

# EFFECTS OF PROBIOTIC ON MORPHOLOGICAL CHANGES IN PORCINE MACROPHAGES DURING IN VITRO CULTIVATION

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*Abstract:* Nowadays, *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium* are frequently used probiotics in porcine nutrition. The probiotics-immunobiotics positively influence function of gastrointestinal tract and can also modulate function of immune system. The probiotics interact with immune cells as macrophages, neutrophils, dendritic cells or other immune cells and stimulate them to produce cytokines or to enhance phagocytosis. The aim of this study was to determine whether interactions of probiotics with porcine monocyte derived macrophages (MDMF) lead to structural changes of these cells during *in vitro* cultivation. We used the light microscopy and our findings suggest that probiotics affected the structural changes of porcine MDMF. Part of MDMF underwent apoptosis or necrosis and it was described the different stadia leading to the cell death. In some MDMF numerous vacuoles are accumulated in cytoplasm. The most pronounced structural changes of MDMF were caused by *Enterococcus faecium*. Finally, interactions of probiotics with MDMF were associated with phagocytosis all used probiotics.

*Key Words:* probiotics, porcine macrophages, apoptosis, necrosis, morphological changes

## INTRODUCTION

Enterococci, Lactobacilli and Bifidobacteria are an essential part of the human and animal gastrointestinal microflora (Pospíšková et al. 2013, Plaza-Diaz et al. 2014) and they are often used as a probiotics in a human and animals nutrition (Borchers et al. 2009). They compete for space and nutrients with potential pathogens (Vieira et al. 2013) and stimulate the differentiation and proliferation of epithelial cells (Duerr, Hornef 2012). They have an immunoregulatory capacities for the prevention and treatment of several gastrointestinal inflammatory disorders (Borchers et al. 2009). Galdeano and Perdigon (2004) shown that after oral feeding of probiotics-fluorescent-labeled lactobacilli were detected in immune cells in Payer's patches and the lamina propria in the small intestine and in immune cells in the crypt and lymph nodules in the colon. It is evident that the probiotic bacteria directly interact with immune cells (dendritic cells, lymphocytes, macrophages, neutrophils, etc.). The interactions of immune cells with probiotic bacteria may lead to phagocytosis. Sun et al. (2007) described that the macrophages may ingest lactobacilli in the strain dependent manner. In addition, some studies also describe effect of probiotics on cell death (Gröbner et al. 2010, Chiu et al. 2010). Question is: can interactions of probiotics with MDMF lead to the structural changes? These information are missing in available literature. Therefore, the aim of this study was described the structural changes in MDMF during short and long time *in vitro* cultivation with probiotics: *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Enterococcus faecium*.

## MATERIAL AND METHODS

### Animals, blood sampling and isolation of PBMC

Ten Large White pigs were used in this study. Four to six months old pigs were kept in the experimental stables of the Veterinary Research Institute, Brno, Czech Republic. Pigs were fed with standard diet. Fifteen mL of peripheral blood were collected from *vena cava cranialis* into sterile pyrogen-free tube containing 25 IU sodium heparin. 1 mL<sup>-1</sup> peripheral blood (Heparin forte Leciva, Zentiva, Czech Republic). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Histopaque 1077 density gradient (Sigma-Aldrich, USA) and re-suspended in complete D-MEM contained 10% normal porcine serum (PS, Gibco, USA) and 100 000 IU · L<sup>-1</sup> penicillin and 100 mg · L<sup>-1</sup> streptomycin (Sigma-Aldrich, USA).

### Cultivation of MDMF

MDMF were derived from PBMC by cultivation for 7 days in complete D-MEM. The cultivation was performed in 24-well plates ( $2 \cdot 10^6$  cells.mL<sup>-1</sup>/well, Tissue Culture Test Plate 24 Wells, TPP, Techno Plastic Products AG, Switzerland) at 37°C in 5% CO<sub>2</sub>. Non-adherent cells were removed after 24 h of cultivation by washing the wells with complete D-MEM with 10% normal porcine serum with penicillin/streptomycin and all subsequent cultivations were performed in this medium.

### *In vitro* cultivation of MDMF with probiotic

*Bifidobacterium bifidum* CCM 3762, *Lactobacillus rhamnosus* CCM 1828 and *Enterococcus faecium* NCIMB 11181 (M74) (all of them from Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) were chosen as probiotic strains. MDMF ( $2,5 \cdot 10^5$  /well) were cultivated *in vitro* with or without probiotics ( $6,25 \cdot 10^5$ ) for 4, 24 and 48 hours at 37°C in 5% CO<sub>2</sub>.

### The light microscopy

Slides were stained panoptically using the Pappenheim method (May-Grünwald-Giemsa stain). They were examined by light microscopy using oil immersion (Olympus IX51 with lens LCACH RC 40x/0.55). Slides were digitalized by camera (Olympus XC50) and cellSens Standard software. Structurally different types of MDMF were assessed by the enumeration of at least 200 cells/slide.

### Statistical analysis

The results were evaluated by Student's pair T-test. The significance of differences in the proportions of structurally different types of MDMF (between treatments and timepoints) during *in vitro* cultivation was tested by the Scheffe's method. *P* values were considered statistically significant if *P*<0.05 and *P*<0.01. The data were processed using STATISTICA 7.1 software (StatSoft CR Ltd, Prague, Czech Republic).

## RESULTS AND DISCUSSION

We are observed a structurally different types of MDMF during *in vitro* cultivation with probiotics. As it can see in Figure 1 (A), a general appearance of MDMF includes a typical morphological features of these cells. Cultivated MDMF possesses an oblong nucleus containing densely stained chromatin. Chromatin was often dispersed. Amount cytoplasm is higher than amount of nucleus. The cytoplasm is mostly without intracytoplasmatic vacuoles. The cells contain abundant pseudopodia on surface. Beside this, the vacuolized forms of these cells were observed. The vacuolized MDMF contain one or more intracellular vacuoles, mostly located around nucleus (Figure 1G) and at the cell periphery (Figure 1H). The cells cultivated with probiotics contain phagocytosed bacteria in their cytoplasm, in contrast to the control. There are tens of phagocytosed probiotics in one cell as it can see in Figure 1F. The fate of cultivated MDMF may be apoptosis or necrosis – lysis. Apoptosis of MDMF includes structurally different stadia: preapoptotic, karyopyknotic, zeiosis and apoptotic bodies. Preapoptotic forms of MDMF contains rounded cells with less pseudopodia and less dispersed nuclear chromatin (Figure 1B). Karyopyknosis was characterized a round shape of the cell without any pseudopodia in the surface. The nucleus is also rounded with non-dispersed, darkly stained nuclear chromatin (Figure 1C). Zeiosis of MDMF was noticeable by nuclear fragmentation into separate darkly stained fragments (Figure 1D). This form precedes the subsequent disintegration into a number

of apoptotic bodies (Figure 1E). These structural changes were characterized for apoptosis of the eukaryotic cells (Kerr et al. 1972). Necrosis – lysis was characterized by a loss of the plasma membrane integrity and disintegration of cell structures, included nucleus with chromatin (Figure 1I).

The *in vitro* interactions of probiotics with MDMF lead to the time dependent structural changes, as it shown in Table 1. The cultivation 48 hours was accompanied by a significant decrease of structurally normal cells ( $P<0.05$ ) and a higher incidence of apoptosis ( $P<0.05$ ) in the case of *Enterococcus faecium*, in contrast to *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and control. Moreover, proportion of vacuolized cells was significantly decreased between 4 hours and 48 hours cultivation in all probiotics and in control (without probiotics) ( $P<0.05$ ). Surprisingly, proportion of necrotic cells decreased during *in vitro* cultivation in all probiotics and in control (significantly in *Enterococcus faecium*,  $P<0.05$ ).

The decrease of necrotic cells after 48 hours cultivation is unexpected, but it can be explained by lysis of MDMF in medium during cultivation. We are used the colorimetric assay for the quantification of cell lysis which is based on the measurement of lactate dehydrogenase activity (LDH) released from the cytosol of damaged cells into the supernatant. The increased LDH concentration confirmed this claim (data not shown). Beside the structural changes, we are also detected phagocytosis of probiotics (Table 2). Normal cell types phagocytosed very frequently, in comparison to the apoptotic and necrotic cells. Moreover, the significant difference was observed in *Enterococcus faecium* in all timepoints relative to *Bifidobacterium bifidum* and *Lactobacillus rhamnosus*. The difference in phagocytosis of bacteria could be associated with adherence of macrophages and formation of their pseudopodia. Nagel et al. (1986) showed that decrease adherence is accompanied by decrease of phagocytosis. Because, this process is associated with ability to form pseudopods essential for phagocytosis (Johnson et al. 1986). Kausal and Kansal (2014) describe increased adherence of peritoneal macrophages to substrate during cultivation with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* and the increase of phagocytosis. This is in contrast to our results. The phagocytosed probiotics observed in apoptotic cells were phagocytosed before start of structural changes as a loss of pseudopods. If phagocytosis of probiotics can induce the apoptotic process and also associated with structural changes of MDMF, it can be object of further studies.

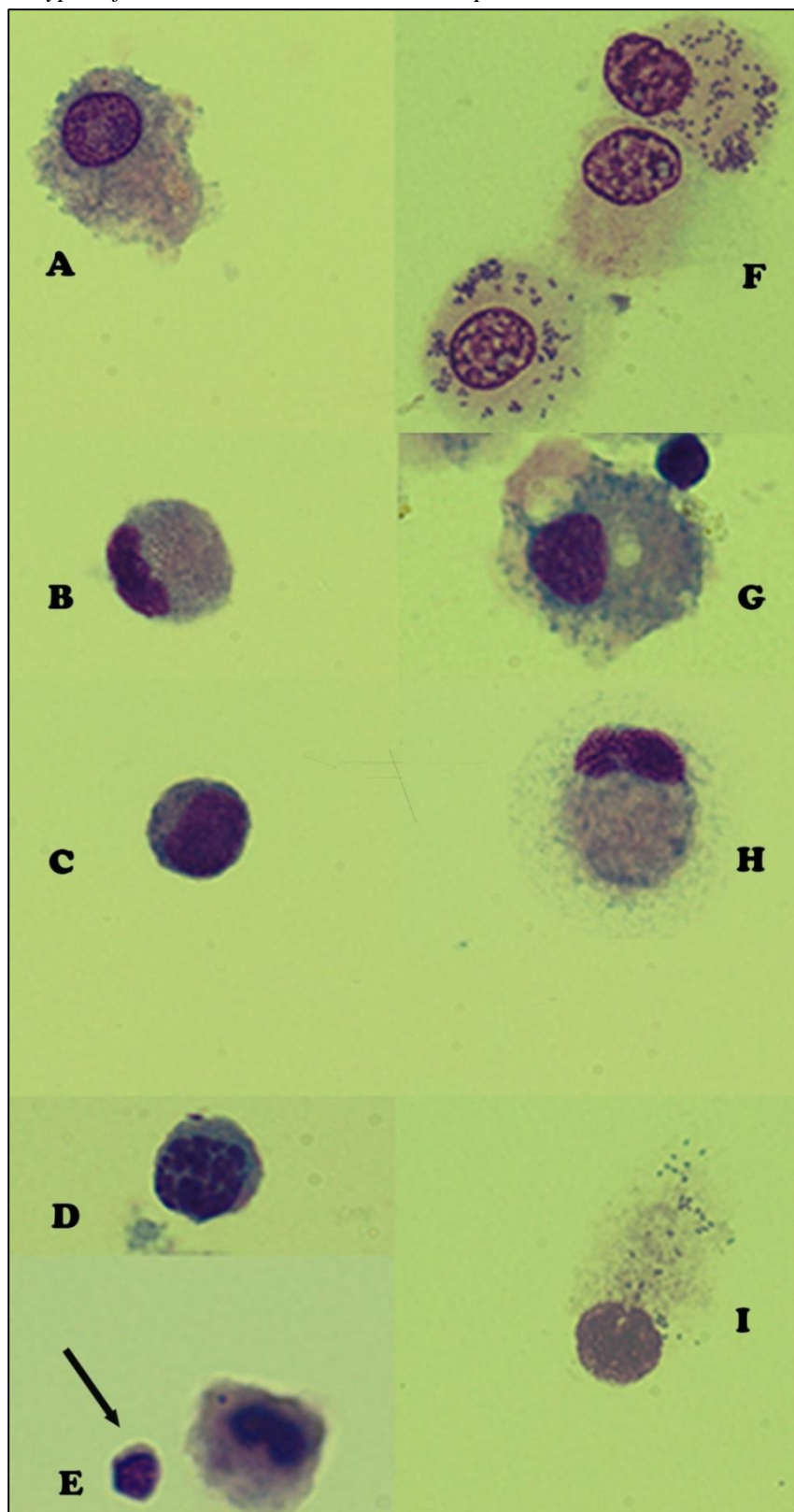
Table 1 Proportion of structural types of MDMF

Stimulation	without stimulation			<i>Bifidobacterium</i>			<i>Lactobacillus</i>			<i>Enterococcus</i>		
	4	24	48	4	24	48	4	24	48	4	24	48
Normal (%)	35.7	62.5	61.8	75.2	27.9	81.6	35.1	46.7	48.5	24.0	35.7	32.5
Preapoptotic (%)	10.7	0.0	20.3	3.1	27.9	5.3	17.9	27.6	18.1	4.8	14.3	20.0
Karyopyknotic (%)	9.2	0.0	9.5	10.1	17.4	6.0	7.5	8.9	12.3	10.3	3.6	27.5
Zeiosis (%)	0.0	0.0	1.2	0.0	2.3	2.6	2.3	1.8	3.0	0.0	0.0	2.5
Apoptotic body (%)	0.0	0.0	0.8	0.0	4.7	0.0	0.0	0.9	1.7	0.0	0.0	12.5
Start of vacuolization (%)	3.6	0.0	0.4	2.2	4.7	0.0	3.1	4.4	2.1	5.1	3.6	0.0
Vacuolized (%)	40.8	31.2	4.6	6.0	9.3	1.5	18.7	5.3	7.2	32.9	10.7	2.4
Necrotic-lysis (%)	0.0	6.3	1.2	3.4	5.8	3.0	15.4	4.5	7.2	23.0	32.1	2.6

Table 2 Proportion of fagocytizing MDMF

Stimulation	Without stimulation			<i>Bifidobacterium</i>			<i>Lactobacillus</i>			<i>Enterococcus</i>		
	4	24	48	4	24	48	4	24	48	4	24	48
Normal (%)	0.0	0.0	0.0	12.0	16.7	9.7	12.6	20.0	15.7	80.0	70.0	69.2
Apoptotic (%)	0.0	0.0	0.0	3.3	17.7	0.0	5.4	16.6	7.2	68.2	100.0	12.0
Necrotic (%)	0.0	0.0	0.0	4.9	5.9	100.0	13.8	25.0	12.8	64.0	30.8	0.0

Figure 1 Structural types of MDMF cultivated in vitro with probiotics



**Legend**

A: monocyte like MDMF, B: preapoptotic MDMF, C: karyopyknotic MDMF, D: zeiosis, E: apoptotic body, F: normal MDMF with phagocytosis of probiotic bacteria, G: start of vacuolization of MDMF, H: vacuolized MDMF.

## CONCLUSION

*Enterococcus faecium* caused the striking structural changes in porcine MDMF during *in vitro* cultivation. In the 4–48 hours cultivation was detected the lowest number of normal MDMF. Most cells exhibited features of apoptosis. As well as apoptosis, in case of phagocytosis, the highest percentage of phagocytosing cells were observed during cultivation with *Enterococcus faecium*.

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## REFERENCES

- Borchers A. T., Selmi C., Meyers F. J., Keen C. L., Gershwin M. E. 2009. Probiotics and immunity. *Journal of Gastroenterology*, 44(1): 26–46.
- Chiu Y. H., Hsieh Y. J., Liao K.W., Peng K.Ch. 2010. Preferential promotion of apoptosis of monocytes by *Lactobacillus casei rhamnosus* soluble factors. *Clinical nutrition*, 29(1): 131–140.
- Duerr C. U., Hornef M. W. 2012. The mammalian intestinal epithelium as integral player in the establishment and maintenance of host-microbial homeostasis. *Seminars in immunology*, 24(1): 25–35.
- Galdeano M. C., Predigón G. 2006. The Probiotic Bacterium *Lactobacillus casei* Induces Activation of the Gut Mucosal Immune System through Innate Immunity. *Clinical and Vaccine Immunology*, 13(2): 219–226.
- Gröbner S., Fritz E., Schoch F., Schaller M., Berger A. C., Bitzer M., Autenrieth I. B. 2010. Lysozyme activates *Enterococcus faecium* to induce necrotic cell death in macrophages. *Cellular and Molecular life sciences*, 67(19): 3331–3344.
- Johnson D. R., Fernandes G., Douglas S. D. 1978. Age related decline in cytoplasmic spreading of mouse peritoneal macrophages. *Developmental and comparative immunology*, 2(2): 347–354.
- Kaushal D., Kansal V. K. 2014. Dahi containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* improves phagocytic potential of macrophages in aged mice. *Journal of food science*, 51(6): 1147–1153.
- Kerr J. F., Wyllie A. H., Currie A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 2(4): 239–257.
- Nagel J. E., Han K., Coon P. J., Alder W. H., Bender B. S. 1986. Age differences in phagocytosis by polymorphonuclear leukocytes measured by flow cytometry. *Journal of leukocyte biology*, 39(4): 399–407.
- Plaza-Diaz J., Gomez-Llorente C., Fontana L., Gil A. 2014. Modulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver by probiotics. *World Journal of Gastroenterology*, 20(42): 15632–15649.
- Pospisková P., Zornikova G., Kolarova M., Sladek Z., Komprda T., Gersiova J. 2013. Effect of probiotics in the pig nutrition on the pathogenic bacteria counts in the gut. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 61(6): 1839–1843.
- Sun J. L., Le G. W., Hou L. X., Wang N. F., Chang G. F., Shi Y. H. 2007. Nonopsonic phagocytosis of *Lactobacilli* by mice Payer's patches' macrophages. *Asia Pacific journal of clinical nutrition*, 16(1): 204–207.
- Vieira A. T., Teixeira M. M., Martins F. S. 2013. The role of probiotics and prebiotics in inducing gut immunity. *Journal List, Frontiers in Immunology*, 4(445): 1–12.