THE EFFECT OF PROBIOTICS ON THE VIABILITY OF THE PORCINE AND HUMAN MONOCYTES

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Abstract: The aim of this study was evaluate the effect of probiotics on the viability of the cells of immune system. For experiment we have chosen the monocytes, which were isolated from porcine and human blood. The population of monocytes was cultivated *in vitro* conditions with probiotics strains such as *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium*. The monocytes were incubated without (control sample) or with probiotics for 2 and 4 hours. The percentage of apoptosis and necrosis of monocytes was analysed by flow cytometry. The results have shown statistically significant differences in proportion of porcine apoptotic monocytes cultivated with *Bifidobacterium bifidum*. *Enterococcus faecium* showed statistically significant effect on necrosis of porcine monocytes. The statistically significant differences in proportion of human apoptotic monocytes were observed in cultivation with *Lactobacillus rhamnosus* and *Enterococcus faecium* and the highest percentage of necrotic cells was seen in human monocytes cultivated with *Bifidobacterium bifidum*. It is obvious that these selected strains of probiotics had immunomodulatory effect on immune cells and induced apoptosis and necrosis of porcine and human monocytes *in vitro* condition.

Key Words: monocytes, probiotics, apoptosis, necrosis, flow cytometry

INTRODUCTION

The immune system includes complex of specific cells. The essential components of innate immunity are monocytes, which play central role in the initiation and resolution of inflammation. Normally, in the bloodstream circulating monocytes are short-lived cells and eventually undergo spontaneous apoptosis. Apoptosis or programmed cell death is an evolutionarily conserved mechanism essential for normal development and defense against pathogens. Apoptosis is characterized by a group of biochemical and morphological changes of cell. During this process is activated the group of enzymes, the caspases. The caspases have a pivotal role in the pathogenesis of inflammatory diseases. Apoptosis of monocytes is blocked during chronic inflammation and the monocytes differentiate locally into macrophages as immune response upon pathogenic challenge (Parihar et al. 2010).

The immune response is caused by antigens, components of pathogenic organisms especially. The major site of defense against potential pathogenic organisms is a gut mucosal immune system (Habil et al. 2011). The intestine is colonized by number of different microorganisms and it represents the largest source of microbial stimulation which exerts harmful and beneficial effects on immune system (Delcenserie et al. 2008). The lumen of the gastrointestinal tract includes pathogenic and commensal bacteria which communicate among themselves. Probiotics are defined as live microorganisms which exert health benefits on human and animals when administered in adequate amounts (Jensen et al. 2015). The most widely strains of probiotics include *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Mansour et al. 2014).

Probiotic bacteria interact with intestinal epithelial cells and mucosal immune cells and can modulate their specific functions (Wells 2011). Recognized mechanisms of the action of probiotics include for example regulation of cytokine production, enhancement of secretion IgA, production of antibacterial substances, maintenance of the intestinal barrier function and competition with pathogenic microorganisms (Yan, Polk 2002). Cytokines induced by probiotics are considered to play key roles in immunoregulation. Specific strains of probiotics induce pro-inflammatory cytokines such as IL-1, IL-6,

TNF-α and IFN-γ and anti-inflammatory cytokines such as IL-10 and TGF-β. These cytokines potently augment the function of macrophages and can be a possible mechanism of their anti-carcinogenic and anti-infectious activity (Shida et al. 2006). Many clinical studies during recent decades demonstrate that some strains of probiotics have beneficial properties for various diseases (Plaza-Diaz et al. 2014). The influence of probiotics on the viability of the cells of the defense system of humans and animals still has not been studied in detail.

Therefore the aim of study was determine whether probiotics, which normally used in human and animals nutrition, can affect the viability of cells of the immune system. We were interested in immunomodulatory effect caused by *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium* which were cultivated with monocytes *in vitro* condition. We focused on detection of apoptosis and necrosis of porcine and human monocytes in short-term cultivation with selected strains of probiotics.

MATERIAL AND METHODS

Animals and volunteers

For isolation of monocytes were used 10 healthy pigs and 10 human volunteers in this study. The Large White pigs were 4 to 6 months old. They were kept in the experimental stables of the Veterinary Research Institute in Brno, Czech Republic and were fed with standard diet.

Blood sampling, isolation and processing of blood leukocytes

Peripheral blood was collected in the morning from pigs and in humans. Porcine peripheral blood (15 mL) were collected from *vena cava cranialis* into sterile flask containing 25 IU/1 mL sodium heparin (Heparin forte Léčiva, Zentiva, Czech Republic) in pyrogen-free 10 mL DPBS (Dulbecco´s Phosphate Buffered Saline, Cambrex, USA). Human peripheral blood (10 mL) was collected from *vena cephalica* into blood collection system (S-MONOVETTE, Sarstedt AG & Co, Germany) containing trisodium citrate solution (0.106 mol/L) and citrate solution (0.5 mL/5 mL).

Human and porcine monocytes were isolated by sedimentation in dextran together with neutrophils. Peripheral blood was mixed with 6% dextran (Dextran clinical grade, MS Biomedicals, France) diluted in DPBS. The suspension of blood leukocytes was washed twice and finally resuspended in complete medium. Complete RMPI-1640 contains the RPMI-1640 (RPMI-1640, Sigma-Aldrich, USA) with 10% fetal calf serum (FCS, Gibco, USA). Complete D-MEM contains D-MEM (Gibco, USA) with 10% normal porcine serum (PS, Gibco, USA). The human cells were resuspended in a complete RMPI-1640 and porcine cells in complete D-MEM.

In vitro cultivation of monocytes with probiotics

Bifidobacterium bifidum CCM 3762, *Lactobacillus rhamnosus* CCM 1828 and *Enterococcus faecium* NCIMB 11181 (M74) (all of them from Czech Collection of Microorganisms, Masaryk University in Brno, Czech Republic) were selected as probiotic strains. Fresh population was analyzed immediately after isolation. The remaining samples were incubated *in vitro* with or without probiotics for 2 and 4 hours at 37 °C in 5% CO_2. Porcine and human monocytes together with neutrophils in amount 1×10^5 were incubated with 2.5×10^5 probiotics in 96-wells plates (Tissue Culture Test Plate 96 Wells, TPP, Techno Plastic Products AG, Switzerland). Porcine cells were incubated in complete D-MEM and human in complete RPMI-1640. The fresh and *in vitro* cultivated cell populations were analyzed by flow cytometry.

Flow cytometry analysis

Flow cytometry (FCM) analysis was used for determination of the differential cell counts and detection of apoptosis and necrosis in monocytes. The measurements were performed on BD LSRFortessa flow cytometer (Becton Dickinson, San Jose, USA). The 800,000 events per sample were acquired. Final dot plots were evaluated using BD FACSDiva software (Becton-Dickinson, San Jose, USA). Apoptotic and necrotic monocytes were analyzed simultaneously after staining with Annexin-V labelled with FITC and propidium iodide (PI). The commercial Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA) was used according to the manufacturer´s instructions.

Statistical analysis

The results were evaluated using paired t-test in statistical program GraphPad Prism v. 5 for the determination of significant sources of variability. The significance of differences in the proportions of apoptotic and necrotic monocytes and significant differences between time-points and between treatments of probiotics during *in vitro* cultivation was tested by the Scheffe´s method. *P* values were considered statistically significant if $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

RESULTS AND DISCUSSION

In this study were observed significant differences *in vitro* cultivation of porcine and human monocytes with selected strains of probiotics. We focused on comparing the percentage of apoptosis and necrosis of monocytes in the control sample (without probiotics) and the sample with probiotics. Percentage of viability was observed initially after 2 hours of cultivation and consequently after 4 hours of cultivation of monocytes with probiotic cultures.

Viability of porcine monocytes

The highest statistically significant difference ($*P<0.05$) was found between the control sample and the cultivation with *Bifidobacterium bifidum* at 2 hours. Other probiotics exhibit no significant differences ($P>0.05$) in cultivation time 2 hours. At 4 hours of cultivation, although the highest percentage of apoptosis showed monocytes cultivated with *Enterococcus faecium*, but there were observed no statistically significant differences (see Figure 1).

At 2 hours of cultivation there were no statistically significant differences in proportion of necrotic monocytes between the control sample and selected strains of probiotics. In contrast, in 4 hours cultivation time was monitored the highest significant difference ($**P<0.01$) in *Enterococcus faecium*. Other probiotic cultures in time 4 hours showed no statistically significant effect on the necrosis of porcine monocytes (see Figure 2).

Viability of human monocytes

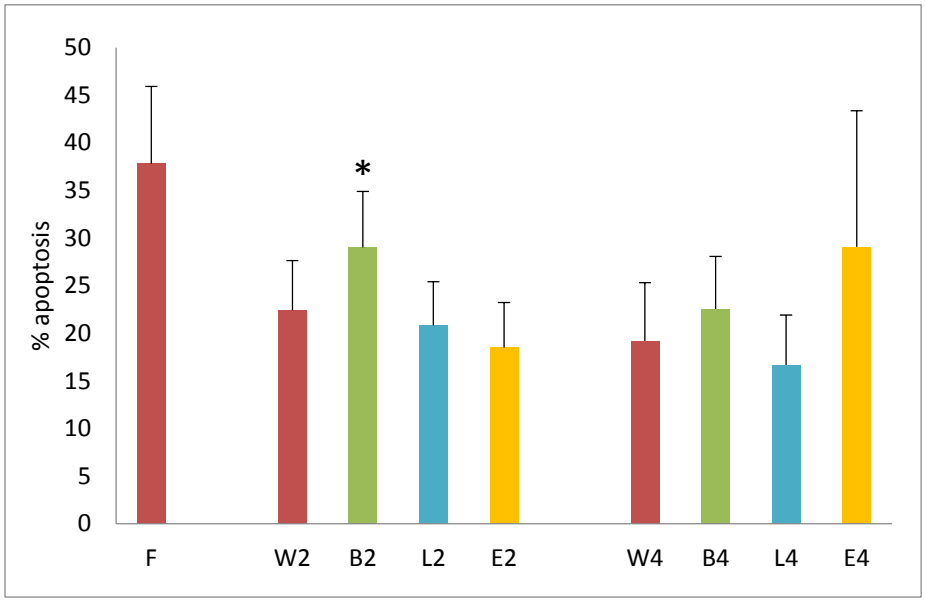
Statistically significant differences in proportion apoptotic monocytes ($***P<0.001$) were observed *Lactobacillus rhamnosus* and *Enterococcus faecium* in 2 hours cultivation time. At 4 hours cultivation were also observed significant differences ($**P<0.01$) for the same strain of probiotics. *Bifidobacterium bifidum* showed no statistically significant effect on apoptosis of human monocytes in both times of cultivation (see Figure 3).

The highest percentage of necrotic cells was seen in monocytes cultured with *Bifidobacterium bifidum*. There was observed a significant difference in the cultivation time 2 hours ($**P<0.01$) and 4 hours ($*P<0.05$). Other probiotic showed no statistically significant differences ($P>0.05$) in both times. Worth mentioning is the high percentage of necrotic monocytes cultivated with *Enterococcus faecium* (see Figure 4).

Chiu et al. (2010) described that *Lactobacillus casei rhamnosus* effectively induced apoptosis of monocytes and lymphocytes and regulated it via expressions of mRNAs (Bcl-2, Bax) and proteins (cytochrome c, caspase 9, caspase 3) by mitochondrial pathway. In comparison to Yan and Polk (2002) showed that *Lactobacillus rhamnosus* supported the survival of intestinal epithelial cells through the activation of the anti-apoptotic and inhibition pro-apoptotic kinase. Khailova et al. (2010) revealed that *Bifidobacterium bifidum* reduced apoptosis in the intestinal ephitelium and concurrently preserved intestinal integrity. Another study demonstrated that *Bifidobacterium bifidum* decreased apoptosis of epithelial cells similar as *Lactobacillus rhamnosus* (Daniluk et al. 2012). *Lactobacillus helveticus* and *Bifidobacterium longum*, in combination as preventive therapy, diminished the apoptosis propensity in the limbic system following a myocardial infarction in rats (Girard et al. 2009). Gröbner et al. (2010) demonstrated that *Enterococcus faecium* induced necrosis of macrophages when exposed to lysozyme *in vitro* and *in vivo*.

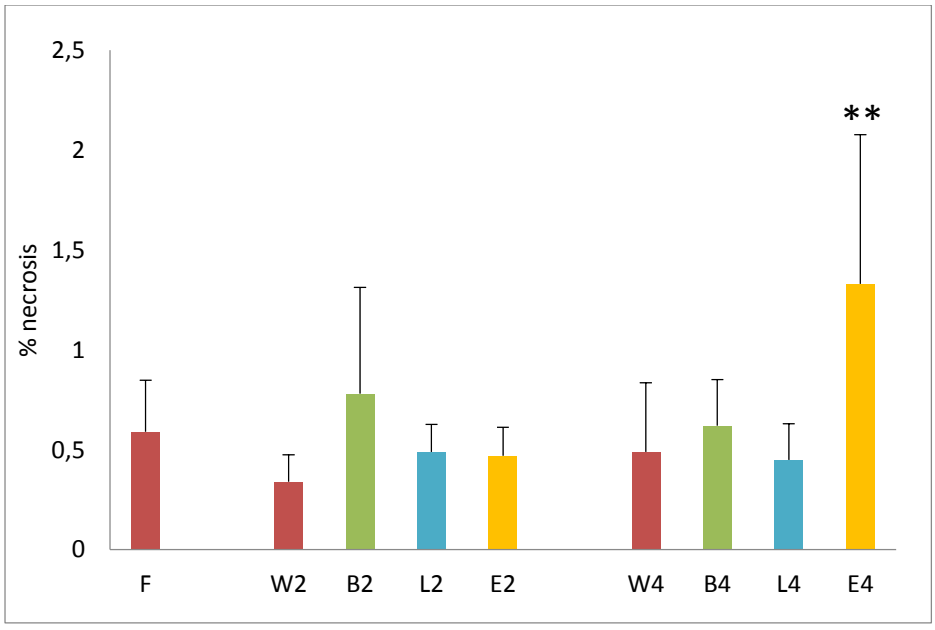
In this study was observed significant influence of *Bifidobacterium bifidum* to increase in apoptosis of porcine monocytes. In contrast, apoptosis of human monocytes was increased by *Lactobacillus rhamnosus* and *Enterococcus faecium*. Necrosis of monocytes was caused by *Enterococcus faecium* in porcine and by *Bifidobacterium bifidum* in human. The different effect of selected strains of probiotics on apoptosis and necrosis of monocytes was a high probability due to interspecies specificity of the immune system.

Figure 1 Apoptosis of porcine monocytes



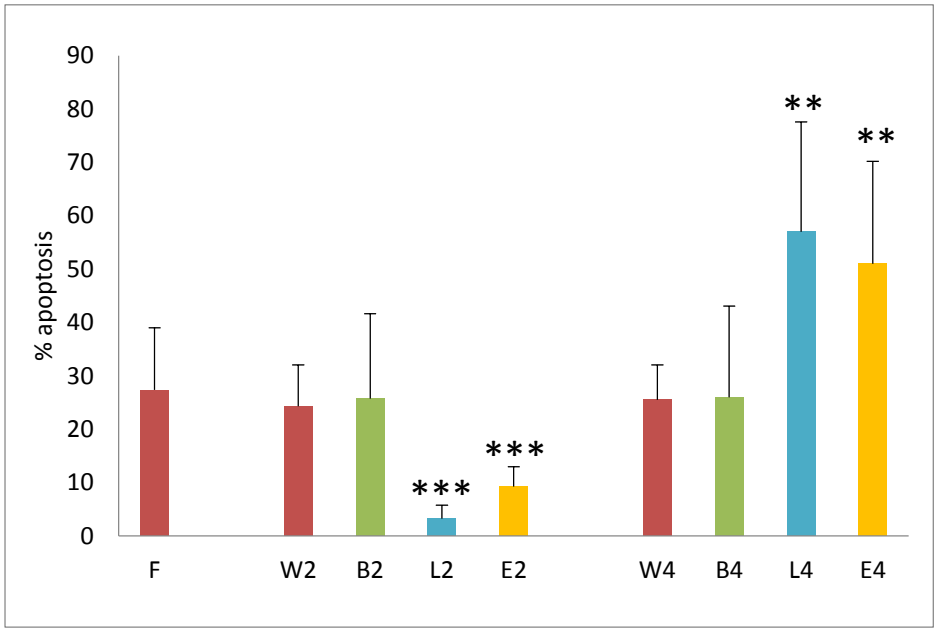
*Legend: Fresh population (F), population without probiotics (W2, W4) and with Bifidobacterium bifidum (B2, B4), Lactobacillus rhamnosus (L2, L4) and Enterococcus faecium (E2, E4) after 2 h and 4 h cultivation in vitro. Data are means in percentages, measured at 0 h, 2 h and 4 h, and signifiant differences are marked by asterisks (*P<0.05, Scheffe's method). The comparisons were made among samples without probiotics relative to 2 h and 4 h samples after cultivation in vitro.*

Figure 2 Necrosis of porcine monocytes



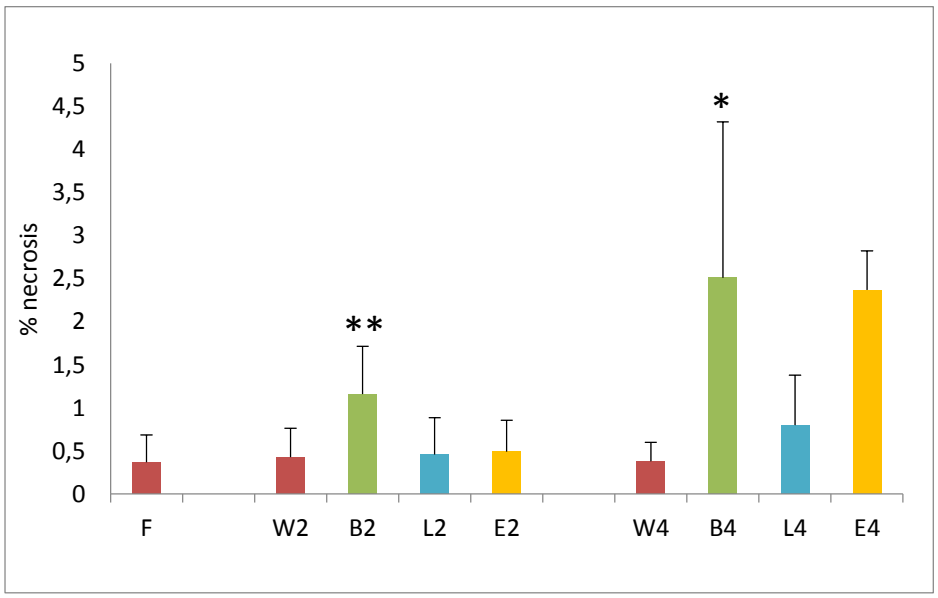
*Legend: Fresh population (F), population without probiotics (W2, W4) and with Bifidobacterium bifidum (B2, B4), Lactobacillus rhamnosus (L2, L4) and Enterococcus faecium (E2, E4) after 0 h, 2 h and 4 h cultivation in vitro. Data are means in percentages, measured at 2 h and 4 h, and signifiant differences are marked by asterisks (**P<0.01, Scheffe's method). The comparisons were made among samples without probiotics relative to 2 h and 4 h samples after cultivation in vitro.*

Figure 3 Apoptosis of human monocytes



*Legend: Fresh population (F), population without probiotics (W2, W4) and with Bifidobacterium bifidum (B2, B4), Lactobacillus rhamnosus (L2, L4) and Enterococcus faecium (E2, E4) after 0 h, 2 h and 4 h cultivation in vitro. Data are means in percentages, measured at 2 h and 4 h, and signifiant differences are marked by asterisks (**P<0.01, ***P<0.001, Scheffe's method). The comparisons were made among samples without probiotics relative to 2 h and 4 h samples after cultivation in vitro.*

Figure 4 Necrosis of human monocytes



*Legend: Fresh population (F), population without probiotics (W2, W4) and with Bifidobacterium bifidum (B2, B4), Lactobacillus rhamnosus (L2, L4) and Enterococcus faecium (E2, E4) after 0 h, 2 h and 4 h cultivation in vitro. Data are means in percentages, measured at 2 h and 4 h, and signifiant differences are marked by asterisks (*P<0.05, **P<0.01, Scheffe's method). The comparisons were made among samples without probiotics relative to 2 h and 4 h samples after cultivation in vitro.*

CONCLUSION

The interaction between selected strains of probiotics and monocytes was examined in this pilot study. It is obvious that *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium* had immunomodulatory effect on immune cells. They induced apoptosis and necrosis of porcine

and human monocytes *in vitro* condition. However, the mechanisms of probiotics with immune cells are incompletely understood. Further *in vivo* studies are necessary for clinical applications.

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