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Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus

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Abstract

Macrophages are an important cellular component of the innate immune system and are normally rapidly recruited and/or activated at the site of virus infection. They can participate in the antiviral response by killing infected cells, by producing antiviral cytokines such as nitric oxide and by producing chemokines and immunoregulatory cytokines that enable the adaptive immune response to recognize infected cells and perform antiviral effector functions. Probiotics, as a part of the normal gut intestinal flora, are important in supporting a functional yet balanced immune system. Improving our understanding of their role in the activation of macrophages and their stimulation of proinflammatory cytokine production in early viral infection was the main goal of this study. Our *in vitro* model study showed that probiotic bacteria, either from the species *Lactobacillus* or *Bifidobacteria* have the ability to decrease viral infection by establishing the antiviral state in macrophages, by production of NO and inflammatory cytokines such as interleukin 6 and interferon-gamma. These effects correlated with the mitochondrial activity of infected macrophages, therefore, the measurements of mitochondrial dehydrogenases activity could be implied as the first indicator of potential inhibitory effects of the probiotics on virus replication. The interactions between probiotic bacteria, macrophages and vesicular stomatitis virus (VSV), markedly depended on the bacterial strain studied.

Keywords: Macrophages; Lactobacillus; Bifidobacteria; Antiviral activity; NO; Inflammatory cytokines

1. Introduction

In host–pathogen interactions, nonspecific and highly specific adaptive mechanisms of immune response have been developed by the host to combat pathogens. Macrophages and dendritic cells are first barrier against infections, located in tissue throughout the body where they sense signals via a variety of receptors for molecules including lipopolisacharide (LPS), mannose, CpG dinucleotides and lipotechoic acid that are conserved in bacteria, including intestinal microflora (Miettinen et al., 2000). These signals promote the secretion of a variety of cytokines that mobilise innate immune reactions and signal T cells to initiate specific responses against pathogens. Feedback communication is additionally directed from T cells to macrophages in the form of interferon-gamma (INF-γ)—a major stimulatory signal for macrophages. It became evident that, under some physiological and pathological conditions, macrophages themselves are an important source of INF-γ (Gessani et al., 1989; Morita et al., 2002). INF-γ activates several macrophage functions including antimicrobial activity that lead to increased eradication of intracellular pathogens. Activation of macrophages is an important step in impeding viral replication in the infected host, as shown for a variety of viruses, including herpes simplex virus (HSV), human immunodeficiency virus (HIV) and vesicular stomatitis virus (VSV) (Born et al., 1993; Ellermann-Eriksen, 2005; Pohjavuori et al., 2004; Bi and Reiss, 1995). Antigen processing and presentation to lymphocytes induces Th1-driven mediated cell response to infection

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but inhibits Th2 cell differentiation and production of anti-inflammatory cytokines such as IL-4, IL-5 and IL-13 (Gessani and Belardelli, 1998). In macrophages exposed to inflammatory stimuli, nitric oxide synthase (iNOS) is induced, which eventually results in production of nitric oxide (NO) around 18 h post-infection. The production of NO and other reactive oxygen species (ROS) is considered to be the second barrier of innate defense mechanisms (Kidd, 2003; Nathan, 1992). NO carries out a variety of tasks for the innate immune system such as the killing of virus-infected cells, tumor cells and parasitic pathogens (Hibbs et al., 1998). The antiviral effects of NO have been well documented in several viral infections (Ellermann-Eriksen, 2005; Paludan et al., 1998; Bi and Reiss, 1995). However, NO causes damage to DNA, proteins and lipids in cells and tissues and could thus be deleterious for the host (Henry et al., 1993; Szabo et al., 1996). Therefore, the final effect of NO should be viewed as the balance between antiviral *versus* toxic effects.

Recent decades have witnessed intense research interest in probiotic lactic acid bacteria – as defined by Metchnikoff (1908) – such as lactobacilli and bifidobacteria, which are most commonly present in fermented food. Several studies have pointed out that orally delivered probiotics can transiently colonize the gastrointestinal (GI) tract and actively communicate with immune system cells, at the local as well as at the systemic level (Acheson and Luccioli, 2004; Cross, 2002; Dugas et al., 1999; Gill, 1998). Recent reports have additionally indicated that surveillance cells of the GI system respond to these *de novo* colonizers by the release of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-12, IL-6 and interferon (INF-γ) or the production of anti-inflammatory/regulatory cytokines (such as the transforming growth factors TGF-β and IL-10) with the specific tumor necrosis factor or transforming growth factors being dependent on the strain of bacterium (Cross et al., 2004; Pohjavuori et al., 2004). Various strains of probiotic lactobacilli, for instance *Lb. rhamnosus* (strain GG), *Lb. reuteri*, *Lb. casei* Shirota and others, have been well characterized in terms of their ability to induce cytokine production following contact with macrophages (Cross et al., 2004; Morita et al., 2002).

Probiotics are undoubtedly important in supporting a functional yet balanced immune systems and further employment of immunomodulatory bacteria in health care can be seen in combating microbial pathogens, including viruses. Several studies involving *Lb. rhamnosus* (strain GG) as a probiotic strain showed a positive effect in treatment of rotavirus gastroenteritis in infants and children admitted to hospital. Immunological analyses of blood samples have shown that probiotic treatment can be associated with significant increases of rotavirus specific IgA titers (Kaila et al., 1995; Majamaa et al., 1995). However, understanding the role of probiotic and protective cultures against viral infections must be investigated in more detail.

This study was, to our knowledge, the first study that aimed to investigate the interactions between macrophages, pathogens and potentially protective probiotic cultures in early immune system defenses against viral infections in cell cultures of pig macrophages, using VSV as a model virus.

2. Materials and methods

2.1. Cells

The 3D4/21 pig alveolar macrophage derived cell line was obtained as previously described by Weingartl et al. (2002).

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich), supplemented with 10% Fetal Calf Serum (Biowhittaker, Europe), L-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (1 mg/ml) at 37 °C in a humidified 5% CO_2 atmosphere in tissue culture flasks until confluent. The cell culture medium was regularly changed. To perform biological assays, the cells were seeded in 96-well plates at a concentration of 6×10^6 viable cells ml^{-1}, as determined by 0.4% trypan blue (Sigma–Aldrich) viability staining, and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2–95% air to reach the monolayer. Just before use, the monolayers were washed twice with DMEM without phenol red and supplements.

2.2. Bacteria

Experiments were carried out using *Lb. paracasei/rhamnosus* Q85, *Lb. paracasei* A14, *Lb. paracasei* F19 and *B. longum* Q46, that showed good adhesive properties to intestinal epithelial cells (unpublished results). All strains were maintained at −40 °C in 20% (v/v) glycerol (Merck, Damstadt, Germany). Prior each of the experiments, bacteria were propagated in 15 ml MRS broth (Oxoid, Basingstoke, England) under anaerobic conditions by the use of Anaerogen (Oxoid) for 24 h at 37 °C.

The number of viable or heat-inactivated bacteria in 1 ml of culture was determined from an internal laboratory standard curve, prepared by measuring optical density at 620 nm of freshly prepared serially diluted cultures versus determination of colony forming units (CFU) of bacteria per ml determined by plating bacteria on De Man, Ragosa, Sharp agar (MRS agar, Fluka, Buchs, Schweiz). The cultures were then centrifuged at 2400 rpm (Centric 3000R, Tehtnica, Slovenia) for 10 min at 24 °C. Bacteria were then washed twice and resuspended in DMEM without phenol red or supplements.

Heat inactivation of bacteria was performed by heat treatment in DMEM without phenol red and supplements in a water bath at 100 °C for 10 min.

The maximum number of bacteria introduced into the antiviral assays was determined as the highest number of bacteria that was not cytotoxic to the macrophage cell line 3D4/21 (results not shown). The value was determined to be 1×10^8 CFU/ml of each strain.

The percentage of adherent bacteria was determined by co-incubation of each bacterial strain with macrophage cell line for 90 min at the 37 °C in a humidified atmosphere of 5% CO_2–95% air. Non-adherent bacteria were removed from the cell cultures by twice washing with PBS. Cells were trypsinized and the percentage of adherent bacteria was determined by agar plate counts of serially diluted suspensions plated on De Man, Ragosa, Sharp agar (MRS agar, Fluka, Buchs, Schweiz).

2.3. Virus

Vesicular stomatitis virus (VSV)—Indiana strain was used in experiments. Virus passages were performed in IPEC-J2 (Intestinal Pig Epithelial Cell Jejunum; a generous gift of Prof. Anthony Blikslager, North Carolina State University) monolayers. Supernatant containing the virus was collected from the flasks when cytopathic effect (CPE) was microscopically observed (24–48 h at 37 °C) and clarified by centrifugation. The virus was stored at −70 °C until used. For the antiviral assay, virus with 6.3 cell culture infective dose 50% units per ml ($CCID_{50}$/ml) was used (100 μl per each well).

2.4. Cytopathic effect (CPE) reduction assay

Washed monolayers of 3D4/21 cells were first incubated with live or heat-inactivated probiotic bacteria (100 μl of each dilution) for 90 min at 37 °C in an atmosphere of 5% CO_2. After incubation, the non-bound bacteria were washed off with DMEM without phenol red and without supplements. Monolayers of 3D4/21 cells were additionally incubated for 24 h under the conditions described above. After the incubation period, the 3D4/21 cell monolayers were washed and challenged with VSV (100 μl of 6.3 $CCID_{50}$/ml). Data were collected at 24 h post-virus infection.

The antiviral effect of probiotic bacteria was determined as percentage of cell survival. CPE was determined after cell fixation and staining with crystal violet in ethanol, rinsed with water and quantified spectrophotometrically after 10% (v/v) acetic acid dye elution at OD_{590}. In the experiments, control wells of cells without virus (0% CPE) and control wells with virus (100% CPE—complete lysis due to virus infection) were included, and the results were expressed as the mean ratios (% ± S.D.) of cell survival in probiotic bacteria treated wells ($n = 8$) to those in the control wells without virus ($n = 8$). To exclude the effect of probiotic bacteria themselves, the OD values in wells treated only with probiotic bacteria were included in the calculation.

2.5. Interferon (INF-γ)

Glycosylated recombinant porcine gamma IFN (rGIFN-γ) was obtained by constructing a tetracycline-inducible expression system in the RK 13 cell line, purified and quantified as previously described by Cencič et al. (1999, 2002).

2.6. Dehydrogenase activity assay—MTT (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay

The MTT assay was used to measure the mitochondrial function, which served as an index of living, metabolically active cells. MTT assay was chosen as indicator of potential beneficial effects of antiviral agents, in our case the probiotic bacteria. The same method was used before successfully in similar studies done on macrophages, HIV and antiviral drug testing by Bergamini et al. (1992) and Zhang et al. (2003). The MTT assay was carried out as previously described by Bergamini et al. (1992). Briefly, after the incubation period (24 h after post-infection with VSV), cell monolayers were washed, MTT in PBS was added to each well and the plates were further incubated at 37 °C in a CO_2 incubator for 75 min. Solubilization of the formazan crystals was achieved by aspiration of 100 μl of supernatant followed by the addition of 100 μl of 10% (v/v) Triton X-100 in acidified isopropanol (2 ml concentrated HCl per 500 ml solvent). The absorbance (OD) at 650 nm was measured using a microplate reader (Multiscan). The percentage of metabolically active cells treated with probiotic bacteria and the percentage of protection from cytopathic effect achieved (cells pre-treated with probiotic bacteria and infected with virus) were then calculated. All data represent the average values for a minimum of three wells of two independent experiments.

2.7. Nitric oxide (NO) measurement

One hundred microliters of each strain of the selected probiotic bacteria at the concentration of 1×10^8 bacteria/ml in DMEM without phenol red and supplements was added to the confluent cell monolayers in P96-well plate before or after VSV infection. Cells were incubated after stimulation with probiotic bacteria and VSV for 48 h at 37 °C in 5% CO_2 in a humidified atmosphere and compared with the cells infected with VSV only. Cells and bacteria alone were separately included in the same plate as negative controls. IFN-γ (1 μg/ml) stimulated cells were used as a positive control. The NO concentration was determined by measuring the amount of released NO_2^- with modified Griess reagent (Sigma–Aldrich) according to the Griess reaction (Green et al., 1982). Briefly, 50 μl of cell culture supernatant was transferred to another P96 plate and 50 μl of modified Griess reagent added to a total volume of 100 μl. After 15 min of incubation at room temperature, optical density (OD) at 540 nm was measured and the concentration of released NO_2^- was extrapolated from the $NaNO_2$ standard curve.

2.8. Pattern of proinflammatory cytokine production

Dot blot was performed to determine the presence of proinflammatory cytokines IFN-γ and IL-6 in the cell free culture supernatants after exposure of 3D4/21 cells to bacteria and virus. The levels of cytokines produced were compared against cytokine levels observed in 3D4/21 in DMEM medium alone (controls). Cell supernatants were loaded under gravity onto the nitrocellulose transfer membrane (Pierce, Rockford, IL) by usage of a dot blot apparatus (BioRad). rGIFN-γ and IL-6 (Sigma–Aldrich) at the concentration of 1 μg/ml were applied to the membrane as the positive controls. The membrane was washed three times with 0.1 ml of TBS (20 mmol/l Tris and 137 mmol/l NaCl, pH 7.6). Non-specific binding sites were blocked with 10% non-fat dry milk in TTBS (Tris–Cl, NaCl, Tween 20) for 16 h at 4 °C. The membranes were additionally washed three times for 10 min at room temperature with TTBS and incubated for 1 h at room temperature with a polyclonal rabbit antihuman IL-6 antibody (Sigma) or polyclonal rabbit antipig INF-γ (VIM, Jouy-en-Josas, France), diluted 1:500 in 5% nonfat dry milk in TTBS. Following subsequent washes,

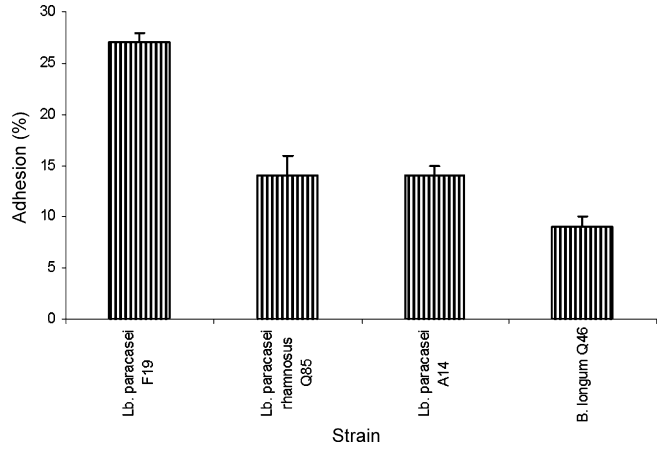

Fig. 1. Adhesion of probiotic strains to macrophages 3D4/21 cell monolayer was examined as described under Section 2 with plate count method. Adhesion is expressed as the percentage of bacteria adhered to the monolayer compared with the amount of bacteria added to the monolayer. Rate of attachment: *Lb. paracasei* F19 (27%) followed equally by *Lb. paracasei rhamnosus* Q85, *Lb. paracasei* A14 (14%) and *B. longum* Q46 (9%).

membranes were incubated for an additional hour at room temperature with the anti-rabbit-horseradish peroxidase-conjugated IgG (Sigma) diluted 1:3000 in 5% nonfat dry milk in TTBS. Membranes were then washed and developed with Supersignal West Pico chemiluminiscent substrate system (Pierce, Rockford, IL). After developing for 1 min, the membranes were exposed to BiomaxMR-1 film (Sigma-Kodak) for 5 min using processing chemicals Kodak GBX developer and fixer system (Sigma).

3. Results

3.1. The inhibition of VSV infection by pre-treatment of cell monolayer is stimulated by lactobacilli and bifidobacteria

All bacteria tested were able to attach to macrophage cell line 3D4/21, but at different rates (Fig. 1). The highest rate of attachment was shown for *Lb. paracasei* F19 (27%) followed equally by *Lb. paracasei rhamnosus* Q85, *Lb. paracasei* A14 (14%) and *B. longum* Q46 (9%). Increased cell survival (protective effect of probiotics) in the 3D4/21 cell line upon VSV challenge was observed upon treatment with live bacteria (Fig. 2) and was dependent on strain and bacterial number applied. Notably macrophage survival (30%) was achieved after exposure to the two highest concentrations used of the *Lb. paracasei* F19 and *Lb. paracasei/rhamnosus* Q85 (1×10^8 and 5×10^7 CFU/ml) (Fig. 2). The same value was achieved for *B. longum* Q46 but at concentration 2.5×10^7 CFU/ml (Fig. 2). When cell monolayers

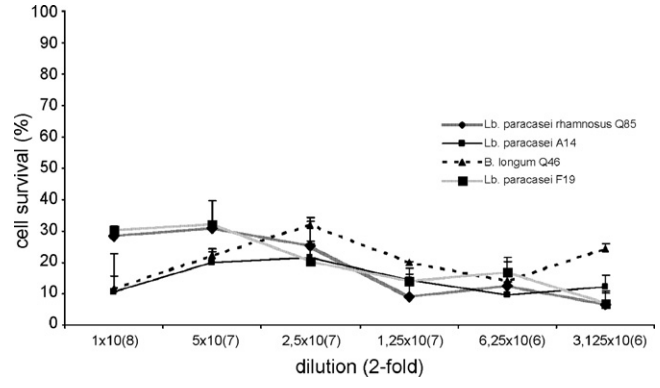

Fig. 2. Correlation between the concentration of bacteria (two-fold dilution with initial concentration of 1×10^8 bacteria/ml) and antiviral protection of macrophages. Antiviral activity of viable probiotic bacteria on VSV infected 3D4/21 macrophages were observed when probiotic strains were applied onto cell monolayers for 90 min. After removal of non-bound bacteria, cells were further incubated for 24 h before VSV infection. Results were collected at 24 h post-infection, expressed as percentage (means ± S.D.) of cell survival, as compared to the control cells, treated with virus only (0% of cell survival).

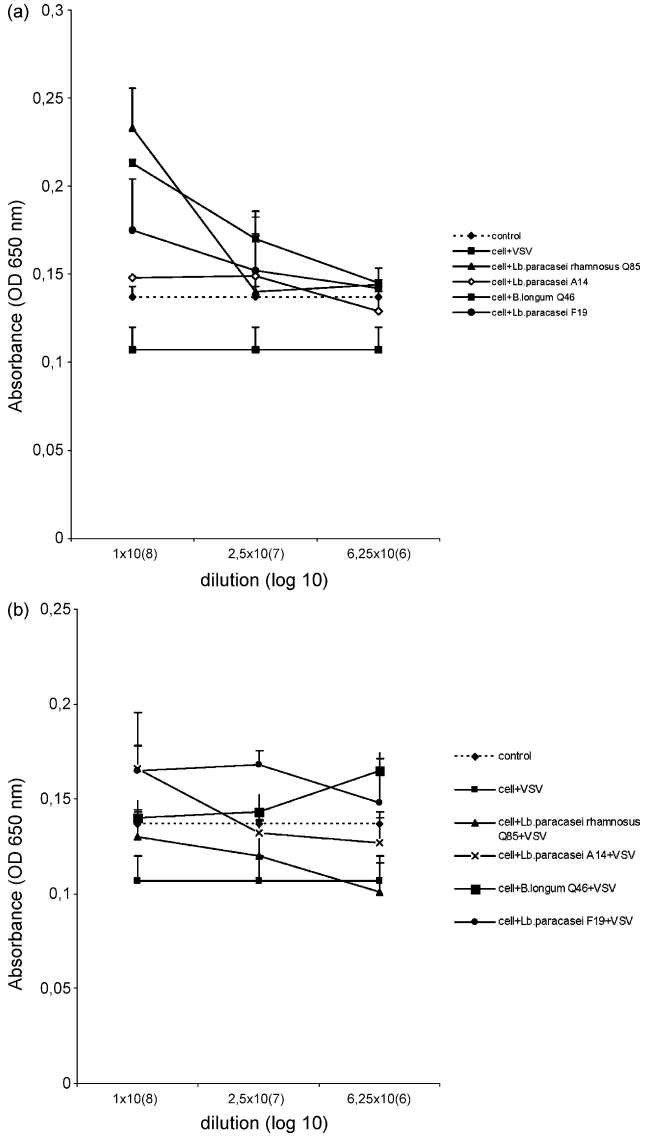

Fig. 3. Dehydrogenase activity of non-infected (a) and VSV-infected (b) macrophages was assessed with MTT assay. After 90 min pre-incubation of cell monolayers (6×10^6 cells/plate) with bacteria (1×10^8, $2{,}5 \times 10^7$ and $6{,}25 \times 10^6$ bacteria/ml) at 37 °C in atmosphere of 5% CO_2, cells were carefully rinsed with PBS to remove excess of the bacteria and the plates were further incubated for 24 h before VSV challenge. At 24 h post-infection the MTT assay was performed as previously described. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (% ± S.D.) of absorbances in treated wells to those in control wells.

were treated with heat-inactivated bacteria no cell survival after infection was observed (0% of cell survival).

3.2. Dehydrogenase activity of VSV-infected and non-infected macrophages is increased by the live and heat-inactivated lactobacilli

All strains of the genus lactobacilli and bifidobacteria increased the metabolic activity in comparison to non-treated cells (control) or cells infected with VSV only (Fig. 3a) at a concentration of 1×10^8 CFU/ml. A gradual decline in the activity of mitochondrial dehydrogenases was observed after decreasing the number of bacterial cells (Fig. 3a and b). As expected, VSV infection notably decreased the dehydrogenase activity of the cells leading to cell death (Fig. 3a and b). Pre-treatment of macrophages with lactobacilli increased the level of dehydrogenase activity above the control cells in the case of *Lb. paracasei* A14, *Lb. paracasei* F19 and *B. longum* Q46 after infection (Fig. 3b). A similar increase in the level of mitochondrial dehydrogenase activity was observed for the following heat-inactivated bacteria (all at the concentration of 1×10^8 CFU/ml): *Lb. paracasei rhamnosus* Q85 for 72.62%, *Lb. paracasei* A14 for 27.37%, *B. longum* Q46 for 26.39% and *Lb. paracasei* F19 for 16.64% as compared to non-treated macrophages 3D4/21 (control). Co-incubation of heat inactivated bacteria with VSV increased dehydrogenase activity in comparison to VSV infected cells only for: *Lb. paracasei rhamnosus* Q85 34.11%, *Lb. paracasei* A14 39,75%, *B. longum* Q46 10.59% and *Lb. paracasei* F19 7.31%. A CpG motif in bacterial DNA that is preserved after heat inactivation of bacteria has been reported to suppress the development of apoptotic morphology and increase the cell viability (Jozsef et al., 2004). Due to that, we could observe increased dehydrogenase activity.

3.3. Production of NO in virus-infected macrophages is synergistically increased by the lactobacilli and bifidobacteria

Pre-treatment of macrophages with live probiotic bacteria increased NO production in macrophages after having been challenged with VSV (Table 1). The highest accumulation of nitrite in the culture medium was obtained in the case of *Lb. paracasei/rhamnosus* Q85 and *B. longum* Q46, followed by the *Lb. paracasei* A14 and *Lb. paracasei* F19, respectively. Moreover, bacteria themselves were capable of stimulation of NO production in 3D4/21 cells in comparison to control (cells only) in the order of *Lb. paracasei* A14, *Lb. paracasei/rhamnosus* Q85, *Lb. paracasei* F19 and *B. longum* Q46 (Table 1, first column). All tested bacteria stimulate NO production in macrophages in a concentration dependent manner with an exception of *Lb. paracasei* A14 where stimulation of NO production was not dependent on a bacterial concentration. Similar results were obtained with the heat-inactivated bacteria (results not shown). The results indicate that VSV contributes significantly to the synergistic increase in NO production for almost all bacteria except for *Lb. paracasei* F19 and for bacterial *Lb. paracasei* A14, but in later case only when the number of applied bacteria is low. On the contrary, the highest synergistic effect was observed for the *B. longum* Q46.

3.4. IFN-γ and IL-6 are produced in macrophages by probiotic bacteria in early response against VSV infection

During the first hours of the virus infection, macrophages produced high amounts of type II interferon, interferon gamma (INF-γ), and inflammatory cytokine IL-6 (Figs. 4 and 5, respectively). Their production was affected by the probiotic bacteria. Activation of 3D4/21 cells following 90 min exposure to live

Table 1
Production of NO_2^- in non-infected and VSV-infected macrophages were assessed after 90 min pre-incubation of cell monolayers (6×10^6 cells/plate) with bacteria (two-fold dilutions from initial concentration of 1×10^8 bacteria/ml) at 37 °C in atmosphere of 5% CO_2

	Control ($mMNO_2^- \pm$ S.D.)	Post-infection with VSV ($mMNO_2^- \pm$ S.D.)
Cells	0.262 ± 0.002	0.267 ± 0.002
Lb. paracasei rhamnosus Q85 (1×10^8 CFU/ml)	0.318 ± 0.0029	0.358 ± 0.0032
Lb. paracasei rhamnosus Q85 (2.5×10^7 CFU/ml)	0.292 ± 0.0006	0.324 ± 0.0036
Lb. paracasei rhamnosus Q85 (6.25×10^6 CFU/ml)	0.281 ± 0.0010	0.294 ± 0.0022
Lb. paracasei A14 (1×10^8 CFU/ml)	0.331 ± 0.0021	0.342 ± 0.0044
Lb. paracasei A14 (2.5×10^7 CFU/ml)	0.280 ± 0.0017	0.309 ± 0.0015
Lb. paracasei A14 (6.25×10^6 CFU/ml)	0.286 ± 0.0013	0.277 ± 0.0039
B. longum Q46 (1×10^8 CFU/ml)	0.298 ± 0.001	0.363 ± 0.0010
B. longum Q46 (2.5×10^7 CFU/ml)	0.270 ± 0.001	0.301 ± 0.003
B. longum Q46 (6.25×10^6 CFU/ml)	0.270 ± 0.001	0.288 ± 0.001
Lb. paracasei F19 (1×10^8 CFU/ml)	0.301 ± 0.003	0.298 ± 0.003
Lb. paracasei F19 (2.5×10^7 CFU/ml)	0.278 ± 0.002	0.283 ± 0.001
Lb. paracasei F19 (6.25×10^6 CFU/ml)	0.276 ± 0.001	0.276 ± 0.003

Following incubation, cells were carefully rinsed with PBS to remove excess of the bacteria and the plates were further incubated for 24 h before VSV challenge. At 24 h after virus infection the NO_2^- assay was performed. Released NO_2^- in cell supernatant was measured by addition of Griess reagent as described under Section 2. The A_{540} was measured, and the results were expressed, for each dilution, by the mean ratios (mM ± S.D.) of absorbances in treated wells to those in control wells.

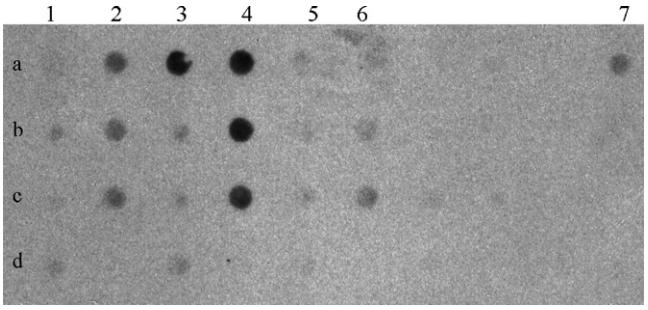

Fig. 4. Induction of major immunoregulatory cytokine interferon gamma (INF-γ) in VSV-infected 3D4/21 cells previously incubated with live and heat-inactivated probiotic bacteria. Macrophages were first incubated with bacteria for 90 min, unbound bacteria were washed off and 24 h later cells were challenged with VSV. Cell supernatants were collected at 24 h post-infection, and dot blot analysis was performed as previously described. Legend—1a: live *Lb. paracasei* A14; 2a–c: live *Lb. paracasei* A14 + VSV; 2d: heat-inactivated *Lb. paracasei* A14; 1b–d: heat-inactivated *Lb. paracasei* A14 + VSV; 3a: live *B. longum* Q46; 4a–c: live *B. longum* Q46 + VSV; 4d: heat-inactivated *B. longum* Q46; 3b–d: heat-inactivated *B. longum* Q46 + VSV; 5a: live *Lb. paracasei* F19; 6a–c: live *Lb. paracasei* F19 + VSV; 6d: heat-inactivated *Lb. paracasei* F19; 5b–d: heat-inactivated *Lb. paracasei* F19 + VSV; 7a: control of purified INF-γ. Neither non-treated macrophages nor only VSV-infected macrophages nor *Lb. paracasei rhamnosus* Q85 infected or non-infected with VSV produced any detectable amount of INF-γ.

probiotic bacteria elicited high production of INF-γ in the case of live *B. longum* Q46 (Fig. 4). Heat-inactivated bacteria were not able to stimulate production of INF-γ, while co-incubation with the VSV led to production of INF-γ in all cases. However, a notable production of IL-6 was achieved by pre-treatment of cells with all bacteria, live as well as heat-inactivated, since the level of IL-6 in cell culture medium increased after viral infection. The highest IL-6 production in macrophages was noted upon stimulation with most of the heat-inactivated bacteria, except with *Lb. paracasei rhamnosus* Q85 (Fig. 5).

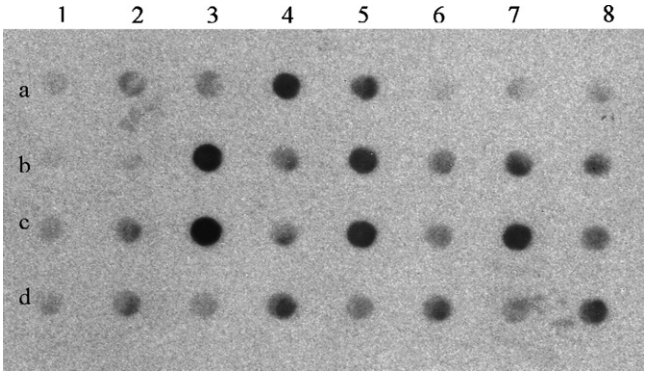

Fig. 5. Induction of major immunoregulatory interleukin (IL-6) by live and heat-nactivated probiotic bacteria in VSV-infected 3D4/21 cells. Macrophages were first incubated with bacteria for 90 min and 24 h later challenged with VSV. Cell supernatants were then collected at 24 h post-infection, and dot blot analysis was performed as previously described. 1a: live *Lb. paracasei rhamnosus* Q85; 2a–c: live *Lb. paracasei rhamnosus* Q85 + VSV; 2d: heat-inactivated *Lb. paracasei rhamnosus* Q85; 1b–d: heat-inactivated *Lb. paracasei rhamnosus* Q85 + VSV; 3a: live *Lb. paracasei* A14; 4a–c: live *Lb. paracasei* A14 + VSV; 4d: heat-nactivated *Lb. paracasei* A14; 3b–d: heat-inactivated *Lb. paracasei* A14 + VSV; 5a: live *B. longum* Q46; 6a–c: live *B. longum* Q46 + VSV; 6d: heat-inactivated *B. longum* Q46; 5b–d: heat-inactivated *B. longum* Q46 + VSV; 7a: live *Lb. paracasei* F19; 8a–c: live *Lb. paracasei* F19 + VSV; 8d: heat-inactivated *Lb. paracasei* F19; 7b–d: heat-inactivated *Lb. paracsei* F19 + VSV. Neither non-treated macrophages nor only VSV infected macrophages produced any detectable amount of IL-6.

4. Discussion

Several studies (Dugas et al., 1999; Gill et al., 2000; Miettinen et al., 1996) have reported the *ex vivo*/*in vitro* cytokine response when co-culturing cells of the innate immune defense system with probiotic bacteria. Moreover, induction of host immunity by such orally derived stimuli has been shown to play an important role in the defense against rapidly growing pathogenic bacteria (Reid and Burton, 2002). In contrast, little is known about the impact of interactions of probiotic bacteria with macrophages on viral infections.

We have evaluated the effects of probiotics on viral infections in the cell culture model of 3D4/21 macrophages and vesicular stomatitis virus (VSV) (Indiana strain) as a model virus. Our results clearly indicate that selected probiotic bacteria, mainly from the genus *Lactobacillus*, can induce defense mechanisms in macrophages against virus infection. We observed enhancement of proinflammatory cytokine production. IL-6 has traditionally been considered as the product of proinflammatory cells (Ng et al., 2003). However, IL-6 is also known to possess several anti-inflammatory characteristics, such as the ability to down-regulate LPS-induced monocyte IL-1 and TNF-α mRNA expression (Miller and McGee, 2002). Induction of NO synthesis throughout iNOS mechanisms in the intestinal epithelia and macrophages is another example of the beneficial actions by which probiotic bacteria in the gastrointestinal tract might counteract enteric pathogens and influence the intestinal barrier permeability. It has been shown that viable *Lactobacillus rhamnosus* GG can induce production of low amounts of NO in murine macrophages, but is not able to induce NO in human colon intestinal cells in the absence of human cytokines (Korhonen et al., 2001). We have also shown in another study that probiotic bacteria can stimulate the production of NO without cytokine priming in cell-free culture medium alone via iNOS (Pipenbaher et al., unpublished results). Probiotic *Lb. rhamnosus* GG (*Lactobacillus* GG) has been found in a previous study to be beneficial in the treatment of viral and antibiotic-associated diarrhea (Vanderhoof, 2001). Furthermore it was reported that HSV-2 infection of murine macrophages induces NF-κB binding to the iNOS promoter (Paludan et al., 1998).

Pre-treatment of cell monolayers with probiotic bacteria for 90 min (the time needed for adherence) and an additional 24 h incubation of monolayers co-cultured with attached bacteria reduced the infectivity of VSV in macrophages. We have shown previously that no differences in CPE reduction were seen between 24 and 48 h incubation (Botić et al., 2007). Furthermore, our results support previous findings where it was shown that a threshold number of probiotic bacteria are needed to obtain beneficial effects (Charteris et al., 1998; Lee et al., 2000). Our results indicate that the number of probiotic bacteria required to induce the response in the macrophages is dependent on the bacterial strain and their adherence ability to the cell surface. It was previously shown that *Lactobacillus* spp. has multiple sur-

face adhesins and up to four adhesins could bind to the mucosal surface at any time (Lee et al., 2000).

Monitoring the antiviral effect of probiotic bacteria was confirmed by the MTT assay that is a well recognized tool for evaluation of cell viability (Bergamini et al., 1992; Zhang et al., 2003). Pre-treatment of cells solely with probiotic bacteria did not result in the decrease of the viability of the cell monolayers; moreover most of the lactobacilli, but not bifidobacteria, stimulated cell metabolism. Results of MTT assays in the case of VSV infection of macrophages showed that live probiotics can limit the destruction of the infected cells as it was previously reported that the positive effect of probiotics as a part of conventional gut flora can influence gene expression in epithelial cells and stimulate their functions (Bry et al., 1996). Based upon the good correlation between inhibition of viral infectivity and cell vital status, this method could be further exploited as a simple test in initial studies on the interaction(s) between antiviral agents and virus infection. Our results support the findings with a similar method applied in monitoring early HIV infection *in vitro* (Born et al., 1993; Zhang et al., 2003).

In the case of heat-inactivated bacteria a CpG motif in bacterial DNA that is preserved after heat inactivation has been reported to suppress the development of apoptotic morphology and increase the cell viability (Jozsef et al., 2004). Heat-inactivated probiotic bacteria in our study could act via the same mechanism in macrophages. Live *Lb. paracasei* F19 has been shown as the most promising candidate against VSV infection in this study, and this finding has been confirmed by the results from adhesion studies showing inhibition of VSV infection of macrophages.

In macrophages exposed to INF-γ the enzyme-inducible nitric oxide is induced, which results in production of nitric oxide (NO). The best results in NO production were obtained in treatment of infected macrophages with *Lb. paracasei/rhamnosus* Q85 and *B. longum* Q46. As in the establishment of the antiviral state there was no activity of *B. longum* Q46 observed: we speculate that there may be different mechanisms used by individual strains of probiotic bacteria in combating viruses or other intracellular pathogens. Previous studies have shown that probiotic bacteria induced a significant increase of NO in co-culture with macrophages but only in the presence of INF-γ (Korhonen et al., 2001). On the contrary, our results indicate that INF-γ is not a key factor in induction of NO production, suggesting that pre-treatment of macrophages with INF-γ is not necessary. The induction of NO production in our experiments was not only limited to viable probiotic bacteria, as the same results were obtained after pre-treatment of macrophages with heat-inactivated bacteria. Our results agree with previous observations where lipotechoic acid, the cell wall component of gram-positive bacteria, was shown to induce NO synthesis in J774 macrophages (Kengatharan et al., 1996), again pointing to the immunomodulatory effect of non-viable probiotic bacteria. It can be concluded that NO production in the early phases of VSV infection is one of the effector mechanisms of the innate immune response inhibiting VSV replication.

In addition, we assayed the production of inflammatory cytokines upon the stimulation of VSV-infected cells by the probiotic bacteria. Cytokine analysis of cell supernatants in our study has shown that only attached live *B. longum* Q46 stimulated INF-γ production in macrophages after 24 h of incubation alone. As in previous cases, the virus co-infection led to the more intense production of INF-γ for all bacteria tested. Other studies have already shown that production of cytokines stimulated by the administration of probiotics is strain-specific (Gill, 1998). This phenomenon may be due to variability in surface structure as it can be concluded from our results that all bacteria tested were not able to induce the production of INF-γ. In killing the pathogenic bacteria, INF-γ was shown to be an important stimulator of macrophage activation (Beckerman et al., 1993).

The ability of the strains tested to upregulate of INF-γ production in our study did not correlate with the achieved antiviral protection. Therefore it can be concluded that INF-γ acts primarily by activating of macrophages, resulting in Th1-cell mediated response to infection. In our study, this response of activated macrophages was observed in induction of another proinflammatory cytokine, IL-6. Secretion of IL-6 by macrophages plays indirect immunoregulatory function in immune response to viral infection (Miettinen et al., 1996). These phenomena should be further explored.

Heat-treated bacterial suspensions are a source of different soluble remnant molecules, among them parts of cell wall and DNA. Pre-treatment of macrophages with heat-inactivated probiotic bacteria resulted in increasing of IL-6 in culture supernatants. It is likely that bacterial DNA rapidly induces an antiviral state and this may explain the very low level of the early INF-γ and high level of other cytokines that have indirect immunoregulatory functions (e.g. IL-6). Potential mechanisms to explain the higher IL-6 production by macrophages upon stimulation with heat-inactivated bacteria may include alternative pathways as described by Rose-John and Neurath (2004). This may be the explanation why antiviral activity in the case of heat-inactivated bacteria in our study was not achieved although we could observe a significant increase in the activity of mitochondrial dehydrogenases and the production of NO and IL-6. Substantial evidence exists that bacterial CpG motifs induce the immune response against infection by wide variety of pathogens and have therapeutic activity as shown in murine models and in human trials (Lammers et al., 2003; Ashkar et al., 2003).

In conclusion, *Lb. paracasei* strains and *B. longum* were found to inhibit virus infectivity. On the basis of previously published results (Botić et al., 2007) and the present study, we suggest several possible mechanisms of action: (1) competition for attachment sites on cell surface between probiotic bacteria and VSV live probiotic bacteria; (2) could stop the destruction of the infected cells by stimulating mitochondrial dehydrogenase activity; or (3) stimulate an innate immune response that in turn inhibits VSV infection; (4) stimulate pro-inflammatory response in macrophages leading to higher cell survival; (5) heat-inactivated bacteria could trigger immune response in macrophages by CpG motifs in their DNA or by parts of their cell wall. Further studies will hopefully help to dissect the mechanisms involved in the antiviral potential of these bacteria.

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