Immunology 1988 **63**, 17–23

Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*

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Accepted for publication 30 July 1987

SUMMARY

This study investigates the effect of feeding fermented milks with *Lactobacillus casei*, *Lactobacillus acidophilus* and a mixture of both micro-organisms on the specific and non-specific host defence mechanisms in Swiss mice. Animals fed with fermented milk for 8 days (100 μg/day) showed an increase in both phagocytic and lymphocytic activity. This activation of the immune system began on the 3rd day, reached a maximum on the 5th, and decreased slightly on the 8th day of feeding. In the 8-day treated mice, boosted with a single dose (100 μg) on the 11th day, the immune response increased further. The feeding with fermented milk produced neither hepatomegaly nor splenomegaly. These results suggest that *L. casei* and *L. acidophilus*, associated with intestinal mucosae, can influence the level of activation of the immune system. The possible clinical application of fermented milks as immunopotentiators is also discussed.

INTRODUCTION

Foods fermented with lactic acid bacteria are important from a historical standpoint, not only for their nutritional value, but also because the fermentation process improves the bioavailability of milk proteins.

In recent years, increased emphasis has been laid on the therapeutic effect of some species of lactobacilli. Bogdanov *et al.* (1977) were the first to observe that *Lactobacillus bulgaricus* possesses a potent anti-tumour activity. There have been many reports on the fact that various lactobacilli have considerable anti-tumour activity, depending on the immunostimulation against experimental and human malignant tumours. The species involved include *L. bulgaricus*, *L. casei*, *L. acidophilus* and products fermented with these lactobacilli (Goldin & Gorbach, 1980; Yasutake *et al.*, 1984; Shahani, Friend & Bailey, 1983; Reddy *et al.*, 1983). Lactic acid bacteria are the starters of dairy products and other foods. Bearing all these facts in mind, it is important to know the effect of the oral administration of these organisms on the immune system. It has been shown that the oral administration of erythrocytes causes the appearance of circulatory antibodies, mainly of the IgA class (Heremans, 1974), and plaque-forming cells (PFC) have been identified in the course of the intragastric stimulation of sheep red blood cells (SRBC) (Andre, Bazin & Heremans, 1973). However, it is clear that the antigenic stimulation by the oral route can also lead to suppression of both humoral and cellular responses: these consequences seem to depend on the form of the antigen, the number of feeds, and the lymphoid organ assayed. Namba *et al.* (1981) showed that lysozyme or digested cell walls presented by the oral route enhance the immune response in guinea-pigs.

In our previous studies, we reported that *L. bulgaricus*, *L. casei* and *L. acidophilus* administered by the oral route can activate peritoneal macrophages (Perdigón *et al.*, 1986a) and the mononuclear phagocytic system (Perdigón *et al.*, 1986c). We also showed that the mixture of *L. casei* and *L. acidophilus* in the absence of fermentation can potentiate the immune response (Perdigón *et al.*, 1986b). We found greater activation of the immune system with these micro-organisms, which are capable of surviving and growing in the intestinal tract.

The present work was carried out in order to find out whether feeding with milk fermented with *L. casei* or *L. acidophilus* or a mixture of both can potentiate the effect that these micro-organisms have on the immune system of mice. An additional aim is to determine whether the prolonged administration of fermented milk produces collateral effects such as hepatomegaly or splenomegaly. The possible use of these micro-

Abbreviations: IgM, immunoglobulin M; K, phagocytic index; MDP, muramyl dipeptide. NADH, nicotine amine adenine dinucleotide, reduced form; NFM, non-fat milk; oNPG, o-nitrophenyl-β-D-galacto-pyranoside; PFC, plaque-forming cells; pNPG, p-nitrophenyl-β-D-gluco-pyranoside; SRBC, sheep red blood cells; $t_{\frac{1}{2}}$, clearance rate of carbon.

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organisms as immunopotentiators, and also in the prevention and treatment of gastrointestinal infections by immunobiological methods, is discussed.

MATERIALS AND METHODS

Animals
Swiss albino mice, each weighing 25–30 g, were obtained from the random-bred closed colony kept by our department at CERELA. The animals were housed in plastic cages and kept at room temperature. Each experimental group for the different type of assays consisted of 32–40 mice (eight to 10 mice for each different day).

Micro-organisms
The micro-organisms used were *L. acidophilus* and *L. casei*, both isolated from human faeces. The cultures were kept freeze-dried and then rehydrated using the following medium: peptone 150 g, tryptone 10·0 g, meat extract 50 g, distilled water 1 litre, pH 7. They were subcultured and cultured for 8 hr at 37° (final log phase) in lactobacillus MRS (Man-Rogola-Sharpe) broth (Oxoid, Hampshire), harvested by centrifugation and washed several times with sterile saline solution. All treatments were administered as a 20% suspension in drinking water. The micro-organisms were resuspended in 10% powder non-fat milk (NFM) and incubated at 37° for 4 hr. The mixture of the fermented milks was made after the 4-hr period of incubation at 37°. The number of viable cells was determined by the agar plate method (Clark *et al.*, 1978) and the cells' protein by the Lowry technique (Lowry *et al.*, 1951).

Drinking and inoculation procedures for the different assays
Mice were housed individually and dosed with 100 μg of cell protein ($2{\cdot}4 \times 10^9$ cells) daily for 8 consecutive days. One group was fed with *L. acidophilus*-fermented milk, another with *L. casei*-fermented milk, and the third with a mixture of both (called fermented milk hereafter). The control group received 10% NFM administered at 20% concentration in the drinking water. The administration of the lactobacilli (100 μg protein daily) cultured in NFM increased the level of protein in the drinking water to 0·6% of the protein content of the diet. All the groups of mice were fed *ad libitum* with a balanced diet.

The feeding schedule described above was used for the examination of enzymatic and *in vitro* phagocytic activity of peritoneal macrophages, as well as for the carbon clearance test. Both assays were effected on the 2nd, 3rd, 5th and 8th days of the feeding period.

The supernatant control groups received the supernatant of fermented milks, centrifuged at 10,000 ***g*** for 10 min, for 5 consecutive days.

For the PFC assays, animals were dosed with 100 μg protein for 8 consecutive days. On Day 8 they were inoculated i.p. with a suspension of 1×10^9 SRBC. Mice were killed on the 2nd, 4th, 5th and 8th days after SRBC inoculation.

For the antibodies assay, mice were fed for 5 days. After they were immunized s.c. with $3{\cdot}3 \times 10^8$ SRBC three times at 24-hr intervals, blood was taken on Days 5, 10 and 15 after the third immunization.

Macrophage collection and culture
Mice were killed by cervical dislocation on the 2nd, 3rd, 5th and 8th days of lactobacilli feeding. Cultured and non-cultured peritoneal macrophages were processed as described by Perdigón *et al.* (1986b).

Enzyme assays
The peritoneal macrophage activity was quantified by assaying lysosomal and non-lysosomal enzymes released in the supernatant of cultured peritoneal macrophages. β-Glucuronidase was assayed by the method of Stossel (1980) and β-galactosidase by the method of Conchie, Findlay & Levvy (1959), using the synthetic substrates p-NPG and o-NPG (Sigma, St Louis, MO), respectively. Lactate dehydrogenase was determined by the oxidation rate of NADH at 340 nm using 2·3 mM pyruvate in 0·05 M phosphate buffer, pH 7·5.

Phagocytosis assays in vitro
The *in vitro* phagocytic activity of peritoneal macrophages was measured with lactobacilli, *Salmonella typhi* and opzonized *S. typhi*. The assays were performed as described by Perdigón *et al.* (1986c).

Phagocytosis assay in vivo
The colloidal carbon clearance was determined as described by Perdigón *et al.*, (1986c). The phagocytic index, K, was calculated by the method of Biozzi *et al.* (1961) and the clearance rate of carbon ($t_{\frac{1}{2}}$) according to Kato, Yokokura & Mutai (1983).

Plaque-forming cells assays
The method described by Jerne & Nordine (1963) and modified by Dresser (1979) was used to determine the number of PFC. The direct method was used for spleen IgM-producing cells against SRBC inoculated i.p. Results were expressed as PFC number/10^6 spleen cells.

Circulating antibodies
Mice were bled from the retro-orbital venous plexus. The serum was diluted and the antibodies titres determined against 1% SRBC and lactobacillus suspension (3×10^9 cells) with haemagglutination and agglutination reaction, respectively.

Time effect of feeding with fermented milk on the immune system
In order to determine the lowest dose of fermented milk needed to produce the stimulation of the immune system, mice were fed for 2, 3, 5 and 8 days. On those days, a carbon clearance test was performed and SRBC were inoculated i.p. to carry out the PFC assay on the 5th day after SRBC inoculation (optimum day of PFC production).

Boosting effect
A group of mice was fed for 8 consecutive days, then boosted with a dose of 100 μg fermented milk on the 11th day. On the 2nd, 5th and 8th days after boosting (13, 16 and 19 days after the first stimulus) the carbon clearance was quantified and SRBC were inoculated for PFC responses.

Secondary effects
Groups of mice of the same weight were fed with fermented milk, sterile milk, and water for 10 consecutive days. Each experimental group consistent of 15 mice. On the 10th day, they

Table 1. Enzymes released from peritoneal macrophages of mice fed with fermented milks at a dose of 100 μg/day of lactobacilli

Day of feeding	Feeding treatment	Mean enzymatic activity ± SD		
		β-Galactosidase*	β-Glucuronidase†	Lactate dehydrogenase‡
2	Fermented milk	18·84 ± 8·4	12·30 ± 4·8	0·024 ± 0·003
	Milk fermented with *L. casei*	32·5 ± 5·5	10·03 ± 3·2§	0·085 ± 0·006
	Milk fermented with *L. acidophilus*	19·90 ± 2·1§	10·03 ± 3·2§	0·025 ± 0·008§
3	Fermented milk	28·65 ± 4·4	8·19 ± 1	0·021 ± 0·009
	Milk fermented with *L. casei*	58·10 ± 8·2	10·03 ± 3·2§	0·240 ± 0·028
	Milk fermented with *L. acidophilus*	19·90 ± 2·1§	10·03 ± 3·2§	0·025 ± 0·008§
5	Fermented milk	49·4 ± 1·7	11·74 ± 2·7	0·042 ± 0·002
	Milk fermented with *L. casei*	37·40 ± 3·8	10·03 ± 3·2§	0·034 ± 0·002
	Milk fermented with *L. acidophilus*	19·90 ± 2·1§	10·03 ± 3·2§	0·025 ± 0·008§
8	Fermented milk	25·6 ± 5	10·00 ± 1·36§	0·029 ± 0·005
	Milk fermented with *L. casei*	37·50 ± 4·5	10·03 ± 3·2§	0·060 ± 0·006
	Milk fermented with *L. acidophilus*	19·90 ± 2·1§	10·03 ± 3·2§	0·025 ± 0·008§

* Enzymatic activity, nmols ONP liberated/hr/10^6 cells.
† Enzymatic activity nmols pNP liberated/hr/10^6 cells.
‡ Enzymatic activity nmols NADH oxidized/min/10^6 cells.
§ Control values: βgal, 19·90 ± 2·1; β-gluc, 10·03 ± 3·2; LDH, 0·025 ± 0·008. Each experimental group consisted of 32–40 mice (eight to 10 mice for each day).

were killed and their bodies, spleens and livers weighed in order to determine if the prolonged administration of fermented milk had produced secondary effects such as hepatomegaly or splenomegaly.

RESULTS

Enzymatic activity in peritoneal macrophages

β-Galactosidase activity of cultured peritoneal macrophages from mice fed with fermented milk was 1·5-fold higher than that of the macrophages of control mice on the 3rd day of feeding, reaching its highest value on the 5th day and decreasing by the 8th. β-Glucuronidase and lactate dehydrogenase activity remained at values similar to the controls (Table 1).

In mice fed with *L. casei*-fermented milk, β-galactosidase enzymatic activity was two to three-fold higher than in the controls, and lactate dehydrogenase three to six times higher. β-Glucuronidase remained at values close to those of controls.

In mice fed with *L. acidophilus*-fermented milk, none of the above enzymes showed differences with the results from control mice. The results were analysed by the Student's *t*-test.

Enhancement of phagocytic activity

In vitro phagocytic activity of peritoneal macrophages from mice treated with fermented milk (Table 2) showed a peak on the 2nd day of administration, with values three to four-fold higher than controls, and maintained values of 50% macrophage-phagocytosing bacteria until the 8th day.

In mice fed with *L. casei*-fermented milk, a peak was reached on the 3rd day, with values three-fold higher than controls up to the 8th day.

In the feeding with *L. acidophilus*-fermented milk, the values obtained were three times higher than controls from the 2nd day onwards.

No significant differences (Student's *t*-test) between opsonized and non-opsonized systems were observed in any of the three treatments.

Effect of fermented milks on the phagocytic function of the reticuloendothelial system in mice

From the 2nd day onwards, the colloidal carbon clearance from circulating blood was rapidly enhanced by the administration of

Table 2. Percentage phagocytosis of peritoneal macrophages in mice*

Phagocytosis system	Feeding treatment	% of phagocytosis: Day of feeding 2	3	5	8	Control
Without antibody †	Fermented milk	74·4±6	54·1±6	53·4±5	48±5	
	Milk fermented with *L. casei*	49±3	65·5±5	49·1±4	35±5	21±3
	Milk fermented with *L. acidophilus*	61±5	64·1±5	52·5±5·6	46·6±6	
With antibody ‡	Fermented milk	74·4±6	59±6	52·5±4	48±5	
	Milk fermented with *L. casei*	49±3	65·5±5	55±7	42±6	33±4
	Milk fermented with *L. acidophilus*	62·5±3·5	67±5·6	52·5±5·6	46·6±6	

* Peritoneal macrophages isolated from the treated mice were incubated with lactobacilli, *S. typhi*† and opsonized *S. typhi* ‡at 37° for 5 and 15 min. The macrophage phagocytosing bacteria were counted microscopically after incubation. Each experimental group consisted of eight to 10 mice for each day.

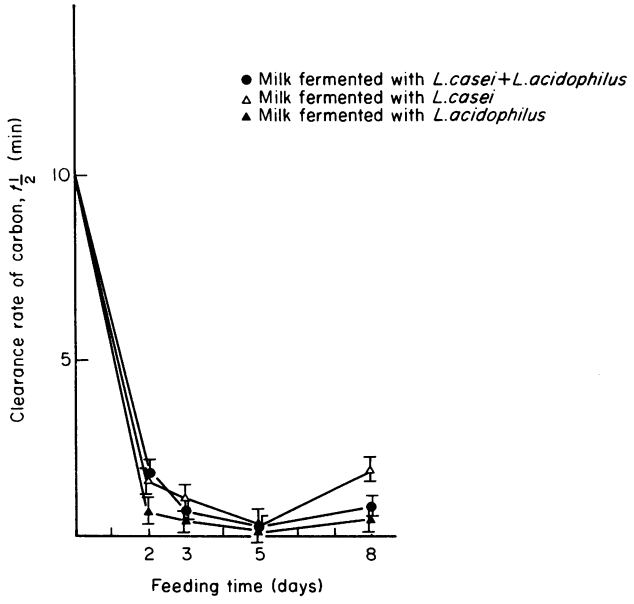

Figure 1. Comparative effect of blood clearance of colloidal carbon in mice fed with milk fermented with *L. casei*, *L. acidophilus*, and a mixture of both at a dose of 100 μg protein/day. On the 2nd, 3rd, 5th, and 8th day throughout the feeding, colloidal carbon was injected into mice, and blood samples taken at 0, 3, 6, 9, 12 and 15 min after injection. The clearance rate of carbon ($t_{\frac{1}{2}}$) was calculated as described previously (Perdigón *et al.* 1986c). Control values for mice fed with NFM (2, 3, 5 and 8 days) were 9·90±0·50. Points and bars represent mean±SD of each group of mice (eight to 10 mice for each day).

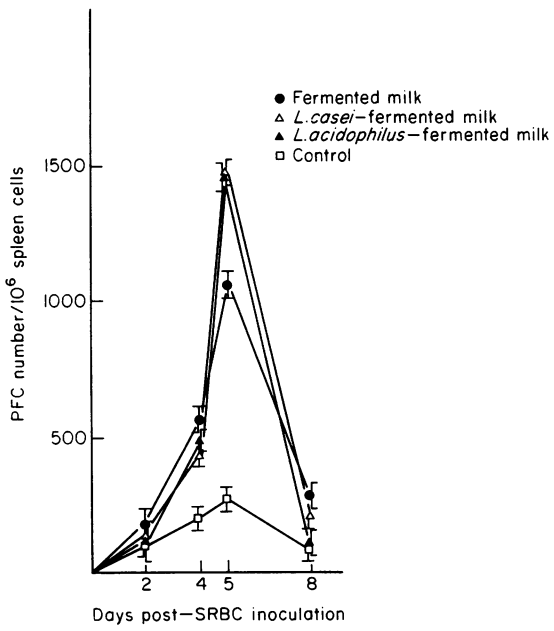

Figure 2. Production of IgM PFC in spleen cells against SRBC in mice. Fermented milks were given at a dose of 100 μg/day for 8 consecutive days. SRBC were inoculated on the 8th day, and the mice killed on the 2nd, 4th, 5th and 8th days following SRBC inoculation. PFC assays were performed as described by Dresser (1979). Points and bars represent mean±SD of each group of mice. The control group was fed with NFM.

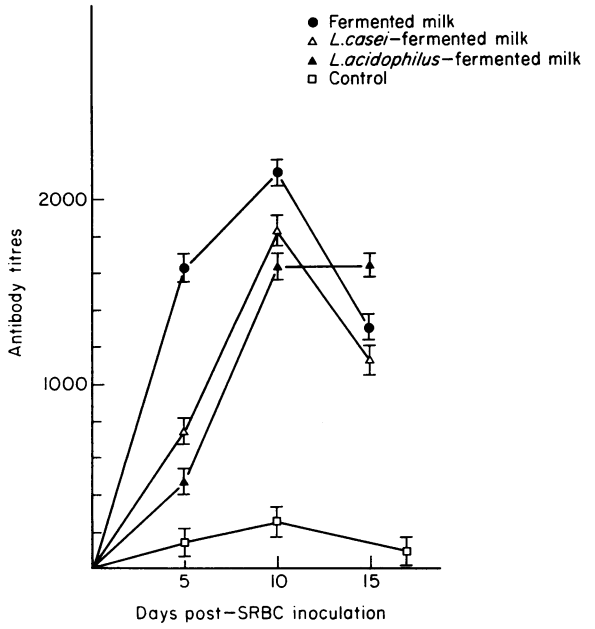

Figure 3. Circulating antibodies from mice fed with milks fermented with *L. casei*, *L. acidophilus*, and a mixture of both (fermented milk) at a dose of 100 μg/day for 5 consecutive days. SRBC were inoculated on the 6th, 7th, and 8th days, and mice bled on the 5th, 10th, and 15th days post-SRBC inoculation. Antibody titres were determined by haemagglutination reaction. The control group was fed with NFM.

the mixture of fermented milk (Fig. 1), reaching phagocytic indices much higher than in control mice. A peak of activity occurred on the 5th day, the clearance rate of carbon ($t_{\frac{1}{2}}$) remaining between 0·31 min and 1·84 min (control mice $t_{\frac{1}{2}}$, 10 min).

In the feeding with *L. casei*-fermented milk, the carbon clearance activity K was highest on the 5th day, decreasing slightly on the 8th day ($t_{\frac{1}{2}}$ between 0·38 min and 1·96 min) (Fig. 1). Treatment with *L. acidophilus*-fermented milk was the most effective, producing $t_{\frac{1}{2}}$ between 0·33 min and 0·64 min from the 2nd up to the 8th day of administration.

Effect of feeding fermented milk on the antibody response to SRBC

The response of spleen IgM-secreting cells to SRBC of mice fed with fermented milk was highest on the 5th day after SRBC inoculation, with values three-fold higher than those of control mice (Fig. 2).

In the feeding with *L. casei*- or *L. acidophilus*-fermented milks, the values of PFC obtained were five times higher than those of control and 1·5-fold higher than with the mixture on the 5th day after SRBC inoculation.

On the 8th day after SRBC inoculation, the values obtained with the mixture were slightly higher than those obtained with either single lactobacillus-fermented milks.

Circulating antibodies

The levels of circulating antibodies are plotted in Fig. 3. The values obtained with the different class of fermented milk were between six-fold and eight-fold higher than control.

Supernatant effect

In order to find out whether the immunostimulation effect observed in mice was caused by the micro-organisms or by some metabolites produced during the fermentation process, similar studies were carried out, this time feeding the mice with the supernatant of the cultures. The results of such treatments are summarized in Table 3.

Effect of feeding time

The effect of feeding time on the stimulation of the immune system is shown in Fig. 4. The lowest dose to produce a good stimulation of the IgM-producing cells and reticuloendothelial system is the one closest to 500 μg, on the 5th day of feeding.

Boosting effect

Figure 4 shows the boosting effect with a 100 μg dose. The number of PFC increased and this high level was maintained until the 8th day after boosting. The clearance rate of carbon was maintained at levels five times lower than in control mice.

Secondary effects

Table 4 summarizes the results of the spleen and liver weights of mice fed with fermented milk, sterile milk or water. No significant differences were observed (Student's *t*-test) between mice fed with fermented milk and control mice.

DISCUSSION

Several studies have shown that lactobacilli constitute one of the dominant groups in the intestinal and fecal flora, and that the harmful effects of putrefactive organisms could be minimized by maintaining the proper lactobacillus flora in the gut (Shahani & Ayebo, 1980). This consideration has contributed significantly to the interest in yoghurt and other cultured milks (Shahani & Chandon, 1979). In recent years, increased emphasis has been placed on the study of the therapeutic effect of lactobacilli and products fermented with lactic acid bacteria upon various human and animal gastrointestinal disorders (Shahani *et al.*, 1983; Reddy *et al.* 1983).

Our results demonstrate that feeding with milk fermented with *L. casei*, *L. acidophilus* or a mixture of both produces a remarkable effect on the immunostimulation in the host. We found that macrophage and lymphocyte activation was much higher in cultures fermented with *L. casei* and *L. acidophilus* or both than in the controls or in the individual or mixed culture without a fermentation process, as previously reported (Perdigón *et al.*, 1986a,b,c). Such enhancement of the immune response might be due to the substances produced by these organisms during the fermentation process, such as some metabolites and casein peptides, bacterial enzymes (proteolytic and bacteriolytic) that improve the digestibility of the milk constituents. This fact is reflected in the results obtained with the supernatant of the fermented milk cultures: we observed an increase in the immune response (see Table 3) independently of the presence of lactobacilli.

The effect of casein and its peptides on the gut-associated lymphoreticular tissue and the appearance of specific cell markers are the first parameters that show the condition of

Table 3. Effect of the supernatants of fermented milks on the immune system of mice

Supernatant from	PFC number/10^6 spleen cells*	Clearance rate of carbon	Circulating antibodies (dilution)‡
L. acidophilus + *L. casei*-fermented milk	497±102	1·83±0·03	1/358±1/140
L. acidophilus-fermented milk	462±81	1·82±0·06	1/760±1/150
L. casei-fermented milk	656±200	7·91±1·21	1/576±1/322
Control	250±60	10·00±0·50	1/256±1/140

The supernatant was obtained as described in the text.

* Mice were fed for 5 days. On the 6th day, SRBC were inoculated. The figures show mean±SD of PFC obtained on the 5th day post-SRBC inoculation (optimum day for PFC production).

† $T_{\frac{1}{2}}$ was determined after 5 days feeding, as described in the text.

‡ Mice were fed for 5 days. On the 6th day, they were inoculated s.c. with SRBC. Antibody titres were determined on the 5th, 10th and 15th days post-SRBC inoculation. The figures represent the higher titres obtained in any of these days.

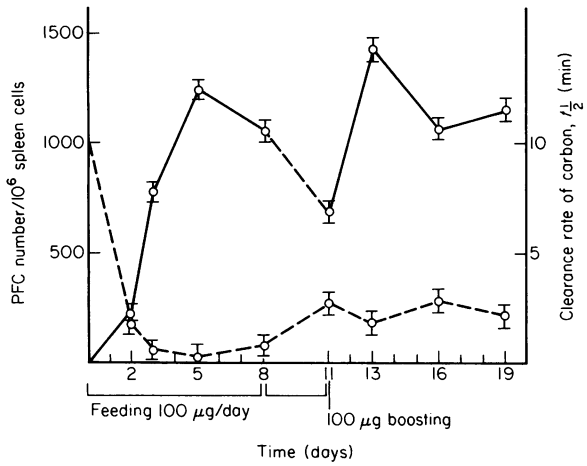

Figure 4. Effect of single boosting (100 μg) with fermented milk in mice previously fed for 8 consecutive days upon the spleen PFC response to SRBC, and carbon-clearance activity. PFC and clearance was determined after 2, 5 and 8 days of feeding mice at a dose of 100 μg/day. Mice were boosted on the 11th day, and the same techniques performed on the 2nd, 5th, and 8th days post-boosting. PFC and clearance assays were performed as described in the text.

Table 4. Colateral effects in mice fed with fermented milk

	Fermented milk	Milk	Water
Δ Body weight (g)	4·29±1·32	4·80±1·61	0·71±0·68
Spleen weight (g)	0·12*±0·01	0·12*±0·02	0·09*±0·04
Liver weight (g)	1·33±0·18	1·43**±0·15	1·24**±0·11

*$P<0·05$; **$P<0·01$ (Students' *t*-test).

Groups of mice of the same weight were fed for 10 consecutive days with fermented milk (100 μg/day), sterile milk or water.

Δ Body weight is the difference between the mice weight on the 1st and 10th day.

Spleen and liver weight are the results obtained after the 10-day feeding.

functional mucosae, and have been demonstrated by Slobodianik *et al.* (1984). It is also known that bacteriolytic enzymes such as lysozyme can release absorbable fragments of peptidoglycan such as muramyl dipeptide (MDP) from the intestinal bacteria, and subsequently could enhance the local and systemic immune response (Namba *et al.*, 1981). We consider that the degradation of milk constituents permits higher absorption and antigenic stimulation. Moreover, this effect is increased by the MDP contained in the cell wall of the lactobacilli strains studied.

We observed that the milks fermented with the mixture of *L. casei* and *L. acidophilus* were less effective in increasing the immune response than the milk fermented with either single lactobacillus. However, the values of macrophagic and lymphocytic activity increased significantly compared to the controls. The reason for the difference between these results is not clear because, although there are beneficial effects between *L. casei* and *L. acidophilus* that improve their survival rate, a competitive interaction has also been found between them which could reduce the viability of bacteria and produce a lower efficiency in the associated culture (Moon & Reinbold, 1976).

Our results show that the prolonged administration of fermented milk by the oral route has no harmful effects (Table 4) such as hepatomegaly, splenomegaly or toxicity on the host. This fact is an advantage as it permits the use of this fermented milk as immunostimulating agents. However, Bloskma *et al.* (1979) reported that some lactobacilli, such as *L. plantarum*, injected i.v., both dead and viable cells, cause an increase in the liver and spleen weight.

The ability of fermented milks to activate immunocompetent cells was persistent (Fig. 4). Mice fed on 8 consecutive days

and then given a single oral booster showed enhanced SRBC and PFC responses and carbon clearance. This may indicate the stimulation of memory cells in the lamina propia and in the Peyer's patches for MDP of lactobacilli.

Clinical applications of fermented milks with the mixture of *L. casei* and *L. acidophilus* in the prevention and treatment of gastrointestinal infections are possible since, although we found a lower effect on the activation of the immune system with this fermented milk, the presence of two lactobacilli strains increases the possibility of colonization of the intestinal tract. This colonization of the gut by lactobacilli (Kotarrsky & Savage, 1979) can inhibit enteropathogenic flora, by producing antibiotic substances (Babel, 1976) or inducing a specific local and systemic immune response in the host.

Although lactobacilli are known to be non-pathogenic micro-organisms, further studies on mucosal immunity are required before using milk fermented with *L. casei* and *L. acidophilus* as an immunobiological method in the treatment of gastrointestinal infections.

ACKNOWLEDGMENTS

The authors wish to thank Dr María Estela Roux for helpful discussions concerning this work.

This work was partially supported by grants from Secretaría de Ciencia y Tecnología, 1985, Argentina. G. Perdigón, M. E. N. de Macías, G. Oliver and A. P. de Ruiz Holgado are members of the Researcher Career of the National Research Council of Argentina.

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