

Effect of *Bifidobacterium bifidum* DSM 20082 Cytoplasmic Fraction on Human Immune Cells

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The objective of the present study was to determine the effect of the soluble cytoplasmic fraction from *Bifidobacterium bifidum* DSM 20082 (Bb) lysate on peripheral blood T cells. In peripheral blood mononuclear cells of healthy subjects, cytotoxic activity, proliferation, apoptosis, and up-regulation of CD8 or CD4 molecules in T cells were examined. When peripheral blood mononuclear cells were stimulated with Bb lysate, the main effect was observed in CD8+ cells as a significant increase of CD8 molecules in a dose-dependent manner, and this behavior was observed at 24, 48, and 72 h after stimulation; in contrast, stimulation with Bb lysate showed no effect on the up-regulation of CD4 molecules in T helper cells. Further Bb lysate did not induce proliferation activity in either CD8+ or CD4+ cells. Bb lysate induced activation of CD8+ cytotoxic activity against autologous monocytes. Around 80% of the cells stimulated with Bb lysate were positive to peanut agglutinin (PNA), suggesting that the stimulated CD8+ cells corresponded to activated/effector cellular populations. When apoptosis was determined, there were no differences between stimulated and non-stimulated cells. Our results indicate

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that Bb lysate is able to increase cytotoxic activity of peripheral CD8⁺ cells, without affecting lymphocyte survival.

Keywords *Bifidobacterium bifidum*, Probiotic, Immune response, CD8 T cells.

INTRODUCTION

As defined by FAO and WHO, probiotics are microorganisms that confer a health benefit to the host (FAO and WHO, 2002; Reid et al., 2003). Probiotics have been used in many clinical trials, from allergies to cancer (Reid et al., 2003), and in vivo studies have reported that probiotics or probiotic-containing diets have anti-tumor effects on colon cancer in carcinogenesis-induced rats (Femia et al., 2002; Marotta et al., 2003). It has also been reported that probiotics enhance Natural Killer (NK) cells lytic activity against a murine lymphoma cell line and delay tumor onset (Takagi et al., 2001). The cytoplasmic fraction of *Bifidobacterium longum* has been reported to have antiproliferative activity in tumor cells due to its ability to induce proliferation of cytotoxic T CD8⁺ cells and to enhance the number of these cells (Lee et al., 2004).

The cytoplasmic extract but not the cell wall extract from probiotic bacteria has an antiproliferative activity upon peripheral blood mononuclear cells (PBMC), suggesting an immunomodulatory action on these cells according to Pessi et al. (1999). In PBMC from infants with cow's milk allergy or IgE-associated dermatitis, probiotics increased INF-gamma production, suggesting beneficial Th1 immunomodulatory signals (Pohjavuori et al., 2004). DNA isolated from *Bifidobacterium* reduced IL1-beta secretion and increased IL-10 in PBMC of healthy individuals (Lammers et al., 2003).

The aim of our work was to determine the effect of the Bb lysate on the cytotoxic activity of peripheral blood CD8⁺ cells, since the role of these cells is essential to activate and maintain cellular immunity against tumors and viral processes.

MATERIALS AND METHODS

Reagents

Phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) against human CD8, Quantum Red (QR) and Fluorescein Isothiocyanate (FITC)-labeled mAb against human CD4, FITC conjugated Peanut agglutinin (PNA), Concanavalin-A (ConA), 7-aminoactinomycin-D (7-AAD) dye, bovine serum albumin (fraction V) (BSA), *p*-formaldehyde, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, β -mercaptoethanol, sodium pyruvate, Roosevelt Park Memorial Institute (RPMI)-1640 cell culture medium, trypan blue dye, and salts were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA). Carboxyfluorescein

diacetate succinimidyl-ester (CFSE) was obtained from Molecular Probes (Eugene, OR, USA).

Preparation of Cytoplasmic Fraction of *Bifidobacterium bifidum*

Human *Bifidobacterium bifidum* DSM 20082 used in this study was kindly provided by F. Gavini (INRA, Villeneuve d'Ascq, France). Bacteria were cultured anaerobically with a GENbox (BioMérieux, Marcy l'Etoile, France) anaerobic jar in Brain Heart infusion (BHI) (Difco, USA) at pH 7.1, 37°C for 16 h. Before use, bacteria were collected by centrifugation at $7000 \times g$ for 15 min at 4°C; the pellet was suspended and 3 three times with phosphate-buffered saline (PBS) (145 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2). The pellet from 10 ml of culture medium was suspended in 1 ml of PBS and bacteria were disrupted by ultrasonic waves, followed by ultra-centrifugation ($150,000 \times g$, at 4°C for 20 min). The supernatant, containing the Bb lysate, was filter-sterilized and kept at -70°C until further use. Control assays were performed using Bb lysate aliquots (1 mg/ml) incubated with 0.05 mg trypsin (Promega sequencing grade, Upsala, Sweden) in 100 µl PBS for 2 h at 37°C, then aliquots were boiled at 100°C for 1 min to inactivate trypsin (trypsinated Bb lysate samples). Protein concentration was determined by a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). All the units are expressed in micrograms of total protein.

Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral venous blood of three healthy volunteers by density gradient centrifugation (1.077) (Lymphoprep Nycomed Pharma, Oslo, Norway) for 30 min at $500 \times g$ at 16°C (Boyum, 1976). Cells were suspended in complete RPMI-1640 medium and plated at a density of 2×10^5 cells/200 µL on 96-well cell culture plates (Nalgene Nunc International, Roskilde, Denmark) and stimulated at different time intervals (24, 48, and 72 h) with different Bb lysate concentrations (from 0 to 50 µg/mL).

Cell Proliferation Assay and Flow Cytometry Conditions

Isolated PBMC were washed twice in RPMI-1640 culture medium and cell viability was determined by the trypan blue dye exclusion method. In this test a cell suspension is mixed with dye and then visually examined whether cells take up or exclude dye, a viable cell has a colorless cytoplasm whereas a nonviable cell has a blue cytoplasm. PBMC were stained with carboxyfluorescein diacetate succinimidyl-ester (CFSE) (Lyons and Parish, 1994). Briefly, 1 ml of PBMC at a density of 10×10^7 cells/mL of RPMI medium, was incubated with 15 µL 0.5 mM CFSE, for 10 min at room temperature in the dark. After

incubation, 8 ml of complete culture medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 M β -mercaptoethanol, 0.1M sodium pyruvate, 100 IU/mL penicillin and 100 μ g/mL streptomycin, was added to the cells. Subsequently, cells were centrifuged, and suspended in 1mL of supplemented culture medium. CFSE-treated PBMC were seeded into 96-well culture plates (Nalgene Nunc International, Roskilde, Denmark) at 2×10^5 cells per well in 200 μ L supplemented RPMI-1640 culture medium at 37°C in a humidified atmosphere containing 5% CO₂. Proliferation assays were performed in the presence of different Bb lysate concentrations (0, 10, 20, 30, 40, and 50 μ g/mL) at different times (24, 48, and 72 h). The cells were harvested and stained with murine Quantum Red fluoro-chrome-conjugated anti-CD4 and Phycoerythrin fluoro-chrome conjugated anti-CD8 mAb's and were analyzed on a FACSCalibur equipped with CellQuest software (Becton-Dickinson, San Jose, CA, USA). Cells undergoing proliferation were gated by forward scatter (FSC) and side scatter (SSC) characteristics. Stimulation with a polyclonal mitogen such as Concanavalin A lectin (1 μ g/mL) was used to demonstrate cell proliferation. The initial undivided CFSE histogram gate was set based on the fluorescence intensity of non-stimulated cells. Cell proliferation was evaluated by half-reduction in the mean fluorescence intensity (MFI) that corresponded to each cell division, determined by flow cytometry. To identify the up-regulation of CD8 molecule on CD8+ lymphocytes, same culture conditions were performed as mentioned above, and MFI of CD8 was determined on histograms from FSC and CD8 dot plots.

Apoptosis

To evaluate membrane conformation of the PBMC and DNA integrity after stimulation with Bb, PBMC were labeled with FITC-CD4 and PE-CD8 mAbs; after mAb staining, the cells were incubated for an additional 20-min period at 4°C in the dark with 7-aminoactinomycin-D. After washing, the cells were analyzed by flow cytometry (FCM), using CellQuest Software (Becton-Dickinson, San Jose, CA, USA). Cells positive to 7-aminoactinomycin-D were considered to be apoptotic cells (Lecoeur *et al.*, 1998).

Isolation of CD8+ Cytotoxic T Cells

After 72 h of stimulation with *Bifidobacterium bifidum* cytoplasmic fraction, 10×10^7 non-adherent PBMC were washed with PBS, centrifuged at $300 \times g$ for 10 min, the pellet was suspended in 80 μ L of PBS, and incubated with 20 μ L of biotin-conjugated mouse anti-human CD4, CD14, CD19, CD36, TCR and Glycophorin A antibodies for 20 min at 4°C; following incubation, cells were washed with PBS, centrifuged at $300 \times g$ for 10 min and the pellet was suspended in 80 μ L of PBS and incubated with 20 μ L of antibiotin-conjugated

microbeads for 20 min at 4°C (Miltenyi, Biotec, Bergisch Gladbach, Germany). The cells were then washed by adding 1 mL of PBS and centrifuged at $300 \times g$ for 10 min, the supernatant was removed and 1 mL PBS was added to the cell pellet. Due to all the cells were already labeled with microbeads antibodies other than CD8 and, isolation of highly pure CD8+ T cells was achieved by depletion of magnetically labeled cells. Up to 95% purity was obtained as determined by flow cytometry using CellQuest software (Becton-Dickinson, San Jose, CA, USA).

Peanut Agglutinin (PNA) Binding Assay

After harvesting and magnetically separating, CD8+ cells were incubated with 10 μ L of a 1:60 dilution of FITC-conjugated PNA for 15 min at 4°C in the dark. To determine the specificity of PNA for CD8+ T cells, control assays were performed by adding 0.2 M lactose to the cells, which indicated negative staining of PNA-recognized cells.

Cytotoxicity Assay

To perform cytotoxicity assay, autologous monocytes (1×10^6) were used as targets, effector CD8+ cells (1×10^6) were suspended in 200 μ L RPMI 1640 supplemented with 1% FBS during the assay. Cytolysis was measured using a cytotoxicity detection kit according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA). Cell-mediated cytotoxicity was determined as a function of lactate dehydrogenase (LDH) enzymatic activity released from the cytosol of damaged cells into the supernatant (Schnyder and Baggiolini, 1978). LDH activity was quantified by monitoring the reduction of tetrazolium salt (yellow) to formazan (red), which was measured at 490 nm in an ELISA reader (Biorad, Hercules, CA, USA). Percentage of cytolysis was calculated using the following equation:

$$\text{Cytotoxicity (\%)} = 100 \times \frac{[(\text{effector and target cell mix} - \text{effector cell control}) - 200 \text{ spontaneous release}]}{(\text{maximum release} - \text{spontaneous release})}$$

Statistical Analysis

All data were collected from triplicates of three different independent assays, which were analyzed with two way ANOVA when dose and time variables were evaluated, and Student *t*-test was performed to determine differences between two groups, using the SigmaStat software, considering $p < 0.05$ as statistically significant.

RESULTS

Up-Regulation of CD8 Molecules on the Cell Surface

When PBMC were stimulated with *Bifidobacterium bifidum*, there was an up-regulation of the CD8 molecule on CD8+ cells, determined as an increase in the mean fluorescence intensity (MFI). Under basal non-Bb lysate stimulated conditions, the mean fluorescent intensity was 150; however, after 24 h of Bb lysate stimulation at a concentration of 40 $\mu\text{g/mL}$, the MFI increased two-fold as compared with non-stimulated cells (Figure 1). Testing a Bb lysate concentration range of 10–50 $\mu\text{g/mL}$ revealed that the MFI increased in a dose dependent-manner, reaching a maximum MFI level (2.5-fold above the basal value) with 40 $\mu\text{g/mL}$ Bb lysate. A similar behavior was observed when cells were stimulated for 48 h and 72 h. The MFI of the 48 h-stimulated cells increased in a dose-dependent manner while a maximum MFI level was observed at 20 $\mu\text{g/mL}$ for the 72 h-stimulated PBMC (Figure 1). Moreover, no changes were observed when cells were treated with trypsinated Bb lysate samples (data not shown) The stimulation of PBMC with 10–50 $\mu\text{g/mL}$ of Bb lysate for different time periods (24–72 h) did not affect the expression of the

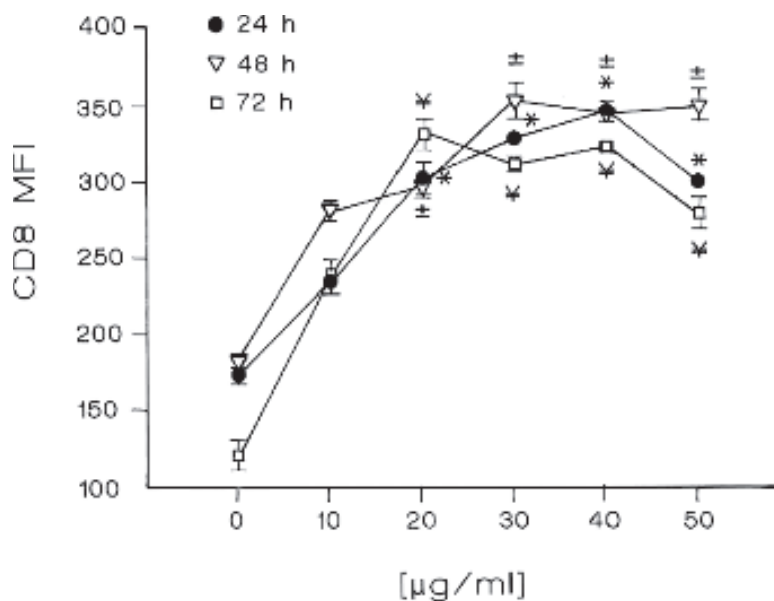


Figure 1: Effect of Bb lysate at different times and doses on the expression of CD8 molecule on CD8+ cells. Peripheral blood mononuclear cells were stimulated at different times with different Bb lysate concentrations. (*) represents statistical differences ($p < 0.05$) in the expression of CD8 molecule on CD8+ cells stimulated with different Bb concentrations at 24h; (\pm) represents statistical differences ($p < 0.05$) in the expression of CD8 molecule on CD8+ cells stimulated with different Bb concentrations at 48h; (¥) represents statistical differences ($p < 0.05$) in the expression of CD8 molecule on CD8+ cells stimulated with different Bb concentrations at 72 h. Concentration of Bb cytoplasmic fraction is represented on the X axis and relative fluorescence units are represented as MFI on the Y axis. $n=3$. (mean \pm SE). There were no statistical differences in response between donors and between time periods.

CD4 molecule on CD4+ cells, revealing no significant differences when compared with control assays (not shown). Similar results were obtained when assays were performed using trypsinated Bb lysate samples (data 228 not shown).

Proliferation Assay

Bb lysate, did not induce cell proliferation of PBMC either during the time periods tested or at the concentrations tested. Similarly, in gated cell populations (CD4+ and CD8+), Bb did not affect T cell proliferation (Figure 2A). As a proliferation control, PBMC were stimulated with an optimal dose of Concanavalin A lectin (1 $\mu\text{g}/\text{mL}$). After 48 h stimulation, cells showed a reduction in fluorescence intensity and two mitoses were observed, indicating that these cells had preserved their proliferating functions (Figure 2B).

Bifidobacterium bifidum-induced Increased Binding of Peanut Agglutinin to CD8+ Cells

The CD8+ cells were purified by magneto-selection, labeled with FITC-PNA, and analyzed by flow cytometry. The non-stimulated CD8+ cells were negative for PNA labelling (Figure 3A). Interestingly, when the cells were stimulated with 40 $\mu\text{g}/\text{mL}$ of Bb lysate, up to 80% of the CD8+ cells were positive to PNA labelling 245 (Figure 3B).

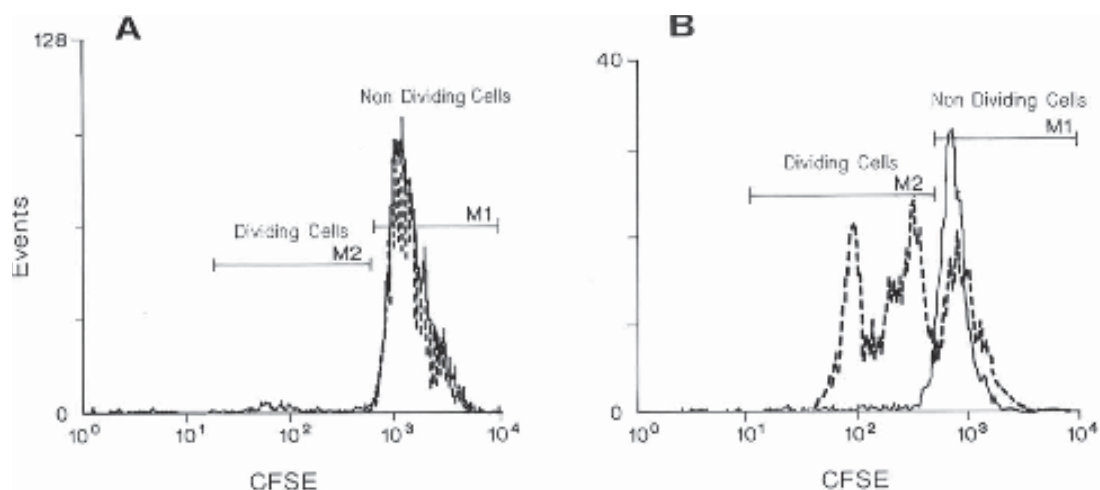


Figure 2: Determination of cell proliferation of peripheral blood mononuclear cells with or without Bb lysate. (A) Total peripheral blood mononuclear cells were stimulated with 30 $\mu\text{g}/\text{ml}$ of Bb lysate and harvested after 72 h of stimulation. Non-stimulated cells (continuous line) and Bb lysate-stimulated cells (dashed line) are shown. No differences were observed. (B) Total peripheral blood mononuclear cells stimulated with 1 $\mu\text{g}/\text{ml}$ of ConA and harvested at 48 h (continuous line) and Bb lysate stimulated cells (dashed line) are shown. A representative experiment of the three experiments performed is shown, all the three experiments showed similar results.

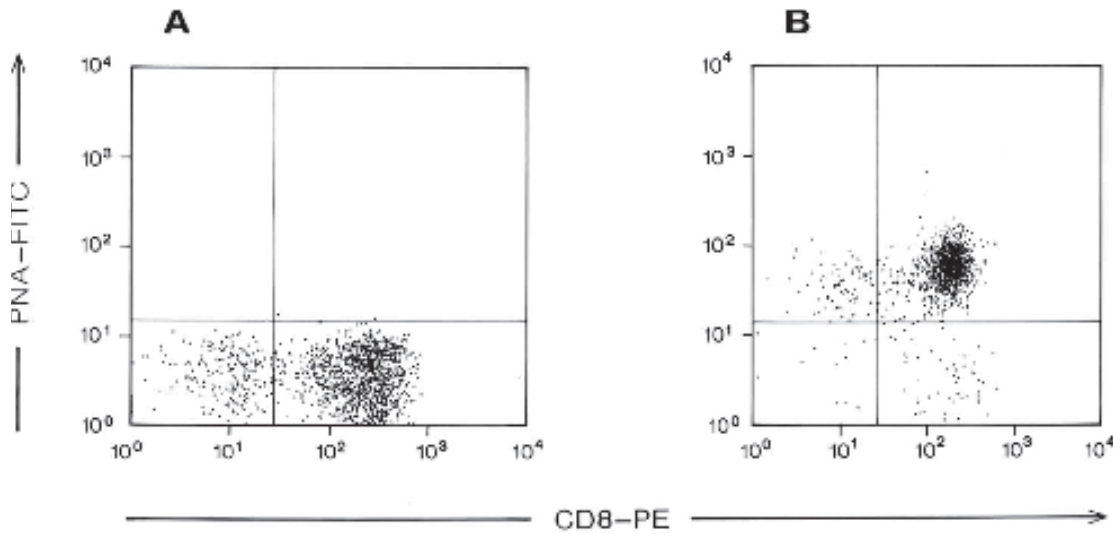


Figure 3: PNA recognizes CD8+ Bb lysate-stimulated cells. Non-adherent cells 430 were magnetically isolated and labeled with FITC-PNA. (A) Non-stimulated cells are shown. (B) Bb lysate-stimulated cells are shown. A representative experiment of the three experiments performed is shown.

Apoptosis

After stimulation with Bb lysate, there were no differences in the binding of 7-aminoactinomycin-D to either CD4+ or CD8+ cells as compared with the non-stimulated cells, suggesting that Bb lysate did not affect cell survival even at high doses (50 µg/mL) (Figure 4A and 4B). Although there seems to be

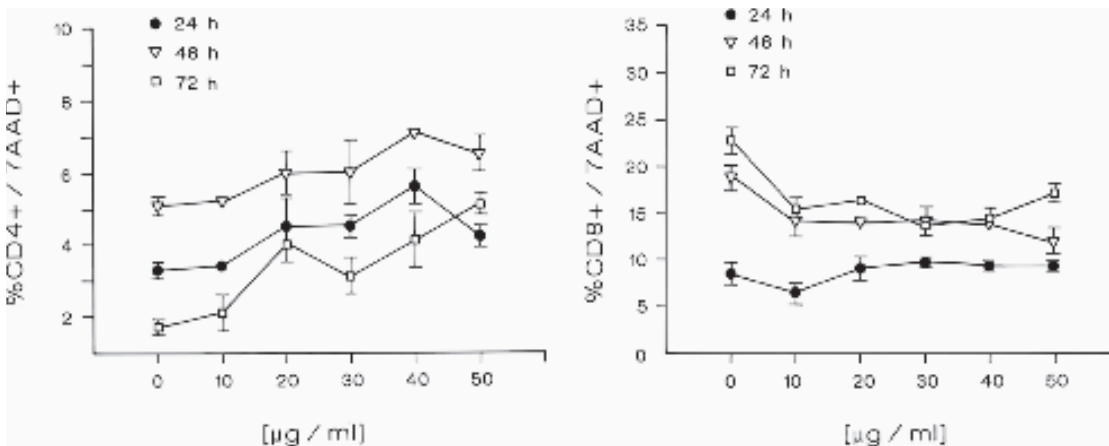


Figure 4: Effect of Bb lysate on CD4+ or CD8+ apoptosis. Cells were stimulated with different Bb concentrations. After each time point, cells were harvested and labeled with Phycoerythrin-conjugated mouse mAb against either human CD4 or CD8 molecules. Thereafter, the cells were incubated with 7-aminoactinomycin-D and analyzed by FCM. The left panel represents the kinetics of CD4+ cells apoptosis, note that there are no differences in apoptosis. Bb lysate did not induce apoptosis in CD8+ cells, even at high concentrations and long time exposures. n=3 (mean ± SE).

a reduction in apoptosis using 10 $\mu\text{g}/\text{mL}$ compared to the non-stimulated cells in the CD4+ cells subset, at all the time periods tested, the difference was not statistically significant.

Cytotoxicity Assay

When CD8+ Bb lysate stimulated cells were exposed to autologous adherent cells (target cells) there was a 7.5-fold increase in cytotoxic activity as compared with non-stimulated CD8+ T cells, indicating that Bb lysate induced activation of CD8+ cells (Figure 5). Twenty micrograms of Bb lysate were chosen because it was the maximum dose which induced the maximum expression of CD8 molecule on CD8. Analysis of adherent cells (target cells) showed that approximately 80% were CD14+ cells, suggesting that the cells were monocytes (data not shown).

DISCUSSION

Little is known about the precise mechanisms of immune regulation mediated by bifidobacteria. There are many clinical trials supporting their use as probiotics for intestinal, allergic, and neoplastic diseases (Reid et al., 2003). We sought to determine the effect of the soluble cytoplasmic fraction on human PBMC from healthy donors. The effect of Bb lysate was observed mainly on CD8+ T cells rather than on CD4+ T cells, and consisted of enhanced cytotoxicity of CD8+ T cells on autologous adherent cells, as shown in Figure 5. CD8+ T cells play an important function in the process of T-cell mediated

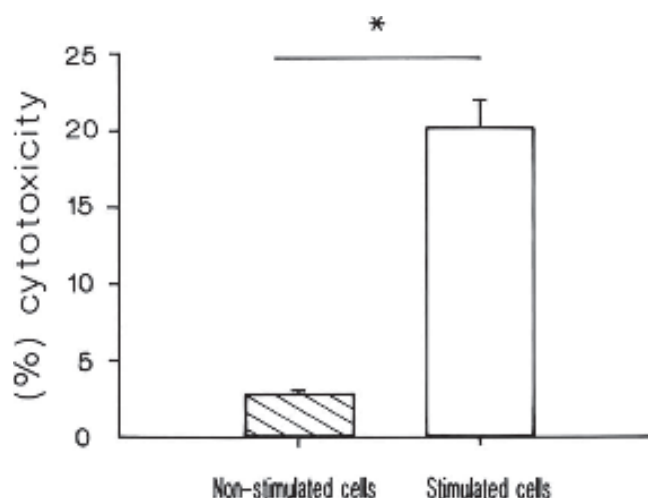


Figure 5: Effect of Bb lysate on cytotoxicity of CD8+ cells. After 72 h and with 20 $\mu\text{g}/\text{ml}$ of Bb lysate stimulation, autologous adherent cells were exposed to CD8+ magnetically purified non-adherent cells for 2 h at 37°C. The release of cytosolic LDH was measured by ELISA. There is a 7.5-fold increase in cytotoxicity comparing Bb stimulated with non-Bb-stimulated cells. (*) $p < 0.05$, $n=3$.

cytotoxicity, which could be restricted by Major Histocompatibility Complex-I presentation; however, the participation of Toll Like Receptor in the function of these CD8+ cells, through the induction of pro-inflammatory cytokines (Karlsson et al., 2002), cannot be ruled out.

Our results confirm that the main PBMC population stimulated by Bb lysate were CD8+ T cells. The non-Bb lysate stimulated CD8+ cells were PNA negative; however, after stimulation with Bb, 80% of the CD8+ cells were positive for PNA binding. The fact that these cells exhibited PNA positive recognition strongly supports the possibility that these cells correspond to memory/effector T cell populations, as has been demonstrated in murine (Harrington et al., 2000) and porcine (Hernandez et al., 2003) CD8+ T cells. PNA shows specificity for the Gal-OH group present in Gal β 1,4GlcNAc of O-glycosidically linked glycans of the CD8, CD43, and CD45 molecules expressed on activated T cells (Casabo *et al.*, 1994; Galvan et al., 1998; Amado et al., 2004; De Maio et al., 1986; Pereira et al., 1976; Wu et al., 1996), suggesting that the soluble cytoplasmic fraction of *Bifidobacterium bifidum* may alter the glycosylation process.

It has been shown that PNA is able to bind also to CD4 cells, however this binding is not able to discriminate between CD4 sub-populations (Galvan et al., 1998). It has been reported that probiotics may promote anti-neoplastic activity through Natural Killer augmentation of the cytotoxic activity (Takaghi et al., 2001). Here, we observed an elevation in the cytotoxic activity of CD8+ cells, which supports the idea that Bb may promote this activity. It is well known that IFN-gamma plays an important role in activation of CD8 cells, and its function in this model cannot be excluded.

Peptidoglycan and lipoteichoic acid from Gram negative bacteria induce an up-regulation of the CD14 molecule, that would prime for cellular activation (Jorgensen et al., 2001); interestingly, we observed an up-regulation of the CD8 molecule after stimulation with Bb lysate, which may explain in part the elevation of the cytotoxic activity, and an indirect mechanism of Bb in this model also has to be considered.

Bb is trypsin-labile, suggesting a proteic nature of the cytoplasmic factors able to activate CD8+ molecule up-regulation, as mentioned in results; however, the activity of RNAses, DNAases or proteases on CD8 cannot be ruled out. In contrast to the results of Lee et al. who used cytoplasmic fraction of *Lactobacillus casei* and *Bifidobacterium longum* (2004), we found that Bb lysate did not promote proliferation of T cells even after 96 h of *in vitro* Bb lysate activation (data not shown), confirming the observations by Pessi et al. (1999), who proposed that probiotic bacteria such as, *Lactobacillus rhamnosus GG*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, and *Streptococcus thermophilus* exert an antiproliferative effect on lymphocytes. In fact, we observed that Bb lysate is able to induce an activation of CD8+ cells without promoting their proliferation. Bb lysate did

not promote apoptosis of CD8+ cells, even at high doses, which suggests that the soluble fraction of Bb does not promote lymphocyte apoptosis, reinforcing the possible use of these probiotic bacteria as a secure alternative for enhancing CD8+ cytotoxic activity, without affecting lymphocyte survival, not only in the gut epithelium but also for systemic immunity (Kato et al., 1999). Similarly Hidaka et al. demonstrated recently that probiotic administration through the parental route modulated immune cells from blood and gut (Hidaka et al., 2007). The practical implication of the effects demonstrated by the cytoplasmic fraction of *Bifidobacterium bifidum* is that this kind of bacteria would be beneficial in cellular immunity processes, such as neoplasias, intracellular bacteria- or viruses-mediated diseases and could be used as a possible probiotic.

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