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Dietary supplementation with *Lactobacillus plantarum* modified gut microbiota, bile acid profile and glucose homoeostasis in weaning piglets

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Abstract

Bile acids (BA) have emerged as signalling molecules regulating intestinal physiology. The importance of intestinal microbiota in production of secondary BA, for example, lithocholic acid (LCA) which impairs enterocyte proliferation and permeability, triggered us to determine the effects of oral probiotics on intestinal BA metabolism. Piglets were weaned at 28 d of age and allocated into control (CON, *n* 14) or probiotic (PRO, *n* 14) group fed 50 mg of *Lactobacillus plantarum* daily, and gut microbiota and BA profile were determined. To test the potential interaction of LCA with bacteria endotoxins in inducing damage of enterocytes, IPEC-J2 cells were treated with LCA, lipopolysaccharide (LPS) and LCA + LPS and expressions of genes related to inflammation, antioxidant capacity and nutrient transport were determined. Compared with the CON group, the PRO group showed lower total LCA level in the ileum and higher relative abundance of the *Lactobacillus* genus in faeces. In contrast, the relative abundances of *Bacteroides*, *Clostridium_sensu_stricto_1*, *Parabacteroides* and *Ruminococcus_1*, important bacteria genera in BA biotransformation, were all lower in the PRO than in the CON group. Moreover, PRO piglets had lower postprandial glucagon-like peptide-1 level, while higher glucose level than CON piglets. Co-administration of LPS and LCA led to down-regulated expression of glucose and peptide transporter genes in IPEC-J2 cells. Altogether, oral *L. plantarum* altered BA profile probably by modulating relative abundances of gut microbial genera that play key roles in BA metabolism and might consequently impact glucose homoeostasis. The detrimental effect of LCA on nutrient transport in enterocytes might be aggravated under LPS challenge.

Key words: ***Lactobacillus plantarum*: Microbiota: Bile acids: Glucose homoeostasis: Piglets**

Bile acids (BA) are amphipathic cholesterol derivatives, which are synthesised in the liver, stored in the gall bladder, secreted into the duodenum when food was ingested, reabsorbed in the terminal ileum and transported back to the liver[1]. Besides the well-known role as detergents for the emulsification and absorption of dietary lipids[2], BA have recently emerged as signalling molecules that regulate epithelial homoeostasis, transportation and barrier function[3]. Particularly, BA exhibit various biological functions in intestinal cells. Chenodeoxycholic acid (CDCA), the primary BA in many species, has been shown to stimulate gut growth in total parenteral nutrition-fed piglets[4]. However, secondary BA, more hydrophobic metabolites of primary BA, are well known for their cytotoxicity[5]. Deoxycholic acid (DCA) could decrease the barrier function of the jejunum and colon in mice[5]. In an *in vitro* study, lithocholic acid (LCA) has been shown to induce the apoptosis of HT-29 and HCT-116 cells[6].

Intestinal cells are exposed to high or low concentrations of BA since the composition varies depending largely on the intestinal microbiota[2]. Most primary BA synthesised from cholesterol in the liver are then conjugated with glycine or taurine[1]. In the distal intestine, deconjugation of BA is carried out by bacteria with bile salt hydrolysis activity and bacteria with 7α dehydroxylation activity perform the biotransformation of cholic acid (CA) and CDCA to DCA and LCA, respectively[7,8]. LCA can also be converted from UDCA under the action of bacteria with 7β dehydroxylation activity[8]. Bile salt hydrolase resides in major intestinal bacteria including *Clostridium*,

Abbreviations: BA, bile acids; CA, cholic acid; CDCA, chenodeoxycholic acid; CON, control group; DCA, deoxycholic acid; FXR, farnesoid X receptor; GLP, glucagon-like peptide; LCA, lithocholic acid; LPS, lipopolysaccharides; PCNA, proliferating cell nuclear antigen; PepT1, peptide transporter-1; PRO, probiotic group; SGLT1, Na-dependent glucose co-transporter 1; TBA, total bile acids.

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Bacteroides, *Lactobacillus* and *Bifidobacterium*[7,9,10]. There is evidence that 7α dehydroxylation is the most important BA transformation, and this action is carried out by *Bacteroides*, *Clostridium*, *Eubacterium*, *Lactobacillus* and *Escherichia*[9,11,12]. Therefore, alteration of gut microbiota may lead to changes in BA composition in the intestine and impact BA signalling and intestinal function.

Growing evidence indicates that probiotics have various beneficial effects such as relief of inflammatory bowel diseases[13,14] and improving immunity[15]. *Lactobacillus plantarum*, a commensal bacterium present in humans, has been proven to increase gut microbiota diversity and promote intestinal barrier function[16–18]. Notably, *L. plantarum* has been reported to inhibit *Clostridium*, a critical bacterium participating BA biotransformation[19]. In a recent *in vitro* study, *L. plantarum* has been shown to play a role in BA metabolism[20]. As elucidated above, variation of BA composition may lead to altered effects on intestinal cells. There is possibility that the beneficial effects of probiotics on gut health are related to changes of BA composition modulated by intestinal microbiota. However, limited data are available targeting whether manipulation of intestinal microbiota through probiotic supplementation impacts BA composition in intestinal tissues. Therefore, the first hypothesis was that oral administration of probiotics could decrease the production of secondary BA through regulating gut microbiota in association with BA metabolism in the intestine of piglets. In addition, young pigs are undeveloped in immune systems and confronted with various pathogenic bacteria, thus frequently suffering from gut disorders especially during weaning transition period[21]. As secondary BA have been shown to disrupt gut barrier function, figuring out the role of secondary BA in exacerbating intestinal cell dysfunction induced by pathogenic bacteria may further prove the importance of diminishing secondary BA in intestinal cells by using probiotics. Therefore, the second hypothesis was that the secondary BA might synergise with bacteria-derived endotoxins to disturb the enterocyte physiology.

Materials and methods

Study design, animals and sampling

Use of animals in the current study was approved by the animal care and use committee of Sichuan Agricultural University. Based on sex and body weight, a total of twenty-eight weaning piglets (Duroc × Landrace × Yorkshire, half male, half female) at 28 d of age were randomly allocated into control (CON) group (7·06 (SEM 0·27) kg, *n* 14) and probiotic (PRO) group (7·14 (SEM 0·25) kg, *n* 14) and housed individually in metal cages. Piglets in both groups had free access to feed (Table 1) and water. Feeds were given to pigs four times per d at 08.00, 12.00, 16.00 and 20.00 hours. *L. plantarum* ($3{\cdot}5 \times 10^{10}$ colony-forming units/g) were obtained from He Tai Hua Biotech Company. At the first meal of each day, piglets in the PRO group were given 50 mg (recommended by the manufacturer) of *L. plantarum* diluted in maize starch, while piglets in the CON group were given the same amount of maize starch without *L. plantarum*. At first feeding on every morning, the maize starch with or without *L. plantarum* was taken out from the freezer and mixed with small amount of feeds and then given to pigs to ensure the complete ingestion of the given *L. plantarum*. The feeding trial lasted 2 weeks. Feed intake was recorded daily. On the morning of days 0, 7 and 14 of the experiment, body weight was recorded and blood samples from anterior vena cava were collected with heparinised tubes. At day 14, fresh faecal samples were collected directly from rectum after stimulating animals to defecate. Then, piglets were transported to the surgical operating room and implanted with catheters in external jugular vein with amoxicillin spread over the surgical site to prevent infection. After 3-d recovery period, overnight-fasted piglets were given feeds and treatments in the morning and feed intake was recorded (CON group: 214·00 (SEM 20·24) g; PRO group: 215·83 (SEM 20·17) g). Blood samples at fasting state (0 min) and at 15, 30, 60, 120 and 180 min postprandial were collected from the jugular catheter. Blood samples were centrifuged at 3000 ***g*** for 10 min, and plasma samples were stored at −20°C. Immediately after the completion of blood collection, six piglets from each group were euthanised. An incision was made on the middle abdomen of piglets, and ileum and liver tissue samples were collected and washed with sterile saline and snap-frozen in liquid N_2. Approximately 2-cm length of duodenum, jejunum and ileum samples were fixed in 4 % paraformaldehyde and used for measurement of morphometry as we previously described in detail[22].

Table 1. Composition and nutrient levels of the basal diet (Percentages)

Ingredients	%	Nutrient levels	%
Extruded maize	50·200	Digestive energy (MJ/kg)	14·110
Extruded soyabean	12·000	Crude protein	19·510
Whey powder (low protein)	12·000	Lys	1·660
Extruded soyabean meal	12·000	Trp	0·240
Fishmeal	3·000	Thr	0·930
Sucrose	2·000	Met + Cys	0·670
Glucose	2·000	Ca	0·710
Sprayed dried plasma protein	4·000	Total P	0·570
Lys	0·435	Available P	0·360
Met	0·161		
Thr	0·128		
Dicalcium phosphate	0·876		
Limestone	0·675		
NaCl	0·300		
Choline chloride	0·100		
Mould inhibitor	0·050		
Ethoxyquin	0·010		
Vitamin premix*	0·035		
Mineral premix†	0·030		
Total	100·000		

* Vitamin premix provided the following per kg of the diet: vitamin A, 36·75 mg; vitamin D_3, 87·5 μg; vitamin E, 6·25 IU; vitamin K, 3·5 mg; thiamine, 3·5 mg; riboflavin, 8·75 mg; niacin, 35 mg; D-pantothenic acid, 17·5 mg; vitamin B_6, 5·25 mg; vitamin B_{12}, 35 μg; D-biotin, 140 μg and folic acid, 1·75 mg.
† Mineral premix provided the following per kg of the diet: Fe, 340 mg; Cu, 25 mg; Mn, 13 mg; Zn, 300 mg; iodine, 0·15 mg and Se, 0·3 mg.

Faecal microbial analysis

DNA was extracted using a stool DNA isolation kit (Omega Bio-Tek Inc.) according to the manufacturer's instructions, and

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DNA concentration and purity were monitored on 1 % agarose gels. The V4 region of the 16S rRNA gene was amplified using 515F and 806R primer (5′-GTGCCAGCMGCCGCGGTAA-3′and 5′-GGACTACHVGGGTWTCTAAT-3′, respectively). Mixture of PCR products was purified with a Qiagen Gel Extraction Kit. Sequencing libraries were generated and subjected for sequencing on the IlluminaHiSeq2500 platform. Paired-end reads were merged using FLASH, and high-quality clean tags were obtained using the QIIME quality-controlled process. Effective tags were obtained after comparing with the reference database and removing the chimera sequences. Sequences were clustered into operational taxonomic units using Uparse software at the 97 % similarity level. The Ribosomal Database Project classifier was used to assign a taxonomic rank to each representative sequence. α-Diversity indices were calculated using QIIME software.

Determination of bile acid composition

All BA standards were obtained from Steraloids Inc. and TRC Chemicals. Ileum tissue samples were collected, weighed and homogenised with acetonitrile:methanol (8:2) containing internal standards and centrifuged at 13 500 rpm and 4°C for 20 min to remove the protein. After centrifugation, the supernatant was freeze-dried with a stopping tray system (Labconco) and then was reconstituted with 1:1 (v/v) of acetonitrile/methanol (80/20, v/v) and ultrapure water and centrifuged at 13 500 ***g*** and 4°C for 20 min. The supernatant was used to determine BA using an ultra-performance liquid chromatography coupled to tandem MS (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp.).

Determination of total bile acids, glucose and glucagon-like peptide-1

The total bile acids (TBA) were measured by using the enzymatic cycling method assay kits, and both TBA and glucose in plasma were measured using a 7020 automatic biochemical analyzer. Plasma glucagon-like peptide (GLP)-1 was measured using an ELISA kit (Millipore) according to the manufacturer's instructions.

Cell culture and treatment

IPEC-J2 cells were grown in the Dulbecco's modified Eagle's medium/F12 medium containing 5 % fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM GlutaMAX (all from Gibco) at 37°C and 5 % CO_2. LCA was dissolved in dimethyl sulphoxide, while lipopolysaccharides (LPS) were dissolved in PBS (LCA, dimethyl sulphoxide and LPS were all purchased from Sigma-Aldrich). Cells were seeded in twelve-well plate. When grown to confluence, 5 μM of LCA or dimethyl sulphoxide was used to treat cells for 12 h and then each well was added with LPS (10 μg/ml) or saline for another 4 h. The concentrations of LCA and LPS used were based on previous studies[22,23]. Cell culture medium was collected for measurement of IL-8. RNA and protein were extracted for analysis of gene and protein expression.

Table 2. Primer sequences for quantitative PCR

Genes		Primer sequence
GAPDH	FW	CAGGTTGTGTCCTCTGACTTT
	RV	ACCCTGTTGCTGTAGCCAAA
FXR	FW	TTTGTGTCGTTTGCGGAGAG
	RV	GTTGCCCCCATTTTTACACTTG
SHP	FW	GCCTACCTGAAAGGGACCAT
	RV	CAACGGGTGTCAAGCCTTTA
FGF19	FW	AAGATGCAAGGGCAGACTCA
	RV	AGATGGTGTTTCTTGGACCAGT
TGR5	FW	CCATGCACCCCTGTTGCT
	RV	GGTGCTGTTGGGTGTCATCTT
ASBT	FW	TACTGGGTTGATGGCGACATGGAT
	RV	TCCAGAGTTGACCCACAGTTTGGT
BSEP	FW	TTTCATTCAGCGCCTGACCA
	RV	ACTCCAATGAGAGGGCTGAC
OST-α	FW	CACCCAGGAGCCTTCTTTAAT
	RV	TGAACTATTCTGCCTTCC
NTCP	FW	TGCCATCTTTCGGTGCTATG
	RV	GTGCTGTGGGAATGGTTTCT
FGFR4	FW	CTACAAGAAAACAAGCAACG
	RV	TGTAGACTCTGTCAAACAAG
β-KLOTHO	FW	GCTAAATCCTCAATCCAGTT
	RV	ATTGAGGACTACCATTCTCA
SOD1	FW	ACCTGGGCAATGTGACTG
	RV	TCCAGCATTTCCCGTCT
CAT	FW	AACTGTCCCTTCCGTGCTA
	RV	CCTGGGTGACATTATCTTCG
IL-8	FW	GCTCTCTGTGAGGCTGCAGTTC
	RV	AAGGTGTGGAATGCGTATTTATGC
IL-6	FW	TCTGGGTTCAATCAGCAGACC
	RV	CTAATCTGCACAGCCTCGAC
IL-1β	FW	AGGCAGATGGTGTCTGTCATC
	RV	AGGATGATGGGCTCTTCTTCAAA
TNF-α	FW	GGCCCAAGGACTCAGATCAT
	RV	TGAGGTACAGCCCATCTGTC
SGLT1	FW	TCATCATCGTCCTGGTCGTCTC
	RV	CTTCTGGGGCTTCTTGAATGTC
GLUT2	FW	ATTGTCACAGGCATTCTTGTTAGTCA
	RV	TTCACTTGATGCTTCTTCCCTTTC
PepT1	FW	CAGACTTCGACCACAACGGA
	RV	TTATCCCGCCAGTACCCAGA

Determination of IL-8 in medium

IL-8 concentration was determined by using an ELISA kit (Meimian, Jiangsu Feiya Biological Technology Co. Ltd) according to the manufacturer's instructions.

Gene expression analysis

Gene expression analysis was performed as we described previously[22]. Total RNA was extracted from tissues and cells using RNAiso Plus (Takara) and then reverse-transcribed to complementary DNA (cDNA) using a PrimeScript™ RT reagent kit (Takara) according to the manufacturer's instructions. The primers are listed in Table 2. The cDNA was subjected to PCR amplification using TB Green™ Premix Ex Taq™ II (Takara) in a CFX96 Real-Time PCR Detection System (Bio-Rad) according to manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method[24] was used to calculate the relative expression of each gene.

Western blotting

Western blotting was performed as we described previously[22]. Briefly, following treatment, cells were washed with PBS for

twice and cellular protein was extracted using RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride protease inhibitor (Beyotime Biotech Inc.) and the concentration was then measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Protein samples were loaded for SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The PVDF membrane was blocked in skimmed milk at room temperature for 1 h and then incubated with primary antibodies (anti-proliferating cell nuclear antigen (PCNA), anti-caspase-3 and anti-β-actin were purchased from Cell Signaling Technology) overnight at 4°C. After incubation with secondary antibody at 4°C for 1 h, visualisation was carried out using the ECL kit (Beyotime Biotech Inc.) in the gel detection system (Bio-Rad).

Statistical analysis

Data from the *in vitro* study and for plasma TBA were analysed by using the two-way ANOVA procedure, and *post hoc* testing was determined by Tukey's test, while data for the rest of the measurements were analysed by using Student's *t* test according to a previous study[25]. Relative abundances of microbiota at different levels were log-transformed before analysis by using the following formula: relative abundance$_{transformed} = \log_{10}$ relative abundance. These procedures were carried out by using GraphPad prism 6.0 software. The glucose and GLP-1 data were analysed using the linear mixed-effects model procedure in SAS 9.4 software.

Results

Growth performance and intestinal morphology

Body weight of piglets was not different ($P > 0{\cdot}10$) between the CON and PRO groups at days 7 and 14 of the experiment phase (Table 3). Average daily feed intake, average daily gain and feed: gain ratio were also not different ($P > 0{\cdot}10$) between groups during the first and second experimental week, though piglets fed *L. plantarum* had numerically 17 % increase in overall average daily gain in comparison with the CON piglets. Villus height, crypt depth and ratios of villus height:crypt depth were also not different ($P > 0{\cdot}10$) between groups (Table 4).

Table 3. Effects of supplementing *Lactobacillus plantarum* on growth performance of weaning piglets (*n* 14)
(Mean values with their standard errors)

	Treatment				
	CON		PRO		
	Mean	SEM	Mean	SEM	*P*
BW (kg)					
7 d	8·34	0·32	8·38	0·35	0·71
14 d	9·62	0·42	10·24	0·45	0·43
1–7 d					
ADFI (g)	222·65	24·32	239·22	30·60	0·68
ADG (g)	153·81	22·92	158·24	28·82	0·91
F:G	1·67	0·11	1·65	0·11	0·90
7–14 d					
ADFI (g)	310·56	25·61	385·10	43·9	0·15
ADG (g)	223·10	15·08	265·27	24·78	0·18
F:G	1·58	0·08	1·52	0·06	0·52
1–14 d					
ADFI (g)	266·61	21·37	312·16	33·95	0·27
ADG (g)	182·02	17·18	211·76	22·61	0·47
F:G	1·67	0·09	1·58	0·06	0·37

CON, control group; PRO, probiotic group; BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; F:G, feed:gain ratio.

Table 4. Effects of supplementing *Lactobacillus plantarum* on intestinal morphology of weaning piglets (*n* 6)
(Mean values with their standard errors)

	Treatment				
	CON		PRO		
	Mean	SEM	Mean	SEM	*P*
Duodenum					
Villus height (μm)	800·41	77·93	900·90	50·54	0·30
Crypt depth (μm)	218·80	15·88	220·69	20·27	0·94
VCR	3·78	0·48	4·24	0·44	0·49
Thickness (μm)	363·09	24·60	368·92	27·54	0·88
Jejunum					
Villus height (μm)	708·46	69·55	644·03	17·43	0·39
Crypt depth (μm)	167·25	10·15	155·37	12·02	0·47
VCR	4·27	0·40	4·29	0·39	0·98
Thickness (μm)	370·85	47·97	360·22	50·13	0·88
Ileum					
Villus height (μm)	591·89	43·56	569·42	29·08	0·67
Crypt depth (μm)	131·49	6·53	133·42	6·60	0·84
VCR	4·56	0·47	4·34	0·34	0·70
Thickness (μm)	743·16	150·05	623·84	67·50	0·46

CON, control group; PRO, probiotic group; VCR, ratio of villus height:crypt depth.

Plasma total bile acid and bile acid profile in ileum tissue

Compared with the CON group, piglets in the PRO group tended ($P < 0{\cdot}10$) to have lower GLCA, while higher CA level (Table 5) in

Table 5. Effects of supplementing *Lactobacillus plantarum* on bile acid profile in the ileum of weaning piglets (*n* 6)
(Mean values with their standard errors)

	CON		PRO		
Items (nmol/g)	Mean	SEM	Mean	SEM	*P*
Primary bile acid					
HCA	38·45	20·73	54·21	21·49	0·699
G-HCA	10·53	4·17	7·82	3·48	0·639
T-HCA	5·37	2·67	13·59	8·52	0·818
CDCA	14·57	7·18	13·55	4·25	0·818
G-CDCA	3·13	1·63	3·64	1·32	0·815
T-CDCA	1·64	0·69	1·66	0·60	0·988
CA	0·04	0·00	0·11	0·03	0·069
T-CA	0·12	0·02	0·08	0·01	0·188
Secondary bile acid					
HDCA	17·30	7·68	14·29	5·54	>0·999
G-HDCA	16·89	11·05	5·15	1·96	0·366
T-HDCA	2·89	1·17	2·17	0·67	0·613
LCA	0·31	0·09	0·15	0·03	0·310
G-LCA	0·06	0·02	0·00	0·00	0·099
T-LCA	0·00	0·00	0·00	0·00	0·691
UDCA	0·20	0·08	0·18	0·08	0·589
G-UDCA	0·03	0·02	0·02	0·01	0·665
T-UDCA	0·07	0·03	0·09	0·02	0·677

CON, control group; PRO, probiotic group; HCA, hyocholic acid; G-, glyco-; T-, tauro-; CDCA, chenodeoxycholic acid; CA, cholic acid; HDCA, heodeoxucholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.

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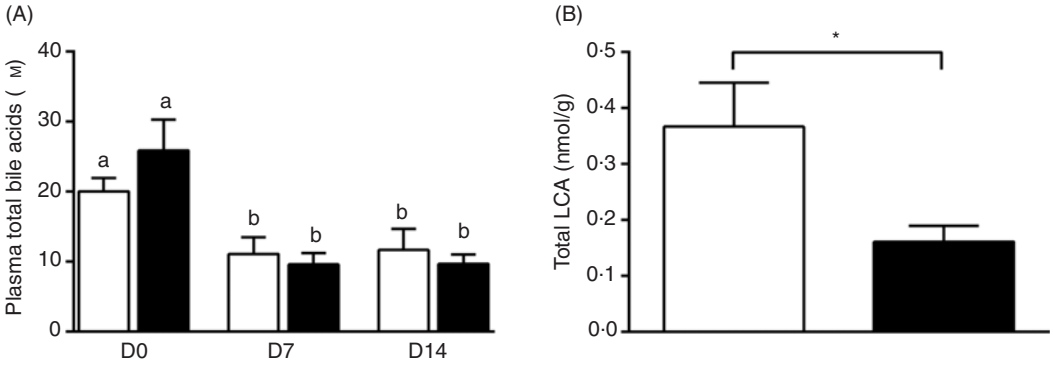

Fig. 1. Effects of supplementing *Lactobacillus plantarum* on plasma total bile acids (A) and total lithocholic acid (LCA) in the ileum (B) of weaning piglets. Values are means with their standard errors, *n* 12 for plasma total bile acids while *n* 6 for total LCA. [a,b] Mean values with unlike letters were significantly different ($P < 0·05$). * $P < 0·05$ probiotic group (PRO) *v.* control group (CON). Total LCA is the sum of LCA, glyco-LCA and tauro-LCA. ▭, CON; ■, PRO; D0, day 0; D7, day 7; D14, day 14.

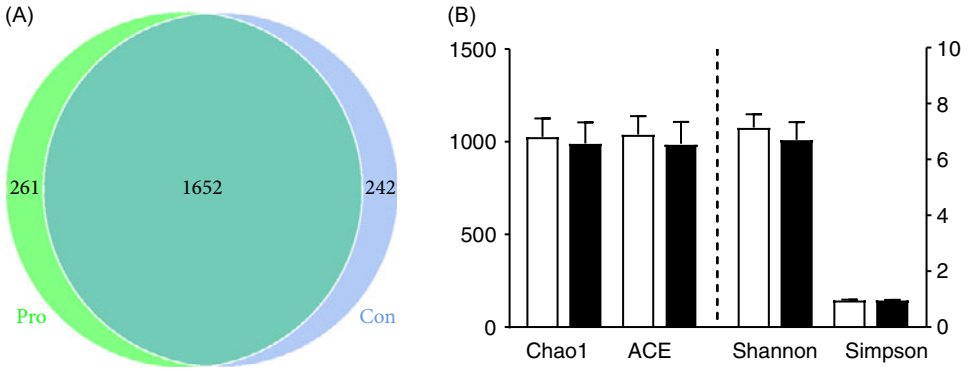

Fig. 2. (Colour online) Venn diagrams for bacterial operational taxonomic units (A) and α-diversity indices (B) in weaning piglets supplemented with *Lactobacillus plantarum* or not. Values are means with their standard errors, *n* 12. ▭, Control group; ■, probiotic group.

ileum tissue. Total LCA level in ileum tissue was significantly lower ($P < 0·05$) in PRO than in CON group (Fig. 1). The other primary and secondary BA were not different ($P > 0·05$) between groups (Table 5). Plasma TBA levels were not affected by oral administration of *L. plantarum* (Fig. 1), whereas they were significantly affected by time, with lower ($P < 0·05$) TBA observed at days 7 and 14 as compared with their baseline levels in both groups.

Faecal microbial profile

In the present study, a total of 5 112 798 reads were obtained with an average of 83 609 sequences per sample. The total number of operational taxonomic units detected was 2152, and the two groups shared the same 1651 operational taxonomic units. The α-diversity results were not different ($P > 0·10$) between groups (Fig. 2). There was a tendency ($P < 0·10$) towards decreased relative abundance of the Tenericutes phylum in the PRO group (Table 6). The relative abundance of the *Lactobacillus* genus tended ($P < 0·10$) to be higher in the PRO than in the CON group. At the genus level, compared with the CON group, the PRO group had higher ($P < 0·05$) relative abundances of *Megasphaera* and *Collinsella* (Table 7), while lower ($P < 0·05$) relative abundances of *Bacteroides*, *Parabacteroides*, *Clostridium_sensu_stricto_1*, *Ruminococcus_1* and *Desulfovibrio* (Fig. 3).

Plasma glucose and glucagon-like peptide-1

Oral administration of *L. plantarum* led to increased ($P < 0·05$) postprandial blood glucose levels (Fig. 4). Notably, at 180 min postprandial, the PRO piglets had significantly higher ($P < 0·05$) blood glucose level than the CON piglets. The AUC of glucose was also higher ($P < 0·05$) in the PRO than in the CON piglets. In contrast, plasma GLP-1 levels tended to decrease ($P < 0·10$) in piglets fed *L. plantarum*. Remarkably, the CON

Table 6. Relative abundances (%) of ten bacteria phyla* (*n* 12) (Mean values with their standard errors)

	CON		PRO		
	Mean	SEM	Mean	SEM	*P*
Firmicutes	55·650	2·284	59·480	2·761	0·314
Bacteroidetes	33·370	2·114	30·980	2·773	0·501
Spirochaetes	1·343	0·283	1·205	0·428	0·328
Actinobacteria	0·931	0·167	1·613	0·353	0·110
Proteobacteria	2·527	0·520	1·883	0·395	0·235
Tenericutes	1·875	0·244	1·194	0·206	0·054
Euryarchaeota	0·544	0·143	0·923	0·255	0·568
Cyanobacteria	0·066	0·012	0·131	0·037	0·217
Planctomycetes	0·046	0·018	0·059	0·024	0·977
Verrucomicrobia	0·003	0·001	0·004	0·001	0·457

CON, control group; PRO, probiotic group.
* Data were log-transformed before statistical analysis.

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Table 7. Relative abundances (%) of twenty bacteria genera* (*n* 12) (Mean values with their standard errors)

	CON		PRO		
Items	Mean	SEM	Mean	SEM	*P*
Lactobacillus	5·59	1·12	9·13	1·59	0·08
Prevotella_9	3·07	0·43	5·39	1·49	0·83
Treponema_2	1·16	0·24	0·75	0·19	0·16
Megasphaera	2·41	0·61	5·93	1·33	0·02
Collinsella	0·24	0·04	0·49	0·10	0·03
Blautia	2·22	0·37	2·35	0·45	0·86
Prevotella_2	1·44	0·26	1·95	0·48	0·67
Prevotellaceae_NK3B31_group	3·24	0·46	2·77	0·56	0·45
Succinivibrio	0·35	0·07	0·28	0·07	0·35
Prevotella_1	1·12	0·27	0·75	0·21	0·22
Phascolarctobacterium	1·76	0·30	2·41	0·62	0·62
Rikenellaceae_RC9_gut_group	2·63	0·45	1·91	0·32	0·27
Ruminococcaceae_UCG-002	2·66	0·32	2·59	0·43	0·60
Lachnospiraceae_XPB1014_group	0·38	0·03	0·77	0·22	0·17
Sarcina	1·68	0·15	2·63	0·44	0·22
Ruminococcaceae_UCG-005	1·49	0·22	1·63	0·38	0·65
Streptococcus	0·63	0·14	0·92	0·22	0·36
Subdoligranulum	0·57	0·08	0·85	0·15	0·10
Alloprevotella	1·23	0·18	0·84	0·11	0·26
Holdemanella	0·38	0·05	0·58	0·12	0·31

CON, control group; PRO, probiotic group.
* Data were log-transformed before statistical analysis.

piglets had higher ($P < 0·05$) plasma GLP-1 levels than the PRO piglets at 60 min and 180 min postprandial. The AUC of GLP-1 also tended ($P < 0·10$) to be lower in the PRO than in the CON piglets.

Gene expression in intestine and liver tissues

As shown in Fig. 5, there were no significant ($P > 0·05$) differences in the relative expression of genes associated with BA metabolism between the CON and PRO groups. The expression of genes related to inflammation and glucose transport was also not affected ($P > 0·05$) by *L. plantarum* consumption (online Supplementary Fig. S1).

IL-8 secretion and gene expression of IL-6 and IL-8 in IPEC-J2 cells

Secretion of IL-8 from IPEC-J2 cells was augmented ($P < 0·05$) by both LPS and LCA treatment (Fig. 6(A)). LPS treatment had no effect ($P > 0·05$) on the relative mRNA abundance of IL-8 (Fig. 6(B)) and IL-6 (Fig. 6(C)), whereas LCA treatment significantly up-regulated mRNA abundance of IL-8 ($P < 0·0001$) and IL-6 ($P < 0·001$).

Expression of antioxidant and transporter gene and protein abundances of proliferating cell nuclear antigen and caspase-3

The relative mRNA expression of catalase and superoxide dismutase 1 was down-regulated ($P < 0·0001$) by LCA treatment (Fig. 7). Interestingly, the mRNA expression of GLUT2 was not affected ($P > 0·05$) by LPS or LCA treatment alone, whereas was down-regulated ($P < 0·05$) by the co-treatment of LPS and LCA as compared with the LCA treatment alone (Fig. 8(A)). Similarly, the mRNA expression of Na-dependent glucose co-transporter 1 (SGLT1) (Fig. 8(B)) and peptide transporter-1 (PepT1) (Fig. 8(C)) was not affected ($P > 0·05$) by LPS or LCA treatment alone, whereas was down-regulated ($P < 0·05$) by the co-treatment of LPS and LCA as compared with the LPS treatment alone. The protein levels of caspase-3 were not affected ($P > 0·05$) by treatment of either LCA or LPS. LPS treatment also had no effect ($P > 0·05$) on protein level of PCNA, whereas LCA treatment alone decreased ($P < 0·05$) the protein abundance of PCNA (Fig. 9).

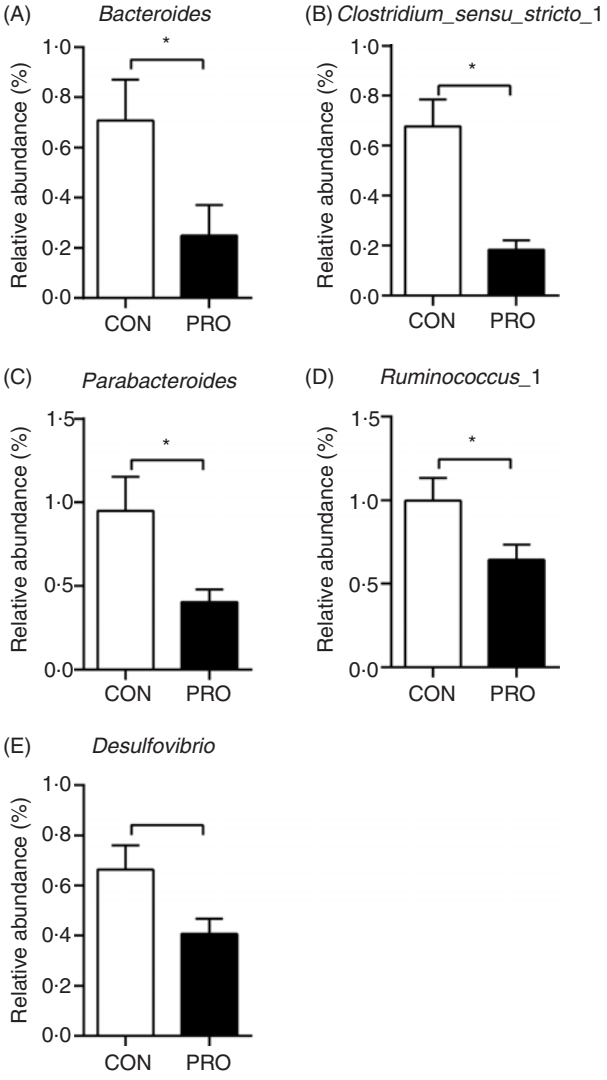

Fig. 3. Effects of supplementing *Lactobacillus plantarum* on faecal bacteria genera in weaning piglets. Values are means with their standard errors, *n* 12. * $P < 0·05$ probiotic group (PRO) *v.* control group (CON).

Discussion

BA play critical roles in the regulation of intestinal physiology[26,27]. The most common secondary BA, LCA and DCA, have been shown to induce cell apoptosis and increase intestinal permeability[5,6,28] and have been correlated with colorectal carcinogenesis[6,29]. Our recent study also revealed the detrimental effect of LCA and DCA on porcine enterocytes including

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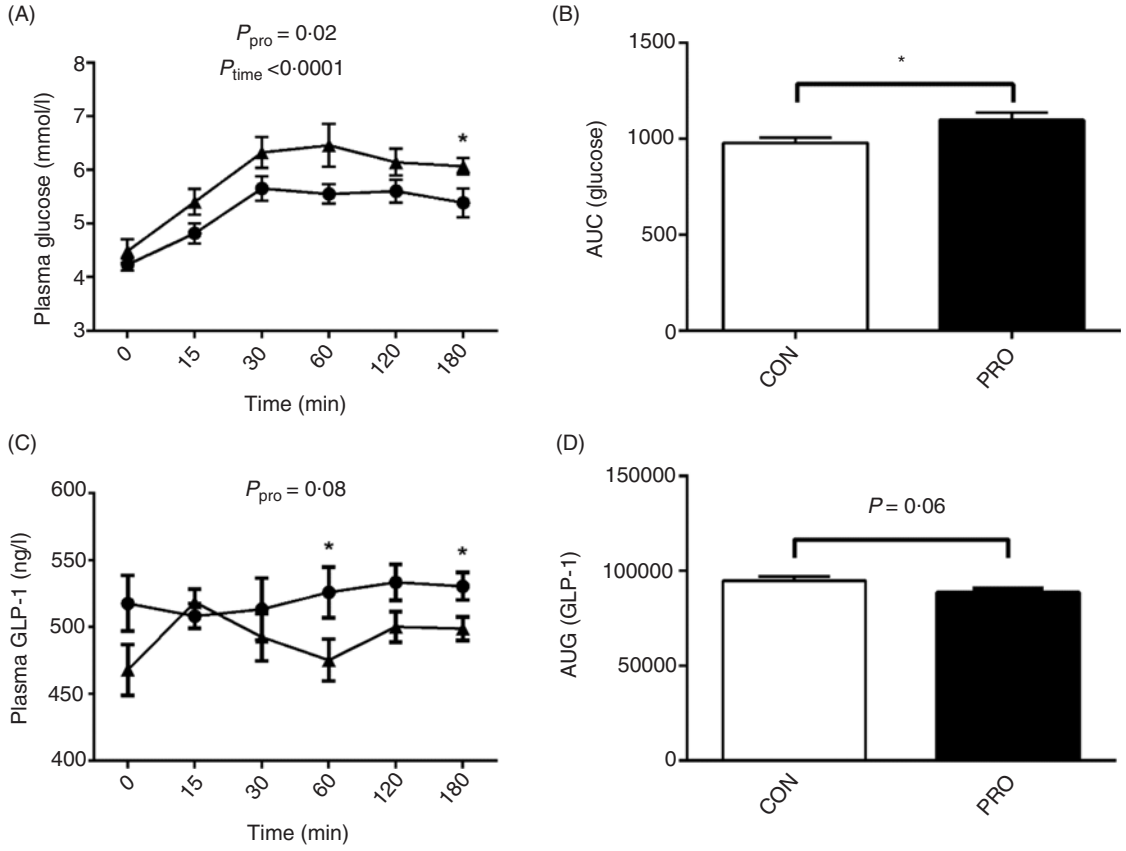

Fig. 4. Effects of supplementing *Lactobacillus plantarum* on plasma glucose (A) and AUC (B) and glucagon-like peptide (GLP)-1 level (C) and AUC (D) in weaning piglets. Values are means with their standard errors, *n* 12. * $P < 0{\cdot}05$ control group (CON) *v.* probiotic group (PRO). —▲—, PRO; —●—, CON.

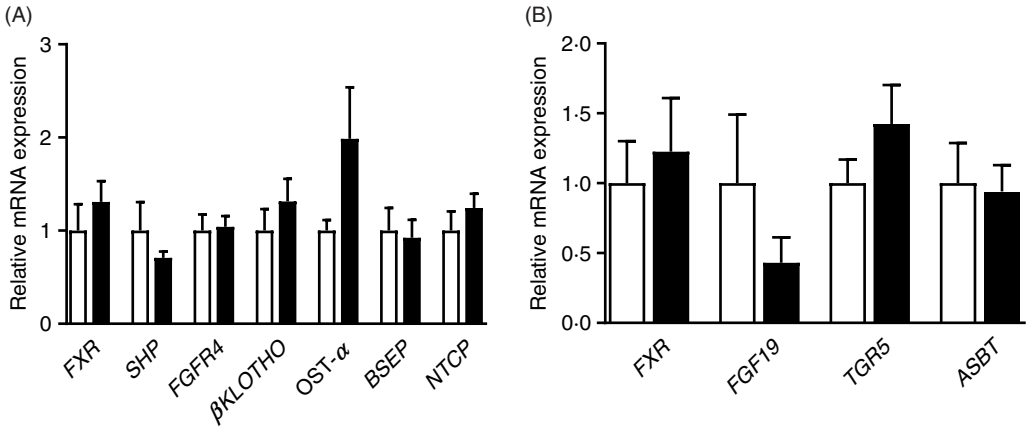

Fig. 5. Effects of supplementing *Lactobacillus plantarum* on expression of genes related to bile acid metabolism in the ileum (A) and liver (B) of weaning piglets. Mean values with their standard errors, *n* 6. ☐, Control group; ■, probiotic group.

suppression of cell proliferation, impairment of tight junction and inducement of inflammatory responses[22]. In light of the importance of gut microbiota in the production of secondary BA[11,12], supplementation of probiotics appeared to be a promising nutritional strategy regulating secondary BA production and may thus aid in the improvement of intestinal health. Hereby, we used piglets as model considering the higher similarity in BA composition between pigs and human when compared with rats and mice[30,31].

In this study, the growth performance of piglets during the first and second week was not different between the CON and PRO groups. This is in line with a previous study in weaning piglets showing that feeding *L. plantarum* had no effects on growth performance in the first 2 weeks but improved the

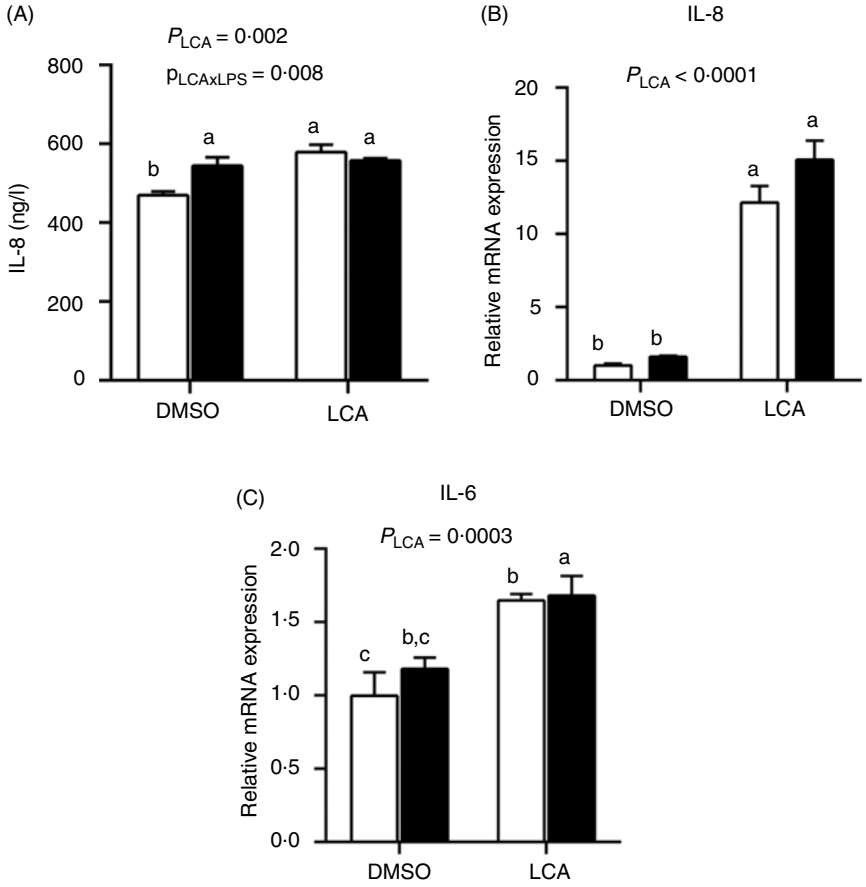

Fig. 6. Effects of lithocholic acid (LCA) on secretion of IL-8 (A) and relative mRNA expression of IL-8 (B) and IL-6 (C) in IPEC-J2 cells in the presence and absence of lipopolysaccharides (LPS). Values are means with their standard errors, *n* 4. [a,b,c] Mean values with unlike letters were significantly different ($P < 0·05$). ▭, –LPS; ■, +LPS.

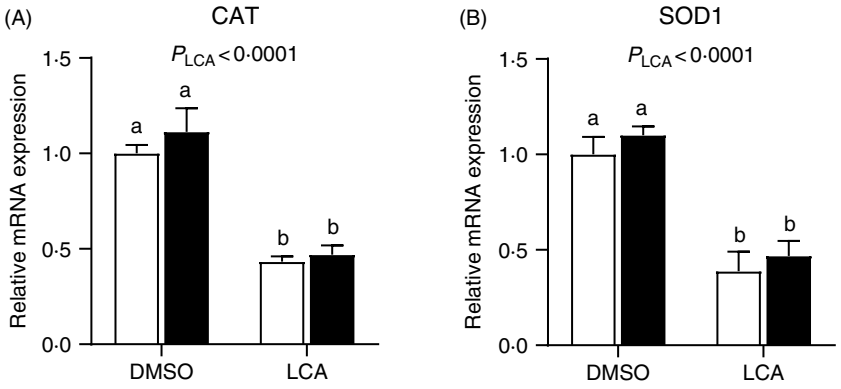

Fig. 7. Effects of lithocholic acid (LCA) on expression of catalase (CAT) (A) and superoxide dismutase 1 (SOD1) (B) in IPEC-J2 cells in the presence and absence of lipopolysaccharides (LPS). Values are means with their standard errors, *n* 4. [a,b] Mean values with unlike letters were significantly different ($P < 0·05$). ▭, –LPS; ■, +LPS; DMSO, dimethylsulfoxide.

average daily gain in the third week[32]. It is therefore inferred that the beneficial effects of *L. plantarum* on growth performance may require longer time.

Plasma TBA levels were not different between the CON and PRO groups, whereas the highest plasma TBA levels were observed at day 0 in both groups, which might be related to the change of food sources. As elucidated in a previous study, sow-reared piglets had higher conjugated BA and unconjugated BA in serum than formula-fed piglets[33], whereas underlying mechanisms need further investigation. After being released into the gastrointestinal tract, most BA can be reabsorbed via BA transporters in the intestine, especially in the ileum[34]. Measuring the BA composition in the ileum allowed us to assess the potential impacts of individual BA on enterocytes.

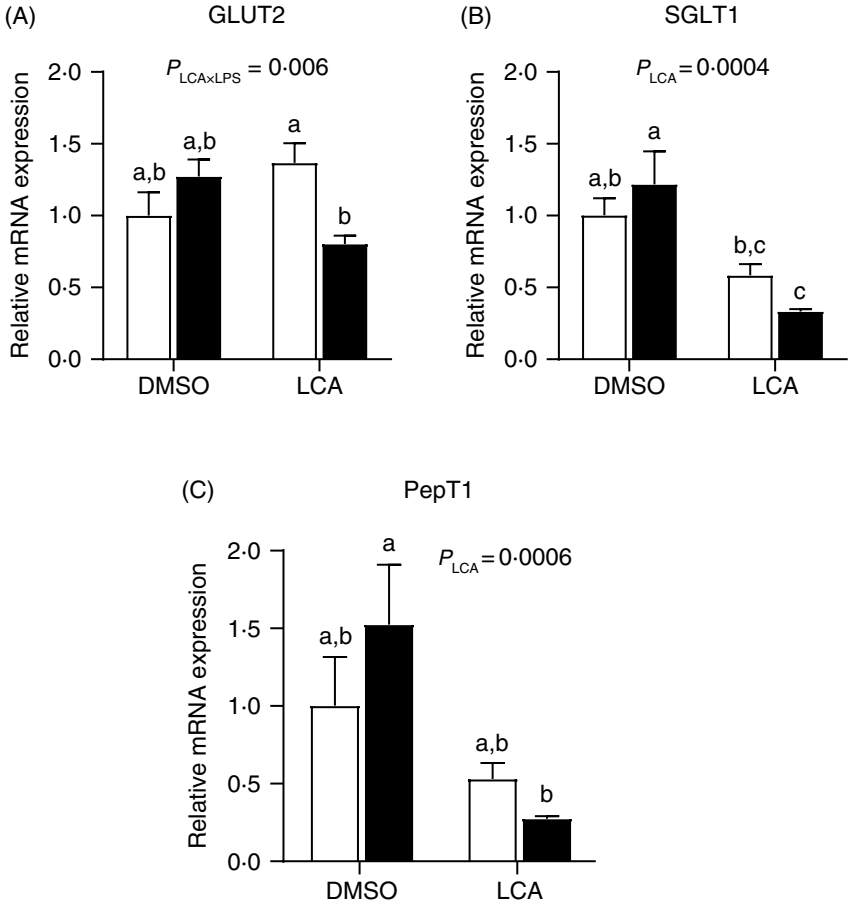

Fig. 8. Effects of lithocholic acid (LCA) on gene expression of GLUT2 (A), sodium-dependent glucose co-transporter 1 (SGLT1) (B) and peptide transporter-1 (PepT1) (C) in IPEC-J2 cells in the presence and absence of lipopolysaccharides (LPS). Values are means with their standard errors, n 4. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). ▭, –LPS; ■, +LPS; DMSO, dimethylsulfoxide.

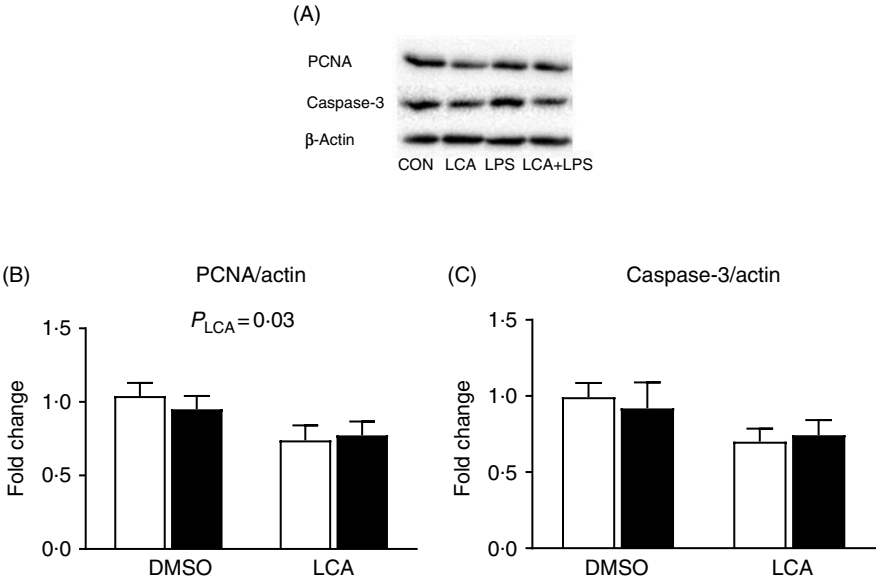

Fig. 9. Effects of lithocholic acid (LCA) on the protein abundances (A) of proliferating cell nuclear antigen (PCNA) (B) and caspase-3 (C) in IPEC-J2 cells in the presence and absence of lipopolysaccharides (LPS). Protein abundances were determined by Western blot and normalised to β-actin. Values are means with their standard errors, n 4. ▭, –LPS; ■, +LPS; DMSO, dimethylsulfoxide.

Interestingly, lower total LCA level was observed in the PRO group. In addition, piglets fed *L. plantarum* tended to have lower GLCA level while higher CA level in the ileum. In the intestine, CDCA and CA are metabolised by intestinal microbiota with 7α dehydroxylation activity to produce LCA and DCA, respectively[10,12]. Neither conjugated nor unconjugated DCA was detected in ileum tissue, which might suggest an extremely low level of DCA reabsorbed. But, the higher CA level in piglets fed *L. plantarum* may indicate dampened biotransformation of CA to form DCA, which might lead to lower DCA accumulation in the other tissues. Given that LCA and its conjugated forms are all ligands to farnesoid X receptor (FXR)[35], we also determined the relative mRNA abundance of central molecules in the FXR-FGF19 signalling pathway and observed no difference between groups, which was probably due to the fact that LCA is a relatively weak FXR agonist when compared with CDCA and DCA[36]. LCA is also a ligand of G protein coupled with bile acid receptor 1 (TGR5) and proposed to activate TGR5 more potent than DCA, CDCA and CA[37,38]. GLP-1, an incretin secreted from enteroendocrine cells in the distal intestine[39,40] with beneficial effects on glucose homoeostasis[41], is an important marker of TGR5 activation[42,43]. We therefore detected the changes of GLP-1 and glucose concentrations in 3 h after feeding. Higher plasma GLP-1 levels in the CON piglets probably indicated that the higher total LCA level maintained the activation of TGR5 and continuous GLP-1 secretion. However, in piglets fed *L. plantarum*, lower GLP-1 might be attributed to dampened TGR5 activation resulted from lower total LCA concentration. Furthermore, in the PRO piglets, the lower GLP-1 level might consequently lead to increased postprandial glucose level.

To elucidate the potential impacts of gut microbiota on alteration of BA composition, we determined the microbiota profile in faecal samples. Despite most BA are reabsorbed in the terminal ileum, the biotransformation of BA mainly occurs in the colon[44] and secondary BA will come into the blood stream via passive reabsorption and recycle in enterohepatic circulation. Faecal microbiota has been shown to have high similarity to colonic microbiota[45], and thus faecal microbiota profile was determined using 16S rRNA gene sequencing to evaluate the change of microbiota involved in BA metabolism. The most direct change lay in the *Lactobacillus* genus which showed tendency towards increase in the PRO piglets. This result confirmed the colonisation of *L. plantarum* supplemented. The PRO piglets also showed higher relative abundances of the *Megasphaera* genus, a SCFA-producing bacterium[46], and the *Collinsella* genus, which have been shown to be positively correlated with T cell response in blood[47]. *Desulfovibrio* facilitates the formation of the hazardous gas hydrogen sulphide and causes colonic inflammation[48,49]. Accordingly, lower relative abundance of *Desulfovibrio* was observed in PRO piglets. Therefore, *L. plantarum* supplementation may benefit the intestine of piglets in different manners. Changes also occurred in gut microbiota related to BA metabolism. Epimerisation of 7α/β hydroxyl group is an important step before the 7α dehydroxylation and formation of LCA and DCA, and *Bacteriodes* and *Ruminococcus* exert important roles in this process[7,12]. It has been shown in a recent study that high-fat diet-induced increase in secondary BA was correlated with the increase in *Ruminococcus*[50]. *Parabacteroides distasonis* treatment in mice was reported to be capable of increasing faecal LCA level[51]. *Clostridium* possesses 7a-dehydroxylation ability, the most important BA transformation that produces DCA and LCA[9,11,12]. It has been shown that *L. plantarum* had antimicrobial activity against species of *Clostridium*[19], which might account for the decrease of *Clostridium* after *L. plantarum* feeding. Therefore, feeding *L. plantarum* inhibited the formation of LCA possibly through reducing the relative abundances of *Bacteroides*, *Ruminococcus*, *Parabacteroides* and *Clostridium*.

High-fat diet-induced increase in intestinal permeability was demonstrated to be accompanied by increased faecal DCA and LCA levels[52]. Our recent study also confirmed the detrimental effects of LCA on tight junction protein expression[22]. In our *in vivo* study, increased LCA accumulation had no significant effects on expression of inflammatory genes in the ileum. Given the role of LCA in disturbing intestinal tight junction and permeability, we propose that LCA may synergise with bacteria endotoxins to augment inflammatory responses in the intestine. Therefore, IPEC-J2 cells were used as an *in vitro* model to explore the effects of LCA and LPS on immunity and gene expression of antioxidant enzymes and nutrient transporters. LCA treatment appeared to suppress the gene expression of IL-6 and IL-8 and led to decreased protein abundances of PCNA, an important marker reflecting cell proliferation[53]. The gene expression of catalase and superoxide dismutase 1, important enzymes that aid in relieving oxidative stresses[54], was also down-regulated by LCA treatment. Secretion of IL-8 and expression of GLUT2 were affected by interaction between LPS and LCA. Additionally, LCA treatment led to down-regulation of SGLT1 and PepT1 expression in the presence of LPS while showed no effect in the absence of LPS. Both SGLT1 and GLUT2 are important for the transport of dietary glucose into enterocytes[55], while PepT1 plays a role in the transport of peptide[56]. These results indicate that LCA may synergise with pathogens to augment inflammation and damage antioxidant activity and nutrient uptake.

In conclusion, our findings indicated that consumption of *L. plantarum* altered BA profile probably by modulating relative abundances of gut microbial genera that play key roles in BA metabolism and might consequently impact glucose homoeostasis. Our *in vitro* study showed that LCA could decrease cell proliferation and expression of antioxidant enzymes, while stimulate inflammatory responses and might interact with LPS to disturb enterocyte nutrient transport.

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The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520001774

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