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Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant

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Summary. The effect of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant on mucosal and tumoral immunity was studied. Milks fermented by *Lb. helveticus* wild type or its non-proteolytic variant were administered orally to mice for different periods (3, 5 and 7 d). The immune response was assessed by analysing the activity of the peritoneal macrophages, the number of cells secreting IgA associated with the gut-associated lymphoid tissue and with the bronchial-associated lymphoid tissue. The number of cells was determined by direct immunofluorescence. The antitumour activity was monitored by studying the regression of the subcutaneously implanted fibrosarcomas. After 3 d feeding of milk fermented by *Lb. helveticus* wild type, the number of sIgA increased significantly at both the intestinal and bronchial levels, indicating that a cellular migration had occurred. This effect was not noticeable when milk fermented by *Lb. helveticus* Protease (−) was orally administered. Both fermented milks (wild type or its variant) exhibited an effect on the activity of the peritoneal macrophages, which might be indirectly correlated to the regression of the fibrosarcoma. Although the mechanism by which the lactic acid bacteria enhance the immune system is not clear, this study clearly suggests that the bioactive compounds released during milk fermentation might contribute to the immunoenhancing properties of these products. By releasing biopeptide, lactic acid bacteria have important implications in modulation of the host's immune response, more specifically its cellular immune response.

Keywords: Probiotics, mucosal and tumoral immunity, bacterial proteolysis, biologically active peptides, nutraceuticals.

Intake of yogurt or fermented milk with a mixture of lactic acid bacteria (LAB) have all led to significant increases in various immune response parameters such as sIgA-producing cells, macrophage activity, specific antibody responses for infections compared to controls. Those products are well known as potent immunomodulators (Perdigon *et al.* 1995, 1999, 2000).

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Increasingly, epidemiological studies, scientific reports and clinical data indicate that consumption of fermented dairy products reduces the risk of certain types of cancer, including tumours of breast, colon, lung and subcutaneously implanted fibrosarcomas (Perdigon *et al.* 1993; Matsuzaki *et al.* 1996; Biffi *et al.* 1997). LAB orally administered can exert a protective effect against infections produced by enteropathogens. A noted survival rate of mice challenged with *Kleibseilla pneumonia* or *Salmonella typhimurium* was reported when mice were administered fermented milks with various LAB (Saucier *et al.* 1992; Perdigon *et al.* 1995). Both antimutagenic activity and protective effect against the infection with *Klebsiella pneumonia* increased during the administration of fermented milk by *Lactobacillus helveticus* (Saucier *et al.* 1992; Matar *et al.* 1997).

Several casein-derived peptides may be implicated in the stimulation of the immune system. These immunopeptides were characterised and found to exert a protective effect against infections and to enhance immune system parameters (Migliore-Sammour *et al.* 1989). In addition, various bioactive peptides derived from bovine casein hydrolysates have been isolated and characterised (Meisel *et al.* 1989). These physiologically active peptides include opiates (Brantl *et al.* 1982), angiotensin converting enzyme inhibitors (Nakamura *et al.* 1996), and platelet aggregating inhibitors (Jollès *et al.* 1986).

LAB possess a variety of proteolytic enzymes that facilitate their growth in milk. During milk fermentation, caseins undergo a slight proteolysis, enough to generate potential bioactive peptides. *Lb. helveticus*, for example, is recognised as possessing efficient protease and peptidase activities with respect to milk proteins. This particular organism has been reported as being highly proteolytic compared with other LAB. In addition, biologically active peptides, opioid and antihypertensive, were shown to be present in milk fermented by *Lb. helveticus* (Matar *et al.* 1996; Nakamura *et al.* 1996). The non-specific release of peptides by bacterial proteases may lead to a peptidic profile, mainly composed of hydrophobic peptides, resistant to hydrolysis, such as proline-rich peptides. The peptidic profile released from milk protein after microbial activity and gastrointestinal enzyme activity is quite different when milk is unfermented, suggesting that bacterial proteolysis offers a potential pool of bioactive peptides (Matar *et al.* 1996; Bellem *et al.* 1999).

This investigation was undertaken to study the immunomodulator effect of *Lb. helveticus* and its non-proteolytic variant. The immunopotentiating effect of *Lb. helveticus* was analysed by studying various parameters related to mucosal immunity, such as the increase of the IgA^+ B cells at the intestinal and bronchial levels, the peritoneal macrophage activity and the effect on fibrosarcoma regression.

Materials and methods

Fermented milk preparation

The strain *Lb. helveticus* R389 (Institut Rosell, Montreal, Canada) was selected for milk fermentation. The culture was grown in MRS broth for 17 h at 37 °C and stored at 4 °C. This culture was used to inoculate 2% (v/v) autoclaved (120 °C, 10 min) reconstituted (12% w/v) low-heat grade, non-fat, non-vitamin A and D added dried milk (DairyTown, NB, Canada). The culture was incubated for 17 h at 37 °C and then used to inoculate milk samples either with *Lb. helveticus* R389 (designed wild type) or with its non-proteolytic variant (designed *Lb. helveticus* protease (−)). The fermented milks was prepared each day of feeding to avoid changes due to storage. *Lb. helveticus* protease (−) was obtained by chemical mutation (Matar *et al.* 1997). It

was characterized by failure to coagulate sterile milk within 18 h at 37 °C. The metabolic characteristics of the variant were indistinguishable from that of the parent strain (API Lactobacilli Characterisation System). The growth profile of *Lb. helveticus* R389 wild type and its non-proteolytic variant was studied during 0, 6, 12, and 24 h by plating on MRS agar medium. During the fermentation by the non-proteolytic strains, the milk was supplemented with 0·4 % yeast extract.

Immune system stimulation studies

The study was carried out by using the *in vivo* experimental model developed by Perdigon and collaborators (1993, 1995). The immunomodulating effect was tested by phagocytic activity of the macrophages, histochemical assays and immunofluorescence tests. BALB/c mice, each weighting 25–30 g were used and assigned randomly. The feeding period and the sampling were as previously described by Perdigon *et al.* (1995), three different lengths of feeding; (3, 5, and 7 d) were applied. Mice were given the conventional balanced diet supplemented with non-fermented or fermented milks (3 ml/mouse per d) orally. Mice were assigned to four experimental groups: two were given non-fermented milk or non-fermented milk supplemented with 0·4 % yeast extract and acted as controls. The other two groups were given milks fermented (12 h) with either *Lb. helveticus* wild type or its non-proteolytic variant. All Milk preparations were replaced twice daily.

At the end of the feeding period, mice were slaughtered and the small intestine and the bronchus tissue were removed and washed out with phosphate-buffered saline. Tissues were prepared for histological evaluation using standard methods. Serial paraffin sections of 4 μm were made for the immunofluorescence assay.

The immunofluorescence test

The number of cells secreting IgA was measured in the histological samples with a direct immunofluorescence assay. The IgA-secreting cells were determined by using anti-mouse IgA α-chain monospecific antibody conjugated with fluorescein isothiocyanate (Sigma-Aldrich, St Louis, MO, USA). After incubation of histological samples with the antibody, the number of fluorescent cells was counted in ten alveolar emplacement of the bronchoalveolar tissue or in ten villi in the small intestine.

Macrophage activity

The peritoneal macrophages were collected after peritoneal lavages. The macrophages were suspended in RPMI 1640 (Sigma-Aldrich) supplemented with fetal bovine serum and gentamicin. The phagocytosis of adherent macrophages was counted after immunofluorescent yeast ingestion with a fluorescent microscope. A detailed description of all these procedures and the phagocytic index determination was given by Valdez & Perdigon (1991).

Induction of fibrosarcoma tumour

The fibrosarcoma tumours were induced by the subcutaneous implantation of methylcholanthrene (MCA) crystals maintained by *in vivo* serial passages (Valdez & Perdigon, 1991). Transplants were made by the subcutaneous inoculation of 5×10^5 viable tumour cells into the left flank of mice receiving either non-fermented milks (controls) or milks fermented with *Lb. helveticus* wild type or its non-proteolytic variant. The controls and fermented milks were given to the mice for several days prior to the fibrosarcomas inoculation. Tumours volume was recorded every 5 d up to 30 d. During this period, mice were fed an *ad libitum* a conventional balanced diet.

Tumour growth was evaluated by calliper measurement of tumour length; tumour volume being determined by the formula of Attia & Weiss (1966):

$$V = 0{\cdot}5 \times d^2 \times D,$$

where V is the tumour volume, d is the shorter diameter and D the longer diameter. The tumour became visible after 2 weeks and reached a volume of 2–3 cm^3 after 20 d. The inhibition rate for tumour growth in mice fed fermented milks (treated) was calculated as follows:

$$\text{Inhibition rate (\%)} = 1 - \frac{\text{mean tumour volume of treated mice}}{\text{mean tumour volume of control mice}} \times 100$$

Statistical analysis

Results were expressed as mean ± SD, and their significance analysed using the Student's t test.

RESULTS

Growth of bacterial strains

Fermentation of milk was ensured by the growth of *Lb. helveticus* R389, which is a lactic acid bacteria with a strong acid tolerance. Similar patterns of growth and medium acidification were observed for *Lb. helveticus* wild-type and its non-proteolytic variant (results not shown). Adding yeast extract to milk fermented by *Lb. helveticus* protease (−), ensured comparable growth with the wild type strain. After 12 h fermentation, the viable cell count of both strains reached 10^8 cfu/ml.

Determination of the IgA B cells associated with the gut-associated lymphoid tissue and the bronchial tissue

As shown by the results in Table 1*a*, oral administration of milk fermented by *Lb. helveticus* R389 wild type increased the number of IgA cells at the level of gut-associated lymphoid tissue (GALT). The most significant increase occurred during the first days of feeding, confirming the dose-dependent effect. Furthermore, this proteolytic strain was able to increase the number of IgA^+ cells in bronchial tissue. The number of the IgA cells in the intestine and the bronchus tissue did not increase when milk fermented by *Lb. helveticus* protease (−) was administered orally (Table 1*b*).

Determination of phagocytic activity of peritoneal macrophages

Table 2 shows that both milk fermented by *Lb. helveticus* wild type and its non-proteolytic variant had an effect on the activity of peritoneal macrophages as tested by *in vitro* immunofluorescent yeast phagocytosis. The activity of both fermented milk preparations was dose-dependent. The maximum phagocytic index was reached after 3 d feeding with milk fermented by *Lb. helveticus* wild type and 7 d feeding with milk fermented by *Lb. helveticus* protease (−).

Determination of the antitumour effect and inhibition of fibrosarcoma in mice

The effects of oral administration of milk fermented by *Lb. helveticus* on the growth, during 30 d, of MCA-induced fibrosarcoma are shown in Table 3. Table 4 shows the percentage of tumour growth after 3 and 7 d feeding of mice with

Table 1. *Effect of oral administration of milk fermented (12 h) by* (*a*) Lb. helveticus *wild type or* (*b*) Lb. helveticus *protease* (−) *on the IgA cell numbers in the intestine and the bronchus associated lymphoid tissues. IgA*$^+$ *B cells were measured by an immunofluorescence test using a monospecific antibody after 3, 5, and 7 d of feeding*

(Values are means for $n = 4 \pm$SD)

Feeding period	Number of IgA cells (intestine)		Number of IgA cells (bronchus)	
	Non-fermented milk[1]	Fermented milk[2]	Non-fermented milk[1]	Fermented milk[2]
	(*a*) *Lb. helveticus* wild type			
3 d	80±5·0	180±3·5**	18±4·0	57±5·2**
5 d	86±3·2	141±1·3**	20±3·4	38±5·2**
7 d	85±2·5	81±3·5	19±1·3	27±3·2*
	(*b*) *Lb. helveticus* protease (−)			
3 d	76·0±3·0	71±6·0	25·3±3·1	23±6·0
5 d	92·5±1·0	81±1·4	22·3±2·0	15±1·15
7 d	75·5±1·5	80·5±5·0	22·66±2·2	18±6·0

[1] Controls were animals given (*a*) uninoculated milk or (*b*) uninoculated milk supplemented with 0·4 % yeast extract.

[2] Test animals were given (*a*) milk fermented by *Lb. helveticus* wild type or (*b*) milk supplemented with 0·4 % yeast extract, fermented by *Lb. helveticus* protease (−). Addition of yeast extract allowed comparable growth in wild-type and protease (−) strains.

Values were significantly different from the corresponding values for controls: $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$.

Table 2. *Effect of oral administration of 12 h milks fermented by* Lb. helveticus *or its non proteolytic variant on the Phagocytic index of peritoneal macrophages of mice*

(Values are means for $n = 4 \pm$SD)

Feeding period	Phagocytic index			
	Non-fermented milk[1]	Milk fermented by *Lb. helveticus* wild type	Non-fermented milk[2] (+yeast extract)	Milk fermented by *Lb. helveticus* protease (−)[3] (+yeast extract)
3 d	2·05±0·38	4·2±1·6**	2·2±0·03	2·91±0·7
7 d	2·33±0·2	2·56±0·9	2·03±0·3	3·19±0·6*

[1,2] Controls are animals given uninoculated milk or uninoculated milk supplemented with 0·4 % yeast extract.

[3] Milk fermented by *Lb. helveticus* protease (−) was supplemented with yeast extract which allowed comparable growth in wild-type and protease (−) strains.

Values were significantly different from the corresponding values for controls[1,2]: $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$.

fermented milks. In all the cases, the feeding of mice occurred prior to the subcutaneous inoculation of fibrosarcomas.

The influence of feeding mice with milk fermented by either *Lb. helveticus* wild type or the non-proteolytic variant prior to inoculation with tumor cells induced a decreased volume of the tumour (Table 3). Since both fermented milks showed an effect on the phagocytic activity of the peritoneal macrophages, one could believe that the antitumour activity and the decrease in growth in fibrosarcoma are closely related to the phagocytic index (Table 2). This effect of feeding mice for 3 d prior to tumor cell inoculation was more effective when the milk was fermented by *Lb. helveticus* wild type rather than the non-proteolytic variant (Table 3); the decrease in the progression in the tumour was significant after 3 d feeding with milk fermented by *Lb. helveticus* wild type and 7 d feeding with milk fermented by *Lb. helveticus* protease (−).

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Table 3. *Effect of previous feeding of mice with milks fermented (12 h) by* Lb. helveticus *wild type and its non-proteolytic variant on fibrosarcomas volume* (cm^3). *Mice were given diets supplemented with fermented milks or the controls for 3 and 7 d prior to the inoculation of* 5×10^5 *tumour cells. The volume and the growth of the methylcholantrene-induced fibrosarcomas were recorded for 10, 15, 20, 25 and 30 d*

(Values are means for $n = 5 \pm$ SD)

Diets (orally administered) prior to tumour implantation	Feeding period	Number of days/volume of fibrosarcomas				
		10 d	15 d	20 d	25 d	30 d
Non-fermented milk[1]		0·34 ± 0·19	1·29 ± 0·5	6·55 ± 1·3	7·3 ± 1·5	6·39 ± 0·82
Milk fermented by	3 d	0·21 ± 0·1**	0·31 ± 0·17*	2·25 ± 0·8*	2·51 ± 1·2*	10·52 ± 2·3
Lb. helveticus wild type	7 d	0·26 ± 0·12	0·54 ± 0·17*	1·2 ± 0·8*	1·16 ± 0·56*	4·05 ± 0·8*
Non-fermented milk + yeast extract[2]		0·30 ± 0·07	0·95 ± 0·06	4·9 ± 0·56	6·5 ± 0·70	6·7 ± 0·5
Milk + yeast extract	3 d	0·28 ± 0·19	1·42 ± 0·18	10·96 ± 3·3	10·98 ± 3·2	8·0 ± 3·8
fermented by	7 d	0·05 ± 0·02*	0·20 ± 0·14*	1·73 ± 0·57*	2·1 ± 1·06*	8·38 ± 2·7
Lb. helveticus protease (−)						

Controls[1,2] are animals given uninoculated milk or uninoculated milk supplemented by 0·4% yeast extract, which allowed growth comparable to that of wild-type strain.

Values were significantly different from the corresponding values for controls[1,2]: $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$.

Table 4. *Effect of feeding mice 12 h milks fermented by* Lb. helveticus *and its non-proteolytic variant on percentage of tumour growth*

(Values are means for $n = 5 \pm$ SD)

Diets (orally administered) prior to tumour implantation	Feeding period	Number of mice with tumour/number of mice after 5 week post tumour implantation	Tumour growth (%)
Non-fermented milk		8/14	57
Milk fermented by	3 d	4/14	28*
Lb. helveticus wild type	7 d	3/13	23*
Milk fermented by	3 d	6/13	46
Lb. helveticus protease (−)	7 d	5/13	38**

Values were significantly different from the corresponding values for control: $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$.

DISCUSSION

Lb. helveticus is commonly used in the manufacture of many fermented dairy products. It exhibits particularly strong protease and peptidase activities compared with other LAB. Numerous studies have also reported the role of *Lb. helveticus* in releasing bioactive peptides during milk fermentation (Nakamura *et al.* 1995; Matar *et al.* 1996). The proteolytic patterns and characteristics of *Lb. helveticus* R389, used during this investigation, have been thoroughly studied in previous works (Matar *et al.* 1996, 1997). Limited proteolysis of milk proteins by this strain had an impact on the pattern and rate of subsequent release of peptides (Matar *et al.* 1996). The antimutagenicity of milk fermented by *Lb. helveticus* R 389 was also shown to be related to the proteolytic activity of this strain. A protease-deficient variant of *Lb. helevticus* R 398 was isolated and characterised, in order to point out the role of proteolysis in the antimutagenic activity of fermented milk (Matar *et al.* 1997). When milk fermented by *Lb. helveticus* protease (−) was supplemented by yeast extract, the fermentative patterns of both strains were not significantly different.

This *in vivo* study aimed to determine the importance of protease activity during milk fermentation by *Lb. helveticus*, particularly the influence on the number of IgA-secreting cells and the regression of subcutaneously implanted fibrosarcomas.

LAB, through the mechanism of fermentation, may release compounds that react with the immune system parameters and induce protective immunity against infections and some tumours (Matar *et al.* 2000). They might be indirectly involved in the modulation of the immune response of the host by contributing, during the fermentation process, to the release of peptides bearing hormone-like activities. Bioactive peptides (casomorphins, immunomodulating and antihypertensive peptides) may produce local effects on the gastrointestinal tract and stimulate the immunocompetent cells acting as immunomodulating agents. Several casein-derived peptides may play a significant role in the stimulation of the immune system. Two hexapeptides derived respectively from human and bovine β-casein were shown to stimulate *in vitro* phagocytosis of sheep red blood cells by peritoneal macrophages and to protect mice against infections (Migliore-Sammour *et al.* 1989). A pepsin-generated hydrolysate of lactoferrin may contain immunostimulating peptides, which can enhance the proliferation of spleen cells (Miyauchi *et al.* 1997). The immunomodulatory peptides from milk proteins might also increase the IFN-γ production, thus acting on the cytokine network (Laffineur *et al.* 1996).

The fermented mixture of LAB was more effective than the non-fermented ones in the potentiation of immune response and inhibition of tumour development (Perdigon *et al.* 1997). The proteolysis that occurred during milk fermentation might be implicated in the immunological mechanisms by which fermented milk exerts its immunomodulatory activity. The peptides released from milk proteins might be responsible for the increase of the IgA number at the intestinal and bronchial levels (Table 1*a*, *b*). These potent metabolites are not generated by the *Lb. helveticus* protease (−) variant, which failed to induce a general activation of the immune system. The number of IgA-secreting cells was correlated to the administered dose (Table 1). This tendency could be due to down-regulatory signals that govern the activity of the immune cells. The dose-dependent pattern of immune stimulation also prevents an increase of harmful inflammatory response (Chouaib *et al.* 1994).

Potent metabolites are released after milk fermentation. Some probiotic preparations are able to promote a local expansion of the IgA cycle, others are able to enhance cellular mobilisation of the IgA^+ cells on the *lamina propria*. The increasing number of T cells, IgA^+ B and $CD4^+$ cells in the *lamina propria* of the intestine, resulting from 'specific interaction' of probiotic mixture with the gut epithelial cells at the Peyer's patches level, might induce an IgA cycle and an increase of the cell population at the bronchial level. This is an important conclusion suggested by Perdigon and colleagues (Perdigon *et al.* 1999; Perdigon & Holgado, 2000) which reinforces our hypothesis on the implication of potent metabolites released during fermentation in the immunostimulation mechanism and sheds light on important controversial mechanistic behaviours of probiotics.

The results obtained with milk fermented by *Lb. helveticus* wild type showed increases in mucosal immunity, as demonstrated by the levels of secretory IgA and an activation of B cells to enter the IgA cycle (Table 1). An immunological communication exists between intestinal and pulmonary mucous tissues including lung, uterus and mammalian membranes (Lamn *et al.* 1995). This connection between mucosal surfaces permits immunity initiated at one anatomical site to protect another mucosal site. The stimulation of an IgA-inductive site (e.g. GALT) is likely to lead to the generalised protection of remote sites such as the nasopharynx,

genital tract, BALT (Bronchus Associated Lymphoid Tissue) and glands (Roux *et al.* 2000). The understanding of the role of the biologically active peptides derived from microbial activity of LAB might be exploited clinically to prevent infections in the mucosal network.

The inhibition of tumour growth, which occurred following prior feeding with milk fermented by *Lb. helveticus* wild type, might be explained by the activation of macrophages through the cellular immunity, which is also responsible for IgA B cell production. *Lb. helveticus* protease (−) is able to stimulate phagocytosis whereas it does not produce a change in IgA B cell numbers. The increase in the phagocytic activity of mice receiving milk fermented by this strain might be indirectly responsible for the regression of the fibrosarcoma volume after 7 d feeding (Table 3). Perdigon *et al.* (1993) reported a close relationship between the activity of peritoneal macrophages and the regression of tumours histologically distant from the immunomodulator penetration route. Milk fermented by *Lb. helveticus* protease (−) enhances phagocytosis, which in turn might be involved in tumour regression. Milk fermented by *Lb. helveticus* wild type might contribute to inhibition of tumours through two mechanisms: activation of the phagocytosis and mediation of the host's immune system (especially activation of type 1 helper T cell and their cytokine INF-γ; Table 3). The increase of tumour regression at 30 d after 3 d feeding of milk fermented by the *Lb. helveticus* wild type and 7 d feeding of *Lb. helveticus* protease (−) might be due to the increase of a general inflammatory response supported by an excess release of some cytokines as reported by Perdigon *et al.* (1993). Those authors concluded that the administration of proper doses is of fundamental importance. In their study, some doses exerted the opposite effect, i.e. enhancing tumour growth; they demonstrated that the only effective dose of *Lb. casei* in the regression of transplantable tumour corresponds to a 2-d feeding period.

The immunomodulating effect of LAB with the host is multifactorial. The knowledge of the mechanism of interaction of these different factors might be very useful in preventing several human diseases. We are currently investigating the particular role of peptidic fractions in immunomodulation and the suppression of transplantable tumours.

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References

Attia, M. A. M. & Weiss, D. W. 1966 Immunology of spontaneous mammary carcinomas in mice. Acquired tumour resistance and enhancement in strain A mice infected with mammary tumour virus. *Cancer Research* **26** 1787–1792

Bellem, M. A. F., Gibbs, B. F. & Lee, B. H. 1999 Proposing sequences for peptides derived from whey fermentation with potential bioactive sites. *Journal of Dairy Science* **82** 486–493

Biffi, A., Coradini, D., Larsen, R., Riva, L. & Di Fronzo, G. 1997 Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line. *Nutrition and Cancer* **28** (1) 93–99

Brantl, V., Teschemacher, H., Bläsig, J., Henschen, A. & Lottspeich, F. 1982 Opioid activities of β-casomorphin. *Life Science* **28** 1903–1909

Chouaib, S., Chatenoud, L., Klatzmann, D. & Fradelizi, D. 1984 The mechanism of inhibition of IL-2 production. II. PGE2 induction of suppressor T lymphocytes. *Journal of Immunology* **132** 1851–1857

Jollès, P., Levy-Toldano, S., Fiat, A. M., Soria, C., Gillessen, D., Thamaidis, A., Dunn, F. W. & Caen, J. P. 1986 Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from κ-casein on platelet function. *European Journal of Biochemistry* **158** 379–382

Laffineur, E., Genetet, N. & Léonil, J. 1996 Immunodulatory activity of β-casein permeate medium fermented by lactic acid bacteria. *Journal of Dairy Science* **79** 2112–2120

Lamn, M., Mazanca, M., Nedrud, J. & Kaetzel, C. 1995 New functions for mucosal IgA. In *Advances in mucosal immunology*, p. 647. (Eds J. Mestecky *et al.*). New York: Plenum Press

Matar, C., Goulet, J., Bernier, R. & Brochu, E. 2000 Bioactive peptides from fermented foods: their role in the immune system. In *Probiotics 3. Immunomodulation by the gut microflora and probiotics*, pp. 193–209 (Eds R. Fuller & G. Perdigon). Netherlands: Kluwer Academic Publishers

Matar, C., Nadathur, S., Bakalinsky, A. & Goulet, J. 1997 Antimutagenic effects of milk fermented by *Lactobacillus helveticus* L89 and a protease-deficient derivative. *Journal of Dairy Science* **80** 1965–1970

Matar, C., Amiot, J., Savoie, L. & Goulet, J. 1996 The effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during *in vitro* digestion. *Journal of Dairy Science* **79** 971–979

Matar, C. & Goulet, J. 1996 β-casomorphin 4 from milk fermented by a mutant of *Lactobacillus helveticus*. *International Dairy Journal* **6** 383–397

Matsuzaki, T., Hashimoto, S. & Yokokura, T. 1996 Effects on antitumor activity and cytokine production in the thoracic cavity by intrapleural administration of *Lactobacillus casei* in tumor-bearing mice. *Medical Microbiology and Immunology* **185** 157–161

Meisel, H., Frister, H. & Schlimme, E. 1989 Biologically active peptides in milk proteins. *Zeitschrift fuer Ernährungswissenschaft* **28** 267–278

Migliore-Sammour, D., Floch, F. & Jollès, P. 1989 Biologically active peptides implicated in immunomodulation. *Journal of Dairy Research* **56** 357–362

Miyauchi, H., Kaino, A., Scinoda, I., Fukuwatari, Y. & Hayasawa, H. 1997 Immunomodulating effect of bovine lactoferrin pepsin hydrolysate on murine splenocytes and Peyer's patch cells. *Journal of Dairy Science* **80** 2330–2339

Nakamura, Y., Yamamoto, N., Sakai, N., Okubo, A., Yamazaki, S. & Takano, S. 1995 Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science* **78** 777–783

Perdigon, G., De Jorrat, M. E., De Petrino, S. F. & Rachid, M. 1993 Antitumor activity of orally administered *Lactobacillus casei*: significance of its dose in the inhibition of a fibrosarcoma in mice. *Food and Agricultural Immunology* **5** 39–49

Perdigon, G., Alvarez, S., Rachid, M., Agüero, G. & Gobbato, N. 1995 Symposium: probiotic bacteria for humans: clinical systems for evaluation of effectiveness. *Journal of Dairy Science* **78** 1597–1606

Perdigon, G., Vintini, E., Alvarez, S., Medina, M. & Medici, M. 1999 Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *Journal of Dairy Science* **82** 1108–1114

Perdigon, G. & Holgado, A. 2000 Mechanisms involved in the immunostimulation by lactic acid bacteria. In *Probiotics 3. Immunomodulation by the gut microflora and probiotics*, pp. 213–229 (Eds R. Fuller & G. Perdigon) Netherlands: Kluwer Academic Publishers

Roux, M. E., del C. Lopez, M. & Florin-Christensen, A. 2000 Mucosal immunity In *Probiotics 3. Immunomodulation by the gut microflora and probiotics*, pp. 12–26 (Eds R. Fuller & G. Perdigon) Kluwer Netherlands: Academic Publishers

Saucier, L., Julien, M., Chéour, F., Letrate, R. & Goulet, J. 1992 Effect of feeding lactic acid bacteria and fermented milk on specific and non-specific immune response of mice infected with *Kleibseilla pneumoniae* AD-1. *Journal of Food Protection* **8** 595–600

Valdez, J. & Perdigon, G. 1991 Piroxicam, indomethacin and aspirin action on murine fibrosarcome effects on tumour-associated and peritoneal macrophages. *Clinical Experimental Immunology* **86** 315–321