

Original article

Immunomodulatory properties of the milk whey products obtained by enzymatic and microbial hydrolysis

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Summary The objective of this study was to investigate the *in vitro* immunomodulatory effect of milk whey through enzymatic hydrolysis, microbial fermentation and two-stage hydrolytic reactions (enzymatic and microbial reactions) by analysing cytokine profiles. Results indicated that the milk whey sample fermented by *Lactobacillus kefiranofaciens* M1 and two-stage hydrolytic reactions (hydrolysed by Alcalase and then fermented by *L. kefiranofaciens* M1) could significantly induce the production of tumour necrosis factor (TNF)- α and IL-12 compared with the milk whey control and enzymatic treatments. Further characterisation of the immunomodulatory factor by membrane filtration and mutanolysin hydrolysis, the stimulatory activity for IL-12 and TNF- α production was found to be reduced and to be correlated positively with the cell wall components in *L. kefiranofaciens* M1. In addition, Th2-polarised splenocytes revealed that *L. kefiranofaciens* M1 had both IL-12 inducing and IL-4 repressing activities. These results suggested that *L. kefiranofaciens* M1 could direct the Th1/Th2 balance toward Th1.

Keywords Alcalase, cytokines, immunomodulation, *Lactobacillus kefiranofaciens*, nautrase, whey.

Introduction

An increasing number of studies suggest that whey proteins may contain bioactive peptide sequences with immunomodulatory properties in their primary sequences. A hydrolysed whey protein isolate, composed of β -lactoglobulin (β -LG), α -lactalbumin (α -LA) and glycomacropeptide (GMP), has been shown to possess a stimulatory effect on lymphocyte proliferation *in vitro* (Mercier *et al.*, 2004). Another peptide, a glycoposphopeptide isolated from cheese whey, has demonstrated a mitogenic activity on splenocytes (Yun *et al.*, 1996). It has also been demonstrated that the peptides issued from whey and milk possess a good immuno-stimulatory effect on keratinocyte growth *in vitro* (Korhonen and Philanto-Leppälä, 2004). Hypersensitivity is another area where the hydrolysis of whey proteins is especially important (Gauthier *et al.*, 2006). The goal of most recent studies has been to design hypoallergenic infant formulas. Prioult *et al.* (2004) have demonstrated that acidic peptides, separated by isoelectric focusing of a tryptic–chymotryptic hydrolysate of β -LG, stimulate splenocyte prolifera-

tion and IFN- γ production *in vitro*. However, the development of whey immunomodulatory peptides is currently limited by the lack of characterization of immunomodulatory peptides from bioactive hydrolysates (HS) and by the absence of clinical data on the physiological effects of these peptides (Gauthier *et al.*, 2006).

One potentially important function of lactic acid bacteria (LAB) is their involvement in development and maintenance of homeostasis in the gut-associated immune system. The immunoregulative effect of LAB was dependent on the LAB strains rather than the LAB species (Fujiwara *et al.*, 2004). Our previous study (Hong *et al.*, 2009) demonstrated that milk kefir supernatants, obtained from kefir fermented for more than 24 h induced the production of pro-inflammatory cytokines in RAW 264.7 cells. Among four LAB isolated from kefir grains and their supernatants, *Lactobacillus kefiranofaciens* M1 and its supernatant had a strong capacity to induce *in vitro* production of tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and IL-12 in RAW 264.7 cells and murine peritoneal macrophages.

Although many studies used different enzymes to produce whey protein hydrolysates with immunomodulatory properties, few investigations used both enzymes

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and microorganisms simultaneously to study hydrolysis of whey proteins. Thus, the objective of this study was to investigate the *in vitro* immunomodulatory effect of milk whey through enzymatic hydrolysis, microbial fermentation and two-stage hydrolytic reactions (enzymatic and microbial reactions) by determining cytokine profiles. The presence of immunomodulatory factors in the milk whey samples was ascertained.

Materials and methods

Preparation of milk whey

Reconstituted skim milk (12% total solids, Anchor, Auckland, New Zealand) was acidified by 1% w/v glucono- δ -lactone (GDL; Sigma Chemical Co., St Louis, MO, USA), which was similar to conditions observed during the manufacture of certain cheese varieties. The milk then was incubated at temperature 42 °C for 40 min. The resultant mixture of casein and whey was immediately filtered through a cheese sieve.

Preparation of enzymatic whey protein

The milk whey was hydrolysed using three different commercial enzymes, Alcalase (3000 U/mg, Trump Chemicals, Taipei, Taiwan), Neutrase (1500 U/mg, Trump Chemicals, Taipei, Taiwan) and pepsin (2500 U/mg, Sigma, St Louis, MO, USA). Hydrolysis conditions for Alcalase and Neutrase were: pH 7.0, temperature 60 °C, ratio enzyme: substrate of 1:100 (mg mL⁻¹), while for pepsin (Schmidt *et al.*, 1995) was: pH 3.0, temperature 37 °C, ratio enzyme: substrate of 1:100 (mg mL⁻¹). After 12-h hydrolysis, the reaction was terminated with heat inactivation at 90 °C for 20 min. Determining food protein degree of hydrolysis was carried out as described by Nielsen *et al.* (2001).

L. kefiranofaciens M1

Lactobacillus kefiranofaciens M1 was isolated and identified using a combination of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rDNA sequencing from kefir grains in our lab (Chen *et al.*, 2008). This strain was proliferated in deMan, Rogosa and Sharp (MRS) broth (Difco Laboratories, Detroit, MI, USA) at 37 °C, and then harvested by washing and resuspending three times in phosphate buffered saline (PBS, 0.1 M, pH 7.2) solution (Amersco, Solon, OH, USA). After washing, the suspension was adjusted to 10⁷ CFU mL⁻¹ in PBS solution. One percent of *L. kefiranofaciens* M1 suspension was then cultured in 10 mL milk whey for 24 h.

Cytokine production in RAW 264.7 cells

The cytokine production in RAW 264.7 cells was carried out as described by Hong *et al.* (2009). In brief, the murine macrophage cell line (RAW 264.7, ATCC TIB71, American Type Culture Collection, Manassa, VA, USA) was cultured in Dulbeccos Modified Eagles Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% low endotoxic fetal calf serum (PAA Laboratories GmbH, Linz, Austria), 2 mM glutamine (Sigma, St Louis, MO, USA) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Sigma, St. Louis, MO, USA). The macrophages were cultured in 95% air with 5% CO₂ at 37 °C in a humidified incubator (Revco, Santa Fe Springs, CA, USA). After proliferation, cell number and viability were assessed by trypan blue dye (0.5% Trypan-blue, Invitrogen, Grand Island, NY, USA) in PBS (0.1 mM, pH 7.3) exclusion on a hemacytometer (American Optical, Buffalo, NY, USA) using a microscope (Optima, Aurora, CO, USA).

After assessment of cell numbers, RAW 264.7 cells were cultured in triplicate at a density of 10⁵ cells mL⁻¹ in 24-well flat-bottomed tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA). The supernatants were then discarded and replaced by heat-inactivated serum (56 °C for 30 min) DMEM. Activation of RAW 264.7 cells was made by adding 5 µL of each whey product hydrolysed by various enzymes or fermented by *L. kefiranofaciens* M1 suspension in 1 mL medium. The positive control was added with 50 ng lipopolysaccharide (LPS, Sigma, St Louis, MO, USA) to 1 mL medium per well. After 24-h incubation at 37 °C, the supernatants were collected and stored at -80 °C until cytokine assays were performed (Aybay and Imir, 1998; Hong *et al.*, 2009).

Cytokine production by murine splenocytes

BALB/c mice (female, 5 weeks old) were purchased from National Laboratory Animal Center (Taipei, Taiwan). The mice were maintained in a temperature- and light-controlled environment with free access to a sterile diet and water. They were acclimatized for 1 week before the study. The splenocytes were isolated from six-week Balb/C female mice (*n* = 6). The mice were killed by CO₂ inhalation. The spleens were removed and placed in individual sterile Petri plates containing 10 mL of Roswell Park Memorial Institute medium 1640 (RPMI medium, Sigma, St Louis, MO, USA), supplemented with 100 µg/mL of penicillin (Wisent, St-Bruno, QC, Canada). The spleens were cut into pieces with a sterile scalpel then pressed through a 70-µm cell strainer (BD Falcon, Bedford, MA, USA) under aseptic conditions. The cells were suspended in 7 mL of complete RPMI and centrifuged (1200 *g*) at room temperature for

5 min. The pellets were resuspended in 10 mL of RPMI with 100 $\mu\text{g mL}^{-1}$ of penicillin and stained with 0.5% trypan blue (0.5% Trypan-blue, Invitrogen, Carlsbad, CA, USA) and counted using a microscope (Optima, Aurora, CO, USA). The cell suspension was then adjusted to 2×10^6 cells mL^{-1} in PRMI medium.

The murine splenocytes (1 mL) were added to the wells of 24-well round-bottomed microplates containing 5 μL of each whey product hydrolysed by various enzymes or fermented by *L. kefiranofaciens* M1. The positive control was added with 50 ng lipopolysaccharide (LPS, Sigma, St Louis, MO, USA) to 1 mL medium per well. After 48-h incubation at 37 °C, the supernatants were collected and stored at -80 °C until cytokine assays were performed.

Anti-allergic study

For the anti-allergic study, ovalbumin (OVA)-primed splenocytes were used. The method was modified the protocol as described by Fujiwara *et al.* (2004). In brief, BALB/c mice were injected intraperitoneally with 1 mg OVA absorbed on 2 mg of $\text{Al}(\text{OH})_3$ (Wako, Tokyo, Japan). Injections were provided twice on day 0 and on day 6. Mice were killed by CO_2 inhalation on day 13 to obtain splenocytes. Splenocytes were suspended at the concentration of 2×10^6 cells mL^{-1} in RPMI medium (Sigma, St Louis, MO, USA) supplemented with 10% FCS and penicillin. Cells were cultured with 1 mg mL^{-1} OVA, in both presence and absence of 5 μL (low dose) and 30 μL (high dose) whey products fermented by *L. kefiranofaciens* M1.

Measurement of cytokine levels

To further determine the concentration of the different cytokines [interferon (IFN)- γ , TNF- α , IL-6, IL-12p40, IL-4 and IL-1 β] in cell culture supernatants, mouse cytokine ELISA kits (R&D System, Minneapolis, MN, USA) were used. The results were expressed as the concentration of each cytokine (pg mL^{-1}).

Characterization of immunomodulatory factor

In order to characterize the immunomodulatory factor, the hydrolysed whey samples were treated with centrifugation, membrane filtration and enzyme digestion, followed by cytokine determination in RAW 264.7 cells and splenocytes. The hydrolysed whey samples were centrifuged (Eppendorf, Hamburg, Germany) at $6000 \times g$ for 30 min and the supernatants were ultra-centrifuged at $13\,000 \times g$ for 30 min. Finally, the supernatants were filtrated using 0.22 μm membranes. To estimate the possible molecular mass of the active substance, the kefir supernatant was subjected to ultra-filtration through YM-30 or YM-10 membranes

(Millipore, Billerica, MA, USA). The cell wall components of M1 were also evaluated by mutanolysin (5 $\mu\text{L mL}^{-1}$, Sigma, St Louis, MO, USA) for the immunomodulatory effects.

Statistical analysis

All results were analysed using the general linear model procedure available from the Statistical Analysis system software package version 8.1 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to detect differences between treatment means. Each experiment was conducted in triplicate.

Results and discussion

Effect of the milk whey products hydrolyzed by various enzymes

The proteolytic activities of different enzymes and *L. kefiranofaciens* M1 (Fig. 1) indicated that the whey protein hydrolysed by Alcalase yielded the highest degree of protein hydrolysis, followed by Neutrase and pepsin. Our results were paralleled with the findings of Sukan & Andrews (1982). They observed that a greater degree of hydrolysis could be achieved with Alcalase than with enzymes such as pepsin and chymotrypsin. These divergences of different enzymes might be due to the distribution in the peptidic fractions reflecting the different cleavage positions of enzymes (Ogino *et al.*, 1999; Hou *et al.*, 2005). Wróblewska *et al.* (2008) also showed Alcalase as a good candidate for industrial production of whey protein hydrolyzates because of its endoprotease content with broad specificity and its low cost.

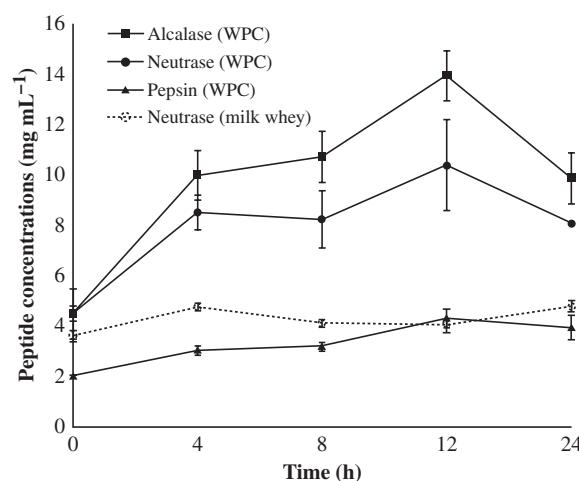


Figure 1 Effects of adding different enzymes and *L. kefiranofaciens* M1 on peptide concentration in the milk whey.

Table 1 lists the effects of the enzymatic whey protein hydrolysates obtained from three enzymes on cytokine production in RAW 264.7 cells. The secretion of TNF- α induced by the milk whey hydrolysed by Alcalase was significantly higher than the milk whey control and other treatments. In contrast, there was no secretion of IL-12, IL-1 β and IFN- γ for all treatments. Similar results were also found on murine splenocytes (Table 2). The Alcalase treatment could significantly induce the production of TNF- α and IL-12 compared with the milk whey control and other treatments. This data can be correlated positively with the whey protein hydrolysis by Alcalase (Fig. 1). No secretion was observed for IL-1 β and IFN- γ on splenocytes. However, our result does not agree with the findings of Pacheco & Sgarbieri (2005). They reported that whey protein concentrate (WPC) Alcalase hydrolysate produced lower stimulation of liver glutathione synthesis compared with WPC and pancreatin and protamex hydrolysates. They also demonstrated a high correlation between glutathione synthesis and immune stimulation. Gauthier *et al.* (2006) reviewed the published reports on the immunomodulating properties of whey peptides and found some conflicting results. Different methodology, raw materials and models might account for these discrepancies.

It is interesting to note that IL-12 stimulated by the milk whey hydrolysed by the enzymes on murine splenocytes (Table 2) was not observed on RAW 264.7 cells (Table 1). Raw 264.7 cells are mouse leukaemic monocyte macrophage cells, while the splenocytes are monocyte including T cells and B cells. Although splenocytes and RAW 264.7 cells share many biological features, it seems that there are differences related to cell types or species with respect to cytokine responses *in vitro*. Diaz-Guerra *et al.* (1996) reported that different

cells elicited different responses in NO synthesis when stimulated with phorbol ester. These findings led us to deduce that the differences in results were because of the different properties of established cell lines and primary culture.

When we compared the effects of the milk whey samples hydrolysed by three enzymes (Tables 1 and 2) and the milk whey control on cytokine production, the levels of TNF- α induced by the whey sample hydrolyzed by Alcalase were statistically higher ($P < 0.05$) than the ones induced by other treatments. The milk whey hydrolysed by Alcalase also stimulated significantly ($P < 0.05$) higher levels of IL-12 on murine splenocytes. Thus, Alcalase was selected for the following study.

Effect of the milk whey hydrolysed by combination of Alcalase and *L. kefiranofaciens* M1

Figure 2 displays that the milk whey sample fermented by *L. kefiranofaciens* M1 or treated by two-stage hydrolytic reactions (hydrolysed by Alcalase and then fermented by *L. kefiranofaciens* M1) could significantly induce the production of TNF- α and IL-12 compared with the milk whey control and the milk whey hydrolysed by Alcalase only on splenocytes and RAW 264.7 cells. Since additional *L. kefiranofaciens* M1 seemingly played an important immunomodulatory factor, we further removed the cells by centrifugation and membrane filtration. Results (Fig. 3) indicated that after removing *L. kefiranofaciens* M1 from the milk whey, both samples, *L. kefiranofaciens* M1 and two-stage hydrolytic reactions, lost their ability to induce the TNF- α and IL-12. Several compounds, including bacteria as well as new metabolites produced during milk whey fermentation, such as bioactive peptides (Matar

Table 1 Effect of the enzymatic whey protein hydrolysates obtained from three enzymes on cytokine production in RAW 264.7 cells

Cytokines (pg/mL)	Milk whey	Alcalase	Neutrase	Pepsin	Blank	LPS
TNF- α	232.19 $\pm$ 22.93 ^d	346.92 $\pm$ 11.19 ^b	295.52 $\pm$ 15.72 ^c	286.26 $\pm$ 38.00 ^c	77.74 $\pm$ 2.92 ^a	612.93 $\pm$ 9.29 ^a
IL-1 β	ND	ND	ND	ND	ND	ND
IFN- γ	ND	ND	ND	ND	ND	ND
IL-12	ND	ND	ND	ND	ND	ND

ND, not detectable.

^{a,b,c,d}Means for each cytokine without a common letter differ significantly ($P < 0.05$).

Table 2 Effect of the enzymatic whey protein hydrolysates obtained from three enzymes on cytokine production in murine splenocytes

Cytokines (pg mL ⁻¹)	Milk whey	Alcalase	Neutrase	Pepsin	Blank
TNF- α	118.64 $\pm$ 22.46 ^b	203.67 $\pm$ 42.29 ^a	65.37 $\pm$ 10.57 ^c	90.60 $\pm$ 14.53 ^b	20.2 $\pm$ 1.23
IL-1 β	ND	ND	ND	ND	ND
IFN- γ	ND	ND	ND	ND	ND
IL-12	99.14 $\pm$ 5.40 ^c	134.59 $\pm$ 1.54 ^a	95.87 $\pm$ 7.71 ^c	111.69 $\pm$ 9.26 ^b	102.96 $\pm$ 12.34 ^{bc}

ND, not detectable.

^{a,b,c}Means for each cytokine without a common letter differ significantly ($P < 0.05$).

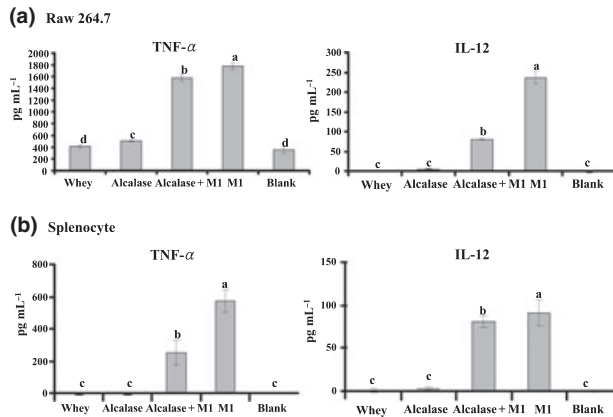


Figure 2 Effect of the milk whey samples obtained from enzymatic hydrolysis and *L. kefirano*faciens M1 fermentation on cytokine production in (a) RAW 264.7 cells and (b) murine splenocytes. Values are means $\pm$ SD. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

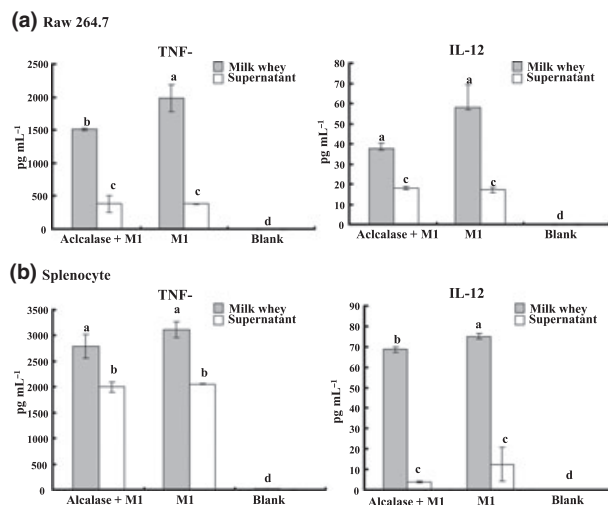


Figure 3 Effect of membrane filtration of the milk whey samples on cytokine production in (a) RAW 264.7 cells and (b) murine splenocytes. Values are means $\pm$ SD. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

et al., 2000; Prioult *et al.*, 2004) and water soluble exopolysaccharides (Monteleone *et al.*, 2002) might be candidates for the immunomodulatory factors. Our findings proved that *L. kefirano*faciens M1 played an important role for the immunomodulatory effects. A couple of investigations reported that the ability of soluble bacterial components to induce TNF- α and IL-12 was weaker than that of intact bacterial cells (Huang *et al.*, 1999; Hessle *et al.*, 2000). Similar to their findings, we also demonstrated that TNF- α and IL-12 stimulated by *L. kefirano*faciens M1 were significantly higher

($P < 0.05$) than by its whey supernatant on Raw 264.7 cells and splenocytes.

In addition, the cytokine levels were also significantly decreased after digestion with mutanolysin (Fig. 4). Mutanolysin is an *N*-acetylmuramidase. It is the muralytic enzyme that cleaves the β -*N*-acetylmuramyl-(1 $\rightarrow$ 4)-*N*-acetylglucosamine linkage of polymer peptidoglycan-polysaccharide on the bacterial cell wall (Sashihara *et al.*, 2006). Our results suggested that the stimulatory activity for IL-12 and TNF- α production originated from the components associated with the cell wall. Our results obtained by membrane filtration (Fig. 3) and the mutanolysin test (Fig. 4) led to the conclusion that the cell wall of *L. kefirano*faciens M1 was the immunomodulatory factor.

Tumour necrosis factor- α is known to enhance the expression of accessory molecules involved in the adhesion of macrophages to the endothelium, increasing the possibility that other memory cells present in the circulatory system are guided to the inflammation site (Vinderola *et al.*, 2006). IL-12 is well-known to play a critical role both in inducing a T helper 1 (Th1)-dominant immune response and in enhancing cellular immunity (Shida *et al.*, 2006). Shida *et al.* (1998) documented that *L. casei* Shirota could promote Th1 cells from naïve CD4 + T cells through secretion of IL-12 by macrophages. Accordingly, the *in vitro* results depicted that *L. kefirano*faciens M1 could induce T helper 2 (Th2) cytokines in murine macrophages and splenocytes, which might inhibit the Th2 response. Thus, the following study analysed cytokine secretion in OVA-sensitized mice fed with the milk whey containing *L. kefirano*faciens M1.

The anti-allergic effects

The secretion levels of IL-12 and IL-4 induced by the milk whey fermented with *L. kefirano*faciens M1 in

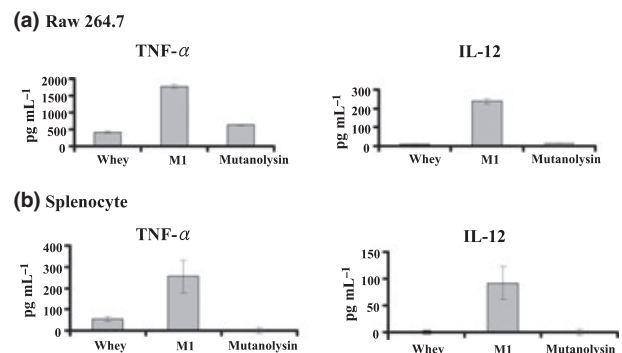


Figure 4 Effect of mutanolysin treatment of the milk whey samples on cytokine production in (a) RAW 264.7 cells and (b) murine splenocytes.

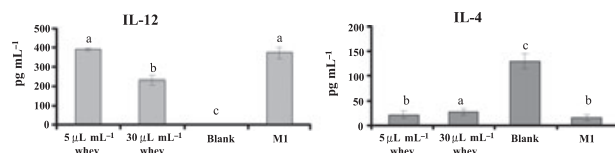


Figure 5 The secretion levels of IL-12 and IL-4 induced by the milk whey fermented with *L. kefiranofaciens* M1 in OVA-sensitized splenocytes. Values are means $\pm$ SD. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

OVA-sensitized splenocytes are shown in Fig. 5. Both the milk whey fermented with *L. kefiranofaciens* M1 and *L. kefiranofaciens* M1 cells induced high amounts of IL-12 from OVA-sensitized splenocytes. The sample of low dosage showed a better IL-12 stimulation than that of high dosage. High secretion levels of IL-4 from OVA-sensitized splenocytes were inhibited by exposing the splenocytes to the fermented whey samples with *L. kefiranofaciens* M1. Similarly, the sample of low dosage showed a better IL-12 stimulation than that of high dosage.

Type I allergies are characterised by an elevated production of IgE and mast cell degranulation. Th2 responses are distinguished by various cytokines, such as IL-4 and IL-5, which promote the production of IgE. In contrast, Th1 inhibits IgE production by secreting IFN- γ . IL-12 produced by antigen-presenting cells is also known to stimulate both natural killer cells and Th1 cells to produce IFN- γ (Fujiwara *et al.*, 2004). The results of *in vitro* (Figs 3 and 4) and Th2-polarised splenocytes (Fig. 5) demonstrated *L. kefiranofaciens* M1 with a high stimulatory activity for IL-12, a strong Th1-skewing cytokine. IL-12 production could stimulate IFN- γ and downregulate IL-4 expression produced by Th2 cells. Similarly, Kato *et al.* (1999) also indicated that LAB were the prime immune cells for Th1 differentiation by secreting IL-12. The anti-allergic effect of LAB, however, was dependent upon the LAB strain rather than the LAB species (Fujiwara *et al.*, 2004) and upon the dosage (Murosaki *et al.*, 1998).

Conclusion

To summarize, the milk whey hydrolysed by Alcalase induced a significantly higher pro-inflammatory cytokine production than by the Neutrase and pepsin. However, the secretion of cytokines induced by the milk whey with *L. kefiranofaciens* M1 was significantly higher than that by Alcalase alone and by the whey control on both cells. The immunomodulatory factor could be the cell wall of *L. kefiranofaciens* M1. In addition, Th2-polarised splenocytes revealed that *L. kefiranofaciens* M1 had both IL-12 inducing and IL-4 repressing activities. These results suggested that *L. kefiranofaciens* M1 could direct the Th1/Th2 balance toward Th1.

Further comparable experiments *in vivo* will be conducted to confirm these results. According to our best knowledge, this is the first report investigating the *in vitro* immunomodulatory effect of milk whey through enzymatic hydrolysis, microbial fermentation, and two-stage hydrolytic reactions by cytokine profiles.

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