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Characterization of immune-active peptides obtained from milk fermented by *Lactobacillus helveticus*

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The objectives of this research were to confirm the effect of compounds derived from milk fermented by *Lactobacillus helveticus* (LH-2) on the nonspecific host defence system, and isolate and characterize the active peptides that mediate the immune response. The cell-free supernatant obtained from the fermented milk and its fractions were tested *in vitro* for immunomodulating activity using murine macrophages (RAW 264·7 cell line). Cytokine production (Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), and Interleukin-1β (IL1-β)), nitric oxide (NO) production and phagocytosis were used as biomarkers. Macrophages stimulated with cell-free supernatant of fermented milk showed higher production of cytokines and NO compared with macrophages stimulated with LPS (Lipopolysaccharide) and a commercial immunomodulator derived from β-casein (f54-59). Phagocytosis was observed by macrophages stimulated with the supernatant. Two of nine fractions collected from the supernatant using size exclusion chromatography produced the highest response when used to stimulate macrophages. The results of the dose-response study of the effect of the fraction with the highest stimulation effect on the production of TNF-α showed a direct correlation between protein concentration and TNF-α release. The fraction contained four novel peptides, three derived from the hydrolysis of β-casein and one from the hydrolysis of α-lactalbumin. These results confirm that fermentation of milk by *Lactobacillus helveticus* (LH-2) results in the production of specific peptides capable of modulating macrophage activity.

Keywords: Immunomodulation, fermented milk, Macrophages, bioactive peptides.

Milk proteins are considered to be the most important source of bioactive peptides that can be released through hydrolysis by proteolytic microorganisms such as lactic acid bacteria (Meisel & Bockelmann, 1999; Matar et al. 2001; LeBlanc et al. 2002, 2004; FitzGerald & Meisel, 2003; Kenny et al. 2003; De LeBlanc et al. 2005, 2008). The immunomodulatory effects of the peptides have been actively studied, particularly those released during milk fermentation with *Lactobacillus helveticus* (Ng & Griffiths, 2002; Vinderola et al. 2007a, b & c). However, only a in a few of those studies have the isolated compounds been characterized to understand their interaction with the innate immune system (Coste et al. 1992; Herbert et al. 1997; Sandre et al. 2001; Vinderola et al. 2007b).

Macrophages play an important role in innate immunity through mechanisms such as nitric oxide production, phagocytosis, and cytokine production (Aderem & Underhill, 1999; Ng & Griffiths, 2002; LeBlanc et al. 2002; Brovko et al. 2003; Vinderola et al. 2007b; de Moreno de LeBlanc et al. 2008). During the course of infection, cytokines such as TNF-α, IL-6, and IL-1 β are produced and transmit signals that alert the various components of the host defence. However, despite the beneficial effects of these pro-inflammatory cytokines for the activation of host defences, various studies have shown that an over-production of these mediators can also lead to vasodilation, increased vascular permeability, hypotension, multiple organ failure and, ultimately, shock and death (Perdigon et al. 2001; Netea et al. 2003). Fermented milk containing bioactive peptides has the potential to provide specific health benefits such as enhancement of the performance of the innate immune system through macrophage activation; however, it is important to acquire a better understanding of the role of these bioactive peptides as inducers of immune responses.

The objectives of this study were to further investigate the immunomodulating effect of cell-free fractions

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of milk fermented by *Lactobacillus helveticus* LH-2 (Ng & Griffiths, 2002; Brovko et al. 2003) by evaluating cytokine production (TNF-α, IL-1β, IL-6), phagocytic activity and nitric oxide production by macrophages, and to isolate and characterize the active peptides present in the cell-free fraction.

Materials and Methods

Fermented Milk Preparation

Lactobacillus helveticus. LH-2 was obtained from the culture collection of the Canadian Research Institute for Food Safety (University of Guelph, Canada) and maintained on Lactobacilli MRS agar (BD Diagnostic Systems) under aerobic conditions at 37 °C.

Reconstituted skim milk (0·1 g/ml) was heat treated at 95 °C for 30 mins and then cooled overnight at room temperature. Isolated colonies of the *Lb. helveticus* strain were used to inoculate the reconstituted milk, which was then incubated anaerobically (BBL GasPak system Becton Dickinson, Oakville, ON, Canada) at 37 °C for 24 h. This was used as a starter culture at a ratio 1 : 10 (v/v) for production of fermented milk by aerobic incubation at the same temperature for 26 h. The fermented milk samples were centrifuged at 16 000 ***g*** for 10 mins at 15 °C (Avanti J-20 XPI, Beckman Coulter, Brea, California, USA). The supernatant was filtered through a 0·2-μm-pore-size filter (Millipore, Bioscience Division, Mississauga, ON, Canada) to remove any bacterial cells present. The protein content of the cell-free supernatant was determined using the Bradford assay (Coomassie Plus Assay Kit, Pierce, Fisher Scientific Ltd., Nepean, ON, Canada) (Kagan et al. 2000). Cell-free supernatant was freeze dried and stored at −80 °C. Even though the Bradford assay may be not the most appropriate method for determination of protein content in hydrolysed protein samples, it was used in this study since no other method was able to quantify protein content less than 10 μg/ml.

Peptide purification

Size exclusion chromatography (SEC). Freeze dried supernatant was reconstituted using 50 mM-sodium phosphate-0·15 M-NaCl buffer (5 times concentrated) and filtered through a 0·45 μm filter (Fisher Scientific Ltd., Nepean, ON, Canada). One hundred microlitres of the sample were loaded on a Superdex 75 column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and eluted with the same buffer at a rate of 1·0 ml/min. The proteins and peptides were monitored at 214 nm using a UV Detector-Spectra system UV 2000-Thermo Finnigan, (San Jose, California, USA). Nine fractions that corresponded to the main peaks were collected and freeze dried to be used for further immune stimulation studies and amino acid sequencing.

Macrophage Stimulation Studies

Samples preparation. To compare the stimulatory effect of the fractions (F1 to F9) obtained from fermented milk by SEC, samples were reconstituted using autoclaved, 18-Ω water and adjusted to a protein content of 3 or 20 μg/ml, as indicated below.

Macrophage stimulation. A RAW 264·7 murine macrophage cell line (ATCC TIB 71) was kindly provided by Dr. Ali Ashkar (McMaster University, Hamilton, ON, Canada). Cells were maintained in RPMI-1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 2 mM L-glutamine, 1% (v/v) penicillin-streptomycin (dual antibiotic solution-10 000 units/ml) and 10% fetal bovine serum (Gibco, Invitrogen, Burlington, ON, Canada). Macrophages were counted and the necessary dilution was made to obtain a density of 5×10^5 cells/ml. Cell suspensions (1 ml) were placed into wells of 6-well plate (Corning No. 3506; Fisher Scientific Canada, Ottawa, ON, Canada) and incubated overnight at 37 °C in 5% CO_2. The medium was removed and replaced by 1 ml of samples and or control diluted in medium (1 : 100 sample to medium). LPS (*Escherchia coli* O111 : B; 0·1 μg/ml) (Sigma-Aldrich, Oakville, ON, Canada) and a commercial immunomodulator from β-casein (f 54-59) (Val-Glu-Pro-Ile-Pro-Tyr) (Sigma-Aldrich) were used as positive controls (Jolles & Migliore-Samour, 1988). Negative controls were prepared by hydrolyzing unfermented milk using an enzyme : substrate (w/w) ratio of 1 : 20 and 1 : 1000 for trypsin and thermolysin (Invitrogen), respectively. Enzymatic hydrolysis was carried out for 24 h at 37 °C and pH 5 followed by centrifugation (92 000 ***g***, 1 h, 20 °C) (Optima L-90K Ultracentrifuge, Beckman Coulter, Fullerton, CA, USA). This supernatant was used in further experiments. Macrophages were also stimulated by cell-free supernatant of fermented milk concurrently with LPS. Stimulated macrophage cells were incubated for 18 h at 37 °C in 5% CO_2 in a CO_2 incubator.

Cytokine quantification

An indirect sandwich Enzyme Linked Immunosorbent Assay (ELISA) was used to quantify the amount of TNF-α, IL-6 and IL-1β in the supernatant. Flat-bottom, high-binding, 96-well plates (Fisher Scientific Canada) were coated with 100 μl monoclonal goat anti-mouse IL-6 antibody (R & D Systems Inc., Minneapolis, MN, USA), anti-mouse IL-1β (R & D Systems Inc.) or anti-mouse TNF-α (Sigma-Aldrich) diluted in carbonate-bicarbonate buffer (pH 8·8). The concentration of monoclonal antibody was 1 μg/ml for IL-6 and IL-1β, and 2 μg/ml for TNF-α. The plates were stored overnight at 4 °C, washed with TBST (10 mM-Tris-HCl, pH 7·5, 150 mM-NaCl, 0·05% Tween-20) and blocked with 200 μl normal donkey serum (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) by incubation at 37 °C for 2 h with shaking

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(200 rpm). Before use plates were washed four times with TBST and patted dry. Samples were added to the wells and after 1 h of incubation at 37 °C, the plate was washed 4 times with TBST. For detection, goat anti-mouse IL-1 β, IL-6, and TNF-α (R & D Systems Inc.) antibodies were used. The concentration of the polyclonal antibodies was 200 ng/ml for IL-1 β, and 400 ng/ml for the other two cytokines. After 1 h of incubation, plates were washed using TBST. Alkaline phosphatase-conjugated donkey anti-goat antibody (Jackson Immuno Research Laboratories Inc.) diluted in TBS (Tris Buffered Saline) pH 8·0 (Sigma-Aldrich) was added (100 µl/well) at a titer of 1 : 10 000. Plates were incubated for 1 h, and washed as described before. For colour development, 100 µl p-nitrophenyl phosphate (Sigma-Aldrich), diluted in the diethanolamine substrate buffer (Fisher Canada) at a concentration of 1 mg/ml, were added. Plates were incubated for 30 mins and stop solution (25 µl 3 M-NaOH) was applied to each well. The absorbance at 405 nm ($A_{405\ nm}$) was measured using a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer Life Sciences Canada, Woodbridge, ON, Canada).

Assay for Nitric Oxide (NO)

Nitric oxide production in culture supernatant was assayed by measuring nitrite (NO_2^-), its stable degradation product, using Griess reagent (Sigma-Aldrich).

RAW 264·7 cells were seeded on a 24-well plate at a concentration of 10^6 cells/ml and allowed to adhere by incubation for 4 h at 37 °C in a CO_2 incubator (Forma Scientific Inc.). Cells were stimulated with LPS (1 µg/ml) or samples (1 : 100 sample to medium) for 24 h. Fifty microliters of supernatant from the cultured cells were mixed with 50 µl Griess reagent and incubated for 15 mins at room temperature. The absorbance was measured at 540 nm in a Victor MultiLabel microplate reader (Perkin Elmer Life And Analytical Sciences, Inc). Sodium nitrite (Sigma-Aldrich) was used as a standard.

Phagocytosis Assay

To quantify the effect of inducers on phagocytic function, the Vybrant™ Phagocytosis assay kit was used (Invitrogen, Burlington, ON, Canada) according to manufacturer instructions. The fluorescence was read using a Wallac 1420 Victor Multilabel counter with excitation and emission wave lengths at 480 and 520 nm, respectively and results were presented as a percent of the Net Positive Reading.

MS analysis and Peptide Sequencing

Active fraction (F5) was spotted on a Matrix-assisted laser desorption/ionization (MALDI) target using 2,5-dihydroxybenzoic acid (Sigma-Aldrich) as the matrix. MALDI mass spectra were acquired on a QStar XL QqTOF (Applied Biosystems, Foster City, CA, USA) equipped with a MALDI 2 source and a nitrogen laser operating at 337 nm. In order to sequence the peptides found in F5, data from MS/MS measurements were searched against the NCBI bovine protein database using the MASCOT server (Matrix Science, Boston, MA, USA) (Perkins et al. 1999).

Statistical analysis

Data from each experiment were evaluated using ANOVA and a subsequent Tukey's test. Differences at $P<0{\cdot}05$ were considered to be significant.

Results

Immunomodulating Activity of Cell-Free Fermented Milk Supernatant was tested by the methods described above. The results obtained suggest that IL-6, TNF-α, and IL-1β production was significantly higher in macrophages stimulated with cell-free supernatant compared to LPS-stimulated macrophages (Fig. 1 & 2). The highest effect was observed for TNF-α since production of this cytokine was 3-fold higher in macrophages stimulated by cell-free fermented milk fraction compared with cells stimulated with LPS. Macrophages stimulated with supernatant concurrently with LPS showed the highest response in terms of IL-6 and IL-1β production. Production of IL-6 in this case was 6-fold higher compared with supernatant-stimulated macrophages. The commercial immunomodulator induced lower cytokine production (TNF-α, IL-6 and IL-1β) compared with cells stimulated with supernatant or supernatant with LPS. Enzymatically hydrolyzed supernatant exhibited a very low effect on cytokine production, confirming that compounds produced during milk fermentation with *Lb. helveticus* are responsible for macrophage activation as these are not present in milk treated with proteolytic enzymes.

The observed phagocytic activity of macrophages stimulated by supernatant from fermented milk was significantly higher than for the commercial immunomodulator, but lower than for macrophages stimulated by LPS (Fig. 3). The commercial immunomodulator is a hexapeptide from human casein (Val-Glu-Pro-Ile-Pro-Tyr) that exerts a direct effect on macrophages by promoting phagocytosis (Parker et al. 1984).

Effect of isolated Peptidic Fractions of fermented milk on macrophages. The effect of the fractions collected by SEC, and adjusted to a protein concentration of 3 µg/ml was evaluated by quantification of cytokine production, phagocytosis and nitric oxide production.

Two fractions (void volume (F1) and a fraction eluted at 15 mins (F5)) produced a higher response in terms of IL-6 and TNF-α release when used to stimulate macrophages compared with all the other fractions (Fig. 4). F5 also induced the highest levels of phagocytosis and NO production compared with all the other fractions since the levels of these biomarkers for the rest of the fractions were

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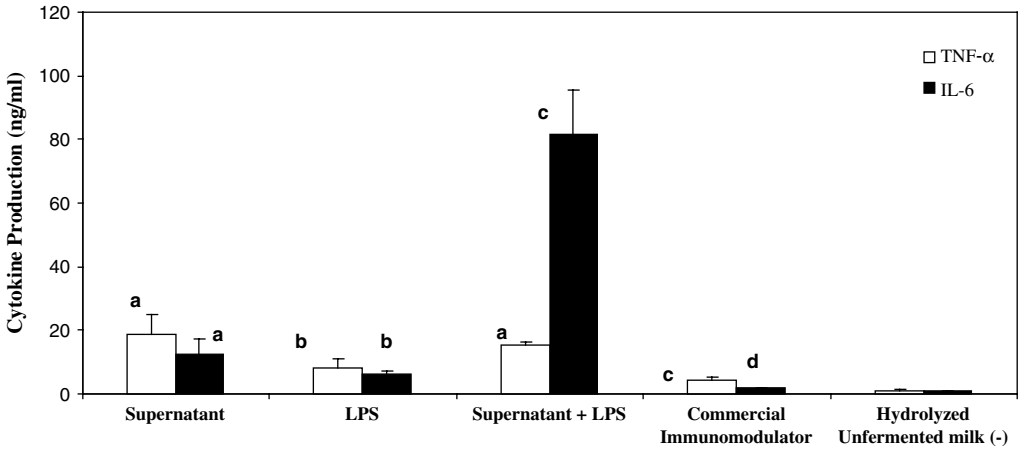

Fig. 1. IL-6 and TNF-α production (ng/ml) by RAW 264·7 cell line stimulated by LPS, supernatant, supernatant+LPS, commercial immunomodulator, and hydrolyzed supernatant. The test was standardized at 100 μg/ml protein per sample to compare activity. The data are the means±the standard deviations of the results of three independent replicate trials. Values with different letters are significantly different $P<0{\cdot}05$.

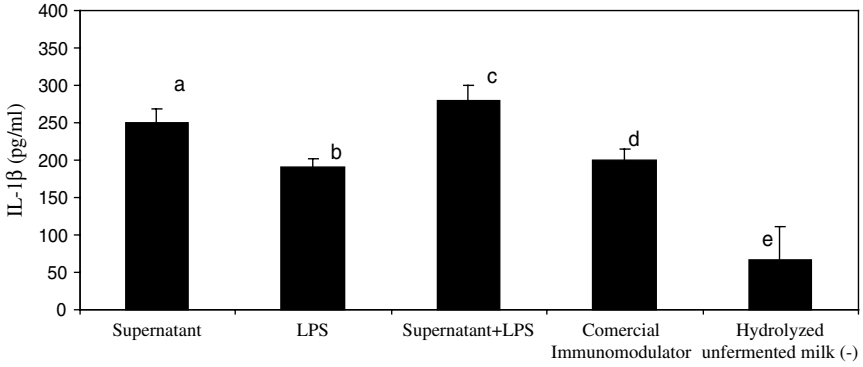

Fig. 2. IL-1β production (pg/ml) by RAW 264·7 cell line stimulated by LPS, supernatant, supernatant+LPS, commercial immunomodulator, and hydrolyzed supernatant. The data are the means±SD of the results of three independent replicate trials. Values with different letters are significantly different $P<0{\cdot}05$.

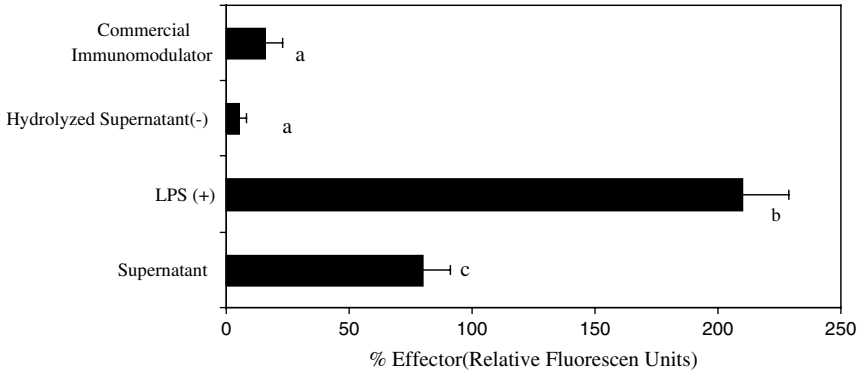

Fig. 3. Phagocytic activity of RAW 264·7 macrophages stimulated by LPS, supernatant, commercial immunomodulator and hydrolyzed supernatant. The data are the means±SD of the results of three independent replicate trials. Values with different letters are significantly different $P<0{\cdot}05$.

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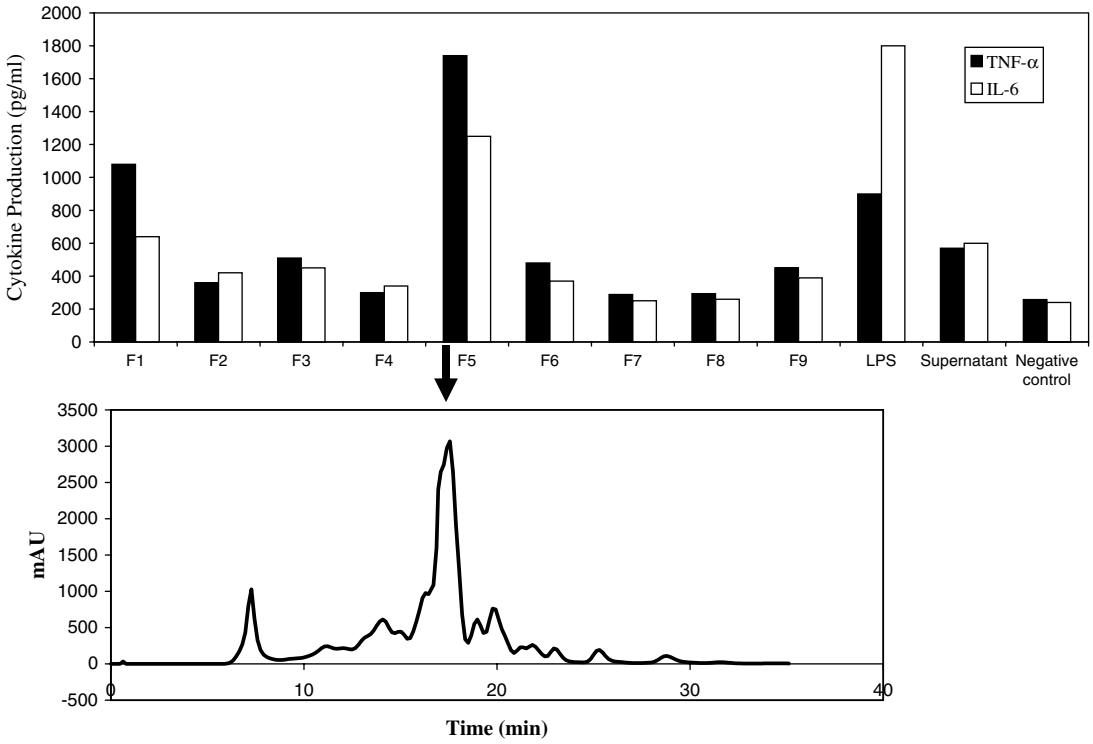

Fig. 4. IL-6 and TNF-α production (pg/ml) by RAW 264·7 cell line stimulated by LPS, cell-free supernatant, hydrolyzed supernatant, and peptidic fractions from supernatant isolated by SEC. The test was standardized at 3 μg/ml protein per sample to compare activity. The data are the means ± SD for three independent replicate trials.

Table 1. Cytokine and nitric oxide production by RAW 264·7 macrophages

	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-1β (pg/ml)	NO (uM)
LPS (+)	6000[a]	12 000[a]	287[a]	33·05[a]
Supernatant	3600[b]	2000[b]	252[a]	28·98[a]
F5	8220[a]	4240[c]	274[a]	31·51[a]
Hydrolyzed Supernatant (-)	300[d]	120[d]	65[b]	5·78[b]
Unfermented Milk	500[d]	150[d]	78[b]	8·94[b]

Protein was standardized at 20 μg/ml. Values with different letters are significantly different $P<0·05$

below the detection limits (data not shown). TNF-α expression in the presence of fraction F5 was 2-fold higher than that obtained with fermented milk supernatant and 16-fold higher than with unfermented milk. However, there was no significant difference for TNF-α production between LPS and F5 stimulated macrophages (Table 1). IL-6 production for macrophages stimulated with F5 was significantly lower than that obtained in the presence of LPS but was more than 2-fold higher than levels of cytokine achieved by addition of the supernatant. There was no significant difference among the analyzed samples in terms of IL-1β and nitric oxide production when macrophages were stimulated with LPS, supernatant or F5 (Table 1).

Samples of the Fraction F5 adjusted to protein concentrations between 2·5–20 μg/ml were evaluated in terms of TNF-α production. Results showed a direct relation between the amount of protein present and TNF-α production (Table 2). Higher concentrations (>20 μg/ml) induced macrophage death.

Sequencing of peptides in F5 fraction

The F5 fraction was analyzed by mass-spectrometry and amino acid sequences of peptides were elucidated. Five peptides were identified in the fraction, which ranged in size from 8 to 16 amino acids (Table 3). Matching of their sequence with bovine proteins (NCBI data base) suggests that four peptides present in the fraction originated from β-casein (f 143-160 and f 192-202), and one from α-lactalbumin (f 115-122).

Discussion

Bioactive peptides produced during fermentation of milk by *Lb. helveticus* have been proposed as an alternative to control bacterial infection because of their potential immune-stimulating and anti-microbial properties (Matar

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Table 2. TNF-α production in response to varying level of protein content (2·5 to 20 μg/ml) in F5

Values are the means ± SD for $n=3$ independent replicate trials

Protein (μg/ml)	TNF-α (pg/ml)
2·5	0·92 ± 0·2
5	2·24 ± 0·88
10	4·57 ± 0·67
20	7·45 ± 0·36

et al. 2001; LeBlanc et al. 2002, 2004; Yasutaka & Kiyoshi, 2002). In contrast with other studies (Vinderola et al. 2007a & b), in our work the pH was not controlled during the fermentation to reproduce the process of production of natural dairy products. In the present study, the potential stimulatory effect of cell-free supernatant from fermented milk and its peptidic fraction F5 on macrophages was investigated by evaluating cytokine (IL-6, TNF-α and IL-1β) production, phagocytosis and nitric oxide production. Cytokines play a central role in coordinating inflammatory responses and in providing signals for cellular differentiation and proliferation (Takahashi et al. 1993). In this study, a cell-free fraction from fermented milk enhanced the production of IL-6, TNF-α, IL-1β by stimulating macrophages. The observed high production of TNF-α and IL-6 by the macrophages was accompanied by increased nitric oxide production and phagocytic activity. Nitric oxide, the synthesis of which is induced by TNF-α, is one of the cytotoxic agents by which macrophages can kill bacteria and other pathogens, as well as tumour cells (Jeffrey et al. 1996; Miettinen et al. 1996).

Further separation of the components of the supernatant according to their size by SEC gave rise to the fraction (F5) that produced a high stimulatory effect on cytokine production by RAW 264·7 cells. The highest response was observed for TNF-α. TNF-α is part of the major host defence components against trauma and infection, and plays an important role in inducing gene expression of several cytokines and nitric oxide synthesis (Urban et al. 1986; Tejada-Simon & Pestka, 1999; Tsao et al. 2004; Ustundag et al. 2005). The results of the dose-response study for the effect of fraction F5 on the expression of TNF-α suggested that the protein concentration for optimal activity was in the range 2·5–20 μg/ml. Higher concentrations of peptides in the sample produced macrophage death, confirming previous observations that moderated production of cytokines have a beneficial effect but high concentration of TNF-α causes tissue injury and shock (Abbas et al. 1994). Thus, preparations of F5 with threshold protein content can induce moderate production of TNF-α and this might have a beneficial effect on macrophage activation.

Five peptides identified and sequenced from fraction F5 were derived from both β-casein and α-la (Table 3). These results confirm our observations from the preliminary tests done on fermentation of the separate fractions of casein and whey. Both preparations stimulated production of IL-6

Table 3. Peptides identified in the F5 fraction of milk fermented with *Lactobacillus helveticus* LH-2 strain after separation by SEC using Superdex 75 column

Peptide	Protein	Sequence assignment
HQPHQPLPPTVMFPPQ	β-Casein	145–160
HQPHQPLPPT	β-Casein	145–154
WMHQPHQPLPPT	β-Casein	143–154
LYQEPVLGPVR	β-Casein	192–202
LDQWLCEK	α-Lactalbumin	115–122

and TNF-α by RAW 264·7 macrophages, although the fermented milk supernatant induced a higher response (results not shown).

Three of the peptides present in the active fraction F5 share the common amino acid sequence of β-casein with variation at N- and C-terminals (f 148-154; f 145-160 and f 143-154) and have a net charge +2. Peptide β-casein (f 192-202) has a net zero charge, and peptide α-la (f 115-122) has a negative net charge, however, in these two last peptides 50% of their residues are hydrophobic (Protein Identification and Analysis Tools- ExPASy Server) (Gasteiger et al. 2005). Peptides identified in F5 have in their structure a high percentage of proline and also contain residues of lysine and histidine (Coste et al. 1992; Christensen et al. 2003).

From all the peptides identified in the present work, only C-terminal fragment of β-casein from 192 to 209 have been reported to have immunostimulating properties (Sandre et al. 2001; Coste et al. 1992). C-terminal fragment of β-casein was obtained by enzymatic hydrolysis of the pure protein using chymosin and isolated by ion-exchange chromatography and HPLC to 80–95% purity. It was tested for mitogenic activity on rat lymph node cells and showed enhanced proliferation of the treated cells at concentration 500 μg/ml. The synthetic analogue of the same peptide produced the similar effect, thus confirming the immunomodulating activity. Later, the effect of the same peptide on phagocytic activity and cytokine production in mouse macrophages was investigated. The stimulatory effect was observed for phagocytic activity, however f193-209 peptide was a very weak inducer of cytokine secretion. Comparing these results with the results obtained in the present study, where F5 was able to up-regulate TNF-α to the same extent as LPS, it can be concluded that peptides isolated from β-casein (f143-154, f145-160, and f148-154) as well as peptide derived from α-la (f115-122) are probably also involved in macrophage activation. In addition the concentration of peptidic fraction used in our study was at least 25 times lower than in previous work, thus confirming the high biological activity of newly obtained peptides. It still remains unclear if all the peptides present in F5 have an impact on its immunomodulatory activity or exert a synergistic effect, or if it is just an individual effect caused by one or a few of

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the sequenced peptides. Further purification and separation of individual peptides was not possible since the concentration of the peptides in F5 was very low.

In summary, this study showed that the cell free supernatant from milk fermented by *Lb. helveticus* LH-2 and its peptidic fraction F5 up-regulate cytokines and nitric oxide production by macrophages as well as stimulating phagocytic activity of these cells. Four novel bioactive peptides derived from β-casein and α-la by fermentation with *Lb. helveticus* LH-2 were identified in this fraction. These effects suggest that F5 might exert an immunostimulating activity through modulation of macrophage functions. It is well-known that the nature and activity of peptides produced by milk fermentation with *Lb. helveticus* is strain specific, unlike in the case of enzymatic hydrolysis of pure casein (Deutsch et al. 2000). Taking advantage of the powerful proteolytic system of this bacterium opens up future opportunities to search for novel food-derived compounds with potential health promoting properties using the approach described in this work.

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