The Effect of Phytonutrient Supplementation on Pig Growth

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Abstract

As the global population continues to increase, the demand for livestock production also rises. This has led to growing interest in efficient approaches to enhance animal growth and development. Phytonutrients are considered a promising alternative to synthetic compounds to improve animal growth performance. The objective of the study was to examine the effects of varying doses of phytonutrient supplementation on pig growth and metabolism. Thirty-two crossbred pigs (approximately 2 months of age, 17.7 kg± 0.82 kg) were randomly assigned to one of four phytonutrient groups (Control: 0 ppm; 1: 62.5 ppm; 2: 125 ppm; 3: 250 ppm) fed ad libitum for 28 days. Body weight was recorded on days -5, -1, 0, 7, 14, 21, and 28. Feed intake was recorded daily, and body composition measured by dual x-ray absorptiometry (DEXA) was obtained on days -1, 14, and 28. Pigs increased ($P<0.0001$) in body weight, irrespective of treatment ($P\leq0.0535$). Control pigs had greater average daily gain (ADG) when compared with other diets ($P<0.001$). Treatment 3 had the highest ($P<0.05$) feed:gain when compared with other treatments. Lean percentage of body weight decreased ($P<0.0001$) while fat mass increased ($P<0.0001$) over the duration of the study. Overall, supplementation of this phytonutrient blend did not show significant improvement to the growth of the pigs.

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General Audience Abstract

As the global population grows, there is increasing pressure to enhance livestock production efficiently. One potential solution is using phytonutrients, natural compounds found in plants, as an alternative to synthetic additives to enhance animal growth. This study explored how different doses of a phytonutrient supplement affects the growth and metabolism of growing pigs. Thirty-two crossbred pigs, approximately two months of age, were assigned to one of four treatment groups (Control: 0 ppm; 1: 62.5 ppm; 2: 125 ppm; 3: 250 ppm) over a 28-day period. Body weight, food intake, and body composition were all measured at various points throughout the study. While pigs did gain weight overall, there was no significant benefit from the phytonutrient supplementation in terms of growth improvement. Notably, pigs on the highest dose had the highest feed to gain ratio (F:G). Additionally, pigs receiving no additional supplement had the highest average daily gain in weight. Overall, phytonutrient supplementation did not lead to noticeable gains in growth performance. The outcome of this study highlights the need for further investigation into alternative growth enhancers in livestock production.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

1,3-BPG = 1,3-bisphosphoglycerate

ADG = Average Daily Gain

AST = Adenosine Triphosphate

ATGL = Adipose Triacylglycerol Lipase

ATP = Adenosine Triphosphate

BIC = Bayesian Information Criterion

BW = Body Weight

CAC = Citric Acid Cycle

cAMP = Cyclic Adenosine Monophosphate

CCO = Cytochrome C Oxidase

CPTI = Carnitine Palmitoyl Transferase 1

CPTII = Carnitine Palmitoyl Transferase 2

CS = Citrate Synthase

DEXA = Dual-Energy X-ray Absorptiometry

DHAP = Dihydroxyacetone Phosphate

ETC = Electron Transport Chain

F-6P = Fructose 6-Phosphate

F:G = Feed to Gain Ratio

FADH = Flavin Adenine Dinucleotide

FDA = Food and Drug Administration

FI = Feed Intake

G6P = Glucose-6-Phosphate

GAP = Glyceraldehyde 3-Phosphate

GDP = Gross Domestic Product

GGT = Gamma-Glutamyl Transferase

GLUT = Glucose Transporter

HSL = Hormone Sensitive Lipase

IACUC = Institutional Animal Care and Use Committee

LAP = Large Animal Profile

LD = Longissimus Dorsi

NADH = Nicotinamide Adenine Dinucleotide

NRC = National Research Council

PDC = Pyruvate Dehydrogenase Complex

PDK = Pyruvate Dehydrogenase Kinase

PEP = Phosphoenolpyruvate

PEPT1 = Peptide Transporter 1

PFK = Phosphofructokinase

PPARα = Peroxisome Proliferator-Activated Receptor α

PPARγ = Peroxisome Proliferator-Activated Receptor γ

SEM = Standard Error Mean

SGLT1 = Sodium-Glucose Transporter 1

Chapter 1

Literature Review

Introduction

The livestock industry is continuously striving to meet the rising global demand for meat production (Henchion et al., 2021; van der Laan et al., 2024). Traditional approaches to enhancing growth in livestock species have included the use of synthetic feed additives (Gonzalez Ronquillo & Angeles Hernandez, 2017). However, consumer preference is increasingly leaning towards more natural and sustainable methods (Lim et al., 2023). This shift has prompted increased research into alternative solutions. Among the strategies currently implemented and those being explored, phytonutrient supplementation has become a promising alternative (Windisch et al., 2008).

Phytonutrients are naturally occurring compounds found in plants that have demonstrated beneficial effects on animal growth and health, serving as a potential alternative to synthetic growth promoters (Kan et al., 2022; Windisch et al., 2008). By leveraging the bioactive properties of phytonutrients, it is possible to achieve improvements in meat production, while aligning with consumer preferences for natural products.

This literature review aims to first explore the current global trends in consumer demand of meat production. To best understand how to enhance or modify growth performance, it is important to examine the underlying metabolic processes, such as carbohydrate, protein, and lipid metabolism. Understanding these dynamics is critical for developing an effective and sustainable feeding method that enhances growth performance while maintaining health integrity from both consumer and animal perspectives.

Livestock Production

Meat consumption and demand has been on a rise, globally, for the past few decades (Daniel et al., 2011; Whitton et al., 2021). There is evidence to suggest that these trends will continue as average gross domestic product (GDP) per capita rises (Whitton et al., 2021). There are relationships between income and per capita meat consumptions that have found increases in meat consumption of low- and middle-income countries, but stagnating or declining trends in rich countries (Parlasca & Qaim, 2022). The decrease or stagnating trends of richer countries can be derived by preference shifts, such as a rising concern about animal welfare (Parlasca & Qaim, 2022) Given that pigs rank among the top animals sacrificed annually for meat, this suggests there is a heightened demand for pig production (Whitton et al., 2021).

In addition to consumption rates rising with the increasing population, other aspects that stands out to the significance of livestock meat would be the economic and social factors. The livestock industry accounts for about 40% of the total agricultural production value, is responsible for about the employment of 1.3 billion people and is considered a significant global asset valued at about $1.4 trillion (Parlasca & Qaim, 2022; Salmon et al., 2020; Thornton, 2010).

While economic and social aspects have previously been mentioned, environmental and animal welfare issues surrounding livestock production have yet to be discussed. The livestock industry accounts for one of the largest users of land, occupying about 70% of all agricultural land (Salmon et al., 2020). In addition to extensive land use, livestock production contributes about 14.5% of total greenhouse gas emissions (Cheng et al., 2022).

Animal welfare is also a top priority for livestock production and consumers. Throughout the production process, animals may experience stress from repeated handling and transport (Broom, 2003). To mitigate these stressors, there are strict regulations enforced by the USDA

Food Safety and Inspection Service, such as animal facility conditions, handling, and slaughtering practices (Edwards-Callaway & Calvo-Lorenzo, 2020). Despite these implications, high production rates can sometimes lead to unavoidable stressors, potentially resulting in adverse effects on animal welfare.

Metabolism

Carbohydrate Metabolism

Glycolysis

Carbohydrates are the primary and the most abundant source of energy in the diets of pigs constituting approximately 60% of total energy intake (Knudsen et al., 2016). Although digestion of dietary carbohydrates begins in the mouth, most of digestion and hydrolysis into monosaccharides occurs in the small intestine (Nafikov & Beitz, 2007). Because of structural differences, monosaccharides have varying absorption rates in the small intestine; however, glucose is absorbed quickly (Chandel, 2021; Drochner, 1993). Glucose is absorbed across the brush-border membrane of the small intestine by secondary active transport via sodium glucose transporter 1 (SGLT1) on the apical side of enterocytes (Gorboulev et al., 2011). Once absorbed into the enterocytes, glucose is passively transported into the bloodstream via glucose transporter 2 (GLUT2), located on the basolateral membrane, to be used throughout the body of the organism (Gorboulev et al., 2011; Sun et al., 2023; Thorens, 2015).

In the liver, glucose that is not immediately used for energy, or in periods of high blood glucose concentration, can be stored as glycogen for later use (Chandel, 2021). Conversely, when blood glucose concentration is low, the liver will break down glycogen into glucose, releasing it back into the bloodstream (Chandel, 2021). Adipose tissue can use glucose for immediate energy or convert it into fat for long-term storage (Luo & Liu, 2016). Lastly, glucose

in muscle tissue can undergo glycolysis and be used immediately for energy or stored as glycogen for later use (Chandel, 2021). Glycolysis acts as a primary pathway for generating adenosine triphosphate (ATP) and serves many essential functions in cellular metabolism.

The glycolytic pathway is initiated when glucose is transported into the cell via glucose transporters (GLUT) (Röder et al., 2014; Thorens, 2015). Once inside the cell, glucose is phosphorylated by the enzyme hexokinase to form glucose-6-phosphate (G6P) (Berg et al., 2019; Chandel, 2021). This step is crucial as it holds glucose within the cell and prevents it from diffusing through the membrane because of its negative charges, marking it as an irreversible step (Berg et al., 2019; Chandel, 2021).

With G6P available, the cell can proceed with the glycolysis pathway. Phosphoglucose isomerase catalyzes the conversion of G6P to fructose 6-phosphate (F-6P). However, since G6P and F-6P are both present primarily in the cyclic forms, additional phosphorylation is required. Therefore, F-6P is then catalyzed by the enzyme phosphofructokinase (PFK) to form fructose 1,6-bisphosphate, which is considered the first committed step because of its irreversible transformation (Berg et al., 2019).

Fructose-1,6-bisphosphate is then cleaved by enzyme aldolase into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Since DHAP is not on the direct pathway of glycolysis, it must be converted to GAP to continue through the pathway. This is done by enzyme triose phosphate isomerase, ultimately resulting in two molecules of GAP (Berg et al., 2019).

The two molecules of GAP are subsequently converted into 1,3-bisphosphoglycerate (1,3-BPG) by glyceraldehyde 3-phosphate dehydrogenase. 1,3-BPG being an acyl phosphate gives it the property of having a high phosphoryl-transfer potential, which will be seen in the

next step. The enzyme phosphoglycerate kinase catalyzes the transfer of the phosphoryl group from 1,3-BPG to ADP, resulting in the products of 3-phosphoglycerate and ATP. As mentioned previously, since there are two molecules of GAP formed, this step generates two molecules of ATP (Berg et al., 2019).

The remaining steps of this pathway involve phosphoglycerate mutase catalyzing the shift of the phosphoryl group from 3-phosphoglycerate forming 2-phosphoglycerate. Next, enolase catalyzes the dehydration of 2-phosphoglycerate forming phosphoenolpyruvate (PEP), which increases the transfer potential of the phosphoryl group. Finally, pyruvate kinase catalyzes the irreversible transfer of a phosphoryl groups from PEP to ADP, forming pyruvate and ATP (Berg et al., 2019).

Citric Acid Cycle

The citric acid cycle (CAC) plays a vital role in cellular respiration, further oxidizing the products of glycolysis to generate high-energy molecules in the mitochondrial matrix. Under aerobic conditions, pyruvate is transported into the mitochondria, where it undergoes an irreversible oxidative decarboxylation by the pyruvate dehydrogenase complex, ultimately forming acetyl CoA (Berg et al., 2019). The CAC begins with the condensation of oxaloacetate and the acetyl group of acetyl-CoA first forming citryl CoA, which is then hydrolyzed to citrate and CoA (Berg et al., 2019). This reaction is catalyzed by the enzyme citrate synthase (CS) (Berg et al., 2019).

Citrate synthase plays a crucial role in the CAC and is a well-established quantitative marker of mitochondrial integrity and function (Chhimpa et al., 2023; Jacobs et al., 2013; Larsen et al., 2012). High CS activity generally indicates efficient mitochondrial energy production, while low citrate synthase activity may indicate mitochondrial disfunction (Chen et al., 2023;

Sumi et al., 2022). Additionally, reduced citrate synthase activity has been associated with aging, suggesting mitochondrial damage and loss of structural integrity (Yeo et al., 2019).

Electron Transport Chain

During the CAC, there is a reduction of NAD+ to NADH and FAD to $FADH_2$ on multiple occasions (Berg et al., 2019). These reduced coenzymes carry high-energy electrons that are crucial for the electron transport chain (ETC) (Nolfi-Donegan et al., 2020). The ETC is comprised of a series of protein complexes and small organic molecules that transfer electrons from NADH and FADH2 to reduce molecular oxygen to water (Berg et al., 2019; Nolfi-Donegan et al., 2020). This process takes place in the mitochondria, resulting in the production of ATP in aerobic organisms (Berg et al., 2019). The flow of electrons through the ETC is coupled with the pumping of protons out of the mitochondrial matrix, creating a proton gradient (Berg et al., 2019; Nolfi-Donegan et al., 2020).

The ETC is composed of five complexes: Complex I (NADH: ubiquinone oxidoreductase), Complex II (succinate dehydrogenase), Complex III (Cytochrome b1 complex), Complex IV (Cytochrome c oxidase), and Complex V (ATP synthase). The ETC begins with Complex I, which accepts electrons from NADH and donates them to ubiquinone, pumping protons into the intermembrane space. Complex II directly oxidizes FADH2 and transfers electrons to ubiquinone without proton pumping. Complex III oxidizes ubiquinol allowing the transfer of electrons to cytochrome c, coupled with additional proton pumping. Cytochrome c oxidase (Complex IV) acts as the terminal enzyme in the ETC, where molecular oxygen is catalyzed and reduced to two molecules of water. Similar to citrate synthase, cytochrome c oxidase acts as a marker for mitochondrial and metabolic function (Jun et al., 2024). This reduction involves the addition of four electron and four protons, producing two molecules of

water and pumping four protons across the membrane, contributing to the proton gradient used by ATP synthase for ATP synthesis via Complex V. (Berg et al., 2019; Nolfi-Donegan et al., 2020).

Protein Metabolism

Protein metabolism begins in the stomach where dietary proteins are denatured and broken down into smaller peptides. The acidic environment of the stomach is highly favorable for protein denaturation, and the primary enzyme, pepsin, is maximally active at a pH of 2. Degradation continues in the small intestine, where pancreatic protease enzymes such as trypsin, chymotrypsin, and carboxypeptidase are excreted through a hepatopancreatic sphincter along with pancreatic bicarbonate. This bicarbonate acts to neutralize stomach acid, creating a larger pH that is optimal for pancreatic protease activity. These enzymes are secreted as inactive zymogens and are converted into active enzymes to further degrade the substrates into smaller peptides and free amino acids (Berg et al., 2019; Goodman, 2010).

Aminopeptidases on the brush border membranes of the small intestine further hydrolyze some of the peptides into free amino acids and di- and tripeptides. These individual amino acids and small peptides are then absorbed by the enterocytes via specific amino acid active transporters, often utilizing sodium-dependent co-transporters. Small peptides, such as di- and tripeptides, are transported via peptide transporters (PEPT1) and further hydrolyzed into amino acids within the enterocytes. Once inside the enterocytes, amino acids enter the bloodstream via facilitated diffusion and are transported to various tissues for utilization or catabolism (Berg et al., 2019; Goodman, 2010).

Amino acids are the building blocks of protein and are classified into essential and non-essential amino acids depending on the body's ability to synthesize them (Berg et al., 2019). Essential amino acids cannot be synthesized by the body and must instead be obtained through the diet (Berg et al., 2019). Amino acids are used to synthesize myofibrillar proteins, which are essential for protein synthesis and muscle growth (Jackman et al., 2017).

The National Research Council (NRC) provides guidelines for nutritional requirements for livestock and domesticated species, including specific recommendations for essential amino acids. These guidelines are in place to formulate balanced diets that support growth, reproduction, and overall health in animals. Growing pigs have greater amino acid requirements to support rapid growth and development (Zhang et al., 2021). Therefore, as pigs approach market weight, their amino acid requirements decrease slightly. According to the NRC, the first limiting amino acids in most swine diets is lysine, meaning it is present in the smallest amount relative to the animals' requirements. The NRC guidelines typically suggest dietary lysine requirement for growing pigs fed ad libitum range from 1.7% to 0.71% of the diet, depending on the growth stage (NRC, 2012). Pigs receiving below their requirements tend to exhibit reduced growth performance and subsequently reduced protein accretion (Remus et al., 2020).

Lipid Metabolism

Lipids are the body's most energy-dense macronutrients, providing almost twice the energy that comes from carbohydrates or proteins. Dietary fats are usually ingested in the form of triglycerides. However, because of their insolubility in water, they must first be emulsified to fatty acids for absorption across the intestinal epithelium. Bile salts help form these triglycerides into micelles, allowing them to be more susceptible to digestion by pancreatic lipases.

Triglycerides are broken down into free fatty acids and monoglycerides by lipases that are then absorbed across the intestinal epithelium into enterocytes. Within the enterocytes, fatty acids and monoglycerides are resynthesized into triglycerides to be packaged into transport particles called chylomicrons. These particles enter the lymphatic system before entering the blood, where they bind to membrane-bound lipoprotein lipases and release free fatty acids and monoglycerides. These are taken up by tissues and resynthesized into triglycerides for storage or energy within the cell (Berg et al., 2019).

A metabolic pathway called lipogenesis occurs in periods of excess carbohydrates and proteins, where they are synthesized into fatty acids and subsequently triglycerides (Imamura et al., 2020). This anabolic pathway occurs predominantly in the liver and adipose tissue. Insulin promotes lipogenesis by increasing the uptake of glucose into cells and activating key lipogenic enzymes. The synthesized fatty acids are esterified into triglycerides and stored in lipid droplets within adipocytes (Kersten, 2001; Trujillo & Scherer, 2006; van der Spek et al., 2012).

Conversely, in periods of energy demand, such as fasting, stored triglycerides in adipose tissue are broken down through a process called lipolysis (Kersten, 2001). Lipolysis is activated by the epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormones. Theses hormones trigger transmembrane receptors that activate adenylate cyclase, increase cyclic AMP concentration, and stimulate protein kinase A, activating the lipases. Released free fatty acids and glycerol into the bloodstream. Hormones such as insulin, glucagon, and catecholamines regulate this process. The free fatty acids are transported to tissues like the liver and muscles, where they are oxidized and ultimately produce acetyl-CoA (Berg et al., 2019; van der Spek et al., 2012).

Fatty acid oxidation, also known as beta-oxidation, is the catabolic process by which fatty acid molecules are oxidized in the mitochondria to generate acetyl-CoA, NADH, and $FADH_2$. These molecules then enter the CAC and ETC to ultimately produce ATP, as discussed earlier. Fatty acids are first activated in the cytoplasm by the enzyme acyl-CoA synthetase, converting them into acyl-CoA. Since long-chain acyl-CoA molecules cannot cross the mitochondrial membrane directly, they are converted to acyl carnitine by enzyme carnitine palmitoyl transferase I (CPT I). Acyl carnitine is further transferred across the inner mitochondrial membrane via translocase. Carnitine palmitoyl transferase II (CPT II) catalyzes the transfer of the acyl group back to CoA on the matrix side of the membrane. This cycle will continue, shortening the fatty acyl chain by two carbon atoms each cycle, until all fatty acid molecules are fully oxidized (Berg et al., 2019; Talley & Mohiuddin, 2024).

Metabolic Flexibility

Maintenance of metabolic homeostasis relies on multiorgan control of available fuel (Smith et al., 2018). Under conditions of high carbohydrate availability, insulin is released into the bloodstream, promoting glucose uptake by skeletal muscle tissues, decreasing the rate of lipolysis in adipose tissue, and stimulating fatty acid and triglyceride synthesis from lipids and glucose (Smith et al., 2018). Because of the decrease in circulating carbohydrates and insulin-glucagon ratio during a fasted state, fatty acid oxidation is preferred for energy (Smith et al., 2018).

The ability to adjust fuel selection in response to changes in metabolic demands and fuel availability is known as metabolic flexibility (Goodpaster & Sparks, 2017). Pyruvate dehydrogenase complex (PDC) is a mitochondrial multi-enzyme complex that plays a crucial

role in metabolic flexibility in mammals, acting as a critical control point of cellular energy metabolism. During periods of energy deprivation, pyruvate dehydrogenase kinase (PDK) suppresses PDC activity through phosphorylation, which limits the conversion of pyruvate to acetyl-CoA in skeletal muscle. This suppression leads to a shift towards fatty acid oxidation for cellular energy, as mentioned previously. In contrast, under conditions of insulin stimulation, PDC activity is activated, facilitating the conversion of pyruvate to acetyl-CoA and promoting glucose oxidation. Failure to effectively switch between metabolic pathways is often a sign of mitochondrial dysfunction (Zhang et al., 2014).

Growth Promotors in the Meat Industry

Beta Adrenergic Agonists

Beta-adrenergic agonists are a class of synthetic drugs that resemble the effects of catecholamines such as norepinephrine, epinephrine, and dopamine when bound to a beta-adrenergic receptor (Almeida et al., 2012; Xanthopoulos et al., 2021) . These receptors are classified into three subtypes: β1, β2, and β3. These subtypes vary by species and tissue type, with β1 being the primary receptor found in swine adipose tissue and skeletal muscle (Almeida et al., 2012; Ritter et al., 2017; Smith, 2024).

For a complete review of the mechanism of beta adrenergic agonists see (Almeida et al., 2012)**.** According to Peterla and Scanes (1990), lipolysis is stimulated by the binding of beta agonist ractopamine, while also inhibiting lipogenesis during an in vitro study (Peterla & Scanes, 1990). This transmembrane signaling pathway is initiated when a beta agonist binds to the receptor and causes an increase in intracellular concentration of cyclic adenosine monophosphate (cAMP) (Almeida et al., 2012; Lövfors et al., 2022). This elevated concentration of cAMP leads

to the activation of the two rate-limiting lipases responsible for lipolysis, adipose triacylglycerol lipase (ATGL) and hormone sensitive lipase (HSL) (Lövfors et al., 2022). The catalyzation of these two rate-limiting lipases are responsible for the breakdown of triglycerides into fatty acids and glycerol as an energy source. Ultimately, the cascades previously mentioned results in a redirection of nutrients from fat growth toward muscle growth (Ritter et al., 2017).

To prevent overstimulation of beta-adrenergic receptors in situations of an excessive exposure of beta agonist, there is an autoregulatory process known as receptor desensitization (Johnson, 2006). Desensitization can occur in three instances: uncoupling of the receptors from adenylate cyclase, internalization of uncoupled receptors, and phosphorylation of internalized receptors (Johnson, 2006). Which of the three mechanisms occur is determined by the duration of beta agonist exposure. Extended exposure will lead to downregulation, where there is a net loss of cellular receptors (Johnson, 2006)

Since the 1990's, synthetic beta adrenergic agonists have been widely used in the livestock industry as a method to increase lean accretion and reduce fat deposition, addressing the rising demand for leaner meat products (Almeida et al., 2012; Bell et al., 1998). The U.S. Food and Drug Administration (FDA) approved the usage of ractopamine hydrochloride in animal feeds as of 2000, followed by zilpaterol hydrochloride in 2006 (FDA, 2000, 2006). Ractopamine plays a large role in swine and cattle production, while zilpaterol is majority in beef cattle production (Ritter et al., 2017). Studies have shown that pigs fed ractopamine have a larger ADG and gain: feed (Armstrong et al., 2004; Marchant-Forde et al., 2003). With greater ADG and better feed efficiency, it provides the industry a more economically efficient effort to raising animals for market.

Despite FDA approval and evidence of positive effects on skeletal muscle growth, consumption of tissues containing β-adrenergic agonists may lead to adverse symptoms such as muscle tremors, muscle pain, and nausea (Bell et al., 1998; Centner et al., 2014; Tang et al., 2018). These negative side effects and concerns have led to some countries enforcing restrictions or bans on meats containing ractopamine (Authority, 2009). As of 2014, approximately 160 countries have implemented restrictions or bans on products containing ractopamine (Centner et al., 2014). As a solution to maintain these effects, but reduce the health risks, researchers have begun investigating a natural supplementation to improve growth amongst livestock species (de Lange et al., 2010).

Natural Alternatives

Phytonutrients are bioactive compounds found in plants that provide health benefits to animals and humans. Botanicals often provide the color, flavor, and/or scent of a plant which makes them very potent compounds. Botanicals have been used as a method for healing for thousands of years. Some examples of plants being used in historical times are from drug recipes taken from Sumerian clay slabs that used plants for medical intervention written 5000 years ago (Ackerknecht, 2016). The clay references the use of over 250 plants to make approximately 12 different medical treatments (Ackerknecht, 2016). While research is limited investigating the effects phytonutrient supplementation has on livestock muscle growth, sufficient research has proven there are positive health effects from phytonutrients. These compounds offer a promising alternative to synthetic compounds used in livestock production.

Many botanicals function mechanistically by influencing cellular metabolism. Botanicals such as chili peppers and cinnamon have been shown to have health benefits by primarily reducing

insulin resistance (Jiang, 2019; Kwon et al., 2009). Chili pepper has an active ingredient of capsaicin (Panchal et al., 2018). Capsaicin was shown to improve homeostasis glucose concentration by reducing insulin resistance (Kwon et al., 2009). When tested *in vivo* and *in vitro* capsaicin has increased glucose uptake in C2C12 muscle cells via activation of AMPK (Kim et al., 2013). Cinnamon has also been used to improve insulin and have an "anti-diabetic" effect (Kim et al., 2006). Cinnamon has been linked to increased insulin sensitivity by activation of the peroxisome proliferator-activated receptors; PPARα and PPARγ (Sheng et al., 2008). Cinnamon has been shown to have an anti-hypertensive effect on patients with metabolic syndrome (Ziegenfuss et al., 2006). A study had investigated fasting blood glucose, systolic blood pressure, and body composition after supplementation of cinnamon or a placebo for 12 weeks. The results had shown that the cinnamon had decreased fasting blood glucose, systolic blood pressure, and improved body composition (Ziegenfuss et al., 2006). A study by Ivanova et al., investigates the effects of phytonutrient, a by-product derived from rose oil production, on productivity, carcass composition and meat quality in pigs (Ivanova et al., 2021). Their findings show that there was improved growth performance, without compromising other measured parameters, amongst groups supplemented with the phytonutrient (Ivanova et al., 2021). Other studies, such as Piran Filho et al., found similar results in feedlot cattle. These animals were supplemented with a phytonutrient for a total of 86 days and resulted in supplemented groups having heavier weights than control groups (Piran Filho et al., 2021). Even with improvements with weight gain, there were no significant differing results for feed efficiency; however, numerically, the supplemented groups had a better average daily gain (Piran Filho et al., 2021).

Summary

As global population continues to increase, meat consumption will follow that trend as well (Daniel et al., 2011). Synthetic compounds, such as beta-adrenergic agonists may have been a promising product to improve production rates, however recent findings show they may no longer be the best option for the industry (Centner et al., 2014). Because of health risks imposed by these synthetic compounds, it is time to discuss natural alternatives with the same effects. Phytonutrients are known to be a promising product in terms of the health benefits it provides humans and animals. With few published studies, it has been proven these compounds may be the next best option to improve production rates without compromising the health of the animal or consumer of the meat products (Manuelian et al., 2021). However, further investigation on the effects of various phytonutrient products is needed to confirm the effectiveness.

Chapter II

The Effect of Phytonutrient Supplementation on Pig Growth

Abstract

As the global population continues to increase, the demand for livestock production also rises. This has led to growing interest in efficient approaches to enhance animal growth and development. Phytonutrients are considered a promising alternative to synthetic compounds to improve animal growth performance. The objective of the study was to examine the effects of varying doses of phytonutrient supplementation on pig growth and metabolism. Thirty-two crossbred pigs (approximately 2 months of age, 17.7 kg± 0.82 kg) were randomly assigned to one of four phytonutrient groups (Control: 0 ppm; 1: 62.5 ppm; 2: 125 ppm; 3: 250 ppm) fed ad libitum for 28 days. Body weight was recorded on days -5, -1, 0, 7, 14, 21, and 28. Feed intake was recorded daily, and body composition via dual x-ray absorptiometry (DEXA) was obtained on days -1, 14, and 28. Pigs increased ($P<0.0001$) in body weight, irrespective of treatment ($P=0.0535$). Control pigs had better average daily gain (ADG) when compared with other diets ($P<0.001$). Treatment 3 had the greatest ($P<0.05$) feed:gain when compared with other treatments. Lean percentage of body weight decreased ($P<0.0001$) while fat mass increased ($P<0.0001$) over the duration of the study. Overall, phytonutrient supplementation did not show significant improvement to the growth of the pigs.

Introduction

Over the past two decades, worldwide meat consumption has surged, and it is projected that demand will continue to increase in the future (Henchion et al., 2021). Skeletal muscle is the main component of meat, and changes in muscle content can significantly impact production economics. Therefore, it is important for meat producers to understand muscle physiology and explore ways to enhance muscle mass while maintaining animal health, consumer safety, and meat quality (de Lange et al., 2010). In addition to optimization of animal genetics, management, and dietary programs, feed supplements can be, and have been, used to increase the yield of skeletal muscle and thereby improve the output per animal (de Lange et al., 2010).

Since the late 1990's, pork producers have used a group of synthetic β-adrenergic receptor agonists as feed supplements to increase lean muscle accretion, decrease fat deposition, and increase feed efficiency in finishing pigs (Almeida et al., 2012). Leading synthetic products on the market for finishing pigs include Paylean (Elanco) and Engain (Zoetis) (Ritter et al., 2017). The efficacy of these supplements has been unprecedented with 10-20% improvement in feed efficiency (Apple et al., 2007). The application also extended to beef cattle, where the effects are even more pronounced (Cônsolo et al., 2015). The use of these products has been a very successful strategy to increase income over feed costs while improving the quality of meat produced. β-adrenergic agonists increase lean muscle accretion mainly by direct effects on skeletal muscle through the binding of specific β-adrenergic receptors (Almeida et al., 2012).

Recent concerns over the food safety and animal welfare have led to the banning of these supplements in many countries (Authority, 2009). In response, several major United States pork producers have committed to not purchasing pigs treated with β-agonists. Therefore, pork

producers have been forced to stop using the supplements and losing the benefits that came with the use.

Currently, there are no natural alternatives to β-agonists; however, there are some natural additives, such as Ambitine (PMI Nutrition) and LeanFuel (Furst-McNess Company), that aim to mitigate losses. These products are simple blends of existing ingredients on the market and have not proven as effective as synthetic β-agonists.

The use of phytochemicals for preventing skeletal muscle loss during aging or improving recovery after injury/exercise, in humans, has been extensively studied (Kan et al., 2022; Monjotin et al., 2022). There are many phytochemicals that can have a marked effect on muscle physiology (Lillehoj et al., 2018). In addition, many of these phytochemicals are approved for use in animal feed by the FDA but are not currently utilized in the feed additive market. Therefore, an opportunity exists for research into novel phytochemical candidates that could specifically targeted and influence muscle mass and function in livestock.

In prior research conducted in collaboration with AVT Natural, we identified a phytochemical formula (ACT F4), which demonstrated significant efficacy in enhancing muscle cell proliferation, differentiation, and fatty acid oxidation in vitro (Rhoads et al., unpublished data). This project proposes further exploration of this formula, with the addition of an additional phytochemical from Nutreco Exploration (Nutreco RF).

This in vivo trial will determine which phytochemical mixture could contribute to a holistic solution for increasing muscle mass in swine, verify that the identified phytochemical formula supplemented in pig feed will increase muscle mass and feed efficiency, and at what dose are these effects maximized. Understanding the potential for alternatives to synthetic compounds currently used in the livestock production could have a significant impact on the

industry. Using natural compounds to maximize feed efficiency could provide a more holistic method of production and ultimately the health of the consumer.

Materials and Methods

Animals and Treatment

All procedures involving pigs were approved by Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (IACUC). A total of 32 crossbred pigs (n=16 barrows, n=16 gilts; 17.7 kg ± 0.82 kg initial body weight), at approximately 2 months of age, were transported to Litton Reaves Hall. Upon arrival, pigs were weighed and randomly placed in individual cages (with individual feeders and waterers) in one of two rooms (8 cages in each; 4 barrows and 4 gilts per room per rep). Both rooms received a 12-hour light and 12-hour dark cycle. All pigs had an acclimation period of 4 days during which they were fed a commercial feed diet ad libitum, providing all essential nutrients at amounts required by the NRC for growing pigs (NRC, 2012). Following the acclimation period, pigs were weighed and randomly assigned to one of four treatment groups (n=4 per group) based on body weights. Four treatments were prepared by hand-mixing a proprietary supplement (Nutreco RF) at 0 ppm (Control), 62.5 ppm (1), 125 ppm (2), 250 ppm (3) into the commercial feed. Pigs continued to have ad libitum access to feed and water. Feed intake was recorded daily by weighing remaining feed each morning (6AM) and subtracting that from the value fed the prior day. Pigs were sacrificed on day 28 via captive bolt and exsanguination.

Room temperatures and humidity were monitored and recorded twice daily at 6AM and 6 PM. Pigs were monitored daily for any signs of distress throughout the entirety of the study. Body weights were obtained for each pig on arrival to Litton Reaves (d-5), one day prior to the start of treatment (d-1), on the start day of treatment (d0), then weekly (d7, d14, d21), and

immediately before sacrifice (d28) using WayPig® Mechanical Market Hog Scale (AH 300) (Ephrata, PA).

Sample Collection

Blood samples were collected one day prior to the start of treatment (d-1), at day 14 of treatment (d14), and immediately before sacrifice (d28). Approximately 10 mL of blood was obtained via venipuncture to the jugular vein using vacutainers containing lithium heparin (BD, Franklin Lakes, NJ) and EDTA (Covidien LLC, Mansfield, MA). Each EDTA vacutainer was centrifuged at 22°C for 10 minutes at 2000 x g and plasma was aliquoted then stored at -20°C for future analysis. The lithium heparin vacutainers containing blood samples were transported to the Virginia Tech Animal Laboratory Services of the Virginia-Maryland College of Veterinary Medicine for blood metabolite analysis, using a large animal profile (LAP). The LAP measured glucose, urea nitrogen, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, AST (GOT), gamma-glutamyl transferase (GGT), total bilirubin, direct bilirubin, indirect bilirubin, CK, sodium, potassium, chloride, CO2, anion gap.

Skeletal muscle biopsies of the *Longissimus Dorsi* (LD) were performed as previously described (Zhao et al., 2018; Kroscher et al., 2022). Samples were collected one day prior to the start of treatment (d-1), two weeks into treatment (d14), and immediately before sacrifice (d28). Pigs were fasted for 8 hours prior to muscle biopsies and placed under general anesthesia using isoflurane. Biopsy sites were shaved and sterilized with Betadine Surgical Scrub (Stamford, CT) and 70% ethanol three times. An incision of approximately 1 cm was made in the skin using a scalpel blade. A 10-gauge X 13.4 cm long Mammotome® Elite Tetherless Vacuum-Assisted Biopsy System Probe (Cincinnati, OH) was inserted through the incision at a 45° angle. For each

biopsy collection, about 0.30 g of muscle was extracted from the LD of each pig. Samples were flash frozen in liquid nitrogen and stored at -80°C. After tissue collection, incision sites were sutured with PGA Violet Braided Polyglycolic Acid Absorbable Suture (Oasis, Mettawa, IL), cleaned with 70 % ethanol, then bandaged with Alushield Aerosol Bandage (VetOne, Boise, ID). Following the conclusion of the study, a portion of the biopsy sample was sent to the Metabolic Phenotyping Core at Virginia Tech for metabolite phenotyping where enzymatic activity and metabolic flexibility were measured. The remaining samples continued storage at -80°C for future analysis.

Metabolic assessment was performed as described previously (Zhao et al., 2018; Fausnacht et al., 2021; Kroscher et al., 2022). Briefly, skeletal muscle samples obtained via biopsy were used by The Metabolism Core at Virginia Tech to assess pyruvate oxidation ([1–14C] pyruvate) with and without the presence of 100 μM palmitic acid. Metabolic flexibility is defined as the precent reduction in pyruvate oxidation in the presence of palmitic acid. Separate portions of the muscle biopsy were stored at -80°C for analysis of enzymatic activity (citrate synthase and cytochrome C oxidase) as described in (Zhao et al., 2018; Fausnacht et al., 2021) .

Following each blood draw and muscle biopsy, pigs remained under general anesthesia while undergoing a scan with dual-energy x-ray absorptiometry (DEXA) (Prodigy Advance, General Electric, Boston, MA). Body composition measurements via DEXA were collected on day -1, day 14, and day 28 of this experiment.

Statistical Analysis

All data were statistically analyzed using a PROC MIXED procedure with repeated measurements in SAS (SAS Inst. Inc., Cary, NC). Normality for all data was ensured by Shapiro-

Wilks test. Covariance structure was determined according to the smallest Bayesian information criterion (BIC) value. Repetition and room were identified as random variables. Model included treatment (Control, 1, 2, 3), time (day or week), and treatment-by-time interactions. Data is reported as least square means ± standard error of the mean (SEM). Differences are considered statistically significant if $P<0.05$.

Results

Growth Parameters

Pigs increased ($P<0.0001$) in body weight (BW) over the period of the study (Figure 1, Panel A). Increasing phytonutrient supplementation tended to have a quadratic effect on BW ($P<.05$; Table 6). There was a treatment by day interaction ($P<0.01$) for BW (Figure 1, Panel B); however, there was no significant effect of treatment for BW (Table 1). There was a significant effect of treatment ($P<0.001$) and week ($P<0.0001$) for average daily gain (ADG) (Figure 1, Panel C and D). Phytonutrient supplementation dosages had a quadratic effect on ADG ($P<.05$; Table 6). Pigs receiving control diet had better ADG when compared with other diets (Figure 1, Panel C). ADG was greatest in week 3 when compared with other weeks, while they remained insignificantly different from one another. There was no significant interaction of treatment by day for ADG (Table 1). There was an effect of treatment ($P<0.05$) and week ($P<0.0001$) for feed intake (FI) (Figure 1, Panel E and F). Week 4 had the highest feed intake when compared with weeks 1, 2, and 3. A weak quadratic effect was observed for FI with increasing supplementation ($P<.05$; Table 6). There was no significant treatment by week interaction on FI (Table 1). There was a significant effect of treatment on feed:gain (F:G) ($P<0.05$) (Figure 1, Panel G) and of week ($P<0.0001$) (Figure 1, Panel H). Increasing supplementation tended to have a linear effect on F:G, with treatment 3 having the highest F:G when compared with the other treatments ($P<.05$; Table 6).

Body Composition

There was a treatment by day interaction ($P<0.01$) as well as a day effect ($P<0.0001$) on fat percentage of BW during the study. Lean percentage of BW decreased ($P<0.0001$) throughout

the duration of the study (Figure 2, Panel B). Across all treatment groups, lean percentage of BW was lowest on day 28 ($P<0.01$) (Figure 3, Panel B). No treatment effect was observed (Table 2).

An increase ($P<0.0001$) of fat mass was observed during the study (Figure 2, Panel C). A treatment by day interaction ($P<0.05$) was also observed. Day 28 had greater fat mass when compared with days -1 and 14 (Figure 3, Panel C). A treatment effect was not observed (Table 2). Throughout the duration of the study, there was an increase ($P<0.0001$) in lean mass (Figure 2, Panel D). While there was a treatment by day interaction ($P<0.05$), there was a uniform increase for all treatments over time, with no significant difference between treatments (Figure 3, Panel D). With increasing supplementation dosages, there tended to be a weak quadratic effect on lean mass ($P<.01$; Table 5).

Blood Parameters

Treatment ($P<0.05$) and day ($P<0.001$) had significant effects on glucose and magnesium concentrations (Figure 4 and 6, respectively). Neither had a treatment by day interaction observed (Table 3). A treatment by day interaction ($P<0.05$) and day effect ($P<0.05$) was observed for both phosphorus and CO_2 (Figure 5 and 7, respectively). There was a greater concentration of phosphorus on day 28 when compared with days -1 and 14 (Figure 5, Panel A). There was an effect of treatment ($P<0.05$) on gamma-glutamyl transferase (GGT) concentration (Figure 8), which tended to decrease linearly ($P<.05$; Table 5). Without respect to treatment, there was a day effect ($P<0.05$) on urea nitrogen, creatinine, total protein, globulin, indirect bilirubin, sodium, albumin, and aspartate transferase (AST) concentrations (Figure 9). No significant ($P>0.05$) difference was observed for calcium, direct bilirubin, potassium, and chloride concentrations (Table 3). There was a quadratic effect observed for magnesium, direct bilirubin, and indirect bilirubin with increasing supplementation dosages ($P<.05$; Table 5).

Skeletal Muscle Metabolism

A treatment by day interaction ($P<0.01$) and day effect ($P<0.0001$) were found for both cytochrome c oxidase (CCO) and citrate synthase (CS) activity. For both CCO and CS, there was a decrease in activity throughout the duration of the study (Figure 10, Panel A and C). There was no significant effect of treatment on CCO or CS activity (Table 4). No significant differences in metabolic flexibility were observed when analyzed by day, treatment, or the interaction between treatment by day (Table 4). Despite the lack of statistically significant findings, it is important to note that treatment groups 1 and 2 exhibited numerically lower percentages of metabolic flexibility compared to both the control and treatment group 3 (Table 4). Additionally, the percentages of metabolic flexibility were numerically highest on day 14 of sample collection compared to days -1 and 33.

Discussion

Beta-adrenergic agonists play a significant role in livestock production because of their effect on improving growth performance. With the rising concern of their impact on animal and meat consumer health, countries are beginning to restrict of ban the use of these synthetic compounds. To keep production levels elevated to reach consumer demand, natural alternatives must be investigated (Lim et al., 2023). This study aimed to evaluate the efficacy of a proprietary blend of phytonutrients supplemented into the feed of growing pigs. The objective was to determine if a phytonutrient compound could mimic effects in growth performance similar to that of synthetic compounds used as feed additives. Overall, the findings of this study proved that this specific proprietary blend had no direct effect at improving growth performance of the growing pigs.

As expected with general growth, average feed intake increased over the duration of the study, considering energy requirements increase as these animals grow (NRC, 2012). However, there was no significant difference between treatment groups. Numerically, the groups on control and diet 3 had a larger feed intake when compared with those of diet 1 and 2, suggesting the increase of supplement dosage played no significant role in the increased feed intake.

Similarly, total body weight gain followed the patterns to that of feed intake. As mentioned previously, as pigs grow, their nutritional requirements and intake increase to support growth and maintenance (NRC, 2012). Pigs in the control and diet 3 groups had a greater weight gain increasing by an average of 24.kg and 22 kg, respectively. Once again, while there was an increase in average body weight gain, it was irrespective of differing treatment groups.

As pigs progress through growth stages, their rate of muscle accretion will begin to slow, even though their overall body size, weight, and maintenance requirements increase (Bikker et

al., 1996). Our findings using DEXA scans are consistent with those of other feeding trials. Lean percentage of body weight gradually decreased throughout the duration of the study, even as the pigs continue to gain weight and their fat percentage of body weight increases. Although lean mass alone was increasing, studies predict pigs in the finishing growth stage will reach a plateau in protein deposition. Any extra energy consumed is further deposited as fat, resulting in a decline in the rate and efficiency of growth (Bikker et al., 1996; Campbell, 1988). Once again, despite noticeable change in these measurements, there was no significant difference between treatments, suggesting that the differing treatment dosages had no effect on this phenomenon.

Blood collection occurred three times throughout the study, once prior to the start of treatment and twice during treatment. While all blood measurements were within normal limits for all pigs and treatments, there were a few parameters that showed significance whether that be by treatment or by day. The first notable parameter that showed a significant result was glucose, by having smaller concentration on the first blood collection when compared with the other two collection periods. The first blood collection occurred the day after arrival to the facility and during their acclimation period, where the pigs were exposed to new dietary regimen and new environments. These stressors can lead to decreased levels in performance parameters such as feed intake, daily gain, or body weight, all of which were observed during this period (Martínez-Miró et al., 2016). With limited feed intake, this can result in lesser blood glucose concentration, which were observed at the initial collection (Martínez-Miró et al., 2016).

Citrate synthase and cytochrome c oxidase are both markers of mitochondrial and metabolic function (Chhimpa et al., 2023; Jun et al., 2024). Activity for both enzymes was measured from skeletal muscle biopsies a total of three occasions. We found a decline in both CCO and CS activity over the course of the study. Despite the significant effect of day on

enzyme activity, there was no significant effect of treatment, indicating the different treatments did not significantly alter these activities although they declined. Decreased activity of these enzymes can be associated with mitochondrial dysfunction, therefore potential causes to the decline should be explored (Chen et al., 2023). It is important to note that upon arrival to the testing facility, pigs were housed in individual cages, where physical activity was limited because of space in comparison to where they were previously housed. For both CCO and CS, a reduction in physical activity can cause a reduction in mitochondrial activity because of the lack of demand for energy production (Jun et al., 2024).

As previously mentioned, our findings did not support our objective because of the lack of significant improvement in growth performance by the proprietary blend. While phytonutrients have promising benefits that suggest they could serve as potential alternatives to synthetic growth-promoting feed additives, our results align with previous findings in that not all natural supplements can achieve the same effects as synthetic compounds (Piran Filho et al., 2021). This may be due in part to differences in bioavailability, inadequate supplement formulation, or improper dosage. Phytonutrients often have lesser bioavailability compared with synthetic compounds, which ultimately could limit their effectiveness (Kan et al., 2022). While supplementation did not play a significant role on the growth outcomes of these pigs, it also did not hinder them from proper growth. Therefore, further research into this blend may still be promising with the proper alterations made.

Even though the proprietary phytonutrient blend did not improve growth performance compared to synthetic beta-adrenergic agonists, our results provide important insight into the effectiveness of natural alternatives. This also highlights the complexity of replacing synthetic compounds with natural alternative and the need for optimized formulations and exploration of

dosage. Future studies should explore additional natural additives and formulations than what are included in this blend.

Conclusion

In this study, we examined the effect of administering a proprietary blend of phytonutrients on pig growth. The goal of our research was to establish a similar effect of synthetic compounds commonly utilized for enhancing growth in livestock animals. Our findings indicate that such supplementation did not positively influence the overall growth in the pigs. While body weight increased over the duration of the study, those pigs receiving treatment did not gain as much mass as those not receiving treatment. Our results also show there was reduced feed efficiency with increasing inclusion of the supplement. No significant differences were found between treatment groups for lean and fat mass accretion. Further research is necessary to determine whether the specific concentrations utilized in this study have a causal relationship with the observed negative growth outcome of the pigs.

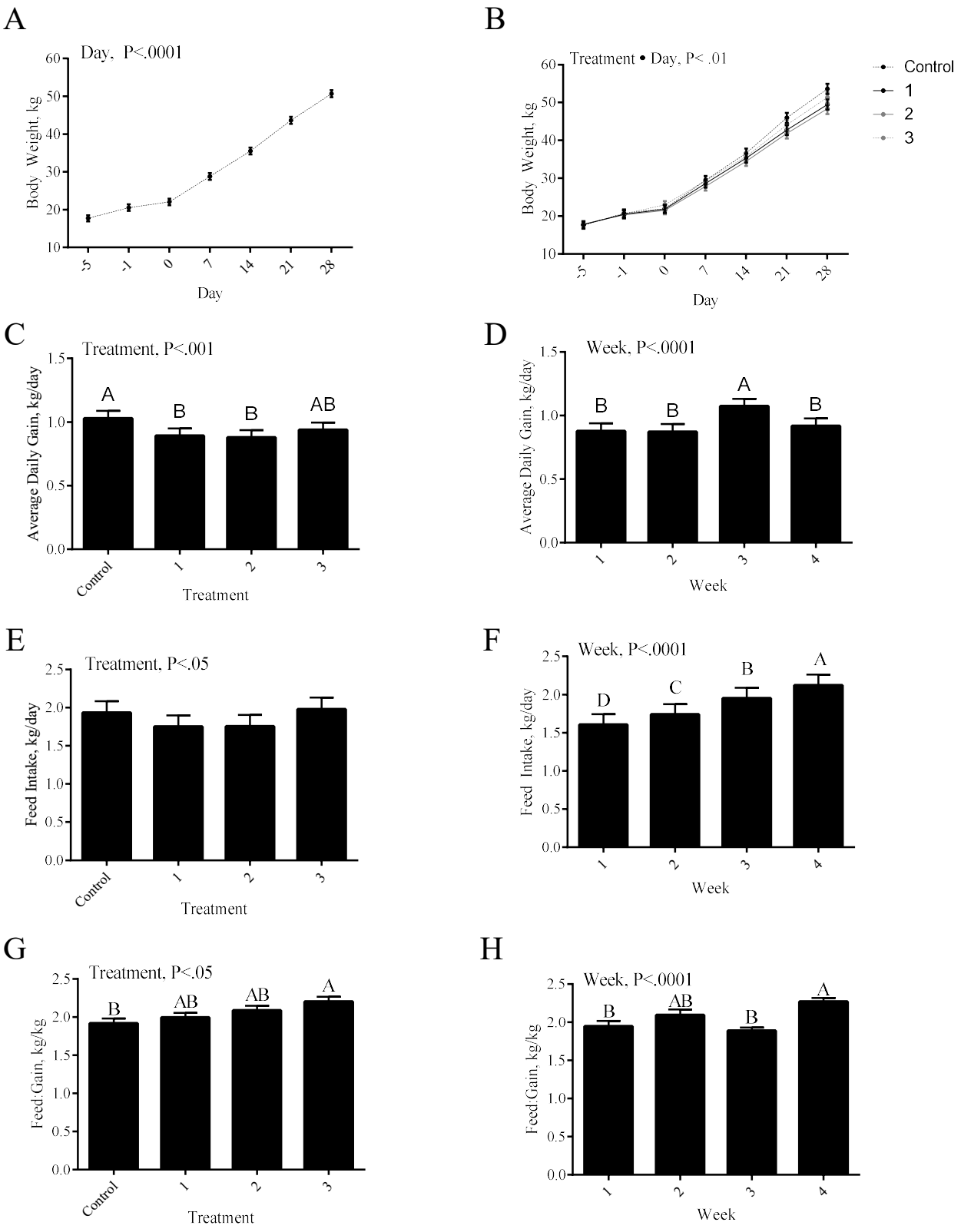

Figure 1 Effects of phytonutrient supplementation on growth parameters in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm,1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Average daily gain, feed intake, and feed:gain are presented on a treatment basis (A, C, and E respectively). Average daily gain, feed intake, and feed:gain are presented on a temporal basis (B, D, and F respectively). Temporal data is presented as the means for each of the four weeks of the study. Body weight (G and H) was recorded on days -5, -1, 0, 7, 14, 21, and 28 of the study. The data presented are LSmeans ± SEM. Differences considered significant when $P<0.05$, n=8. LSmeans without a common letter are different at $P<0.05$.

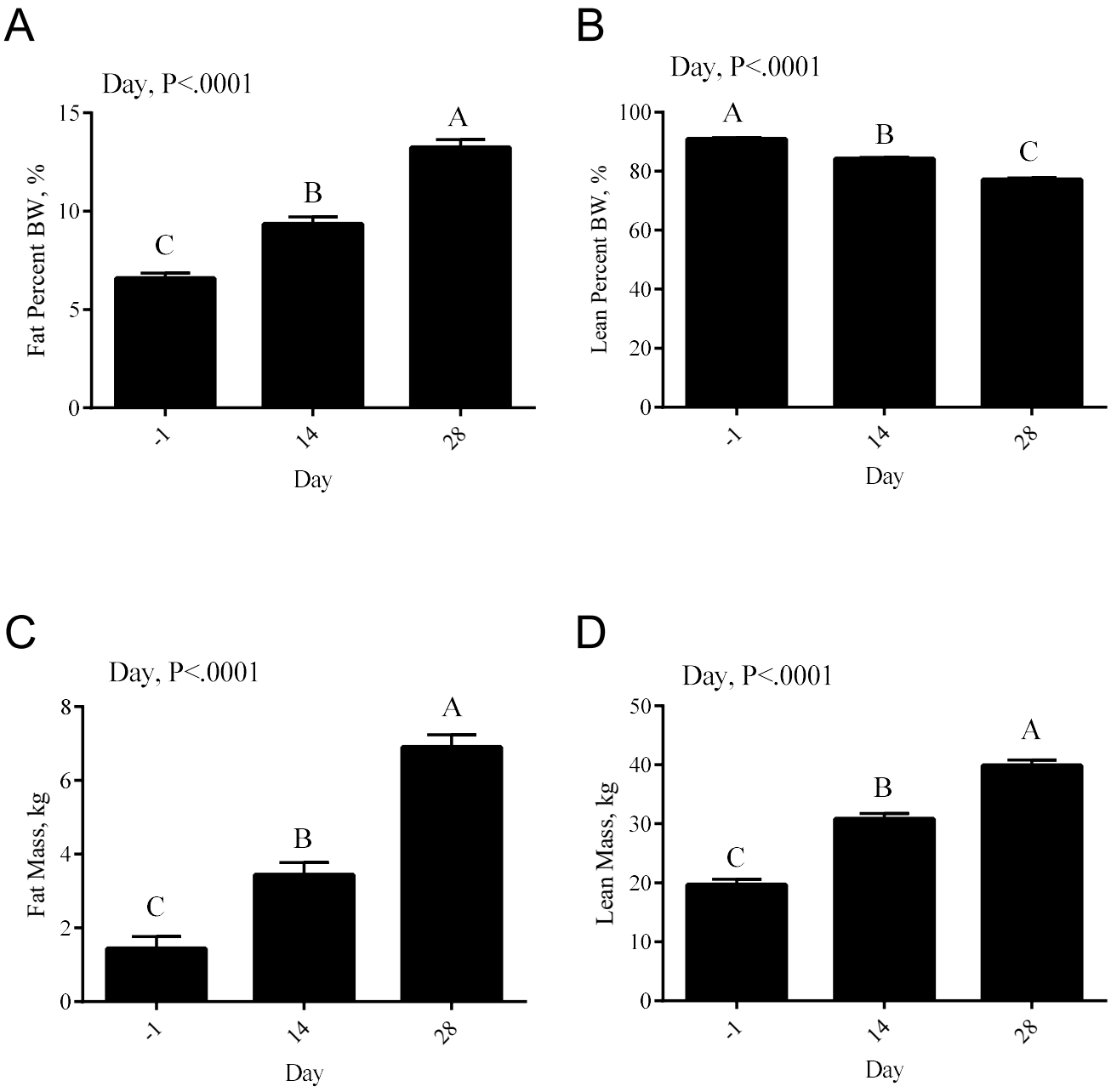

Figure 2 Effects of phytonutrient supplementation on body composition in growing pigs. Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Fat mass as a percentage of BW (A), lean mass as a percentage of BW (B), fat mass (C), and lean mass(D) measurements were obtained by DEXA on days -1, 14, and 28. The data presented are LSmeans ± SEM of a day effect. Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

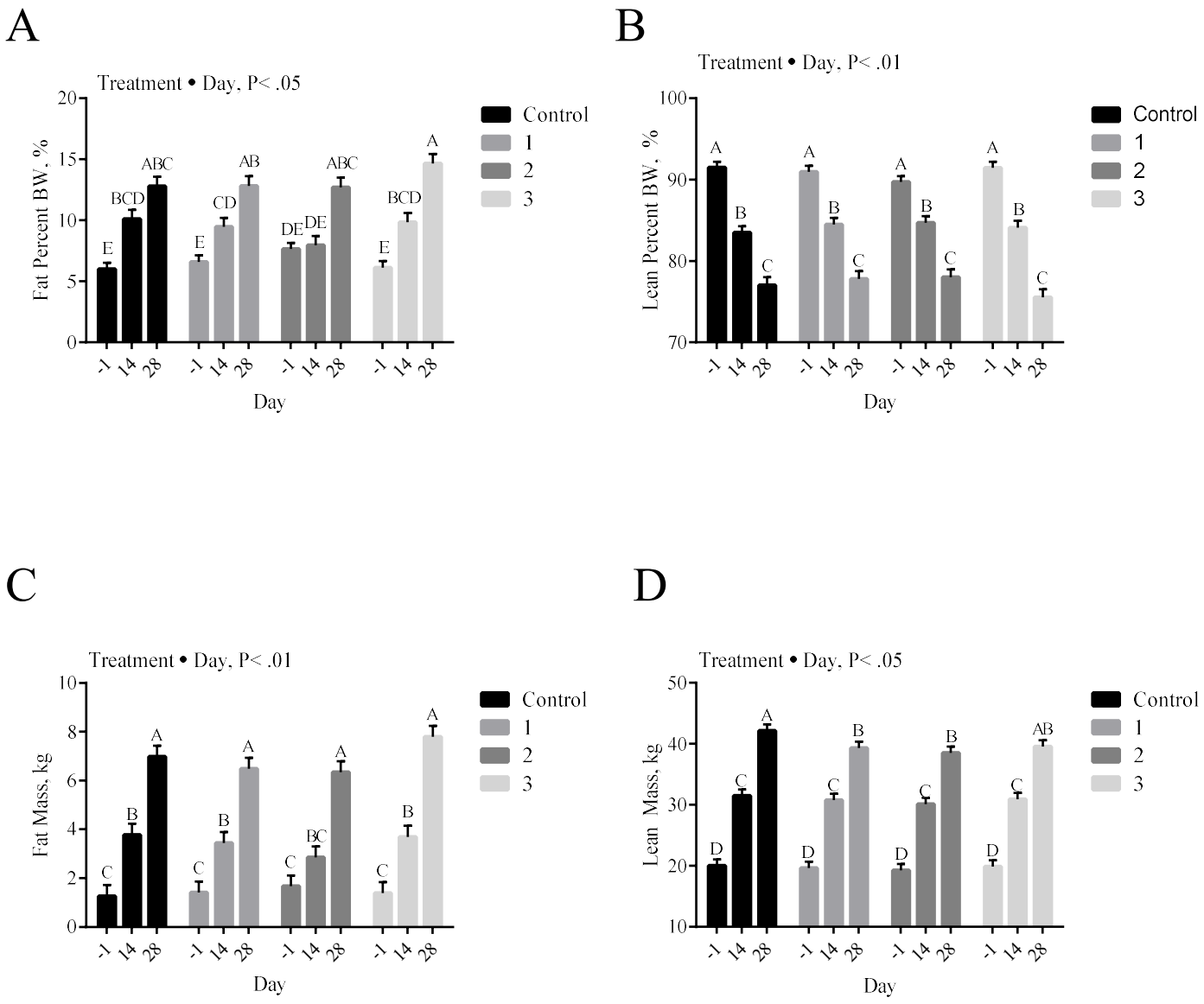

Figure 3 Effects of phytonutrient supplementation on body composition in growing pigs. Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Fat mass as a percentage of BW (A), lean mass as a percentage of BW (B), fat mass (C), and lean mass(D) measurements were obtained by DEXA on days -1, 14, and 28. The data presented are LSmeans ± SEM of a treatment by day interaction. Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

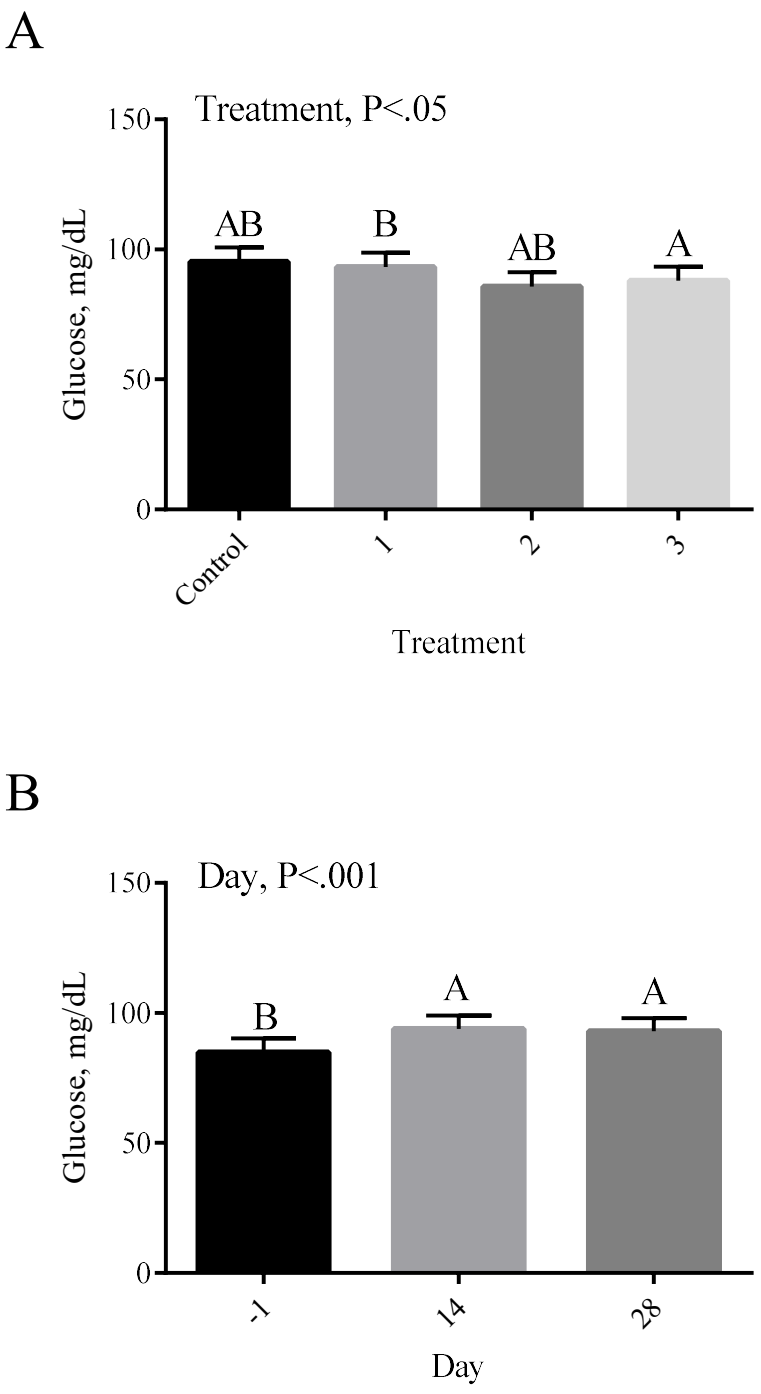

Figure 4 Effects of phytonutrient supplementation on blood glucose in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of glucose with a treatment effect (A) and a day effect (B). Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

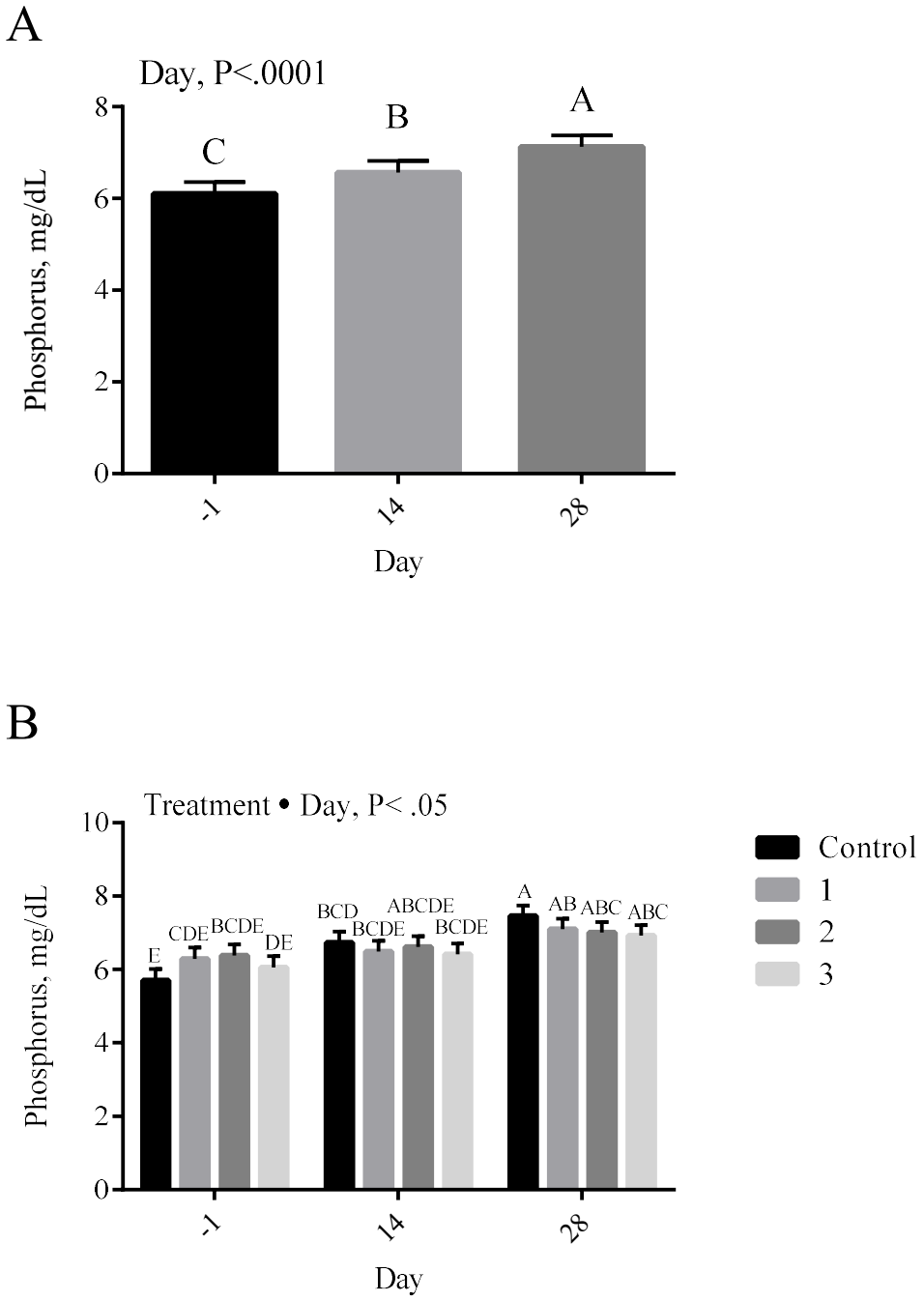

Figure 5 Effects of phytonutrient supplementation on blood phosphorus in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of phosphorus with a day effect (A) and a treatment by day interaction (B). Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

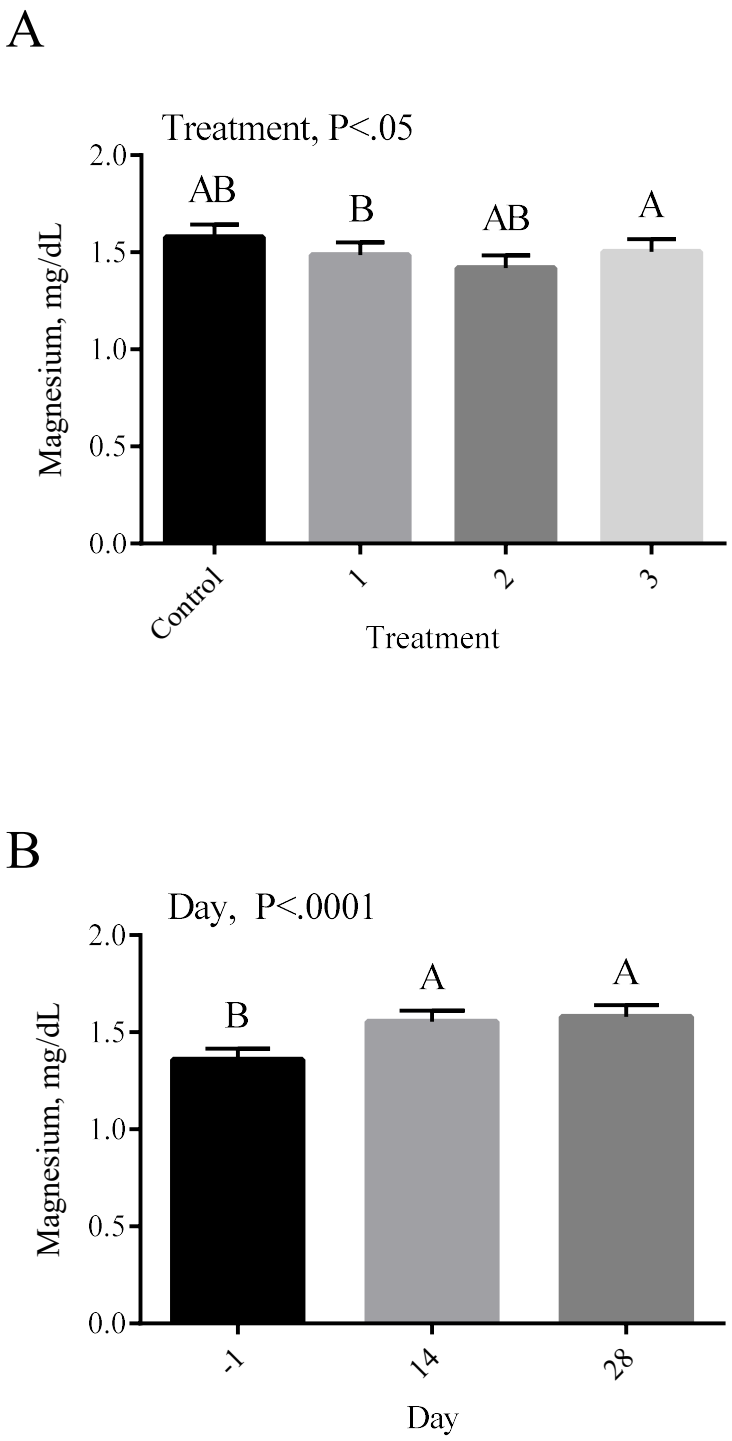

Figure 6 Effects of phytonutrient supplementation on blood magnesium in growing pigs. Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of magnesium with a treatment effect (A) and a day effect (B). Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

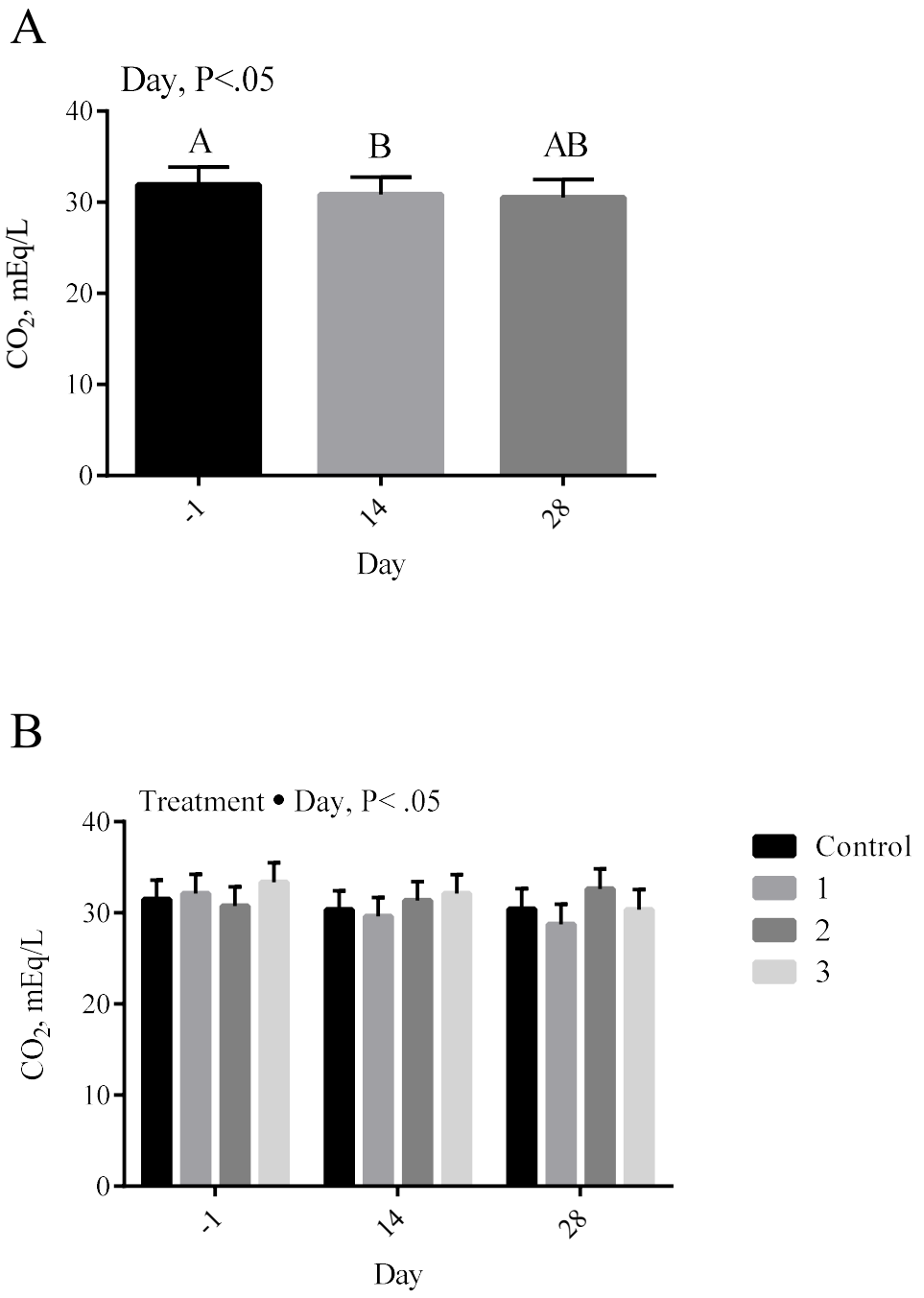

Figure 7 Effects of phytonutrient supplementation on blood CO_2 in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of CO_2 with a day effect (A) and a treatment by day interaction (B). Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

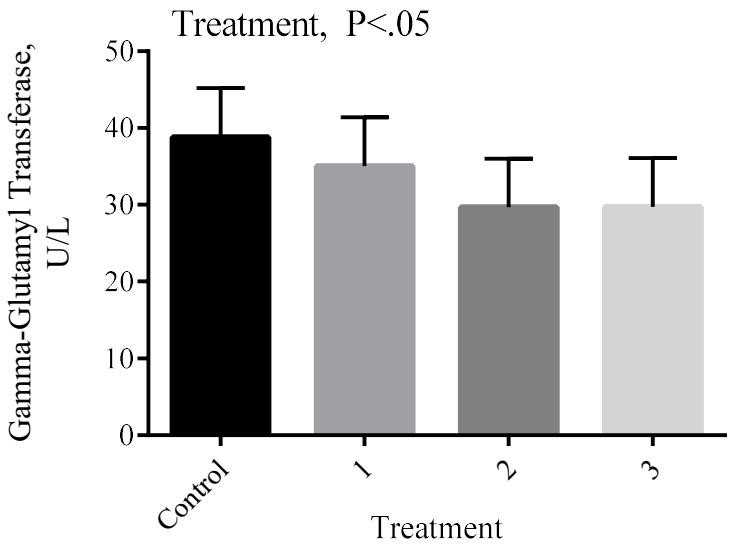

Figure 8 Effects of phytonutrient supplementation on blood gamma-glutamyl transferase in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of GGT with a treatment effect. Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

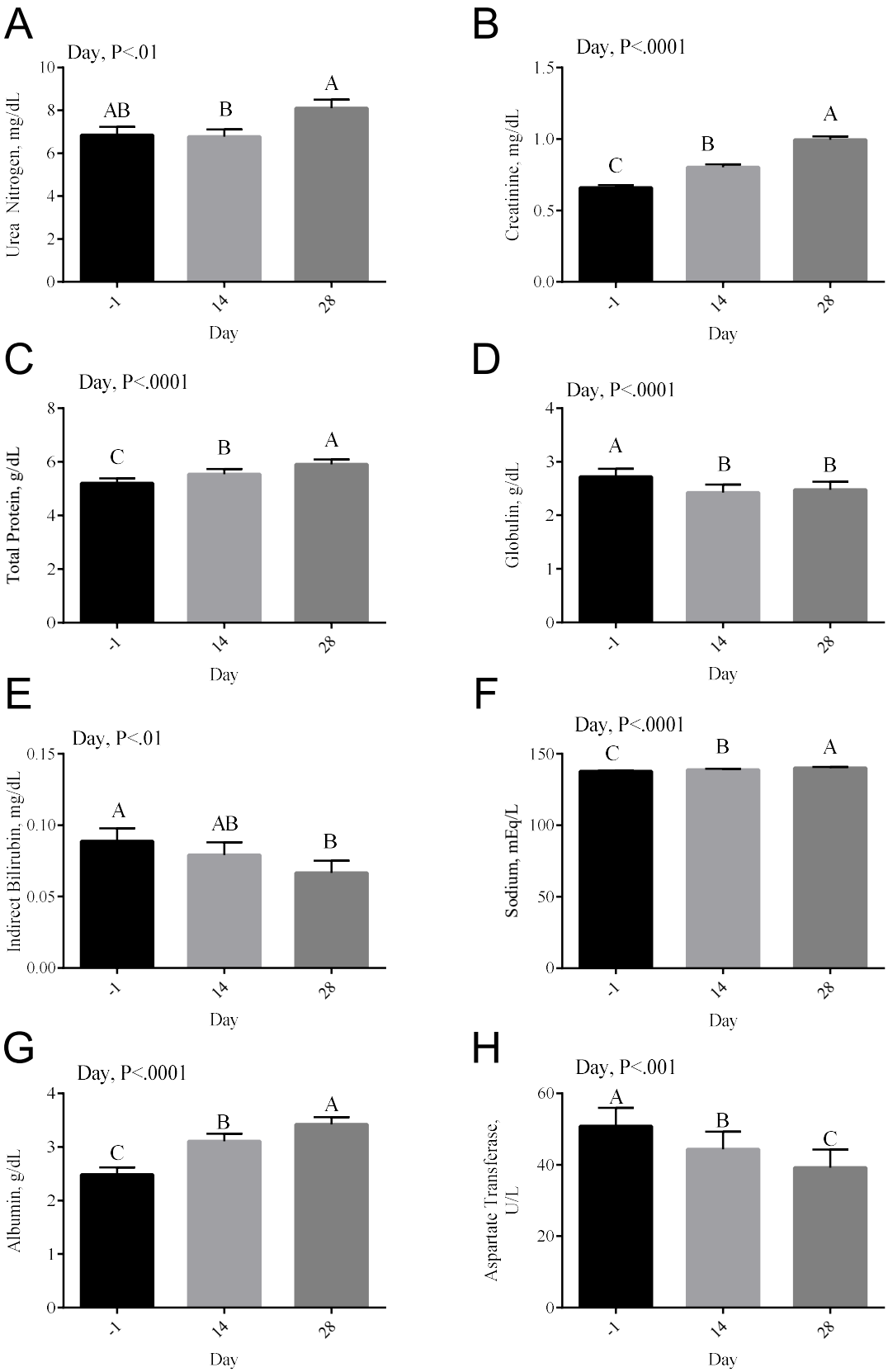

Figure 9 Effects of phytonutrient supplementation on blood parameters in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans ± SEM of variables with a day effect. Differences are considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

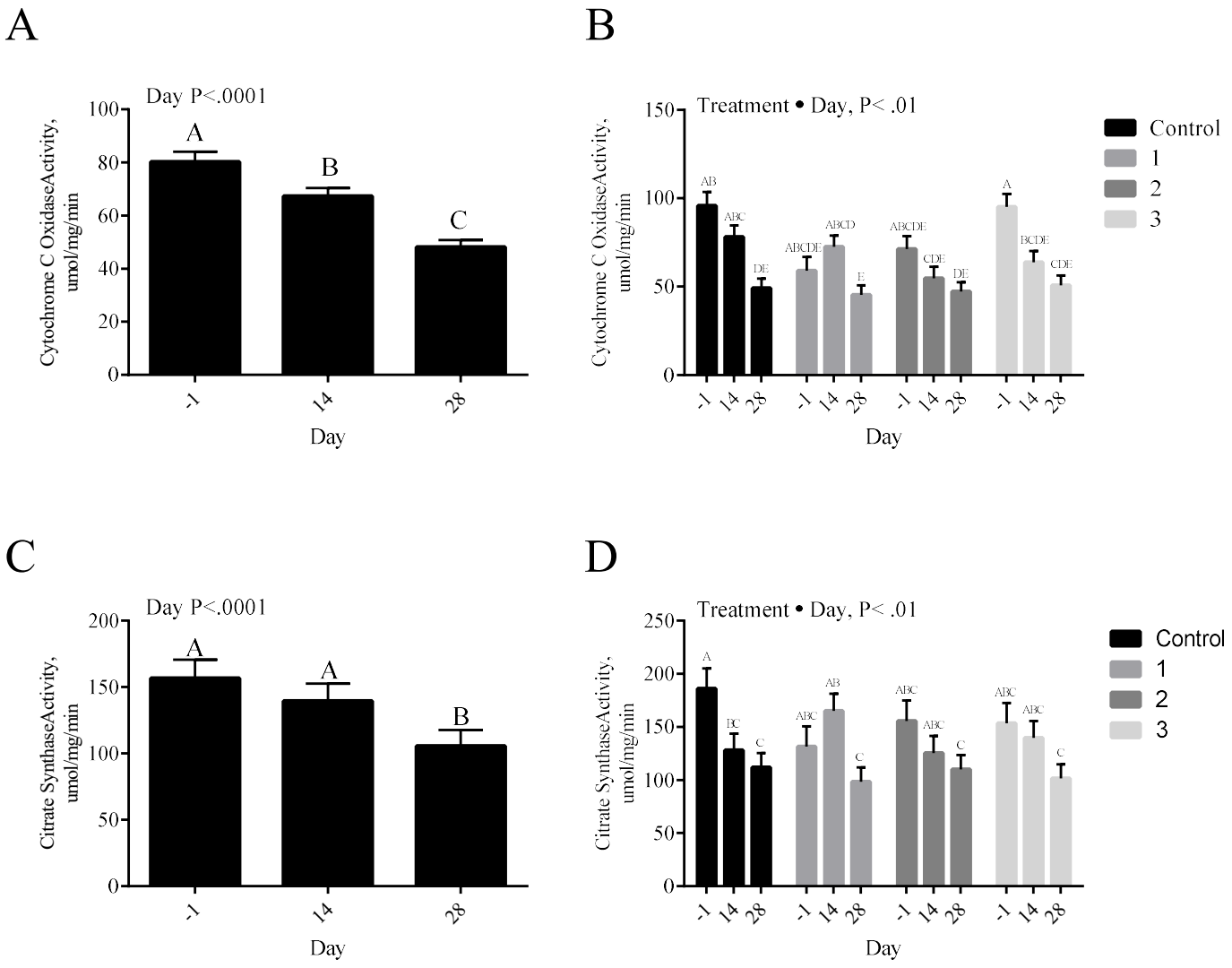

Figure 10 Effects of phytonutrient supplementation on skeletal muclse enzymatic activities in growing pigs.
Pigs were randomly assigned one of four phytonutrient supplementaion groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Skeletal muscle samples were obtained via biopsy from the longissimus dorsi (LD) on days -1, 14, and 28 for Cytochrome C Oxidase activity (A and B) and Citrate Synthase activity (C and D). The data presented are LSmeans ± SEM of a day effect (A and C, respectively) and of treatment by day interaction (B and D, respectively). Differences considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

Table 1 Effects of phytonutrient supplementation on growth parameters in growing pigs.

	Week 1[1]				Week 2[1]				Week 3[1]				Week 4[1]				P-Value			
Variable	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	SEM	Trt	Day	Trt·Day
BW[6] (kg)	27.40^{G}	26.50^{G}	25.70^{G}	27.20^{G}	34.50^{F}	33.20^{F}	32.40^{F}	33.60^{F}	43.90^{CDE}	40.60^{E}	39.70^{E}	42.00^{DE}	51.50^{A}	47.40^{ABC}	46.20^{BCD}	49.20^{AB}	1.64	0.0535	<.0001	0.0045
ADG[7] (kg/day)	0.97	0.87	0.83	0.84	0.92	0.87	0.87	0.84	1.24	0.96	0.97	1.12	0.99	0.87	0.85	0.96	0.08	0.0009	<.0001	0.2889
Feed Intake (kg/day)	1.65	1.54	1.53	1.69	1.82	1.62	1.68	1.83	2.05	1.84	1.83	2.08	2.20	2.00	1.97	2.30	0.16	0.0424	<.0001	0.8643
Feed:Gain (kg/kg)	1.77	1.90	1.99	2.13	2.03	1.96	2.05	2.34	1.73	1.97	1.94	1.92	2.15	2.14	2.36	2.43	0.12	0.0211	<.0001	0.4535

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. The data presented are LSmeans of a treatment by day interaction. Differences considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

[2]Control: 0 ppm
[3]1: 62.5 ppm
[4]2: 125 ppm
[5]3: 250 ppm
[6]Body Weight
[7]Average Daily Gain

Table 2 Effects of phytonutrient supplementation on body composition in growing pigs.

	Day -1[1]				Day 14[1]				Day 28[1]				P-Value			
Variable	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	SEM	Trt	Day	Trt·Day
Fat Percent BW (%)	5.99^{E}	6.60^{E}	7.64^{DE}	6.13^{E}	10.11^{BCD}	9.46^{CD}	7.96^{DE}	9.84^{BCD}	12.79^{ABC}	12.83^{AB}	12.71^{ABC}	14.66^{A}	0.69	0.721	<.0001	0.0104
Lean Percent BW (%)	91.48^{A}	90.97^{A}	89.7^{A}	91.5^{A}	83.48^{B}	84.49^{B}	84.7^{B}	84.1^{B}	77.04^{C}	77.80^{C}	78^{C}	75.7^{C}	0.84	0.884	<.0001	0.0086
Fat Mass (kg)	1.26^{C}	1.42^{C}	1.67^{C}	1.4^{C}	3.77^{B}	3.45^{B}	2.85^{BC}	3.7^{B}	6.98^{A}	6.49^{A}	6.35^{A}	7.80^{A}	0.45	0.253	<.0001	0.0462
Lean Mass (kg)	20.00^{D}	19.66^{D}	19.28^{D}	19.88^{D}	31.50^{C}	30.80^{C}	30.12^{C}	30.96^{C}	42.15^{A}	39.35^{B}	38.56^{B}	39.58^{AB}	1.00	0.077	<.0001	0.0041

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) where they were fed ad libitum for 28 days. Fat mass as a percentage of BW, lean mass as a percentage of BW, fat mass, and lean mass measurements were obtained by DEXA[6]. The data presented are LSmeans of a treatment by day interaction. Differences considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

[2]Control: 0 ppm

[3]1: 62.5 ppm

[4]2: 125 ppm

[5]3: 250 ppm

[6] Dual-energy X-ray Absorbtiometry

Table 3 Effects of phytonutrient supplementation on blood parameters in growing pigs.

	Day -1[1]				Day 14[1]				Day 28[1]				P-Value			
Variable	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	SEM	Trt	Day	Trt·Day
Glucose (mg/dL)	91.22	89.82	78.52	79.87	97.35	97.32	89.77	90.99	97.33	92.96	88.89	92.78	5.94	0.0443	0.0002	0.7999
Urea Nitrogen (mg/dL)	6.98	5.99	6.76	7.65	6.98	5.61	6.51	8.02	8.73	8.36	6.51	8.80	0.76	0.1371	0.0043	0.2126
Creatinine (mg/dL)	0.68	0.63	0.69	0.64	0.78	0.84	0.79	0.80	0.98	1.05	0.98	0.96	0.04	0.7835	<.0001	0.1474
Phosphorus (mg/dL)	5.70^{E}	6.29^{CDE}	6.38^{BCDE}	6.05^{DE}	6.74^{BCD}	6.49^{BCDE}	6.61^{ABCDE}	6.41^{BCDE}	7.46^{A}	7.10^{AB}	7.01^{ABC}	6.93^{ABC}	0.30	0.7568	<.0001	0.0245
Calcium (mg/dL)	10.87	11.23	10.95	10.59	11.01	11.09	10.89	10.88	11.06	11.04	11.06	11.04	0.14	0.4099	0.2084	0.3136
Magnesium (mg/dL)	1.43	1.31	1.31	1.38	1.66	1.56	1.48	1.52	1.64	1.59	1.47	1.61	0.07	0.0221	<.0001	0.4906
Total Protein (g/dL)	5.22	5.23	5.21	5.15	5.62	5.49	5.48	5.59	5.95	5.92	5.85	5.94	0.21	0.9438	<.0001	0.8106
Albumin (g/dL)	2.51	2.59	2.51	2.31	3.18	3.12	3.00	3.13	3.48	3.43	3.32	3.45	0.16	0.6664	<.0001	0.1777
Globulin (g/dL)	2.76	2.66	2.68	2.79	2.44	2.38	2.45	2.44	2.45	2.49	2.51	2.46	0.17	0.9589	<.0001	0.68
AST (GOT) (U/L)[6]	50.88	55.49	49.49	47.46	41.62	42.99	50.37	42.62	42.62	37.53	36.79	40.68	5.98	0.952	0.0006	0.1878
GGT (U/L)[7]	41.91	35.54	31.92	29.02	39.29	35.17	28.79	28.79	35.29	34.42	28.29	31.42	6.53	0.0426	0.2771	0.0938
Direct Bilirubin (mg/dL)	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.01	0.6267	0.5282	0.6792
Indirect Bilirubin (mg/dL)	0.09	0.10	0.09	0.08	0.06	0.08	0.09	0.07	0.06	0.06	0.06	0.08	0.01	0.3538	0.0042	0.1611
Sodium (mEq/L)	137.83	138.20	137.70	137.45	138.95	138.83	138.70	139.33	140.70	139.81	140.45	139.83	0.82	0.9527	<.0001	0.5818
Potassium (mEq/L)	4.51	4.36	4.23	4.40	4.48	4.34	4.35	4.46	4.61	4.28	4.11	4.54	0.14	0.2246	0.8436	0.665
Chloride (mEq/L)	101.12	100.63	99.50	99.63	100.50	100.63	99.75	100.38	100.25	100.75	100.12	100.37	0.55	0.4082	0.9007	0.6403
CO_2 (mEq/L)	31.49	32.12	30.74	33.37	30.37	29.62	31.37	32.12	30.41	28.74	32.62	30.37	2.13	0.5064	0.0382	0.048

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. Blood samples were collected on days -1, 14, and 28. The data presented are LSmeans of a treatment by day interaction. Differences considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

[2]Control: 0 ppm

[3]1: 62.5 ppm

[4]2: 125 ppm

[5]3: 250 ppm

[6]Aspartate transferase (Glutamic-oxaloacetic transaminase) (Units/Liter)

[7]Gamma-glutamyl transferase (Units/Liter)

Table 4 Effects of phytonutrient supplementation on enzymatic activities and metabolic flexibility in growing pigs.

	Day -1[1]				Day 14[1]				Day 28[1]				P-Value			
Variable	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	Control[2]	1[3]	2[4]	3[5]	SEM	Trt	Day	Trt·Day
Cytochrome C Oxidase Activity (umol/mg/min)	95.82^{AB}	59.00^{ABCDE}	71.22^{ABCDE}	95.10^{A}	78.04^{ABC}	72.51^{ABCD}	54.69^{CDE}	63.78^{BCDE}	49.17^{DE}	45.32^{E}	47.13^{DE}	50.81^{CDE}	6.48	0.05	<.0001	0.0041
Citrate Synthase Activity (umol/mg/min)	186.21^{A}	131.59^{ABC}	155.67^{ABC}	153.49^{ABC}	127.99^{BC}	165.32^{AB}	125.60^{ABC}	139.67^{ABC}	111.99^{C}	98.48^{C}	110.17^{C}	101.47^{C}	16.17	0.7	<.0001	0.0075
Metabolic Flexibility (%)	40.31	43.07	36.39	44.62	45.85	41.99	43.21	49.95	43.49	38.68	32.22	39.35	6.96	0.32	0.1321	0.8579

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) where they were fed ad libitum for 28 days. Skeletal muscle samples were obtained via biopsy from the longissimus dorsi (LD) on days -1, 14, and 28 for cytochrome c oxidase activity, citrate synthase activity, and metabolic flexibility. The data presented are LSmeans ± SEM. Differences considered significant when $P<0.05$, n=8 per group. LSmeans without a common letter are different at $P<0.05$.

[2]Control: 0 ppm

[3]1: 62.5 ppm

[4]2: 125 ppm

[5]3: 250 ppm

Table 5 Linear and quadratic responses to treatment in growing pigs at day 28.[1]

	Treatment[2]				Contrast[3]			
Variable	Control	1	2	3	Linear	R-Squared	Quadratic	R-Squared
Glucose (mg/dL)	97.33	92.96	88.89	92.78	N.S.	0.0443	N.S.	0.24506
Urea Nitrogen (mg/dL)	8.73	8.36	6.51	8.80	N.S.	0.0919	N.S.	0.3427
Creatinine (mg/dL)	0.98	1.05	0.98	0.96	N.S.	0.1467	N.S.	0.1543
Phosphorus (mg/dL)	7.46^{A}	7.10^{AB}	7.01^{ABC}	6.93^{ABC}	N.S.	0.2743	N.S.	0.3113
Calcium (mg/dL)	11.06	11.04	11.06	11.04	N.S.	0.0063	N.S.	0.0087
Magnesium (mg/dL)	1.64	1.59	1.47	1.61	N.S.	0.0988	0.0412	0.3800
Total Protein (g/dL)	5.95	5.92	5.85	5.94	N.S.	0.0562	N.S.	0.1576
Albumin (g/dL)	3.48	3.43	3.32	3.45	N.S.	0.0543	N.S.	0.2016
Globulin (g/dL)	2.45	2.49	2.51	2.46	N.S.	0.0114	N.S.	0.1029
AST (GOT) (U/L)[4]	42.62	37.53	36.79	40.68	N.S.	0.0086	N.S.	0.2518
GGT (U/L)[5]	35.29	34.42	28.29	31.42	0.0249	0.4158	N.S.	0.4308
Direct Bilirubin (mg/dL)	0.03	0.03	0.03	0.02	N.S.	0.0121	<0.0001	0.0956
Indirect Bilirubin (mg/dL)	0.06	0.06	0.06	0.08	N.S.	0.2711	<0.0001	0.3131
Sodium (mEq/L)	140.70	139.81	140.45	139.83	N.S.	0.1731	N.S.	0.1743
Potassium (mEq/L)	4.61	4.28	4.11	4.54	N.S.	0.1165	N.S.	0.3270
Chloride (mEq/L)	100.25	100.75	100.12	100.37	N.S.	0.0143	N.S.	0.0176
CO_2 (mEq/L)	30.41	28.74	32.62	30.37	N.S.	0.2033	N.S.	0.2683
Cytochrome C Oxidase Activity (umol/mg/min)	49.17^{DE}	45.32^{E}	47.13^{DE}	50.81^{CDE}	N.S.	0.0705	N.S.	0.13105
Citrate Synthase Activity (umol/mg/min)	111.99^{C}	98.48^{C}	110.17^{C}	101.47^{C}	N.S.	0.1194	N.S.	0.12377
Metabolic Flexibility (%)	43.49	38.68	32.22	39.35	N.S.	0.2250	N.S.	0.40862
Fat Percent BW (%)	12.79^{ABC}	12.83^{AB}	12.71^{ABC}	14.66^{A}	N.S.	0.3112	N.S.	0.35707
Lean Percent BW (%)	77.04^{C}	77.80^{C}	78^{C}	75.7^{C}	N.S.	0.2150	N.S.	0.33251
Fat Mass (kg)	6.98^{A}	6.49^{A}	6.35^{A}	7.80^{A}	N.S.	0.2154	N.S.	0.3811
Lean Mass (kg)	42.15^{A}	39.35^{B}	38.56^{B}	39.58^{AB}	N.S.	0.3329	0.0044	0.57493

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. The data presented represent treatment effect on day 28. Differences considered significant when $P<0.05$, n=8 per group.

[2]Control: 0 ppm; 1:62.5 ppm; 2:125 ppm; 3:250 ppm

[3]Assessed using Day 28 data.

[4]Aspartate transferase (Glutamic-oxaloacetic transaminase) (Units/Liter)

[5]Gamma-glutamyl transferase (Units/Liter)

N.S.: Not Significant

Table 6 Linear and quadratic responses to treatment in growing pigs at week 4.[1]

	Treatment[2]				Contrast[3]			
Variable	Control	1	2	3	Linear	R-Squared	Quadratic	R-Squared
BW[4] (kg)	51.50^{A}	47.40^{ABC}	46.20^{BCD}	49.20^{AB}	N.S.	0.15162	0.00230	0.54206
ADG[5] (kg/day)	0.99	0.87	0.85	0.96	N.S.	0.06684	0.04540	0.36726
Feed Intake (kg/day)	2.20	2.00	1.97	2.30	N.S.	0.12836	0.01240	0.45858
Feed:Gain (kg/kg)	2.15	2.14	2.36	2.43	0.02290	0.41426	N.S.	0.41749

[1]Pigs were randomly assigned one of four phytonutrient supplementation groups (Control; 0 ppm, 1; 62.5 ppm, 2; 125 ppm, 3; 250 ppm) fed ad libitum for 28 days. The data presented represent treatment effect on week 4. Differences considered significant when $P<0.05$, n=8 per group.

[2]Control: 0 ppm; 1: 62.5 ppm; 3: 250 ppm

[3]Assessed using week 4 data

[4]Body Weight

[5]Average Daily Gain

N.S.: Not Significant

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