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Original article

LBP gene methylation involved in mRNA expression and resistance to *E. coli* F18 in weaned piglets

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Abstract

Lipopolysaccharide binding protein (LBP) plays an important role in recognizing and regulating endotoxin. In this study, we aimed at clarifying the relationship between the methylation of *LBP* gene and it's expression, to identify mechanisms involved in resistance to *E. coli* F18 in Sutai weaned piglets. *LBP* expression was detected by real-time PCR in duodenum and jejunum tissues from *E. coli* F18-sensitive or -resistant piglets. The *LBP* methylation status of the regions with many CG sites upstream of the transcription start site was analyzed by Bbisulfite Sequencing PCR (BSP) +Miseq in jejunum and duodenum tissue. The results showed that *LBP* expression was significantly higher in the sensitive group than the resistant group in duodenum tissue ($p<0.05$). There was a negative correlation between the methylation of CpG islands upstream of the *LBP* transcription start site and its expression; the methylation at two CpG sites in particular was significantly correlated with reduced *LBP* expression (CpG-1 and CpG-2; $p<0.05$ and $p<0.01$, respectively). These indicated that the methylation of CpG-1 and CpG-2 sites in the *LBP* region is involved in the regulation of *LBP* expression, and may provide key contributions to resisting *E. coli* F18 in Sutai weaned piglets.

Key words: *LBP* gene, methylation analysis of the *LBP* region, *E.coli* F18 strain, weaned piglets

Introduction

Lipopolysaccharide binding protein (LBP) is a glycoprotein of 58-60 ku, and can be detected as a soluble factor in human and animal sera, and is mainly synthesized by liver cells (Jiang 1997). LBP can promote the degradation of lipopolysaccharide multimers into monomers, and may facilitate the association of LPS to cluster of differentiation antigen 14 (CD14), enhancing the sensitivity of target cells to LPS. In macrophages, transmembrane signal transmission by CD14 and toll-like receptor 4-myeloid differentiation protein 2 (TLR4-MD2) triggers intracellular signal transduction and cytokine production, which lead to the initiation of a series of inflammatory reactions. Meanwhile, LBP can promote the combination of LPS and lipoproteins, accelerating the clearance of LPS in vivo (Feng et al. 2007). It was found

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that the N-terminal domain of LBP functions mainly bind to LPS, resulting in a reduction of the proinflammatory effect of LPS and competitive inhibition of the increased sensitivity to LPS induced by LBP (Han et al. 1994). LPS can combine with LBP to form a LPS-LBP complex, which is discriminated by CD14 receptors, establishing a key step in mediating the production of an inflammatory response. Therefore, the genetic mutation of the *LBP* gene may be related to some diseases. Hubacek et al. (2001) found that a polymorphism of the human *LBP* gene, Cys98Gly, was associated with susceptibility to systemic infection. Liu et al. (2008) detected the existence of the 292G/A and 1168G/A mutations in Yorkshire and Duroc pigs, and found that the polymorphism was related to general resistance to disease .

Methylation of DNA is an important epigenetic regulation mechanism, whereby methyl groups add to DNA segments, which means 5-cytosine is converted to 5-methylcytosine (5mC) by methtyltransferases especially in regions with a high frequency of CpG sites (CpG islands). It was found that an active DNA methyltransferase existes in adult Drosophila, and the presence of asymmetric methylation confirm that sequence specificity is exhibited at corresponding sites in the genomic DNA (Chitra et al. 2015). DNA methylation occurs mainly in CpG island-rich promoter regions, and methylation of these regions, hence hinders the binding of transcription factors to the promoter, thereby inhibiting gene transcription (Wang and Xu 2014). Studies have also shown that methylation in the promoter region of genes that lack CpG islands can also negatively correlate with expression of those genes (Han et al. 2011, Balasubramanian et al. 2012).

Enterotoxigenic Escherichia coli F18 and K88 (F4) are the most common and dangerous pathogens in pig breeding industries. These pathogens can cause yellow scour, white scour, diarrhea, edema, and other diseases in piglets (Van den Broeck et al. 2000). Gram-negative bacteria (G^-) include Escherichia coli, and more than 20 other kinds of pathogenic bacteria. All these bacteria have a common characteristic, that the major constituent of the cell wall outer membrane is lipopolysaccharide (LPS), or endotoxin, which is the main factor that determines G^- bacterial infection, and has a close relationship with many other diseases in humans. It has been shown that the lipopolysaccharide binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) play an important role in the identification and regulation of endotoxin in the body (Zheng et al. 2001). As G^- bacteria, *E. coli* F18 releases large amounts of endotoxin (LPS) after death, which initiates a multistage kinase cascade, activating the MAPK signal pathway and strongly stimulating inflammatory responses (Dziareke et al. 1996). Based on previous research describing the biological function of the *LBP* gene, we hypothesized that the *LBP* gene may be associated with resistance to *E. coli* F18.

In previous studies, we established Escherichia coli-resistant and -sensitive populations of Sutai (Duroc × Meishan) pigs, we analyzed the production of functional adhesin through the type V secretion system, combined with receptor binding experiments, to further determine and verify the resistance/sensitivity to the *E. coli* F18 strain among these pig populations (Wu et al. 2007). We uesd Agilent double labeled gene expression profiling to analyze differential gene expression in the duodenum of *E. coli* F18-resistant and -sensitive pig populations (data presented in NCBI Gene Expression Omnibus, accession number GSE26854), and we determined that the glycosphingolipid biosynthesis pathway and the major functional genes of this pathway had a regulatory role in the resistance to *E. coli* F18. At present, the hereditary basis and regulative mechanism of anti-*E. coli* F18 are unknown in weaned piglets of Chinese native breeds, and mechanisms of resistance to *E. coli* F18 in native pig breeds and exotic pig breeds might be different (Bao et al. 2012). In this study, BSP (bisulfite sequencing PCR) +Miseq was used to detect the methylation status of regions with many CG sites of the *LBP* geneupstream of the transcription start site in duodenum and jejunum tissues in *E. coli* F18-resistant and -sensitive Sutai weaned piglets, and the correlation of methylation status with *LBP* mRNA expression was determined. The aim of this study is to investigate the correlation between *LBP* gene methylation and mRNA expression, in order to provide a theoretical basis for further study on the function of the *LBP* gene and its involvement in mechanisms of resistance to *E. coli* F18 in pig populations.

Materials and Methods

Ethics Statement

The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee with permit number: SYXK (Su) IACUC 2012-0029. All experimental procedures involving piglets were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Table 1. Primer Sequences.

Primer	Sequence (5'–3')	Length (bp)
BSP PCR primer	F:TTATTTTTGTAGTTTTGTTTTAATTGTATTA R: TCCCTTCTAAAAAAACAAATTTTCTC	556
LBP RT-PCR primer	F: ATATCGAATCTGCGCTCCGA R: TTGATGCCAACCATTCTGTCC	136
GAPDH RT-PCR primer	F: ACATCATCCCTGCTTCTACTGG R: CTCGGACGCCTGCTTCAC	187

Experimental Materials and Sample Collection

Enterotoxigenic Escherichia coli (ETEC) F18-resistant and -sensitive Sutai piglets were obtained from the Center of Sutai Pig Breeding (Suzhou City, Jiangsu Province, China). All the piglets were maintained under the same piggery conditions, the environmental temperature was controlled at 25~28. Around the time of weaning (35 days old), we selected forty full-sib healthy piglets from 8 families with *E. coli* F18-resistant population when the piglets are easy to infect with ETEC F18, the birth weights and weaning weights were similar, reared under the same conditions. Using the adhesion test in intestinal epithelial cells to select and verify *E. coli* F18 resistance/susceptibility. Anaerobic bacteria were washed with phosphate buffer saline (PBS) three times, and the bacterial concentration was adjusted to approximately 1×10^9 CFU/ml. After adding 1% mannose (w/v) to 0.5 ml of wild type or recombinant bacteria, the suspensions were incubated at 37°C for 30 min, mixed with 0.5 ml of small intestinal cells, incubated at 37°C for 30 min, and finally centrifuged at $900 \times g$ for 5 min. At this time, 50 fl of the preparation was extracted after being suspended in PBR (0.24 g KH_2PO_4, 1.44 g Na_2HPO_4, 0.42 g KCl, 9 g NaCl, 0.25 g $CaCl_2$ and distilled water was added to 1 l), and the mixture (50 µl) was deposited on a glass slide, air dried, heat-fixed and stained with methylene blue for 5 min (Wu et al. 2007). Villi were then taken with an oil immersion lens at 1000 × magnification and the adhesion of bacteria was evaluated quantitatively by counting the mean number of bacteria adhering along a 50 fm villous brush border at 20 randomly selected sites for each piglet, after which the mean bacterial adhesion was calculated. Adhesion of fewer than five and more than 30 bacteria per 250 mm brush border length was noted as resistant or susceptible respectively (Coddens et al. 2007). Finally, we obtained four resistant and four sensitive piglets for further studies. After the pig was electrically stunned, it was venously bloodlet within 15 s to avoid suffering. The duodenum and jejunum tissues were then collected and frozen in liquid nitrogen, and subsequently stored in a low-temperature freezer (-80°C).

Bioinformatic Analysis

According to our previous research we obtained the promoter sequence in the 3-kb region upstream of the *LBP* gene (Yu et al. 2012). BSP primers were designed using the MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi).

Bisulfite Sequencing PCR Methylation Analysis

Duodenum and jejunum tissues were used to extract Genomic DNA through standard phenol/chloroform extraction. Using an EpiTect bisulfite kit from Qiagen (Valencia, CA, USA) to convert DNA, according to the instructions. Touchdown PCR amplification reactions of bisulfite-treated DNA (BST-DNA) was carried out: 3 µl DNA template, 3 µl 10 × PCR buffer, 2 µl Mg^{2+} (25 mmol/l), 1 µl forward and reverse BSP primer (10 µM/l), 1 µl dNTPs (10 mmol/l), 0.8 µl Taq polymerase (5 U/µl), and water added to 50 µl. The following run protocol was used: 4 min at 98°C, then 20 cycles of 45 s at 94°C, 45 s at 66°C (reduced by 0.5°C with each cycle) and 1 min at 72°C; 20 cycles of 45 s at 94°C, 45 s at 56°C and 1 min at 72°C, then 10 min at 72°C for extension. The products (about 500 bp) were separated by electrophoresis on 1% agarose gels and were sent for sequencing by the Illumina Miseq platform.

Real-time PCR

Total RNA was extracted from duodenum and jejunum tissues (50-100 mg) according to Trizol reagent's instructions (TaKaRa Biotechnology Dalian Co., Ltd, China). Precipitated RNA was resuspended in 20 µl RNase-free H_2O and stored at -80°C. The RNA quality and quantity were assessed by agarose

gel electrophoresis and ultraviolet (UV) spectrophotometry, respectively. RNA was converted to cDNA through reverse transcription, The ABI Prism 7500 sequence-detection system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (TaKaRa) were used to quantify cDNA, the reaction system containing 2 µl cDNA, 0.4 µl each of forward and reverse primer (10 µmol/l), 10 µl SYBR Green Real-time PCR Master Mix (2×), 0.4 µl ROX Reference Dye II (50×), and 6.8 µl ddH_2O. The Real-time PCR reactions were amplified for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 62°C. A triplicate sample was tested in three independent experiments to ensure the reproducibility of the data. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. All primers (Table 1) were synthesized by Invitrogen Biotechnology.

Statistical Analysis

Methylation sequencing results were analyzed by QuMA software for analysis (http://quma.cdb.riken.jp/), the $2^{-\Delta\Delta Ct}$ (Shaw et al. 2007) method was used to process real-time PCR results, Statistical analyzes were carried out using SPSS 17.0 software. Correlation analysis was used for methylation level and mRNA expression by Pearson correlation.

Results

Bioinformatic Analysis

Meth Primer analysis showed that the porcine *LBP* gene did not contain CpG islands in the region 3000 bp upstream of the transcription start site (Fig. 1). Therefore, we analyzed the methylation status of the *LBP* gene in the region with many CG sites upstream of the transcription start site (approximately -1636 to -2091 bp), and designed primers for further analysis.

Amplification of Target Fragment

Bisulfite-modified DNA was amplified by PCR and separated by electrophoresis on 1% agarose gels. The size of the single product amplified was ofthe expected length for the target fragment, confirming the specificity of the PCR primers and demonstrating the product could be used for bisulfite sequencing PCR.

Differential Expression of *LBP* Gene in Duodenum and Jejunum Tissues between *E.coli* F18-Resistant and -Sensitive Sutai Piglets

For *E. coli* F18-resistant and -sensitive groups, we compared the expression of the *LBP* gene in the main tissues (duodenum and jejunum) that are impacted by *E. coli* F18. The expression level of *LBP* in the duodenum tissue from the sensitive group is significantly higher than in the duodenum tissue from the resistant group (Fig. 2; $p<0.05$). There were no significant differences in *LBP* expression in the jejunum tissue from the resistant and the sensitive groups.

Analysis of *LBP* Gene Single Locus Methylation Level

The methylation level of the region of the *LBP* gene analyzed is shown in Fig. 3. Methylation levels in the duodenum tissue of the resistant and sensitive groups were 84.42% and 85.15%, respectively (Fig. 3); the methylation levels in the jejunum tissue of the resistant and sensitive groups were 83.34% and 85.01%, respectively (Fig. 3). These results indicated that the methylation level of a single CpG site in the *LBP* gene in different tissues is relatively consistent. We observed an overall negative correlation between methylation levels of specific CpG sites and *LBP* mRNA expression, where CpG-1 and CpG-2 showed the most negative correlation with *LBP* gene expression ($p<0.05$, $p<0.01$, respectively; Fig. 4).

Discussion

LBP is the key factor that recognizes G^- endotoxin and initiates the immune response to pathogen insult. LBP binds with lipid A of LPS with high affinity, and forms a LPS-LBP complex (Wright et al. 1990). This complex can stimulate stimulates neutrophils, macrophages, and monocytes through the CD14 receptor to produce cytokines, resulting in enhanced cellular adhesion and initiation of inflammatory responses, and triggers a series of physiological reactions (Abrahamson et al. 1997). In our experimental model we used populations of Sutai piglets that are resistant and sensitive to *E. coli* F18 to detect and analyze differential gene expression of *LBP* in the duodenum and jejunum tissues. The results indicated that the expression level of *LBP* in the duodenum of the sensitive group is significantly higher than the resistant group ($p<0.05$). The intestinal tract is not only import-

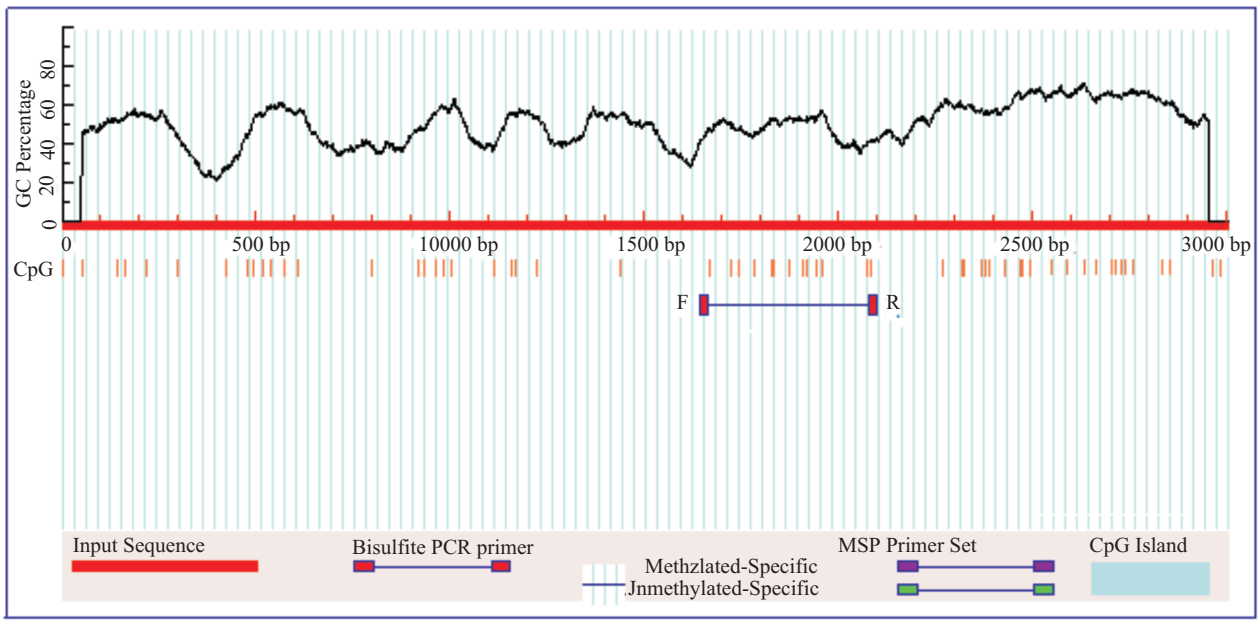

Fig. 1. Bioinformatics analysis of CpG-rich areas in the 3 kb region upstream of the porcine *LBP* gene.

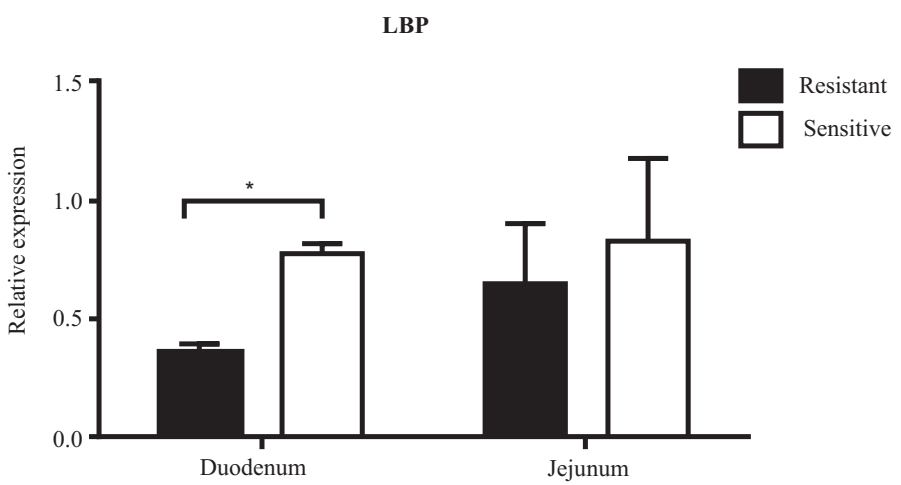

Fig. 2. *LBP* gene expression in intestinal tissue of *E. coli* F18 -resistant and -susceptible piglets.

ant for digestion and absorption of nutrients, but it is also the largest immune organ. *E. coli* F18 strain can cause diarrhea and edema, mainly through interactions of fimbriae and the brush border receptor of piglets mucosal epithelial cells, which facilitate bacterial colonization, reproduction, production of enterotoxin, and finally inducing diarrhea. Studies have shown that the presence of LPS and its subsequent recognition and binding is important in mediating inflammatory responses. LBP can regulate LPS to induce an anti-inflammatory response, but is also involved in mediating anti-pathogen responses by facilitating the clearance of bacteria through phagocytic action of other mononuclear cells, leading to the timely clean up of LPS and G^- bacteria in vivo (Wei 2011). As the expression level of *LBP* was elevated in the duodenum of sensitive piglets, we concluded that LBP may fail to clear LPS, and that G^- bacteria may remain in the intestine, leading to diarrhea and edema in sensitive piglets.

Promoters are often located at 5'flanking regions, methylation in these regions is one of the most usual epigenetic phenomena inhibiting gene expression. Normally, the change of DNA methylation occurs in the CpG islands (Kang et al. 2002). DNA methylation modulates transcriptional factor (TF) activity by regulating TF binding sites (TFBS) located in the regions with many CG sites, causing gene expression to be activated or inhibited (Beamer et al. 1998, Hubacek et al. 2001). Research has reported that promoter

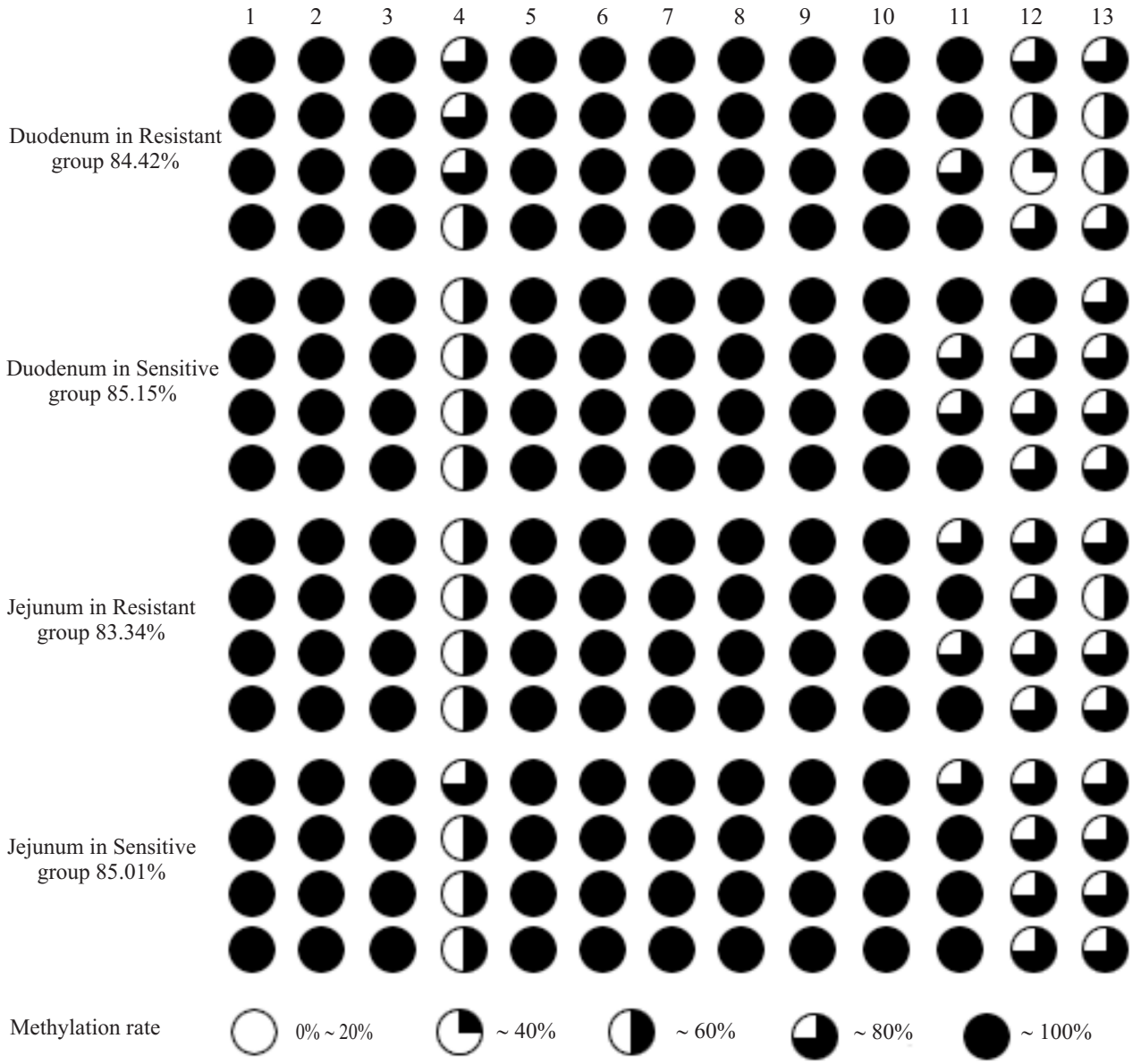

Fig. 3. Methylation level of the *LBP* gene fragment amplified from duodenum and jejunum tissues of *E. coli* F18-resistant and -susceptible piglets.

methylation is usually in a highly dynamic process during the development process (Baker-Andresen et al. 2014), which is particularly evident in embryonic development (Laurent et al. 2010), and it plays an important role in the reprogramming of the genome (Haaf 2006, Thurston et al. 2007). In this study, we investigated the region 3000 bp upstream of the *LBP* gene, and found that there were no CpG islands in this region. Therefore, we analyzed the methylation status of the region of the *LBP* gene with many CG sites upstream of the transcriptional start site, using BSP+Miseq to analyze the methylation status. Figure. 3 shows that the methylation level of the *LBP* gene region is relatively constant, methylation levels in different tissues were between 80-85%. Correlation analysis found that the methylation level of the *LBP* gene and mRNA expression exhibited a negative correlation, indicating that methylation of the *LBP* gene region may inhibit the expression of the *LBP* gene to a certain degree.

Barrera et al. (2012) reported that different CpG islands have different genomic elements, which have important and definite functions in gene regulation, and only a few CpG islands may be pivotal for regulation of gene expression, indicating that not every site of methylation can change gene expression (Mikeska et al. 2007). This indicated, while CpG island contains many CpG sites, the methylation level of each site will not necessarily lead to changes in mRNA expression; only methylation at a few key sites can lead to gene

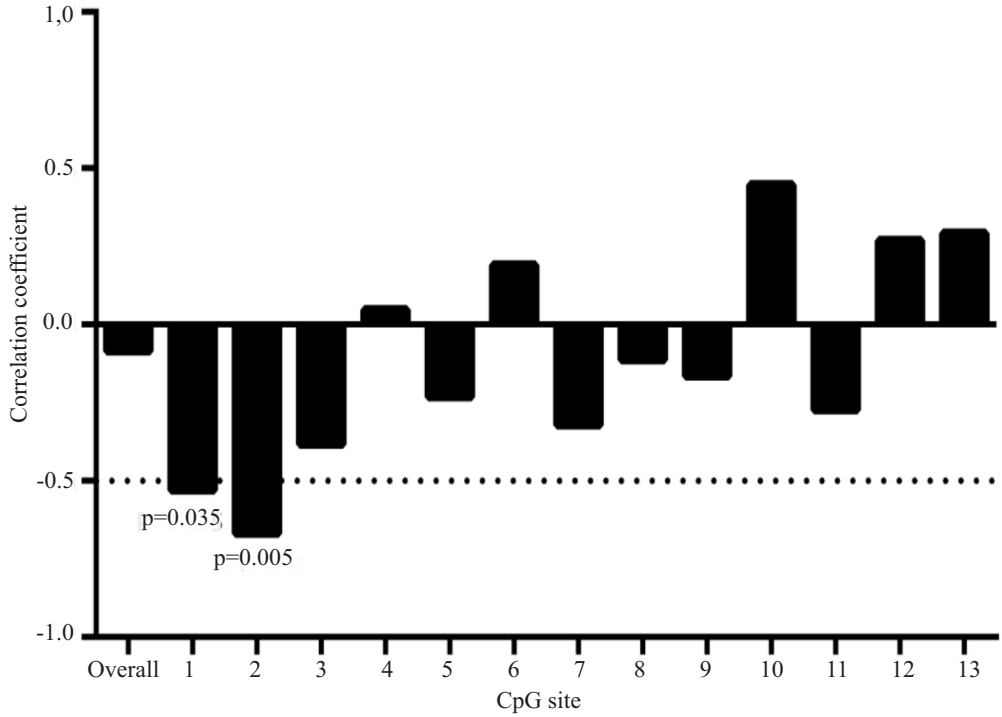

Fig. 4. Correlation analysis between methylation level of different CpG sites upstream of the *LBP* transcriptional start site and *LBP* mRNA expression.

silencing, and the methylation of other CpG sites may not effect gene expression (Xie et al. 2012). It was found that the region of the *LBP* gene we analyzed for methylation contained 13 CpG sites, among which methylation of CpG-1 was negatively correlated with *LBP* expression ($p<0.05$), and methylation of CpG-2 exhibited an extremely significant negative correlation with *LBP* expression ($p<0.01$). Therefore, we suggest that CpG-1 and CpG-2 may be the key sites of methylation that can regulate *LBP* gene expression. Since the other 11 sites had no direct correlation between mRNA expressions, these may only play supplementary roles in regulation of *LBP* expression. Because the sample sizes of this study are not large, and only from duodenum and jejunum tissues, we need to further verify the relationship between the methylation of promoter and *LBP* gene expression in additional samples to detect the methylation level of the *LBP* gene in other tissues. In this study, we analyzed the methylation level of 13 CpG sites upstream of the transcriptional start site of the *LBP* gene; it remains to be determined if there are other CpG sites within the promoter region of the *LBP* gene that could regulate *LBP* mRNA expression.

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