Effects of plant extract and natural substance food additives on stress and immune response in weaning piglets

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SUMMARY

Physiological effects of \textit{Chlorella vulgaris}, alginate, two mixtures of essential oils and inulin on immune and stress response were investigated in piglets fed a diet with the respective supplement from weaning at day 28. The experiment was performed at an experimental (EF) and a commercial farm (CF). At day 39 transcript levels of molecular expression markers for immune response (Toll-like receptor 4, \textit{TLR4}, inducible nitric oxide synthase, \textit{iNOS}), oxidative stress response (glutathione S-transferase A1, \textit{GSTA1}), cellular stress response (heat shock protein 70.1, \textit{HSP70.1}) and for general translation activity (eukaryotic release factor 1, \textit{eRF1}) in ileal mucosa and ileocolic lymph nodes were measured by quantitative RT-PCR. In addition, expression of \textit{eRF1} in liver was analysed. Down-regulation of \textit{GSTA1} and up-regulation of \textit{eRF1} in ileal mucosa from inulin fed piglets suggested an increased metabolic activity and reduced oxidative stress response. Farm had strong effect on expression level of the studied genes ($P<0.001$). Up-regulated \textit{TLR4} and \textit{iNOS} in ileocolic lymph nodes of piglets kept at CF indicated an elevated immune response of the animals in comparison with the EF. Moreover, up-regulated \textit{GSTA1} and \textit{eRF1} in ileal mucosa suggested an increased metabolic activity associated with increased oxidative stress response.

Keywords: plant extracts, natural substances, food additives, real-time RT-PCR, gene expression, stress response, swine

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INTRODUCTION

Nutritional factors during early development have important effects on growth, body composition and body functions (e.g., Koletzko et al. 1998). In this context, nutritional factors may account for variable stress response, which may be more or less effective at counteracting e.g. oxidative damage. A redox imbalance attributable to an overproduction of reactive oxygen species in relation to the capacity of protective defense mechanisms of cells are thought to be associated to the development and progression of neurodegenerative, as well as cardiovascular disease and cancer (Klatt and Lamas 2000; Go et al. 2003; Abrescia and Golino 2005). Therefore, “novel” dietary bioactive molecules have to be proven for their stress-associated physiological side effects. Here, we describe effect of five plant extracts and natural substances (PENS; *Chlorella vulgaris* powder, Na-alginate, two mixtures of essential oils and inulin) on metabolic activity and immune and stress response in ileum and liver of weaning piglets using molecular expression markers.

The used PENS (*Chlorella vulgaris* powder, Na-alginate, two mixtures of essential oils and inulin) are known for their potential to modify microbial gut population and to have strong antibacterial effects (Janczyk et al. 2009a, b; Lallés et al. 2009). Inulin is an α-D-glucopyranoside-[β-Dfructofuranosyl]_{n-1}-β-D fructofuranoside (G\_F\_n) or a β-D-fructopyranosyl-[β-D-fructofuranosyl]_{n-1}-β-D-fructofuranoside and the main natural reservoir are chicory and topinambur (*Helianthus tuberosum*). Dietary administration of inulin resulted in increase of faecal lactobacilli and bifidobacteria and in decrease of clostridia, Gram-positive cocci and *Bacteroides* (Mitsouka et al. 1987; Kleessen et al. 1997; Harmsen et al. 2002). Na-alginate is isolated from number of brown seaweed species. Feeding humans and rats high levels of alginate has been shown to reduce the bioavailability of β-carotene (Riedl et al. 1999) and minerals (Harmuth-Hoene et al. 1980; Bosscher et al. 2001). Alginate is considered to influence digestibility and availability of nutrients from the diet (Terada et al. 1995; Bach Knudsen 2001, Drochner et al. 2004). For the unicellular microalgae *Chlorella vulgaris* immune-modulating and anti-cancer properties have been reported (Justo et al. 2001; Konishi et al. 1985; 1990; 1996; Morimoto et al. 1995; Noda et al. 1996; Singh et al. 1999; Tanaka et al. 1984; 1986; Yusukawa et al. 1996). Essential oils are volatile, aromatic mixtures, mainly consisting of terpenes and phenylpropane derivatives. They are present in many parts of plants (e.g., leaves), where they protect the plant against bacteria and parasites. Many essential oils have strong antibacterial effects *in vitro* (Burt 2004). Active components of essential oils are known to have antioxidative (Grassmann et al. 2001) and anti-inflammatory effects (Peana et al. 2002; Santos and Rao 2000). Plant extracts affected the microbiota in the digestive tract of early-weaned piglets by increasing the number of lactobacilli and the ratio of lactobacilli and enterobacteria in the jejunum and cecum (Manzanilla et al. 2004; Castillo et al. 2006) and they had positive effects on digestibility of nutrients and growth performance (Stoni et al. 2006; Cho et al. 2006). In the
present study, limonene, eugenol, and pinene were chosen to be combined in a feed additive for weaned piglets. These substances are well known for their antibacterial properties (Filipowicz et al. 2003; Friedman et al. 2002). They were also selected because of known antioxidative (Grassmann et al. 2001), and anti-inflammatory (Kim et al. 2003), but also for their immuno-modulatory (Raphael and Kuttan 2003) and relaxant and spasmyloytic effects (Camara et al. 2003; Reiter and Brandt 1985). All of these effects might be especially beneficial to piglets during the stressful weaning period.

The PENS were supplemented to the starter diet in concentrations according to supplier’s recommendations. To study potential stress-associated physiological side effects of the selected PENS the transcript levels of expression markers were analyzed by real time RT-PCR of RNA isolated from liver, ileocolic lymph nodes and ileal mucosa under consideration of their physiological function in nutrient absorption, immune response and energy metabolism. The experiment was performed at an experimental (EF) and a commercial farm (CF) in order to investigate possible effects of different sanitary regimes. As marker genes we selected genes of metabolic pathways involved in animal response to biotic or external (stressful) stimuli, representing indicators for their stressful effects, because of an elevated stimulus-associated expression. As molecular expression markers we chose the inducible nitric oxide synthase (iNOS) and the Toll-like receptor 4 (TLR4) for immune response; the glutathione S-transferase A1 (GSTA1) for oxidative stress response; and the heat shock protein 70.1 (HSP70.1) for cellular stress response. As a general expression marker for translation activity we used the eukaryotic release factor 1 (eRF1), an ubiquitously expressed translation termination factor.

**MATERIAL AND METHODS**

**Animals, diets, PENS and experimental design**

The experiment was performed using a total of 96 male weaning piglets of German Landrace. Each 48 piglets were weaned at the CF and at the EF at 28 days of age (8.6 ± 0.69 and 6.5 ± 0.35 kg body weight (BW), respectively). At each farm, 8 piglets were housed in a pen as a group. Commencing at weaning piglets were offered a barley-wheat based starter diet (reference diet, RF, Janczyk et al. 2009b) or the RF supplemented with *Chlorella vulgaris* powder (RF+CV), Na-alginate (RF+A), one of the two mixtures of essential oils (RF+EO1 and RF+EO2), and inulin (RF+I), respectively. The experimental diets were formulated to provide 12.5 g/kg lysine and 10.0 MJ/kg energy.

The PENS were supplemented to the starter diet according to supplier’s recommendations in the following concentrations: 0.1% of Na-alginate (Bio-algeen®, Schulze&Hermsen GmbH, Dahlenburg, Germany); 1.0% of *Chlorella vulgaris* as a bullet-milled micro algae powder (IGV GmbH, Nuthetal/Bergholz-
Rehbrücke, Germany; Janczyk et al. 2009a). The essential oil mixture 1 contained 25 g limonene, 5 g eugenol and 12 g pinene per kg product on organic and inorganic carrier (provided by Delacon Biotechnik GmbH; Austria). The mixture 2 contained the same oils but closed in microcapsules. Each mixture was added to RF at level of 0.04%. Finally, inulin (Raftiline® HP, Orafti S.A., Oreye, Belgium) was used in a concentration of 1.5% as similar concentration has been reported to have an effect on porcine gastrointestinal microbiota and short chain fatty acids (Branner et al. 2004; Loh et al. 2006).

The piglets were fed the respective diets twice daily, at 8.00 am and 4.00 pm in amounts allowing ad libitum feed intake for 11 days. Water was supplied via automatic nipples ad libitum.

For the purposes of the gene expression study, four pigs from each group (pen) were randomly chosen and sacrificed at age of 39 days. In order to insure no effects of direct nutrient intake the feed was withdrawn 16 to 18 h before sampling. The piglets were transferred from each farm to the preparation building within 15 minutes. Pigs were anesthetised with intra muscular injection of azaperone (1 mg/kg, Stresnil®, Janssen-Cilag GmbH, Neuss, Germany) and ketamine (300mg/kg, Ursotamin®, Serumwerke Bernburg, Bernburg, Germany) and finally euthanized by intracardial injection of T61® (Intervet, Unterschleißheim, Germany). Ileum, ileocolic lymph nodes and caudal lobe of liver were dissected. Ileum was flushed with sterile 0.9% saline and mucosa was collected. All samples were immediately frozen in liquid nitrogen and stored at –80 °C until analysis of mRNA abundance of the selected expression markers was performed.

The study protocol was approved by the Animal Care and Use Committee of the Ministry of Nutrition, Agriculture, Forestry, and Fishery of the State Mecklenburg Vorpommern, Schwerin, State Mecklenburg-Vorpommern, Germany (permission no. VI 522a-7221.31-1-018/99).

RNA extraction
After homogenization in Trizol® reagent the total RNA was extracted from tissue samples using RNeasy Easy Kit® (QIAGEN, Hilden, Germany) according to manufacturers´ protocol. The quality and quantity of RNA samples were assessed by measuring the optical density of each sample at 260 and 280 nm on NanoDrop™ (NanoDrop Technologies, Wilmington, DE, USA) and by agarose electrophoresis. Gene expression analyses were carried out using representative group specific RNA pools from liver and intestine. RNA pools of each tissue and group were made up of equal amounts of RNA aliquots of the individual undiluted RNA samples.

Real-time RT-PCR
Synthesis of first strand cDNA was performed with MMLV-RT (Promega, Madison, USA) and Poly-T primers using 2µg pooled total RNA of the corresponding group. Quantitative analysis of PCR products was carried out in
the LightCycler instrument (Roche, Mannheim, Germany) according to optimized PCR protocols essentially as described by Schwerin et al. (2002). Gene specific primers, annealing temperature and fluorescence acquisition temperature are given in Table I. For all the assays a standard curve was generated using DNA standard dilutions ($10^1$, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ copies) of the corresponding PCR product. Fluorescence signals recorded on-line during amplification were subsequently analyzed using the “Second Derivative Maximum” method of the LightCycler Data Analysis software (Roche, Mannheim, Germany). The copy number of the housekeeping gene GAPDH was measured to normalize for equal RNA amounts. The mRNA abundance was analyzed in triplicate.

Statistical analysis
Data was given in copy number/10ng total RNA. The relative transcript level was calculated by relating the data to the mean signal of the analogous control (reference diet) group. Relative transcript levels were compared applying t-test using the GLM procedure of SAS (SAS 2002).

RESULTS
Effect of PENS supplemented starter diet on immune and stress response associated gene expression in ileum mucosa of weaning piglets
In Figures 1A (EF) and 1B (CF) relative ileal transcript levels of the cellular stress, oxidative stress and immune response markers investigated in this study (HSP70.1, GSTA1 and iNOS, respectively) and of translation activity marker (eRF1) of weaning piglets fed PENS supplemented starter diet between living days 28 and 39 are shown in relation to piglets fed reference diet. The mRNA abundance of the corresponding control group fed the reference diet was set to 100 %.

Considering expression of the oxidative stress marker GSTA1 only piglets fed RF+I had significantly lower transcript level of this gene in comparison with piglets fed the RF. Feeding of inulin reduced the GSTA1 transcript level down to 35 % at EF ($P<0.037$) and down to 76 % at CF ($P < 0.107$) compared to the corresponding control group. At CF, inulin-affected down-regulation of GSTA1 coincided with up-regulation of the translation activity marker eRF1 in ileal mucosa in comparison with piglets fed RF (128 %, $P<0.017$). In addition, we recorded significantly higher mRNA abundance of iNOS in ileal mucosa from piglets fed RF+A at EF (236 %, $P<0.022$) and RF+EO1 at CF (177%, $P<0.044$). The higher iNOS transcript level in ileal mucosa from piglets fed RF+A at EF coincided with significantly higher iNOS transcript level in ileocolic lymph node (146 %, $P<0.003$). Transcript level of all other expression markers did not show any significant differences in ileocolic lymph nodes (iNOS, TLR4, HSP70.1) and liver (eRF1) between piglets fed RF+A, RF+EO1, RF+EO2 and RF+CV, and RF, neither at EF nor at CF (Figures 2A and 2B).
In ileocolic lymph nodes GSTA1 was expressed only at a very low level (less than 100 copies per 10 ng total RNA) and therefore it was not involved in the final analysis.

**Figure 1.** Mean relative mRNA abundance of cellular stress response (HSP70.1), oxidative stress response (GSTA1), immune response (iNOS) and translation activity markers (eRF1) in ileal mucosa of piglets (age 39 days, each n = 4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), *Chlorella vulgaris* powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d. Piglets were kept at experimental farm. Transcript levels are shown in relation to piglets fed RF. The mRNA abundance of this control group was set 100%. Real time RT-PCR was carried out in the LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. *P* value represents result of corresponding t-test in comparison with the RF.

Abbreviations: GSTA1, glutathione S-transferase; iNOS, inducible nitric oxide synthase; eRF1, eukaryotic release factor 1; HSP70.1, heat shock protein 70.1.

**Effect of farms (experimental vs. commercial farm) on immune and stress response associated gene expression in ileum of weaning piglets**

Figure 3A presents transcript levels of the cellular stress, oxidative stress, immune response (HSP70.1, GSTA1, iNOS) and translation activity marker genes (eRF1) in ileal mucosa of weaning piglets fed different PENS and kept at the two different farms. The EF was assumed to possess higher hygienic conditions than the CF (Janczyk et al. 2009b). Weaning piglets kept at CF had up to four times higher GSTA1 transcript levels in ileal mucosa in all groups except for the RF+A in comparison with EF (RF - 1.79x, RF+EO1 - 2.9x, RF+EO2 - 4.0x, RF+CV - 2.69x, RF+I - 3.91x). This corresponded to the eRF1
mRNA abundance in ileal mucosa, which was higher in all feeding groups at CF in comparison to EF. However, transcript levels of *iNOS* and *HSP70.1* in ileal mucosa did not differ significantly between the corresponding groups at both farms. In contrast, mRNA abundance of both genes and of the immune response marker *TLR4* was significantly higher in ileocolic lymph nodes from all groups at CF in comparison with EF (Figure 3B). In addition, hepatic expression level of *eRF1* was higher in all groups at CF in comparison to EF except for the RF+I.

Figure 2. Mean relative mRNA abundance of cellular stress response (*HSP70.1*), oxidative stress response (*GSTA1*), immune response (*iNOS*) and translation activity markers (*eRF1*) in ileal mucosa of piglets (age 39 days, each n=4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), *Chlorella vulgaris* powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d. Piglets were kept at commercial farm. Transcript levels are shown in relation to piglets fed RF. The mRNA abundance of this control group was set 100%. Real time RT-PCR was carried out in the LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. *P* value represents result of corresponding t-test in comparison with the RF. Abbreviations: *GSTA1*, glutathione S-transferase; *iNOS*, inducible nitric oxide synthase; *eRF1*, eukaryotic release factor 1; *HSP70.1*, heat shock protein 70.1
Figure 3. Mean relative mRNA abundance of cellular stress response (HSP70.1) and immune response marker (iNOS, TLR4) in ileocolic lymph nodes and translation activity marker (eRF1) in liver of piglets (age 39 days, each n=4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), Chlorella vulgaris powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d. Piglets were kept at experimental farm. Transcript levels are shown in relation to piglets fed RF. The mRNA abundance of this control group was set 100%. Real time RT-PCR was carried out in the LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. P value represents result of corresponding t-test in comparison with the RF. Abbreviations: iNOS, inducible nitric oxide synthase; eRF1, eukaryotic release factor 1; HSP70.1, heat shock protein 70.1; TLR4, Toll-like receptor 4.

DISCUSSION

In the present study we investigated physiological effects of feeding five plant extracts and natural substances (PENS) on immune and stress response in weaning piglets. The PENS chosen for the study were Chlorella vulgaris powder, Na-alginate, two mixtures of essential oils and inulin. They are all known to modify microbial gut population and/or to have strong antibacterial effects (e.g., Justo et al. 2001; Drochner et al. 2004; Harmsen et al. 2002; Castillo et al. 2006). Here, their effects on expression of marker genes were studied. Transcript levels of molecular expression markers for immune response (inducible nitric oxide synthase, iNOS), oxidative stress response (glutathione S-transferase A1, GSTA1), cellular stress response (heat shock protein 70.1,
HSP70.1) and for general translation activity (eukaryotic release factor F1, eRF1) were measured by real-time RT-PCR in ileal mucosa collected after 11 days of feeding the corresponding PENS. In addition, expression of iNOS, HSP70.1 and Toll-like receptor 4 (TLR4) involved in immune response was analyzed in ileocolic lymph nodes, and transcript level of eRF1 was studied in liver.

Figure 4. Mean relative mRNA abundance of cellular stress response (HSP70.1) and immune response marker (iNOS, TLR4) in ileocolic lymph nodes and translation activity marker (eRF1) in liver of piglets (age 39 days, each n = 4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), Chlorella vulgaris powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d. Piglets were kept at commercial farm. The mRNA abundance of this control group was set 100 %. Real time RT-PCR was carried out in the LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. P value represents result of corresponding t-test in comparison with the RF. Abbreviations: iNOS, inductible nitric oxide synthase; eRF1, eukaryotic release factor 1; HSP70.1, heat shock protein 70.1; TLR4, Toll-like receptor 4.

The eukaryotic release factor 1 terminates protein biosynthesis by recognizing stop codons at the A site of the ribosome and stimulating peptidyl-tRNA bond hydrolysis at the peptidyl transferase center and serves therefore as ubiquitously expressed marker for protein biosynthesis. The eRF1 has been remarkably conserved during evolution and is essential in the translation termination process (Frolova et al. 1994; Guenet et al. 1999; Inagaki et al. 2000). Metabolic activity of weaning piglets as indicated by hepatic eRF1
transcript levels did not differ between piglets fed the reference diet and piglets fed one of the PENS supplemented diet in both farms. However, piglets from EF fed RF+I exhibited a significantly higher mRNA abundance of eRF1 in ileal mucosa than piglets fed RF. It could indicate a higher inulin-associated mucosal activity of ileum. In contrast, no elevated or changed transcript levels of eRF1 was observed in the other test groups (Na-alginate, Chlorella vulgaris and essential oils) indicating that these PENS, at least at the tested concentrations, did not affect ileal mucosal activity. Up-regulation of eRF1 in ileal mucosa in inulin fed piglets was associated with down-regulation of GSTA1. GSTA1 is one of the glutathione S-transferases from a family of enzymes catalyzing the addition of the tripeptide glutathione to endogenous and xenobiotic substrates with electrophilic functional groups. The function of the glutathione S-transferases is to counteract oxidative stress (e.g. lipid peroxidation). It has been shown in rats that the decrease of the activity of antioxidant enzymes and phase II metabolizing enzymes such as glutathione-S-transferase is strongly associated with an increased microsomal lipid peroxidation (Khan et al. 2001). Under consideration of the physiological function of both marker genes we conclude that 1.5% inulin possesses a promoting effect on ileal metabolism in pigs. No health promoting activity of inulin could be observed as transcript levels of all other expression markers (iNOS, TLR4, HSP70.1 in lymph nodes and eRF1 in liver) showed no differences in comparison with the control group, neither at EF nor at CF.

Figure 5. Mean mRNA abundance (copy number/10ng total RNA) of cellular stress response (HSP70.1), oxidative stress response (GSTA1), immune response (iNOS) and translation activity markers (eRF1) in ileal mucosa of piglets (age 39 days, each n =4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), Chlorella vulgaris powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d and kept at an experimental (EF, empty bars) and a commercial farm (CF, gray bars). Real time RT-PCR was carried out in the
LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. 

Figure 6. Mean mRNA abundance (copy number/10ng total RNA) of cellular stress response (HSP70.1) and immune response marker (iNOS, TLR4) in ileocolic lymph nodes and translation activity marker (eRF1) in liver of weaning piglets (age 39 days, each n =4) fed starter diet (RF) supplemented with Na-alginate (RF+A), essential oil mixture 1 and 2 (RF+EO1, RF+EO2), Chlorella vulgaris powder (RF+CV) and inulin (RF+I) for 11 days since weaning at 28 d and kept at an experimental (EF, empty bars) and a commercial farm (CF, gray bars). Real time RT-PCR was carried out in the LightCycler® using specific primers and Light Cycler DNA Master SYBR Green I® (Roche, Mannheim, Germany). External DNA standard dilutions were generated from corresponding PCR products. Copy number of the housekeeping gene RPL was measured to normalize for equal RNA amounts. Bars represent mean values and SD obtained in two independent analyses of each individual. 

P value represents result of corresponding t-test in comparison with the RF. Abbreviations: GSTA1, glutathione S-transferase; iNOS, inducible nitric oxide synthase; eRF1, eukaryotic release factor 1; HSP70.1, heat shock protein 70.1; TLR4, Toll-like receptor 4.

The inducible NO synthase (iNOS) catalyses formation of reactive nitrogen induced by bacteria, bacterial endotoxines (e.g. LPS) and host cytokines (TNF-a, IL-1b, IFN-g). Its expression is therefore linked to immune response. Macrophages are important for early immune responses to invading microorganisms and the production of nitric oxide (NO) is vital for this
function. The iNOS shows antioxidative properties at low concentrations and it is involved in formation of cytotoxic, reactive nitrogen compounds at high concentrations (Fehr et al. 1997; Massa et al. 1998). Toll-like receptors (TLRs) are membrane proteins present on many cells involved in innate immunity such as macrophages, dendritic cells, neutrophils, mucosal epithelial cells, and endothelial cells. Ten different mammalian TLRs have been identified and all have distinct specificities for microbial ligands. Upon recognition of a microbial product, TLRs initiate a signalling pathway to activate innate immunity. TLR4 recognizes gram-negative bacterial- and Chlamydia-associated lipopolysacharride (LPS) and HSP60 (Akira et al. 2001; Akira 2003; Byrd-Leifer et al. 2001). Heat shock proteins of about 70 kDa (HSP70) are induced in virtually all eukaryotic cells by hyperthermia or other cellular stressors. Although specific functions of the different HSP70 are not yet identified, HSP70 are thought to be involved in mediating the thermotolerance and the ability of the cell to survive injury and oxidative stress, and autoimmune antigenic stimulation (Lindquist and Craig 1988; Craig and Gross 1991; Hightower 1991; Gething and Sambrook 1992; DeNagel and Pierce 1993).

Expression levels of marker genes in the three analysed tissues suggested no ileal metabolism and/or health promoting effects of Na-alginate, essential oils mixtures 1 and 2, and Chlorella vulgaris, at least in the used concentrations. Other than expected, in both farms piglets fed diet supplemented with these PENS did not exhibit lower expression levels of immune and stress response markers or higher level of the translation marker in comparison with piglets fed the reference diet. To study putative health promoting effects of these PENS in more detail, other than the recommended concentrations of the suppliers should be analyzed in corresponding feeding experiments.

Comparing mean transcript levels of the marker genes between both farms, a strong farm effect on expression level of the studied genes was noticed. In all feeding groups at CF higher transcript levels of the immune response markers TLR4 (146 - 191 %) and iNOS (134 - 271 %), and the cellular stress response marker HSP70.1 (161 - 330 %) were observed in ileocolic lymph nodes in comparison with the EF. Up-regulated expression level of these markers in the lymph nodes indicates an elevated immune and cellular stress response in piglets kept at CF probably due to higher microbial load in comparison with the experimental farm (Janczyk et al. 2009b). In addition, in ileal mucosa from piglets from almost all groups (except for RF+A) at CF higher transcript levels of the oxidative stress response marker GSTA1 (179 - 400 %) and the translation activity marker eRF1 (145 - 184 %) were observed in comparison with the EF. Furthermore, mRNA abundance of the cellular stress and immune response markers HSP70.1 and iNOS, respectively, did not differ between corresponding groups of both farms. Thus, in indicates higher metabolic activity in ileal mucosa from piglets kept at CF. This assumption is further supported by observed higher daily weight gains of piglets from the corresponding groups at CF in comparison with the EF (data not shown).
In summary, the presented results underline the possible impact of inulin on the metabolic activity and stress response at intestinal mucosal level in regard to its assumed promoting effects on digestibility of nutrients and growth performance. However, no metabolism and/or health promoting effects of Na-alginate, the essential oils and *Chlorella vulgaris* used in the tested concentrations could be observed. To study putative health promoting effects of these PENS in more detail, further feeding experiments with different concentrations of PENS are necessary.

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