Effects of avilamycin and essential oils on mRNA expression of apoptotic and inflammatory markers and gut morphology of piglets

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ABSTRACT: In this study 120 piglets were allotted to 3 dietary treatments, negative control group, one group receiving a blend of essential oils (EO) derived from oregano, anise and citrus peels (40 mg/kg diet), and a positive control group treated with avilamycin as growth promoting feed additive (40 mg/kg diet). On day 22 of the experiment, 12 representative animals from each treatment group were sacrificed and tissue samples were collected for quantitative real time-PCR analysis and gut tissue morphology. EO and avilamycin decreased the gene expression of the transcriptional factor NFκB and the apoptotic marker TNFα significantly in the ileum and jejunum, respectively. The expression of the proliferation marker Cyclin D1 was also significantly decreased by both substances in the colon, mesenteric lymph nodes and spleen. The colonic crypt depth was reduced by avilamycin, and also numerically by the essential oils. These changes correlated with the up-regulation of the apoptosis factor Caspase 3. Histomorphometry revealed a smaller size of ileal Peyer's patches through the use of both feed additives, which correlated significantly with lower expression rates of NFκB. In conclusion, the results suggest that EO and avilamycin relieved weaning piglets from an immune defence stress in a similar way.

Keywords: piglets; essential oils; avilamycin; gene expression; gut morphology

Essential oils have recently attracted significant interest as feed additives for piglets and poultry, possibly being able to replace the use of antibiotic growth promoters which have been prohibited in the European Union since 2006. In general, the antimicrobial action of essential oils has been demonstrated widely (Sivropoulou et al., 1996; Si et al., 2006) as their potential has to positively affect zootechnical performance (Rodehutscord and Kluth, 2002; Westendarp, 2005). In a recent piglet study, it was shown that beneficial effects on growth rate and feed intake, on parameters of intestinal microbial activity and nutrient digestion were similar in animals supplied with essential oils derived from oregano, anise and citrus peels, compared to those of the antimicrobial feed additive avilamycin (Kroismayr et al., 2007; Zitterl-Eglseer et al., 2007) However, there is still a considerable lack of information available about the interplay between the primary effects of such feed additives, mainly localized in the gut content, and the responses of the host animal in terms of inflammation, tissue growth and gut tissue morphology. In this context, this paper presents reactions of the mRNA expres-

sion rates of selected marker genes involved in inflammatory response, apoptosis, cell proliferation and cell cycle, as well as gut morphology, in piglets treated with essential oils and avilamycin as growth promoting feed additives.

MATERIAL AND METHODS

In the present study, 120 (65 male and 55 female) weaned piglets (crossbred Large White × Pietrain, 28 days of age, mean body weight 8.2 ± 2.3 kg) were used to investigate the effects of two feed additives (a blend of essential oils v. avilamycin) on mRNA expression of marker genes and gut histology.

The study comprised 3 consecutive experimental time replicates, using 30, 45, and 45 animals, respectively. Within experimental replicates, piglets were grouped into blocks, each containing 3 animals according to genetics (litter), sex and initial live weight. The animals in each block were allotted to one of the three treatments: (1) basal feed with no additions of potentially growth enhancing agents like antibiotics, organic acids, probiotics, or excessive contents of zinc and copper (negative control group), (2) basal feed supplemented with a blend of essential oils (essential oils group), and (3) basal feed supplied with the antimicrobial feed additive avilamycin (avilamycin group). These three treatments were allotted to each of the 3 pens of a rearing unit. In total, each dietary treatment was represented by 3 pens containing 10, 15 and 15 animals for experimental time replicates 1, 2, and 3, respectively, resulting in a total of 40 animal blocks.

The animals were housed under conventional conditions (air-conditioned room containing 3 pens with identical construction (slatted floor, heated lying area, height adjustable nipple drinker, cup drinker and a round feeding hopper)). Animals were fed a common pre-starter and starter diet consisting mainly of maize, wheat, barley and soya extracts to meet nutritional requirements (pre-starter diet, experimental day 1 to 7: 15.2 MJ ME/kg and 20.3% CP; starter diet until experimental day 22: 14.8 MJ ME/kg and 19.5% CP). Feed and water were offered *ad libitum* to the piglets throughout the experiment.

The essential oils group received the botanical product Biomin® P.E.P. 1000 (Biomin GmbH, Austria), which is permitted in the European Community (EC) to be used as a feed additive for piglets (Reg. EC No. 1831/2003). It contains essential oils derived from oregano (*Origanum vulgare*), anise (*Pimpinella anisum*) and citrus peels (*Citrus sinensis*) with chicory (*Cichorium intybus*) powder acting as a carrier substance. The product contains as its main active ingredients the phenolic monoterpenes carvacrol, thymol, anethole and the cyclic monoterpene – hydrocarbon limonene. The product was added at 2 g per kg fresh mater (FM) of the finished diets (corresponding to 40 ppm essential oils). For the avilamycin group, Maxus100 (Elanco Animal Health Ltd.) was used. It is based on the active ingredient avilamycin which was permitted for use as AGP in EC during the course of the experiment (2005). Maxus 100 contains 10% avilamycin and it was added to the diets at levels of 0.4 g per kg FM (corresponding to 40 ppm avilamycin).

On the 22nd day of each experimental replicate, 4 blocks of animals (2 females and 2 castrated males) with body weights ranging closest to the median of the pen were sacrificed in order to obtain samples of tissues for further investigations (mean body weight of sacrificed animals = 13.7 ± 1.7 kg.) Further information about the formulation of diets, analysed nutrient contents and animal husbandry were published in Kroismayr et al. (2007).

Sample collection and preparation

Whole blood was collected at bleeding, using a blood sampling vacutainer containing EDTA (Greiner bio-one GmbH, Frickenhausen, Germany) to prevent coagulation. Tubes were kept cold on crushed ice. Blood containing EDTA was diluted 1/1 (v/v) with lysis buffer (830 mg NH_4Cl, 3.7 mg Na-EDTA, 100 mg KCl in 100 ml H_2O pH 7.4) to lyse red blood cells and subsequently centrifuged for 10 min at 220 g. The first cell pellet containing all white blood cells was re-suspended in lysis buffer and centrifugation was repeated. Leukocytes were transferred to 350 µl RNA extraction buffer (Macherey-Nagel, Düren, Germany) and the supernatant was discarded. Hereinafter, this sample is referred to as white blood cells (WBC).

Tissue samples were collected from the stomach (exterior corpus region), jejunum (mid part), ileum (15 cm from distal end), colon (at flexura centralis), mesenteric lymph nodes (one entire node), liver (central region), kidney (cortex region), spleen (distal end), brain, and muscle (*m. longissimus dorsi*, central cut). Samples were immediately snap frozen

and preserved in liquid nitrogen for the later mRNA expression analysis.

Total RNA was extracted from tissue samples following a slightly modified phenol-based extraction method (Fleige et al., 2006). Nucleic acid concentrations were measured in triplicate at 260 nm using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). RNA integrity was verified by an optical density OD_{260}/OD_{280} absorption ratio > 1.90.

Real-time one-step qRT-PCR

To get exact samples with similar quantities of RNA fluid and reacting agents, a pipetting robot epMotion 5075 (Eppendorf, Hamburg, Germany) was used to prepare RT-PCR templates containing RNA samples and master mix. Therefore, 25 ng mRNA in 1 µl volume was mixed with master mix components: 0.3 µl iScript (Bio-Rad, Munich, Germany), 0.225 µl of forward and reverse primer (Table 1) synthesized by MWG Biotech (Ebersberg, Germany), 7.5 µl 2 × SYBR Green (Bio-Rad) and up to 14 µl of RNAse free water.

Relative mRNA concentration was measured using the one-step quantitative RT-PCR (qRT-PCR) in an ep realplex cycler (Eppendorf, Hamburg, Germany). One-step qRT-PCR was performed with 40 cycles and product specific annealing temperature. Realplex analysis software (Eppendorf, Hamburg, Germany) was used to calculate the crossing points (CP) of the samples. The CP is defined as the point at which fluorescence rises above background fluorescence (Pfaffl et al., 2002). This means that the higher the CP, the lower the RNA content in the original sample. A relative quantification was conducted using Histon H3 as a reference gene and marker genes as listed in Table 1.

According to the relative quantification method (ΔΔCP by Livak and Schmittgen, 2001), mRNA expression of single marker genes were first normalized (ΔCP) by the constant reference gene expression (Histon H3) method, according to:

$$\Delta CP_{(\text{Histon H3 – marker gene})} = CP_{(\text{Histon H3})} - CP_{(\text{marker gene})}$$

As a second step, ΔΔCP values were calculated for each marker gene investigation in the essential oils (EO) or asilanycil groups. Therefore the average ΔCP value from the negative control group animals was subtracted from ΔCP values of treated animals, according to:

$$\Delta\Delta CP\ (\text{EO/avilamycin}) = \Delta CP_{(\text{Histon H3 – marker gene})}\ (\text{EO/avilamycin}) - \text{Ø}\Delta CP_{(\text{Histon H3 – marker gene})}\ (\text{negative control group})$$

Positive and negative ΔΔCP values in the groups with feed additives in the diets indicate differences in gene expression compared to the negative control group. Values are expressed as 2^{x} –folds. The marker genes screened in this study are known for activities as follows: NFκB: transcription factor; TNFα: pro-inflammatory and apoptosis factor; IL10: anti-inflammatory factor; Caspase 3: apoptotic marker; Cyclin D1: proliferation and cell cycle marker; IGF1: insulin-like growth factor and general cell survival factor; Histon H3: housekeeping and reference gene.

Morphological investigations

Slices of approximately 2–3 mm were collected from fresh tissue samples of the jejunum, ileum, and colon derived for PCR analysis (see above). First, the tissues were washed thoroughly in physiological NaCl-solution, then they were fixed in a

Table 1. Sequences of forward and reverse primers for one-step qRT-PCR

Gene	Forward primer	Reverse primer
Histon H3	act ggc tac aaa agc cgc tc	act tgc ctc ctg caa agc ac
TNFα	ccc cca gaa gga aga gtt tc	ttg gcc cct gaa gag gac
IGF1	tgc ttc cgg agc tgt gat c	agc tga ctt ggc agg ctt ga
IL10	act tta agg gtt acc tgg gtt g	gta gac acc cct ctc ttg ga
NFκB	ggt gga gaa ctt tga gcc tc	cca gag acc tca tag ttg tcc
Caspase3	tgt gtg ctt cta agc cat gg	agt tct gtg cct cgg cag
CyclinD1	tcc tgt gct gcg aag tgg a	ggt cca ggt agt tca tgg c

Table 2. Gene expression of NFκB, TNFα and IL10 in investigated tissues

Primer	Tissue	Treatment		
		essential oils[1]	avilamycin[1]	SEM
NFκB	stomach	–0.31	–0.32	0.17
NFκB	jejunum	–0.02	0.26	0.11
NFκB	ileum	–1.12*	–1.53*	0.26
NFκB	colon	–0.59(*)	–0.53(*)	0.12
NFκB	mesent. lymph nodes	–1.06*	–1.83*†	0.15
NFκB	WBC	0.52(*)	1.10*†	0.22
NFκB	liver	–0.57*	–0.37	0.11
NFκB	spleen	–0.99	0.40	0.24
NFκB	kidney	–0.05	0.03	0.19
NFκB	muscle	0.04	0.19	0.11
NFκB	brain	–0.55	–0.61	0.47
TNFα	stomach	–0.67	–0.12	0.26
TNFα	jejunum	–1.20*	–0.85*	0.18
TNFα	ileum	–0.34	–0.89(*)	0.25
TNFα	colon	–0.82	–0.24	0.17
TNFα	mesent. lymph nodes	0.10	0.32	0.11
TNFα	WBC	0.02	–1.14*(†)	0.34
TNFα	liver	0.16	0.59	0.15
TNFα	spleen	0.04	0.18	0.07
TNFα	kidney	–0.63	–0.68	0.22
TNFα	muscle	0.26	0.34	0.12
TNFα	brain	–0.83	–0.82	0.37
IL10	mesent. lymph nodes	–0.45	–0.23	0.16
IL10	spleen	0.01	0.25	0.10

*linear contrast of means compared to the negative control group ($P < 0.05$)
(*)linear contrast of means compared to the negative control group ($P < 0.1$)
†linear contrast of means between essential oils and avilamycin group ($P < 0.05$)
(†)linear contrast of means between essential oils and avilamycin group ($P < 0.1$)
[1]values are expressed as $2^{\times}$-folds compared to the negative control group

3.7% formalin solution over night. On the following day, the tissue samples were dehydrated and embedded in paraffin.

For morphological analyses, samples were cut (with LEICA RM 2145) to the thickness of 6 µm and stained with haematoxylin and eosin (HE) according to Mayer (Luna, 1969). Sliced samples were examined with a microscope (Zeiss Axioskop 2 plus, Göttingen, Germany) and digital microphotographs were taken (Zeiss Axio Cam MR, Göttingen, Germany). The microphotos were evaluated with a graphic program (Zeiss Axio Vision 3.1, Göttingen, Germany).

Measurements of villi height and width in the jejunum and ileum were done on three well dissected preparations (3 replicates per sample gut tissue and animal). This meant that the central lymph canal (*Vas lymphaticum centrale*) had to be visible over a large area and the overall length of the villi was as long as possible. The villus height was measured between the top of the villus and the beginning of the *lamina muscularis mucosae*. The width of the villi was measured at the broadest zone of each villus. Measurements of crypt depth in the colon were done on three well prepared crypts for each slide.

The surface of Peyer's patches in the ileum was measured by taking the 3 largest patches from each slide as they are most representative.

Statistical analysis

The GLM procedure of SAS (SAS, 2002) was used to determine treatment effects by analysis of variance (ANOVA) using a randomised complete block design. The statistical model comprised a 2 factorial design using dietary treatment and animal block. Treatment means were compared statistically by the Tukey-Kramer test. The result tables present the mean values of the treatments and the pooled standard error of the means (SEM). Statistically significant ($P < 0.05$) differences between means are indicated by superscripts. Furthermore, linear contrasts were done for a pairwise comparison of the means (essential oils vs. control; avilamycin vs. control, essential oils vs. avilamycin). The Pearson's correlations coefficients were calculated between gut morphology and expression rates of NFκB, TNFα, Caspase 3, Cyclin D1 and IGF1 in the respective tissues.

RESULTS AND DISCUSSION

The mRNA expression of the reference gene Histon H3 was not affected by any dietary treatment in all 11 tissues investigated. The gene expression of IL10 could be quantified in mesenteric lymph nodes and spleen only, whereas the respective measurements of Caspase 3, NFκB, TNFα, Cyclin D1 and IGF1 worked in all tissues except for kidney (no Caspase 3) and white blood cells (WBC) (no Cyclin D1 or IGF1).

PCR analysis revealed treatment effects on the expression of the transcriptional factor NFκB and pro-inflammatory cytokine TNFα (Table 2). Compared to negative controls, NFκB expression was significantly ($P < 0.05$) decreased by essential oils and avilamycin in the ileum. The same trend of down-regulation ($P < 0.1$) was observed in colonic tissue. Avilamycin caused significantly ($P < 0.05$) lower NFκB expression in mesenteric lymph nodes and WBC than in the essential oils and negative control groups, while essential oils led to lower ($P < 0.05$) NFκB expression in mesenteric lymph nodes and slightly lower values ($P < 0.1$) in WBC compared to the negative controls. Additionally, essential oils decreased ($P < 0.05$) NFκB expression in the liver compared to the negative control group.

TNFα mRNA expression was lowered by both feed additives ($P < 0.05$) in the jejunum, while avilamycin also slightly decreased ($P < 0.1$) this cytokine in the ileum in comparison to the negative control group. In WBC avilamycin led to significantly lower ($P < 0.05$) TNFα expression compared to the negative control group and slightly decreased contents ($P < 0.1$) compared to the essential oils group.

Table 3. Gene expression of Caspase 3 in investigated tissues

Primer	Tissue	Treatment		
		essential oils[1]	avilamycin[1]	SEM
Caspase 3	stomach	–0.53	–0.26	0.19
Caspase 3	jejunum	–0.51	–1.02	0.26
Caspase 3	ileum	–0.66	–0.25	0.17
Caspase 3	colon	0.45	0.63*	0.11
Caspase 3	mesent. lymph nodes	0.24	0.34	0.09
Caspase 3	WBC	–0.13	0.52	0.28
Caspase 3	liver	–2.42	–3.12	0.32
Caspase 3	spleen	–0.09	–0.47(*)	0.10
Caspase 3	muscle	0.43	0.53(*)	0.11
Caspase 3	brain	–0.42	–1.16	0.36

*linear contrast of means compared to the negative control group ($P < 0.05$)

(*)linear contrast of means compared to the negative control group ($P < 0.1$)

[1]values are expressed as $2^{\times}$-folds compared to the negative control group

Table 4. Gene expression of Cyclin D1 and IGF1 in investigated tissues

Primer	Tissue	Treatment		
		essential oils[4]	avilamycin[4]	SEM
Cyclin D1	stomach	1.30	1.79	0.42
Cyclin D1	jejunum	–0.48	–0.54[1]	0.13
Cyclin D1	ileum	–0.50	–0.25	0.11
Cyclin D1	colon	–0.67*	–0.62*	0.13
Cyclin D1	mesent. lymph nodes	–2.96*	–4.86*[2]	0.42
Cyclin D1	spleen	2.12*	3.20*	0.40
Cyclin D1	kidney	–1.37	–0.23	0.44
Cyclin D1	brain	–1.79	–0.51	0.11
Cyclin D1	muscle	0.07	0.15	0.52
IGF1	stomach	–0.64	0.65	0.26
IGF1	jejunum	–0.11	–0.5	0.17
IGF1	ileum	–1.18[1]	–1.15[1]	0.17
IGF1	colon	0.23	0.10	0.12
IGF1	mesent. lymph nodes	0.38	–0.08	0.13
IGF1	liver	–1.86	–2.19	0.26
IGF1	spleen	0.59	1.27*	0.19
IGF1	kidney	3.46[1,3]	0.27	0.49
IGF1	brain	0.31	–0.57	0.15
IGF1	muscle	0.38	–0.02	0.28

*linear contrast of means compared to the negative control group ($P < 0.05$)
[1]linear contrast of means compared to the negative control group ($P < 0.1$)
[2]linear contrast of means between essential oils and avilamycin group ($P < 0.05$)
[3]linear contrast of means between essential oils and avilamycin group ($P < 0.1$)
[4]values are expressed as $2^{\times}$-folds compared to the negative control group

The apoptosis factor Caspase 3 was affected only by avilamycin (Table 3). While an increased expression rate was observed in the colon ($P < 0.05$) and in tendency also in the muscle ($P < 0.1$), it was slightly ($P < 0.1$) down-regulated in the spleen.

Table 4 presents the effect of essential oils and avilamycin on the expression of Cyclin D1 and IGF1. Compared to the negative control group, both feed additives decreased ($P < 0.05$) Cyclin D1 expression in the colon, mesenteric lymph nodes and spleen. In mesenteric lymph nodes, avilamycin additionally led to significantly lower ($P < 0.05$) expression of this marker than in the essential oils group. Avilamycin also slightly decreased ($P < 0.1$) Cyclin D1 expression in the jejunum in comparison with the negative control group. Both treatments showed a tendency to decrease IGF1 expression in the ileum ($P < 0.1$). In the spleen, this factor was expressed at a significantly ($P < 0.05$) higher level in the avilamycin group than in the negative control group. A similar result could be seen in kidney where essential oils led to slightly higher ($P < 0.1$) IGF1 contents than in the other two groups.

Morphological investigations showed no influence of any feed supplement on villi or crypts in the jejunum, caecum or colon (Table 5) generally. Only colonial crypts of animals treated with avilamycin were less deep than those of the negative control group animals ($P < 0.05$). Measurements of the surface of ileal Peyer's patches showed that both treatments led to a significantly ($P < 0.05$) smaller size compared to the negative control group (essential oils: –32%; avilamycin –28%).

Table 6 presents Pearson's correlation coefficients between gut morphology and expression rates of NFκB, TNFα, Caspase 3, Cyclin D1 and IGF1 in

Table 5. Effects of avilamycin and essential oils on gut histology

	Treatment			
	negative control	essential oils	avilamycin	SEM
Jejunum				
Villi length (mm)	0.70	0.66	0.68	0.020
Villi width (mm)	0.15	0.14	0.16	0.005
Ileum				
Villi length (mm)	0.67	0.62	0.66	0.019
Villi width (mm)	0.16	0.16	0.16	0.004
Colon				
Crypt depth (mm)	0.48	0.45	0.43*	0.009
Width between Crypts (mm)	0.03	0.03	0.03	0.002
Peyer's patches (Ileum)				
Area (mm^2)	0.50[a]	0.34[b]	0.36[b]	0.028

*linear contrast of means compared to the negative control group ($P < 0.05$)
[a,b]Tukey-Kramer test $P < 0.05$

the respective tissues. Ileal villi length and colonic crypt depth were correlated with Caspase 3 expression rates ($r = 0.33$, $P < 0.05$ and $r = –0.45$, $P < 0.01$), as well as the surface of lymph cells in Peyer's patches with NFκB ($r = 0.47$, $P < 0.01$). No statistically evident correlations were found between gene expression rates and other parameters of gut morphology analyzed here.

In the present piglet study, dietary additions of essential oils or avilamycin showed a beneficial impact on the animals' overall gastrointestinal health and productivity. Results reported elsewhere (Kroismayr et al., 2007; Zitterl-Eglseer et al., 2007) suggested an improved zootechnical performance (higher feed intake and weight gain), lower intestinal microbial activity (lower bacterial colony counts and microbial products such as volatile fatty acids and biogenic amines), as well as an overall improvement of apparent digestibility of dry matter and crude protein through both feed additives. These effects might be reflected by concomitant changes in the mRNA expression of inflammatory and apoptotic marker genes as well as in gut morphology. For this reason, expression rates of marker genes were assessed in 11 different tissues using the quantitative real-time reverse transcriptase polymerase chain reaction (PCR).

The real-time, fluorescence-based reverse transcriptase PCR is one of the enabling technologies of the genomic age and has become the method of choice for the detection of mRNA (Bustin, 2000). Reverse transcription is the process of making a double stranded DNA molecule from a single stranded RNA template. The real time PCR system is based on the detection and quantification of a fluorescent dye. The methods of real-time PCR data analysis may be broadly carried out as 'absolute' or 'relative' quantification. Relative quantification determines changes in the mRNA levels of a gene across multiple samples and expresses it relative to the levels of another mRNA (reference) gene (Bustin, 2002). It is an adequate tool to investigate small physiological changes in gene expression levels.

NFκB is the key transcriptional factor of many pro-inflammatory cytokines (Baldwin, 1996). It is found essentially in all cell types and is involved in the activation of a large number of genes in response to infections, inflammation and other stressful situations such as AIDS, asthma and cancer (Karin and Ben-Neriah, 2000; Baldwin, 2001). NFκB may also promote apoptosis (van Antwerp et al., 1996). Essential oils and avilamycin decreased NFκB expression in mesenteric lymph nodes and there was a similar tendency in the ileum, colon and liver. This effect suggests that the inflammatory activity in these tissues was lower compared to the untreated controls. Most of other tissues remained unaffected except for an unexplained up-regulation of NFκB in the blood cells.

TNFα is a potent cytokine produced by many cell types in response to inflammation, infection,

injury and other environmental challenges (Baud and Karin, 2001). It activates the transcription factor NFκB (Shaulian and Karin, 2001). According to Beg and Baltimore (1996), TNFα is one of the prime signals inducing apoptosis of cells, which is amplified by concomitant activation of the transcription factor NFκB (Wang et al., 1996). In the intestinal tract in particular, TNFα is known to mediate mucosal inflammation (Playford and Ghosh, 2005). In the present study, essential oils and avilamycin resulted in the down-regulation of TNFα expression rates in WBC, mesenteric lymph nodes, jejunum and ileum, while the other tissues remained largely unaffected. These reactions coincided well with the effects observed for NFκB and support the hypothesis that pro-inflammatory reactions were less pronounced in treated animals, the more so as the expression rates of IL10, an anti-inflammatory cytokine mainly active in lymphoid tissues (Moore et al., 2001), remained unchanged. The down-regulation of both TNFα and NFκB in the intestinal tract and mesenterial lymph nodes suggests a lower immune defence activity due to the antimicrobial action of both feed additives. In the case of essential oils, the antimicrobial activity against various bacterial strains such as *E. coli, Salmonella, Staphylococci* or *Clostridia* has been widely demonstrated, especially for oregano essential oils (Deans and Ritchie, 1987; Mahmoud, 1994; Sivropoulou et al., 1996; Dorman and Deans, 2000; Shan et al., 2007). In this context, the down-regulation of TNFα in WBC and of NFκB in liver give further support to the concept that both feed additives relieved the animal from immune defence activities and possibly also the liver from detoxification stress (e.g. biogenic amines; Kroismayr et al., 2007).

Caspase 3 plays a key role in promoting apoptosis (Heczko et al., 2001). It is known to be up-regulated at a high pathogen pressure e.g. from *Salmonella* or *Shigella* (Watson, 1997). In the present study, Caspase 3 was not modified by avilamycin except for the up-regulation in the colon and some tendency of minor changes in muscle (up-regulation) and in the spleen (down-regulation), the relevance of which is unclear. With essential oils, no effect was visible in any of the tissues investigated. These results suggest that the animals were not subject to major challenges from specific pathogens. Consequently, the decline in pro-inflammatory reactions as indicated by the down-regulation of NFκB and TNFα in splanchnic tissues of treated animals seems to reflect variations in immune responses within the normal range of pathogen pressure in virtually healthy individuals. This corresponds to the observation that the piglets studied here developed well with zootechnical performance data varying within normal margins (Kroismayr et al., 2007). It might also explain why in our study no correlation could be observed between the expression rates of Caspase 3 and NFκB in the jejunum ($r = 0.15$), which is in accordance with Sehm et al. (2006). In most of the other tissues, however, the respective correlation was partially highly significant (stomach: $r = 0.63$, $P < 0.01$; mesenteric lymph nodes: $r = -0.20$, not significant; WBC: $r = 0.90$, $P < 0.01$; liver: $r = -0.43$, $P < 0.05$; spleen: $r = 0.08$, not significant; muscle: $r = 0.51$, $P < 0.01$).

Cyclin D1 is a marker of cell proliferation in the gut and is induced by growth factors like IGF1 (Albanese et al., 1999). IGF1 is so-called "survival gene" controlling the general metabolic activity of cells and is a potent mediator of the action of growth hormone. (Elsaesser et al., 2002) It is up-regulated under stressful conditions and in tumour cells (von Wichert et al., 2000). Essential oils and avilamycin tended to reduce the expression of IGF1 in the ileum and lowered that of Cyclin D1 in the colon and mesenteric lymph nodes. Furthermore, avilamycin tended to reduce the expression of Cyclin D1 in the jejunum. The coincidental down-regulation of TNFα and NFκB in these tissues might give rise to the suspicion that lower gene expression rates of Cyclin D1 and IGF1 also reflect relief from an immune defence stress in these tissues. In the spleen, however, Cyclin D1 was significantly up-regulated, for unknown reasons. Furthermore, essential oils tended to produce a selective up-regulation of IGF1 in kidneys, which might suggest a cellular stress possibly induced by the excretion of absorbed essential oils and their metabolites, respectively (Zitterl-Eglseer et al., 2007). However, this observation failed to be statistically significant and requires verification.

Collectively, brain and muscle tissues did not respond to essential oils or avilamycin in terms of the gene expression of Cyclin D1, IGF 1, NFκB and TNFα. This is not surprising as both tissues are hardly exposed to these feed additives and are not involved in immune defence activities.

Morphometric investigations of the jejunal tissue showed that none of the feed additives applied in the present study affected the length or width of villi. Generally, villi were a little longer in the jeju-

Table 6. Pearson's correlation coefficients between gut morphology and expression rates of NFkB, TNFα, Caspase 3, Cyclin D1 and IGF1 in the respective tissues

	NFkB	TNFα	Caspase 3	Cyclin D1	IGF1
Jejunum					
Villi length (mm)	–0.05	–0.07		0.11	–0.01
Villi width (mm)	0.16	0.13		–0.07	0.06
Ileum					
Villi length (mm)	–0.21	–0.21	0.33*	0.14	0.21
Villi width (mm)	–0.10	–0.04	–0.16	–0.05	–0.17
Colon					
Crypt depth (mm)	–0.01	–0.25	–0.45**	–0.05	–0.13
Width between crypts (mm)	–0.05	–0.38*	–0.26	–0.21	–0.27
Peyer's patches					
Surface of cells (mm^2)	0.47**	0.27	0.25	0.17	0.21

*$P < 0.05$; **$P < 0.01$

num than in the ileum, which is in accordance with former reports (Görke, 2000; Mekbungwan and Yamauchi, 2004). The ileum villi length correlated with Caspase 3 expression rates to a statistically significant extent, possibly reflecting a pronounced cell turnover in general. The colonic crypt depth was reduced by avilamycin, and also numerically by essential oils. These changes corresponded to the up-regulation of Caspase 3 as supported by statistically significant correlations (Table 6). In addition, the colonic Cyclin D1 expression was down-regulated in animals treated with essential oils and avilamycin. Interestingly, both feed additives raised the apparent digestibility of crude protein (Kroismayr et al., 2007). This is a typical reaction to growth promoting feed additives such as antibiotics and organic acids and is commonly regarded to reflect a lower bacterial growth due to an improved pre-caecal digestion of nutrients and hence a reduced influx of fermentable matter into the hind gut (e.g. Kirchgessner et al., 1995; Roth et al., 1998). These findings give rise to the hypothesis that the lower hindgut fermentation activity is associated with a decrease in cell proliferation (Sehm et al., 2006). Furthermore, such interactions with microbial activity might explain why the correlations of tissue morphology with Caspase 3 expression rates were the opposite for the ileum and colon, respectively.

The smaller surface of ileal Peyer's patches of animals treated with essential oils or antibiotics suggests lower activation, which corresponds to the statistically significant correlation with NFκB expression rates (Table 6). This might serve as a direct evidence for a lower need of the immune defence activity in the gut due to the antimicrobial action of either avilamycin or essential oils.

CONCLUSION

Using essential oils or avilamycin as a growth promoting feed additive affected the expression of marker genes involved in the immune response and cell cycle activity especially in splanchnic tissues and WBC, while other internal organs and muscle tissues remained largely unaffected. The results suggest mainly a relief from the immune defence activity in the gastrointestinal tract. This is in accordance with concomitant improvements in zootechnical performance, gut microbiology including fermentation products and apparent nutrient digestion induced by both feed additives as reported elsewhere (Kroismayr et al., 2007; Zitterl-Eglseer et al., 2007). Collectively, these results draw a consistent picture of essential oils producing gastrointestinal effects comparable to those of the antibiotic feed additive avilamycin. This coincidence may be best explained by the assumption of a similar mode of primary action, namely an overall antimicrobial effect in the gastrointestinal tract.

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