Evaluation of dietary factors limiting caloric and nutritional efficiency in pork production

A THESIS

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Dedication

I dedicate this dissertation to my parents, Shi-Long Hung and Shih-Chung Shao, who have loved me unconditionally and been providing persistent support and encouragement to me during the challenges of graduate school and life in the US.

Abstract

Swine nutrition research goes beyond diet formulation and feeding pigs. When considering a systems approach, swine nutrition research is indispensable to improve food security, animal well-being, animal health, sustainability, and the environmental impact of production systems. The One Health concept has emerged to ensure optimal health outcomes toward animals, humans, and the environment. This can be achieved in the swine industry by optimizing nutrient utilization of pigs and removing antimicrobial growth promoters (AGPs) from diets. With the aim to improve pig production performance, four studies were conducted to investigate dietary factors that limit caloric and nutritional efficiency of pigs. Research in chapters 2 and 3 indicated that lipid peroxidation has detrimental effects on animal health and growth performance of swine and poultry fed iso-caloric diets, whereas adding antioxidants enhance lipid stability preventing further lipid peroxidation of supplemental lipids, reducing incidence of oxidative stress in pigs. Research in chapter 4 revealed that diets with increased viscosity induced by dietary fiber changed intestinal morphology and digestive enzyme activities with decreased energy and nutrient digestibility in pigs. Research in chapter 5 characterized growth-related responses in the intestine of pigs fed AGPs, shedding light on the potential mechanisms of growth-promoting effects of antimicrobials that could guide the discovery of AGP alternatives. In this dissertation, a novel approach combining swine nutrition research and digestive physiology was adopted to pinpoint dietary factors limiting nutrient utilization and growth in pigs. The main findings from this research on feeding lipids, dietary fiber, and AGPs will help develop sustainable feeding programs for pigs and achieve One Health.

Table of Contents

List of Tables

Lists of Figures

List of Abbreviations

AA – Amino Acid

ACN – Acetonitrile

ADF – Acid Detergent Fiber

ADG – Average Daily Gain

ADFI – Average Daily Feed Intake

AGPs – Antimicrobial Growth Promoters

AID – Apparent Ileal Digestibility

AOCS – American Oil Chemists' Society

BW – Body Weight

AnV – Anisidine Value

BA – Bile Acid

BHA – Butylated hydroxyanisole

BHT – Butylated hydroxytoluene

CEL – Cellulose

CF – Crude Fiber

CI – Confidence Interval

Cu – Cupper

$CuSO_4$ – Cupric Sulfate

CMC – Carboxymethylcellulose

CP – Crude Protein

CSBM – Corn-Soybean Meal

DC – Dansyl Chloride

DDE – 2,4-Decadienal

DDGS – Distillers Dried Grains with Solubles

DE – Digestible Energy

DF – Dietary Fiber

DFM – Direct-Fed Microbials

DM – Dry Matter

EE – Diethyl Ether Exact

EQ – Ethoxyquin

EU – European Union

FA – Fatty Acids

GE – Gross Energy

G:F – Gain to Feed Ratio

GI – Gastrointestinal

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

GPx – Glutathione Peroxidase

H&E – Hematoxylin and Eosin

4-HNE – 4-Hydroxynonenal

HQ – 2-Hydrazinequinoline

HSI – Hepatosomatic Index

IL – Interleukin

LCA – Lithocholic Acid

LC-MS – Liquid Chromatography–Mass Spectrometry

MDA – Malonaldehyde

MRT – Mean Retention Time

MIU – Moisture, Insolubles, and Unsaponifiables

ME – Metabolizable Energy

NE – Net Energy

NIR – Near Infra-Red

NDF – Neutral Detergent Fiber

NSP – Non-Starch Polysaccharides

OSI – Oil Stability Index

PAS-AB – Periodic Acid Schiff-Alcian Blue

PDIFF – *p*-Values for Differences

PG – Propyl Gallate

PV – Peroxide Value

PWD – Post-Weaning Diarrhea

Se – Selenium

SCFA – Short-Chain Fatty Acids

TBARS – Thiobarbituric Acid Reactive Substances

TBHQ – tert-Butylhydroquinone

TDF – Total Dietary Fiber

TiO_2 – Titanium Dioxide

TNFα – Tumor Necrosis Factor

US – United States

WHC – Water-Holding Capacity

WBC – Water-Binding Capacity

Zn – Zinc

ZnO – Zinc Oxide

Chapter 1 – Literature review

Swine nutrition and One Health perspectives in pork production

Nowadays, some of the our major challenges are the growing demand for animal-derived products, the use of crops and grains used for intensive animal production instead of as human food, environmental degradation, and climate change (Nabarro and Wannous, 2014; Mottet et al., 2017). As the human population grows with increasing economic prosperity, increased demand for meat and animal protein is expected (Nabarro and Wannous, 2014). It is estimated that more than 600 million people in the world will be undernourished by 2030 (FAO, 2018) and food production must double to combat food insecurity by 2050 (United Nations, 2009). Pork is the most widely consumed meat globally, and the projected global pork consumption by 2029 is around 127 million tonnes, which means that 20% more pork will be needed compared with the current pork production (OECD/FAO, 2020). Indeed, current agricultural production systems are not fully efficient and sustainable (FAO, 2018).

Large quantities of human-edible crops are used as sources of energy and nutrients in swine diets today. It is estimated that animal feed utilizes 36% of the total calories of global crop production (Cassidy et al., 2013). Given that 3.2 kg of human-edible ingredients is needed to produce 1 kg of boneless meat in non-ruminant systems (Mottet et al., 2017), using non-human edible ingredients as alternative feedstuffs supports the sustainability of pork production. Moreover, prices of cereal grains and oil seeds are projected to increase (Zijlstra and Beltranena, 2013; Woyengo et al., 2014), which challenges profitability for pork producers. Hence, there is an increasing interest in studying alternative feedstuffs from the agricultural industry to decrease production costs, replace human-edible feed ingredients in animal diets, and contribute toward sustainability (Shurson, 2017; van Hal et al., 2019).

Energy represents the greatest cost and component of swine diets and is essential for all physiological and productive functions in pigs. In North America, about 39 and 9 million tonnes of biofuel co-products and rendered by-products, respectively, are produced annually, and these non-human-edible co- and by-products are used as alternative feedstuffs to meet the needs of pigs, decrease feed cost, and minimize

environmental impact (Meeker, 2006; Popp et al., 2016). However, alternative feedstuffs contain variable amounts of anti-nutritional factors and digestible energy and nutrients (Zijlstra and Beltranena, 2013), which makes diet formulation a challenge for achieving optimal growth performance of pigs.

Alternative feedstuffs often undergo thermal processing to achieve long-term storage stability or improve hygiene, but thermal treatment can accelerate unavoidable chemical reactions, such as Maillard reactions and lipid peroxidation, that decrease the nutritional and energy value of alternative feedstuffs, leading to poor growth performance (Almeida et al., 2013; Rosero et al., 2015; Gurbuz, 2017). Common rendered lipids added to swine diets up to 5% are poultry fat, tallow, choice white grease, and recycled restaurant grease (Cromwell, 2006). The effects of feeding fats and oils subjected to thermal stress as energy sources on growth performance and oxidative status of pigs have not been systematically evaluated. In addition to lipid peroxidation, many alternative feedstuffs contain high amount of dietary fiber (DF) which decreases energy digestibility (Le Goff and Noblet, 2001), growth performance, and carcass yield when fed to pigs (Agyekum and Nyachoti, 2017). Therefore, understanding how lipid peroxidation and DF influence growth performance will advance our knowledge necessary for sustainable pig farming.

Under the theme of sustainability, feeding antimicrobial growth promoters (AGPs) is neither socially acceptable nor environmentally sustainable to ensure swine production efficiency. The use of antibiotics and pharmacological levels of copper (Cu) and zinc (Zn) as AGPs in swine diets (Hill et al., 2000; Hill et al., 2001) has contributed to the development of antimicrobial-resistant bacteria (Cogliani et al., 2011; Looft et al., 2012), and the spread of resistant genes in enterobacteria in contaminated groundwater and soil (Vahjen et al., 2015; Jensen et al., 2016). Hence, research is also needed to understand the modes of action of AGPs and identify effective AGP alternatives.

Actions should be taken to improve nutrient utilization efficiency of swine diets containing alternative feedstuffs, and minimizing negative environmental impacts associated with feeding animals. A systems approach in swine nutrition research helps understand interdependent dietary factors influencing whole-animal growth performance, improving efficiency and sustainability in pork production. Achieving these

improvements further drives positive impacts on humans and the environment following the concept of "One Health," which acknowledges an inextricable connection between humans, animals, and the environment while emphasizing optimal health outcomes at the animal-human-ecosystem interface (Nabarro and Wannous, 2014). This dissertation integrates dietary factors and biological outcomes associated with growth performance of pigs, thereby contributing knowledge necessary for resolving some of the challenges of alternative feeding approaches (Figure 1.1). Finally, the contribution of this research goes beyond sustainable pig production, it helps achieve One Health.

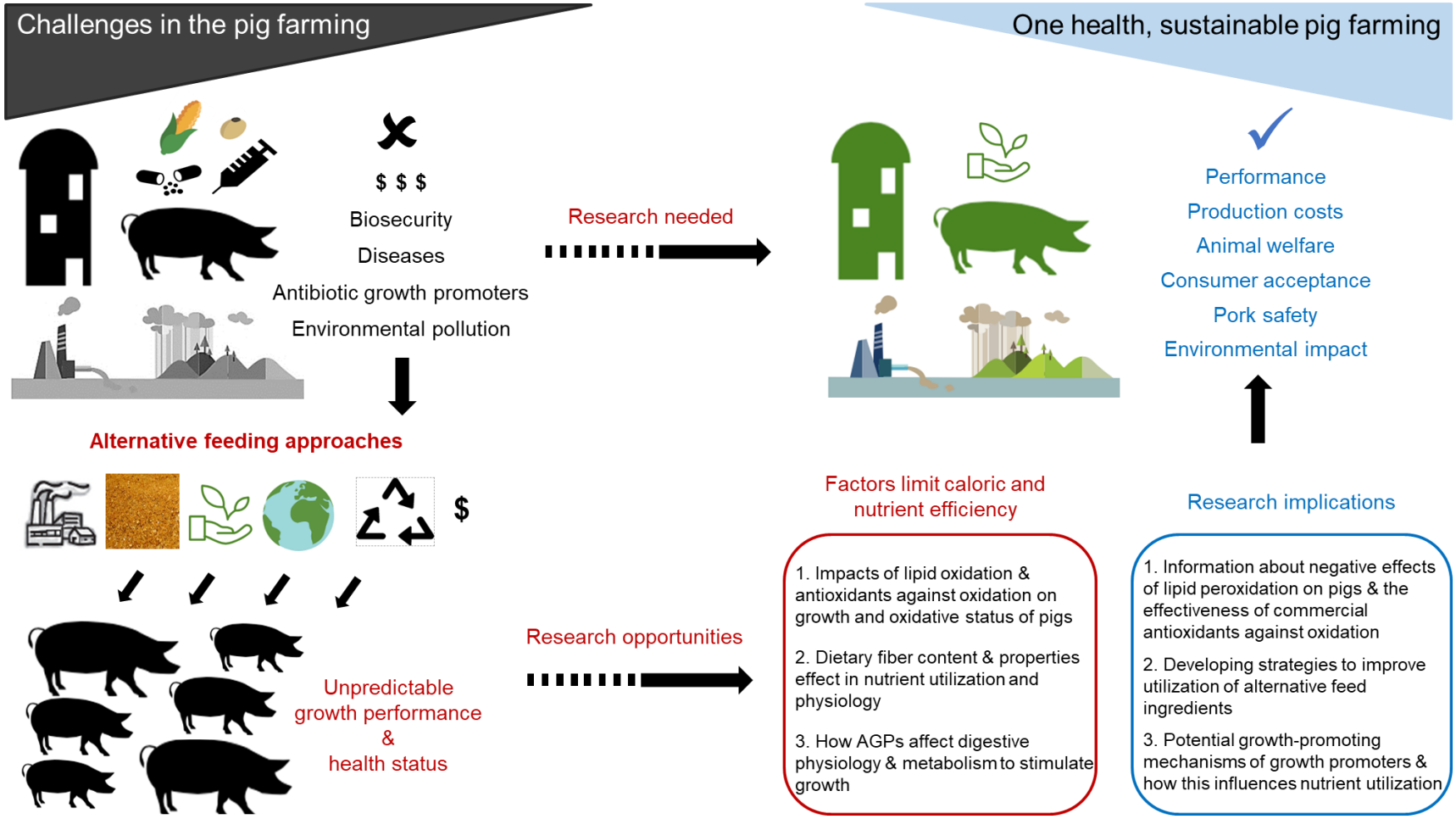

Figure 1.1. Sustainable pig farming as a solution to overcome challenges

Dietary lipid quality and its nutritional value in poultry and swine diets

The role of lipids in animal nutrition

The nutritional benefits of adding lipids to animal diets include increasing energy density and providing essential/bioactive fatty acids (i.e., linoleic and α-linolenic acid) to support animals' growth, reproduction, and health (NRC, 2012; Rosero et al., 2016). Lipids in diets are primarily in the chemical form of triacylglycerols that consist of a single glycerol molecule and three fatty acids (FA) (Botham and Mayes, 2015). The sources of triacylglycerols in animal diets are of plant (e.g., grains, oilseeds, and oils) and animal origin (e.g., fats, meat meal, and poultry meal). Moreover, by-products of biodiesel, ethanol, and oilseed industries (e.g., crude glycerin, acid oils, distillers corn oils and soap stock) can be used as dietary energy sources. The FA profile of lipids is particularly important because it influences energy content, energy digestibility, lipid characteristics and quality, animal health and performance, and carcass quality (Wood et al., 2004; Kerr et al., 2015; Shurson et al., 2015).

Supplementing lipids in diets often improves growth rate and feed efficiency of growing animals compared with feeding non-lipid supplemented diets. Supplemental lipids improves feed efficiency of animals, largely due to their high energy values and "extra" caloric effect (Campbell, 2005). The extra caloric effect of supplemental lipids enables animals to obtain greater metabolizable energy (ME) and net energy (NE) than animals fed isocaloric diets without added lipids (Nitsan et al., 1997). Llpstein and Bornstein (1975) presumed that the extra caloric effect promotes animal growth by reducing the heat increment (diet-induced thermogenesis) in animals. Rijnen et al. (2004) reported that heat increment induced by crude fat is lesser than the one induced by proteins and carbohydrates. Therefore, the caloric efficiency of lipids is greatest compared with other dietary energy components (Rijnen et al., 2004; Patience, 2012). Hence, adding lipids to diets is an effective approach to increase gross energy of diets and improve energy utilization of isocaloric diets, increasing growth rate of animals under the scenario of heat stress and energy-dependent phase of growth.

Chemical structure of lipids, such as chain length and the degree of saturation of FAs, significantly affects digestibility and energy value of lipids in poultry and swine

(Stahly, 1984; Wiseman et al., 1991; Wiseman et al., 1998). Short-chain and medium-chain FAs are easily digested (apparent digestibility that can be as high as 95%), absorbed, and metabolized (Stahly, 1984; Cera et al., 1990). The digestion and absorption of lipids containing high amount of unsaturated FAs are greater than of saturated FAs in poultry and swine (Stahly, 1984; Wiseman, 1984). The esterification and re-esterification of unsaturated FAs are greater than of saturated FAs, resulting in a faster absorption of unsaturated FAs than of saturated FAs in the intestine (Ockner et al., 1972). Therefore, the digestibility and utilization of vegetable oils are greater than animal fats, and increasing the ratio of unsaturated to saturated FAs improves digestible energy (DE) value of lipids in a positive curvilinear manner in poultry and swine (Stahly, 1984; Wiseman, 1984). Although unsaturated FAs improve the energy value of lipids, they are relatively unstable compared with saturated FAs, developing oxidation and off flavor that can decrease their feeding value (Belitz et al., 2009; Kerr et al., 2015).

Free FAs refer to those FAs that are not bound to any molecules nor in the form of triacylglycerols, and their content in the diet is another factor influencing lipid utilization and consequently energy value of lipids fed to animals. The digestion and absorption of free FAs are lesser than those of triacylglycerols (Sklan, 1979). In pigs, the digestibility, DE, and ME content of animal fats and vegetable oils are decreased by increasing free FA content (Powles et al., 1993; Jørgensen and Fernández, 2000). Similarly, increasing free FA content regardless of lipid source linearly decreased apparent ME in broilers (Wiseman et al., 1991; Wiseman et al., 1992). Hence, free FA content is important and can be used with FA profiles to predict energy content of lipids. For example, DE kcal/kg = {36.898 – [0.005 × Free FA, g/kg] – [$7.330^{(-0.906 \times \text{unsaturated to saturated FA ratio})}$]} / 4.184 (Wiseman et al., 1998; NRC, 2012). This equation can be further applied to calculate ME as 98% of DE and NE as 88% of ME, respectively (Van Milgen et al., 2001).

Although energy content of various lipid sources can be estimated by the Wiseman et al. (1998) equation, recent studies have shown that predicted DE values of specialty lipid products fed to pigs can be overestimated by as much as 4,400 kcal/kg (Kerr et al., 2016; Kerr and Shurson, 2017). The current energy prediction equations have been summarized by Shurson et al. (2021) indicating that some equations are too

simplistic, resulting in inaccurate prediction of energy content. Furthermore, none of energy prediction equations consider lipid oxidation, which is a factor that reduces lipid quality and digestibility (Shurson et al., 2015; Shurson et al., 2021). Hence, prediction equations should be revised to potentially include more factors that will improve the estimation of DE, ME, and NE of various lipids used in swine diets.

The majority of research on the role of dietary lipids in animal nutrition has mainly focused on energy content, and growth performance when feeding high-quality lipids (Kerr et al., 2015). Different lipid sources are manufactured differently and are variable in FA profile, resulting in various quality and energy values used in feed. Lipid quality, in terms of the extent of lipid oxidation, although it has not been adequately addressed in previous research, it can have a detrimental effect on nutritional value and physiological response (Kerr et al., 2015; Shurson et al., 2015).

Lipid peroxidation and its effects on lipid quality and animal health and performance

Lipid peroxidation is a term to describe complex chemical reactions between FA and free radicals, reactive species, and oxygen. The presence of heat, light, enzymes, and metals can accelerate the oxidative process in lipids (Belitz et al., 2009; Shahidi and Zhong, 2010). Lipid peroxidation is responsible for the deterioration of lipids (Shurson et al., 2015). Dietary lipids, especially containing unsaturated FA, are prone to oxidative degradation because of low C-H bond energy at the carbon with double bonds (Shahidi and Zhong, 2010). Autoxidation is the most common pathway of lipid peroxidation. Lipid peroxidation comprises initiation, propagation, and termination as shown in figure 1.3. In addition to the autoxidation pathway, lipids are subjected to photooxidation and thermal oxidation associated with free radicals. Photooxidation is triggered by the excitation of a photosensitizer by light and yields energy transfer to lipid molecules that ultimately elicit oxidative process (Shahidi and Zhong, 2010). Thermal oxidation can change chemical properties of lipids and accelerates oxidative reactions (Falade and Oboh, 2015).

The initiation phase of lipid peroxidation requires the presence of initiators, such as heat, light, and transition metals to remove a hydrogen from the FA and generate the first radicals. Lipid hydroperoxides are formed during propagation as primary peroxidation products, which are unstable and subsequently form secondary peroxidation

products (i.e., aldehydes, hydrocarbons, epoxy, alcohol, or ketone group). In the termination, radicals react with each other or with antioxidants as stable non-radical products (i.e., tertiary peroxidation products) (Shahidi and Zhong, 2010). Because lipid peroxidation is a dynamic process with diverse peroxidation products produced and degraded in each phase, accurately quantifying the extent of lipid peroxidation is challenging.

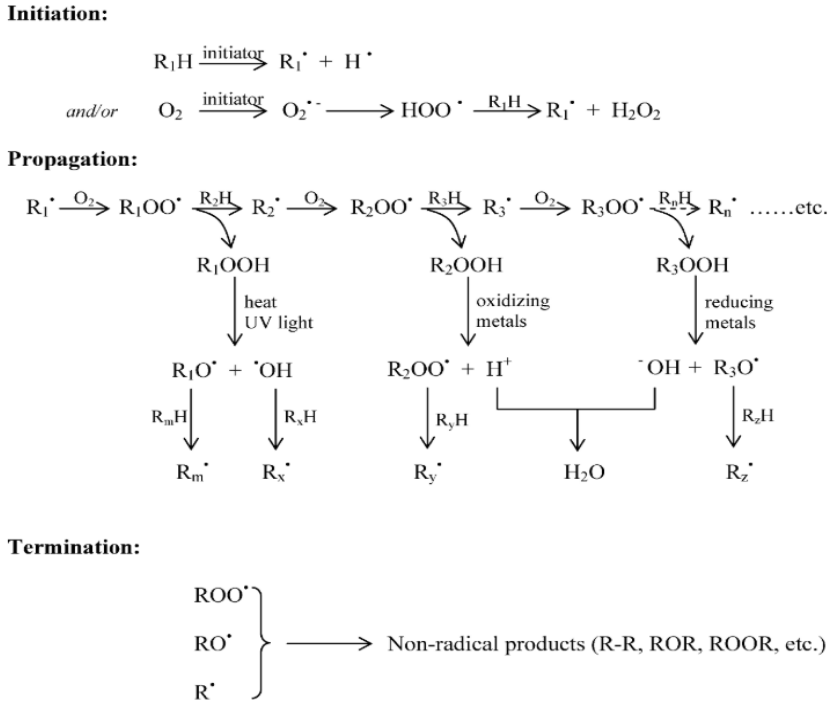

Figure 1.2. Lipid autoxidation induced lipid peroxidation (Shahidi and Zhong, 2010) – Published by Chemical Society Reviews.

Oxidative degradation further deteriorates nutritional value of lipids through degrading essential FAs, fat-soluble vitamins and natural antioxidants, and producing toxic compounds. Several polyunsaturated FAs-derived aldehydes have been identified (Wang et al., 2016), and of those, acrolein, malondialdehyde, and 4-Hydroxynonenal (4-HNE) have been intensively studied due to their negative effects on cellular redox homeostasis that in turn have been associated with aging, cancer, cardiovascular diseases, and inflammation in humans (Pizzimenti et al., 2013; Shoeb et al., 2014). Because of the adverse biological effects of aldehydes, lipid peroxidation should be a major consideration when diets containing lipids.

Several studies have been conducted to characterize the effect of peroxidized lipids on several parameters related to growth performance. Peroxidized lipids fed to poultry and pigs increase oxidative stress, morbidity, and mortality (Liang et al., 2015; Hanson et al., 2016; Chang et al., 2019), and impair immune functions (Dibner et al., 1996; Liang et al., 2015). Additionally, peroxidation products can react with proteins and decrease their bioavailability (Nielsen et al., 1985). Feeding peroxidized lipids decreases apparent fecal digestibility of energy and protein in poultry (Kamran et al., 2020) and pigs (Yuan et al., 2007; Rosero et al., 2015; Lindblom et al., 2018), resulting in the reduction of lipid and nitrogen retention (Engberg et al., 1996; Lindblom et al., 2018). As a result, consumption of peroxidized lipids has been reported to decrease growth performance in poultry (Engberg et al., 1996; Liang et al., 2015) and swine (Liu et al., 2014a; Hanson et al., 2016; Lindblom et al., 2018; Chang et al., 2019). However, not all studies have reported changes in energy, nutrient digestibility, and growth performance of feeding peroxidized lipids (Racanicci et al., 2008; Upton et al., 2009; Açıkgöz et al., 2011; Liu et al., 2014b; Hung et al., 2019). These inconsistent observations on nutrient digestibility and growth performance from feeding peroxidized lipids to animals suggest that further evaluation is needed.

Assessing lipid quality and its relation to feeding value for poultry and swine

Lipid quality is commonly assessed by color, FA profile, FA content, iodine value, saponification value, moisture, insoluble, and unsaponifiable content of oils and fats (Table 1.1) (Azain, 2001; Meeker, 2006; Ehr et al., 2015; Shurson et al., 2015). These measurements are useful for trading specifications, but do not provide information regarding oxidative status of lipids that mainly influence feeding value and animal performance (Kerr et al., 2015; Shurson et al., 2015). To maximize performance of fast-growing pigs, nutritionists are interested in maximizing the available energy from supplemental lipids. Moving towards sustainability in animal production, various lipid sources are used or mixed in diets. This practice highlights the importance of assessing lipid quality because unknown quality of lipid mixture may negatively impact on energy value of lipids, health and growth performance of animals. By assessing lipid quality, appropriate practices can be implemented to manage the use of low-quality lipids fed to animals.

Table 1.1. Common lipid quality indices used for trading specifications[1]

Items	Descriptions	Recommended levels[2]
Color	Graded based on the Fat Analysis Committee standard, ranging from 1 (light) to 45 (dark).	Not reported
Fatty acid profile	Relative amounts of individual fatty acids.	Various
Free fatty acid	Amount of fatty acids not bound to the glycerol backbone in a triglyceride.	≤ 10 - 15%
Total fatty acids	The total of both free fatty acids and fatty acids combined with glycerol.	≥ 90%
Iodine value	Amount of fatty acids not bound to the glycerol backbone in triacylglycerols.	Various
Moisture (M)	Amount of moisture in a lipid.	≤ 0.5 - 1%
Insolubles (I)	Amount of sediment in a sample, such as fiber, hair, hide, bone, and soil.	≤ 0.5%
Unsaponifiables (U)	A measure of material in the lipid that will not saponify (form a soap) when mixed with caustic soda (NaOH or KOH). Examples include sterols, hydrocarbons, pigments, fatty alcohols, and vitamins.	≤ 1%
Total MIU	The sum of moisture, insoluble, and unsaponifiable content	≤ 2%
Non-elutable materials	Reflects the total amount of non-nutritional material, including moisture, impurities, unsaponifiable material, glycerol, and oxidized and polymerized fats.	Not reported
Titer	The solidification temperature of lipids in a source or mix, which is an important characteristic in producing soaps or fatty acids.	Various

[1]Modified and summarized from Azain, 2001; Meeker, 2006; Kerr et al., 2015; Shurson et al., 2015

[2]The recommendation is based on Meeker, 2006

Lipid peroxidation assays (Table 1.2) have been developed and used to evaluate lipid quality by quantifying lipid peroxidation products (i.e., indicative tests) or measuring the susceptibility of lipids to oxidation under pro-oxidative conditions (i.e., predictive tests). Because lipid peroxidation is a very dynamic and complex process, the limitations of each assay should be acknowledged. Shahidi and Zhong (2005) and Shurson et al. (2015) comprehensively reviewed common assays for lipid peroxidation and discussed their limitations, summarized in table 1.2. Liu et al. (2014b) reported that the active oxygen method was positively correlated with thiobarbituric acid reactive substances (TBARS) and anisidine value (AnV) (r = 0.51 and 0.53). Peroxide value was positively correlated with TBARS, hexanal, and 2,4-decadienal (DDE) (r = 0.75, 0.76, and 0.61, respectively). The oil stability index was negatively correlated with AnV (r = -0.57), suggesting the more resistance of lipids to oxidation, the lesser aldehydes formation measured by AnV.

Table 1.2. Assays measuring lipid peroxidation and their limitations[1]

Tests	Compounds/Measurements	Limitations
Indicative tests		
Peroxide value (PV)	Peroxides and hydroperoxides	Products are unstable and may underestimate peroxidation during continuous oxidation conditions. Peroxides may be undetectable in lipids exposed to > 150°C.
Thiobarbituric acid reactive substances (TBARS)	Malondialdehyde	Not specific. 2-alkenals and 2,4-alkedienals can react with thiobarbituric acid.
Anisidine value (AnV)	Aldehydes	Not specific. 2-alkenals and 2,4-alkedienals can react with p-anisidine under acid conditions.
Conjugated dienes and trienes	Compounds formed after rearrangement of double bonds	Less sensitivity than PV. Carotenoids are absorbed in the same wavelength range, which can cause misleading results.
TOTOX value	2 × PV + AnV or TBARS	No scientific basis. Increases the lack of specificity inherent with AnV or TBARS and PV.
Carbonyls	Aldehydes and ketones	Lack of specificity and tendency to be influenced by non-carbonyl compounds.
Hexanal	Secondary products formed during the oxidation of linoleic and other n-6 FA	Volatile at high temperatures and may provide a misleading indication of the extent of peroxidation.
2,4-decadienal (DDE)	Aldehyde derived from linoleic acid	Complicated and expensive assay requiring gas chromatography-mass spectrophotometry.
4-hydroxynonenal (4-HNE)	Aldehyde formed during lipid peroxidation of n-6 polyunsaturated FA	Complicated and expensive assay.
Triacylglycerol dimers and polymers	Polymeric compounds formed during the termination phases	Measured with size exclusion chromatography. Limited information on their use in evaluating lipid quality and effects on animal health.
Non-elutable materials	Estimates the non-elutable material of lipid after a correction for glycerol	Not commonly seen in the literature. Collectively measures most degraded chemical structures of lipids by Gas–liquid chromatography.
Predictive tests		
Active oxygen method	Measurement of PV of lipids under bubbling air at 97.8°C repeatedly	PV is a poor indicator for oxidation. Outdated and labor-intensive methods. Difficult to make inter-laboratory comparisons due to modified procedures.
Oil stability index (OSI)	Formation of volatile acids by monitoring the change in electrical conductivity	OSI is better than a single indicative test, but the potential loss of volatile acids may cause an error.
Oxygen bomb method	Changes of oxygen pressure by oxidation in a bomb	Not limited to dietary lipids. Required the oxygen bomb machine and labor-intensive methods.

[1]Table is modified and summarized from Shurson et al., 2015

Research on the development of accurate lipid peroxidation measurements is active because no single test can accurately estimate the extent of lipid peroxidation. The kinetics of individual aldehyde production as markers for the extent of lipid peroxidation has been studied in heated vegetable oils. Wang et al. (2016) found that 4-HNE concentration increased linearly within 6 hours of heating soybean oil at 185 °C ($r = 0.99$), suggesting that 4-HNE can be a marker for lipid peroxidation. Recently, C9-C11 unsaturated aldehydes, such as 4-HNE, DDE, 2-decenal and 2-undecenal have been shown to be useful measurements for estimating lipid peroxidation and predicting growth responses of poultry and pigs fed oxidized lipids (Yuan et al., 2020). Indeed, some assays for measuring lipid peroxidation presented in table 1.2 (e.g., measuring PV, TBARS, and volatile compounds) are commonly used even though they are not as specific as individual aldehyde detection for assessing the extent of lipid peroxidation. Currently, selecting two or more assays that can measure primary, secondary, or end peroxidation products provides a reasonable assessment for lipid quality (Shahidi and Zhong, 2005a; Shurson et al., 2015).

Studies have reported that 4-HNE and DDE concentrations negatively correlated with lipid digestibility, DE:GE and ME:DE ratio of pigs fed soybean oils (Lindblom et al., 2018; Overholt et al., 2018), suggesting reduced energy utilization in peroxidized lipids. Furthermore, because secondary aldehydes are partially absorbed and metabolized in the liver, consuming diets containing these products results in toxicity and imbalance in antioxidant defense (Kanazawa and Ashida, 1998; Lykkesfeldt and Svendsen, 2007), growth reduction (Hanson et al., 2016; Chang et al., 2019), and oxidative stress in the liver and intestine (Takahashi and Akiba, 1999; Tavárez et al., 2011; Rosero et al., 2015; Lindblom et al., 2019). Studies have examined the relationship among several lipid peroxidation assays with markers of oxidative stress. However, the correlation coefficients between values of peroxidation measured using several assays (e.g., PV, AnV, 4-HNE) and oxidative stress markers in blood and liver (e.g., TBARS, protein carbonyls, 8-hydroxy-2’deoxy-guanosine, glutathione peroxidase activity) with growth performance were generally low (Overholt et al., 2018; Lindblom et al., 2019).

The ultimate goal of measuring lipid peroxidation in swine nutrition is to provide information about the feeding value of peroxidized lipids and determine if a tolerance

level of feeding peroxidized lipids exists. Different sources of lipids are used in animal diets as part of sustainable feeding approaches. There is a need to establish guidelines for using dietary lipids varying in quality and estimating their impact on growth performance and oxidative status of animals. However, various lipid peroxidation measurements demonstrate only a fraction of the oxidation products that can potentially be produced, making it difficult to infer and conclude on the impacts of feeding oxidized lipids to pigs and poultry from published studies. A meta-analysis approach (Chapter 3) provides an unbiased and systematic evaluation on feeding value of peroxidized lipids in swine and poultry diets.

Supplementation of dietary antioxidants to ameliorate lipid peroxidation

Feed and dietary lipids are subjected to oxidative damage during processing and storage under hot and humid environments. Exogenous antioxidants are often added to dietary lipids to minimize their oxidative deterioration and stabilize shelf life and quality. Antioxidants can be classified by either their origin (natural or synthetic), or by their mechanisms of action (primary or secondary). Synthetic antioxidants are relatively more stable and effective against lipid peroxidation than natural antioxidants, so they are often added to food and feed to preserve quality (Crane et al., 2000; Shahidi and Zhong, 2005). Primary antioxidants donate hydrogen to block a chain reaction and promote the formation of stable radicals, whereas secondary antioxidants decrease the rate of oxidation by chelating metals, reducing primary antioxidants, degrading hydroperoxides, and scavenging oxygen (Shahidi and Zhong, 2010). It is important to note that antioxidants delay or prevent oxidation, but do not reverse the oxidative process after the reaction has occurred (Belitz et al., 2009).

Commercial synthetic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin, tert-butylhydroquinone (TBHQ), and propyl gallate (PG), are used in animal feeds (Table 1.3). Among them, ethoxyquin was banned in European Union (EU) since 2017 because it may cause genetic mutations. Each synthetic antioxidant exhibits different features, such as thermal stability, effective dosage, and synergistic actions (Shahidi and Zhong, 2005). Therefore, a combination of several antioxidants is recommended and considered an effective, affordable way to

prevent lipid peroxidation (Omura, 1995; Yen et al., 1997; Mahmoud Allam and Aly Mohamed, 2002).

The effects of adding antioxidants to diets on animal growth performance have been evaluated, but results have been inconsistent. The addition of antioxidants to diets has prevented growth reduction in poultry (Dibner et al., 1996; Lu et al., 2014a) and pigs (Fernández-Dueñas, 2009; Harrell et al., 2010; Boler et al., 2012) compared with those treatments without antioxidants. However, results from other studies showed no effect of supplemental dietary antioxidants for preventing detrimental effects on growth performance (Anjum et al., 2002; Tavárez et al., 2011; Chang and van Heugten, 2016; Silva-Guillen et al., 2020). Although the effectiveness of adding antioxidants on growth performance is still debatable, oxidized lipids without supplemental antioxidants can be further oxidized, potentially causing detrimental effects on growth performance.

In the feed industry, distillers corn oil is a novel alternative lipid source, which has become more attractive than choice white grease for swine diets due to some swine producers wanting to use all-vegetable diets because of perceived swine virus contamination in animal-derived by-products. However, distiller corn oil is more susceptible to oxidation than choice white grease due to its greater concentrations of unsaturated FAs, especially linoleic acid. Independently of whether antioxidants are added in the process of obtaining distillers corn oil, the resulting product is susceptible to oxidation. The effectiveness of adding commercial antioxidants to peroxidized distillers corn oil to prevent further lipid peroxidation and growth depression of pigs has not been extensively studied (Fernández-Dueñas, 2009; Harrell et al., 2010; Boler et al., 2012). It is meaningful to evaluate the effects of commercial antioxidants on growth and oxidative status of pigs fed peroxidized distillers corn oil.

Table 1.3. Commercially available synthetic antioxidants used in feed industry[1]

Antioxidants	**Features**	**Maximum diet inclusion levels**
Butylated hydroxyanisole, BHA	Poor thermal stability Can be used in combination with BHT Often added to feed ingredients, such as cereal grains, fish meal, fats, and oils Frequently used by the poultry industry	200 ppm (US) 150 ppm (EU)
Butylated hydroxytoluene, BHT	Good thermal stability	200 ppm (US) 150 ppm (EU)

	Often added to feed ingredients, such as cereal grains, fish meal, fats, and oils Can be used in combination with BHA	
Ethoxyquin, EQ	Poor thermal stability Can be used in combination with PG Protects against lipid peroxidation and stabilizes carotene, xanthophylls, and vitamins Often added to feed ingredients, such as cereal grains, fish meal, fats, and oils Frequently used by the poultry industry	150 ppm (US) Banned (EU)
Propyl gallate, PG	Poor thermal stability May form undesirable dark brown complex Often used in animal fats Can be used in combination with BHA, BHT, EQ, and TBHQ	100 ppm (US) Livestock: 40 ppm (EU)
Tertiary butylhydroxyquinone, TBHQ	Good thermal stability, but relatively expensive Often added to feed ingredients, such as cereal grains, fish meal, and lipids Effective against lipid peroxidation in oil than BHA and BHT Widely used in combination with BHA and BHT	100 ppm (US) 30 ppm and 150 ppm when supplemented with BHA and BHT (EU)

[1]Summarized from (Sanhueza et al., 2000; Shahidi and Zhong, 2005; Merrill et al., 2008; Błaszczyk et al., 2013; EFSA, 2015; Salami et al., 2015; Ezerskis, 2015; ESFA, 2018; EFSA, 2020)

Challenges of feeding high-fiber ingredients to pigs

Quantity and functionalities of dietary fiber and their implications in swine nutrition

Carbohydrates are the primary energy source of swine diets, and dietary carbohydrates plus lipids can fulfill the caloric needs of pigs (NRC, 2012). To support economic and environmental sustainability of pork production, there is a trend to increase the inclusion of low-cost cereal co-products in swine diets (Zijlstra and Beltranena, 2013; Woyengo et al., 2014). Because not all carbohydrates in feeds are digestible, those "that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine…promote beneficial physiological effects including laxation, and/or blood cholesterol and glucose attenuation" are defined as dietary fiber (DF) (AACC, 2001). Indeed, DF is a simple term to describe a group of carbohydrates with complex characteristics regarding chemical structure, physical properties, and physiological effects that have significant impact on health and growth performance of animals (Wenk, 2001; Choct, 2015; Agyekum and Nyachoti, 2017). The characteristics of DF in cereal co-products can limit the efficiency of energy and nutrient utilization by

pigs. Therefore, knowing quantity and functionality of DF and their effects on digestive physiology would aid nutritionists formulating swine diets with high-fiber feedstuffs.

Crude fiber (CF) was the first method developed to quantify DF by measuring an ingredient's organic residue after treating it with sulfuric acid and sodium hydroxide (Henneberg and Stohmann, 1859). Crude fiber has been widely used for fiber analysis and feed formulation, especially in poultry nutrition, and CF content is required to be on the label of feed sold in the U.S. However, CF underestimates DF content because the method measures mainly lignin, with partial recovery of cellulose and hemicelluloses in the sample. Because of the incomplete recovery of cell wall components in CF, measuring CF content underestimates DF content of feedstuffs and is an inappropriate indicator when studying biological outcomes to fiber ingestion (Mertens, 2003). In 1963, Van Soest and his colleagues developed detergent methods for quantifying DF (Van Soest, 1963; Van Soest et al., 1991). Van Soest detergent fiber analysis is commonly applied in swine and ruminant nutrition for measuring insoluble components of DF in neutral and acid detergents. Acid detergent fiber (ADF) estimates the sum of lignin and cellulose, whereas neutral detergent fiber (NDF) estimates the sum of lignin, cellulose, and hemicelluloses contents. Thus, ADF and NDF determinations allow nutritionists to quantify DF more precisely than using CF.

Previous research has shown that DF content decreases energy and nutrient digestibility of pigs, and the accuracy of using NDF to predict energy digestibility of growing pigs is greater than using CF and ADF (Noblet and Perez, 1993). Each additional 1% NDF can decrease energy digestibility by 0.8 – 1.0% (Noblet and Perez, 1993). Studies have reported that increasing dietary NDF linearly decreased energy and nutrient digestibility in growing pigs (Schulze et al., 1994; Moeser and Van Kempen, 2002). Although detergent methods improve the accuracy of DF analysis, the relationship between values of detergent DF and the effects of DF on nutrient digestion, absorption, and metabolism do not directly correspond to the measured values. The greatest limitation of measuring detergent DF is that does not quantify soluble non-starch polysaccharides (NSP) (e.g., soluble hemicelluloses, pectins, gums, and β-glucan) which also impact nutrient digestion and absorption (Grieshop et al., 2001). From a practical standpoint, ADF and NDF values help estimate the digestibility of energy and nutrients,

but not to the extent of fully explaining digestive physiological effects of DF on nutrient transporter expression, mucin secretion, and susceptibility to infection in the gut, among other.

Analyzing intrinsic plant cell-wall polysaccharides as per the NSP method is another way to describe DF, but the NSP method cannot measure resistant starch (Englyst et al., 1994; Englyst et al., 2007). Currently, analyzing total dietary fiber (TDF) is more accurate for measuring DF than quantifying CF, ADF, and NDF (Mertens, 2003). Analyzing TDF uses two procedures: an enzymatic-gravimetric procedure that estimates the sum of soluble and insoluble NSP and lignin; and an enzymatic-chemical procedure that quantifies NSP with (Theander et al., 1994; Choct, 2015) or without resistant starch, uronic acids, and lignin (Englyst et al., 1994; Grieshop et al., 2001). However, TDF methods (AOAC 985.29 or 991.43) do not completely represent the entire fiber components of a feed ingredient unless other fiber components (e.g., complete recovery of resistant starch, fructo-oligosaccharides, inulin, and galacto-oligosaccharides) are analyzed and added to the TDF value. Furthermore, TDF analysis is expensive, labor-intensive, time-consuming, and less reproducible than detergent methods, so it is not widely implemented. Alternatively, the application of the near infra-red (NIR) technique provides a rapid estimation of types and composition of NSP in cereal grains (Blakeney and Flinn, 2005). However, the accuracy of estimating DF by NIR is largely dependent upon multivariate calibration and sufficient reference data.

Results from both *in vitro* and *in vivo* studies have shown that increasing TDF content decreases digestibility of energy, dry matter, and nitrogen (Zhang et al., 2013; Jha et al., 2015; Huang et al., 2017; Pu et al., 2020). Total DF of corn DDGS, soybean hulls, and wheat straw was negatively correlated with *in vitro* dry matter digestibility ($r = -0.98$) (Huang et al., 2017), suggesting that TDF content could be used to estimate the amount of digestible nutrients of ingredients. Urriola et al. (2010) reported that 29% and 21% of TDF in distillers co-products are digestible and fermentable in the small intestine and hindgut, respectively. These results implied that dietary energy is partially obtained from digestible and fermentable TDF. However, a regression analysis indicates that TDF content is a poor predictor ($R^2 = 0.02$) of the amount of DF being utilized for energy in the intestine of pigs because TDF content is not correlated with digestible TDF content in

the intestine (Figure 1.3). Hence, the information on fiber quantity *per se* is inadequate to predict DF effect on digestible nutrients.

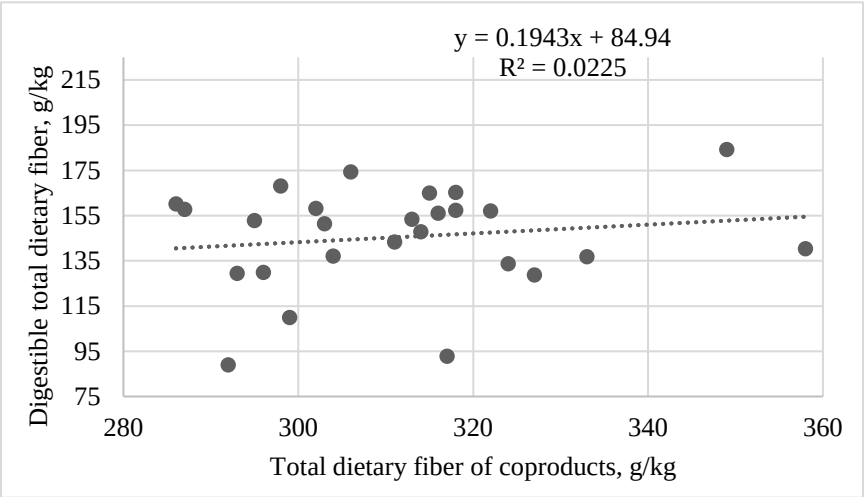

Figure 1.3. The relationship between total dietary fiber (TDF) content of co-products and digestible TDF in the intestine of growing pigs. Data for digestibility and content of TDF from Urriola et al.(2010).

Currently, considerable attention has been devoted to studying the physicochemical properties of DF (Blackwood et al., 2000), including solubility, hydration capability, viscosity, fermentability, and cation-exchange capacity. Physicochemical properties of DF can affect the kinetics of nutrient digestion, absorption and, subsequently, nutrient utilization, hindgut fermentation, and overall animal health status (Blackwood et al., 2000; Lentle and Janssen, 2008; Lentle and Janssen, 2010).

Due in part to differences in solubility in water among DF sources, the mean retention time (MRT) of solids and liquids in the GI tract is different (Schop et al., 2019; Schop et al., 2020), and DF solubility could further affect other physicochemical properties of DF, such as viscosity and fermentability through water insolubility of DF. Williams et al. (2019) stated that the solubility *per se* is not enough to explain DF functionalities because various degrees of viscosity and fermentability can be observed in soluble and insoluble DF. Hydration is the water-holding or -binding capacity (WHC or WBC, respectively) that are used interchangeably to describe the interaction between water and DF. Water-binding capacity is positively correlated with soluble fiber content

(Slama et al., 2019) and may affect the rheological properties of digesta, thereby affecting the kinetics of digestion and absorption in pigs (Schop et al., 2020). Viscosity of DF represents the ability to thicken or form viscous gels in a solution and is often attributed to soluble NSP (Dikeman and Fahey, 2006). Feeding viscous DF can increase MRT and digesta viscosity, decrease nutrient digestion, plasma glucose at 60 minutes post-feeding, and growth performance; and impair health status of pigs by promoting expansion of certain enteric pathogens (McDonald et al., 2001; Hopwood et al., 2006; Owusu-Asiedu et al., 2006). Fermentability of DF is likely responsible for health benefits due to the influences of gut microbiota and the production of microbial fermentation metabolites, such as acetate, propionate and butyrate (Williams et al., 2001; Jha and Berrocoso, 2016). Finally, cation-exchange capacity describes the ability of fiber to bind hydrogen ions, minerals, and organic molecules to exhibit buffering capacity, which can help maintain pH in the GI tract appropriate for microbial activity (McBurney et al., 1983; Van Soest et al., 1991).

The effects of physicochemical properties of dietary fiber on nutrient utilization and intestinal function

Traditional animal nutrition studies have focused on evaluating effects of DF quantity on DE and ME values, carcass parameters, and odor and volume of manure. However, solely relying on DF content is insufficient to understand the role of DF on nutrient utilization and intestinal function in pigs (Bach Knudsen, 2001). Physicochemical properties of DF, especially viscosity and fermentability, are key to understanding the physiological impacts of DF (Jha and Berrocoso, 2016; Jha et al., 2019). Fiber sources exhibit a wide range of viscosity and fermentability that can change gastric emptying rate and digesta flow, nutrient digestion and absorption, and production of short-chain FA (Dikeman and Fahey, 2006; Zijlstra et al., 2012; Zhu et al., 2013), thereby affecting GI physiology, and health status (Zijlstra et al., 2012; Molist et al., 2014).

Viscosity is less of a concern in insoluble DF feed ingredients, as soluble NSP are likely responsible for increased digesta viscosity (Figure 1.5; Hopwood et al., 2004). Viscosity of digesta can alter nutrient digestibility, digestive enzyme activities, glycemic

responses, and blood and liver lipid concentrations (Gallaher et al., 1993; Dikeman and Fahey, 2006). The viscous environment in the intestinal lumen may create a barrier that limits the interaction between enzymes and chyme, or nutrients and transporters in enterocytes, leading to limited digestion and absorption (Jha and Berrocoso, 2015). An *in vitro* study showed that pectin and guar gum increased viscosity of the duodenal content supernatant and decreased activities of amylase, trypsin, and lipase (Isaksson et al., 1982). Similarly, pigs fed refined pectin presented decreased lipase and trypsin activities in the digesta (Pluschke et al., 2018). Furthermore, increasing digesta viscosity decreased nutrient digestibility in broilers (Smits et al., 1997; Maisonnier et al., 2001) and pigs (Mosenthin et al., 1994; Hopwood et al., 2004; Buraczewska et al., 2007). In addition, high viscosity can change the intestinal structure through increasing mucosal cell proliferation and villi crypt depth (Gee et al., 1996). Luminal viscosity appears to change the intestinal secretory activity by increasing goblet cell numbers and mucin secretion (Piel et al., 2005; Ito et al., 2009), but the mechanisms involved in these responses remain unclear. Although the intestine adapts to different types of fiber fractions by changing expression of nutrient transporters (Saqui-Salces et al., 2017; Wu et al., 2018), whether and how viscosity of DF affects nutrient transporters remains to be evaluated.

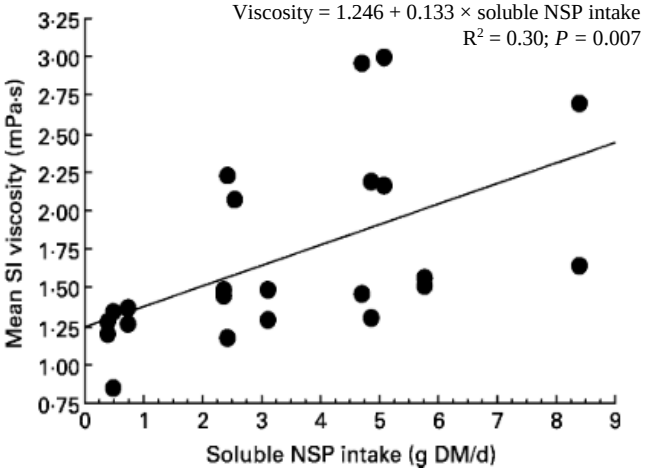

Figure 1.4. Linear relationship between soluble NSP intake and viscosity of small intestinal digesta in newly weaned pigs (Adapted from Hopwood et al., 2004) – Published by British Journal of Nutrition.

Undigested carbohydrates in the small intestine are the main substrates for microbial fermentation in the large intestine. The extent of DF fermentation depends on

chemical components, physicochemical properties, MRT, and fiber-degrading bacteria present (Jha and Berrocoso, 2015; Agyekum and Nyachoti, 2017). Soluble DF has greater fermentability than insoluble DF (Agyekum and Nyachoti, 2017). Fiber fermentation yields short-chain FA, which are not only used as an energy source in colonocytes, but also metabolized by hepatic cells and skeletal cells (Wong et al., 2006; Hu et al., 2018). The energy produced from short-chain FA provides up to 30% of the maintenance energy requirement of growing pigs (Yen et al., 1991; Varel and Yen, 1997). Fermentable DF provides pronounced prebiotic effect, which promotes the growth of activity of beneficial bacteria as probiotics in the GI tract that may improve host health (Slavin, 2013; Fouhse et al., 2015). The importance of prebiotic effect of DF is because it may be an attractive way to maximize swine health and performance through maintaining a healthy gut microbial ecosystem (Lindberg, 2014).

Because of the complexity of DF and the variability of physicochemical properties based on each feedstuff or fiber source, identifying the drivers of gastrointestinal responses associated to feeding high-fiber diets is difficult. Recently, our research found that growing pigs fed similar amounts of NDF by introducing different sources of fiber resulted in different responses on goblet cell area and gene expression of nutrient sensors, transporters, cytokines, and cell differentiation markers in the ileum (Saqui-Salces et al., 2017; Ferrandis Vila et al., 2018). This suggests that the relationship between fiber ingestion and its responses in digestive physiology of pigs is not solely explained by NDF content and may be explained by the physicochemical properties, as we explored in Chapter 4.

The challenges of removing antimicrobial growth promoters from diets in swine production

Antimicrobial growth promoters and non-antibiotic feed additives use in swine diets

The growth-promoting effect of dietary antibiotics was first identified in broilers by Moore et al. (1946), and a few years later, several antibiotics were also used as antimicrobial growth promoters (AGPs) in pig diets (Luecke et al., 1951). The effectiveness of feeding AGPs on growth performance has been documented in many

studies at universities, research facilities, and commercial farms (Cromwell, 2002; Cromwell, 2012; Helm et al., 2019). The rationale behind the use of in-feed AGPs is prophylaxis, improvement of growth performance, and reduced mortality and morbidity, thereby maximizing production efficiency (Cromwell, 2002). However, non-therapeutic use of antibiotics as AGPs in animal production raised major concerns related to promotion of bacterial antimicrobial resistance (AMR) (Cogliani et al., 2011; Looft et al., 2012). Hence, the ban on all in-feed AGPs in Europe and the Veterinary Feed Directive regulations in the U.S. were implemented to ensure animal and public health by limiting the use of antibiotics for treatment in production animals, thus decreasing the drive to develop antimicrobial-resistant traits in the microbes.

Due to legislation and consumer pressure for antibiotic-free animal production, alternatives to AGPs are attractive for their use in sustainable pork production. The ideal candidates to replace AGPs should provide similar growth performance and health benefits when included in swine diets. Several non-antibiotic feed additives, such as antimicrobial peptides, acidifiers, pharmacological doses of zinc and copper, direct-fed microbials (DFM), yeast, essential oils, enzymes, and antimicrobial lipids have been used as potential growth promoters in diets, and a brief summary of their benefits and limitations is presented in table 1.4.

The oral administration of antibiotics as AGPs to pigs was commonly used in the suckling and post-weaning periods (Lekagul et al., 2019). Clearly, studies on identifying effective AGPs alternative have been conducted in nursery pigs. Unfortunately, the effectiveness of feeding AGP alternatives is inconsistent and the magnitude of the response observed from most products has not as matched feeding AGPs (Thacker, 2013). Gabler and Schweer (2018) conducted a systematic review to evaluate growth responses of nursery pigs fed AGP alternatives. They concluded that alternative products may improve growth performance, but 63% of the trials did not show improvement in gain efficiency (Figure 1.6), suggesting the inconsistent effectiveness of using AGPs alternatives to promote growth performance of nursery pigs. Indisputably, a lack of knowledge on the mechanisms of action of AGPs hampers the progress of identifying effective AGPs alternatives (Dibner and Richards, 2005; Niewold, 2007; Gadde et al., 2017; Helm et al., 2019). Because the modes of action of AGP alternatives are different

and the effectiveness of each alternative may differ under certain conditions, a combination of different additives with husbandry and biosecurity approaches may provide a greater likelihood to achieve a better outcome in the absence of feeding AGPs (de Lange et al., 2010; Gadde et al., 2017; Liu et al., 2018).

Theoretical mechanisms of action of AGPs

Many growth promoters do not directly provide energy or nutrients, but they may have value in supporting caloric and nutritional efficiency by demonstrating their effects on antimicrobial properties and metabolic modifications to the host and bacteria. The possible modes of action of AGPs on growth promotion of animals have been proposed in many review papers (Anderson et al., 2000; Cromwell, 2002; Gaskins et al., 2002; Dibner and Richards, 2005; Niewold, 2007; Gadde et al., 2017). At least five hypotheses can be drawn from these reviews: 1) inhibition of subclinical infections, 2) reduction of growth-depressing microbial metabolites, 3) reduced competition of nutrients for microbial use, 4) enhanced energy and nutrient utilization, and 5) decreased inflammation.

Antimicrobial properties of AGPs as a direct effect for growth promotion remains debatable. The argument is that AGPs are fed in sub-therapeutic doses which are lower than the minimum inhibitory concentration (MIC) (Niewold, 2007; Niewold, 2014). Consequently, sub-therapeutic doses of AGPs are unlikely to inhibit growth of the target microorganism after incubation. Furthermore, some studies observed no changes on short-chain FA in the large intestine and feces of pigs fed AGPs (Yu et al., 2017b; Trudeau et al., 2018; Helm et al., 2019), suggesting that growth promotion is not directly attributed to the alteration of anaerobic bacterial metabolism. Nevertheless, growth-promoting effects of AGPs is absent in germ-free animals and feeding AGPs below MIC has shown changes in *in-vivo* parameters, such as bacterial growth profile, production of virulence factors, and gut microbiome population (Kim et al., 2012; Looft et al., 2014b; Looft et al., 2014a; Broom, 2017). Therefore, effects of AGPs on gut microbiota and subsequent metabolic modifications that are metabolically important for growth and health response might be worth to further investigation.

Table 1.4. Summary of the benefits and limitations of using non-antibiotic feed additives in diets to improve growth performance of pigs

Feed additives	Benefits	Limitations	References
Antimicrobial peptides	Antimicrobial properties Specific to pathogenic bacteria Activated systemic and local immune systems Decreased incidence of diarrhea Maintained growth performance under a challenge model	High cost A narrow spectrum antibacterial activity Only few studies conducted *in vivo* Inconsistent results The exact mode of action is not confirmed yet	(Wu et al., 2012; Yoon et al., 2012; Thacker, 2013; Yoon et al., 2013)
Acidifiers (organic and inorganic acids)	Antimicrobial properties Gastric acidification in young pigs Improved proteolytic enzymes activity and protein digestion Modulated gut microbiome Increased growth performance	Unknown mode of action Inconsistent results Pathogenic bacteria (e.g., *E. Coli*) may develop resistance to acidic environments A blend of several acids required to maximize positive outcomes	(Partanen and Mroz, 1999; Kiarie et al., 2016; Gadde et al., 2017; Liu et al., 2018; Gómez-García et al., 2019)
Cu (100-250 ppm) and Zn (1000-3000 ppm)	Bacteriostatic and bactericidal properties Maintained intestinal barrier integrity Reduced post-weaning diarrhea Increased growth stimulation	Heavy metal pollution in soil and water Development of antibiotic-resistance bacteria Pharmacological supplementation will be disallowed in EU and other countries	(Hill et al., 2000; NRC: NRC, 2012; EFSA, 2014; Dębski, 2016; Poole, 2017; Liu et al., 2018)
Direct-fed microbials (DFM) and yeast	Improved nutrient digestibility Decreased pH and toxic metabolites in the lumen Alleviated immune challenge Improved gut health Increased growth performance	Not all DFM are thermostable Lost their activity after feed processing No standard procedures to estimate yeast content and its components to distinguish uniqueness of products Inconsistent results	(Stein and Kil, 2006; Buntyn et al., 2016; Liao and Nyachoti, 2017; Liu et al., 2018; Shurson, 2018)
Essential oils	A wide spectrum of antibacterial activity Antioxidant capacity Improved nutrient digestibility Increased growth performance	Antimicrobial properties are mainly based on *in vitro assays* Unstable during feed processing Product variability Various types and doses lead to inconsistent results Interactions between acidifiers and DFM are unclear	(Thacker, 2013; Zeng et al., 2015; Liu et al., 2018)
Enzymes (e.g., lysozyme, carbohydrases)	Targeting specific substrates in feed Increased nutrient digestibility Decreased variability of ingredients between batches Modulated gut microbiome	Moisture, retention time in the GI tract, pH, endogenous enzymes, substrates, and temperature may limit effectiveness Efficacy is dependent on feed ingredients	(de Lange et al., 2010; Ravindran, 2013; Thacker, 2013;

	Decreased incidence and severity of diarrhea	Inconsistent results	Kiarie et al., 2016; Gadde et al., 2017)
Medium chain fatty acids (MCFAs) and monoglycerides	Antiviral and antimicrobial properties Unlikely develop resistance to MCFAs and monoglycerides Immunomodulatory activity Increased growth performance	Required utilizing medium-chain triglycerides as a carrier and lipases to cleavage active compounds *in vivo* Efficacy is dependent on specific free FA Individual and blend of MCFAs could have different responses	(Yoon et al., 2018; Jackman et al., 2020)

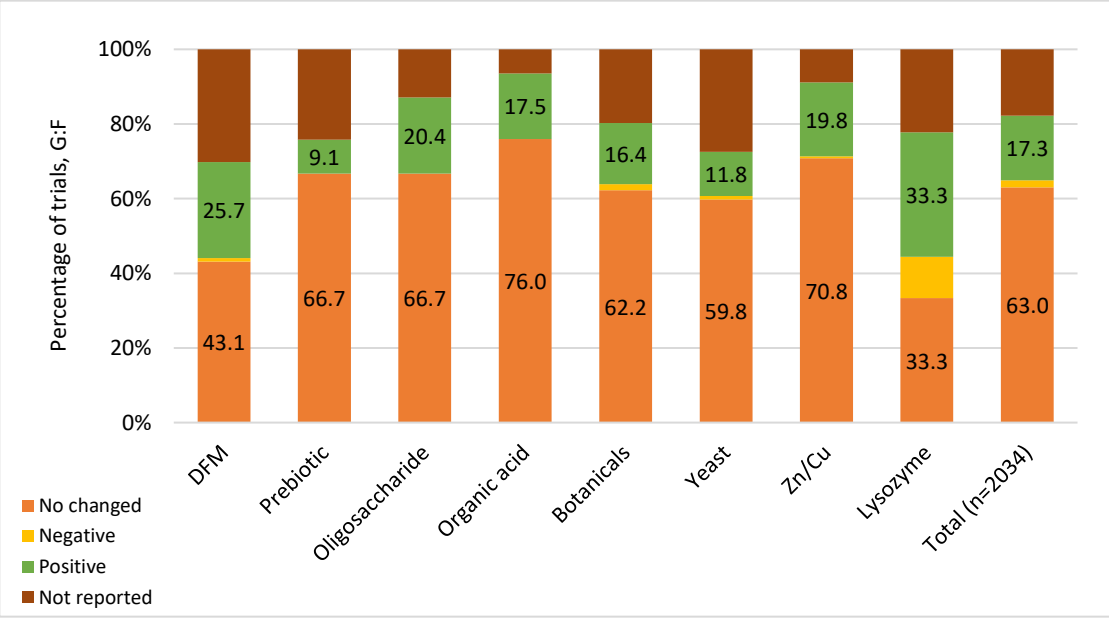

Figure 1.5. Percentage of trials reporting a no change, negative, positive, or not reported in G:F relative to control. (Adapted from Gabler and Schweer, 2018) – Presented in 78th Minnesota Nutrition Conference.

Antibiotics, such as β-lactams, cyclines, macrolides, and streptogramins used as growth promoters have been shown to provide anti-inflammatory effects (Niewold, 2007; Niewold, 2014), and studies have reported co-occurrence of growth-promoting effects and anti-inflammatory effects in poultry and pigs fed cycline antibiotics (Khadem et al., 2014; Soler et al., 2016). Compared with pigs fed control diet, those fed sub-therapeutic doses of oxytetracycline and chlortetracycline had reductions in several inflammatory parameters, including serum haptoglobin, amyloid A, and α-1-acid glycoprotein (Soler et al., 2016), and the gene abundance of cytokine chemokine ligand 2 and β-defensin 2 in the ileum (Helm et al., 2019), suggesting a lower level of inflammation. Nevertheless, these associations between growth improvements and changes in microbial metabolism and reduced local and systematic inflammation in the host need to be validated in future studies.

An improvement in gain efficiency of pigs fed AGPs (Cromwell, 2002; Gaskins et al., 2002) suggests that the growth promotion of AGPs is likely attributed to changes in nutrient utilization and metabolic functions. Feeding diets (14% crude protein) containing 20 ppm tylosin and avilamycin to finishing pigs increased nitrogen retention by 10% and 8% compared with feeding control diets (Roth and Kirchgessner, 1993). Feeding tylosin at 40 ppm to growing pigs resulted in greater energy and dry matter digestibility than in pigs fed control diets (Kirchgessner et al., 1995). In-feed antibiotic cocktails (olaquindox, oxytetracycline, and kitasamycin) added at 50 ppm, increased concentrations of serum amino acids and mRNA expression of intestinal amino-acid transporters in pigs, suggesting an improvement on amino acid sensing, uptake, and utilization (Yu et al., 2017a). Furthermore, pigs fed antibiotic cocktails (22.7 ppm of chlortetracycline, 22.7 ppm of sulfamethazine, and 11.4 ppm of penicillin) showed increased serum concentration of insulin-like growth factor 1, which is involved in anabolic processes and muscle growth (Hathaway et al., 1996; Hathaway et al., 1999). Recently, Trudeau et al. (2018) reported that tylosin induced changes in bile acid metabolism and increased fecal hyodeoxycholic acid concentrations, which was previously reported to be associated with increased protein synthesis in skeletal muscle (Ipharraguerre et al., 2018). Results from proteomics studies indicated that pigs fed diets with AGPs has increased abundance of metabolism related enzymes (glycolytic and gluconeogenic enzymes and cytochrome c

oxidase) in the liver, suggesting post-absorptive changes on nutrient metabolism (Helm et al., 2019). These results suggest that feeding AGPs might trigger a holistic improvement on energy and nutrient utilization, leading to growth-promoting effects. In order to better understand how antibiotics act as growth promoters, a broad-spectrum integrative study design rather than a trial-and-error approach is needed.

Pharmacological levels of copper and zinc as AGPs and their impact on One Health

Trace minerals, such as Cu and Zn, are indispensable nutrients because of their role in many physiological functions (e.g., synthesis of hemoglobin and several enzymes and nutrient metabolism). The requirements of Cu and Zn is 6 to 3 ppm and 100 to 50 ppm for nursery and grow-finishing pigs, respectively (NRC, 2012). In addition to being essential in swine diets, pharmacological levels of Cu (250 ppm) and Zn (2500 ppm) were also identified as a growth promoter for pigs by Barber et al. (1955) and Poulsen (1995), respectively. Although either pharmacological Cu or Zn supplementation improves growth performance of piglets, the growth-promoting effect of the combination of pharmacological Cu and Zn is inconclusive (Hill et al., 2000; Pérez et al., 2011). For purposes of post-weaning diarrhea prevention and growth promotion, a common feeding practice is to supplement pharmacological Zn (1000 to 3000 ppm) from zinc oxide (ZnO) in nursery pig diets up to phase II and then replace Zn by pharmacological Cu supplementation (75 to 250 ppm) from cupric sulfate ($CuSO_4$) for the remaining nursery period (Menegat et al., 2019).

Although the modes of action for growth-promoting effect of pharmacological Cu and Zn supplementation are not fully elucidated, it has been hypothesized that antibacterial properties of Cu and Zn may contribute to growth-promoting effect (NRC, 2012; EFSA, 2014; EFSA, 2016; Blaabjerg and Poulsen, 2017). Antibacterial properties could decrease pathogen load, microbial catabolism of nutrients, decomposition of bile salts, and concentrations of toxic metabolites (Corpet, 2000). These changes could increase the amount of available nutrients for growth and improve nutrient utilization (Corpet, 2000; Klasing, 2007). Compared with basal Cu, pharmacological Cu supplementation decreased total leukocyte count and increased growth in conventional piglets but not in germ-free piglets (Shurson et al., 1990). Feeding pharmacological Zn to piglets decreased bacterial activity (ATP accumulation) in digesta (Højberg et al., 2005)

and the rate of gastric and intestinal bacteria growth, measured as qPCR and turbidity test (Starke et al., 2013). These results support that pharmacological Cu and Zn supplementation act as an antimicrobial agent to increase growth rate.

Feeding both AGPs with pharmacological Cu or Zn to nursery pig diets showed an additive manner on growth performance (Hill et al., 2001; Cromwell, 2002), suggesting that growth promotion is independent of antimicrobial effects. Interestingly, $CuSO_4$ and ZnO are not the most bioavailable mineral sources, but they are effective in promoting growth, suggesting their potential luminal effects in the GI tract (Pérez et al., 2011; Dębski, 2016). Post-weaning pigs often experience intestinal and immune system dysfunctions, thereby impairing health and growth performance (Moeser et al., 2007; Campbell et al., 2013; Moeser et al., 2017). Supplementation with Cu-exchanged montmorillonites at 1500 ppm improved gain efficiency and decreased plasma concentrations of diamine oxidase and ileal concentrations of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) in weaned pigs, suggesting improved gut barrier function and alleviated local inflammation (Song et al., 2013). Several studies also reported that pharmacological Zn supplementation improved intestinal barrier function and alleviated gut inflammation of weaned pigs as demonstrated by increasing transepithelial electrical resistance, reducing gut permeability, and counteracting pro-inflammatory cytokine-induced inflammation (Hu et al., 2013; Song et al., 2015a; Song et al., 2015b; Zhu et al., 2017). Robust immune responses regulate anabolic and catabolic processes (i.e., muscle protein degradation) re-partitioning dietary nutrients to the immune response, at the expense of growth (Klasing, 2007; Johnson, 2012). Pharmacological Cu and Zn supplementation maintains gut homeostasis, which partially explains the growth-promoting effect of Cu and Zn.

However Cu and Zn supplementation as growth promoter in diets should be revised because this practice might increase the risk of AMR, which threatens human and animal health (Hao et al., 2014). Pharmacological Cu and Zn supplementation driving the development of AMR in bacteria has been identified. The AMR development occurs by co-selection potential because the coding for antibiotic-, Cu-, and Zn-resistant genes are close in the bacterial DNA and co-transfer of both genes may occur (Slifierz et al., 2015; Blaabjerg and Poulsen, 2017; Poole, 2017). Pharmacological Cu supplementation caused

the selection of Cu-resistant *Enterococcus faecium* and macrolide- and glycopeptide-resistant *Enterococcus faecium* in swine feces (Hasman et al., 2006). Pharmacological Zn supplementation increased copy numbers for tetracycline- and sulfonamide-resistant genes in intestinal bacteria (Vahjen et al., 2015) and prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in nasal swabs of nursery pigs (Slifierz et al., 2015). Livestock-associated MRSA due to AGPs, Cu, and Zn supplementation is a global concern (Hao et al., 2014; Poole, 2017).

Pharmacological Cu and Zn supplementation also causes concern for One Health due to their pollution in the environment that further acts as a reservoir for AMR (EFSA, 2014; EFSA, 2016). One objective of the One Health approach is to combat AMR, which causes at least 700,000 deaths each year and could increase to 10 million by 2050 if no precautions are taken (WHO, 2019). Most Cu and Zn in feed will be excreted in the feces when pharmacological dose of Cu and Zn are supplemented. It is unequivocal that heavy metals in soils and water are mainly from industrial and agricultural practices and will induce the selection pressure for increasing the bacterial resistome (Berg et al., 2010). In addition to environmental reservoirs of AMR, applying manure with elevated Cu and Zn levels as fertilizer can cause toxicity in plants like corn, soybean, peanuts, and rice (Borkert et al., 2008). Soil contaminated with heavy metals can inhibit plant growth by influencing photosynthesis and chlorophyll biosynthesis (Adrees et al., 2015). However, the risk of application of swine manure to a land is largely dependent upon particular metals of manure, soil sensitivity/types and tillage frequency. An environmental risk assessment indicated that soil with long-term exposure (50 years) to Cu and Zn derived from pigs could exceed the predicted no-effect concentration, suggesting that risks of Cu and Zn contaminated land could occur in some systems in the near future (Monteiro et al., 2017).

The risks of AMR and environmental pollution can be managed through lowering dietary Cu and Zn supplementation (Jondreville et al., 2003; EFSA, 2014; EFSA, 2016). The EU animal feed legislation states that the maximal Cu concentration for piglets up to 25 kg of BW is 170 ppm and the maximal Zn supplementation will be limited to 150 ppm by 2022. This regulation aims to reduce AMR and environmental pollution from swine production, in alignment with the One Health movement. However, swine industry

stakeholders lack of effective alternatives to maintain pig performance without feeding pharmacological Cu and Zn supplementation or AGPs. Thus, there has never been a more urgent time for better understand biological responses associated with growth-promoting effect of pigs fed AGPs (Chapter 5). Swine nutritionists might help control the spread of AMR by removing pharmacological supplementation and achieve the performance target of pigs by knowing the mechanisms of growth-promoting effects.

Summary, aim, and outline of the dissertation

Higher demand for animal protein is expected as economic well-being improves and the global population grows over the next 30 years. The swine industry is one of the players to meet the high demand for animal protein through continuous increasing pork production. However, producing more pork using a conventional approach may not be economically and environmentally sustainable, or promote animal health and wellbeing. The current feeding schemes, although effective to ensure growth performance and production efficiency, do not align with the core values of One Health of achieving optimal health on humans, animals, and the environment. While the U.S. pork industry is committed to One Health (NPB, 2016), several nutritional challenges to feeding pigs sustainably need to be addressed.

First, alternative dietary energy sources for pigs can be obtained from agro-industrial by- and co-products. However, rendered lipids, distillers corn oil, and many other lipid sources are subjected to lipid peroxidation that impairs energy utilization by animals. Studies on the impact of feeding diets containing peroxidized lipids on pigs' growth performance and oxidative status are inconsistent. Secondly, although cereal co-products and ethanol by-products provide a large amount of dietary energy to pigs, DF in these alternative feedstuffs tends to limit caloric efficiency, impair nutrient digestibility and growth performance of pigs. It remains unclear which characteristics of DF limit the capability of pigs to utilize energy and nutrients from alternative feedstuffs. Thirdly, it is imperative to identify products or feeding strategies that mimic the growth-promoting effects of AGPs without increasing the risk of AMR and environmental pollution. Assessing a broad spectrum of biological responses to feeding AGPs to pigs might reveal mechanisms of action for growth promotion.

It is important to maintain profitability of pig farming while moving into sustainable production and attempting to achieve One Health. Thus, the objective of this dissertation was using systems approach to address some of the current nutritional challenges by investigating dietary factors influencing caloric and nutritional efficiency to improve growth performance without increasing the negative environmental impacts of pork production and cost (Figure 1.2).

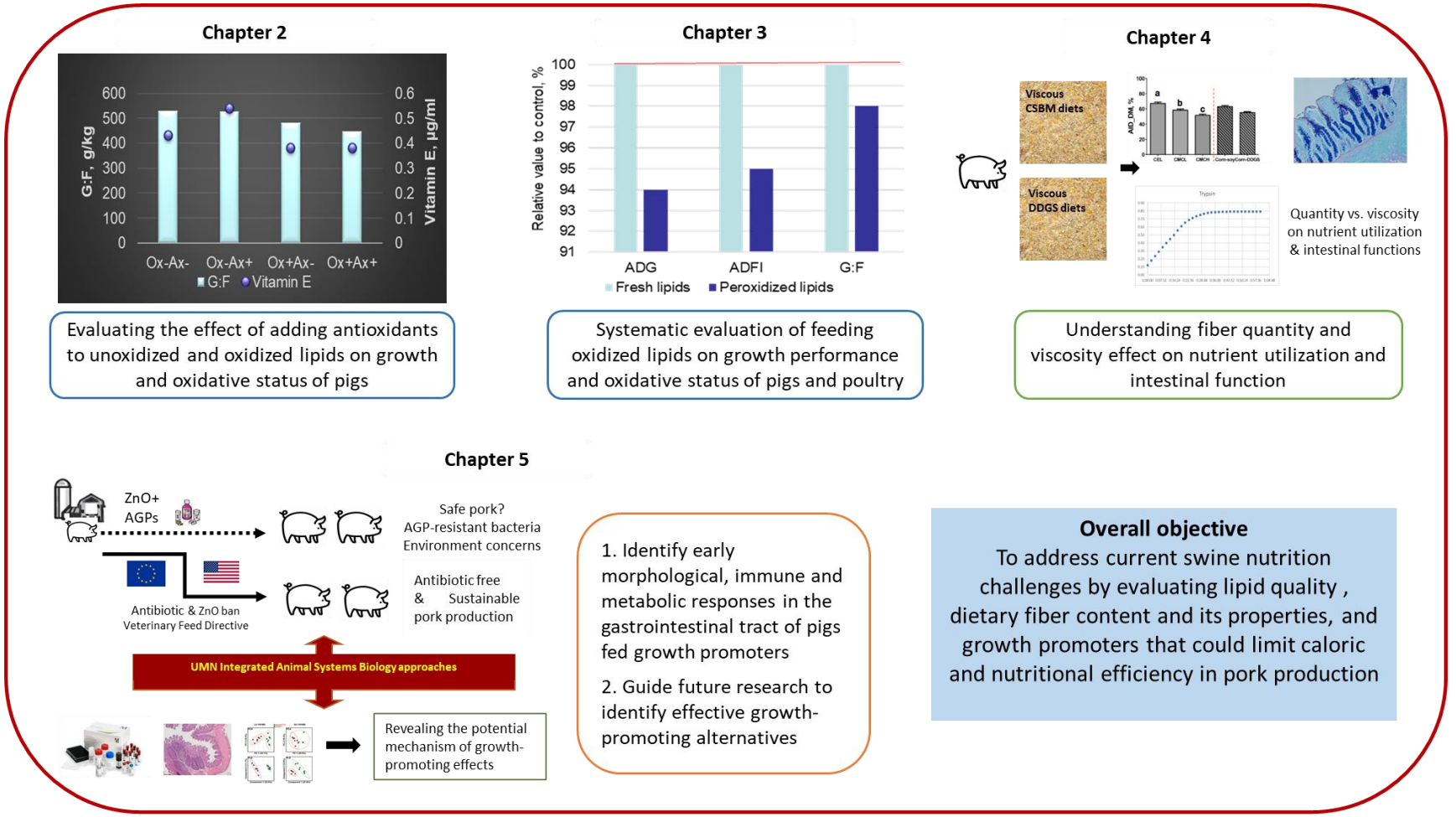

Figure 1.6. Organization of dissertation chapters and overall objective

Chapter 2 – Effect of supplemental antioxidants after oil oxidation on growth performance and oxidative status of pigs

Addition of tert-butylhydroquinone (TBHQ) to maize oil reduces lipid oxidation but does not prevent reductions in serum vitamin E in nursery pigs

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Synopsis

Background: Maize oil is abundantly used in foods and feeds and is highly susceptible to oxidation. Consequently, commercially available antioxidants should be evaluated for effectiveness against lipid oxidation in swine diets. Our study was conducted to evaluate growth performance of nursery pigs fed oxidized maize oil and to determine effects of using antioxidants on oxidative status in a 2 × 2 factorial design. Two hundred eight weaned pigs were blocked by initial BW into 13 blocks, resulting in 4 pigs per pen and 13 pens per treatment. Dietary treatments included 6% unoxidized or oxidized maize oil, and 0 or 60 mg/kg of tert-butylhydroquinone (TBHQ), which was added after lipid oxidation. Data for growth performance were collected from 5 time periods of a two-phase feeding program (Phase 1 = d 0 to 12 and Phase 2 = d 13 to 34). Serum and liver samples were collected from one pig per pen, which had initial BW closest to average BW to determine oxidative status on d 34. **Results:** Oxidized maize oil was heated for 12 h at 185 °C with 12 L/min of air, yielding a peroxide value (PV) of 5. 98 mEq O_2/kg and TBARS of 0.11 mg MDA eq/g. Addition of TBHQ to diets containing oxidized maize oil decreased PV by 37% and increased the oil stability index by 69%. Final BW, ADG, ADFI, and G:F of pigs were not different among the four dietary treatments. However, pigs fed oxidized maize oil tended ($P < 0.08$) to increase hepatosomatic index by 5% compared with those fed unoxidized oil, and this was not affected by adding TBHQ. The serum vitamin E concentration of pigs fed oxidized maize oil was less ($P < 0.03$) than pigs fed unoxidized oil, but this reduction was not reversed by adding TBHQ. Finally, the serum and liver selenium concentration were not different among the treatments. **Conclusions:** The addition of TBHQ did not affect growth performance and vitamin E status in pigs fed moderately oxidized maize oil, but TBHQ reduced lipid oxidation, enhanced the oil stability, and appeared to reduce oxidative stress.

Keywords: growth performance, lipid oxidation, maize oil, nursery pigs, TBHQ, vitamin E

Background

Maize oil is an abundant source of vegetable oil used in human foods and animal feeds. Maize oil provides 8,579 kcal/kg of ME (NRC (National Research Council), 2012), which is comparable to soybean oil (8,574 kcal/kg), and greater than canola oil (8,384 kcal/kg) and palm kernel oil (7,119 kcal/kg) when added to swine diets. The addition of lipids to animal feeds not only increases energy density, but also enhances the absorption of fat-soluble vitamins and improves feed efficiency and palatability (Azain, 2001). The U.S. ethanol industry produces about 1.84 billion kg of distillers maize oil annually, which is used in swine and poultry feeds as well as biodiesel feedstock (USDA-ARS, 2018). However, recent studies have reported that distillers maize oil sources vary in the extent of peroxidation (Kerr et al., 2016; Lindblom et al., 2017). Because maize oil contains high concentrations of unsaturated fatty acids, which can range from 81% to 95% of total fatty acids (Xu et al., 2010; Kerr et al., 2016), it is highly susceptible to peroxidation when storage and processing conditions involve exposure to oxygen, heat, and pro-oxidant metals (Belitz et al., 2009).

Several negative effects of feeding peroxidized lipids to poultry and swine have been summarized (Hung et al., 2017), including reduced energy digestibility (Inoue et al., 1984; Engberg et al., 1996; Rosero et al., 2015), growth rate (Tavárez et al., 2011; Rosero et al., 2015), feed intake (DeRouchey et al., 2004), feed efficiency (Hanson et al., 2016; Chang et al., 2017), and impaired immune function and oxidative status (Boler et al., 2012; Hanson et al., 2016). Furthermore, some studies observed that feeding diets containing peroxidized lipids increased the number of pigs medicated (Chang et al., 2017) and mortality rate in broilers (Anjum et al., 2004). Therefore, there is a tremendous need to find approaches to prevent lipid peroxidation of fats and oils used in animal feeds to minimize these negative effects on animal health and growth performance.

Although maize oil contains significant amounts of natural antioxidant compounds (tocopherols, tocotrienols, phytosterols, steryl ferulates, and carotenoids) (Winkler-Moser and Breyer, 2011), synthetic antioxidants are considered to be more effective and stable during processing (Crane et al., 2000). Several commonly used commercial antioxidant products include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin, tert-butylhydroquinone (TBHQ), and propyl gallate.

Compared to BHA and BHT, TBHQ has superior protection in vegetable oil due to its stability at high temperatures (Mahmoud Allam and Aly Mohamed, 2002; Shahidi and Zhong, 2005b). In addition, a synergistic effect has been observed for TBHQ when used in combination and other antioxidants (e.g., citrate, BHA, and BHT) to increase the thermal stability and smoke point of fats and oils (Yen et al., 1997; Mahmoud Allam and Aly Mohamed, 2002). The addition of TBHQ and a blend of ethoxyquin and TBHQ have been shown to significantly reduce lipid peroxidation of distillers' maize oil when stored under hot (38.6 °C) and humid (94% relative humidity) conditions for 28 d (Hanson et al., 2015). Nevertheless, the potential benefits of feeding lipids containing synthetic antioxidants have not been extensively evaluated, and responses have been inconsistent. Dietary antioxidants tended to improve G:F in finishing barrows during the first 28 d, but not for the entire feeding period (Boler et al., 2012). Therefore, we hypothesized that the addition of TBHQ to maize oil supplemented in nursery pig diets would prevent further lipid peroxidation and ameliorate the potential negative effects on growth performance and oxidative stress. The objective of this study was to investigate the effects of adding TBHQ to unperoxidized and peroxidized maize oil on growth performance and oxidative status of nursery pigs.

Materials and Methods

The University of Minnesota Institutional Animal Care and Use Committee approved the experimental design and animal use under protocol 1304-30545A.

Animals and experimental design

This experiment was conducted at the University of Minnesota West Central Research and Outreach Center in Morris, MN. Crossbred barrows and gilts (n = 208) were weaned at 21 days of age (initial BW = 7.6 ± 0.6 kg) and fed experimental diets during a 34-d feeding period, using a two-phase feeding program (Phase 1 = d 0 to 12 and Phase 2 = d 13 to 34). Pigs were housed in 2 identical nursery rooms and stratified by BW into 13 blocks. Pens of pigs (4 pigs/pen) within block were assigned randomly to 1 of 4 treatments in a 2 × 2 factorial arrangement, resulting in 13 pens per treatment. Each pen (2.4 m × 1.2 m) consisted of plastic grated flooring with a dry feeder (4 feeder spaces) and a nipple drinker with cup.

Maize oil treatments

Refined, deodorized, bleached maize oil (Stratas Foods, LLC, Memphis, TN) was purchased and used in this study. This maize oil source had an initial peroxide value (PV) of 0.05 mEq O_2/kg, and 0.04% free fatty acids. Half of the amount of fresh maize oil was subjected to oxidation by heating it at 185 °C for 12 h with 12 L/min continuous flow of compressed air. The other half of fresh maize oil was not heated (unoxidized). After heating exposure, both oxidized and unoxidized maize oil samples were stored in barrels at − 20 °C to prevent further oxidation. At 7 d prior to weaning and the start of the experiment, half of the oil samples from oxidized and unoxidized oil were mixed with Rendox® CQ (Kemin Industries, Des Moines, IA) to supply 60 mg/kg of TBHQ prior to mixing experimental diets. Hence, the TBHQ was added after the heating process. The oxidized and unoxidized maize oil batches, with and without TBHQ, were moved to the feed mill and added at 6% to the phase 1 diets (Table 2.1). After mixing the phase 1 diets, maize oil samples were stored in the feed mill for 15 d until mixing the phase 2 diets.

Maize oil samples were retained during manufacture of phase 1 and 2 diets at -20 °C until the end of the growth performance study, and were subsequently analyzed for peroxidation at the University of Missouri Agricultural Experiment Station Chemistry Laboratory using standard AOCS procedures (American Oil Chemists' Society (AOCS), 2013) for PV (method Cd 8-53), thiobarbituric acid reactive substances (TBARS), P-anisidine value (AnV), and oil stability index (OSI). The TBARS value was modified from AOCS procedure (method Cd 19-90), which uses malonaldehyde (MDA) as a standard as described by Pegg (2005) (Pegg, 2005). The OSI at 110 °C was determined by the AOCS official method (method Cd 12b-92) (American Oil Chemists' Society (AOCS), 2013). The AnV is a relative measurement to determine the concentration of aldehydes in lipid after hydroperoxides were decomposed, and was determined using method Cd 18-90 at Barrow-Agee Laboratory (Memphis, TN).

Experimental diets

Diets were isocaloric and consisted of maize and soybean meal with 6% unperoxidized or peroxidized maize oil in combination with 0 or 60 mg/kg of TBHQ (Rendox® CQ). Phase 1 diets contained dried whey and soy protein isolate to minimize the inclusion and antigenic effects of soybean meal. Zinc oxide (3600 mg/kg) and

antibiotics (tiamulin - 38.5 mg/kg and chlortetracycline - 440 mg/kg diet) were also included in in Phase 1 diets to minimize post-weaning health concerns, such as enteric diseases and diarrhea. All diets were formulated to exceed the nutritional requirements of weaned pigs suggested by NRC (2012), with an additional safety margin of 5% for lysine, methionine + cysteine, tryptophan, and vitamin E. All pigs were provided ad libitum access to their assigned experimental diets, which were fed in meal form in both nursery phases.

Feed samples were retained, frozen at -20°C, and analyzed by following AOAC official method (AOAC, 2012) for dry matter (method 930.15), crude fat (method 920.39), crude fiber (method 978.10), crude protein (method 990.03), and ash (method 942.05) at Minnesota Valley Testing Laboratories (New Ulm). Lysine, methionine, cysteine, threonine, and tryptophan were analyzed (method 982.30) at the University of Missouri Agricultural Experiment Station Chemistry Laboratory (Columbia).

Data and sample collection

Individual pigs and feeders in each pen were weighed on days 0, 5, 12, 19, 26, and 34 (end of trial) post-weaning to calculate pen average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F). For blood and tissue collection, focal pigs (13 pigs/treatment) were selected based on the individual pig closest to the mean initial BW in each pen. Blood (10 mL) was collected via jugular venipuncture using trace element-free serum tubes (Cat #368380; Becton Dickinson, Franklin Lakes, NJ) on d 34 (fed state). Blood samples were placed on ice for clotting after collection and centrifuged at 1400 ×g for 10 min at 4 °C. Serum was transferred into microcentrifuge tubes and frozen at − 20 °C for subsequent selenium (Se) and vitamin E analysis. At the end of trial (d 34), all focal pigs were euthanized by captive bolt followed by exsanguination. Intact livers were excised, weighed individually, and the hepatosomatic index (HSI) was calculated as wet liver weight (kg)/body weight (kg). Subsequently, portions of the liver were harvested and stored at − 80 °C until analysis of Se and vitamin E concentration.

Selenium and vitamin E analysis in serum and liver

The Se and vitamin E concentrations in serum and liver were analyzed at the Michigan State University Diagnostic Center for Population and Animal Health (East

Lansing). One gram of liver tissue was digested overnight in 2 mL nitric acid, and Se concentrations were determined according to the procedure of Wahlen et al. (2005) of using an inductively coupled plasma mass spectrometer (Agilent 7500ce, Agilent Technologies, Inc., Santa Clara, CA). For vitamin E analysis, liver samples were weighed and homogenized in distilled, deionized water (1:4 w/v). Serum samples and liver homogenates were mixed with equal volumes of hexane and a solution of BHT in ethanol (10% w/v). Mixtures were centrifuged at 1900 ×g for 10 min, and a known aliquot of the hexane layer was removed and dried under vacuum. Samples were dissolved in a chromatographic mobile phase (7:2:1, acetonitrile, methylene chloride, methanol) and analyzed by HPLC (Separation Module 2690) using an analytical column (Waters Symmetry C18, 3.5 mm, 4.6 μm × 75 mm) with detection by UV absorbance at 292 nm (Waters, Milford, MA). Trans-β-APO-8″-carotenal was used as an internal standard.

Statistical analysis

Data were analyzed for overall structure, absence of outliers, and normal distribution using the PROC UNIVARIATE procedure of SAS (SAS Institute, Cary, NC). Experimental data were analyzed as a randomized complete block design using PROC MIXED of SAS. Pen was considered as the experimental unit for all responses. The statistical model included fixed effects of extent of maize oil oxidation, time, antioxidant, as well as 2- and 3-way interactions. Random effects included block and pen. Data were analyzed for the effect of time by REPEATED measures, and an unstructured covariance matrix was used. Data for liver weight, HSI, as well as hepatic and serum vitamin E and Se were analyzed with a similar treatment structure, but the effect of time was not included in the model. Interactions were removed from the model when there was no significant effect. All results were reported as least squares means. Multiple comparisons among treatments were performed using PDIFF and adjusted by the Tukey option for multiple comparisons of means. Significant differences were declared at $P < 0.05$ and statistical trends at $P < 0.10$.

Results

General observations

Due to poor health, 8 pigs either died or were removed from 3 of the 4 experimental treatments (3 pigs from unoxidized oil without TBHQ, 3 pigs from

unoxidized oil with TBHQ, and 2 pigs from oxidized oil without TBHQ), and none died or were removed for the treatment of oxidized oil with TBHQ, so these pigs were not included in the data set. No outliers were identified and no data required removal. However, there were signs of acute diarrhea and growth depression between d 5 and d 12 post-weaning. Therefore, based on the attending veterinarian's recommendation, all pigs were treated with neomycin sulfate (22 mg/kg BW) for 3 d, then tiamulin hydrogen fumarate (23 mg/kg BW) for 5 d by water medication to reduce the impact of diarrhea on the growth performance responses. However, after administering the water medication, suboptimal health continued for this group of pigs, which affected the growth performance responses observed in the study.

Oxidation analysis of maize oil

Maize oil samples were analyzed for concentrations of PV, TBARS, AnV, and OSI. The PV of fresh, unoxidized maize oil was 1.85 mEq O_2/kg, which was greater than the manufacturer specification (0.05 mEq O_2/kg; Table 2.2). Maize oil heated at 185 °C for 12 h with air resulted in greater concentrations of PV, TBARS, and AnV than unheated maize oil, which indicated that the heating process and temperature used achieved oxidation. For the maize oil used in phase 1 diets, the PV of the oxidized oil with TBHQ added after heating was 63% less than the oxidized maize oil without TBHQ. However, TBARS and AnV concentrations were not affected by addition of TBHQ to both oxidized and unoxidized maize oil. Oil stability index was 20.85 h in the unoxidized maize oil and 16.05 h in the oxidized maize oil. When TBHQ was added to unoxidized and oxidized maize oil, the OSI increased by 36% and 69%, respectively.

The concentration of PV, TBARS, and AnV of the maize oil used in the phase 2 diets did not change dramatically due to the addition of TBHQ. Nevertheless, adding TBHQ in both unoxidized and oxidized maize oil increased the time of OSI more than 2 times compared with no addition of TBHQ. After mixing phase 1 diets, the oil was stored in the feed mill until mixing phase 2 diets, so the OSI was expected to be reduced during storage. The OSI of unoxidized maize oil decreased from 20.85 h in phase 1 to 10.25 h in phase 2, but adding TBHQ to unoxidized maize oil resulted in the OSI decreasing by only 5 h. Furthermore, the OSI in the oxidized maize oil without TBHQ decreased by 71% (16.05 to 4.65 h,) while the OSI of the oxidized maize oil with TBHQ decreased by 59%

(27.2 to 11.15 h). These results indicate that TBHQ was partially effective in stabilizing both oxidized and unoxidized maize oil based on changes in OSI.

Effect of oxidized maize oil and TBHQ on growth performance

There were no differences in initial BW among four dietary treatments (Table 2.3). Feeding diets containing oxidized maize oil, with or without TBHQ addition, did not affect BW ($P > 0.48$) on d 5, 12, 19, 26, and final BW on d 34. Similarly, the ADG in any of the feeding periods was not affected by feeding diets containing oxidized maize oil, with or without the addition of TBHQ, and there was no interaction between maize oil oxidation and antioxidant ($P > 0.44$; Table 2.4). Furthermore, there was no effect of feeding diets containing oxidized maize oil, with or without TBHQ, on ADFI on d 5, 12, 19, 26, and overall ($P > 0.27$; Table2.5). As a result of no differences in ADG and ADFI among dietary treatments, there were no effects of dietary treatments on G:F (Table 2.6).

Effect of oxidized maize oil and TBHQ on oxidative stress

There was no interaction between maize oil oxidation and antioxidant on liver and serum oxidative status (Table 2.7). Liver weight, as well as liver Se and vitamin E concentrations, were not affected by feeding oxidized maize oil with or without the addition of TBHQ. However, the HSI tended to be greater ($P = 0.08$) for pigs consuming oxidized maize oil compared with those fed unoxidized maize oil, but was not affected by adding TBHQ to maize oil. In serum, the Se concentration was not different in pigs consuming oxidized maize oil ($P > 0.40$) compared with unoxidized maize oil, with or without the addition of TBHQ ($P > 0.32$). In contrast, pigs fed oxidized maize oil had reduced ($P = 0.03$) serum vitamin E concentrations compared with those consuming the diet with unoxidized oil, but adding TBHQ to unoxidized and oxidized maize oil did not significantly increase serum vitamin E concentrations in pigs.

Discussion

Peroxide value, TBARS, AnV, and OSI are common assays used to characterize lipid oxidation of fats and oils in the feed industry (Kerr et al., 2015). However, each assay measures only a fraction of various types of oxidation products, which accumulate and degrade over time (Shurson et al., 2015). Therefore, multiple oxidation measures are required for a more comprehensive assessment of the extent of oxidation of fats and oils (Shurson et al., 2015). Results from the current study showed that PV, TBARS, and AnV

content was increased when maize oil was heated at 185 °C for 12 h with air, and OSI was decreased, which is in agreement with results from previous studies (Liu et al., 2014c; Hanson et al., 2016; Lindblom et al., 2018). The TBARS content of oxidized maize oil produced in our study was 2.5 times greater than the value reported by Kerr et al. (2015) and Hanson et al. (2016) using similar thermal processing conditions. The AnV of oxidized maize oil in our study was similar to that reported by Hanson et al. (2016), but lower than the value reported by Kerr et al. (2015). Wang et al. (2016) reported that the kinetic profile of TBARS reached a plateau of 10.75 mg MDA/kg oil after heating oil at 185 °C for 2 h, but our TBARS value was about 10-fold greater than this value after heating at 185 °C for 12 h. In contrast, the oxidized maize oil evaluated in our study contained a lower PV than that reported in other studies (Chang and van Heugten, 2016; Chang et al., 2017), while the OSI of oxidized maize oil was greater than reported by others (Kerr et al., 2015; Hanson et al., 2016; van Heugten et al., 2016). These results show the difficulty of characterizing the extent of oxidation of lipids because high TBARS infers extensive production of secondary aldehydes from oxidation, but the lower PV and greater OSI values of oxidized maize oil in our study relative to other studies suggests that less oxidation occurred than reported in other studies. Because there were no effects of feeding oxidized maize oil on growth performance of pigs in our study, the PV, AnV, TBARS, and OSI values obtained for oxidized maize oil were not great enough to cause negative performance effects over a 34-day feeding period for nursery pigs.

Synthetic antioxidants are commonly used to increase the oxidative stability of lipids in human foods and feed ingredients. Merrill et al. (2008) reported that addition of TBHQ, alone or in combination with other antioxidants, was effective in increasing the stability of high-oleic vegetable oils. We observed that the addition of TBHQ after heating to oxidized maize oil resulted in a lower PV value and greater OSI than maize oil without TBHQ supplementation, which confirms that TBHQ is effective in stabilizing maize oil and increases its resistance to oxidation. Belitz et al. (2009) explained that antioxidants delay the rate of oxidation, but do not reverse oxidation if it has occurred. Because TBHQ was added to maize oil after the heating process in the current study, and oil used in phase 2 diets had longer storage time, the magnitude of oxidation of maize oil was greater when added to phase 2 diets than the oil used in phase 1 diets. Therefore,

adding TBHQ to oxidized maize oil reduced the magnitude of further lipid oxidation but did not completely prevent it.

Animal physiological status, types of lipids fed, level of oxidation, and multiple combinations of synthetic or natural antioxidants result in variable growth performance responses of nursery pigs fed oxidized lipids. Results from a recent meta-analysis study showed that reductions in growth performance responses from feeding oxidized lipids to pigs and broilers were highly variable, but average reductions in ADG, ADFI, and G:F were 5%, 3%, and 2% respectively, compared with feeding unoxidized lipids (Hung et al., 2017). Based on the extent of maize oil oxidation and the heating process used in our experiment, we expected to observe differences in growth performance. However, Chang and van Heugten reported that ADG, ADFI, and G:F were not affected by feeding oxidized maize oil with PV equal to 8.8 mEq O_2/ kg diet (Chang and van Heugten, 2016). Similarly, no differences in ADG and ADFI were observed in pigs fed highly oxidized maize oil with PV of 8.1 mEq O_2/ kg in the diet (Chang et al., 2017). In previous work, G:F declined linearly with increasing lipid oxidation (Chang et al., 2017), but results from the current study showed no dietary effects on G:F. The oxidized maize oil fed in our study had a PV less than 0.4 mEq O_2 /kg diet and OSI greater than 4.5 h, which may be considered to be mildly oxidized compared with lipids fed in previous studies. However, PV is not a definitive, comprehensive indicator of the extent of oxidation of lipids. The lack of differences in growth performance responses from feeding oxidized maize oil in the current study suggests that the PV, TBARS, AnV, and OSI values of oil may be considered acceptable when evaluating lipid oxidation of maize oil sources. Unfortunately, the health status of the pigs in our study was suboptimal, and acute post-weaning diarrhea resulted in growth depression. As a result, the suboptimal health status of pigs may have compromised our ability to detect differences in growth performance responses from feeding oxidized and unoxidized diets. The overall ADG of pigs fed the unoxidized maize oil in the current study was 360 g/d, which was 5% less than the ADG responses reported by Hanson et al. (2016) using the same research facilities and thermal processing conditions to produce oxidized maize oil.

Pigs fed oxidized maize oil in our study had similar feed intake compared with pigs fed oxidized maize oil in our previous study (Hanson et al., 2016). Several studies

have shown that rancid flavor from oxidized lipids (Dibner et al., 1996; Belitz et al., 2009), aldehyde odor (Esterbauer et al., 1991; Halliwell and Chirico, 1993), and reduced palatability of oxidation products (Nwanguma et al., 1999) are possible reasons for reduced feed intake when feeding diets containing oxidized lipids. Furthermore, Dibner et al. (2011) reported that lipid oxidation reduced gross energy content by 35%, and oxidized vegetable oil had lower nutrient and energy digestibility in poultry (Inoue et al., 1984; Engberg et al., 1996), suggesting that oxidation reduces the nutritional value of lipids. Our results indicate that the presence of lipid oxidation products in the maize oil fed in the current study were not great enough to cause a reduction in feed intake.

Results from our study showed that adding TBHQ to maize oil was effective in minimizing lipid oxidation by reducing PV up to 37% and OSI up to 69% compared to not using TBHQ in the stored maize oil. However, it is not clear whether TBHQ supplementation to unoxidized and oxidized maize oil has beneficial effects on pigs. Although adding TBHQ to maize oil reduced further lipid oxidation, it had no benefit on growth performance. Chang and van Heugten reported no differences in nursery pig growth performance when an antioxidant blend of ethoxyquin, BHT, and BHA was added at 60 mg/kg to an oxidized maize oil diet (Chang and van Heugten, 2016). Likewise, McGill et al. (2011a) observed no differences in ADFI and ADG when broilers consumed oxidized lipid diets supplemented with ethoxyquin at 150 mg/kg. In contrast, adding an antioxidant blend of ethoxyquin and propyl gallate at 135 mg/kg to oxidized soybean oil diets resulted in mitigation of growth rate and feed efficiency reductions in pigs, which was comparable to those fed a maize-soybean meal diet without antioxidants (Lu et al., 2014b), as well as improvements in feed intake and growth rate in broilers (Tavárez et al., 2011). Further studies are needed to evaluate differences in growth performance responses of pigs when adding various types of synthetic antioxidants to oxidized lipid diets. However, it is important to recognize that the U.S. Food and Drug Administration strictly regulates the use of synthetic antioxidants in foods and animal feeds. The maximum usage rate of TBHQ is to not exceed 0.02% of the lipid content of food or feed. The maximum inclusion level for ethoxyquin is 150 mg/kg, and 200 mg/kg for BHA and BHT (Salami et al., 2016). These restrictions are based on safety concerns from excessive use because metabolites of antioxidants can be cytotoxic (Okubo et al.,

2003) and can result in DNA damage (Shahidi and Zhong, 2005b). In addition, oxidative stress can be triggered by inappropriate antioxidant usage (Poljsak et al., 2013) and an overdose of antioxidants can lead to an oxidant-antioxidant imbalance (Poljsak and Milisav, 2012; Salami et al., 2016). Therefore, prudent use of synthetic antioxidants in animal feeds is warranted to avoid adverse physiological effects from excessive use.

The trend for increases in HSI observed in pigs fed oxidized maize oil in our study is consistent with previous findings (Huang et al., 1988; Liu et al., 2014a). An increase in liver size relative to body weight has been used as an indicator of toxicity (Juberg et al., 2006), and studies have shown that feeding oxidized lipid diets increases HSI (Eder, 1999). This response may be a result of increased synthesis of microsomal enzymes to mitigate toxicity (Huang et al., 1988). Toxic oxidized products can be absorbed and transported to liver (Kanazawa et al., 1985), and broilers that consumed oxidized lipids have been shown to have increased hepatocyte proliferation (Shermer et al., 1995). Increased hepatocyte proliferation may be related to cytotoxicity of oxidized products because elevated plasma alanine transaminase has been observed in pigs fed oxidized soybean oil (Lu et al., 2014b). Studies have also shown that liver weight was positively correlated with active oxygen method, AnV, and TBARS (Liu et al., 2014a), and negatively correlated with growth rate in swine (Lu et al., 2014b).

The reduction in serum vitamin E concentrations from feeding oxidized maize oil in our study is consistent with reports from several other studies (Engberg et al., 1996; Boler et al., 2012; Varady et al., 2012; Chang and van Heugten, 2016; Hanson et al., 2016), but it may be inappropriate to compare changes in vitamin E concentrations among studies due to varying levels of oil oxidation used. However, our results are in agreement with results reported by Hanson et al. (2016), which used similar experimental conditions and found serum vitamin E concentration to be 0.3 μg/mL in nursery pigs fed diets containing 6% oxidized maize oil. The physiological antioxidant defense system requires involvement of vitamin E (non-enzymatic antioxidant) and Se (structural component of glutathione peroxidase) (Kerr et al., 2015). On average, pigs fed oxidized lipids have a 46% reduction in serum vitamin E content compared with pigs fed unoxidized lipids (Hung et al., 2017). Our findings indicate that even with moderately low oxidation of the maize oil fed to weaned pigs, the reduction in serum vitamin E

concentrations were not recovered by adding TBHQ to maize oil. There are 3 possible explanations for this reduction in serum vitamin E. First, high metabolic demands reduce serum vitamin E concentration because it interacts with free radicals produced from oxidation to minimize the negative effects of endogenous oxidative stress (Boler et al., 2012). Second, thermally oxidized lipids decrease the concentration of tocopherols (Varady et al., 2012; Liu et al., 2014c). Lastly, thermally oxidized lipids have decreased α-tocopherol digestibility (Eder et al., 2002), resulting in animals absorbing less vitamin E. Unlike vitamin E, there were no oxidation and antioxidant differences in serum and liver Se concentrations. Hanson et al. reported no effect of increasing dietary levels of oxidized maize oil on liver Se concentration, but there was a trend for reduced Se concentration in serum (Hanson et al., 2016). Similar to our results, Tabatabaei et al., (2008) found no differences in serum and liver concentration of Se in rats fed either fresh or oxidized sunflower oil. Therefore, serum vitamin E concentration appears to be a more sensitive and appropriate marker than Se for evaluating oxidative status of pigs fed oxidized maize oil.

Conclusions

Heating maize oil at 185 °C with 12 L/min of continuous air for 12 h achieved moderate oxidation, and the addition of TBHQ was partially effective in preventing further oxidation as measured by oil stability index of unoxidized and oxidized maize oil. The PV, TBARS, and AnV values of the oxidized maize oil fed in this study may serve as a general guide for estimating threshold levels of oxidation products that do not reduce growth performance of weaned pigs. However, the long-term effects of feeding this source of oxidized maize oil are unknown because diets were only fed for 34 days, but the trend for greater HSI and reduced serum vitamin E concentration in pigs fed oxidized maize oil suggests potential negative health and performance effects. These effects were not prevented by the addition of TBHQ to oxidized maize oil. The addition of TBHQ to maize oil partially protected oils against further oxidation but did not affect growth performance of nursery pigs.

Tables and Figures

Table 2.1. Ingredient and nutrient composition of experimental diets (as-fed basis)

Phase	Phase 1 (Weaning – d 12)				Phase 2 (d 13 – d 34)			
Maize oil	Unoxidized		Oxidized		Unoxidized		Oxidized	
Antioxidant[1]	-	+	-	+	-	+	-	+
Ingredient composition, %								
Maize	36.67	36.67	36.67	36.67	55.34	55.34	55.34	55.34
Soybean meal, 47.5% CP	18.01	18.01	18.01	18.01	34.04	34.04	34.04	34.04
Unoxidized maize oil[2]	6.00	.	.	.	6.00	.	.	.
Unoxidized maize oil + TBHQ	.	6.00	.	.	.	6.00	.	.
Oxidized maize oil[3]	.	.	6.00	.	.	.	6.00	.
Oxidized maize oil + TBHQ	.	.	.	6.00	.	.	.	6.00
Dried whey	24.31	24.31	24.31	24.31	.	.	.	.
Soy protein isolate	10.00	10.00	10.00	10.00	.	.	.	.
Zinc oxide	0.50	0.50	0.50	0.50	.	.	.	.
Antibiotics[4]	0.57	0.57	0.57	0.57	.	.	.	.
Vitamin/mineral premix[5]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Lys	0.43	0.43	0.43	0.43	0.53	0.53	0.53	0.53
DL-Met	0.26	0.26	0.26	0.26	0.21	0.21	0.21	0.21
L-Thr	0.12	0.12	0.12	0.12	0.17	0.17	0.17	0.17
Monocalcium phosphate	1.03	1.03	1.03	1.03	1.16	1.16	1.16	1.16
Limestone	1.25	1.25	1.25	1.25	1.28	1.28	1.28	1.28
Salt	0.35	0.35	0.35	0.35	0.77	0.77	0.77	0.77
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Calculated composition								
ME[6], kcal/kg	3619	3619	3619	3619	3556	3556	3556	3556
Crude protein, %	23.88	23.88	23.88	23.88	21.56	21.56	21.56	21.56
NDF, %	4.82	4.82	4.82	4.82	7.83	7.83	7.83	7.83
Ether extract, %	8.07	8.07	8.07	8.07	8.44	8.44	8.44	8.44
Linoleic acid, %	3.88	3.88	3.88	3.88	4.26	4.26	4.26	4.26
ATTD[7] P, %	0.39	0.39	0.39	0.39	0.33	0.33	0.33	0.33
Ca, %	0.90	0.90	0.90	0.90	0.81	0.81	0.81	0.81
SID[8] Lys, %	1.56	1.56	1.56	1.56	1.42	1.42	1.42	1.42
SID Met/Cys %	0.86	0.86	0.86	0.86	0.78	0.78	0.78	0.78
SID Thr, %	0.91	0.91	0.91	0.91	0.83	0.83	0.83	0.83
SID Trp, %	0.26	0.26	0.26	0.26	0.23	0.23	0.23	0.23
Lactose, %	17.50	17.50	17.50	17.50	0.00	0.00	0.00	0.00
Vitamin E, IU/kg	22.00	22.00	22.00	22.00	22.00	22.00	22.00	22.00
g SID Lys:Mcal ME	4.31	4.31	4.31	4.31	3.99	3.99	3.99	3.99
g ATTD P:Mcal ME	1.08	1.08	1.08	1.08	0.93	0.93	0.93	0.93
Ca:ATTD P	2.31	2.31	2.31	2.31	2.45	2.45	2.45	2.45
SID Met+Cys:SID Lys	55.13	55.13	55.13	55.13	54.93	54.93	54.93	54.93
SID Thr:SID Lys	58.33	58.33	58.33	58.33	58.45	58.45	58.45	58.45
SID Trp:SID Lys	16.67	16.67	16.67	16.67	16.20	16.20	16.20	16.20
Analyzed composition, %								

Dry matter	91.14	91.33	91.13	91.17	88.39	88.18	88.21	88.10
Ether extract	4.72	5.01	4.49	3.81	4.12	4.22	3.79	3.77
Crude fiber	2.15	2.04	2.04	1.88	3.02	3.26	2.94	2.73
Ash	7.38	7.20	7.06	7.05	5.46	5.01	6.02	6.01
Crude protein	22.89	23.72	23.98	23.50	21.28	21.22	22.78	21.60
Lysine	1.62	1.80	1.72	1.83	1.52	1.54	1.59	1.67
Methionine	0.50	0.50	0.54	0.51	0.46	0.41	0.49	0.45
Cysteine	0.32	0.33	0.32	0.33	0.31	0.31	0.34	0.34
Threonine	0.99	1.05	1.03	1.06	0.93	0.94	0.99	1.01
Tryptophan	0.32	0.32	0.33	0.35	0.29	0.28	0.30	0.27

[1]Antioxidant, Rendox CQ (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg in maize oil.

[2]Stratas Foods, LLC.

[3]Maize oil heated for 12 h at 185 °C with a constant air flow rate of 12 L/min.

[4]Antibiotics added were 0.175% Denagard (tiamulin 22 g/kg; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) and 0.4% Aureomycin 50G (chlortetracycline 110 mg/kg; Zoetis, Inc., Florham Park, NJ), which provided 38.5 mg tiamulin per kg of diet, and 440 mg chlortetracycline per kg of diet.

[5]Premix for nursery pigs (< 22.5 kg) at 0.5% dietary inclusion rate provided the following nutrients per kilogram of feed: 11,023 IU of vitamin A as retinyl acetate; 2,756 IU of vitamin D_3; 22 IU of vitamin E as dl-alpha tocopheryl acetate; 4.41 mg of vitamin K as menadione dimethylpyrimidinol bisulfite; 9.92 mg of riboflavin; 55.11 mg of niacin; 33.07 mg of pantothenic acid as D-calcium pantothenate; 992 mg of choline as choline chloride; 0.06 mg of vitamin B_{12}; 14.3 mg of pyridoxine; 1.65 mg of folic acid; 2.20 mg of thiamine; 0.33 mg of biotin; 2.20 mg of iodine as ethylenediamine dihydroiodide; 0.30 mg of selenium as sodium selenite; 299 mg of zinc as zinc sulfate; 299 mg of iron as ferrous sulfate; 19.8 mg of copper as copper sulfate; and 17.6 mg of manganese as manganese oxide.

[6]ME = metabolizable energy

[7]ATTD = apparent total tract digestible

[8]SID = standardized ileal digestible

Table 2.2. Lipid oxidation indices of maize oil used in the experiment

Maize oil[1]	**Unoxidized**		**Oxidized**	
Antioxidant[2]	-	+	-	+
Oil used in phase 1 diets				
Peroxide value, mEq O_2/kg[3]	1.85	1.99	5.98	3.75
TBARS, mg MDA/g[4,5]	0.06	0.06	0.11	0.12
AnV[6]	3.0	3.1	134.9	131.9
Oil stability index, h	20.85	28.30	16.05	27.20
Oil used in phase 2 diets				
Peroxide value, mEq O_2/kg	1.95	2.00	5.99	5.89
TBARS, mg MDA/g	0.06	0.06	0.11	0.11
AnV	3.1	3.3	140.8	142.0
Oil stability index, h	10.25	23.15	4.65	11.15

[1]Oils were sampled after manufacturing phase 1 and 2 diets (15 d after mixing phase 1 diets), and stored at -20 °C until analysis at the University of Missouri Agricultural Experiment Station Chemistry Laboratory
[2]Antioxidant = Rendox CQ (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg in maize oil.
[3]Milliequivalents peroxide per kg of oil. W/W% = g/100 g of sample
[4]TBARS = thiobarbituric acid reactive substances
[5]MDA = malondialdehyde
[6]AnV = p-anisidine value

Table 2.3. Effects of feeding maize oil and TBHQ on body weight of nursery pigs

Maize oil	**Unoxidized**		**Oxidized**	
Antioxidant[1]	-	+	-	+
Body weight, kg				
Initial	7.6	7.6	7.6	7.6
Day 5	7.6	7.6	7.6	7.5
Day 12	9.9	9.8	9.8	9.4
Day 19	12.5	12.3	12.3	11.7
Day 26	15.8	15.5	15.5	14.8
Day 34	20.5	20.1	20.2	19.2
Pooled-SEM		0.60		
P-value				
Oxidation		0.53		
Antioxidant		0.48		
Oxidation × Antioxidant		0.73		
Day		< 0.01		
Day × Oxidation		0.26		
Day × Antioxidant		0.09		
Day × Oxidation × Antioxidant		0.84		

[1]Rendox® CQ, (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg of lipid.

Table 2.4. Effects of feeding maize oil and TBHQ on average daily gain of nursery pigs

Maize oil	**Unoxidized**		**Oxidized**	
Antioxidant[1]	-	+	-	+
Average daily gain, g				
Day 0-5	16	2	1	-9
Day 5-12	324	310	322	265
Day 12-19	356	361	351	329
Day 19-26	474	462	465	444
Day 26-34	629	628	634	601
Overall (day 0-34)	360	353	355	326
Pooled-SEM			14	
P-value				
Oxidation			0.25	
Antioxidant			0.20	
Oxidation × Antioxidant			0.44	
Day			< 0.01	
Day × Oxidation			0.99	
Day × Antioxidant			0.84	
Day × Oxidation × Antioxidant			0.89	

[1]Rendox® CQ, (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg of lipid.

Table 2.5. Effects of feeding diets containing maize oil and TBHQ on feed intake of nursery pigs

Maize oil	Unoxidized		Oxidized	
Antioxidant[1]	-	+	-	+
Average daily feed intake, g				
Day 0-5	99	97	90	82
Day 5-12	395	369	374	311
Day 12-19	704	703	711	716
Day 19-26	734	689	703	683
Day 26-34	999	943	964	972
Overall (day 0-34)	586	560	568	553
Pooled-SEM	19			
P-value				
Oxidation	0.50			
Antioxidant	0.27			
Oxidation × Antioxidant	0.79			
Day	< 0.01			
Day × Oxidation	0.83			
Day × Antioxidant	0.81			
Day × Oxidation × Antioxidant	0.82			

[1]Rendox® CQ, (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg of lipid.

Table 2.6. Effects of feeding diets containing maize oil and TBHQ on gain efficiency of nursery pigs

Maize oil	**Unoxidized**		**Oxidized**	
Antioxidant[1]	-	+	-	+
Gain:Feed, g/kg				
Day 0-5	-38	-199	-308	-344
Day 5-12	858	941	882	843
Day 12-19	519	525	499	458
Day 19-26	648	680	665	654
Day 26-34	658	682	669	619
Overall (day 0-34)	529	526	481	446
Pooled-SEM	58			
P-value				
Oxidation	0.27			
Antioxidant	0.73			
Oxidation × Antioxidant	0.78			
Day	< 0.01			
Day × Oxidation	0.80			
Day × Antioxidant	0.97			
Day × Oxidation × Antioxidant	0.97			

[1]Rendox® CQ, (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg of lipid.

Table 2.7. Serum and tissue parameters of nursery pigs fed maize oil and TBHQ

Maize oil	**Unoxidized**		**Oxidized**		**SEM**	***P*-values**		
Antioxidant[1]	**-**	**+**	**-**	**+**		**OX[2]**	**AX[3]**	**OX × AX[4]**
Liver								
Weight, g	583	605	633	621	33	0.32	0.88	0.60
HSI[5]	2.96	3.08	3.17	3.20	0.09	0.08	0.42	0.65
Se, μg/g, dry wt.	2.55	2.45	2.53	2.56	0.06	0.44	0.61	0.31
Vitamin E, μg/g, dry wt.[6]	29.12	26.61	26.12	24.88	2.66	0.38	0.48	0.81
Serum								
Se, ng/mL	173	163	164	164	6	0.40	0.32	0.39
Vitamin E, μg/mL	0.43	0.54	0.38	0.38	0.05	0.03	0.23	0.26

[1]Rendox® CQ, (active ingredient is TBHQ; Kemin Industries, Des Moines, IA) was added at 1,000 mg/kg of lipid.
[2]OX = oxidation
[3]AX = antioxidant
[4]OX × AX = interaction effect between oxidation and antioxidant
[5]HSI = hepatosomatic index = liver weight as % of BW.
[6]Vitamin E was measured in α-tocopherol equivalents.

Chapter 3 – A systematic review of the impact of peroxidized lipids on growth performance and oxidative status in poultry and swine

Peroxidized lipids reduce growth performance of poultry and swine: A meta-analysis

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Synopsis

Animal performance is affected by feeding peroxidized lipids. Nevertheless, inaccuracies and limitations of common lipid peroxidation analyses are associated with inconsistent results re- garding animals consuming diets with peroxidized lipids. A comprehensive meta-analysis was conducted to determine the effects of feeding dietary peroxidized lipids on growth performance and oxidative status in poultry and swine. A total of 29 publications with 42 poultry and 23 swine observations were analyzed. Concentration of dietary thiobarbituric acid reactive substances (TBARS) and peroxide value (PV), along with ADG, ADFI, G:F, and serum or plasma con- centrations of vitamin E and TBARS were obtained from publications when reported. The relative impact of feeding peroxidized lipids was calculated as a percentage of ADG, ADFI, and G:F re- lative to responses from feeding isocaloric diets containing unperoxidized lipids. Data were analyzed for outliers, general distribution, and correlations among variables. Overall, feeding peroxidized lipids to both species resulted in a 5% reduction in ADG, a 3% reduction in ADFI, and G:F was reduced by 2% compared with feeding unperoxidized lipids. The difference in the average magnitude of reduction in ADG, compared with less average magnitude of reduction in ADFI, suggests that factors other than caloric intake (i.e., oxidative stress) contribute to reduced ADG when feeding peroxidized lipids. Both species fed peroxidized lipids had reduced serum or plasma vitamin E content (52%) and increased TBARS concentration (120%) relative to animals fed unperoxidized lipids, suggesting that feeding peroxidized lipids contributes to increased oxidative stress. Dietary PV was negatively correlated with ADG ($r = -0.81$, $P < 0.01$) for poultry, whereas for swine, dietary TBARS was negatively correlated with ADG ($r = -0.58$, $P = 0.04$), but there were large prediction errors for poultry (MSE = 0.87) and swine (MSE = 12.79). In conclusion, these results suggest that feeding peroxidized lipids reduce growth performance of poultry and swine, but the magnitude of reduction varies among ex- periments due to differences in fatty acid profiles among types of lipid sources, time and tem- perature of peroxidation conditions, and relative growth responses among studies. More accurate peroxidation measurement methods need to be developed to accurately estimate the negative impacts of feeding peroxidized lipids on animal growth performance.

Key words: growth performance, lipid quality, meta-analysis, peroxidation, poultry, swine

Introduction

Many sources of lipids are added to poultry and swine diets to increase caloric density, improve palatability and pellet quality, reduce dustiness, and provide essential fatty acids (Azain, 2001; Rocha et al., 2012). However, cost, energy content, and quality vary substantially among lipid sources. Common quality indices used to evaluate lipids include color, fatty acid profile, free fatty acid content, iodine value, saponification value, titer, as well as concentrations of free fatty acids, insoluble, moisture, nonelutable material, total fatty acids, and unsaponifiables (Kerr et al., 2015). While many of these lipid quality measures provide useful information about the characteristics of lipids, they do not directly provide an assessment of the extent of lipid peroxidation. Concentration of unsaturated fatty acids, heat, oxygen, moisture, and pro-oxidant metals affect peroxidation of lipids (Belitz et al., 2009). Dietary lipids are commonly exposed to these pro-oxidant conditions during processing, storage, and extent and time of exposure to these conditions determines the extent of peroxidation (Dibner et al., 2011; Song and Shurson, 2013). During peroxidation, fatty acids are converted into numerous products including peroxides, aldehydes, ketones, acids, esters, hydrocarbons, epoxides, polymers, lactones, furans, and aromatic compounds (Spiteller et al., 2001; Seppanen and Saari Csallany, 2002; Belitz et al., 2009; Rocha et al., 2012).

Several indicative and predictive assays can be conducted to assess lipid peroxidation of various types of lipids, but none of these methods provide a complete assessment of peroxidative damage because numerous and chemically diverse compounds are produced, and subsequently degraded to other compounds during the peroxidation process (Shurson et al., 2015). Among all peroxidation indicator assays available, peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) have been the used because these assays are relatively simple and inexpensive to conduct, but they are inaccurate as single indicators of the extent of peroxidation (Shurson et al., 2015). Despite the limitations of using PV as an accurate peroxidation indicator, some

animal nutritionists consider a lipid source to be of inferior quality (rancid) if the PV of a lipid exceeds 20 meq O_2/kg lipid (DeRouchey et al., 2004). Results from an industry survey have shown that the PV of fats and oils can range from 0.1 to 180.8 meq O_2/kg lipid among 610 samples, suggesting large variability in extent of peroxidation among dietary lipids (Dibner, 2013; personal communication). Furthermore, significant amounts of frying oils from restaurants are recycled and directly added to animal feed, or are blended with rendered animal fats to produce animal-vegetable blends (Kerr et al., 2015; van Heugten et al., 2016). Due to prolonged heating of frying oils at high temperatures, they become highly peroxidized and may have PV as high as 248 meq O_2/kg (Rosero et al., 2015).

Because each peroxidation measure provides only partial information of the extent of lipid damage, they have been of limited value in predicting animal growth performance (Kerr et al., 2015). Several studies have shown that feeding peroxidized lipids reduces feed efficiency (McGill et al., 2011a; McGill et al., 2011b; Tavárez et al., 2011), growth rate (Boler et al., 2012; Liu, 2012; Rosero et al., 2015), and energy digestibility (Inoue et al., 1984; Engberg et al., 1996), while increasing oxidative stress (Boler et al., 2012; Liu, 2012), mortality (Tahashaki and Akiba, 1999; Anjum et al., 2004; van Heugten et al., 2016), and impairing immune function (Dibner et al., 1996; Liang et al., 2015; van Heugten et al., 2016). However, these responses, both in direction and magnitude, have not been observed consistently across experiments. Therefore, the objective of this study was to compile and summarize the effects of feeding isocaloric diets containing unperoxidized and peroxidized lipids on growth performance and oxidative status of poultry and swine.

Materials and Methods

Data collection and management

Publications with growth performance data from poultry and swine fed peroxidized lipids were obtained by searching online databases (e.g., Agricola, Google Scholar, and Web of Science), scholarly journal archives, and conference proceedings using key- words (i.e., peroxidized lipids; lipid peroxidation; growth performance; broilers; and swine). Additional publications were identified by retrieving citations within many of the published papers. The main criteria for inclusion of data from experiments in

this meta-analysis were: (1) animals were fed isocaloric diets containing peroxidized and unperoxidized lipids; and (2) growth performance responses (i.e., gain:feed) were reported regardless of whether the responses were positive or negative.

Twenty six journal articles (Oldfield et al., 1963; L'Estrange et al., 1966; Cabel et al., 1988; Lin et al., 1989; Engberg et al., 1996; Wang et al., 1997; Tahashaki and Akiba, 1999; Anjum et al., 2002; Anjum et al., 2004; DeRouchey et al., 2004; Yuan et al., 2007; Racanicci et al., 2008; Upton et al., 2009; Açıkgöz et al., 2011; Bayraktar et al., 2011; McGill et al., 2011a; McGill et al., 2011b; Tavárez et al., 2011; Zhang et al., 2011; Boler et al., 2012; Rocha et al., 2012; Liu et al., 2014a; Ehr et al., 2015; Liang et al., 2015; Rosero et al., 2015; Hanson et al., 2016), 1 abstract (Harrell et al., 2010), and 2 conference proceedings (Inoue et al., 1984; van Heugten et al., 2016) were obtained to provide a total of 29 publications. Some publications evaluated the effects of multiple diets containing peroxidized lipids relative to the effect of similar diets containing unheated lipids. This resulted in 42 comparisons for poultry and 23 for swine. In poultry, 4 comparisons from turkeys were merged into analysis with data from broilers because they included lipids, level of lipid peroxidation, and dietary inclusion rates similar to broiler studies. Sources of dietary lipids included animal fats, vegetable oils, and animal-vegetable blends. Processing temperature and time to induce peroxidation (reported in 82% of the publications (L'Estrange et al., 1966; Cabel et al., 1988; Engberg et al., 1996; Tahashaki and Akiba, 1999; Anjum et al., 2002; Anjum et al., 2004; DeRouchey et al., 2004; Racanicci et al., 2008; Upton et al., 2009; Harrell et al., 2010; McGill et al., 2011a; McGill et al., 2011b; Tavárez et al., 2011; Boler et al., 2012; Rocha et al., 2012; Liu et al., 2014a; Ehr et al., 2015; Liang et al., 2015; Rosero et al., 2015; Hanson et al., 2016; van Heugten et al., 2016) ranged from 27 to 185 °C (mean = 74 °C for poultry experiments and mean = 121 °C for pig experiments) for 7–1968 h (mean = 949 h for poultry experiments and mean = 108 h for pig experiments)). Of the 65 comparisons analyzed, 100% reported dietary PV, and 34% reported dietary TBARS concentration among all swine and poultry studies. Both TBARS and p-Anisidine value (AnV) assays can be used to estimate concentrations of some secondary lipid peroxidation products, but TBARS is preferred and often used because it is simple, more accurate and sensitive than AnV for some animal fats and animal-vegetable blends (Nuchi et al., 2009). Since the p-

Anisidine is more sensitive to unsaturated aldehydes compared with saturated aldehydes, AnV can provide misleading indications of the extent of peroxidation if lipids generate more unsaturated than saturated aldehydes during peroxidation (Shahidi and Zhong, 2005a; Barriuso et al., 2013). Additionally, AnV were not often reported (reported by 7 publications) in the experiments analyzed in this study, and thus, TBARS data were the focus of analysis instead of AnV.

The PV of lipid sources, primarily in vegetable oil, used to mix diets ranged from 1 to 448 meq O_2/kg lipid (mean = 167.1 and 54.0 meq O_2/kg lipid for poultry and swine experiments). The TBARS content, mainly in vegetable oil, ranged from 4.4 to 162.0 mg malondialdehyde (MDA) eq/kg lipid (mean = 39.3 mg MDA eq/kg oil for pigs, n = 13), and this measure was only reported in 2 publications involving poultry. Within all comparisons from 29 publications, unperoxidized dietary lipids were used in all control diets, while dietary lipids with different level of peroxidization were included in treatment group diets.

From each comparison, data from the following variables were obtained and converted (if needed) to the same units to standardize comparisons, and included: reported processing temperature and time, dietary PV and TBARS concentrations, supplemental dietary vitamin E and selenium concentrations, initial age of poultry or initial body weight of swine, average daily gain (ADG), average daily feed intake (ADFI), gain: feed (G:F), and serum or plasma concentrations of α-tocopherol and TBARS. Concentration of PV and TBARS in the final diets were calculated from lipid inclusion level% × PV or TBARS of lipid sources because there were no experiments that reported lipid peroxidation analyses of diets. Data were tabulated in a spreadsheet (Excel 2016, Microsoft Corporation; Redmond, WA) with a separate row for each comparison. Within each comparison, dependent variables were expressed as a percentage relative to animals fed the same dietary lipid source which was unperoxidized.

Statistical analysis

The UNIVARIATE and CORR procedures of SAS (v9.4; SAS Inst. Inc., Cary, NC) were used to summarize the data collectively and separately for poultry and swine. All variables within the database were tested for normal distribution and the presence of outliers using the UNIVARIATE procedure of SAS. Means, standard deviations,

correlation coefficients, P values of correlations, and the coefficient of determination were reported. The effect of consuming peroxidized lipids was analyzed using R Studio (R version 3.2.4) with packages "Meta" (Schwarzer, 2007) to estimate the mean value and 95% confidence interval (CI). In brief, we calculated standard deviation from standard error and replicates among studies, then package "Meta" computed the mean and 95% CI by each input standard deviation. Studies were considered as random effects. Additionally, PROC REG of SAS procedure was applied to predict the magnitude of change on relative ADG, ADFI, and G:F based on the association between growth performance and PV or TBARS in poultry and swine. Data on growth performance, oxidative status, correlation analysis, and regression analysis were weighted by the inverse of the standard error (Sauvant et al., 2008) to improve the precision of the analysis. Significance was declared at $P < 0.05$, and values between $0.05 \leq P \leq 0.10$ were considered trends.

Results and Discussion

Overall database structure

Data from two publications were not included in this meta-analysis. One of these publications evaluated fish oil that was peroxidized not only by heating and continuous air injection, but also by adding $FeSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ to accelerate the peroxidation process. Consequently, the heated fish oil contained a TBARS value that was several order magnitudes greater than TBARS in lipid sources in any other publications (Yuan et al., 2007). The other excluded publication (Rosero et al., 2015) used a commercial ELISA kit to determine the concentration of MDA in soybean oil, but it is not clear how the results from the ELISA assay compare with the generally accepted assay protocol from American Oil Chemists' Society (AOCS; method Cd 19–90). After conversion of MDA concentration data to TBARS, the calculated TBARS concentration for heated soybean oil was higher than all other published values. The AOCS assay for TBARS measures the reaction of MDA and thiobarbituric acid by spectrophotometer at wavelength 530–535 nm, but thiobarituric acid also reacts with other chemicals, such alkanals, amino acids, and ketones, to produce interference color (Guillén-Sans and Guzmán-Chozas, 1998; Jardine et al., 2002). Therefore, the concentration of MDA

cannot be considered equivalent to TBARS values that were calculated and presented as mg MDA equivalents/kg lipid.

Growth performance

The fact that feeding peroxidized lipids decreases growth performance of poultry and swine is well accepted, but the magnitude of the responses from consuming diets containing peroxidized lipids on animal growth performance are debatable because inconsistent results have been observed among publications (Table 3.1). Feeding peroxidized lipids reduces G:F of animals depending on peroxidation conditions and sources of lipids (Cabel et al., 1988; Tahashaki and Akiba, 1999; Anjum et al., 2004; McGill et al., 2011a; McGill et al., 2011b; Tavárez et al., 2011; Ehr et al., 2015; Hanson et al., 2016), but not in others (Oldfield et al., 1963; Racanicci et al., 2008; Upton et al., 2009; Açıkgöz et al., 2011; Bayraktar et al., 2011; Rocha et al., 2012; Liu et al., 2014b; Rosero et al., 2015; van Heugten et al., 2016). These inconsistent responses are likely related to the variation in fatty acid profiles among lipid sources, dietary inclusion rate of lipids, extent of peroxidation, methods employed to induce peroxidation, inaccurate quantitative methods of lipid peroxidation, and inadequate replication among studies.

Significance of growth performance responses varied among experiments when comparing the 65 relative responses from feeding peroxidized and unperoxidized lipids to poultry and swine (Tables 3.2 and 3.3). The mean value for ADG was 95% (CI = 93–97%), ADFI was 97% (CI = 96–98%), and G:F was 98% (CI = 97 to 99%) for poultry and swine fed dietary peroxidized lipids relative to controls. The magnitude of reduction from feeding diets containing peroxidized lipids relative to diets with unperoxidized lipids for ADG (5 vs. 6%), ADFI (2 vs. 5%), and G:F (3 vs. 2%) were similar for poultry and swine.

A combined analysis of the response of poultry and swine to peroxidized lipids suggests that the proportion of decrease in ADG is the result of significant reduction in ADFI (Oldfield et al., 1963; Engberg et al., 1996; Anjum et al., 2002; Anjum et al., 2004; DeRouchey et al., 2004; Tavárez et al., 2011; Boler et al., 2012) independent of caloric content of the diets because all diets in these comparisons were isocaloric. The decrease in ADFI when feeding peroxidized lipids may be related to an undesirable odor or flavor of aldehydes generated during peroxidation (Esterbauer et al., 1991; Halliwell and

Chirico, 1993). The overall reduction in G:F among studies may be related to the oxidative stress and physiological changes resulting from feeding peroxidized lipids. Although specific mechanisms involving the physiological effects of feeding peroxidized lipids on growth performance responses have not completely elucidated, studies have reported that dietary peroxidized lipids reduce energy and nutrient digestibility (Inoue et al., 1984; Engberg et al., 1996), impaired immune function (Dibner et al., 1996), cause enterocyte dysfunction, and induce metabolic oxidative stress (Rosero et al., 2015). A comprehensive gene expression analysis of pigs fed peroxidized lipids showed a significant increase in liver catabolism of fatty acids by induction of peroxisome proliferator-activated receptor alpha (Liu et al., 2014a). Altogether, observations from this meta-analysis suggest that the effects of peroxidized lipids are negligible or detrimental, but apparently never beneficial. Similarly, chicks fed peroxidized rice bran also had poor growth performance (Anjum et al., 2004). Further studies are needed to accurately measure specific peroxidation compounds and their mechanistic role in reducing growth rate, feed consumption, and energy and nutrient utilization efficiency.

Correlation and prediction between growth performance and lipid peroxidation

Dietary peroxidation measures that accurately predict reduction in growth performance have not been established. Lipid hydroperoxides are produced initially during lipid peroxidation, but they subsequently react to form numerous secondary and tertiary products (Spiteller et al., 2001; Seppanen and Saari Csallany, 2002; Belitz et al., 2009). Consequently, using a single measurement of lipid quality appears to be insufficient to predict growth performance of animals fed peroxidized lipids. Numerous assays are available to assess peroxidation of dietary lipids. For instance, PV and TBARS are commonly reported, but other assays and bio- markers such as conjugated dienes, hexanal, 4-hydroxynonenal (4-HNE), and 2,4-decadienal can be measured. However, neither their independent nor combined effects on growth performance and health of animals are well established. In our analysis, dietary PV was negatively correlated with ADG in poultry ($r = -0.81$, $P < 0.01$; Table 3.4), and the decrease in the ADG appeared to be linear with increasing dietary PV ($P < 0.01$; Fig. 3.1). Similarly, dietary TBARS content was negatively correlated with ADG ($r = -0.58$, $P = 0.04$; Table 2.5) in swine, and appeared to be linear ($P < 0.05$; Fig. 3.2). Because dietary TBARS content was only

reported in two publications for broilers, it was impossible to determine the correlation between TBARS and growth performance of poultry. While these significant linear correlations suggest that PV is associated with growth rate in poultry, and dietary TBARS concentration is associated with growth rate in swine, these correlations were influenced strongly by a few extreme data points. Therefore, these results should be interpreted and considered with caution. For example, the coefficient of determination between TBARS and ADG in swine is 0.33, which means only 33% of the variance in ADG is predictable from dietary TBARS values. When the extreme data point (dietary TBARS = 16.20 mg MDA eq/kg) was excluded from the analysis, or when an outlier value for TBARS (65.88 mg MDA eq/kg) was included in the analysis, the coefficient of determination decreased ($0 \leq R^2 \leq 0.1$). Similarly, while our analysis showed that PV was significantly correlated with ADG of poultry fed lipids exposed to low temperatures, it does not indicate that PV is a better measurement than other procedures. In turkey poults, feeding dietary lipid with high PV did not result in a reduction of ADG (Rocha et al., 2012). The PV has been the most common measurement used to estimate the extent of lipid peroxidation over the past several decades, but does not infer that it is an accurate comprehensive measure of peroxidation, and this bias limits the effectiveness of this meta-analysis approach to assess the utility of using other peroxidation indicator measures (Inoue et al., 1984; Cabel et al., 1988; Lin et al., 1989; Tahashaki and Akiba, 1999; Anjum et al., 2002; Anjum et al., 2004; Upton et al., 2009; McGill et al., 2011b; McGill et al., 2011a; Tavárez et al., 2011; Rosero et al., 2015; Hanson et al., 2016). To facilitate the development of predictive models, researchers should report more comprehensive data on the composition, processing conditions, and the extent of peroxidation using multiple peroxidation measures in the future experiments.

Processing temperature of lipid peroxidation

Interestingly, the temperature used to peroxidize lipids had a significant negative correlation with ADG and ADFI in swine, but not in poultry (Tables 3.4 and 3.5). Lower temperatures (mean = 74 ± 30° C) were used to create peroxidation of experimental lipids in many of the poultry experiments compared with those used in the swine experiments (mean = 121 ± 49 °C). As a result, the total concentration of peroxidation compounds may have been greater in peroxidized lipids fed in the swine studies, which subsequently

may have resulted in a greater negative association with reduced ADG and ADFI. Peroxides are relatively unstable during long-term peroxidation, are degraded at high temperatures (Shahidi and Zhong, 2005), and result in an initial increase in PV, which later declines in lipids exposed to temperature greater than 80 °C (DeRouchey et al., 2004; Danowska-Oziewicz and Karpinska-Tymoszczyk, 2005). Conversely, other researchers have reported that PV increases continuously in lipids during peroxidation at lower tempera- tures (≤ 65°C) (Wanasundara and Shahidi, 1994; Naz et al., 2005; Winkler-Moser and Breyer, 2011; Chen et al., 2014). However, relative changes in PV during the peroxidation process is also dependent on the concentration of unsaturated fatty acids in a lipid source, which are more susceptible to peroxidative damage than saturated fatty acids (Tavárez et al., 2011; Liu et al., 2014c; van Heugten et al., 2016). Therefore, while it may appear that there is a species dependent response to PV, our results indicate that PV has little, if any value for predicting growth performance responses when feeding peroxidized lipids to swine and poultry. Perhaps multifactorial models could be developed which include several peroxidation indicators, fatty acid profile of lipids, and time and temperature used during the peroxidation process to accurately predict growth performance when feeding diets containing peroxidized lipids.

Lipid peroxidation assays as predictors of animal performance

Currently, nutritionists lack practical guidelines for estimating growth performance responses from adding peroxidized lipids to poultry and swine diets. Some researchers have suggested maximal thresholds of acceptable levels of peroxidation products determined by PV. Azain suggested that lipids with PV below 5 meq O_2/kg are "not currently rancid," implying that such lipids are of acceptable quality (Azain, 2001). Conversely, Gray and Robinson suggested that lipids with PV greater than 20 meq O_2/kg are "definitely rancid," but did not provide experimental data to support these recommendations (Gray and Robinson, 1941). DeRouchey et al. suggested that feeding diets with PV levels greater than 2.4 meq O_2/kg can result in reduced ADG and ADFI of pigs (DeRouchey et al., 2004). However, the worst growth performance response in their experiment was achieved when pigs were fed diets with a PV less than 1 meq O_2/kg due to extending heating lipids to 11 days (DeRouchey et al., 2004). Liu et al. (2014a) also showed substantial reductions in ADG when diets contained low PV values (< 1.2 meq

O_2/kg) compared to pigs fed unheated lipids. Furthermore, feeding dietary lipid with PV value greater than 2.5 meq O_2/kg to turkeys reduced ADG, especially after feeding them for long periods of time (> 12 wk) (Jankowski et al., 2000). However, characterizing the quality of lipids based solely on PV can be misleading. Contrary to the concept of a maximum threshold of lipid peroxidation, under which animal performance is minimally affected, results from our analysis suggest that the negative responses between increasing dietary lipid peroxidation and growth performance are linear (Table 3.6). This association is supported by other studies showing that as the extent of lipid peroxidation increases, apparent fecal digestibility of lipid, gross energy, feed intake, and weight gain in nursery pigs is linearly reduced (van Heugten et al., 2016), as well as apparent metabolizable energy in broilers (Wiseman et al., 1992). These results further emphasize the need to identify analytical methods and peroxidation biomarkers that are strongly associated with physiological and metabolic parameters of animals fed peroxidized lipids. In fact, a recent publication by Wang et al. (2016) suggested that 4-HNE is a useful marker of *in vivo* peroxidation, and had a greater correlation with the duration of thermal stress compared with other aldehydes in soybean oil. This potential relationship needs to be evaluated when feeding peroxidized lipids to swine and poultry.

Biological responses to peroxidized lipids

The oxidative status of animals generally declines as indicators of peroxidation (e.g., TBARS or protein carbonyls) increase (Zhang et al., 2011; Boler et al., 2012; Hanson et al., 2016). Consequently, concentrations of vitamin E in serum or tissues decline with increasing levels of dietary peroxidized lipids for pigs (Boler et al., 2012; Liu et al., 2012; Hanson et al., 2016), sows (Nielsen et al., 1973), rats (Liu and Huang, 1995; Eder, 1999; Brandsch and Eder, 2004) and broilers (Tahashaki and Akiba, 1999; Tavárez et al., 2011). This effect may be partly related to the degradation of vitamin E in peroxidized lipids resulting in reduced intake (Liu, 2012), altered uptake, or increased metabolic utilization (Liu and Huang, 1995). Conversely, tissue or serum concentrations of TBARS increase with dietary peroxidized lipids for swine (Boler et al., 2012), rats (Liu and Huang, 1995), and broilers (Lin et al., 1989; Tahashaki and Akiba, 1999; Anjum et al., 2004). Moreover, greater dietary lipid peroxidation increases the concentration of MDA in the jejunal mucosa of nursery pigs, and reduces the mucosal total antioxidant

capacity (Rosero et al., 2015). In the current study, the effect of feeding peroxidized lipids on measures of oxidative status varied (Table 3.7). The serum or plasma content of vitamin E was 52% (CI = 33 to 71%, n = 22) and TBARS was 120% (CI = 113 to 127%, n = 15) for poultry and swine fed diets with peroxidized lipids relative to animals fed unperoxidized lipids. Upton et al. (2009) showed that the activity of glutathione peroxidase (GPx) increased in the liver of broilers fed peroxidized poultry fat. These authors suggested that the production of GPx increased in response to greater peroxide yield and higher oxidative stress in the peroxidized lipid (Upton et al., 2009; Bayraktar et al., 2011). Vitamin E may contribute to other free radical scavenging systems to combat oxidative stress (Bayraktar et al., 2011). Thus, birds fed peroxidized lipids with a high concentration of α-tocopherol acetate did not have elevated the GPx activity (Bayraktar et al., 2011). In turkeys, feeding a high vitamin E diet with peroxidized soybean oil reduced the concentration of hydroperoxides in the jejunum (Rocha et al., 2012). Ultimately, the increased concentration of circulating TBARS accompanied by reduced circulating concentrations of vitamin E suggests that oxidative stress may have occurred when feeding dietary peroxidized lipids in these studies.

Dietary constituents that can be used to predict changes in metabolic oxidative status have been evaluated, but the relationships of these dietary markers with changes in metabolic markers and growth performance are not clear. In swine, the serum or plasma TBARS concentration was negatively correlated with vitamin E in serum or plasma ($r = -0.69$, $P = 0.01$; Table 3.5), which was similar to correlations reported by others (Sadrzadeh et al., 1994; Liu and Huang, 1995; Yanik et al., 1999). Moreover, serum or plasma TBARS concentration was positively associated with dietary TBARS ($r = 0.68$, $P = 0.02$) and dietary PV ($r = 0.68$, $P = 0.01$), but the associations between serum or plasma vitamin E content and conditions of lipid peroxidation were not observed (Table 3.5). Nevertheless, similar associations were not observed in poultry because few poultry publications reported serum or plasma concentrations of TBARS and vitamin E, and as a result, a correlation model could not be developed. Although the biomarkers indicate that oxidative stress likely occurred in these studies, their use as predictors of subsequent growth performance appears to be limited. For example, the circulating concentration of vitamin E failed to show a significant correlation with ADG, ADFI, and G:F in both

swine and poultry fed peroxidized lipids. However, serum or plasma vitamin E showed a positive tendency with ADFI (r = 0.53, P = 0.08) in swine. Additionally, serum or plasma concentration of TBARS tended to be negatively correlated with ADG (r =− 0.53, P = 0.08) and ADFI (r = −0.55, P = 0.07) in swine. Song et al. (2013) and Song et al. (2014) observed that feeding peroxidized DDGS did not increase TBARS in serum and longissimus muscle area because other dietary components (e.g., methionine and taurine) appeared to overcome the negative effects of lipid peroxidation instead of vitamin E. Hanson et al. (2016) observed that serum TBARS tended to increase when serum concentrations of selenium and α-tocopherol were reduced. It appears that a comprehensive targeted metabolomics analysis is warranted to identify more reliable biomarkers that can be used to accurately predict animal growth performance responses when feeding peroxidized lipids.

The efficacy of antioxidant against lipid peroxidation

Some researchers have suggested that supplemental dietary antioxidants may alleviate some of the negative effects of peroxidized lipids on growth performance. Growth rate (Tavárez et al., 2011) and gain efficiency (Cabel et al., 1988; McGill et al., 2011a) improved by 2–4% when ethoxyquin was incorporated into broiler diets formulated with either poultry fat or animal-vegetable blend containing a PV of approximately 7 meq O_2/kg diet. Similarly, Oldfield et al. (1963) reported that adding dl-α-tocopheryl acetate or ethoxyquin to swine diets with peroxidized menhaden fish oil resulted in complete recovery of ADG and ADFI to levels similar to control animals. Lin et al. (1989) reported a similar recovery when α-tocopherol or other antioxidants (butylated hydroxytoluene and butylated hydroanisole) were added to broiler diets containing peroxidized sunflower oil. Açıkgöz et al. (2011) concluded that a mild oxidative stress can be mitigated by supplementing dl-α-tocopheryl acetate to decrease MDA concentration and increase superoxide dismutase activity in broilers. These results suggest that inadequate dietary antioxidants contribute to growth depression when animals consume diets containing peroxidized lipids, and partial or complete recovery from these negative effects can be achieved by supplementing dietary antioxidants, such as dl-α-tocopheryl acetate. Supplemental levels vitamin E in the diets with peroxidized lipids was positively correlated with ADG in poultry (r = 0.53, P = 0.03), but not swine

(Tables 3.4 and 3.5). These associations were supported by previous publications that antioxidants may offset the growth depression associated with feeding peroxidized lipids (Oldfield et al., 1963; Lin et al., 1989; Harrell et al., 2010). However, the ameliorative effects of antioxidants on animal growth are inconsistent among studies (Wang et al., 1997; Harrell et al., 2010; McGill et al., 2011b; Boler et al., 2012). In spite of positive benefits observed from supplementing diets with antioxidants (e.g., ethoxyquin and propyl gallate), some studies failed to show a significant improvement on G:F (McGill et al., 2011b; Tavárez et al., 2011; Boler et al., 2012). As the results, further studies are needed to reach a consensus about the effectiveness of adding antioxidants to diets to prevent further lipid peroxidation and enhance growth performance.

Conclusions

Feeding peroxidized lipids significantly decreases growth rate, feed intake, and gain efficiency in poultry and swine, but the magnitude of reduction varies between species. While the specific modes of action remain unknown, these growth performance reductions may be related to the lack of adequate antioxidant capacity of the diet and oxidative stress status. There is a dearth of knowledge regarding the limitations and accuracy of using individual peroxidation indicators and predictive assays to define the extent of lipid peroxidation in animal feed ingredients and diets. While PV and TBARS have been widely used as indicators of lipid peroxidation historically, they are inadequate to accurately predict broiler and swine performance for many reasons. Furthermore, the concept of maximum thresholds of peroxidation is inappropriate because reductions in growth performance with increasing lipid peroxidation is linear. Thus, multifactorial and metabolomics-based approaches must be utilized to identify the most accurate measures of lipid peroxidation, as well as more appropriate biological indicators for prediction of animal growth performance reductions when feeding peroxidized lipids to poultry and swine.

Tables and Figures

Table 3.1. Summary of growth performance responses from feeding peroxidized lipids to poultry and swine

Items	Response to dietary peroxidized lipids				
	Number of observations	Significant increase[1]	Significant decrease[1]	No difference[2]	Not reported
ADG	65	0	27	33	5
ADFI	65	0	12	48	5
G:F	65	0	17	48	0

[1]$P < 0.05$, relative to isocaloric diet with unperoxidized lipid.
[2]$P > 0.05$, relative to isocaloric diet with unperoxidized lipid.

Table 3.2. Summary of 42 comparisons of growth and serum responses from feeding peroxidized lipids to poultry

Reference[1]	Lipid Source[1]	Temp, °C[2]	Time, h[2]	PV, meq O_2/ kg diet[3]	ADG, %[4]	ADFI, %[4]	G:F, %[4]	Serum/plasma Vitamin E, %[4]	Serum/plasma TBARS, %[4,5]
a	beef	90	52	10.9	100	99	101	.	.[6]
b	soy	.	.	5.9	97	96	102	61	.
b	soy	.	.	15.5	87	93	94	62	.
b	soy	.	.	22.0	86	93	92	48	.
b	soy	.	.	11.7	94	103	90	106	.
b	soy	.	.	30.9	61	76	79	50	.
b	soy	.	.	44.0	50	70	70	23	.
c	poultry	80	.	2.1	.	.	99	.	.
c	poultry	80	.	4.1	.	.	99	.	.
c	poultry	80	.	7.2	.	.	89	.	.
d	sunflower	.	.	22.0	95	98	97	.	.
e	vegetable	30	336	17.2	92	93	99	61	.
f	poultry	.	.	26.8	89	92	92	.	.
g	soy	60	.	10.0	92	99	93	15	157
g	soy	60	.	10.0	80	91	89	20	175
h	soy	27	240	0.98	97	99	98	.	.
h	soy	27	240	1.5	94	99	95	.	.
i	soy	27	240	1.0	96	99	97	.	.
j	poultry	115	210	1.6	100	98	102	.	.
k	poultry	80	.	3.0	.	.	102	.	.
k	poultry	80	.	3.0	.	.	105	.	.
l	soy	95	72	7.0	94	98	96	42	97
m	A-V blend	59	.	3.5	97	99	98	.	.
m	A-V blend	59	.	7.1	98	99	98	.	.
n	A-V blend	59	.	3.5	99	100	99	.	.
n	A-V blend	59	.	7.1	99	99	98	.	.
o	sunflower	75	.	8.9	99	101	98	.	.
o	sunflower	75	.	8.9	98	98	99	.	.
p	sunflower	75	.	6.0	100	101	102	.	.
p	sunflower	75	.	6.0	100	103	98	.	.
q	A-V blend	.	.	5.0	99	101	98	.	.
r[7]	soy	100	1440	3.9	105	99	107	.	.
r[7]	soy	100	1440	8.8	103	103	100	.	.

r[7]	soy	100	1440	3.9	108	101	108	.	.
r[7]	soy	100	1440	8.8	101	100	101	.	.
s	soy	60	1968	1.0	100	98	101	.	.
s	soy	60	1968	3.1	100	98	101	.	.
s	soy	60	1968	5.0	97	101	96	.	.
s	soy	60	1968	7.1	96	99	97	.	.
s	soy	60	1968	9.0	98	102	96	.	.
t	corn	95	72	10.0	96	98	98	.	.
t	corn	185	12	0.42	97	99	97	.	.
Mean ± SD		74 ± 30	949 ± 833	8.9 ± 8.9	95 ± 11	97 ± 7	97 ± 7	49 ± 27	143 ± 41
Adjusted mean[8]					95	98	97	50	130
95% CI[9]					93-97	97-99	96-98	19-81	83-177

[1][a]L'Estrange et al., 1966, [b]Inoue et al., 1984, [c]Cabel et al.,1998, [d]Lin et al., 1989, [e]Engberg et al., 1996, [f]Wang et al., 1997, [g]Tahashaki et al., 1999, [h]Anjum et al., 2002, [i] Anjum et al., 2004, [j]Racanicci et al., 2008, [k]Upton et al., 2009, [l]Tavarez et al., 2011, [m]McGill et al., 2011a, [n]McGill et al., 2011b, [o]Açikgöz et al., 2011, [p]Bayraktar et al., 2011, [q]Zhang et al., 2011, [r]Rocha et al., 2012, [s]Liang et al., 2015, [t]Ehr et al., 2015.

Lipid source = source of supplemental lipid in diet, A-V blend = blend of animal fat-vegetable oil, poultry = poultry fat, sunflower = sunflower oil, vegetable = vegetable oil, soy = soybean oil, and corn = corn oil.

[2]Processing temperature and processing hours are those used in the preparation of peroxidized lipids.

[3]Peroxide value (PV) of diet containing peroxidized lipid was calculated as: dietary lipid inclusion level, % × PV of lipid source, or as reported by authors.

[4]The values presented represent the percentage of the variable relative to control responses. All response variables were calculated by dividing the mean of the animals fed diets with peroxidized lipids by those fed unperoxidized lipids and multiplying by 100.

[5]TBARS = thiobarbituric acid reactive substances.

[6]A period indicates that this variable was not reported or estimable from the publication.

[7]Data from turkeys.

[8]Adjusted mean was weighted by the inverse of the standard error.

[9]CI = confidence interval.

Table 3.3. Summary of 23 comparisons of growth and serum responses from feeding dietary peroxidized lipids to swine

Citation	Source[1]	Processing temp, °C[2]	Processing hours[2]	TBARS, mg MDA eq/kg diet[3,4]	PV, meq O_2/ kg diet[3,4]	ADG, %[5]	ADFI, %[5]	G:F, %[5]	Serum/plasma Vitamin E, %[5]	Serum/plasma TBARS, %[3,5]
Oldfield et al., 1963	fish	room temp	.	4.9	1.6	94	90	104	.	.
Oldfield et al., 1963	fish	room temp	.	16.2	6.1	65	77	84	.	.
DeRouchey et al., 2004	CWG	80	264	.	0.1	91	91	100	.	.
DeRouchey et al., 2004	CWG	80	216	.	0.1	97	91	106	.	.
DeRouchey et al., 2004	CWG	80	168	.	6.3	95	92	104	.	.
DeRouchey et al., 2004	CWG	80	120	.	2.4	100	98	102	.	.
Harrell et al., 2010	corn	.	.	.	7.5	95	95	100	.	.
Boler et al., 2012	corn	95	.	.	7.5	93	95	95	46	113
Liu et al., 2014a	beef	95	72	0.4	2.9	105	110	94	97	103
Liu et al., 2014a	poultry	95	72	1.1	5.7	101	102	97	74	107
Liu et al., 2014a	corn	95	72	1.6	15.1	92	89	103	29	121
Liu et al., 2014a	canola	95	72	7.0	23.9	75	90	84	49	123
Liu et al., 2014a	poultry	185	7	0.4	0.2	77	87	89	70	103
Liu et al., 2014a	corn	185	7	0.9	0.2	77	84	91	19	115
Liu et al., 2014a	beef	185	7	0.3	0.3	86	89	96	90	102
Liu et al., 2014a	canola	185	7	4.5	1.2	76	79	97	60	120
Rosero et al., 2015	soy	80	144	.	2.8	95	102	99	.	.
Rosero et al., 2015	soy	80	216	.	3.5	93	95	99	.	.
Rosero et al., 2015	soy	80	288	.	3.1	91	93	100	.	.
Van Heugten et al., 2016	corn	80	288	.	8.8	103	103	100	.	.
Hanson et al., 2016	maize	185	12	1.4	0.3	100	102	99	60	105
Hanson et al., 2016	maize	185	12	2.8	0.39	98	102	96	60	103
Hanson et al., 2016	maize	185	12	4.2	0.5	92	95	97	31	108
Mean ± SD		121 ± 49	108 ± 103	3.5 ± 4.4	4.4 ± 5.7	91 ± 10	94 ± 8	97 ± 6	57 ± 24	110 ± 8
Adjusted mean[7]						94	95	98	54	109
95% CI[8]						92-96	93-97	96-100	40-68	105-113
Mean ± SD, poultry and swine[9]						93 ± 11	96 ± 7	97 ± 6	53 ± 25	117 ± 22
Adjusted mean of both species						95	97	98	52	120

95% CI of both species	93-97	96-98	97-99	33-71	113-127
Significance	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

[1]Source = source of supplemental lipid in diet, fish = fish oil, CWG = choice white grease, corn = corn oil, beef = beef tallow, poultry = poultry fat, canola = canola oil, soy = soybean oil, maize = maize oil.

[2]Processing temperature and processing hours are the preparation of test dietary lipids.

[3]TBARS = thiobarbituric acid reactive substances; PV = peroxide value.

[4]Peroxide value of diet containing peroxidized lipid was calculated as: fat inclusion level, % x peroxide value of fat source or reported as stated by researchers.

[5]The values presented represent the percentage of the variable relative to controls. All response variables were calculated by dividing the mean of the animals fed diets with peroxidized lipids by those fed unperoxidized lipids and multiplying by 100.

[6]A period indicates that this variable was not reported or estimable from the research reports.

[7]Adjusted mean was weighted by the inverse of the standard error.

[8]CI = confidence interval.

[9]The mean value of poultry and swine was calculated from table 2 and 3.

Table 3.4. Correlation matrix among measures of dietary lipid peroxidation, oxidative status, and growth performance in poultry[1]

Items	Dietary PV[2]	Processing temp.[3]	Serum / plasma vitamin E	Serum / plasma TBARS[2]	ADG[2]	ADFI[2]	G:F[2]	Initial age[4]	Suppl. dietary vitamin E	Suppl. dietary Se	Processing time[3]	Processing temp. × time
Diet PV	1 . 42											
Processing temp.	-0.12 0.49 33	1 . 33										
Serum / plamsa vitamin E	-0.22 0.55 10	-0.33 0.67 4	1 . 10									
Serum / plasma TBARS	0.97 0.14 3	-0.97 0.14 3	-0.93 0.24 3	1 . 3								
ADG	**-0.81** **<0.01** **37**	0.36 0.06 28	0.35 0.32 10	-0.72 0.48 3	1 . 37							
ADFI	**-0.79** **<0.01** **37**	0.22 0.26 28	0.40 0.26 10	-0.74 0.47 3	**0.95** **<0.01** **37**	1 **.** 37						
G:F	**-0.77** **<0.01** **42**	0.33 0.06 33	0.35 0.33 10	-0.87 0.33 3	**0.96** **<0.01** **37**	**0.82** **<0.01** **37**	1 **.** 42					
Initial age	-0.10 0.55 38	-0.06 0.76 29	-0.36 0.31 10	0.97 0.15 3	0.18 0.31 33	0.18 0.32 33	0.09 0.58 38	1 . 38				
Suppl. vitamin E	0.10 0.65 24	0.37 0.07 24	1.00 . 2	. . 1	**0.53** **0.03** **17**	0.26 0.31 17	0.32 0.15 22	-0.01 0.97 18	1 . 22			
Suppl. dietary Se	**0.69** **0.04** **9**	0.07 0.86 9	1.00 . 2	. . 1	-0.16 0.76 6	**-0.87** **0.03** **6**	0.47 0.21 9	**0.96** **<0.01** **9**	**0.93** **<0.01** **9**	1 **.** 9		
Processing time	-0.02 0.94 18	-0.12 0.58 18	1.00 . 2	. . 1	**0.47** **0.05** **18**	0.45 0.06 18	0.09 0.72 18	-0.29 0.23 18	**0.66** **0.04** **10**	0.44 0.38 6	1 . 18	
Processing temp.× time	0.01 0.97 18	0.07 0.79 18	1.00 . 2	. . 1	**0.70** **<0.01** **18**	**0.54** **0.02** **18**	0.37 0.14 18	-0.30 0.23 18	**0.66** **0.04** **10**	0.57 0.23 6	**0.92** **<0.01** **18**	1 . 18

[1]The Pearson correlation coefficient: r value, p value, and number of observations are listed in the top, middle, and bottom of each row.
[2]Abbreviations: PV = peroxide value, TBARS = thiobarbituric acid reactive substances, ADG = average daily gain, ADFI = average daily feed intake, and G:F = gain:feed.
[3]Processing temp.= temperature used to heat dietary lipids, and processing time = time of cooking oil for lipid peroxidation.
[4]This is to evaluate whether young or old animals responded differently to peroxidized lipids.

Table 3.5. Correlation matrix among measures of dietary lipid peroxidation, oxidative status, and growth performance in swine[1]

Items	Dietary TBARS[2]	Dietary PV[2]	Processing temp.[3]	Serum / plasma vitamin E	Serum / plasma TBARS[2]	ADG[2]	ADFI[2]	G:F[2]	Initial BW[4]	Suppl. dietary vitamin E	Suppl. dietary Se	Processing time[3]	Processing temp.× time
Diet TBARS	1 . 13												
Diet PV	0.29 0.33 13	1 . 23											
Processing temp.	-0.11 0.74 11	-0.41 0.07 20	1 . 20										
Serum / plasma vitamin E	-0.49 0.13 11	-0.34 0.28 12	-0.06 0.83 12	1 . 12									
Serum / plasma TBARS	**0.68** **0.02** **11**	**0.68** **0.01** **12**	-0.30 0.34 12	**-0.69** **0.01** **12**	1 . 12								
ADG	**-0.58** **0.04** **13**	-0.15 0.49 23	**-0.55** **0.01** **20**	0.44 0.15 12	-0.53 0.08 12	1 . 23							
ADFI	-0.46 0.11 13	0.01 0.99 23	**-0.58** **0.01** **20**	0.53 0.08 12	-0.55 0.07 12	**0.90** **<0.01** **23**	1 . 23						
G:F	-0.47 0.10 13	-0.25 0.25 23	-0.39 0.09 20	-0.03 0.94 12	-0.02 0.94 12	**0.68** **<0.01** **23**	0.28 0.19 23	1 . 23					
Initial BW	**0.72** **0.01** **13**	0.11 0.62 22	-0.13 0.57 20	-0.24 0.45 12	0.10 0.76 12	-0.10 0.67 22	-0.13 0.56 22	-0.07 0.77 22	1 . 22				
Suppl. dietary vitamin E	. . 8	-0.21 0.50 13	**-0.62** **0.03** **13**	-0.28 0.46 9	0.06 0.89 9	0.23 0.46 13	0.06 0.84 13	0.47 0.10 13	0.37 0.22 13	1 . 13			
Suppl. dietary Se	. . 8	-0.10 0.74 13	0.15 0.63 13	0.28 0.46 9	-0.06 0.89 9	-0.09 0.77 13	0.06 0.85 13	0.01 0.99 13	**-1.00** **<0.01** **13**	-0.37 0.22 13	1 . 13		
Processing time	0.13 0.71 11	0.09 0.72 19	**-0.81** **<0.01** **19**	0.13 0.70 11	0.28 0.41 11	**0.47** **0.04** **19**	0.41 0.08 19	**0.50** **0.03** **19**	0.48 0.04 19	**0.87** **<0.01** **12**	. . 12	1 . 19	
Processing temp.× time	0.15 0.67 11	0.10 0.67 19	**-0.81** **<0.01** **19**	0.12 0.73 11	0.26 0.44 11	**0.48** **0.04** **19**	0.44 0.06 19	**0.50** **0.03** **19**	0.48 0.04 19	**0.85** **<0.01** **12**	. . 12	**1.00** **<0.01** **19**	1 . 19

[1]The Pearson correlation coefficient: r value, p value, and number of observations are listed in the top, middle, and bottom of each row.
[2]Abbreviations: PV = peroxide value, TBARS = thiobarbituric acid reactive substances, ADG = average daily gain, ADFI = average daily feed intake, and G:F = gain:feed.
[3]Processing temperature= temperature used to heat dietary lipids, and lipid processing time = time of cooking oil for lipid peroxidation.
[4]This is to evaluate whether animal body weight responded differently to peroxidized lipids.

Table 3.6. Linear regression to estimate the magnitude of change on growth performance from lipid peroxidation[1]

Measurements, %[2]	Equation[3]	n	MSE	R-square	*P*-value
Poultry					
ADG	104.18-0.99 × (Dietary PV, meq O_2/ kg diet)	37	0.87	0.65	< 0.01
ADFI	102.45-0.54 × (Dietary PV, meq O_2/ kg diet)	37	0.19	0.62	< 0.01
G:F	102.62-0.61 × (Dietary PV, meq O_2/ kg diet)	42	0.40	0.59	< 0.01
Swine					
ADG	91.64-1.79 × (Dietary TBARS, mg MDA eq./ kg diet)	13	12.79	0.33	0.04
ADFI	94.88-1.18 × (Dietary TBARS, mg MDA eq./ kg diet)	13	7.85	0.28	0.11
G:F	96.31-0.71 × (Dietary TBARS, mg MDA eq./ kg diet)	13	1.61	0.22	0.10

[1]Abbreviations: ADG = average daily gain, ADFI = average daily feed intake, G:F = gain:feed.
[2]The ADG, ADFI, and G:F were presented as percentage of the variable relative to controls.
[3]Dietary PV and dietary TBARS were calculated from the dietary lipid inclusion level, % × PV and TBARS of lipid source.

Table 3.7. The effects of feeding dietary peroxidized lipids on oxidative status in poultry and swine

Items	Response to dietary peroxidized lipids, no. of experiments					
	Number of observations	Significant increase[1]	significant decrease[1]	Non-significant difference[2]	Not reported	Unknown[3]
Vitamin E[4]	65	0	17	5	43	0
TBARS[5]	65	4	0	13	44	4
GPx[6]	65	2	1	4	58	0

[1]$P < 0.05$, relative to isocaloric diet with unperoxidized lipid.
[2]$P > 0.05$, relative to isocaloric diet with unperoxidized lipid.
[3]Unable to recognize the difference between each treatment from the publications.
[4]Serum, plasma, or tissue level of α-tocopherol.
[5]Serum, plasma, or tissue level of thiobarbituric acid reactive substances.
[6]Glutathione peroxidase activity of liver and plasma.

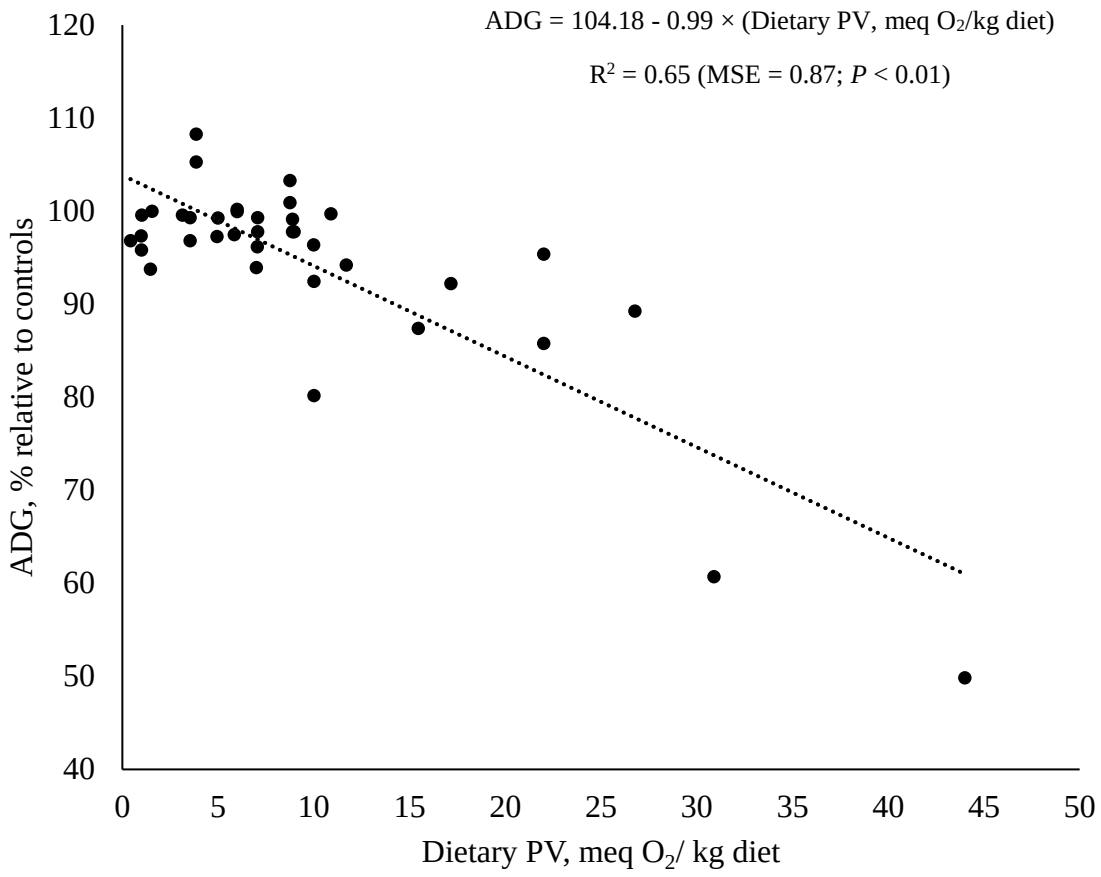

Figure 3.1. Linear regression between dietary peroxide value (PV) and average daily gain (ADG) of poultry.

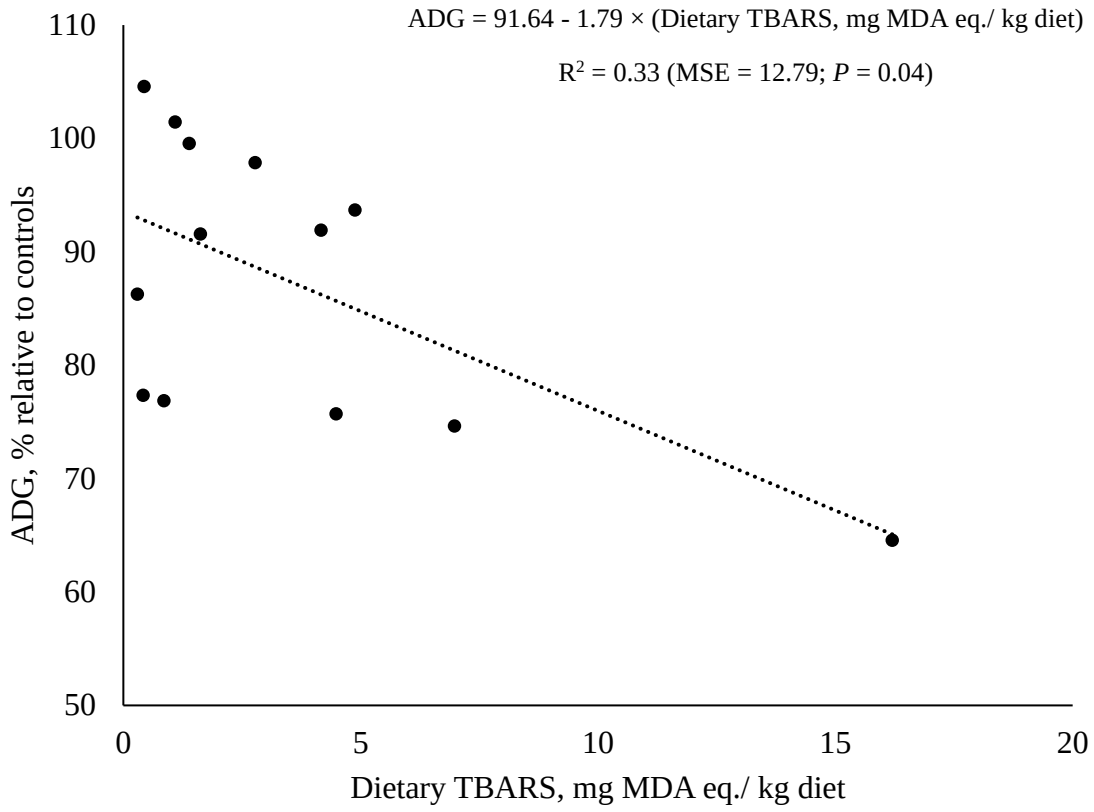

Figure 3.2. Linear regression between dietary thiobarbituric acid reactive substances (TBARS) and average daily gain (ADG) of swine.

Chapter 4 – Evaluation the influence of quantity and physicochemical properties of dietary fiber in digestive function

Decreased nutrient digestibility due to viscosity is independent of the amount of dietary fiber fed to growing pigs

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Synopsis

Fibre content and its effect on chyme viscosity are associated with changes in the digestive system of humans and pigs. However, it is unclear if fibre content and viscosity affect nutrient utilization and intestinal function independently or interactively. Therefore, we evaluated apparent ileal digestibility (AID) of nutrients and intestinal function in 36 ileal-cannulated barrows fed for 29 days either corn-soy (CSBM) or high-fibre CSBM+30% distillers dried grains with solubles (CSBM+DDGS) modified to three levels of viscosity by adding 5% non-viscous cellulose (CEL), 6.5% medium-viscous carboxymethylcellulose (MCMC) or 6.5% high-viscous CMC (HCMC). Digesta was collected on days 27 and 28 and intestinal samples on day 29. Feeding CMC, regardless of fibre content, increased viscosity of whole digesta ($P = 0.003$) and digesta supernatant ($P < 0.0001$) compared with CEL. Feeding CSBM+DDGS or CMC decreased AID of dry matter ($P = 0.003$; $P < 0.0001$) and crude protein ($P = 0.02$; $P < 0.0001$) compared with CSBM or CEL. Feeding CMC regardless of fibre content increased crypt depth in jejunum ($P = 0.02$) and goblet cell area in ileum ($P = 0.004$) compared with CEL. Adding DDGS or CMC did not affect villus height and gene expression of jejunal monosaccharide and amino-acid transporters. Feeding HCMC, regardless of fibre content, elevated amylase activity by 46% and 50% in jejunal ($P = 0.03$) and ileal digesta ($P = 0.01$) compared with CEL. In summary, diets with increased viscosity decreased nutrient digestibility and induced intestinal changes that were independent of the amount of fibre fed.

Keywords: Dietary fiber functionality; Nutrient utilization; Digestive physiology; Monogastric animals

Introduction

Dietary fibre (DF) intake has been associated with health benefits like lowering risk for obesity, metabolic diseases, cancers, chronic diseases, and supporting gut health (Lattimer and Haub, 2010; Slavin, 2013). Continuous consumption of DF is important for maintenance of gut mucosal integrity, supporting a favourable microbiome, and

improving immune response for humans and animals (Slavin, 2013; Agyekum and Nyachoti, 2017; Jha et al., 2019). In addition, there is increasing interest for incorporating various DF-rich alternative feedstuffs to swine for optimizing intestinal function and health status, as well as reducing diet costs without compromising growth performance (Molist et al., 2014; Agyekum and Nyachoti, 2017; Jha et al., 2019). In pigs, typical rations are corn and soybean meal-based diets that contain about 8% neutral detergent fibre (NDF), whereas the inclusion of DF-rich ingredients can elevate dietary NDF to about 20%, depending on the inclusion rate and composition of ingredients (Saqui-Salces et al., 2017; Ferrandis Vila et al., 2018). Feeding high-fibre diets (e.g., 20% NDF) can induce intestinal adaptation characterized by changes in intestinal cell turnover, morphology(Jin et al., 1994) and increased goblet cells (Saqui-Salces et al., 2017; Ferrandis Vila et al., 2018) compared with typical corn and soybean meal-based diets. Overall, a common effect of including high levels of DF in human and pig diets is the reduction of nutrient digestibility (Baer et al., 1997; Moeser and Van Kempen, 2002; Agyekum and Nyachoti, 2017).

Dietary fibre is often analysed through measuring crude fibre, acid or neutral detergent, and total dietary fibre methods, which quantify indigestible carbohydrates in feedstuffs. These analytical methods have limited capability to characterize the physiological effects of DF. The physical-chemical properties of DF – fermentability, solubility and viscosity provide useful characterization of nutritional and physiological responses to DF (Guillon and Champ, 2000; Dikeman and Fahey, 2006). Research has shown that viscosity and fermentability of DF are factors that modulate digestive physiology by affecting digesta passage rate, energy and nutrient digestibility (Hooda et al., 2011), and fermentation kinetics that affect the production of short-chain fatty acids (SCFA) in pigs (Jha and Zijlstra, 2018). Viscosity likely plays a role in influencing small intestine digestion and absorption function, metabolism of nutrients, and production performance in animals (Inborr, 1994; Smits et al., 1997; Guillon and Champ, 2000; McDonald et al., 2001; Dikeman and Fahey, 2006; Molist et al., 2014). Viscosity may also affect microbial fermentation in the large intestine, affecting an important source of energy for colonic cells, although not the major contributor to satisfy energy need of pigs (Jha et al., 2019). It is estimated that energy produced from SCFA contributes up to 15%

of the maintenance energy requirements of growing pigs (Dierick et al., 1989). In this study, we have focused on the function of the small intestine. Although both fibre content and viscosity are likely important for gastrointestinal physiological responses to diet, there is a dearth of information regarding which of these factors plays a dominant role and whether they interact in influencing small intestinal function.

In the current study, we focused on evaluating the roles of DF content and viscosity on changes in nutrient digestibility and intestinal responses. We hypothesized that increased viscosity would cause greater effects on nutrient digestibility and changes on intestinal physiology than the DF content. In order to increase DF content and viscosity independently, corn dried distillers grains with solubles (DDGS) and carboxy-methylcellulose (CMC), a viscous and non-fermentable polysaccharide, were used in the experimental diets to change DF content and viscosity, respectively. These dietary treatments allowed us to evaluate the effect of DF content and viscosity on nutrient digestibility, intestinal morphology, expression of nutrient transporters, and digestive enzyme activities.

Materials and Methods

The Institutional Animal Care and Use Committee at the University of Minnesota reviewed and approved the animal use protocol (#1703-34701A) for the present study.

Animals, diets and experimental design

A total of 36 barrows (initial body weight (BW) = 26.5 ± 3.9 kg) from Topigs females (Landrace × Yorkshire, Winnipeg, MB, Canada) sired by Duroc boars (Compart's Boar Store Inc., Nicollet, MN) were housed individually in metabolism crates equipped with a stainless steel feeder and a nipple drinker at the UMN Southern Research and Outreach Centre (Waseca, MN). Pigs were fitted with a T-cannula with an inner diameter of 1.6 cm at approximately 10 cm from the ileocecal valve. After 14 d post-surgery, pigs were allotted to 6 blocks of 6 pigs with similar initial BW. Within each block, pigs were assigned to 1 of 6 dietary treatments in a 2 × 3 factorial arrangement with 2 basal diets – corn-soybean meal (CSBM) and CSBM + 30% DDGS diets and 3 levels of viscosity: non-viscous cellulose (CEL) at 5% inclusion, medium-viscous carboxymethylcellulose (MCMC) at 6.5% inclusion, and high-viscous CMC (HCMC) at 6.5% inclusion (Table 4.1). Cellulose (Ticalose 100 cellulose powder) and CMC

(Ticalose® CMC 6000 and 15000) were purchased from TIC Gums (White Marsh, MD). Medium- and high-viscous CMC had a minimum viscosity of 4000 and 7500 mPa·s, respectively. All diets were formulated to meet or exceed the nutritional requirements of growing pigs according to the National Research Council nutrient requirements of swine (NRC (National Research Council), 2012). Titanium dioxide (TiO_2) was included at 0.5% as an indigestible marker in diets for calculation of digestibility. Daily feed allowance was calculated based on 3 × maintenance energy requirement of growing-finishing pigs (824.25 × kJ/kg $BW^{0.60}$), and was fed in two equal meals at 0800 h and 1600 h. All pigs were provided *ad libitum* access to water from nipple drinkers throughout the experiment.

Sample collection

Pigs were fed their respective experimental diets for 29 d. Ileal digesta samples were collected for 8 h on day 27 and day 28, starting at 0800 h until 1600 h and using a 225 mL plastic bag attached to the cannula barrel using a cable tie. Bags were removed every 30 min or whenever full. Ileal digesta samples from the 2-d collection were pooled into 1 L wide-mouth bottles and a 50 mL digesta subsample was used for determining viscosity. Ileal digesta samples were stored at -20ºC immediately until further analysis.

On day 29, pigs were weighed and euthanized by captive bolt followed by exsanguination, and the gastrointestinal tract was removed immediately. Digesta samples (5 mL) from the jejunum and ileum were collected, snap-frozen in liquid nitrogen and stored at –80°C for the analysis of enzymatic activity. Tissue segments (2 cm in length) of jejunum (1 m distal to the pyloric sphincter) and ileum (15 cm proximal to the ileocecal valve) were collected and fixed overnight in Carnoy's solution for histological evaluation. About 2 g of jejunal samples were collected, snap-frozen in liquid nitrogen, and then stored at –80°C until analysis for gene expression. All samples were identified with numbers and identifiers were only associated to the respective treatment at the time of statistical analysis.

Chemical analysis

Pooled frozen ileal digesta samples were thawed and kept on ice, mixed thoroughly, sub-sampled, and lyophilized. Dried digesta samples were ground to pass a 1 mm screen. Diet and digesta samples were analysed following AOAC official method (AOAC, 2012) for crude protein (CP, method 990.03) and diethyl ether exact (EE,

method 996.01) at the University of Missouri Experiment Station and Chemical Laboratories. Moisture (method 930.15), ash (method 942.05) and NDF (Ankom NDF method A200) were analysed at the University of Minnesota. The gross energy (GE) in all samples was determined using a bomb calorimeter (model 6400; Parr Instrument Co., Moline, IL). The concentration of TiO_2 in diets and ileal digesta was analysed as described by Myers et al. (2004).

Viscosity measurement

Ileal digesta samples were thawed on ice prior to viscosity measurement using an Advanced Rheometric Expansion System with stress-controlled Rheometer (TA Instruments, New Castle, DE). The measurement was according to the method described by Shelat et al. (2015) with minor modifications. A vane rotor with grooved cup was used for whole digesta viscosity measurement. Digesta samples were loaded into a temperature-controlled cup (39 °C) and pre-warmed for 5 min before measurement. The peak hold test was performed at 0.1 s^{-1} shear rate for 2 min. The steady shear measurements were performed for shear rates ranging from 0.1 to 100 s^{-1}. All measurements were performed at 39 °C to approximate the body temperature of pigs. For measuring the viscosity of the digesta supernatant, supernatants were obtained after centrifugation at 3,500 × *g* for 10 min. Approximately 15 mL of digesta supernatant were loaded into the rheometric system. Steady shear flow measurements were conducted using a concentric cylinder geometry with a cone DIN rotor (30 mm diameter) at a gap of 500 µm with shear rates ranging from 0.1 to 100 s^{-1}. The viscosity of whole digesta exhibited non-Newtonian flow behaviour and therefore was fitted using the power-law model below according to Holdsworth (Holdsworth, 1971) $\eta = K \times \gamma^{n-1}$, where η = viscosity (mPa·s), K = consistency constant, γ = shear rate, and n = power-law index or flow behaviour index.

Histological analysis and goblet cell quantitation

Intestinal tissue samples were fixed, trimmed, dehydrated, and embedded in paraffin. Slides with 5 µm tissue sections were stained with periodic acid-Schiff with Alcian blue (PAS-AB; Newcomer Supply, Middleton, WI) following the manufacturer's instructions. Histological analysis was performed as previously described by Saqui-Salces et al. (2017). Well-oriented villi and crypts were measured in 10 randomly chosen

fields per slide at 100 × magnification and tissue area occupied by goblet cells was measured in five randomly chosen fields per slide at 200 × magnification under light microscopy (Olympus BX53, Center Valley, NJ) using the CellSense image software (Olympus, Center Valley, NJ). Data presented are the means of the average of the fields per pigs in each treatment.

Digestive enzyme activity analysis

Jejunal and ileal digesta were thawed on ice. Digesta (1 g) was extracted by adding 2 mL ice-cold PBS (1×), vortex, and then subjected to centrifugation (3000 × *g* for 15 min). α-Amylase, trypsin, and chymotrypsin activities were determined using commercial kits (Pluschke et al., 2018) (Biovision K711-100, K771-100, and K352-100; California, USA) following the manufacturer's instructions.

Gene expression analysis

Total RNA from jejunal tissue was isolated using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE), and 500 ng of RNA were reverse transcribed using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA). The expression of genes of interest was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA). The PCR conditions were initial activation at 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s, denaturation, and annealing at 60 °C for 60 s. The primer sequences are shown in Table S1. Relative gene expression was calculated using the primer efficiency values as described by Pfaffl (2001). The target gene expression was normalized to the gene expression of the reference gene, *GAPDH*, and the target gene expression of each sample was normalized to the mean of the control group (CSMB+CEL).

Apparent ileal digestibility (AID) and its calculation

The AID of nutrients was measured using TiO_2 as an indicator to normalize feed intake. The TiO_2, CP, EE, ash, and dry matter (DM) in diets and digesta samples were determined as previously described in the *chemical analysis* section. The AID of nutrients were calculated based on the equation: AID, % = [1 − (N digesta /N diet) × (M

diet /M digesta)] × 100, where N digesta and N diet are the nutrient concentrations (g/kg) in digesta and diet DM respectively, and M diet and M digesta are the TiO_2 concentrations (g/kg) in diet and digesta DM, respectively.

Statistical analysis

Statistical power and sample size analyses were performed using SAS (SAS Institute Inc., Cary, NC). A priori sample size calculation indicated that a minimal number of 6 pigs per group was required to achieve significance in a two-way analysis of variance. Considering nutrient digestibility of DM as a primary outcome of this study, retrospective power analysis showed a power of 0.85 was reached on the main effect of fibre and viscosity using GLMPOWER procedure of SAS based on α level of 0.05. Data were analysed using the MIXED procedure with each pig as the experimental unit. The model included fibre content of basal diets (i.e., CSBM vs. CSBM+DDGS), viscosity (i.e., CEL, MCMC, HCMC) and their two-way interaction as fixed effects, and block was considered as random effect. Treatment means were calculated using the LSMEANS statement and least-square means were compared using the PDIFF statement with Tukey-Kramer adjustment. Linear and quadratic polynomial contrasts were performed to evaluate dose response to increase viscosity on BW, ADG, and nutrient digestibility. Polynomial contrast coefficients were adjusted for unequally spaced treatments using the IML procedure. Pearson correlation analysis was carried out to determine the relationship between viscosity, digestibility, enzymatic activities, and nutrient transporters. The power-law model parameters for whole digesta viscosity (k and n) were estimated for each pig using PROC NLIN. Differences were considered statistically significant if $P < 0.05$ and a trend if $P < 0.1$.

Results

General observations

Pigs consumed all of their assigned rations and no signs of diarrhoea were observed throughout the study. General growth performance and health of pigs fed the experimental diets were normal for pigs of this age, feeding regime, and cannulation conditions. After ileal digesta collection on day 28, three pigs were removed from the study (one in CSBM+CEL, one in CSBM+MCMC, and one in DDGS+MCMC) due to undefined health issues.

Viscosity of ileal digesta and growth performance

Inclusion of CMC increased the viscosity of whole digesta ($P = 0.003$) and digesta supernatant ($P < 0.0001$) in the ileum, while increased fibre content from adding DDGS to diets did not affect digesta viscosity (Table 4.2). The viscosity of whole digesta fitted a power-law index model. There were no differences for the consistency constant among the treatments, indicating that the viscosity of whole digesta was driven by the power law index when shear rate was fixed. Digesta viscosity resulting from inclusion of CMC (MCMC or HCMC) in the diets had greater ($P = 0.003$) power-law index than that of digesta of the CEL group regardless of fibre content, indicating that digesta from CMC-fed pigs showed thicker flow behaviour than digesta from CEL-fed pigs. Similarly, inclusion of CMC in diets resulted in greater viscosity of digesta supernatant ($P < 0.0001$) than CEL group regardless of fibre content. Inclusion of HCMC had higher digesta viscosity ($P = 0.04$) than MCMC, but fibre content did not interact with viscosity (i.e., the addition of CMC) to alter digesta viscosity. These results provided a proof of concept that viscous CMC, but not DDGS fibre, increased the viscosity of whole digesta and digesta supernatant in the small intestine of pigs. Thus, our design allowed evaluating the two variables – fibre content and viscosity.

For pig growth performance, average daily gain (ADG) and final BW declined quadratically ($P = 0.01$) with increasing viscosity (Table 4.3). Inclusion of MCMC and HCMC in the diets decreased ($P = 0.0002$) ADG by about 200 g and 165 g, respectively, compared with CEL regardless of fibre content. No interactions of viscosity and fibre content were observed. Pigs fed diets containing MCMC and HCMC had 14% and 9% lower ($P = 0.0004$) final BW than those fed CEL because ADG directly affects final BW. Because pigs were fed two times daily with the amount of feed equivalent to three times the maintenance energy requirement, the decreased growth performance is most likely attributed to reduced digestibility of nutrients and energy.

Apparent ileal digestibility

As anticipated, both fibre content and viscosity negatively affected nutrient digestibility (Figure 4.1). Increased fibre content from adding DDGS (CSBM+DDGS diets) decreased AID of DM ($P = 0.0003$), EE ($P = 0.06$), and CP ($P = 0.02$) compared with CSBM diets. Pigs fed diets containing MCMC and HCMC had lower AID of DM (*P*

< 0.0001), ash (P < 0.0001), EE (P = 0.01), and CP (P < 0.0001) compared with pigs fed diets containing CEL regardless of fibre content, and no differences were observed between MCMC and HCMC treatments. Apparent ileal digestibility of DM, ash, EE, and CP decreased linearly (P < 0.0001, P < 0.0001, P = 0.002, and P < 0.0001) with increasing viscosity from adding MCMC and HCMC. These results suggested that viscosity and fibre content independently decreased nutrient digestibility in the small intestine.

Intestinal epithelial responses to DF and viscosity

Feeding diets with increased viscosity resulted in longer villi (P = 0.06), deeper crypts (P = 0.02) in the jejunum, and deeper crypts (P = 0.09) in the ileum compared with those in the CEL group (Table 4.4). However, feeding diets with increased fibre content did not affect tissue morphology of the small intestine. The goblet cell area in the ileum of pigs fed diets with CMC inclusion was 52% greater (P = 0.004) compared with the ileum of pig in the CEL group. Pigs fed MCMC and HCMC had similar goblet cell area in the ileum. As for morphology, increased fibre content had no impact on goblet cell area in the small intestine, nor did it interact with viscosity to modify morphology and goblet cells area. These results suggest that viscosity, rather than the amount of DF, is a primary cause of the adaptation of intestinal epithelium.

The gene expression of monosaccharides and amino acid transporters in the jejunum were not influenced by viscosity, fibre content, or their interaction (Table 4.5).

Digestive enzymes activities

Increased viscosity from dietary inclusion of MCMC and HCMC increased amylase activities by 40% and 46% in the jejunal digesta (Figure 4.2A; P = 0.03), and by 11% and 50% in the ileal digesta, respectively (Figure 4.2D; P = 0.01) compared with enzymatic activities in the digesta of the CEL group (Figure 4.2). No differences were observed for activity of trypsin and chymotrypsin in the jejunum or ileum. No interactions between viscosity and fibre content were observed for digestive enzymatic activities in the digesta of jejunum and ileum.

Correlation analysis for viscosity effect

To further evaluate the relationships between the parameters measured in the study, we performed a correlation analysis. Several significant correlations were observed

among saccharide and AA transporters, nutrient digestibility, ADG, and digesta viscosity (Figure 4.3). Average daily gain was positively correlated with AID of EE (r = 0.37; P = 0.03) and CP (r = 0.47; P = 0.006). Digesta viscosity was negatively correlated with AID of DM (r = -0.60; $P < 0.001$), ash (r = -0.56; P = 0.001), CP (r = -0.57; $P < 0.001$), and ADG (r = -0.52; P = 0.002). These correlations suggest a positive association between nutrient digestibility and growth performance and a negative association between viscosity and nutrient digestibility.

Discussion

In the present study, increasing DF content and viscosity were achieved by adding DDGS and CMC. Dietary inclusion of CMC is a practical way to manipulate digesta viscosity without changes on other physical-chemical properties of DF as demonstrated in poultry (Smits et al., 1997) and swine studies (McDonald et al., 2001; Piel et al., 2005; Hooda et al., 2011). Regarding digesta viscosity, the current study showed that dietary inclusion of CMC increased viscosity of whole digesta and digesta supernatant. Many studies have only measured viscosity in the digesta supernatant (Smits et al., 1997; McDonald et al., 2001; Piel et al., 2005; Hooda et al., 2011). However, the rheological behaviour of the digesta supernatant is not representative of the whole digesta (Takahashi and Sakata, 2004). Likewise, the majority of experiments that have evaluated the effects of fibre or viscosity on intestinal function were conducted using soluble DF sources (Ito et al., 2009; Hooda et al., 2011; Pluschke et al., 2018). This presents a disadvantage because most common DF sources (e.g., whole-wheat flour, cereal grain bran, vegetables, DDGS, wheat middlings, rice bran) contain high quantities of insoluble DF. Diets and feedstuffs are a mixture of soluble and insoluble non-starch polysaccharides (NSP) that can be degraded throughout the gastrointestinal tract, changing the viscoelastic properties of the digesta (Dikeman et al., 2006). Thus, analysing the effects of insoluble DF on viscosity and intestinal responses is needed. In our study, the addition of DDGS increased insoluble DF by 7% but this amount did not affect digesta viscosity as CMC addition did, suggesting that fibre solubility may be a stronger determinant of digesta rheological properties than the amount of insoluble fibre.

Although the use of ileal-cannulated pigs in digestibility trials is not an ideal model to assess growth performance, we observed substantial decrease in ADG and final

BW of pigs fed diets with increased viscosity even when pigs consumed the same amount of feed and metabolizable energy among dietary treatments. Similar observations have been reported previously in both broilers (Langhout et al., 1999; Langhout et al., 2000; Ayres et al., 2019) and pigs (McDonald et al., 2001; Owusu-Asiedu et al., 2006; Hooda et al., 2011). We also observed that digesta viscosity was negatively correlated with ADG, which is in agreement with observations made in broilers (Ayres et al., 2019) and pigs (Hooda et al., 2011). Increased viscosity impairs nutrient digestibility and energy utilization that could be responsible for reduction of ADG and final BW (Smits et al., 1997; Langhout et al., 2000; Owusu-Asiedu et al., 2006). Our results indicate that increased digesta viscosity is detrimental to growth performance.

A reduction in nutrient digestibility indicates less nutritional value and net nutrient supply from the diet that could be used for body functions. Feeding high-fibre diets can decrease nutrient digestibility in humans (Baer et al., 1997) and pigs (Moeser and Van Kempen, 2002; Agyekum and Nyachoti, 2017). In agreement with previous studies, our results showed that pigs consuming increased amount of DF had decreased AID of DM and CP compared with pigs fed CSBM diets. Several mechanisms contributing to this negative effect of DF on nutrient utilization have been proposed. Among them are reduced enzymatic activity because of complex starch-protein-cell wall matrix that may limit nutrient digestion (Jha et al., 2015; Jha and Berrocoso, 2015; Agyekum and Nyachoti, 2017), decreased retention time because of insoluble DF leading to less time for digestion (Agyekum and Nyachoti, 2017), and the formation of a gel-viscous barrier limiting enzymatic action (Jha and Berrocoso, 2015; Agyekum and Nyachoti, 2017; Bedford, 2018). Also, increasing DF content and its associated viscosity would likely increase specific endogenous losses, including sloughed cells, mucins, and intestinal and pancreatic secretions (Montoya et al., 2016); thereby decreasing apparent nutrient digestibility (Smits et al., 1997). However, the approaches used in this study did not allow the measurement of these losses.

Although most studies in broilers (Smits et al., 1997; Langhout et al., 1999; Langhout et al., 2000; Maisonnier et al., 2001) and pigs (Mosenthin et al., 1994; Hopwood et al., 2004; Owusu-Asiedu et al., 2006; Buraczewska et al., 2007) have reported negative effects of viscosity on nutrient digestibility, other studies have reported

no detrimental effects of viscosity on nutrient digestibility in pigs (Piel et al., 2005; Hooda et al., 2011). This discrepancy may result from the diet composition. Semi-purified diets are highly digestible compared with cereal-based diets, and have been used in the studies that reported no negative effect of viscosity on nutrient digestibility (Piel et al., 2005; Hooda et al., 2011). It is important to note that the lack of a standard procedure measuring digesta viscosity limits our capacity for analysing and concluding from the literature because of methods and value variations. Therefore, a consensus is needed for the procedure to measure digesta viscosity in the near future. We proposed the methods used in this study to be used as standard, as they consider the complex composition and rheology of digesta.

The effect of DF on goblet cells and mucin production has been reported in murine (Ito et al., 2009; Hino et al., 2012), chicken (Langhout et al., 1999), and pig models (Piel et al., 2005; Chen et al., 2013; Saqui-Salces et al., 2017; Ferrandis Vila et al., 2018). However, it is unclear whether this response is directly related to DF content or to its rheological properties. In the present study, an increase in goblet cells was in accordance with previous publications (Piel et al., 2005; Ito et al., 2009). More importantly, we found that viscosity but not fibre content, induced this change in the ileum, suggesting that the rheological property of the digesta is the main driver for goblet cell expansion and mucin production in the distal small intestine. Although mucin produced from goblet cells has a protective and beneficial effect for the host, mucin is considered as a non-dietary antagonist because when it is in excess, nutrient digestibility decreases (Montoya et al., 2016) and represents energy and nutrient loses for production animals (Montagne et al., 2004). The number of goblet cells and amounts of mucins secretion that provide protection without resulting in production loses is still undetermined.

Results from a recent study revealed that gene expression of nutrient transporters and enzymes in the jejunum are involved in the changes of nutrient digestibility of growing pigs (Vigors et al., 2016). A few studies have shown that feeding DF or certain NSP increases gene expression of intestinal nutrient transporters, mainly *SGLT1, GLUT2, GLUT5, and PEPT1* (Chen et al., 2015; Saqui-Salces et al., 2017; Wu et al., 2018), suggesting an adaptation in response to nutrient availability in the gut. In the current

study, several AA transporters in addition to monosaccharide and peptide transporters were analysed, but neither fibre content nor viscosity affected the gene expression of nutrient transporters in the jejunum. This discrepancy may be due to the concentration of DF used in the previous studies, the age of pigs and the nutritional composition and the methods of measurement of DF of the diets.

Digestibility may also be determined by changes in digestive enzyme secretion. Our results suggested that activities of amylase is affected by viscosity rather than DF content, which is in agreement with the finding that soluble DF intake decreases macronutrient digestion by reducing digestive enzyme activities (Isaksson et al., 1982; Espinal-Ruiz et al., 2014; Pluschke et al., 2018). Nevertheless, previous *in vitro* and *in vivo* studies have shown different responses on the effect of viscosity on digestive enzyme activities. Increasing viscosity by NSP in starch-digestive enzyme suspensions depressed glucose diffusion in a dialysis tube, suggesting the suppression of enzymatic hydrolysis and nutrient diffusion by viscosity (Sasaki and Kohyama, 2012). Isaksson et al. demonstrated that increased viscosity by adding pectin to jejunal juice of humans reduced activities of trypsin and amylase (Isaksson et al., 1982). In contrast, the activities of amylase, protease, and lipase in the pancreas and on pancreatic-biliary secretion were increased in rats fed viscous guar gum for 14-d compared with control (Ikegami et al., 1990), and increased amylase activity has been reported in pigs fed pectin for 21-d compared with those fed control diets (Pluschke et al., 2018). Because of the very different models and compounds used in the literature, to define the effect of chyme viscosity on digestive enzymatic activity requires further research.

Some limitations to this study merit consideration. The experimental procedures (i.e., cannulation, housed individually, and fixed amount of daily feed) used in the study deviate from the norm for raising pigs. Experimental conditions and the number of animals limit our capability to make conclusions on growth performance. Other factors such as feed intake and solubility of nutrients influence retention time of solids and liquids in the gastrointestinal tract, thereby changing the kinetics of nutrient flow (Schop et al., 2019). Although soluble DF and diet viscosity might influence retention time (Van et al., 1993), how digesta viscosity regulates retention time, thereby altering nutrient digestibility cannot be discerned in the current study. The impact of retention time on

nutrient digestibility of complex diets requires further evaluation. The viscosities achieved for HCMC and MCMC diets, and the corresponding AID, were not as different as expected. This limited our capability to estimate the broader effects of viscosity on nutrient digestibility. Because of the number of treatments and range of non-equally spaced viscosity, inferences on dose response effects are not conspicuous, although the linear and quadratic responses shed some light on dose effects. Finally, we used CMC, a soluble, non-fermentable fibre, to manipulate viscosity. However, the effects observed in this study may not apply to all soluble DF sources because not all soluble DF are viscous, such as inulin, fructooligosaccharides, and wheat dextrin (Slavin, 2013).

In conclusion, the current results support the hypothesis that viscosity has a significant impact on digestive function. Although increased DF content and viscosity independently decreased nutrient digestibility, the content of DF fed had no effect on ADG, final BW, intestinal morphology, goblet cell area, and digestive enzymatic activities. In contrast, increased digesta viscosity regardless of fibre content resulted in decreased ADG and final BW, deeper crypts, and greater goblet cell area and greater amylase activity, suggesting that viscosity is the dominant factor that affects intestinal digestive physiology. Results from our study emphasize the need for considering the variable viscosity properties of high-fibre ingredients in particular ingredients high in viscous DF, used in human food and formulating animal diets to more closely predict effects on nutrient digestibility and improve DF utilization.

Tables and Figures

Table 4.1. Ingredient and nutrient composition of experimental diets (as-fed basis)[1]

Fiber content	CSBM			DDGS		
Viscosity	CEL	MCMC	HCMC	CEL	MCMC	HCMC
Ingredient composition, %						
Corn, yellow dent	54.80	52.51	52.51	29.72	27.88	27.88
Soybean meal	33.77	34.36	34.36	29.36	29.51	29.51
Corn DDGS	-	-	-	30.00	30.00	30.00
Soybean oil	3.47	4.17	4.17	3.24	3.93	3.93
Monocalcium phosphate	1.08	1.08	1.08	0.54	0.54	0.54
Limestone	0.88	0.88	0.88	1.14	1.13	1.13
Salt	0.25	-	-	0.25	-	-
Premix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50
CEL[3]	5.00	-	-	5.00	-	-
MCMC[4]	-	6.25	-	-	6.25	-
HCMC[5]	-	-	6.25	-	-	6.25
Calculated nutrient composition						
ME, MJ/kg	13.81	13.81	13.81	13.81	13.81	13.81
Crude Protein	20.25	20.33	20.33	24.34	24.25	24.25
Standardized ileal digestible (SID) Lys, %	1.0	1.01	1.01	0.98	0.98	0.98
SID Thr, %	0.66	0.58	0.58	0.73	0.73	0.73
SID Met+Cys, %	0.58	0.58	0.58	0.68	0.68	0.68
SID Trp, %	0.22	0.22	0.22	0.22	0.22	0.22
Acid detergent fiber, %	8.70	9.92	9.92	11.37	12.58	12.58
Neutral detergent fiber, %	13.70	14.80	14.80	20.00	21.09	21.09
Standardized total tract digestible P, %	0.30	0.30	0.30	0.33	0.33	0.33
Analyzed nutrient composition, %						
Dry matter	88.32	88.97	88.79	89.73	89.48	89.81
Crude protein	20.05	20.82	19.66	23.96	23.22	24.30
Ether extract	4.80	5.04	5.07	6.80	7.04	6.96
NDF	11.01	11.49	11.35	18.34	19.36	
Ash	6.24	5.93	5.79	6.28	7.09	7.12
Titanium dioxide	0.39	0.42	0.46	0.48	0.51	0.47

[1]Two basal diets: corn-soybean meal (CSBM) or CSBM plus 30% distillers dried grains with solubles (CSB+DDGS) with three levels of viscosity (i.e., non-viscous cellulose, CEL; medium-viscous carboxymethylcellulose, MCMC; high-viscous CMC, HCMC).

[2]The premix provided the following per kilogram of complete diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 30 IU; vitamin K_3, 3 mg; vitamin B_{12}, 0.012 mg; riboflavin, 4 mg; niacin, 40 mg; pantothenic acid, 15 mg; choline chloride, 400 mg; folic acid, 0.7 mg; thiamin, 1.5 mg; pyridoxine, 3 mg; biotin, 0.1 mg; Zn, 105 mg; Mn, 22 mg; Fe, 84 mg; Cu, 10 mg; I, 0.50 mg; Se, 0.35 mg.

[3]CEL = cellulose (Ticalose® 100 cellulose powder).

[4]MCMC = medium-viscosity CMC (Ticalose® CMC 6000).

[5]HCMC = high-viscosity CMC (Ticalose® CMC 15000).

Table 4.2. Rheological characteristics of ileal digesta from pigs fed experimental diets containing CMC and DDGS[1]

Fiber content	CSBM			DDGS			SEM[2]	*P*-values[3]		
Viscosity	CEL	MCMC	HCMC	CEL	MCMC	HCMC		F	V	F × V
Whole digesta[4]										
K	5.10	4.71	5.03	4.05	3.52	7.48	1.47	0.95	0.32	0.38
n	0.19^{b}	0.37^{a}	0.39^{a}	0.21^{a}	0.18^{ab}	0.40^{a}	0.05	0.24	0.003	0.11
Digesta supernatant, mPa·s										
Viscosity	1.28^{c}	6.98^{b}	9.24^{a}	1.53^{c}	6.97^{b}	11.25^{a}	1.25	0.48	.0001	0.69

^{a,b,c}Different superscripts in a row indicate values are different ($P < 0.05$).

[1]Two basal diets: corn-soybean meal (CSBM) or CSBM plus 30% distillers dried grains with solubles (CSB+DDGS) with three levels of viscosity (i.e., non-viscous cellulose, CEL; medium-viscous carboxymethylcellulose, MCMC; high-viscous CMC, HCMC).

[2]Standard error of the mean.

[3]Factorial arrangement of treatments fiber (F) and viscosity (V) main effects with 18 and 12 observations respectively, and fiber and viscosity interaction with (F × V) with 6 observations per treatment.

[4]The dynamic whole digesta viscosity was fitted a power law model according to Holdsworth[(23)]: $\eta = K \times \gamma^{n-1}$, where η = viscosity, K = consistency constant, γ = shear rate and n = power law index.

Table 4.3. Body weight and average daily gain in pigs fed diets differing in fiber content and viscosity[1]

Fiber content	CSBM			DDGS			SEM[2]	*P*-values[3]				
Viscosity	CEL	MCMC	HCMC	CEL	MCMC	HCMC		F	V	F × V	Linear	Quadratic
Initial BW, kg	26.68	26.16	26.40	26.33	26.78	26.92	1.73	0.46	0.08	0.47	NA	NA
Final BW, kg	56.44[a]	48.67[b]	49.29[b]	56.34[a]	48.29[b]	52.30[b]	2.45	0.38	.0004	0.59	.0006	0.01
ADG, kg	0.84[a]	0.61[b]	0.64[b]	0.83[a]	0.62[b]	0.70[b]	0.45	0.49	.0002	0.67	.0003	0.01

[a,b]Different superscripts in a row indicate values are different ($P < 0.05$).

[1]Two basal diets: corn-soybean meal (CSBM) or CSBM plus 30% distillers dried grains with solubles (CSB+DDGS) with three levels of viscosity (i.e., non-viscous cellulose, CEL; medium-viscous carboxymethylcellulose, MCMC; high-viscous CMC, HCMC).

[2]Standard error of the mean.

[3]Factorial arrangement of treatments fiber (F) and viscosity (V) main effects with 18 and 12 observations respectively, and fiber and viscosity interaction with (F × V) with 6 observations per treatment. Polynomial (linear and quadratic) contrasts were used to determine the effect of viscosity.

Table 4.4. Intestinal morphology and goblet cell area in pigs fed diets differing in fiber content and viscosity[1]

Fiber content	CSBM			DDGS			SEM[2]	*P*-values[3]		
Viscosity	CEL	MCMC	HCMC	CEL	MCMC	HCMC		F	V	F × V
Jejunum										
Villus height, µm	312.76	326.73	384.02	333.06	340.53	375.32	24.73	0.68	0.06	0.82
Crypt depth, µm	99.51[b]	99.73[b]	127.09[a]	109.99[b]	101.16[b]	127.85[a]	9.64	0.60	0.02	0.86
Villus:crypt ratio	3.37	3.44	3.25	3.24	3.50	3.19	0.13	0.84	0.66	0.94
Goblet cell area, %[4]	3.55	4.17	4.03	3.39	3.49	3.88	0.52	0.45	0.64	0.85
Ileum										
Villus height, µm	307.69	333.29	376.50	351.26	358.18	367.81	20.93	0.25	0.12	0.42
Crypt depth, µm	209.44	257.15	267.66	238.24	250.60	244.11	17.47	0.97	0.09	0.23
Villus:crypt ratio	1.53	1.34	1.47	1.54	1.49	1.55	0.08	0.23	0.35	0.69
Goblet cell area, %[4]	7.59[b]	12.84[a]	12.26[a]	8.08[b]	11.02[a]	11.68[a]	1.22	0.53	.004	0.66

[a,b]Different superscripts in a row indicate values are different ($P < 0.05$).

[1]Two basal diets: corn-soybean meal (CSBM) or CSBM plus 30% distillers dried grains with solubles (CSB+DDGS) with three levels of viscosity (i.e., non-viscous cellulose, CEL; medium-viscous carboxymethylcellulose, MCMC; high-viscous CMC, HCMC).

[2]Standard error of the mean.

[3]Factorial arrangement of treatments fiber (F), viscosity (V) main effects with 18 and 12 observations respectively, and fiber and viscosity interaction with (F × V) with 6 observations per treatment.

[4]Goblet cells area was defined as area of positive cells for PAS-AB staining / mucosal area × 100.

Table 4.5. Relative mRNA expressions of nutrient transporters in the jejunum of pigs fed diets differing in fiber content and viscosity[1]

Fiber content	CSB			DDGS			SEM[2]	*P*-values[3]		
Viscosity	CEL	MCMC	HCMC	CEL	MCMC	HCMC		F	V	F × V
Sugar transporters[4]										
GLUT2	1.00	1.03	1.02	0.92	0.96	0.98	0.05	0.17	0.74	0.94
GLUT5	0.99	1.00	0.96	0.97	0.96	0.88	0.09	0.50	0.66	0.92
SGLT1	1.00	1.04	0.98	0.90	1.01	0.98	0.07	0.41	0.56	0.70
Amino acid transporters[5]										
TAT1	1.00	1.04	0.98	0.90	1.00	0.99	0.08	0.48	0.63	0.74
b0,$^{+}$AT	1.00	1.01	1.00	0.86	1.00	0.95	0.08	0.34	0.65	0.75
B0AT1	1.00	1.05	0.98	0.83	1.03	0.98	0.10	0.48	0.53	0.07
LAT2	1.00	1.05	0.97	0.91	1.01	1.00	0.08	0.66	0.65	0.74
CAT1	1.00	1.04	0.97	0.89	1.00	0.97	0.07	0.39	0.53	0.70
ASCT2	1.00	1.12	0.96	0.88	1.01	0.97	0.09	0.32	0.33	0.71
PEPT1	1.00	1.11	1.03	0.90	1.06	1.03	0.07	0.43	0.21	0.77

[1]Two basal diets: corn-soybean meal (CSBM) or CSBM plus 30% distillers dried grains with solubles (CSB+DDGS) with three levels of viscosity (i.e., non-viscous cellulose, CEL; medium-viscosity carboxymethylcellulose, MCMC; high-viscosity CMC, HCMC).

[2]Standard error of the mean.

[3]Factorial arrangement of treatments fiber (F), viscosity (V) main effects with 18 and 12 observations respectively, and fiber and viscosity interaction with (F × V) with 6 observations per treatment.

[4]*GLUT2* = solute carrier family 2 member 2 (*SLC2A2*), *GLUT5* = solute carrier family 2 member 5 (*SLC2A5*), *SGLT1* = Sodium-dependent glucose cotransporter 1 (*SLC5A1*).

[5]*AT1* = T-type amino acid transporter 1 (*SLC16A10*), b0,$^{+}$AT = b(0,$^{+}$)-type amino acid transporter 1 (*SLC7A9*), *B0AT1* = B(0,+)-Type Amino Acid Transporter 1 (*SLC6A19*), *LAT2* = Large neutral amino acids transporter small subunit 2 (*SLC7A8*), *CAT1* = Cationic amino acid transporter 1 (*SLC7A1*), *ASCT2* = Alanine, Serine, Cysteine Transporter 2 (*SLC1A5*), *PEPT1* = peptide transporter solute carrier family 15 member 1 (*SLC15A1*).

Table 4.6. Genes of interest and primer sequences used in this study

Gene[1]	Forward	Reverse
GLUT2	TTTTGGGTGTTCCGCTGGAT	GAGGCTAGCAGATGCCGTAG
GLUT5	TGTGTGGCTCCTGGTAACAC	TCGGCCATGTTCGATTCCTT
SGLT1	CCCAAATCAGAGCATTCCATTCA	AAGTATGGTGTGGTGGCCGGTT
TAT1	GGATTTCTGCTCGGATTCAT	AACAAAGGACAACACCTCCAA
b0,$^{+}$AT	GAACCCAAGACCACAAATC	ACCCAGTGTCGCAAGAAT
B0AT1	ACAACAACTGCGAGAAGGACTC	GCAGGTCAAACCCGTTGATAAG
LAT2	CGGAGACTGGTTCTGGAGAG	AGTTGACCCATGTGAGGAGC
CAT1	GAGCAAGACCAAACTCTCCTTC	AGCCTATCAGCATCCACACTG
ASCT2	GATTGTGGAGATGGAGGATGTGG	TGCGAGTGAAGAGGAAGTAGATGAGA
PEPT1	TTGTGGCTCTGTGCTACCTG	TCCGTTGTGGTCGAAGTCTG
GAPDH	ATCCTGGGCTACACTGAGGAC	AAGTGGTCGTTGAGGGCAATG

[1]*GLUT2* = solute carrier family 2 member 2 (*SLC2A2*), *GLUT5* = solute carrier family 2 member 5 (*SLC2A5*), *SGLT1* = sodium-dependent glucose cotransporter 1 (*SLC5A1*), *TAT1* = T-type amino acid transporter 1 (*SLC16A10*), b0,$^{+}$AT = b(0,$^{+}$)-type amino acid transporter 1 (*SLC7A9*), *B0AT1* = B(0,+)-type amino acid transporter 1 (*SLC6A19*), *LAT2* = large neutral amino acids transporter small subunit 2 (*SLC7A8*), *CAT1* = cationic amino acid transporter 1 (*SLC7A1*), *ASCT2* = alanine, serine, cysteine transporter 2 (*SLC1A5*), *PEPT1* = peptide transporter solute carrier family 15 member 1 (*SLC15A1*), *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase

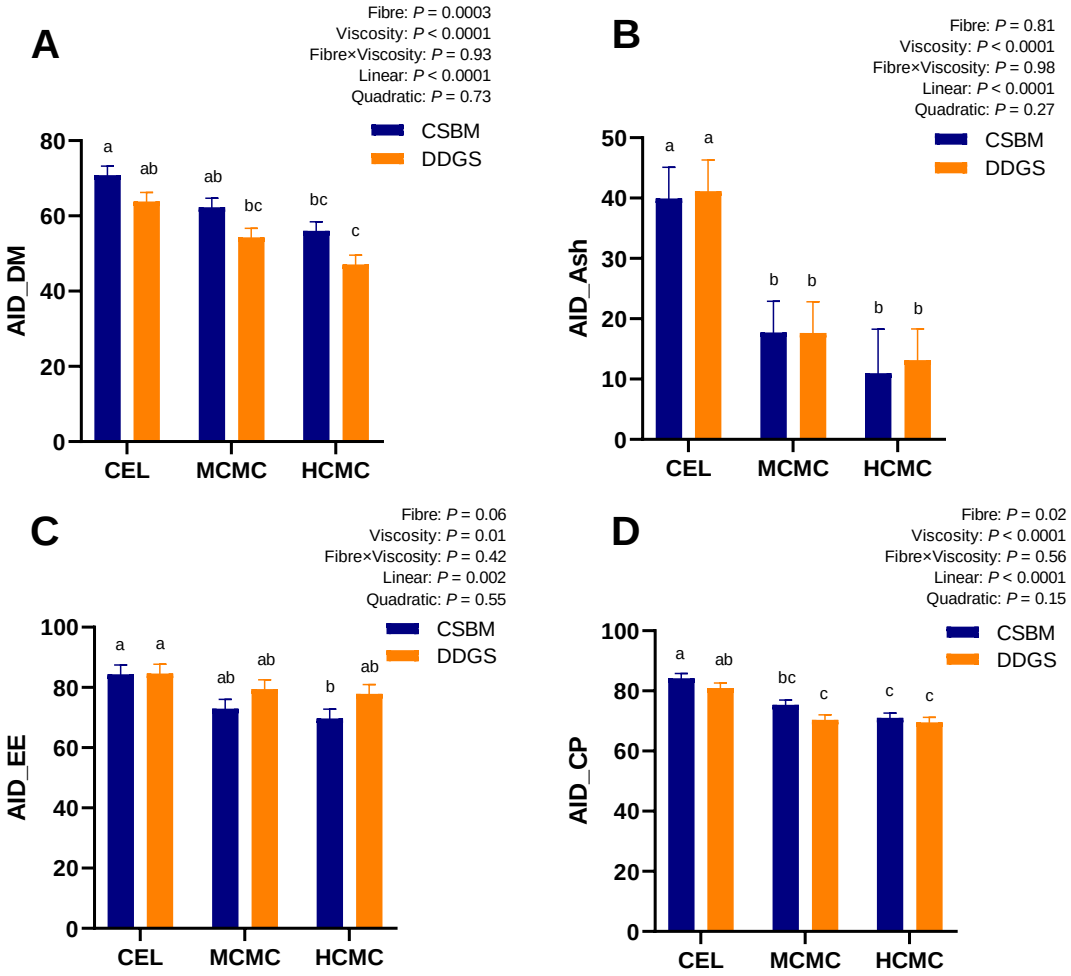

Figure 4.1. Apparent ileal digestibility (AID) of dry matter (A), ash (B), ether extract (C), and crude protein (D) in pigs fed diets differing in fiber content and viscosity. Bars represent LS means ± SEM, n = 6. Polynomial (linear and quadratic) contrasts were used to determine the effect of viscosity. CSBM = corn-soybean meal, DDGS = CSBM plus 30% DDGS, CEL = non-viscous cellulose, MCMC = medium-viscous CMC, HCMC = high-viscous CMC. [a,b,c]Different letters indicate values are different ($P < 0.05$).

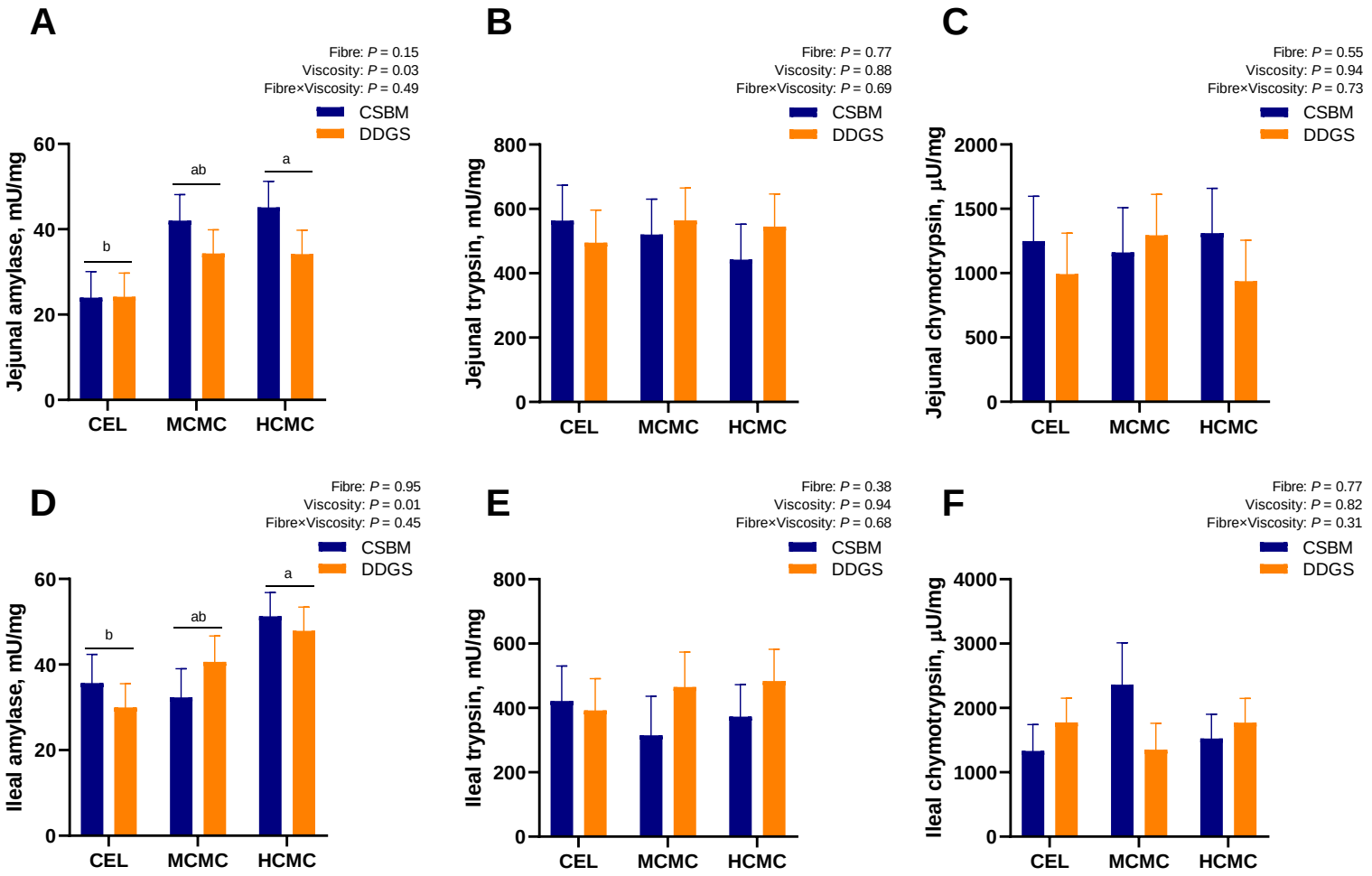

Figure 4.2. Enzymatic activities in the intestinal digesta of pigs fed diets differing in fiber content and viscosity. Bars represent LS means ± SEM, n = 6. CSBM = corn-soybean meal, DDGS = CSBM plus 30% DDGS, CEL = non-viscous cellulose, MCMC = medium-viscous CMC, HCMC = high-viscous CMC. [a,b]Different letters indicate differences ($P < 0.05$) among viscosity treatments regardless of fibre content.

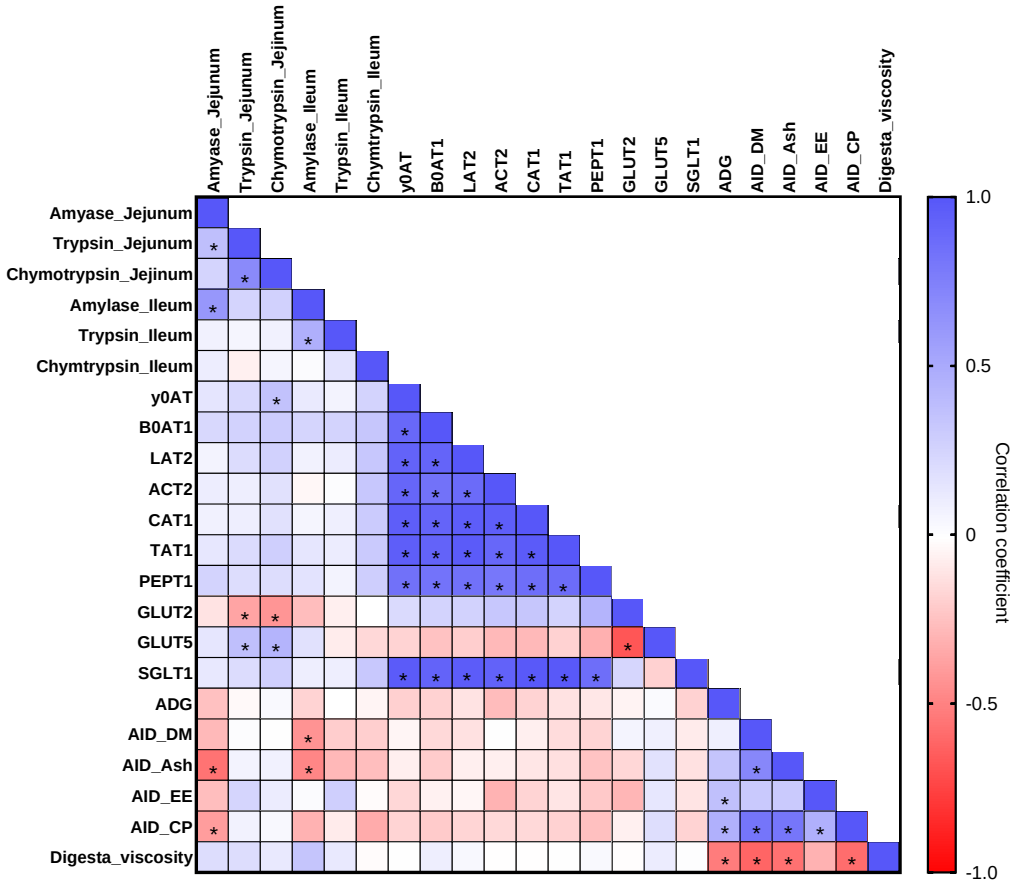

Figure 4.3. Pearson correlation analysis is expressed in a heatmap. $P < 0.05$ is indicated with *

Chapter 5 – An exploratory study to investigate the influence of antimicrobial growth promoters supplementation on morphological, immune, and metabolic responses in the gastrointestinal tract

Analysis of gastrointestinal responses revealed both shared and specific targets of zinc oxide and carbadox in weaned pigs

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Synopsis

Antibiotics and pharmacological zinc supplementation were commonly used as growth promoters for several decades in the swine industry before being limited because of public health and environmental concerns. Further, the physiological and metabolic responses associated with their growth promotion effects are unclear. To characterize these responses induced by pharmacological zinc supplementation (2500 mg/kg) and carbadox (55 mg/kg), 192 post-weaning pigs were fed basal and test diets for 43 days. Compared with basal, pharmacological zinc and carbadox independently improved growth performance. Pharmacological zinc increased gastric mucosa thickness compared with basal zinc, while carbadox increased intestinal villus:crypt ratio compared with non-carbadox. Pharmacological zinc and carbadox independently reduced interleukin (IL)-1β concentration compared with basal zinc and non-carbadox. Pharmacological zinc increased IL-1RA:IL-1 ratio by 42% compared with basal zinc, while carbadox tended to increase the IL-10 and IL10:IL-12 ratio compared with non-carbadox. Carbadox increased fecal concentrations of histidine and lysine compared with non-carbadox. The independent effect of pharmacological zinc and carbadox on morphology and nutrient metabolism, and their shared effect on immunity may contribute to the additive effect on growth promotion. These results further confirmed the concept that growth promotion is multifactorial intervention. Therefore, elucidating growth-promoting effects and searching for alternatives should include wide-spectrum evaluation.

Keywords: animal production; carbadox; cytokines; gastrointestinal tract; gut metabolites; zinc oxide

Introduction

Antimicrobial growth promoters (AGPs) and pharmacological doses of zinc (Zn, 1000–3000 mg/kg diet) from zinc oxide (ZnO) were the most common approaches to prevent the adverse effects of weaning in piglets. However, the non-therapeutic use of antibiotics as AGPs and pharmacological doses of Zn in weaned pigs is of concern because they may cause the selection of multi-drug resistant bacteria (Cogliani et al., 2011), and enterobacteria have been shown to develop resistant genes (Vahjen et al.,

2015). In addition, the excretion of Zn in manure can contaminate groundwater and soil (Dębski, 2016; Jensen et al., 2016). Therefore, the European Union banned all in-feed AGPs in animal production in 2006 and will only allow the inclusion of dietary Zn concentrations of 150 mg/kg for supplementation to meet the piglets' nutritional requirement by 2022. The United States (US) implemented the Veterinary Feed Directive in 2017, which prohibited the use of medically important antibiotics as growth promoters in animal diets (FDA, 2015). Preventing post weaning diarrhea (PWD) and maintaining health and optimal growth performance of weaned pigs are priorities for pork producers. With the restriction of using AGPs and pharmacological doses of Zn in diets, various alternatives, such as probiotics, prebiotics, organic acids, enzymes, phytogenic compounds, and trace minerals, have been the subject of evaluation as growth promoters (Gadde et al., 2017). A recent review summarizing results from more than 2000 published trials showed limited improvements in weight gain of pigs fed diets using these alternative growth promoters (Gabler and Schweer, 2018). Although the effects of AGPs and pharmacological doses of Zn on growth performance have been widely reported (Thrasher et al., 1969; Yen et al., 1985; Poulsen, 1995; Hill et al., 2001; Sales, 2013), the mechanisms involved in these effects have not been fully identified (Niewold, 2014).

Several mechanisms of action of growth-promoting responses of Zn and AGPs have been proposed. Pharmacological Zn supplementation increased plasma ghrelin levels, altered gut microbiota, and maintained the intestinal mucosal barrier and immune system (Højberg et al., 2005; Yin et al., 2009; Shen et al., 2014). These responses have been suggested to contribute to growth promotion. Moreover, growth-promoting effects of AGPs may be attributed to antimicrobial properties, modification of gut microbiota, and a direct or indirect influence on inhibition of inflammatory responses (Niewold, 2014; Yu et al., 2017). However, in the industry these treatments are commonly used in combination, not separately. Whether pharmacological Zn supplementation and AGPs used in combination share common targets and mechanisms to exert their growth-promoting effects remains unclear. Animal growth is strongly related to nutrient digestion, utilization, and metabolism. Immunomodulation can also influence host growth response via its metabolic costs. Considering the significance of the gastrointestinal (GI) tract in these functions, our objective was to identify early morphological, immune and

metabolic responses in the GI tract when feeding a pharmacological dose of Zn or carbadox, alone or in combination, in the diets of weaned pigs.

Materials and Methods

All chemicals used were purchased from Sigma-Aldrich unless otherwise stated. The feeding experimental procedures were conducted in the Cargill Animal Nutrition Innovation Campus (Elk River, MN, USA), approved and supervised by Cargill Animal Nutrition Committee on Animal Use for Research and Scientific Purposes in accordance with Directive 2010/63/EU. All other analysis and procedures were performed at the University of Minnesota (St. Paul, MN, USA) and performed in accordance with the University's regulation and guidelines.

Animals and Experimental Design

A total of 192 crossbred barrows and gilts (BW = 5.9 ± 1.1 kg) were weaned at 21 days of age and fed four experimental diets during a 43-day feeding period, using a three-phase feeding program (Phase 1 = day 0 to day 7, Phase 2 = day 7 to day 21, and Phase 3 = day 21 to day 43, post weaning). Pigs were housed individually in 4 identical nursery rooms and stratified by sex and BW into 8 blocks. The experimental design consisted of a 2 × 2 factorial arrangement of treatments in a randomized complete block design. The first factor was dietary concentration of Zn to meet the requirement (basal Zn; 150 mg/kg diet) and pharmacological dose (2500 mg Zn/kg diet). The second factor was carbadox (Mecadox; Phibro Animal Health, Fairfield, NJ, USA) added to the diets at a concentration of 0 or 55 mg/kg diet. The pharmacological dose of Zn was provided from ZnO and was removed in the third phase of the feeding program. All diets were formulated to meet or exceed the nutritional requirements of weaned pigs suggested by Cargill Nutrition System (Table 5.1). Pigs were provided ad libitum access to their assigned experimental diets and water during the entire study.

Data and Sample Collection

Experimental diet samples were collected and analyzed by Cargill Animal Nutrition with Near Infrared Spectroscopy at the Cargill Animal Nutrition Innovation Center (Elk River, MN) for ash, crude protein, ether extract, neutral detergent fiber, and moisture. Focal pigs (n = 10/treatment) were selected by statisticians with consideration

of BW, sex, and room. In order to characterize early physiological changes, focal pigs were euthanized by CO_2 on day 7 post-weaning (28 d of age) for collecting tissue samples. About 20 mL of gastric contents was collected and stored at −20 °C for gastric acidity determination. Samples of gastric antrum and corpus, jejunum (1 m distal to the pyloric sphincter), and ileum (15 cm proximal to the ileocecal valve) were collected and fixed in 4% buffered formaldehyde (Fisher Scientific, Hampton, VA, USA) for histological evaluation. About 2 g of ileal digesta from focal pigs and feces from rectum (n = 44 pigs/treatment) were also collected, snap frozen, and stored at −80 °C for analysis of targeted metabolites.

Gastric Acid Titration

Gastric acid was analyzed using the protocol as described by Waghray et al. (2010). Briefly, thawed gastric contents were centrifuged at 3000× *g* for 15 min at 4 °C, and the supernatant was recovered for acid titration with an automatic titrator (TitroLine easy, Schott Instruments GmbH, Germany). The supernatants were diluted 10 × with 0.9% saline solution and titrated with 0.01 N sodium hydroxide (NaOH) solution using the titrator. Each sample was analyzed in triplicate and the volume of NaOH used to titrate to a final pH of 7.4 was recorded. Gastric acidity was expressed as mEq H+/mL and calculated by the formula: ((volume of NaOH of sample − volume of NaOH of blank) × normality of the titrating solution)/sample volume) × sample dilution factor.

Histological Analysis

Tissue samples were fixed overnight in 4% buffered formaldehyde and trimmed before processing in a STP 120 Spin Tissue Processor (Thermo Fisher Scientific, Waltham, MA, USA). Tissues were then embedded in paraffin, and tissue blocks were sectioned at 5 µm thickness and mounted on charged slides. Deparaffinized slides were stained with hematoxylin and eosin (H&E, Newcomer Supply, Middleton, WI, USA). Stomach antrum and corpus sections were also stained with periodic acid–Schiff with Alcian blue (PAS-AB, Newcomer Supply, Middleton, WI, USA) following the manufacturer's instructions after rehydration. Histological analysis was performed as previously described Saqui-Salces et al. (2017). Briefly, the total mucosal height of corpus and antrum were measured from the tip of the glands to the muscularis mucosa. Intestinal villus height was defined as the length of a line drawn at the center of the villus

from the crypt neck to the tip of the villus. Villus width was measured as the distance between the epithelial cells at the middle point between the tip and the base of the villus. Crypt depth was measured as the distance at the center of the crypt from the villus neck toward the muscularis mucosa up to the point where epithelial cells were observed. Peyer's patches were defined as round to oval follicular nodes. Well-oriented mucosal glands and villi were measured in 10 randomly chosen fields per slide at 100 × magnification and the area of each Peyer's patch was measured at 40 × magnification under light microscopy (Olympus BX53, Center Valley, NJ, USA), using the CellSense image software for measuring (Olympus, Center Valley, NJ, USA). Data presented are the means of the 10 fields for each pig in each treatment.

Tissue Cytokine Analysis

Tissue cytokine analysis was performed as previously described by (Ferrandis Vila et al., 2018). Briefly, tissue protein was extracted after homogenization of ileal samples in lysis buffer containing deoxycholic acid (12.7 mM), Igepal CA-630 (1%), Tris-HCl (50 mM), NaCl (150 mM), and protease inhibitor cocktail (1X, Halt protease inhibitor Cocktail, Thermo Fisher Scientific, Rockford, IL, USA) adjusted to pH 7.4. Homogenized samples were centrifuged at 12,000× *g* for 15 min at 4 °C and total protein was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) and stored at −80 °C until cytokine analyses. A Multiplex Map Kit (Porcine cytokine/chemokine Magnetic Bead Panel, Merck Millipore, Darmstadt, Germany) was used to quantify 13 porcine cytokines, including 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 (TNFα). The concentration of each cytokine was expressed per mg of total tissue protein.

Quantitative Analysis of Free Amino Acids, Fatty Acids, and Bile Acids in Ileal Digesta and Feces

Sample preparation. Ileal fluid was obtained by centrifuging the digesta, and the fluid was further diluted 5 times with 50% aqueous acetonitrile (ACN) followed by centrifugation at 18,000× *g* for 10 min to obtain the supernatants. Fecal samples were soaked in 50 volumes (*v*/*w*) of 50% aqueous ACN overnight at 4 °C, extracted by

vortexing and sonication for 10 min, and centrifuged at 18,000× *g* for 10 min to remove the insoluble fraction (Trudeau et al., 2018).

Chemical derivatization. For free AA analysis, samples were derivatized by dansyl chloride (DC) (Ma et al., 2019). Briefly, 5 μL of sample or standard was mixed with 5 μL of 100 μM *p*-chlorophenylalanine (internal standard), 50 μL of 10 mM sodium carbonate, and 100 μL of DC solution (3 mg/mL in acetone). The mixture was incubated at 60 °C for 15 min, centrifuged at 18,000× *g* for 10 min, and the supernatant was transferred into a vial for further analysis. For FA analysis, samples were derivatized by 2-hydrazinequinoline (HQ) (Lu et al., 2013). Briefly, 2 μL of sample was added into a 100 μL of freshly prepared ACN solution containing 1 mM 2,2′-dipyridyl disulfide, 1 mM triphenulphosphine, and 1 mM HQ. The reaction mixture was incubated at 60°C for 30 min, chilled on ice, and then mixed with 100 μL of ice-cold deionized water. After centrifugation at 18,000× *g* for 10 min, the supernatant was transferred into a vial for analysis. The DC and HQ derivatives in the reaction mixture were analyzed using Liquid Chromatography–Mass Spectrometry (LC–MS).

LC–MS analysis. The analysis was performed as described previously (Ma et al., 2019). A 5 μL aliquot prepared from the sample of ileal fluid or fecal extract was injected into an Acquity Ultra-Performance Liquid Chromatography system (Waters, Milford, MA) and separated in a BEH C18 column. For BA analysis, 10 mM NH4OAc (pH = 9) (A), 95% ACN and 5% water with 10 mM NH4OAc (pH = 9) (B) were used as mobile phase. For AA analysis, 0.1% formic acid (A) and ACN containing 0.1% formic acid (B) were used as mobile phase. Fatty acid analysis was conducted using 0.05% (*v*/*v*) aqueous acetic acid containing 5 mM ammonium acetate (A), 95% (*v*/*v*) aqueous ACN containing 0.05% acetic acid, and 5 mM ammonium acetate (B) as the mobile phase. The eluent from LC was introduced into a mass spectrometer (Q-TOF-MS, Waters) for accurate mass measurement and ion counting. For electrospray ionization, capillary voltage and cone voltage were maintained at 3 kV and 30 V for positive-mode detection or at 3 kV and 35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), with argon used as the collision gas. Sodium formate solution (mass-to-charge (m/z) ratio: 50–1000) was used to calibrate the mass

spectrometer and was monitored by the intermittent injection of the lock mass leucine enkephalin ([M + H]+ = 556.2771 m/z) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynxTM software in centroided format. Additional structural information was obtained by tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 40 eV.

Data analysis. The concentration of individual metabolites was determined by calculating the ratio between the peak area of compound and the peak area of internal standard, and a standard curve was fitted using QuanLynx software (Waters, Milford, MA, USA).

Statistical Analysis

To calculate sample size, growth performance was considered the primary outcome and a power calculation was performed in SAS (v9.3; SAS Inst. Inc., Cary, NC, USA) using an effect size of 29.7 g for ADFI and 26.7 g for ADG, α level of 0.05, and a power of 80%. The calculation indicated a minimal number of pigs per group of 38. Forty-eight pigs were used per treatment to account for possible losses along the experiment. The normality test was evaluated using the procedure (PROC) Univariate procedure of SAS, and data were analyzed as a randomized complete block design using the PROC Mixed procedure. Because pigs were housed individually, pigs were considered as experimental unit for all measurements and were used as a subject for repeated measurements for data analysis of growth performance responses. Fixed effects included dietary concentration of Zn, addition of carbadox, time, and two- and three-way interactions for growth performance responses. Block was considered as a random effect. In contrast to growth performance, the fixed effect of time was removed from the statistical models for other responses, including gastric acidity, histological analysis, tissue cytokines analysis, and quantification of AA, FA, and BA analysis. Data for growth performance were analyzed using repeated measurements in the model. All mean values were reported as least squares means. Multiple comparisons among treatments were performed using *p*-values for differences (PDIFF) and adjusted by Tukey for multiple comparisons of means. Additionally, the PROC correlation (CORR) procedure of SAS was applied to identify correlations between metabolites and ADG. Significant differences were declared at $p < 0.05$ and statistical trends at $p < 0.10$.

Ethical Approval

The project has been approved by the Cargill Animal Nutrition Animal Care and Use Committee—Elk River (Project identification code: 2577-1829N).

Results

Effects of Dietary Zn and Carbadox on Growth Performance

The diets were formulated to fulfil the swine nutritional requirement (basal Zn, 150 mg/kg diet or with pharmacological Zn dose, 2500 mg/kg diet). Both diets were fed with or without carbadox (55 mg/kg diet). For overall growth performance responses, no interaction between Zn concentration and carbadox was observed (Figure 5.1). The time effect was significant ($p < 0.05$) for body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F), but there were no interactions of Zn × carbadox × time.

After the study period, pharmacological Zn supplementation numerically increased the BW of pigs by 3% compared with basal Zn, while carbadox supplementation increased ($p < 0.01$) the BW of pigs by 8% compared with non-carbadox treatments. Pigs fed diets containing the pharmacological dose of Zn plus carbadox were 12% heavier ($p < 0.05$) than pigs fed basal Zn without carbadox (control) (Figure 5.1A). For the 43-day feeding period, we observed that pharmacological Zn supplementation and carbadox treatments increased ADG by 7% ($p < 0.01$) and 13% ($p < 0.01$), respectively, compared with basal Zn and non-carbadox treatments (Figure 5.1B). Moreover, the combination of pharmacological Zn supplementation with carbadox resulted in an increase of ADG by 20% ($p < 0.01$) compared with pigs fed the control diet. These results indicated that pharmacological Zn dose and carbadox have an additive effect on growth rate.

Theoretically, ADFI is directly related to ADG responses in weaned pigs (Collins et al., 2017). The overall results showed that pharmacological Zn supplementation tended to increase ADFI by 3% ($p = 0.06$) compared with the basal Zn, whereas carbadox addition to the diet increased ADFI by 7% ($p < 0.01$) compared with no carbadox added (Figure 5.1C). Additionally, the combination of pharmacological Zn supplementation and carbadox increased ADFI by 10% compared with feeding the control diet. For overall

gain efficiency, pharmacological Zn supplementation and carbadox treatments improved G:F by 14% ($p < 0.01$) and 9% ($p = 0.02$), respectively, compared with basal Zn and non-carbadox treatments (Figure 5.1D). Furthermore, the combination of pharmacological Zn supplementation with carbadox improved G:F by 25% compared with pigs fed the control diet ($p < 0.01$). These results suggested a potentiating effect of pharmacological Zn dose and carbadox on gain efficiency.

Effects of Dietary Zn and Carbadox on Gastric Acid Production and GI Morphology

Gastric and intestinal morphology were evaluated (Figure 5.2 and Table 5.2). There were no interactions between Zn concentration and carbadox on gastric morphology and gastric acid production (Table 5.2). Gastric morphology (Figure 5.2A,B) analysis showed that pigs fed pharmacological dose of Zn had a 14% and 11% greater ($p < 0.01$) mucosal height of the corpus and antrum, respectively, compared with those fed basal dose of Zn. In contrast, the addition of carbadox did not affect gastric mucosa. Both pharmacological Zn supplementation and carbadox treatments had no effects on the acidity of gastric contents, suggesting that the increase in the mucosa of the corpus was not related to changes in acid secretion.

The villus width and villus height of the jejunum and ileum of pigs were not affected by pharmacological Zn supplementation and carbadox treatments. However, interactions were observed for crypt depth of the jejunum and ileum, with pigs fed the carbadox diet having shallower crypts when in combination with the basal dose of Zn, but not with the pharmacological dose of Zn. More importantly, pigs fed diets containing carbadox had an 11% increase ($p < 0.01$) in villus to crypt ratio in the jejunum and a 12% increase of this ratio ($p = 0.03$) in the ileum compared with those fed diets without carbadox. The area of Peyer's patches in the ileum was not affected by dietary treatments, suggesting Zn concentration and carbadox may not directly influence the development of Peyer's patches at 7 days post-weaning.

Effects of Dietary Zn and Carbadox on Ileal Cytokines

The ileal concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, and tumor necrosis factor α (TNFα) were below the limit of detection of the cytokine panel, and thus, only the concentrations of 10 cytokines are reported (Table 5.3). For pro-inflammatory cytokines, pharmacological Zn supplementation and

carbadox treatments reduced the concentration of IL-1β compared with basal Zn supplementation ($p = 0.03$) and non-carbadox treatments ($p < 0.01$). The concentration of IL-18 in pharmacological Zn supplementation was reduced numerically by 11% ($p = 0.09$) compared with basal Zn. Furthermore, Zn concentration and carbadox resulted in an interaction effect ($p = 0.01$) on the concentration of IL-6. The addition of carbadox reduced the concentration of IL-6 in pigs fed a pharmacological dose of Zn ($p < 0.05$), but not in pigs fed a basal dose of Zn.

For anti-inflammatory cytokines, carbadox treatments tended to increase the concentration of IL-10 ($p = 0.09$) compared with non-carbadox treatments. Another interaction between Zn concentration and carbadox ($p = 0.03$) was observed in the concentration of IL-1RA. Adding carbadox reduced the concentration of IL-1RA ($p < 0.05$) in pigs fed a pharmacological dose of Zn, but the response was not observed in pigs fed basal dose of Zn. Finally, the ratio of anti- to pro-inflammatory cytokines showed that pharmacological Zn supplementation increased the IL-1RA:IL-1 ratio by 42% ($p = 0.04$) and carbadox treatments tended to increase IL-10:IL-12 ($p = 0.07$) compared with a basal dose of Zn and non-carbadox treatments.

Effects of Dietary Zn and Carbadox on Free Amino Acid (AA), Bile Acid (BA), and Fatty Acid (FA) in Ileal Digesta

Free AA, BA, and FA in ileal digesta were not affected by pharmacological Zn supplementation or carbadox (Table 5.4). However, the carbadox treatments tended to increase the concentrations of essential AA (i.e., Leu/Ile, Thr, and Trp) and palmitic acid in ileal digesta ($p = 0.09$, 0.07, 0.08, and 0.09, respectively) compared with non-carbadox treatments. Because AA, FA, and BA participate in a variety of physiological and metabolic functions, the correlation analysis can be useful for interpreting whether the changes of those metabolites may be related to animal growth performance responses or not. The correlations between metabolites of ileal digesta and ADG from 0–7 days post weaning were examined, but no significant correlations between ADG and BA and FA were observed. However, essential AA including Arg, His, Leu/Ile, Met, Thr, Trp, and Val in the ileal digesta were positively correlated with ADG (r = 0.38, 0.36, 0.40, 0.37, 0.44, 0.46, and 0.41, respectively, $p < 0.05$; Table 5.3).

Effects of Dietary Zn and Carbadox on Free AA, BA, and FA in Feces

Carbadox treatments selectively increased the concentration of some essential AA, including His ($p = 0.03$) and Lys ($p = 0.03$), as well as non-essential AA, including Ala ($p = 0.01$), Glu ($p < 0.01$), Ser ($p = 0.04$) in feces, while pharmacological Zn supplementation had no effects on fecal AA concentrations (Table 5.5). Neither pharmacological Zn supplementation nor the carbadox treatments affected the concentration of BA in feces. Similarly, pharmacological Zn supplementation and the carbadox treatments did not alter the fecal concentration of short-chain FA. However, feces from pigs fed diets containing pharmacological Zn concentrations had a greater concentration of C18:3 ($p = 0.01$) than feces from pigs fed the basal dose of Zn. Furthermore, the carbadox treatments increased fecal C14:1 ($p < 0.01$), C16:1 ($p = 0.02$), and C18:3 ($p = 0.04$) compared with the non-carbadox treatments. The correlation analysis between fecal metabolites and ADG from 0–7 days post weaning showed that Ala, Asp, Glu, Gly, C14:1 C18:1, C18:2 and C18:3 were positively correlated with ADG (r = 0.40, 0.32, 0.36, 0.34, 0.50, 0.18, 0.32 and 0.34, respectively, $p < 0.05$; Table 5.5).

Discussion

Post-weaning diarrhea is a common health issue associated with growth depression in nursery pigs, and was usually controlled by the addition of a pharmacological dose of Zn in diets, mainly in the form of ZnO (Poulsen, 1995; Hill et al., 2000; Sales, 2013). The North Central Regional-42 Committee on Swine Nutrition has reported that adding Zn up to 3000 mg/kg in swine diets improved post-weaning growth performance in a dose-dependent manner, especially for pigs older than 28 days (Hill et al., 2001). Additional growth-promoting benefits have also been observed when carbadox was added in combination with pharmacological Zn supplementation in diets for nursery pigs (Mahan et al., 2000; Hill et al., 2001). The results of the present study reconfirmed these growth performance responses when supplementing diets with ZnO (2500 mg/kg) and carbadox independently and in combination for nursery pigs, and no severe PWD and obvious illness were observed during the trial. More importantly, the influences of Zn and carbadox on GI morphology, intestinal immune status, and metabolites of digesta and feces were examined to identify their associations with growth performance, and summarized in figure 5.3.

Gastric morphology is commonly evaluated by histological examination of corpus and antral mucosa, two main regions with distinctive cell types and functions. The corpus mucosa contains oxyntic glands secreting mucus, gastric acid, ghrelin, and enzymes, while the antral mucosa glands secrete mucus and gastrin (Ghoshal and Bal, 1989; Feher, 2017). In the present study, Zn effects on the growth of gastric mucosa were observed, but the exact mechanisms behind this observation required further investigation. A similar effect of Zn on intestinal mucosal growth has been reported. Potential mechanisms induced by pharmacological Zn supplementation include increased insulin-like growth factor-1 in blood and increased ghrelin secretion in pigs post-weaning (Yin et al., 2009), which both can stimulate intestinal mucosa growth (Carlson et al., 2004; Li et al., 2006; Yin et al., 2009). In contrast to the observation on gastric mucosa, intestinal villus height was not affected by pharmacological Zn supplementation in the present study. Increased villus height effects were reported in some studies when diets containing pharmacological doses of Zn were fed to weaned pigs (Li et al., 2011; Shen et al., 2014), but not in other studies (Hedemann et al., 2006; Upadhaya et al., 2018).

In contrast to the effects of pharmacological Zn supplementation on gastric and intestinal morphology, carbadox treatments did not affect stomach mucosa, but decreased crypt depth and increased villus:crypt ratio in the small intestine. This observation is in agreement with the results from feeding AGPs to broilers (Markovicv et al., 2009). As an antimicrobial, carbadox mainly targets the gut microbiome in the host. Decreases in the richness of gut microbial taxa as well as individual species, such as *E. coli*, have been observed in pigs fed diets containing carbadox (Looft et al., 2014b). It has been reported that the gut microbiota can change intestinal morphology. For example, enterotoxigenic *E. coli* that causes PWD can significantly reduce villus:crypt ratio of weaned pigs (Gao et al., 2013). Therefore, the carbadox treatment used in the current study may have resulted in decreased crypt depth and increased intestinal villus:crypt ratio through modifications of gut bacterial communities. The intestinal stem cells reside in the crypt and produce transient amplifying cells that can differentiate into different cell lineages (Clatworthy and Subramanian, 2001). Therefore, shallow crypts with unchanged villi height, indicated by increased villus:crypt ratio in the jejunum and ileum, may indicate stable, low cell turnover that results in lower energy requirements for intestinal

cell maintenance and regeneration, leading to sparing energy and amino acids for overall growth.

Niewold (2007, 2014) suggested that the growth-promoting effects of AGPs are likely due to the inhibition of the intestinal inflammatory responses (Niewold, 2007; Niewold, 2014). The status of inflammatory responses can be evaluated effectively by the individual cytokines and the ratios among cytokines. For example, the IL-10:IL-12 ratio indicates the balance between anti-inflammatory and pro-inflammatory signals (Couper et al., 2008). Weaning is commonly associated with intestinal inflammation in piglets, as indicated by the upregulation of mRNA expression of IL-1β, IL-6, and TNF-α (Pié et al., 2004). We analyzed the concentration of anti- and pro-inflammatory cytokines in the intestine to directly address the intestinal response that cannot be discerned from serum measurements (Pei et al., 2019). In the current study, both pharmacological Zn supplementation and carbadox treatments reduced the concentration of IL-1β, which could be beneficial for animal growth because the nutritional cost of inflammation is responsible for the animal's growth potential (Klasing, 2007). Pharmacological Zn supplementation increased IL-1RA:IL-1 ratio, suggesting an ability of Zn to alleviate pro-inflammatory responses because of a negative correlation between IL-1RA:IL-1 ratio and inflammatory bowel disease (Casini-Raggi et al., 1995; Ludwiczek et al., 2004). Carbadox tended to increase the IL-10:IL-12 ratio, which suggests the ability of carbadox to reduce Th1 cytokines (pro-inflammatory cytokines) (Couper et al., 2008). Overall, these results suggest that pharmacological Zn supplementation and carbadox alleviate host intestinal immune responses by reducing pro-inflammatory cytokines and stabilizing the anti- to pro-inflammatory cytokines ratio, which may contribute to less metabolic cost for immune responses and result in more energy and nutrients being available for growth.

Amino acids, FA, and BA are the major functional metabolites in the GI tract because they serve as the sources of energy and nutrients for growth and also regulate inflammation and signaling. Our analysis focused on metabolites instead of microbiome composition in the GI tract and addressed functional changes induced by pharmacological Zn and carbadox. The correlation analysis in the current study confirmed the relevance of their concentration, especially AA concentrations, in ileal digesta and feces to growth performance. Interestingly, essential AA in ileal digesta,

including Leu/Ile, Val, Trp, Thr, Arg, and His, had greater correlations with ADG than other AA. In contrast, in feces, non-essential AA, including Ala, Gly, Glu, and Asp, were better correlated with ADG than essential AA. The correlation of essential AA in ileal digesta with growth is not unexpected based on their nutritional value, but the correlation of nonessential AA in feces with growth is not a well-established fact and requires further investigation. Nevertheless, the causes of correlation between fecal AA and growth should be different from correlations between ileal AA and growth responses in considerations of the distinct physiological role and composition of microbiota.

Analyzing the influence of Zn concentration and carbadox on these metabolites in ileal digesta and feces revealed two prominent features: (1) carbadox treatments caused far more metabolic changes than pharmacological Zn supplementation, and (2) many metabolic changes from the carbadox treatments were only observed in feces, but not in ileal digesta. Because the large intestine is the physiological connection between the ileal digesta and feces, these features clearly suggest that anti-microbial activity of carbadox in the large intestine may be responsible for the changes in fecal metabolites.

Microbial protein constitutes a large proportion of fecal protein in piglets (Laplace et al., 1985). The increases of certain free AA in fecal samples from pigs fed carbadox in the current study should be not interpreted as a reduction in protein digestibility. Instead, it should be considered a consequence of increased microbial fermentation of proteins or increased microbial synthesis of AA in the large intestine, considering that the protein concentrations were similar between experimental diets within each phase. This phenomenon has been observed in mouse studies, in which fecal Val, Leu, Ile, and Phe levels were increased by feeding diets containing ciprofloxacin, while Arg and Lys were increased by feeding vancomycin-imipenem compared with no antibiotic treatment (Choo et al., 2017). The exact mechanisms for these selective effects of carbadox on fecal AA remain to be determined. However, the microbial synthesis of essential AA in pigs, including Lys, Phe, Leu, Ile, and Val, has been reported previously (Torrallardona et al., 2003), and the increased expression of the microbial genes encoding aromatic AA has also been observed in the pigs fed antibiotic cocktails (Looft et al., 2014a). It is important to mention that the colonocytes may have potential to absorb a small amount of AA due to the presence of AA transporters (van der Wielen et al., 2017).

Therefore, all of these activities can increase nutrient and energy utilization in nursery pigs and provide the substrates for producing other functional metabolites, including AA, short-chain FAs, organic acids, phenolic and indole compounds (Dai et al., 2011).

Short-chain FAs and secondary BAs are two groups of microbial metabolites commonly examined in antibiotic studies. Selective effects of antibiotic treatments on short-chain FAs and secondary BAs have been observed in previous studies. For example, Looft et al. (2014b) found that feeding carbadox increased the relative abundance of *Prevotella*, a genus of bacteria involved in producing short-chain FAs, in the microbiome of weaned pigs, while Trudeau et al. (2018) reported that feeding with tylosin did not affect short-chain FAs, but selectively increased hyodeoxycholic acid, a secondary BA in feces. In the current study, neither short-chain FAs nor BAs were affected by pharmacological Zn supplementation and carbadox treatments. The inverse correlation between ADG and ileal lithocholic acid (LCA), a secondary BA, was observed. LCA exposure has been shown to impede Th1 cell activation *in vitro* (Pols et al., 2017). However, this correlation might not be able to explain the observed changes in cytokines because LCA is a very minor secondary BA in ileal digesta in comparison to other BAs. Interestingly, selective medium-chain and long-chain FAs were affected by the treatments. Pharmacological Zn supplementation increased pentadecanoic acid (C15:0) and α-linolenic acid (C18:3), while carbadox addition increased caproic acid (C6:0), myristoleic acid (C14:1), palmitoleic acid (C16:1), and α-linolenic acid (C18:3) in feces. These metabolic changes can likely be attributed to microbial metabolism, but the exact mechanisms remain to be determined.

Overall, the independent and additive effects of pharmacological Zn supplementation and carbadox on growth performance of weaned pigs provided proof of concept that their mode of action on growth promotion occur likely through eliciting different responses. Our findings demonstrated that pharmacological Zn and carbadox effects on morphology, immune, and metabolic status of the GI tract were different. Pharmacological Zn supplementation affected gastric mucosa while carbadox addition affected intestinal mucosa and modified the presence of targeted metabolites, and both influenced intestinal immune status (Figure 5.3). These results further confirmed the concept that beneficial effects of growth-promoting agents are multifactorial, including

GI morphology, immune response, and microbial metabolism, suggesting that prospective alternatives for AGPs and a pharmacological dose of Zn should manifest similar effects on these parameters.

Tables and Figures

Table 5.1. Ingredient and nutrient composition of experimental diets (as-fed basis)

Phase	Phase 1 (Weaning – day 7)				Phase 2 (day 7 – 21)				Phase 3 (day 21 – 42)	
Zn	150		2500		150		2500		150	
Carbadox	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Ingredient composition, g/kg										
Corn	318	298.4	311.5	292	386.7	367.1	380.3	360.7	426.8	411.5
Wheat	200	200	200	200	200	200	200	200	200	200
Wheat middlings	.	.	.	.	.	.	.	.	75	75
Soybean meal	150	150	150	150	200.0	200	200	200	250	250
Calcium carbonate	7.4	7.4	7.4	7.4	7.6	7.6	7.6	7.6	9.2	9.2
Monocalcium phosphate	11.5	11.5	11.5	11.5	12.9	12.9	12.9	13	12.3	12.3
Soybean protein concentrate	73.3	74.7	73.7	75.1	54	55.4	54.5	55.8	.	.
Poultry fat blend	24.8	32.9	27.4	35.6	19.2	27.4	21.9	30	14.3	19.4
Trp 100%	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	.	.
DL-Met	2.6	2.6	2.6	2.6	2.7	2.8	2.7	2.8	1.1	1.2
L-Lys HCl	5.1	5	5.1	5	5.8	5.8	5.8	5.8	3.2	3.2
Thr	2.3	2.3	2.3	2.3	2.7	2.7	2.7	2.7	1.2	1.3
L-Val	0.7	0.7	0.7	0.7	1.1	1.2	1.2	1.2	.	.
Plasma - Dried	30	30	30	30		.	.	.	.	.
Salt	2.3	2.2	2.3	2.2	5.6	5.6	5.6	5.6	4.6	4.5
Whey permeate	169.6	169.6	169.6	169.6	98.9	98.9	98.9	98.9	.	.
Vitamin/Mineral1 Premix	2.6	2.6	2.6	2.6	2.4	2.4	2.4	2.4	2.4	2.4
ZnO	0	0	3.3	3.3	0	0	3.3	3.3	.	.
Mecadox[2]	0	10	0	10	0	10	0	10	0	10
Calculated composition, g/kg										
CP	202.6	202.2	202.5	202	194.1	193.7	194	193.6	189.4	188.4
Fat	40.6	48	43	50.4	37.3	44.7	39.7	47.1	35.4	40
Ash	61.5	61.4	64.7	64.6	59.6	59.5	62.9	62.7	54.1	53.9
NDF	62.4	61.1	62	60.7	68.3	67	67.8	66.6	89.3	88.2
Moisture	93.3	90.8	92.5	90	103.3	100.8	102.5	100	118.5	116.5
Analyzed composition, g/kg										
CP	189.3	207.2	199.1	208.9	177.9	190.1	187.7	197.2	179.2	178.5
Ether extract	41.8	44	48.4	49.4	35.7	39.2	46.5	46.4	35.8	38.6
Ash	50.2	53.5	50.4	52.2	43	44.1	45.8	44.1	44.4	43.2
NDF	78.7	83.6	84	85.6	87.9	89.9	90.8	91.5	104.2	105.3
Moisture	105.5	102	103.1	101.8	113.9	113.5	108.9	108.3	122.6	120.8

[1]Premix supplied the following nutrients per kilogram of diet: 60 IU Vitamin E Equivalent, 10,000 IU Vitamin A, 1,600 Vitamin D3 Equivalent, 1.5 mg Thiamine, 8 mg Riboflavin, 50 mg Niacin, 60 mg Pantothenic Acid, 2 mg Pyridoxine, 1.1 mg Folic Acid, 35 µg Vitamin B12, 3.5 mg Menadione, 75 µg Ethoxyquin, 18 mg Copper, 110 mg Iron, 0.7 mg Iodine, 50 mg Manganese, 110 mg Zinc, 0.3 mg Selenium

[2]Provided 5500 mg/kg carbadox, Phibro Animal Health Co., Fairfield, NJ.

Table 5.2. Gastric acid production and gastrointestinal (GI) morphology of post-weaning pigs fed diets containing a pharmacological dose of Zn and carbadox

Zn	150		2500		SE[1]	*P*-values		
Carbadox	No	Yes	No	Yes		Zn	AB[2]	Zn × AB[3]
Stomach								
Mucosal height of corpus, µm	671.5	606.7	736.3	723.2	27.52	<0.01	0.13	0.33
Mucosal height of antrum, µm	496.8	489.6	561.8	534.5	15.11	<0.01	0.25	0.50
Gastric acid, mEq/mL	0.065	0.054	0.058	0.052	0.006	0.49	0.19	0.71
Jejunum								
Villus width, µm	93.4	85.0	90.7	93.0	5.35	0.56	0.50	0.25
Villus height, µm	535.6	500.8	551.6	572.3	23.74	0.07	0.77	0.25
Crypt depth, µm	143.1	113.9	139.3	137.7	5.63	0.08	0.01	0.02
Villus:crypt ratio	3.74[a]	4.40[b]	3.99[ab]	4.17[ab]	0.09	0.94	<0.01	0.06
Ileum								
Villus width, µm	71.1	69.3	68.2	73.5	2.7	0.81	0.51	0.19
Villus height, µm	280.4	286.9	287.8	308.6	16.3	0.38	0.41	0.67
Crypt depth, µm	111.0[b]	90.1[a]	98.2[ab]	99.4[ab]	5.4	0.75	0.07	0.05
Villus:Crypt ratio	2.64	3.22	2.93	3.10	0.17	0.61	0.03	0.22
Peyer's patch, µm[2]	163,673	169,555	171,699	163341	9816	0.93	0.90	0.47

[a,b,c] Values in the same row with different superscript differ ($p < 0.05$); [1] SE = pooled standard error of means, n = 10 pigs/treatment; [2] AB = Carbadox; [3] Interaction effect between Zn concentration and Carbadox.

Table 5.3. Concentration of ileal cytokines of pigs fed diets containing a pharmacological dose of Zn and Carbadox[1]

Zn	150		2500		SE[2]	*P*-values		
Carbadox	No	Yes	No	Yes		Zn	AB[3]	Zn × AB[4]
IFNγ	96.70	85.24	91.06	84.39	10.04	0.75	0.38	0.81
IL-1α	1.19	1.18	1.16	0.90	0.19	0.38	0.43	0.46
IL-1β	85.27	44.33	51.78	34.48	9.75	0.03	< 0.01	0.23
IL-1RA	13.21[ab]	12.89[ab]	18.1[b]	11.24[a]	1.48	0.28	0.02	0.03
IL-1RA:IL-1	0.20	0.32	0.39	0.35	0.05	0.04	0.41	0.13
IL-4	19.74	15.07	15.39	8.48	7.05	0.46	0.43	0.88
IL-6	1.27[a]	2.29[ab]	3.32[b]	1.34[a]	0.68	0.28	0.33	0.01
IL-8	270.47	234.71	224.13	170.08	49.36	0.12	0.20	0.80
IL-10	0.36	0.54	0.31	0.58	0.12	0.98	0.09	0.69
IL-12	2.67	2.36	2.89	2.12	0.56	0.98	0.34	0.68
IL-10:IL-12	0.14	0.26	0.15	0.23	0.05	0.83	0.07	0.67
IL-18	931.05	889.50	856.91	765.01	57.45	0.09	0.25	0.66

[a,b,c] Values in the same row with different superscript differ ($p < 0.05$); [1] Units are pg/mg total protein for all cytokines except for IL-4, which is fg/mg total protein; [2] SE = pooled standard error of means, n = 10 pigs/treatment; [3] AB = Carbadox; [4] Interaction effect between Zn concentration and Carbadox.

Table 5.4. Effect of Zn concentration and carbadox on amino acid (AA), fatty acid (FA), and bile acid (BA) in ileal digesta

Zn	150		2500		SE[1]	*P*-values			ADG (r Value)[4]
Carbadox	No	Yes	No	Yes		Zn	AB[2]	Zn × AB[3]	
Amino acids, mg/g									
Alanine	0.257	0.252	0.221	0.270	0.030	0.78	0.51	0.41	0.33
Arginine	0.321	0.282	0.290	0.595	0.108	0.20	0.23	0.12	0.38
Asparagine	0.035	0.081	0.070	0.080	0.027	0.53	0.28	0.49	0.32
Aspartic acid	0.131	0.169	0.149	0.220	0.034	0.31	0.11	0.64	-
Glutamic acid	1.011	1.087	0.965	1.112	0.174	0.95	0.53	0.84	-
Glutamine	0.123	0.142	0.113	0.184	0.035	0.64	0.20	0.45	0.33
Glycine	0.211	0.255	0.191	0.204	0.035	0.31	0.42	0.66	-
Histidine	0.070	0.073	0.064	0.093	0.015	0.63	0.29	0.38	0.36
Leucine/Isoleucine	0.195	0.216	0.152	0.265	0.039	0.94	0.09	0.25	0.40
Lysine	0.729	0.612	0.687	0.818	0.132	0.54	0.96	0.35	-
Methionine	0.021	0.028	0.016	0.033	0.009	0.94	0.19	0.57	0.37
Phenylalanine	0.259	0.257	0.192	0.289	0.038	0.64	0.22	0.20	-
Proline	0.201	0.163	0.157	0.199	0.024	0.87	0.92	0.11	0.30
Serine	0.086	0.128	0.113	0.163	0.036	0.39	0.21	0.91	0.38
Threonine	0.073	0.103	0.075	0.133	0.023	0.45	0.07	0.55	0.44
Tryptophan	0.043	0.063	0.047	0.095	0.019	0.35	0.08	0.46	0.46
Tyrosine	0.304	0.294	0.236	0.391	0.069	0.84	0.30	0.24	0.35
Valine	0.174	0.192	0.141	0.241	0.035	0.83	0.11	0.26	0.41
Fatty acids, mg/g									
Acetic acid	0.376	0.159	0.148	0.221	0.069	0.20	0.27	0.03	-
Propionic acid	0.115	0.008	0.002	0.001	0.054	0.35	0.32	0.34	-
Butyric acid	0.638	0.018	0.010	0.013	0.311	0.32	0.33	0.33	-
C6:0	0.130	0.093	0.098	0.085	0.020	0.32	0.22	0.53	-
C8:0	0.008	0.007	0.009	0.008	0.001	0.35	0.45	0.71	-
C10:0	0.002	0.002	0.009	0.010	0.002	0.24	0.73	0.73	-
C12:0	0.060	0.061	0.049	0.029	0.027	0.42	0.73	0.70	-
C14:0	0.035	0.061	0.024	0.035	0.015	0.25	0.23	0.65	-
C14:1	0.009	0.005	0.004	0.006	0.003	0.57	0.78	0.30	-
C15:0	0.009	0.016	0.008	0.009	0.003	0.24	0.25	0.41	-
C16:0	1.944	2.371	2.214	2.419	0.186	0.38	0.09	0.55	-
C16:1	1.044	0.895	0.498	0.825	0.309	0.33	0.78	0.45	-
C17:0	0.019	0.042	0.024	0.023	0.011	0.52	0.34	0.30	-
C17:1	0.089	0.075	0.035	0.065	0.025	0.21	0.75	0.39	-
C18:0	4.162	3.314	3.999	3.425	0.423	0.95	0.10	0.75	-
C18:1	7.775	8.369	7.792	9.257	2.250	0.83	0.62	0.84	-
C18:2	6.029	5.992	5.467	6.364	1.674	0.95	0.79	0.77	-

C18:3	0.830	0.890	0.770	0.870	0.310	0.90	0.79	0.96	-
Bile acids, µg/g									
LCA	0.092	1.001	0.166	0.067	0.397	0.27	0.30	0.20	−0.32
CDCA	7.67	16.47	14.50	2.85	6.980	0.63	0.84	0.15	-
CA	0.144	4.615	0.404	0.202	1.513	0.17	0.15	0.12	-
GDCA	0.047	3.809	0.088	0.309	1.835	0.35	0.29	0.34	-
TCDCA	4.13	11.05	5.72	2.78	4.278	0.44	0.64	0.26	-
DCA	1.33	1.57	2.45	1.21	0.804	0.64	0.54	0.36	-
GCA	0.242	10.031	0.584	0.514	3.038	0.14	0.12	0.11	-
GCDCA	14.31	26.85	13.87	17.42	12.86	0.70	0.54	0.73	-
TDCA	1.991	1.202	1.830	2.496	0.954	0.56	0.95	0.45	-
TCA	1.499	3.347	0.500	0.210	1.597	0.20	0.63	0.50	-
HDCA	25.24	6.33	38.67	22.72	14.84	0.25	0.17	0.91	-

[1]SE = pooled standard error of means, n = 10 pigs/treatment; [2] AB = Carbadox; [3] Interaction effect between Zn concentration and Carbadox; [4] Pearson correlation coefficient between metabolite and ADG (day 0 – 7). The reported number mean $p < 0.05$.

Table 5.5. Effect of Zn concentration and carbadox on AA, FA, and BA in feces

Zn	150		2500		SE [1]	*p*-values			ADG (r Value)[4]
Carbadox	No	Yes	No	Yes		Zn	AB[2]	Zn × AB[3]	
Amino acids, mg/g									
Alanine	0.056	0.081	0.063	0.069	0.007	0.77	0.01	0.13	0.40
Arginine	0.006	0.010	0.011	0.008	0.002	0.60	0.69	0.10	-
Asparagine	0.001	0.002	0.001	0.001	0.001	0.60	0.38	0.32	-
Aspartic acid	0.050	0.054	0.052	0.059	0.006	0.55	0.31	0.79	0.32
Glutamic acid	0.357	0.493	0.387	0.490	0.046	0.73	<0.01	0.68	0.36
Glutamine	0.007	0.009	0.007	0.009	0.002	0.94	0.20	0.90	-
Glycine	0.048	0.076	0.049	0.055	0.009	0.29	0.07	0.22	0.34
Histidine	0.011	0.047	0.010	0.013	0.009	0.05	0.03	0.07	-
Leucine/Isoleucine	0.148	0.179	0.148	0.172	0.017	0.83	0.10	0.85	-
Lysine	0.315	0.426	0.351	0.386	0.033	0.94	0.03	0.25	-
Methionine	0.026	0.030	0.025	0.029	0.003	0.85	0.21	0.94	-
Phenylalanine	0.128	0.156	0.121	0.132	0.020	0.42	0.32	0.68	-
Proline	0.071	0.073	0.083	0.074	0.009	0.47	0.71	0.58	-
Serine	0.050	0.060	0.052	0.059	0.005	0.88	0.04	0.72	-
Threonine	0.028	0.034	0.029	0.035	0.003	0.85	0.07	0.93	-
Tryptophan	0.022	0.018	0.014	0.016	0.005	0.28	0.77	0.55	-
Tyrosine	0.175	0.178	0.158	0.180	0.024	0.76	0.61	0.70	-
Valine	0.089	0.109	0.090	0.104	0.009	0.83	0.06	0.77	-
Fatty acids, mg/g									
Acetic acid	3.549	3.310	3.593	3.721	0.270	0.39	0.85	0.49	0.31
Propionic acid	2.020	2.028	2.286	2.399	0.250	0.14	0.78	0.81	-
Butyric acid	4.482	3.856	4.225	4.844	0.474	0.44	0.99	0.19	-
C6:0	0.753	0.828	0.631	1.313	0.163	0.22	0.01	0.04	-
C8:0	0.016	0.016	0.015	0.017	0.001	0.91	0.45	0.42	-
C10:0	0.009	0.011	0.011	0.013	0.001	0.11	0.14	0.76	-
C12:0	0.214	0.182	0.197	0.164	0.023	0.43	0.15	0.97	-
C14:0	0.350	0.351	0.443	0.442	0.049	0.06	1.00	0.98	-
C14:1	0.020	0.031	0.023	0.035	0.005	0.30	< 0.01	0.89	0.50
C15:0	2.149	1.967	2.931	2.724	0.326	0.02	0.55	0.97	-
C16:0	2.931	2.592	3.317	2.975	0.205	0.06	0.10	0.99	-
C16:1	1.705	2.107	1.536	2.065	0.216	0.60	0.02	0.75	-
C17:0	1.430	0.838	2.000	1.591	0.341	0.05	0.14	0.79	-
C17:1	2.465	3.213	3.370	3.805	0.876	0.35	0.46	0.84	-
C18:0	4.603	3.862	4.658	4.034	0.347	0.74	0.05	0.86	−0.23
C18:1	15.629	17.527	17.140	19.417	1.396	0.20	0.11	0.89	0.18
C18:2	5.462	5.976	6.034	7.150	0.567	0.10	0.13	0.57	0.32
C18:3	0.756	0.907	0.982	1.248	0.114	0.01	0.04	0.59	0.34

Bile acids, µg/g									
LCA	185.07	182.63	160.94	233.36	25.754	0.61	0.18	0.15	-
CDCA	8.310	11.720	12.719	10.573	3.952	0.68	0.87	0.48	-
CA	4.070	6.159	7.064	4.808	1.049	0.44	0.94	0.04	-
GDCA	2.327	2.720	2.962	3.199	0.398	0.16	0.42	0.84	-
TCDCA	1.152	1.754	1.207	1.534	0.331	0.80	0.16	0.68	-
DCA	6.933	10.936	10.364	8.194	2.903	0.91	0.75	0.29	-
GCA	0.516	0.651	0.588	0.516	0.100	0.72	0.72	0.24	-
GCDCA	0.781	0.972	0.965	1.014	0.138	0.39	0.36	0.59	-
TDCA	0.308	0.424	0.288	0.719	0.196	0.48	0.16	0.42	-
TCA	0.048	0.080	0.078	0.069	0.019	0.61	0.54	0.26	-
HDCA	129.23	120.74	140.32	131.94	17.58	0.53	0.63	1.00	-

[1]SE = pooled standard error of means, n = 44 pigs/treatment; [2] AB = Carbadox; [3] Interaction effect between Zn concentration and Carbadox; [4] Pearson correlation coefficient between metabolite and ADG (day 0–7). The reported number mean $p < 0.05$.

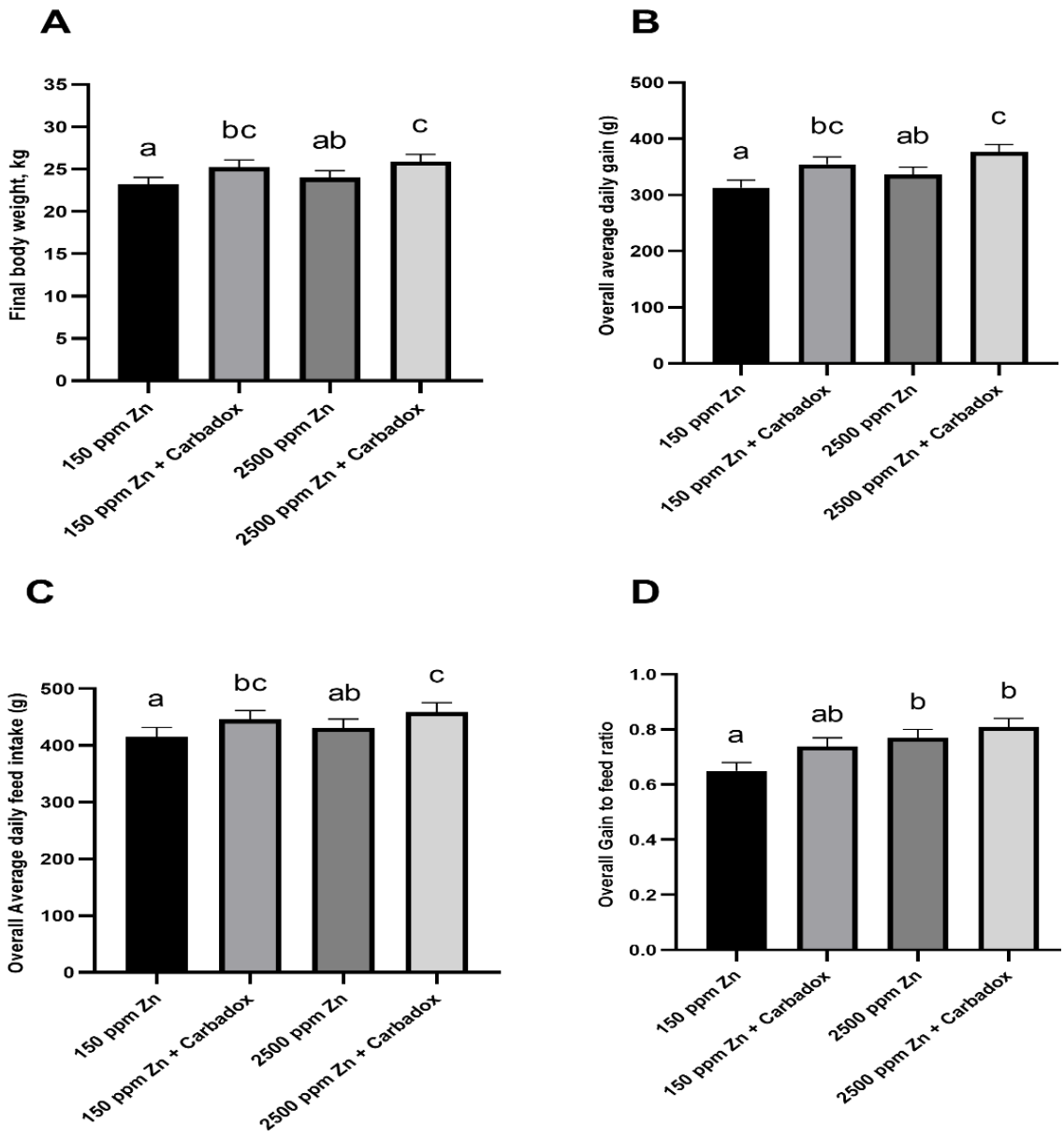

Figure 5.1. Final body weight (A), overall average daily gain (B), feed intake (C), and gain to feed ratio (D) of weaned pigs fed diets containing a pharmacological dose of Zn (2500 ppm) and carbadox. Different letters indicate significant differences ($p < 0.05$). Bars represent least squares means ± standard error of the mean.

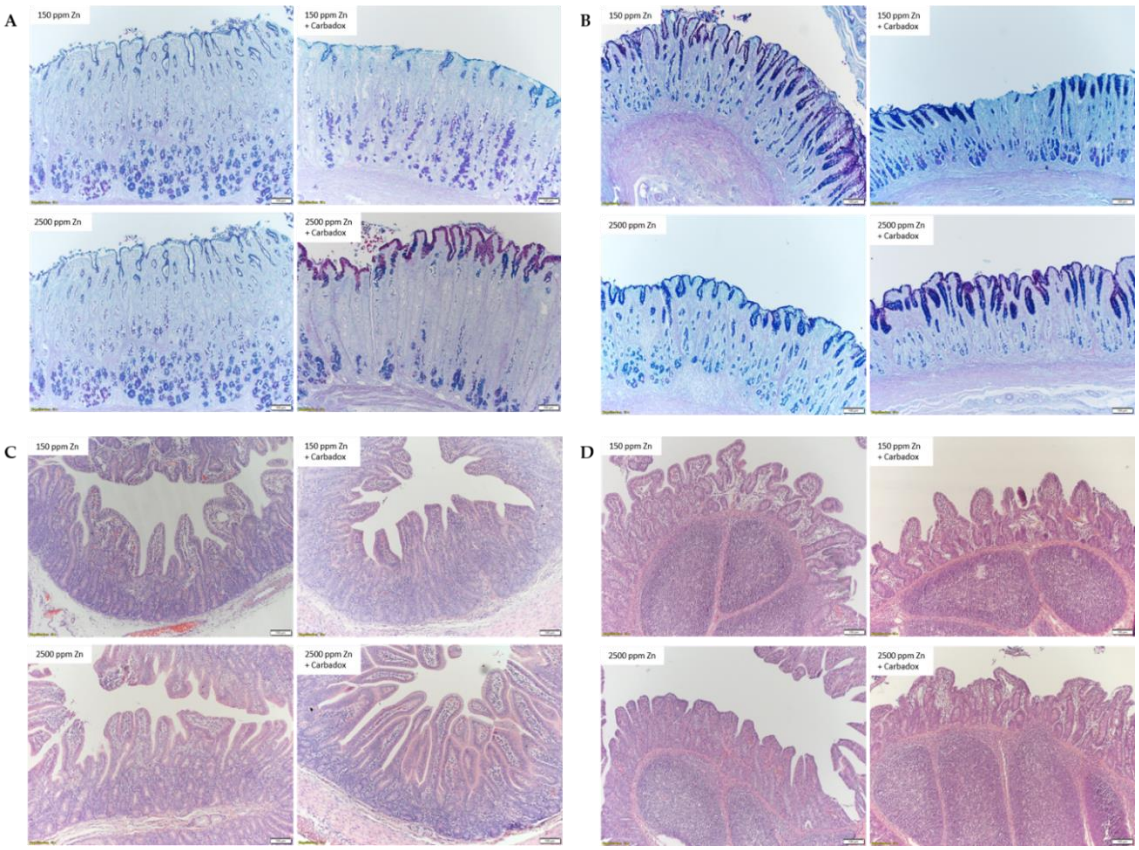

Figure 5.2. Representative images of gastric corpus (A) and antrum (B) stained with Periodic acid–Schiff/Alcian blue and jejunum (C) and ileum (D) stained with hematoxylin and eosin (H&E). Scale bar: 100 µm.

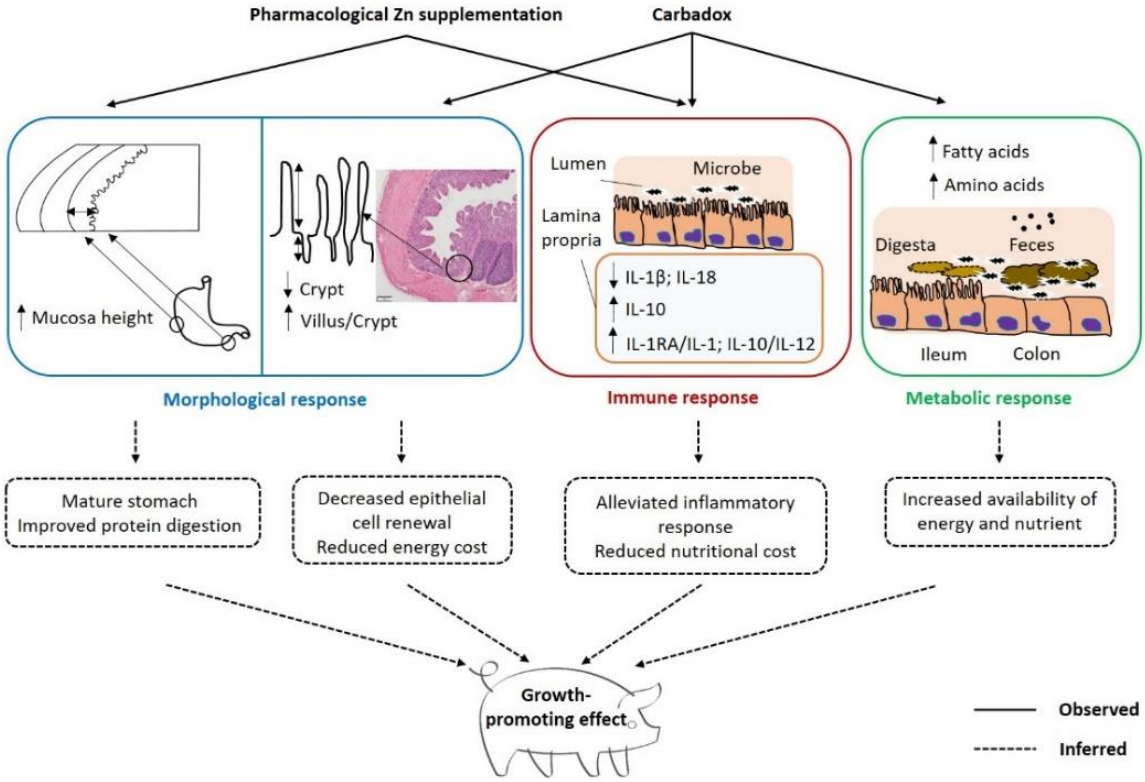

Figure 5.3. Summary the morphological, immune and metabolic responses of pharmacological Zn supplementation and carbadox to post-weaning pigs.

Chapter 6 – Overall summary and implications

Global meat consumption continues to increase and increased demand for pork is expected. Expanding swine production using conventional, resource-intensive farming (i.e., highly dependent on human-edible ingredients and antimicrobial growth promoters (AGPs)) to increase production goes against One Health principles. Under the theme of sustainable pig farming, growth performance and sustainability are equally important. Swine nutrition research that aims at increasing the efficiency of dietary energy and nutrient utilization of pigs fed non-human edible ingredients can increase production efficiency without AGPs, which creates a positive impact on One Health. A systems approach was applied in this dissertation to integrate various dietary interventions with growth, physiological, and metabolic response of pigs to provide knowledge necessary for improving caloric and nutritional efficiency in sustainable pork production.

Adding antioxidants is one method to preserve quality and feeding value of lipids. In Chapter 2, a study was designed to evaluate growth performance and systemic oxidative status of nursery pigs fed 6% unoxidized or oxidized distillers corn oil as an alternative energy source, with or without supplementation of 60 ppm tert-butylhydroquinone (TBHQ). The addition of TBHQ to distillers corn oil partially protected the oils against further peroxidation but did not improve animal growth performance or affect oxidative status of nursery pigs. This study provided information about the effectiveness of using commercial antioxidants in reducing lipid peroxidation in distillers corn oil.

In Chapter 3, a meta-analysis was conducted to evaluate the effects of feeding peroxidized lipids on growth performance and oxidative status in published studies involving 42 poultry and 23 swine comparisons. This study showed that feeding peroxidized lipids has a detrimental effect on growth performance and oxidative status of poultry and swine. Additionally, increasing dietary peroxide value (PV) was negatively associated with decreases in ADG in poultry, and increasing dietary thiobarbituric acid reactive substances (TBARS) was negatively correlated with reduced ADG in swine, but the prediction equation generated by PV or TBARS are not robust enough for nutritionists to estimate growth reduction. Results from this study highlight that the magnitude of growth reduction of animals fed peroxidized lipids differs among studies

because of the use of various lipid sources with different FA profiles, oxidative conditions for diet preparation, and measurements for determining lipid peroxidation. Finally, lipid peroxidation has strong implications for lipid quality, caloric efficiency, growth performance and overall health of animals. Therefore, when adding lipids to diets to improve gain/feed efficiency of animals, the extent of lipid peroxidation must be considered.

In Chapter 4, a study was conducted to evaluate whether DF content and viscosity of DF affect nutrient utilization and intestinal function of growing pigs independently or interactively. We found that increasing DF content and viscosity independently decreased nutrient digestibility of diets. Dietary treatments of increasing DF viscosity induced a series of changes in the intestine involving morphology, goblet cell area, and digestive enzyme activities. Results of this study showed that decreased nutrient digestibility and intestinal changes due to diets with increased viscosity were independent of the amount of DF fed. These results underscore the importance of considering viscosity of feedstuffs, especially in development of precision diet formulation to optimize caloric and nutritional efficiency of the diet. Additionally, data on decreased digestibility due to DF content and viscosity can be applied to further improve modelling digestion kinetics to better estimate energy and nutrient utilization of pigs fed fibrous ingredients, which could improve the use of alternative feedstuffs in swine production.

In Chapter 5, we evaluated the potential mechanisms of the growth-promoting effect of carbadox and pharmacological Zn supplementation by studying the physiological and metabolic responses in the GI tract of weaned pigs. We observed that pharmacological Zn supplementation and carbadox induced morphological changes in stomach and small intestine, respectively. Dietary pharmacological Zn supplementation and carbadox resulted in a shared effect on alleviating pro-inflammatory responses. Carbadox supplementation selectively changed AA and FA concentrations in digesta and feces. We suggested that our results provide evidence that the growth-promoting effect of carbadox and pharmacological Zn supplementation is likely multifactorial and additive due to their shared and separate effects. Considering the regulatory pressure to remove AGPs from animal diets, the mechanistic responses in the GI tract observed in this study

may provide insights for the development or identification of AGP alternatives that mimic growth-promoting effects of AGPs.

In summary, using integrated animal systems analysis is a cutting-edge approach to improve the nutritional efficiency of alternative feed ingredients, eliminate AGPs in diets, and minimize the environmental impact of pig farming, thereby developing sustainable feeding programs. Improving efficiency of sustainable feeding requires information about what factors limit energy and nutrient utilization of currently used inexpensive alternative feedstuffs for pigs. This dissertation integrates nutrition information with physiological responses to demonstrate how various dietary factors influencing nutrient utilization and growth in pigs. Based on what we discovered, we encourage nutritionists:

1) thinking beyond energy value of supplemental lipids and being aware the negative outcomes of using peroxidized lipids, which can decrease nutritional value of dietary lipids, induce oxidative stress, and impair caloric and growth efficiency of pigs

2) measuring viscosity of DF in addition to DF quantity in alternative feed ingredients when applicable, which may account for reduced nutrient availability to achieve precision diet formulation

3) focusing on biological responses (i.e., tissue morphology, immunomodulation, and metabolism in the GI tract) and functional benefits of feeding AGPs (i.e., caloric and nutritional efficiency) when evaluating AGP alternatives.

The approach and findings of this dissertation contribute to improving caloric and nutritional efficiency of pork production, showing a commitment of swine nutrition research to help improve food security while protecting our ecosystem.

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