Intestinal Expression of TFF and Related Genes During Postnatal Development in a Piglet Probiotic Trial

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Key Words

TFF • Trefoil factor • Weaning • Intestinal development • Cytokines • Epidermal growth factor receptor • Transforming growth factor- α • Tumor necrosis factor- α • Cyclooxygenase 2

Abstract

Trefoil factor family (TFF) peptides provide protective and reparative effects by enhancing epithelial integrity and promoting mucosal restitution. TFF peptide expression is induced after mucosal damage. These processes are of central physiological relevance during the postnatal intestinal development and are strongly influenced during the weaning period. In piglets, weaning at early maturation stages frequently causes mucosal inflammation. The aim of this study was to evaluate postnatal intestinal TFF expression in a piglet probiotic trial. Low intestinal TFF2 expression was measured at early maturation stages. Weaning, however, was associated with a distinct response of increased TFF2 expression, indicating an important role in enhancing mucosal integrity. In the distal jejunum and ileum weaning could as well be associated with increased TFF3 mRNA lev-

els. Differential TFF1 expression was not detected. Furthermore, TFF2 localization studies in different intestinal loci were performed by means of immunohistochemistry. Expression of selected genes (TGFA, EGFR, Cox-2) known to promote TFF signaling showed differential expression pattern as well, thereby providing further functional background. Furthermore, the expression patterns of EGFR observed in this study contribute to an advanced view of previous findings of EGFR regulation mainly obtained in rodents. An upregulated EGFR expression during early postnatal development suggests a local relevance to porcine intestinal maturation. However, a feed supplementation with the probiotic strain *Enterococcus faecium* did not influence TFF expression.

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Introduction

Postnatal intestinal development is a complex process resulting in essential morphological and functional changes of the mucosa. These alterations are controlled by intrinsic programming, endogenous hormones and ex-

trinsic luminal factors. The weaning phase is one of the important developmental periods characterized by massive exposure to novel antigens enhancing modifications of the small intestine. Epithelial cells lining the gastrointestinal mucosa are composed of distinct cell types, each of which contributes in a unique way to mucosal defense and the maintenance of barrier integrity [1]. Restoration of epithelial continuity following injury or environmental challenges requires a regulatory network controlling diverse mechanisms of epithelial repair like gene expression, cell migration, proliferation and reestablishment of cell-cell contacts [2].

Important candidates for such a local epithelial restoration network comprise TFF peptides, a family of small secreted peptides consisting of three members: TFF1, TFF2 and TFF3. TFFs share a common structural motif, the so called trefoil domain, which is defined by six conserved cysteine residues forming characteristic disulfide bonds [3, 4], making the peptides resistant against gastric acid and proteases [5]. TFFs are known to be expressed in gastrointestinal epithelial cells of various organisms and are often co-secreted with mucins by mucus-producing cells. Recent studies have uncovered a role of TFFs in maintaining intestinal epithelial integrity based on restitution, wound healing, apoptosis, cell motility and adhesion [6-11]. In modulating mucosal immune responses at the level of leukocyte recruitment [12] and tissue repair functions, TFFs have a major impact on maintenance of healthy mucosal surfaces. They are regulated by both pro-inflammatory [13] and anti-inflammatory cytokines [14]. One of the important local cytokines likely regulating TFF2 and TFF3 during mucosal repair is the transforming growth factor- α (TGFA) [15, 16]. TGFA is thought to be the main endogenous epidermal growth factor receptor (EGFR) ligand in the intestine controlling cell proliferation and cell migration of gastrointestinal epithelial cells [17-19]. Like EGF, TFF3 is able to actively support epithelial repair through their mitogenic activity [11]. In addition, pathways of TFF2 and TFF3 have been indirectly connected to EGFR through cyclooxygenase 2 (Cox-2)-dependent signaling affecting *in vitro* proinvasive properties [20].

Cox-2 participates in early stages of mucosal repair by promoting cytoprotective prostaglandin synthesis [21, 22]. Like TFF2, rapid Cox-2 up-regulation was reported in response to injury or inflammation to restore mucosal integrity. Moreover, activation of Cox-2 by TFF peptides is an important determinant for their cytoprotective activity [21]. On the other hand, cytokines may also have inhibitory effects on TFF expression. The pro-inflamma-

tory factor tumor necrosis factor α (TNF), a key contributor to many forms of mucosal injury [15, 23, 24] induces a transcriptional repression of TFF3 through NF κ B activation *in vitro*.

All these factors have been discussed in relation to regulatory TFF pathways [15] and their study in parallel to TFF analysis will provide further functional background to understand the mode of TFF action.

In previous studies, porcine TFF2 expression and localization was observed by means of immunohistology [25], however, TFF2 expression has not been quantified in the context of postnatal intestinal development and the weaning period. In respect to their beneficial functions during gastrointestinal healing, TFFs represent potential candidates to act as luminal regulators in response to the high microbial and nutritional challenges arising during intestinal postnatal development and weaning. During this period accelerated epithelial growth and a simultaneous high antigen exposure due to adjustment to ingested nutrients occurs. This makes piglets highly vulnerable to disease and leads to reduced performance in food industry. In addition, piglets represent a well established animal model to investigate human gastrointestinal disease [26, 27] and to understand signaling pathways related to mucosal development.

In this context, probiotics are increasingly employed in animals and humans for the treatment and prevention of intestinal disease. Depending on the probiotic strain, an influence on many components of the epithelial barrier function is described in the literature, such as epithelial tight junctions, toll-like receptors or regulation mediated by inflammatory cytokines [28-30]. These diverse mechanisms proposed to mediate beneficial effects of probiotics also include promotion of the production of protective substances by the epithelium. These substances include mucins [31-34], some of which are co-expressed with TFFs [35], so that a potential impact of a probiotic treatment on TFF expression has to be considered.

In this respect, our aim was to study the developmental gene expression pattern of TFF peptides and selected pathway-related genes (TGFA, EGFR, Cox-2 and TNF) and to evaluate the local relevance on growth and maturation in piglets, especially regarding the weaning period. Therefore, quantitative real-time PCR and immunoblotting were employed to detect and quantify TFF1, TFF2 and TFF3 transcripts and peptides in distal jejunum, ileum and ascending colon of piglets. Furthermore, it was examined if a probiotic treatment with the strain *E. faecium* NCIMB 10415 might affect gastrointestinal health by influencing TFF expression.

Materials and Methods

Animals and Administration of Probiotics

The study was approved by the local animal welfare committee of the Federal Ministry of Consumer Protection, Food and Agriculture (No. G0037/02). Litters of crossbreed piglets (EUROC x Pietrain) and respective EUROC hybrid sows (EUROC^F x Landrace, Hülseberger Zuchtschweine, Pinneberg, Germany) were housed separately in pens with farrowing crates until weaning (end of d28). Piglets had ad libitum access to water, to grounded pre-starter feed from d15 to d28 and to pelleted starter feed from d29 to d56 of age. Both basal diets for piglets were based mainly on wheat and soybean meal and were formulated to fulfill NRC recommendations (National Research Council, 1998). Litters were randomly assigned to 2 groups, either receiving (probiotic group) or not receiving (control group) a probiotic *E. faecium* NCIMB 10415 supplement (Cylactin, batch no. ED0231, Cerbios-Pharma, Barbengo, Switzerland). Once daily, from directly after birth until 34 days of age, piglets of the probiotic group received orally 1 ml of *E. faecium* NCIMB 10415 (4.5×10^9 cfu/ml deionized water). The control piglets got a placebo (solely containing equivalent amounts of the primary component of the respective probiotic product coating/ml deionized water). Additionally, *E. faecium* NCIMB 10415 was provided as a feed supplement in the diet of the probiotic group. The median concentrations of the probiotic in the deployed feed batches was 9.4×10^6 cfu/g feed and 2.8×10^6 cfu/g feed in prestarter and starter feed, respectively (Taras, data not shown). The probiotic strain was not detectable in control feed.

Samples

From each treatment group, a total of five litters was randomly chosen and one piglet per litter with median body weight was selected at the end of days 7, 14, 28, 31, 35 and 56 ($n = 5$ piglets per treatment at each of the 6 time points resulting in a total of 60 animals). These piglets were sedated with Ketamin and Azaperon, and after narcosis with Pentobarbital the abdominal cavity was opened. After euthanasia of the animal with Pentobarbital intestinal tissue sections of approximately 2 cm length were taken from distinct regions of the distal jejunum (65 cm prior to the jejuno-ileal junction), ileum (right after the jejuno-ileal junction) and ascending colon (10 cm after the begin of the first centrifugal loop).

Sample preparation and RNA/Protein extraction

In order to obtain representative measurements in each intestinal region, we cut 3 cross sections of approximately 2 mm out of the 2 cm segment of frozen intestine. These 3 sections were pooled and lysed in extraction buffer by using an automated homogenizer (FastPrep Instrument, Qbiogene/MP Biomedicals, Heidelberg, Germany). Subsequently total RNA was isolated using NucleoSpin RNA/Protein (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Yield of total RNA was quantified photometrically at 260 nm with NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was assessed by electrophoresis on a formaldehyde-containing 1% (w/v)

agarose gel, ethidium bromide staining and judging 28S/18rRNA.

To confirm TFF2 expression pattern on protein level, the intestinal tissue was homogenized in RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) sodium-deoxycholat, 1% (v/v) Triton X-100, 1 mM Na_3VO_4 , 1 mM NaF, 1% (w/v) SDS and 5 mM Pefabloc (Merck, Darmstadt, Germany). The homogenate was centrifuged at $16.000 \times g$ at 4 °C for 5 minutes to remove insoluble material, followed by determination of protein concentration in the supernatant by using 2-D Quant Kit (Amersham Biosciences, Buckinghamshire, United Kingdom).

Reverse Transcription and Quantitative Real-Time PCR

Isolated total RNA was treated with RQ1 RNase-Free DNase (Promega, Mannheim, Germany) in order to remove residual genomic DNA. Total RNA (2 µg) was reverse transcribed using 200 U RevertAidTM Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Fermentas, St. Leon-Rot, Germany), 3.75 µM random hexamers (Amersham Biosciences), 1 mM dNTPs (Fermentas) and 1X of the supplied RT buffer in a total volume of 60 µl. Control samples were treated under the same specified conditions, but without M-MLV RT in order to monitor the absence of genomic DNA.

Quantitative cDNA analysis was carried out using the SYBR Green chemistry (Quanta ce Ltd, London, United Kingdom) on a real-time PCR Cycler (Rotor-Gene RG-3000, Corbett Research, Sydney, Australia). The following real-time PCR protocol was applied: denaturation at 95 °C for 10 minutes, three-step amplification including denaturation at 95 °C for 15 sec, annealing at specified temperature (table 1) for 20 sec and extension at 72 °C for 30 sec, a subsequent melting curve (50-99 °C) with continuous fluorescence measurement and final cooling to room temperature.

For mRNA quantification, a calibration curve was established by serial dilutions of known amounts of the specific PCR product, which were amplified simultaneously with the samples. Sample-to-sample variation was normalized measuring 18S rRNA levels in each sample under the same conditions. Ratios of mRNA of interest and 18S rRNA were calculated for further evaluation of gene expression data. Primer sequences and GenBank accession numbers of corresponding mRNA sequences are listed in table 1. All resulting amplicons were sequenced to verify transcript identity. Calculations were performed using Rotor-Gene 4.6 software and Excel (Microsoft Corp., Redmond, USA).

Western Blotting

Pooled protein samples of all animals within the trial groups (50 µg or 20 µg for detection of TFF2 or TFF3, respectively) were separated on a 15% (w/v) polyacrylamide gel under reducing conditions. Subsequently proteins were transferred onto Immobilon-P[®] Transfer PVDF membranes (Millipore, Schwalbach, Germany) for TFF3 detection or Nitrocellulose Blotting Membrane (Sartorius, Goettingen, Germany) for TFF2 detection by electro blotting for 15 min at 3.1 mA/cm² and fixed on the membrane with 0.25% (v/v) glutaraldehyde (Sigma-Aldrich, Munich, Germany). In order to minimize un-

Gene	GenBank accession	Primer ^a	Oligonucleotide Sequences (5'-3')	T _m ^b	Product size, bp
TFF1	AM283538	S AS	CCA TGG AGC ACA AGG TGA AGG GTG GAA GCA CCA CGG GA	59 °C	200
TFF2	AM283539	S AS	CAA GAG TCT GAG GAG TGC GTC A GAC ATG GGG AAG AAG C AC C	59 °C	143
TFF3	F14493	S AS	GGG AGT ATG TGG GCC TGT C AGG TGC ATT CTG TTT CCT GC	55 °C	174
18S rRNA	AY265350	S AS	AAT CGG TAG TAG CGA CGG AGA GGG ACA AGT GGC GTT C	59 °C	275
COX-2	AF207824	S AS	ATG ATC TAC CCG CCT CAC AC GCA GCT CTG GGT CAA ACT TC	60 °C	279
EGFR	AY117054	S AS	ACT GGA CAT CCT GAA AAC CG TAG CAC AGG TTT CGG TTT CC	63 °C	239
TGF α	X71014	A AS	TGT CCC ATT TTA ATG ACT GCC TCA CAG TGT TTT CCG ACC TG	59 °C	271
TNF α	NM_214022	S AS	TCA AAC CTC AGA TAA GCC CG AGT GAG GAA AAC GTT GGT GG	60 °C	214

Table 1. Synthetic oligonucleotides designed for quantitative real-time PCR (qRT-PCR).

^aS, sense primer; AS, antisense primer, ^bannealing temperature.

specific antibody binding, membranes were incubated overnight at 4°C in 2% (w/v) ECL blocking agent (Amersham Biosciences) in PBST (PBS with 0.05% (v/v) Tween 20) and subsequently incubated with polyclonal rabbit anti-mouse TFF2 antibody [35] at 1:5000 dilution overnight at 4°C. Donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) was used at a ratio of 1:60.000 for 1 h. For TFF3 detection monoclonal anti-human TFF3 antibody [36] was used at 1:100 dilution overnight at 4°C. Donkey anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) was used at a ratio of 1:20.000 for 1 h. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences) and exposed to X-ray film (Hyperfilm ECL, Amersham Biosciences). Recombinant human TFF2 (2ng) expressed in *E. coli* (Peprotech GmbH, Hamburg, Germany) and 6µg of total protein extracted from porcine gastric tissue were used as positive controls for TFF2. Recombinant human TFF3 (0.5 µg) [10] was used as a positive control for TFF3. The specificity of antigen staining was assessed by exchanging the first antibody by pre-immune rabbit serum (DakoCytomation, Hamburg, Germany) for anti-TFF2 and Mouse IgG1 (DakoCytomation) for anti-TFF3.

Immunohistochemistry

Several pieces of the different parts of the intestine were fixed in Bouin's solution (4% (w/v) picric acid, 2.5% (w/v) cupper(II)acetate, 3.5% formaldehyde) overnight at room temperature. Fixed pieces were washed in 70% (v/v) ethanol, then dehydrated and embedded in paraffin. Paraffin sections of approximately 5µm were cut and mounted on silane coated slides. The slides were heated for 45 min at 58°C. Subsequently, the sections were deparaffinized in xylene, rehydrated through graded ethanol and rinsed in distilled water and PBS (pH 7.4). To block the endogenous peroxidase activity the sections were incubated twice with 3% (v/v) hydrogen peroxide in methanol for 15 min at room temperature, and rinsed 3x with PBS (pH 7.4). Unspecific binding was blocked with 2% (v/v) normal goat serum in PBS-T (DakoCytomation) (1.5 h at 37°C). Sections

were then incubated overnight at 4°C with the primary TFF2 antibody diluted 1:12.000 in PBS (pH 7.4). Control sections were processed simultaneously with rabbit IgG fraction (DakoCytomation) instead of the primary antibody. After intensive washing steps (3 x 15 min, PBS-T, on shaker) bound primary antibody was marked by a dextran polymer carrying secondary antibody and horseradish peroxidase molecules (EnVision, DakoCytomation; 30 min, room temperature). Diaminobenzidin (DAB plus, DakoCytomation; 1-4 min, under visual control) was taken as chromogen and hemalum as counterstain.

Statistical Analysis

Data are represented as mean values + SEM (standard error of the mean) of five individual animals. Significance of the differences between time points and the trial groups was evaluated by Mann-Whitney-U-Test using SPSS for Windows 12.0 (Chicago, USA). $P \leq 0.05$ was considered significant. Statistical analysis was performed separately for the probiotic-treated and the placebo group because of lower variance of gene expression levels within trial groups than between the groups.

Results

Detection of TFF Transcripts

In order to investigate gene expression pattern of TFFs during postnatal development, mRNA was quantified by means of quantitative reverse transcription PCR (qRT-PCR). Selected transcripts were analyzed in three different intestinal regions of pre- and postweaning piglets, namely the distal jejunum, ileum and ascending colon of piglets aged 7, 14, 28, 31, 35 and 56 days.

TFF1, TFF2 and TFF3 transcripts were detected in different amounts in all investigated tissues. Sequencing confirmed the identity of each amplicon. For the first time porcine TFF1 and TFF2 partial cDNA sequences have

Fig. 1. Expression of TFF2 mRNA during postnatal development measured by qRT-PCR in different intestinal regions: A) distal jejunum, B) ileum, C) ascending colon. The control and probiotic trial groups are labeled as grey or black bars, respectively. Bars represent the mean + SEM of results obtained from separate RNA preparations from 5 individual animals at each time point. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. In distal jejunum and ileum expression levels before weaning (d7-28) were significantly lower than postweaning levels in both trial groups. In ascending colon expression on preweaning d7 differed significantly from postweaning time points in both groups, while d14 had significantly lower expression levels only in control animals. Comparing the 2 trial groups, there is no significant difference in TFF2 expression between controls and probiotic treated animals (not indicated).

been reported (GenBank accession numbers - TFF1: AM283538, TFF2: AM283539). Sequence identity of TFF1 partial cDNA compared to other species was 71% for human, 65% for mouse and 69% for rat. TFF2 partial

Fig. 2. A) Representative Western Blot analysis of TFF2 peptide from equal amounts of porcine jejunal protein samples from control (1) and probiotic-treated (2) 7d old piglets, control (3) and probiotic-treated (4) 35d old piglets and control (5) and probiotic-treated (6) 56d old piglets. As positive controls 6 μ g of protein prepared from porcine gastric tissue (8) as well as 2 ng of recombinant human TFF2 (9) (Peptrotech GmbH, Hamburg, Germany) were used. Lane 7 was left unused. B) Densitometric analysis of three TFF2 Western blots using GeneTools Software after separation by polyacrylamide gel electrophoresis under reducing conditions. Each bar represents the mean + SEM of the antibody response calculated by integration of the area under the corresponding peak, which was normalized with the signal from porcine gastric tissue. Protein and mRNA levels both reflect tendencies towards enhanced TFF2 expression at late stages of development (35d and 56d, also see Fig. 1 A).

cDNA identity was 82% for human, 79% for mouse and 79% for rat.

TFF2 Expression

Mean total transcript concentrations of TFF2 were decreasing towards distal regions of the intestinal tract. Reverse transcription reactions with balanced efficiencies yielded 0.4fg/ μ l cDNA in distal jejunum, 0.2fg/ μ l cDNA in ileum and 0.009fg/ μ l cDNA in ascending colon. An initial constant increase of TFF2 mRNA was observed in the distal jejunum with highest mean levels on day 35 (d35, Fig. 1A). In the control group a 6-fold increase of mean gene expression levels was observed comparing d7 and d35. In the probiotic treated animals, a 28-fold difference between d7 and d35 or d56 was notable. In

Fig. 3. Immunohistochemical localization of TFF2 protein in the intestinal epithelium of the pig at day 7, 35 and 56 after weaning: A-C) Ileum, D-F) Jejunum, G-I) Colon; small pictures: negative controls; chromogen: DAB (brown), counterstain: hemalum; magnification $\times 100$, scale bars represent $100\mu\text{m}$.

ileum, a progressive increase in TFF2 gene transcription was observed as well. Highest mean expression values were found on d56, displaying a 15-fold or 8-fold increase compared to d7 in the probiotic treated and control group, respectively (Fig. 1B). Differences in transcript concentrations remained unaffected by the probiotic treatment.

In ascending colon TFF2 expression showed a similar developmental profile, as gene expression increased from d7 onwards to 13-fold and 16-fold elevated transcript concentrations in the control and probiotic group, respectively (Fig. 1C). However, variability of expression was higher between the individual animals compared to the distal jejunum and ileum. This may depend on the lower absolute levels of mRNA present in this tissue.

In order to determine if these differences in TFF2 expression can be verified on protein level, TFF2 peptide

was detected by Western blotting. Immunoblots of samples derived from the distal jejunum as well as from gastric tissue revealed a single protein band of ~ 12 kDa corresponding to TFF2 isolated from porcine gastric tissue and a recombinant hTFF2 standard (Fig. 2A). Increasing amounts of TFF2 protein were detectable during development and after weaning (Fig. 1A, 2B), thereby reflecting observed changes in TFF2 expression measured by qPCR. Densitometric quantification of Western blot signals from pooled jejunal tissue samples resulted in an increase in signal intensity comparing d7 and later maturation stages in the control and probiotic group, respectively.

Statistical analysis revealed that a probiotic treatment with *E. faecium* did not affect porcine TFF2 expression at any developmental stage within the three intestinal regions.

Fig. 4. TFF3 mRNA expression measured by qRT-PCR. The control and probiotic trial groups are labeled as grey or black bars, respectively. Bars represent the mean + SEM of results obtained using separate RNA preparations from 5 individual animals at each time point. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. In distal jejunum and ileum, significantly lower expression levels were found at specific preweaning time points (d7-28) compared to expression on postweaning time points d31 and d35 as indicated.

Localization studies on TFF2 showed specific immunoreactivity at all developmental stages as shown for d7, d35 and d56. Furthermore, all of the studied intestinal regions (distal jejunum, ileum and ascending colon) showed distinct staining as illustrated in Fig. 3 (A-I). TFF2 was localized exclusively in the enterocytes of the intestinal mucosa. The protein was predominantly abundant in the surface epithelium lining the upper portion of the intestinal villi. A faint staining was also visible in the profound parts of the crypts except for the ileum, where only the cells lining the lumen were positive for TFF2. No immunostaining was observed when the primary antibody was replaced with rabbit IgG1 (Fig. 3, small pictures).

TFF3 Expression

Mean absolute TFF3 transcript concentrations ranged from 14fg/ μ l cDNA in the distal jejunum and ascending colon to 5fg/ μ l cDNA in ileum.

In the distal jejunum, a significant 2-fold increase of TFF3 expression was observed comparing pre- and postweaning piglets in the control group (d28 and d35), as well as in the probiotic group (d14 and d35). The same change was measured in ileal tissue samples of probiotic treated piglets (d28 and d35). From weaning to the mature stages no developmentally related changes in TFF3 expression occurred in the ascending colon (Fig. 4A-C). The probiotic treatment did not influence TFF3 expression significantly in any of the studied tissues. TFF3 expression was also demonstrated on protein level (Fig. 5) in samples from distal jejunum, ileum and ascending colon of a 14 day old individual animal. Bands on the TFF3 Western Blots were on the same level like recombinant hTFF3 used as a positive control (data not shown).

Fig. 5. Western blot analysis of TFF3 in protein samples from (1) jejunum, (2) ileum and (3) ascending colon of a 14d old individual piglet. As a positive control 0.5 μ g recombinant human TFF3 was used (data not shown).

TFF1 Expression

In all investigated intestinal regions TFF1 mRNA levels were frequently undetectable (data not shown). Because of transcript concentrations below the detection limit of the assay, TFF1 expression was not further investigated.

Fig. 6. TGFA mRNA expression measured in separate RNA preparations from 5 individual animals at each time point. Bars represent the mean + SEM of expression levels. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. In distal jejunum and ileum elevated expression levels were observed during early development. Expression in distal jejunum and ileum significantly decreased already within the preweaning period, so that no difference was observed between preweaning d28 and postweaning levels. In ascending colon no significant differences were measured.

Fig. 7. EGFR mRNA expression quantified by qRT-PCR. Bars represent the mean + SEM of results obtained using separate RNA preparations from 5 individual animals at each time point. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. In all of the studied tissues higher levels of EGFR mRNA were measured at indicated preweaning time points. In ileum, however, expression shortly before weaning (d28) was significantly lower than the 3 days after weaning (d31).

Gene Expression Pattern of TFF-pathway Related Genes

A) Transforming Growth Factor- α (TGFA).

In distal jejunum the developmental gene expression profile of TGFA (Fig. 6A) showed highest expression values on d7, decreasing to up to 10-fold and 14-fold lower values on d28 to d56 in the control and probiotic group, respectively. This profile of differential expression was as

well reflected in ileal samples (Fig. 6B), displaying a 4-5-fold decreased gene expression level on d56 compared to d7. No age-related differences in mRNA expression occurred in the ascending colon (Fig. 6C).

B) Epidermal Growth Factor Receptor (EGFR). EGFR gene expression analysis in distal jejunum revealed a gradual 3-fold and 4-fold reduction from 7d to 56d in control and probiotic treated animals, respectively (Fig.

Fig. 8. Cox-2 mRNA expression measured by qRT-PCR. Bars represent the mean + SEM of expression levels in separate RNA preparations from 5 individual animals at each time point. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. Cox-2 expression was highest at early developmental stages. Expression declined to significantly lower levels during the preweaning period in jejunum and ileum. Expression levels on preweaning d28 did not show significant differences to postweaning d31 and d35.

7A). In ileum, control animals also showed a significantly decreased (2-fold) EGFR expression comparing d7 and d28 to d35. However, this expression profile was not reflected in ileal samples of probiotic treated animals, which showed a significant 1.6-fold increased EGFR expression shortly after weaning comparing preweaning (d28) and postweaning (d35) time points (Fig. 7B). In ascending colon, a significant 2-fold decreased EGFR expression was detected in both trial groups comparing mean

Fig. 9. TNF mRNA expression measured by qRT-PCR. Bars represent the mean + SEM of results obtained using separate RNA preparations from 5 individual animals at each time point. Within trial groups, bars with stars indicate a significant difference between preweaning time points and postweaning d31 and/or d35 ($p \leq 0.05$). One, two or three stars indicate significant differences between preweaning time points and d31, d35 or both, respectively. In ascending colon, gene expression of TNF was significantly higher at postweaning days, compared to early developmental stages as indicated.

values of 7d and 35d old piglets. In the probiotic group expression values remained significantly reduced at mature stages (d56) (Fig. 7C).

C) *Cyclooxygenase-2 (Cox-2)*. In distal jejunum elevated Cox-2 mRNA expression was observed on d7 and d14 in probiotic treated animals. Comparing d7 and d56 in control and probiotic treated animals a 7-fold and 8-fold reduction was observed, respectively (Fig. 8A). In ileal samples a 3-fold and 7-fold reduction was assessed

in the control and probiotic group, respectively (Fig. 8B). This expression profile was again reflected by a 4-fold reduction of Cox-2 mRNA expression in colon of probiotic animals, although this tendency was statistically not significant in corresponding control animals (Fig. 8C).

D) Tumor necrosis factor- α (TNF). TNF mRNA expression in distal jejunum was characterized by generally variable expression levels. However, a significant 3-fold increase in jejunal TNF expression was found comparing d7 and d56 in the probiotic group (Fig. 9A). In ileum no differences in expression with an at least 2-fold altered expression level were found (Fig. 9B).

In colon, a significant developmental expression profile was observed. From early developmental stages to weaning, expression levels increased 2- and 4-fold in the control and probiotic group, respectively (Fig. 9C). Except minor differences in ileum, the probiotic treatment did not influence TNF expression levels.

Discussion

Along the entire mammalian gastrointestinal tract expression of TFF peptides and generation of a TFF-containing mucus layer by mucus producing cells was reported [37]. In pigs TFF2 is apparently expressed on the entire luminal surface of the gastrointestinal tract at rather high concentrations [25]. Expression of TFF2 *in vivo* has been documented in various organisms, each having a unique tissue specific pattern. In humans, TFF2 expression is restricted to the stomach, duodenum and ducts of the pancreas [37] and more specifically, duodenal TFF2 expression is observed in mucus cells of Brunner glands but not in the epithelial lining [38]. Surprisingly, Thim et al. found lowest concentrations of TFF2 in porcine stomach and duodenum compared to distal intestinal regions [39]. Our results in porcine gastric tissue, however, are concordant to high protein expression of TFF2 in the stomach reported in other species. In a previous study with pigs by Rasmussen et al., immunostaining revealed TFF2 expression in pancreas, in mucous cells of the glands of the stomach, in Brunner's glands of the duodenum, and in epithelial cells of the crypts of Lieberkühn in the jejunum and ileum, but not in duodenal goblet cells [25]. Localization of TFF2 in this study is in agreement with the studies by Rasmussen et al. in terms that goblet cells of the intestinal epithelium did not express TFF2. Furthermore, Rasmussen et al. reported the expression of TFF2 in columnar cells in the lower parts of the crypts from jejunum and ileum. In our study the

staining was much more intensive in the upper enterocytes of intestinal villi and a positive immunoreaction in columnar cells in the lower part of the ileal crypts was not observed. However, Rasmussen et al. used a different TFF2 antibody, pigs of a different strain (LYY) and with a weight ranging between 20 and 25 kg rather than defining distinct time points for taking samples. Also, differences in the study design and feeding conditions have to be considered. Eventually, a localization of TFF2 in intestinal epithelial cells underlines possible TFF functions in restitution and the maintenance and re-establishment of epithelial cell continuity.

A low total level of TFF2 mRNA expression in ascending colon was observed in the study presented here. Thus, absence of immunostaining in this intestinal region reported by Rasmussen et al. [25] may be due to a TFF2 level below the detection limit. Target amplification by PCR leads to enhanced sensitivity, which most likely enabled the detection of differences in expression levels in colon in this study.

Transcription of TFF2 changed markedly during postnatal development in piglets suggesting a potential significance of intestinal TFF2 expression in pigs. Most pronounced changes occurred in distal jejunum. Expression was characterized by consistently low levels on d7, although one extreme value in the control group contributed to higher variance of mRNA levels in this trial group. This was possibly the reason for a weak protein detection on d7 in the control group by Western blot analysis, while in the probiotic group no signal was observed. The generally enhanced TFF2 expression after weaning in all studied tissues points to a natural role in managing challenges in the postweaning period like epithelial response to novel food antigens and accelerated epithelial growth [40, 41].

In humans TFF3 is mainly expressed in goblet cells of the small intestine and colon, and in Brunner's glands of the small intestine [42, 43]. An upregulated expression of TFF3 around the weaning period was measured in rat intestine [44]. This is concordant with our results in piglets, showing a 2-fold increased TFF3 expression in distal jejunum and ileum comparing specific pre- and postweaning time points.

In the human gastrointestinal tract TFF1 is known to be expressed in the upper gastric mucosal cells [37] while it has been reported to be absent in the small intestine or present in very low amounts in the duodenum only [45, 46]. In this study, gene expression levels were below the detection limit of the applied assay. Therefore in piglets TFF1 appears to play no relevant functional

role in the intestinal regions analyzed in this study.

The distinct expression pattern, especially that of TFF2 in distal parts of the small intestine as well as in colon contributes to a gain of knowledge about an essential local physiological regulation, which is important for selecting possible targets for future therapeutical approaches. Besides the search for therapeutics for the treatment of inflammatory bowel disease in humans, there is also a need to discover substances supporting piglets to tolerate the high challenges in food industry. This fact gained importance after the European ban of using antibiotics as growth promoters in 2006. TFF peptides represent interesting candidates for this purpose because of their role as gastrointestinal healing peptides modulating epithelial restitution and repair processes. Also the natural response to weaning associated challenges observed in this study supports this approach. The use of exogenous TFFs in the treatment of intestinal disease was already assessed in some studies using animal models. Oral administration of TFF-secreting *Lactococcus lactis* proved to be very effective in prevention and healing of experimentally induced acute and chronic colitis in mice, decreasing the morbidity and mortality of the animals [47]. In addition, oral administration of TFF2 and TFF3 in an animal model of gastric injury in rats proved to have a notable protective effect through a surface mucosal defense [48].

Remarkably, in ascending colon, TFF2 as well as TNF expression is elevated after weaning compared to early time points (d7). This underlines a possible role of TFFs in the response to weaning associated inflammatory processes. In a recent study based on gene expression analysis of pro-inflammatory cytokines, weaning in piglets was as well associated with a transient inflammation of the gut [49].

However, the regulation of postnatal intestinal development is known to be influenced by multiple factors modulating intestinal growth, which are also associated with TFF signaling pathways [16, 50, 51]. Therefore, our following results add additional information to the functional intestinal cellular network that is part of a TFF-mediated response.

Growth factors like TGFA and epidermal growth factor (EGF) are ligands for EGFR and are regarded as important factors in postnatal development of the small intestine, particularly as they are present in human breast milk [50]. This growth factor system seems to be involved in the global and intrinsic control of gut morphogenesis and homeostasis. In the context of gut epithelial development in different species there is a general agreement

about tissue localization of EGFR and the growth promoting effects of its ligands on the adult gastrointestinal tract. However, in fetal and postnatal stages considerable differences in EGFR function and expression exist between humans and rodents. In humans the amount of EGFR tends to decrease before birth which might be attributed to an advanced degree of organ maturation that is achieved *in utero* [52, 53]. In rodents however, EGF does not only act as a fetal promoting agent but seems to be one of the few factors mediating redifferentiation of epithelial cells during postnatal adaption of intestinal function at weaning [52]. Gallo-Payet et al. characterized EGFR during postnatal development of mouse small intestine and observed low expression at birth and during milk feeding and elevated expression at weaning [54]. This argues against a potential importance of EGFR during early murine postnatal development [50]. Another study in mice suggested the involvement of EGF in redifferentiation of epithelial cells during postnatal adaptation at weaning [55]. In contrast, our results show highest gene expression levels of intestinal EGFR in jejunum at early developmental stages (d7). This finding points to a local relevance of EGFR in early porcine intestinal development underlining differences in regulation of EGFR expression between humans and rodents. In ileum, control animals as well show a less distinct, but significantly reduced expression comparing early stages (d7 and d14) and later stages of development, which was not reflected in probiotic animals. Here, a significantly enhanced EGFR expression was observed comparing pre- and postweaning time points (d28 and d31), which points to a potential immunomodulatory function of the probiotic strain. In order to address the functional importance of this observation, further studies are required. The significantly reduced EGFR expression comparing pre- and postweaning time points in porcine ascending colon correlates with a study in mice showing highest EGFR expression immediately after birth in the maturing colon [56].

TGFA is known to be one of the important factors mediating intestinal wound healing. However, scarce reports exist about the regulation of TGFA expression in the mucosa [57]. Several studies reported a rapid induction of TGFA expression in models of gut inflammation and mucosal injury [58-61], suggesting a potential relevance for TGFA in weaning-associated inflammation. Recently, TGFA expression was studied in colon and duodenum during postnatal development in rats [62, 63], but to our knowledge this is the first study profiling postnatal TGFA expression in distal small intestine in pigs.

According to our results in distal jejunum and ileum a significantly upregulated TGFA expression at early stages (d7 and d14) compared to constant weak expression levels from 28d onwards support the potential relevance of EGFR and its major ligand TGFA in early postnatal development. A reported increase of TGFA expression between weaning and the adult stage in proximal colon of rodents [62] is not reflected by our results in pigs. An induction of TGFA expression at weaning was not observed in any intestinal region.

A similar gene expression profile similar to TGFA was also observed for Cox-2. Significantly higher expression in early stages of maturation (d7 and d14) indicates relevance in early development, but during weaning no or only slight regulation was observed. Although it is suggested that an upregulation of Cox-2 is at least partly responsible for cytoprotective effects of TFFs in response to epithelial injury [21], this was not reflected by a concordant differential expression during postnatal development in this study.

An approach to prevent and treat gastrointestinal disease is the use of probiotics, which are as well applied as food additives in pig industry. The WHO defines “probiotics” as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host [64]. Recently, various mechanisms were proposed by which probiotics influence the host. These include competition with different bacteria, alteration of bacterial metabolism and stimulation of the immune system [30, 65-67]. However, these mechanisms are specific for each probiotic strain and underlying mechanisms of probiotic action are yet not well understood [68].

There is evidence that probiotics mediate epithelial barrier function by their ability to increase mucin synthesis [69]. In a rat animal model the basal luminal mucin content increased by 60% after treatment with a mixture of different probiotic strains [34]. Since TFFs are mucin-

associated molecules and are co-expressed with some mucins [70, 71] the potential impact of probiotic treatment on TFF expression was studied. However, the probiotic treatment using the strain *E. faecium* did not have a significant influence on TFF expression in any of the studied intestinal regions. Further studies using other probiotic strains may generate different results.

In summary, it has been demonstrated that TFF2 as well as TFF3 expression is differentially regulated during development in the porcine intestine. TFF2 was significantly upregulated after weaning in the mucosa. Selected cytokines and growth factors associated with TFF signalling pathways also show differential expression patterns pointing towards significance for early developmental processes as a complex local growth factor network. As recently suggested by Dürer et al. both TFF peptides and EGFR ligands act synergistically and optimize speed and precision during restitution of mucous epithelia [11]. It appears that especially TFF2 probably in concert with EGFR ligands exerts an influence on mucosal reconstruction during postnatal adaptation at weaning.

However, the chosen probiotic treatment did not show any significant influence on such local growth factor systems.

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