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Down-regulation of monocarboxylate transporter 1 (*MCT1*) gene expression in the colon of piglets is linked to bacterial protein fermentation and pro-inflammatory cytokine-mediated signalling

Carmen Villodre Tudela[1], Christelle Boudry[2], Friederike Stumpff[3], Jörg R. Aschenbach[3], Wilfried Vahjen[1], Jürgen Zentek[1] and Robert Pieper[1]*

[1]*Department of Veterinary Medicine, Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Strasse 49, D-14195 Berlin, Germany*
[2]*Animal Science Unit, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés 2, 5030 Gembloux, Belgium*
[3]*Department of Veterinary Medicine, Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, D-14163 Berlin, Germany*

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Abstract

The present study investigated the influence of bacterial metabolites on monocarboxylate transporter 1 (*MCT1*) expression in pigs using *in vivo*, *ex vivo* and *in vitro* approaches. Piglets (*n* 24) were fed high-protein (26 %) or low-protein (18 %) diets with or without fermentable carbohydrates. Colonic digesta samples were analysed for a broad range of bacterial metabolites. The expression of *MCT1*, *TNF-α*, interferon γ (*IFN-γ*) and *IL-8* was determined in colonic tissue. The expression of *MCT1* was lower and of *TNF-α* and *IL-8* was higher with high-protein diets ($P<0·05$). *MCT1* expression was positively correlated with L-lactate, whereas negatively correlated with NH_3 and putrescine ($P<0·05$). The expression of *IL-8* and *TNF-α* was negatively correlated with L-lactate and positively correlated with NH_3 and putrescine, whereas the expression of *IFN-γ* was positively correlated with histamine and 4-ethylphenol ($P<0·05$). Subsequently, porcine colonic tissue and Caco-2 cells were incubated with Na-butyrate, NH_4Cl or TNF-α as selected bacterial metabolites or mediators of inflammation. Colonic *MCT1* expression was higher after incubation with Na-butyrate ($P<0·05$) and lower after incubation with NH_4Cl or TNF-α ($P<0·05$). Incubation of Caco-2 cells with increasing concentrations of these metabolites confirmed the up-regulation of *MCT1* expression by Na-butyrate (linear, $P<0·05$) and down-regulation by TNF-α and NH_4Cl (linear, $P<0·05$). The high-protein diet decreased the expression of *MCT1* in the colon of pigs, which appears to be linked to NH_3- and TNF-α-mediated signalling.

Key words: Dietary protein: Monocarboxylate transporter 1: Pigs: TNF-α: Ammonia

Dietary and endogenous proteins that enter the large intestine of humans or monogastric animal species can be fermented by indigenous bacteria to form metabolites such as NH_3, hydrogen sulphide, branched-chain fatty acids, phenols and indols, which may promote intestinal disorders[1–4]. For example, protein level and quality in pig diets have been shown to be related to diarrhoea and shedding of enterotoxigenic *Escherichia coli* after a challenge[5,6]. A promising approach to reduce the formation of harmful metabolites from protein fermentation in the large intestine is the inclusion of fermentable carbohydrates in the diet[4,7,8]. This approach can be used to direct bacterial fermentation activity towards utilisation of carbohydrates instead of proteins, yielding lower luminal concentrations of branched-chain fatty acids and NH_3 and higher levels of SCFA.

Among SCFA, butyrate is of specific interest, as it serves as a source of energy for colonocytes and plays a crucial role in the epithelial maintenance of gut barrier function[9]. In addition to non-ionic diffusion, carrier-mediated uptake of butyrate from the large-intestinal lumen can also occur through monocarboxylate transporter 1 (*MCT1*) expression. Increased formation of butyrate from the fermentation of carbohydrates has been shown to increase the expression of *MCT1* in pigs[10,11]. MCT1 can also be induced by ketone bodies, pyruvate and lactate in addition to butyrate[12,13]. Activation by butyrate probably occurs through a butyrate response element in the promoter region of the *MCT1* gene[14]. In contrast, the expression of the *MCT1* gene is down-regulated in colonocytes during transition to malignancy[15]. Moreover, interferon γ (IFN-γ) and TNF-α signalling down-regulates

Abbreviations: BW, body weight; CP, crude protein; IFN-γ, interferon γ; MCT1, monocarboxylate transporter 1; SBP, sugarbeet pulp.

* **Corresponding author:** R. Pieper, fax +49 3 838 55938, email robert.pieper@fu-berlin.de

MCT1 expression during inflammation, leading to butyrate deficiency in intestinal epithelial cells[16].

Whether metabolites derived from intestinal protein fermentation could influence the expression of *MCT1* in pigs is yet unknown. We have previously demonstrated that increased formation of protein-derived metabolites in the large intestine increases the expression of pro- and anti-inflammatory cytokines, tight junction proteins and epithelial response to histamine[17–19]. Of these activities, the pro-inflammatory response may suggest that *MCT1* expression is potentially down-regulated by high-protein diets. We hypothesised that such down-regulation of *MCT1* expression, if present, could be dependent on the levels of both dietary protein and fermentable carbohydrates, because these nutrients induce microbial metabolites that, in turn, influence cytokine expression. In addition to testing this core *in vivo* hypothesis, we also used *ex vivo* (Ussing chamber) and *in vitro* (Caco-2 cells) approaches to further study the influence of NH_3 and TNF-α on the expression patterns of *MCT1* and cytokines.

Materials and methods

All procedures involving pig handling and treatments were approved by the State Office of Health and Social Affairs 'Landesamt für Gesundheit und Soziales Berlin' (LAGeSo Reg. no. 0389/12).

Animals, diets and sampling

A total of twenty-four piglets (Euroc × Pietrain) with a mean body weight (BW) of 7·4 (SE 1·0) kg were weaned at 25 d of age and randomly assigned to one of four dietary treatment groups in a 2 × 2 factorial arrangement, balancing for sex and BW. Diets (Table 1) were formulated to meet or exceed the nutrient requirements of the weaning pig[20]. The sources of dietary crude protein (CP) were soyabean meal, fishmeal and potato protein. Fermentable carbohydrates were supplied as sugarbeet pulp (SBP). Water and feed were provided *ad libitum*. Feed intake and BW of pigs were recorded weekly, and health status was checked daily.

Piglets were euthanised on experimental day 21 ± 1 by an intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®; Intervet) after sedation with 20 mg/kg BW of ketamine hydrochloride (Ursotamin®; Serumwerk Bernburg AG) and 2 mg/kg BW of azaperone (Stresnil®; Janssen-Cilag). Following euthanasia, digesta contents and tissue samples from the colon ascendens were taken and immediately stored at −80°C until further analysis.

Chemical analyses

Weende proximate nutrients (ash, CP and diethyl ether extract) and starch were determined using standard procedures[21]. Total dietary fibre was analysed using a commercial kit (Megazyme K-TDF; Megazyme), as described previously[22]. Trace mineral content in feedstuff was determined by atomic absorption spectrometry using an AAS Vario 6 spectrometer (Analytik Jena) after hydrolysis of samples in concentrated HCl.

D- and L-Lactate, NH_3, SCFA, biogenic amines, phenols and indols were determined in the digesta samples, as described previously[17,22,23].

Table 1. Ingredients and chemical composition of the experimental diets

	Low CP		High CP	
	Low SBP	SBP	Low SBP	SBP
Ingredients (g/kg as fed)				
Maize	450	341	300	300
Wheat	335	300	352	222
Soyabean meal (49 % CP)	110	120	140	140
Fishmeal	30	30	100	110
Potato protein	30	30	75	75
Sugarbeet pulp	-	120	–	120
Monocalcium phosphate	9	9	5	5
Limestone	9	8	5	3
Mineral and vitamin premix*	12	12	12	12
Salt	1	1	1	1
Soya oil	5	20	5	7
Lys HCl	3	3	–	–
Trp	1	1	–	–
TiO_2	5	5	5	5
Metabolisable energy (MJ/kg)	13·4	13·2	13·5	13·3
Analysed composition (g/kg DM)				
Ash	52	55	53	60
CP	183	179	256	255
Diethyl ether extract	33	38	29	33
Total dietary fibre	192	273	216	315
Insoluble dietary fibre	143	215	169	244
Soluble dietary fibre	48	58	47	71
Starch	450	376	350	308
Ca	8·7	8·6	8·7	8·5
P	5·8	5·5	6·5	6·5
Mg	1·8	2·1	1·7	2·0
Na	2·0	2·0	2·1	2·2
Analysed composition (mg/kg DM)				
Zn	103	98	85	93
Mn	97	99	90	95
Cu	15	12	11	11
Fe	203	365	157	358
Calculated composition (g/kg DM)				
Lys	12·5	13·0	17·4	18·1
Met	3·6	3·6	5·7	5·8
Thr	7·6	7·8	11·9	12·2
Trp	3·0	3·1	3·3	3·3

CP, crude protein; SBP, sugarbeet pulp.
*Mineral and vitamin premix (Spezialfutter Neuruppin GmbH) containing per kg DM: 130 g Na (as NaCl); 55 g Mg (as magnesium oxide); 210 mg retinol; 3000 μg cholecalciferol; 8000 mg DL-α-tocopherol; 300 mg menadione; 250 mg thiamin; 250 mg riboflavin; 400 mg pyridoxine; 2000 μg vitamin B_{12}; 2500 nicotinic acid; 100 mg folic acid; 25 000 μg biotin; 1000 mg pantothenate; 80 000 mg choline chloride; 5000 mg Fe (as iron (II) carbonate); 1000 mg Cu (as copper (II) sulphate); 5000 mg Zn (as zinc oxide); 6000 mg Mn (as manganese (II) oxide); 45 mg I (as calcium iodate); 35 mg Se (as sodium selenite).

Ussing chamber experiments

Segments of the ascending colon from six piglets (age 54 (SE 2) d) fed a standard weaning diet (containing 18% CP) were collected immediately after euthanasia and settled in a pre-warmed modified Ringer buffer solution, oxygenated with 95% O_2–5% CO_2. The composition of the buffer was 111 mM-NaCl, 5 mM-KCl, 1·5 mM-$CaCl_2$, 1·2 mM-$MgCl_2$, 0·6 mM-NaH_2PO_4, 2·4 mM-Na_2HPO_4, 25 mM-$NaHCO_3$, 10 mM-glucose

and 2 mM-mannitol, and the pH was adjusted to 7·4. The epithelium was stripped from the serosa and muscular layers, and subsequently mounted on Ussing chambers (exposed area 1·31 cm^2). For each pig, six chambers were prepared with two chambers per pig (including control) assigned to each treatment, resulting in a total of six chambers with tissues from three pigs per treatment. The apical and basolateral surfaces of the tissues were rinsed with 15 ml of oxygenated buffer solution and maintained at 37°C in water-jacketed reservoirs. Electrical measurements were obtained using a microcomputer-controlled voltage/current clamp (K. Mussler Scientific Instruments), as described previously[18]. The transepithelial potential difference in response to bipolar 50 μA current pulses generated for 200 ms, and tissue conductance (G_t) was calculated according to Ohm's law. After equilibration for approximately 15–30 min, tissues were short-circuited by clamping the voltage at 0 mV. The test substrates Na-butyrate and NH_4Cl were added to the mucosal and TNF-α to the serosal side of the tissue to give a final concentration of 20 mmol/l, 20 mmol/l and 50 ng/ml, respectively, with osmolality adjusted to 295 (SE 10) mOsmol/l. After 1 h of incubation, the exposed portion of the tissue was harvested and immediately stored in RNAlater RNA Stabilization Reagent (Qiagen GmbH) at −80°C until use.

Caco-2 cell experiments

To study the influence of different concentrations of Na-butyrate, NH_4Cl and TNF-α on the expression of *MCT1* and cytokines, *in vitro* cell-culture experiments using Caco-2 cells were performed. Caco-2 cells were grown routinely in a T-75 plastic flask at 37°C in an atmosphere of 5 % CO_2, and maintained in Eagle's minimum essential medium (LGC Standards) supplemented with 20 % fetal bovine serum, 100 units penicillin/ml and 100 μg streptomycin/ml (Biochrom). Confluent Caco-2 cells were subcultured in a ratio of 1:10 using 0·25 %/0·02 % trypsin–EDTA (Biochrom). For the present experiment, cells were seeded on twenty-four-well plates at a density of 10^5 cells/well and allowed to differentiate for 21 d, with fresh medium replacement three times per week. All cells used in the experiments were between passage numbers 19 and 21. Caco-2 cells were treated with buffer of the same composition as that used for electrophysiological measurements indicated above (pH 7·2). Buffer media contained increasing concentrations of Na-butyrate (10, 20 and 50 mmol/l), NH_4Cl (5, 10 and 20 mmol/l) or TNF-α (25, 50 and 100 ng/ml). Osmolality was adjusted to 295 (SE 10) mOsmol/l. After 1 h of incubation, cells were rinsed with the buffer solution. The buffer was immediately replaced with RNAlater RNA Stabilization Reagent, the cells were scraped from the surface, and the solution was stored at −80°C for the analyses of *MCT1*, *IL-8* and *TNF-α* gene expression.

Gene expression analysis

Analysis of gene expression was performed as described previously with slight modifications[17,24]. Briefly, total RNA from colonic tissue and Caco-2 cells was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel GmbH & Company KG). RNA quality and quantity were determined on an Agilent 2100 Bioanalyzer (Agilent). Subsequently, 100 ng of total RNA were reverse-transcribed into complementary DNA in a final volume of 20 μl using the SuperScript® III Reverse Transcriptase First-Strand complementary DNA Synthesis System (Invitrogen). The complete reaction mix was incubated for 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C in a Sure Cycler 8800 (Agilent Technologies). Primers for *MCT1*, *TNF-α*, *IFN-γ*, *IL-8*, 60S ribosomal protein L19 (*RPL19*), β_2-microglobulin, succinate dehydrogenase subunit A (*SDHA*), β-actin and TATA box-binding protein (*TBP*) were designed based on published sequences. Porcine (*RPL19* and *SDHA*) and human (β-actin and *TBP*) housekeeping genes were selected for data normalisation. Primer information and annealing temperatures are summarised in Table 2. Quantitative real-time PCR was performed in a total volume of 25 μl, which contained 12·5 μl Brilliant II SYBR Green QPCR Master Mix with Low ROX (Agilent Technologies), 0·5 μl of each primer (10 μM), 10·5 μl of water and 1 μl complementary DNA. The real-time quantitative PCR was performed on a Stratagene MX3000p (Stratagene) with general cycling conditions as follows: one cycle of denaturation at 95°C for 15 min, followed by forty cycles with 30 s denaturation at 95°C, 30 s annealing and 30 s extension at 72°C. Gene expression data were normalised, and times-fold expression was calculated based on mean C_t values of the housekeeping genes using real-time PCR efficiency according to the method proposed by Pfaffl[25].

Statistical analysis

Data from the *in vivo* study were analysed using general linear model procedures in SPSS (version 21.0; SPSS, Inc.) with SBP and CP and their interaction as sources of variation. Pearson's correlation analysis was performed to determine the correlation between colonic metabolite concentrations and relative gene expression data in pigs. The effects of increasing concentrations of selected metabolites on gene expression in Caco-2 cells were analysed by ANOVA and polynomial (linear, quadratic and cubic) contrasts, corrected for unequal spacing of treatment concentrations. Electrophysiological data from Ussing chambers and gene expression data from the *ex vivo* and *in vitro* experiments were analysed by one-way ANOVA followed by Tukey's honestly significant difference test. For non-normally distributed data, the Kruskal–Wallis test was applied to determine group differences, and groups were separated using the Mann–Whitney test. Differences at $P<0·05$ were considered significant. Data are presented as means with their standard errors, unless otherwise stated.

Results

During the *in vivo* study, all pigs remained in good health condition, and no clinical signs of diarrhoea or health impairment were observed. BW gain, feed intake and final BW did not differ significantly between the treatments, although pigs fed SBP diets had numerically lower BW gain and final BW (data not shown).

Table 2. List of the primers used in the present study

Targets	Species	Sequences of primers (5′ to 3′)	A_T (°C)	References
β_2-Microglobulin	Porcine	F: CCCCGAAGGTTCAGGTTTAC R: CGGCAGCTATACTGATCCAC	60	Martin *et al.*[24]
SDHA	Porcine	F: CAAACTCGCTCCTGGACCTC R: CCGGAGGATCTTCTCACAGC	60	Martin *et al.*[24]
RPL19	Porcine	F: GCTTGCCTCCAGTGTCCTC R: GCGTTGGCGATTTCATTAG	60	Pieper *et al.*[17]
MCT1	Porcine	F: GGAGACCAGTATAGACGCTGC R: CTCCTCCTCTTTGGGGCTTC	60	Present study
IL-8	Porcine	F: GGTCTGCCTGGACCCCAAGGAA R: TGGGAGCCACGGAGAATGGGTT	60	Present study
TNF-α	Porcine	F: CAAGCCACTCCAGGACCCCCT R: AGAGTCGTCCGGCTGCCTGT	60	Present study
IFN-γ	Porcine	F: TCCAGCGCAAAGCCATCAGTG R: ATGCTCTCTGGCCTTGGAACATAGT	58	Present study
β-Actin	Human	F: TTGCCGACAGGATGCAGAAGGA R: AGGTGGACAGCGAGGCCAGGAT	60	Present study
TBP	Human	F: TGCACAGGAGCCAAGAGTGAA R: CACATCACAGCTCCCCACCA	60	Present study
TNF-α	Human	F: GGCCTCTGTGCCTTCTTTTG R: CCTCAGCAATGAGTGACAGT	60	Present study
IL-8	Human	F: GGTGCAGTTTTGCCAAGGAG R: TGGGGTGGAAAGGTTTGGAG	60	Present study
MCT1	Human	F: CAGCAGAATCTCCGGACCAG R: TACCCTTCAGCCCCATGGAT	60	Present study

A_T, annealing temperature; F, forward; R, reverse; *SDHA*, succinate dehydrogenase subunit A; *RPL19*, 60S ribosomal protein L19; *MCT1*, monocarboxylate transporter 1; *IFN-γ*, interferon γ; *TBP*, TATA box-binding protein.

Metabolite concentrations in the colonic digesta have been described previously[22]. Briefly, pigs fed dietary SBP had higher concentrations of lactate and acetate and lower propionate concentration in the colonic digesta ($P<0·05$), whereas pigs fed diets high in CP exhibited higher concentrations of butyrate, NH_3 and biogenic amines ($P<0·05$). Inclusion of SBP in high-CP diets led to lower concentrations of some protein-derived metabolites, such as NH_3, cadaverine, *p*-cresol and indole ($P<0·05$), but the values were still higher as with low-CP diets. Other metabolites such as putrescine, histamine, phenol, 4-ethylphenol or 3-methylindol were unaffected. Lactate concentration was slightly lower ($P<0·10$) in high-CP diet-fed pigs, whereas inclusion of both CP and SBP led to slightly higher ($P<0·10$) levels of SCFA.

The relative gene expression of *MCT1*, *IL-8*, *TNF-α* and *IFN-γ* in the colon of pigs fed the different diets is given in Table 3. The expression of *MCT1* was lower ($P<0·05$) in pigs fed high-CP diets, irrespective of SBP. In contrast, the expression of *IL-8*, *TNF-α* and *IFN-γ* was higher ($P<0·05$) in pigs fed high-CP diets. The inclusion of dietary SBP had no effect on these gene expression patterns. In addition, no interaction was observed, indicating that dietary CP level was the main factor that influenced the expression of *MCT1*, *IL-8*, *TNF-α* and *IFN-γ*, irrespective of dietary SBP inclusion.

Pearson's correlation analysis was performed to determine the correlation between the metabolite concentrations in the colonic digesta and the relative gene expression of *MCT1*, *IL-8*, *TNF-α* and *IFN-γ* in colonic tissue (Table 4). A positive correlation ($P<0·05$) occurred between *MCT1* expression and L-lactate concentration, whereas NH_3 and putrescine concentrations were negatively correlated with *MCT1* expression ($P<0·05$). *IL-8* gene expression was negatively correlated with L-lactate ($P<0·05$) and positively linked with NH_3, putrescine, phenol and 4-ethylphenol ($P<0·05$). *TNF-α* expression also negatively correlated with L-lactate and positively correlated with acetate, NH_3, putrescine and histamine ($P<0·05$). Finally, *IFN-γ* expression was only positively correlated with histamine and 4-ethylphenol concentrations ($P<0·05$).

Table 3. Relative gene expression (fold change) of monocarboxylate transporter 1 (*MCT1*), *IL-8*, *TNF-α* and interferon γ (*IFN-γ*) in the colon of pigs fed diets high or low in dietary crude protein (CP) with or without the addition of sugarbeet pulp (SBP)

(Mean values with their standard errors; *n* 6)

	Low CP		High CP			*P**		
Genes	Low SBP	High SBP	Low SBP	High SBP	SEM	CP	SBP	CP×SBP
MCT1	1·00	1·15	0·57	0·56	0·13	<0·05	NS	NS
IL-8	1·34	1·14	3·36	3·65	1·69	<0·05	NS	NS
TNF-α	1·09	0·90	1·57	1·74	0·13	<0·05	NS	NS
IFN-γ	1·08	1·01	1·62	1·96	0·32	<0·05	NS	NS

* *P* values indicate the main effects for CP and SBP, respectively.

Table 4. Pearson's correlation of the microbial metabolite concentrations in the colonic digesta with the relative gene expression of monocarboxylate transporter 1 (*MCT1*), *IL-8*, *TNF-α* and interferon γ (*IFN-γ*) in the colonic tissue of pigs fed diets high or low in dietary crude protein with or without the addition of sugarbeet pulp*

	MCT1	*IL-8*	*TNF-α*	*IFN-γ*
L-Lactate	0·53	−0·50	−0·62	–
Acetate	–	–	0·49	–
NH_3	−0·54	0·46	0·43	–
Putrescine	−0·42	0·45	0·53	–
Histamine	–	–	0·49	0·55
Phenol	–	0·49	–	–
4-Ethylphenol	–	0·52	–	0·76

*Only significant correlations ($P<0·05$) are indicated.

Colonic tissue from pigs fed commercial standard diets was mounted on Ussing chambers and mucosally exposed to different metabolites. Changes in short-circuit current were found to be higher ($P<0·05$) after the mucosal addition of NH_4Cl compared with control chambers and chambers treated with Na-butyrate and TNF-α, where practically no biologically relevant change occurred (data not shown). The expression of *MCT1*, *IL-8*, *TNF-α* and *IFN-γ* was subsequently analysed. *MCT1* gene expression was higher ($P<0·05$) after Na-butyrate treatment and lower ($P<0·05$) after exposure to NH_4Cl and TNF-α compared with the untreated controls (Fig. 1(A)). The expression of *IL-8* was only increased ($P<0·05$) after exposure to TNF-α, whereas all the other treatments had no effect (Fig. 1(B)). The expression of *TNF-α* was only increased ($P<0·05$) after tissue exposure to NH_4Cl, whereas Na-butyrate or TNF-α had no effect (Fig. 1(C)). Finally, Na-butyrate decreased ($P<0·05$) *IFN-γ* gene expression, whereas all the other treatments did not (Fig. 1(D)).

Increasing concentrations of Na-butyrate, NH_4Cl and TNF-α were used to study the possible dose-dependent effects in Caco-2 cells (Table 5). The expression of *MCT1* was increased with Na-butyrate treatment and decreased with NH_4Cl and TNF-α treatments (linear, $P<0·05$), respectively. Linear and quadratic effects ($P<0·05$) were also observed for *IL-8* gene expression after Na-butyrate and TNF-α treatments, respectively. Similarly, *TNF-α* gene expression showed a quadratic response ($P<0·05$) towards increasing concentrations of NH_4Cl, and a linear and quadratic response ($P<0·05$) after exposure to TNF-α.

Discussion

Previous studies[10,11,26] have shown that diets high in resistant starch or soluble fibres up-regulate *MCT1* expression in the large intestine of pigs. However, to date, the influence of dietary protein and protein-derived bacterial metabolites on *MCT1* expression is largely unknown. In the present study, high CP

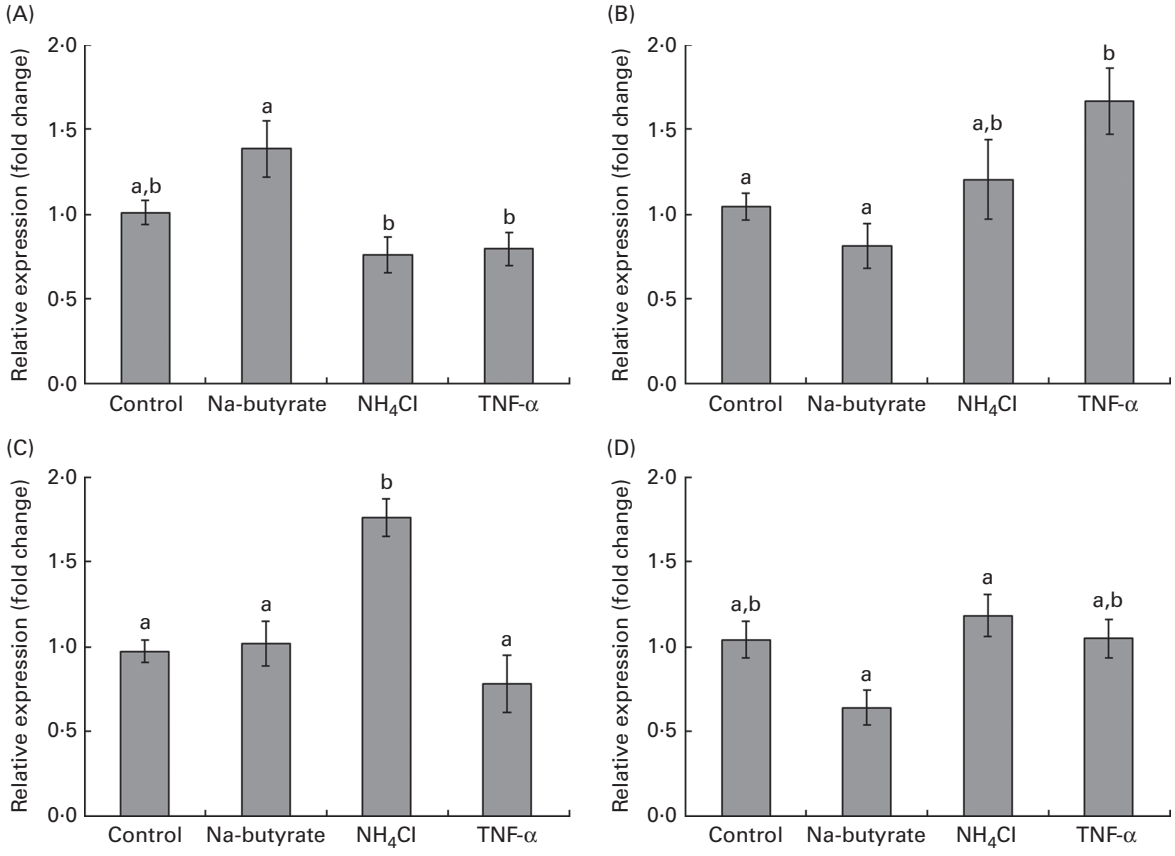

Fig. 1. Relative gene expression (fold change) of (A) monocarboxylate transporter 1 (*MCT1*), (B) *IL-8*, (C) *TNF-α* and (D) *IFN-γ* in colonic tissue after the mucosal addition of Na-butyrate, NH_4Cl or TNF-α, respectively. Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

Table 5. Relative gene expression (fold change) of monocarboxylate transporter 1 (*MCT1*), *IL-8* and *TNF-α* in Caco-2 cells after the addition of different concentrations of Na-butyrate, NH_4Cl or TNF-α, respectively
(Mean values with their standard errors; *n* 6).

	Substance (concentration in incubation medium)							
	Na-butyrate (mmol/l)							
Genes	0	10	20	50	SEM	Linear	Quadratic	Cubic
MCT1	1·05	1·33	1·44	1·62	0·05	<0·05	NS	NS
IL-8	0·95	0·51	0·50	0·64	0·04	<0·05	<0·05	NS
TNF-α	1·02	0·82	0·82	1·20	0·14	NS	NS	NS
	NH_4Cl (mmol/l)							
	0	5	10	20				
MCT1	0·95	0·93	0·91	0·72	0·09	<0·05	NS	NS
IL-8	0·95	0·95	0·99	1·06	0·27	NS	NS	NS
TNF-α	1·20	0·57	0·59	0·87	0·26	NS	<0·05	NS
	TNF-α (ng/ml)							
	0	25	50	100				
MCT1	1·04	1·09	0·65	0·73	0·09	<0·05	NS	NS
IL-8	1·19	48·75	44·34	49·36	7·46	<0·05	<0·05	NS
TNF-α	0·91	6·87	6·52	4·89	2·73	<0·05	<0·05	NS

dietary level led to the down-regulation of *MCT1* expression in the colon of pigs, irrespective of dietary SBP inclusion. The fact that no interaction between CP and SBP was observed could be either due to data variability or the number of animals per treatment group (*n* 6). In contrast, inclusion of SBP into high-CP diets did not reduce the levels of protein-derived metabolites to similar levels as with low-CP diets[22]. Previous findings have also shown that gene expression of cytokines in the colon increased with high-CP diets, irrespective of dietary fermentable carbohydrate inclusion[17]. This is, to a certain extent, in contrast to our initial hypothesis; however, it can be concluded that in the present study, the decreased expression of *MCT1* coincided with increased luminal availability of protein fermentation products such as NH_3, and the concomitantly increased pro-inflammatory cytokine expression in the colonic mucosa.

The role of MCT1 in the transport of butyrate into colonocytes has been well studied. MCT1 is present on the luminal membrane of the human and pig colon, and is involved in the uptake of butyrate by both proton co-transport and anion-exchange mechanisms, as has been discussed in the literature[4,27,28]. It has been demonstrated that butyrate uptake is enhanced at acidic pH and inhibited or reduced by structural analogues such as acetate, propionate, L-lactate or pyruvate[4,27]. In addition to its role as a colonic fuel, butyrate is involved in the maintenance of colonic homeostasis by regulating the expression of genes linked to cellular processes, including proliferation, differentiation or apoptosis[29].

Previous studies on human colonic AA/C1 cells have revealed that butyrate increases MCT1 mRNA and protein expression[13]. Other studies have shown that MCT1 promoter activity is stimulated by Na-butyrate in a dose-dependent way, and that this is related to the NF-κB signalling pathway[14]. Coherent with these results, earlier studies have found higher *MCT1* expression in pigs fed high levels of fermentable carbohydrates that promoted higher levels of colonic butyrate[10,26]. However, the sole inclusion of SBP to a diet without an increase in colonic butyrate concentration was not able to increase *MCT1* expression in the present study. In contrast to previous studies where SBP inclusion stimulated butyrate formation in the colon[30,31], such an increase was not observed in the present study where only lactate and acetate concentrations were affected by the inclusion of dietary SBP.

If butyrate specifically promotes *MCT1* expression in the colon, the question arises why higher butyrate absorption was not accompanied by an increment in *MCT1* expression when induced by a diet high in CP. Although SCFA are mainly derived from saccharolytic fermentation, they can also be derived from amino acids, such as lysine or histidine, through reductive deamination[32]. However, apart from butyrate, our diets high in CP also increased the luminal concentrations of NH_3 and biogenic amines. The latter were inversely associated with the expression of *MCT1*, and positively correlated with pro-inflammatory cytokines. An up-regulation of pro- and anti-inflammatory cytokines in the colon of pigs has also been observed in a previous study when high-protein diets were fed[17]. Interestingly, chronic intestinal inflammation (inflammatory bowel disease) is well known to coincide with reduced *MCT1* expression in the intestinal mucosa[16]. Furthermore, treatment with the pro-inflammatory cytokines TNF-α and IFN-γ in colonic HT29 cells down-regulated *MCT1* mRNA and protein expression[16]. The present results suggest that NH_3 and other protein-derived metabolites present in the lumen may entail inflammatory responses in the colonic mucosa, which negatively influence the expression of *MCT1*. This may provide an interesting link between impaired protective effects of butyrate on the colonic epithelium and pro-inflammatory conditions in the colon of pigs fed high-protein diets. In fact, the higher butyrate concentration in the colonic lumen of piglets fed a

high-protein diet might, in part, even be a consequence of decreased butyrate absorption due to decreased *MCT1* expression. When assuming that butyrate is accumulated in the lumen because of decreased uptake by colonocytes, it follows that the intracellular concentration of butyrate in colonocytes might have been unchanged or even lower on a high-protein diet despite higher luminal concentrations. Such concept of 'cellular butyrate starvation' upon inflammatory down-regulation of *MCT1* expression is well established as a mechanism contributing to the progression of inflammatory bowel disease[16].

To further assess the individual effects of NH_3, Na-butyrate and TNF-α on the expression of *MCT1* and cytokines in the target tissue, the pig colonic mucosa was mounted on Ussing chambers. In agreement with previous reports, Na-butyrate resulted in a higher expression of *MCT1*, whereas TNF-α had an inhibitory effect[13,16]. Butyrate has anti-inflammatory properties in human intestinal epithelial cells, which is probably mediated by the inhibition of the NF-κB pathway[33,34]. Furthermore, colonic and systemic immunoreactivity was reduced after a long-term feeding experiment with resistant starch in pigs, possibly due to an increased luminal concentration of butyrate[35]. In contrast, NH_3 is a putatively toxic metabolite derived from amino acid fermentation in the large intestine. Some studies have confirmed the potential toxicity on the gut epithelium with adverse effects on both colonocyte metabolism and barrier function[1,3,19]. There is also evidence for NH_3 as a precursor of cancer and inflammatory bowel diseases in humans[36–38]. NH_3 has been shown to interfere with oxidative metabolism in colonocytes[39]. Moreover, it has been associated with a mucosal inflammatory response, which, in turn, can also reduce butyrate oxidation[40]. Therefore, a reduced expression of *MCT1* under inflammatory conditions may result in reduced uptake and oxidation of butyrate. In support of the *in vivo* findings, colonic tissue treated with NH_4Cl exhibited lower *MCT1* and up-regulated *TNF-α* gene expression. Similarly, colonic tissue had also reduced *MCT1* expression after treatment with TNF-α, while *IL-8* was markedly increased in parallel. Although no direct effect of TNF-α on MCT1 promoter activity or butyrate uptake has been reported previously in Caco-2 cells[14], the present results reinforce the notion that MCT1 is down-regulated by pro-inflammatory cytokines.

Finally, increasing concentrations of the same substrates were used to study dose-dependent effects in Caco-2 cells. The regulation of *MCT1* by Na-butyrate and TNF-α is in agreement with the results obtained from the *ex vivo* experiments and previous studies[13,14,16]. The stimulation of Caco-2 cells with increasing concentrations of Na-butyrate increased *MCT1* expression, whereas treatment with NH_4Cl and TNF-α had opposite effects, supporting the idea of the regulation of *MCT1* gene expression by protein-derived metabolites such as NH_3 and pro-inflammatory signalling. However, the underlying mechanisms need further elucidation.

In conclusion, the present study shows that diets high in CP reduce the expression of *MCT1* in the colon of pigs, even when higher concentrations of butyrate are present in the gut lumen. This effect seems to be related to an inflammatory response of the colonic mucosa triggered by metabolites derived from the bacterial fermentation of protein. While we provide data that NH_3 is one important fermentation product involved in the down-regulation of *MCT1* gene expression, the present correlation analyses from the *in vivo* study suggest that other metabolites (e.g. putrescine) could have also been involved. Further studies are required to investigate the complete portfolio of luminal protein-derived fermentation products that affect *MCT1* gene expression and their precise mechanism of action.

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