

Dietary calcium concentration and cereals differentially affect mineral balance and tight junction proteins expression in jejunum of weaned pigs

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Abstract

Ca plays an essential role in bone development; however, little is known about its effect on intestinal gene expression in juvenile animals. In the present study, thirty-two weaned pigs (9.5 (SEM 0.11) kg) were assigned to four diets that differed in Ca concentration (adequate *v.* high) and cereal composition (wheat–barley *v.* maize) to assess the jejunal and colonic gene expression of nutrient transporters, tight junction proteins, cytokines and pathogen-associated molecular patterns, nutrient digestibility, Ca balance and serum acute-phase response. To estimate the impact of mucosal bacteria on colonic gene expression, Spearman's correlations between colonic gene expression and bacterial abundance were computed. Faecal Ca excretion indicated that more Ca was available along the intestinal tract of the pigs fed high Ca diets as compared to the pigs fed adequate Ca diets ($P < 0.05$). High Ca diets decreased jejunal zonula occludens 1 (*ZO1*) and occludin (*OCN*) expression, up-regulated jejunal expression of toll-like receptor 2 (*TLR2*) and down-regulated colonic *GLUT2* expression as compared to the adequate Ca diets ($P < 0.05$). Dietary cereal composition up-regulated jejunal *TLR2* expression and interacted ($P = 0.021$) with dietary Ca on colonic *IL1B* expression; high Ca concentration up-regulated *IL1B* expression with wheat–barley diets and down-regulated it with maize diets. Spearman's correlations ($r > 0.35$; $P < 0.05$) indicated an association between operational taxonomic units assigned to the phyla Bacteroidetes, Firmicutes and Proteobacteria and bacterial metabolites and mucosal gene expression in the colon. The present results indicate that high Ca diets have the potential to modify the jejunal and colonic mucosal gene expression response which, in turn, interacts with the composition of the basal diet and mucosa-associated bacteria in weaned pigs.

Key words: Calcium: Intestinal mucosa: Gene expression: Dietary cereal: Juvenile pigs

In addition to the well-established critical role that sufficient Ca intake plays in skeletal development and health^(1,2), there has been growing evidence that dietary Ca has further benefits, commencing in the intestinal lumen, where Ca was shown to exert anti-inflammatory and cytoprotective effects when it was available at higher luminal concentrations^(3–5). These effects were linked to the high buffering property of

Ca, which abrogated negative aspects of intense fermentation in the intestinal lumen on mucosal integrity in rodent models⁽⁵⁾. Moreover, luminal Ca is essential for intestinal epithelial function, because it affects the composition of intestinal plasma membranes and para- and transcellular Ca transport either directly via the modulation of signalling pathways or indirectly via the modification of the vitamin D endocrine

Abbreviations: ATTD, apparent total tract digestibility; *GLUT2*, solute carrier family 2 (facilitated glucose transporter), member 2; *INFG*, interferon- γ ; LPS, lipopolysaccharide; *OCN*, occludin; OTU, operational taxonomic units; *SGLT1*, solute carrier family 5 (sodium/glucose cotransporter), member 1; *SMCT*, sodium-coupled monocarboxylate transporter; *TGF β 1*, transforming growth factor- β 1; *TLR*, toll-like receptor; *ZO1*, zonula occludens 1.

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response^(6,7). In adult rodents, the colon was proposed as the main intestinal site of action for Ca⁽⁸⁾. In a recent study with weaned pigs, however, we found that the main responding intestinal site was the small intestine and not the colon⁽⁹⁾. Overall, Ca effects on the intestinal mucosa in the juvenile phase have not been explored in detail, and there is little information available about the potential effects of high Ca intake on the intestinal digestive, absorptive, immunal and barrier functions of young animals. Young mammals, like pigs, are very vulnerable to intestinal perturbations in the immediate phase after weaning because of the immaturity of their mucosal immunity, digestive capacity and microbiota^(10,11), and they may benefit from the anti-inflammatory and cytoprotective effects of extra dietary Ca. The composition of the dietary complex carbohydrate fractions may mitigate the effect of high dietary Ca supply either by stimulating intestinal Ca absorption or by complexing Ca intestinally⁽⁹⁾. Whole grains are typical sources of dietary fibre in human and animal diets; however, common dietary cereals, such as wheat, barley and maize, largely differ in their cellulose and hemicellulose fractions⁽¹²⁾. Therefore, potential Ca effects on intestinal mucosal functions should be investigated when considering differences between the complex carbohydrate fractions of the basal diet to account for the interaction between dietary Ca concentration and dietary cereals. Thus, we hypothesised that a dietary Ca intake that is much higher than the actual requirement may reduce intestinal pro-inflammatory cytokine expression and also up-regulate the expression of nutrient transporters and tight junction proteins in the small and large intestines of young pigs and that dietary cereal composition would interact with dietary Ca concentration in intestinal gene expression.

Aside from direct regulation^(10,13), dietary ingredients modulate the intestinal mucosal response by altering the intestinal microbiota and their metabolites which are generated during the fermentation of diet-derived substrates⁽¹⁴⁾. Consequently, dietary composition, including carbohydrate fractions and micronutrient concentrations, can be targeted to selectively stimulate bacteria with known health-promoting effects for the host^(14–18). Due to their ultimate proximity to the enterocytes, it is accepted that intestinal mucosa-associated microbiota have a significant impact on mucosal gene and protein expression⁽¹⁴⁾. Considering these findings, we further hypothesised that the mucosal expression of genes would be connected to specific mucosa-associated bacteria and luminal microbial metabolites independently of the composition of the diet fed to the animals.

To test our hypotheses, the present study was designed to investigate the effect of dietary Ca concentration on the expression of candidate genes that target nutrient absorption, barrier function and mucosal inflammatory response in the jejunum and colon of male weaned pigs. In addition, indirect estimates of digestion and absorption, which were based on apparent total tract digestibility (ATTD) and the retention of nutrients, were applied to study the influence of Ca on intestinal nutrient uptake, and serum acute-phase proteins were determined as markers of systemic inflammation. To evaluate potential Ca × dietary cereal interactions on intestinal mucosa

and nutrient digestion, two basal diets with contrasting cereals (wheat–barley *v.* maize) were formulated. The relationship between mucosal gene expression and mucosa-associated bacteria and luminal microbial metabolites was assessed for the colon using recently published data^(17,18).

Materials and methods

The experimental design and methods have been described in detail recently^(17–19).

Animals and experimental design

All procedures involving animal handling and treatment were approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the national authority according to §8ff of the Law for Animal Experiments, Tierversuchsgesetz – TVG (GZ 68.205/0222-II/3b/2011). In the present study, weaned barrows (*n* 32; (Landrace × Large White) × Piétrain; average body weight 9.5 (SEM 0.11) kg; weaning age 28 d) were randomly allocated to four diets (*n* 8/diet) comprising either wheat and barley or maize as cereals and containing either adequate or high concentrations of Ca (Table 1). The experiment consisted of nine blocks with eight pigs each, with two pigs per run being fed the same diet. In each run, pigs had *ad libitum* access to the experimental diets for 15 d from day 8 post-weaning (experimental day 1). We used castrated male pigs in order to be able to collect urine and faeces separately.

Diets met or exceeded the current nutrient requirements for pigs between 10 and 20 kg⁽²⁰⁾ (Table 1) and were formulated to be isoenergetic and isonitrogenous. Because of the higher fat concentration in maize, diets differed in their fat source and thus their fatty acid composition. Wheat–barley diets contained 3% soya oil, whereas maize diets contained only 1.5% soya oil. Diets adequate in Ca were formulated to meet 100% of the pigs' Ca and P requirements, and high Ca diets were formulated to contain Ca and P at a concentration of 190% of the Ca and P requirement for 10–20 kg pigs⁽²⁰⁾. The calculated Ca and P concentration of adequate Ca diets amounted to 8.0 g Ca and 6.1 g P/kg diet (DM basis), whereas the calculated Ca and P concentration of high Ca diets were 15.1 g Ca and 11.7 g P/kg diet (DM basis). Thus, the calculated Ca:P ratio was 1.3:1 for all diets so that any potential Ca effects on the intestinal mucosa would not be compromised by a diverging Ca:P ratio, as reported in rats⁽⁴⁾. Microbial phytase was added to the diets to compensate for the different intrinsic phytase activities in wheat, barley and maize⁽²¹⁾. Representative diet samples were taken in each experimental block. Feed leftovers (spillage and feed left in the trough) were collected each morning at 08.00 hours before fresh feed was provided. Animals had free access to demineralised water. Pigs were weighed at the commencement, on day 9 and day 15 (end) of the experiment. One pig that had been fed the wheat–barley diet high in Ca was removed from the experiment because it developed meningitis at the commencement of the experiment.

Table 1. Ingredients (as-fed basis) and analysed chemical composition of the experimental diets^(16–18)

Ingredient (g/kg)	Wheat–barley diet		Maize diet	
	Adequate Ca	High Ca	Adequate Ca	High Ca
Maize	–	–	494.0	494.0
Wheat	352.0	352.0	–	–
Soyabean meal	280.0	280.0	335.0	335.0
Barley	182.0	182.0	–	–
Dextrose	50.0	50.0	50.0	50.0
Talkum	33.2	2.7	34.3	4.0
Saccharose	30.0	30.0	30.0	30.0
Soya oil	30.0	30.0	15.0	15.0
Limestone	13.0	19.5	12.5	19.3
Salt	10.0	10.0	10.0	10.0
Monocalcium phosphate	7.5	31.5	8.5	32.0
Vitamin–mineral premix*	6.3	6.3	6.3	6.2
Lys	3.5	3.5	2.4	2.5
L-Thr	1.5	1.5	1.0	1.0
D,L-Met	1.0	1.0	1.0	1.0
Phytase (FTU/kg)†	500	500	500	500
Analysed chemical composition (DM basis, g/kg)				
DM	929	917	921	929
Crude protein	222	223	222	218
Crude ash	94	96	95	96
Crude fibre	36	37	36	36
Xylose	16	18	11	12
β-Glucan	9	9	1	1
Neutral-detergent fibre	117	122	112	104
Acid-detergent fibre	85	79	83	68
Total starch	302	310	299	301
Ca	8.2	14.8	8.4	14.1
P	6.0	11.9	6.6	11.7
Ca:P ratio	1.37	1.24	1.27	1.21
Gross energy (Mcal/kg)	4.00	4.01	4.05	3.99
Calculated chemical composition (DM basis, g/kg)‡				
Metabolisable energy (MJ/kg)	15.11	15.15	15.22	15.27
Metabolisable energy (Mcal/kg)	3.61	3.63	3.63	3.65

* Provided per kg of complete diet: 10 000 IU (3000 µg) of vitamin A, 2222 IU (56 µg) of vitamin D₃, 62.5 mg of vitamin E, 1.67 mg of vitamin B₁, 4.45 mg of vitamin B₂, 2.22 mg of vitamin B₆, 0.022 mg of vitamin B₁₂, 2.2 mg of vitamin K, 22.2 mg of niacin, 11.11 mg of pantothenic acid, 500 mg of choline chloride, 0.05 mg of biotin, 0.56 mg of folic acid, 25 mg of vitamin C, 44 mg of Mn (as MnO), 89 mg of Zn (as ZnSO₄), 153 mg of Fe (as FeSO₄), 13 mg of Cu (as CuSO₄), 0.44 mg of Se (as Na₂SeO₃), 1.67 mg of I (as Ca(IO₃)₂).

† Included 500 phytase units (FTU) per kg of complete diet.

‡ Calculated according to Gesellschaft für Ernährungsphysiologie⁽¹⁹⁾.

Balance and digestibility study

After 9 d of dietary treatment, pigs' faeces and urine were quantitatively collected from day 10 to day 14 for determination of nutrient digestibility and N, Ca and P balances. The total amounts of faeces and urine produced were recorded during collection. Fresh faecal samples were collected several times daily and frozen at –20°C. Urine was collected in buckets containing 25 ml of 18.75 M-H₂SO₄ (VWR International) to minimise microbial fermentation for 24 h, weighed and filtered. Representative subsamples (250 ml) were pooled over the 4 d collection period, kept at 4°C during the collection days and stored at –20°C until analysis.

Sampling of blood and jejunal and colonic mucosa

On day 15 of the experiment, 3–4 h after receiving fresh feed, animals were anaesthetised (Narketan, 10 ml/kg body weight; Ketamine HCl; Vétoquinol AG and Stresnil, 3 ml/kg body weight; Azaperone; Biokema SA); blood was collected by cardiac puncture into serum collection tubes (Primavette; KABE Labortechnik) and placed on ice until further processing.

Blood samples were centrifuged at 1811 g for 10 min at 4°C (Eppendorf Centrifuge 5810 R; Eppendorf) and stored at –20°C until analysis. Immediately after blood withdrawal, pigs were euthanised by an intracardiac injection of T61 (10 ml/kg, Embutramide; MSD Animal Health) and exsanguinated. The abdominal cavity was opened and the entire gastrointestinal tract was removed. For candidate gene expression, the mid-jejunum (30 cm in the middle of the jejunum) and mid-colon (the middle of the colon after division into three equal segments) were selected as important intestinal sites for digestion and fermentation, respectively. Gut pieces were opened at the mesentery, and digesta were removed. Gut tissues were thoroughly washed in ice-cold PBS, and the mucosa was scraped off using a glass plate. Mucosa samples were immediately snap-frozen in liquid N₂ and stored at –80°C.

Gene expression profiling

Total RNA was extracted and the mRNA expression in jejunal and colonic tissue were determined using quantitative

reverse-transcription PCR, which has been described recently⁽¹⁹⁾. The amplification of target genes for sugar transporters (solute carrier family 2 (facilitated glucose transporter), member 2 (*GLUT2*) and solute carrier family 5 (sodium/glucose cotransporter), member 1 (*SGLT1*)), monocarboxylate transporters (monocarboxylate transporter 1 (*MCT1*) and sodium-coupled monocarboxylate transporter (*SMCT*)), tight junction proteins (occludin (*OCLN*) and zonula occludens 1 (*ZO1*)), cytokines (*IL1B*, *IL6*, *IL10*, interferon- γ (*IFNG*), *TNFA* and transforming growth factor- β 1 (*TGFBI*)) and pathogen-associated molecular patterns (toll-like receptor (*TLR*)2 and *TLR4*) as well as house-keeping genes (β -actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β -2 microglobulin (*B2M*) and ornithine decarboxylase antizyme 1 (*OAZ1*); see online supplementary Table S1) were performed on a ViiA 7 Real-Time PCR system (Life Technologies). Reaction conditions for complementary DNA synthesis and quantitative PCR using the fluorescent DNA stain EvaGreen (Biotium) have been described elsewhere⁽¹⁹⁾. Reverse transcription controls (RT minus) were included in order to control for residual DNA contamination, and experiments were performed in duplicates. Data were normalised to the expression of the three most suitable house-keeping genes (*ACTB*, *GAPDH* and *OAZ*), which were identified using the geNorm algorithm⁽²²⁾. Relative gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method⁽²³⁾.

Chemical analysis

Before proximate analysis, faecal samples were thawed at 4°C and homogenised per pig, and a subsample was taken for DM determination. The remaining faeces were freeze-dried. Diet and freeze-dried faeces were ground into a particle size of less than 1 mm before analysis. Chemical analyses of diets and faeces were performed using classical methods⁽²⁴⁾ and commercial enzymatic kits, which have been described by Metzler-Zebeli *et al.*^(17,19). Porcine-specific commercial ELISA kits were used to analyse serum concentrations of the acute-phase proteins haptoglobin and serum amyloid A (GenWay). Serum acute-phase proteins were selected on the basis of previously published data⁽²⁵⁾. Ca and P in serum was determined by enzymatic colorimetric analysis using an autoanalyser for clinical chemistry (Cobas 6000/c501; Roche Diagnostics GmbH).

Calculations

The ATTD of nutrients was calculated as:

$$\text{ATTD (\%)} = \left(\frac{\text{Daily nutrient intake} - \text{daily nutrient excretion in faeces}}{\text{daily nutrient intake}} \right) \times 100.$$

Daily nutrient excretion in faeces was defined as the amounts of Ca, P and N minerals present in the faeces and urine per d. Daily apparent retention was calculated as the difference between daily nutrient intake and daily nutrient excretion in faeces and urine.

Statistical analyses

To compare the differences between dietary Ca concentrations and cereals, data were subjected to ANOVA using the procedure PROC MIXED of SAS (Statistical Analysis System version 9.3; SAS Institute, Inc.). Fixed effects in the model included the main effects of Ca concentration, dietary cereal, their two-way interaction, the experimental run and the random effect of pig (n 31) nested within sow (n 16). The pig was considered the experimental unit. The Kenward–Rogers method was used to approximate df (ddfm = kr). Data of mucosal gene expression collected from the same pig but at various gut sites were considered as spatial repeated measures using various variance–covariance matrix structures to take into consideration close associations among repeated measures. Gut site repeated measures and corresponding two-way and three-way interactions were analysed. Because there were significant gut site effects on the expression of all genes of interest, except *TLR2* expression, gut sites were also individually analysed. Moreover, data on body weight, average daily gain and feed intake from the same pig but on different days of the experimental period were analysed using time-repeated measures and corresponding interactions. Means were reported as least squares means with their standard errors. Differences were considered significant at $P \leq 0.05$ and as trend at $0.05 < P \leq 0.10$.

Linear discriminant analysis by JMP Pro (version 10.0; SAS Institute, Inc.) was applied to demonstrate potential relationships between diets and mucosal gene expression in the jejunum and colon using gene expression as the covariate and diet as the categorical variable. To illustrate the associations between colonic gene expression and mucus-associated bacteria and their metabolites, Spearman's rank correlation analysis was performed in JMP. For the relation between mucosal gene expression and mucus-associated bacteria, Spearman's rank correlation for the fifty most abundant operational taxonomic units (OTU) (published in Mann *et al.*⁽¹⁷⁾) and gene expression were estimated. In Mann *et al.*⁽¹⁸⁾, Roche/454-sequencing paired reads were analysed using the software package mothur⁽²⁶⁾, and OTU were assigned based on a sequence similarity threshold of 97%. Due to the short reads generated by Roche/454-sequencing, the cut-off of 97% was taken as a proxy for species-level divergence⁽²⁷⁾ so that the overall species diversity would not be overestimated, which might have been the case with a more stringent cut-off of 98 or 99%. Obtained OTU were blasted against National Center for Biotechnology Information GenBank numbers to give an approximation of species classification (closest reference strains). Also, Spearman's rank correlations were computed for the relationships between colonic bacterial metabolites (published by Metzler-Zebeli *et al.*⁽¹⁷⁾) and gene expression. SCFA concentrations in colonic digesta were analysed using GC, whereas lipopolysaccharides (LPS) were measured using a pyrochrome *Limulus* amoebocyte lysate assay. The *Limulus* amoebocyte lysate assay determines total LPS and does not differentiate between the LPS of different immune reactivities present in the sample.

Results

Dietary composition, feed intake and growth performance

Chemical analyses showed that Ca concentrations were 0.2–0.3 g/kg diet (DM basis) higher for adequate Ca diets and 0.3–1.0 g/kg diet lower for high Ca diets as compared to the calculated dietary Ca concentration (Table 1). Consequently, actual dietary Ca concentrations corresponded to 103, 186, 104 and 176% of the Ca requirement of 10 kg pigs for the wheat–barley diet adequate in Ca, the wheat–barley diet high in Ca, the maize diet adequate in Ca and the maize diet high in Ca, respectively. The P concentration of the maize diet adequate in Ca was 0.6 g P/kg diet (DM basis) higher, whereas the other three diets closely met the calculated P values (Table 1). Thus, actual P concentrations corresponded to 98, 195, 108 and 192% of the P requirement of 10 kg pigs for the wheat–barley diet adequate in Ca, the wheat–barley diet high in Ca, the maize diet adequate in Ca and the maize diet high in Ca, respectively. Dietary Ca:P ratios were 1.37:1, 1.24:1, 1.27:1 and 1.21:1 for the wheat–barley diet adequate in Ca, the wheat–barley diet high in Ca, the maize diet adequate in Ca and the maize diet high in Ca, respectively (Table 1). As indicated by the time × cereal interaction ($P=0.032$), pigs that were fed the maize-based diets had a greater body weight and a 60 g higher average daily gain ($P<0.05$) than pigs that were fed the wheat–barley-based diets, even though the daily feed intake was similar among the feeding groups (Table 2).

Apparent total tract digestibility and nitrogen and mineral balances

To establish the actual effect of dietary Ca concentration and cereal composition on digestive and absorptive processes, we determined the ATTD of DM, organic matter, protein, fat, ash, xylose, β -glucan, Ca and P as well as Ca, P and N retention (Tables 3 and 4). When maize was the cereal source, pigs had a 1.5, 5.5 and 7.3% higher ($P<0.05$) ATTD of DM, ash and Ca, and they also tended to have greater ($P<0.1$) ATTD of organic matter and fat as compared to the pigs fed wheat–barley-based diets (Table 3). The dietary Ca concentration influenced the pigs' ATTD of DM and ash, resulting in 1.8 and 13% higher ($P<0.001$) ATTD of DM and ash with the high and adequate Ca diets, respectively. The ATTD of protein

(Table 3), N intake, and faecal and urinary N excretion (Table 4) did not differ among the diets; however, daily N retention tended ($P=0.074$) to be greater by 1.7 g in the pigs that were fed the maize diet as compared to the wheat–barley diet.

Daily Ca ($P<0.001$) and P ($P<0.001$) intake was greater by 4 and 4.8 g with the high Ca diets as compared to the adequate Ca diets, respectively (Table 4). The pigs fed the high Ca diets excreted more ($P<0.001$) Ca and P via faeces than those fed the adequate Ca diets, whereas only urinary P excretion increased ($P<0.001$) with the high Ca diets as compared to the adequate Ca diets. Although total daily absorption of Ca and P were higher with the high Ca diets as compared to the adequate Ca diets, high Ca diets slightly decreased ($P=0.037$) the ATTD of Ca by 6.4% as compared to the adequate Ca diet (Table 3). The pigs fed the high Ca diets had a 2.7 and 2.3 g higher ($P<0.001$) total daily Ca and P retention than the pigs fed the adequate Ca diets, respectively (Table 4). The pigs fed maize diets had a 1.1 g higher ($P=0.009$) Ca retention as compared to those fed the wheat–barley diets. Cereal source did not influence P retention, but P intake was 0.8 g higher ($P=0.042$) in the pigs fed maize diets than it was in the pigs fed the wheat–barley diets.

Serum parameters

Acute-phase response was not stimulated by either dietary Ca concentration or cereal composition, as was indicated by the similar serum haptoglobin concentrations among the pigs and serum amyloid A concentrations that were below the detection limit (Table 3). There was a cereal × Ca interaction ($P=0.028$), which shows that high Ca concentration reduced serum Ca concentration when it was added to the wheat–barley diets, whereas with maize diets, it increased the serum Ca concentration. Serum P concentration was raised ($P<0.001$) in the pigs fed the high Ca diets as compared to those fed adequate Ca diets, but the effect was smaller when pigs were fed the maize diets instead of the wheat–barley diets, as was indicated by the cereal × Ca interaction ($P=0.049$).

Mucosal gene expression in the jejunum and colon

Generally, mucosal expression of *SGLT1*, *GLUT2*, *MCT1* and *SMCT* was higher ($P<0.05$) in the jejunum than in the colon (see online supplementary Table S2). Mucosal expression of

Table 2. Body weight, average daily feed intake (ADFI) and average daily gain (ADG) of weaned pigs fed wheat–barley or maize diets adequate or high in calcium

(Mean values with their standard errors; n 8 per diet, except wheat–barley diet high in calcium n 7)

	Wheat–barley diet		Maize diet		SEM	<i>P</i>		
	Adequate Ca	High Ca	Adequate Ca	High Ca		Cereal	Ca	Cereal × Ca
Body weight (kg)*								
Day 1	9.6	9.4	9.6	9.5	0.25	0.260	0.453	0.816
Day 15	16.9	16.4	18.0	17.3	0.51			
ADG (g)†	488	466	559	518	23.0	0.013	0.189	0.696
ADFI (g)†	679	631	725	702	37.4	0.132	0.357	0.737

* Data on body weight were analysed using time-repeated measures over time, day $P<0.001$, time × Ca $P=0.282$, time × cereal $P=0.032$ and time × Ca × cereal $P=0.680$.

† Data on ADG and ADFI for experimental days 1–8 and 9–15 can be found in Metzler-Zebeli *et al.*⁽¹⁹⁾. Data on ADG and ADFI were analysed using time-repeated measures.

Table 3. Apparent total tract digestibility (ATTD) of nutrients and serum haptoglobin, calcium and phosphorus concentration in weaned pigs fed wheat–barley or maize diets adequate or high in calcium

(Mean values with their standard errors; *n* 8 per diet, except wheat–barley diet high in calcium *n* 7)

	Wheat–barley diet		Maize diet		SEM	<i>P</i>		
	Adequate Ca	High Ca	Adequate Ca	High Ca		Cereal	Ca	Cereal × Ca
ATTD (%)								
DM	86.5	87.7	87.4	89.9	0.66	0.027	0.009	0.348
Organic matter	88.4	88.2	88.7	90.4	0.67	0.074	0.290	0.144
Ash	59.0	73.5	65.8	78.2	1.73	0.003	<0.001	0.550
Fat	69.3	73.9	74.4	75.0	1.66	0.077	0.136	0.242
Protein	86.0	85.2	86.1	87.4	0.87	0.195	0.766	0.231
Xylose	44.8	52.1	31.0	36.5	8.74	0.104	0.472	0.919
β-Glucan	97.4	97.6	97.0	92.8	1.76	0.152	0.262	0.232
Ca	73.2	67.4	80.5	73.5	2.90	0.031	0.037	0.831
P	72.9	76.7	77.0	77.8	1.58	0.116	0.152	0.355
Serum haptoglobin (mg/l)	246	226	205	204	86.3	0.721	0.904	0.914
Serum Ca (mmol/l)	3.1 ^a	2.9 ^{a,b}	2.8 ^b	2.9 ^{a,b}	0.07	0.170	0.300	0.028
Serum P (mmol/l)	1.9 ^b	2.8 ^a	2.5 ^{a,b}	2.9 ^a	0.13	0.009	<0.001	0.049

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

ZO1 was higher in the jejunum than in the colon, whereas that of *OCN* was lower in the jejunum than in the colon ($P < 0.05$; see online supplementary Table S2). Except for *TLR4* and the cytokines *IL10* and *INFG*, whose mucosal expression showed the opposite effect, the expression of *IL1B*, *IL6* and *TGFB1* ($P < 0.05$) as well as that of *TNFA* ($P < 0.1$) was higher in the colon than in the jejunum (see online supplementary Table S2). Mucosal expression of *TLR2* was similar in jejunal and colonic tissue. The principal effects of differences in dietary Ca intake occurred on the jejunal expression of tight junction proteins and *TLR2*. Expression of *OCN* ($P < 0.031$) and *ZO1* ($P < 0.044$) was down-regulated in the jejunal mucosa of the pigs fed the high Ca diets as compared to those fed the adequate Ca diets (Fig. 1(a) and (b)). Mucosal expression of

TLR2, in turn, was up-regulated ($P < 0.016$) in the jejunum of the pigs fed the high Ca diets and was three to four times higher than that of the pigs fed the adequate Ca diets (Table 5). Also, the basal diet affected *TLR2* expression; there was greater ($P < 0.039$) *TLR2* expression in the jejunal mucosa of the pigs fed the wheat–barley diets as compared to those fed the maize diets. There was a tendency for a Ca × cereal interaction ($P < 0.092$) for *INFG* expression, indicating that with the wheat–barley diet, high Ca concentration down-regulated jejunal *INFG* expression, whereas high Ca concentration up-regulated its expression with the maize diets. Dietary Ca concentration and basal diet did not affect sugar and monocarboxylate transporter expression in the jejunal mucosa of the pigs, whereas there was a trend ($P = 0.076$)

Table 4. Nitrogen, calcium and phosphorus balances in weaned pigs fed wheat–barley or maize diets adequate or high in calcium

(Mean values with their standard errors; *n* 8 per diet, except wheat–barley diet high in calcium *n* 7)

	Wheat–barley diet		Maize diet		SEM	<i>P</i>		
	Adequate Ca	High Ca	Adequate Ca	High Ca		Cereal	Ca	Cereal × Ca
N balance (g/d)								
N intake	29.2	26.8	29.3	29.6	1.26	0.268	0.405	0.268
Faecal N excretion	4.1	4.0	4.1	3.8	0.29	0.633	0.483	0.760
Urinary N excretion	4.3	4.1	3.8	4.3	0.34	0.667	0.707	0.291
Total N excretion	8.4	8.1	7.9	8.1	0.54	0.597	0.884	0.615
N absorption	25.2	25.2	22.8	25.2	1.12	0.175	0.449	0.201
N retention	20.8	18.7	21.4	21.5	0.91	0.074	0.291	0.239
Ca balance (g/d)								
Ca intake	6.8	10.9	7.0	12.0	0.43	0.142	<0.001	0.301
Faecal Ca excretion	1.8	3.6	1.4	3.2	0.35	0.296	<0.001	0.917
Urinary Ca excretion	0.59	0.44	0.40	0.49	0.12	0.555	0.834	0.318
Total Ca excretion	2.4	4.0	1.8	3.7	0.39	0.269	<0.001	0.695
Ca absorption	5.0	7.3	5.6	8.7	0.38	0.013	<0.001	0.282
Ca retention	4.4	6.8	5.2	8.2	0.39	0.009	<0.001	0.450
P balance (g/d)								
P intake	5.0	9.0	5.4	10.0	0.33	0.042	<0.001	0.458
Faecal P excretion	1.3	2.1	1.2	2.2	0.14	0.804	<0.001	0.444
Urinary P excretion	0.12	1.21	0.12	1.37	0.21	0.683	<0.001	0.698
Total P excretion	1.4	3.3	1.4	3.6	0.23	0.600	<0.001	0.403
P absorption	3.6	6.9	4.2	7.7	0.29	0.028	<0.001	0.640
P retention	3.5	5.7	4.1	6.4	0.32	0.820	<0.001	0.868

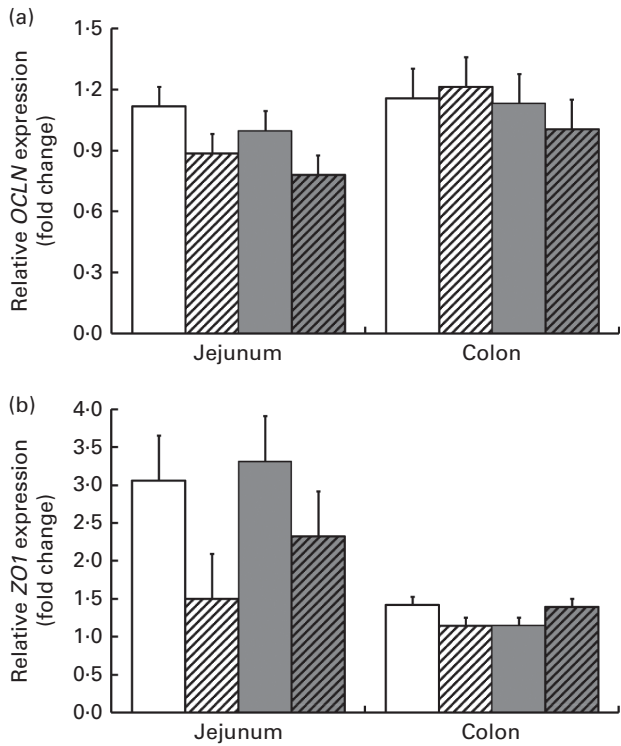


Fig. 1. Relative expression of (a) occludin (*OCLN*) and (b) zonula occludens 1 (*ZO1*) in the jejunal and colonic mucosa of weaned pigs fed wheat–barley or maize diets adequate or high in Ca. Values are means with their standard errors; *n* 8 per diet, except wheat–barley diet high in calcium *n* 7, are represented by vertical bars. (a) Main effects on *OCLN* expression: jejunum, calcium *P* = 0.031, cereal *P* = 0.269, calcium × cereal *P* = 0.0.942; colon, calcium *P* = 0.816, cereal *P* = 0.443, calcium × cereal *P* = 0.543; gut site effect *P* = 0.028, gut site × calcium *P* = 0.509, gut site × cereal *P* = 0.443, gut site × calcium × cereal *P* = 0.843. (b) Main effects on *ZO1* expression: jejunum, calcium *P* = 0.044, cereal *P* = 0.371, calcium × cereal *P* = 0.638; colon, calcium *P* = 0.884, cereal *P* = 0.921, calcium × cereal *P* = 0.950, gut site *P* < 0.001, gut site × calcium *P* = 0.028, gut site × cereal *P* = 0.341, gut site × calcium × cereal *P* = 0.619. □, wheat–barley diet adequate in calcium; ▨, wheat–barley diet high in calcium; ■, maize diet adequate in calcium; ▩, maize diet high in calcium.

for a down-regulation of *SMCT* expression in the colonic mucosa of the pigs fed the high Ca diets as compared to those fed the adequate Ca diets (Table 6). As indicated by the Ca × cereal interaction (*P* = 0.021), the mucosal *IL1B* expression responded differently to the dietary Ca concentration depending on the cereal source fed to the pigs. Mucosal *IL1B* expression was up-regulated by high Ca concentration when combined with a wheat–barley diet, but it was down-regulated when combined with a maize diet as compared to the respective adequate Ca diets. There was a gut site × Ca interaction (*P* < 0.028) for *ZO1* expression, which indicates that *ZO1* expression was down-regulated by high Ca diets in the jejunum but not in the colon (Fig. 1), although no further gut site × Ca, gut site × cereal or gut site × Ca × cereal interaction could be found for the expression of the other target genes (data not shown).

The linear discriminant analysis of diets and mucosal gene expression in the jejunum (Fig. 2(a)) and the colon (Fig. 2(b)) showed that diet modulated mucosal gene expression and separated the effects of the diets into two

and four clusters, respectively. In the jejunum, diets clustered completely separately from one other. The jejunal expression of *TLR2*, *TNFA*, *INFG*, *TGFB1* and *OCLN* discriminated best for the wheat–barley diet with adequate Ca concentration, whereas the expression of *TLR4* and *SMCT* was more associated with the wheat–barley diet high in Ca. The maize diet adequate in Ca, in turn, correlated to jejunal *IL1B* expression, and the maize diet high in Ca correlated to the jejunal expression of *MCT1*, *GLUT2*, *SGLT1*, *IL10*, *IL6* and *ZO1*. In the colon, the wheat–barley diet adequate in Ca clustered separately, whereas the other three diets formed one cluster according to their overlapping 95% CI. In the colon, the wheat–barley diet adequate in Ca was clearly related to the mucosal expression of *TGFB1*, which distinguishes it from the other three diets.

Spearman's rank correlations between bacteria and mucosal candidate gene expression in the colon

To identify potential associations between the mucosa-attached bacteria, microbial metabolites and mucosal gene expression in the colon, we performed Spearman's rank correlation. Only Spearman's rank correlation coefficients showing a significant correlation are presented (Tables 7 and 8). Correlations ranged from *r* ± 0.36 to ± 0.62, and correlations were stronger for microbial metabolites. Out of the fifty most abundant OTU, twelve mucosa-associated OTU positively or negatively correlated to the colonic expression of sugar and monocarboxylate transporters, cytokines, pathogen-recognition receptors and tight junction proteins (Table 7). Microbial metabolites (SCFA and LPS) positively or negatively correlated with the colonic expression of cytokines, *TLR* and *GLUT2* (Table 8).

Discussion

The present results demonstrated that dietary Ca concentration modified the jejunal expression of genes related to mucosal permeability and pathogen-associated molecular patterns in young barrows. In addition, dietary cereal composition appeared to interact with the Ca effects on mucosal gene expression response, as was indicated by the distinguishable patterns exhibited by the two Ca concentrations and basal diets in the linear discriminant analysis scores plots, particularly for the jejunum, and as was indicated by the observed Ca × cereals interactions. Correlation coefficients substantiated the link between mucosa-associated bacteria and microbial metabolites and colonic gene expression response. Because we tried to keep the dietary Ca:P ratio similar between the four experimental diets, the effects of Ca and P cannot be seen separately in the present study. Moreover, we would like to point out that we used castrated male pigs, and results obtained in the present study thus might not be totally transferable to young female pigs or intact males, as the P requirements of pigs between 10 and 20 kg differ between the sexes^(21,28).

To explore the potential Ca effects on digestion and mucosal response in the small and large intestines of young pigs, it was critical to maintain contrasting Ca and P concentrations

Table 5. Relative expression of cytokines and pathogen-recognition receptors in jejunal and colonic mucosa of weaned pigs fed wheat–barley or maize diets adequate or high in calcium

(Mean values with their standard errors; *n* 8 per diet, except wheat–barley diet high in calcium *n* 7)

	Wheat–barley diet		Maize diet		SEM	<i>P</i>		
	Adequate Ca	High Ca	Adequate Ca	High Ca		Cereal	Ca	Cereal × Ca
Jejunum								
<i>IL1B</i>	0.85	0.74	0.77	0.78	0.101	0.879	0.628	0.580
<i>IL6</i>	0.39	0.42	0.52	0.43	0.112	0.533	0.766	0.604
<i>IL10</i>	2.63	2.88	2.14	2.36	0.388	0.197	0.560	0.974
<i>INFG</i>	3.37 ^a	2.24 ^b	1.82 ^b	2.80 ^{a,b}	1.581	0.396	0.899	0.092
<i>TNFA</i>	1.31	1.24	1.59	1.56	0.298	0.320	0.868	0.937
<i>TGFB1</i>	0.92	0.73	1.05	1.01	0.270	0.464	0.669	0.768
<i>TLR2</i>	0.88	3.33	0.43	1.22	0.582	0.039	0.016	0.172
<i>TLR4</i>	1.98	2.70	1.76	1.97	0.374	0.211	0.228	0.510
Colon								
<i>IL1B</i>	2.73 ^b	5.95 ^a	3.99 ^{a,b}	3.30 ^b	0.793	0.390	0.123	0.021
<i>IL6</i>	1.72	1.93	2.20	1.97	0.249	0.297	0.965	0.381
<i>IL10</i>	1.40	1.69	1.87	1.64	0.178	0.244	0.862	0.157
<i>INFG</i>	0.98	1.51	1.06	1.31	0.311	0.844	0.222	0.671
<i>TNFA</i>	1.86	1.96	2.31	1.64	0.261	0.815	0.285	0.153
<i>TGFB1</i>	1.43	1.47	1.65	1.30	0.135	0.868	0.252	0.156
<i>TLR2</i>	1.21	1.69	1.31	1.46	0.202	0.735	0.130	0.423
<i>TLR4</i>	1.35	1.55	1.60	1.48	0.157	0.559	0.802	0.318

INFG, interferon- γ ; *TGFB1*, transforming growth factor- β 1; *TLR*, toll-like receptor.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

along the gastrointestinal tract with the two dietary concentrations of Ca and, as a consequence, also of P, and according to the results of the faecal excretion of Ca and P, we were successful in doing so. Both elements are necessary for the formation of the Ca-phosphate complex in the intestinal lumen⁽⁵⁾. In rats, the beneficial cytoprotective effects of high Ca diets in the intestinal lumen have been principally linked to the buffering properties of this Ca-phosphate complex, and the colon has been identified as the main intestinal site that responds to Ca action^(4,5). In line with our previous study⁽⁹⁾, the intestinal site where we observed a stronger Ca effect on mucosal gene expression was the small intestine; fewer Ca effects were visible for the colonic mucosa. In contrast to our assumption that high dietary Ca intake would

up-regulate the expression of genes related to mucosal barrier function, we found a substantial down-regulation of *OCN* and *ZO1* expression in the jejunum with the high Ca diets as compared to the adequate Ca diets, whereas the expression of *OCN* and *ZO1* in the colon was unaffected by the dietary Ca concentration. Bearing in mind the potential translational modification of mRNA, findings of a down-regulation in the expression of genes that encode for tight junction proteins in the jejunum might be counterproductive in the juvenile phase, given that proper barrier function has been suggested as a key mechanism for the development of intestinal disease⁽²⁹⁾. However, we did not see an enhanced serum acute-phase response or intestinal translocation of LPS⁽¹⁷⁾, which would have been a sign of higher intestinal

Table 6. Relative expression of nutrient transporters related to sugar and fermentation acid absorption in the jejunum and the colon of weaned pigs fed wheat–barley or maize diets adequate or high in calcium

(Mean values with their standard errors; *n* 8 per diet, except wheat–barley diet high in calcium *n* 7)

	Wheat–barley diet		Maize diet		SEM	<i>P</i>		
	Adequate Ca	High Ca	Adequate Ca	High Ca		Cereal	Ca	Cereal × Ca
Jejunum								
<i>GLUT2</i>	0.96	0.93	1.14	1.06	0.135	0.271	0.705	0.866
<i>SGLT1</i>	7.21	7.79	8.43	8.71	1.305	0.421	0.740	0.910
<i>MCT1</i>	1.50	1.31	1.23	1.70	0.355	0.876	0.696	0.363
<i>SMCT</i>	1.06	1.36	1.11	1.26	0.168	0.885	0.186	0.647
Colon								
<i>GLUT2</i>	0.07	0.09	0.21	0.03	0.061	0.206	0.475	0.130
<i>SGLT1</i>	1.10	0.94	1.08	0.94	0.096	0.884	0.131	0.928
<i>MCT1</i>	1.00	0.82	0.77	0.92	0.098	0.556	0.878	0.108
<i>SMCT</i>	1.16	0.95	1.08	0.94	0.507	0.631	0.076	0.722

GLUT2, solute carrier family 2 (facilitated glucose transporter), member 2; *SGLT1*, solute carrier family 5 (sodium/glucose cotransporter), member 1; *MCT1*, monocarboxylate transporter 1; *SMCT*, sodium-coupled monocarboxylate transporter.

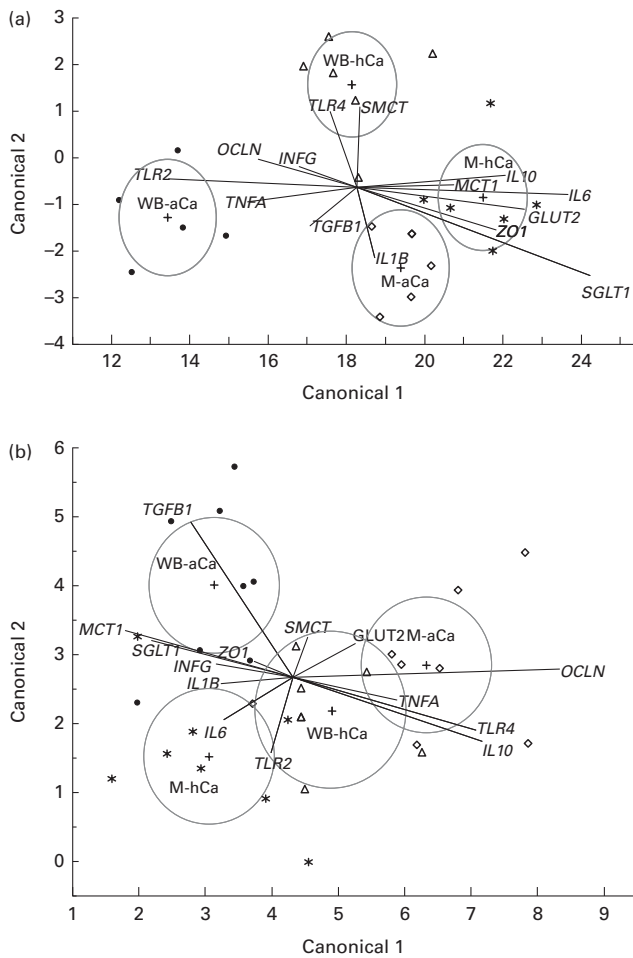


Fig. 2. Linear discriminant analysis of the wheat–barley and maize diets adequate or high in calcium and data of gene expression in (a) the jejunum and (b) the colon of weaned pigs (n 31). ●, Wheat–barley diet adequate in calcium (WB-aCa); △, wheat–barley diet high in calcium (WB-hCa); ◇, maize diet adequate in calcium (M-aCa); *, maize diet high in calcium (M-hCa); +, treatment mean. Circles indicate 95% confidence intervals. *TLR*, toll-like receptor; *OCLN*, occludin; *INFG*, interferon- γ ; *SMCT*, sodium-coupled monocarboxylate transporter; *TGF β 1*, transforming growth factor- β 1; *MCT1*, monocarboxylate transporter 1; *GLUT2*, solute carrier family 2 (facilitated glucose transporter), member 2; *ZOI*, zonula occludens 1; *SGLT1*, solute carrier family 5 (sodium/glucose cotransporter), member 1.

permeability. Thus, it is possible that alterations in gene expression were not translated into functional protein. We did not scrutinise Ca absorptive pathways in the present study; however, the down-regulation in the expression of *ZOI* and *OCLN* also may have been related to the intestinal availability and absorption of Ca^(30–32). In pigs, the main site of active Ca absorption is the proximal small intestine⁽³³⁾. Transport proteins expression at the apical site of the enterocyte strongly regulates active transcellular Ca uptake, which is adjusted to luminal Ca availability^(33,34). The latter has also been proposed to influence the rate of paracellular Ca transport by modifying the gene expression of tight junction proteins which form paracellular channels for selective ion transfer between neighbouring epithelial cells in the small intestine^(31,32,35). Evidence is growing for the cooperative functioning of active and passive Ca transport pathways

via the regulation of the expression of tight junction proteins by transcellular calbindin-D_{9K} protein expression⁽³²⁾. Diets high in Ca were reported to reduce active transcellular Ca uptake via the down-regulation of membrane Ca channels and calbindin proteins expression in the upper small intestine⁽³⁵⁾. Further studies should therefore look more closely at the link between intestinal transcellular and paracellular Ca transfer pathways in juvenile animals, such as weaned pigs, and explore whether there is an association between the regulation of paracellular Ca transport and the barrier function of the intestinal mucosa, thereby focusing not only on gene expression but also on functional protein expression.

Intestinal monocarboxylate transporters expression is substrate induced^(36,37). Therefore, lower substrate availability due to the binding of SCFA to Ca-phosphate may explain the down-regulated expression of *SMCT* in the colonic mucosa of the pigs fed the high Ca diets as compared to those fed the adequate Ca diets. Colonic SCFA concentrations were not altered by dietary Ca concentration⁽¹⁷⁾. The present findings are in line with the trend of a lower ileal *MCT1* expression which was recently observed in weaned pigs fed high Ca diets as compared to those fed low Ca diets⁽⁹⁾. The beneficial cytoprotective effects of high Ca diets, such as the down-regulation of pro-inflammatory cytokine expression, which have been suggested by previous studies in rats^(3,4) and weaned pigs⁽⁹⁾ were not as clearly visible. However, present findings demonstrated that the cereal composition of the diet interacted with the Ca effect on the mucosal expression of pro-inflammatory cytokines, as indicated by the jejunal *INFG* expression and colonic *IL1B* expression. In previous research in rodents^(3,4,8) and weaned pigs⁽⁹⁾, animals were fed semi-purified diets based on purified starch and casein. Thus, interactions between dietary Ca \times composition of the basal diet (corn starch–casein diets *v.* cereal–soyabean meal-based diets) may explain the diverging mucosal gene expression responses between previous studies and the present experiment. Moreover, differences in the dietary Ca and P concentrations may have also played a role in the varying results between the present and previous studies^(4,8,9). For instance, in our earlier study in weaned pigs, dietary Ca concentrations were lower compared to those in the present study; they corresponded to 60 and 125% of the daily Ca requirement, respectively⁽⁹⁾.

High Ca diets improved the ATTD of DM, which may be mostly a result of improved ash digestibility, as indicated by the similar ATTD of organic matter as compared to adequate Ca diets. Maize, wheat and barley differ in their major nutrient fractions (i.e. carbohydrates, dietary fibre, proteins and fat), which leads to differences in their major nutrient digestibilities^(38,39) and systemic nutrient metabolism⁽¹⁹⁾. The higher ATTD of DM and N retention was therefore very likely the reason for the enhanced growth of the pigs fed the maize diets as compared to the pigs fed the wheat–barley diets. Despite the fact that dietary fat concentration was small, it is possible that diverging fatty acid profiles caused by the different dietary fat origins may have influenced the digestibility of Ca, because dietary Ca forms insoluble Ca soaps with fatty

Table 7. Spearman's correlation coefficients for colonic gene expression and mucosa-associated bacterial operational taxonomic units (OTU) in the colon of pigs fed wheat–barley or maize diets adequate or high in calcium

	Closest reference strain (GenBank accession no.)*	Gene	<i>r</i>	<i>P</i>
OTU 1	<i>Lactobacillus johnsonii</i> (JN012223)	<i>SMCT</i>	−0.36	0.050
OTU 2	<i>Lactobacillus amylovorus</i> (EF120375)	<i>SMCT</i>	0.36	0.049
OTU 3	<i>Lactobacillus mucosae</i> (AB425938)	<i>IL1B</i>	−0.39	0.034
OTU 3	<i>Lactobacillus mucosae</i> (AB425938)	<i>TGFB1</i>	−0.56	0.001
OTU 3	<i>Lactobacillus mucosae</i> (AB425938)	<i>TNFA</i>	−0.46	0.011
OTU 4	<i>Lactobacillus delbrueckii</i> (AB680073)	<i>TGFB1</i>	−0.37	0.046
OTU 5	<i>Prevotella copri</i> (AB649279)	<i>ZO1</i>	0.37	0.043
OTU 8	<i>Prevotella copri</i> (AB649279)	<i>SGLT1</i>	−0.52	0.003
OTU 18	<i>Prevotella copri</i> (NR_040877)	<i>TLR2</i>	−0.41	0.026
OTU 27	<i>Lachnospiraceae</i> bacterium DJF_RR61 (EU728764)	<i>MCT1</i>	−0.42	0.022
OTU 27	<i>Lachnospiraceae</i> bacterium DJF_RR61 (EU728764)	<i>SMCT</i>	−0.44	0.015
OTU 89	<i>Eubacterium</i> sp. F1 (EU281854)	<i>SMCT</i>	0.38	0.037
OTU 155	<i>Prevotellaceae</i> bacterium DJF_VR15 (EU728784)	<i>TLR2</i>	−0.38	0.043
OTU 351	<i>Helicobacter rappini</i> (AY034817)	<i>OCNL</i>	−0.38	0.038
OTU 351	<i>Helicobacter rappini</i> (AY034817)	<i>ZO1</i>	−0.37	0.045
OTU 624	<i>Campylobacter lanienae</i> (JX912520)	<i>TGFB1</i>	0.52	0.003
OTU 624	<i>Campylobacter lanienae</i> (JX912520)	<i>TNFA</i>	0.47	0.009
OTU 2498	<i>Prevotella ruminicola</i> (AF218618)	<i>TGFB1</i>	0.46	0.012

SMCT, sodium-coupled monocarboxylate transporter; *TGFB1*, transforming growth factor-β 1; *ZO1*, zonula occludens 1; *SGLT1*, solute carrier family 5 (sodium/glucose cotransporter), member 1; *TLR2*, toll-like receptor 2; *MCT1*, monocarboxylate transporter 1; *OCNL*, occludin.

*Mucosa-associated OTU are published in Mann *et al.*⁽¹⁸⁾ and were blasted against National Center for Biotechnology Information GenBank numbers. A sequence similarity of 97% was used as a cut-off for assigning sequences to OTU.

acids in the small intestine^(2,40). Likewise, the diverging hemicellulose and cellulose profiles of the maize *v.* wheat–barley diets might have altered Ca and P absorption because of changes in intestinal digesta viscosity or the complexing of Ca by complex carbohydrates^(41–43). Furthermore, wheat–barley and maize diets differ in their digestible and resistant starch fractions⁽¹⁷⁾, which could change the jejunal expression of the apical and baso-lateral sugar transporters *SGLT1* and *GLUT2*, respectively, but this was not the case in the present study. Instead, the colonic expression of *GLUT2* was down-regulated by the high *v.* adequate Ca concentration but only in the pigs fed maize diets. Overall, we could not find dietary Ca × cereal interactions for indirect estimates of digestion and absorption, which suggests that Ca effects were similarly directed by, and hence mostly independent of, the cereal composition of the basal diet.

A noteworthy observation of the present study was that the Ca and P balance data suggested that the wheat–barley diet adequate in Ca did not contain sufficient amounts of available P. This was particularly visible when comparing the results for urinary excretion and retention from the pigs fed the wheat–barley diet adequate in Ca with those fed the wheat–barley diet high in Ca. It indicates that the absorbed dietary Ca could not be sufficiently used by the pigs fed the wheat–barley diet adequate in Ca. The pigs fed the wheat–barley diets adequate in Ca excreted more Ca via urine than the pigs fed the corresponding diet high in Ca, although it should have been vice versa. Likewise, P retention increased more than Ca retention in the pigs fed the wheat–barley diet adequate in Ca as compared to the pigs fed the wheat–barley diet high in Ca. Diets were formulated according to current recommendations for the nutrient requirements for pigs^(20,21), and microbial phytase was added to increase the

bioavailability of phytate-P. Dietary Ca and P analysis confirmed that the analysed dietary Ca and P concentrations were very similar as compared to the calculated dietary concentrations. Thus, the dietary Ca and P concentrations in the adequate diet should have met the actual dietary requirements of Ca and available P for 10–20 kg pigs. However, diet analysis also showed that the Ca:P ratio in the wheat–barley diet adequate in Ca was wider, at 1:37:1, as compared to the wheat–barley diet high in Ca, which had Ca and P concentrations in a ratio of 1:24:1. This diverging Ca:P ratio could have contributed to the present results for urinary Ca

Table 8. Spearman's correlation coefficients for colonic gene expression and bacterial metabolites in the colonic digesta of pigs fed wheat–barley or maize diets adequate or high in calcium

Bacterial metabolite*	Gene	<i>r</i>	<i>P</i>
Acetate	<i>INFG</i>	0.36	0.044
Acetate	<i>TNFA</i>	0.37	0.043
Propionate	<i>GLUT2</i>	0.36	0.044
Iso-butyrate	<i>IL1B</i>	−0.36	0.047
Iso-butyrate	<i>TLR4</i>	−0.43	0.017
Valerate	<i>TLR2</i>	−0.46	0.011
Iso-valerate	<i>TLR4</i>	−0.42	0.020
Caproate	<i>TLR2</i>	−0.60	<0.001
Lipopolysaccharide	<i>IL1B</i>	−0.53	0.002
Lipopolysaccharide	<i>IL6</i>	−0.60	<0.001
Lipopolysaccharide	<i>IL10</i>	−0.58	<0.001
Lipopolysaccharide	<i>TNFA</i>	−0.62	<0.001
Lipopolysaccharide	<i>TGFB1</i>	−0.48	0.006
Lipopolysaccharide	<i>TLR4</i>	−0.38	0.036

INFG, interferon-γ; *GLUT2*, solute carrier family 2 (facilitated glucose transporter), member 2; *TLR*, toll-like receptor; *TGFB1*, transforming growth factor-β 1.

*Bacterial metabolite concentrations are published in Metzler-Zebeli *et al.*⁽¹⁷⁾.

excretion and Ca and P retention when comparing the wheat–barley diet adequate in Ca to the wheat–barley diet high in Ca. Aside from the ratio between Ca and available P being too wide, high concentrations of dietary protein also could have interfered with bone mineralisation⁽⁴⁴⁾ by changing the acid–base balance⁽⁴⁵⁾; this is particularly the case when diets contain rather low concentrations of P, which is the current practice today^(20,21). Recommendations for the P requirements of pigs have been constantly reduced over the past two decades, mostly because of environmental and economic concerns^(46,47). Because microbial phytase was supplemented to the pigs’ diet and wheat contains substantial intrinsic phytase activity, the role of dietary phytate may have been less pronounced, which would explain the lower availability of dietary P with the wheat–barley diets adequate in Ca. Ion-binding capacity and rheological properties, such as viscosity, of dietary complex carbohydrates as well as microbial mineral demands on P and Ca for the fermentation of complex carbohydrates should be mentioned as further factors that may have influenced intestinal Ca and P availability^(9,41–43,48). For instance, negative effects of dietary supplementation of 5% microcrystalline cellulose on the apparent retention of Ca, Mg, P and trace elements were previously reported by our group, which indicated an increased daily requirement for minerals for growing pigs⁽⁴³⁾.

The intestinal microbiota interacts with the host mucosa, thereby modulating mucosal gene expression⁽¹⁰⁾. In the present study, the up-regulated *TLR2* expression in the jejunum of the pigs fed high Ca diets suggested a higher mucosal abundance of gram-positive bacteria in the jejunum as compared to the pigs fed the adequate Ca diets. Also, the maize diets up-regulated jejunal *TLR2* expression as compared to the wheat–barley diets, probably because of the same reason. TLR belong to the family of pathogen-recognition receptors and are involved in the recognition of microbe-associated molecular patterns. *TLR2* interacts with the cell wall components of gram-positive bacteria, including lipoteichoic acids, peptidoglycan and lipoproteins⁽⁴⁹⁾. We did not quantify the bacterial microbiota at the jejunal mucosa. However, results from the stomach mucosa (Pars non-glandularis) and from gastric and ileal digesta indicated changes in the gram-positive bacterial composition as a result of dietary Ca and cereal composition. For instance, the pigs fed the high *v.* adequate Ca diets had considerably more lactobacilli-related OTU at the Pars non-glandularis of the stomach⁽¹⁸⁾. Accordingly, we observed an increase in the abundance of bifidobacteria in the gastric and ileal digesta of the pigs fed maize as compared to those fed wheat–barley diets⁽¹⁷⁾. Correlation coefficients for the colon further demonstrated that OTU which were related to the main phyla Bacteroidetes, Firmicutes and Proteobacteria, according to 97% sequence similarity as the cut-off for species level divergence, affected colonic gene expression. Because monocarboxylate transporters have different affinities for lactic acid and SCFA⁽⁵⁰⁾, differences between the main fermentation metabolites of mucosa-associated OTU 1, 2, 27 and 89, with the closest reference strains being *Lactobacillus johnsonii*, *Lactobacillus amylovorus*, *Lachnospiraceae* bacterium and *Eubacterium* sp., respectively, likely explained the correlations

with colonic monocarboxylate transporters expression. The closest reference strains to OTU 3 and 4, *Lactobacillus mucosae* and *Lactobacillus delbrueckii*⁽¹⁸⁾, have been shown to have immunomodulatory capacities⁽⁵¹⁾ which might have been reflected in the negative relation between OTU 3 and 4 and the colonic expression of pro-inflammatory cytokines and *TGFB1*, respectively. The modification of *TGFB1* expression has been proposed as one possible mechanism related to immunological tolerance towards microbial components in the intestine⁽⁵²⁾. Known probiotic immunomodulins include lactic acid, surface proteins of lactobacilli, peptidoglycan-derived neuropeptides, capric acid and histamine^(51,53). By contrast, OTU 624, with its closest reference strain being *Campylobacter lariena*, might have been recognised by the immune system as a potential threat, and the colonic expression of *TGFB1* and *TNFA* were therefore up-regulated. However, it should always be kept in mind that the taxonomic assignment of OTU to their closest reference strains is only an approximation of species classification. Present negative correlations between colonic cytokines genes and *TLR4* gene expression and luminal LPS concentrations, in turn, may suggest that the present LPS was mostly of a non-harmful nature⁽⁵⁴⁾. Sustained exposure to low concentrations of LPS is also known to induce the tolerant states that are typically associated with the decreased expression of pro-inflammatory cytokines⁽⁵⁵⁾, and this may also explain the negative correlations observed here. In addition, new data suggest that intestinal alkaline phosphatase play important protective roles in the detoxification of free LPS⁽⁵⁶⁾. Further research focusing on LPS immune reactivity, intestinal alkaline phosphatase and LPS tolerance is needed to establish the role of mucosal and systemic immune responses to luminal LPS in pigs.

In conclusion, high Ca diets increased intestinal Ca and P concentrations and modified the jejunal and colonic mucosal gene expression response. The observed effects of Ca × cereal interaction on intestinal cytokine expression emphasise that the basal diet may intensify or alleviate certain Ca-related physiological effects. Overall, it appears that there is a stronger Ca effect in the small intestine than in the colon, which is in line with our previous study⁽⁹⁾. Shifts in bacterial composition caused by the diets might have been reflected in up-regulated jejunal *TLR2* expression, whereas Spearman’s correlations supported the close relationship between mucosal bacterial community and gene expression in the colon.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114515000380>

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