**The name of the journal: Animal**

**Supplementary material**

**Antibiotic-induced alterations of the gut microbiota and microbial fermentation in protein parallel the changes in host nitrogen metabolism of growing pigs**

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**Supplementary Table S1** *Ingredients composition and nutrient levels of the experimental diets for growing pigs (as-fed basis)*

|  |  |
| --- | --- |
| Items | Composition, % |
| Corn (8.0% CP) | 67.7 |
| Soybean meal (45.2% CP) | 20.0 |
| Fish meal (66.2% CP) | 1.00 |
| Limestone | 0.72 |
| Dicalcium phosphate | 1.10 |
| Soy protein concentrate | 2.00 |
| Soybean oil | 2.00 |
| Salt | 0.30 |
| Choline chloride (50%) | 0.10 |
| L-Lysine·HCl (98.5%) | 0.54 |
| DL-Methionine (99%) | 0.23 |
| L-Threonine (98.5%) | 0.21 |
| L-Tryptophan (98%) | 0.05 |
| L-Valine (98.5%) | 0.10 |
| L-Isoleucine (99%) | 0.06 |
| L-Leucine (99%) | 0.13 |
| L-Phenylalanine (99%) | 0.09 |
| L-Histidine·HCl (99%) | 0.07 |
| Fine rice bran | 2.32 |
| Choline chloride | 0.30 |
| Vitamin and mineral premix1 | 1.00 |
| Nutrient levels, % |  |
| NE2, MJ/kg | 9.92 |
| CP | 17.42 |
| EE | 4.81 |
| ADF | 4.60 |
| NDF | 14.20 |
| Ash | 4.83 |
| Ca | 0.63 |
| P | 0.53 |
| AA composition3, % |  |
| TLysine | 1.25 |
| TMethionine | 0.49 |
| TThreonine | 0.82 |
| TTryptophan | 0.24 |
| TValine | 0.89 |
| TIsoleucine | 0.71 |
| TLeucine | 1.58 |
| TPhenylalanine | 0.92 |
| THistidine | 0.49 |
| TArginine | 1.12 |
| TTyrosine | 0.58 |
| TCysteine | 0.26 |
| Methionine + Cysteine | 0.74 |

NE = net energy; EE = ether extract.

1 Supplied the following per kg of diet: 8 000 IU, vitamin A; 2400 IU, vitamin D3; 20 mg, vitamin E; 15 mg, pantothenic acid; 5 mg, vitamin B6; 0.3 mg, biotin; 3 mg, folic acid; 0.03 mg, vitamin B12; 40 mg, ascorbic acid; 120 mg, Fe; 25 mg, Cu; 20 mg, Mn; 150 mg, Zn; 0.5 mg, I; 0.30 mg, Se.

2 Values for NE were calculated according to Noblet *et al*. (1994), the contents of EE, CP, ADF, NDF, Ash, Ca, and P were analyzed.

3 T = total.

**Supplementary Table S2** *List of primers for pigs used in this study*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Forward primer (5'-3') | Reverse primer (5'-3') | References1 | Annealing temp. (°C) |
| Total bacteria | GTGSTGCAYGGYYGTCGTCA | ACGTCRTCCMCNCCTTCCTC | Maeda et al., 2003 | 60 |
| Firmicutes | GGAGYATGTGGTTTAATTCGAAGCA | AGCTGACGACAACCATGCAC | Guo et al., 2008 | 60 |
| Bacteroidetes | GGARCATGTGGTTTAATTCGATGAT | AGCTGACGACAACCATGCAG | Guo et al., 2008 | 60 |
| *Clostridium* cluster IV | GCACAAGCAGTGGAGT | CTTCCTCCGTTTTGTCAA | Matsuki et al., 2004 | 60 |
| *Escherichia coli* | CATGCCGCGTGTATGAAGAA | CGGGTAACGTCAATGAGCAAA | Huijsdens et al., 2002 | 60 |
| *Lactobacillus* | AGCAGTAGGGAATCTTCCA | ATTCCACCGCTACACATG | Khafipour et al., 2009 | 60 |
| *Clostridium* cluster XIVa | CGGTACCTGACTAAGAAGC | AGTTTYATTCTTGCGAACG | Bartosch et al., 2014 | 60 |
| *Bifidobacterium* | TCGCGTCYGGTGTGAAAG | GGTGTTCTTCCCGATATCTACA | Walker et al., 2011 | 60 |
| *Ruminococcus* | GAAAGCGTGGGGAGCAAACAGG | GACGACAACCATGCACCACCTG | Verma et al., 2010 | 60 |
| *ETEC K88 fimbriae* | GCACATGCCTGGATGACTGGTG | CGTCCGCAGAAGTAACCCCACCT | Chen et al., 2014 | 60 |
| *sta* | GAAACAACATGACGGGAGGT | GCACAGGCAGGATTACAACA | Lee etal., 2008 | 60 |

ETEC = *Enterotoxigenic Escherichia coli*; sta = Heat-stable enterotoxin 1.

**1References:**

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Verma R, Verma AK, Ahuja V and Paul J 2010. Real-time analysis of mucosal flora in patients with inflammatory bowel disease in India. Journal of Clinical Microbiology 48, 4279-4282.

Chen XY, Woodward A, Zijlstra RT and Ganzle MG 2014. Exopolysaccharides synthesized by Lactobacillus reuteri protect against Enterotoxigenic Escherichia coli in piglets. Applied and Environmental Microbiology 80, 5752-5760.

Lee SI, Kang SG, Kang ML and Yoo HS 2008. Development of multiplex polymerase chain reaction assays for detecting enterotoxigenic Escherichia coli and their application to field isolates from piglets with diarrhea. Journal of Veterinary Diagnostic Investigation 20, 492-496.

**Supplementary Table S3** *Effects of antibiotics on growth performance in growing pigs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Treatment1 | | SEM | *P*-value |
| CON | ANTI |
| ADFI, kg/d | 1.45 | 1.52 | 0.050 | 0.32 |
| Initial BW, kg | 18.42 | 18.74 | 0.638 | 0.72 |
| Final BW, kg | 30.99 | 32.77 | 1.247 | 0.33 |
| Total BW Gain, kg | 12.57 | 14.03 | 0.835 | 0.24 |
| ADG, kg/d | 0.60 | 0.67 | 0.040 | 0.24 |
| F:G | 2.46 | 2.31 | 0.103 | 0.32 |

ADFI = average daily feed intake; BW = body weight; ADG = average daily weight gain; F: G = feed intake: weight gain.

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).

**Supplementary Table S4** *Major bacteria taxonomic groups in growing pigs before antibiotics treatment*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Treatment1 | | SEM | *P*-value |
| CON | ANTI |
| Ileum digesta | log10 gene copies/ng DNA sample | | | |
| Total bacteria | 10.06 | 9.99 | 0.033 | 0.43 |
| Firmicutes | 9.43 | 9.52 | 0.072 | 0.63 |
| Bacteroidetes | 7.43 | 7.53 | 0.229 | 0.86 |
| *Bifidobacterium* | 5.49 | 5.25 | 0.185 | 0.60 |
| *Lactobacillus* | 8.24 | 8.26 | 1.738 | 0.99 |
| *Ruminococcus* | 7.81 | 7.85 | 0.275 | 0.81 |
| *Clostridium* cluster IV | 5.17 | 5.41 | 0.152 | 0.50 |
| *Clostridium* cluster XIVa | 5.81 | 5.55 | 0.178 | 0.55 |
| *Escherichia coli* | 6.17 | 5.98 | 0.087 | 0.36 |
| *ETEC K88 fimbriae* | 5.02 | 5.13 | 0.048 | 0.60 |
| *sta* | 1.75 | 1.73 | 0.124 | 0.53 |
| Feces | log10 gene copies/ng DNA sample | | | |
| Total bacteria | 11.03 | 10.91 | 0.185 | 0.79 |
| Firmicutes | 9.39 | 9.39 | 0.249 | 0.99 |
| Bacteroidetes | 9.88 | 9.50 | 0.185 | 0.79 |
| *Bifidobacterium* | 5.44 | 5.45 | 0.085 | 0.92 |
| *Lactobacillus* | 7.93 | 8.20 | 0.240 | 0.64 |
| *Ruminococcus* | 8.04 | 8.20 | 0.134 | 0.92 |
| *Clostridium* cluster IV | 7.78 | 7.46 | 0.174 | 0.42 |
| *Clostridium* cluster XIVa | 7.35 | 7.26 | 0.185 | 0.85 |
| *Escherichia coli* | 7.71 | 7.62 | 0.115 | 0.78 |
| *ETEC K88 fimbriae* | 5.74 | 5.81 | 0.033 | 0.31 |
| *sta* | 2.30 | 2.03 | 0.161 | 0.45 |

ETEC = *Enterotoxigenic Escherichia coli*; sta = Heat-stable enterotoxin 1.

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).

**Supplementary Table S5** *Concentrations of metabolic profiles in ileal digesta and feces before antibiotics treatment in pigs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Items** | Treatment1 | | SEM | *P*-Value |
| CON | ANTI |
| SCFA, μmol/g wet content |  |  |  |  |
| In ileal digesta |  |  |  |  |
| Acetate | 7.21 | 6.50 | 0.805 | 0.69 |
| Propionate | 1.71 | 1.63 | 0.173 | 0.49 |
| Isobutyrate | 0.16 | 0.22 | 0.049 | 0.47 |
| Butyrate | 0.46 | 0.38 | 0.077 | 0.79 |
| Isovalerate | 0.13 | 0.16 | 0.055 | 0.74 |
| Valerate | 0.33 | 0.38 | 0.063 | 0.31 |
| BCFA | 0.39 | 0.38 | 0.084 | 0.55 |
| Total SCFA | 10.04 | 9.32 | 1.007 | 0.75 |
| In feces |  |  |  |  |
| Acetate | 23.42 | 27.30 | 3.206 | 0.60 |
| Propionate | 11.78 | 13.69 | 1.474 | 0.56 |
| Isobutyrate | 2.08 | 1.88 | 0.301 | 0.87 |
| Butyrate | 4.24 | 3.76 | 0.517 | 0.68 |
| Isovalerate | 1.41 | 1.01 | 0.226 | 0.50 |
| Valerate | 1.93 | 1.43 | 0.341 | 0.90 |
| BCFA | 3.49 | 2.99 | 0.616 | 0.32 |
| Total SCFA | 45.26 | 48.11 | 5.523 | 0.82 |
| Amines, μmol/g wet content |  |  |  |  |
| In ileal digesta |  |  |  |  |
| Methylamine | 0.12 | 0.09 | 0.012 | 0.25 |
| Tryptamine | 0.20 | 0.19 | 0.007 | 0.80 |
| Putrescine | 1.99 | 1.60 | 0.170 | 0.28 |
| Cadaverine | 4.71 | 5.13 | 0.423 | 0.68 |
| Tyramine | 0.18 | 0.14 | 0.010 | 0.12 |
| Spermidine | 2.15 | 2.79 | 0.259 | 0.23 |
| Spermine | 0.59 | 0.53 | 0.058 | 0.33 |
| Total amines | 9.96 | 10.46 | 0.694 | 0.74 |
| In feces |  |  |  |  |
| Methylamine | 0.34 | 0.25 | 0.059 | 0.48 |
| Tryptamine | 0.31 | 0.38 | 0.044 | 0.46 |
| Putrescine | 3.18 | 3.95 | 0.424 | 0.41 |
| Cadaverine | 8.00 | 6.67 | 0.420 | 0.51 |
| Tyramine | 0.49 | 0.50 | 0.019 | 0.70 |
| Spermidine | 4.12 | 4.65 | 0.102 | 0.12 |
| Spermine | 0.31 | 0.34 | 0.041 | 0.82 |
| Total amines | 15.14 | 17.54 | 1.953 | 0.67 |
| Ammonia, mg/g wet content |  |  |  |  |
| Ileal ammonia | 5.52 | 6.00 | 0.310 | 0.68 |
| Fecal ammonia | 26.15 | 25.86 | 7.450 | 0.77 |

SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids*.*

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).

**Supplementary Table S6** *Effects of antibiotics on blood parameters in growing pigs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Treatment1 | | SEM | *P-*value |
| CON | ANTI |
| Glucose, mM | 4.91 | 4.93 | 0.091 | 0.83 |
| Glucagon, ng/L | 101.72 | 97.41 | 5.801 | 0.60 |
| Insulin, µg/L | 212.11 | 212.62 | 22.104 | 0.99 |
| Albumin, g/L | 34.53 | 35.33 | 0.292 | 0.23 |
| Globulin, g/L | 23.43 | 21.66 | 0.457 | 0.16 |
| Albumin/Globulin | 1.65 | 1.53 | 0.031 | 0.51 |
| TG, mM | 0.28 | 0.24 | 0.033 | 0.49 |
| TC, mM | 1.73 | 1.84 | 0.032 | 0.97 |
| HDLC, mM | 0.59 | 0.64 | 0.044 | 0.85 |
| LDLC, mM | 1.27 | 1.25 | 0.021 | 0.14 |
| LDH, IU/L | 528.00 | 547.30 | 10.030 | 0.75 |
| Total protein, g/L | 55.23 | 57.42 | 0.543 | 0.06 |
| Urea nitrogen, mM | 2.71 | 4.22 | 0.152 | 0.02 |

TG = triglyceride; TC = cholesterol; HDLC = high density lipoprotein cholesterol; LDLC = low density lipoprotein cholesterol; LDH = lactate dehydrogenase.

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).

**Supplementary Table S7** *Effects of antibiotics on blood hormone parameters in growing pigs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Treatment1 | | SEM | *P*-value |
| CON | ANTI |
| GH, ng/mL | 35.31 | 37.02 | 2.244 | 0.56 |
| IGF-1, µg/L | 200.79 | 212.91 | 16.444 | 0.58 |
| Motilin, ng/L | 586.34 | 573.22 | 28.173 | 0.73 |
| Gastrin, ng/L | 140.33 | 150.64 | 10.171 | 0.45 |
| GIP, ng/L | 67.12 | 65.53 | 3.662 | 0.73 |
| Ghrelin, ng/L | 3353.44 | 2976.79 | 235.273 | 0.25 |
| PYY, pg/mL | 719.13 | 671.34 | 80.684 | 0.66 |
| 5-HT, ng/mL | 681.62 | 725.63 | 28.203 | 0.35 |
| CCK, ng/L | 220.54 | 238.72 | 9.602 | 0.26 |
| Secretin, ng/L | 185.22 | 194.23 | 8.831 | 0.54 |
| GLP-1, pg/mL | 108.41 | 106.12 | 6.832 | 0.79 |

GH= growth hormone; IGF-1= Insulin-like growth factor 1; GIP=gastric inhibitory polypeptide; PYY= peptide YY; 5-HT= 5-hydroxytryptamine; CCK= cholecystokinin; GLP-1= glucagon-like peptide 1.

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).

**Supplementary Table S8** *Effects of antibiotics on the enzyme activity terminal in ileal digesta in growing pigs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Items | Treatment1 | | SEM | *P-*value |
| CON | ANTI |
| Trypsin, U/mgprot | 2554.12 | 2611.64 | 133.131 | 0.76 |
| Chymotrypsin, U/mgprot | 6.72 | 7.44 | 0.932 | 0.62 |
| Lipase, U/gprot | 398.41 | 370.43 | 24.494 | 0.43 |
| Amylase, U/gprot | 264.12 | 282.12 | 18.462 | 0.50 |

1 Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI) (n = 8).



**Supplementary Figure S1****Effects of antibiotics on the terminal ileal digesta (A) and feces (B) pH value at 1, 4, 8, 12h after morning feeding in growing pigs.** Pigs were fed a basal diet (CON) or basal diet supplemented with antibiotics (ANTI). Asterisks indicate statistically significant difference between CON and ANTI at the same point. \**P* < 0.05.