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FLAX-SEED OIL AND *LACTOBACILLUS PLANTARUM* SUPPLEMENTATION MODULATE TLR AND NF- κ B GENE EXPRESSION IN ENTEROTOXIGENIC *ESCHERICHIA COLI* CHALLENGED GNOTOBIOTIC PIGS

Mária CHYTILOVÁ^{1,2}, Radomíra NEMCOVÁ¹, Soňa GANCARČIKOVÁ¹,
Dagmar MUDROŇOVÁ¹ and Ludmila TKÁČIKOVÁ^{1*}

¹Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice, Slovak Republic; ²Current address: Department of Experimental Medicine, Faculty of Medicine, P. J. Šafárik University in Košice, Košice, Slovak Republic

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The present study analyses the effect of flax-seed oil rich in n-3 polyunsaturated fatty acids (PUFAs), the probiotic strain *Lactobacillus plantarum* – Bio-cenol™ LP96 and their combination on the expression level of selected Toll-like receptor (TLR) genes (TLR2, TLR4, TLR5, TLR9) and their downstream molecules (myeloid differentiation factor 88, MyD88; nuclear factor- κ B, NF- κ B) in the jejunum of gnotobiotic pigs challenged with enterotoxigenic *Escherichia coli* (ETEC). The results show that both immunomodulators are able to modulate the RNA level of at least one of the target molecules and thus regulate pathogen-induced inflammation. We confirmed that not only probiotic lactobacilli or flax-seed oil alone but also their synergistic action has great potential in the prevention and treatment of porcine colibacillosis. The results give an insight into one of the possible mechanisms by which natural agents, such as probiotic lactobacilli and flax-seed oil, exert their immunoregulatory properties during pathogen-induced inflammation.

Key words: Flax-seed oil, lactobacilli, ETEC, gene expression, TLR, NF- κ B

Toll-like receptors (TLRs) represent a large family of evolutionarily conserved receptors, which play an important role in protection against invading microorganisms. After pathogen recognition, TLRs trigger signalling cascades leading to the activation of nuclear factor- κ B (NF- κ B), which in turn activates genes involved in the inflammatory response (Akira et al., 2006). In the context of an enterotoxigenic *Escherichia coli* (ETEC) infection, increased expression of several TLRs (TLR2, TLR3, TLR4, TLR5, TLR8) in porcine intestinal cells after bacterial or lipopolysaccharide (LPS) stimulation (Moue et al., 2008) has been reported. Similarly, altered expression of at least one TLR (TLR4, TLR5, TLR9)

*Corresponding author; E-mail: Ludmila.Tkacikova@uvlf.sk; Phone: 00421 (915) 984-603

has been observed in porcine blood mononuclear cells in response to LPS and other bacterial ligands (CpG, lipoteichoic acid, peptidoglycan) (Raymond and Wilkie, 2005).

Apart from playing a role in pathogen recognition, TLRs also react with structural components of health-promoting probiotic microorganisms that possess various immunomodulatory effects (Takeda et al., 1997; Kitazawa et al., 1998, 2001; Sato et al., 2004; Iliev et al., 2005). In intestinal dendritic cells, recognition of commensal-bacterial molecular patterns via TLRs (especially TLR2, TLR4, TLR9) leads to their maturation and to the release of cytokines, which coordinate the differentiation of naive T-helper cells (Th0) into a mature Th1, Th2 or Th3/Treg subpopulation (Rescigno et al., 2001; Rachmilewitz et al., 2004). Up-regulation of TLR gene expression and cytokine production, induced by probiotic lactobacilli (*Lactobacillus bulgaricus* and *L. gasseri*), has also been observed in immature intestinal tissue and gut-associated lymphoid tissue of pre-suckling newborn swine (Tohno et al., 2006). Unlike pathogens, probiotic bacteria do not cause inflammation, because they can regulate the immune response via a complex of mechanisms. This has been confirmed in human monocyte-like cells, where preventive treatment with genomic DNA of probiotic lactobacilli (*L. plantarum*), followed by LPS stimulation, led to the reduction of some TLRs, inhibition of NF- κ B and mitogen-activated protein kinase (MAPK) signalling pathways, and induction of TLR-negative regulators (Kim et al., 2012).

Similarly, substances like polyunsaturated fatty acids (PUFAs) are also able to affect TLR and NF- κ B levels (Lee et al., 2003a, 2003b; Weatherill et al., 2005; Lu et al., 2007). Furthermore, it has been demonstrated that PUFAs (especially n-3 PUFAs) can potentiate the immunomodulatory properties of probiotic lactobacilli via stimulation of bacterial adhesion to the intestinal wall (Ringø et al., 1998; Bomba et al., 2003; Nemcová et al., 2012). This mechanism is not fully understood yet; however, Kankaanpää et al. (2001) have found that n-3 PUFAs are able to modulate the composition of the gastrointestinal flora through their direct effect on the binding sites on epithelial cells. Based on this background and our previous results from the same experiment concerning cytokine immune response (Chytilová et al., 2013), we decided to analyse the effect of both immunomodulators (flaxseed oil rich in n-3 PUFAs and the probiotic strain of *L. plantarum* – Bioceno1TM LP96) alone and in combination on the gene expression level of selected TLRs (TLR2, TLR4, TLR5 and TLR9), their main adaptor molecule MyD88 and transcription factor NF- κ B in the jejunum of gnotobiotic pigs challenged with ETEC O8: K88ab: H9:ent⁻.

Materials and methods

The experiment with newborn gnotobiotic piglets was carried out at the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine

and Pharmacy, Košice, Slovakia. The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol (number 2931/09-221) and the animals were handled and sacrificed in accordance with the guidelines established by the ethics committee. The experiment was carried out on 18 gnotobiotic piglets of the Slovak White × Landrace breed. Gnotobiotic piglets were prepared and reared exactly as described in our previous study (Chytilová et al., 2013). The piglets did not receive colostrum and were fed autoclaved milk substitute (Sanolac Ferkel, Germany) six times daily, *ad libitum*.

Flax-seed oil and bacterial strains

Flax-seed oil (cultivar Flanders) was obtained and analysed by AGRITEC (Czech Republic) and it contained 45.78% of lipids in dry matter basis. The composition of lipids was as follows: palmitic acid (C16:0) – 5.08%; stearic acid (C18:0) – 3.67%; oleic acid (C18:1) – 18.39%; linoleic acid (C18:2) – 16.08%; linolenic acid (C18:3) – 56.78%.

The *L. plantarum* – Biocenol™ LP96 strain was isolated from the gut contents of healthy suckling piglets. The strain was characterised by strong adherence to epithelial cells from the porcine intestine, by inhibitory activity against *Escherichia coli* O8:K88ab:H9 *in vitro*, and production of hydrogen peroxide and organic acids (Nemcová et al., 1997). These properties as well as the significant immunomodulatory action of this strain were subsequently confirmed during *in vivo* experiments in pigs (Nemcová et al., 2012; Chytilová et al., 2013). The strain was grown in de Man, Rogosa and Sharpe broth (MRS; Merck, Germany) for 18 h at 37 °C. The optical density at 640 nm of the bacterial culture was adjusted to give approximately 1×10^9 colony forming units (CFU)/mL (Specol EK, Carl Zeiss, Jena, Germany).

The *E. coli* O8:K88ab:H9 strain, without enterotoxin production, was obtained from the Laboratory of Immunology and Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic. An overnight broth culture of *E. coli* (1 mL) was inoculated into 50 mL Trypticase soy broth (Oxoid Unipath Ltd., Basingstoke, UK) and cultivated at 37 °C in a water bath shaker (JULABO SW 2C, Labor Technik GMBH Selbach, Germany) for approximately 2 h to an optical density 0.5 at 640 nm (corresponding to 1×10^8 CFU/mL). Subsequently, the bacterial culture was diluted in isotonic saline to obtain a final concentration of 1×10^5 CFU/mL.

Experimental design

The newborn germ-free piglets were divided into four groups: K (control, n = 3), L (*Lactobacillus plantarum* – Biocenol™ LP 96, n = 5), MK (flax-seed oil, n = 5), and LMK (*L. plantarum* and flax-seed oil, n = 5). The piglets from the LMK and L groups were inoculated orally with 2 mL of *L. plantarum* (1×10^9 CFU/mL)

each day. In addition, flax-seed oil was administered to the animals of Groups LMK and MK once a day at a dose of 0.5 mL. At the age of five days, the piglets from all groups were challenged orally with 2 mL of *E. coli* O8:K88ab:H9:ent⁻ (1×10^5 CFU/mL). All piglets from all groups were sacrificed on day 9 (the 4th day after *E. coli* inoculation) by intracardiac euthanasia with 1 mL/kg b. w. T61[®] (Intervet International BV, Boxmeer, The Netherlands). The gastrointestinal tract was immediately removed and the cranial mid-part of the jejunum was used for further gene expression analysis.

Gene expression analysis

Intestinal tissues (40 mg) were homogenised and total RNA was isolated using PureZOL[™] (BioRad, USA). RNA quality and purity was determined, measuring absorption at 260/280 nm using a Nanodrop (NanoDrop Technologies, Inc., USA). Before cDNA synthesis, all RNA samples were treated with a TURBO DNA-free[™] Kit (Ambion). One µg of RNA was used to generate first-strand cDNA using RevertAid Premium Reverse Transcriptase (Fermentas Inc., Germany), with a blend of oligo(dT)₁₈ and random hexamer primers (Fermentas).

Table 1
Sequences of primers used in RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	References
TLR2	ACATGAAGATGATGTGGGCC	TAGGAGTCCTGCTCACTGTA	Moue et al. (2008)
TLR4	CTCTGCCTTCACTACAGAGA	CTGAGTCGTCTCCAGAAGAT	Moue et al. (2008)
TLR5	TTTCTGGCAATGGCTGGACA	TGGAGGTTGTCAAGTCCATG	Moue et al. (2008)
TLR9	GTGGAAGTGTGGGCATC	CACAGCACTGAGCTTTGT	Moue et al. (2008)
MYD88	TGAAGCAGCAGCAGGAGGCA	TCGCTGGGGCAGTAGCAGATGA	This study
NF-κB	GGAGCTGGTGGAGGCCCTGA	GCCTTGTGGAGGCAGGCGAG	This study
β-actin	CATCACCATCGGCAACGA	GCGTAGAGGTCCTTCTGATGT	Moue et al. (2008)

Primers for the amplification of TLR2, TLR4, TLR5, TLR9 and β-actin genes were designed according to Moue et al. (2008) (Table 1). Primers for the amplification of MYD88 and NF-κB genes were designed using the gene sequences of MYD88 (NM001099923.1) and NF-κB (NM001048232.1) from the GenBank database and using Primer-Blast software. RT-qPCRs were performed in an iCycler iQ (BioRad, USA) in 25 µL reaction volume containing 1 × iQ[™] SYBR Green Supermix (0.2 mM dNTP, 3 mM MgCl₂, BioRad), 6 µL nuclease-free water, 0.4 µM forward and reverse primer and 6 µL of cDNA. All reactions were performed in triplicates. The reaction conditions were as follow: 95 °C 3 min, 40 cycles [94 °C 15 s, 58 °C (for TLRs) or 60 °C (for MYD88 and NF-κB) 30 s, 72 °C 30 s], 72 °C 15 min followed by melting curve analysis to confirm ampli-

fication of a specific product. β -actin was used as a housekeeping gene for internal control. Each assay included a negative control without cDNA template. Relative expression levels of target and β -actin mRNA were evaluated by the $2^{\Delta\Delta CT}$ method.

Statistical analysis

Data were evaluated with GraphPadPrism version 3.00 by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test.

Results

In this study we examined if the application of flax-seed oil rich in n-3 PUFAs and the probiotic strain *L. plantarum* alone or in combination alters gene expression of TLRs and their downstream molecules in ETEC-challenged gnotobiotic pigs. Based on our previous microbiological observations from the same experimental conditions (Nemcová et al., 2012), confirming that combined treatment had the most significant effects in the jejunum, we used samples from the same part of the intestine for the gene expression analysis.

In our study, RT-qPCR showed that the preventive administration of *L. plantarum* (L, 0.11 ± 0.02) and its combination with flax-seed oil (LMK, 0.22 ± 0.03) to gnotobiotic pigs followed by ETEC infection led to significant down-regulation of the TLR2 mRNA level compared to the control group (K, 1.02 ± 0.13) and the group fed with flax-seed oil alone (MK, 0.96 ± 0.05) ($P < 0.001$) (Fig. 1a). On the other hand, piglets in the MK group had a significantly higher amount of jejunal TLR4 mRNA (1.54 ± 0.23) compared with the control (1.01 ± 0.12) and piglets from the other experimental groups (L, 1.03 ± 0.10 ; LMK, 0.88 ± 0.06) ($P < 0.001$) (Fig. 1b). However, simultaneous administration of both immunomodulators (LMK group) resulted in the decrease of TLR4 transcript to below the basal level (LMK, 0.88 ± 0.06), although this was not statistically significant. Similar results were found in the case of TLR9, where flax-seed oil supplemented to the diet increased the amount of this transcript in the jejunum of gnotobiotic pigs (1.43 ± 0.28) (MK vs. K, $P < 0.05$; MK vs. L and LMK, $P < 0.001$) (Fig. 1c). In the LMK group we found a slight down-regulation of TLR9 (0.81 ± 0.11) and a similar result was obtained in pigs fed with *L. plantarum* (0.87 ± 0.13), although without statistical significance in both groups. In the case of TLR5, all three types of preventive administration resulted in a significant down-regulation of this transcript in comparison with the control piglets (L, 0.78 ± 0.11 ; L vs. K, $P < 0.05$), (MK, 0.84 ± 0.11 ; MK vs. K, $P < 0.05$), with a maximum effect in the group given the combined treatment (LMK, 0.53 ± 0.08 ; LMK vs. K, $P < 0.001$; LMK vs. L, $P < 0.05$; LMK vs. MK, $P < 0.01$) (Fig. 1d). Furthermore, we found a significant suppression of MyD88 gene expression in the group

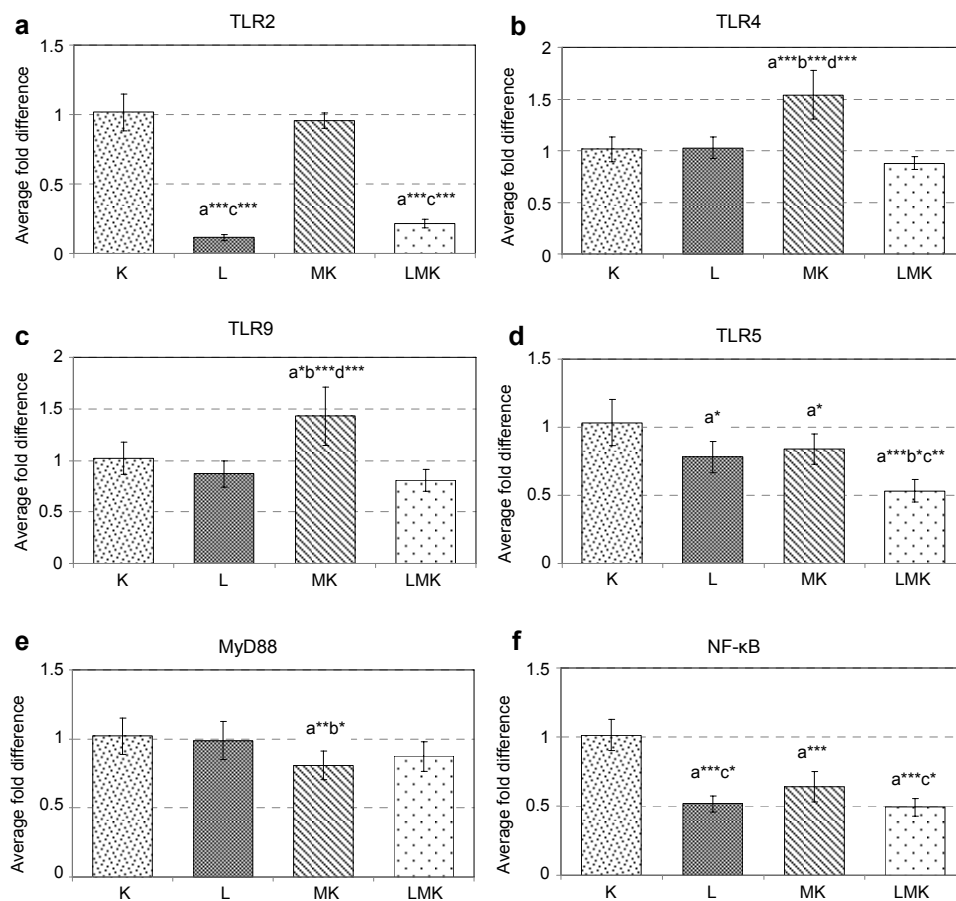


Fig. 1. TLRs (a–d), MyD88 (e) and NF-κB (f) mRNA expression levels normalised to β-actin (Average fold difference) in ETEC O8:K88ab:H9:ent⁻ challenged pigs (n = 5) pretreated with *L. plantarum* BiocenolTM-LP96 (L), flax-seed oil rich in n-3 PUFAs (MK) or their combination (LMK). K – control, L – *L. plantarum*, MK – flax-seed oil, LMK – *L. plantarum* + flax-seed oil, ^a – significantly different from Group K, ^b – significantly different from Group L, ^c – significantly different from Group MK, ^d – significantly different from Group LMK, * – P < 0.05, ** – P < 0.01, *** – P < 0.001

with flax-seed oil pre-treatment (0.81 ± 0.10 ; MK vs. K, $P < 0.01$; MK vs. L, $P < 0.05$). This tendency was also found in the L (0.99 ± 0.14) and LMK groups (0.87 ± 0.11) (Fig. 1e). In the case of transcription factor NF-κB, the most significant decrease of this transcript was observed in the jejunum of pigs fed a diet supplemented with *L. plantarum* (0.52 ± 0.06), and also in the group simultaneously administered both tested immunomodulators (0.49 ± 0.06) (L and LMK vs. K, $P < 0.001$; L and LMK vs. MK, $P < 0.05$) (Fig. 1f).

Discussion

TLRs play an important role in the defence against invading microorganisms. After pathogen recognition, TLRs trigger signalling cascades leading to the activation of NF- κ B, which in turn activates genes involved in the inflammatory response (Akira et al., 2006). In the present study we demonstrated that the application of *L. plantarum*, flax-seed oil and their combination alters the RNA level of at least one TLR, their main adaptor molecule MyD88 and transcription factor NF- κ B in the jejunum of gnotobiotic pigs, where the most important pathophysiological changes in ETEC infections take place.

In this study, RT-qPCR showed that the administration of *L. plantarum* to gnotobiotic pigs followed by ETEC infection resulted in a significant down-regulation of TLR2, TLR5 and NF- κ B. In addition, the same group (L) manifested a similar trend in the case of TLR9. Currently, only a few studies are known which deal with the immunomodulatory effect of probiotic lactobacilli at the TLR level during porcine colibacillosis. There is an *in vitro* study by Kim et al. (2012), where the pre-treatment of THP-1 cells with *L. plantarum* gDNA and their subsequent stimulation with LPS resulted in suppression of TLR2, TLR4, TLR9 and NF- κ B transcripts. On the other hand, in their *in vivo* study Li et al. (2012) showed that the preventive administration of *L. rhamnosus* ACTT 7469 to weanlings for one week followed by ETEC infection led to a significant up-regulation of TLR2 and to a statistically not significant reduction of TLR4 and TLR5 mRNA in the jejunum of the pigs treated. However, differences between the results of each experimental study are due to various factors such as the type of experimental scheme (*in vitro* or *in vivo*), the type and concentration of the probiotic strain applied, and the developmental level of pigs (sucklings/weanlings) in the case of *in vivo* studies.

Furthermore, unlike the L group in our study we also found a significant decrease of the MyD88 mRNA level in pigs after flax-seed oil stimulation, despite the fact that the expression of this adaptor protein is lower in the gut of newborn swine than in other tissues (Tohno et al., 2007). On the other hand, preventive administration of flax-seed oil led to the increase of TLR4 and TLR9 gene expression. The balance between saturated and unsaturated fatty acids, which can be altered by the types of dietary fat consumed, can also affect the induction of TLR-mediated immune response (Weatherill et al., 2005). Several studies have demonstrated that saturated fatty acids activate TLR2 and TLR4, whereas unsaturated fatty acids inhibit the agonist-induced activation of TLRs (Lee et al., 2001, 2003a, 2004; Weatherill et al., 2005). We suppose that the increased expression of TLR4 detected in our study on gnotobiotic pigs (MK) may be affected by saturated fatty acids, which were also present in flax-seed oil. The immunomodulatory effect may also be influenced by other components in the flax-seed oil, e.g. by phytosterols. Brüll et al. (2010) demonstrated TLR2 activa-

tion in human peripheral blood mononuclear cells by plant stanols and plant sterols. On the other hand, the same authors did not confirm the activation of TLR4. These discrepancies could be due to different experimental conditions (*in vivo/ex vivo* study), different cell types (intestinal cells/blood cells) and/or the different form of tested substances (flax-seed oil/pure stanols and sterols). However, in our study, flax-seed oil alone (MK) and particularly its combination with *L. plantarum* (LMK) administered to gnotobiotic pigs challenged with ETEC resulted in a significant decrease of TLR5 and NF- κ B mRNA level. In addition, in the LMK group we found a similar trend in the case of TLR4 and TLR9 transcripts.

Even though there are several studies focusing on the effect of probiotic lactobacilli on TLR levels during porcine colibacillosis (Kim et al., 2012; Li et al., 2012), this area of the study is not sufficiently covered, and in the case of combined treatment with n-3 PUFAs studies are fully absent. However, the results presented here are in correlation with cytokine gene expression analysis from a previous experiment (Chytilová et al., 2013), for example: down-regulation of TLR2, TLR5 and NF- κ B, and decrease of pro-inflammatory cytokine mRNA (IL-1 α , IL-8) found in the L and LMK groups; increase of TLR4 transcript, induced by flax-seed oil, and up-regulation of both inflammatory mediators (IL-1 α , IL-8) detected in the jejunum of the same piglets. Moreover, significant results detected in the LMK group are consistent with the results of previous bacteriological analysis on the same piglets (Nemcová et al., 2012), which confirmed that supplementation of flax-seed oil rich in n-3 PUFAs resulted in significantly higher numbers of *L. plantarum* and lower numbers of ETEC adhered to the jejunal mucosa.

In conclusion, we confirmed that the application of *L. plantarum* and flax-seed oil rich in n-3 PUFAs can modulate mRNA levels of some TLRs, their main adaptor molecule MyD88 and transcription factor NF- κ B in various ways, and thus regulate pathogen-induced inflammation in the jejunum of gnotobiotic pigs. These results clearly confirm that not only immunomodulators alone (*L. plantarum* or flax-seed oil monotherapy) but also their synergistic action has a great potential in the prevention and treatment of porcine colibacillosis. Since only few studies on similar subjects have been published, these results can contribute to our understanding of the molecular mechanisms underlying the mode of action of these immunomodulators.

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