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Dietary arginine supplementation alleviates immune challenge induced by *Salmonella enterica* serovar Choleraesuis bacterin potentially through the Toll-like receptor 4-myeloid differentiation factor 88 signalling pathway in weaned piglets

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Abstract

The present study evaluated whether dietary arginine (Arg) supplementation could attenuate immune challenge induced by *Salmonella enterica* serovar Choleraesuis C500 (S.C500) through the Toll-like receptor (TLR) 4-myeloid differentiation factor 88 (Myd88) signalling pathway in weaned piglets. A total of thirty-six weaned pigs were randomly allocated into six groups with six replicates per group. Pigs were subjected to three dietary treatments (namely two groups per treatment) in the first week (0–7 d) and fed with diets containing 0, 0·5 and 1·0 % L-Arg, respectively. On day 8, pigs were injected intramuscularly either with S.C500 or sterile saline. Serum samples were collected at day 8 (before injection), and at 1, 3 and 10 d post-injection, pigs were killed for evaluation of tissue gene expression following the last blood collection. Piglets fed the diets with 0·5 or 1·0 % Arg supplementation had a higher concentration of serum Arg ($P<0·05$). S.C500-challenged piglets had higher ($P<0·05$) serum antibody levels during the days 9–18. Weight gain and feed intake were decreased remarkably ($P<0·01$) after the injection of S.C500, and 0·5 or 1·0 % Arg supplementation tended to alleviate the inhibition. The S.C500 challenge significantly enhanced ($P<0·05$) serum C-reactive protein (CRP), interferon-γ and IL-12 concentrations, but Arg supplementation attenuated ($P<0·05$) the increase in CRP level. The mRNA expression of TLR4, TLR5, Myd88, p65 NF-κB and TNF-α was up-regulated ($P<0·05$) by the S.C500 challenge in different tissues, but was down-regulated ($P<0·05$) by Arg supplementation. In conclusion, Arg supplementation could inhibit the excessive activation of the TLR4-Myd88 signalling pathway and thus attenuated the negative effects caused by the immune challenge of S.C500.

Key words: Arginine: *Salmonella* Choleraesuis C500: Weaned piglets: Toll-like receptor 4-myeloid differentiation factor 88 signalling pathway

Arginine (Arg) is one of the essential amino acids for weaned piglets, but is present only in low amounts in breast milk[1–3]. Sufficient Arg in the diet is indispensable to optimise the health status of weaned piglets[4–6]. Previous studies have shown that Arg is an important immunomodulator both in animals and humans[7,8]. Arg modulates immune functions through regulating a variety of hormones and cytokines, such as glucagons, growth hormone, insulin-like growth factor, prolactin, IL-1β, IL-2 and TNF-α[7,9]. In addition, dietary Arg supplementation has been shown to alleviate a variety of infections and immune challenges[10–13].

Toll-like receptor (TLR) 4 is an important member of pattern recognition receptors expressed on cell surfaces, which activates the innate immunity system by recognising the pathogen-associated molecular patterns[14]. TLR4 transmits signals to NF-κB and mitogen-activated protein kinases (MAPK) through two signalling pathways, namely the myeloid differentiation primary response gene 88 (Myd88)-dependent pathway and the Toll/IL-1 receptor (TIR) domain-containing adaptor protein-inducing interferon (IFN)-β-dependent pathway, resulting in the production of IFN-γ and inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12[15,16]. The transcription of TLR4 and inducible NO synthase was activated after bacterial infections or lipopolysaccharide (LPS) injection[17–19], indicating that NO may be involved in resistance to immune stress. Since Arg is a unique substrate for the synthesis of NO in

Abbreviations: Arg, arginine; CRP, C-reactive protein; IFN, interferon; ILN, inguinal lymph node; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MLN, mesenteric lymph node; Myd88, myeloid differentiation factor 88; OD, optical density; OD%, optical density percentage; S.C500, *Salmonella enterica* serovar Choleraesuis C500; TLR, Toll-like receptor.

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animals, we supposed that the Arg-modulated immune response may be associated with the regulation of the TLR4 signalling pathway.

The present study was conducted to investigate the effects of dietary Arg supplementation on immune challenge in weaned pigs. Moreover, the potential mechanisms behind the Arg-regulated immune responses were investigated on a molecular basis.

Materials and methods

Animal care and diets

The animal protocol for the present study was approved by the Animal Care and Use Committee of Sichuan Agricultural University. A total of thirty-six crossbred male piglets (Duroc × (Landrace × Yorkshire)) weaned at 21 (SEM 1) d (7·19 (SEM 0·63) kg) were randomly allocated into six groups (n 6). Pigs were housed individually in metabolism cages (1·5 m × 0·7 m × 1·0 m). Each cage was equipped with a feeder and a nipple waterer to allow pigs to access feed and water *ad libitum*. The basal diet (Table 1) was formulated according to the National Research Council's[20] requirements for all nutrients. The room temperature was maintained at 25–27°C.

Experimental design

Before the experiment, all pigs were fed with a basal diet for 4 d (adaptation period). Piglets were randomly allocated into six groups with six replicates per group (n 6). Pigs were subjected to three dietary treatments (namely two groups per treatment) in the first week (0–7 d) and fed with diets containing 0, 0·5 and 1·0 % synthetic L-Arg, respectively. On day 8, all pigs were sampled for blood and then injected intramuscularly either with 4 ml (3×10^{10} colony-forming units (CFU)/ml, four times of recommended immune dose in the specification) *Salmonella enterica* serovar Choleraesuis C500 (S.C500; Qilu Animal Health Corporation) or the same amount of sterile 0·9 % NaCl solution (control). S.C500 is a kind of attenuated *Salmonella enterica* serovar Choleraesuis bacterin that contains the antigen O (LPS) and antigen H (flagellum) of *S. enterica* serovar Choleraesuis. S.C500, which was dissolved in a sterile 0·9 % NaCl solution to an appropriate concentration (3×10^{10} CFU/ml) according to the specification. The dose of S.C500 was chosen based on the result of our preliminary study, which showed that it significantly induced the production of *Salmonella* antibody in piglets. At 1 and 3 d post-injection, pigs were sampled for blood, and at 10 d post-injection, pigs were again sampled for blood and killed for evaluation of tissue gene expression. Body weight and feed intake were measured at 08.00 hours on days 0, 8 and 18.

In addition, piglets with the S.C500 challenge were fed separately from those injected with sterile saline for avoiding mutual infection. Piglets were fed in the Animal Center of Animal Nutrition Institute, Sichuan Agricultural University (Ya'an, China).

Table 1. Ingredient composition of the basal diet (as-fed basis)*

Ingredients	%
Maize (7·8 % CP)	30·0
Extruded maize (7·8 % CP)	25·5
Extruded full-fat soyabean (35·5 % CP)	18·0
Soyabean meal (46 % CP)	6·0
Fishmeal (62 % CP)	6·7
Maize protein powder (55 % CP)	0·8
Whey powder (3 % CP)	8·0
Sucrose	2·0
Choline chloride	0·15
Dicalcium phosphate	0·57
Limestone	0·80
Salt	0·30
L-Lys HCl (78 %)	0·35
DL-Met (98·5 %)	0·15
L-Thr (98 %)	0·14
L-Trp (98 %)	0·04
Vitamin and mineral premix†	0·50
Nutrient composition	
Digestible energy (calculated; kJ/kg)	14 393
CP (analysed)	19·17
Ca (analysed)	0·85
Total P (analysed)	0·60
Digestible Lys (calculated)	1·35
Digestible Met + Cys (calculated)	0·79
Digestible Thr (calculated)	0·88
Digestible Trp (calculated)	0·26
Digestible Arg (calculated)	1·14

CP, crude protein; Arg, arginine.

*In the 0·5 and 1 % Arg diets, 0·5 and 1 % maize were replaced by Arg, respectively.

† The vitamin and mineral premix provided the following amounts per kg complete diet: vitamin A, 0·92 mg; vitamin D_3, 0·008 mg; vitamin E, 20 mg; vitamin K_3, 1 mg; vitamin B_{12}, 0·03 mg; riboflavin, 5 mg; niacin, 20 mg; pantothenic acid, 15 mg; folic acid, 0·5 mg; thiamin, 1·5 mg; pyridoxine, 2 mg; biotin, 0·1 mg; Zn, 100 mg; Mn, 50 mg; Fe, 100 mg; Cu, 10 mg; I, 0·30 mg; Se, 0·30 mg.

Blood sampling and analyses

Blood samples were collected in the morning of days 8, 9, 11 and 18, and were centrifuged at 3000 ***g*** for 15 min. The isolated serum samples were stored at −20°C until analysis. The interval of blood sampling was based on the determined concentration of antibody in serum samples after the S.C500 challenge (data not shown).

Serum Arg concentration was assayed using the amino acid automatic analyser L8800 (Hitachi). Frozen serum samples were thawed at 4°C and 3 ml of 10 % (w/v) solution of sulfosalicylic acid were added into 1 ml serum sample and centrifuged (12 000 ***g*** for 1 h) at 4°C. The Arg concentration of deproteinised serum was determined by ion-exchange chromatography.

Serum antibody of *Salmonella* was assayed using a commercially available IDEXX Swine Salmonella Ab kit (Beijing IDEXX Laboratories Company Limited). The optical density (OD) percentage (OD%) was used to express the levels of antibody in the serum: $OD\% = (OD_{sample} - OD_{blank\ control}/OD_{positive\ control} - OD_{blank\ control})$.

Serum C-reactive protein (CRP) was assayed using a commercially available porcine CRP ELISA kit (R&D Systems China Company Limited). The minimum detectable concentration of porcine CRP was 0·1 ng/ml and the intra-assay CV was <10 %. Serum IFN-γ was assayed using a commercially available porcine IFN-γ ELISA kit (R&D Systems). The minimum detectable concentration of IFN-γ was 6·1 pg/ml and the intra-assay CV was <10 %. Serum IL-12 was assayed using a commercially available porcine IL-12 ELISA kit (R&D

Systems). The intra-assay CV was <10% and the minimum detectable concentration of IL-12 was 9·0 pg/ml.

Tissue sample collection

All piglets were slaughtered at the end of the trial after anaesthesia. The liver, spleen, lung, kidney, mesenteric lymph node (MLN) and inguinal lymph node (ILN) were removed immediately and frozen in liquid N_2 after snipping, and then stored at −80°C until total RNA extraction.

Total RNA extraction and RT reaction

Total RNA was extracted using Trizol Reagent (TaKaRa Biotechnology Company Limited) treated with diethylprocarbonate (DEPC)-treated water (Invitrogen Life Technologies). RNA was spectrophotometrically quantified (A_{260}) and its integrity verified by agarose gel electrophoresis. Reverse transcription using the PrimeScript™ RT reagent kit (TaKaRa Biotechnology) was carried out according to the manufacturer's instructions.

Real-time PCR

Expression levels of TLR4, TLR5, Myd88, p38 MAPK, p65 NF-κB and TNF-α in the liver, spleen, lung, kidney, MLN and ILN were analysed by real-time PCR using SYBR Premix Ex Taq reagents (TaKaRa Biotechnology) and CFX-96 Real-Time PCR detection System (Bio-Rad Laboratories, Inc.). The PCR system consisted of 5 μl SYBR Premix Ex Taq™ (2×), 1 μl forward primers (4 μM), 1 μl reverse primers (4 μM), 2 μl double distilled water and 1 μl complementary DNA in a total volume of 10 μl. Cycling conditions were 95°C for 10 s, followed by forty cycles of 95°C for 5 s, annealing temperature (Table 2) for 25 s and 72°C for 15 s. A melting curve analysis was generated following each real-time quantitative PCR assay to check and verify the specificity and purity of all PCR products. Each sample was amplified in triplicate. The primers used are presented in Table 2. The standard curve of each gene was run in duplicate and three times for obtaining reliable amplification efficiency. The correlation coefficients (r^2) of all standard curves were >0·99 and the amplification efficiency was between 90 and 110%. Target gene mRNA concentration was normalised to the mRNA concentration of the reference gene β-actin.

Statistical analysis

Growth performance (during 0–7 d) and serum parameter (on d 8) were analysed by one-way ANOVA using general linear model (GLM) procedures of SAS version 8.1 (SAS Institute). Other data including growth performance (during 8–18 d), serum parameters (on 9, 11 and 18 d) and relative gene expressions were analysed by two-way ANOVA using GLM procedures of SAS version 8.1. The statistical model included the effects of the immune challenge (S.C500 or saline), Arg supplementation levels (0, 0·5 and 1%) and their interaction. Data for relative gene expressions were calculated using the Pfaffl[21] method before statistical analysis. Results are expressed as means with their standard errors. Differences were considered significant when $P<0·05$.

Results

Concentrations of serum arginine and antibody of Salmonella

The data of serum Arg and antibody of *Salmonella* are shown in Table 3. Compared with the basal diet, supplementation of 0·5 or 1·0% Arg significantly increased serum Arg concentration ($P<0·05$). The S.C500 challenge resulted in an acute reduction of serum Arg concentration on day 11 ($P<0·01$). There was no significant interaction effect of Arg supplementation and S.C500 challenge on Arg concentration.

S.C500-challenged piglets showed an increase in serum S.C500 antibody levels on days 9 ($P<0·05$), 11 ($P<0·01$) and 18 ($P<0·01$). On the other hand, supplementation with Arg and the interaction of Arg and S.C500 challenge had no obvious effects on serum S.C500 antibody level.

Growth performance

There were no significant differences in average daily gain, average daily feed intake and feed:gain ratio among the

Table 2. Primer sequences used for real-time PCR

Gene name	Primer sequences 5′–3′	Product length (bp)	Annealing temperature (°C)	Accession no.
TLR4	F: TCAGTTCTCACCTTCCTCCTG R: GTTCATTCCTCACCCAGTCTTC	166	62·0	GQ503242.1
TLR5	F: GAGTCTTTCGCCATCTGACTG R: GAGAGGAGCTGGTTTCTGGATAG	127	55·9	FJ754217.1
Myd88	F: GATGGTAGCGGTTGTCTCTGAT R: GATGCTGGGGAACTCTTTCTTC	148	56·5	AB292176.1
p38 MAPK	F: CTTACGGATGACCCACGTTCAGT R: GCTCACAGTCTTCATTCACAGC	127	56·5	XM001929490.1
p65 NF-κB	F: GTGTGTAAAGAAGCGGGACCT R: CACTGTCACCTGGAAGCAGAG	139	56·5	EU399817.1
TNF-α	F: CGTGAAGCTGAAAGACAACCAG R: GATGGTGTGAGTGAGGAAAACG	121	62·5	EU682384.1
β-Actin	F: TCTGGCACCACACCTTCT R: TGATCTGGGTCATCTTCTCAC	114	55·9	DQ178122

TLR, Toll-like receptor; *Myd88*, myeloid differentiation factor 88; *MAPK*, mitogen-activated protein kinase.

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Table 3. Effects of arginine (Arg) supplementation and *Salmonella enterica* serovar Choleraesuis C500 (S.C500) challenge on the concentration of Arg and the antibody of *Salmonella* in serum
(Mean values with their standard errors, day 8, *n* 12; days 9, 11, 18, *n* 6)

	S.C500 (−)			S.C500 (+)				Analysis by effect (*P*)		
Response	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	SEM	Arg	S.C500	Arg × S.C500*
Free amino Arg (nmol/ml)										
Day 8†	68·6	87·2	117·4	–	–	–	9·0	0·021	–	–
Day 9	110·1	160·6	189·2	98·4	153·1	156·5	13·9	0·000	0·094	0·225
Day 11	157·7	201·4	210·9	102·9	160·1	173·7	15·7	0·000	0·000	0·767
Day 18	135·0	183·5	193·1	113·2	191·6	173·1	13·5	0·001	0·299	0·447
Antibody of *Salmonella* (%)‡										
Day 8†	2·0	2·2	1·7	–	–	–	0·6	0·753	–	–
Day 9	3·3	1·7	2·3	3·5	5·4	5·5	0·6	0·711	0·048	0·416
Day 11	2·4	1·4	3·3	27·7	11·1	14·9	2·3	0·104	0·000	0·128
Day 18	2·7	2·8	4·1	50·5	29·1	36·7	4·0	0·131	0·000	0·116

* Arg × S.C500 interaction effect.
† The serum samples of day 8 were collected before the administration of S.C500.
‡ The levels of the antibody are expressed by the value of optical density percentage (OD%). $OD\% = (OD_{sample} - OD_{blank\ control}/OD_{positive\ control} - OD_{blank\ control})$.

three dietary treatments during 0–7 d (pre-challenge); S.C500 challenge resulted in a remarkable reduction of average daily gain ($P<0·05$) and average daily feed intake ($P<0·01$). However, dietary supplementation of 0·5 or 1·0 % Arg trended to alleviate the loss of average daily gain and average daily feed intake (Table 4).

Serum C-reactive protein, IL-12 and interferon-γ levels

As shown in Table 5, Arg supplementation significantly increased serum IFN-γ concentration ($P<0·05$), but had no influence on serum CRP and IL-12 levels on day 8 (pre-challenge). The administration of S.C500 significantly increased serum CRP (on days 9 and 11, $P<0·01$), IFN-γ (on days 11 and 18, $P<0·05$ and $P<0·01$) and IL-12 concentrations (on day 11, $P<0·05$), and trended to enhance the concentration of IFN-γ on day 9 ($P=0·09$) and CRP on day 18 ($P=0·09$). Dietary supplementation of Arg increased the concentration of IFN-γ (on day 9, $P<0·05$), and significantly alleviated the increase in CRP (on day 9 and 11, $P<0·05$), IFN-γ and IL-12 (on days 11 and 18) after the administration of S.C500. Furthermore, Arg supplementation and S.C500 challenge had a significant interaction effect on CRP (on days 9 and 11, $P<0·05$), IL-12 (on day 9, $P<0·01$) and IFN-γ (on day 9, $P<0·05$).

Expression of Toll-like receptor 4, 5, myeloid differentiation factor 88, p38 mitogen-activated protein kinases, p65 NF-κB and TNF-α in tissues

The data for mRNA expression of TLR4, TLR5, Myd88, p38 MAPK, p65 NF-κB and TNF-α are shown in Table 6. The expression of TLR4 mRNA was increased after the administration of S.C500 in the spleen ($P<0·05$), liver ($P<0·01$) and lung ($P<0·05$). Arg supplementation decreased TLR4 mRNA abundance in the liver ($P=0·064$), MLN ($P=0·01$) and ILN ($P<0·01$).

TLR5 mRNA abundance in the lung ($P < 0·01$) and MLN ($P=0·055$) was enhanced after the administration of S.C500. Arg supplementation decreased TLR5 mRNA abundance in the spleen ($P < 0·01$) and MLN ($P < 0·01$). Furthermore, Arg supplementation and S.C500 challenge had a significant interaction effect on TLR5 mRNA abundance in the ILN ($P < 0·05$) and MLN ($P < 0·01$).

The S.C500 challenge increased Myd88 mRNA abundance in the liver ($P<0·01$). Arg supplementation induced a significant decrease in Myd88 mRNA abundance in the liver ($P<0·01$), lung ($P<0·01$), MLN ($P<0·05$) and ILN ($P<0·05$). There was no obvious interaction effect of Arg and S.C500 administration on the mRNA expression of Myd88.

Arg supplementation caused mRNA expression of NF-κB to decrease ($P<0·05$) in all tissues except the ILN, and the S.C500 challenge increased NF-κB mRNA abundance in the liver ($P<0·05$) and MLN ($P<0·05$). Arg and S.C500 challenge had an interaction effect on the mRNA expression of NF-κB in the spleen ($P<0·05$), liver ($P=0·079$) and lung ($P=0·061$).

The mRNA expression of TNF-α was increased remarkably in the liver ($P<0·01$), kidney ($P<0·01$), lung ($P<0·01$) and

Table 4. Effects of arginine (Arg) supplementation and *Salmonella enterica* serovar Choleraesuis C500 (S.C500) challenge on the performance of weaned piglets (as-fed basis)*†

	S.C500 (−)			S.C500 (+)				Analysis by effect (*P*)		
Response	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	SEM	Arg	S.C500	Arg × S.C500‡
ADG (g)										
Day 1–7	147	163	197	–	–	–	12	0·117	–	–
Day 8–17	311	363	378	265	282	275	14	0·338	0·011	0·704
Day 1–17	253	280	315	234	237	243	10	0·878	0·012	0·482
ADFI (g)										
Days 1–7	243	265	262	–	–	–	11	0·700	–	–
Days 8–17	412	428	453	315	350	372	13	0·194	0·002	0·764
Days 1–17	337	352	387	276	308	324	11	0·812	0·381	0·011
Feed:gain										
Days 1–7	1·63	1·59	1·35	–	–	–	0·09	0·192	–	–
Days 8–17	1·31	1·20	1·21	1·20	1·26	1·36	0·04	0·112	0·615	0·732
Days 1–17	1·36	1·28	1·26	1·23	1·31	1·35	0·03	0·956	0·823	0·059

ADG, average daily gain; ADFI, average daily feed intake.
* S.C500 was injected on day 8.
† 1–7 d, *n* 12; 8–17 d; 1–17 d, *n* 6.
‡ Arg × S.C500 interaction effect.

Table 5. Effects of arginine (Arg) supplementation and *Salmonella enterica* serovar Choleraesuis C500 (S.C500) challenge on serum C-reactive protein (CRP), IL-12 and interferon (IFN)-γ levels in weaned piglets*

(Mean values with their standard errors, day 8, *n* 12; days 9, 11, 18, *n* 6.)

	S.C500 (−)			S.C500 (+)				Analysis by effect (*P*)		
Response	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	SEM	Arg	S.C500	Arg × S.C500†
Day 8‡										
CRP (ng/ml)	0·85	0·83	0·82	–	–	–	0·03	0·957	–	–
IL-12 (pg/ml)	92·1	123·3	117·6	–	–	–	9·7	0·177	–	–
IFN-γ (pg/ml)	173·8	279·3	268·9	–	–	–	20·1	0·023	–	–
Day 9										
CRP (ng/ml)	0·74	0·71	0·81	2·34	1·53	1·59	0·12	0·032	0·000	0·023
IL-12 (pg/ml)	95·7	131·8	123·1	133·7	96·4	107·9	4·7	0·480	0·540	0·009
IFN-γ (pg/ml)	185·5	213·2	363·6	285·9	331·2	307·6	17·1	0·040	0·090	0·045
Day 11										
CRP (ng/ml)	0·67	0·61	0·77	2·22	1·57	1·69	0·13	0·044	0·000	0·047
IL-12 (pg/ml)	98·6	107·4	117·4	147·8	126·3	121·7	5·0	0·581	0·024	0·160
IFN-γ (pg/ml)	174·7	234·6	293·8	366·3	285·1	191·4	19·3	0·718	0·040	0·136
Day 18										
CRP (ng/ml)	0·71	0·68	0·56	0·95	0·82	0·77	0·06	0·510	0·090	0·951
IL-12 (pg/ml)	117·5	134·8	125·3	146·7	127·2	136·8	4·5	0·821	0·245	0·337
IFN-γ (pg/ml)	205·6	269·4	330·3	618·0	424·9	439·6	39·5	0·494	0·004	0·215

* S.C500 was injected on day 8.
† Arg × S.C500 interaction effect.
‡ The serum samples of day 8 were collected before the administration of S.C500.

Table 6. Effects of arginine (Arg) supplementation and *Salmonella enterica* serovar Choleraesuis C500 (S.C500) challenge on the relative mRNA expression of Toll-like receptor (TLR) 4, TLR5, myeloid differentiation factor 88 (Myd88), p38 mitogen-activated protein kinase (MAPK), p65 NF-κB and TNF-α in the tissues of weaned piglets*

(Mean values with their standard errors, *n* 4)

	S.C500 (−)			S.C500 (+)				Analysis by effect (*P*)		
Response	0 % Arg/kg†	0·5 % Arg/kg	1·0 % Arg/kg	0 % Arg/kg	0·5 % Arg/kg	1·0 % Arg/kg	SEM	Arg	S.C500	Arg × S.C500‡
TLR4										
Spleen	1·00^{a,b}	0·47^{a}	0·63^{a,b}	2·22^{c}	1·71^{b,c}	0·69^{a,b}	0·19	0·164	0·039	0·319
Liver	1·00^{a}	0·84^{a}	1·02^{a}	1·92^{b}	1·15^{a}	1·34^{a}	0·11	0·064	0·004	0·189
Lung	1·00^{a}	1·44^{a,b}	1·10^{a}	3·74^{b}	2·72^{a,b}	2·85^{a,b}	0·35	0·852	0·031	0·482
Kidney	1·00	1·11	1·80	1·82	0·74	0·70	0·35	0·645	0·565	0·560
MLN	1·00^{a}	0·65^{a}	0·96^{a}	1·15^{b}	0·76^{a}	0·81^{a}	0·08	0·010	0·234	0·138
ILN	1·00^{a}	0·33^{b}	0·67^{a,b}	1·35^{c}	0·47^{a,b}	0·51^{a,b}	0·13	0·001	0·218	0·163
TLR5										
Spleen	1·00^{a}	0·48^{b}	0·55^{a,b}	1·51^{c}	0·61^{a,b}	0·75^{a,b}	0·11	0·001	0·282	0·113
Liver	1·00^{a,b}	0·97^{a,b}	0·96^{a,b}	1·34^{b}	0·68^{a}	0·92^{a,b}	0·09	0·274	0·976	0·341
Lung	1·00^{a,b}	1·12^{a}	0·43^{a,b}	3·03^{c}	1·03^{a,b}	1·67^{b}	0·29	0·233	0·006	0·084
Kidney	1·00	0·91	1·27	1·79	1·11	0·90	0·30	0·531	0·838	0·587
MLN	1·00^{a}	0·90^{a,b}	0·85^{a,b}	1·48^{c}	0·69^{b}	0·68^{b}	0·10	0·001	0·055	0·001
ILN	1·00^{a}	1·10^{a,b}	0·86^{a}	1·62^{b}	0·78^{a}	1·33^{a,b}	0·09	0·155	0·101	0·048
Myd88										
Spleen	1·00^{a,b}	0·78^{a}	1·03^{a,b}	1·12^{b}	1·01^{a,b}	0·98^{a,b}	0·04	0·175	0·174	0·271
Liver	1·00^{a}	0·63^{a}	0·79^{a}	1·63^{b}	1·03^{a}	1·02^{a}	0·09	0·009	0·003	0·359
Lung	1·00^{b}	0·58^{a}	0·73^{a}	1·01^{b}	0·87^{a}	0·71^{a}	0·05	0·002	0·817	0·888
Kidney	1·00	0·92	1·04	1·12	0·79	0·96	0·04	0·084	0·684	0·337
MLN	1·00^{a}	0·78^{a}	0·86^{a}	1·23^{b}	0·84^{a}	0·87^{a}	0·05	0·015	0·297	0·752
ILN	1·00^{a}	0·86^{a}	1·25^{a,b}	1·55^{b}	0·85^{a}	1·18^{a,b}	0·08	0·021	0·186	0·081
P38 MAPK										
Spleen	1·00	0·90	0·78	1·05	0·93	0·87	0·04	0·119	0·455	0·951
Liver	1·00	0·81	0·73	1·10	0·83	0·79	0·08	0·052	0·104	0·304
Lung	1·00	0·86	0·76	0·94	0·84	0·98	0·07	0·427	0·898	0·273
Kidney	1·00	1·02	1·03	1·26	0·83	0·82	0·05	0·063	0·365	0·051
MLN	1·00	1·16	0·97	1·59	0·93	1·11	0·05	0·768	0·621	0·391
ILN	1·00	0·78	0·85	0·83	0·77	0·78	0·03	0·091	0·108	0·496
P65 NF-κB										
Spleen	1·00^{a,b}	1·03^{a,b}	0·89^{a,b}	1·13^{b}	0·88^{a}	0·90^{a}	0·04	0·035	0·492	0·036
Liver	1·00^{a}	0·80^{a}	0·90^{a}	1·66^{b}	1·04^{a}	0·87^{a}	0·08	0·012	0·023	0·079
Lung	1·00^{b}	0·63^{a}	0·56^{a}	0·93^{a,b}	0·62^{a}	0·66^{a}	0·05	0·016	0·305	0·061
Kidney	1·00^{a,b}	0·91^{a,b}	0·96^{a,b}	1·21^{b}	0·76^{a}	0·84^{a,b}	0·05	0·044	0·820	0·174
MLN	1·00^{a}	0·77^{a}	0·73^{a}	1·53^{b}	1·01^{a}	0·87^{a}	0·09	0·034	0·044	0·474
ILN	1·00^{a,b}	0·85^{a}	0·86^{a,b}	0·91^{b}	0·87^{a,b}	0·87^{a,b}	0·06	0·113	0·411	0·617
TNF-α										
Spleen	1·00^{b}	0·78^{a,b}	0·60^{a}	1·27^{c}	0·76^{a,b}	0·84^{a,b}	0·06	0·012	0·078	0·488
Liver	1·00^{a}	1·00^{a}	0·99^{a}	2·21^{b}	1·44^{a}	1·18^{a}	0·12	0·027	0·001	0·029
Lung	1·00^{a}	1·19^{a}	1·02^{a}	2·00^{b}	1·57^{a,b}	1·51^{a,b}	0·12	0·530	0·003	0·304
Kidney	1·00^{a}	1·03^{a}	0·95^{a}	2·99^{b}	1·55^{a}	1·45^{a}	0·22	0·114	0·007	0·122
MLN	1·00^{a}	1·02^{a}	0·96^{a}	2·41^{b}	1·31^{a}	0·85^{a}	0·15	0·010	0·011	0·012
ILN	1·00^{a}	1·05^{a,b}	0·83^{a}	1·68^{b}	0·81^{a}	0·94^{a}	0·06	0·027	0·375	0·038

MLN, mesenteric lymph node; ILN, inguinal lymph node.
^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* Tissues were collected in the morning of the day.
† The control group of relative gene expression.
‡ Arg × S.C500 interaction effect.

MLN ($P<0·05$) post-injection with S.C500. However, Arg supplementation decreased TNF-α mRNA abundance obviously in the spleen ($P<0·05$), liver ($P<0·05$), MLN ($P=0·01$) and ILN ($P<0·05$). Arg and S.C500 challenge had an interaction effect on the mRNA expression of TNF-α in the liver ($P<0·05$), MLN ($P<0·05$) and ILN ($P<0·05$).

Discussion

Flagellum and LPS are the main virulence factors of *S. enterica* serovar Choleraesuis, which can cause an inflammatory reaction[19,22–24]. In order to evaluate whether Arg supplementation could alleviate immune challenge through the TLR4-Myd88 signalling pathway in weaned pigs, a model for inducing immune stress in pigs by injecting *S. enterica* serovar Choleraesuis bacterin was used. The bacterin used in the present experiment was a kind of attenuated bacterin that still contained virulence factors. It was shown that serum *Salmonella* antibody, CRP levels and TNF-α mRNA were significantly increased after being challenged with S.C500. The results indicated that the immune challenge model of *S. enterica* serovar Choleraesuis bacterin injection was successful.

In the present study, serum Arg concentration was increased upon dietary Arg supplementation, while the S.C500 challenge significantly decreased serum Arg concentration, indicating that pigs consumed more Arg after the immune challenge.

As an essential amino acid, Arg promotes protein synthesis, maintains normal functions of the enteric canal in the intestinal tract, decomposes to important biological regulators such as polyamine, accelerates the release of growth hormone and insulin-like growth factor, stimulates the proliferation of T-lymphocytes, and enhances the phagocytosis of macrophages and the cytoactivity of natural killer cells[25,26]. Due to the functions of Arg, it was easy to understand why piglets need more Arg under the condition of immune challenge.

Before the S.C500 challenge, Arg supplementation had no significant effect on the growth performance of weaned piglets, which is consistent with the finding of Liu *et al.*[27]. However, Kim *et al.*[4] reported that dietary supplementation with 0·2 and 0·4 % Arg to milk-fed piglets improved growth performance. The difference may be due to the Arg concentration used in the diet. In the present study, the digestible Arg level of the basal diet (1·14 %) was adequate for maintaining the growth of weaned piglets in a normal physiological condition. The S.C500 challenge resulted in a remarkable reduction of growth performance, and the dietary supplementation of 0·5 or 1·0 % Arg alleviated the loss of average daily gain and average daily feed intake. The results from the present study are, however, consistent with previous findings that dietary Arg supplementation is helpful to piglets suffering from infection and disease[27].

In the present study, we supposed that the administration of S.C500 up-regulates the expression of pro-inflammatory cytokines in the TLR4-Myd88 signalling pathway and Arg exerts its protective effects through influencing the TLR4-Myd88 signalling pathway. In the present study, serum CRP, IFN-γ and IL-12 concentrations, and the mRNA abundance of TNF-α were acutely increased after the S.C500 challenge. The results are consistent with previous reports that over-production of pro-inflammatory cytokines and their mRNA abundance had a negative effect on pigs[28,29]. Pie *et al.*[28] reported that inflammatory cytokines such as IL-6, IL-12 and TNF-α mRNA increased in the intestine of piglets after weaning, and it may contribute to both anatomical and functional intestinal disorders. In the present study, Arg supplementation repressed the production of serum CRP, IFN-γ and IL-12, and down-regulated the mRNA abundance of TNF-α after the S.C500 challenge, indicating that Arg has protective effects on pigs under the immune challenge. In addition, the protective effects of Arg on animals have also been observed in other studies[30,31]. Liu *et al.*[27] reported that dietary supplementation of Arg exerted beneficial effects in alleviating the gut mucosal injury of LPS-challenged piglets.

The underlying mechanism of the fact that Arg regulates cytokine production and consequently displays protective effects on piglets was unknown. It is noteworthy that we found Arg to be closely related to the TLR4-Myd88 signalling pathway that mediates an innate immune response. It has been demonstrated in previous studies[16] that the TLR4-Myd88 signalling pathway is involved in inflammation. TLR4 can form a homodimer by itself or form a heterodimer with TLR5[32], and transmit the signal to the downstream molecule Myd88 after combining with ligands such as LPS and bacterial flagella[15,33], and finally transmit to NF-κB and MAPK to produce an immunological reaction[16]. p38 MAPK and p65 NF-κB are the family members of MAPK and NF-κB, respectively, and they are the main signalling molecules in the TLR4-Myd88 signalling pathway of their family[34–36]. The cytokines that were produced post-activation of the signalling pathway mainly consist of IL-1β, IL-6, IL-12, TNF-α and IFN-γ[15,16]. Overactivation of this signalling pathway would aggravate an inflammatory reaction and then have negative effects on the organs of animals. The results of the present study showed that mRNA levels of TLR4, TLR5, Myd88 and p65 NF-κB to the spleen, liver, lung, MLN and ILN were up-regulated to different degrees when post-challenged with S.C500, but the addition of Arg attenuated the harmful effects. However, how Arg interacts with the TLR4 signalling pathway is yet unknown.

Overactivation of the TLR4 signalling pathway was accompanied by the overexpression of inducible NO synthase[17,18]. Inducible NO synthase is one kind of enzyme that is expressed in quantity when animals have an external stimulus such as LPS and bacterial infection; its main function is to decompose Arg into NO[19,37]. This means that in such circumstances, the animal's organs need more NO to resist the damages brought by external challenges. Wu *et al.*[38] found that NO could down-regulate TLR4 gene expression when it is overactivated in rats, and Arg is the only zymolyte of NO synthesis in the animal's organ[39]. So we deduce that an important reason for piglets under an immune challenge to accelerate Arg consumption is that the organs need the decomposition of additional Arg into NO to restrain the overactivation of the TLR4-Myd88 signalling pathway, but whether Arg and the TLR4 signalling pathway are associated with NO needs further confirmation.

In addition, the possible reason why mRNA expression of p38 MAPK did not change significantly is that the biological functions of MAPK were mainly dependent on phosphorylation and dephosphorylation[40,41] instead of being regulated by gene expression levels in this signalling pathway.

In conclusion, dietary Arg supplementation was helpful to attenuate the reduction of weight gain and feed intake caused by the administration of *S. enterica* serovar Choleraesuis bacterin. It is possible that the protective effects of Arg on piglets challenged with S.C500 are associated with a decrease in CRP and the inhibition of the excessive activation of the TLR4-Myd88 signalling pathway through the down-regulation of TLR4, TLR5, Myd88, p65 NF-κB and TNF-α mRNA abundance of tissues.

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