
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*

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Abstract

Numerous experiments have demonstrated that feeding a lower protein diet decreases protein fermentation in the gastrointestinal tract (GIT) and reduces the incidence of post-weaning diarrhea (PWD). However, there is a lack of holistic evidence underpinning the relationship between feeding a lower protein diet and PWD in relation to physiological responses and protein fermentation in the GIT. The scope of this article, therefore, will: (i) focus on the impact of dietary protein levels on selected indices of GIT health in weaned pigs without and with experimental infection with an enterotoxigenic strain of *Escherichia coli*; and (ii) attempt to conduct regression analysis to examine the relationships between dietary-origin protein intake, nitrogen fermentation indices, fecal consistency and the incidence of PWD. We used datasets generated from a series of four intensive experiments in weaned pigs. The collective results derived from these datasets indicate that restriction of daily protein intake to less than 60 g through feeding a lower protein diet for as little as 7 days after
weaning reduced the incidence of PWD commensurate with a reduction in protein fermentation indices.

Keywords: antimicrobial compounds; diet; pig; post-weaning diarrhea; protein

Introduction

Weaning of piglets is primarily characterized by a gap in the protective immune system due to depleted passive immunity and under-developed components of innate immune function. Concomitant changes in nutritional, psychological and environmental conditions add additional complexity to the compromised immune status, which can make the newly weaned pig vulnerable to intestinal malfunction and enteric pathogen-associated diarrhea (Pluske et al. 1997). To assist in overcoming enteric pathogen-associated morbidity and (or) mortality that often occurs at weaning, the pig industry has used or is still currently using, depending on rules and regulations specific to each country, prophylactic and (or) therapeutic levels of antimicrobial compounds (AMC) such as ZnO, CuSO₄ and (or) in-feed antibiotics, which in general reduce gastrointestinal disorders such as post-weaning diarrhea (PWD) and therefore improve productivity (Heo et al. 2013).

Due to potential transfer of antibiotic resistance to humans, where for example an epidemiological study reported that the use of avoparcin in diets for meat animals increased vancomycin-resistant Enterococcus faecium in humans (Witte 2000), there was and continues to be, much discussion worldwide about the continued use of in-feed AMC. The use of in-feed AMC in pig feeds has been voluntarily banned in Sweden (1986), Denmark (1998) and Switzerland (1999), with forced bans in the European Union (2006) and Republic of Korea (2011), but many countries still permit their use. Nonetheless, recent years have seen a marked increase in the number of weaner pig studies focused on searching for alternatives to AMC. Numerous dietary additives and nutritional strategies have been proposed and evaluated, including organic acids (Tsiloyiannis et al. 2001), prebiotics (Pierce et al. 2006), probiotics (Zeyner & Boldt 2006), essential oils (Huang et al. 2010), functional feed additives (Marquardt et al. 1999; Bhandari et al. 2009) and manipulating dietary carbohydrates
Pieper et al. 2012. For these feed additives and dietary strategies, several review articles have been published (e.g., Pluske et al. 2002; Halas et al. 2007; Stein 2007; De Lange et al. 2010; Kim et al. 2012; Heo et al. 2013).

In this regard and in the past, some pork producers would restrict-feed their weaner diet and (or) feed a grower- and (or) finisher-diet of lower specifications to weaner pigs to reduce the risk of PWD (Ball & Aherne 1982). This practice led to the notion that feeding a lower protein diet could possibly mitigate PWD in the weaning transition period, although post-weaning production was generally compromised by such a practice. Scientific research has tested this hypothesis and numerous authors have previously demonstrated that feeding a lower protein diet reduced protein fermentation in the gastrointestinal tract (GIT) and simultaneously decreased the incidence of PWD (Le Bellego & Noblet 2002; Nyachoti et al. 2006; Htoo et al. 2007; Wellock et al. 2008; Yue & Qiao 2008; Heo et al. 2009; Opapeju et al. 2009; Kim et al. 2011). Moreover, there is supporting evidence that: (i) feeding a high protein diet immediately after weaning causes protein maldigestion (Högberg & Lindberg 2004) as weanling pigs have an underdeveloped digestive system to adequately digest and absorb dietary proteins (Cranwell 1995); (ii) feeding a lower protein diet significantly decreases total nitrogen intake, ileal dietary-origin nitrogen flow (Heo et al. 2010b) and protein fermentation indices in the small intestine (Nyachoti et al. 2006; Heo et al. 2008, 2009; Wellock et al. 2008); and (iii) feeding a lower protein diet improves fecal consistency (Nyachoti et al. 2006; Wellock et al. 2006, 2008; Yue & Qiao 2008).

Despite considerable research on feeding a lower protein diet to prevent PWD in weaned pigs, there is a lack of holistic evidence underpinning how a lower protein diet reduces PWD in relation to the metabolic and physiological responses of the GIT. Therefore, establishing relationships between dietary protein intake and protein fermentation indices such as plasma urea nitrogen (PUN) and ammonia-N (NH₃-N) in the GIT, along with the incidence of PWD, will provide objective data to support the notion that increased dietary protein intake increases the incidence of PWD due to increased protein fermentation in the GIT. In this paper, an opportunity existed using previously published experimental data, where weaned pigs were fed either a high- or a low-protein diet without
and with oral infection with an enterotoxigenic *Escherichia coli* (ETEC; Heo *et al*. 2008, 2009, 2010a,b,c), to examine statistically relationships between dietary-origin protein intake, nitrogen fermentation indices, fecal consistency and the incidence of PWD.

**Materials and Methods**

These studies were reviewed and approved by the Murdoch University Animal Ethics Committee and the Animal Ethics Committee of the Department of Agriculture and Food, Western Australia.

**Experimental design and procedures**

For the current paper, we used datasets generated from a series of four intensive experiments in weaned pigs. The advantage of using these datasets in this paper is that they are all derived from the same experimental ETEC infection model with pigs of the same genotype that were fed and housed under similar conditions.

*Experiment 1*

The first experiment examined the effects of dietary protein level and zinc oxide supplementation on the incidence of PWD, performance and GIT characteristics in weaner pigs challenged with ETEC (Heo *et al*. 2010a,c). Briefly, the experiment was conducted as a split plot design (*n* = 12 pigs per treatment combination) with the whole plot being challenge or no challenge with ETEC and the dietary treatments used as subplots and arranged in a completely randomized 2 × 2 factorial design, with the factors being: (i) two dietary protein levels (251 g/kg vs. 192 g/kg crude protein (CP)); and (ii) addition or no addition of 2500 ppm ZnO. Ninety-six (1:1, male : female gender ratio) individually housed pigs (Large White × Landrace) weighing 7.2 ± 0.69 kg (mean ± SEM) and weaned at 21 days were used. Pigs were allocated to the treatments based on their initial body weight (BW) and gender. Pigs were offered the experimental diets on an *ad libitum* basis for 3 weeks, and had free access to water at all times. Feed intake was recoded on a weekly basis as feed disappearance from the feeder. PWD, NH₃-N and PUN were measured. At the end of the second week, eight pigs of median BW and
the same gender ratio per treatment were selected from the non-challenged groups, and were euthanized to harvest digesta material and organs.

**Experiment 2**

The second experiment examined the effects of feeding a lower protein diet to weaned pigs on PUN, fecal NH$_3$-N, the incidence of PWD and performance after weaning without experimental ETEC infection (Heo et al. 2008). Briefly, the experiment was conducted as a completely randomized design with five combinations of diet and feeding-duration treatments ($n = 12$ pigs per treatment). The five treatments were: (i) a high protein diet (243 g/kg CP) fed for 14 days after weaning; (ii) a low protein diet (173 g/kg CP) fed for 5 days after weaning; (iii) a low protein diet fed for 7 days after weaning; (iv) a low protein diet fed for 10 days after weaning; and (v) a low protein diet fed for 14 days after weaning. At the conclusion of the various feeding treatments, all pigs then received a second-stage diet (215 g/kg CP). None of the diets contained AMC. Sixty (1:1, male : female gender ratio) individually housed pigs (Large White × Landrace) weighing 6.1 ± 0.13 kg (mean ± SEM) weaned at approximately 21 days were used. Pigs were offered the experimental diets on an ad libitum basis for 4 weeks, and water was available at all times. Feed intake was recorded on a daily basis as feed disappearance from the feeder. Fecal consistency, NH$_3$-N and PUN were measured.

**Experiment 3**

The third experiment examined the effects of feeding a lower protein diet on indices of protein fermentation and the incidence of PWD in weaned pigs challenged with ETEC (Heo et al. 2009). This experiment was a 3 × 2 factorial arrangement of treatments. The factors comprised three feeding regimens associated with different combinations of feeding duration and diet crude protein levels, described as: (i) a high protein diet (256 g/kg CP) fed for 14 days after weaning; (ii) a low protein diet (175 g/kg CP) fed for 7 days after weaning; and (iii) a low protein diet fed for 14 days after weaning; and either infection or non-infection with ETEC at 72, 96 and 120 h after arrival, respectively. Seventy-two female pigs (Large White × Landrace) weaned at 21 days of age with initial BW of 5.9 ± 0.12 kg (mean ± SEM) were used. Pigs were allocated to their experimental treatments based on
initial BW and block within room in the animal facility. Pigs were offered the experimental diets on
an ad libitum basis for 4 weeks, and water was available at all times. Feed intake was recorded on a
daily basis as feed disappearance from the feeder. Fecal consistency, NH₃-N and PUN were
measured.

Experiment 4

The fourth experiment examined the effect of feeding a lower protein diet on protein content
(N × 6.25) in the GIT and the incidence of PWD in pigs challenged with ETEC (Heo et al. 2010b).
Briefly, the experiment was designed as a 2 × 2 × 2 factorial arrangement of treatments (n = 6), with
the respective factors being: (i) two dietary protein levels (239 g/kg vs. 190 g/kg); (ii) without and
with challenge of an ETEC (serotype O149; K91; K88); and (iii) time of feeding after weaning (7
days vs. 14 days after weaning). Forty-eight male pigs (Large White × Landrace) aged 21 days and
weighing 6.9 ± 0.11 kg (mean ± SEM) were used. Pigs were allocated to their experimental feeding
regimen based on initial BW and block within the room in the animal facility. The pigs were offered
their respective experimental diets ad libitum for 2 weeks. Feed intake was recorded on a daily basis as
feed disappearance from the feeder. Fecal consistency, NH₃-N and PUN were measured. Pigs were
euthanized at the end of each feeding period for harvesting of digesta material and organs.

Infection procedure

Pigs were infected with ETEC (β-hemolytic serotype O149; K91; K88; toxins LT, STα, STβ) at 72, 96
and 120 h after weaning. At 72 h after weaning, freshly prepared ETEC broth was orally inoculated to
initiate infection without adverse effect (3–8 mL of 10⁷–10⁸ colony-forming units based on health
status of pigs). Then, an 8–10 mL ETEC broth with 10⁷–10⁸ colony-forming units were inoculated at
96 and 120 h for clinical expression of PWD. The inoculation procedure, which involved mild manual
restraint and giving the broth per os with a 20-mL syringe, took 15–30 s to perform.

Fecal consistency score and the incidence of diarrhea
Feces were visually assessed daily for 2 weeks after weaning to determine fecal consistency scores and the incidence of PWD. Fecal consistency was determined using procedures previously described (Heo et al. 2008).

**Plasma urea nitrogen and ammonia-N**

Plasma urea nitrogen (PUN) was determined using an enzymatic (urease) kinetic method (Randox Laboratories Ltd, Crumlin, Co., Antrim, UK). The assay was performed on an automated analyzer (RX Daytona; Randox Laboratories Ltd, Crumlin, Co., Antrim, UK). The metabolite was determined in the Clinical Pathology Laboratory at Murdoch University (WA, Australia). The ammonia-N (NH$_3$-N) concentration in fresh fecal samples was measured according to a method described by Weatherburn (1967).

**Statistical methods**

Using the collective data set generated from these four experiments, multiple regression analysis was attempted to establish relationships between daily protein intake, protein fermentation indices and the incidence of PWD. However, multiple regression analysis could not be performed because: (i) not all pigs in each experiment succumbed to PWD, meaning that some pigs had to be removed from the dataset; (ii) of those pigs with PWD, not all pigs had PUN value analyzed (Heo et al. 2008, 2009, 2010a,c) and ileal protein flow measured (Heo et al. 2010b); (iii) some pigs were euthanized on day 7 and some on day 14 (Heo et al. 2010a,b,c); and (iv) the experimental unit was on an individual basis for two experiments (Heo et al. 2008, 2010a,c) and was on a pen basis for the other two experiments (Heo et al. 2009, 2010b). Subsequently, linear-plateau statistical relationships were established between selected contributing factors to PWD such as dietary-origin protein intake (g/day) and fecal NH$_3$-N and PUN. In this analysis, average daily protein intake was calculated based on average daily feed intake (either individual basis or pen basis) and dietary protein levels, and then linear-plateau regression analyses were conducted using a Nutritional Response Model (Version 1.1; Vedenov & Pesti 2008).
Results and Discussion

Post-weaning diarrhea and *E. coli*

One of the most noticeable clinical signs of intestinal malfunction and morbidity in the post-weaning period is PWD, which concurrently can cause dehydration, depression, a growth check, weight loss, reduction in feed intake and (or) even death within the first 2 weeks after weaning (Hampson 1987; Pluske *et al.* 1997, 2002; Madec *et al.* 2000; Heo *et al.* 2013). Although it is a multifactorial disease and its precise pathogenesis remains unclear, PWD is typically associated with fecal shedding of β-hemolytic ETEC that particularly proliferate in the small intestine of both healthy and unhealthy pigs in the immediate post-weaning period. In this regard, studies examining relationships between diet and PWD often use an ETEC oral challenge model to mimic the responses observed under commercial conditions, and indeed many studies have confirmed that an experimental ETEC infection predisposes and increases the incidence of PWD in pigs (e.g. McDonald *et al.* 1999; Montagne *et al.* 2004; Kim *et al.* 2011; Liu *et al.* 2013). The ETEC attach (i.e. via fimbrial adhesins, afimbrial adhesins and outer membrane proteins) to specific receptors on the small-intestinal enterocytes and produce one or more enterotoxins such as LT (heat-labile toxins) or ST (heat-stable toxins; variants STa and STb). The LT toxins increase secretion of sodium, chloride and hydrogen carbonate ions into the lumen, while the ST toxins reduce the absorption of liquid and salts (Hampson 1987; Bolduan *et al.* 1988). In both cases, the result is hyper-secretion of water and electrolytes into the small intestine that exceeds the ability of the colon to reabsorb them, thus causing diarrhea and a range of other effects, including (but not all-encompassing) dehydration, reduced feed intake, reduced nutrient digestibility, reduced growth and even death (Hampson 1994; Madec *et al.* 2000). In addition, Spitz *et al.* (1995), Muza-Moons *et al.* (2004) and McLamb *et al.* (2013) demonstrated that intestinal barrier function is possibly disrupted because of destabilization
and dissociation of tight junction protein from the epithelial cells and stimulation of mast cells due to *E. coli* infection.

**Impact of dietary protein levels on the GIT environment**

Although there are other dietary factors such as type and concentration of non-starch polysaccharides that are associated with proliferation of pathogenic bacteria in the GIT (Kim *et al*. 2012), dietary protein content and type (e.g. Jeaurond *et al*. 2008) are known to play crucial roles in the maintenance of intestinal health and the incidence of PWD (Kim *et al*. 2012; Rist *et al*. 2013). Commercial diets for weaned pigs have traditionally been formulated to contain 210–240 g/kg of dietary crude protein to support maximum lean growth of the modern genotypes. However, and following bans on the use of AMC in some European countries, reducing dietary protein level was proposed as an alternative strategy to the use of in-feed AMC. This is because not all dietary proteins are absorbed and available for body protein deposition (e.g. Högb erg & Lindberg 2004), and instead a portion of dietary protein (20–40% of that consumed) escapes enzymatic digestion in the small intestine. These undigested dietary proteins along with endogenous proteins contained in mucus, enzymes and sloughed cells enter the large intestine, which encourages proliferation of nitrogen utilizing bacteria (Piva *et al*. 1996; Reid & Hillman 1999). Especially, bacterial groups known as saccharo-proteolytic microbes including *E. coli*, Proteus and Clostridia can rapidly proliferate when ideal conditions are provided such as an increase in the protein : carbohydrate ratio in the dietary chyme (Weijers & van de Kamer 1965; Abe *et al*. 1981; Nollet *et al*. 1999).

Consequently, bacterial fermentation of proteins in the large intestine generates numerous unfavorable toxic compounds, including some branched-chain fatty acids, indole, phenols, ammonia and biogenic amines (Williams *et al*. 2001), which have been implicated in the aetiology of PWD (e.g. Bolduan *et al*. 1988; Aumaitre *et al*. 1995; Gaskins 2001; Pluske *et al*. 2002; Heo *et al*. 2008; Kim *et al*. 2008, 2012; Rist *et al*. 2013). For instance, work by Lin and Visek (1991) and Nousiainen (1991) demonstrated that NH3 in the GIT could damage mucosal architecture and inhibit its digestive
and secretory functions. Therefore, strategies to reduce the microbial protein fermentation in the GIT by reducing dietary protein levels, or reducing the amount of fermentable dietary proteins by using highly digestible ingredients, can be beneficial to improve the GIT health of piglets in the immediate post-weaning period. The proposed mechanisms of action of feeding a lower protein diet as a means of managing PWD is illustrated in Figure 1.

**Relationships between average daily protein intake and fecal NH₃-N content in weanling pigs**

The linear-plateau regression analysis (Figs 2, 3) clearly demonstrated that average daily protein intake was positively correlated to both fecal NH₃-N ($Y = 485.2 - 3.33 \times (72.83 - x)$, $R^2 = 0.72$, residual standard deviation (RSD) = 29.87, $P < 0.001$) and PUN contents ($Y = 5.43 - 0.087 \times (63.67 - x)$, $R^2 = 0.84$, RSD = 0.70, $P < 0.001$), respectively. As average daily feed intake did not differ between dietary protein levels across the studies (Heo et al. 2009, 2010a,b,c; $P > 0.05$), this finding supports the general hypothesis that feeding a high protein diet increases protein fermentation indices such as fecal NH₃-N content compared with feeding a lower protein diet. Nevertheless, the dataset suggests that under the experimental ETEC infection conditions examined, fecal NH₃-N and plasma urea concentrations reached a plateau at 72 g and 64 g dietary protein intake per day, respectively.

**Relationships between average daily CP intake and the incidence of PWD**

The relationships between average daily protein intake and the incidence of PWD are presented in Figure 4. The statistical analysis was conducted individually for each experiment as the pigs' health status and ETEC infections, which are the contributing factors to the incidence of PWD, were heterogeneous among the four experiments. The results showed that the incidence of PWD was highly dependent on daily protein intake levels. Although all pigs ate less than 60 g dietary protein per day in Experiment 3 (Heo et al. 2009) and had a linear relationship between daily dietary protein intake and incidence of PWD, pigs in Experiments 1, 2 and 4 (Heo et al. 2008, 2010b,c) developed PWD when eating more than 60 g of dietary protein daily. This finding suggests that PWD could be reduced when dietary protein intake is restricted to less than 60 g/day/pig, or therefore pigs should be fed 350 g/day
with an ideal lower protein diet (e.g. approximately 180 g/kg or less) for at least 7 days. This is a comparable result to the report by Sørensen et al. (2009) that protein restriction increased fecal dry matter content and reduced diarrhea in 7-week-old weaner pigs (167 g vs. 94 g/kg digestible protein).

Conclusions

The collective results derived from these datasets indicate that restriction of daily protein intake to less than 60 g through feeding a lower protein diet for as little as 7 days after weaning reduced the incidence of PWD commensurate with a reduction in protein fermentation indices. These results confirm that feeding a lower protein diet is a viable alternative strategy to the use of in-feed AMC, and indeed many commercial post-weaning diets now manufactured in Europe for example and which are devoid of AMC routinely have dietary CP levels of 165–175 g/kg. Nonetheless, further research would be required: (i) to explore the role of other dietary components (e.g. sources of dietary proteins and dietary fiber, protein : carbohydrate ratio) on PWD; (ii) to investigate the influences of feeding a lower protein diet on specific bacterial populations in the GIT of newly weaned pigs; and (iii) to explore the best combination of other purported or proven antimicrobial substances (e.g. chito-oligosaccharide, organic acids, herbs, essential oils) with a lower protein diet that efficiently and cost-effectively reduces PWD and improves GIT health.

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Figure 1. Schematic representation of the proposed mechanisms of action of feeding a lower protein diet as a means of managing post-weaning diarrhea (PWD). Adapted from Opapeju (2010) with some modifications.
Figure 2. A linear-plateau relationship between average daily protein intake after experimental enterotoxigenic strain of *Escherichia coli* (ETEC) infection and faecal ammonia-N (NH$_3$-N) content in weanling pigs. Datasets were used from Heo *et al.* (2009, 2010b,c). RSD = residual standard deviation.
Figure 3. A linear-plateau relationship between average daily protein intake after experimental *Escherichia coli* (ETEC) infection and plasma urea nitrogen content in weaner pigs. Datasets were used from Heo *et al.* (2008, 2009, 2010b,c). RSD = residual standard deviation.
Figure 4. Relationships between average daily protein intake after experimental Escherichia coli (ETEC) infection and the incidence of post-weaning diarrhea (PWD) in weaner pigs across experiments. HP = pigs fed high protein diet, LP = pigs fed low protein diet, HP + E = pigs fed HP with ETEC infection, LP + E = pigs fed LP with ETEC infection (Experiments 1, 2, 3 and 4 refer to Heo et al. (2010c), Heo et al. (2008), Heo et al. (2009) and Heo et al. (2010b), respectively).

\( \times \), HP + E; \( \triangle \), HP; \( \bullet \), LP + E; \( \ast \), LP.