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Effect of probiotics on the viability of porcine and human neutrophils *in vitro*

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ABSTRACT: *Lactobacillus*, *Bifidobacterium* and *Enterococcus* cultures are increasingly used as probiotics for humans and pigs. The aim of this study was to investigate if co-cultivation of porcine and human neutrophils with probiotics can lead to increased apoptosis *in vitro*. Ten adult Large white pigs and 10 healthy human donors were used in this study. Neutrophils were isolated by dextran sedimentation and cultivated with and without the lactic acid bacteria *Bifidobacterium bifidum*, *Lactobacillus rhamnosus*, and *Enterococcus faecium* for 2, 4, 24 and 48 h. Early and late apoptosis was measured using flow cytometry, and cell lysis was detected based on lactate dehydrogenase activity (LDH). A significant ($P < 0.05$; $P < 0.01$) increase in apoptotic neutrophils and LDH was observed at 24 h and 48 h *in vitro*. All probiotics exerted their greatest effects on the early apoptosis of porcine neutrophils, while the effects of *L. rhamnosus* were most pronounced on late apoptosis and those of *B. bifidum* on LDH release of human neutrophils. The increased neutrophil apoptosis caused by probiotic bacteria can be beneficial for more efficient efferocytosis and faster resolution of inflammation and tissue regeneration. In conclusion, we have demonstrated that the interaction of *B. bifidum*, *L. rhamnosus*, and *E. faecium* with human and porcine neutrophils leads to their apoptosis.

Keywords: pig; early; late; apoptosis; cell lysis; *Bifidobacterium*; *Lactobacillus*; *Enterococcus*

In recent years, interest in the therapeutic and preventive effects of probiotics has been rapidly and significantly growing worldwide. Probiotics include a wide variety of bacterial genera, which include lactic acid bacteria (LAB). Species of these genera can be found in the gastrointestinal tracts of humans and animals, as well as in fermented foods, and include *Lactobacillus*, *Bifidobacterium* and *Enterococcus*, among others (Klein et al. 1998).

Lactobacillus, *Bifidobacterium* and *Enterococcus* cultures are thus increasingly used as probiotics in pharmaceuticals and in foods, and there is a growing consensus regarding the beneficial effects of these probiotics on human and swine health (Reuter 2001; Macha et al. 2004; Mitsuoka 2014; Yang et al. 2015).

Probiotics may have a positive effect upon intestinal homeostasis, which is maintained by balancing the rate between cell proliferation and cell loss (Ramachandram et al. 2000). Uncontrolled apoptosis is often the main mechanism in the pathogenesis of chronic inflammation. A high rate of intestinal epithelial apoptosis leads to severe necrotising enterocolitis (Clark et al. 2005), and has been observed in the group of bowel disease disorders, for example Crohn's disease (Teahon et al. 1992). Administration of lactobacilli or bifidobacteria has been shown to reduce apoptosis in intestinal

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epithelial cells induced by such pro-inflammatory cytokines as tumour necrosis alpha (TNF-α), interleukin 1 beta (IL-1β) and interferon gamma (IFN-γ) (Daniluk et al. 2012). Another study showed that culture of *Lactobacillus rhamnosus* GG with human colon cells activates the anti-apoptotic Akt/protein kinase B (Yan and Polk 2002). Dykstra et al. (2011) observed that *Lactobacillus plantarum* strain 299v reduced caspase-3- and caspase-7-dependent apoptotic pathways in intestinal epithelial cells. On the other hand, some studies describe pro-apoptotic effects of probiotics on cells. For example, Song et al. (2015) observed that membrane proteins isolated from heat-killed *Lactobacillus plantarum* L67 can stimulate apoptotic signals and then consequently induce programmed cell death in HT-29 cells. Sakatani et al. (2016) showed that polyphosphate derived from *Lactobacillus brevis* induced apoptosis in SW620 cells by activating the extracellular signal-regulated kinase pathway.

In the intestine, probiotics interact also with immunocompetent cells using the mucosal interface. Galdeano and Perdigon (2006) showed that after oral intake of lactobacilli these probiotics were detected in immune cells within Payer's patches and in *lamina propria mucosae* in the small intestine, as well as in immune cells in the crypt and lymph nodes in the colon. At these sites, probiotics may modulate apoptosis also in immune cells. Carol et al. (2006) showed that co-culture of inflamed mucosa with *Lactobacillus casei* significantly reduced the release of interleukin-6, which is associated with an increased proportion of apoptotic lymphocytes. Another study showed that *L. brevis* induced immune cell apoptosis through ceramide generation via neutral sphingomyelinase (Angulo et al. 2011).

Neutrophils are abundant cells in conditions of intestinal inflammation such as inflammatory bowel diseases in humans or colitis in swine. In this case, neutrophils are recruited to the site of inflammation within minutes and cross the epithelium into the intestinal lumen. The trans-epithelial migration is associated with mucosal injury and increased intestinal permeability (Weber et al. 2014), which, in turn, is associated with increased translocation of bacteria across the epithelium and invasion of the mucosa. These invading bacteria are then phagocytosed by neutrophils (Fournier and Parkos 2012). A massive infiltration of neutrophils into mucosa also has the potential of causing severe tissue destruction. Indeed, neutrophils may undergo necrosis and subsequently release cytotoxic granule contents into surrounding tissues. Therefore, neutrophils undergo apoptosis and apoptotic neutrophils are quickly removed via efferocytosis. Apoptosis and efferocytosis of neutrophils are essential for resolving inflammation and for mucosal homeostasis (Martin et al. 2014).

Some of the effects of probiotics on the biological features of neutrophils are known. Ingestion of probiotic bacteria enhances the phagocytic capacity of blood neutrophils (Arunachalam et al. 2000), triggers respiratory burst (Donnet-Hughes et al. 1999) and increases the expression of receptors involved in phagocytosis, especially complement receptor 3 (Fang et al. 2000). Nevertheless, little is known about the effect of probiotics on neutrophil apoptosis. As mentioned above, probiotics may affect cell lifespan. Therefore, we hypothesised that the interaction of probiotics with neutrophils will lead to their apoptosis and necrosis. The aim of this study was thus to investigate the effects of probiotics on the lifespan of neutrophils. We selected for this purpose three LAB as probiotics: *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium*.

MATERIAL AND METHODS

Animals and donors. Ten Large white pigs and 10 healthy human donors were used in this study. The 4–6-month-old gilts were kept in experimental stables at the Veterinary Research Institute, Brno, Czech Republic. The animals were fed a standard diet. The experimental tie-stall used in this study is certified, and animal care conformed to a good care practice protocol. All experimental procedures were approved by the Central Commission for Animal Welfare of the Czech Republic.

Seven healthy women (24–50 years of age) and three healthy men (25–49 years of age) were recruited for the study. Written informed consent was obtained from all participants and the study was approved in accordance with the ethical standards of the Declaration of Helsinki.

Blood sampling, isolation and processing of blood leukocytes. Peripheral blood was collected from the *vena cava cranialis* in pigs and from the *vena cephalica* in humans. Porcine peripheral blood (15 ml) was collected into sterile pyrogen-free tubes containing 25 IU sodium heparin/1 ml of peripheral

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blood (Heparin, Leciva, Zentiva, Czech Republic). Human peripheral blood (10 ml) was collected into a blood collection system (S-Monovette, Sarstedt AG and Co, Germany) containing trisodium citrate solution (0.106 mol/l) and citrate solution (0.5 ml/5 ml). Porcine and human leukocytes were isolated by sedimentation in dextran, as described previously by Zelnickova et al. (2006).

Bacterial strains and growth conditions. Lyophilised *Bifidobacterium bifidum* (CCM 3762, *B. bifidum*), *Lactobacillus rhamnosus* (CCM 1828, *L. rhamnosus*), and *Enterococcus faecium* M74 (CCM 6226, *E. faecium*) (all from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) bacteria were chosen as the probiotic strains. The used probiotics belong to the first (*B. bifidum*) or second biosafety risk group (*L. rhamnosus* and *E. faecium*). All lyophilised bacteria were cultivated on Petri dishes in an incubator under appropriate conditions. *B. bifidum* was cultivated for 72 h under anaerobic conditions at 37 °C. BSM-Broth medium with a supplement for selective isolation and identification of bifidobacteria (Sigma Aldrich, St Louis, USA) was used. *L. rhamnosus* was cultivated anaerobically in Lactobacillus MRS Agar (Himedia Laboratories, Mumbai, India) for 72 h at 37 °C. *E. faecium* was cultivated in COMPASS Enterococcus Agar (Biokar Diagnostics, Beauvais Cedex, France) for 48 h at 44 °C.

***In vitro* cultivation of neutrophils with probiotics**. Processed and cultivated neutrophils (porcine and human) were divided into two aliquots. The first aliquot was analysed as a fresh population immediately after isolation. The second one was incubated *in vitro* with probiotics for 2, 4, 24 and 48 h at 37 °C in 5% CO_2. Human and porcine neutrophils (1×10^5) were incubated with 2.5×10^5 probiotic bacteria in 96-well plates (Tissue Culture Test Plate 96 Wells, TPP, Techno Plastic Products AG, Switzerland). A DEN-1B densitometer (bioSAN, Riga, Latvia) was used to measure cell and probiotic concentrations. Human neutrophils were cultivated in RPMI 1640 medium (Sigma-Aldrich, USA), while D-MEM (Gibco, USA) was used for porcine neutrophils. Both media were supplemented with 10% serum (human-foetal calf, porcine-normal porcine; Gibco, USA).

Flow cytometry analysis. Flow cytometry analysis was used to detect early and late apoptosis of neutrophils co-cultivated with probiotics. Measurements were taken using a BD LSRFortessa flow cytometer (Becton Dickinson, San Jose, USA). Eight hundred thousand events were acquired per sample. Final dot plots were evaluated using BD FACSDiva software (Becton-Dickinson). Early and late apoptotic neutrophils were analysed simultaneously after staining with Annexin-V labelled with fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by Vermes et al. (1995) but with a slight modification as described by Ren et al. (2001). Annexin V-negative/PI-negative neutrophils were regarded as live cells. Annexin V-positive/PI-negative neutrophils were considered as early apoptotic cells. Annexin V-positive/PI-positive neutrophils were represented as late apoptotic or secondary necrotic cells (Ren et al. 2001). The gating strategy for flow cytometry detection of early and late apoptosis of neutrophils co-cultivated with probiotics is presented in Figure 1.

Transmission electron microscopy. The samples of neutrophils for ultrathin sectioning were fixed in 3% glutaraldehyde in cacodylate buffer, post-fixed in 2% OsO_4 solution in phosphate buffer, dehydrated in 50%, 70%, 90% and 100% acetone and embedded in Epon-Durcupan mixture (Epon 812 Serva, Germany; Durcupan, ACM Fluka, Switzerland). The sections were cut using a UC 7 ultramicrotome (Leica, Austria), then contrasted using 2% uranyl acetate and 2% lead citrate and analyzed at 80 kV under an EM Philips 208 S Morgagni (FEI, Czech Republic) transmission electron microscope.

Quantification of porcine and human neutrophil cytolysis. Cell death and cell lysis were quantified based on the measurement of lactate dehydrogenase (LDH) activity, as indicated by its release from the cytosol of damaged cells into the supernatant. The Cyto Tox96 Non-Radioactive Assay (Promega, USA) was used according to the manufacturer's instructions to measure LDH. A Synergy H1 microtiter plate reader (BioTek, USA) at a wavelength of 490 nm was used to measure the assay values. All measurements were performed in duplicate.

Statistical analysis. Statistical analysis of the obtained data was carried out using multifactorial analysis of variance (ANOVA) by Scheffe's method. Data were processed using STATISTICA 7.1 software (StatSoft CR Ltd, Prague, Czech Republic). Differences between the mean values for different treatment groups were considered significant at $P < 0.01$ or $P < 0.05$.

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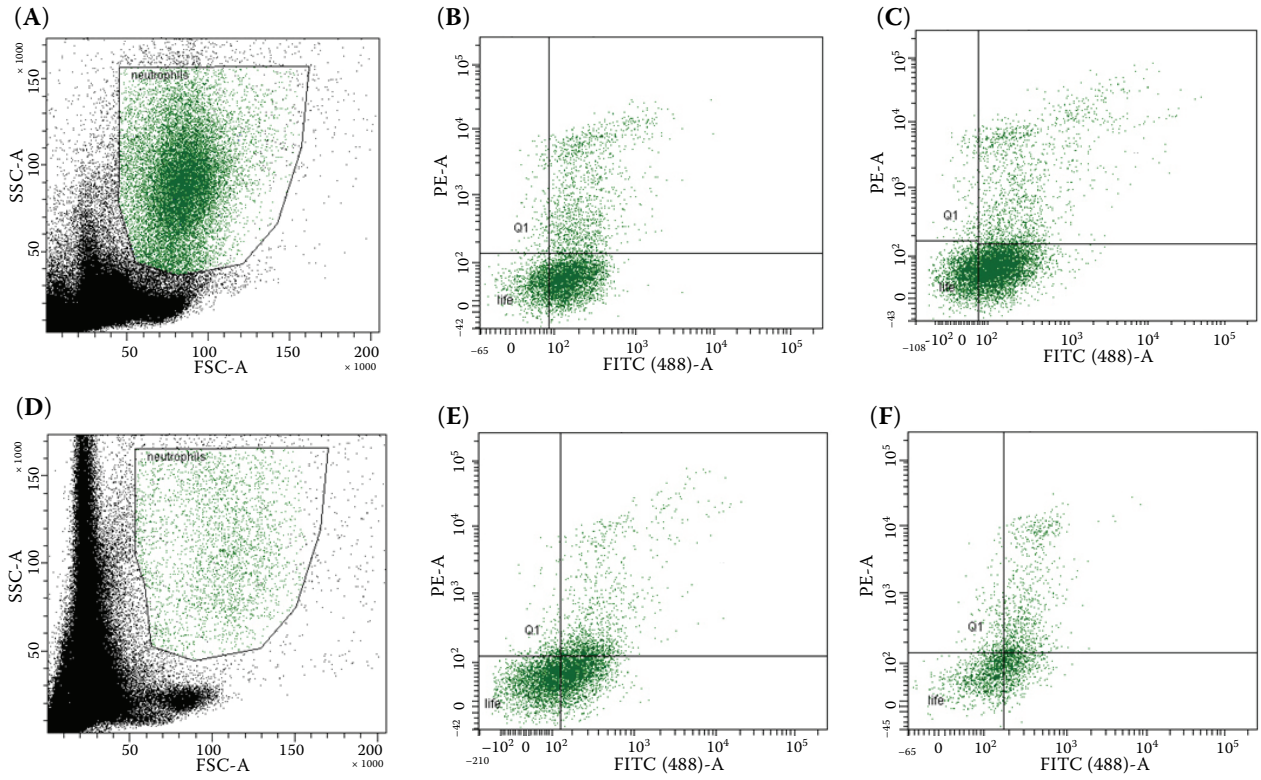

Figure 1. Gating strategy for detection of porcine (**A**–**C**) and human (**D**–**F**) neutrophils in early and late apoptosis by flow cytometry after co-cultivation with probiotics (representative dot plots)

Dot plots (**A**) (porcine) and (**D**) (human) show the distribution of cells differentiated by their forward-scatter (FSC) and side-scatter (SSC) parameters. Neutrophils are highlighted in green. Porcine neutrophils were co-cultivated with *Enterococcus faecium* and human neutrophils with *Bifidobacterium bifidum*. Dot plots (**B** and **C**) (porcine) and (**E** and **F**) (human) present typical flow cytometric profiles for staining with Annexin-V labelled with fluorescein isothiocyanate (FITC) and propidium iodide (PE-A), showing viable neutrophils (lower left quadrants), early apoptotic neutrophils (lower right quadrants), and late apoptotic neutrophils (upper right quadrants). (**B**) and (**E**) present 24 h co-cultivation, and (**C**) and (**F**) present 48 h co-cultivation with probiotics

RESULTS

In vitro cultivation of neutrophils with probiotics

In this study, we observed the effects of probiotics on the viability of porcine and human neutrophils. Structurally normal cells predominated in the population of freshly isolated neutrophils. Only a small proportion of neutrophils showed structural changes typical of early and late apoptosis. Early apoptotic neutrophils were identified by flow cytometry as AnnexinV$^+$ and PI$^-$ cells (7.3% in porcine and 7.0% in human neutrophils). In addition, some neutrophils were detected as late apoptotic (secondary necrotic; AnnexinV$^+$ and PI$^+$; 8.4% in swine and 6.4% in humans, respectively). The gating of these cells in flow cytometry is presented in Figure 1.

Subsequent co-cultivation of neutrophils with probiotics *in vitro* resulted in an increase in apoptotic cells. By transmission electron microscopy, we observed structurally normal neutrophils with segmented nuclei and pseudopodia. Some contained phagocytosed probiotic bacteria in various stages of digestion within the cytoplasm (Figure 2A). We also observed early apoptotic neutrophils with typical structural changes (Figure 2B) characterised by a rounded shape without pseudopodia, rounded nuclei with moon-shaped condensed chromatin and vacuoles in the cytoplasm. Late apoptotic and/or secondary necrotic neutrophils were characterised by loss of plasma membrane integrity and disintegration of cell structures (Figure 2C).

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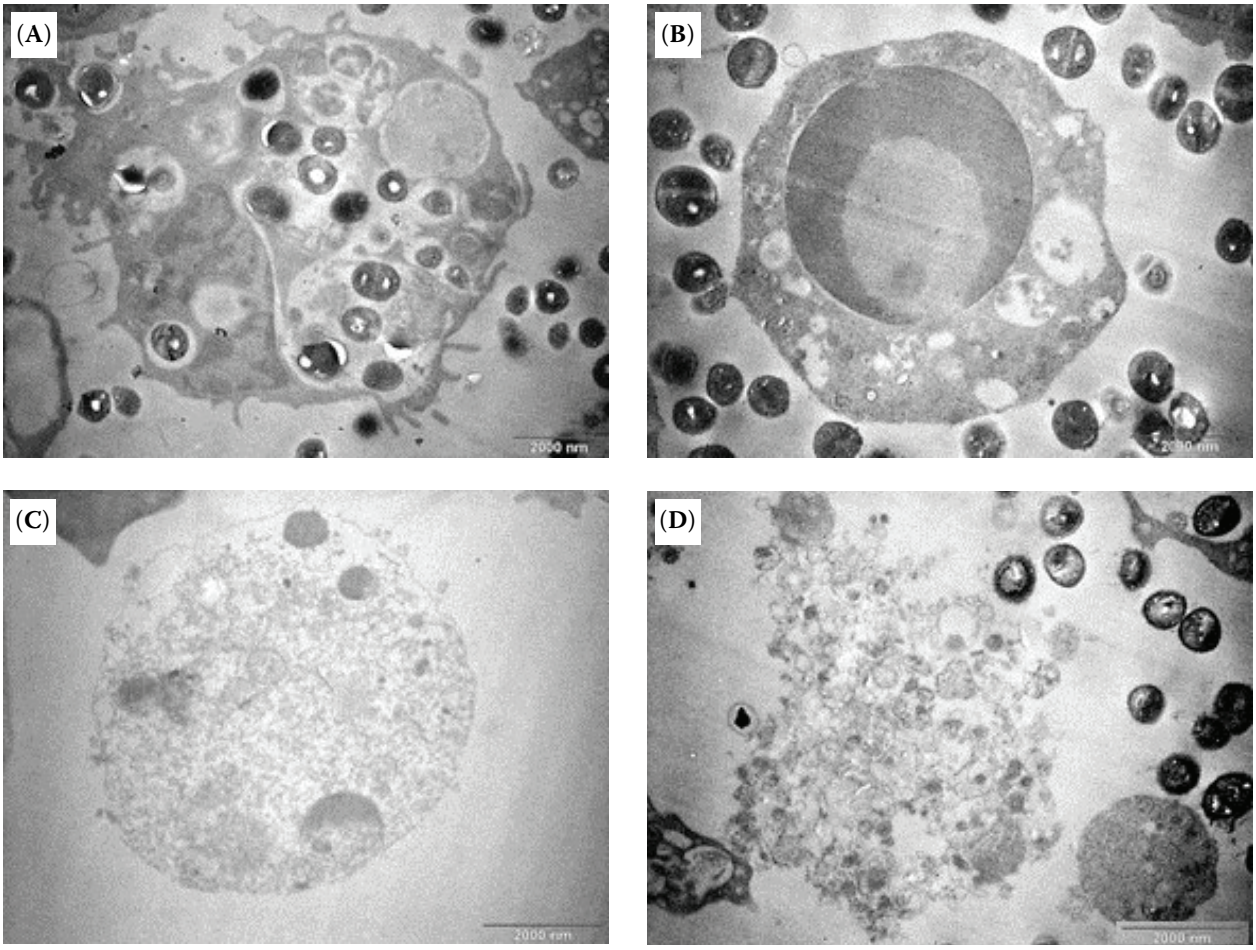

Figure 2. Transmission electron microscopy images of neutrophils co-cultivated with probiotics (*Enterococcus faecium*): (**A**) Normal viable neutrophil with phagocytosed *E. faecium*, (**B**) early apoptotic neutrophil, (**C**) late-apoptotic neutrophil, (**D**) necrotic neutrophil (lysis). Magnification × 10 000

Completely disintegrated lysed cells were also seen (Figure 2D).

Effect of probiotics on neutrophil apoptosis

In the population of neutrophils not exposed to probiotics, we observed a time-dependent increase in apoptotic cells. A short period of cultivation with probiotics, however, led to an insignificant increase in the proportion of apoptotic cells in comparison to neutrophils cultivated without probiotics (Figures 3 and 4).

In porcine neutrophils, the interaction of probiotics with neutrophils resulted in an increase in early apoptotic cells after 24 h in the cases of *L. rhamnosus* and *E. faecium* ($P < 0.01$) and after 48 h in the cases of *L. rhamnosus* ($P < 0.05$), *B. bifidum*, and *E. faecium* ($P < 0.01$). In human neutrophils, we observed an increase in early apoptotic cells only in the case of *L. rhamnosus* ($P < 0.01$) after 24 h. In contrast, a lower proportion of apoptotic neutrophils was detected in the cases of *B. bifidum* ($P < 0.01$), *L. rhamnosus* ($P < 0.05$) and *E. faecium* (non-significant) in comparison to neutrophils cultivated without probiotics (Figure 4).

As described for early apoptosis, the numbers of late apoptotic neutrophils increased in a time-dependent manner during *in vitro* cultivation with or without probiotics. As is evident from Figure 5, in porcine neutrophils we detected no significant differences in the proportions of late apoptotic neutrophils during co-cultivation with probiotics. In human neutrophils, on the other hand, we observed a non-significant increase in late-apoptotic cells for all probiotics after 24 h and 48 h of co-cultivation.

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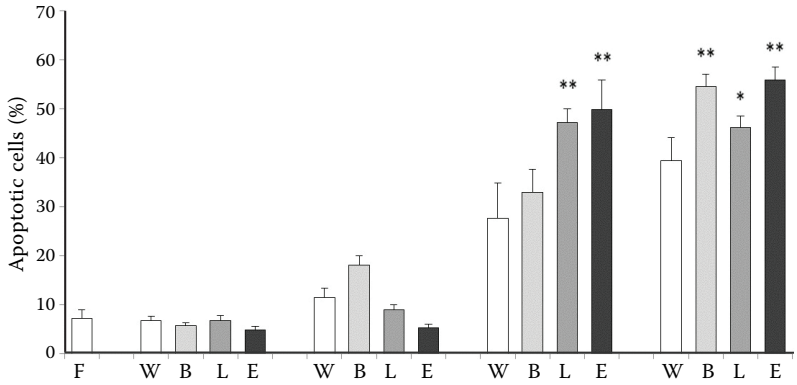

Figure 3. Early apoptosis of porcine neutrophils co-cultivated with probiotics for 2–48 h *in vitro.* Data are presented as mean ± SE
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics
*$P < 0.05$, **$P < 0.01$

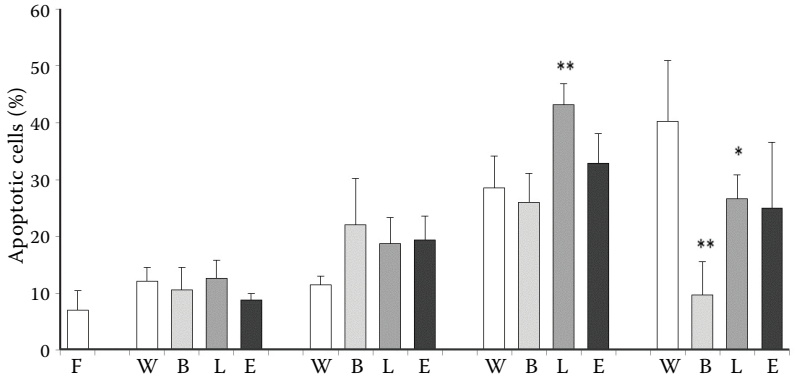

Figure 4. Early apoptosis of human neutrophils co-cultivated with probiotics for 2–48 h *in vitro.* Data are presented as mean ± SE
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics
*$P < 0.05$, **$P < 0.01$

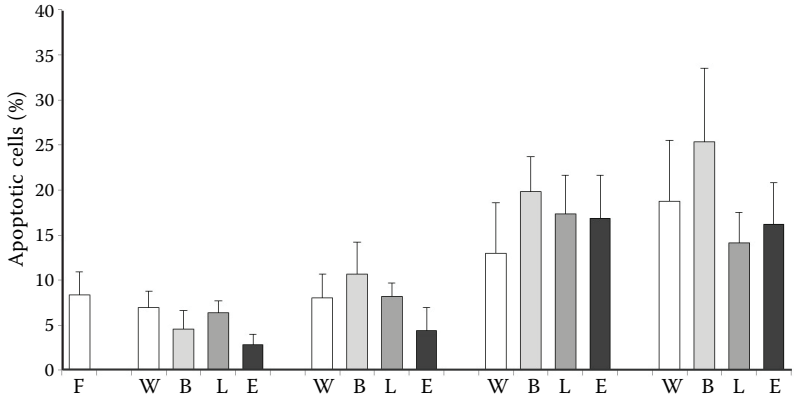

Figure 5. Late apoptosis of porcine neutrophils co-cultivated with probiotics for 2–48 h *in vitro*. Data are presented as mean ± SE
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics

Only in the case of *B. bifidum* was a significant increase in late apoptotic neutrophils observed after 48 h (Figure 6).

Effect of probiotics on neutrophil lysis

We detected a time-dependent but non-significant increase in LDH activity in porcine neutrophils cultivated with or without probiotics (Figure 7). In contrast to porcine neutrophils, human neutrophils exhibited significantly increased LDH activity after exposure to all probiotics after 24 h and 48 h of co-cultivation (Figure 8).

DISCUSSION

In this study, we detected apoptosis and necrosis (lysis) in porcine and human neutrophils after treatment with the probiotics *B. bifidum, L. rhamnosus* and *E. faecium*, which are essential constituents of the human and animal gastrointestinal microflora (Plaza-Diaz et al. 2014). Neutrophil granulocytes are inherently short-lived cells and undergo constitutive apoptosis during aging *in vitro* (Payne et al. 1994). In this study, we observed apoptosis in neutrophils cultivated with and without probiotics in a time-dependent manner. Under normal *in vivo* physiological conditions, however, apoptotic neu-

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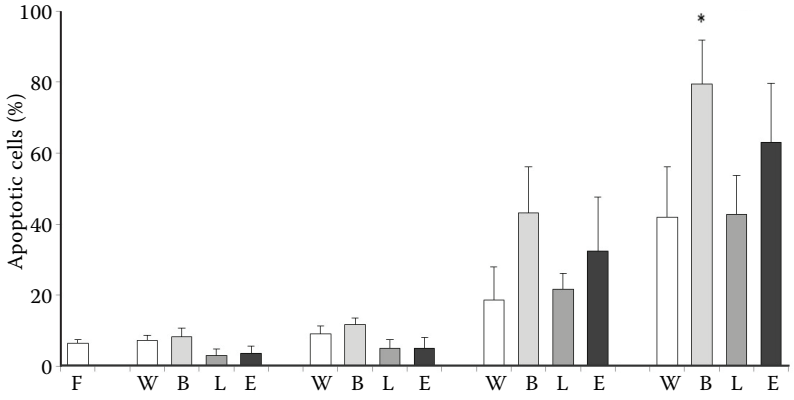

Figure 6. Late apoptosis of human neutrophils co-cultivated with probiotics for 2–48 h *in vitro.* Data are presented as mean ± SE
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics
*$P < 0.05$

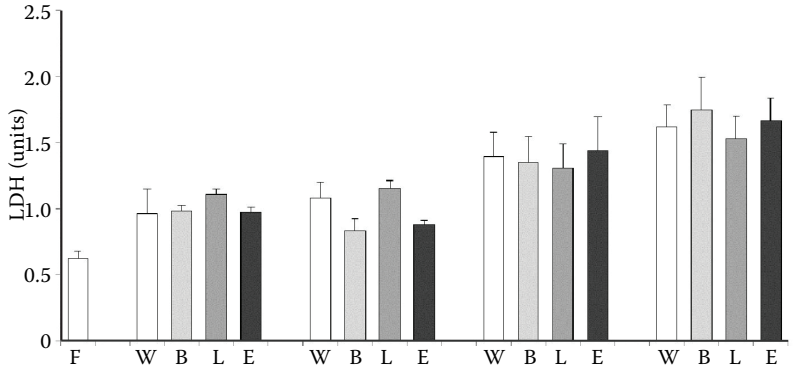

Figure 7. Lactate dehydrogenase activity of porcine neutrophils co-cultivated with probiotics *in vitro*. Data are presented as mean ± SE of units
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics

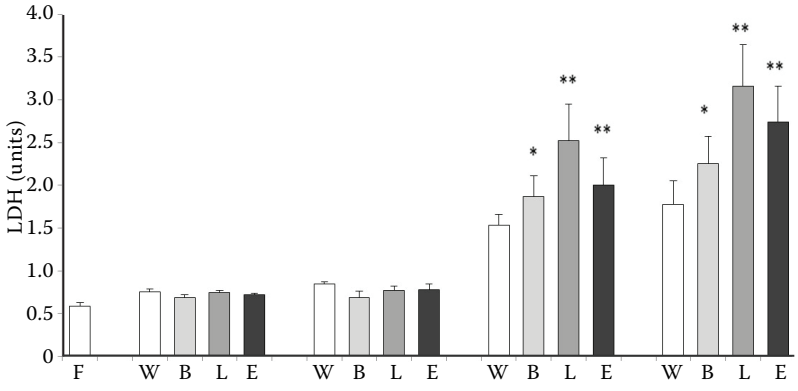

Figure 8. Lactate dehydrogenase activity of human neutrophils co-cultivated with probiotics *in vitro.* Data are presented as mean ± SE of units
B = neutrophils co-cultivated with *Bifidobacterium bifidum*, E = *Enterococcus faecium*, F = fresh population after dextran sedimentation without probiotics, L = *Lactobacillus rhamnosus*, W = neutrophils cultivated without probiotics
*$P < 0.05$, **$P < 0.01$

trophils are phagocytosed by macrophages. As both macrophages and efferocytosis are lacking *in vitro*, a loss of cell membrane integrity gradually occurs in apoptotic neutrophils and these cells become late apoptotic and/or secondary necrotic (Kelly et al. 2003). We therefore observed both early and late apoptotic neutrophils during cultivation *in vitro* with and without probiotics.

In the controls, apoptosis in porcine and human neutrophils ranged from 15% to 55% during cultivation. Similar as well as contrasting results have been reported in the literature. For example, Elyoussoufi et al. (2013) reported 5% and 10% apoptotic cells after 2 h and 4 h incubation, respectively, in cultivated human neutrophils. Sulowska et al. (2005) observed about 15–23% apoptotic neutrophils after 8 h of cultivation *in vitro*. In contrast, Payne et al. (1994) observed 50% apoptotic human neutrophils after 6 h and 90% apoptotic human neutrophils after 12 h of incubation under the same conditions as in this study. In porcine neutrophils, Matalova et al. (2003) observed 25% apoptotic cells after 24 h of cultivation. It is clear that human neutrophils undergo spontaneous apoptosis *in vitro* faster compared to porcine neutrophils.

All probiotics used in this study significantly increased the early apoptosis of porcine neutrophils. Moreover, *L. rhamnosus* increased late ap-

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optosis and *B. bifidum* increased LDH release of human neutrophils during co-cultivation. Chiu et al. (2010) described soluble bacterial factors secreted by *Lactobacillus casei rhamnosus* which induced apoptosis of blood monocytes and lymphocytes. Song et al. (2015) reported that membrane proteins (12 and 15 kDa fractions) from heat-inactivated *Lactobacilli* induced apoptosis in HT-29 cells. Dolgushin et al. (2008) demonstrated an apoptogenic effect of *Lactobacillus* ssp. and *Bifidobacterium ssp.* on neutrophils in the mucous membrane of the female genital tract. It has also been observed that probiotic *Enterococcus lactis IW5* induces apoptosis in HeLa cells (Nami et al. 2015). Similarly, the *Enterococcus faecalis* vaginal strain, which has probiotic properties, induces apoptosis in AGS, HeLa, MCF-7 and HT-29 human cancer cell lines (Nami et al. 2014).

Induction of apoptosis may be a comparably faster process in the cases of pathogenic bacteria. It has been shown that soluble factors from *S. aureus* may induce apoptosis of neutrophils after 60 min *in vitro* (Lundqvist-Gustafsson et al. 2001).

We observed the phagocytosis of probiotics by neutrophils in our study. The phagocytosis of some bacteria may delay or induce apoptosis in neutrophils (for more details, see DeLeo 2004). In this study, no effect of probiotics on neutrophil apoptosis was observed after 2 h or 4 h of co-cultivation. Such periods thus seem to be too short for probiotics to have an effect on neutrophil vitality. This suggests, therefore, that phagocytosis of probiotic bacteria may induce apoptosis only after longer periods of co-cultivation. By comparison, a significant induction of neutrophil apoptosis has been recorded after 18 h in pathogenic bacteria (*S. aureus*, *E. coli*) (Williams et al. 1996; Yamamoto et al. 2002).

In this study, different dynamics of early and late apoptosis and LDH release were observed in porcine versus human neutrophils. As compared to the control, a higher proportion of early apoptotic cells was observed in porcine neutrophils after 24 h and 48 h of co-cultivation with probiotics. In human neutrophils, we detected a lower proportion of early apoptotic cells after 48 h of co-culture with all probiotics. At the same time, however, late apoptotic neutrophils increased after co cultivation with all probiotics. The early apoptosis of neutrophils reached its peak after approximately 48–52 h of *in vitro* co-culture. On the contrary, late apoptosis increased up to 68–72 h (Hebert et al. 1996; Ren et al. 2001). Therefore, we observed a gradual increase in early apoptosis at 24 to 48 h in the control and in porcine neutrophils cultivated with probiotics. These early apoptotic neutrophils do not reach the peak and only a few of them become late apoptotic. Similarly, a low number of late apoptotic cells subsequently lyse and release LDH. A similar situation was observed in the human neutrophil control samples. On the contrary, for neutrophils co-cultured with probiotics early apoptosis increased up to 24 h and subsequently decreased after 48 h of cultivation compared to the control. Early apoptosis of human neutrophils culminated just after 24 h of cultivation with probiotics. Therefore, during subsequent cultivation it can be expected that the proportion of late apoptotic neutrophils and magnitude of LDH release are increased as was observed after 48 h of cultivation.

Differences in early apoptosis after cultivation of neutrophils with individual probiotics were observed in this study. In the available literature, the effect of probiotic bacteria on the early apoptosis of neutrophils is unknown. However, a lower proportion of early apoptotic porcine and human neutrophils can be observed in response to co-culture with *B. bifidum* in comparison to types of bacteria. For example, *B. bifidum* increases the expression of cyclooxygenase-2 (COX-2) (Khailova et al. 2010). COX-2 is involved in apoptosis regulation and suppresses neutrophil apoptosis (Jinzhou et al. 2008). On the other hand, *Lactobacillus* significantly increased COX-2 expression in intestinal cells (Otte et al. 2008) and this can lead to apoptosis.

It is evident that human neutrophils undergo spontaneous apoptosis during cultivation *in vitro* with and without probiotics more quickly than do porcine neutrophils, lose cell membrane integrity, and exhibit higher LDH activity. The reason for the different susceptibilities of human and porcine neutrophils is unknown. Differences between porcine and human mononuclear cells are described in the literature (Fairbairn et al. 2011), but there is no data describing differences between neutrophils. We assume that interspecies heterogeneity, distinct lifespans, different isolation conditions, different media may play a role.

In conclusion, we have demonstrated for the first time that the interaction of *B. bifidum*, *L. rhamnosus* and *E. faecium* with human and porcine neutrophils leads to their apoptosis. An increase in

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neutrophil apoptosis can be beneficial in terms of more efficient efferocytosis and faster resolution of inflammation and tissue regeneration. It must be stated, however, that transmigrated tissue neutrophils differ from the blood neutrophils used in this study, especially in terms of surface receptor expression, and they respond differently to certain stimuli (Bekkering and Torensma 2013). Therefore, a detailed study of probiotic interactions with tissue neutrophils is needed.

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