ORIGINAL ARTICLE

Stimulation of nitric oxide, cytokine and prostaglandin production by low-molecular weight fractions of probiotic *Lactobacillus casei* lysate

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Abstract

OBJECTIVES: Major medical indications of probiotic bacteria are conditions associated with the gastrointestinal tract. They exhibit not only the local but also systemic effects, the molecular mechanisms of which are poorly understood. We hypothesized that the action at remote sites of the body could be at least partially attributed to substances of the low molecular mass released from digested bacteria and able to cross the intestinal barrier. The aim of the study was the analysis of immunobiological properties of bacterial lysates and characterization of chemical constituents participating on this mode of action. **METHODS:** *Lactobacillus casei* probiotic strain DN-114001 was employed. Lysates were prepared by passing bacteria through a French press (1500 psi) followed by lyophilisation. The fractions were prepared by the microfiltration of the crude lysate using the 3-, 10-, 30-, 50-, and 100-kDa cutoff filters (Amicon® Ultra 0.5 ml, Millipore Corp.). This procedure completely removes biologically active bacterial macromolecules such as peptidoglycan (PGN), lipoteichoic acid (LTA) and lipopolysaccharide (LPS). Effects of microfiltrates on the in vitro production of nitric oxide (NO), cytokines, and prostaglandin E2 (PGE2) were investigated in rat peritoneal cells. **RESULTS:** The original crude lysate (≤10 μg/ml) activated the biosynthesis of NO, PGE2, and secretion of cytokines. The amount of the lysate needed for the preparation of microfiltered fractions exhibiting immunostimulatory effects was 10-fold higher (100 μg/ml). The molecules with the molecular mass ≤3 kDa were responsible for approximately 45% and 83% of the NO- and PGE2-enhancing activities of the crude lysate, respectively. The microfiltered fractions of the lysate also enhanced secretion of interleukin-6 and tumor necrosis factor-α but not that of interleukin-10 and interferon-γ. **CONCLUSION:** The *Lactobacillus casei* probiotic strain DN-114001 contains low molecular mass (≤3 kDa) molecules possessing immunostimulatory properties. Their chemical nature remains to be identified.

Abbreviations:

AUC	- area under curve
CL	- crude lysate
CLF	- centrifuged and 0.22μm-filtered crude lysate (CL)
FBS	- foetal bovine serum
IFN-γ	- interferon-γ
IL-	- interleukin-
LAL	- Limulus amebocyte lysate
LPS	- lipopolysaccharide
LTA	- lipoteichoic acid
MDP	- muramyl dipeptide
MF	- microfiltrates prepared from CLF
MF3	- microfiltrates containing molecules ≤3 kDa
MF10	- microfiltrates containing molecules ≤10 kDa
MF30	- microfiltrates containing molecules ≤30 kDa
MF50	- microfiltrates containing molecules ≤50 kDa
MF100	- microfiltrates containing molecules ≤100 kDa
NO	- nitric oxide
PGE2	- prostaglandin E2
PGN	- peptidoglycan
TNF-α	- tumour necrosis factor-α

INTRODUCTION

Major medical indications of probiotic bacteria are conditions associated with the gastrointestinal tract (Culligan *et al.* 2009). The mechanisms of the health beneficial effects of probiotics are poorly understood but they are supposed to be attributed to enhancing the intestinal barrier functions (Štětinová *et al.* 2010; Wang *et al.* 2011), decreasing the attachment and consequent colonization of the intestine by pathogens, and lowering the intestinal pH (Damaskos & Kolios 2008; Ewaschuk & Dieleman 2006; Guandalini 2008).

Typical feature of probiotic bacteria is activation of the immune system which has been suggested as one of the mechanisms contributing to their therapeutic effectiveness (Williams 2010). Lactobacilli, major representatives of probiotics, have been found to stimulate production of nitric oxide (NO) and secretion of cytokines such as interleukin-1β (IL-1β), IL-2, IL-6, IL-10, IL-12, IL-18, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Cross *et al.* 2004; Helwig *et al.* 2006; Kim *et al.* 2007; Maassen *et al.* 2000; Marcinkiewicz *et al.* 2007; Morita *et al.* 2002; Niers *et al.* 2005; Shida *et al.* 2006).

A number of probiotic bacteria have been found to exhibit not only the local but also systemic effects. Applied orally, *L. gaseri* increases the IL-12 and decreases the transforming growth factor beta (TGF-β) production in mouse splenocytes (Yazdi *et al.* 2010). Oral administration of *L. acidophilus* and *L. paracasei* enhances the phagocytic activity of mouse peritoneal macrophages and secretion of IFN-γ and IL-10 in concanavalin A-primed splenocytes (Paturi *et al.* 2008). While orally applied viable *L. acidophilus* and *L. casei* enhance the mitogen-activated IL-6 and IL-12 (the latter one also IFN-γ) production by mouse peritoneal cells, *L. helveticus, L. gasseri and L. reuteri* have the opposite, *i.e.* suppressive influence on IL-6 and IFN-γ secretion (Tejada-Simon *et al.* 1999). Lactobacilli provide protection against many Gram-positive and Gram-negative bacterial pathogens (Jebur 2010). For example, *L. casei* strain DN-114001 decreases the duration of common respiratory tract infections (Cobo Sanz *et al.* 2006; Guillemard *et al.* 2010; Turchet *et al.* 2003). *L. casei* strain CRL 431 has favorable effects on the immune parameters associated with acute liver injury (Haro *et al.* 2009). Furthermore, a randomized clinical trial has shown that orally ingested *L. casei* strain Shirota prevents the recurrence of superficial bladder cancer (Aso *et al.* 1995). Interestingly, probiotic *L. casei* might be able to contribute to the prevention against colorectal cancer by decreasing the levels of certain forms of xenobiotic-metabolizing enzymes (Matušková *et al.* 2011).

Not only live and dead bacteria but also the lysate preparations possess the beneficial therapeutic effects. A composite lysate from eight bacterial species (colifagina) (Vetrano *et al.* 2008) as well as a simple lysate from *Escherichia coli* (Konrad *et al.* 2003) ameliorate the severity of experimental colitis in mice. Orally administered lysozyme lysate from *L. bulgaricus* (deodan) exhibits the antitumor activity in mice and humans and decreases the mortality of mice experimentally infected with *Klebsiella pneumoniae* and *Listeria monocytogenes* (Popova *et al.* 1993). A significant decline in the virulence of *E. coli* O157:H7 has been observed after the treatment with lysates of *L. acidophilus* (Kim *et al.* 2006). Lysates from the probiotic *Enterococcus lactis* and *L. acidophilus* have the cytoprotective potential against the drug induced liver injury (Sharma *et al.* 2011).

The aim of our experiments was to elucidate whether the bacterial lysates possess the immunomodulatory properties, and to find out what type of chemical constituents are responsible for this mode of action.

MATERIAL AND METHODS

Animals and cells

Female Wistar rats, 8–11 wks old, were purchased from Velaz (Prague, CZ). Animals were kept in transparent plastic cages maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France) under controlled 12/12 h light/dark cycle (lights on 06.00 a.m.), temperature (22±2 °C), and relative humidity (50±10%) conditions. Animals, killed by cervical dislocation, were intraperitoneally injected with 16 ml of sterile saline. Pooled peritoneal cells collected from 3–7 animals were washed, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar) in 100-μl volumes, 2×10^6 cells/ml. The total mixed peritoneal cell population was maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator. Complete RPMI-1640 culture medium contained 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/mL gentamicin, and 5×10^{-5}

M 2-mercaptoethanol (all Sigma-Aldrich, Prague, CZ). Each experimental variant was run in duplicate. The animal welfare and all experimental procedures have been approved by the Institution Animal Ethics Committee.

Preparation of bacterial lysate

Lysates were prepared according to the principles reviewed by Chisti and Moon-Young (1986). Briefly, lactobacilli resuspended in distilled water were disrupted by passing three times through a French press (1500 psi). 1 g of the lysate originated from approximately 4×10^{12} bacteria. To kill possible remnants of viable bacteria, the lysate was heated to 60 °C for 30 min. Resulting lysates were lyophilized and diluted to a working concentration of 30 mg/ml. The sterility of all components was verified by both aerobic and anaerobic cultivation before the use. These samples are designated as crude lysates (CL). To further enhance the requirement for bacteria- and gross debris-free samples, the CL was centrifuged and passed through 0.22 μm filters (CLF lysate variant).

Centrifugal microfiltration of lysate

Following our recently described procedure (Zídek *et al.* 2012; Zídek and Kmoníčková 2012), the biologically active bacterial macromolecules (LPS, PGN, LTA) were removed from the CLF samples using the Amicon® Ultra 0.5 mL Centrifugal devices (Millipore Corp., Billerica, MA). The filter membrane of 1 cm^2 active area is made of the Millipore Ultracel® low binding regenerated cellulose. The filters with molecular weight cutoffs of 3, 10, 30, 50, and 100 kDa were used. According to the producer recommendations, the spin times were 30 min for 3 kDa, 15 min for 10 kDa, and 10 min for 30, 50, and 100 kDa cutoff microfiltration units.

Bacterial macromolecules (PGN, LTA)

Bacillus subtilis LTA (lot No. 12111102-022M4108V) and PGN (lot No. 017067/1-R017067/1V) were purchased from Sigma. Stock solutions were prepared in phenol red-free RPMI-1640 culture medium (without FBS).

Nitric oxide (NO) assay

The cells were cultured 24 h in presence of bacterial macromolecules (LPS, PGN, LTA), applied either alone or in the presence of the rat or mouse recombinant IFN-γ (R&D Systems, Minneapolis, MN), respectively. The concentration of nitrites in supernatants of cells was taken as a measure of NO production (Marletta *et al.* 1988). It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert the absorbance to μM nitrite.

Cytokine and prostaglandin E_2 assay

The cells were cultured 24 h in presence of LPS, PGN, and LTA applied alone. Concentration of cytokines (TNF-α, IFN-γ, IL-6, IL-10) and prostaglandin E_2 in cell supernatants was determined using ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis

Analysis of variance (ANOVA), subsequent Dunnetts´s multiple comparison test and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

RESULTS

Effects of lysate microfiltrates on NO production

Both CL (crude lysate) and CLF (centrifuged plus 0.22 μm-filtered CL) activated the high output production of NO by rat peritoneal cells (Figure 1A). Irrespective of the type of molecular weight cutoff microfiltration (*i.e.* 3 kDa, ≤10 kDa, ≤30 kDa, ≤50 kDa, and ≤100 kDa), the resulting fractions (MF) stimulated the production of NO as well. The effects of MFs were less prominent than those of CL and CLF (Figure 1A). The NO-enhancing effects of all distinct MFs were very similar. Yet, the differences among them were statistically significant ($F_{/4,30/}$=4.60, p=0.005). The significance proved to be due to the slightly higher activity of the MF100 fraction in comparison to the fractions containing molecules with lower molecular weight ($F_{/1,12/}$=12.76, p=0.0038; as compared to the fraction MF3). Evaluated by the AUC analysis, the effectiveness of MFs to stimulate NO was approximately 30–45% of appropriate control *i.e.* the CLF counterpart (Figure 1B). The tendency towards the enhanced effectiveness in dependence on the presence of molecules with higher molecular weight was observed.

Effects of lysate microfiltrates on cytokine secretion

All preparations differentially and dose-dependently stimulated secretion of the pro-inflammatory cytokines TNF-α and IL-6 (Figure 2). The most effective was the crude lysate (CL) which also stimulated the production of IFN-γ and IL-10. Its prominent ability to activate the IFN-γ production was most apparent at the concentration of 0.1 μg/ml. It was less effective at concentrations >10 μg/ml.

The CLF variant of lysate (*i.e.* centrifuged plus 0.22 μm-filtered CL) was much less effective than was the CL one (Figure 2). The 10-fold higher concentration (1 μg/ml) was required to augment the TNF-α and IL-6 secretion. The IL-10 secretion was only mildly enhanced with the concentration of 10 μg/ml.

The microfiltration of CLF led to the attenuation of imunostimmulatory potential of the original lysate (Figure 2). When applied at concentration of 100 μg/ml, both 3 kDa and 100 kDa cutoff microfiltrates (MF3 and MF100, respectively) were highly potent to stimulate

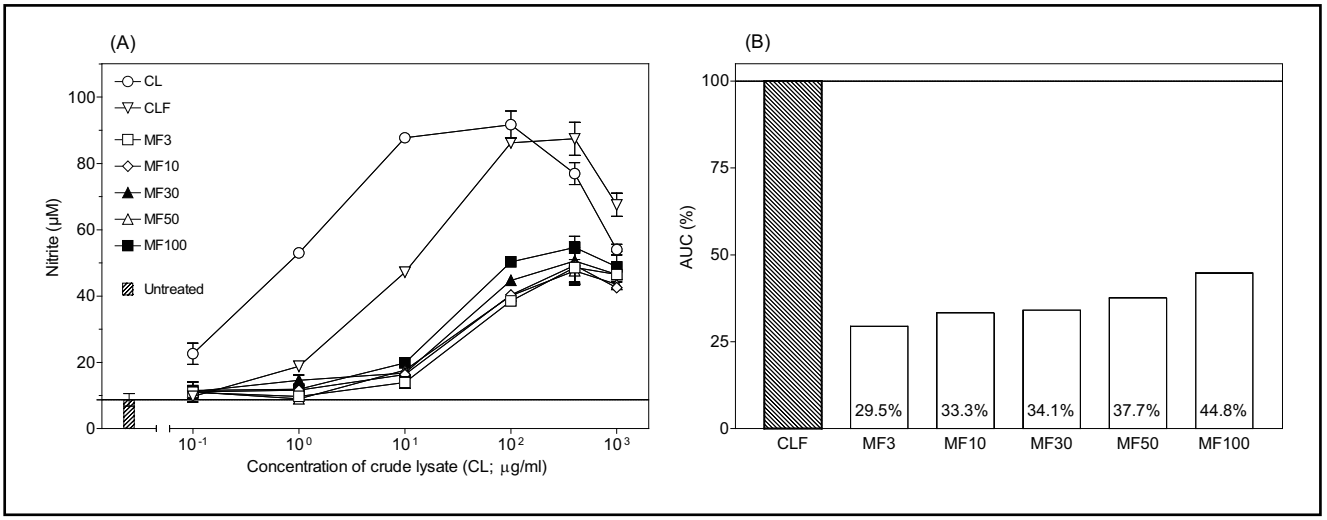

Fig. 1. Production of NO by *L. casei* lysate and its microfiltration fractions (MF) differing in the content of molecules with specified molecular weight (A). The MFs were prepared by the microfiltration of the indicated concentrations of the lysate variant CLF using the 3 kDa to 100 kDa cutoffs, respectively. NO was determined after the 24-h culture of rat (n=4) peritoneal cells using the Griess reagent. Each point is mean±SEM. The data are representative of two identical experiments. Relative efficacy of the preparations to induce NO production was evaluated in terms of the area under curve (AUC). The AUC of the CLF preparation was taken as 100% (B). In order to eliminate the bias that may be caused by the attenuated NO-enhancing ability of samples applied at concentrations > 100 μg/ml (see Figure 1A), the AUC analysis has been based on the data up to the concentrations ≤100 μg/ml.

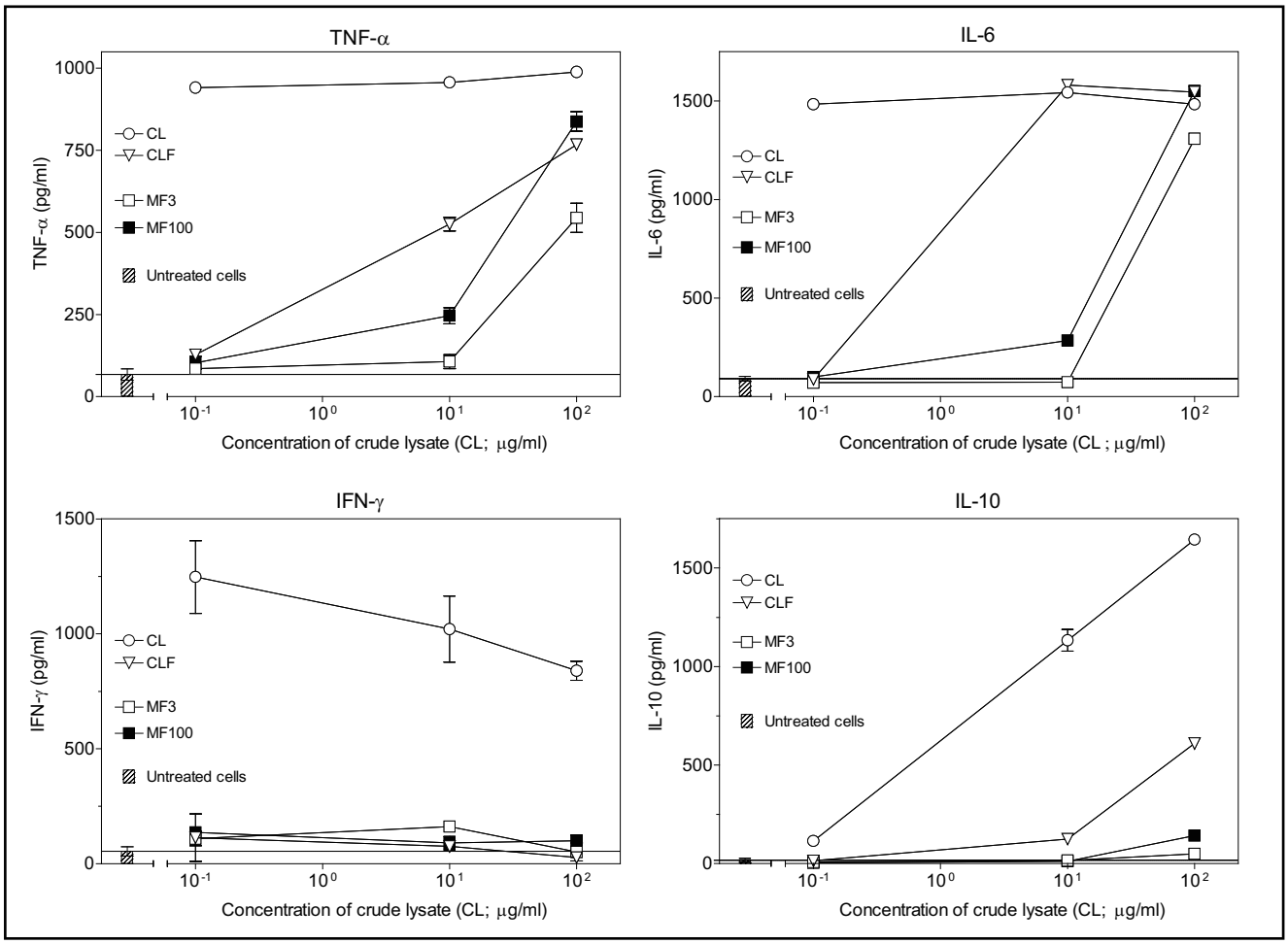

Fig. 2. Secretion of cytokines by rat (n = 5) peritoneal cells upon the 24-h exposure to *L. casei* lysate and its microfiltration fractions (MF) differing in the content of molecules with specified molecular weight. The MF3 and MF100 were prepared from the indicated concentrations of the lysate variant CLF that was treated by the 3 kDa and 100 kDa cutoff microfiltration, respectively. Concentration of cytokines was assayed by ELISA. Each point is mean±SEM. The data are representative of two identical experiments.

secretion of TNF-α and IL-6. However, only MF100 enhanced significantly, though only marginally, the IL-10 secretion ($p<0.01$).

The ability to stimulate IFN-γ was confined to the crude lysate (CL) merely.

Effects of lysate microfiltrates on prostaglandin E_2 production

All lysate samples induced the formation of PGE_2 (Figure 3). The highest effectiveness was exhibited by the crude lysate (CL) that showed prominent activity at as low concentration as 0.1 μg/ml. The effects of CL and CLF (*i.e.* centrifuged plus 0.22 μm-filtered CL) lysate variants were nearly identical when applied at the 10 μg/ml concentration. This concentration turned out to be a minimum requirement for the activation of PGE_2 production by the MF fractions obtained by microfiltration of CLF. Stimulatory effects of relatively high concentration of all MFs (100 μg/ml) resembled closely the effects of the CL and CLF. Slight but statistically significant differences among individual MFs to augment PGE_2 production were observed. The onset of significant enhancement was found after the 10 μg/ml dose of MF50 and MF100 ($p<0.05$, and $p<0.05$, respectively). The MF3 and MF10 were ineffective at this concentration.

Effects of peptidoglycan and lipoteichoic acid on production of NO

PGNs and LTAs of *B. subtilis and S. aureus* origin enhanced production of NO (Figure 4). The microfiltration treatment of PGN and LTA stock solutions, including the passage through the 100 kDa cutoff centrifugal units, led to the complete disappearance of NO-stimulatory effects.

DISCUSSION

Probiotic bacteria do not disrupt the intestinal mucosal integrity and do not translocate to the spleen, liver, kidney and blood (Pan *et al.* 2011; Paturi *et al.* 2008). Despite of it, they exhibit not only the local but also systemic effects. The mechanisms of local action of probiotics are only insufficiently understood. Even less known are the mechanisms of their systemic health benefits.

Bacteria are phagocytosed and intracellulary digested by splenocytes, monocyte/macrophages and dendritic cells (Cai *et al.* 2010; Maassen *et al.* 2000). This process depends on the expression of TLR2 receptors (Matsuzaki *et al.* 2004; Shida *et al.* 2006). It may lead to the release of immunobiologically active components of bacterial cell walls and/or other cell structures. The immunomodulatory effects of various preparations of lactic acid bacteria including crude lysates have been suggested to be mainly due to the complex macromolecules of bacterial cell walls such as peptidoglycans (PGNs) and lipoteichoic acids (LTAs). PGNs possess multiple immunomodulatory functions (Bhatt *et al.* 2009; Chow *et al.* 2009; Kengatharan *et al.* 1998). They are absorbed probably undigested (Forestier *et al.* 1999) from the intestinal lumen (Lichtman *et al.* 1991) and translocated systemically. The intestinal permeability of PGNs is facilitated by the PGN recognition protein-3 (PGlyRP3) (Bu *et al.* 2010). Also LTAs possess prominent immunostimulatory activities (Zídek *et al.* 2010). There are, however, no data on possible transintestinal transport of LTAs. Their contribution to the systemic effects of probiotics is considered unlikely (Yipp *et al.* 2002).

One of the possibilities of systemic effects could be the transport of locally induced immune mediators to

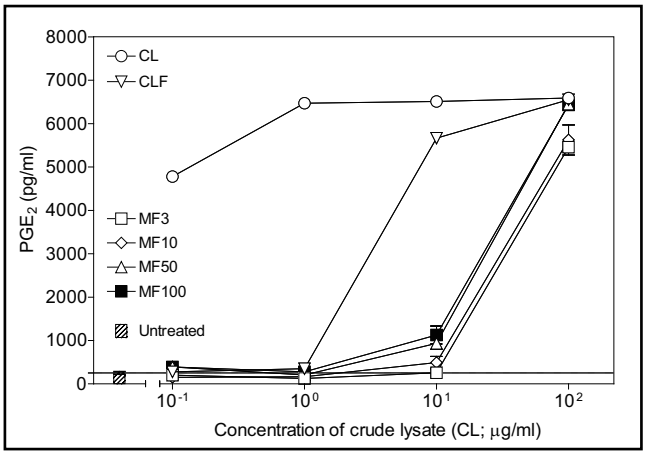

Fig. 3. Production of PGE_2 by rat (n = 3) peritoneal cells upon the 24-h exposure to *L. casei* lysate and its microfiltration fractions (MF) differing in the content of molecules with specified molecular weight. The MFs were prepared by microfiltration of the indicated concentrations of the lysate variant CLF using the 3 kDa to 100 kDa cutoffs, respectively. Concentration of PGE_2 was assayed by ELISA. Each point is mean±SEM. The data are representative of two identical experiments.

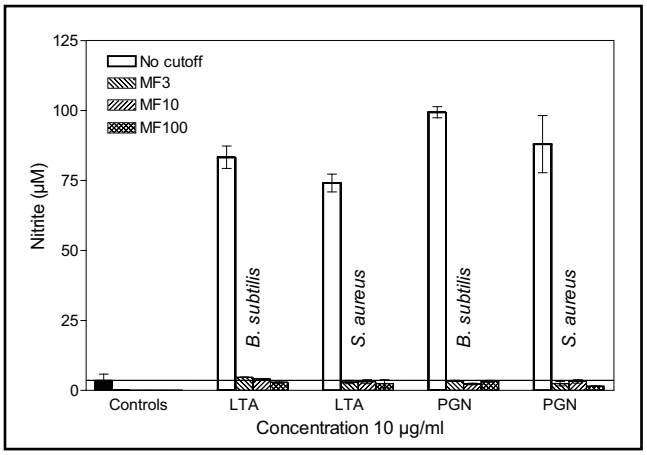

Fig. 4. Production of NO by rat (n = 3) peritoneal cells cultured 24 h in the presence of peptidoglycans (PGN), lipoteichoic acids (LTA). The samples of PGN and LTA treated with specified cutoff microfilters were completely devoid of the NO-stimulatory activity. Columns are means±SEM. The data are representative of two identical experiments.

remote sites of the body. Cytokines that are primarily produced in the intestine can be found in plasma and lung, although in much lower amount than in the original site of the induction (Narita *et al.* 2004).

The digestion of bacteria has been shown to lead to the release of low-molecular mass fragments of bacterial macromolecules such as muropeptides (Billot-Klein *et al.* 1997; Vermeulen & Gray 1984). These findings have become a rational background for our hypothesis that molecules of low molecular mass participate on the systemic effects of probiotics. It may be supposed that these molecules possess higher intestinal permeability and systemic distribution than PGN and LTA with very high molecular size.

Our present data prove that the low-molecular weight fractions of bacterial lysates do possess the immunobiological activity of the original lysate. It should be underlined that the microfiltration fractions are devoid of all traces of PGN and LTA as well as of LPS (Zídek & Kmoníčková 2012; Zdek *et al.* 2012). It has been observed that the immunostimulatory properties of microfiltrates are almost entirely due to chemical species with the molecular size ≤3 kDa. The dose-response experiments indicate that approximately 4×10^7 bacteria are needed for preparation of microfiltrates which contain the amount of compound(s) sufficient to induce production of immune mediators. The chemical nature of species remains to be identified. Anyhow, the participation of MDP, the minimum constituent responsible for biological activities of PGNs (Adam *et al.* 1975; Hoedemakers *et al.* 1994) is highly improbable, because it has not been found in the preparations of digested bacteria (Billot-Klein *et al.* 1997; Vermeulen & Gray 1984). The present findings encourage further studies that should ascertain whether the immunomodulatory effects are bound to the low-molecular weight compounds of probiotic lactobacilli merely or are a common property of other bacteria.

CONCLUSION

Live bacteria, heat-killed ones and lysates thereof are known to activate a number of immune mediators. The present results provide unequivocal evidence showing that the stimulatory effects of lysate preparations are significantly contributed to by chemical species of the molecular mass ≤3 kDa.

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Potential Conflicts of Interest: *None disclosed.*

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