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ARTICLE

# Low dietary protein and high carbohydrate infant formula affects the microbial ecology of the large intestine in neonatal rats

Wenguang Fan, Haiwei Ren, Yingying Cao, Yonggang Wang, and Guicheng Huo

**Abstract:** The aim of this study was to investigate the effects of a low dietary protein and high carbohydrate infant formula on the large intestine of neonatal rats. A total of 24 neonatal Sprague–Dawley rats (14-days-old) were randomly assigned to the low protein, high carbohydrate infant formula-fed group (I group) and a human breast milk-fed group (H group). After 7 days, we selected 6 rats at random from each group to study. No significantly different microbial colonization patterns were observed in the 2 groups at the phylum level. At the family level, *Enterobacteriaceae* and *Bacteroidaceae* were the dominant bacteria in I and H rats. While *Bacteroides* was the most abundant bacteria at the genus level, no significant difference was observed between the 2 groups. Methanoic acid, acetate, and butyrate increased in concentration in the I group compared with the H group. Protease activities, ammonia, and indole in the large intestine were lower in I rats than H rats. A significant increase in the expression of GADPH and decrease in the expression of aquaporin 8, aminopeptidase A, cathepsin F precursor, and ubiquitin carboxyl-terminal hydrolase FAF-Y were observed in I rats compared with H rats. These results suggest that a low protein diet could modulate the microbial ecology in the large intestine of neonatal rats.

*Key words:* Sprague–Dawley rats, low protein and high carbohydrate infant formula, microbiota, metabolites, gene expression.

**Résumé :** Le but de cette étude était d'examiner les effets d'une préparation pour nourrissons pauvre en protéines et riche en glucides sur le gros intestin de rats nouveau-nés. Un total de 24 rats nouveau-nés SD (âgés de 14 jours) ont été distribués au hasard dans les groupes nourris à la préparation pour nourrissons pauvre en protéines et riche en glucides (I) et ou nourris avec du lait maternel humain (H). Après 7 jours, les auteurs ont choisi 6 rats au hasard dans chacun des groupes. Aucune différence significative des différents patrons de colonisation microbienne n'était observée entre les 2 groupes à l'échelle du phylum. À l'échelle de la famille, les *Enterobacteriaceae* et les *Bacteroidaceae* étaient dominantes chez les rats des groups I et H. Alors que les *Bacteroides* étaient les plus abondantes à l'échelle du genre, aucune différence significative n'était observée entre les 2 groupes. Les concentrations d'acide méthanoïque, d'acétate et de butyrate étaient accrues chez le groupe I comparativement au groupe H. L'activité des protéases, l'ammoniac et l'indole du gros intestin étaient plus faibles chez les rats du groupe I que ceux du groupe H. Une augmentation significative de l'expression de la GAPDH et une diminution significative de l'expression de l'aquaporine 8, de l'aminopeptidase A, du précurseur de la cathepsine F et de l'ubiquitine carboxy-terminal hydrolase FAF-Y étaient observées chez les rats du groupe I comparativement à ceux du groupe H. Ces résultats suggèrent qu'une diète pauvre en protéines pourrait moduler l'écologie microbienne dans le gros intestin des rats nouveau-nés. [Traduit par la Rédaction]

*Mots-clés :* rats SD, préparation pour nourrissons pauvre en protéines et riche en glucides, microbiote, métabolites, expression génique.

## Introduction

The physiological role of the gastrointestinal ecology has become an important subject in the field of nutrition research in recent years. In addition, the microbial ecosystem of the gastrointestinal tract (GIT) is influenced by various factors, and variation in diet composition has been identified as one of the most important determinants. When altering the diet, the change in the intestinal fermentation activities and microbial ecology could be significant to the nutrition of the host organism. Improving intestinal health

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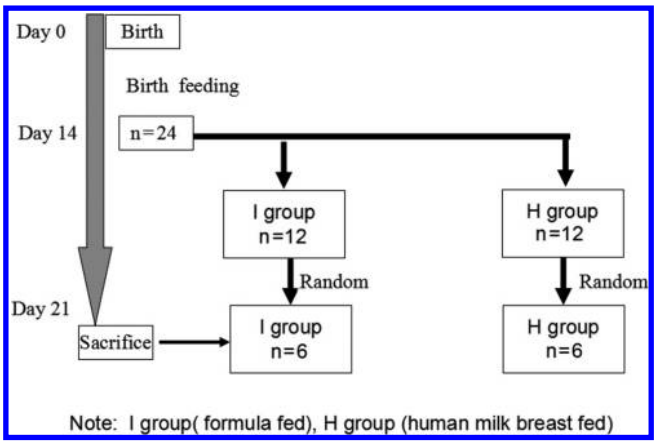
by the use of dietary ingredients that beneficially affect the intestinal microbial composition and activity has been the focus of a number of studies. Protein and carbohydrate resources are 2 important dietary ingredients that have been studied.

High protein diets increase the luminal concentrations of and epithelial exposure to toxic metabolites, thereby increasing the risk of intestinal disorders (Davila et al. 2013; Heo et al. 2015). High protein fermentation may favor the growth of enteropathogenic *Escherichia coli* (Rojas and Stein 2013), and bacterial protein fermentation occurs in the large intestine and to some extent in the proximal GIT. Protein-derived metabolites may also induce impaired barrier function by altering mucus composition and tight junctions (Fairbrother et al. 2005). Diets high in fermentable protein reduce the activity of the large intestinal epithelial amiloride-sensitive sodium channel, which has been shown to be related to increased liquid feces (Richter et al. 2014). Proteins are broken down into peptides and amino acids by proteinases and the unabsorbable dietary proteins produce nitrogen sources in the large intestine. Ammonia has toxigenic and damaging effects on the intestinal epithelium in humans (Davila et al. 2013). A previous study showed that fecal ammonia concentration increased with higher dietary protein levels, increasing the incidence of weaning diarrhea (Heo et al. 2015). Ammonia was shown to indirectly increase apoptosis and promote higher cell proliferation by interfering with the oxidative metabolism of short-chain fatty acids (SCFA) in colonocytes (Bikker et al. 2007). Ammonia was also shown to negatively influence the expression of the gene *MCT1* and promote pro-inflammatory signaling expression (Villodre Tudela et al. 2015). The primary site of phenol and indole formation is in the large intestine, and phenol has been shown to increase epithelial permeability (Hughes et al. 2008). Taking in to account the results discussed above, excessive protein fermentation in the intestine could be regarded as one factor for the development of enteric diseases.

The strategy of using a low protein diet has been widely accepted (Bikker et al. 2006). A low (<18%) dietary protein level was shown to reduce the risk of weaning diarrhea (Heo et al. 2013, 2015) and may also increase the ratio of fecal lactobacilli to enterobacteria (Wellock et al. 2006). However, some studies showed no effect of the protein level on the number of different microbial groups (Hermes et al. 2009). On the basis of this knowledge, dietary inclusion of fermentable carbohydrates may be a promising approach to reduce the effects of harmful protein fermentation (Jeaurond et al. 2008), since this strategy shifts fermentation processes toward the utilization of carbohydrates rather than proteins (Kim et al. 2008). Fermentable carbohydrate was shown to increase the number of lactobacilli in the small intestine, and enhanced the stability and diversity of the bacterial community in the colon of weaned piglets as well as suppressed potential pathogenic proteolytic bacteria (Konstantinov et al. 2004). Carbohydrate fermentation

**Fig. 1.** Experimental study design.




was also observed to increase SCFA production (Pieper et al. 2012). Although several studies have investigated the effects of the inclusion of fermentable carbohydrates in low protein diets, there is still much to be discovered with respect to its effect on the intestinal microbial ecology of neonatal rats.

In this study, we hypothesized that the intake of a low protein (1.8 g/100 kcal) and high carbohydrate (10.2 g/100 kcal) infant formula would affect the microbial ecology of the large intestine of neonatal rats with respect to (*i*) the microbiota of the large intestine, (*ii*) the fermented metabolites, and (*iii*) the expression of genes involved in carbohydrate and protein metabolism. The present study was conducted using a combination of 454 barcoded pyrosequencing, performance liquid chromatography, and RT-PCR. The use of diets with low levels of high-quality protein and higher levels of carbohydrates may help us to produce an infant formula composition closer to that of human milk.

## Materials and methods

### Animal experiments

The animal experiment was as shown in Fig. 1. In brief, intervention was given for 7 days. The study groups were weaned rats fed infant formula (I group, $n = 12$) and weaned rats fed human breast milk (H group, $n = 12$). After 7 days, we selected 6 rats at random from each group to study. The pups in the I group were fed a standard moist diet (Fan et al. 2014) consisting of a porridge made by adding warm water to an infant formula at a final proportion of 65 g of infant formula to 15 mL of water. The pups in the H group were fed human breast milk, which was purchased from Harbin City Maternal and Child Health Care Center, Heilongjiang Province. The H and I groups were administered a 1.0 mL diet every 4 h, including during the night. Each group had a separate feeding needle. The pups were weighed and measured daily for weight loss or gain. This study was carried out at the Northeast Agricultural University (NEAU), Harbin City. Pilot experiments were performed to optimize all the experimental procedures, including handling and treatment of rats. The experimental protocol was approved by the Institutional Animal Care and Use Committee of North-

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**Table 1.** Primers for real-time RT-PCR analysis of gene expression in the large intestine of neonatal rats.

| No. | Gene name | Forward primer | Reverse primer |
|---|---|---|---|
| 1 | 18S | 5′-TCCGACTTTCGTTCTTGATTAATG-3′ | 5′-TGGACCGGCGCAAGAC-3′ |
| 2 | GADPH | 5′-GGCTCTGTGCTCCTCCCTGTTCTA-3′ | 5′-TGCCGTTGAATTGCCGTGGG-3′ |
| 3 | Aquaporin 8 | 5′-GCTTCTCTGTCACCGTGGATATC-3′ | 5′-AAGGCTCGTGCGGGATTC-3′ |
| 4 | Aminopeptidase A | 5′-GGAAGGATCCAGGGGTTGGTG-3′ | 5′-ATCGGGCGCGTGAATGAT-3′ |
| 5 | Cathepsin F precursor | 5′-GGAGCTGTGGAAGAAGACCC-3′ | 5′-ACACTGGCCCTGGTTTTTGA-3′ |
| 6 | Ubiquitin carboxyl-terminal hydrolase FAF-Y | 5′-TTTGGAAACAGTTCAGGCTTTG-3′ | 5′-AGCCGGGTGTCCTAAAGCTT-3′ |

east Agricultural University under the approved protocol No. SRM-06.

### Microbial diversity in the large intestine

The microbiota present in large intestine was assessed by 454 barcoded pyrosequencing. First, the full contents of the large intestinal samples were placed into sterile polypropylene centrifuge tubes and kept frozen at −80 °C until DNA extraction. DNA was extracted according to the protocol described by the E.Z.N.A. Soil DNA kit (OMEGA). Afterwards, PCR amplification and pyrosequencing were performed. The universal bacterial primer set 27F (AGAGTTTGATCCTGGCTCAG) and 533R (TTACCGCGGCTGCTGGCAC), covering the V1–V3 regions, was selected to construct community libraries through tagged pyrosequencing. PCR reactions were carried out in 20 μL reaction volumes and contained 10 ng of DNA template, 2 μL of dNTPs (2.5 mmol/L), 0.4 μL of each primer (5 μmol/L), and 0.4 U of FastPfu Polymerase with the appropriate FastPfu Buffer (4 μL) and de-ionized ultrapure water (to 20 μL). The protocol was optimized with low cycles for better accuracy and reliability of the subsequent data analysis. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. PCR products were purified using a MiniElute PCR purification kit (AXYGEN) and quantified using an ABI GeneAmp® 9700 system. Parallel tagged sequencing was performed via MiSeq Sequencing by Majorbio. The program Mothur was used to perform data preprocessing. Operational taxonomic units at a 97% sequence identity were used to cluster the sequences. All sequences were classified at the phylum, family, and genus level according to the program Mothur using the default setting.

### Measurement of protease activities in the large intestine

The protease activities in the large intestine content was measured according to a previous study (Andriamihaja et al. 2010). Colonic luminal contents were mixed with borate buffer (pH 7.4), after which the samples were centrifuged at 200*g* for 5 min to maintain the bacteria in the supernatant. Luminal contents (0.75 mg) were incubated for 30 min at 37 °C in 50 μL of borate buffer containing 0.48 mg of purified caseins. The reaction was halted with ice-cold trichloroacetic acid solution and was then centrifuged (12 000*g*, 5 min). Next, 20 μL of the supernatants were mixed with 20 μL of NaOH solution (0.3 mol/L). Absorbance of peptides was then measured by spectrophotometry at 650 nm by using a Bio-Rad kit, with bovine serum albumin as a standard curve. Aliquots of the mixture corresponding to 0.025 mg of large intestine content were incubated and treated using the same protocol. Time and protease activities showed a correlation for a period of up to 60 min. Lastly, at 4 °C, protease activities represented 5% ± 2% of the activities measured at 37 °C ($n$ = 3). Protease activities were assessed by the release of peptides.

### Measurement of SCFA contents in the large intestine

SCFA production was analyzed during fermentation by high-performance liquid chromatography (HPLC) (Pescuma et al. 2010). HPLC was performed using a Knauer Smartline RI detector fitted with a BioRad Aminex HPX-87H column (300 mm × 7.8 mm, Hercules, California, USA). The operating conditions were as follows: 5 mmol/L $H_2SO_4$ was used as eluent at a flow rate of 0.6 mL/min for 30 min, with an internal temperature of 50 °C. For the quantification of SCFA, calibration curves for each compound were constructed using pure standards at different concentrations.

The contents of the large intestine (1 g) were dissolved in 10 mL of a phosphate buffer solution, then 2 mL of supernatant, 4 mL of barium hydroxide (1.8%), and 4 mL of zinc sulfate (2%) were added. The mixture was stirred using a swirl device and allowed to naturally precipitate, and was then centrifuged for 10 min at 13 000 rev/min. A total of 200 μL of the supernatants were used for assays.

### Ammonia and indole measurements in the large intestine

For ammonia measurements, the contents were mixed with 10 mL of perchloric acid (4%) per gram of content and centrifuged at 10 000*g* for 15 min (Pescuma et al. 2010). The supernatant was neutralized and ammonia was measured after centrifugation with 15 μL of supernatant using a Sigma Ammonia Assay kit. Indole was detected by HPLC as described in a previous (Murray and Adams 1988).

### RNA extraction and reverse transcription

Frozen tissue (0.5 g) was homogenized in 5 mL of TRIzol reagent (Invitrogen) and total RNA was isolated according to the manufacturer's recommendations. RNA integrity and quantity were analyzed using an Agilent 2100 Bioanalyzer. The 28S ribosome : 18S ribosome peak areas ratio was ≧1.80 for all samples, indicating that little degradation of the RNA occurred (Wang et al. 2008). RNA reverse transcription was performed according to the instructions provided with the Promega Reverse Transcriptase.

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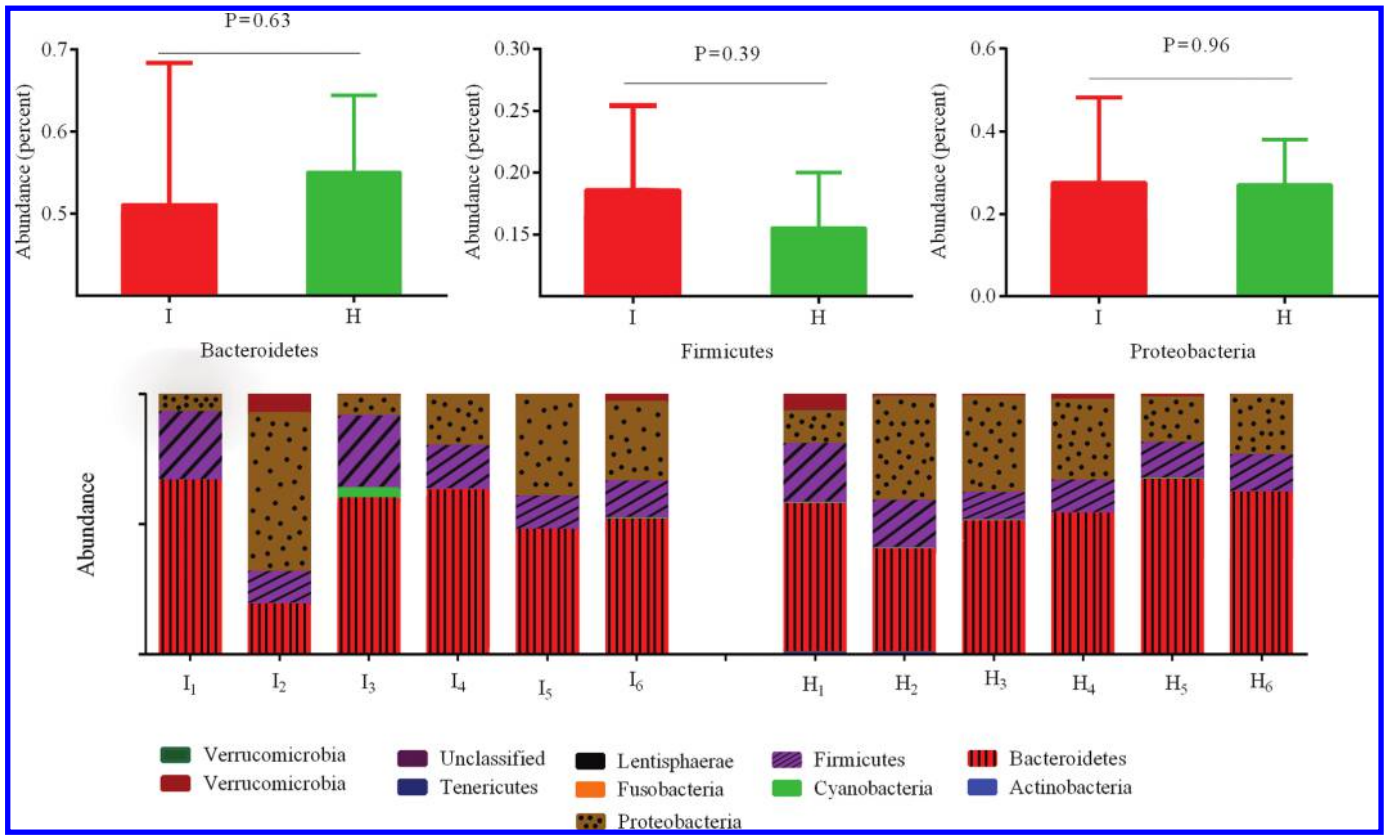
**Fig. 2.** The mean abundance (top graph) of the intestinal flora at the phylum level in the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. Bacterial phyla (bottom graphs) present in the large intestine of rats fed a low protein, high carbohydrate infant formula and rats fed human breast milk. Values are the mean ± standard deviation of $n = 6$ repetitions. [Colour online.]




**Real-time PCR quantification of related gene expression**

The PCR primers used to quantify the gene expression in the large intestine are shown in Table 1. Real-time PCR quantification of gene expression was performed using a LightCycler 480 instrument. The PCR amplification was performed in duplicate with a reaction volume of 100 μL, which contained 10 μL of buffer (10×), 8 μL of dNTPs (2.5 mmol/L), 5 μL of each primer (20 pmol), 0.5 μL of *Taq* DNA polymerase, and 5 μL of cDNA template. The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. The 18S rRNA gene was used as an internal standard (house-keeping gene). The specificity of PCR amplifications was verified by melting curve analysis and agarose gel electrophoresis.

**Statistical analyses**

The results were expressed as the means ± SE together with the number of independent experiments. The data were analyzed using *t* test. A *P* value of <0.05 was considered statistically significant between means. Prism 6.1 software was used to generate graphs.

## Results

**Diversity of intestinal microbiota**

Most sequences in the entire I and H samples belonged to the 3 most abundant bacterial phyla, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, with less *Actinobacteria* observed (Fig. 2). Differences were found in the proportions of the phyla, with *Firmicutes* and *Proteobacteria* being more represented in I rats than H rats (18.55% vs. 15.52% and 37.52% vs. 26.98%, respectively), whereas *Bacteroidetes* was less abundant in I rats than H rats (51.04% vs. 55.03%). No significant differences were observed among the *Firmicutes* ($P = 0.39$), *Bacteroidetes* ($P = 0.63$), and *Proteobacteria* ($P = 0.96$) phyla. The ratio of *Firmicutes* to *Bacteroidetes* in the I group was lower than in the H group (0.282 vs. 0.363); this proportion decreased in the low-calorie diet. The abundance of the bacterial families in I and H rats is shown in Fig. 3. *Enterobacteriaceae* and *Bacteroidaceae* were the dominant bacteria families in the I and H rats. The abundance of *Ruminococcaceae* ($P = 0.01$) was significantly different between the I and H rats. At the genus level, *Bacteroides* was the most abundant (Fig. 4), but no significant difference ($P = 0.24$) was observed between it and other groups. The secondary genus in both the I and H groups was *Escherichia–Shigella*. As a well-known group of probiotics, *Lactobacillus* was more represented in I rats than in H rats ($P = 0.44$). *Clostridium* was less abundant in I rats than H rats ($P = 0.11$). *Enterococcus* was more abundant in I rats than H rats, and it was significantly different in the I rats compared with the H rats ($P = 0.0142$).

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**Fig. 3.** The mean abundance of the intestinal flora at the family level in the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. Values are the mean ± standard deviation of $n$ = 6 repetitions. [Colour online.]

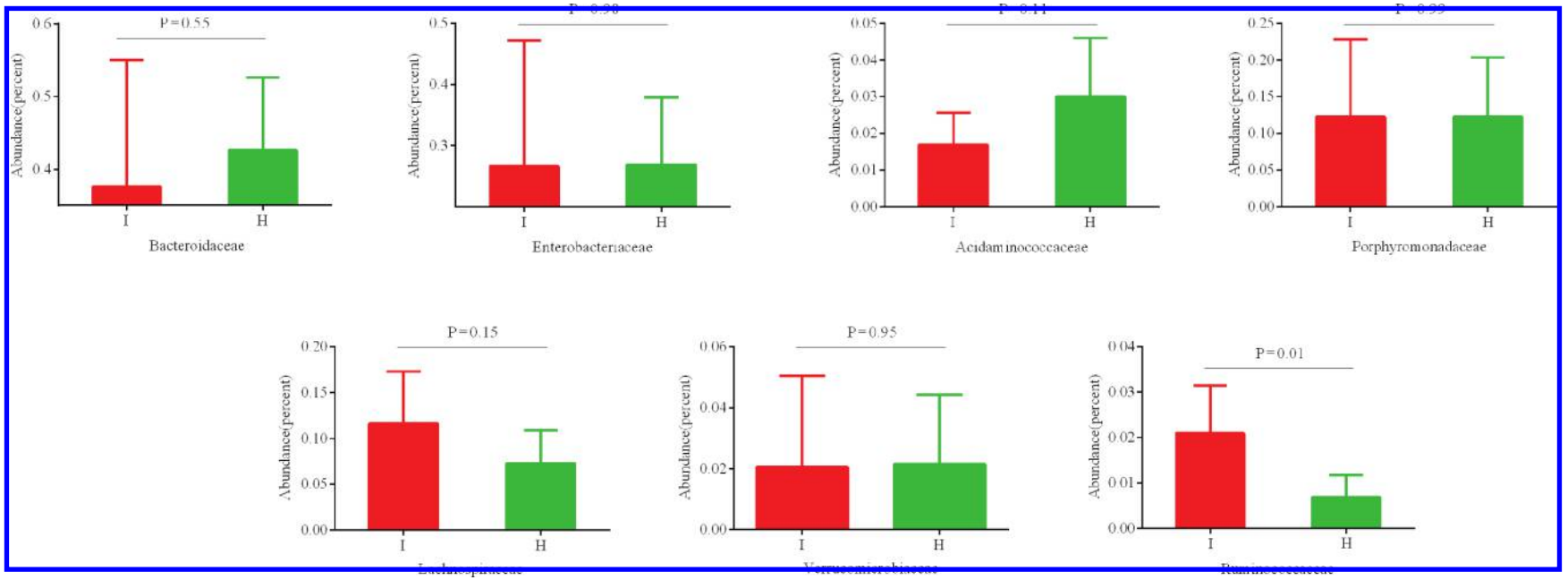

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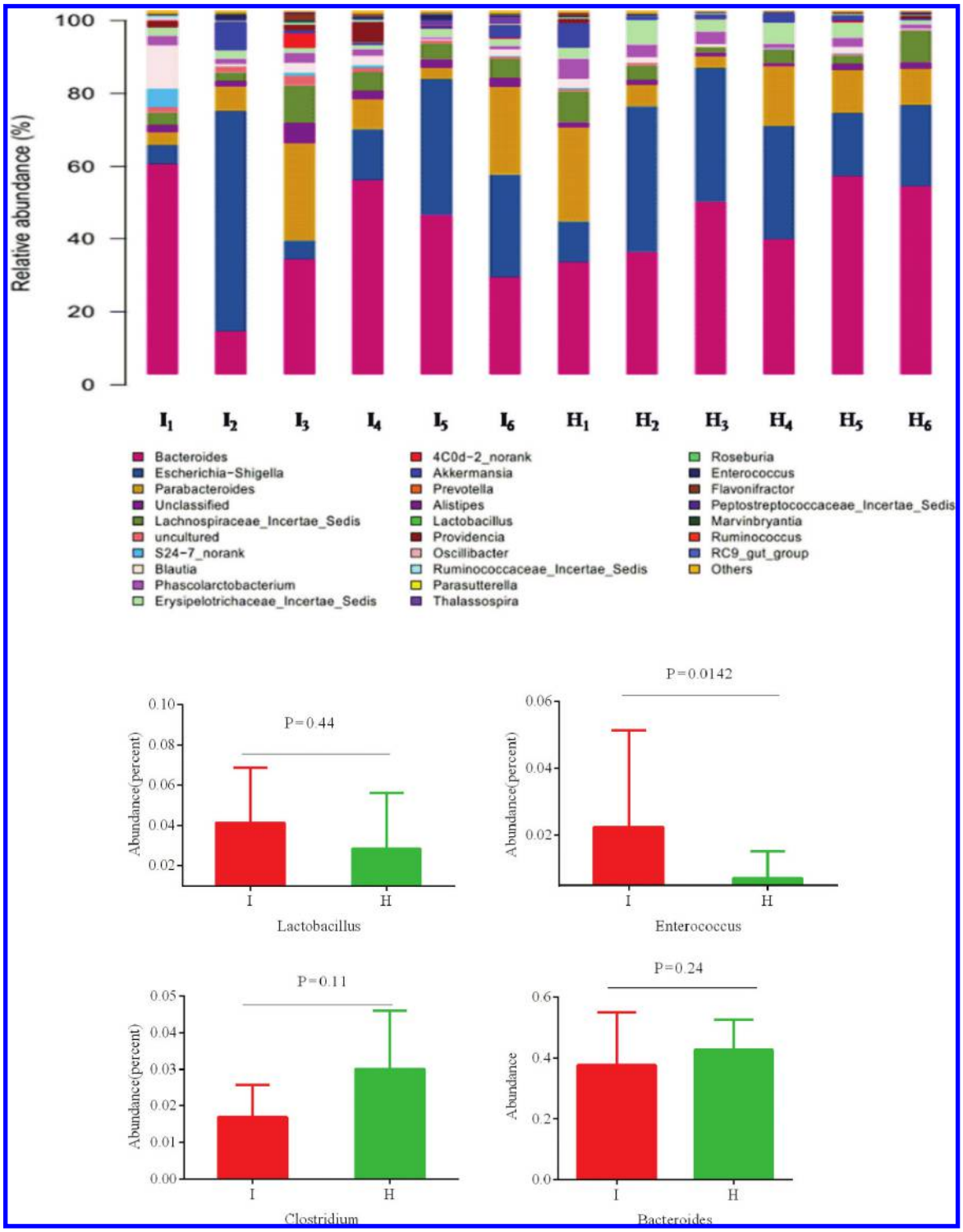
**Fig. 4.** Relative abundance (top graph) of the intestinal bacteria at the genus level in the large intestine of the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. The mean abundance (bottom graphs) of the intestinal flora at the genus level in the I and H groups. Values are the mean ± standard deviation of $n$ = 6 repetitions. [Colour online.]




**Effect of formula on protease activities in the large intestine**

Protease activities in the large intestine after the ingestion of the formula diet decreased when compared with the activities measured in H group (Fig. 5). Our results showed that there was a significant difference in protease activities between the I and H rats ($P$ = 0.014). A total of 60.21 ± 0.03 mg of peptides were released per 30 min in I animals, and a total of 60.98 ± 0.26 mg of peptides were released per 30 min in the H group.

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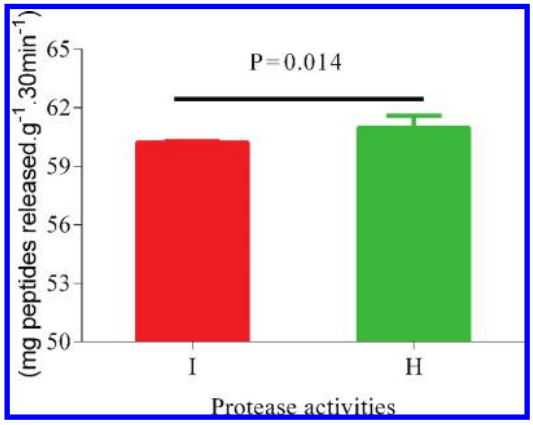
**Fig. 5.** Protease activities measured in the large intestine of the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. Values are the mean ± standard deviation of $n = 6$ repetitions. [Colour online.]




### Diet-induced changes in SCFA concentrations

SCFA concentrations in the I and H groups are showed in Fig. 6. The concentrations of methanoic acid, acetate, propionate, butyrate, and lactate in the I group were higher than in the H group. Of the 5 major SCFA, methanoic acid, acetate, and butyrate increased in concentration (by 4.28 μmol/g, $P = 0.02$; 56.3 μmol/g, $P = 0.01$; and by 8.14 μmol/g, $P = 0.02$; respectively) for the I group compared with for H group. Propionate and lactate in the I and H groups were not significantly different ($P = 0.19$ and $P = 0.11$, respectively).

### Effect of diet on ammonia and indole concentrations

Ammonia and indole were detected in all large intestines from the I and H groups (Fig. 7). The amount of ammonia in the large intestine was lower for I than H rats (10.14 vs. 10.22 mol/kg), but there was no significant difference between the mean ammonia concentrations between the I and H rats ($P = 0.13$). The results of indole concentration measurements in the I and H groups was the same as that observed for ammonia, the amount of indole in the large intestine was also lower for I than H rats (5.47 vs. 5.57 mol/kg), but there was no significant difference between mean indole concentrations in I and H rats ($P = 0.31$).

### Effect of diet on the related genes involved in carbohydrate and protein metabolism

A difference in the expression of genes was observed between the large intestines of I rats compared with H rats (Table 2). A significant increase in GADPH expression ($P = 0.029$) was observed in I rats compared with the H rats. A significant decrease in the expression of aquaporin 8 ($P = 0.018$), aminopeptidase ($P = 0.021$), cathepsin F precursor ($P = 0.019$), and ubiquitin carboxyl-terminal hydrolase FAF-Y ($P = 0.016$) was observed in I rats compared with H rats.

## Discussion

In the present study, a low protein diet (1.8 g/100 kcal) infant formula was used to investigate the effects of intestinal microbial ecology in neonatal rats compared with human-milk fed rats. We observed that for rats from the formula and human milk groups, which were maintained under the same living circumstances, the level of protein with high carbohydrates in formula had immediate consequences on gut ecology. The formula did not affect the diversity of the large intestinal microbiota of rats, although the abundance of several genera was modulated, as well as some metabolites. Five genes in the large intestine that are involved in carbohydrate and protein metabolism also changed. There is increasing interest in improving intestinal health by the use of a low protein diet to beneficially affect the microbial composition and activity. A low dietary protein level (<18%) or total daily intake of less than 60 g was used to reduce the risk of post-weaning diarrhea (Heo et al. 2013, 2015). Ingesting low protein diets (13%) may increase the ratio of fecal lactobacilli to enterobacteria (Wellock et al. 2006). In addition, a low protein diet (16%) may modulate the microbial composition and metabolites of the hindgut after an extended period of time (Zhou et al. 2016).

The effect of dietary protein on human and animal gut microbiota has received a great deal of attention over the years. In our study, *Firmicutes* and *Proteobacteria* were well-represented in the I rats compared with the H rats, whereas *Bacteroidetes* was less abundant, but no significant difference was observed. At the family level, *Enterobacteriaceae* and *Bacteroidaceae* were the dominant bacteria, and *Ruminococcaceae* was significantly different between the I and H rats. *Bacteroides* was the most abundant division at the genus level. Although no significant differences were observed for the *Lactobacillus* and *Clostridium* genera, a significant difference was observed in the *Enterococcus* populations between the I and H groups. Although our findings were consistent with a previously reported study (Zhou et al. 2016), the dietary treatment in this study only modulated several genera without drastically changing the diversity of the gut microbiota, in contrast with a previous report (Liu et al. 2014). *Bacteroides* are known to utilize protein in the large intestine and in the proximal GIT (Dai et al. 2011), while *Clostridium* has been observed to degrade protein and produce potentially toxic products (Bikker et al. 2007). As a member of the lactic acid bacterial group, *Lactobacillus* can promote healthy living in humans and animals (Kleerebezem et al. 2003), but the present study showed that the low protein diet decreased the abundance of *Lactobacillus*. The findings of our study will require further validation.

Protease activities increase the speed of degradation of proteins and peptides. We observed that when the protein level decreased, there was a corresponding decrease in the production of ammonia and indole. Protein fermentation products, such as ammonia and indole, have been primarily associated with toxigenic and damaging effects on the intestinal epithelium (Davila et al. 2013). Recent results suggested that ammonia also triggers inflammatory responses (Villodre Tudela et al. 2015). The concentrations of SCFAs in

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**Fig. 6.** Short-chain fatty acid concentrations measured in the large intestine of the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. Values are the mean ± standard deviation of $n = 6$ repetitions. [Colour online.]

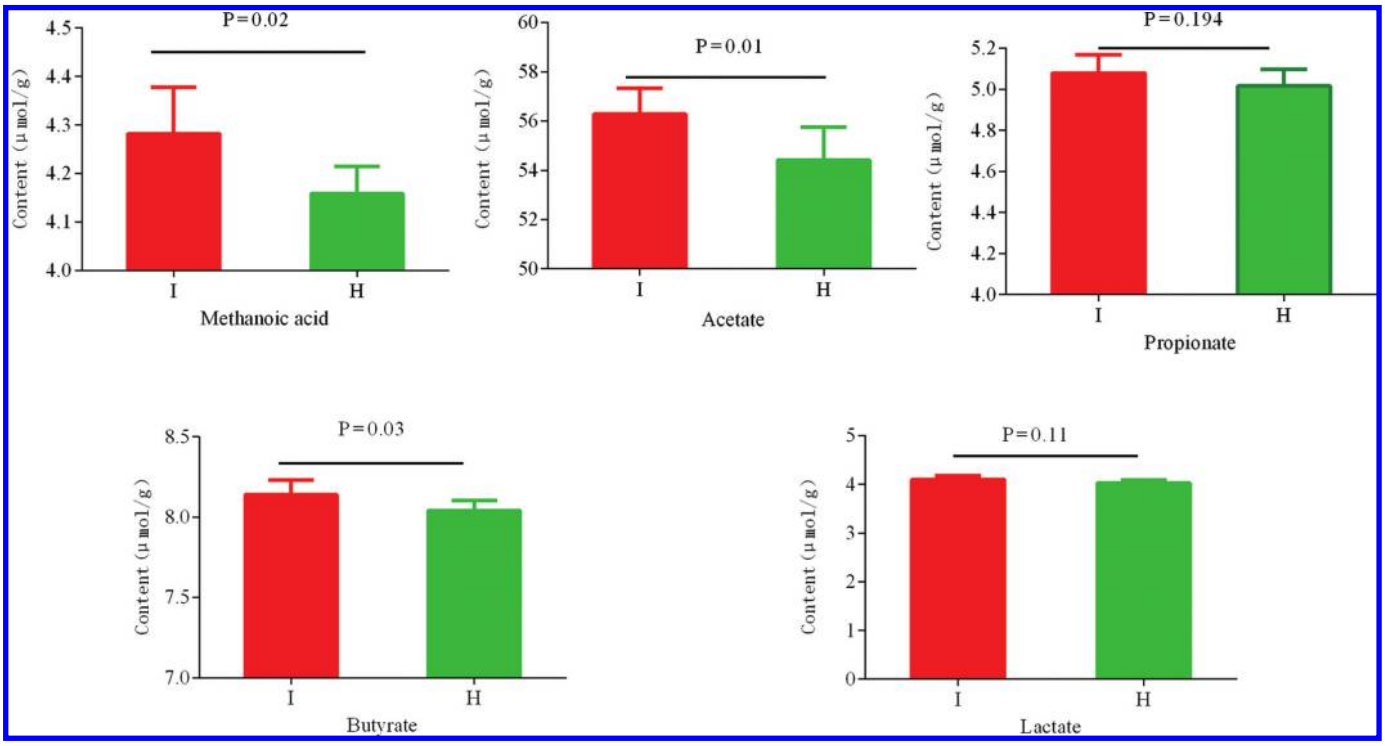


**Fig. 7.** Ammonia and indole measured in the large intestine of the low protein, high carbohydrate infant formula fed (I) and human breast milk fed (H) groups. Values are the mean ± standard deviation of $n = 6$ repetitions. NS, not significant. [Colour online.]

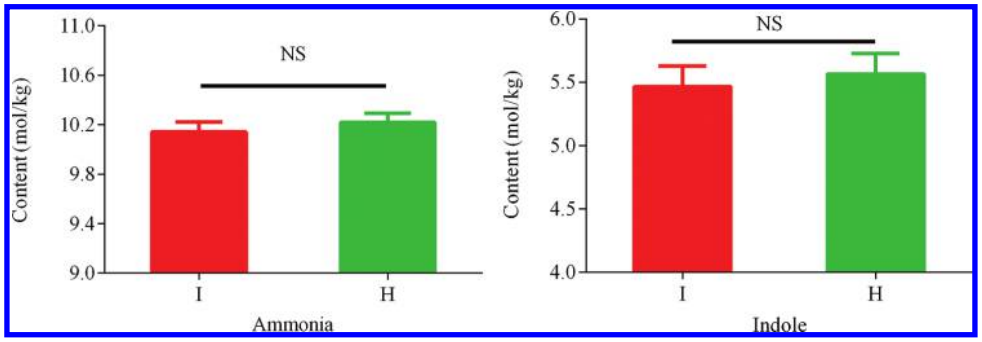


**Table 2.** Expression of genes involved in carbohydrate and protein metabolism in the infant formula fed (I) group compared with human breast milk fed (H) group.

| No. | Gene name | *P* value | Change |
|---|---|---|---|
| 1 | GADPH | 0.029 | +21 |
| 2 | Aquaporin 8 | 0.018 | −35 |
| 3 | Aminopeptidase | 0.021 | −29 |
| 4 | Cathepsin F precursor | 0.019 | −33 |
| 5 | Ubiquitin carboxyl-terminal hydrolase FAF-Y | 0.016 | −38 |

the I group were higher than in the H group. Of the 5 major SCFA, methanoic acid, acetate, and butyrate significantly increased in concentration in the I group compared with the H group. SCFAs are known as energy substrates (Kambashi et al. 2014), and as the main energy source for colonocytes, butyrate was observed to be primarily metabolized by epithelial cells (Hamer et al. 2008). SCFAs could stimulate cell epithelial proliferation and barrier function, modulate the immune response, and regulate the inflammatory response (Plöger et al. 2012). A significant increase in GADPH expression was observed in I rats compared with H rats, whereas the expression of aminopeptidase, cathepsin F precursor, and ubiquitin carboxyl-terminal hydrolase FAF-Y, which are involved in protein and peptide degradation, was decreased in I rats compared with H rats (Wilkinson 1997). Aquaporin-8 can stimulate water permeation across the gut (Flach et al. 2004), which may lead to diarrhea that frequently occurs in early weaned piglets (Ou et al. 2007).

The use of fermentable carbohydrates seems to be a promising approach to beneficially affect the composition

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and activity of the intestinal microbiota (de Lange et al. 2010), and the addition of fermentable carbohydrates to the diet can reduce the protein fermentation (Jeaurond et al. 2008). Thus, a low dietary protein and high carbohydrate formula was investigated in the present study. A previous study observed lower fecal concentrations of ammonia after the inclusion of fermentable carbohydrates that resulted from a shift from protein to carbohydrate fermentation (Aschenbach et al. 2006), which was consistent with the present study. *Lactobacillus* was not easily affected, but the number of *Lactobacillus* was increased. Many studies have shown that supplemental oligosaccharides have a reducing effect on potential harmful bacteria, such as *E. coli* (Houdijk et al. 2002) and *Enterobacteria* spp. (Libao-Mercado et al. 2009). Overall there is growing evidence that supplementation of the diet with fermentable carbohydrates can be an effective strategy in reducing both intestinal protein fermentation and potential pathogenic bacteria.

In conclusion, in the present study we investigated the effects of a low protein infant formula on the microbial composition, metabolomic profiles, and the expression of genes related to protein and carbohydrate degradation in the large intestines of neonatal rats. We found that a low protein diet affected the microbial ecology. However, the impact of a low protein diet on animal health should be evaluated in future studies.

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## References

Andriamihaja, M., Davila, A.M., Eklou-Lawson, M., Petit, N., Delpal, S., Allek, F., et al. 2010. Colon luminal content and epithelial cell morphology are markedly modified in rats fed with a high-protein diet. Am. J. Physiol.: Gastrointest. Liver Physiol. **299**(5): G1030–G1037. doi:10.1152/ajpgi.00149.2010. PMID:20689060.

Aschenbach, J.R., Schwelberger, H.G., Ahrens, F., Fürll, B., and Gäbel, G. 2006. Histamine inactivation in the colon of pigs in relationship to abundance of catabolic enzymes. Scand. J. Gastroenterol. **41**(6): 712–719. doi:10.1080/00365520500419540. PMID:16716971.

Bikker, P., Dirkzwager, A., Fledderus, J., Trevisi, P., le Huërou-Luron, I., Lallès, J.P., and Awati, A. 2006. The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. J. Anim. Sci. **84**(12): 3337–3345. doi:10.2527/jas.2006-076. PMID:17093226.

Bikker, P., Dirkzwager, A., Fledderus, J., Trevisi, P., Huërou-Luron, I.L., Lallès, J.P., and Awati, A. 2007. Dietary protein and fermentable carbohydrates contents influence growth performance and intestinal characteristics in newly weaned pigs. Livest. Sci. **108**(1–3): 194–197. doi:10.1016/j.livsci.2007.01.057.

Dai, Z.L., Wu, G., and Zhu, W.Y. 2011. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Front. Biosci. **16**(1): 1768. doi:10.2741/3820.

Davila, A.M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P.H., Sanz, Y., and Tomé, D. 2013. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacol. Res. **69**(1): 114–126. doi:10.1016/j.phrs.2013.01.003. PMID:23318949.

de Lange, C.F.M., Pluske, J., Gong, J., and Nyachoti, C.M. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest. Sci. **134**(1–3): 124–134. doi:10.1016/j.livsci.2010.06.117.

Fairbrother, J.M., Nadeau, É., and Gyles, C.L. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. **6**(1): 17–39. doi:10.1079/AHR2005105. PMID:16164007.

Fan, W., Tang, Y., Qu, Y., Cao, F., and Huo, G. 2014. Infant formula supplemented with low protein and high carbohydrate alters the intestinal microbiota in neonatal SD rats. BMC Microbiol. **14**: 279. doi:10.1186/s12866-014-0279-2. PMID:25403909.

Flach, C.F., Lange, S., Jennische, E., and Lönnroth, I. 2004. Cholera toxin induces expression of ion channels and carriers in rat small intestinal mucosa. FEBS Lett. **561**(1–3): 122–126. doi:10.1016/S0014-5793(04)00139-5. PMID:15013762.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., and Brummer, R.J. 2008. The role of butyrate on colonic function. Aliment. Pharmacol. Ther. **27**(2): 104–119. PMID:17973645.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J., and Nyachoti, C.M. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. **97**(2): 207–237. doi:10.1111/j.1439-0396.2012.01284.x.

Heo, J.M., Kim, J.C., Yoo, J., and Pluske, J.R. 2015. A between-experiment analysis of relationships linking dietary protein intake and post-weaning diarrhea in weanling pigs under conditions of experimental infection with an enterotoxigenic strain of *Escherichia coli*. Anim. Sci. J. **86**(3): 286–293. doi:10.1111/asj.12275. PMID:25231832.

Hermes, R.G., Molist, F., Ywazaki, M., Nofrarías, M., Gomez de Segura, A., Gasa, J., and Pérez, J.F. 2009. Effect of dietary level of protein and fiber on the productive performance and health status of piglets. J. Anim. Sci. **87**(11): 3569–3577. doi:10.2527/jas.2008-1241. PMID:19648494.

Houdijk, J.G., Hartemink, R., Verstegen, M.W., and Bosch, M.W. 2002. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. Arch. Anim. Nutr. **56**(4): 297–307. doi:10.1080/00039420214346.

Hughes, R., Kurth, M.J., McGilligan, V., McGlynn, H., and Rowland, I. 2008. Effect of colonic bacterial metabolites on Caco-2 cell paracellular permeability in vitro. Nutr. Cancer, **60**(2): 259–266. doi:10.1080/01635580701649644. PMID:18444159.

Jeaurond, E.A., Rademacher, M., Pluske, J.R., Zhu, C.H., and de Lange, C.F.M. 2008. Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs. Can. J. Anim. Sci. **88**(2): 271–281. doi:10.4141/CJAS07062.

Kambashi, B., Boudry, C., Picron, P., and Bindelle, J. 2014. Forage plants as an alternative feed resource for sustainable pig production in the tropics: a review. Animal, **8**(8): 1298–1311. doi:10.1017/S1751731114000561. PMID:24673804.

Kim, J.C., Mullan, B.P., Hampson, D.J., and Pluske, J.R. 2008. Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract. Br. J. Nutr. **99**(6): 1217–1225. PMID:18042308.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., et al. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100**(4): 1990–1995. doi:10.1073/pnas.0337704100. PMID:12566566.

Konstantinov, S.R., Favier, C.F., Zhu, W.Y., Williams, B.A., Klüss, J., Souffrant, W.B., et al. 2004. Microbial diversity studies of the

porcine gastrointestinal ecosystem during weaning transition. Anim. Res. **53**(4): 317–324. doi:10.1051/animres:2004019.

Libao-Mercado, A.J.O., Zhu, C.L., Cant, J.P., Lapierre, H., Thibault, J.N., Sève, B., et al. 2009. Dietary and endogenous amino acids are the main contributors to microbial protein in the upper gut of normally nourished pigs. J. Nutr. **139**(6): 1088–1094. doi:10.3945/jn.108.103267. PMID:19403708.

Liu, X., Blouin, J.M., Santacruz, A., Lan, A., Andriamihaja, M., Wilkanowicz, S., et al. 2014. High-protein diet modifies colonic microbiota and luminal environment but not colonocyte metabolism in the rat model: the increased luminal bulk connection. Am. J. Physiol.: Gastrointest. Liver Physiol. **307**(4): G459–G470. doi:10.1152/ajpgi.00400.2013. PMID:24970777.

Murray, K.E., and Adams, R.F. 1988. Determination of simple phenols in faeces and urine by high-performance liquid chromatography. J. Chromatogr. B: Biomed. Sci. Appl. **431**: 143–149. doi:10.1016/S0378-4347(00)83077-7. PMID:3235525.

Ou, D., Li, D., Cao, Y., Li, X., Yin, J., Qiao, S., and Wu, G. 2007. Dietary supplementation with zinc oxide decreases expression of the stem cell factor in the small intestine of weanling pigs. J. Nutr. Biochem. **18**(12): 820–826. doi:10.1016/j.jnutbio.2006.12.022. PMID:17475461.

Pescuma, M., Hébert, E.M., Mozzi, F., and Font de Valdez, G. 2010. Functional fermented whey-based beverage using lactic acid bacteria. Int. J. Food Microbiol. **141**(1–2): 73–81. doi:10.1016/j.ijfoodmicro.2010.04.011. PMID:20483186.

Pieper, R., Kröger, S., Richter, J.F., Wang, J., Martin, L., Bindelle, J., et al. 2012. Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. J. Nutr. **142**(4): 661–667. doi:10.3945/jn.111.156190. PMID:22357743.

Plöger, S., Stumpff, F., Penner, G.B., Schulzke, J.D., Gäbel, G., Martens, H., et al. 2012. Microbial butyrate and its role for barrier function in the gastrointestinal tract. Ann. N. Y. Acad. Sci. **1258**: 52–59. doi:10.1111/j.1749-6632.2012.06553.x. PMID:22731715.

Richter, J.F., Pieper, R., Zakrzewski, S.S., Günzel, D., Schulzke, J.D., and Van Kessel, A.G. 2014. Diets high in fermentable protein and fibre alter tight junction protein composition with minor effects on barrier function in piglet colon. Br. J. Nutr. **111**(6): 1040–1049. doi:10.1017/S0007114513003498. PMID:24229735.

Rojas, O.J., and Stein, H.H. 2013. Concentration of digestible, metabolizable, and net energy and digestibility of energy and nutrients in fermented soybean meal, conventional soybean meal, and fish meal fed to weanling pigs. J. Anim. Sci. **91**(9): 4397–4405. doi:10.2527/jas.2013-6409. PMID:23893994.

Villodre Tudela, C., Boudry, C., Stumpff, F., Aschenbach, J.R., Vahjen, W., Zentek, J., and Pieper, R. 2015. Down-regulation of monocarboxylate transporter 1 (MCT1) gene expression in the colon of piglets is linked to bacterial protein fermentation and pro-inflammatory cytokine-mediated signalling. Br. J. Nutr. **113**(4): 610–617. doi:10.1017/S0007114514004231. PMID:25656974.

Wang, J., Chen, L., Li, P., Li, X., Zhou, H., Wang, F., et al. 2008. Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. J. Nutr. **138**(6): 1025–1032. PMID:18492829.

Wellock, I.J., Fortomaris, P.D., Houdijk, J.G.M., and Kyriazakis, I. 2006. The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs. Anim. Sci. **82**(3): 327–335. doi:10.1079/ASC200643.

Wilkinson, K.D. 1997. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. FASEB J. **11**(14): 1245–1256. PMID:9409543.

Zhou, L., Fang, L., Sun, Y., Su, Y., and Zhu, W. 2016. Effects of the dietary protein level on the microbial composition and metabolomic profile in the hindgut of the pig. Anaerobe, **38**: 61–69. doi:10.1016/j.anaerobe.2015.12.009. PMID:26723572.

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