The impact of specialty protein ingredients on the growth performance and health of nursery pigs

by

Leigh Ann Ruckman

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John F. Patience, Major Professor
Kenneth J. Stalder
Alejandro Ramirez

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

AB	Antibiotic
ADD	Specialty protein additive
ADFI	Average daily feed intake
ADG	Average daily gain
aEE	Acid-hydrolyzed ether extract
ANF	Anti-nutritional factor
AOAC	Association of Official Analytical Chemists
BD	Backfat depth
BW	Body weight
Ca	Calcium
cDNA	Complementary deoxyribonucleic acid
CLDN	*Claudin*
CTC	Chlortetracycline
CV	Coefficient of variation
d	Day
DEP	Hyperimmunized dried egg protein
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Dressing percentage
dpi	Days post-inoculation
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay

ESBM	Enzymatically-treated soybean meal
ETEC	Enterotoxigenic *Escherichia coli*
F:G	Feed:gain ratio
G:F	Gain:feed ratio
GC	Gas chromatography
GE	Gross energy
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
HCW	Hot carcass weight
IAV	Influenza A virus
IFN	Interferon
Ig	Immunoglobulin
IgY	Egg-yolk antibody
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
Ile	Isoleucine
LBP	Lipopolysaccharide-binding protein
LD	Loin depth
LP	Lean percent
Lys	Lysine
M	Molar
Mcal	Megacalorie

MDA	Malondialdehyde
ME	Metabolizable energy
min	Minute
MOA	Mode of action
mRNA	Messenger ribonucleic acid
n	Sample size
N	Nitrogen
Na	Sodium
NDO	Non-digestible oligosaccharide
NE	Net energy
OCLN	*Occludin*
OEE	Oregano essential oils
P	Probability
P	Phosphorus
PBS	Phosphate-buffered solution
PC	Phytogenic compound
PCA	Phytogenic compound and acidifier
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus type 2
PEDV	Porcine epidemic diarrhea virus
PRRSV	Porcine reproductive and respiratory syndrome virus
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPL19	*Ribosomal protein-L19*
RT-qPCR	Real-time quantitative polymerase chain reaction
SBM	Soybean meal
SDPP	Spray-dried plasma protein
SEM	Standard error of the mean
SID	Standardized ileal digestible
STTD	Standardized total tract digestible
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
Thr	Threonine
TI	Trypsin inhibitor
TJ	Tight junction
TNF-α	Tumor necrosis factor-α
Trp	Tryptophan
TSAA	Total sulfur amino acids
V:C	Villi height:crypt depth ratio
Val	Valine
VFA	Volatile fatty acid
VTM	Vitamin and trace mineral
WBC	Water-binding capacity
WHC	Water-holding capacity
ZO	*Zonula occluden*

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ABSTRACT

The pork industry is interested in feed additives, such as specialty protein ingredients, that can be fed to pigs in order to help mitigate the effects of weaning on pig performance and gastrointestinal tract (GIT) health and function. Functional specialty proteins, such as spray-dried plasma protein (SDPP) and hyperimmunized dried egg protein (DEP), have been identified as potential alternatives to dietary antibiotics due to their biologically active components (such as immunoglobulins) that can aid in pathogen inhibition in the GIT. However, the industry's understanding of these protein's mode of action and their impact in commercial environments is lacking. Reducing the inclusion of soybean meal (SBM), which contains anti-nutritional factors, in early nursery diets has been identified as a way to mitigate the effect of weaning stress in pigs. Enzymatically-treated SBM (ESBM) has reduced anti-nutritional factor concentrations and improved nutrient digestibility compared to SBM, but this ingredient has yielded inconsistent results in performance studies. Further, there is a lack of research investigating this ingredient's mode of action, so its true value to producers is unknown.

Therefore, the primary objective of the research reported in this thesis was to investigate the impact of specialty protein ingredients on nursery pig performance and specific markers of GIT health and function. In order to achieve these objectives, two experiments were conducted with the specific objectives to 1) compare under commercial conditions the effects of including SDPP or DEP in the diet, with or without subtherapeutic levels of antibiotics in the phase 1 and 2 nursery diets, on growth performance and markers of intestinal physiology and function (experiment 1), and 2) determine the impact of diets in which ESBM replaced increasing amounts of SBM on growth performance, intestinal structure and barrier integrity, inflammation, and oxidative status in newly weaned pigs (experiment 2).

In experiment 1, (Chapter 2) the inclusion of either SDPP or DEP in the diet improved the growth rate and feed intake of weaned pigs when antibiotic-free diets were fed during phases 1 and 2. However, when antibiotic-positive diets were fed, the inclusion of SDPP or DEP failed to alter the growth performance of the pigs compared to that of the SBM control. Due to the low dietary inclusion of DEP versus SDPP (0.2% or 3% in phase 1; 0.1% or 2% in phase 2), feeding DEP was a cost-effective method to improve performance in the antibiotic-free diets. The pigs fed the SDPP and DEP required fewer individual medical treatments than pigs fed the control diet, indicating a positive effect on their overall health. Both SDPP and DEP were shown to beneficially modulate the inflammatory response in the GIT of pigs and slightly improve ileal morphology measures. Overall, this study provided novel data, collected under commercial conditions, about the impact of specialty proteins, in the absence or presence of antibiotics.

In experiment 2 (Chapter 3), there was a linear decrease in final body weight and overall growth rate and feed intake of pigs due to feeding 14% or 21% ESBM (in phase 1; 7 or 10.5% in phase 2) compared to the control (0% ESBM) or the lowest ESBM diet (7 or 3.5% in phases 1 and 2). The inclusion of ESBM did not have any impact on feed efficiency. However, feeding ESBM, independent of inclusion level, improved the overall fecal score of pigs compared to the control, likely due to the reduction in the dietary concentration of the antigenic proteins glycinin and β-conglycinin and non-digestible oligosaccharides in ESBM. Further, feeding ESBM did beneficially modulate oxidative stress measures, improve intestinal barrier integrity markers, and increase volatile fatty acid production in the small intestine.

Overall, this thesis research provided valuable and novel data to the swine industry regarding the use of specialty protein ingredients in nursery diets and how these proteins may impact pig performance and markers of GIT health and function. These data show that functional

specialty proteins (such as SDPP and DEP) are beneficial in antibiotic-free feeding systems; however, a pork producer that is using in-feed antibiotics may not see the same improvements in pig performance when feeding these proteins. Further, increasing the inclusion of ESBM and decreasing SBM levels may benefit the GIT health of pigs, but performance will be impaired when feeding higher ESBM levels. In order to maximize the feeding value of specialty proteins, the industry needs further investigation into the dietary use of these proteins to fully understand their potential mode of action and impact on pig performance and health in commercial nursery environments.

CHAPTER 1. LITERATURE REVIEW

Introduction

The transition which occurs at the time of weaning is the most significant and stressful event in a pig's life, due to changes in diet, environment, and social hierarchy. These stressors, as well as exposure to unfamiliar pathogens, result in poor growth performance and feed intake while impairing gastrointestinal tract (GIT) health and function (Lallès et al., 2004; Moeser et al., 2006). Further, weaned pigs are highly susceptible to enteric pathogens and disease due to a still developing immune system and gut (de Lange et al., 2010). These issues have heightened the pork industry's interest in high-quality feed ingredients, specifically specialty proteins, that can improve weaned pig performance and positively affect GIT health (Pluske et al., 2013).

Functional proteins are a category of specialty protein ingredients that have been identified as a means to mitigate the detrimental effects that weaning has on young pigs. Spray-dried plasma protein (SDPP) and hyperimmunized dried egg protein (DEP) are two functional proteins that have been shown to improve growth and feed intake in newly weaned pigs (Marquardt et al., 1999; Tran et al., 2014). Further, several authors have reported improvements in intestinal barrier integrity and immune modulation due to feeding these ingredients (Torrallardona, 2010; Li et al., 2015). Due to the beneficial effects of these functional proteins, it has been suggested that SDPP and DEP could be used to limit the use of growth-promoting antibiotics in nursery pig diets.

Enzymatically-treated soybean meal (ESBM) is a specialty protein ingredient that is fed to weaned pigs as a way to decrease the inclusion of conventional soybean meal (SBM) in nursery diets. Though SBM is the main protein source in swine diets, it contains harmful anti-

nutritional factors (ANF) that detrimentally impact piglet performance and health (Yang et al., 2007). Therefore, further processing methods have been developed to produce ESBM, which has been shown to improve growth compared to SBM (Zhu et al., 1998; Zhou et al., 2011). However, the pork industry has a poor understanding of the mechanisms behind the improved piglet performance and additional effects on GIT health and function.

The objectives of this review are 1) to characterize the pigs' phenotypic response to weaning, 2) to discuss the benefits and concerns of in-feed antibiotics in nursery pig diets, 3) to describe functional protein ingredients (specifically SDPP and DEP) while detailing their impact on pig performance and potential modes of action (MOA), and 4) to define the ANF in SBM and describe the impact of conventional SBM and ESBM on piglet performance and health.

Weaning stress in pigs

The impact of weaning stress on nursery pig performance and health has been well documented in the literature. Newly weaned pigs encounter several types of stressors, including abrupt dietary changes, new social and housing environments, and exposure to previously unknown pathogens (Moeser et al., 2006). The weaning transition is associated with poor voluntary feed intake for the first 24-48 hours, resulting in decreased energy intake and a subsequent reduction in growth (Brooks et al., 2001). Poor feed intake can affect the rate of cell production in intestinal crypts and cause atrophy of the villi (Pluske et al., 1997). Villi height and surface area have been directly correlated with absorptive capabilities of the gut (Montagne et al., 2007).

Young pigs are susceptible to a variety of gastrointestinal disorders after weaning, but the most prevalent is postweaning diarrhea. Typically, intestinal infection by enterotoxigenic *Escherichia coli* (ETEC) is the main cause of post-weaning diarrhea (Li et al., 2019; Becker et

al., 2020). The ETEC use fimbrial adhesions to bind to the intestinal epithelium and proliferate in the gut. These bacteria release enterotoxins that can damage the epithelium and disrupt the water-electrolyte balance and the process of fluid absorption, resulting in watery diarrhea (Sun and Kim, 2017; Li et al., 2019). These toxins may also alter intestinal permeability or incite inflammatory immune responses in the GIT (Li et al., 2019; Becker et al., 2020). Severe post-weaning diarrhea can increase the mortality rate of weaned pigs, resulting in considerable economic losses for producers (Becker et al., 2020).

The intestinal epithelium and mucosal barrier are major physical defense mechanisms of the GIT immune system, but barrier permeability is increased through the weaning transition (Hu et al., 2013; France and Turner, 2017). Tight junction protein complexes maintain the paracellular permeability between epithelial cells, but disruption of these complexes by enterotoxins or pro-inflammatory cytokines can increase intestinal permeability (Pluske et al., 2018). This increases the risk of pathogens and/or toxins translocating into the body, which could potentially activate a nutrient and energy expensive immune response (Huntley et al., 2018). The inflammatory response is a crucial part of intestinal immunity as it signals the recruitment and activation of immune cells in response to pathogens or toxins (Pié et al., 2004). However, uncontrolled inflammation can impact growth as nutrients and energy are partitioned away from growth (Huntley et al., 2018). Weaning has been shown to increase the production of several pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Pié et al., 2004; Hu et al., 2013).

Antibiotic use in nursery pig diets

Historically, one of the most common methods of improving weaned pig performance and reducing postweaning mortality has been the dietary inclusion of antibiotics (Patience,

2019). The growth promoting nature of antibiotics was first discovered through poultry research in the 1940s and was quickly utilized in the production of other livestock species (Gustafson and Bowen, 1997). Today, antibiotics have become a staple component of nursery diets and may be included at therapeutic or subtherapeutic levels (Jacela et al., 2009). Therapeutic levels are considered dose levels of antibiotics that are used to treat and/or prevent disease, whereas subtherapeutic levels are focused more on improving growth rate, feed conversion, and mortality or morbidity of pigs (Cromwell, 2002).

In a review by Cromwell (2002), over 1,000 studies (occurring between 1950 and 1985) that evaluated the impact of subtherapeutic antibiotics on growth performance of pigs were summarized. Several antibiotics were tested through these experiments, including chlortetracycline, carbadox, lincomycin, tiamulin, and tylosin (Zimmerman, 1986). Cromwell (2002) reported that dietary antibiotics improved the average daily gain (ADG) of young pigs (7 to 25 kg) by 16% and feed conversion by 7%. During the grow-finish period (24 to 89 kg), growth rate and feed conversion were improved by only 4 and 2%, respectively. Dritz et al. (2002) summarized more recent antibiotic evaluation studies and reported a 5% increase in ADG and no change in feed:gain (F:G) when subtherapeutic antibiotics were fed to nursery pigs; further, antibiotics did not impact ADG or F:G during the grow-finish period. Therefore, subtherapeutic doses of antibiotics may provide some benefit throughout the entire growing period, but the benefit is most substantial during the nursery phases (Cromwell, 2002).

The growth-promoting MOA of antibiotics remains unknown, but several potential mechanisms have been proposed (Gaskins et al., 2006). Antibiotics are known to have bacteriostatic and/or bactericidal activity and can be used to inhibit or reduce the proliferation of pathogenic bacteria in the GIT (Zimmerman, 1986; Jacela et al., 2009). This inhibition of

bacterial infections, both clinical and subclinical, can reduce microbial use of nutrients and the abundance of growth-depressing metabolites and toxins (Gaskins et al., 2006). Further, antibiotic-fed pigs have been shown to have thinner intestinal walls and reduced epithelial cell turnover than those not fed antibiotics, indicating that the use of subtherapeutic antibiotics may allow for enhanced nutrient uptake and use (Gaskins et al., 2006).

Though subtherapeutic antibiotics are proven to be beneficial in nursery diets, there has been a growing push for the pork industry to reduce or eliminate antibiotic use in swine diets (Olsen et al., 2018). There has been global concern that continuous subtherapeutic antibiotic use has created antimicrobial-resistant bacteria that could be transmitted to humans through meat consumption. This could potentially lead to human diseases that cannot be treated with antibiotics (Thacker, 2013). In 2017, the FDA implemented the Veterinary Feed Directive to regulate antibiotic use by prohibiting the feeding of medically important antibiotics for growth promoting purposes or without veterinary supervision (FDA, 2012).

Functional protein ingredients

Due to these concerns regarding antibiotic use in swine diets, the pork industry has tried to identify and evaluate feed ingredients that could be used to reduce antibiotic inclusion in nursery diets. Functional proteins are one category of specialty protein ingredient that have been identified as a potential alternative. These ingredients are included in the diet to provide more than just nutritive value, as they can also alter the health or GIT structure and function of pigs. Commonly utilized functional animal proteins include milk proteins (such as dried whey, dry skim milk, or casein), blood products such as SDPP, egg proteins, or fish meal (Pettigrew, 2006).

Spray-dried plasma protein

Spray-dried plasma protein is a highly digestible protein ingredient that has been used in nursery diets since the late 1980s (Pérez-Bosque et al., 2016). It is most commonly produced from porcine or bovine sources but may also be made from avian species (Zhang et al., 2016). The SDPP contains a variety of bioactive components that are known to positively impact growth performance and GIT health of weaned pigs (Pierce et al., 2005; Tran et al., 2014). Further, the high palatability of SDPP can ease the dietary transition to solid feed and increase feed intake after weaning (van Dijk et al., 2001a).

Production of spray-dried plasma protein

The SDPP is a by-product of the meatpacking industry. The multi-step production process begins when whole blood is collected at the slaughterhouse and treated with anticoagulant. The blood is then centrifuged and filtered to remove the red and white blood cells from the plasma fraction. The spray-drying process occurs at a high pressure and temperature (approximately 80°C), converting the plasma into a powder while preserving and concentrating the bioactive components (Gerber et al., 2014; Pérez-Bosque et al., 2016; Blázquez et al., 2020).

There have been concerns within the pork industry that feeding SDPP, specifically porcine, could transmit pathogens to other pigs. This concern mainly stems from the North American porcine epidemic diarrhea virus (PEDV) outbreak in 2013 (Gerber et al., 2014). However, extensive research has been conducted to evaluate this risk, and most studies have demonstrated the efficacy of the spray-drying process to inactivate viral and microbial pathogens (Blázquez et al., 2020). Gerber et al. (2014) and Opriessnig et al. (2014) reported no transmission of PEDV when feeding SDPP that was made from the blood of PEDV positive pigs or PEDV-inoculated SDPP. Further, Polo et al. (2005) and Pujols et al. (2008) demonstrated that SDPP

inoculated with other porcine viruses, such as porcine circovirus type 2 (PCV-2) or porcine reproductive and respiratory syndrome virus, was unable to transmit the viruses when fed to pigs. In 2014, a case study was performed to investigate the outbreak of PEDV on U.S. Midwestern pig farms that were feeding ingredients that came from porcine origin, including porcine SDPP (Neumann et al., 2014). Farms that had experienced a PEDV outbreak and fed porcine ingredients were identified, and the sources of the ingredients were tracked. Farms that had fed diets including ingredients from the same sources but had not reported a PEDV outbreak were identified as control farms. Overall, the case study evaluated 43 case farms and 418 control farms. The results of the case study indicated that SDPP was not positively associated with PEDV outbreaks on farms feeding this ingredient.

It should be noted, however, that there may still be a risk of disease transmission when feeding SDPP to pigs. Patterson et al. (2010) reported transmission of PCV-2 to naïve pigs when SDPP, produced from the blood of PCV-2 infected pigs, was administered through oral gavage. A different author reported that weaned pigs were infected with PEDV after inoculation with SDPP samples that had tested positive for the virus (Pasick et al., 2014). The pigs showed minimal clinical symptoms of PEDV (such as diarrhea and fever), but the SDPP-inoculated pigs shed the virus for over 9 days post-inoculation (dpi) and infected healthy pigs that were introduced to the group on dpi 7. Case studies by Pasma et al. (2016) and Aubry et al. (2017) investigated the links between initial PEDV outbreaks in Canadian swine herds and the feeding of PEDV-positive SDPP, which had been imported from the U.S., and reported a strong association between the two variables. Though Pasick et al. (2014) had also reported that pigs inoculated with complete feed that contained PEDV-positive SDPP were not infected with the virus, it has been speculated that SDPP inclusion in complete commercial feed may still pose a

threat to pigs. In a report for National Pork Board, Sampredo et al. (2015) suggested that the apparent inactivation of viruses in bioassay testing may not be equivalent to the biological inactivation of a virus as the assays do have detection limits. Even SDPP with a low level of viral presence and activity, which may be undetected in a smaller laboratory setting, could cause infection when fed in commercial-sized production. Therefore, the debate regarding the biosecurity of SDPP is controversial and there are producers who prefer to not feed SDPP to pigs.

Impact of spray-dried plasma protein on pig performance

Since its development, numerous studies have been performed to evaluate the efficacy of SDPP in nursery diets. The majority of these studies report a beneficial impact on growth performance when SDPP is fed, especially during the first two weeks after weaning (van Dijk et al., 2001a; Torrallardona, 2010). A recent SDPP review states that the average improvement in d 0-7 ADG and average daily feed intake (ADFI), due to feeding SDPP, was 36% and 17%. From d 7-14 postweaning, feeding SDPP resulted in a 2% and 3% improvement in ADG and ADFI (Balan et al., 2020). A review by Torrallardona (2010) suggested that the optimal dietary inclusion of SDPP in the first week after weaning is 4-8%. However, this level has been disputed as other authors have suggested higher (6-8% SDPP) or even lower (3% SDPP) optimal dietary inclusions to maximize growth performance (Gatnau and Zimmerman, 1992; Coffey and Cromwell, 1995). The benefit of feeding SDPP may also be dependent on the composition of the control diet and nutritive value of the protein source against which the SDPP is being evaluated (Torrallardona, 2010).

Recent studies have compared SDPP against soybean protein sources, such as SBM or soy protein concentrate, or other animal protein sources like fish meal. Crenshaw et al. (2017)

reported that feeding either 2.5 or 5% SDPP, in comparison to a SBM and soy protein concentrate control diet, increased ADG and ADFI while improving gain:feed (G:F) during the first two weeks postweaning. Further, other studies have also reported improvements in ADG and ADFI when feeding 5-88% SDPP in comparison to a soy protein concentrate control diet (Pierce et al., 2005; Tran et al., 2014; Pujols et al., 2016). While utilizing an ETEC K88 challenge model, Bosi et al. (2004) fed pigs diets containing either 6% fish meal or 6% SDPP for two weeks postweaning. It was reported that feeding SDPP increased ADG and ADFI compared to fish meal, with a tendency to improve final body weight (BW).

Feeding SDPP has been proposed as a potential way to limit or reduce the use of subtherapeutic antibiotics in nursery diets. Though SDPP has rarely been shown to increase growth more than antibiotics, several studies have reported equal performance between the two treatments (Pérez-Bosque et al., 2016). Studies that have evaluated SDPP in both the presence and absence of antibiotics have reported no interactions between SDPP and antibiotics, suggesting that the effects of both are additive and independent of one another (Torrallardona, 2010). It has been hypothesized that these additive effects are due to the dual-protection that SDPP offers against both bacteria and viruses, rather than just the antimicrobial effect of antibiotics (Pérez-Bosque et al., 2016).

The potential for SDPP to provide protection against multiple pathogen types could explain the increased benefit of feeding SDPP in unsanitary or commercial housing environments. Zhao et al. (2007) evaluated 6% SDPP in nursery diets fed to pigs housed in rooms that were either cleaned or uncleaned between nursery turns. There was an interaction tendency for G:F between SDPP and housing conditions, as pigs housed in the unsanitary room had a greater response to SDPP than those housed in the cleaned room. Similarly, Coffey and

Cromwell (1995) did not report any differences in d 0-28 performance when comparing 8% SDPP and a spray-dried skim milk control in a clean, experimental nursery environment. When the same treatments were fed in a commercial nursery environment, SDPP increased ADG and improved F:G compared to the control diet.

These opposing responses to SDPP due to different housing environments support the idea that inconsistent methodology or reporting in experiments evaluating potential antibiotic alternatives can change the outcome and interpretation of the study. Olsen et al. (2018) presented a list of methodology and experimental components that need to be described in studies evaluating antibiotic alternatives in order to provide context to the reader. These components include providing the genetic background and vaccine and medication history of pigs, characterizing the herd health status, inclusion of a negative control diet against which to compare experimental treatments, analysis of experimental diets and ingredients, and clearly describing the experimental design. When evaluating the studies by Zhao et al. (2007) and Coffey and Cromwell (1995), clearly characterizing the herd health statuses through diagnostic testing or documenting changes in health status, mortality, or morbidity would have provided future researchers with more context to interpret how SDPP may impact weaned pigs in those environments or pathogen exposures.

Mode of action of spray-dried plasma protein

There are currently two main proposed MOA to explain the improvements in weaned pig performance when feeding SDPP. The first proposed MOA is that SDPP is highly palatable to young pigs, thus increasing feed intake in the first few weeks after weaning (Torrallardona, 2010). In 1994, Ermer et al. demonstrated that weaned pigs who were given free choice between diets containing 8.5% porcine SDPP or 20% dried skim milk preferred the SDPP diet. Increased

feed intake can improve GIT morphology by reducing the extent of villus atrophy in weaned pigs, thus increasing the amount of surface area available for nutrient absorption (Pluske et al., 1997). Tran et al. (2014) reported an increase in duodenal villus height when feeding diets with 5% SDPP. Other authors have reported no impact on villi height when feeding SDPP, however, indicating that SDPP does not have a significant trophic effect on intestinal villi (van Dijk et al., 2001b; Nofrarías et al., 2006).

The second and more widely accepted MOA is that the bioactive components found in SDPP provide protection against enteric pathogens and enterotoxins through inhibition of pathogen adhesion to the epithelial lining and improved immunocompetence (Bosi et al., 2004; Torrallardona, 2010; Peace et al., 2011; Tran et al., 2014). The SDPP is made up of several functional and bioactive proteins, such as immunoglobulins, albumin, glycoproteins, and bioactive peptides (Bah et al., 2013; Kar et al., 2016). However, Pierce et al. (2005) reported that the immunoglobulin G (IgG)-rich fraction of SDPP is the main bioactive component responsible for improvements in growth performance. Both IgG and glycoproteins have the ability to bind to receptors or surface factors of pathogenic bacteria and viruses in the GIT, thus preventing the pathogens from adhering to the epithelium and damaging barrier integrity or releasing harmful toxins (Nollet et al., 1999; Corl et al., 2007; Torrallardona, 2010).

The proposed mechanism of pathogen adhesion inhibition is supported by research showing that feeding SDPP can improve young pig performance and reduce diarrhea during bacterial or viral enteric disease challenges (van Dijk et al., 2002; Bosi et al., 2004; Corl et al., 2007). Peace et al. (2011) and Zhang et al. (2016) both reported improvements in intestinal barrier integrity when feeding SDPP. Further, SDPP supplementation may improve the immunocompetence of pigs, indicated by increased anti-inflammatory and reduced pro-

inflammatory cytokine production in the GIT (Bosi et al., 2004; Gao et al., 2011; Zhang et al., 2016). This immune modulatory effect may also explain reports that feeding SDPP can reduce the concentration of reactive oxygen species in serum, as the production of these molecules has been linked to the immune response (Lugrin et al., 2014; Muller et al., 2018).

Hyperimmunized dried egg protein

The pork industry utilizes several by-products of the egg-laying industry as functional protein ingredients for nursery pigs. Eggs are known to be an excellent source of highly digestible protein and fat, as well as essential vitamins and minerals (Zhang et al., 2015). Though results are inconsistent, spray-dried whole egg and spray-dried egg yolk have been shown to improve piglet performance (Song et al., 2012). Similar to SDPP, eggs contain biologically active components that can counteract pathogen activity in the GIT. Egg-yolk antibodies (IgY) are the most prolific of these bioactive components and have been identified as a potential alternative to dietary antibiotics (Li et al., 2015).

Egg-yolk antibodies

The IgY are the maternal form of antibodies in avian species and are transferred from the serum of birds to the embryo through the egg yolk. The purpose of IgY is to protect the developing embryo from pathogens through passive immunization. Therefore, continuously feeding IgY to weaned pigs can provide artificially-acquired passive immunity during crucial postweaning periods (Li et al., 2015; Abbas et al., 2019). The roles of IgY and mammalian IgG are biologically similar, but structural differences between the two affect their effectiveness against pathogens. The IgY lack the hinge region that exists in IgG, resulting in decreased flexibility of IgY but increased specificity (Warr et al., 1995). Therefore, IgY that are produced

against specific pathogens have a stronger binding affinity than IgG produced for the same pathogen (Ikemori et al., 1993).

Researchers are still investigating the exact MOA that IgY uses to counteract enteric pathogens, but four mechanisms have been proposed: inhibition of adhesion, agglutination, opsonization followed by phagocytosis, and toxin neutralization (Li et al., 2015; Abbas et al., 2019). However, most authors agree that the main mechanism of IgY is its ability to inhibit the adhesion of pathogens to the intestinal epithelium. The IgY has been shown to bind to exposed factors on the pathogen surface, such as the fimbriae on *Escherichia coli* (*E. coli*) K88, and effectively block the binding of these pathogens to epithelial receptors (Wang et al., 2019). Without adherence to the epithelium, pathogenic bacteria are unable to release damaging and diarrhea-inducing toxins into the intestine of pigs (Marquardt et al., 1999; Wang et al., 2019).

Production of hyperimmunized dried egg protein

Due to the strong binding affinity of IgY against specific pathogens, methods have been developed to stimulate the production of highly-specific IgY in hens. First, hens are hyperimmunized against bacterial or viral pathogens that are known to affect young pigs (Schade et al., 2005). In most studies that have evaluated specific IgY and pigs, hens are hyperimmunized using the fimbrial antigens of various *E. coli* strains, such as K88 or F18 (Wang et al., 2019). However, commercially available specific-IgY products may be produced by hyper immunizing hens against a wide variety of pathogens, including *E. coli* K88, *E. coli* K99, *Salmonella spp.*, porcine rotavirus, transmissible gastroenteritis virus, and porcine circovirus (Torrallardona and Polo, 2016). Once eggs are collected from these hens, the egg yolk can be dried to create a concentrated source of specific IgY. The resulting DEP can be fed to weaned pigs to improve

postweaning growth performance and GIT health (Marquardt et al., 1999; Owusu-Asiedu et al., 2003a; Li et al., 2015).

Impact of hyperimmunized dried egg protein in pigs

In 1991, Wiedemann reported reduced diarrhea in weaned pigs that were fed an *E. coli* K88-specific DEP. Since then, more studies have been conducted to evaluate the impact of DEP on weaned pig performance, diarrhea, mortality, and GIT health and integrity. Studies that have evaluated DEP using a disease challenge that matches the IgY specificity have shown the greatest response to the protein (Li et al., 2015).

For example, Wang et al. (2019) fed either a *E. coli* K88-specific DEP or non-immunized egg-yolk powder to weaned pigs that were challenged with *E. coli* K88. It was reported that feeding the DEP reduced the adherence of *E. coli* K88 to the intestinal mucosa and decreased the expression of diarrhea-inducing enterotoxins in colonic digesta compared to the non-immunized egg-yolk powder. This was supported by results that the diarrhea scores did not differ between infected pigs fed the DEP and uninfected pigs that were not fed an IgY product. However, infected pigs that were fed the non-immunized egg-yolk powder developed severe diarrhea compared to the uninfected control group. The DEP also modulated the ETEC-associated inflammatory response, resulting in similar mucosal expression of pro-inflammatory cytokines (TNF-α, IL-6, and IL-22) as the uninfected pigs. The TNF-α and IL-6 are typically upregulated in response to bacterial infections, while IL-22 is produced to help maintain intestinal barrier homeostasis against specific pathogens (Gao et al., 2013; Wang et al., 2019).

In two separate studies, Owusu-Asiedu et al. (2003a,b) reported reductions in diarrhea score, mortality, and *E. coli* K88 shedding when feeding an *E. coli* K88-specific DEP, compared to non-immunized egg-yolk powder, to weaned pigs challenged with *E. coli* K88. This author

also reported improvements in overall ADG and villus height after feeding the DEP (Owusu-Asiedu et al., 2003a,b). Similarly, Yokoyama et al. (1997) showed that *E. coli* F18 infected pigs that were fed an *E. coli* F18-specific DEP had decreased diarrhea rates and increased BW gain compared to infected pigs not fed DEP.

Most results demonstrate the benefit of feeding DEP when pigs are challenged with the same bacterial strain for which the IgY is specific. However, commercially-raised pigs will be exposed to a wide variety of pathogens rather than just one specific disease. Feeding an *E.coli* K88-specific DEP to non-challenged nursery pigs did not impact growth performance compared to the control treatment (Heo et al., 2015). Torrallardona and Polo (2016) and Crenshaw et al. (2017) both reported no improvement in performance due to feeding DEP (which was hyperimmunized for multiple pathogen types) in unsanitary, commercial nurseries. However, these authors did show that feeding 5-6% SDPP improved d 0-14 ADG, ADFI, and final BW compared to the control and DEP treatment. Due to the decreased specificity of IgG compared to IgY, the impact of SDPP on growth performance may be greater than DEP when pigs are exposed to multiple pathogens in commercial conditions (Warr et al., 1995).

Enzymatically-treated soybean meal

The most commonly fed protein source in swine diets is SBM. Soybean meal contains high concentrations of the essential amino acids lysine and tryptophan, making it a complementary amino acid source to most cereal grains, especially corn (Dilger et al., 2004; Stein et al., 2008). However, SBM does contain high enough concentrations of ANF to detrimentally impact the growth performance and intestinal health of weaned pigs (Li et al., 1991; Yang et al., 2007).

Production of soybean meal

Several types of SBM are used in commercial pig production, and the processing methods of each vary slightly. The most common is dehulled, solvent-extracted SBM, which will be referred to as conventional SBM in this review. After the harvest and storage of raw soybeans, SBM production begins with the cleaning, drying, and cracking and dehulling of raw soybeans. The soybeans are heated before flaking and expanding the beans to prepare for extraction of the oil with a hexane solvent. Once the oil has been extracted, the SBM is desolventized and toasted using steam at approximately 100-105ºC for 15-30 minutes. Lastly, the SBM is cooled and ground into meal before being incorporated into swine diets (Lusas, 2004; Stein et al., 2016). Co-products from conventional SBM production, such as soybean hulls or soybean oil, can also be used in diets for swine and other livestock (Stein et al., 2008). Due to the removal of the fibrous soybean hulls, the dehulled SBM contains higher crude protein and fat levels than non-dehulled SBM (NRC, 2012; Stein et al., 2016).

Another common type of SBM is non-dehulled, solvent-extracted meal. Though the hull is not removed from the soybean during processing, the oil is still extracted using a hexane solvent in this type of SBM (Lusas, 2004). The inclusion of soybean hulls increases the fiber content of the diet, but also reduces the digestibility of amino acids and energy compared to dehulled SBM (Dilger et al., 2004; Stein et al., 2016). Typically, dehulled, solvent-extracted SBM contains 46-48% crude protein and 1.5% fat. However, non-dehulled, solvent-extracted SBM contains 42-44% crude protein and 1.2% fat (Cromwell et al., 1999; NRC, 2012; Pedersen et al., 2016; Stein et al., 2016). Further, the concentration of phosphorus is reported to be higher in dehulled SBM than non-dehulled, but there are conflicting results regarding which type has higher phosphorus digestibility (Cromwell et al., 1999; Stein et al., 2008; Stein et al., 2016).

Dehulled, expelled SBM may also be utilized in swine diets. This type of SBM contains higher fat and energy levels than conventional SBM, as oil is removed using the less efficient process of mechanical expelling (Stein et al., 2008). Dehulled, expelled SBM contains, on average, 44-45% crude protein and 4-6.6% fat (NRC, 2012; Stein et al., 2016).

Anti-nutritional factors in soybean meal

Anti-nutritional factors can be defined as substances naturally-produced by plant species that interfere with nutrient digestion and utilization, resulting in a negative impact on livestock health and production (Makkar, 1993). Several types of ANF are present in raw soybeans, such as antigenic proteins, oligosaccharides, and trypsin inhibitor (TI). However, the concentration in SBM can be affected by the initial heating and processing methods (Liener, 1994).

The main antigenic proteins in SBM, glycinin and β-conglycinin, are storage globulins that are known to be allergenic to pigs (Zhao et al., 2010). Young pigs are particularly susceptible to these antigenic proteins as they cause transitory hypersensitivity in the GIT, typically lasting 7-10 days after ingestion (Engle, 1994). This hypersensitivity results in a T-helper 2 (Th2) cell type immune response wherein Th2 lymphocytes increase their secretion of anti-inflammatory cytokines, such as IL-4 or IL-6, to aid in B lymphocyte proliferation and differentiation (Sun et al., 2008b). These B lymphocytes then secrete IgE and other immunoglobulins that can mediate the allergic response (Sun et al., 2008a). In addition to immune activation, the ingestion of purified antigenic soy proteins has been shown to damage enterocytes and reduce gut barrier integrity (Zhao et al., 2010; Zhao et al., 2014; Zhao et al., 2015). Damage to intestinal villi has also been reported in nursery pigs fed SBM, which could potentially reduce a pig's nutrient absorption capability (Li et al., 1991). Studies in which

purified antigenic soy proteins were fed to young pigs resulted in decreased performance and increased diarrhea (Sun et al., 2008a; Wu et al., 2016).

Soybean meal contains non-digestible oligosaccharides (NDO) that have been categorized as ANF due to their detrimental impact on weaned pig performance (Zhang et al., 2003). Oligosaccharides make up approximately 10% of the carbohydrates in SBM and include sucrose (3-8%), stachyose (1-6%) and raffinose ($\leq$ 1.5%; Choct et al., 2010). Sucrose may cause diarrhea in newborn pigs due to insufficient endogenous sucrase activity until the second week of age; however, sucrose is considered a highly digestible energy source in nursery pig diets (Aherne et al., 1969; Mavromichalis et al., 2001). Stachyose and raffinose (both galactooligosaccharides) are the main NDO that are considered to be ANF as pigs do not possess the endogenous enzyme α-galactosidase that is needed to break the α-1,6 and α-1,2 linkages (Krause et al., 1994). The NDO remain undigested until they are fermented by microbes in the cecum or colon, resulting in the production of gases that may cause bloating and discomfort in non-ruminants (Choct et al., 2010). However, it has also been suggested that NDO could have a prebiotic effect in the GIT when they are fermented by microbiota (Smiricky-Tjardes et al., 2003). Microbial fermentation produces volatile fatty acids, such as acetate, butyrate, and propionate, that can be used as an energy source for the host and potentially lower GIT pH (Gao et al., 2019). Many pathogenic bacteria, such as *E. coli*, cannot thrive in acidic conditions, so NDO could benefit pigs undergoing an enteric disease challenge (Tran et a., 2016). Zhang et al. (2003) reported increased volatile fatty acid production and reduced diarrhea when purified stachyose was fed to weaned pigs.

Raw soybeans contain several heat-labile ANF, including TI and lectin (Liener, 1994). Trypsin inhibitors are proteins that reduce proteolytic enzyme activity, thus reducing the

digestibility of protein in soybeans (Herkelman et al., 1992). The majority of TI in soybeans are Kunitz TI, but Bowman-Birk TI are also present (Liener, 1994). Bowman-Birk TI are more heat resistant than Kunitz TI, so the extrusion step of SBM production must reach a temperature of 150ºC to fully inactivate the TI (Webster et al., 2003). Lectins are glycoproteins that bind to both the intestinal epithelium and carbohydrate-containing molecules. Once bound, lectins can damage the epithelium by disrupting brush border enzymes and causing villus atrophy (Liener, 1994; Palacios et al., 2004). The majority of these heat-labile ANF are inactivated in SBM, but residual concentrations may still be present (Liener, 1994).

Use of soybean meal in swine diets

In order to minimize the negative effects of SBM and its ANF on piglet growth and intestinal health, the inclusion of SBM is typically limited in phase 1 nursery diets. A swine genetics company recommends that the dietary inclusion of SBM does not exceed 20% for 5.5-7.5 kg pigs (PIC, 2016). As pigs age and develop a tolerance for soy proteins, specifically glycinin and β-conglycinin, the inclusion of SBM can be increased up to 32% in the nursery diet (Engle, 1994; PIC, 2016).

The impact of SBM on nursery pig performance has been inconsistent in the literature. Li et al. (1991) and Friesen et al. (1993) both reported decreased d 0-14 ADG and ADFI and impaired feed efficiency when weaned pigs were fed diets containing 38 or 40% SBM compared to a milk protein-based control diet. In a different experiment, Friesen et al. (1993) fed four increasing inclusions of SBM (0 to 22.5%) to weaned pigs. Pig performance was not impacted from d 0-14 but increasing SBM inclusion did linearly improve overall G:F. The author hypothesized that this improvement in overall feed efficiency was due to the development of oral tolerance to soy protein.

In a recent study utilizing older nursery pigs (11-25 kg), Cemin et al. (2020) fed three increasing inclusions of SBM (27.5 to 37.5%) over the course of four experiments. Three of the experiments took place in commercial research facilities, while one was completed at a university facility. In one of the commercial nursery experiments, the author reported a linear decrease in final BW, ADG, and ADFI when SBM inclusion was increased; SBM inclusion did not impact these parameters in the other experiments. However, in all four experiments, linear improvements in G:F were observed as SBM inclusion increased. These results further support the idea that young pigs will develop an oral tolerance to soybean proteins as they age, allowing for increased consumption without sacrificing growth performance (Engle, 1994).

Further processing of soybean meal

The dietary limitations of SBM in phase 1 and 2 nursery diets require nutritionists to utilize other high-quality protein sources. Animal and milk proteins have long been a preferred protein of choice in nursery diets as these are highly digestible ingredients that can improve growth compared to SBM-based control diets (Pettigrew, 2006). However, these protein ingredients can be quite expensive, so further processing methods for SBM have been developed to reduce the concentration of ANF (Min et al., 2009).

One type of further processed SBM is ESBM. This ingredient is produced by dehulling raw soybeans, defatting them using a hexane-solvent, and treating the meal with a proprietary blend of enzymes for several hours. Upon completion of the enzyme treatment, the enzymes are deactivated and the ESBM is dried and milled to a powder-like consistency (Goebel, 2010). The enzyme blends typically include proteases and carbohydrases (such as α-galactosidase or sucrase), but may also include phytase (Goebel and Stein, 2011). Phytate is the main storage form of phosphorus in grains and oil seeds, but it cannot be utilized by pigs as they lack the

endogenous form of phytase needed to digest the phytate (Acosta and Patience, 2019). Therefore, treatment of ESBM with exogenous phytase can increase phosphorus digestibility in the resulting product (Goebel and Stein, 2011). Overall, the proprietary enzyme treatment of ESBM has been shown to drastically decrease the concentration of antigenic proteins and NDO, with a slight reduction in the residual TI concentration (Table 1.1).

Impact of enzymatically-treated soybean meal in pigs

In the few published studies that have evaluated the impact of ESBM on growth performance, most agree that feeding ESBM to reduce conventional SBM inclusion improves weaned pig performance. However, the optimum dietary inclusion level of ESBM is unknown. Zhu et al. (1998) reported improvements in overall ADG and F:G when 3.5, 7, or 10.5% ESBM were fed over a four week study, compared to a 0% ESBM control diet. Similarly, Zhou et al. (2011) reported increased final BW, ADG, and ADFI when feeding 5, 10, or 15% ESBM compared to the 0% ESBM control; the 10 or 15% ESBM diets also improved F:G compared to the control diet. Although orthogonal contrasts were not performed in this study, it appeared that the increasing inclusion of ESBM and decreasing SBM improved growth performance in a linear manner (Zhou et al., 2011). In two separate studies, Ma et al. (2019a,b) reported improvements in overall ADG and G:F by feeding 9% ESBM in phase 1 (d 0-14) and 7.5% ESBM in phase 2 (d 14-28), compared to a 0% ESBM control diet. These growth improvements may be partially explained by increased crude protein and energy digestibility in pigs fed ESBM versus a SBM control diet (Cervantes-Pahm et al., 2010; Zhou et al., 2011; Ma et al., 2019b).

However, not all studies have reported improved performance when feeding ESBM. Jones et al. (2018a) reported linear decreases in final BW, ADG, and ADFI of weaned pigs when feeding increasing levels of ESBM (6.7 to 20% ESBM in phase 1; 5 to 15% ESBM in phase 2)

and decreasing SBM. Further, a different experiment by Jones et al. (2018b) resulted in decreased overall ADG and ADFI, but improved G:F, when pigs were fed 15% ESBM in comparison to either a SBM or fish meal based control diet.

The mechanisms by which ESBM may improve weaned pig performance compared to conventional SBM are poorly understood as very few studies have investigated this. However, it has been hypothesized that these improvements are due to the reduced concentration of ANF (Zhou et al., 2011). Compared to a SBM control diet, feeding ESBM has been shown to impact the immune response of weaned pigs by decreasing the mucosal concentration of TNF-α, a pro-inflammatory cytokine (Ma et al., 2019a). Serum IgA, IgG, and IgM levels were also increased when ESBM was fed, indicating that immune function and development are improved when less antigenic proteins are present in the diet (Ma et al., 2019a,b). This author reported increases in villus height and tight junction protein expression in pigs fed ESBM; this is likely due to a reduced hypersensitivity response to glycinin and β-conglycinin (Zhao et al., 2014; Ma et al., 2019a,b). Further, the improved intestinal barrier integrity and morphology could partially explain the reduced rate of diarrhea in these pigs (Pluske et al., 1997; Ma et al., 2019a,b).

The main purpose of feeding ESBM to weaned pigs is to decrease the dietary inclusion of conventional SBM, resulting in the reduced concentration of harmful ANF in the complete diet. The detrimental effects of ANF, specifically antigenic soy proteins and NDO, on weaned pig performance and GIT health and function, have previously been described in this review. Therefore, the improvements associated with feeding ESBM, that are described in the previous paragraphs, may be caused by decreasing the dietary inclusion of SBM rather than increasing the ESBM inclusion. Even when experimental diets are carefully formulated to ensure that the ingredient composition is identical (except for the ingredients being evaluated), some level of

confounding is unavoidable due to the decrease in SBM that occurs as ESBM inclusion increases.

Conclusion

In conclusion, weaning stress can drastically impair the growth and feed intake of young pigs while disrupting their GIT health and function. Traditionally, antibiotics have been included in nursery diets due to promote growth and feed intake in young pigs while mediating GIT disorder and disease. However, emerging concerns of antimicrobial resistant bacteria and increased government regulation of in-feed antibiotics has prompted the pork industry to look for other high-quality ingredients, such as specialty proteins, that can improve pig performance and GIT health. Both SDPP and DEP are functional specialty proteins that have been identified as potential alternatives for antibiotics in nursery diets. The performance and health benefits of feeding SDPP have been well-documented, but some producers are hesitant to feed SDPP due to biosecurity concerns. The DEP is a newer protein ingredient that has been shown to improve performance and reduce diarrhea in ETEC challenge models, but the industry is lacking an understanding of this ingredient's impact in commercial conditions. Another type of specialty protein is ESBM. The concentration of ANF is reduced in ESBM, so it can be used to decrease the inclusion of conventional SBM in nursery diets. Feeding ESBM has been shown to improve pig performance, but the mechanisms behind this improvement are unknown.

Therefore, the overall objective of this thesis research was to investigate the impact of specialty protein ingredients on nursery pig performance and health. The specific objectives of this thesis are 1) to compare the effects of feeding SDPP or DEP, with or without subtherapeutic levels of antibiotics, on growth performance and markers of intestinal physiology and function in nursery pigs raised in commercial conditions, and 2) to determine the impact of diets in which

ESBM replaced increasing amounts of SBM on growth performance, intestinal structure and barrier integrity, inflammation, and oxidative status in newly weaned pigs. It was hypothesized that feeding the specialty proteins SDPP and DEP in antibiotic-free diets or increasing the inclusion of ESBM and decreasing SBM would positively impact the growth performance of weaned pigs while beneficially modulating markers of GIT health and function.

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Table 1.1. The concentration of anti-nutritional factors in conventional soybean meal (SBM) or enzymatically-treated soybean meal (ESBM).

	Ma et al., 2019a		Goebel and Stein, 2011			Cervantes-Pahm and Stein, 2010	
Anti-nutritional factor	SBM	ESBM	SBM	ESBM	ESBM + phytase[1]	SBM	ESBM
Trypsin inhibitor, TIU/mg[2]	3.9	0.8	5.7	2.4	1.8	4.0	2.1
Glycinin, mg/g	57.0	0.3	420.0	3.3	0.1	23.0	5.3
β-conglycinin, mg/g	16.0	0.2	130.0	0.004	0.01	15.0	0.001
Stachyose, mg/g	54.0	1.3	37.8	2.7	2.0	51.7	7.1
Raffinose, mg/g	12.0	0.6	10.5	4.3	2.1	10.8	1.6
Sucrose, mg/g	61.0	5.0	57.8	2.0	2.0	78.1	ND[3]

[1] The ESBM was produced using an enzyme mixture that included phytase.
[2] TIU = trypsin inhibitor unit
[3] ND = not detected

CHAPTER 2. THE IMPACT OF PORCINE SPRAY-DRIED PLASMA PROTEIN AND DRIED EGG PROTEIN HARVESTED FROM HYPER-IMMUNIZED HENS, PROVIDED IN THE PRESENCE OR ABSENCE OF SUBTHERAPEUTIC LEVELS OF ANTIBIOTICS IN THE FEED, ON GROWTH AND INDICATORS OF INTESTINAL FUNCTION AND PHYSIOLOGY OF NURSERY PIGS

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Leigh A. Ruckman[*], Amy L. Petry[*], Stacie A. Gould[*], and John F. Patience[*]

[*]Department of Animal Science, Iowa State University, Ames, IA 50011

Abstract

The objective of this experiment was to compare the effects of spray-dried plasma protein (**SDPP**) and dried egg protein (**DEP**), without (**AB-**) or with (**AB+**) in-feed antibiotics, on growth performance and markers of intestinal health in nursery pigs raised under commercial conditions. This 42-d experiment utilized 1,230 pigs [4.93 ± 0.04 kg body weight (BW); approximately 15-18 d of age]. Pens were randomly assigned to 1 of 6 dietary treatments that were arranged as a 2 × 3 factorial of in-feed antibiotics (AB- vs AB+) and a specialty protein additive [none (**CON**), porcine SDPP, or DEP]. Diets were fed in 4 phases with phases 3 and 4 as a common diet across all treatments. Specialty protein additives were fed in phases 1 (0-13 d; 3% SDPP and 0.20% DEP) and 2 (13-26 d; 2% SDPP and 0.10% DEP). Antibiotics were fed in phases 1-3 [662 mg chlortetracycline (CTC)/kg, 28 mg carbadox/kg, 441 mg CTC/kg,

respectively). Ileal tissue and blood samples were collected from 48 pigs (8 per treatment) on d 20. Data were analyzed using PROC MIXED of SAS (9.4) with pen as the experimental unit; protein additives, antibiotics and their interaction were fixed effects and block was a random effect. The pigs experienced naturally occurring health challenges in weeks 2 and 4. In the AB- diets, SDPP and DEP increased average daily gain (ADG; $P = 0.036$) and average daily feed intake (ADFI; $P = 0.040$) compared to CON; in the AB+ diets, neither SDPP nor DEP increased ADG or ADFI compared to CON but SDPP did increase these parameters over DEP. The SDPP and DEP diets decreased the number of individual medical treatments compared to CON ($P = 0.001$). The AB+ increased ileal mucosal interleukin (IL)-1 receptor antagonist ($P = 0.017$). Feeding DEP reduced the concentration of mucosal IL-1β compared to CON, but not SDPP ($P = 0.022$). There was a trend for SDPP and DEP to increase villus height:crypt depth compared to CON ($P = 0.066$). Neither antibiotics or protein additive affected serum malondialdehyde concentration or ileal mRNA abundance of *claudin-3* or *4*, *occludin*, or *zonula occludens-1* ($P > 0.10$). In conclusion, SDPP and DEP improved growth performance of weaned pigs in the absence of antibiotics but neither improved growth compared to CON when feeding standard antibiotic levels. The specialty proteins had a positive effect on health; specialty proteins and antibiotics were able to modulate some markers of intestinal inflammation and morphology.

Keywords: functional protein, in-feed antibiotics, IgY, intestinal inflammation and morphology, spray-dried plasma protein, weaned pig

Introduction

The weaning process exposes pigs to a multitude of stressors such as dietary and environmental changes, social stress, and an unpredictable array of pathogens. The combination of these stressors typically results in reduced growth rate and feed intake as well as impaired

function and integrity of the gut (Lallès et al., 2004; Pluske, 2013; Li et al., 2019). Further, the immune system of a weaned pig is still undergoing development, increasing their susceptibility to enteric pathogens that can cause diarrhea or other gastrointestinal tract (GIT) disorders (Lallès et al., 2007).

In order to combat these performance and health issues, and to reduce mortality and morbidity during the post-weaning period, antibiotics have been used at sub-therapeutic and therapeutic levels in the feed for over five decades (Patience, 2019). However, growing concerns about antimicrobial resistance to antibiotics, consumer demands and government regulation of antibiotics in livestock diets have prompted the pork industry to seek dietary methods to reduce or eliminate antibiotic use during the nursery stage (Olsen et al., 2018).

Spray-dried plasma protein (**SDPP**), either from a porcine or bovine source, has been used in nursery diets since the late 1980s and been shown to improve performance and reduce diarrhea in weaned pigs (Peace et al., 2011; Tran et al., 2014). It has been hypothesized that these improvements are the result of increased feed intake, the protective effects of the constituent immunoglobulin rich fraction, and modulation of the immune response and gut barrier structure (Pierce et al., 2005; Peace et al., 2011). This proposed mode of action, as well as reports that SDPP improves performance of unhealthy and/or environmentally challenged pigs, suggests that SDPP could be used to limit or reduce antibiotics in nursery diets (Torrallardona et al., 2002). However, the cost of this ingredient and a desire by some producers to reduce animal products in their feed have prompted the industry to look for alternatives to SDPP (Patterson et al., 2010; Gerber et al., 2014).

Dried egg protein (**DEP**), specifically egg-yolk antibodies, has garnered attention recently as a promising SDPP alternative. It has been used to protect weaned pigs against

diarrhea and enteric diseases since the early 1990s (Wiedemann et al., 1991). This product is produced by drying eggs harvested from hens that are hyper-immunized against specific bacterial antigens known to challenge young pigs (Schade et al., 2005; Li et al., 2015). The resulting ingredient is a concentrated source of egg-yolk immunoglobulin proteins (IgY) that could aid in immune modulation, reduce diarrhea, and improve performance of weaned pigs (Pettigrew, 2006). Supplementing IgY has improved growth performance in enterotoxigenic *Escherichia coli* (ETEC) challenge models (Owusu-Asiedu et al., 2003a; Pozzebon da Rosa et al., 2015). However, the results have been inconsistent in non-challenge studies (Heo et al., 2015; Torrallardona and Polo, 2016). The pork industry needs to have a better understanding of both the impact of IgY under commercial nursery conditions and their mode of action to determine if it can be a practical alternative to SDPP.

Therefore, the objective of this experiment was to compare under commercial conditions the effects of including SDPP or DEP in the diet, with or without subtherapeutic levels of antibiotics in the phase 1 and 2 nursery diets, on growth performance and markers of intestinal physiology and function. It was hypothesized that the SDPP and DEP would improve pig performance in the reduced antibiotic diets, and that this improvement could be mediated by changes in gut integrity and structure, oxidative status, and gut inflammation.

Materials and methods

All experimental procedures employed in this experiment adhered to principles for the ethical and humane use of animals for research according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and were approved by the Iowa State University Institutional Animal Care and Use Committee (S-18-129).

Animals, housing, and management

This 42 d experiment was conducted in June and July, 2018 in one room of a commercial wean-to-finish research barn located in central Iowa. A total of 1,230 crossbred pigs (PIC 359 × PIC 1050; PIC, Hendersonville, TN) were weaned at 15-18 days of age with a mean body weight (BW) of 4.93 ± 0.04 kg. At weaning, the pigs were vaccinated for porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Circumvent PCV-MG2, Merck Animal Health, Madison, NJ). The pigs were housed in a tunnel-ventilated barn and each pen was equipped with a 4-space dry self-feeder, dish waterer, and fully slatted concrete floors. Pigs had *ad libitum* access to feed and water for the entirety of the experiment. Treatment diets were delivered quantitatively to individual pens using an automatic feed delivery system designed specifically for research purposes (Big Dutchman, Holland, MI).

Sixty pens were utilized for a total of 10 replicates per dietary treatment. The barn was blocked by location within the barn into 10 blocks to balance any potential effect due to position within the barn; dietary treatments were randomly assigned within each block. Each pen housed 20 or 21 mixed sex pigs; both sex and the number of pigs per pen were equalized within block.

Experimental treatments and design

Experimental treatments were offered during phase 1 and 2 of the nursery feed budget (Tables 2.1 and 2.2) and were arranged as a 2 × 3 factorial comparing in-feed antibiotics [AB; none (**AB-**) vs standard (**AB+**) levels] and a specialty protein additive [ADD; none (**CON**), porcine SDPP, or DEP]. The antibiotics were included in the diets at the expense of corn; phase 1 included 662 mg of chlortetracycline (CTC)/kg (Aureomycin 100, Zoetis, Florham Park, NJ) and phase 2 contained 28 mg of carbadox/kg (Mecadox 10, Phibro Animal Health Corporation, Teaneck, NJ). Due to the health challenges experienced by the pigs, the phase 3 diets contained 441 mg of CTC/kg (Chlormax 50, Zoetis, Florham Park, NJ); however, they did not contain the

specialty proteins (Table 2.3). Phase 4 contained no antibiotics nor specialty proteins. In the DEP diet, Globimax JS (EW Nutrition, Des Moines, IA) was added to CON at the expense of corn at 0.20% in phase 1 and 0.10% in phase 2. The SDPP (AP 920, APC Inc., Ankeny, IA) was added to the diet at 3.0% in phase 1 and 2.0% in phase 2. During diet formulation, no nutritive value was assigned to DEP due to its very low inclusion level; SDPP was formulated into the experimental diets according to its nutrient profile as provided by the manufacturer. Phase 3 and 4 were common diets fed across all treatment groups. Phase 1 and 2 were fed in pelleted form based on a pre-determined feed budget: 2.3 kg of phase 1 (d 0 to 13) and 5.5 kg of phase 2 (d 13 to 26). Phase 3 was fed from d 26 to 40 and phase 4 was fed from d 40 to 42, both in mash form.

All diets in this study were formulated to meet or exceed nutrient requirements of the pigs (NRC, 2012). The CON diets contained 20% soybean meal (SBM) in phase 1, 25% in phase 2, and 28% in phases 3 and 4; the DEP diets contained the same inclusion of SBM as CON in phase 1 and 2. The SDPP diets were formulated to contain less SBM than CON and contained only 14.2% and 21.9% SBM in phases 1 and 2, respectively. All diets were formulated to contain the same levels of standardized ileal digestible (SID) lysine, regardless of protein source (phase 1: 1.50% SID lysine; phase 2: 1.35% SID lysine; phase 3 and 4: 1.26% SID lysine). Other potentially limiting amino acids were formulated according to target SID amino acid to SID lysine ratios (NRC, 2012). To the greatest extent possible, all basal ingredients, other than SBM and the experimental ingredients, were included in the diets at the same levels across treatments to minimize the risk of confounding the experimental outcomes. The DEP was weighed out on an analytical scale and delivered to the commercial mill prior to diet manufacturing.

Medical treatments and health status characterization

Pigs were individually treated with ceftiofur (Excede, Zoetis, Florham Park, NJ) or enrofloxacin (Enroflox 100, Norbrook Laboratories, Newry, Northern Ireland) as indicated by

clinical observation. Pigs that did not respond to medical treatment were removed from the study. During the experiment, individual medical treatments were recorded by product, pen, day, and dosage. The pen, date, BW at removal, and cause were recorded for all mortalities and removals.

Under the direction of a veterinarian and in response to observed diarrhea, lethargy, and respiratory symptoms, medication was also delivered through the water as required: gentamicin sulfate (Gen-Gard, Agrilabs, St. Joseph, MO; 13.2 mg gentamicin sulfate/L of water; d 4-7 and d 11-14), electrolytes (Blue 2, TechMix LLC, Stewart, MN; 7.8 mL of stock solution/L of water; d 14-18 and d 25-28), aspirin (AniPrin LQ-PM, AniMed, Winchester, KY; 7.8 mL of 12% aspirin solution/L of water; d 32-36), and penicillin (Penicillin G Potassium USP, Quo Vademus LLC, Kenansville, NC; 396,258 units of penicillin G/L of water; d 36-39).

Diagnostic necropsies were performed on 2 pigs on d 11 to confirm exposure to specific pathogens (Table 2.4). Oral fluids were collected on d 32 from 3 pens per treatment according to Prickett et al. (2008) to characterize the pathogens present in the barn (Olsen et al., 2018). All diagnostic tests, including diagnostic necropsies, were conducted at the Veterinary Diagnostics Lab (Iowa State University, Ames, IA). If a tissue or oral fluid sample was positive for a specific pathogen, the entire barn was considered to have exposure to that pathogen.

Data and sample collection

Pigs were weighed by pen at the beginning of the experiment, and at the end of the three weigh periods (d 13, 26, and 42) to determine average daily gain (ADG). Feed intake was recorded for the same periods to determine average daily feed intake (ADFI) and to calculate gain:feed (G:F). Weights and removal dates of pigs were recorded and ADG and ADFI were calculated according to pig days on test.

On d 20, 8 pigs per treatment were randomly selected for necropsy from the 8 heaviest pens on each treatment (using d 13 pen weight) to maximize the uniformity of necropsied pigs. Prior to euthanasia, blood was collected by jugular venipuncture into a 10 mL vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Blood samples were placed on ice and allowed to clot prior to centrifugation at 2,000 × g for 15 min at 4°C. The resulting serum was stored at -80°C for later analysis. The pigs were then euthanized by captive bolt stunning followed by exsanguination. Ileal tissue was collected 10 cm proximal to the ileocecal junction, rinsed with phosphate buffered solution (PBS), snap-frozen in liquid nitrogen, and stored at -80°C for later analysis. Ileal mucosal scrapings were collected, snap-frozen in liquid nitrogen, and stored at -80°C. A segment of mid-ileum (approximately 60 cm proximal to the ileal-cecal junction) was collected and fixed in 10% neutral buffered formalin.

Diet sample analysis

Feed samples were taken directly from five feeders per dietary treatment at the end of each phase, pooled within phase and treatment, and homogenized before being stored at -20°C. Diets were ground to 1 mm particle size using a Wiley Mill (Variable Speed Digital ED-5 Wiley Mill; Thomas Scientific, Swedesboro, NJ), dried to a constant weight at 60°C, and analyzed in duplicate for dry matter (DM; method 930.15; AOAC, 2007), ash (method 942.05; AOAC, 2007), acid-hydrolyzed ether extract (aEE; method 2003.06; AOAC, 2007), and nitrogen (N; method 990.03; AOAC, 2007; TruMac; LECO Corp., St. Joseph, MI). An ethylenediaminetetraacetate sample (9.56% N; determined to have 9.54 ± 0.05% N) was used for standard calibration and crude protein was calculated as N × 6.25. The intra-assay coefficient of variation (CV) for DM, ash, aEE, and N was 0.8%, 1.0%, 4.8%, and 0.9%, respectively. Diet samples were analyzed for total amino acids at the Agricultural Experiment Station Chemical Laboratories (University of Missouri, Columbia, MO). The SID levels of amino acids were

calculated using the assayed total amino acid values and the SID coefficient for each ingredient in the formulation (NRC, 2012).

Oxidative stress and inflammatory measures

Ileal mucosal samples (50 mg) were homogenized in 4.5 mL of PBS buffer, which contained detergent (0.1%; Triton X-100, Fisher Scientific, Fair Lawn, NJ) and a protease inhibitor cocktail (1:100 ratio to PBS; Sigma-Aldrich, St. Louis, MO) before centrifugation at 10,000 × g for 15 min at 4°C. The supernatant was analyzed for cytokines by an external laboratory (Eve Technologies Corporation, Calgary, AB, Canada) using a multiplex assay. The assay included granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and tumor necrosis factor-α (TNF-α). Malondialdehyde (MDA), a marker of oxidative stress, was measured in serum using a thiobarbituric acid reactive substances (TBARS) kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI) as previously described (Armstrong and Browne, 1994; Yagi, 1997). The intra-assay CV was 4.9% and the assay sensitivity was 0-50 μM MDA.

RNA isolation and real-time quantitative PCR

Total ribonucleic acid (RNA) of ileal tissue was isolated using a commercial kit (RNeasy Plus Mini Kit, Qiagen, Carlsbad, CA) and Qiagen Tissuelyser II (Germantown, MD). The RNA was treated with a deoxyribonuclease enzyme to prevent genomic deoxyribonucleic acid (DNA) contamination (DNA-free DNA removal kit, Invitrogen, Carlsbad, CA). The RNA concentration was quantified using a spectrophotometer (ND-100; NanoDrop Technologies Inc., Rockland, DE) and all samples had 260:280 nm ratios above 1.8. Complimentary DNA (cDNA) was transcribed from 0.8 μg RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and cDNA samples were diluted 10-fold with nuclease-free water.

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) in triplicate. The gene-specific primers (Table 2.5) were diluted to 10 µM with nuclease-free water. Each 20 µL reaction included 10 µL of SYBR Green Supermix, 1 µL of each forward and reverse primer, 3 µL of cDNA and 5 µL of nuclease-free water. A no-reverse transcriptase negative control and a pooled cDNA reference sample were included on each plate. The SYBR Green fluorescence was quantified using a RT-qPCR detection system (iQ5; Bio-Rad Laboratories Inc.) and the following cycling conditions: 5-min initial denaturation at 95°C followed by 40 RT-qPCR cycles (95°C for 30 s, 55 or 60°C for 30 s, and 72°C for 30 s) and a dissociation curve to verify the amplification of a single RT-qPCR product. Optical System Software (iQ5, version 2.0; Bio-Rad Laboratories Inc.) was used to analyze amplification plots and cycle threshold values for each reaction were obtained. The messenger RNA (mRNA) abundance was normalized to a reference gene (ribosomal protein- L19) and the pooled sample. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calculate fold change. The intra-assay CV was less than 4.6% for all RT-qPCR analysis.

Intestinal morphology

Ileal tissues (fixed in 10% neutral buffered formalin) were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin, and mounted on glass slides (Iowa State University Veterinary Diagnostics Lab, Ames, IA). Images of the slides were taken at 10x power using a DP80 Olympus Camera mounted on an OLYMPUS BX 53/43 microscope (Olympus Scientific, Waltham, MA). Eight villi and crypt pairs per ileal sample were measured using OLYMPUS CellSens Dimension 1.16 software. The ratio of villus height to crypt depth was calculated for each pair (V:C ratio).

Economic analysis

The overall cost of gain ($/kg of BW gain) was calculated for each pen using the cost of complete diets (manufactured in June and July 2018) and individual medical treatments. Using retail costs from a veterinary supplier, the medical treatments were determined to be $0.65 per 0.5 mL of ceftiofur or $0.48 per 1.0 mL of enrofloxacin. The cost of water medications or vaccinations were not included in the economic analysis.

Statistical analysis

Data were analyzed using one of two mixed models. Model 1 assumed that residuals were normally distributed with a compound symmetry (CS) dependent covariance structure [$N(0, I\ CS\sigma_e^2)$]. This model was used to analyze growth performance data by weigh period.

$$Model\ 1\text{: } Y_{ijklm} = \mu + \tau_i + \upsilon_j + \tau_i \upsilon_j + a_k + \rho_l + \tau_i \rho_l + \upsilon_j \rho_l + \tau_i \upsilon_j \rho_l + e_{ijklm}$$

where Y_{ijklm} is the observed value for the mth experimental unit (pen) within the lth period in the kth block within the jth level of protein additive and ith level of antibiotic; μ is the general mean; τ_i is the fixed effect of the ith antibiotic (i = AB-, AB+); υ_j is the fixed effect of jth protein additive (j = CON, SDPP, DEP); $\tau_i \upsilon_j$ is the interaction term of antibiotic × additive; a_k is the random effect of the kth block (k = 1 to 10); ρ_l is the fixed effect of period (l = 1 to 3); $\tau_i \rho_l$ is the interaction term of antibiotic × period; $\upsilon_j \rho_l$ is the interaction term of additive × period; $\tau_i \upsilon_j \rho_l$ is the interaction term of antibiotic × additive × period; and e_{ijklm} is the associated variance as described by the model for Y_{ijklm} (m = 1 through 60), assuming $a_l \sim N(0, I\ \sigma_a^2)$, and $e_{ijklm} \sim N(0, I\ CS\sigma_e^2)$, where *I* is the identity matrix.

Model 2 assumed that residuals were independent and normally distributed [$N(0, I\ \sigma_e^2)$]. The following mixed model was used to analyze all data except for growth performance by weigh period.

$$Model\ 2: Y_{ijkl} = \mu + \tau_i + \upsilon_j + \tau_i \upsilon_j + a_k + e_{ijkl}$$

where Y_{ijkl} is the observed value for the lth experimental unit (pen) within the kth block in the jth level of protein additive and ith level of antibiotic; μ is the general mean; τ_i is the fixed effect of the ith antibiotic (i = AB-, AB+); υ_j is the fixed effect of jth additive (j = CON, SDPP, DEP); $\tau_i \upsilon_j$ is the interaction term of antibiotic × additive; a_k is the random effect of the kth block (l = 1 to 10); and e_{ijkl} is the associated variance as described by the model for Y_{ijkl} (l = 1 through 60), assuming $a_k \sim N(0, I\sigma_a^2)$ and $e_{ijkl} \sim N(0, I\ \sigma_e^2)$, where *I* is the identity matrix.

Normality and homogeneity of the studentized residuals were verified using the UNIVARIATE procedure of SAS 9.4 (SAS Inst., Cary, NC). Statistical outliers, defined as occurring greater than three standard deviations from the mean, were identified and removed from the analysis. All data and models were analyzed using the MIXED procedure. The CS covariance structure was selected as the best fit for model 1 according to Bayesian Information Criterion for all dependent variables. Fisher's Least Significant Difference test was used to separate least squares means and differences were considered significant if $P < 0.05$ and trends if $0.05 \geq P < 0.10$.

Results

Animal health

The pigs experienced multiple health challenges throughout the experimental period (Table 2.4). During the second week, 2 pigs were submitted for necropsy and diagnosed with porcine rotavirus (groups A, B, and C) and *Salmonella* (species not identified). In week 4, analysis of oral fluids confirmed the presence of porcine reproductive and respiratory syndrome virus (PRRSV; wild type 1-7-4 ORF5) and influenza A virus (IAV; H3 and N2 strains). Overall,

mortality was 2.0% and morbidity (pigs removed due to illness or injury) was 6.3%. Therefore, the total removal rate was 8.3%.

There was no AB × ADD interaction for the number of medical treatments or total removals ($P > 0.10$; Table 2.6). The AB levels did not impact the number of medical treatments ($P > 0.10$). The inclusion of SDPP and DEP reduced the number of medical treatments compared to CON ($P = 0.001$). Neither AB nor ADD impacted the number of total removals ($P > 0.10$).

Growth performance

Overall, AB+ increased ADG ($P = 0.020$; Table 2.7) and ADFI ($P = 0.002$) in comparison to AB-. However, there were no differences for final BW or G:F ($P > 0.10$). Feeding SDPP resulted in the greatest increase in ADG ($P = 0.044$) and ADFI ($P = 0.026$), followed by DEP and then CON; similarly, there was a trend for SDPP to increase final BW compared to CON, but not DEP ($P = 0.077$).

The CON (AB+) diet improved overall ADG and ADFI but not G:F compared to CON (AB-) diets ($P < 0.05$; Table 2.7). There was an AB × ADD interaction for overall ADG ($P = 0.036$) and ADFI ($P = 0.040$) with a trend for an interaction for final BW ($P = 0.061$). The SDPP and DEP increased ADG, ADFI and final BW compared to CON in the AB- diets, but not in the AB+ diets. In the AB+ diets, SDPP increased ADG and ADFI over DEP.

Considering growth performance by period, the AB × ADD × period interaction was not significant for any growth parameters ($P > 0.10$), therefore only the main effects of AB or ADD are presented (Table 2.8). The AB+ diet increased G:F compared to AB- in period 1 but did not differ in periods 2 or 3 ($P = 0.018$). Similarly, feeding SDPP increased G:F compared to CON and DEP in period 1, but ADD did not have an impact in subsequent periods ($P = 0.019$).

Oxidative stress and inflammatory measures

The inclusion of AB or ADD did not impact the levels of serum MDA ($P > 0.10$; Table 2.9). There was no effect of AB or ADD, or their interaction, on the following ileal mucosa cytokines: IFNγ, IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, or IL-12 ($P > 0.10$). The concentrations of GM-CSF and TNF-α were not detectable in any of the samples. Feeding DEP resulted in the lowest concentration of IL-1β compared to CON, with SDPP being intermediate between them ($P = 0.022$). The concentration of IL-1RA was increased by feeding AB+ compared to AB- ($P = 0.017$). An AB × ADD interaction was observed for IL-18 as the concentration did not differ in the AB- diets but was significantly increased in the CON diet compared to SDPP and DEP when AB+ was fed ($P = 0.012$).

Ileal gene transcription

The mRNA abundance of *claudin-3* (*CLDN3*), *CLDN4*, *occludin* (*OCLN*), or *zonula-occludens-1* (*ZO-1*) was not impacted by AB or ADD ($P > 0.10$; Table 2.10). No AB × ADD interactions were observed for the mRNA abundance of these genes ($P > 0.10$).

Morphology of gut

There was a trend for DEP to increase villus height compared to CON, with SDPP being intermediate between them (P = 0.098; Table 2.11). In AB- diets, there was a trend for SDPP to result in shallower crypts compared to CON with DEP being intermediate between them; however, in AB+ diets, neither SDPP nor DEP impacted crypt depth compared to CON (P = 0.098). There was a trend for feeding SDPP and DEP to result in a larger V:C ratio than CON (P = 0.066).

Economic analysis

When AB- diets were fed, the CON and DEP diets resulted in the lowest overall cost of gain compared to SDPP; however, in AB+ diets, feeding SDPP and DEP increased the cost of gain compared to CON (P = 0.060; Table 2.12). Further, feeding DEP in AB+ diets increased the cost of gain compared to feeding DEP in AB- diets.

Discussion

The proposed mode of action for SDPP is that it increases feed intake by improving feed palatability and benefits intestinal health by reducing intestinal permeability and modulating immune responses (Ermer et al., 1994; Peace et al., 2011; Tran et al., 2014). These beneficial effects have been attributed to the immunoglobulin G (IgG)-rich fraction of SDPP (Pierce et al., 2005). The DEP is a concentrated source of IgY, the main circulating antibody in chickens that has a similar biological role as mammalian IgG (Abbas et al., 2019). These immunoglobulins can counteract pathogen activity in the GIT of weaned pigs by inhibiting adhesion to the intestinal epithelium, possibly reducing other symptoms of enteric diseases such as increased intestinal inflammation or permeability (Bosi et al., 2004; Abbas et al., 2019). Therefore, SDPP and DEP could be beneficial to pigs that are overcoming weaning stress and exposure to new pathogens. In this study, we evaluated the impact of SDPP or DEP, with or without in-feed antibiotics, on growth performance and intestinal permeability, inflammation, and morphology of nursery pigs raised under commercial conditions.

When AB- diets were fed, SDPP and DEP increased overall ADG and ADFI and tended to increase final BW compared to the CON diet. Therefore, these specialty proteins could be beneficial to pork producers that are trying to reduce their usage of in-feed antibiotics. Improved performance from feeding SDPP in antibiotic-free studies has been reported in

multiple publications, but the benefits of SDPP tend to be more pronounced during the first 2 weeks after weaning (van Dijk et al., 2001; Pérez-Bosque et al., 2016). Further, SDPP has been shown to benefit growth in commercial environments or during pathogen challenges (Coffey and Cromwell, 1995; Bosi et al., 2004). The growth response to feeding DEP or purified IgY to weaned pigs has been more inconsistent in the literature. Torrallardona and Polo (2016) and Crenshaw et al. (2017) reported that DEP (0.2% or 0.44%) did not improve growth in antibiotic-free diets compared to the control diet and was outperformed by diets containing 5% or 6% SDPP. The specificity of the pathogens that a hen was hyperimmunized against can have a large impact on the efficacy of IgY (Li et al., 2015). Studies that have used a disease challenge model, such as ETEC, and have supplemented IgY that is specific to these pathogens, have seen greater growth responses compared to the control diet (Owusu-Asiedu et al., 2003a; Pozzebon da Rosa et al., 2015). However, the pathogen specificity of the DEP used in this trial is unknown.

When AB+ diets were fed, the SDPP and DEP did not have an impact on overall growth performance compared to CON. Therefore, these specialty proteins do not provide as large of a benefit in feeding systems that are using standard antibiotic levels. Bikker et al. (2004) reported that the d 0-14 growth response to SDPP, compared to the control, was greater when feeding antibiotic-free diets rather than antibiotic-positive diets. It was hypothesized that SDPP provided more benefit to pigs fed antibiotic-free diets because pathogenic bacteria were more likely to colonize their GIT compared to pigs fed medicated diets (Bikker et al., 2004). To our knowledge, there are no published studies that have evaluated DEP using a factorial arrangement with antibiotics.

In this study, feeding CON with AB+ compared to AB- increased ADG and ADFI. A proposed mode of action for antibiotics is improved performance by inhibiting bacterial

infections in the GIT, which can reduce microbial use of nutrients and abundance of growth-depressing metabolites and toxins (Cromwell et al., 2002; Gaskins et al., 2006). The growth promoting ability of antibiotics has been well-documented in the literature, but the magnitude of response to antibiotics can be influenced by medication type and dosage or health status of pigs (Cromwell et al., 2002; Jacela et al., 2009).

During this experiment, the pigs faced multiple naturally-occurring health challenges. Porcine rotavirus and *Salmonella*, both diagnosed on d 11, are associated with watery diarrhea and depressed growth and feed intake (Turner et al., 2002; Corl et al., 2007). On d 32, PRRSV and IAV were diagnosed after severe lethargy and coughing were observed. Despite these severe health challenges, mortality and morbidity were only 2% and 6.3%, respectively. A previous nursery trial that took place in the same facility reported 1.8% mortality and 6.1% morbidity with a naturally occurring PRRSV challenge, so the rates in our study were not abnormal (Olsen et al., 2018). The number of administered medical treatments was quite high, but pigs that were fed SDPP or DEP required 25% fewer medical treatments than those fed CON. To our knowledge, there is no published literature that reports the impact of SDPP or DEP on the number of administered medical treatments.

Pro-inflammatory cytokines play a crucial role in the GIT immune system by recruiting and activating cells to signal an immune response against pathogens (Pié et al., 2004). However, uncontrolled inflammatory responses result in increased intestinal permeability and decreased growth performance, probably due to the partitioning of nutrients and energy away from growth to support the immune response (Huntley et al., 2018). In this study, feeding DEP significantly reduced the mucosal concentration of pro-inflammatory IL-1β compared to CON. The IgY in DEP can block the adhesion of pathogens to the epithelial lining, preventing or reducing the need

for an inflammatory response and overproduction of IL-1β (Wang et al., 2019). The mucosal concentration of pro-inflammatory IL-18 was increased by CON when AB+ diets were fed, but the concentration was not affected by the other diets. It has been reported that the mRNA abundance of IL-18 by porcine immune cells is increased during bacterial infections to increase resistance (Foss et al., 2001). However, antibiotics are known to reduce microbial infections and so the increased mucosal IL-18 in pigs fed CON with AB+ diets cannot be explained. Feeding AB+ diets increased the mucosal concentration of anti-inflammatory IL-1RA compared to AB- diets. The IL-1RA can prevent overactivation of the immune response by inhibiting the pro-inflammatory IL-1 family, so this outcome points to an immune modulating effect of antibiotics (Netea et al., 2015).

A physical defense mechanism of the GIT immune system is the intestinal epithelial barrier and mucous layer (Gao et al., 2013; France and Turner, 2017). Paracellular permeability between epithelial cells is maintained by tight junction (TJ) proteins, but the mRNA abundance of these proteins is decreased in weaned pigs (Hu et al., 2013). Disruption of the TJ proteins and increased paracellular permeability can result in the translocation of pathogens or endotoxins from the lumen into the body, which may activate the GIT immune response (Awad et al., 2017). The mRNA abundance of CLDN3, CLDN4, OCLN, or ZO-1 was not affected by antibiotics or protein additive in this study. Zhang et al. (2016) reported that feeding SDPP increased the abundance of ZO-1 and CLDN1 but did not change abundance of OCLN. To the authors knowledge, there are no published studies that report the impact of DEP on the mRNA abundance of TJ proteins. It is known that pro-inflammatory cytokines, such as IFNγ and TNF-α, can disrupt the regulation of TJ proteins (Al-Sadi et al., 2009). Further, these cytokines have been shown to reduce the mRNA abundance of OCLN and ZO-1 (Youakim and Ahdieh, 1999;

Mankertz et al., 2000). Therefore, the lack of differences in the mucosal concentration of IFNγ could partially explain why we did not observe altered abundance of TJ proteins (Hu et al., 2013).

The weaning process causes villus atrophy in the small intestine, resulting in less surface area for nutrient digestion and absorption (Montagne et al., 2007). Villus atrophy without crypt hyperplasia is caused by decreased feed intake after weaning as this slows the crypt-cell production rate and reduces the number of new enterocytes that migrate to the villi tip (Pluske et al., 1997). Feeding DEP tended to increase villus length compared to the CON diet, but there was no change in crypt depth. The SDPP numerically increased villus length compared to CON and tended to reduce crypt depth in the AB- diets. Therefore, feeding the SDPP and DEP diets tended to increase the V:C ratio, indicating that these pigs had more absorptive capabilities than those fed the CON diet (Nabuurs et al., 1993). Further, since crypt hyperplasia was not observed in pigs fed the CON diet, it is likely that the villus atrophy occurred due to decreased feed intake. Improvements in the ileal V:C ratio from feeding SDPP or DEP have been reported in other publications (Owusu-Asiedu et al., 2003b; Nofrarías et al., 2006).

In conclusion, the SDPP and DEP improved growth rate and feed intake of pigs in AB- diets. When AB+ diets were fed, the SDPP and DEP did not improve growth performance compared to CON. Further, SDPP and DEP reduced the number of required medical treatments. These results made DEP a cost-effective solution to improve growth in AB- diets, but SDPP and DEP increased the cost of gain when AB+ diets were fed. The mucosal cytokine results indicate that specialty proteins and antibiotics can modulate the intestinal immune response of weaned pigs. Feeding specialty proteins had a slight beneficial impact on ileal morphology. These results support our hypothesis that feeding specialty proteins in antibiotic-free diets would improve pig

performance, and the modulation of intestinal inflammation and gut morphology could partially explain these improvements. This experiment provides novel data about the impact of specialty proteins on weanling pig performance and intestinal health when differing levels of antibiotics are fed in a commercial setting. Both SDPP and DEP could provide benefit to pork producers that are reducing their dietary antibiotic usage, but further research is still needed to characterize the mode of action of DEP and determine the full extent of its impact on weaned pigs.

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Table 2.1. Ingredient and nutrient composition of experimental diets (as-fed basis): phase 1[1,2].

	AB-			AB+		
Item	CON	SDPP	DEP	CON	SDPP	DEP
Ingredient composition, %						
Corn	30.98	33.94	30.78	30.68	33.64	30.48
Oat Groats	15.00	15.00	15.00	15.00	15.00	15.00
Soybean meal	20.00	14.21	20.00	20.00	14.21	20.00
Whey permeate	15.00	15.00	15.00	15.00	15.00	15.00
Dried yeast 1[3]	5.00	5.00	5.00	5.00	5.00	5.00
Dried yeast 2[4]	3.08	3.08	3.08	3.08	3.08	3.08
Fish meal, menhaden	5.00	5.00	5.00	5.00	5.00	5.00
Spray-dried plasma protein[5]	-	3.00	-	-	3.00	-
Dried egg protein[6]	-	-	0.20	-	-	0.20
Choice white grease	3.00	3.00	3.00	3.00	3.00	3.00
L-lysine HCl[7]	0.50	0.45	0.50	0.50	0.45	0.50
MHA[8]	0.23	0.19	0.23	0.23	0.19	0.23
L-threonine	0.15	0.11	0.15	0.15	0.11	0.15
L-tryptophan	0.03	0.03	0.03	0.03	0.03	0.03
Monocalcium phosphate 21%	0.46	0.35	0.46	0.46	0.35	0.46
Limestone	0.82	0.90	0.82	0.82	0.90	0.82
Salt	0.45	0.45	0.45	0.45	0.45	0.45
Nursery VTM premix[9]	0.18	0.18	0.18	0.18	0.18	0.18
Choline chloride 60%	0.12	0.12	0.12	0.12	0.12	0.12
Chlortetracycline[10]	-	-	-	0.30	0.30	0.30
Calculated nutrients						
SID Lys[11], %	1.50	1.50	1.50	1.50	1.50	1.50
SID TSAA:Lys[12]	0.55	0.55	0.55	0.55	0.55	0.55
SID Thr:Lys	0.60	0.60	0.60	0.60	0.60	0.60
SID Trp:Lys	0.18	0.18	0.18	0.18	0.18	0.18
Ca, %	0.85	0.85	0.85	0.85	0.85	0.85
STTD P[13], %	0.45	0.45	0.45	0.45	0.45	0.45
ME[14], Mcal/kg	3.48	3.51	3.48	3.48	3.51	3.48
NE[15], Mcal/kg	2.35	2.38	2.35	2.35	2.38	2.35
Analyzed nutrients						
Dry matter, %	88.91	89.48	89.41	89.14	89.49	89.30
Ash, %	6.08	5.90	6.19	6.04	6.08	5.91
Crude protein, %	22.41	22.34	23.20	22.70	22.41	22.95
aEE[16], %	7.22	6.88	7.24	7.37	6.92	7.38
SID Lys, %	1.47	1.45	1.44	1.46	1.41	1.45
SID TSAA, %	0.80	0.79	0.81	0.81	0.79	0.83
SID TSAA:Lys	0.54	0.54	0.56	0.55	0.56	0.57
SID Thr, %	0.88	0.86	0.84	0.90	0.86	0.89
SID Thr:Lys	0.60	0.59	0.58	0.62	0.61	0.61
SID Trp, %	0.24	0.26	0.26	0.25	0.28	0.25
SID Trp:Lys	0.16	0.18	0.18	0.17	0.20	0.17
SID Ile, %	0.86	0.80	0.84	0.87	0.79	0.85
SID Ile:Lys	0.59	0.55	0.58	0.60	0.56	0.59

Table 2.1. continued.

	AB-			AB+		
Item	CON	SDPP	DEP	CON	SDPP	DEP
SID Val, %	0.98	0.99	0.95	0.99	0.96	0.97
SID Val:Lys	0.67	0.68	0.66	0.68	0.68	0.67

[1] Phase 1 was fed from approximately d 0-13. The feed budget was 2.27 kg/pig.
[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]
[3] Dried yeast 1 is Proplex DY (Archer Daniels Midland Company, Decatur, IL).
[4] Dried yeast 2 is Proplex T (Archer Daniels Midland Company, Decatur, IL).
[5] Spray-dried plasma protein is AP 920 (APC Inc., Ankeny, IA).
[6] Dried egg protein is Globimax JS (EW Nutrition, Des Moines, IA).
[7] L-lysine HCl = L-lysine hydrochloride
[8] MHA = methionine hydroxy analogue
[9] The vitamin and trace mineral (VTM) premix provided per kg of complete diet: 0.21 ppm Cr as Cr_2O_3, 10 ppm Cu as $CuSO_4$, and Cu-MHA chelate, 0.31 ppm I as calcium iodate, 82 ppm Fe as $FeSO_4$, 21 ppm Mn as MnO and Mn-MHA chelate, 0.31 ppm Se as selenium yeast, 170 ppm Zn as ZnO and Zn-MHA chelate, 1,701 IU vitamin D3, 11,337 IU vitamin A, 45.3 IU vitamin E, 4.53 mg menadione, 0.23 mg biotin, 1.7 mg folic acid, 51 mg niacin, 15.6 mg pyridoxine, 28.3 mg pantothenic acid, 8.5 mg riboflavin, 39.7 mg vitamin B12, 514.4 FTU phytase (AxtraPhy, Danisco Animal Nutrition, Marlborough, UK). Premix also contained per kg of complete diet 0.06 g of *bacillus*-based direct-fed-microbial ($1.6x10^3$ CFU/g).
[10] Chlortetracycline (Aureomycin 100, Zoetis, Florham Park, NJ) was added to the diet at 662 mg/kg.
[11] SID = standard ileal digestible
[12] TSAA = total sulfur amino acids (Met + Cys)
[13] STTD = standardized total tract digestible
[14] ME = metabolizable energy
[15] NE = net energy
[16] aEE = acid-hydrolyzed ether extract

Table 2.2. Ingredient and nutrient composition of experimental diets (as-fed basis): phase 2[1,2].

	AB-			AB+		
Item	CON	SDPP	DEP	CON	SDPP	DEP
Ingredient composition, %						
Corn	51.43	52.70	51.33	51.31	52.58	51.21
Oat Groats	5.00	5.00	5.00	5.00	5.00	5.00
Soybean meal	25.00	21.86	25.00	25.00	21.86	25.00
Whey permeate	5.00	5.00	5.00	5.00	5.00	5.00
Dried yeast 1[3]	5.00	5.00	5.00	5.00	5.00	5.00
Dried yeast 2[4]	1.45	1.45	1.45	1.45	1.45	1.45
Spray-dried plasma protein[5]	-	2.00	-	-	2.00	-
Dried egg protein[6]	-	-	0.10	-	-	0.10
Choice white grease	3.00	3.00	3.00	3.00	3.00	3.00
L-lysine HCl[7]	0.50	0.44	0.50	0.50	0.44	0.50
MHA[8]	0.18	0.16	0.18	0.18	0.16	0.18
L-threonine	0.14	0.11	0.14	0.14	0.11	0.14
L-tryptophan	0.02	0.02	0.02	0.02	0.02	0.02
Monocalcium phosphate 21%	1.05	0.97	1.05	1.05	0.97	1.05
Limestone	1.24	1.30	1.24	1.24	1.30	1.24
Salt	0.70	0.70	0.70	0.70	0.70	0.70
Nursery VTM premix[9]	0.16	0.16	0.16	0.16	0.16	0.16
Choline chloride 60%	0.12	0.12	0.12	0.12	0.12	0.12
Carbadox[10]	-	-	-	0.125	0.125	0.125
Calculated nutrients						
SID Lys[11], %	1.35	1.35	1.35	1.35	1.35	1.35
SID TSAA:Lys[12]	0.55	0.55	0.55	0.55	0.55	0.55
SID Thr:Lys	0.60	0.60	0.60	0.60	0.60	0.60
SID Trp:Lys	0.18	0.18	0.18	0.18	0.18	0.18
Ca, %	0.80	0.80	0.80	0.80	0.80	0.80
STTD P[13], %	0.40	0.40	0.40	0.40	0.40	0.40
ME[14], Mcal/kg	3.41	3.42	3.41	3.41	3.42	3.41
NE[15], Mcal/kg	2.37	2.39	2.37	2.37	2.39	2.37
Analyzed nutrients						
Dry matter, %	88.38	88.53	88.52	88.32	88.47	88.61
Ash, %	5.90	5.40	5.60	5.55	5.81	5.25
Crude protein, %	20.56	21.69	20.99	21.30	21.22	20.90
aEE[16], %	6.44	6.27	6.69	6.07	6.61	6.62
SID Lys, %	1.32	1.39	1.35	1.33	1.33	1.27
SID TSAA, %	0.68	0.75	0.70	0.70	0.74	0.67
SID TSAA:Lys	0.52	0.54	0.52	0.53	0.56	0.53
SID Thr, %	0.76	0.82	0.76	0.75	0.82	0.70
SID Thr:Lys	0.58	0.59	0.56	0.56	0.62	0.55
SID Trp, %	0.23	0.26	0.25	0.25	0.26	0.24
SID Trp:Lys	0.17	0.19	0.19	0.19	0.20	0.19
SID Ile, %	0.78	0.83	0.78	0.80	0.77	0.74
SID Ile:Lys	0.59	0.60	0.58	0.60	0.58	0.58

Table 2.2 continued.

	AB-			AB+		
Item	CON	SDPP	DEP	CON	SDPP	DEP
SID Val, %	0.85	0.96	0.86	0.87	0.89	0.81
SID Val:Lys	0.64	0.69	0.64	0.65	0.67	0.64

[1] Phase 2 was fed from approximately d 13-26. The feed budget was 5.45 kg/pig.
[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]
[3] Dried yeast 1 is Proplex DY (Archer Daniels Midland Company, Decatur, IL).
[4] Dried yeast 2 is Proplex T (Archer Daniels Midland Company, Decatur, IL).
[5] Spray-dried plasma protein is AP 920 (APC Inc., Ankeny, IA).
[6] Dried egg protein is Globimax JS (EW Nutrition, Des Moines, IA).
[7] L-lysine HCl = L-lysine hydrochloride
[8] MHA = methionine hydroxy analogue
[9] The vitamin and trace mineral (VTM) premix provided per kg of complete diet: 0.19 ppm Cr as Cr_2O_3, 9 ppm Cu as $CuSO_4$, and Cu-MHA chelate, 0.28 ppm I as calcium iodate, 73 ppm Fe as $FeSO_4$, 19 ppm Mn as MnO and Mn-MHA chelate, 0.28 ppm Se as selenium yeast, 151 ppm Zn as ZnO and Zn-MHA chelate, 1,512 IU vitamin D3, 10,077 IU vitamin A, 40.3 IU vitamin E, 4.03 mg menadione, 0.20 mg biotin, 1.5 mg folic acid, 45 mg niacin, 13.9 mg pyridoxine, 25.2 mg pantothenic acid, 7.6 mg riboflavin, 35.3 mg vitamin B12, 457.2 FTU phytase (AxtraPhy, Danisco Animal Nutrition, Marlborough, UK). Premix also contained per kg of complete diet 0.06 g of *bacillus*-based direct-fed-microbial ($1.6x10^3$ CFU/g).
[10] Carbadox (Mecadox 10, Phibro Animal Health Corporation, Teaneck, NJ) was added in diet at 28 mg/kg.
[11] SID = standard ileal digestible
[12] TSAA = total sulfur amino acids (Met + Cys)
[13] STTD = standardized total tract digestible
[14] ME = metabolizable energy
[15] NE = net energy
[16] aEE = acid-hydrolyzed ether extract

Table 2.3. Ingredient and nutrient composition of experimental diets (as-fed basis): phase 3-4[1].

	Phase 3	Phase 4
Item	CON	CON
Ingredient composition, %		
Corn	50.99	51.39
Corn DDGS[2]	15.00	15.00
Soybean meal	28.14	28.14
Choice white grease	2.56	2.56
Lysine sulfate 54.6%	0.69	0.69
DL-methionine	0.14	0.14
L-threonine	0.08	0.08
L-tryptophan	0.04	0.04
Monocalcium phosphate 21%	0.18	0.18
Limestone	1.10	1.10
Salt	0.43	0.43
VTM premix[3]	0.15	0.15
Vitamin E (20,000)	0.05	0.05
Copper chloride	0.03	0.03
Phytase[4]	0.01	0.01
Chlortetracycline[5]	0.40	-
Calculated nutrients		
SID Lys[6], %	1.26	1.26
SID TSAA:Lys[7]	0.58	0.58
SID Thr:Lys	0.62	0.62
SID Trp:Lys	0.20	0.20
Ca, %	0.59	0.59
STTD P[8], %	0.35	0.35
ME[9], Mcal/kg	3.44	3.44
NE[10], Mcal/kg	2.40	2.40
Analyzed nutrients		
Dry matter, %	88.38	88.53
Ash, %	5.90	5.40
Crude protein, %	20.56	21.69
aEE[11], %	6.44	6.27
SID Lys, %	1.34	1.31
SID TSAA, %	0.75	0.67
SID TSAA:Lys	0.56	0.51
SID Thr, %	0.74	0.85
SID Thr:Lys	0.55	0.65
SID Trp, %	0.25	0.22
SID Trp:Lys	0.19	0.17
SID Ile, %	0.75	0.76
SID Ile:Lys	0.56	0.58

Table 2.3. continued.

	Phase 3	Phase 4
Item	CON	CON
SID Val, %	0.83	0.83
SID Val:Lys	0.62	0.63

[1] Phase 3 was fed from approximately d 26-40 and phase 4 was fed from d 40-42. Phases were common diets across all treatments.

[2] DDGS = distiller's dried grains with solubles

[3] The vitamin and trace mineral (VTM) premix provided per kg of complete diet: 11,000 IU of vitamin A, 1,650 IU of vitamin D, 33 IU of vitamin E (dl-alpha tocopheryl acetate), 11 IU of vitamin E (d-alpha tocopheryl acetate), 4.4 mg of vitamin K, 0.027 mg of vitamin B_{12}, 5.5 mg of riboflavin, 38.5 mg of niacin, 22 mg of pantothenic acid, 0.22 mg of biotin, 1.10 mg of folic acid, 0.88 mg of pyridoxine, 0.395 mg of Co as $CoCO_3$, 0.016 g of Cu as CuO or $CuSO_4$, 0.22 mg of I as ethylenediamine dihydroiodide (EDDI) or CaI_2, 0.15 g of Fe as $FeSO_4$, 0.03 g of Mn as MnO or $MnSO_4$, 0.3 mg of organic Se as selenium yeast, and 0.15 g of Zn as ZnO or $ZnSO_4$.

[4] Phytase (Optiphos 2000, Huvepharma, Sofia, Bulgaria) included in the diet to provide 250 FTU/kg.

[5] Chlortetracycline (Chlormax 50, Zoetis, Florham Park, NJ) added in diet at 441 mg/kg.

[6] SID = standard ileal digestible

[7] TSAA = total sulfur amino acids (Met + Cys)

[8] STTD = standardized total tract digestible

[9] ME = metabolizable energy

[10] NE = net energy

[11] aEE = acid-hydrolyzed ether extract

Table 2.4. Results of diagnostic testing throughout experiment (d 0-42).

Day[1]	Pathogen[2]	Result[3]	Testing method[4]
11	PEDV	Negative	Fecal PCR
11	PDCoV	Negative	Fecal PCR
11	TGEV	Negative	Fecal PCR
11	Porcine Rotavirus[5]	Positive	Fecal PCR
11	*Salmonella*[6]	Positive	Intestinal culture
32	PRRSV[7]	Positive	Oral fluid PCR
32	IAV[8]	Positive	Oral fluid PCR

[1] Day of sample collection.

[2] PEDV = porcine epidemic diarrhea virus, PDCoV = porcine deltacoronavirus, TGEV = transmissible gastroenteritis virus, PRRSV = porcine reproductive and respiratory syndrome virus, IAV = influenza A virus

[3] On d 11, 2 pigs showing symptoms of diarrhea, lethargy and gauntness were selected for necropsy. On d 32, oral fluids were collected and tested from 18 pens, spaced equidistantly the barn. If a sample was positive for a specific pathogen, the whole barn was considered to have exposure to that pathogen.

[4] PCR = polymerase chain reaction

[5] Pigs were positive for Rotavirus group A, B, and C.

[6] *Salmonella* species were not identified.

[7] PRRSV strain was wild type 1-7-4 ORF5.

[8] Pigs were positive for influenza H3 and N2.

Table 2.5. Primers used for real-time quantitative polymerase chain reaction (RT-qPCR).

Gene[1]	Primer sequence, 5'→3'[2]	Product size, base pair	GenBank accession	Annealing Temperature, °C
CLDN3	F: TTGCATCCGAGACCAGTCC	85	NM_001160075	60
	R: AGCTGGGGAGGGTGACA			
CLDN4	F: CAACTGCGTGGATGATGAGA	140	NM_001161637	60
	R: CCAGGGGATTGTAGAAGTCG			
OCLN	F: TCGTCCAACGGGAAAGTGAA	95	NM_001163647	55
	R: ATCAGTGGAAGTTCCTGAACCA			
ZO-1	F: CTCTTGGCTTGCTATTCG	197	XM_003353439	55
	R: AGTCTTCCCTGCTCTTGC			
RPL19	F: AACTCCCGTCAGCAGATCC	147	AF_435591	55
	R: AGTACCCTTCCGCTTACCG			

[1] *CLDN3 = claudin-3*; *CLDN4 = claudin-4*; *OCLN = occludin*; *ZO-1 = zonula occludens-1*; *RPL19 = ribosomal protein-L19*

[2] F = forward primer; R = reverse primer

Table 2.6. The effects of in-feed antibiotics and specialty protein additives on medical treatments and removals[1,2,3].

	AB		ADD			Pooled	*P*-value[4]	
Item	AB-	AB+	CON	SDPP	DEP	SEM	AB	ADD
Medical treatments, proportion[5]	0.86	0.78	0.98[a]	0.74[b]	0.73[b]	0.08	0.157	0.001
Removals, proportion[6]	0.09	0.08	0.08	0.08	0.09	0.02	0.396	0.832

[1] Data are least square means; n = 10 pens per treatment with 20 or 21 pigs per pen, totaling 1,230 pigs; 42 d growth experiment

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Means within a row without a common superscript (a-b) differ significantly ($P < 0.05$).

[4] The AB × ADD interaction was tested but was not significant for either variable ($P > 0.10$).

[5] Medical treatments were calculated as the total number of medical treatments administered per pen divided by number of pigs allotted to pen.

[6] Removals were calculated as the total number of pigs removed from study (found dead or removed for illness or injury) divided by number of pigs allotted to pen.

Table 2.7. The effects of in-feed antibiotics and specialty protein additives on overall growth performance and feed efficiency of pigs[1,2,3].

	AB-			AB+			Pooled	*P*-value		
Item[4]	CON	SDPP	DEP	CON	SDPP	DEP	SEM	AB	ADD	AB × ADD
Pens per treatment	10	10	10	10	10	10	-	-	-	-
Pigs per treatment, initial	205	205	205	205	205	205	-	-	-	-
Pigs per treatment, final[5]	179	180	177	182	181	180	-	-	-	-
Start BW (d 0)	4.9	4.9	4.9	4.9	4.9	4.9	0.02	0.247	0.372	0.262
End BW (d 42)	15.3	16.0	16.2	16.0	16.4	15.8	0.44	0.196	0.077	0.061
ADG, kg	0.24^{a}	0.25^{bc}	0.26^{bc}	0.26^{bc}	0.27^{b}	0.25^{c}	0.01	0.020	0.044	0.036
ADFI, kg	0.36^{a}	0.38^{b}	0.38^{bc}	0.39^{bc}	0.40^{c}	0.38^{b}	0.01	0.002	0.026	0.040
G:F	0.66	0.67	0.67	0.67	0.67	0.66	0.01	0.984	0.429	0.345

[1] Data are least square means; n = 10 pens per treatment with 20 or 21 pigs per pen, totaling 1,230 pigs; 42 d growth experiment; growth calculations included pig days to account for morbidity and mortality.

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain:feed ratio

[5] Number of pigs removed for necropsy during the experiment: AB-CON: 9; AB-SDPP: 8; AB-DEP: 8; AB+CON: 8; AB+SDPP: 8; AB+DEP: 9

Table 2.8. The effects of in-feed antibiotics and specialty protein additives on growth performance and feed efficiency of pigs by weigh period analyzed as a mixed model with a time dependent variance structure[1,2,3,4].

Item[5]	AB		ADD			Pooled SEM	*P*-value[6]					
	AB-	AB+	CON	SDPP	DEP		AB	ADD	Period	AB × Period	ADD × Period	AB × ADD
BW, kg						0.2	0.006	0.003	<0.001	0.575	0.642	0.010
d 0	4.9	4.9	4.9	4.9	4.9							
d 13	6.2	6.4	6.2	6.4	6.2							
d 26	9.7	10.0	9.6	10.1	9.9							
d 42	15.9	16.1	15.7	16.2	16.0							
ADG, kg						0.01	0.017	0.022	<0.001	0.670	0.687	0.032
d 0-13	0.10	0.11	0.10	0.11	0.10							
d 13-26	0.27	0.28	0.27	0.29	0.28							
d 26-42	0.37	0.37	0.37	0.37	0.37							
ADFI, kg						0.02	0.002	0.019	<0.001	0.893	0.955	0.051
d 0-13	0.14	0.15	0.14	0.15	0.15							
d 13-26	0.40	0.42	0.40	0.43	0.41							
d 26-42	0.56	0.57	0.56	0.57	0.57							
G:F						0.01	0.088	0.010	<0.001	0.018	0.019	0.073
d 0-13	0.67^{x}	0.72^{y}	0.67^{ab}	0.73^{c}	0.69^{a}							
d 13-26	0.67^{x}	0.67^{x}	0.65^{b}	0.68^{ab}	0.68^{ab}							
d 26-42	0.66^{x}	0.65^{x}	0.68^{ab}	0.65^{b}	0.65^{b}							

[1] Data are least square means; n = 10 pens per treatment with 20 or 21 pigs per pen, totaling 1,230 pigs; 42 d growth experiment; growth calculations included pig days to account for morbidity and mortality.

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Weigh periods: period 1 (d 0-13), period 2 (d 13-26), and period 3 (d 26-42)

[4] Within a dependent variable, means without a common superscript (x-y or a-c) differ significantly ($P < 0.05$).

[5] BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain:feed ratio

[6] The AB × ADD × period interaction was tested but was not significant for any of the variables ($P > 0.10$).

Table 2.9. The effects of in-feed antibiotics and specialty protein additives on oxidative stress and ileal mucosa cytokines[1,2,3].

	AB-			AB+			Pooled	*P*-value		
Item	CON	SDPP	DEP	CON	SDPP	DEP	SEM	AB	ADD	AB × ADD
Malondialdehyde, μM	16.60	14.46	13.45	16.23	16.65	16.09	1.35	0.184	0.481	0.492
Cytokines[4], ng/g										
IFNγ	21.41	21.82	18.91	25.26	23.00	22.82	2.72	0.166	0.624	0.838
IL-1α	5.26	4.56	3.36	4.16	4.96	4.85	0.93	0.705	0.684	0.320
IL-1β	16.97	15.54	11.65	23.16	22.23	11.27	3.45	0.113	0.022	0.473
IL-1ra	10.25	10.25	8.79	15.18	11.75	12.54	1.65	0.017	0.422	0.577
IL-2	1.12	1.25	1.10	1.27	1.12	1.18	0.14	0.757	0.923	0.596
IL-4	1.13	1.24	1.14	1.37	1.09	0.78	0.20	0.559	0.332	0.313
IL-6	0.77	0.86	0.62	1.07	0.83	0.94	0.15	0.111	0.636	0.425
IL-8	256.91	243.68	200.88	271.47	226.15	215.31	31.26	0.881	0.203	0.840
IL-10	0.66	0.72	0.41	0.82	0.65	0.67	0.11	0.194	0.139	0.266
IL-12	7.93	6.72	5.86	7.41	6.78	6.98	1.03	0.784	0.429	0.699
IL-18	38.03[a]	44.92[a]	37.20[a]	124.73[b]	57.23[a]	49.75[a]	15.62	0.002	0.019	0.012

[1] Data are least square means; n = 8 replicates per treatment; serum and ileal tissue samples were collected on d 20

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] Inerferon-γ (IFNγ); interleukin-1α (IL-1α); interleukin-1β (IL-1β); interleukin-1 receptor antagonist (IL-1ra); interleukin-2 (IL-2); interleukin-4 (IL-4); interleukin-6 (IL-6); interleukin-8 (IL-8); interleukin-10 (IL-10); interleukin-12 (IL-12); interleukin-18 (IL-18)

Table 2.10. The effects of in-feed antibiotics and specialty protein additives on relative ileal gene mRNA abundance[1,2,3].

	AB		ADD			Pooled	*P*-value[5]	
Gene[4]	AB-	AB+	CON	SDPP	DEP	SEM	AB	ADD
CLDN3	1.64	1.38	1.20	2.00	1.33	0.35	0.505	0.187
CLDN4	1.60	1.17	1.22	1.37	1.56	0.36	0.267	0.969
OCLN	1.38	1.08	1.09	1.34	1.25	0.24	0.477	0.541
ZO-1	1.08	0.99	0.93	1.04	1.15	0.16	0.916	0.779

[1] Data are least square means; n = 8 replicates per treatment; ileal tissue samples were collected on d 20

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] *CLDN3*: *claudin-3*; *CLDN4*: *claudin-4*; *OCLN*: *occludin*; *ZO-1*: *zonula occludens-1*

[5] The AB × ADD interaction was tested but was not significant for either variable ($P > 0.10$).

Table 2.11. The effects of in-feed antibiotics and specialty protein additives on ileal morphology[1,2,3].

	AB-			AB+			Pooled	*P*-value		
Item	CON	SDPP	DEP	CON	SDPP	DEP	SEM	AB	ADD	AB × ADD
Villus height, µm	321.9	353.4	371.2	341.1	359.2	355.2	14.9	0.810	0.098	0.499
Crypt depth, µm	270.7	244.3	262.3	258.8	267.0	256.6	10.3	0.802	0.550	0.098
Villi height:crypt depth	1.3	1.6	1.5	1.4	1.5	1.5	0.1	0.700	0.066	0.313

[1] Data are least square means; n = 8 replicates per treatment; ileal tissue samples were collected on d 20 and fixed in 10% neutral-buffered formalin before histology slides were made

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

Table 2.12. The effects of in-feed antibiotics and specialty protein additives on overall cost of gain ($/kg of gain) of pigs[1,2,3,4,5,6,7,8].

	AB-			AB+			Pooled	*P*-value		
	CON	SDPP	DEP	CON	SDPP	DEP	SEM	AB	ADD	AB × ADD
Cost of gain[9], $/kg	0.61	0.65	0.59	0.59	0.65	0.62	0.01	0.915	<0.001	0.060

[1] Data are least square means; n = 10 pens per treatment with 20 or 21 pigs per pen, totaling 1,230 pigs; 42 d growth experiment

[2] AB = in-feed antibiotics [none (AB-) vs standard (AB+) levels]; ADD = specialty protein additive [none (CON), spray-dried plasma protein (SDPP), or dried egg protein (DEP)]

[3] Phase 1 was fed from approximately d 0-13 and the feed budget was 2.27 kg/pig. Phase 2 was fed from approximately d 13-26 and the feed budget was 5.45 kg/pig. Phase 3 was fed from approximately d 26-40 and phase 4 was fed from d 40-42.

[4] Cost of phase 1 diets ($/tonne of feed): AB-CON: 418.23; AB-SDPP: 531.41; AB-DEP: 461.74; AB+CON: 436.81; AB+SDPP: 551.49; AB+DEP: 480.74

[5] Cost of phase 2 diets ($/tonne of feed): AB-CON: 260.29; AB-SDPP: 349.18; AB-DEP: 280.68; AB+CON: 289.36; AB+SDPP: 376.92; AB+DEP: 310.15

[6] Cost of phase 3 diet ($/tonne of feed): 241.93 (common diet)

[7] Cost of phase 4 diet ($/tonne of feed): 230.77 (common diet)

[8] Means within a row without a common superscript differ significantly ($P < 0.05$).

[9] Cost of gain was calculated using the cost of experimental diets and medical treatments. Diet cost was calculated using only the cost of ingredients and mixing.

CHAPTER 3. THE EFFECTS OF ENZYMATICALLY-TREATED SOYBEAN MEAL ON GROWTH PERFORMANCE AND INTESTINAL STRUCTURE, BARRIER INTEGRTY, INFLAMMATION, OXIDATIVE STATUS, AND VOLATILE FATTY ACID PRODUCTION OF NURSERY PIGS

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Leigh A. Ruckman[*], Amy L. Petry[*], Stacie A. Gould[*], Brian J. Kerr[†] and John F. Patience[*]

[*]Department of Animal Science, Iowa State University, Ames, IA 50011

[†]USDA-ARS National Laboratory for Agriculture and the Environment, Ames, IA 50011

Abstract

The objective of this experiment was to determine the impact of diets containing increasing amounts of enzymatically-treated soybean meal (ESBM) but decreasing amounts of soybean meal (SBM) on growth performance, intestinal structure and barrier integrity, inflammation, and oxidative status in weaned pigs. A total of 480 pigs [6.3 $\pm$ 1.2 kg body weight (BW)] were blocked by initial BW and pens (n = 12 per treatment) were randomly allotted to 1 of 4 dietary treatments. Diets were fed in 3 phases (d 0-14, 14-28, and 28-35) over a 35-d period. The 4 dietary treatments consisted of a negative control diet (**NC**), the NC with 7.0% ESBM (**ESBM1**), the NC with 14.0% ESBM (**ESBM2**), and the NC with 21.0% ESBM (**ESBM3**). Soybean meal was reduced proportionately in each treatment. In phase 2, ESBM inclusion was decreased by 50% (3.5%, 7.0%, and 10.5% ESBM, respectively); phase 3 was a common diet

and contained no ESBM. Fecal score was visually ranked weekly using a 4-point scale. Intestinal tissue, digesta and blood samples were collected from 48 pigs (1 per pen) on d 10. Data were analyzed using PROC MIXED of SAS (9.4) with pen as the experimental unit; diet and block were considered fixed effects. Linear and quadratic contrasts were used to determine the effect of increasing ESBM. Overall, ESBM2 and ESBM3 decreased final BW, ADG, and ADFI compared to NC and ESBM1 (diet, $P < 0.05$; linear, $P < 0.05$). Overall fecal score (diet, $P < 0.05$) and fecal DM ($P < 0.05$) were improved by feeding ESBM diets compared to NC. Volatile fatty acid (VFA) concentration of acetate, propionate, butyrate, and total VFA in ileal contents increased as ESBM inclusion increased ($P < 0.05$). Colonic VFA concentration was not impacted ($P > 0.10$). Total antioxidant capacity was increased by ESBM ($P < 0.05$). The concentration of mucosal interleukin-4 increased as the inclusion of ESBM increased (linear, $P < 0.05$). Messenger ribonucleic acid abundance of *occludin* and *zonula-occludens-1* in ileal tissue was increased by ESBM1 or ESBM2 ($P < 0.05$). In conclusion, increasing the dietary levels of ESBM over 7% had a negative impact on nursery pig performance, but ESBM positively impacted fecal score. Feeding ESBM improved oxidative status and intestinal barrier integrity while increasing ileal VFA production but had minimal impact on intestinal inflammation or morphology. Further research is needed to determine the optimal inclusion level of ESBM.

Keywords: health, oxidative status, weaned pig, gut health, swine, cytokines

Introduction

The transition at weaning exposes pigs to multiple new stressors, leaving them susceptible to low feed intake and reduced growth, gastrointestinal tract (GIT) disorders, and impaired intestinal function and integrity (Lallès et al., 2004; Moeser et al., 2007; Li et al., 2020). Further, a suppressed immune system and still-developing GIT can increase weaned pigs'

vulnerability to pathogens and enteric disease (de Lange et al., 2010; Becker et al., 2020). These issues have generated greater interest in feeding strategies that will positively affect intestinal health and function of weaned pigs.

Soybean meal (SBM) is frequently the main protein source in swine diets. The processing of raw soybeans into SBM includes heat treatment, which inactivates the majority of trypsin inhibitor and urease (Herkelman et al., 1992; Woyengo et al., 2017). However, the concentration of other anti-nutritional factors (ANF) in SBM may be high enough to negatively impact the growth and intestinal health of young pigs (Li et al., 1991; Yang et al., 2007). Glycinin and β-conglycinin, the main antigenic proteins found in SBM, cause a hypersensitive immune response in the GIT of weaned pigs, resulting in abnormal morphology of the small intestine and reduced absorptive capacity (Li et al., 1991). Further, the non-digestible oligosaccharides (NDO) in soybeans (specifically stachyose and raffinose) can cause diarrhea while reducing growth (Zhang et al., 2003). These dietary components limit the use of SBM in early nursery diets, leading to greater use of protein sources of animal origin that are highly digestible but also quite expensive (Min et al., 2009). Thus, further processing methods for SBM have been developed to diminish the concentration of ANF.

Enzymatically-treated SBM (ESBM) is produced by treating SBM with proprietary blends of microbial enzymes (typically including proteases and carbohydrases) for several hours (Goebel and Stein, 2011). The resulting ingredient has reduced levels of several ANF, and improved digestibility of amino acids and crude protein compared to conventional SBM (Cervantes-Pahm and Stein, 2010; Ma et al., 2019b). The ESBM is considered a high-quality protein source for weaned pigs as it has been reported that replacing conventional SBM with ESBM in nursery pig diets can improve gain and feed conversion (Zhu et al., 1998; Zhou et al.,

2011). However, the impact of ESBM on the intestinal health and function of weaned pigs is still largely unknown.

The objective of this experiment was to determine the impact of diets in which ESBM replaced increasing amounts of SBM on growth performance, intestinal structure and barrier integrity, inflammation, and oxidative status in newly weaned pigs. It was hypothesized that replacing conventional SBM with ESBM would improve growth performance of pigs while beneficially modulating markers of intestinal structure and barrier integrity, immune status, and oxidative status.

Materials and methods

All experimental procedures adhered to the principles for the ethical and humane use of animals for research according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and were approved by the Iowa State University Animal Care and Use Committee (IACUC-19-073).

Animals, housing, and experimental design

A total of 480 pigs [6.34 ± 1.18 kg body weight (BW); L337 × Camborough, PIC, Hendersonville, TN] were weaned at approximately 21 d and transported to the Iowa State University Swine Nutrition Farm (Ames, IA). Upon arrival, pigs were ear-tagged, weighed individually, and vaccinated against K88+ and F18 *Escherichia coli* via a water-delivered vaccine (Entero Vac and Edema Vac, Arko Laboratories, Jewell, IA). Pigs were blocked by initial body weight and pens were randomly assigned to 1 of 4 dietary treatments. Each pen housed 10 pigs and there were 12 pens per treatment. Sexes were not separated by pen, but similar numbers of barrows and gilts were assigned to each pen within a block. Each pen (1.2 m

× 2.4 m) had a wire mesh floor and was equipped with a four-space dry self-feeder and two nipple waterers to provide ad libitum access to feed and water.

Dietary treatments and feeding

Experimental diets were fed in 3 phases over 35 d (Tables 3.1 and 3.2). Phase 1 was fed from d 0-14, phase 2 was fed from d 14-28, and phase 3 was fed from d 28-35. The dietary treatments consisted of a negative control diet (**NC**) with no ESBM, NC diet with 7.0% ESBM (HP 300, Hamlet Protein Inc., Findlay, OH) and reduced SBM (**ESBM1**), NC diet with 14.0% ESBM and a larger reduction of SBM (**ESBM2**), and NC with 21.0% ESBM and the elimination of SBM (**ESBM3**). In phase 2, the inclusion of ESBM in the ESBM1, ESBM2, and ESBM3 diets was decreased by 50% (3.5%, 7.0%, and 10.5% ESBM, respectively). The phase 3 diet was a common diet and contained no ESBM. The NC diets contained 25.75% SBM in phase 1, 28.84% in phase 2, and 31.41% in phase 3. The ESBM1, ESBM2, and ESBM3 diets contained 16.97%, 8.35%, and 0% SBM in phase 1 and 24.40%, 19.95%, and 15.46% SBM in phase 2, respectively.

All diets were formulated to meet or exceed the pigs' nutrient requirements for each phase (NRC, 2012). Because the experiment was designed to evaluate a specific ingredient, it was important to minimize changes in the levels of all other ingredients in the formulation while at the same time, as much as possible, also maintaining constant energy and nutrient levels; otherwise, the results of the study could be confounded. Therefore, the levels of most ingredients across treatments within phase were identical. The only exceptions were corn, SBM, and ESBM; very small differences in the levels of select synthetic amino acids and limestone were necessary to maintain constant calcium and standardized ileal digestible (SID) amino acid concentrations. All diets were fed in mash form.

Medical treatments

When required, and according to the farm protocol, pigs were individually treated with ceftiofur (Excede, Zoetis, Florham Park, NJ); animals not responsive to medical treatment were removed from the study. Individual medical treatments were recorded by pen, day, and dosage. Under the direction of a veterinarian, sodium salicylate (Oral-Pro Sodium Salicylate Concentrate, Aurora Pharmaceutical, Northfield, MN; 78 mL of sodium salicylate concentrate/L of water) was added to the water on d 13-17 and d 34-35 to treat lethargy and respiratory symptoms.

Data and sample collection

Pigs were individually weighed on d 0, 14, 28 and 35 to determine average daily gain (ADG). Feed disappearance was also recorded on each weigh day to determine average daily feed intake (ADFI) and to calculate gain:feed ratio (G:F). Weights and removal dates of pigs were recorded and ADG and ADFI were calculated according to pig days on test. Fecal consistency was scored by pen on d 7, 14, 21, 28, and 35 using the following categorical scale: 1 = solid, 2 = semi-solid, 3 = semi-liquid, and 4 = liquid. Fecal score was assessed independently by two people, and an average score of these two was recorded for each pen during each week.

Blood collection and necropsies were performed on d 10. This time point was selected so that sampling occurred during the peak of the late post-weaning period and gut adaptation to the weaning diet. One pig from each pen (12 per treatment) was selected, and blood samples were collected by jugular venipuncture into two 10 mL vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to obtain plasma and serum. Plasma tubes were centrifuged at 1,000 × g for 10 min at 4°C and serum tubes were centrifuged at 1,500 × g for 15 min at 4°C before aliquots were harvested and stored at -80°C.

Pigs were subsequently euthanized by captive bolt stunning followed by exsanguination. Ileal tissues were collected 10 cm proximal to the ileocecal junction. Digesta was collected and tissues were rinsed with phosphate buffered solution (PBS) before both were snap-frozen in liquid nitrogen and stored at -80°C. Two tissue samples were collected from the mid-ileum, rinsed with PBS and fixed in 10% neutral buffered formalin for 24 h and then transferred to 70% ethanol. Digesta was collected from the mid-colon, snap-frozen in liquid nitrogen, and stored at -80°C. The pH and temperature of ileal and colonic digesta were measured using a portable pH meter (pH 150 Meter Kit, Oakton Instruments, Vernon Hills, IL). Fresh ileal and colonic digesta samples were collected into tubes and stored on ice.

Feed samples were collected during the manufacturing of the diets. Multiple subsamples of each diet were collected throughout each batch of feed and homogenized before being stored at -20°C. On d 11-13, fecal samples were collected via grab sampling from each pen and stored at -20°C until further processing.

Chemical analysis

Diets were ground to 1 mm particle size (Variable Speed Digital ED-5 Wiley Mill; Thomas Scientific, Swedesboro, NJ), dried at 60°C to a constant weight, and analyzed in duplicate for dry matter (DM; method 930.15; AOAC, 2007), ash (method 942.05; AOAC, 2007), acid-hydrolyzed ether extract (aEE; method 2003.06; AOAC, 2007), and nitrogen (N; method 990.03; AOAC, 2007; TruMac; LECO Corp., St. Joseph, MI). Crude protein (CP) was calculated as N × 6.25 with ethylenediaminetetraacetate (EDTA; 9.56% N; determined to have 9.56 ± 0.03% N) used for standard calibration. An isoperibolic bomb calorimeter was used to determine gross energy (GE; model 6200; Parr Instrument Co., Moline, IL); benzoic acid (6318 kcal GE/kg; Parr Instrument Co., Moline, IL), determined to contain 6323 ± 7 kcal GE/kg, was used as the calibration standard. The SID amino acid levels of the diets were calculated using the

assayed total amino acid concentration (Agricultural Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO) and the ingredient's SID coefficient for individual amino acids (NRC, 2012). Diets were analyzed for Ca and total P (Eurofins US, Des Moines, IA) using inductively coupled plasma optical emission spectrometry (AOAC 984.27, 927.02, 985.01, and 965.17). Fecal samples were thawed, homogenized, and then dried to a constant weight at 60°C. Dried feces were ground using a mortar and pestle and analyzed in duplicate for DM (method 930.15; AOAC, 2007). The coefficient of variation (CV) threshold for repeating an assay was 1% for DM, ash, CP, GE, and fecal DM and 5% for aEE.

The concentration of volatile fatty acids (VFA) was measured in ileal and colonic digesta in triplicate. Colonic digesta (1 g) was diluted with 5 mL of deionized water and mixed overnight on a rocking platform before centrifugation at 20,000 × g for 20 min at 4°C. The supernatant (1 mL) was placed into a gas chromatography (GC) vial with 0.3 g of NaCl and 100 µL of phosphoric acid. Ileal digesta (2 g) was centrifuged at 20,000 × g for 20 min at 4°C before the supernatant (1 mL) was placed into a new tube with 100 µL of phosphoric acid. The tubes were centrifuged at 4,000 × g for 10 min at 4°C and the supernatant (1 mL) was placed into a GC vial with 0.3 g of NaCl. The prepared samples were frozen at -20°C and sent to an external laboratory (USDA-ARS-MWA-NLAE, Ames, IA) for GC analysis (Agilent 7890A Gas Chromatograph, Agilent Technologies Inc., Wilmington, DE) using methods previously described by Kerr et al. (2015). The total VFA concentration is the sum of acetate, propionate, and butyrate concentration and is expressed as mM of VFA/L of digesta. The molar proportions of VFA (%) were calculated using individual and total VFA concentration: [(mM $VFA_{individual}$/L ÷ mM VFA_{total}/L) × 100]. The CV threshold was less than 15% for all VFA analyses.

The water-holding capacity (WHC) of feed was measured in triplicate using a modified protocol from Giger-Reverdin (2000). Dried and ground feed (0.5 g) was soaked in 50 mL of deionized water for 24 h. The sample was filtered using a fritted funnel (40-60 µm porosity) for 1 h and the remaining wet sample was weighed. The WHC was calculated using the following equation (WHC = g of retained water/g of dry feed) and was expressed as mL of water per g of DM. The CV threshold was less than 5% for WHC. The water-binding capacity (WBC) of ileal and colonic digesta was measured in triplicate using a modified protocol from Serena et al. (2008). Fresh digesta (2 g) was centrifuged at 10,000 × g for 20 min at 4°C to separate the liquid and solid components. The liquid fraction was removed by suction immediately after centrifugation and again 12 h later. The solid fraction was weighed, and the WBC was calculated using the wet weight (WW) and dry weight (DW) of the digesta [WBC= (WW-DW) ÷ DW].

Oxidative status, lipopolysaccharide-binding protein, and mucosal cytokines

Ileal tissue samples (100 mg) were sonicated in 1 mL of buffer (RIPA buffer, Sigma-Aldrich, St. Louis, MO), containing 1 mM of EDTA disodium salt, before centrifugation at 1,600 × g for 10 min at 4°C. The total protein concentration of the resulting lysate was measured using a Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Woltham, MA). Malondialdehyde (MDA) was measured in plasma and ileal tissue lysate using the colorimetric protocol of a thiobarbituric acid reactive substances (TBARS) kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI) as previously described (Armstrong and Browne, 1994; Yagi, 1997). The MDA concentration of ileal tissue lysate was expressed as µM MDA/µg protein.

Total antioxidant capacity (TAC) was measured in plasma (diluted 1:10 with provided assay buffer) using a commercially-available colorimetric assay (Antioxidant Assay Kit, Cayman

Chemical Company, Ann Arbor, MI) as previously described (Miller et al., 1993). Lipopolysaccharide-binding protein (LBP) was measured in serum (diluted 1:300 with provided assay buffer) using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (LBP various species ELISA kit, Hycult Biotech, Uden, The Netherlands). The CV threshold was less than 5% for total protein, MDA, and TAC values and less than 10% for LBP values.

Ileal mucosal homogenates were prepared using methods previously described by Becker et al. (2020). The homogenates were sent to a commercial laboratory (Eve Technologies Corporation, Calgary, AB, Canada) and analyzed for cytokines using a multiplex immunoassay that utilized laser bead technology. The assay included granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and tumor necrosis factor-α (TNF-α).

RNA isolation and real-time quantitative PCR

Ileal tissues (25-40 mg) were homogenized in a lysis buffer (RNeasy Plus Mini Kit, Qiagen, Carlsbad, CA) using the Qiagen Tissuelyser II (Germantown, MD) to isolate total ribonucleic acid (RNA). The RNA was treated with a deoxyribonuclease enzyme to prevent genomic deoxyribonucleic acid (DNA) contamination (DNA-free DNA removal kit, Invitrogen, Carlsbad, CA). A spectrophotometer (ND-100; NanoDrop Technologies Inc., Rockland, DE) was used to quantify RNA concentration and all samples had 260:280 nm ratios above 1.8. Complimentary DNA (cDNA) was synthesized from isolated RNA (0.8 μg) using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and all cDNA samples were diluted 10-fold with nuclease-free water.

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). The gene-specific

primers (Table 3.3) were diluted to 10 µM with nuclease-free water. Each 20 µL reaction included 10 µL of SYBR Green Supermix, 1 µL of each forward and reverse primer, 3 µL of cDNA and 5 µL of nuclease-free water. Each plate included a no-reverse transcriptase negative control and a pooled cDNA reference sample. A RT-qPCR detection system (iQ5; Bio-Rad Laboratories Inc.) was used to quantify SYBR Green fluorescence with the following cycling conditions: 5-min initial denaturation at 95°C followed by 40 RT-qPCR cycles (95°C for 30 s, 55 or 60°C for 30 s, and 72°C for 30 s) and a dissociation curve to verify the amplification of a single RT-qPCR product. Optical System Software (iQ5, version 2.0; Bio-Rad Laboratories Inc.) was used to analyze amplification plots and cycle threshold values for each reaction were obtained. The messenger RNA (mRNA) abundance was normalized to a reference gene (ribosomal protein- L19) and the pooled sample. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calculate fold change. The CV threshold was less than 5% for all RT-qPCR analysis.

Intestinal morphology

Ileal tissues (fixed in 70% ethanol) were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin, and mounted on glass slides (Iowa State Veterinary Diagnostics Lab, Ames, IA). A DP80 Olympus Camera mounted on an OLYMPUS BX 53/43 microscope (Olympus Scientific, Waltham, MA) was used to take images of slides at 10x power. Ten villi and crypt pairs per ileal sample were measured using OLYMPUS CellSens Dimension 1.16 software. The ratio of villus height to crypt depth was calculated for each pair (V:C ratio).

Statistical analysis

Data were analyzed using the following mixed models. Model 1 assumed that residuals were normally distributed with an unstructured (UN) dependent covariance structure

[$N(0, I\ UN\sigma_e^2)$]. The following mixed model was used to analyze data for fecal score and growth performance by phase.

$$Model\ 1: Y_{ijkl} = \mu + \tau_i + \rho_k + \tau_i \rho_k + a_j + e_{ijkl}$$

where Y_{ijkl} is the observed value for l[th] experimental unit (pen) within the k[th] period in the j[th] block within the i[th] level of diet; μ is the general mean; τ_i is the fixed effect of the i[th] diet (i = NC, ESBM1, ESBM2, ESBM3); a_j is the fixed effect of the j[th] block (j = 1 to 12); ρ_k is the fixed effect of period (k = 1 to 3); $\tau_i \rho_k$ is the interaction term of diet × period; and e_{ijkl} is the associated variance as described by the model for Y_{ijkl} (l = 1 through 48), assuming $e_{ijkl} \sim N(0, I\ UN\ \sigma_e^2)$ where I is the identity matrix.

Model 2 assumed that residuals were independent and normally distributed [$N(0, I\ \sigma_e^2)$]. The following mixed model was used to analyze all data except for fecal score and growth performance by phase.

$$Model\ 2: Y_{ijk} = \mu + \tau_i + a_j + e_{ijk}$$

where Y_{ijk} is the observed value for the k[th] experimental unit (pen) within the j[th] block in the i[th] level of diet; μ is the general mean; τ_i is the fixed effect of the i[th] diet (i = NC, ESBM1, ESBM2, ESBM3); a_j is the fixed effect of the j[th] block (j = 1 to 12); and e_{ijk} is the associated variance as described by the model for Y_{ijk} (k = 1 through 48), assuming $e_{ijk} \sim N(0, I\ \sigma_e^2)$ where I is the identity matrix.

Normality and homogeneity of the studentized residuals from the reported models were verified using the UNIVARIATE procedure of SAS 9.4 (SAS Inst., Cary, NC). Statistical outliers, defined as being greater than three standard deviations from the mean, were identified and removed from the analysis. All data and models were analyzed using the MIXED procedure. The UN covariance structure was selected as the best fit for model 1 according to Bayesian

Information Criterion for all dependent variables. Linear and quadratic orthogonal polynomial contrasts were applied to determine the effects of increasing levels of ESBM. Fisher's Least Significant Difference test was used to separate least square means and differences were considered significant if $P < 0.05$ and trends if $0.05 \geq P < 0.10$.

Results

Health and fecal score

The total removal rate of pigs, including mortalities and removals due to illness, was 1.3%. Pigs fed NC and ESBM2 required more medical treatments than pigs fed ESBM1, and ESBM3 was intermediate between them (diet, $P = 0.038$; Table 3.4). Feeding NC increased the overall fecal score compared to ESBM1, ESBM2 and ESBM3 (diet, $P = 0.003$; Table 3.5). No diet × period interactions were observed for fecal score ($P > 0.10$).

Growth performance

Overall, pigs fed NC and ESBM1 had greater final BW compared to ESBM2 and ESBM3 (diet, $P = 0.001$; Table 3.6). This pattern was also observed for overall ADG (diet, $P = 0.001$) and ADFI (diet, $P = 0.001$), but G:F was not impacted ($P > 0.10$). There was a decrease in final BW, overall ADG and ADFI as the inclusion of ESBM increased (linear, $P < 0.001$).

In phase 1, NC and ESBM1 increased BW more than ESBM3 with ESBM2 being intermediate, but NC and ESBM1 improved BW compared to ESBM2 and ESBM3 in phase 2 and 3 (diet × period, $P = 0.039$; Table 3.7). Feeding NC increased G:F more than ESBM2 and ESBM3 in phase 1, while ESBM1 was intermediate; however, ESBM1 did increase G:F more than ESBM3 (diet × period, $P = 0.008$). In phase 2, ESBM1 increased G:F more than ESBM3 but not NC or ESBM2. The ESBM3 increased G:F compared to NC or ESBM1 in phase 3, with

ESBM2 being intermediate. No diet × period interactions were observed for ADG or ADFI (P > 0.10).

Fecal and digesta characteristics

Fecal DM increased as the inclusion of ESBM in the diets was increased (linear, P <0.001; Table 3.8). The WBC of ileal digesta was increased in pigs fed NC and ESBM3 compared to pigs fed ESBM1 and ESBM2 (quadratic, P = 0.058) but diet did not affect the WBC of colonic digesta (P > 0.10). Further, diet did not impact the pH of ileal or colonic digesta (P > 0.10).

Volatile fatty acids

In the ileum, increasing the inclusion of ESBM increased the concentration of acetate, butyrate, and total VFA (linear, P < 0.05; Table 3.9). Increasing ESBM linearly and quadratically increased propionate concentration in the ileum (linear, P = 0.006; quadratic, P = 0.003). The molar proportion (%) of ileal acetate was not affected by diet (P > 0.10), but the proportion of propionate was increased by NC and ESBM3 compared to ESBM1 and ESBM2 (quadratic, P = 0.029). The molar proportion of butyrate in the ileum was increased by ESBM3 compared to NC, ESBM1 and ESBM2 (diet, P = 0.023; linear, P = 0.019).

Diet did not affect the concentration of acetate, propionate, butyrate, or total VFA in the colon (P > 0.10). Feeding ESBM1 and ESBM3 increased the molar proportion of acetate and decreased butyrate in the colon more than ESBM2 but not NC (diet, P < 0.05). The molar proportion of propionate in the colon tended to decrease as the inclusion of ESBM increased (linear, P = 0.097).

Oxidative status, mucosal cytokines, and lipopolysaccharide-binding protein

The concentration of LBP in serum was not impacted by diet (P > 0.10; Table 3.10). The total antioxidant capacity of plasma was increased as the inclusion of ESBM increased (linear, P

= 0.002). The MDA concentrations in plasma or ileal tissue were not impacted by diet (P > 0.10). There was no effect of diet on the following ileal mucosa cytokines: IFNγ, IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, or IL-12 (P > 0.10). The concentration of TNF-α was not detectable in any of the samples. The concentration of IL-4 increased as the inclusion of ESBM increased (linear, P = 0.005). The NC diet tended to increase GMC-SF concentration compared to ESBM1 and ESBM2, but not ESBM3 (quadratic, P = 0.081). The ESBM2 diet tended to increase IL-1RA concentration compared to NC and ESBM1 with ESBM3 being intermediate (diet, P = 0.092). The concentration of IL-18 was increased by NC and ESBM2 compared to ESBM1, but not ESBM3 (diet, P = 0.098).

Ileal tissue gene transcription

Ileal mRNA abundance of claudin-3 (CLDN3) and CLDN4 was not altered by diet (P > 0.10; Table 3.11). Feeding ESBM2 increased the abundance of occludin (OCLN) compared to ESBM3, with NC and ESBM1 being intermediate (quadratic, P = 0.049). The abundance of zonula-occludens-1 (ZO-1) was increased by ESBM1 compared to NC and ESBM3, but not ESBM2 (quadratic, P = 0.013).

Gut morphology

Villus height in the ileum was not impacted by diet (P > 0.10; Table 3.12). However, crypt depth was slightly decreased by increasing the inclusion of ESBM3 (linear, P = 0.069). No difference in the V:C ratio was observed (P > 0.10).

Discussion

Overall, feeding increasing levels of ESBM decreased final BW, ADG, and ADFI in a linear fashion, but had no impact on feed efficiency. The BW of pigs was decreased by feeding 21% ESBM during phase 1 or feeding greater than 7% ESBM in phase 2. Jones et al. (2018a)

reported results similar to our study, as feeding increasing levels of ESBM (6.7 to 20% ESBM in phase 1; 5 to 15% ESBM in phase 2) linearly decreased final BW, ADG, and ADFI. Further, 15% ESBM has been shown to decrease ADG and ADFI compared to the control diet, but feed efficiency was improved (Jones et al., 2018b). Contrary to our results, Zhu et al. (1998) and Zhou et al. (2011) both reported improved performance by feeding 3.5, 7, and 10.5 or 5, 10, and 15% ESBM, respectively. Other studies have reported increased ADG and G:F by feeding 9% ESBM and decreasing SBM (Ma et al., 2019a,b). These growth improvements are attributed to the increased nutrient digestibility and the reduced concentration of ANF in ESBM (Zhou et al., 2011).

In the current experiment, the reduced growth rate and feed intake that resulted from feeding increased ESBM was unexpected, but there are possible explanations. Increasing the inclusion of ESBM did not impact feed efficiency, suggesting that the decrease in ADG was due to reduced ADFI. Reduced feed intake could be attributed to the increased WHC of diets as the inclusion of ESBM increased. The WHC is a measure of a feedstuff's ability to hold water within its matrix (Giger-Reverdin, 2000). As feed particles move through the GIT and absorb water, the feed can swell and limit consumption (Anguita et al., 2007). It has been shown that the ADFI of pigs decreases as the WHC of feed increases (Kyriazakis and Emmans, 1995; Ndou et al., 2013). Further, Zhang et al. (2001) reported that the transit rate of digesta decreased in diets containing 19% ESBM compared to diets with 23.5% SBM or 19% ESBM plus 1% stachyose. Feed intake has been inversely linked to the transit rate of digesta through the GIT, so feeding ESBM could slow this transit rate and limit feed intake of pigs (Ratanpaul et al., 2019).

We observed improvements in overall fecal score and fecal DM when ESBM was included in the diet, indicating that the ESBM could reduce diarrhea in weaned pigs. The

ESBM1 diet also reduced the number of medical treatments required, but higher levels provided no benefit. Ma et al. (2019a) reported that feeding ESBM reduced the incidence of diarrhea in weaned pigs compared to conventional SBM. These results are likely due to the reduced concentration of antigenic proteins and NDO in ESBM. The hypersensitivity associated with glycinin and β-conglycinin in SBM has been linked to malabsorption of nutrients and diarrhea in young pigs (Zhang et al., 2003; Sun et al., 2008a). Further, pigs do not possess the endogenous enzymes that are needed to digest the galactooligosaccharides stachyose and raffinose (Dersjant-Li and Peisker, 2010). Makinde et al. (1996) hypothesized that decreased digestion and absorption of soybean carbohydrates in the small intestine would increase fermentation in the colon, affecting the osmolarity of the colonic contents and decreasing water absorption (Grahofer et al., 2016).

Although the NDO in SBM could contribute to increased diarrhea, it has been suggested that the NDO could also act as a prebiotic in the GIT of growing pigs (Smiricky-Tjardes et al., 2003). Undigested carbohydrates can be fermented by bacteria in the GIT, producing VFA that serve as an energy source for the host. The majority of microbial fermentation occurs in the cecum and colon, but limited fermentation takes place in the small intestine as well (Choct et al., 2010). Cervantes-Pahm and Stein (2010) reported that SBM has increased concentrations of stachyose and raffinose compared to ESBM. However, our results show a linear increase in acetate, butyrate, propionate, and total VFA concentration in ileal digesta associated with an increase in the inclusion level of ESBM and a reduction in the SBM content. Therefore, increasing the inclusion of ESBM may slow the digesta transit rate, giving microbes in the ileum more time to ferment NDO and other non-starch polysaccharides to produce VFAs (Zhang et al., 2001; Zhang et al., 2003). In contrast, ESBM inclusion did not impact the colonic concentration

of individual or total VFAs compared to SBM. The pH of colonic digesta was not changed by ESBM, which can be explained by the lack of differences in VFA concentration. Zhang et al. (2003) observed no differences in total VFA production in the ileum or colon when feeding diets with 0% SBM, 0% SBM plus 1% stachyose, or 20% SBM, indicating that the concentration of NDO in a complete corn-SBM diet is not high enough to have a prebiotic effect.

Weaning can cause oxidative stress due to an imbalance between the production of antioxidants and scavenging of reactive oxygen species (ROS), potentially reducing the activity of antioxidant enzymes and causing damage to DNA, lipids, or protein (Betteridge, 2000; Yin et al., 2014). Malondialdehyde is a product of lipid peroxidation that indicates oxidative damage to lipids by ROS (Del Rio et al., 2005). Total antioxidant capacity represents the ability of endogenous and dietary antioxidants to prevent oxidative damage (Ghiselli et al., 2000). We observed no change in MDA concentration in plasma or ileal tissue when feeding ESBM, however, the TAC of plasma linearly increased as the inclusion of ESBM increased and SBM decreased. Feeding ESBM has been shown to reduce MDA concentration and improve TAC in the serum of weaned pigs (Ma et al., 2019a,b). It was also reported that ESBM increased serum superoxide dismutase and glutathione peroxidase activity, indicating that feeding ESBM may alleviate oxidative stress and improve TAC by increasing antioxidant enzyme activity (Betteridge, 2000; Ma et al., 2019a,b).

The production of ROS and subsequent oxidative stress have also been linked to the immune response (Lugrin et al., 2014). The production of cytokines to signal and modulate an inflammatory response is a crucial step in activating the GIT immune system (Pié et al., 2004). In our study, there was a tendency for the ESBM treatments to decrease the ileal mucosal concentration of GM-CSF. The GM-CSF has a pro-inflammatory role and activates ROS-

producing neutrophils, further explaining the modulation of oxidative stress by ESBM (Shiomi et al., 2016). The mucosal concentration of IL-4 was linearly increased as the inclusion of ESBM increased. The IL-4 is an anti-inflammatory cytokine that helps regulate the hypersensitivity reaction caused by glycinin and β-conglycinin by stimulating the production of immunoglobulin (Ig) E and differentiation of type 2 helper T-cells (Sun et al., 2008b). Multiple studies have reported increases in serum or mucosal IL-4 concentration when pigs are fed purified glycinin, making our results unexpected because the concentration of glycinin is reduced in ESBM (Sun et al., 2008b; Wu et al., 2016).

The intestinal epithelial barrier acts in a defensive role to prevent antigens, pathogens, and toxins from translocating through the lumen into the body (Awad et al., 2017). The paracellular permeability of the epithelium is maintained by transmembrane tight junction (TJ) proteins. A decrease in the mRNA abundance of TJ proteins, which typically occurs during weaning or an immune response, can indicate disruption of TJ protein complexes and increased permeability of the epithelial barrier (Hu et al., 2013). In our study, the highest mRNA abundance of *OCLN* or *ZO-1* was observed in the ESBM1 or ESBM2 treatments compared to NC or ESBM3, resulting in a quadratic response. These improvements in TJ protein mRNA abundance are likely due to the reduced concentration of glycinin and β-conglycinin in ESBM. However, the reduced mRNA abundance of *OCLN* and *ZO-1* after feeding the ESBM3 diet was unexpected and cannot be explained by this study. The ESBM3 diet should have contained the lowest levels of glycinin and β-conglycinin, resulting in an improved mRNA abundance of TJ proteins compared to NC. Similar to our study, Ma et al. (2019a) reported increased protein abundance of *OCLN* and *ZO-1* after feeding 9% ESBM. Further, Zhao et al. (2014) reported decreases in the mRNA abundance of *OCLN* and *ZO-1* when intestinal porcine epithelial cells

were treated with glycinin or β-conglycinin. The mechanisms causing this reduction are largely unknown but may be linked to the increased apoptosis of enterocytes when antigenic proteins are fed (Bojarski et al., 2004; Zhao et al., 2010).

The main epithelial cells lining the villi in the small intestine are absorptive enterocytes, so villus atrophy results in less surface area for nutrient absorption to occur (Yang and Liao, 2019). Villus atrophy without crypt hyperplasia typically occurs after weaning due to decreased feed intake and slowed production of crypt-cells (Pluske et al., 1997). However, the antigenic proteins in SBM can cause villus atrophy and crypt hyperplasia, indicating an increased rate of cell loss on the villi (Li et al., 1991; Pluske et al., 1997). Improvements in duodenal morphology after feeding ESBM have been reported, but the changes in ileal morphology have been inconsistent (Ma et al., 2019a,b). Though our results showed no impact of ESBM on villus height or V:C in the ileum, there was a tendency for increasing ESBM to reduce crypt depth. Feed intake decreased as the inclusion of ESBM increased, possibly resulting in villus atrophy without crypt hyperplasia. Therefore, the antigenic proteins in SBM may have impacted villus height but treatment differences could not be differentiated.

In conclusion, the pigs that were fed the two highest levels of ESBM (14 or 21% in phase 1; 7 or 10.5% in phase 2) had decreased overall BW, ADG, and ADFI compared to pigs fed the control and lowest ESBM diets. However, the inclusion of ESBM did not have an impact on the overall feed efficiency of pigs. Our hypothesis was partially incorrect, as increasing the inclusion of ESBM linearly decreased growth performance rather than improving it. This response may be driven by reductions in feed intake due to increased WHC of ESBM diets. However, the inclusion of ESBM did beneficially modulate markers of oxidative stress and intestinal health and function. Feeding ESBM improved overall fecal score and increased fecal DM, indicating

that ESBM could reduce diarrhea in weaned pigs. Further, the ESBM appeared to increase ileal fermentation of carbohydrates due to increased VFA production, but this response did not occur in the colon. Oxidative status and intestinal barrier integrity were improved by ESBM, but the impact on intestinal inflammation and morphology was minimal. This research identified key aspects of intestinal health and function that may be improved by replacing portions of soybean meal with ESBM. However, further research is needed to determine the ideal inclusion level of ESBM to optimize growth performance while benefiting various aspects of GIT physiology and function.

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Table 3.1. Ingredient and nutrient composition of experimental diets (as-fed basis): phase 1[1,2,3].

	Phase 1			
Item	NC	ESBM1	ESBM2	ESBM3
Ingredient composition, %				
Corn	36.49	38.27	39.91	41.27
Soybean meal[4]	25.75	16.97	8.35	-
Oat groats	12.50	12.50	12.50	12.50
Whey permeate	15.00	15.00	15.00	15.00
Milk casein	3.00	3.00	3.00	3.00
Enzymatically-treated soybean meal[5]	-	7.00	14.00	21.00
Corn oil	3.00	3.00	3.00	3.00
L-lysine HCl	0.51	0.51	0.50	0.49
DL-methionine	0.28	0.28	0.27	0.27
L-threonine	0.27	0.26	0.25	0.24
L-tryptophan	0.02	0.03	0.03	0.04
L-valine	0.10	0.08	0.05	0.03
Monocalcium phosphate 21%	0.57	0.57	0.57	0.57
Limestone	1.44	1.47	1.50	1.52
Salt	0.58	0.58	0.58	0.58
Vitamin premix[6]	0.24	0.24	0.24	0.24
Trace mineral premix[7]	0.20	0.20	0.20	0.20
Phytase[8]	0.05	0.05	0.05	0.05
Calculated nutrients				
Total Lys, %	1.53	1.53	1.53	1.53
SID Lys[9], %	1.40	1.40	1.40	1.40
SID TSAA:Lys[10]	0.58	0.58	0.58	0.58
SID Thr:Lys	0.61	0.61	0.61	0.61
SID Trp:Lys	0.18	0.18	0.18	0.18
NDF[11], %	6.65	6.42	6.19	5.96
Ca, %	0.85	0.85	0.85	0.85
STTD P[12], %	0.43	0.43	0.43	0.43
ME[13], Mcal/kg	3.41	3.42	3.44	3.45
NE[14], Mcal/kg	2.46	2.50	2.54	2.58
Analyzed nutrients				
Dry matter, %	88.83	89.91	90.99	91.06
Ash, %	5.74	5.78	6.17	6.07
Crude protein, %	19.91	19.57	20.23	20.86
aEE[15], %	5.72	5.76	5.97	6.04
Total Lys, %	1.47	1.34	1.54	1.34
SID Lys, %	1.35	1.23	1.41	1.23
SID TSAA, %	0.71	0.76	0.74	0.81
SID TSAA:Lys	0.51	0.58	0.55	0.65
SID Thr, %	0.83	0.85	0.83	0.85
SID Thr:Lys	0.59	0.65	0.61	0.68
SID Trp, %	0.22	0.23	0.22	0.23
SID Trp:Lys	0.16	0.18	0.16	0.18

Table 3.1. continued.

	Phase 1			
Item	NC	ESBM1	ESBM2	ESBM3
SID Ile, %	0.74	0.78	0.80	0.83
SID Ile:Lys	0.53	0.60	0.59	0.66
SID Val, %	0.86	0.95	0.93	0.95
SID Val:Lys	0.61	0.73	0.69	0.76
GE[16], Mcal/kg	3.91	3.94	3.94	3.99
Ca, %	0.70	0.85	0.91	0.87
Total P, %	0.56	0.55	0.57	0.56
WHC[17], mL/g of dry matter	1.08	1.14	1.22	1.40

[1] Phase 1 was fed from d 0-14.
[2] All diets were formulated to meet or exceed nutrient requirements of the pigs (NRC, 2012).
[3] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal
[4] Dehulled, solvent-extracted soybean meal
[5] HP 300 (Hamlet Protein Inc., Findlay, OH).
[6] The vitamin premix provided per kg of complete diet: 7,350 IU vitamin A, 840 IU vitamin D3, 60 IU vitamin E, 3.6 mg vitamin K, 13.2 mg riboflavin, 67.2 mg niacin, 32.4 mg pantothenic acid, and 60 μg vitamin B_{12}.
[7] The trace mineral premix provided per kg of complete diet: 160 ppm Fe as $FeSO_4$, 160 ppm Zn as $ZnSO_4$, 9 ppm Mn as $MnSO_4$, 12 ppm Cu as $CuSO_4$, 0.3 ppm I as $C_2H_{10}I_2N_2$ or KIO_3, and 0.3 ppm Se as Na_2SeO_4 or Na_2SeO_3.
[8] Quantum Blue 5 G (AB Vista Feed Ingredients, Marlborough, Wiltshire, UK) was added at 0.05% for 2,500 FTU/kg.
[9] SID = standard ileal digestible
[10] TSAA = total sulfur amino acids (Met + Cys)
[11] NDF = neutral detergent fiber
[12] STTD = standardized total tract digestible
[13] ME = metabolizable energy
[14] NE = net energy
[15] aEE = acid-hydrolyzed ether extract
[16] GE = gross energy
[17] WHC = water-holding capacity

Table 3.2. Ingredient and nutrient composition of experimental diets (as-fed basis): phase 2 to 3[1,2,3].

	Phase 2				Phase 3
Item	NC	ESBM1	ESBM2	ESBM3	NC
Ingredient composition, %					
Corn	50.81	51.75	52.69	53.68	61.81
Soybean meal[4]	28.84	24.40	19.95	15.46	31.41
Oat groats	5.00	5.00	5.00	5.00	-
Whey permeate	7.50	7.50	7.50	7.50	-
Milk casein	0.85	0.85	0.85	0.85	-
Enzymatically-treated soybean meal[5]	-	3.50	7.00	10.50	-
Corn oil	3.00	3.00	3.00	3.00	3.00
L-lysine HCl	0.50	0.50	0.50	0.50	0.42
DL-methionine	0.26	0.26	0.26	0.26	0.22
L-threonine	0.25	0.24	0.24	0.23	0.19
L-tryptophan	0.02	0.02	0.02	0.02	-
L-valine	0.12	0.11	0.10	0.09	0.05
Monocalcium phosphate 21%	0.52	0.52	0.52	0.52	0.57
Limestone	1.29	1.30	1.32	1.33	1.23
Salt	0.56	0.56	0.56	0.56	0.61
Vitamin premix[6]	0.24	0.24	0.24	0.24	0.24
Trace mineral premix[7]	0.20	0.20	0.20	0.20	0.20
Phytase[8]	0.05	0.05	0.05	0.05	0.05
Calculated nutrients					
Total Lys, %	1.43	1.43	1.43	1.43	1.37
SID Lys[9], %	1.30	1.30	1.30	1.30	1.23
SID TSAA:Lys[10]	0.58	0.58	0.58	0.58	0.58
SID Thr:Lys	0.61	0.61	0.61	0.61	0.60
SID Trp:Lys	0.18	0.18	0.18	0.18	0.18
NDF[11], %	7.48	7.37	7.25	7.14	8.21
Ca, %	0.75	0.75	0.75	0.75	0.70
STTD P[12], %	0.38	0.38	0.38	0.38	0.35
ME[13], Mcal/kg	3.40	3.41	3.42	3.42	3.40
NE[14], Mcal/kg	2.48	2.50	2.52	2.54	2.50
Analyzed nutrients					
Dry matter, %	88.66	88.96	89.35	89.88	87.47
Ash, %	5.49	5.67	5.62	5.66	5.28
Crude protein, %	18.64	18.57	19.32	19.07	17.44
aEE[15], %	6.15	6.15	6.08	5.94	6.41
Total Lys, %	1.43	1.43	1.50	1.47	1.39
SID Lys, %	1.30	1.30	1.37	1.33	1.24
SID TSAA, %	0.63	0.73	0.79	0.76	0.75
SID TSAA:Lys	0.48	0.56	0.58	0.57	0.60
SID Thr, %	0.86	0.79	0.75	0.81	0.80
SID Thr:Lys	0.66	0.61	0.55	0.61	0.65
SID Trp, %	0.20	0.20	0.20	0.21	0.21
SID Trp:Lys	0.15	0.15	0.15	0.16	0.17

Table 3.2. continued.

	Phase 2				Phase 3
Item	NC	ESBM1	ESBM2	ESBM3	NC
SID Ile, %	0.67	0.69	0.73	0.70	0.69
SID Ile:Lys	0.52	0.53	0.53	0.53	0.56
SID Val, %	0.84	0.83	0.88	0.89	0.87
SID Val:Lys	0.65	0.64	0.64	0.67	0.70
GE[16], Mcal/kg	3.90	3.91	3.94	3.94	3.86
Ca, %	0.79	0.76	0.81	0.75	0.72
Total P, %	0.50	0.49	0.51	0.50	0.49
WHC[17], mL/g of dry matter	1.40	1.49	1.44	1.46	1.21

[1] Phase 2 was fed from d 14-28 and phase 3 was fed from d 28-35. Phase 3 was a common diet across all treatments.
[2] All diets were formulated to meet or exceed nutrient requirements of the pigs (NRC, 2012).
[3] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 3.5% enzymatically-treated soybean meal; ESBM2: 7% enzymatically-treated soybean meal; ESBM3: 10.5% enzymatically-treated soybean meal
[4] Dehulled, solvent-extracted soybean meal
[5] HP 300 (Hamlet Protein Inc., Findlay, OH).
[6] The vitamin premix provided per kg of complete diet: 7,350 IU vitamin A, 840 IU vitamin D3, 60 IU vitamin E, 3.6 mg vitamin K, 13.2 mg riboflavin, 67.2 mg niacin, 32.4 mg pantothenic acid, and 60 μg vitamin B_{12}.
[7] The trace mineral premix provided per kg of complete diet: 160 ppm Fe as $FeSO_4$, 160 ppm Zn as $ZnSO_4$, 9 ppm Mn as $MnSO_4$, 12 ppm Cu as $CuSO_4$, 0.3 ppm I as $C_2H_{10}I_2N_2$ or KIO_3, and 0.3 ppm Se as Na_2SeO_4 or Na_2SeO_3.
[8] Quantum Blue 5 G (AB Vista Feed Ingredients, Marlborough, Wiltshire, UK) was added at 0.05% for 2,500 FTU/kg.
[9] SID = standard ileal digestible
[10] TSAA = total sulfur amino acids (Met + Cys)
[11] NDF = neutral detergent fiber
[12] STTD = standardized total tract digestible
[13] ME = metabolizable energy
[14] NE = net energy
[15] aEE = acid-hydrolyzed ether extract
[16] GE = gross energy
[17] WHC = water-holding capacity

Table 3.3. Primers used for real-time quantitative polymerase chain reaction (RT-qPCR).

Gene[1]	Primer sequence, 5'→3'[2]	Product size, base pair	GenBank accession	Annealing Temperature, °C
CLDN3	F: TTGCATCCGAGACCAGTCC R: AGCTGGGGAGGGTGACA	85	NM_001160075	60
CLDN4	F: CAACTGCGTGGATGATGAGA R: CCAGGGGATTGTAGAAGTCG	140	NM_001161637	60
OCLN	F: TCGTCCAACGGGAAAGTGAA R: ATCAGTGGAAGTTCCTGAACCA	95	NM_001163647	55
ZO-1	F: CTCTTGGCTTGCTATTCG R: AGTCTTCCCTGCTCTTGC	197	XM_003353439	55
RPL19	F: AACTCCCGTCAGCAGATCC R: AGTACCCTTCCGCTTACCG	147	AF_435591	55

[1] *CLDN3 = claudin-3*; *CLDN4= claudin-4*; *OCLN = occludin*; *ZO-1 = zonula occludens-1*; *RPL19 = ribosomal protein-L19*
[2] F = forward primer; R = reverse primer

Table 3.4. The effect of increasing enzymatically-treated soybean meal on the number of medical treatments[1,2,3].

						P-value		
Item	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
Medical treatments, proportion[4]	0.30[a]	0.17[b]	0.36[a]	0.25[ab]	0.05	0.038	0.840	0.787

[1] Data are least square means; n = 12 pens per treatment with 10 pigs per pen, totaling 480 pigs; 35 d growth experiment

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal. The inclusion of ESBM decreased by half in phase 2. Phase 3 was a common diet with no ESBM.

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] Medical treatments were calculated as the total number of medical treatments administered per pen divided by number of pigs allotted to pen.

Table 3.5. The effect of increasing enzymatically-treated soybean meal on weekly fecal score[1,2,3,4].

					Pooled	*P*-value[5]	
Item	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Diet × Period
Fecal score[6]					0.2	0.003	0.409
d 7	2.0	1.3	1.7	1.5			
d 14	1.3	1.2	1.0	1.1			
d 21	1.2	1.1	1.0	1.1			
d 28	1.2	1.1	1.0	1.0			
d 35	1.1	1.0	1.0	1.0			
Overall	1.4[a]	1.1[b]	1.2[b]	1.1[b]			

[1] Data are least square means; n = 12 pens per treatment with 10 pigs per pen, totaling 480 pigs; 35 d growth experiment

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal. The inclusion of ESBM decreased by half in phase 2. Phase 3 was a common diet with no ESBM.

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] Dietary phases: phase 1 (d 0-14), phase 2 (d 14-28), and phase 3 (d 28-35).

[5] Period was significant for this variable ($P < 0.001$).

[6] Fecal scoring: 1 = solid, 2 = semi-solid, 3 = semi-liquid, 4 = liquid; pens were independently scored by two people and the score was averaged.

Table 3.6. The effect of increasing enzymatically-treated soybean meal on overall growth performance and feed efficiency of pigs[1,2,3,4].

						P-value		
Item[5]	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
Pens per treatment	12	12	12	12	-	-	-	-
Pigs per treatment, initial	120	120	120	120	-	-	-	-
Pigs per treatment, final[6]	106	108	106	106	-	-	-	-
Start BW (d 0)	6.3	6.3	6.4	6.3	0.01	0.324	0.712	0.141
Final BW (d 35)	20.0[a]	20.1[a]	19.0[b]	18.8[b]	0.3	0.001	<0.001	0.588
ADG, kg	0.38[a]	0.39[a]	0.35[b]	0.35[b]	0.01	0.001	<0.001	0.383
ADFI, kg	0.53[a]	0.54[a]	0.50[b]	0.50[b]	0.01	0.001	<0.001	0.646
G:F	0.71	0.71	0.71	0.70	0.004	0.302	0.108	0.323

[1] Data are least square means; n = 12 pens per treatment with 10 pigs per pen, totaling 480 pigs; 35 d growth experiment; growth calculations included pig days to account for removed pigs.

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal. The inclusion of ESBM decreased by half in phase 2. Phase 3 was a common diet with no ESBM.

[3] Dietary phases: phase 1 (d 0-14), phase 2 (d 14-28), and phase 3 (d 28-35).

[4] Means within a row without a common superscript differ significantly ($P < 0.05$).

[5] BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain:feed ratio

[6] 12 pigs were removed from each treatment for necropsy on d 10.

Table 3.7. The effect of increasing enzymatically-treated soybean meal on growth performance and feed efficiency of pigs by phase analyzed as a mixed model with a time dependent variance structure[1,2,3,4].

Item[5]	NC	ESBM1	ESBM2	ESBM3	Pooled SEM	*P*-value[6]	
						Diet	Diet × Period
BW, kg					0.4	0.017	0.039
d 0	6.3[a]	6.3[a]	6.4[a]	6.3[a]			
d 14	9.1[b]	9.2[b]	8.9[bc]	8.6[c]			
d 28	16.0[d]	15.9[d]	15.1[e]	14.7[e]			
d 35	20.0[f]	20.1[f]	19.0[g]	18.8[g]			
ADG, kg					0.02	0.015	0.292
d 0-14	0.20	0.21	0.18	0.17			
d 14-28	0.48	0.47	0.43	0.42			
d 28-35	0.58	0.59	0.56	0.59			
ADFI, kg					0.02	0.018	0.396
d 0-14	0.26	0.26	0.24	0.22			
d 14-28	0.67	0.67	0.62	0.61			
d 28-35	0.89	0.88	0.84	0.86			
G:F					0.01	0.243	0.008
d 0-14	0.82[a]	0.81[ab]	0.78[bc]	0.76[c]			
d 14-28	0.71[de]	0.72[d]	0.71[de]	0.69[ef]			
d 28-35	0.65[g]	0.65[g]	0.67[fg]	0.68[f]			

[1] Data are least square means; n = 12 pens per treatment with 10 pigs per pen, totaling 480 pigs; 35 d growth experiment; growth calculations included pig days to account for removed pigs.

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal. The inclusion of ESBM decreased by half in phase 2. Phase 3 was a common diet with no ESBM.

[3] Dietary phases: phase 1 (d 0-14), phase 2 (d 14-28), and phase 3 (d 28-35).

[4] Within a dependent variable, means without a common superscript (a-g) differ significantly ($P < 0.05$).

[5] BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain:feed ratio

[6] Period was significant for all variables ($P < 0.001$).

Table 3.8. The effect of increasing enzymatically-treated soybean meal on fecal and digesta characteristics[1,2,3].

						P-value		
Item	NC	EBSM1	EBSM2	EBSM3	SEM	Diet	Linear	Quadratic
Fecal analyses								
Dry matter, %	20.40[a]	20.91[a]	22.63[b]	24.62[c]	0.57	<0.001	<0.001	0.204
Ileal digesta analyses								
WBC[4], mL/g of dry digesta	1.39	0.99	0.73	1.37	0.27	0.237	0.797	0.058
pH	6.23	6.32	6.20	6.45	0.17	0.668	0.435	0.622
Colonic digesta analyses								
WBC[4], mL/g of dry digesta	0.23	0.21	0.15	0.19	0.05	0.701	0.437	0.599
pH	5.84	5.66	5.85	5.69	0.09	0.245	0.479	0.905

[1] Data are least square means; n = 12 replicates per treatment; fecal samples were collected from each pen on d 11-13; digesta samples were collected on d 10

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] WBC = water-binding capacity

Table 3.9. The effect of increasing enzymatically-treated soybean meal on volatile fatty acid (VFA) concentration and molar proportions in digesta[1,2,3].

						P-value		
Item	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
Ileal digesta VFA, mM/L								
Acetate	3.27[a]	4.06[a]	4.50[ab]	6.98[b]	0.86	0.029	0.005	0.329
Propionate	0.10[a]	0.04[a]	0.08[a]	0.23[b]	0.03	0.002	0.006	0.003
Butyrate	0.02[a]	0.02[a]	0.02[a]	0.06[b]	0.01	0.029	0.015	0.071
Total	3.47	4.08	4.67	6.70	0.95	0.100	0.018	0.436
Ileal digesta VFA molar proportion, %								
Acetate	95.80	97.98	95.89	96.02	0.78	0.161	0.671	0.187
Propionate	3.48	1.29	2.15	3.53	0.80	0.146	0.773	0.029
Butyrate	0.53[a]	0.66[a]	0.52[a]	1.11[b]	0.15	0.023	0.019	0.137
Colonic digesta VFA, mM/L								
Acetate	74.73	82.58	69.75	80.81	9.05	0.717	0.892	0.857
Propionate	19.30	22.37	19.13	20.05	3.50	0.895	0.948	0.752
Butyrate	10.93	7.85	9.21	8.62	1.55	0.533	0.415	0.407
Total	103.08	109.31	96.77	103.05	11.85	0.897	0.808	0.998
Colonic digesta VFA molar proportion, %								
Acetate	73.04[ab]	75.64[a]	71.30[b]	76.97[a]	1.39	0.030	0.239	0.278
Propionate	18.96	17.68	19.11	16.10	0.93	0.103	0.097	0.362
Butyrate	8.58[ab]	6.57[a]	9.46[b]	6.61[a]	0.80	0.034	0.409	0.605

[1] Data are least square means; n = 12 replicates per treatment; ileal and colonic digesta samples were collected on d 10

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

Table 3.10. The effect of increasing enzymatically-treated soybean meal on lipopolysaccharide binding protein, markers of oxidative status, and mucosal cytokines[1,2,3].

						P-value		
Item	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
Serum measures								
LBP[4], μg/mL	21.7	21.3	22.2	20.8	2.8	0.986	0.888	0.863
Plasma measures[5]								
MDA, μM/μL	12.95	14.34	14.05	12.67	1.01	0.554	0.790	0.161
TAC, mM trolox	4.09[a]	4.11[a]	4.58[b]	4.49[b]	0.11	0.006	0.002	0.617
Ileal tissue measures[5]								
MDA, μM/μg protein	0.63	0.62	0.68	0.52	0.05	0.194	0.276	0.149
Ileal mucosa measures[6], ng/g								
GM-CSF	0.61	0.39	0.44	0.48	0.07	0.175	0.282	0.081
IFNγ	20.33	19.08	19.01	18.98	1.46	0.895	0.532	0.680
IL-1α	1.75	1.39	1.59	1.56	0.15	0.383	0.551	0.260
IL-1β	18.01	15.86	16.18	16.45	2.96	0.941	0.715	0.658
IL-1RA	8.46	8.43	11.61	9.26	0.98	0.092	0.213	0.244
IL-2	1.33	1.20	1.29	1.31	0.10	0.802	0.939	0.472
IL-4	0.63[a]	0.65[a]	1.17[b]	1.01[b]	0.12	0.009	0.005	0.484
IL-6	2.34	2.21	2.26	2.22	0.07	0.568	0.343	0.542
IL-8	491.27	394.24	426.54	448.03	45.11	0.447	0.622	0.180
IL-10	0.41	0.36	0.42	0.40	0.04	0.764	0.883	0.725
IL-12	4.75	3.73	4.64	4.34	0.58	0.605	0.908	0.537
IL-18	180.04	138.52	180.88	167.79	13.12	0.098	0.924	0.286

[1] Data are least square means; n = 12 replicates per treatment; all tissue and blood samples were collected on d 10

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] LBP = lipopolysaccharide-binding protein

[5] MDA = malondialdehyde; TAC = total antioxidant capacity

[6] Granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon-γ (IFNγ); interleukin-1α (IL-1α); interleukin-1β (IL-1β); interleukin-1 receptor antagonist (IL-1RA); interleukin-2 (IL-2); interleukin-4 (IL-4); interleukin-6 (IL-6); interleukin-8 (IL-8); interleukin-10 (IL-10); interleukin-12 (IL-12); interleukin-18 (IL-18)

Table 3.11. The effect of increasing enzymatically-treated soybean meal on relative ileal gene messenger ribonucleic acid (mRNA) abundance[1,2,3].

						P-value		
Gene[4]	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
CLDN3	1.20	1.69	1.25	0.84	0.45	0.679	0.538	0.570
CLDN4	1.13	1.65	1.15	0.98	0.29	0.313	0.429	0.188
OCLN	1.59	2.22	2.44	0.87	0.59	0.191	0.369	0.049
ZO-1	1.17	1.86	1.58	1.13	0.29	0.091	0.771	0.013

[1] Data are least square means; n = 12 replicates per treatment; ileal tissue samples were collected on d 10

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

[4] *CLDN3: claudin-3; CLDN4: claudin-4; OCLN: occludin; ZO-1: zonula occludens-1*

Table 3.12. The effect of increasing enzymatically-treated soybean meal on ileal morphology[1,2,3].

						P-value		
Item	NC	ESBM1	ESBM2	ESBM3	SEM	Diet	Linear	Quadratic
Villus height, µm	342.7	365.2	341.8	322.1	15.5	0.294	0.228	0.182
Crypt depth, µm	244.8	234.1	242.4	222.6	6.9	0.120	0.069	0.517
Villi height:crypt depth	1.5	1.7	1.5	1.5	0.1	0.387	0.598	0.431

[1] Data are least square means; n = 12 replicates per treatment; ileal tissue samples were collected on d 10 and fixed in 10% neutral-buffered formalin for 48 hours followed by 70% ethanol before histology slides were made

[2] NC: negative control, 0% enzymatically-treated soybean meal; EBSM1: 7% enzymatically-treated soybean meal; ESBM2: 14% enzymatically-treated soybean meal; ESBM3: 21% enzymatically-treated soybean meal

[3] Means within a row without a common superscript differ significantly ($P < 0.05$).

CHAPTER 4. INTEGRATIVE SUMMARY

General discussion

Weaning is a critical time during a pig's life when they are exposed to several stressors (such as dietary, social, and environmental) and unfamiliar pathogens. The culmination of these stressors is reduced growth and feed intake directly after weaning, as well as disruptions in gastrointestinal (GIT) health and increased susceptibility to enteric pathogens (Lallès et al., 2004; Moeser et al., 2006). The pork industry has increased its interest in identifying feed ingredients, specifically specialty proteins, that can be fed to mitigate the detrimental effects of weaning, rather than exacerbating the stress (Pluske et al., 2013). In-feed antibiotics have typically been included in nursery pig diets for their growth promoting and antimicrobial effects. However, antimicrobial resistance concerns and increased government regulation of in-feed antibiotics have prompted the industry to look for potential alternatives (Gaskins et al., 2006; Olsen et al., 2018).

Functional proteins, such as spray-dried plasma protein (SDPP) and hyperimmunized dried egg protein (DEP), are a class of specialty proteins that can beneficially alter GIT health and modulate immune responses (Pettigrew, 2006). Feeding SDPP is known to improve pig performance and positively impact the GIT, but some producers are hesitant to feed this protein due to biosecurity and disease transmission concerns (Gerber et al., 2014; Tran et al., 2014). The DEP has been identified as a potential alternative to both antibiotics and SDPP in nursery diets. The efficacy of DEP has been shown during *Escherichia coli* (*E. coli*) challenge studies, but the industry is lacking an understanding of this ingredient's true mode of action (MOA) and impact under commercial conditions (Crenshaw et al., 2017; Wang et al., 2019). Enzymatically-treated

soybean meal (ESBM) is another specialty protein that may be used to prevent the escalation of weaning stress in pigs. This ingredient is a further processed form of conventional soybean meal (SBM), in which the concentrations of harmful antinutritional factors (ANF) have been reduced (Cervantes-Pahm and Stein, 2010). Feeding ESBM may improve performance when it is used to reduce dietary SBM inclusion, but results are inconsistent. There is also a lack of research investigating the MOA of this ingredient in the GIT (Zhou et al., 2011; Ma et al., 2019a,b).

Therefore, the primary objective of this thesis research was to investigate methods in which these specialty proteins could be fed to pigs to help them overcome the stressors of weaning and improve performance and markers of GIT health and function. In order to achieve this objective and develop a better understanding of how specialty proteins may impact nursery pig performance and health, two experiments were performed to determine 1) if specialty protein ingredients could be a potential alternative to in-feed antibiotics in early nursery diets, and 2) how specialty proteins can be used to lower the inclusion of ANF-containing SBM in nursery diets.

Specifically, experiment 1 (Chapter 2) compared the effects of feeding SDPP or DEP, with or without subtherapeutic antibiotics in the phase 1 and 2 diets, on growth performance and markers of intestinal health and function in nursery pigs raised in a commercial environment. The factorial design of this experiment was a novel method of evaluating SDPP and DEP in feeding programs with differing antibiotic statuses, as there are very few published studies that have evaluated these proteins with or without in-feed antibiotics. The specific objective of experiment 2 (Chapter 3) was to determine the impact of diets in which ESBM replaced increasing amounts of SBM on growth performance, intestinal structure and barrier integrity, inflammation, and oxidative status in newly weaned pigs. There are few studies that directly

evaluate how feeding ESBM to reduce SBM inclusion in the diet affects various markers of GIT health and physiology. Further, there are none that evaluate these markers using a titration of increasing ESBM and decreasing SBM levels. In this thesis, it was hypothesized that feeding the specialty proteins SDPP and DEP in antibiotic-free diets or increasing the inclusion of ESBM and decreasing SBM would positively impact the growth performance of weaned pigs while beneficially modulating markers of GIT health and function.

The results of the aforementioned experiments have shown that feeding specialty proteins can impact the growth performance of weaned pigs, but this impact is inconsistent and not always beneficial to the pig. In experiment 1, feeding both SDPP or DEP improved the rate of gain and feed intake of pigs when antibiotics were not included in the phase 1 and 2 diets. When the diets contained antibiotics, neither SDPP nor DEP impacted piglet growth performance. These results agree with previous studies that reported performance improvements when feeding SDPP. Some studies that have evaluated DEP in commercial nurseries have seen no response to the protein (Crenshaw et al., 2017; Balan et al., 2020). In experiment 2, feeding the two highest levels of ESBM (14 or 21% in phase 1; 7 or 10.5% in phase 2) linearly decreased the final body weight and overall rate of gain and feed intake of pigs compared to those fed the control (0% ESBM) or lowest ESBM diet (7 or 3.5% in phases 1 and 2). The linear decrease in overall growth performance was unexpected as multiple studies have reported improvements in performance when feeding ESBM, even in titration studies (Zhu et al., 1998; Zhou et al., 2011). However, upon further evaluation of the literature, it was revealed that the growth response to ESBM has been somewhat inconsistent and others have reported similar reductions in performance (Jones et al., 2018a,b).

The data from this thesis show conflicting results regarding the impact of specialty proteins on weaned pig performance, but these discrepancies may be due to the differing MOA of the specialty protein ingredients and the housing environment in which the proteins were tested. The beneficial effects of feeding SDPP and DEP are both attributed to their immunoglobulin (Ig) content, as both IgG and IgY have been shown to inhibit pathogen adhesion to the intestinal epithelium, therefore reducing the proliferation of enteric pathogens and the release of harmful enterotoxins (Bosi et al., 2004; Wang et al., 2019). The benefit of feeding ESBM is likely due to the decreased dietary inclusion of SBM and subsequent ANF when ESBM levels are increased in the diet, rather than any intrinsic component of ESBM itself (Zhou et al., 2011). This reduction in ANF in the complete diet could reduce the hypersensitivity response or diarrhea associated with high concentrations of ANF in early nursery diets (Ma et al., 2019a,b).

During the evaluation of SDPP and DEP in experiment 1, the pigs were housed in a commercial research nursery with 20-21 pigs per pen and were naturally challenged with multiple enteric pathogens (such as porcine rotavirus and *Salmonella*). Although the true impact of these health challenges cannot be determined (due to the lack of a nonchallenged control), this housing environment likely provided a more commercially relevant model in which to evaluate SDPP and DEP with or without the use of in-feed antibiotics. Since the pathogen challenge likely reduced pig performance and health more than just the normal effects of weaning, there would be more potential for the SDPP and DEP to improve these parameters compared to the control diet. Antibiotics are known to have bactericidal and bacteriostatic effects, so the data showing that SDPP and DEP did not impact performance when antibiotics were fed supports this theory (Gaskins et al., 2006). Contrary to this study, experiment 2 was conducted in the nursery

facilities of a university research farm and with a lower housing density (10 pigs per pen). These pigs did not undergo any significant health challenges that would have further exacerbated any performance and/or GIT health issues associated with weaning. Even though the ANF in SBM may have resulted in a hypersensitivity response in pigs, the lower-stress housing environment may have reduced the potential for the increasing ESBM and decreasing SBM levels to mitigate weaning stress. Further, Olsen et al. (2018) suggested that the response to antibiotic alternative ingredients is less pronounced in studies that house pigs in smaller groups, presumably due to the situation being less stressful than that of larger group sizes.

Although the performance responses to specialty proteins differed between the two experiments, this thesis research further supported the belief that specialty proteins can beneficially modulate markers of GIT health and function. Both DEP and SDPP were able to modulate intestinal inflammation and improve gut morphology, indicating a positive effect on GIT health. Also, the reduced number of individual medical treatments that were required for pigs fed SDPP or DEP points to a pathogen-inhibition effect of both proteins (Abbas et al., 2019; Balan et al., 2020). In experiment 2, the inclusion of ESBM did beneficially modulate measures of oxidative stress, reduce GIT inflammation, increase volatile fatty acid production in the small intestine, and reduce the overall diarrhea score. Several of these results represent novel data since, to the best of our knowledge, they have not been previously published in the scientific literature; studies on the MOA of specialty proteins have been relatively rare (especially in the instance of DEP and ESBM). In commercial production, it is likely that a combination of several animal and plant-based specialty proteins will be included in nursery diets. Although it cannot be determined through this research, we can hypothesize that nursery diets containing combinations

of SDPP, DEP, and ESBM would provide even greater benefit to the GIT health and function of weaned pigs.

In conclusion, the research in this thesis has shown that specialty proteins provide some degree of benefit in the gut of pigs, but that their overall impact on growth performance can vary based on the composition of the experimental diets and the animal's environment. Our hypothesis was proven to be partially correct. Feeding the specialty proteins SDPP, DEP, and ESBM were shown to beneficially modulate markers of GIT health and function; however, growth performance was only improved by feeding SDPP or DEP in antibiotic-free diets. For producers and nutritionists, the decision to include specialty proteins in nursery diets will be impacted by several factors, such as herd health status, cost of the diet, and the antibiotic-status of the feeding program. The results of experiment 1 demonstrated that producers who are raising pigs in reduced or antibiotic-free systems may see benefit from the dietary inclusion of SDPP or DEP. However, in traditional systems that are antibiotic-positive, these specialty proteins will not provide the same value, at least not under the conditions of this trial. In experiment 2, the results indicated that feeding ESBM to reduce SBM in nursery diets can improve aspects of GIT health and physiology but increasing the inclusion of ESBM beyond 7 or 3.5% in phase 1 and 2 diets, respectively, may be detrimental to piglet growth performance. Unless pigs are exposed to high-stress situations, including ESBM in the diet may not provide enough benefit to be a cost-effective ingredient.

Recommendations for future research

The research results presented in this thesis support the belief that specialty protein ingredients can provide some sort of benefit to weaned pigs, whether it be through improved growth performance or beneficial modulation of GIT health markers. However, continued

research into the MOA of these proteins will provide the pork industry with a better understanding of their mechanisms and how to best feed specialty proteins to maximize their value in nursery diets. During future evaluations of SDPP and DEP, researchers should consider feeding a combined SDPP + DEP treatment to determine if there are additive effects between the two proteins. In commercial production, diets will contain more than just one functional specialty protein, and so it will be crucial to understand how these proteins interact. Due to the highly pathogen-specific nature of IgY and less specific nature of IgG (which is found in SDPP), a combination of SDPP and DEP may be more beneficial against the wide variety of pathogens to which commercial production would expose pigs (Warr et al., 1995). In order to increase the value of ESBM in nursery diets, there needs to be further exploration of the MOA of this ingredient and how it impacts growth in large, commercial-scale experiments. Further, more research is needed to determine an optimal inclusion of ESBM to maximize performance. Future studies may benefit from feeding lower inclusions of ESBM in the phase 1 and 2 nursery diets, as this ingredient would typically be fed at approximately 3-8% in the diet.

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