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ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 914–921
Journal of Nutritional Biochemistry

A moderate threonine deficiency affects gene expression profile, paracellular permeability and glucose absorption capacity in the ileum of piglets☆

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Received 6 February 2009; received in revised form 29 June 2009; accepted 2 July 2009

Abstract

High dietary threonine extraction by the digestive tract suggests that threonine contributes to maintain gut physiology. In the present study, we evaluated the impact of a low (6.5 g of threonine/kg diet; LT group) or a control well-balanced threonine diet (9.3 g of threonine/kg diet; C group) given to piglets for 2 weeks on ileal permeability and Na+-dependant glucose absorption capacity in Ussing chambers. The paracellular permeability was significantly increased in the ileum of LT compared to C piglets (P=.017). The Na+-dependent glucose absorption capacity showed a nonsignificant increase in the LT piglets. In addition, we analysed ileal gene expression profiles in the LT and C groups using porcine multitissue cDNA microarrays. Compared to the C piglets, the expression of 324 genes was significantly modified in the ileum of the LT piglets: 214 genes were overexpressed (145 annotated) and 110 were down-expressed (79 annotated). Among them, some are involved in immune and defense responses, energy metabolism and protein synthesis. Furthermore, microarray analysis highlights changes in the expression of the gene encoding for the sodium/glucose cotransporter (SGLT1) and of genes involved in the regulation of paracellular permeability (ZO-1, cingulin and myosin light chain kinase). In conclusion, our results indicate that a moderate threonine deficiency affects intestinal functionality.

Keywords: Threonine; Small intestine; Paracellular permeability; Gene expression

1. Introduction

Although the small intestine represents less than 5% of whole-body mass, it accounts for 25% of whole-body energy expenditure and for 20–50% of total protein turnover in pigs [1]. This high metabolic activity generates important amino acid (AA) requirements. In order to meet its requirement, the small intestine extracts part of dietary AA [2,3]. Among essential AA, threonine is extracted in greater proportion by the small intestine [3–5], suggesting that threonine is involved in intestinal functionality and maintenance. However, the metabolic fate and the functional role of threonine in the small intestine are still unclear.

The high rate of intestinal threonine extraction could be associated with protein synthesis [4] and especially with the synthesis of mucins [6–8] whose threonine content ranges from 13% to 26% of total AA [9–11]. Threonine deficiency could also impact on other functions of the small intestine. We previously demonstrated that feeding young piglets with a low threonine supply (70% of recommendations), which corresponds to a moderate deficiency, for 2 weeks induced a villous atrophy associated with a reduction in aminopeptidase N activity in the ileum [12]. Because villous atrophy is frequently associated with functional disturbances, further work was needed to determine the effect of threonine deficiency on small intestine physiology. In rats and pigs, villous atrophy caused by starvation or malnutrition, such as occurring after weaning, increased mucosal permeability and glucose absorption capacity [13–15]. Therefore, the first objective of the present study was to determine whether a moderate threonine deficiency modified small intestinal physiology. We focused on the distal part of the small intestine where we observed structural modifications. To do so, we evaluated the effect of the threonine dietary content on ileal paracellular permeability and glucose absorption capacity in Ussing chambers.

In addition, we used porcine cDNA microarrays to evaluate the impact of dietary threonine supply on global gene expression profile in the piglet ileum. With this methodology, our second objective was to identify, without *a priori*, the functions potentially affected by an inadequate dietary threonine supply. Within the frame of our work,

☆ Part of the results was presented at the Xth International Symposium on Digestive Physiology in Pigs, May 2006, Vejle, Denmark. It was published as a short paper (Hamard A, Mazurais D, Boudry G, Le Huërou-Luron I, Sève B, Le Floc'h, N. Physiological aspects and ileal gene expression profile of early-weaned piglets fed a low threonine diet. *Livestock Science*, 2007;108:17–19).

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doi:10.1016/j.jnutbio.2009.07.004

the microarray study is more exploratory and hypothesis generating than explanatory. This is particularly interesting considering the scarcity of knowledge about the implication of this AA in aspects of the small intestine physiology other than mucin synthesis.

2. Materials and methods

2.1. Animals and feeding

The experiment was conducted under the guidelines of the French Ministry of Agriculture for animal care. Seven pairs of Pietrain×(Large White×Landrace) piglets from the INRA experimental herd (Saint-Gilles, France) were weaned at 7 days of age. These pairs were constituted of littermates with close body weights (2.5±0.06 kg). From weaning, piglets were placed into individual stainless-steel cages in a room maintained at 30°C.

Within each pair, one piglet received a control well-balanced diet (C group) and the other one a low threonine diet (LT group). The composition of the diets is presented in Table 1. Protein was supplied by skimmed-milk powder and a soluble fish protein concentrate. Those raw materials set the basal threonine level in both diets. A free AA mixture without threonine was added to comply with the ideal protein pattern for weaned piglets [16]. Free threonine was added only in the C diet. The nitrogen content of the LT diet was adjusted by addition of aspartic acid and ammonium citrate. Aspartic acid was chosen as an amino nitrogen source. This dispensable AA is massively extracted by the small intestine [17] but seems to have no physiological impact such as described for glutamine or glutamic acid. Threonine content was 9.3 g/kg in the C diet and 6.5 g/kg in the LT diet. Diets provided 250 g of crude protein (N×6.25) and 15 MJ of digestible energy per kilogram [10.9 MJ of net energy (NE)].

The meals were prepared as a mash (powdered diet–warm water, 2:1) just before distribution. The daily amount of diet was adjusted to the metabolic weight (600 kJ NE/kg body weight$^{0.75}$) and given in four equal meals. The piglets were offered 50% of this daily intake the first 2 days. Water was offered *ad libitum* throughout the experiment. Piglets were weighed on experimental days 1, 4, 6, 8, 11 and 13.

2.2. Slaughtered procedure

After 2 weeks of experiment and 3 h after the last meal, piglets were killed with a lethal dose of pentobarbital immediately followed by exsanguination. The gastrointestinal tract was quickly removed. The small intestine, from the Treitz ligament to the ileo-caecal junction, was weighed empty of contents and the length was measured. It was divided into three parts of equal length, the proximal jejunum, the distal jejunum and the ileum. In the middle of each part, 3-cm segments were collected in phosphate-buffered formalin (10%, pH 7.6) for morphometric measurements. A 20-cm segment of the ileum was sampled in bicarbonate Ringer's solution (in millimoles per liter: 145 Na^+, 128 Cl^-, 0.32 PO_4^{3-}, 2 Ca^{2+}, 1 Mg^{2+}, 25 HCO_3^-, 1 SO_4^{2-}, 6.3 K^+; pH 7.4) for measurements made in Ussing chambers. Small (1 cm) pieces of the ileum were collected, rinsed with sterile saline and stored in RNAlater (Ambion, USA) at −20°C until RNA extraction.

Table 1
Ingredients and nutritional values of the experimental diets

	Diet	
	Low threonine (LT)	Control (C)
Ingredients, g/kg diet		
Skimmed milk powder	250	250
Soluble fish protein concentrate	74.3	74.3
Free amino acids mix[a]	54.9	54.9
Maltodextrins	430.15	430.44
Sunflower oil	62.37	62.37
Ammonium citrate tribasic	30	30
Bicalcium phosphate	49	49
Trace element and vitamin premix[b]	10	10
L-Aspartic acid	39.28	36.48
L-Threonine	–	2.51
Chemical analysis[c]		
Dry matter, %	92.9	92.8
Crude protein (N×6.25), %	24.4	25
Net energy, MJ/kg diet	10.9	10.9
Threonine, %	0.65	0.93

[a] Supplying the following amount of free amino acids (g/kg diet): L-lysine HCl, 3.53; L-tryptophane, 0.85; L-leucine, 1.86; L-isoleucine, 1.35; L-valine, 1.39; L-phenylalanine, 1.42; L-glutamate monoNa/glutamic acid (50/50), 35.3; glycine, 9.2.

[b] Supplying the following amount of vitamins and minerals (per kilogram of diet): Ca, 1.82 g; Fe, 200 mg; Cu, 40 mg; Zn, 200 mg; Mn, 80 mg; Co, 4 mg; Se, 0.6 mg; I, 2 mg; vitamin A, 30,000 UI; vitamin D_3, 6000 UI; vitamin E, 80 UI; vitamin B_1, 4 mg; vitamin B_2, 20 mg; panthotenic acid, 30 mg; vitamin B_6, 20 mg; vitamin B_{12}, 0.1 mg; vitamin PP, 60 mg; folic acid, 4 mg; vitamin K_3, 4 mg; biotin, 0.4 mg; choline, 1600 mg; vitamin C, 200 mg.

[c] Values are means from analysis except for net energy which was calculated according to Noblet et al. (1994) (cited in Ref. [53]).

2.3. Ileal morphometry

After fixation in phosphate-buffered formalin during 24 h at 4°C, samples were washed and stored in ethanol/water (75:25, v/v). They were stained with Schiff's reagent after dehydration according to the technique of Goodlad et al. [18]. Villous/crypt units were isolated from intestinal samples by microdissection and mounted on a glass slide in acetic acid (45%). Villous height and crypt length, width and surface were measured using image analysis (LUCIA software, Laboratory Imaging, Czech Republic). Mean values of these parameters were determined on 30 villi and crypts per sample.

2.4. Measurements of ileal glucose absorption capacity and paracellular permeability in Ussing chambers

Immediately after sampling, the seromuscular layer of ileal segments was removed by microdissection. Briefly, a circular incision of the muscular layer was performed around the segment using scissors, then the muscular layer was gently peeled back from the mucosa using tweezers. The mucosa was then opened along the mesenteric border, cut into flat sheets, then mounted in Ussing chambers with an exposed area of 1.13 cm^2. They were bathed on each side with a bicarbonate Ringer's solution with 16 mM glucose and 16 mM mannitol on the serosal and mucosal sides, respectively, and maintained at 38°C. The short-circuit current (I_{SC}) and the transepithelial resistance were measured as previously described [19]. A first set of Ussing chambers was used to estimate paracellular permeability through measuring the flux of fluorescein isothiocyanate (FITC) dextran 4000 Da (FD4) as a model molecule. This molecule was added on the mucosal side at the final concentration of 0.375 mg/ml. Its transport was monitored by sampling 500 µl of bathing solution from the serosal side at 30-min intervals for 120 min. The solution was replaced by fresh medium to maintain a constant volume within the chamber. The concentrations of FD4 in the serosal side were measured by fluorometry. In a second set of Ussing chambers, Na^+-dependent glucose absorption capacity was evaluated. Increasing amounts of D-glucose were added to the mucosal buffer every 5 min, resulting in final concentrations of 2, 4, 8, 16 and 32 mM. The addition of glucose on the mucosal side was osmotically balanced by the addition of mannitol on the serosal side. Maximal variation of the short-circuit current (ΔI_{SC}) was recorded at each concentration, and V_{max} and K_m for Na^+-dependent glucose absorption were then calculated.

2.5. RNA Extraction

Total RNA was extracted from ileal samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Concentrations of RNA were quantified by measuring absorbance at 260 nm (Multiskan Spectrum, Thermo Labsystems, France), and RNA integrity was checked using Agilent 2100 bioanalyser (Agilent Technologies, Germany).

2.6. Microarray analysis and data processing

Transcriptomic analyses were performed using nylon microarrays obtained from the Resource Center GADIE (UMR LREG, INRA, France) and encompassing 8960 clones from a multitissue porcine cDNA library (AGENAE, INRA, France). The 8960 clones spotted on the arrays represented 8800 genes, of which 60% are annotated. These arrays are recorded on the GEO Platform under the accession number GPL3729.

Labelling of cDNA complex probes, hybridization and washes were performed according to the procedures described by Mazurais et al. [20]. Briefly, after their extraction from ileum samples, total purified RNA was retro-transcripted in the presence of [α-^{33}P]dCTP for labelling. After array image acquisition (BAS 5000, Fuji), quantification of hybridization signals revealed the expression level of each of the 8960 clones (BZ Scan). Then, the expression level of each clone was first log transformed to yield normal distribution and then median centred to minimize technical variability. We selected clones which displayed significant differential expression between the C and LT groups at $P<.01$ for null hypothesis using variance analysis (GeneANOVA, CNRS, UPRESA 8087, France) [21]. The selected clones were submitted to hierarchical clustering with the Gene Cluster software [22].

2.7. Real-time PCR

Reverse transcription was performed with 2 µg of total DNAse-treated RNA (High capacity cDNA archive kit; Applied Biosystems, USA). The primers were designed using Primer Express Software (Applied Biosystems) based on *Sus scrofa* published nucleotide sequences (Iccare) and are described in Table 2. Real-time PCR was carried out on an ABI PRISM 7000 SDS thermal cycler (Applied Biosystem). Real-time PCR was performed in 25 µl of PCR buffer (SYBRGreen PCR Master Mix, Applied Biosystems) with 500 nM of each primer, 5 µl of optimized concentration of the RT reaction and 2 U of Uracyl DNA Glycosylase (Invitrogen, France). Forty cycles of PCR consisting of

Table 2
Forward and reverse primers used in RT-PCR reactions[a]

Gene	Protein name	Forward primer	Reverse primer	Accession no.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	CATCCATGACAACTTCGGCA	GCATGGACTGTGGTCATGAGTC	AF017079
TJP1	Tight junction protein ZO-1	AGGCGATGTTGTATTGAAGATAAATG	TTTTTGCATCCGTCAATGACA	CK453343
SGLT1	Sodium/glucose cotransporter 1	CCCAAATCAGAGCATTCCATTCA	AAGTATGGTGTGGTGGCCGGTT	DY417361
CGN	Cingulin	GTTAAAGAGCTGTCCATCCAGATTG	CTTAGCTGGTCTTTCTGGTCATTG	DN116728

[a] The primers were designed using Primer Express Software (Applied Biosystems) based on *Sus scrofa* published nucleotide sequences (Iccare; http://bioinfo.genopole-toulouse.prd.fr/Iccare/).

denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min were performed. Amplification product specificity was checked by dissociation curve analyses. To determine the efficiency of each primer set, a standard curve was done with serial dilutions of a pool of samples' RT products. Then for each sample, the amount of the target RNA was determined by comparison with the corresponding standard curve [23]. Finally, the amount of the target RNA was calculated relative to the GAPDH transcript level of the same sample.

2.8. Statistical analysis

For all measurements, except for transcriptomic analysis (see Microarray Analysis and Data Processing), analysis of variance was performed using the General Linear Model procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). The effects of pair (litter) and dietary threonine supply were tested using the residual variation between piglets as the error. All the results are presented as least square means±S.E.M. Differences were considered significant at $P<.05$ for null hypothesis. Trends to $.05<P<.1$ are presented and discussed when relevant for physiological interpretation.

3. Results

3.1. A threonine moderate deficiency did not affect growth rate

As expected, the average feed intakes were not significantly different between pair-fed C and LT piglets (Table 3). Threonine intake was significantly reduced by 29% in the LT piglets compared to the C piglets ($P<.0001$). The low threonine supply affected neither final body weight nor body weight gain.

3.2. A threonine moderate deficiency induced ileal villous hypotrophy

The weight and length of the small intestine were not altered by the low threonine supply (data not shown). In the proximal and distal jejunum, no modification of the mucosa morphology was observed (Table 4). In the ileum, villous height was not significantly different between LT and C piglets despite the fact that it tended to be reduced in LT piglets compared to the C piglets ($P=.06$). In accordance with this result, villous surface was reduced by 18% in LT piglets compared to the C piglets ($P<.01$).

3.3. A threonine moderate deficiency increased glucose absorption capacity

Measurements performed in Ussing chambers showed no significant difference in Na+-dependent glucose absorption capacity, measured as the I_{SC} variation (ΔI_{SC}) to graded glucose addition, between C and LT piglets. However, as illustrated by a higher dose–response curve (Fig. 1), if V_{max} was not significantly different between C and LT piglets, it increased by 81% in the ileum of LT piglets compared to the C piglets ($P=.1$; Table 5), and K_m did not change between the two groups.

3.4. A threonine moderate deficiency modified epithelial barrier function

The paracellular permeability measured in Ussing chambers was 89% increased in the ileum of LT piglets compared to the C piglets ($P=.017$; Fig. 2). Moreover, despite no statistical significance, the reduced threonine supply decreased transepithelial resistance by 30% (Fig. 3).

3.5. A threonine moderate deficiency affected ileal transcriptome

A 30% reduction of dietary threonine supply significantly affected the expression of 324 genes ($P<.01$): 214 genes were overexpressed (145 annotated) and 110 were down-expressed (79 annotated) in LT piglets. Differentially expressed genes ($P<.01$) are listed in Supplemental Tables 1 and 2. The fold changes of down-expressed genes in LT piglets ranged between 0.42 and 0.78. Fold changes for over-expressed genes ($P<.01$) ranged between 1.51 and 3.00 except for SGLT-1 whose expression was 4.9-fold increased in the LT group.

Differentially expressed genes were classified according to their biological process ontology determined from the Uniprot/Swiss-Prot database and the QuickGO Gene Ontology browser (http://www.ebi.ac.uk/ego/). Some genes were not classified in a functional group and for some others no informative annotation was available (Supplemental Tables 1 and 2 in Appendix A).

Feeding a reduced threonine supply for 2 weeks increased the expression of genes involved in immune and inflammatory responses such as the complement C1s subcomponent (C1S), the MHC class I antigen (HLA-B), the T-cell differentiation antigen CD6 (CD6), the C-C motif chemokine 16 (CCL16) and chemokine receptors (IL17RB, CCR4, DARC). We also noted the overexpression of genes coding the selenoprotein W (SEPW1), the beta-defensin 129 (DEFB129), the microsomal glutathione S-transferase 1 (MGST1) or the mucin 1 (MUC1); these proteins play a crucial role in antimicrobial or antioxidative defenses.

Feeding a low threonine diet also affected the expression of genes involved in cell turnover. The gene encoding IGF2 was overexpressed, whereas several genes acting as inhibitor of cell proliferation (BTG1 protein, BTG1; Pin2-interacting protein X1, PINX1; Forkhead box protein C1, FOXC1) were down-expressed in the ileum of LT piglets. The expression of two genes involved in the induction of apoptosis, the BH3 interacting domain death agonist (BID) and the death-associated protein kinase 1 (DAPK1), was increased.

The expression of genes coding the sodium/potassium/calcium exchanger 4 (SLC24A4), the phospholemnan (PXYD1), the amiloride-sensitive sodium channel beta-subunit (SCNN1B) as well the Y+L AA transporter 1 (SLC7A7) and the sodium/glucose cotransporter 1 (SGLT-1) was significantly increased in the ileum of LT piglets. The increase in SGLT-1 mRNA expression was confirmed by RT-PCR (2.04-fold, $P<.05$) (Fig. 4). This could indicate modifications in the transport of ions and nutrients. Modifications in the expression of genes involved in the intracellular protein transport were also observed. For example, genes encoding the kinectin (KTN1), the centractin (ACTR1B), the transmembrane protein 9 precursor (TMEM9), the Golgin subfamily A member 5 (GOLGA5), the importin alpha-1 subunit (KPNA1) were overexpressed, whereas genes coding the adapter-related protein complex 3 delta 1 subunit (AP3D1), the charged multivesicular body protein 1a (PCOLN3), the vacuolar protein sorting-associated protein 33B (VPS33B) or the kinesin-like protein KIF2 (KIF2A) were down-expressed in the ileum of LT piglets.

Table 3
Growth performance of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet) or a low threonine diet (LT: 6.5 g threonine/kg diet) for 2 weeks

	Diet		S.E.M.	P
	C	LT		
Initial weight, kg (Day 0)	2.52	2.51	0.01	ns
Final weight, kg (Day 14)	4.35	4.29	0.08	ns
BW gain, kg/day	0.169	0.171	0.004	ns
Feed intake, g/kg $BW^{0.75}$ per day	51.7	51.8	0.74	ns
Thr intake, g/kg $BW^{0.75}$ per day	0.48	0.34	0.006	<.0001

Piglets fed the LT diet exhibited increased ileal expression of genes involved in cell adhesion (tight junction protein ZO-1, TJP1; cingulin, CGN; paxillin, PXN; cadherin EGF LAG seven-pass G-type receptor 2, CELSR2; plectin 1, PLEC1; collagen alpha 1, CO9A1; integrin α5, ITGA5) and communication (ephrin A-4, EFNA4; gap junction β5, GJB5) as well as in cytoskeleton organisation (neurofilament triplet M protein, NEFM; tropomodulin, TMOD1; tropomyosin 1, TPM1; Wiskott–Aldrich syndrome protein interacting protein homolog, WASIP). The significant increase in the expression of ZO-1 and cingulin (CGN) was confirmed by RT-PCR analysis: the relative levels of ZO-1 and CGN mRNA were 26% and 36% higher in LT piglets (Fig. 4), although differences did not reach significance. Lack of significance could be explained by a high variability between individuals and would need to be confirmed using a larger number of animals.

LT piglets also displayed modifications in the expression of genes involved in transcriptional and translational processes of protein synthesis. For example, genes coding the DNA-directed RNA polymerase II 140-kDa polypeptide (POLR2B), the RNA polymerase-associated protein 1 (PAF1), the transcription initiation factor IIE alpha subunit (GTF2E1) and the transcription initiation factor IIB (GTF2B) were overexpressed. On the contrary, the eukaryotic translation initiation factor 2-alpha kinase 4 (GCN2), known to inactivate eIF2, and the eukaryotic translation initiation factor 4A-binding protein 1 (EIF4EBP1), known to inactivate eIF4, were down-expressed. The expression profile of these genes could be indicative of an increase in protein synthesis rate. The LT diet also induced modifications of transcription factors regulating expression of specific target genes (KLF9, ZNF644, ZNF169, ZFP161, ZFP37, ZNF429). Most of the genes involved in mRNA splicing were down-regulated (PRMT5, RBM9, SF1, SFRS5, SRRM1, STRAP, LSM2). Genes involved in RNA metabolism such as mRNA stability (SERBP1) or mRNA degradation (EDC3) were also differentially expressed in the ileum of LT piglets.

The LT diet altered the ileal expression of genes involved in the cellular protein metabolism. Apart from genes involved in regulation of translation (noticed above), we identified genes involved in protein folding (Dnaj homolog subfamily B member 9, DNJB9; peptidyl-prolyl *cis-trans* isomerase, PPIF; prefoldin subunit 2, PFDN2; torsin A, TOR1A) and protein catabolism (STIP1 homolog and U box-containing protein 1, STUB1; mitochondrial processing peptidase beta subunit, MPPB; F-box/wd-repeat protein 4, FBXW4; CAAX prenyl protease 1 homolog, ZMPSTE24; ubiquitin carboxyl-terminal hydrolase BAP1, BAP1; proteasome subunit beta type 3, PSMB3; probable E3 ubiquitin-protein ligase TRIP12, ubiquilin, UBQLN1, etc.).

Finally, we also showed the differential expression of genes involved in fatty acid metabolic process (carnitine *O*-acetyl transferase, CACP; carnitine *O*-palmitoyltransferase I, CPT1B; peroxisomal-coenzyme A synthase, FAT2; peroxisomal 3,2-*trans*-enoyl-coenzyme A isomerase, PECI; fatty acid-binding protein, epidermal, FABP5; dihydroxyacetone phosphate acyltransferase, GNPAT), in generation of energy (ATP synthase O subunit, ATP5O; NADH-ubiquinone oxidoreductase 13kDa-B subunit, NDUFA5) or in signal transduction (calcitonin receptor precursor, CALCR; GTPase-activating protein, GAP; calcium/calmodulin-dependent protein kinase type II beta chain, CAMK2B; insulin receptor substrate 1, IRS1; phosphatidylinositol 4-kinase alpha, PIK4CA; phosphatidylinositol-4phosphate 5-kinase type I gamma, PIP5K1C; tyrosine-protein kinase JAK1; mitogen-activated protein kinase 8, JNK1, etc.).

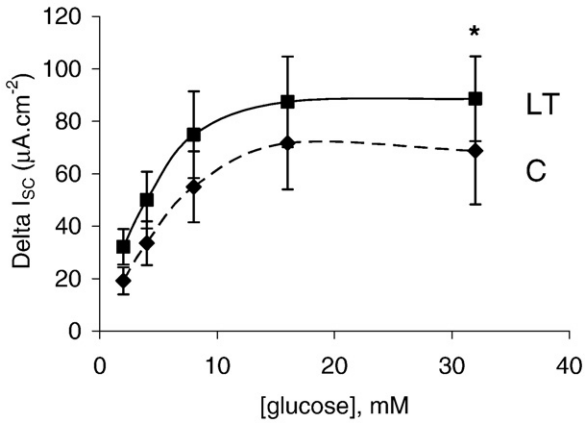

Fig. 1. Variation of ΔI_{SC} in response to increasing dose of glucose, in the ileum of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet; dotted line) or a low threonine diet (LT: 6.5 g threonine/kg diet; full line) for 2 weeks. Tissues were mounted in Ussing chambers and graded doses of glucose were added to the mucosal side every 5 min, osmotically balanced on the serosal side by mannitol. The maximal increase in I_{SC} (ΔI_{SC}) after addition of each dose of glucose was recorded. Values are LSmeans±S.E.M., n=7. *Difference between LT and C piglets, P<.05.

Table 4
Small intestinal morphology of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet) or a low threonine diet (LT: 6.5 g threonine/kg diet) for 2 weeks

	Diet		S.E.M.	P
	C	LT		
Jejunum proximal				
Villous height, µm	623	653	36	ns
Villous surface, µm^2	105,008	99,303	7389	ns
Crypt depth, µm	149	145	6	ns
Jejunum distal				
Villous height, µm	568	586	39	ns
Villous surface, µm^2	89,384	86,907	6189	ns
Crypt depth, µm	161	156	7	ns
Ileum				
Villous height, µm	591	518	23	.06
Villous surface, µm^2	81,668	67,197	2589	.007
Crypt depth, µm	150	146	4	ns

4. Discussion

We evaluated the impact of a moderate threonine deficiency: threonine supply was reduced by 30% in LT piglets compared to the C piglets. This distinguishes our work from others which tested severe deficiency below 50% of threonine recommendations [7,8]. Compared to severe threonine deficiency that can be suspected to induce indirect effects, a moderate threonine deficiency caused physiological

Table 5
Glucose-induced changes in short-circuit current in the ileum of early weaned piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet) or a low threonine diet (LT: 6.5 g threonine/kg diet) for 2 weeks

	Diet		S.E.M.	P
	C	LT		
V_{max}, µA/cm^2	68.98	124.83	19.54	.10
K_m, mM	4.93	4.10	0.91	.55

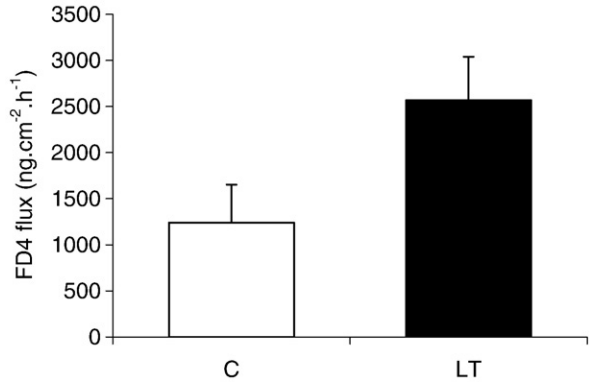

Fig. 2. FITC dextran 4000 Da flux (ng/cm^2 per hour) across the ileum of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet, black bar) for 2 weeks. Tissues were mounted in Ussing chambers. FITC dextran 4000 (FD4) was added on the mucosal side at the final concentration of 0.375 mg/ml. Its transport was monitored by sampling solution from the serosal side at 30-min intervals for 120 min. After measuring FD4 concentrations in the samples, the flux over the 120-min period was calculated. Values are LSmeans±S.E.M., n=7. *Difference between LT and C piglets, P<.05.

variations in threonine availability. Moreover, when analyzing the plasma AA profile, we previously showed that threonine was the only AA for which plasma concentrations remained lower in LT than in C piglets throughout the experimental period [12], confirming that threonine was the first limiting AA. Therefore the modifications we observed can be attributed specifically to threonine deficiency.

Despite the fact that the difference was not significant in the present experiment, we showed that a low threonine supply induced ileal villous hypotrophy, which is in accordance with what we reported in a previous experiment [12]. After the piglets were fed a moderate threonine-deficient diet for 2 weeks, villous atrophy was not observed in the proximal part of the intestine [12]. This does not exclude structural alterations that might have occurred earlier. As observed in weaned piglets [24], villous height recovery could occur in the proximal part of the intestine quite early, before reaching the ileum later. Ileal villous hypotrophy was associated with alterations of functionality. Indeed, a novel finding of the present study is that a 30% reduced threonine supply enhanced ileal paracellular permeability as measured by the mucosa-serosa FD4 flux. Such an increase was previously reported in piglets encountering nonoptimal nutritional conditions, receiving total parenteral nutrition [25], submitted to 48 h

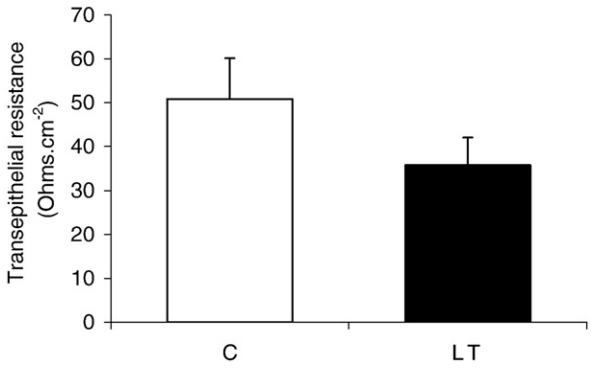

Fig. 3. Transepithelial resistance (Ω/cm^2) in the ileum of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet) for 2 weeks. Tissues were mounted in Ussing chambers and the transepithelial resistance measured after 20 min of equilibrium. Values are LSmeans ±S.E.M., n=7.

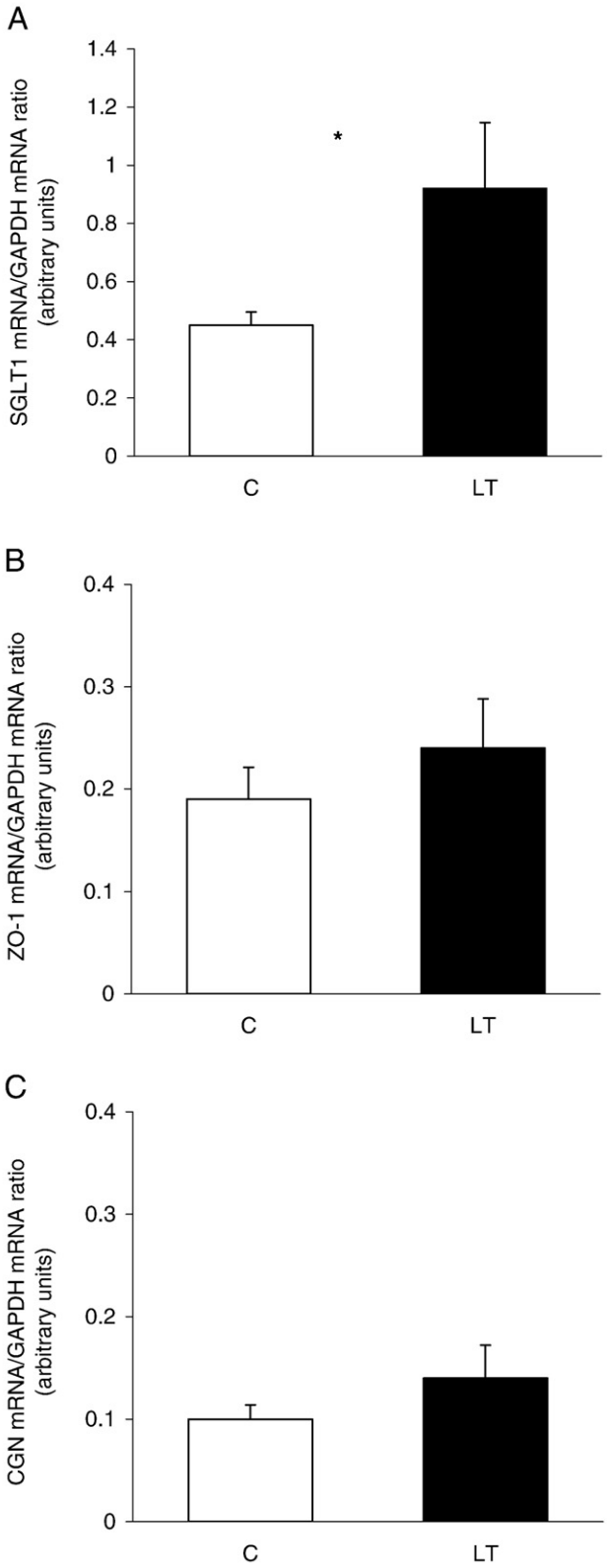

Fig. 4. Relative mRNA abundance of the sodium/glucose cotransporter 1 (SGLT-1, A), the tight junction protein (ZO-1, B) and cingulin (CGN, C) in the ileum of piglets pair-fed either a well-balanced control diet (C: 9.3 g threonine/kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet, black bar) for 2 weeks. Target gene was expressed relatively to GAPDH level. Values are LSmeans±S.E.M., n=7. *Difference between LT and C piglets, P<.05.

of fasting [26] or in response to undernutrition associated with weaning [15].

Increased paracellular permeability reflects a reduction in epithelial barrier selectivity and, consequently, a greater susceptibility to antigens' passage across the intestinal epithelium even if not associated with clinical signs [14]. Piglets fed the LT diet presented neither diarrhea nor a feverish episode. They consumed all their feed and their weight gain was not affected. These results are in accordance with our previous observations [12,27] in which a 30% reduction in threonine supply did not induce important alterations in piglet growth and in the development of piglets and their intestine. This can be explained by the low growth rate of the experimental piglets (170 g/day) compared to suckling piglets (200 to 250 g/day). The potential of growth was not fully expressed in our experiment because the energy intake was lower than in suckling piglets. Together with the shortness of the experimental period, this may have limited the reduction of growth rate that could occur with marginal threonine deficiency. Moreover, the good sanitary and nutritional experimental conditions have probably minimized the incidence of gut permeability and morphology modifications on piglet growth and health.

Despite the absence of clinical signs of inflammation, the analyses performed with cDNA microarrays showed that genes coding the complement C1s subcomponent (C1S), the MHC class I antigen (HLA-B), the T-cell differentiation antigen CD6 (CD6), the C-C motif chemokine 16 (CCL16) or chemokine receptors (IL17RB, CCR4, DARC) were overexpressed in the ileum of LT piglets. This might reflect an immune response to the passage of antigens through the intestinal epithelium. For example, the overexpression of genes coding chemokines and chemokine receptors characterises an inflammatory state [28]. CCL16 is known to be a powerful proinflammatory chemokine that is expressed in ulcerative colitis [29]. Moreover, feeding the LT diet induced increased expression of genes encoding for mucins [30], *S*-glutathione-transferase 1, the selenoprotein W or a defensin. These proteins play a crucial role in intestinal protection [31–33]. Overexpression of MUC1 mRNA is of particular interest because threonine utilisation by the gut is generally associated with mucin synthesis (MUC2 and MUC3 were not represented on our microarrays). Mucin production is increased during infection [34] or as a result of inadequate nutritional conditions [35].

Microarray analysis revealed transcriptional modifications of factors controlling the paracellular permeability (ZO1, cingulin and MLCK). Cingulin and ZO1 are important components of the tight junction which is the major element of the paracellular pathway. These two proteins belong to the complex structure coupling the transmembrane sealing protein (occludin and claudins) and the actin network [36]. They play a pivotal role in the structural and functional organization of the tight junction. Impaired intestinal permeability has been associated with lower expression of ZO-1 in pathophysiological conditions [37–39]. The role of cingulin in the regulation of paracellular permeability remains to be confirmed. Myosin light chain kinase (MLCK) allows the phosphorylation and the contraction of the perijunctional actomyosin ring leading to increased paracellular permeability [40]. Overexpression of these genes is expected to be associated with decreased paracellular permeability in the ileum of LT piglets, which is apparently inconsistent with the physiological data we obtained with Ussing chambers. Indeed, genes encoded for ZO1 and cingulin were up-expressed in LT piglets. The physiological consequences of these results need to be discussed with caution knowing that, firstly, ZO1 and cingulin overexpression was not statistically confirmed by RT-PCR and, secondly, that modification of a biological process results from regulations occurring at different levels: transcription or depletion of one or few genes, translation or post-translational modifications. Furthermore, a delay may exist between modifications in mRNA expression and physiological response. So, we hypothesized that cingulin and ZO1 overexpression and MLCK down-expression observed in the ileum of LT piglets could indicate an attempt to restore epithelial barrier function in response to functional changes.

Restoration of barrier function implied different processes such as cell proliferation and migration [41]. Integrins play a crucial role in these processes. In our experiment, several genes encoding for actors of the integrin signalling pathway (PAK4, MLCK and WIP, integrin α5, paxillin) were differentially expressed in the ileum of LT piglets compared to the C piglets. The gene coding the integrin α5 was overexpressed in the ileum of LT piglets. The increase in mRNA expression of integrin α5 promotes cell adhesion to fibronectin and cell migration in various cell types [42–45]. In the intestine, the role of integrin α5 in cell proliferation, notably during repetitive deformation [46,47], has been explored. The fixation between the integrin and extracellular matrix proteins leads to the recruitment of proteins such as the paxillin to the cellular membrane and the subsequent activation of p21-activated kinases such as PAKs involved in cytoskeletal rearrangement [48]. Genes coding the paxillin and the PAK4 isoform were overexpressed in the ileum of LT piglets. Finally, the gene coding the WIP, an important actin-binding protein that participates in the deformation of the actin network for migration [49], was overexpressed. Overall, the expression profile of these genes may prefigure the activation of the integrin pathway and supports the hypothesis of barrier restoration.

The overexpression of the SGLT-1 gene associated with the increased glucose absorption capacity measured in Ussing chambers demonstrated that threonine deficiency stimulated glucose absorption via an increase in SGLT-1 transporter expression. Indeed, the lack of effect on K_m indicated no change in the affinity of the transporter for its substrate. The trend for an increase in V_{max} could be due to either an increase in the number of SGLT-1 transporters or to an increase in Na^+-K^+-ATPase activity. An increase in glucose absorption has already been observed in other situations such as a 48-h fast [26] or after weaning [15]. Glucose is a major source of energy for body tissues and notably for the small intestine in young animals [50]. So we hypothesized that an increase in glucose absorption capacity reflects an increased energy demand in the small intestine, or in peripheral tissues, or in both of LT piglets. In support of our hypothesis, two genes involved in energy generation were also differentially expressed: the gene coding the ATP synthase O subunit, a component of the mitochondrial proton-translocating ATP synthase complex, and the gene coding the NADH-ubiquinone oxidoreductase 13-kDa B subunit from the mitochondrial respiratory chain complex I. Additionally or otherwise, it appears that the contribution of glucose to intestinal energy production depends on age. Darcy-Vrillon et al. [51] showed that the capacity of cultured porcine enterocytes to use glucose was high during the first week of life and decreased during the second week when the small intestine used mainly AA. Therefore that change in energy supplier may have been delayed in LT piglets. The effect of threonine deficiency on energy metabolism in the small intestine is probably an indirect effect. Indeed, threonine is not degraded in this tissue but mainly used for protein synthesis [4]. Thus, the effect of threonine deficiency on energy metabolism in the small intestine cannot be explained by the lower provision of threonine as an energetic substrate.

We showed that a low threonine supply induced structural and functional alterations. These modifications could result from an alteration in protein synthesis rate. In accordance with this hypothesis, Wang et al. [8] found that protein synthesis rate was reduced in the small intestine of piglets receiving either 50% or more than 100% of daily threonine recommendations. In our experiments with only 30% reduced threonine supply, we found no alteration [27]. The use of transcriptomic analysis allowed us to identify genes coding regulatory

factors of protein synthesis that were differentially expressed in the ileum of LT piglets. The down-regulation of genes coding the eukaryotic translation initiation factor 2-alpha kinase 4 (GCN2) and the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) is of particular interest. These genes are implied in the down-regulation of mRNA translation. Firstly, GCN2 prevents the formation of the 43S preinitiation complex (Met-tRNA, GTP and eIF2) by phosphorylating the translation initiation factor eIF2α. Secondly, 4E-BP1 inhibits the assembly of the eIF4E-mRNA complex to the 40S ribosomal subunit by binding to the eukaryotic initiation factor 4E (eIF4E). These two factors are assumed to be implied in the down-regulation of protein synthesis by AA starvation. For example, *in vitro* leucine deprivation induced activation of these factors and, consequently, inhibition of the initiation phase of mRNA translation [52]. In our study, the down-regulation of these genes was expected to be associated with an increase or an attempt to increase protein synthesis rate through a feedback regulation process. Regarding the lack of effect on fractional synthesis rate that we previously reported [27], we may hypothesize that the down-expression of GCN2 and 4E-BP1 in the ileum of pigs fed the LT diet could be a mechanism for preserving protein synthesis under the condition of moderate threonine deficiency.

In conclusion, this study demonstrates for the first time that a 30% reduced threonine supply for 2 weeks induced increased paracellular permeability and glucose absorption capacity. Moreover, transcriptomic analysis showed that a moderate threonine deficiency altered ileal gene expression profiles. Because some of the differences reported in this article did not reach a statistical significant level, these differences need to be confirmed using for instance a greater number of animals. Nevertheless, these transcriptional modifications opened new pathways of investigation. Notably, the increase in the expression of genes involved in immune and defense functions associated with the increased paracellular permeability suggests that threonine may be essential to preserve intestinal integrity. Therefore the response of the piglets to a reduced threonine supply should be evaluated in aggression situations in order to provide irrefutable evidence for a protective role of this AA on a stressed intestine. Finally, we assume that, when young piglets were fed a moderate threonine-deficient diet, a number of adaptive mechanisms aimed at preservation of the intestinal development, but that functionality may be affected in the first place, before tissue and animal growth.

Acknowledgments

We would like to thank the GADIE Center (UMR314, LREG INRA-CEA, Jouy-en-Josas Cedex, France) for producing the porcine microarray. We also thank Veronique Romé, Cécile Perrier and Romain d'Inca for technical assistance, and Yves Lebreton, Francis Le Gouevec and Vincent Piedvache for animal care.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2009.07.004.

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