spleen (Nagata *et al.* 2000; Weiner, 2000). Thus, the stimulation of the immune system involves activation of immunocompetent cells: macrophages, T lymphocytes (T helper (cluster determinant (CD)4+) and T cytotoxic-suppressor cells (CD8+))and B lymphocytes, their proliferation and production of cytokines. Cytokines are autocrine-, paracrine-, and endocrine-regulatory glycoproteins that interact with specific cell receptors and have pleiotropic effects. They affect both the activation state, proliferation, maturation, and function of cells from the immune system, as well as cell functions in major organs (Cavaillon, 1996). Interferon (IFN)-γ is an essential cytokine in immune reactions (Trinchieri & Perussia, 1985).

Studies in healthy volunteers have shown that ingestion of yoghurt containing live bacteria effectively elevates 2–5A synthetase activity in blood mononuclear cells (BMC) (Solis & Lemonnier, 1991; Aattouri & Lemonnier, 1997), an enzyme induced by IFN and considered to play an important role in their action (Samuel, 1991). *In vitro*, human BMC produce IFN-γ and other cytokines when incubated with lactic-acid bacteria, in particular those used for yoghurt processing (Solis & Lemonnier, 1993). Studies in the mice have suggested that feeding fermented milk or lactic-acid bacteria may affect the immune system (for review, see Meydani & Ha, 2000), for example modify macrophage phagocytosis (Perdigon *et al.* 1988) and immunoglobulin (i.e. immunoglobulin (Ig) G-2a) secretion (Conge *et al.* 1980). Such functions can be controlled by IFN-γ (Street & Mosmann, 1991; Murray, 1990). Recently, Gill *et al.* (2000) have observed that spleen cells from mice

Abbreviations: BMC, blood mononuclear cells; CD, cluster determinant; Con A, concanavalin A; IFN, interferon; Ig, immunoglobulin; PP, Peyer's patches.

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fed lactic-acid bacteria, produce higher amounts of IFN-γ in response to stimulation with concanavalin A (Con A) than cells from control mice. However, the role of lymphoid organs in the production of IFN-γ in response to these bacteria or to fermented milk has not been investigated in relation to immune blood cells. To evaluate the possible effects of feeding fermented milk, we studied the production of IFN-γ by cells from PP, spleen and blood, as well as the proliferation of cells from these organs and their number of immune cells.

Materials and methods

Animals and diets

Specific pathogen-free inbred female Wistar-Furth rats (4-week-old) were obtained from Iffa-Credo (L'Arbresle, France) and housed in transparent plastic cages with stainless-steel wire lids (four rats per cage). The rats were maintained at 21 ± 1°C and 60–80 % relative humidity, with a 12 h light–dark cycle. After feeding for 2 weeks on a basal diet, rats were subdivided at random into two groups and fed the basal diet for 4 weeks (Table 1) (Potier de Courcy *et al.* 1989) supplemented with 350 g milk or yoghurt/kg diet. The mixture was prepared just before distribution every 2 d. The rats were given free access to food and water. Yoghurt (total number of bacteria in the yoghurt was 2×10^7 bacteria/ml) and milk were obtained from the Centre de Recherche International Daniel Carasso (Le Plessis Robinson, France) in liquid form (Table 2). All procedures were in accordance with the Institute's guide for the care and use of laboratory animals.

Bacterial strain

Lactobacillus bulgaricus, *Streptococcus thermophilus* (strains 100158 and 001158 respectively) were used for *in vitro* studies and for yoghurt processing, yoghurt being a milk fermented by these two symbiotic species and containing abundant numbers of viable lactic-acid bacteria, and *in vitro* studies. Strains were provided by the Centre de Recherche International Daniel Carasso. For *in vitro* studies bacteria were cultured in Manose–Rugose–Sharpe medium, in separate cultures, and were washed three times with RPMI 1640 medium (Gibco, Cergy Pontoise, France) without antibiotics and without fetal calf serum before use.

Table 1. Composition of the basal diet used

Ingredient	Level in the diet (g/kg)
Casein	140
DL-Methionine	1
Cellulose	20
Peanut oil	25
Rapeseed oil	25
Sucrose–starch (1:3, w/w)	734
Mineral mixture*	45
Vitamin mixture†	10

* Mineral mixture (g/kg): $CaHPO_4.2H_2O$ 380, K_2HPO_4 240, $CaCO_3$ 180, $MgSO_4.7H_2O$ 90, NaCl 69, MgO 20, $FeSO_4.7H_2O$ 8·6, $ZnSO_4.H_2O$ 5, $MnSO_4.H_2O$ 5, $CuSO_4.5H_2O$ 1, NaF 0·8, $CrK(SO_4)_2.12H_2O$ 0·50, KI 0·04, $(NH_4)_6Mo_7O_2.4H_2O$ 0·02, $CoCO_3$ 0·02, Na_2SeO_3 0·02.

† Vitamin mixture (mg/kg): retinyl acetate 150, cholecalciferol 62·5, tocopherol acetate 5000, menadione 100, thiamin HCl 1000, riboflavin 1000, niacin 4500, D-pantothenate Ca 3000, pyridoxine HCl 1000, inositol 5000, D-biotin 20, folic acid 200, cyanocobalamin 1·35, ascorbic acid 10 000, para-aminobenzoic acid 5000, choline chloride 75 000. Sucrose was added to a total of 1 kg.

Table 2. Composition of milk and yoghurt used

Ingredient	Milk	Yoghurt
Protein (g/kg)	34	34
Carbohydrate (g/kg)	45	36
Lipid (g/kg)	50	46
Energy (MJ/kg)	3·10	3·08
Chlorine (mmol/kg)	27	38
Sodium (mmol/kg)	10	20
Potassium (mmol/kg)	39	49
Magnesium (mmol/kg)	2·8	5·7
Calcium (mmol/kg)	31	41

Preparation of cellular suspensions

All procedures were performed under aseptic conditions. The rats were anaesthetised with 40 mg pentobarbital/kg (Sanofi, Paris, France).

Peripheral blood. Blood (10 ml) was withdrawn from the abdominal aorta into a heparinised syringe. The BMC fraction was obtained by density gradient centrifugation on Ficoll Hypaque (Pharmacia Biotech, Orsay, France) (Boyum, 1968). BMC were washed with RPMI 1640 (Gibco) supplemented with fetal calf serum (50 ml/l) (Gibco) and 100 μg gentamicin/ml (Gibco); this medium was used in all experiments unless otherwise indicated. The red blood cells were removed by incubating BMC for 8 min with 0·16 M-NH_4Cl in ice and washed twice with medium.

Spleen cells. The spleen was removed from each rat and placed in sterile Petri dishes in medium, passed through a cell strainer (Falcon, Paris, France), red blood cells were removed by the same method as used for BMC. Then, the cells were washed twice with medium.

Peyer's patches. All PP were carefully excised from the whole length of the small intestine, removed and transferred to Petri dishes containing medium. PP were slit by a surgical blade and teased gently. The cellular suspensions were passed through a cell strainer and then through a sterilised gauze band to remove cell debris and washed twice with medium. Cells were counted at the end of the preparation of cellular suspensions, and the proportion of viable cells was determined by Trypan Blue exclusion before culture.

Proliferation assays

A single-cellular suspension of BMC, spleen cells or PP cells was incubated in a flat bottom, ninety-six-well culture plate (Falcon) at 2×10^5 cells/well in complete medium (RPMI 1640 (Gibco) supplemented with fetal calf serum (100 ml/l), 2 mM-L-glutamine, 100 U penicillin/ml, 100 μg

streptomycin/ml, 5×10^{-5} M-2-mercaptoethanol and 100 μg gentamicin/ml). Triplicate wells were challenged with a dose of 2×10^3, 2×10^4, 2×10^5 or 2×10^6 bacteria/well for 48 h. The cells were pulsed with $27{\cdot}7 \times 10^4$ Bq [6 − ^{3}H]thymidine (specific activity 185 GBq/mmol; Amersham, Les Ulis, France) in a volume of 20 μl per well. The cells were harvested on glass filter paper 18 h later and the incorporation of [^{3}H]thymidine into DNA was measured with an Inotech counter (Automatic filter counting system IBN-384, Dottikon, Switzerland). Several concentrations of Con A were tested from 1 to 10 μg/ml, to determine an optimal concentration (Fig. 1).

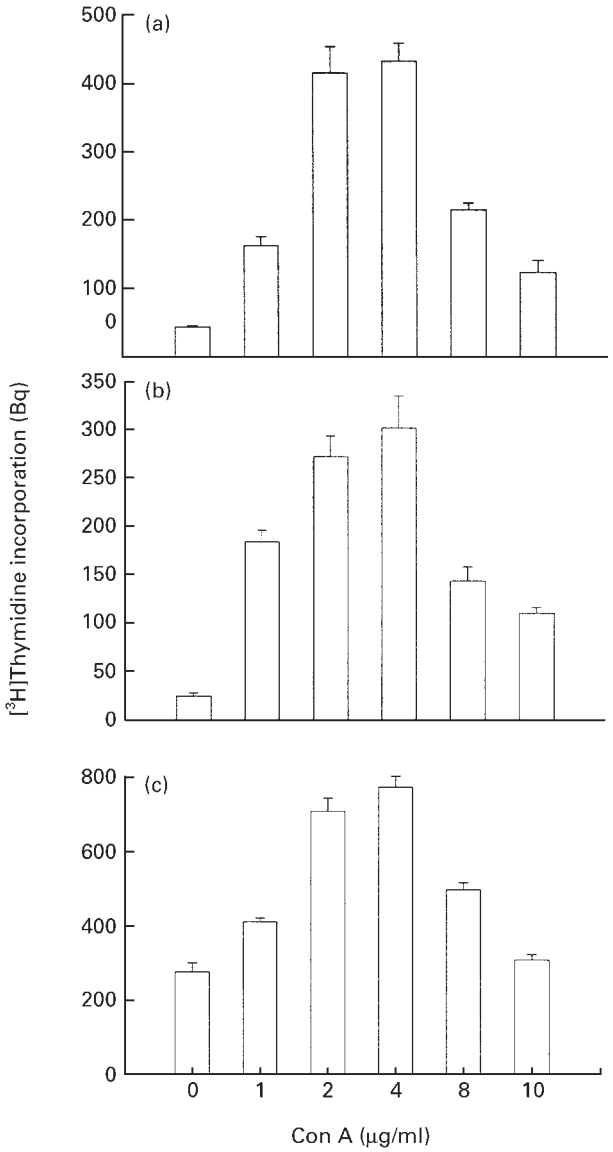

Fig. 1. Determination of optimal concanavalin A (Con A) concentration: proliferative response of (a) Peyer's patches cells, (b) blood mononuclear cells and (c) spleen cells to Con A. Cells were obtained from rats fed basal diet. They were incubated (2×10^6 cells/ml) with different quantities of Con A for 48 h and pulsed with [^{3}H]thymidine. For details of diet and procedures, see Table 1 and p. 368. Values are means for four rats per group with standard errors shown by vertical bars.

Determination of optimal in vitro *conditions of interferon-γ production*

To determine the optimal conditions for IFN-γ production, two concentrations of cells (1×10^6 and 2×10^6 cells/ml) were incubated for 24 or 48 h with different concentrations of bacteria (2×10^4, 2×10^5, 2×10^6 or 2×10^7 bacteria/ml). The optimal production of IFN-γ was obtained with 2×10^5 bacteria/ml in the case of BMC and PP and spleen cells, when incubated for 48 h. Thus, on the basis of these results, the following conditions were kept in all subsequent experiments: 2×10^6 cell/ml, 2×10^5 bacteria/ml and incubation time 48 h.

Cell cultures

BMC, spleen cells or PP cells (100 μl; 1×10^6 and 2×10^6/ml) were cultured in a total volume of 200 μl in ninety-six-well plates in complete medium. Single-cell suspensions were incubated with several densities of bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus* (1:1), over a range of 2×10^4 to 2000×10^4). Cultures were incubated for 48 h at 37°C in air – CO_2 (95:5, v/v), with either bacterial mixture or 2 μg Con A/ml (optimal concentration). At the end of the incubations, cell-free supernatant fractions were obtained and stored at −80°C until assayed for IFN-γ.

Interferon-γ assays

Interferon-γ concentration was determined by ELISA using a kit from Holland Biotechnology (ref. 13281-019; Life Technologie, Paris, France) according to the manufacturer's instructions. A linear dose–response curve was obtained between 250 and 10 000 pg/ml. The intra-assay CV was 8·5 %, the inter-assay CV was 11·9 %.

Fluorescence staining and flow cytometric analysis

The expression of cell surface markers was investigated by indirect immunofluorescence. Briefly, BMC, spleen cells or PP cells (10^6 cells/ml) were incubated with mouse monoclonal antibodies to characterise B lymphocytes (OX12, mouse IgG2a antibody to rat κ light chain; 0·6 μg/10^6 cells), T cytotoxic–suppressor (OX8, mouse IgG1 anti-CD8; 0·4 μg/10^6 cells) and macrophages–monocytes (ED1, mouse IgG1, anti-CD68; 0·2 μg/10^6 cells) for 1 h at 4°C. Analysis of each cell suspension included an isotype negative control in which cells were incubated with mouse IgG2a (F10-89-4; 0·2 μg/10^6 cells) or mouse IgG1 (F8-11-13; 0·5 μg/10^6 cells). After two washings with PBS supplemented with 50 ml fetal calf serum/l and 15 mmol sodium azide/l, the cells were treated for 30 min at 4°C, with F(ab′)$_2$ rabbit anti-mouse IgG R-phycoerythrin conjugate (10 μg/10^6 cells) in the case of staining B lymphocytes and macrophages–monocytes, and with streptavidin conjugated to R-phycoerythrin in the case of staining T cytotoxic–suppressor. To stain T lymphocytes and T helper cells, a direct assay with a phycoerythrin-conjugated mouse anti-rat IG (OX19, mouse IgG1, anti-CD5; 10 μg/10^6 cells) and a fluorescein isothiocyanate-conjugated

mouse anti-rat immunoglobulin (W3/25, mouse IgG1, anti-CD4; 0·5 μg/10^6 cells), were used respectively. Fluorescein isothiocyanate-conjugated mouse IgG1 (2 μg/10^6 cells) or phycoerythrin-conjugated mouse IgG1 (10 μg/10^6 cells) were used as negative controls for direct assay. Cells were incubated for 30 min at 4°C and washed twice. As indicated earlier, the antibodies used were able to identify differentiated cells, however, unlabelled cells (undifferentiated cells) that could not be reacted with the used antibodies remained unidentified and thus could not be counted.

The stained surfaces were fixed in paraformaldehyde (10 ml/l) and analysed in a FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France). All antibodies were purchased from Serotec (Oxford, Oxon., UK).

Statistics

Data were analysed by ANOVA or *t* test using the procedures of Statistical Analysis Systems, version 6.03 (SAS Institute Inc., Cary, NC, USA). $P<0·05$ was taken to indicate statistical significance. The results are reported as mean values with their standard errors.

Results

Rats fed for 4 weeks on either milk- or yoghurt-supplemented diets consumed the same amount of food (19 (SEM 0·35) g/d). As calculated from the composition of the diet, the rats fed yoghurt consumed about $1·4 \times 10^9$ bacteria/d. Weight gain was not significantly different between the two groups (3·25 (SEM 0·17) g/d).

Lymphoid cell surface markers

BMC, spleen cells and PP cells isolated from rats fed for 4 weeks on either milk- or yoghurt-supplemented diets were first analysed for surface markers of lymphoid cells, i.e. B lymphocytes, T lymphocytes, T lymphocyte subsets and monocyte–macrophage cells. The number of cells per PP was significantly higher in the yoghurt-supplemented group than in the milk-supplemented group ($P<0·05$). The increase in cell number was entirely explained by a significant ($P<0·05$) increase in the number of B lymphocytes from $24·0 \times 10^6$ (SEM $0·9 \times 10^6$) in rats fed milk to $33·0 \times 10^6$ (SEM $2·6 \times 10^6$) in rats fed yoghurt. The proportions of the other cell types in PP were unaffected by the consumption of yoghurt. No significant changes were detected in the number of cells per spleen and in the proportions of the spleen and blood lymphocytes, lymphocyte subsets and macrophages–monocytes between the two groups (Table 3).

In vitro *proliferation of blood mononuclear cells, spleen cells and Peyer's patches cells*

Cells isolated from blood, spleen and PP of rats fed for 4 weeks either with milk- or yoghurt-supplemented diets were incubated *in vitro* in the presence of Con A or lactic-acid bacteria, and their capacity to proliferate measured as the incorporation of [^{3}H]thymidine after 48 h incubation. Lymphocytes from the two groups proliferate in response to Con A, this response was greater for cells from rats fed the yoghurt diet (Table 4). As shown in Fig. 2, lymphocytes from rats fed the milk-supplemented diet did not proliferate in response to bacteria. Feeding yoghurt for 4 weeks significantly increased the proliferative response of BMC, spleen cells and PP cells to the lactic-acid bacteria. An optimal effect was obtained for 2×10^5 or 2×10^6 bacteria/ml: cell proliferation was increased 122-, 43- and 21-fold for spleen, PP and blood cells respectively ($P< 0·001$ for each tissue).

In vitro production of interferon-γ by blood mononuclear cells, spleen cells and Peyer's patches cells

BMC, spleen cells and PP cells from the rats fed for 4 weeks either with the milk- or yoghurt-supplemented diets were incubated *in vitro* in the presence of either lactic-acid bacteria or a mitogen (Con A), and the concentrations of IFN-γ in the supernatant fractions of the culture media measured by ELISA.

Table 3. Effect of feeding rats with yoghurt *v.* milk on the proportion of immune cells†
(Mean values with their standard errors for four rats per group)

		Cells labelled (%)‡									
		B lymphocytes		T lymphocytes		T helper lymphocytes		T cytotoxic–suppressor lymphocytes		Macrophages–monocytes	
	Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Peyer's patches	Milk	59·8	1·5	16·5	1·1	14·1	1·1	4·5	1·5	3·0	0·7
	Yoghurt	81·8***	3·3	17·7	1·1	15·1	1·4	5·0	0·7	3·5	1·5
Blood	Milk	19·0	2·6	68·5	2·5	54·0	2·6	23·5	2·2	3·7	1·3
	Yoghurt	20·8	2·9	71·9	1·2	55·9	1·9	23·6	2·8	4·4	1·4
Spleen	Milk	43·6	2·6	42·6	3·6	37·0	2·7	19·5	2·3	4·0	0·6
	Yoghurt	44·0	4·5	43·1	3·5	37·8	4·4	17·8	1·0	4·0	0·7

† For details of diets and procedures, see Tables 1 and 2 and p. 368.
‡ The percentage of cells labelled was determined by indirect and direct immunofluorescence B lymphocytes (B), cytotoxic–suppressor lymphocytes (CD8) monocytes–macrophages (CD68) and T lymphocytes (CD5) helper lymphocytes (CD4) respectively.
Mean value was significantly different from those of the milk group: ***$P<0·001$.

Table 4. Effect of feeding rats with yoghurt *v.* milk on the *in vitro* proliferative response to concanavalin A†‡

(Mean values with their standard errors for eight rats per group)

		[^{3}H]Thymidine incorporation (Bq)‡			
		Cell alone		Con A (2 μg/ml)	
	Group	Mean	SEM	Mean	SEM
Peyer's patches	Milk	43	3	465	21
	Yoghurt	40	5	1675***	75
Blood	Milk	21	2	224	13
	Yoghurt	24	6	876***	49
Spleen	Milk	141	11	657	55
	Yoghurt	138	7	14651***	134

† For details of diets and procedures, see Tables 1 and 2 and p. 368.
‡ Cells were obtained from rats fed on diets enriched with milk or yoghurt for 4 weeks. They were incubated (2×10^6 cells/ml) with Con A for 48 h and pulsed with [^{3}H]thymidine. Con A, concanavalin A.
Mean values were significantly different from those of the milk-fed groups: ***$P<0{\cdot}05$.

IFN-γ was not detected in supernatant fraction cells incubated in absence of bacteria or of Con A. In contrast, a high production of IFN-γ was observed in presence of bacteria or Con A in both groups of rats. Comparison between the two groups (milk and yoghurt) showed that IFN-γ production in the yoghurt group was doubled after mitogen or bacterial stimulation in both spleen cells and PP cells. However, results obtained from BMC did not reveal any diet effect of diet on IFN-γ production (Table 5).

Discussion

Our results demonstrate that a stimulatory effect on intestinal and systemic immune functions is observed when rats are fed a diet containing the live lactic-acid bacteria of yoghurt compared with a diet enriched with milk. This significant effect was characterised by a higher proliferative response of lymphocytes from the PP, the spleen and blood, by an elevated production of IFN-γ from the PP and spleen cells, and by a greater number of PP B lymphocytes. This work also shows rat cells studied *ex vivo* have a similar response to that previously obtained with mouse and human cells (Solis & Lemonnier, 1991, 1993; Muscettola *et al.* 1994; Aattouri & Lemonnier, 1997) and that rats are a convenient animal model allowing studies of immune functions of PP.

The milk used to prepare yoghurt, and the milk used for the milk-enriched diet, came from the same batch, thus the differences observed between the two groups of rats are due to the presence of bacteria in the yoghurt or/and to the fermentation process which might have generated active products. We believe that it is the bacteria which are responsible for the observed effects: in our *in vitro* experiments they stimulated cell proliferation as well as the production of IFN-γ. It has also been shown that IFN-γ induction is attributable to bacterial cell walls (Solis & Lemonnier, 1993). Muramyl dipeptide, a fragment of peptidoglycans (the major component of lactic-acid bacteria cell wall), stimulates the production of IFN-γ by CD4+ lymphocytes (Aattouri & Lemonnier, 1997). In addition, orally administered muramyl peptide can induce

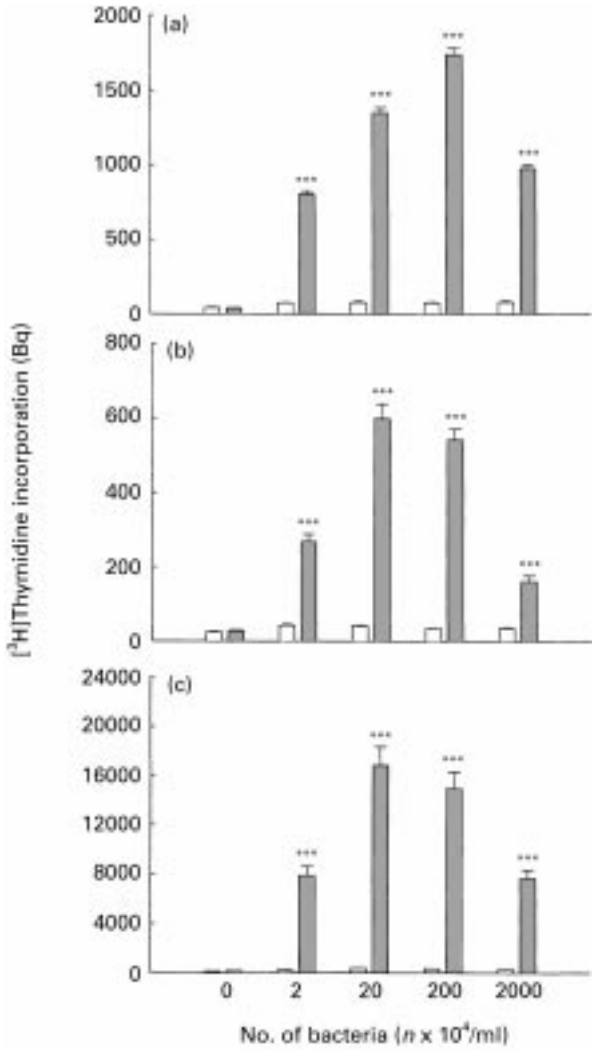

Fig. 2. Effect of feeding rats with milk *v.* yoghurt on the proliferative response, proliferative response of (a) Peyer's patches cells, (b) blood mononuclear cells and (c) spleen cells to the lactic bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (1:1) Cells were obtained from rats fed on diets enriched with milk (□) or yoghurt (▨) for 4 weeks. They were incubated (2×10^6 cells/ml) with different quantities of bacteria for 48 h and pulsed with [^{3}H]thymidine. Optimal concentrations were determined to be 20×10^4 and 200×10^4 bacteria/ml. For details of diets and procedures, see Tables 1 and 2 and p. 368. Values are means for eight rats per group with standard errors shown by vertical bars. Mean values were significantly different from the milk-fed group at each concentration of bacteria added in the media: ***$P<0{\cdot}001$.

endogenous cytokines (Okutomi *et al.* 1990). Thus, lactic-acid bacteria, which are sensitive to lysozyme digestion, may liberate peptidoglycan fragments *in situ*, which can then induce immunomodulating effects at the intestinal level.

Lactic-acid bacteria enter the host's body through the feeding process. It is known that lactic-acid bacteria are able to be in contact with the immune system of the gut-associated lymphoreticular tissue, which is largely represented by the PP. Ingested antigens are known to be first taken up by active pinocytic cells, i.e. M cells, which cover the dome region of PP, and are then delivered to

Table 5. Effect of feeding yoghurt *v.* milk on *in vitro* interferon-γ production†‡
(Mean values with their standard errors for eight rats per group)

		Interferon-γ concentrations (pg/ml)				
			Lactic bacteria (2×10^5)		Con A (2 μg/ml)	
	Group	Cells alone	Mean	SEM	Mean	SEM
Peyer's patches	Milk	ND	329	57	741	50
	Yoghurt	ND	731***	77	1464***	170
Blood	Milk	ND	3772	899	5177	1228
	Yoghurt	ND	4170	1102	5640	1027
Spleen	Milk	ND	11110	1619	13966	2027
	Yoghurt	ND	26692*	7364	28051*	6014

ND, not detected.
Mean values were significantly different from those of the milk-fed groups: *$P<0{\cdot}05$, ***$P<0{\cdot}001$.
† For details of diets and procedures, see Tables 1 and 2 and p. 368.
‡ *In vitro* production of interferon-γ by 2×10^6 cells/ml in different sites after 48 h incubation in the presence of either 2×10^5 cells/ml lactic bacteria or Con A, from rats fed on diets enriched with milk or yoghurt for 4 weeks.

their underlying T and B cell zones (Bienestock & Befus, 1980; Neutra *et al.* 1987). Thus, bacteria and their walls are generally presented to M cells and then to lymphocytes, whether the bacteria were still viable or whether they were killed in the digestive tract. Indeed, these lymphocytes obtained from rats fed yoghurt, when incubated in presence of lactic-acid bacteria, produced high levels of IFN-γ and demonstrated an enhanced proliferative response. Thus, these cells have been sensitised by the bacteria contained in yoghurt, a mechanism that may lead to an enhanced production of IFN-γ by T and natural killer cells (Lefèvre *et al.* 1996). Interferon-γ is known to stimulate the production of interleukin 2 which is involved in B and T lymphocyte proliferation (Nakanishi *et al.* 1992). Thus, IFN-γ could be implicated in this in increased proliferative response to bacteria. The higher number of B lymphocytes observed in the PP of rat fed lactic-acid bacteria may be in accordance with a greater *in vivo* proliferative process.

Lymphocytes from PP are known to be released from the intestine to reach the systemic circulation (Guy-Grand *et al.* 1978; Ruedl *et al.* 1993) and the effects of antigen feeding on this phenomenon are documented (Mowat, 1987). In rats that were fed lactic-acid bacteria, proliferation of blood and spleen lymphocytes was higher *in vitro* when incubated in presence of lactic-acid bacteria. Thus, the circulating activated cells from the PP could explain the higher proliferative response observed in the spleen and in the blood of lactic-acid bacteria-fed rats.

The diet containing lactic-acid bacteria did not induce any change in IFN-γ production by BMC and cell proliferation was less elevated than that observed for cells from PP and the spleen. This is not surprising since the blood contains a small and labile pool of responsive lymphocytes (Westermann & Pabst, 1990). However, in yoghurt-fed healthy human subjects, a higher activity of 2–5A synthetase in circulating BMC has been observed (Solis & Lemonnier, 1991; Aattouri & Lemonnier, 1997). Circulating 2–5A synthetase might be a more sensitive marker than an *in vitro* response. Moreover, the activity of 2–5 A synthetase can be induced by other interferons that were not measured in our present study.

The exposure of intestinal immune cells (PP may not be the exclusive site of action) to lactic-acid bacteria *in vivo* caused elevated *in vitro* proliferation and the production of IFN-γ by addition of lactic-acid bacteria in the culture medium. As expected, Con A, a polyclonal activator of T cells, enhanced the production of IFN-γ from incubated PP and spleen cells obtained from rats fed the milk-supplemented diet. Furthermore, this non-specific response was elevated by ingestion of lactic-acid bacteria, since IFN-γ production doubled when the rats received the lactic-acid bacteria diet. These results demonstrate that feeding live lactic-acid bacteria contained in yoghurt exerts an immunoadjuvant effect on the host.

One major function of T helper cells is to provide signals necessary for activation, proliferation and differentiation of B cell. These effects depend on the cytokines being secreted at the time of T–B cell interaction. Interferon-γ is able to provide help to B cell in the production of the IgG2a isotype (Coffman *et al.* 1988). Interferon-γ induces expression of class II of major histocompatibility complex determinants on various cell types such as macrophages and epithelial intestinal cells. Interferon-γ treatment increases antigen presentation, induces activation of oxidative metabolism and inhibits the growth of intracellular parasites in macrophages (Murray, 1990). The known effects of IFN-γ could explain or contribute to the observed effects of feeding lactic-acid bacteria or fermented milk on macrophage phagocytosis and production of IgG2a (Conge *et al.* 1980; Vesely *et al.* 1985; Perdigon *et al.* 1988). Previous studies have shown that laboratory animals fed diets supplemented with fermented milks are partially protected against *Salmonella* (Hitchins *et al.* 1985; De Simone *et al.* 1988; Perdigon *et al.* 1990). Moreover, pretreatment with IFN-γ protects mice against *S. typhimurium* (Gould & Sonnenfeld, 1987). Thus, it is suggested that the stimulation of IFN-γ by consumption of live lactic-acid bacteria might contribute to protection against potentially harmful intestinal microflora and enteropathogenic micro-organisms.

In conclusion, this present study demonstrates that oral administration of lactic-acid bacteria stimulates the production of IFN-γ in PP and spleen cells in healthy rats and elevates the proliferation of lymphocytes from PP,

spleen and blood. This present study suggests that beneficial consequences might be obtained by an increase in resistance to some infections.

Acknowledgements

We thank the Centre Daniel Carasso for supplying bacterial preparations and EU STD3 program no. TS3-CT94-0282 for their financial support of this work. We are also grateful to M. Dubarry, M. Rautureau and J.F. Huneau for their very helpful suggestions and to R. Medeiros for his advice in editing the manuscript.

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