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June 14, 2018

Dr. Geoffrey Wong
Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notification of GraINzyme® Phytase for Use in Swine Feed by Agrivida, Inc.

Dear Dr. Wong,

Under the Final Rule for the notification of self-determination of "Generally Recognized As Safe" (GRAS) for novel animal feed additives (21 CFR Parts 20, 25, 170 et al., Federal Register, vol. 81, No. 159; August 17, 2016) Agrivida, Inc. is hereby submitting a notification of the conclusion of Agrivida, Inc. that the use of the 6-phytase enzyme, GraINzyme® Phytase, in the feed of swine is GRAS. This enzyme releases phosphate groups from phytin and phytate that are present in plant based feed ingredients, thereby improving the availability of phosphorus in animal feeds.

Based upon scientific procedures and information, Agrivida, Inc. had previously concluded that the use of GraINzyme® Phytase in poultry feed is GRAS and the Center for Veterinary Medicine has reviewed information supporting this conclusion and had no further questions (AGRN#21) related to this conclusion. Agrivida, Inc. has conducted further scientific investigation of the safety and functionality of the GraINzyme® Phytase in swine, the results of which support our conclusion of the GRAS status of this product for use in swine feed.

Accompanying this letter is a CD that contains files in PDF format. One of these contains a description of the studies conducted and results that support Agrivida, Inc.'s conclusion on the GRAS status of GraINzyme® Phytase. A folder on the disk contains PDF files of the literature cited in the document for which web links are not available and that support the scientific principles underlying our conclusions on the GRAS status of GraINzyme® Phytase for use in swine.

The complete data and original information that are the basis of this GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours at our offices located at 78E Olympia Avenue, Medford, MA 01801.

Sincerely,

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

A phytase feed enzyme produced by *Zea mays* expressing a phytase gene derived from *Escherichia coli* K12

SUMMARY of DATA SUPPORTING a NOTIFICATION of GRAS STATUS for USE in SWINE FEED

Submitting Company:

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801

Submitted by:

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June 14, 2018

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1.0 Signed statements and certification

1.1. Submission of a GRAS notice

Agrivida, Inc. is hereby submitting a GRAS notice in accordance with §170.225(c) of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016) for a phytase enzyme for use in the feed of swine to improve the digestibility of phosphorus from phytic acid in feed.

1.2. Name and address of notifier

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801 USA
Tel: 781-391-1262

Person responsible for the dossier:
James Ligon, PhD
Agrivida, Inc.
VP, Regulatory Affairs and Stewardship
1023 Christopher Drive
Chapel Hill, NC 27517 USA
Tel: 919-675-6666; Email: jim.ligon@agrivida.com

1.3 Name of the notified substance

The substance that is the subject of this GRAS notice is a 6-phytase enzyme (E.C. 3.1.3.26) that is produced in the grain of *Zea mays*. The trade name of the phytase product is GraINzyme® Phytase.

1.4 Conditions of use of the notified substance

The GraINzyme® Phytase product is considered GRAS for use as a feed additive in the feed of poultry (AGRN #21, 2017). This GRAS notice is for the purpose of extending the use of GraINzyme® Phytase in the feed of swine in order to increase the availability of phytin bound phosphorous in the feed. The recommended inclusion rate of the GraINzyme® Phytase in swine feed is 500 FTU to 4,500 FTU/kg feed where one FTU (phytase activity unit) is the amount of enzyme that releases 1 µmole of inorganic phosphorus per minute from phytate.

1.5 Statutory basis for conclusion of GRAS status

The conclusion that the GraINzyme® Phytase enzyme is GRAS for use in swine feeds is based on scientific procedures in accordance with §170.30(a) and (b) of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016).

1.6 Substance is exempt from premarket approval

It is the opinion of Agrivida, Inc. that the GraINzyme® Phytase is exempt from the requirement for premarket approval under the Food, Drug and Cosmetic Act based on our conclusion that it is GRAS for its intended use in the feed of swine.

1.7 Data availability

The data that is the basis for the conclusion that the GraINzyme® Phytase is GRAS for its intended use will be made available to FDA either during or after its evaluation of the GRAS notice. Upon request of the FDA, Agrivida, Inc. will make all relevant data available for review or copying during customary business hours at its office in Woburn, MA. In addition, upon request by the FDA, Agrivida, Inc. will produce copies of requested information either in paper or suitable electronic form and provide these to the FDA.

1.8 Confidential business information in this GRAS notice

Agrivida, Inc. considers some information in this notice to be confidential business information under the Freedom of Information Act, 5 U.S.C 552. The information in this notice that is considered by Agrivida, Inc. to be confidential business information is identified by shaded text (e.g., CBI).

1.9 Certification

Agrivida, Inc. hereby certifies that to the best of its knowledge, this GRAS notice includes all relevant information, both favorable and unfavorable, that is pertinent to the safety and functionality of the GraINzyme® Phytase for its use in the feed of swine.

1.10 Signatory person

The following person will sign the GRAS notice on behalf of Agrivida, Inc.:

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

_____ Date: 14 June 2018

1.11 Authorization to send trade secrets

If necessary, Agrivida, Inc. authorizes FDA Center for Veterinary Medicine to send information from this notification, including information considered by Agrivida, Inc. to be trade secret or CBI, to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture.

2.0 Identity, method of manufacture, specifications and technical effect

2.1 Identification of the notified substance

The GraINzyme® Phytase product developed by Agrivida, Inc. is produced by genetically modified *Zea mays* (corn or maize). The gene that produces the phytase enzyme was derived from the native *E. coli* strain K12 *appA* phytase gene. Expression of the phytase gene is directed by endosperm and embryo specific monocot derived promoters such that the GraINzyme® Phytase is produced only in the grain of *Z. mays*. Detailed information about the production organism, enzyme, manufacturing process and safety of the GraINzyme® Phytase for use in poultry nutrition was submitted in the GRAS notice for the use of GraINzyme® Phytase produced by *Z. mays* in the feed of poultry (AGRN #21, 2017).

2.2 Method of manufacture

The Phy02 phytase is produced by maize event PY203 that was genetically engineered to contain copies of the *phy02* phytase gene under the regulation of monocot derived, seed specific promoters. This results in the production of the Phy02 phytase protein in the grain of maize with little or no production in the leaves, stalks, or other tissues. The method of production of the commercial Phy02 phytase product employs the same agronomic practices as is typically used for the production of maize grain. These include planting seed of maize event PY203 that contains copies of the Phy02 gene into soil in the spring once the soil temperature has reached the appropriate temperature for seed germination, management of the crop using common agricultural practices for the cultivation of maize that may include the application of chemical fertilizers and crop protection chemicals such as herbicides and insecticides that are approved for use on maize, and harvesting by mechanical maize harvesters with a sheller to produce whole maize grain. Alternatively, the Phy02 producing maize event PY203 can be grown in a greenhouse with controlled temperature using common practices for the cultivation of maize in a greenhouse. It is well recognized that using these practices it is possible to produce maize grain in a greenhouse that is nutritionally equivalent to that produced in a field environment.

The whole grain containing the Phy02 phytase is dried to a moisture content of less than 15% and is stored in dry, secure grain storage bins prior to being milled to a coarse maize meal (< 3 mm diameter). Once the Phy02 grain is milled it is packaged into a secure, labeled container that is either a double paper bag with sewn seams containing approximately 20 kg of product or a large heavy plastic tote containing 1 ton of product. The amount of Phy02 phytase produced in the grain of maize event PY203 is in the range of 4,000 to 7,000 units of phytase activity (FTU) per gram. It is expected that 100g to 1kg of the Phy02 phytase product is sufficient to treat one ton of animal feed in order to deliver an effective dose of phytase to improve phosphorus digestibility.

Since the Phy02 phytase product consists of milled maize grain containing the Phy02 phytase protein, its nutrient composition is the same as that of typical maize

grain. The addition of relatively small quantities of the Phy02 phytase product to typical corn/soy based diets will replace an equally small amount of the maize that is normally a component of the diet and this substitution will not alter the nutrient composition of the feeds.

3.0 Target animal exposure and safety factor calculation

The GraINzyme® Phytase product is currently GRAS for use in poultry feeds and the purpose of this GRAS notice is to extend its use to swine feeds. The recommended dose range of the product in swine feed is 500 to 4,500 FTU/kg feed. As described in §6.5 of this document the Phy02 phytase enzyme of GraINzyme® Phytase is substantially equivalent to the phytases in two commercial phytase products referred to herein as Commercial Phytase 1 and 2 (CP1 and CP2; Quantum® and Quantum Blue®, respectively). Since the GraINzyme® Phytase has the same enzymatic characteristics and is nearly identical to the phytase enzymes in these products, the NOAEL established for the CP1 phytase derived from a 90-day study with rats (EFSA, 2008) can be justifiably used to calculate the safety margin for GraINzyme® Phytase in swine. Based on the NOAEL of 2,000 mg TOS/kg bwt/day for the CP1 phytase (EFSA, 2008), equivalent to 462,000 FTU/kg rat body weight/day, and the typical daily intake of feed from the NRC feeding tables (NRC, 2012) and from the GraINzyme® Phytase swine tolerance study, the safety margins for swine were calculated and these are presented in Table 1. Using the growth and feed intake data from the NRC, the calculated safety margin for GraINzyme® Phytase fed at 4,500 FTU/kg feed is 1,621 based on a NOAEL for the equivalent of 462,000 FTU/kg body weight. Similarly, if the calculation is based on the pig body weight and feed intake data from the GraINzyme® Phytase tolerance study in swine (§6.4 of this document), a safety margin of 2,734 fold is determined.

A third approach to determining the safety margin of the GraINzyme® Phytase is by using the specific activity of the GraINzyme® Phytase of approximately 150 FTU/mg protein as described in Appendix 7 (§8.7.2.1 Determination of specific activity of Phy02). Using the NOAEL of 2000 mg TOS/kg bwt/day determined for the nearly identical CP1 phytase (EFSA, 2008) a safety margin of 1,786 is determined (Table 1).

In conclusion, using three different methods to determine the safety margin of the GraINzyme® Phytase, safety margins of 1,621, 2,734, and 1,786 were calculated for swine consuming feed treated with 4,500 FTU/kg of GraINzyme® Phytase. These different methods determined that the maximum daily intake of the GraINzyme® Phytase in swine based on a diet including 4,500 FTU GraINzyme® Phytase/kg feed is 285 FTU/kg body weight/day for weaned piglets or 1.9 mg phytase/kg body weight/day (Table 1). Based on this it is clear that the inclusion of GraINzyme® Phytase at up to 4,500 FTU/kg of swine feed is well within a reasonable level of safety relative to the NOAEL established for the nearly identical CP1 phytase enzyme.

Table 1. Safety margin calculations for GraINzyme® Phytase at 4,500 FTU/kg feed based on: 1) the NRC feeding tables for weaned piglets (NRC, 2012), 2) the results of the GraINzyme® Phytase tolerance study after 43 days of feeding, and 3) the established NOAEL for CP1 phytase of 2000 mg TOS/kg body weight/day and the specific activity of GraINzyme® Phytase of approximately 150 FTU phytase activity/mg GraINzyme® Phytase protein.

Calculation Basis		Body weight (kg)	Typical Feed Intake (kg feed/day)	GraINzyme® Phytase highest recommended dose	Highest expected phytase intake		Safety Margin
				FTU/kg feed	FTU/day	FTU/kg bwt/day	
1	Weaned piglets (NRC, 2012)	15.0	0.95	4,500	4,275	285	1,621
2	GraINzyme® Phytase Tolerance Study	27.7	1.04	4,500	4680	169	2,734
3	GraINzyme® Phytase Specific Activity	27.7	1.04	4,500	31.2 mg phy/day	1.12 mg phy/kg bw/day	1,786

4.0 Self-limiting levels of use

The GraINzyme® Phytase product is not intended for inclusion in human food and it will be marketed in labeled containers that state that the product is to be used only for inclusion in poultry and swine feeds. Therefore, according to §170.240 of 21 CFR Parts 20, 25, 170 *et. al* (Federal Register, Vol. 81, No. 159, August 16, 2016) there is no requirement to establish a self-limiting level of use for the GraINzyme® Phytase product.

The GraINzyme® Phytase is produced by maize genetically engineered with the *phy02* phytase gene derived from *Escherichia coli* strain K12 to produce the GraINzyme® Phytase in the grain. Typically grain derived from the maize production host contains between 4,000 and 7,000 FTU/g of grain. Other than the presence of the GraINzyme® Phytase, the GraINzyme® Phytase containing maize grain is nutritionally equivalent to normal maize grain that is used as a major feed ingredient in the feed of swine. The presence of the GraINzyme® Phytase in maize grain does not affect the taste, palatability or other organoleptic properties of the grain. Therefore, the maximum amount of GraINzyme® Phytase product that might be theoretically consumed by an animal is equal to the total amount of maize meal included in the feed. In the case of swine feed based on a maize/soybean meal diet, the maize meal typically comprises between 50 and 60% of the total feed. Accordingly, the maximum amount of GraINzyme® Phytase that might be consumed by swine is equivalent to the amount of GraINzyme® Phytase contained in the maize meal of the diet assuming that all of the maize meal was GraINzyme® Phytase product. However, since the GraINzyme® Phytase product will be marketed in either 20 kg bags or 1 ton totes with a product label that directs the user to add the appropriate amount of the product when mixing the feed, the likelihood that a feed would be prepared using the GraINzyme® Phytase product to replace all of the maize meal in the diet is very remote. Assuming that a 1 ton tote of GraINzyme® Phytase product was used in place of normal maize meal to make a swine feed, the maximum amount of feed that could be produced would be less than 2 tons. In the unlikely event that this transpired, the resulting feed would not be expected to cause adverse effects on the swine that consume it. Phytase is an enzyme whose only enzymatic activity is the sequential removal of phosphate moieties from phytic acid with the ultimate production of inositol. If large amounts of phytase were included in a feed it would be expected that most or all of the phytic acid in the diet would be converted to inositol with the concomitant release of phosphate and once all phytic acid had been converted to inositol there would be no substrate for the phytase which would thereafter cease to have any function in the gastrointestinal tract. One study has been reported in which a corn/soybean meal based feed was treated with high levels of the maize expressed NOV9X phytase that is the phytase contained in the phytase product CP1. The swine in these studies that received up to 49,500 FTU NOV9X phytase/kg of feed demonstrated good performance without any signs of toxicity (Nyannor *et al*., 2007). The GraINzyme® Phytase is substantially equivalent to the CP1 phytase (§6.5).

Based on the above, it is expected that if in the unlikely event that grain from GraINzyme® Phytase expressing maize were to be substituted for all of the maize in a typical maize/soybean meal swine diet that it would not adversely affect the performance of the swine nor would it cause any safety concerns for the animals. Additionally, the meat derived from such animals would not be expected to contain GraINzyme® Phytase protein or to be unsafe for human consumption.

5.0 Experience based on common use prior to 1958

The GraINzyme® Phytase product was not in use prior to 1958 and Agrivida, Inc.'s conclusion of GRAS status for the use of this product in swine feed is not based on its common use prior to 1958. Agrivida's conclusion that the GraINzyme® Phytase product is GRAS for use in swine feed is based on scientific principles. Therefore, the requirement to provide evidence of its use prior to 1958 is not applicable.

6.0 Safety and Functionality of the GraINzyme® Phytase in Swine Feed

6.1. Safety of the maize production host

Maize is the largest cultivated crop in the world and is widely cultivated in most areas of the world. In 2017/18 the global production of maize grain was 1,317 million metric tons (MT), including the 384 million MT produced in the U.S. from planting over 91 million acres (USDA FAS, 2018). In the U.S., maize is grown in almost every state.

In industrialized countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and (2) as a raw material for wet- or dry-milled processed products such as high fructose maize syrup, oil, starch, glucose, dextrose and ethanol. By-products of the wet- and dry- mill processes are also used as animal feed. These processed products are used as ingredients in many industrial applications and in human food products. Most maize produced is used as animal feed or for industrial purposes, but maize remains an important food staple in many developing regions, especially sub-Saharan Africa and Central America, where it is frequently the mainstay of human diets (Morris 1998).

Maize is a very familiar plant that has been rigorously studied due to its use as a staple food/feed and the economic opportunity it brings to growers. The domestication of maize likely occurred in southern Mexico between 7,000 and 10,000 years ago (Goodman, 1988). While the putative progenitor species of maize have not been recovered, it is likely that teosinte played an important role in contributing to the genetic background of maize. Although grown extensively throughout the world, maize is not considered a persistent weed or a plant that is difficult to control. Maize, as we know it today, cannot survive in the wild because the female inflorescence (the ear) is covered by a husk thereby restricting seed dispersal, it has no seed dormancy, and is a poor competitor in an unmanaged ecosystem. The transformation from a wild, weedy species to one dependent on humans for its survival most likely evolved over a long period of time through plant breeding by the indigenous inhabitants of the western hemisphere. Today, virtually all maize varieties grown in the U.S. are hybrids, a production practice that started in the 1930's (Wych, 1988). Maize hybrids are developed and used based on the positive yield increases and plant vigor associated with heterosis, also known as hybrid vigor (Duvick, 1999).

Conventional plant breeding results in desirable characteristics in a plant through the unique combination of genes already present in the plant. However, there is a limit to genetic diversity with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of highly desirable traits that are profitable to growers.

Given the long history of the safe use of maize grain and its by-products and maize silage as food and feed ingredients, maize and its grain are considered to be

generally recognized as safe (GRAS). Therefore, it is concluded that maize and grain produced by it are safe for consumption by humans and animals and that its cultivation does not present any threats to the environment. Pariza and Foster (1983) developed a decision tree to determine the safety of food and feed enzyme preparations that was updated by Pariza and Johnson (2001) and Pariza and Cook (2010). A key tenet of this decision tree is that since enzymes by themselves are not toxic, the primary consideration of the safety of a food enzyme preparation is the safety of the production organism. In the case where the production organism is a plant that has a long history of safe use as a food ingredient, the enzyme preparation from such a plant is considered to be safe and nontoxic. Based on the decision tree for establishing the safety of food enzyme preparations by Pariza and coauthors (Pariza and Foster, 1983; Pariza and Johnson; 2001; Pariza and Cook, 2010) and on the established long history of safe use of maize for food and feed, the Phy02 enzyme preparation that is the subject of this document is considered to be safe for its intended use in animal feed.

6.1.1 Source of the maize line

The *phy02* genes responsible for the production of Phy02 phytase in maize were initially transformed into a maize line named (b) (4) maintained by the U.S. National Plant Germplasm System (NPGS, 1995) that is also known by the name (b) (4). The resulting T_0 plants containing the *phy02* genes were subsequently crossed with a second maize line, (b) (4) (b) (4) Several other backcrosses with the *phy02* gene progeny were made to maize line (b) (4) in order to increase the percentage of the genome from this line in the Phy02 producing lines.

6.1.2 Origin of the gene encoding phytase Phy02

The native *E. coli appA* phytase gene was optimized using Gene Site-Saturation Mutagenesis (Short, 2001) to generate a gene encoding the NOV9X phytase with increased thermotolerance. Thermotolerance is a desirable trait for commercial feed enzymes since many animal feeds are produced by a pelleting process that involves a heat treatment that inactivates thermolabile enzymes. The Phy02 phytase gene was derived from the NOV9X gene by further optimization to create additional specific amino acid substitutions for improved thermotolerance and sensitivity to digestion in the gastric environment. The NOV9X phytase is the active phytase in the commercial phytase product CP1 that is produced by the yeast *Pichia pastoris* and that was approved by FDA-CVM for inclusion in animal diets since 2008.

6.1.3 Characteristics of the Phy02 Expression Construct.

A transformation gene cassette containing three copies of the Phy02 phytase gene, each with a different monocot derived promoter and (b) (4) terminator was constructed in plasmid (b) (4). The genetic elements of plasmid (b) (4) that was used to transform maize are shown in Figure 1. The individual genetic elements within plasmid (b) (4) are described in Table 2. This plasmid was transformed by *Agrobacterium*-mediated transformation into immature maize

embryo tissue as described by Negrotto *et al.* (2000) and transformants were selected based on the presence of the plant selectable marker *manA* gene on the transformed DNA fragment that encodes the enzyme phosphomannose isomerase (PMI). The PMI enzyme enables maize tissue to grow on mannose as a sole source of carbon (Negrotto *et al.*, 2000). The *pmi* gene has been used as a selectable gene in several genetically modified maize varieties that have completed review by the USDA, FDA, and EPA for food and feed safety, including maize events 5307 and Mir604 maize with resistance to corn rootworm, lepidoptera resistant Mir162, and α-amylase expressing 3272, all products of Syngenta Seeds. Maize plants containing the Phy02 phytase gene were cultivated and were demonstrated to produce more than 4000 units of phytase activity (FTU) per gram of grain. The transformation event chosen as a development candidate was designated event PY203.

Figure 1. Plasmid map of (b) (4) that was used in the transformation of maize to create the phytase producing event PY203.

Table 2. Description of the genetic elements in the (b) (4) containing three copies of the Phy02 phytase gene that was used to transform maize and generate event PY203.

(b) (4)

6.1.4 Genetic characterization of maize event PY203

The genetic characterization of maize event PY203 and the insertions containing the *phy02* genes was described in detail in the GRAS notice for the use of GraINzyme® Phytase in poultry feed (AGRN #21, §2.4 Characterization of the maize Phy02 expression host, pg. 15). Southern hybridization experiments demonstrated that event PY203 contains two T-DNA insertions that were designated locus (b) (4) and locus (b) (4) (AGRN #21, §2.4.1 Determination of number of DNA insertions, pg. 15-17) and confirmed the absence of DNA fragments from the transformation vector backbone in the genome of PY203 (AGRN #21, §2.4.2 Screening for plasmid backbone fragments, pg. 17-18). The genomic DNA of these two genetic loci, including the complete T-DNA and genomic maize flanking DNA were sequenced and characterized (AGRN #21, §2.4.3, pg. 18-20). Analysis of the sequence revealed that locus (b) (4) contains a complete T-DNA insertion containing three copies of the *phy02* gene and the *pmi* selectable marker gene and that it is located in maize chromosome 8. Similar analysis of locus (b) (4) revealed that it is truncated and contains two copies of the *phy02* gene but is lacking the third copy and the *pmi* gene. Locus (b) (4) was determined to be located in maize chromosome 2. The genetic stability of the two *phy02* gene loci in the Phy02 phytase producing maize event PY203 were evaluated by two different methods in four different backcross (BC) generations in an inbred genetic background (AGRN #21, §2.4.4 Genetic stability of the inserts over multiple generations, pg. 20-23.) and the results demonstrated that both insertions were stable over the four backcross generations that were examined. The results of the genetic characterization of the two *phy02* gene containing loci in maize event PY203 did not reveal any issues or concerns regarding the safety of consumption of grain derived from event PY203.

6.1.5 Taxonomy of *Zea mays*

The taxonomy of maize is described by OECD (2003) as follows:
Family: Poaceae
Subfamily: Panicoideae
Tribe: Maydeae

Western Hemisphere:
Genus *Zea*[1]
Section *ZEA*

- *Zea mays* L. (maize)
- *Zea mays* subsp.*mays* (L.) Iltis (maize, $2n^2$ =20)
- *Zea mays* subsp. *mexicana* (Schrader) Iltis (teosinte, 2n = 20))
  - race Nobogame[3]
  - race Central Plateau[3]
  - race Durango[3]
  - race Chalco[3]
- *Zea mays* subsp. *parviglumis* Iltis and Doebley (teosinte, 2n = 20)
- var. *parviglumis* Iltis and Doebley (=race Balsas)
- var. *huehuetenangensis* Doebley (=race Huehuetenango)

[1]Iltis and Doebley, 1980; Doebley, 1990. [2]diploidy number. [3]Sánchez-González *et al*., 2018.

6.1.6 History of safe use of *Zea mays*

There is a long history of safe use of maize for food and feed that is described in §6.1.

6.1.7 Absence of toxicity

Grain derived from maize has been used as food and feed for thousands of years without incident. The history of safe use of maize grain is described in and §6.1 above. Based on the long history of safe use of maize, it is accepted to be GRAS and to be nutritious and nontoxic.

6.1.8 Summary

As a staple food and feed crop for thousands of years, maize is widely considered to be safe for food, feed, and the production of food and feed ingredients.

6.2 Safety of *Escherichia coli* K12

6.2.1 Introduction

This discussion addresses the safety of *E. coli* K12 strain MG1655, which is the donor organism of the phytase gene (CGSC, 1997). It is worth noting that only the coding sequence of a single gene (i.e. the *appA* phytase gene) was used from *E. coli* K12 strain to produce the *phy02* gene that was used to transform maize.

6.2.2 Taxonomy of *E. coli*.

Escherichia coli has been used extensively in studies of physiology, genetics, and biochemistry, making this species one of the most well studied bacterial species. *Escherichia coli* belongs to the family Enterobacteriaceae and is ubiquitous in water, soil, and the normal intestinal flora in humans and other animals (Bettelheim, 1992). Enterobacteriaceae are Gram-negative, oxidase-negative, straight, rod-shaped bacteria that do not produce spores. They are chemoorganotrophic and are capable of both respiratory and fermentative metabolism. Growth temperatures range from 22-39ºC. Currently, there are 29 recognized genera and over 100 named species of Enterobacteriaceae (Brenner, 1992).

6.2.3 Laboratory use of *E. coli* K12.

E. coli strains have been used for the last 70 years in the study of bacterial physiology and genetics. Historically, wild-type strain K12 was used in early studies on conjugation and recombination (Swartz, 1996). The use and study of strain K12 continued to predominate due to its use in the study of recombination and the generation and mapping by conjugation of a large number of mutants in metabolic pathways that aided both the studies of bacterial genetics and physiology. Since *E.*

coli K12 has been used extensively in research and in many laboratories for decades without causing any harm, *E. coli* K12 is generally recognized as safe.

6.2.4 Safety assessment of *E. coli* K12.

Although there has been no indication over the past 70 years of intensive laboratory study that strain K12 has the ability to cause disease or has toxigenic potential, it has been only recently that studies in regard to this issue have been carried out.

These studies have focused primarily on the determination of the presence or absence of known virulence factors, i.e., properties of an organism that may contribute to its pathogenic potential, since in recent years it has become apparent that certain *E. coli* strains clearly have the potential to cause disease.

In a study of *E. coli* strains including representatives of the K12 strain, polymerase chain reaction (PCR) amplification demonstrated the absence of defined virulence genes that are present in known pathogenic isolates of this genus (Kuhnert, 1997). The authors concluded that the K12 strains commonly used in the laboratory are devoid of virulence factors and should be considered nonpathogenic.

A more direct study of the pathogenic potential of K12 strains was conducted using both a BALB/c mouse and chick gut model. In this study, the strains were found to be unable to express long-chain lipopolysaccharide (O-antigen) and were serum-sensitive (i.e. susceptible to complement killing). In addition, they were unable to persist or survive in selected mouse tissues or the gut. In the chick model, the strains were unable to invade the spleen, which is a hallmark of *E. coli* strains able to cause systemic infections. The authors concluded that K12 strains do not possess the recognized pathogenic mechanisms and should be considered nonpathogenic (Chart, 2000).

As mentioned above, *E. coli* K12 was the predominant organism of choice for recombinant DNA research because of the large amount of information about recombination and biochemical genetics that was developed using this strain. This information resulted in the NIH Guidelines (prepared by the National Institute of Health) listing strain K12 as safe for recombinant use, as detailed in Appendix C-II of the NIH guidelines (NIH, 2013).

In summary, the following demonstrates that *E. coli* K12 is officially recognized as, and considered a safe organism with no demonstrated toxigenic or pathogenic properties, including:

- The long-term use of *E. coli* K12 in numerous laboratories with no reports of illness or disease as a result of its use;

- The absence of genes encoding defined virulence factors as determined by PCR and other molecular methods;

- The lack of pathogenic potential in both a mouse and chick animal model; and

- The inclusion of this strain in the RG1 classification by the NIH Office of Biotechnology Activities and the Recombinant DNA advisory committee.

6.3 Safety of human consumption of meat produced by animals treated with GraINzyme® Phytase

The meat derived from animals that consume feed treated with GraINzyme® Phytase is safe for human consumption and does not present any human safety concerns. The GraINzyme® Phytase is an enzyme and enzymes are proteins. The dietary fate of the GraINzyme® Phytase in animals that consume feed treated with it is the same as that of all other proteins in the animal's diet that are digested into the constituent amino acids of the dietary proteins. As part of an Early Food Safety Evaluation for the GraINzyme® Phytase that was submitted to FDA/CFSAN (FDA/CFSAN, 2015), Agrivida, Inc. demonstrated that the GraINzyme® Phytase enzyme is sensitive to digestion in a simulated gastric environment. Therefore, the GraINzyme® Phytase is expected to be digested in the gastro-intestinal tracts of animals and is not expected to be absorbed intact into the blood of animals that consume it or to be deposited into the tissues of the animals, including the meat. The safety of phytase feed additives for humans that consume meat from animals that consume feed treated with phytases is further supported by the fact that phytases have been included in the feed of swine for decades without any adverse effects on human safety.

6.4 Tolerance study with GraINzyme® Phytase in weaned piglets

A study to demonstrate tolerance of the GraINzyme® Phytase in swine was conducted under Good Laboratory Practices (GLP) by (b) (4)). In this study 40 weaned piglets were divided randomly into 20 pens with two piglets in each pen. The pigs were fed a standard corn-soybean meal based, unpelleted, mash feed that met all nutrient requirements of the NRC (NRC, 2012) including a pre-starter feed from days 0 – 13 and a starter feed from day 14 to the end of the study at day 43. The feed provided to pigs in 10 pens was amended by the addition of 60,000 FTU GraINzyme® Phytase/kg while the pigs in the other 10 pens were provided feed without phytase. The body weight of individual pigs and the weight of feed consumption by pen were determined at days 0, 13 and 43 of the study. At 43 days, blood samples were collected and the pigs were euthanized and necropsied. The blood samples were analyzed by the (b) (4) for albumin, glucoses, phosphorus, alanine transaminase, creatine phosphokinase, red blood cells, hematocrit, mean corpuscular volume, blood platelets, white blood cells, hemoglobin, neutrophils, eosinophils, basophils, lymphocytes, and monocytes. The final report from this study is presented in Appendix 1. The phytase units (FTU/kg) were measured in

the feeds and it was determined that the prestarter and starter feeds contained approximately 45,000 FTU/kg rather than the target of 60,000 FTU/kg feed (Appendix 2).

The pigs in both the control and GraINzyme® Phytase treated groups were generally healthy and grew well during the course of the study. There were no statistically relevant differences (P>0.05) between the two groups of pigs in body weight, average daily weight gain, average daily feed intake or feed efficiency (G:F) throughout the study (Table 3).

Table 3. Growth performance of pigs in the control and GraINzyme® Phytase treated groups of pigs from day 0 to day 43 and at stages of the experiment including 0-13, 13-43, and 0-43 days, including body weight (BW), average daily weight gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F).

	Day 0 BW	Day 13 BW	Day 0-13 ADG	Day 0-13 ADFI	Day 0-13 G:F
Unit	lb	lb	lb/d	lb/d	lb:lb
Control	10.29	17.39	0.546	0.736	0.738
GraINzyme	10.16	17.22	0.543	0.723	0.751
SEM	0.05	0.56	0.045	0.042	0.030
P Values					
Treatment	0.12	0.84	0.96	0.82	0.77
Block	0.0001	0.35	0.92	0.95	0.52
		Day 43 BW	Day 13-43 ADG	Day 13-43 ADFI	Day 13-43 G:F
Unit		lb	lb/d	lb/d	lb:lb
Control		61.05	1.455	2.320	0.628
GraINzyme		61.07	1.462	2.349	0.623
SEM		1.50	0.035	0.063	0.008
P values					
Treatment		0.99	0.90	0.75	0.66
Block		0.25	0.23	0.62	0.13
			Day 0-43 ADG	Day 0-43 ADFI	Day 0-43 G:F
Unit			lb/d	lb/d	lb:lb
Control			1.181	1.841	0.641
GraINzyme			1.184	1.857	0.637
SEM			0.035	0.055	0.007
P values					
Treatment			0.95	0.84	0.70
Block			0.41	0.75	0.08

A comparison of the results of hematological analyses of pigs in the control and GraINzyme® Phytase treated groups shows that there were no statistically relevant (P>0.05) differences between the groups except in the case of alanine aminotransferase (Table 4). For alanine aminotransferase the GraINzyme® Phytase

treated group was higher than that of the control group, but both values were within the range considered to be normal for 9 week old pigs. Compared to the normal ranges for these analytes in 9 week old pigs as defined by the Iowa State University, Dept. of Veterinary Pathology (Ames, IA) (ISU, 2017) and the Merck Manual (Merck, 2017a and 2017b), both the control and GraINzyme® Phytase treated groups had phosphorus values that were slightly higher than the normal ranges for this element. The creatine kinase level in the control group was slightly higher than the upper limit of the normal range from ISU. In the case of hematocrit and mean platelet volume, the values for the control and GraINzyme® Phytase treated groups were statistically equivalent and slightly above the upper limit of the reference range whereas the mean cell hemoglobin concentration was slightly lower for both groups. Since there were no statistically relevant differences between the control and GraINzyme® Phytase treated groups for all analyses except alanine aminotransferase and since only a few analytes for both the control and treated groups were slightly out of the ranges considered to be normal for pigs of this age, these results support the conclusion that the inclusion of GraINzyme® Phytase in feed up to 45,000 FTU/kg is safe and does not impede the healthy growth of pigs.

Table 4. Results of hematological analyses of pigs at 43 days. As a reference, normal ranges for these analytes as reported by the Department of Veterinary Pathology, Iowa State University (Ames, IA; ISU, 2017) for 9-week-old pigs and by Merck (2017a and 2017b) are also presented.

	Glucose	ALT	ALB	P	CK
Units	mg/dl	IU/L	gm/dl	mg/dl	IU/L
ISU Ref. Intervals	65-150	25-90	3.0-4.5	4.5-9.0	100-2500
Merck Ref. Intervals	85-150	31-58 U/L	1.9-3.9	5.3-9.6	2.4-22.5 U/L
Control	116.5	49.90	3.790	10.69	2859
GraINzyme	113.8	57.35	3.760	10.11	2152
SEM	2.0	1.92	0.074	0.24	615
P Values					
Treatment	0.36	0.023	0.78	0.12	0.44
Block	0.74	0.81	0.97	0.86	0.86

ALT = Alanine aminotransferase; ALB = Albumin; P = Phosphorus; CK = Creatine Kinase

	WBC	Neut	Lymp	Mono	Eos	Baso	Luc	RBC
Units	$x10^3$/ul							
ISU Ref. Intervals	11.4-28.9	2.0-10.4	5.3-17.9	<3.7	<1.3	<0.4	NA	5.88-8.19
Merck Ref. Intervals	11-22	2-15	3.8-16.5	<1	<1.5	<0.5	NA	5-8
Control	15.55	4.69	9.22	0.870	0.653	0.0725	0.115	7.116
GraINzyme	15.42	4.11	9.39	1.064	0.683	0.0735	0.102	7.122
SEM	0.83	0.26	0.56	0.108	0.052	0.0102	0.017	0.083
P Value								
Treatment	0.91	0.19	0.83	0.23	0.70	0.95	0.61	0.96
Block	0.94	0.33	0.89	0.89	0.41	0.75	0.58	0.71

WBC = White Blood cell; Neut = Neutrophil; Lymp = Lymphocyte; Mono = Monocyte; Eos = Eosinophil; Baso = Basophil; Luc = Absolute Leukocyte; RBC = red blood cells

	Hemo	HCT	MCV	MCH	MCHC	RDW	Plate	MPV
Units	gm/dl	%	fl	pg	gm/dl	%	$x10^3$/ul	fl
ISU Ref. Intervals	11.2-14.7	32.3-42.6	47.5-59.2	16.3-20.6	33.3-35.8	8.0-15.0	119-523	6.5-11.9
Merck Ref. Intervals	10-16	36-43	50-68	17-21	30-34	NA	200-500	NA
Control	13.19	46.51	65.40	18.53	28.34	15.20	221.8	12.74
GraINzyme	12.96	45.90	64.45	18.21	28.25	14.86	221.3	12.05
SEM	0.16	0.59	0.52	0.20	0.13	0.23	17.9	0.58
P Value								
Treatment	0.33	0.48	0.23	0.28	0.66	0.32	0.98	0.42
Block	0.35	0.65	0.43	0.68	0.54	1.00	0.25	0.46

Hemo = Hemoglobin; HCT = Hematocrit; MCV = mean cell volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration; RDW = Red blood cell distribution width; Plate = Platelets; MPV = Mean platelet volume

6.5 Substantial Equivalence of Phy02 Phytase to Two Commercial Phytases

Several commercial phytase enzymes are derived from the AppA phytase of *Escherichia coli*. These include CP1 (Quantum®) and CP2 (Quantum Blue®) (AB Vista), Phyzyme® (DuPont), and OptiPhos® (Huvepharma). CP1 phytase was derived from the native AppA phytase of *E. coli* by Gene Site Saturation Mutagenesis (Short, 2001) to generate an enzyme with increased thermotolerance. Thermotolerance is a desirable trait for commercial feed enzymes since many animal feeds are produced by a pelleting process that involves a heat treatment that inactivates thermolabile enzymes. CP1 was first used commercially as a phytase feed additive for poultry in 2007. Further modifications to the gene encoding CP1 phytase (NOV9X) were developed to further improve performance of the enzyme and this product, named CP2, was introduced into the marketplace in 2012 and it is currently ranked second in the global phytase feed enzyme market (Harvey, 2018). The Phy02 phytase gene was derived from the NOV9X gene by further optimization to create additional specific amino acid substitutions for improved thermotolerance and sensitivity to digestion in the gastric environment. Based upon information presented herein, the Phy02 phytase is substantially equivalent to the CP1 and CP2 phytases that have been used commercially as feed additives for poultry and swine for over ten years.

At the amino acid level, the Phy02 phytase shares a high level of identity with both the CP1 and CP2 phytases (Figure 2). The NOV9X phytase has 8, and the Phy02 phytase has 16, amino acid substitutions relative to the AppA phytase from *E. coli* that consists of 410 amino acids (Table 5). The Phy02 phytase differs from the NOV9X phytase by 12 amino acids and the two phytases share 97.1% amino acid identity. The Phy02 phytase differs from the CP2 phytase by 13 amino acids with 96.8% amino acid identity between these two phytases (Table 5). These minor sequence variations are smaller than the variations observed between *E. coli* derived phytases and other phytases derived from other organisms (e.g., the *Aspergillus niger* phytase marketed as Natuphos® and the *Peniophora lycii* phytase marketed as Ronozyme®). Because the CP1 phytase has been shown to be substantially equivalent to these more divergent enzymes, which collectively have already been shown to be safe and efficacious in swine (Guggenbuhl, et al., 2007), it is expected that other *E. coli* variant enzymes will be at least substantially equivalent to the CP1 phytase (Pariza and Cook, 2010) and therefore also safe and efficacious in swine.

The Phy02 phytase produced in three typical product batches of maize event PY203 has been characterized as described in Appendix 7. All results and data support the conclusion that the Phy02 phytase enzyme produced in maize is the expected size based on its gene coding sequence and that the Phy02 enzyme is substantially equivalent to the CP1 and CP2 phytases. The characterization demonstrates that the Phy02 phytase produced in maize contains the expected amino acid sequence and it is not glycosylated (Appendix 7). Examination of Phy02 phytase for the potential to

catalyze other enzymatic reactions than the removal of phosphate from phytate demonstrated that it is primarily a phytase with no capability of catalyzing other enzymatic reactions (Appendix 7).

Figure 2. Alignment of the amino acid sequences of the *E. coli* AppA, CP1 (Quantum®), CP2 (Quantum Blue®) and Phy02 phytases. The amino acid sequences of the mature *E. coli* AppA (Accession no. EFE63517), CP1, CP2, and Phy02 phytases are aligned using the Clustal W (v. 1.83) multiple sequence alignment protocol. Amino acids that differ from the AppA sequence are shown in bold red colored font and the amino acids that do not vary among all four phytases are indicated as asterisks beneath the alignment. The consensus phytase active site (RHGxRxP) is underlined.

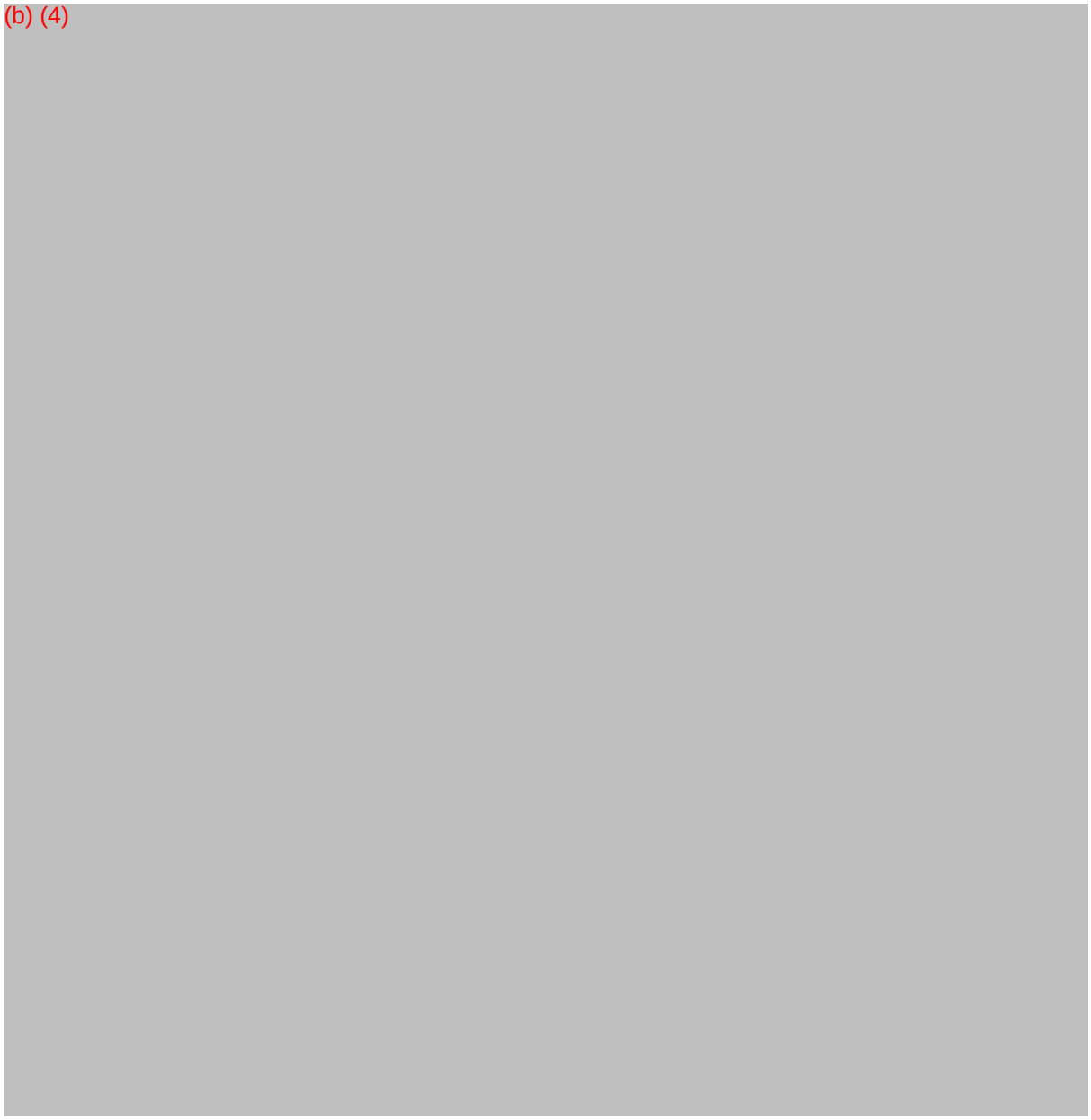


Table 5. Comparison of the amino acid sequences of the *E. coli* AppA, CP1, CP2, and Phy02 phytases. The number of different amino acids and percent amino acid identity (in parentheses) among these phytases are presented.

Phytase	AppA	CP2	CP1	Phy02
AppA	0 (100%)	17 (95.9%)	8 (98.0%)	16 (96.1%)
CP2	17 (95.9%)	0 (100%)	11 (97.3%)	13 (96.8%)
CP1	8 (98.0%)	11 (97.3%)	0 (100%)	12 (97.1%)
Phy02	16 (96.1%)	13 (96.8%)	12 (97.1%)	0 (100%)

Agrivida, Inc. has demonstrated that the apparent size of the Phy02 phytases produced in maize event PY203 and in a microbial host are identical and of the expected size. Protein extracts were prepared from three typical product batches of Phy02 phytase consisting of ground corn meal. The production of the three product batches is described in Appendix 7. A sample of purified Phy02 produced by a microbial host and the protein extracts from the three typical Phy02 product batches were analyzed by SDS-PAGE followed by staining with Coomassie-Blue. The results demonstrate that the three Phy02 product batches contain a prominent protein of the same size as the purified Phy02 protein that is not present in protein extracts of grain from a conventional control variety of maize (Figure 3).

Agrivida, Inc. has also demonstrated that the Phy02 and CP1 phytases have the same apparent molecular size. Purified Phy02 phytase protein was prepared from grain of maize event PY203 and was similarly compared to purified CP1 phytase by SDS-PAGE analysis (Figure 4). The apparent sizes of both the Phy02 and CP1 phytases were demonstrated to be approximately 46,000 kDa, the expected size for both proteins.

Figure 3. Coomassie-Blue stained SDS-PAGE gel containing protein extracts from three Phy02 phytase product batches (AV_Phy02_0043, #43; AV_Phy02_0049, #49; and AV_Phy02_0050, #50), extract from grain of a conventional, non-phytase engineered maize variety (Wild-type), and purified Phy02 phytase protein produced by a microbial production host (Microbial). Protein size markers were run in the left lane and their associated sizes are indicated on the left of the gel.

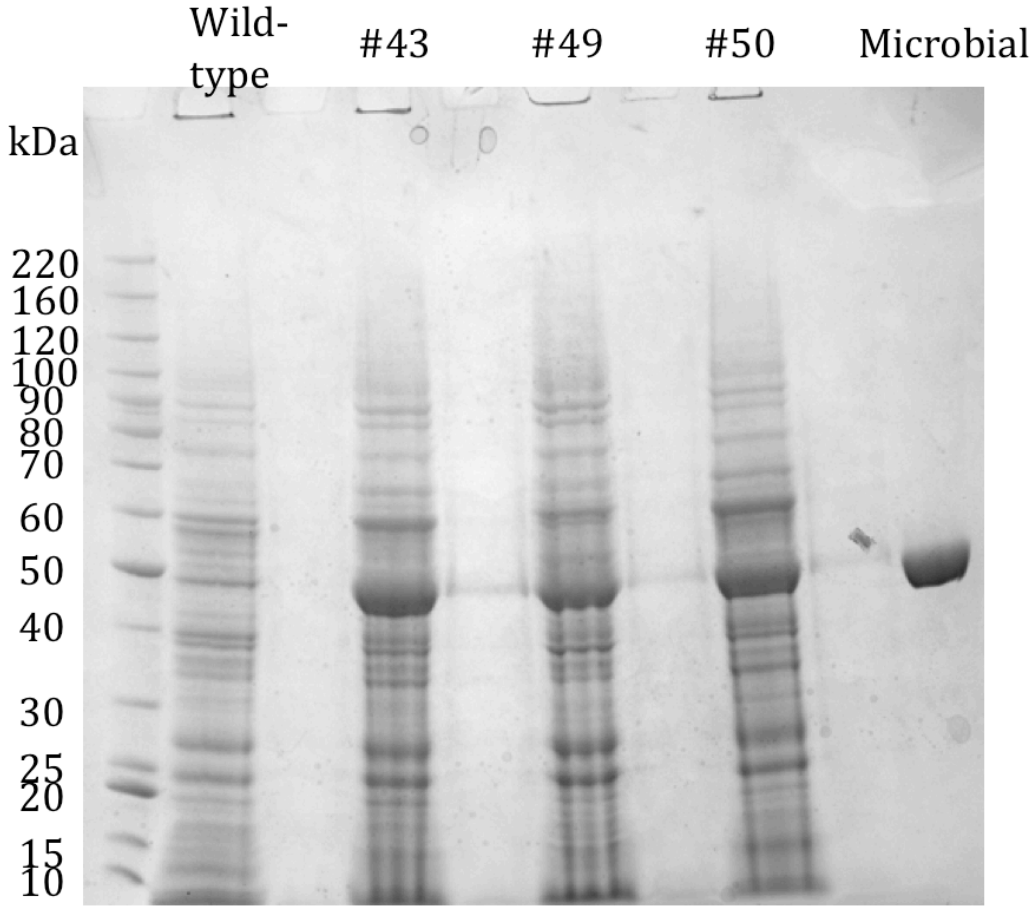
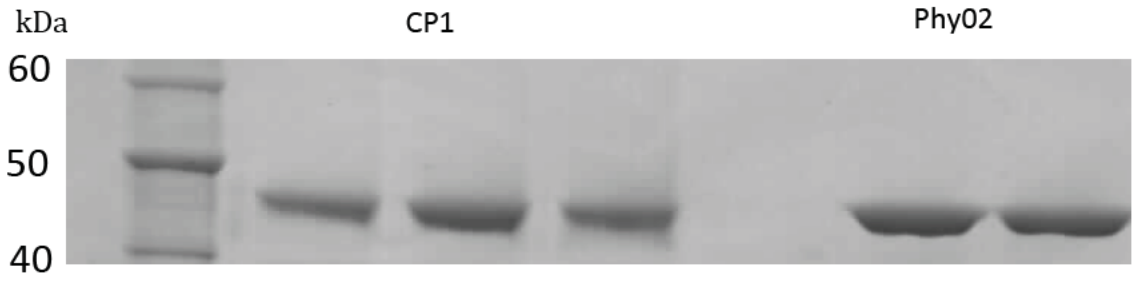


Figure 4. Coomassie-Blue stained SDS-PAGE gel containing purified samples of CP1 phytase (lanes 2, 3, and 4) and the Phy02 phytase (lane 6 and 7). Protein molecular size standards of 40, 50, and 60 kDa are shown in lane 1.



The near identity of the Phy02, CP1, and CP2 phytases at the amino acid level is reflected in nearly identical enzyme kinetic characteristics of these three phytases for the enzymatic removal of phosphate from phytate (Table 6). The measured k_{cat} and K_m values for these phytases in a phytase enzymatic reaction using phytic acid as the substrate at 37°C are as follows: CP1 > Phy02 > CP2. The specificity constants (K_{cat}/K_m) for Phy02 and CP1 phytases are similar but, due to a relatively lower K_m, the Phy02 phytase has a slightly higher specificity constant (Table 6). These results demonstrate that Phy02, CP1, and CP2 phytases share very similar enzymatic kinetic characteristics in phytase enzymatic reactions using phytate as the substrate and provides further support that these three phytases are substantially equivalent to one another.

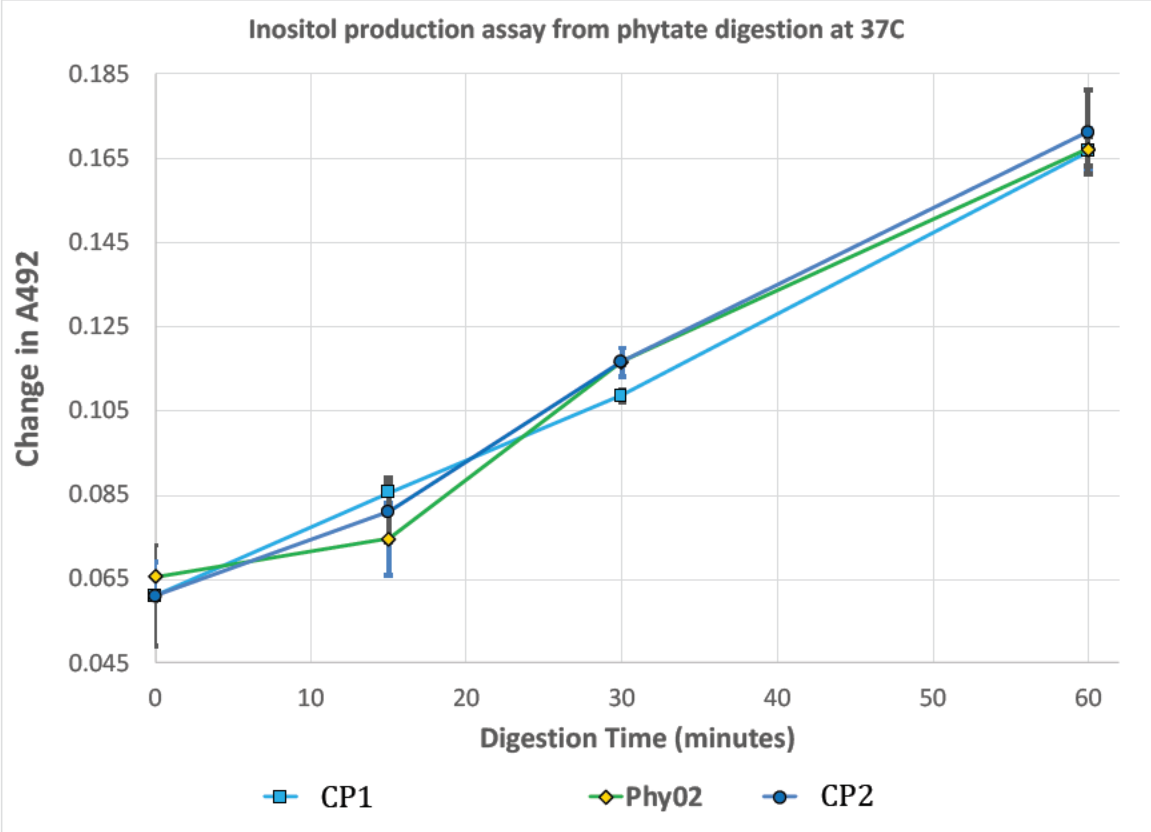
Table 6. Enzyme kinetic parameters measured for three phytases derived from the native *E. coli* AppA phytase including CP1, Phy02, and CP2.

	CP1	**Phy02**	**CP2**
k_{cat} (turnover/s)	767.3	548.8	261.1
K_m (mM)	0.789	0.515	0.494
k_{cat} /K_m (turnover/s/mM)	972.2	1065.5	529.0

The native *E. coli* AppA phytase and all phytases derived from it, including the Phy02, CP1, and CP2 phytases, are classified as 6-phytases (Griener, 2000), meaning that the enzyme preferentially removes the phosphate at the 6 position of phytate first, followed by the subsequent removal of the remaining phosphate groups until eventually all phosphate groups are removed to produce inositol. The production of inositol from phytate at 37°C by the Phy02, CP1, and CP2 phytases was evaluated

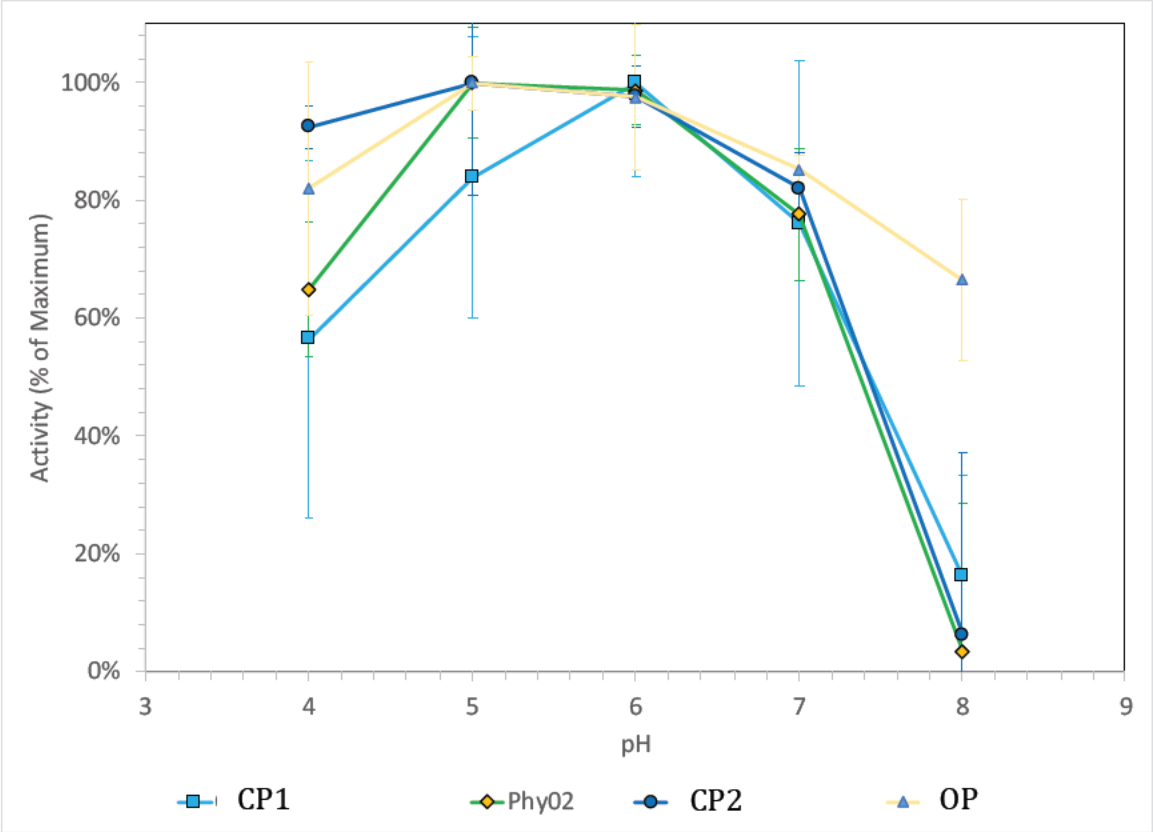
over time in identical reaction conditions. The results demonstrate that all three of these *E. coli* derived phytases produce inositol at nearly identical rates (Figure 5). This result provides further confirmation of the substantial equivalence of these three closely related phytase enzymes.

Figure 5. Production of inositol from phytate by the Phy02, CP1, and CP2 phytases in identical reaction conditions. Inositol was measured using *myo*-inosital assay reagents from (b) (4) and monitored spectrophotometrically by absorbance at 492 nm.



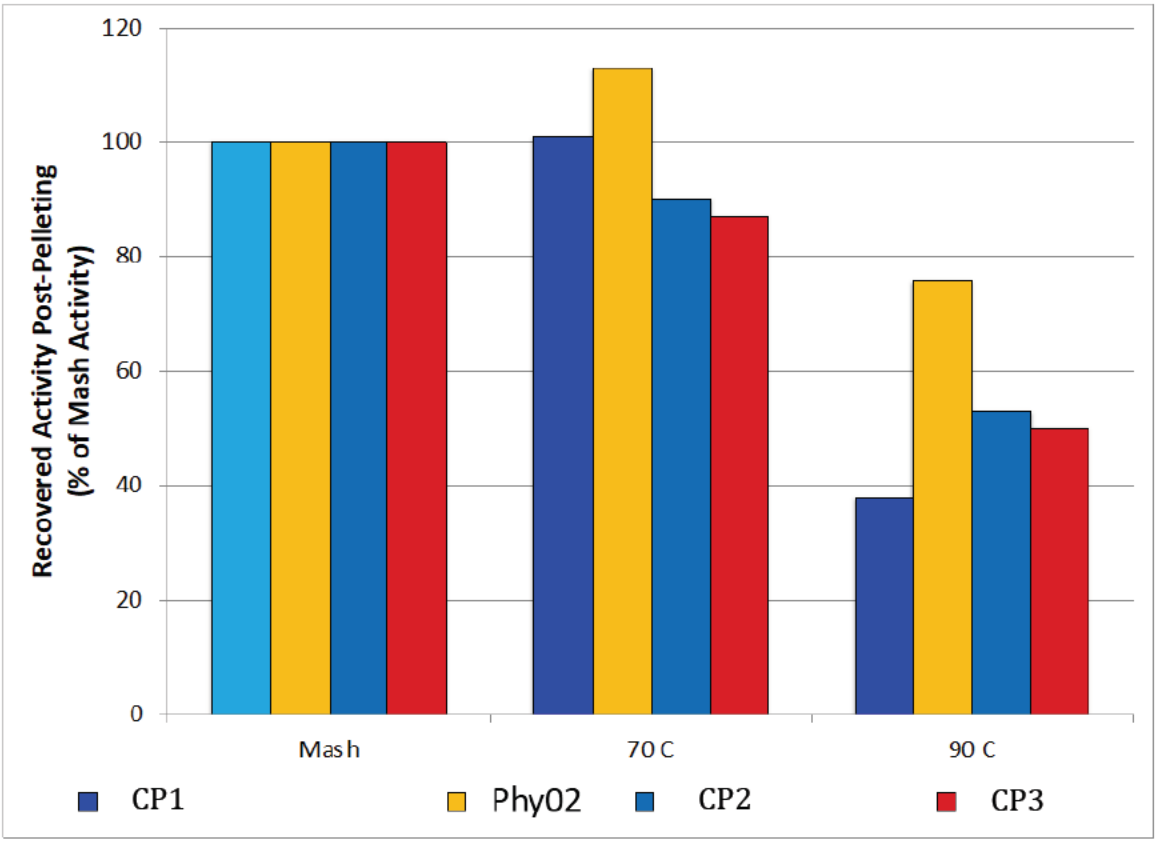
In addition to having similar enzyme kinetic characteristics for phytase reactions, the Phy02 phytase is substantially equivalent to the CP1 and CP2 phytases in other key characteristics. A comparison of the phytase activity of the Phy02, CP1, CP2, and OptiPhos® phytases demonstrated that all four of these AppA derived phytase enzymes demonstrate similar levels of phytase activity over a range of pH (Figure 6). The Phy02 phytase demonstrated a level of phytase activity equivalent or not significantly different from the other phytases at all pHs.

Figure 6. The phytase activity of four phytases derived from the AppA phytase of *E. coli*, including Phy02, CP1, CP2, and OP (OptiPhos®, another *E. coli* derived commercial phytase) at different pHs. The activity of each phytase is presented for each pH as a percent of its maximum activity. The range of standard deviation for each measurement is also presented.



The Phy02, CP1, CP2, and Phyzyme® (DuPont) phytases were mixed into a typical corn/soybean meal mash diet at a rate of 3000 FTU/kg and the diets were pelleted in a typical pressurized steam pelleting mill at different conditioning temperatures to assess the thermal tolerance of the different phytases. After pelleting at 70°C the phytase activity recovered in the pellets was very similar for Phy02, CP1, and CP2 treated feeds (Figure 7). For the feed pellets produced using a conditioning temperature of 90°C, the Phy02 phytase treated feed retained about 75% of the original mash phytase activity, much more than that of the other phytase treated feeds.

Figure 7. Corn-soybean meal based animal feeds in a mash form were treated with 3000 FTU/kg feed of Phy02, CP1, CP2, or CP3 (Phyzyme®) phytase prior to pelleting at 70 and 90°C. The amount of recovered phytase activity in the resulting feed pellets as a percentage of the activity in the mash feed prior to pelleting is presented.



As noted above, the Phy02, CP1, and CP2 phytases are all derived from the native *E. coli* AppA phytase. These three phytases have nearly identical amino acid sequences, differing from each other by only a small number of amino acids. The Phy02 and CP1 phytases have been shown to be of similar size on an SDS-PAGE gel. These three phytases share very similar phytase enzyme kinetics, similar phytase activities over a range of pH, and similar thermotolerance in feed pelleting and are considered safe and effective for use in poultry. In addition, all of these phytases have been demonstrated to improve the phosphorus digestibility of feeds and improve animal performance when included in the feed of poultry and swine. In summary, this information demonstrates and supports a conclusion that the Phy02 phytase is substantially equivalent to the CP1 (Quantum®), and CP2 (Quantum Blue®) phytases that have been used commercially as feed additives for poultry and swine for over ten years.

6.6 Conclusions of the Safety of GraINzyme® Phytase

The 10X tolerance study in which weaned pigs were grown for 43 days on diets containing 45,000 FTU GraINzyme® Phytase/kg of feed described in §6.4 demonstrates that the growth and weight gain of the treated pigs was no different from that of the control group whose feed did not contain phytase. Furthermore, comparison of the hematological analyses of the pigs from the GraINzyme® Phytase treated and control groups at the end of the study (43 days) showed no differences that would indicate that the high dose of GraINzyme® Phytase would cause any negative safety issues. In the course of the necropsies performed on the pigs from both the GraINzyme® Phytase treated and control groups there were no indications of health or safety issues in tissues of the treated group relative to the controls. The results of this study are similar to those in a published report by Nyannor et al. (2007) in which Nov9X Phytase (CP1, Quantum®) was expressed in corn grain in a manner similar to the system that Agrivida uses to produce Phy02. When fed to swine in a diet treated with up to 49,500 FTU/kg of corn-expressed NOV9X phytase Nyannor et al. (2007) saw good performance and an absence of toxicity or ill effects. The safety of the NOV9X phytase that is substantially equivalent to the GraINzyme® Phytase Phy02 (§6.5) was also reviewed in a published report from the European Food Safety Authority (EFSA, 2008) and this provides further support for the safety of the GraINzyme® Phytase. The results of other swine feeding studies using GraINzyme® Phytase that demonstrated good growth and performance of the animals without indications of toxicity or abnormalities have also been published (Broomhead *et al*., 2017; Lee *et al*., 2016, Lee *et al*., 2017a; Lee *et al*., 2017b). The results of studies conducted by Agrivida, Inc. described herein taken together with the above cited published reports support a conclusion that the inclusion of GraINzyme® Phytase in the feed of pigs at up to 45,000 FTU/kg is safe and effective and does not impede the growth or normal development of the pigs.

Agrivida, Inc. has concluded as described in §6.5 that the Phy02 phytase is substantially equivalent to two commercial phytases, CP1 (Quantum®) and CP2 (Quantum Blue®). CP1 phytase has been used commercially in poultry and swine feeds since 2007 and CP2 since 2012. CP1 and CP2 phytases have been used safely and successfully in poultry and swine feeds for many years (Beaulieu et al., 2007; Guggenbuhl et al., 2007; Hughes et al., 2008 and 2009; Laird et al., 2016; Veum et al., 2006). In addition, phytase enzymes produced from a modified *E. coli appA* phytase gene by the production host *Pichia pastoris* are listed as safe and functional enzymes for use in poultry and swine feeds in the Official Publication of the Association of American Feed Control Officials (AAFCO, 2015). Although the Phy02 phytase is produced by a different production organism, *Z. mays*, this production organism has a very long history of safe use and consumption (see §6.1.6) and so the Phy02 phytase should be considered to be as safe as the CP1 and CP2 phytases. Based on the conclusion that the Phy02 phytase is substantially equivalent to the CP1 and CP2 phytases, it is logical and reasonable to conclude that the Phy02 phytase is as safe

and functional as a feed additive in poultry and swine feeds as are the CP1 and CP2 phytase products.

6.7 Enzyme Functionality Studies

In order to demonstrate the functionality and efficacy of the GraINzyme® Phytase in swine diets four independent feeding trials were performed with weaned piglets. In all trials the animal performance and bone mineral characteristics of animals fed a feed with reduced P levels were compared to animals fed a typical corn/soybean meal diet with adequate P and Ca (Positive Control, PC) and to animals fed a diet deficient in P (Negative Control, NC). In three of the four trials Ca was also reduced in the Phy02 phytase treated and NC feed. It is recognized that phytase also causes an increase in Ca availability due to its effect of limiting Ca chelation by phytate (Adeola and Cowieson, 2011; González-Vega et al., 2015). Therefore, Ca was reduced as well as P in order to maintain an appropriate nutritional ratio of Ca to P. These trials and the results derived from them are discussed here.

6.7.1 Swine Study 1 – (b) (4)

A total of 60 weanling pigs (30 barrows and 30 gilts) that were the offspring of L 359 males and C-46 females ((b) (4) were included in this study that lasted 28 days. At the start of the trial the pigs were 5 weeks of age and had a body weight of 10.78 ± 0.67 kg. There was 1 pig per pen and 10 replicate pigs (5 barrows and 5 gilts) per treatment. The six treatment groups in the study consisted of: 1) a positive control (PC) group that received a complete feed designed to contain all nutrients at levels recommended by the NRC (2012), 2) a negative control (NC) group that received the same diet as the PC except that the levels of Ca and P were reduced from those of the PC group by 0.2 and 0.18%, respectively, and 3) four treatment groups that were fed the NC diet supplemented with 500, 1,000, 2,000, or 4,000 FTU of GraINzyme® Phytase per kg of feed. All diets included titanium oxide as an indigestible marker at 0.4%. The pigs were offered their respective diets in an unpelleted, mash form and on an *ad libitum* basis and water was freely available through out the trial. A list of all feed ingredients and amount included and the composition of proximate nutrients by analysis are presented in the final study report in Appendix 3. The phytase activities measured in the different mash feed preparations are presented in Appendix 2.

The amount of feed added to each pen was recorded and on the last day of the trial, feeders were emptied and the amount of feed left in each feeder was recorded and subtracted from total feed allotments to calculate feed disappearance in each pen. Pig weights were recorded at the beginning of the experiment and on the last day of the experiment. During the last 3 days of the experiment, a fecal sample was collected from all pigs daily by anal stimulation. The fecal samples from the 3 days were pooled for each pig, dried in a forced air oven, and ground through a 1 mm screen. A subsample was then analyzed for titanium dioxide, dry matter (DM), ash, Ca, and P. On the last day of the experiment, all pigs were euthanized via captive bolt penetration and the right femur was removed. The bones were soaked in ether

for three days to remove the bone marrow. Bone weights were recorded, and bones were analyzed for dry matter (2hr at 135˚C) and total bone ash (24hr at 600˚C).

All data were analyzed using the Proc Mixed procedure of SAS® (version 9.3, SAS Institute; Cary, USA). Orthogonal contrasts were used to determine the responses to inclusion of graded levels of phytase to the negative control diet. Means were calculated using the LS Means statement in SAS. The pig was the experimental unit and an alpha level of 0.05 was used for the determination of significance among means.

The functionality of the GraINzyme® Phytase in the swine diets is supported by numerous aspects of the resulting data. In the case of apparent total tract digestibility (ATTD) of P and Ca, digestibility increased in a GraINzyme® Phytase dose dependent manner (linear, $P < 0.01$). The PC group had significantly greater ($P < 0.01$) ATTD for both P and Ca compared to the NC group, whereas the animals receiving 500 FTU GraINzyme® Phytase/kg feed demonstrated ATTD for both P and Ca that was statistically equivalent ($P > 0.05$) to that of the PC group (Table 7, Figure 8). The results in bone ash and bone P weight followed a similar pattern. The PC group and the group receiving 1000 FTU GraINzyme® Phytase/kg feed had significantly greater ($P < 0.01$) bone ash and bone P weights than the NC group. Bone ash and bone P weights increased in a GraINzyme® Phytase-dose dependent manner (linear, $P < 0.01$; Table 7, Figure 9).

There was a significant reduction ($P < 0.01$) in final body weight of pigs fed the negative control diet compared with pigs fed the positive control diet (21.54 vs. 28.40 kg; Table 7). In the case of average daily weight gain (ADG), there was also a significant reduction ($P < 0.01$) for pigs fed the negative control diet compared with pigs fed the positive control diet (383 vs. 600 g/d, Table 7, Figure 10). The same pattern was observed for average daily feed intake (ADFI), where pigs fed the negative control diet consumed less feed ($P < 0.05$) when compared with pigs fed the positive control diet (848 vs. 1029 g/d, Table 7). As a consequence, there was also a reduction ($P < 0.01$) in the feed efficiency (G:F) for pigs fed the negative control diet compared with pigs fed the positive control diet (0.482 vs. 0.584, Table 7, Figure 10).

Growth performance (final BW, ADFI, ADG, and G:F) increased linearly ($P < 0.01$) as the concentration of GraINzyme® Phytase added to the negative control diet increased. Adding 500 FTU/kg of GraINzyme® Phytase to the negative control diet resulted in pigs having an ADG (480 vs. 600 kg) and G:F (0.529 vs. 0.584) that was not significantly different ($P > 0.05$) from that of the PC group (Table 7, Figure 10). Adding 1000 FTU GraINzyme® Phytase/kg feed resulted in increased ($P < 0.01$) final BW, ADG, and G:F as compared to NC group and not significantly different ($P > 0.01$) than the PC group.

In summary, the performance data from swine in this study, including final body weight, average daily gain and feed efficiency, support the functionality of the Phy02 phytase in swine diets. Inclusion of the GraINzyme® Phytase in a low phosphorus and calcium basal diet demonstrated a dose response with improved weight gain and feed efficiency with increasing doses of GraINzyme® Phytase. In addition, the functionality of the GraINzyme® Phytase was demonstrated by improved apparent total tract digestibility of both P and Ca and by improved ash, Ca, and P weight in the right femur. Altogether, the results of this study clearly demonstrate the functionality of the GraINzyme® Phytase in improving phosphorus availability and nutrition in swine.

Table 7. Growth performance, apparent total tract digestibility (ATTD) of Ca and P, and bone mineralization of pigs fed diets containing 0 to 4000 FTU/kg of GraINzyme Phytase for 28 days[1]

	Treatments						Pooled SEM	*P*-Value	
	Positive Control	Negative Control	500 FTU	1000 FTU	2000 FTU	4000 FTU		Treatment	Linear
Initial BW, kg	10.77	10.81	10.82	10.81	10.74	10.74	0.22	1.000	0.766
Final BW, kg	28.40^{a}	21.54^{c}	24.41^{bc}	26.55^{ab}	26.87^{ab}	29.40^{a}	0.84	<.0001	<.0001
ADG, g/d	600.14^{ab}	382.54^{c}	480.47^{bc}	562.00^{ab}	575.89^{ab}	637.22^{a}	30.40	<.0001	<.0001
ADFI, g/d	1,028.61^{ab}	847.74^{b}	955.71^{ab}	1,025.39^{ab}	1,070.18^{a}	1,118.46^{a}	47.73	0.003	0.0002
G:F	0.584^{a}	0.482^{b}	0.529^{ab}	0.549^{a}	0.553^{a}	0.568^{a}	0.015	0.0001	0.0002
ATTD Ca, %	65.96^{a}	51.61^{b}	65.49^{a}	72.36^{a}	74.96^{a}	73.53^{a}	2.88	<.0001	<.0001
ATTD P, %	53.64^{b}	40.82^{c}	52.53^{b}	50.29^{b}	63.82^{a}	63.05^{a}	1.64	<.0001	<.0001
Bone ash[2], %	42.71^{a}	33.48^{d}	35.79^{cd}	37.73^{bc}	40.30^{ab}	42.58^{a}	0.79	<.0001	<.0001
Bone ash, g	14.80^{a}	7.49^{e}	8.89^{de}	10.58^{cd}	12.26^{bc}	14.31^{ab}	0.57	<.0001	<.0001
Bone Ca, %	34.50	34.13	34.15	34.31	34.80	34.16	0.51	0.9131	0.852
Bone Ca, g	5.10^{a}	2.57^{d}	3.05^{cd}	3.64^{bc}	4.27^{ab}	4.91^{a}	0.22	<.0001	<.0001
Bone P, %	16.96	16.48	16.76	16.64	16.94	16.81	0.25	0.7425	0.370
Bone P, g	2.51^{a}	1.24^{d}	1.50^{cd}	1.76^{bc}	2.08^{ab}	2.42^{a}	0.11	<.0001	<.0001

[a-e] Means within a row lacking a common superscript letter are different ($P < 0.05$).
[1]Data are means of 10 observations per treatment, except for the negative control, which had only 9 observations.
[2]Bone ash as percent of the weight of dried defatted bone.

Figure 8. Apparent total tract digestibility (ATTD) of phosphorus and calcium in the feces of pigs in the positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).

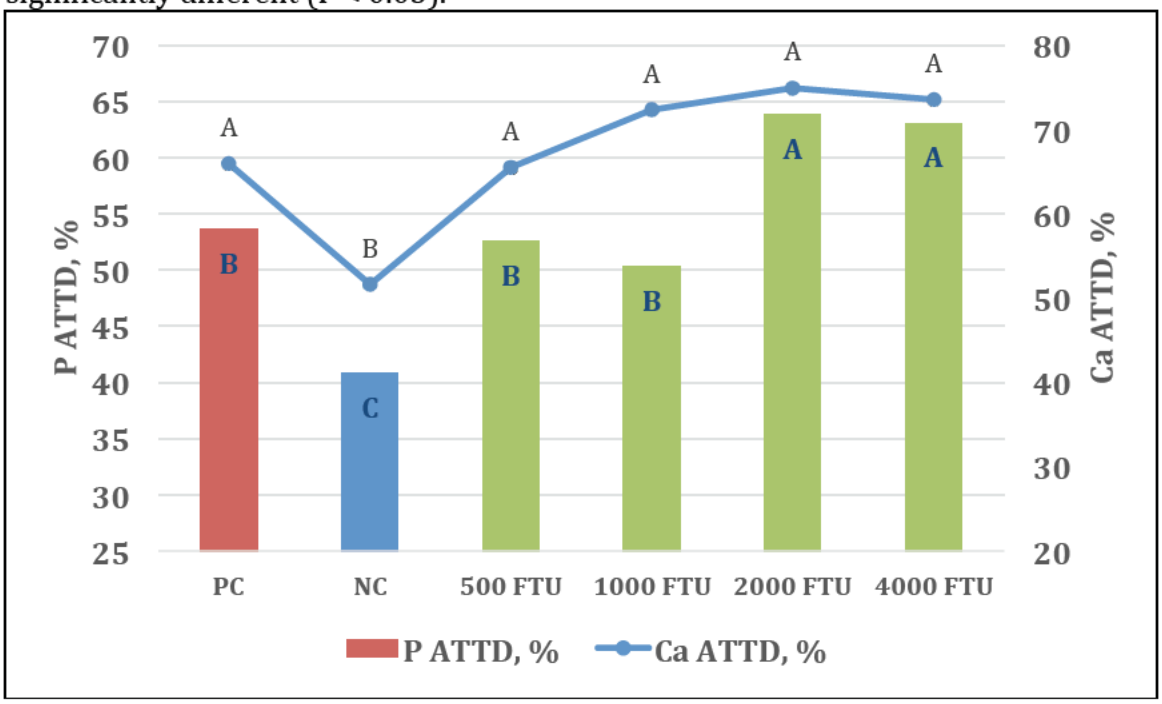


Figure 9. Percent and weight of bone ash of the right femur of pigs in the positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).

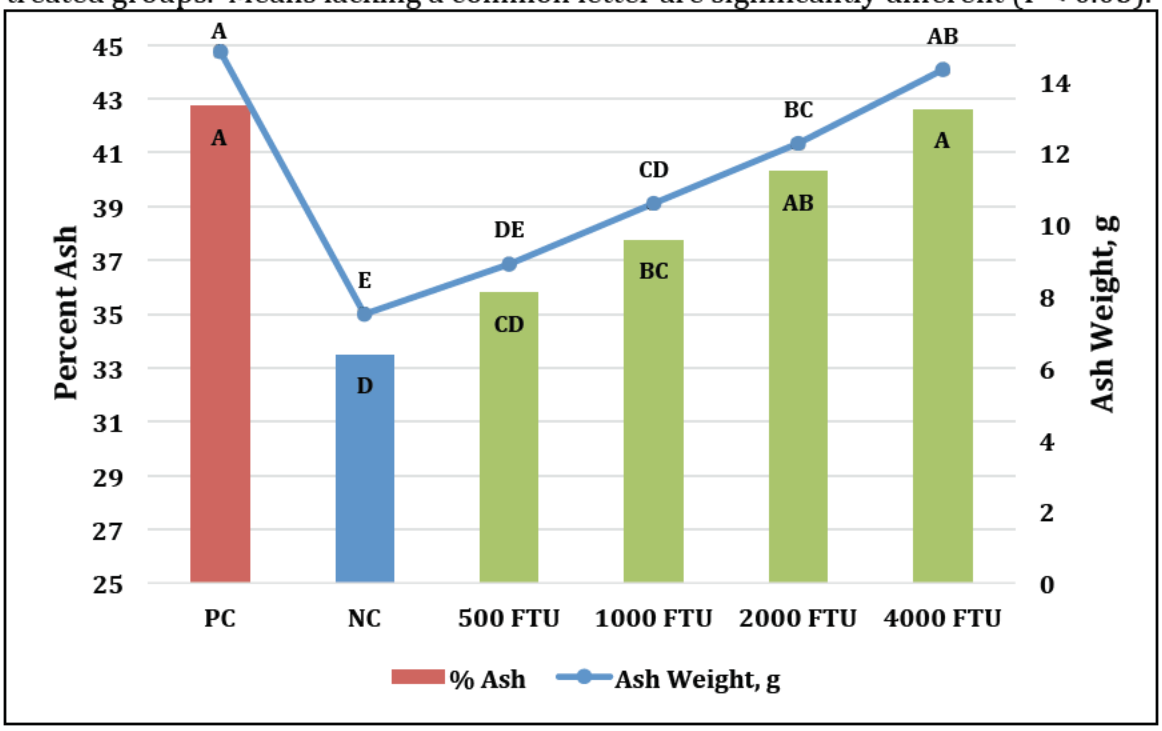
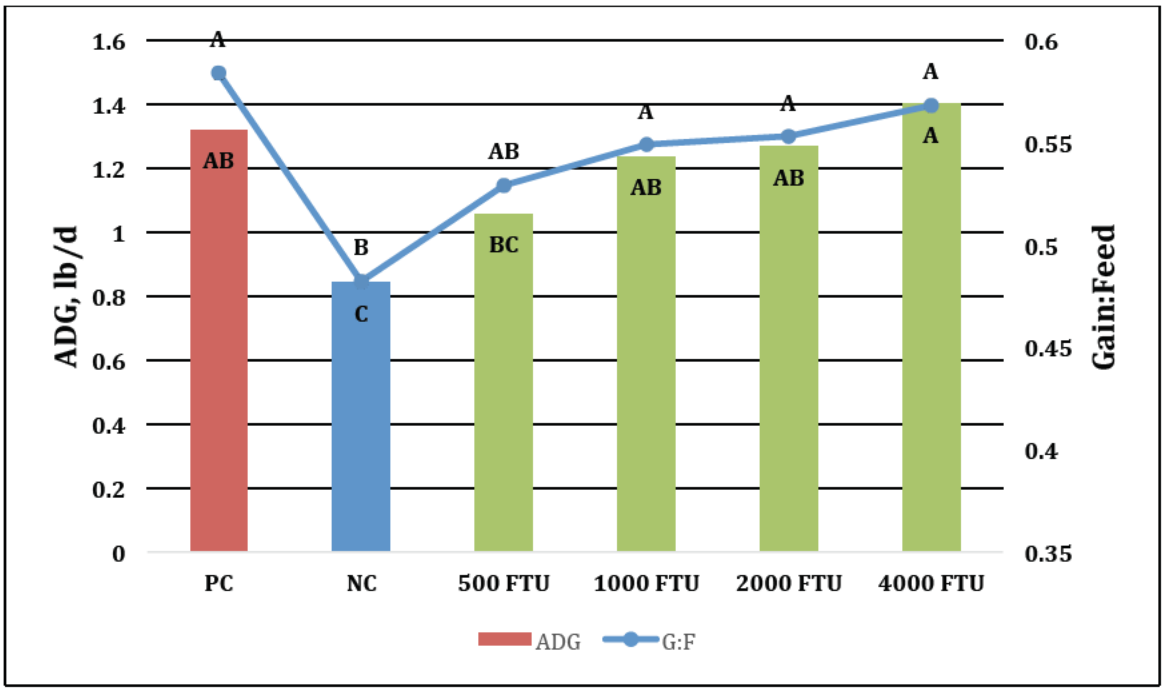


Figure 10. Average daily weight gain (ADG) and feed efficiency (Gain:Feed) of pigs in the positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).



6.7.2 Swine Study 2 – (b) (4)

A second swine feeding trial with GraINzyme® Phytase was conducted at the (b) (4) using a similar trial protocol as that used in Swine Study 1 (§6.7.1). The study started with 5 week old pigs that were 2 weeks post-weaning. There were 48 pigs in the study, 24 barrows and 24 gilts, with one pig per pen and 8 pens per treatment for a total of 48 pens. The trial lasted for 28 days and the treatments consisted of the following: 1) a positive control (PC) group that received a complete feed designed to contain all nutrients at levels recommended by the NRC (2012), 2) a negative control (NC) group that received the same diet as the PC except that the levels of Ca and P were reduced from those of the PC group by 0.2 and 0.15%, respectively, 3-4) two treatment groups that were fed the NC diet supplemented with 500 or 1000 FTU/kg of the commercial phytase Axtra® Phy (DuPont), 5-6) two treatment groups that were fed the NC diet supplemented with 500 or 1000 FTU/kg GraINzyme® Phytase. The same measurements (except for fecal digestibility) were made in this study as in Study 1 also conducted at the University of Illinois (§6.7.1). A list of all feed ingredients and amount included and the composition of proximate nutrients by analysis are presented in the final study report in Appendix 4.

As was shown in swine Study 1, the results of this study demonstrate the functionality of the GraINzyme® Phytase. For bone ash and bone P and Ca weights, the PC group and the group treated with 500 FTU/kg GraINzyme® Phytase/kg had weights that were significantly greater ($P < 0.01$) than those of the NC group (Table 8, Figure 11). The performance and bone ash/mineralization results for the 500 FTU/kg GraINzyme® Phytase and Axtra® Phy treated groups were statistically equivalent ($P > 0.05$). Average daily gain of the pigs receiving 500/kg FTU GraINzyme® Phytase/kg feed were numerically greater than, but not statistically different from, the NC body weights, whereas the 1000 FTU/kg GraINzyme® Phytase group was statistically equivalent to the PC group (Table 8, Figure 12).

Table 8. Performance and Bone ash, Ca, and P in the right femur of pigs fed experimental diets.

	Treatment						SEM	*P*-value
	Positive control	Negative control	Axtra® Phy 500 FTU	Axtra® Phy 1,000 FTU	GraINzyme 500 FTU	GraINzyme 1,000 FTU		
Initial BW, kg	11.26	11.31	11.04	11.23	11.21	11.18	0.32	0.780
ADG, kg	0.75^{a}	0.56^{c}	0.70^{ab}	0.65^{abc}	0.61^{bc}	0.67^{abc}	0.04	<0.001
ADFI, kg	1.25	1.20	1.33	1.19	1.13	1.32	0.06	0.107
G:F	0.60^{a}	0.47^{c}	0.53^{abc}	0.55^{ab}	0.55^{ab}	0.52^{bc}	0.02	<0.001
Final BW, kg	32.26^{a}	26.99^{c}	30.67^{ab}	29.53^{abc}	28.41^{bc}	29.81^{abc}	1.16	<0.001
Bone Ash, g	16.71^{a}	7.35^{d}	11.15^{c}	13.63^{b}	9.56^{c}	11.16^{c}	0.52	0.001
Bone Ash, %	49.98^{a}	41.64^{d}	44.91^{bcd}	48.52^{ab}	42.80^{cd}	46.11^{bc}	0.88	0.001
Bone Ca, g	6.10^{a}	2.67^{d}	4.03^{c}	4.82^{b}	3.47^{c}	4.05^{bc}	0.21	0.001
Bone Ca, %	36.53	36.39	36.08	36.75	36.28	36.28	0.36	0.863
Bone P, g	2.83^{a}	1.23^{d}	1.88^{c}	2.25^{b}	1.60^{c}	1.87^{c}	0.09	0.001
Bone P, %	16.96	16.67	16.85	17.11	16.72	16.74	0.18	0.710

$^{a-d}$ Means within a row lacking a common superscript letter are different ($P < 0.05$).

Figure 11. Bone ash weight and bone phosphorus weight in the right femur of pigs in the positive (PC) and negative (NC) control groups and groups treated with 500 and 1000 FTU/kg Axtra® Phy (AX) or GraINzyme® Phytase (GZ). Means lacking a common letter are significantly different (P < 0.05).

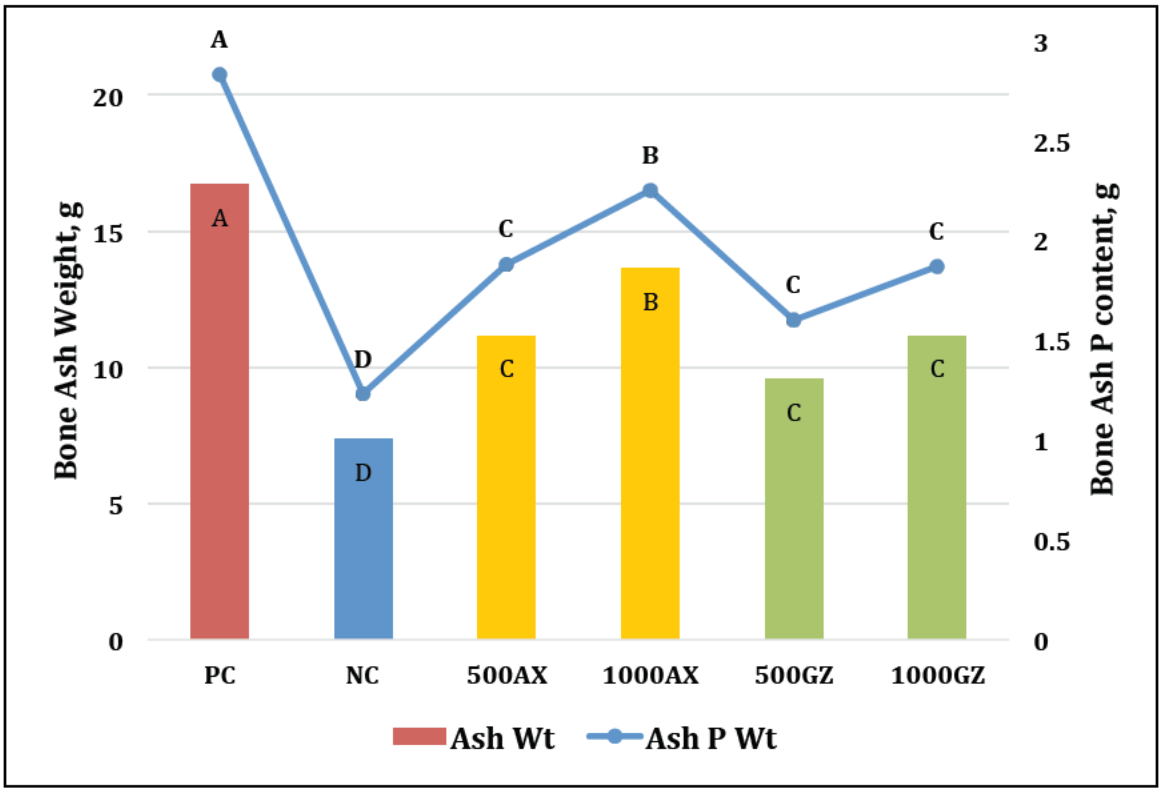
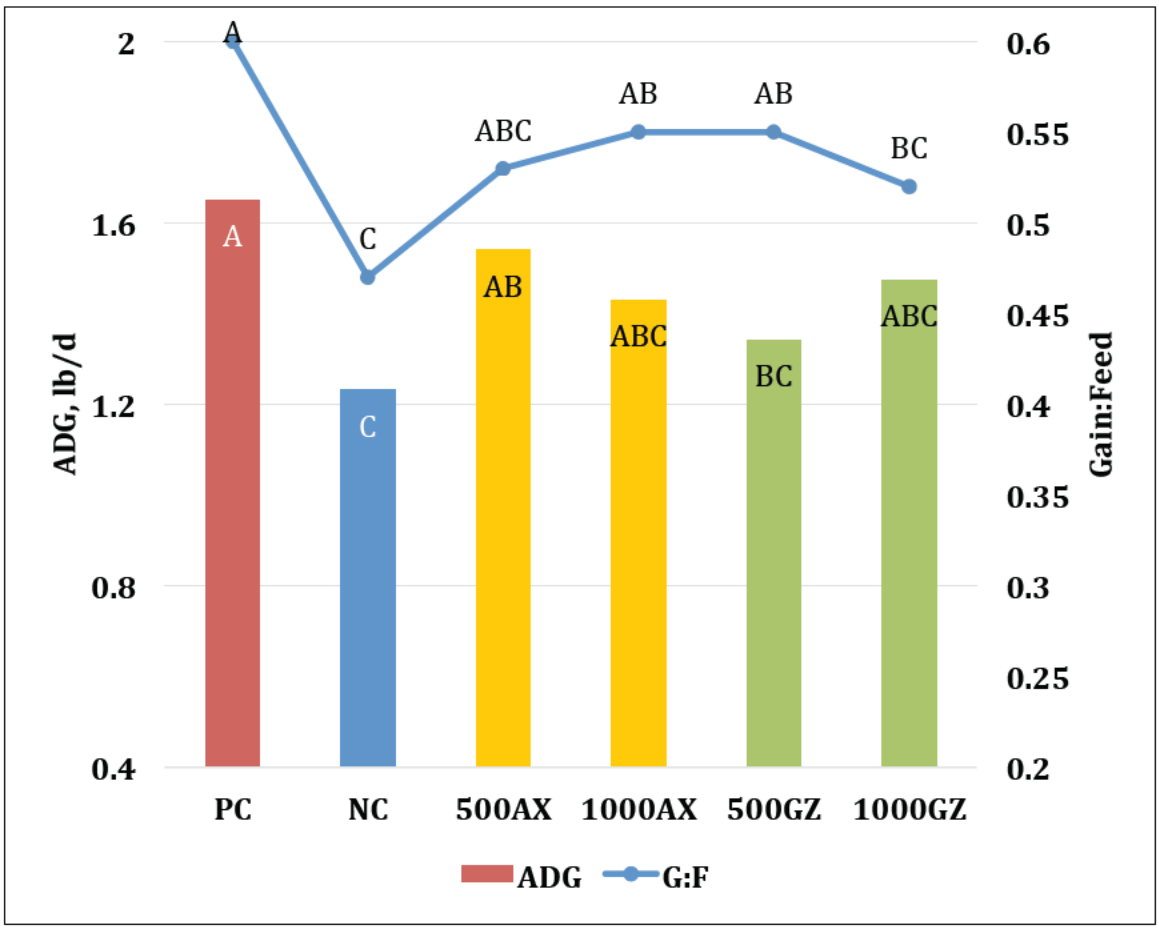


Figure 12. Average daily weight gain (ADG) and feed efficiency (Gain:Feed) of pigs in the positive (PC) and negative (NC) control groups and groups treated with 500 and 1000 FTU/kg Axtra® Phy (AX) or GraINzyme® Phytase (GZ). Means lacking a common letter are significantly different ($P < 0.05$).



6.7.3 Swine Study 3 – (b) (4)

A swine feeding trial with GraINzyme® Phytase was conducted by (b) (4) (b) (4) A group of 360 weaned piglets (barrows and gilts) was received and placed on a transition diet for 7 days. At the end of the transition (acclimation) phase, the pigs were weighed and randomized into sixty pens with each pen containing six pigs. The pigs were fed different base diets in three 14-day phases (P2, P3, and P4). The pigs in each pen were fed one of six treatment diets that included:

1) Positive control (PC) diet that received a complete feed designed to contain all nutrients at levels recommended by the NRC (2012). This diet contained 0.40, 0.32 and 0.32% available P for Phases 2, 3 and 4, respectively.

2) Negative control (NC) diet that was the same as the PC diet except that the level of P was reduced from those of the PC group to contain 0.250, 0.174, and 0.174% available P for Phases 2, 3 and 4, respectively.
3-6) GraINzyme® Phytase supplemented diets that were the same as the NC diet but that were supplemented with 500, 1,000, 2,000 or 4,000 FTU GraINzyme® Phytase/kg feed.

The 4th Phase basal diets also contained chromium oxide (Cr_2O_3) at level of 0.4% to serve as an indigestible marker for determining apparent P digestibility. All diets were pelleted using a flow rate and steam pressure such that the pelleting temperature did not exceed 165°F (74°C). A complete list of ingredients of the diets and proximate nutrient analysis is presented in the complete study report (Appendix 5) and the phytase activity measured in each feed after pelleting is presented in Appendix 2.

Each pig and all feed was weighed at the start and end of the study and at each phase change. On day 35 of the study, fecal samples were collected from four pigs in each pen and pooled to create one sample per pen. These were analyzed to determine apparent P digestibility. At the end of the live phase (Study day 42) all pigs were humanely euthanized using a captive bolt gun. Four pigs from each pen were randomly selected for the collection of the 3rd and 4th metacarpals from their right front foot that were removed and subsequently analyzed to determine bone breaking strength and percent ash.

The apparent P digestibility after 35 days on treatment was not significantly different between the PC and NC groups (Table 9). However, addition of only 500 FTU/kg of the GraINzyme® Phytase to the NC basal diet resulted in a significant increase ($P < 0.01$) in apparent P digestibility compared to the PC and NC groups. There was a linear increase ($P < 0.01$) in apparent P digestibility as GraINzyme® Phytase dose increased, with the 4,000 FTU/kg treatment group demonstrating almost twice the apparent P digestibility as the PC group (73.17 vs. 38.33%; Table 9). For bone breaking strength and ash weight, the PC group was significantly greater than ($P < 0.01$) the NC group, whereas the 500 FTU/kg GraINzyme® Phytase group was significantly greater than ($P < 0.01$) the NC group and the 1000 FTU/kg group was statistically equivalent ($P > 0.05$) to the PC group (Table 9 and Figures 13 and 14).

Throughout the trial, the body weights, average daily gain, and average daily feed intake of the PC treatment group were significantly greater ($P < 0.01$) than those of the NC group and statistically equivalent ($P > 0.05$) to the 500 FTU/kg phytase group (Table 9 and Figure 15). For each of these categories, the values increased with the GraINzyme® Phytase dose level (linear $P < 0.01$) and 4000 FTU/kg GraINzyme® Phytase dose group had statistically greater ($P < 0.01$) overall body weight, average daily gain and feed intake than those of the PC group (Table 9 and Figure 15). The results of this trial clearly demonstrate the functionality of the

GraINzyme® Phytase product in increasing the availability of phosphorus in the diets of swine when fed a corn-soybean meal diet that is deficient in available phosphorus.

Table 9. Effects of positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme Phytase on individual phase (0, 14, 28, and 41 days) and overall performance data and P digestibility, bone breaking strength and bone ash.

	Body Weight (lb)				Avg Daily Gain (lb/day)			
Trt	0d	14d	28d	41d	0-14d	14-28d	28-41d	0-41d
PC	14.59	22.48^{a}	35.00^{b}	57.13^{bc}	0.56^{b}	0.88^{ab}	1.70^{ab}	1.04^{bc}
NC	14.58	21.23^{b}	31.76^{c}	50.35^{d}	0.48^{c}	0.74^{c}	1.43^{c}	0.87^{d}
500 FTU	14.57	22.43^{a}	34.03^{b}	55.30^{c}	0.56^{b}	0.82^{bc}	1.61^{b}	0.99^{c}
1000 FTU	14.60	22.70^{a}	35.15^{b}	57.35^{bc}	0.58^{ab}	0.88^{ab}	1.71^{a}	1.04^{bc}
2000 FTU	14.53	23.12^{a}	35.40^{ab}	57.57^{b}	0.61^{ab}	0.88^{ab}	1.71^{a}	1.05^{b}
4000 FTU	14.57	23.40^{a}	37.19^{a}	60.36^{a}	0.63^{a}	0.97^{a}	1.78^{a}	1.12^{a}
Std Err	0.138	0.359	0.636	0.793	0.022	0.036	0.031	0.019
				P values				
Linear*	0.9276	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Overall	0.9996	0.0026	<.0001	<.0001	0.0002	0.0021	<.0001	<.0001

	Avg Daily Feed Intake (lb/day)				Feed Conversion (feed/gain)			
Trt	0-14d	14-28d	28-41d	0-41d	0-14d	14-28d	28-41d	0-41d
PC	0.70^{a}	1.34^{bc}	2.55^{bc}	1.51^{bc}	1.26^{abc}	1.53	1.62^{a}	1.52
NC	0.62^{b}	1.18^{d}	2.08^{d}	1.28^{d}	1.32^{c}	1.60	1.57^{a}	1.52
500 FTU	0.72^{a}	1.29^{c}	2.46^{c}	1.47^{c}	1.28^{bc}	1.69	1.66^{a}	1.58
1000 FTU	0.72^{a}	1.39^{b}	2.62^{bc}	1.55^{b}	1.24^{abc}	1.60	1.66^{a}	1.56
2000 FTU	0.73^{a}	1.38^{b}	2.66^{b}	1.56^{b}	1.20^{ab}	1.58	1.69^{ab}	1.56
4000 FTU	0.73^{a}	1.52^{a}	2.94^{a}	1.70^{a}	1.16^{a}	1.58	1.79^{b}	1.60
Std Err	0.018	0.027	0.062	0.025	0.034	0.064	0.043	0.036
				P values				
Linear*	0.0021	<.0001	<.0001	<.0001	0.0014	0.4682	0.0014	0.2598
Overall	0.0007	<.0001	<.0001	<.0001	0.0364	0.6765	0.0248	0.6335

	Fecal P Digestibility	Bone Breaking Strength		De-fatted Bone Weight		Bone Ash	
Trt	%	gr	kg	gr	gr (DM)	gr	%
PC	38.33^{d}	44845^{a}	44.84^{a}	3.92^{b}	3.37^{b}	1.56^{b}	46.46^{ab}
NC	31.53^{d}	31038^{c}	31.04^{c}	3.16^{c}	2.71^{d}	1.11^{d}	40.68^{d}
500 FTU	56.95^{c}	39617^{b}	39.62^{b}	3.74^{b}	3.15^{c}	1.41^{c}	44.87^{c}
1000 FTU	57.99^{c}	44934^{a}	44.93^{a}	3.91^{b}	3.36^{b}	1.51^{b}	44.90^{c}
2000 FTU	65.32^{b}	44587^{ab}	44.59^{ab}	3.86^{b}	3.32^{bc}	1.52^{b}	45.84^{bc}
4000 FTU	73.17^{a}	44989^{a}	44.99^{a}	4.24^{a}	3.64^{a}	1.72^{a}	47.33^{a}
Std Err	2.442	1831	1.831	0.076	0.065	0.030	0.402
				P values			
Linear*	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Overall	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

^{a,b,c,d} LS Means with different superscripts are significant different ($P < 0.05$).

* Orthogonal contrasts within NC diets, not including PC treatment.

Figure 13. Bone breaking strength and weight of positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).

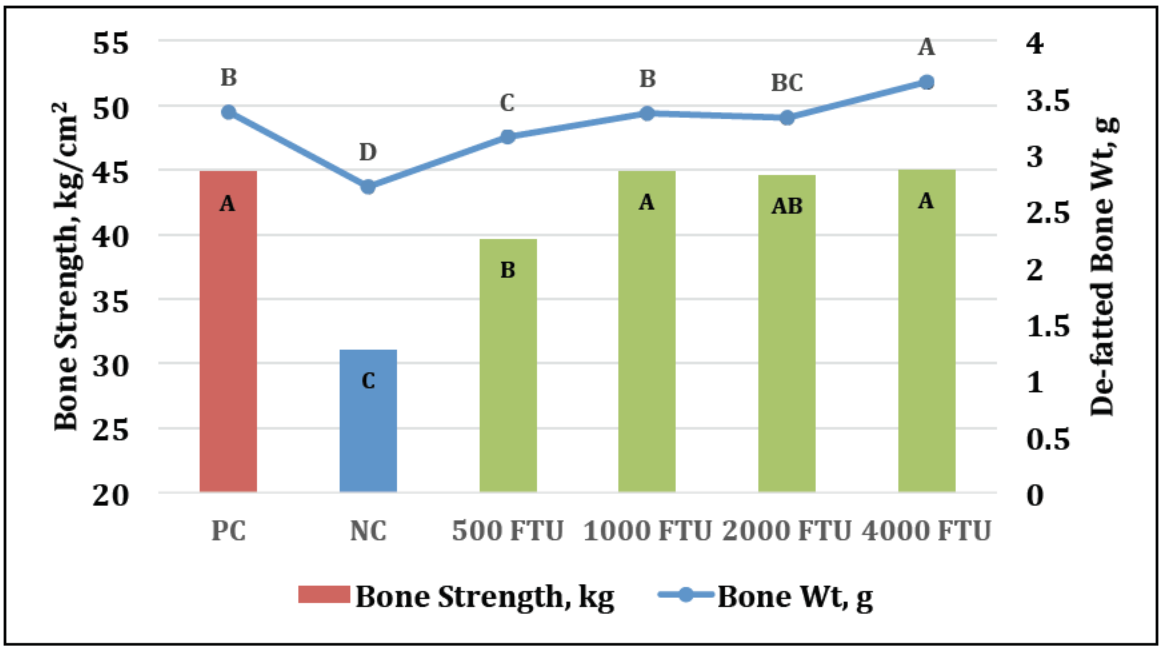


Figure 14. Bone ash weight and percent of positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).

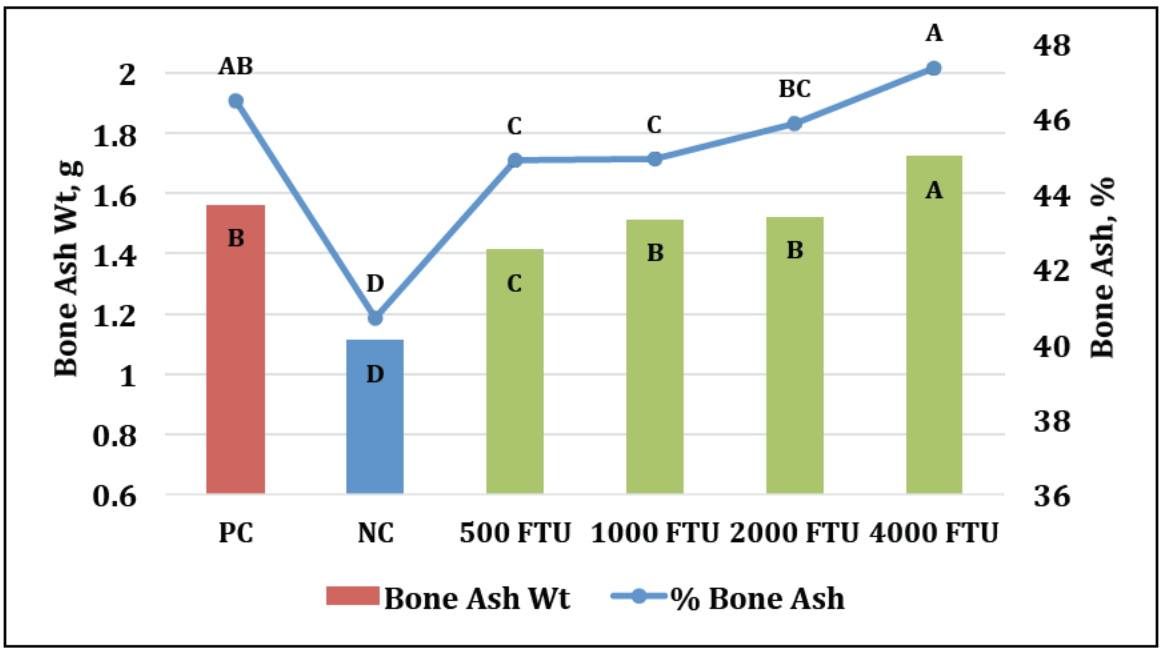
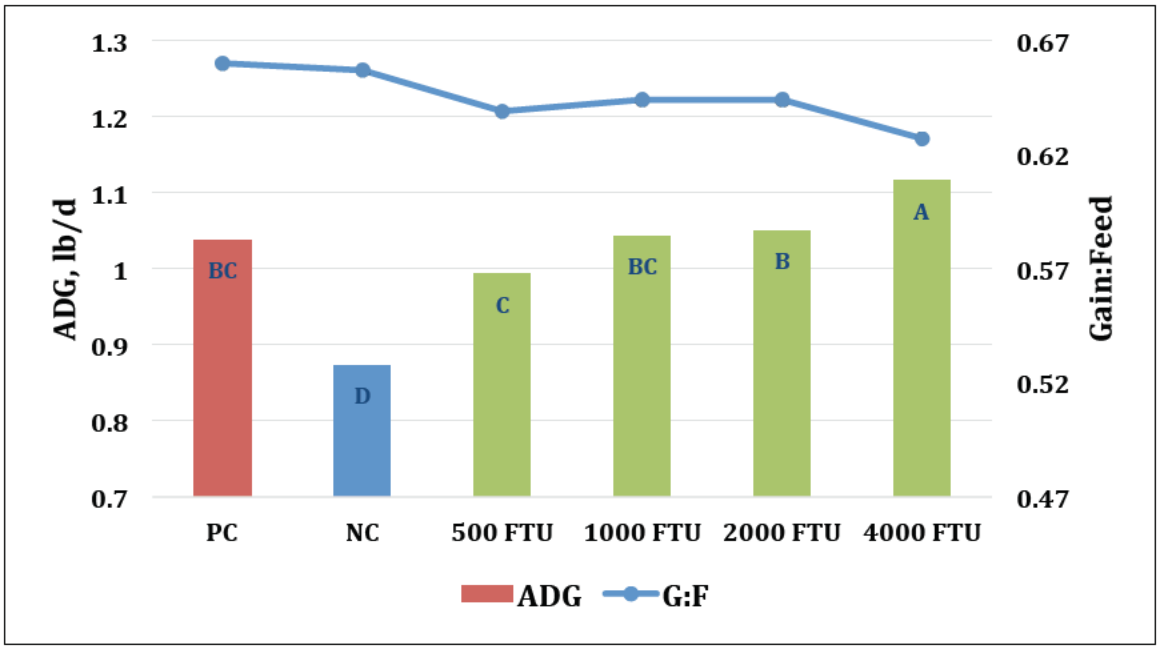


Figure 15. Average daily weight gain (ADG) and weight gain:feed intake (Gain:Feed) of positive (PC) and negative (NC) controls and 500 to 4000 FTU/kg GraINzyme® Phytase treated groups. Means lacking a common letter are significantly different (P < 0.05).



6.7.4 Swine Study 4 – (b) (4)

A fourth swine trial was conducted at the (b) (4) that included 288 weaned piglets (barrows and gilts) that were 21 days old. The pigs were individually weighed and sorted at weaning and allowed a 7-day adaption phase. To avoid the confounding effect of initial weight, pigs were assigned to 8 blocks of 36 pigs. There were a total of 8 replicates per treatment with pigs housed 6 pigs/pen. Pigs remained in the same pens throughout the experiment. The six treatment groups in the study consisted of: 1) a positive control (PC) group that received a complete feed designed to contain all nutrients at levels recommended by the NRC (2012), 2) a negative control (NC) group that received the same diet as the PC except that the levels of Ca and P were reduced from those of the PC group by 0.1 and 0.15%, respectively, 3-5) three treatment groups that were fed the NC diet supplemented with 500, 1,000, or 1,500 FTU of GraINzyme® Phytase per kg of feed, and 6) a treatment group that was fed a NC diet supplemented with a commercial phytase control, Ronozyme® HiPhos (DSM), at 500 FTU/kg feed. All feeds used in the study were unpelleted, mash feeds and the study was divided into four dietary phases. During phase 1 (7d) all animals received a common diet, without phytase, as an acclimation period and phases 2 (7d) and 3 and 4 (each 14d) in which the animals received one of the six diets described above. A list of all feed ingredients

and amount included and the composition of proximate nutrients by analysis are presented in the final study report in Appendix 6.

At the start of the study and at the end of each phase throughout the study, individual pig weights and pen feed intake was measured in order to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F) for each phase. At study termination, after 35 days of treatment, the pigs were euthanized and the front left feet were removed for subsequent isolation of the metacarpal for bone ash determination. The data were analysed using the MIXED procedures of SAS in which the treatment was the fixed effect. An orthogonal contrast was used to determine the effects of increasing levels of GraINzyme® Phytase on performance and bone characteristics. Probability values of $P \leq 0.05$ were considered as a statistically significant difference, with $0.05 < P \leq 0.10$ considered a statistical trend.

In all phases, pigs fed the positive control diet had numerically improved ADG when compared to those fed the negative control diet and ADG increased linearly during phase 2 ($P < 0.05$), phase 4 ($P < 0.01$) and for the overall study ($P < 0.01$) with increasing levels of GraINzyme® Phytase from 0 to 1,500 FTU/kg of diet (Table 10). The linear improvement in ADG with increasing GraINzyme® Phytase during the combined phase 2 and phase 3 periods approached significance ($P = 0.10$, Table 10). Average daily gain in pigs fed the highest dose of GraINzyme® Phytase (1,500 FTU/kg of diet) was higher in phase 4 ($P < 0.08$) when compared to those fed the negative control diet. Overall ADG was higher ($P = 0.10$) in pigs fed all GraINzyme® Phytase levels compared to those fed the negative control diet (Table 10; Figure 16). ADG in pigs fed the commercial control phytase product at 500 FTU/kg was similar to ADG observed in pigs fed the PC and GralNzyme® Phytase at 500 FTU/kg diet ($P > 0.10$; Table 10). However, overall ADG in commercial phytase group was not significantly different than ADG of NC group ($P > 0.10$, Table 10). As might be expected based on ADG, body weight (BW) at study completion increased with increasing dietary level of GraINzyme® Phytase from 0 to 1,500 FTU (linear, $P < 0.05$; Table 10).

ADFI was similar among all treatments in all phases ($P > 0.35$) with the exception of phase 4 where ADFI increased linearly ($P < 0.01$) with increasing level of GraINzyme® Phytase from 0 to 1,500 FTU/kg of feed (Table 10). ADFI in phase 4 also tended to be higher in pigs fed 1000 or 1500 FTU/kg GralNzyme® Phytase when compared to those fed the NC diet ($P < 0.10$).

Feed efficiency was similar among pigs fed the negative control and positive control diets. However, the G:F increased linearly with increasing GraINzyme® Phytase from 0 to 1,500 FTU/kg of diet in phase 3, the combined phase 2 and 3 periods, and for the overall study ($P < 0.05$; Table 10). The G:F was also numerically higher in pigs fed the highest level of GraINzyme® Phytase in all phases when compared to pigs fed the NC diet. The G:F was also numerically higher in pigs fed the highest

level of GraINzyme® Phytase in all phases with the exception of phase 4 when compared to those fed the PC diet (Table 10). The performance data after 21 and 35 days on treatments are presented graphically in Figures 17 and 18, respectively.

The effect of GraINzyme® Phytase on metacarpal bone characteristics (Table 11; Figure 16) indicates that bone length and bone ash weight tended to increase linearly with increasing level of GraINzyme® Phytase from 0 to 1,500 FTU/kg of feed ($P < 0.10$). Percent bone ash increased both linearly (Table 11; $P < 0.001$) with pigs fed 1,000 FTU/kg GraINzyme® Phytase being the highest within pigs fed NC diet. Pigs fed the highest dose of GraINzyme® Phytase (1,500 FTU/kg of diet) had similar bone ash weight ($P > 0.05$) compared to those fed the PC diet (Table 11; Figure 16). Bone ash weight and percent of bone ash were higher in pigs fed 500 FTU/kg of GraINzyme® Phytase or the commercial phytase control when compared to those fed the NC diet but lower when compared to pigs fed the PC diet ($P < 0.05$). However, pigs fed 500 FTU/kg GraINzyme® Phytase had higher percent bone ash ($P < 0.05$) than the equivalent dose of the commercial phytase control treatment. The results of this trial clearly demonstrate the functionality of the GraINzyme® Phytase product in increasing the availability of phosphorus in the diets of swine when fed a corn-soybean meal diet that is deficient in available phosphorus.

Table 10. Effect of GraINzyme® Phytase and Ronozyme® HiPhos on growth performance in nursery pigs

	Positive	Negative	GraINzyme® Phytase			HiPhos		*P* - Value	
FTU/kg	Control	Control	500 FTU	1,000 FTU	1,500 FTU	500 FTU	SEM	Treatment	Linear[1]
ADG, kg/d									
Phase 2	0.172	0.143	0.176	0.180	0.187	0.170	0.014	0.3694	0.0412
Phase 3	0.419	0.401	0.430	0.438	0.434	0.412	0.019	0.6901	0.2013
Phase 4	0.633[ab]	0.579[b]	0.640[ab]	0.634[ab]	0.682[a]	0.635[ab]	0.023	0.0788	0.0044
Phase 2&3	0.337	0.315	0.345	0.352	0.351	0.331	0.015	0.5256	0.1016
Overall	0.455[ab]	0.421[b]	0.463[a]	0.465[a]	0.483[a]	0.453[ab]	0.015	0.1016	0.0063
BW, kg									
Initial	6.67	6.70	6.66	6.73	6.64	6.64	0.28	0.9846	0.8205
End of phase 2	7.88	7.70	7.89	7.99	7.95	7.83	0.28	0.8578	0.2530
End of phase 3	13.75	13.32	13.91	14.12	14.02	13.60	0.47	0.6930	0.1799
End of phase 4	22.61	21.43	22.87	23.00	23.56	22.49	0.67	0.1846	0.0135
ADFI, kg/d									
Phase 2	0.273	0.209	0.256	0.243	0.246	0.240	0.020	0.3520	0.2792
Phase 3	0.707	0.661	0.697	0.629	0.631	0.646	0.045	0.6582	0.3956
Phase 4	0.992[abc]	0.913[c]	0.999[abc]	1.018[ab]	1.056[a]	0.952[bc]	0.033	0.0603	0.0041
Phase 2&3	0.563	0.511	0.550	0.501	0.503	0.511	0.033	0.6041	0.5999
Overall	0.734	0.671	0.730	0.707	0.724	0.687	0.028	0.5198	0.2752
G:F									
Phase 2	0.672	0.674	0.685	0.750	0.750	0.722	0.046	0.5870	0.1282
Phase 3	0.623	0.621	0.631	0.705	0.712	0.653	0.041	0.2419	0.0312
Phase 4	0.665	0.638	0.641	0.637	0.654	0.667	0.017	0.6936	0.5962
Phase 2&3	0.624	0.627	0.639	0.709	0.717	0.662	0.035	0.1382	0.0172
Overall	0.644	0.630	0.638	0.666	0.679	0.663	0.019	0.4122	0.0416

[abc] Least Square means with different superscripts tend to be different (P ≤ 0.10).

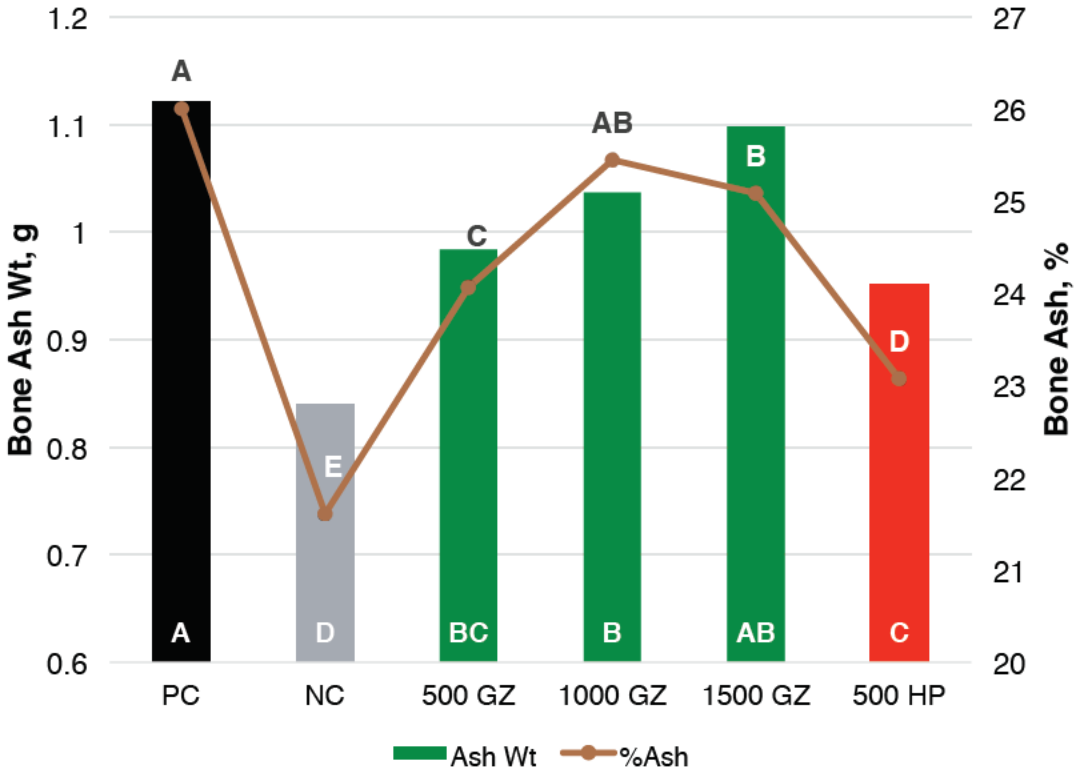
[1] Orthogonal contrast analysis for Negative Control and GralNzyme® Phytase treatments (500, 1,000, and 1,500 FTU/kg).

Table 11. Effect of GraINzyme® Phytase and Ronozyme® HiPhos on metacarpal bone characteristics in nursery pigs

	Positive Control	Negative Control	GraINzyme® Phytase			HiPhos		*P* - Value	
FTU/kg			500 FTU	1,000 FTU	1,500 FTU	500 FTU	SEM	Treatment	Linear[1]
Length, mm	36.87	36.31	36.57	36.75	37.00	36.39	0.31	0.3862	0.0627
Width, mm	9.248	9.113	9.148	9.048	9.365	9.300	4.149	0.4510	0.2403
Fresh bone, g	4.314^{ab}	3.872^{c}	4.091^{bc}	4.090^{bc}	4.393^{a}	4.134^{b}	0.110	0.0030	0.6360
Ash, g	1.122^{a}	0.840^{d}	0.984^{bc}	1.037^{b}	1.098^{ab}	0.951^{c}	0.029	<0.0001	0.0878
Ash, %	26.010^{a}	21.610^{e}	24.070^{c}	25.450^{ab}	25.090^{b}	23.080^{d}	0.284	<0.0001	<0.0001

[a-e] Least Square means with different superscripts are significantly different (P < 0.05).
[1] Orthogonal contrast analysis for Negative Control and GralNzyme® Phytase treatments (500, 1,000, and 1,500 FTU/kg).

Figure 16. Bone ash weight and percent ash in the femur of pigs in the positive (PC) and negative (NC) controls and 500, 1000 and 1500 FTU/kg GraINzyme® Phytase (GZ) and 500 FTU/kg Ronozyme® HiPhos (HP) treated groups at the end of the study (35 days on treatment).



A-E; Least Square means with different superscripts are significantly different ($P < 0.0001$).

Figure 17. Average daily gain (ADG) and feed efficiency (Gain:Feed) of pigs in the positive (PC) and negative (NC) controls and 500, 1000 and 1500 FTU/kg GraINzyme® Phytase (GZ) and 500 FTU/kg Ronozyme® HiPhos (HP) treated groups after 21 days on treatment.

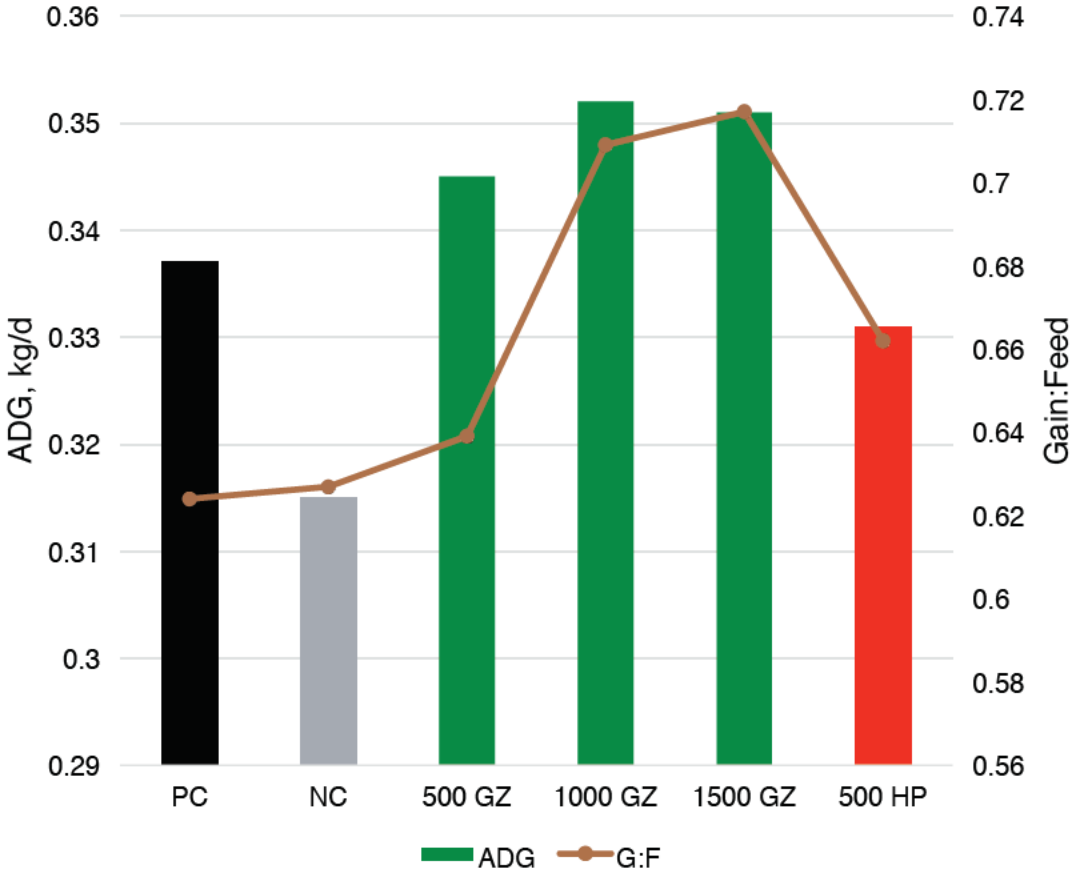
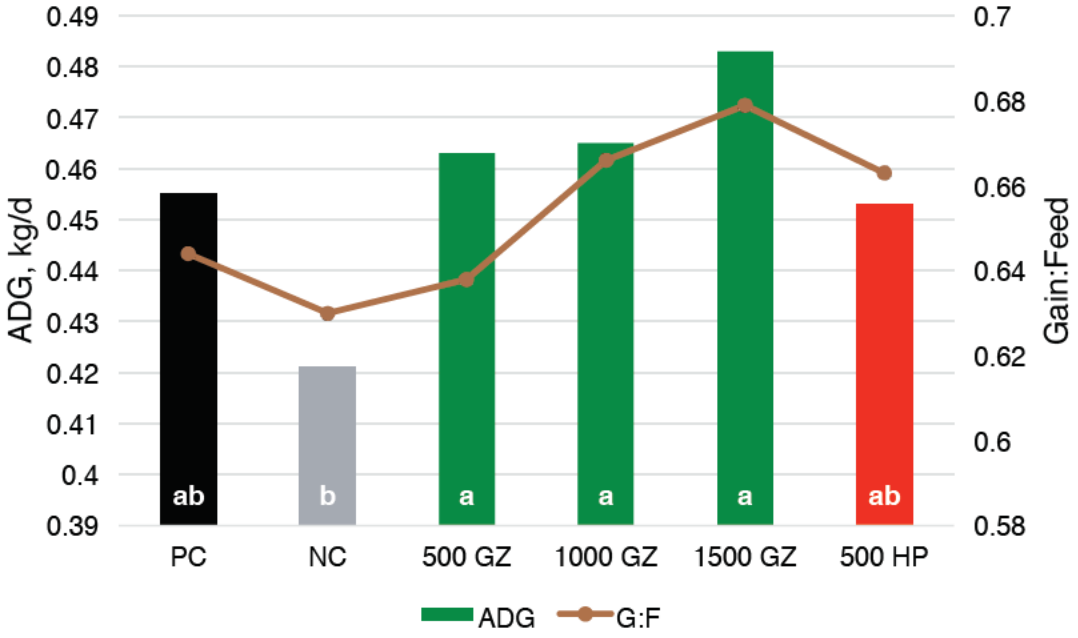


Figure 18. Average daily gain (ADG) and feed efficiency (Gain:Feed) of pigs in the positive (PC) and negative (NC) controls and 500, 1000 and 1500 FTU/kg GraINzyme® Phytase (GZ) and 500 FTU/kg Ronozyme® HiPhos (HP) treated groups through the end of the study (35 days on treatment).



ab: Least Square means with different letter tend to be different (P = 0.10).

6.7.5 Conclusions on the Functionality of GraINzyme® Phytase

In each of the four swine feeding studies, the performance of pigs fed a diet low in available phosphorus but supplemented with increasing doses of GraINzyme® Phytase was compared to that of a group of pigs receiving a diet with adequate available phosphorus and calcium (positive control, PC) and to another group that received a diet low in available phosphorus and calcium without phytase supplementation (negative control, NC). In Study 1 conducted at the (b) (4) (b) (4) the PC group had significantly greater means for ADG, G:F, bone ash weight and percent ash compared to the NC group. In the case of all of these measurements, there was a dose response with increasing doses of GraINzyme® Phytase and the dose at which the GraINzyme® Phytase was statistically the same as the PC group was 500 FTU/kg for ADG and G:F (Figure 10) and 2000 and 4000 FTU/kg, respectively, for percent bone ash and ash weight (Figure 9). In Study 2, also conducted at the (b) (4), the PC group had significantly greater means for ADG, G:F, bone ash weight and percent ash compared to the NC group. In the case of all of these measurements, there was a dose response with increasing doses of GraINzyme® Phytase and the dose at which the GraINzyme® Phytase was statistically equivalent to the PC group was 500 and 1000 FTU/kg for G:F and ADG, respectively (Figure 12). Although none of the GraINzyme® Phytase doses were equivalent to the means of the PC group, the means were significantly greater than those of the NC group, except for the lowest dose for percent ash. In addition, for all measurements in this study the 500 FTU/kg GraINzyme® Phytase group was statistically equivalent to the commercial phytase control at an equivalent dose. In Study 3 conducted by (b) (4) the PC group had significantly greater means for ADG, bone ash weight, percent ash, and bone strength compared to the NC group (Figure 13, 14 and 15). In this study there were no significant differences among means of any treatments for G:F. In the case of all of these measurements, there was a dose response with increasing doses of GraINzyme® Phytase and the dose at which the GraINzyme® Phytase was statistically equivalent to the PC group was 500 FTU/kg for ADG, 1000 FTU/kg for bone ash weight and bone breaking strength, and 2000 FTU/kg for percent ash weight (Figure 13, 14 and 15). In Study 4 conducted at the (b) (4) the means for the PC group were significantly greater than those of the NC group for bone ash weight and percent bone ash. In the case of ADG the means for the PC groups were numerically greater than those of the NC group but were not significantly different (Figure 16). In the case of all of these measurements, there was a dose response with increasing doses of GraINzyme® Phytase and the dose at which the GraINzyme® Phytase was equivalent to the PC group was 500 FTU/kg for ADG, 1000 FTU/kg for percent ash weight and 1500 FTU/kg for bone ash weight. The means for ADG, bone ash weight, and percent bone ash for the group receiving 500 FTU/kg GraINzyme® Phytase were equivalent to the commercial phytase control at an identical dose. The statistical relationships among treatment groups for all measurements that are discussed above are summarized in Table 12.

In summary, the results of four swine feeding trials demonstrate the functionality of the GraINzyme® Phytase in releasing phosphorous from phytate in the diet and making it nutritionally available to the animals. In general the addition of GraINzyme® Phytase to swine diets resulted in improvements in ADG, G:F, bone ash weight and percent bone ash. In all studies the increase in these performance parameters was in a dose dependent manner relative to the amount of GraINzyme® Phytase included in the feed. In addition, in Study 2 and Study 4 that contained a commercial phytase control treatment, the performance and bone characteristics of pigs receiving GraINzyme® Phytase at 500 FTU/kg were equivalent to the commercial phytase controls at an equivalent dose. The results of these studies confirm the functionality of the GraINzyme® Phytase in swine feeds.

Agrivida, Inc. has concluded as described in §6.5 that the Phy02 phytase is substantially equivalent to two commercial phytases, CP1 and CP2 (Quantum® and Quantum Blue®, respectively). CP1 phytase has been used commercially in poultry and swine feeds since 2007 and CP2 since 2012. CP1 and CP2 phytases have been used effectively in poultry and swine feeds for many years (Beaulieu et al., 2007; Guggenbuhl et al., 2007; Hughes et al., 2008 and 2009; Laird et al., 2016; Veum et al., 2006). In addition, phytase enzymes produced from a modified *E. coli appA* phytase gene by the production host *Pichia pastoris* are listed as safe and functional enzymes for use in poultry and swine feeds in the Official Publication of the Association of American Feed Control Officials (AAFCO, 2015). Based on the conclusion that the Phy02 phytase is substantially equivalent to the CP1 and CP2 phytases, it is logical and reasonable to conclude that the Phy02 phytase is as safe and functional as a feed additive in poultry and swine feed as are the CP1 and CP2 phytase products.

6.8 Identification of information that is inconsistent with the conclusion that GraINzyme® Phytase is GRAS for use in swine

In this GRAS notification, Agrivida, Inc. has presented all information in its possession and or that Agrivida, Inc. is aware of that is relevant and pertinent to its conclusion that the use of the GraINzyme® Phytase in swine is GRAS. Agrivida, Inc. has no information nor is it aware of any information that is inconsistent with, or contradicts, this conclusion of GRAS status for the use of GraINzyme® Phytase in the feed of swine.

6.9 Confidential Business Information in this GRAS notice

This GRAS notice contains information that Agrivida, Inc. considers to be confidential (CBI). The information within this document that is considered by Agrivida, Inc. to be confidential business information is identified by shaded text (e.g., CBI).

Table 12. A summary and comparison of the statistical relationships among means for animal performance and bone ash in four swine feeding studies. Alphabetic statistical indicators within a measurement that do not have a letter in common are statistically (ABC; P<0.05) or numerically (ab; P = 0.10) different.

	Measurement	NC	PC	Dose Level of GraINzyme Phytase (FTU/kg)					CC (500 FTU/kg)	PC>NC?	Dose of GZ=PC	Dose of GZ=CC	GZ dose response?
				500	1000	1500	2000	4000					
Study 1	ADG	C	AB	BC	AB	-	AB	A	-	+	500	NA	+
U. of IL	G:F	B	A	AB	A	-	A	A	-	+	500	NA	+
	Ash Wt	E	A	DE	CD	-	BC	AB	-	+	4000	NA	+
	Ash %	D	A	CD	BC	-	AB	A	-	+	2000	NA	+
Study 2	ADG	C	A	BC	ABC	-	-	-	AB	+	1000	500	+
U. of IL	G:F	C	A	AB	BC	-	-	-	ABC	+	500	500	-
	Ash Wt	D	A	C	C	-	-	-	C	+	None	500	+
	Ash %	D	A	CD	BC	-	-	-	BCD	+	None	500	+
Study 3	ADG	D	BC	C	BC		B	A	-	+	500	NA	+
SRS	G:F	No Significant Differences Among Treatments							-	NA	NA	NA	-
	Ash Wt	D	B	C	B	-	B	A	-	+	1000	NA	+
	Ash %	D	AB	C	C	-	BC	A	-	+	2000	NA	+
	Bone Str	C	A	B	A	-	AB	A	-	+	1000	NA	+
Study 4	ADG	b	ab	a	a	a	-	-	ab	-	500	500	+
U. of AR	G:F	No Significant Differences Among Treatments								NA	NA	NA	+
	Ash Wt	D	A	BC	B	AB	-	-	C	+	1500	500	+*
	Ash %	E	A	C	AB	B	-		D	+	1000	<500[1]	+

CC = Commercial phytase control at 500 FTU/kg; GZ = GraINzyme® Phytase; NA = non-applicable; NC = negative control; PC = positive control

[1]500 FTU/kg GraINzyme Phytase response is statistical greater than 500 FTU/kg of commercial phytase control

7. 0 References

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8.0 Appendices

8.1 Appendix 1

Study report for a 10X tolerance dose swine feeding trial

Study Title
GraINzyme phytase Phy02 10x Tolerance Swine Study

Study Number
16-17

Sponsor
Agrivida
200 Boston Ave., Suite 2975
Medford, MA 02155

Sponsor Representative
Jon Broomhead, PhD

Study Initiation Date
July 19, 2016

Study Completion Date
October 03, 2017

Performing Laboratories

Processing
(b) (4)
(b) (4)

Phytase Testing
Agrivida
200 Boston Ave., Suite 2975
Medford, MA 02155

Blood Chemistry and Hematology
(b) (4)

Proximate Analysis
(b) (4)

Study Investigator
(b) (4)

(b) (4)

Animal Facility Location
(b) (4)

GLP Compliance Statement

Study title: GraINzyme phytase Phy02 10x Tolerance Swine Study

This study was conducted in compliance with United States Good Laboratory Practice (GLP) regulation 9 CFR Part 58, Good Laboratory Practice for Nonclinical Laboratory studies, with the following exceptions:

1. Feed and text article mixing
2. Laboratory testing:
    a. Hematology testing
    b. Serum testing
    c. Proximate testing
    d. Phytase testing
3. Stability testing is not reported as part of this study (See Deviation 7).
4. Test article characterization results were not provided to be included as part of this study.

The quality assurance statement including dates of quality assurance inspections can be found in Appendix 3.

(b) (4)

02Oct17
Date

03 Oct 17
Date

Personnel involved with this study were:

(b) (4)

Study Materials and Equipment

Beuthanasia-D Special Euthanasia Solution; (b) (4)

Corvac™ integrated serum separator tube (SST); Covidien, Mansfield, MA
Excede® for Swine; Pharmacia & Upjohn Company, Division of Pfizer Inc., New York, NY
Fatal Plus®; Vortech Pharmaceuticals Ltd., Dearborn, MI
RTI Nursery 1 Low Zn No Phytase – 53714-6 (Lot Code #B01076428; Prestarter); Hubbard Feeds Inc., Mankato, MN
RTI Nursery 2 Low Zn No Phytase – 53715-6 (Lot Code #B01095697; Starter); Hubbard Feeds Inc., Mankato, MN
RTI Nursery 2 Low Zn No Phytase – 53715-6 (Lot Code #B01076429; Starter); Hubbard Feeds Inc., Mankato, MN
Smart Weigh ACE200 Digital Scale; Chestnut Ridge, NY
Starter feed from Volga Ag Center RTI Hubbard Mix
Tanita BWB-800A Scale (Serial #0281); Tanita Corporation of America, Inc., Arlington Heights, IL
Traceable™ Dew-Point/Wet-Bulb/Humidity Thermometer; Fischer Scientific™, Pittsburgh, PA
Whirl-Pak® bags; Nasco, Fort Atkinson, WI
12 mL Syringes; Monoject™, Covidien™, Mansfield, MA
18G x 1" injection needles; Monoject™, Covidien™, Mansfield, MA
20 G x 1" multi-sample blood collection needles; Monoject™, Covidien™, Mansfield, MA

Study Methods

Animal Care and Use. Prior to study initiation, animal use and procedures were evaluated and approved by the (b) (4) .

Animal (Source and) Arrival. On August 17, 2016, a total of forty-one (41) approximately 21 day-old female and castrated male mixed breed pigs arrived in good condition at the (b) (4) (see Deviation #1, dated 17Aug16 and Note-to-File #1, dated 17Aug16.). The pigs were sourced by (b) (4) ; herd free of tuberculosis, brucellosis, and pseudorabies) from a herd determined to be negative for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Epidemic Diarrhea Virus (PEDV), Transmissible Gastroenteritis Coronavirus (TGEV), Porcine Delta Corona Virus (PDCoV), and *Mycoplasma hyopneumoniae* (*M. hyo*) via polymerase chain reaction (PCR) testing. Upon arrival, pigs were double-ear tagged with uniquely numbered ear tags, and 0.5 mL Excede® was administered intramuscularly (IM) in the right neck for prevention of respiratory disease related to shipping (see Deviation #3, dated 21Oct16 and Note-to-File #1, dated 17Aug16). No abnormalities were observed at the time of arrival.

Test Article (Investigational Veterinary Product) Receipt, Storage, and Handling. On July 1 and August 2, 2016, a total of 11.65 kg and 2.5 kg, respectively, of *GraINzyme*™

Phytase Av_Phy02_0083/84 arrived in good condition at the (b) (4) . The product was stored at ambient temperature at the facility until used in mixing the test article on August 4, 2016. Additionally, a total of 4.5 kg of *GraINzyme*™ Phytase Av_Phy02_0086 arrived in good condition at the (b) (4) main office on September16, 2016, and was stored at ambient temperature at the facility until used in mixing additional test article on September 19, 2016 (see Deviation #5, dated 21Oct16).

On August 4 and September 19, 2016, a total of 14.15 kg of *GraINzyme*™ Phytase Av_Phy02_0083/84 and 4.5 kg (8.8 lbs) of *GraINzyme*™ Phytase Av_Phy02_0086, respectively, were transported at ambient temperature to the (b) (4) for mixing (see Deviation #'s 4 and 5, dated 21Oct16, and Note-to-File #3, dated 21Sep16). On August 4, 2016, a total of 4.4 kg *GraINzyme*™ Phytase Av_Phy02_0083/84 was placed in the mixer with 550 lbs of pre-starter feed (b) (4) ; was mixed for a period of 9 minutes, samples were taken, and the mixture was bagged. Additionally, 9.6 kg of *GraINzyme*™ Phytase Av_Phy02_0083/84 was mixed with a total of 1200 lbs of starter feed (b) (4) (i.e., 600 lbs of feed added, then Phytase, then 600 lbs of feed), mixed for 9 minutes, samples were collected, and the mixture was bagged. Similarly, on September 19, 2016, a total of 8.8 lbs of *GraINzyme*™ Phytase Av_Phy02_0086 was mixed with 500 lbs of starter feed (b) (4) mixed for 10 minutes, samples were collected, and the mixture was bagged. All bags were labeled as either pre-starter or starter A (control feed) or pre-starter or starter B (feeds containing Phytase) (see Note-to-File #7, dated 04Nov16). Feeds were labeled as either A or B to ensure that personnel feeding the animals remained blinded to treatment group assignment. The remaining 0.15 and 0.5 kg of Phytase Av_Phy02_0083/84 and Av_Phy02_0086, respectively, were returned to (b) (4) after use, and were subsequently shipped to the Sponsor on November 2, 2016.

Prior to and following each Phytase/feed mixing procedure, the mixing system was flushed. Briefly, 500 lbs of rolled corn was placed in the mixer, mixed for 9 minutes, flushed through the system, and bagged. Following Phytase mixing, the rolled corn was again placed in the mixer, mixed for 8 - 10 minutes, flushed through the system, and was bagged for disposal. Bags of rolled corn used to flush the system on August 4 and September 19, 2016, were disposed of in the local landfill on August 4 and September 29, 2016, respectively (see Note-to-File #10, dated 07Nov16).

Bags of mixed feed were transported to the (b) (4) facility and utilized, according to protocol. While at the test facility bags were stored on pallets at room temperature in the clean hallway, just outside of the animal room. On August 30 and September 29, 2016, remaining open bags of pre-starter and starter feeds, respectively, were taped closed and placed on a pallet. The pallets were subsequently wrapped and transported to the (b) (4) for storage until destruction (see Note-to-File #'s 2, 5, and 10 dated 30Aug16, 24Oct16, and 18Nov16, respectively). All remaining feed was incinerated on 02Nov16- 04Nov16 and 07Nov16 –14 Nov16 (see Note-to-File #10, dated 18Nov16).

Randomization. At the time of arrival, all pigs were weighed, ranked by weight, and randomly divided into 2 groups of 20 pigs per group using Microsoft® Excel® to ensure animals weights were equally represented in both treatment groups. After assignment to one of the two groups, either Group A or B, pigs were ranked by weight within their respective groups, and were sequentially divided into 10 pairs per group to ensure relative uniformity of weight in each pen. Once paired, the pairs were randomly assigned to one of 20 pens using Microsoft® Excel®. Prior to the mixing of the feeds, the treated and untreated feeds were assigned a letter code, A or B, by drawing slips of paper out of a hat (See Note-to-File #12). Letter 'A' was assigned to the untreated/control feeds. Letter 'B' was assigned to the treated/10x feeds. See **Table 1** for animal ID, Pen, and Group assignments.

Table 1. Assignments of Pigs by Pen and Group

ID	Pen	Group	ID	Pen	Group
274	4	A	270	1	B
301	4	A	294	1	B
269	5	A	284	2	B
291	5	A	296	2	B
292	8	A	293	3	B
297	8	A	264	3	B
280	10	A	298	6	B
279	10	A	262	6	B
283	11	A	263	7	B
285	11	A	281	7	B
271	14	A	261	9	B
272	14	A	282	9	B
265	16	A	277	12	B
278	16	A	275	12	B
295	17	A	287	13	B
300	17	A	290	13	B
267	18	A	268	15	B
276	18	A	289	15	B
299	20	A	273	19	B
288	20	A	286	19	B

Blinding. All personnel performing animal observations or laboratory assays were blinded to treatment assignment.

Housing. All pigs were housed in a facility that was Biosafety Level 2 (BSL2) compliant. The pigs were placed into one of 20 pens labeled with the appropriate treatment code (i.e., either Group A or Group B), according to the randomization, in Room #5 of the (b) (4) Facility. Pens were approximately 5' x 7' in size, on elevated, slatted flooring. Each pen was equipped with a single nipple waterer, a stainless steel feeder that provided approximately 5 linear feet of feeder space, a mat, and a heat lamp. Pens were set up in such a way as to ensure that there was no contact between feeders (see Appendix 1, (b) (4) Facility Diagram).

Ambient temperature (^{o}F) and relative humidity (%) were recorded daily using a calibrated digital Dew-Point/Wet-Bulb/Humidity Thermometer.

Animal Feeding and Test Article Administration. Upon arrival, all animals were given free choice access to the pre-starter rations. Pigs in Group B were fed rations containing approximately 60,000 FTU/kg of Phytase, while pigs in Group A were fed the same base rations without Phytase (see Note-to-File #7, dated 04Nov16). The pre-starter rations were fed on August 17 – 29, 2016, and starter rations were fed beginning on the morning of August 30, 2016, through the remainder of the trial (see Deviation #6, dated 24Oct16). The phytase rations mixed on August 4 and September 19, 2016, were fed on August 30 – September 22, 2016, and September 23 – 29, 2016, respectively (see Note-to-File #3, dated 21Sep16). Feeds containing phytase were stored separately from control feeds, to prevent any potential cross-contamination.

Feed and water were available *ad libitum* throughout the duration of the trial. Feed was placed in feeders and levels were checked daily and replenished, as needed, to ensure access to feed at all times. Feeders were weighed prior to being filled, each addition of feed was weighed prior to being added to feeders, and all weights were recorded (see Note-to-File #6, dated 24Oct16). On August 30, 2016, feeders were weighed with the remaining pre-starter feed and were subsequently emptied (see Note-to-File #2, dated 30Aug16). Empty feeders were then filled with the appropriate starter ration. Upon trial completion on September 29, 2016, feeders were again weighed with the remaining starter feed, prior to being emptied. All remaining feeds were bagged for disposal and destruction by incineration (see Deviation #2, dated 26Sep16 and Note-to-File #10, dated 18Nov16).

Feed Weights. All feeds were weighed prior to being added to the feeders using either the Tanita or Easy Weigh digital scale. Scales were check-weighted with certified weights for accuracy before and after each weighing event. Feeds were weighed into totes labeled with the corresponding feed identification (i.e., either A or B), prior to being added to the feeders (see Note-to-File #6, dated 24Oct16). Specifically, feeds were weighed into totes on August 22, 29, and 30, 2016, and September 02, 05, 06, 08, 12, 14, 16, 18, 19, 21 – 23, 25, 26, and 28, 2016. Additionally, all remaining feed was weighed upon study completion on September 29, 2016 (see Deviation #2, dated 26Sep16 and Note-to-File #8, dated 04Nov16).

Daily Health Observations. All pigs were observed for general health once daily by an (b) (4) employee beginning on the day of arrival and continuing through the duration of the trial. Individual animal abnormalities were recorded and reported to an (b) (4) veterinarian, as appropriate. During the course of the study, the following health observations were observed.

No abnormalities were noted for any animals in Group A for the duration of the study. On August 31, 2016, animal #284 was reported with a mild lameness in Group B. Between September 27 and September 29, 2016, animal #286 was noted to have a hernia (which was found to be a distension of the urethra upon necropsy) in Group B. Also in Group B, between September 27 and September 29, 2016, animal #293 was reported to have a left hock swelling, coughing, sneezing and epistaxis. No treatments were given to any animals during the duration of the study.

Blood Sample Collection and Testing. Whole blood was collected from all enrolled pigs a total of 2 times throughout the trial {i.e., at the time of study initiation on August 17, 2016, [Study Day 0 (D0)] and prior to necropsy on September 29, 2016, [Study Day 43 (D43)] (see Deviation #2, dated 26Sep16). Briefly, piglets were manually restrained and a total of approximately 12 – 14 mL of whole blood was collected [i.e., approximately 10 mL into 12 mL serum separator tubes (SST) and approximately 2 mL into 3 mL ethylene diamine tetra-acetic acid (EDTA) tubes] using 20 G x 1" multi-sample blood collection needles. EDTA tubes were inverted several times to ensure thorough mixing of blood and anticoagulant, and all tubes were labeled with the animal identification number and were transported to the (b) (4) Laboratory for processing. SST tubes were centrifuged at approximately1500 x g for approximately15 minutes at 2-7°C, serum was aliquoted into 2 aliquots per sample, and were frozen at ≤-20°C. One aliquot was submitted to (b) (4) for blood chemistry analysis, including albumin, alanine aminotransferase (ALT), creatinine kinase (CK), glucose, and phosphorous. EDTA tubes were shipped overnight on ice to (b) (4) for hematology testing including total white blood cell count (WBC), WBC differential (i.e., neutrophil, lymphocyte, monocyte, eosinophil, basophil), absolute large unstained cells (LUC), red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, and mean platelet volume (MPV).

Feed Sample Collection and Testing Summary. Feed samples were collected multiple times throughout the trial, according to protocol. See **Table 2** below for sample dates, number of samples collected, test performed, and performing laboratory. (See Note-to-File #13, dated 02Oct17). For each collection, approximately 500g of feed was collected into 18 oz Whirl-Pak® bags. Samples were stored and shipped at ambient temperature to the Sponsor for phytase testing and/or retention or submission to SGS North America for proximate analysis including moisture, crude ash, dry matter content, crude fat, crude fiber, and crude protein (see Note-to-File #4, dated 24Oct16).

Table 2. Feed Sample Collection Log

Date of Sample Collection	Feed Collected	Number of samples collected	Date samples shipped to laboratory	# of samples to laboratory	Test(s) performed*	Performing/Retain Laboratory
August 04, 2016	Pre-starter, B (treated)	10	08Aug16	10	Phytase	Agrivida
August 04, 2016	Starter, B (treated)	10	08Aug16	10	Phytase	Agrivida
August 05, 2016	Pre-starter, A (untreated)	2	08Aug16	1	Phytase	Agrivida
			08Aug16	1	Retain	Agrivida
August 05, 2016	Starter, A (untreated)	2	08Aug16	1	Phytase	Agrivida
			08Aug16	1	Retain	Agrivida
August 31, 2016	Pre-starter, B (treated)	2	31Aug16	1	Proximates	(b)
			31Aug16	1	Retain	Agrivida
August 31, 2016	Starter, B (treated)	2	31Aug16	1	Proximates	(b)
			31Aug16	1	Retain	Agrivida
August 31, 2016	Pre-starter, A (untreated)	2	31Aug16	1	Proximates	(b)
			31Aug16	1	Retain	Agrivida
August 31, 2016	Starter, A (untreated)	2	31Aug16	1	Proximates	(b)
			31Aug16	1	Retain	Agrivida
September 19, 2016	Starter, B (treated)	13	20Sep16	1	Proximates	(b)
			20Sep16	11	Phytase	Agrivida
			20Sep16	1	Retain	Agrivida
September 19, 2016	Starter, A (untreated)	3	20Sep16	1	Proximates	(b)
			20Sep16	1	Phytase	Agrivida
			20Sep16	1	Retain	Agrivida

On August 04, 2016, a total of 20 samples (i.e., 10 each of pre-starter and starter feeds) of feed containing phytase were collected during the mixing process. Similarly, 4 samples (i.e., one of each for phytase testing and one of each for retention) of each of the untreated pre-starter and starter feeds were collected on August 05, 2016. Feed samples collected after mixing were shipped to the Sponsor on August 08, 2016. The sponsor completed testing for phytase content for data on uniformity of the feed mixing process.

A total of 8 feed samples (i.e., 2 each of pre-starter feeds A and B and starter feeds A and B) were collected on August 31, 2016. One sample of each was subsequently submitted to SGS on 31Aug16, one each of pre-starter B and starter B were shipped to the Sponsor on 31Aug16, and one each of pre-starter A and starter A were stored at (b) (4) at room temperature.

On September 19, 2016, a total of 12 samples of starter feed B (with phytase) were collected during the mixing process, and 3 samples of starter feed A were collected on September 20, 2016. One sample of each of the starter feeds A and B were submitted to SGS, and the remaining samples (n = 13) were shipped to the Sponsor on September 20, 2016.

Animal Body Weights. Animal body weights were determined for all pigs a total of three times throughout the study (see **Table 11** in the Results Section). On August 17,

2016 (D0), August 30, 2016 (D13) and September 29, 2016 (D43), the pigs were weighed using the Tanita digital scale (see Deviation #2, dated 26Sep16). The scale was check-weighted with certified weights for accuracy before and after each weighing event. Necropsy. On September 29, 2016, all pigs were euthanized via intravenous (IV) injection of a barbiturate overdose and necropsied (see Deviation #2, dated 26Sep16). Carcasses were examined for abnormalities or indication of toxicity including, but not limited to, kidney and bladder changes. Samples of joint fluid were collected from 2 pigs (i.e., Pig #'s 279 and 263 in Groups A and B, respectively) using an 18G x 1in needle and 12.0 mL syringe. Abnormal tissues were collected into 10% formalin jars and stored at ambient temperature, while joint fluid was maintained in syringes and stored in a refrigerator. All samples were retained at (b) (4) until further notice (see Note-to-File #9, dated 07Nov16).

Animal Disposition. On August 17, 2016, Pig #266 was removed from the trial due to extreme difficulty collecting blood, and custody was transferred to (b) (4) (see Note-to-File #1, dated 17Aug16). All remaining pigs (n = 40) were euthanized and necropsied on September 29, 2016, and the carcasses were buried (see Deviation #2, dated 26Sep16).

Study Forms.

The data capture forms in **Table 3** below were used for data collection during the animal trial and are part of the study records. The origin of the form and the form number or form ID are included in the table.

Table 3. Study data capture forms utilized in the collection of raw data during the animal portion of this trial.

Form Title	Form Origin	Form Number/ID
Abbreviation Definitions	(b) (4)	
Amendment / Deviation	(b) (4)	
Animal Arrival Form	(b) (4)	
Animal Disposition Form	(b) (4)	(b) (4)
Animal Weight Form	(b) (4)	
Chain of Custody	(b) (4)	
Daily Swine Husbandry/Activity Log	(b) (4)	
Dosage Administration and Product Use Record – Single Day	(b) (4)	
Error Correction Codes	(b) (4)	
Feed Mixing Record Form		
Feed Sample Collection Form		
Feed Weight Record		
General Health Observations		
Incineration Transfer Record		
Necropsy Report Form		
Note-to-File		
Personnel Signature Form		
Pre-Study Training Form		
Sample Check-off and Processing		
Sample Collection Form		
Sample Collection Form		
Scale Check Weight Form		
Test Article/Study Material Receipt, Use, and Disposition Record		

Results

Results include feed ration testing results, blood testing results, feed consumption results and necropsy results. Feed ration testing results include phytase uniformity testing and proximate testing. Blood testing results include hematology and blood chemistry. Feed consumption results include animal weights, feed weights, and a summary of feed consumption analysis. Necropsy results include tissue samples that were collected due to abnormal appearance. Tissue testing was not performed.

Feed Ration Testing.

Treated and untreated feeds were tested for phytase uniformity/content after mixing. For each of the treated feeds, Group B feed rations, ten (10) samples were collected through the bagging processes so the uniformity of phytase in the feed could be determined. Two samples of each untreated feed ration, group A feed rations, were collected to verify the absence of phytase in untreated samples. One sample of the untreated rations was for retain, however to provide additional data the retain samples of untreated feed rations were tested.

The treated pre-starter samples (Group B pre-starter samples) contained 21762 - 57695 FTU/kg phytase. The coefficient of variation (%CV) of the treated pre-starter feeds was 24%. The untreated pre-starter feed (Group A pre-starter samples) contained no phytase. The treated starter feed samples from the first mixing on August 04, 2016 (Group B Batch 1 Starter samples) contained 38683 – 51589 FTU/kg phytase. The coefficient of variation of the treated starter feed mixed on August 04, 2016 was 12%. The untreated starter feed (Group A Batch 1 Starter samples) contained no phytase. The treated starter feed from the second mixing on September 19, 2016 (Group B Batch 2 Starter samples) contained 17856 – 51160 FTU/kg phytase. The coefficient of variation for the treated feeds from the second mixing was 25%. The untreated feed from the second mixing contained no phytase. The average and range of phytase in the feed rations are in Table 4. The phytase results and standard deviations of phytase content measured in each feed sample are in Table 5. The sample results from the treated feed rations were plotted in a line graph to show the variation between samples collected through the bagging process. The line graphs for each set of ten (10) samples collected through the feed bagging process are in Figures 1-3. Results of phytase testing are in **Table 4** and **Table 5**.

Proximate testing was completed on treated and untreated feeds. Proximate testing included testing for percentage moisture, percentage crude ash, percentage crude fat, percentage crude fiber, and percentage crude protein. The laboratory reported results on an as received basis and a dry matter basis. The percentage moisture in all feed rations was 8.74-10.18%. The average percentage moisture of feed rations was 9.55% with a coefficient of variation between rations was 6.71%. The percentage of crude ash in feed rations on a dry matter basis was 6.02-6.64%. The average percentage of crude ash on a dry matter basis was 6.38% with a coefficient of variation between rations of 3.19%. The percentage of crude fat on a dry matter basis was 4.78-5.67%. The average percentage crude fat was 5.15% with a coefficient of variation of 6.41% between rations. The percentage of crude fiber on a dry matter basis was 2.39-2.89%. The average percentage crude fiber was 2.61% with a coefficient of variation between rations of 7.78%. The percentage of crude protein in ratios was 19.733-24.78. The average percentage of crude

protein was 21.71% with a coefficient of variation between rations of 7.94%. The results of proximate testing are in **Table 6**.

Table 4. Average Phytase content and range of phytase in feed rations.

Average Phytase Homogeneity in Feed Rations		
Feed ID	**FTU/kg**	**Range**
Prestarter B	44,926	21,762 to 57,695
Prestarter A	-2,693	-2,768 to -2,617
Starter B Batch 1	44,134	38,683 to 51,589
Starter A Batch 1	-2,989	-3,197 to -2,781
Starter B Batch 2	35941	17856 to 51160
Starter A Batch 2	679	243 to 1114

Table 5. Phytase Concentration/ Homogeneity in Feed Rations

Pre-Starter B Bag 2 Top	51685	4910	
Pre-Starter B Bag 2 Bottom	57695	4888	24%
Pre-Starter B Bag 3 Middle	32385	7199	

Pre-Starter B Bag 4	49929	5347	
Pre-Starter B Bag 5	21762	2405	
Pre-Starter B Bag 6	46736	5669	
Pre-Starter B Bag 7	42942	3281	
Pre-Starter B Bag 8	46262	3185	
Pre-Starter B Bag 9	43289	3164	
Pre-Starter B Bag 10 Top	56574	3884	
Pre-Starter A Phytase Test	-2617	N/A	
Pre-Starter A Retention	-2768	N/A	
Starter Phytase Feed Batch 1 Sample ID	**FTU/Kg (average)**	**Standard Deviation**	
Starter B Bag 3 Top	41606	2868	
Starter B Bag 3 Bottom	41101	4033	
Starter B Bag 7	45901	2357	
Starter B Bag 8	38683	3253	
Starter B Bag 13 Top	51589	2195	12%
Starter B Bag 13 Bottom	51525	7477	
Starter B Bag 16	51542	6993	
Starter B Bag 19	39863	4172	
Starter B Bag 21 Top	38694	4289	
Starter B Bag 21 Bottom	408312	2914	
Starter A Phytase Test	-3197	N/A	
Starter A Retention	-2781	N/A	
Starter Phytase Feed Batch 2 Sample ID	**FTU/kg (average)**	**Standard Deviation**	
Batch 2 Starter B Trt 1	51160	7449	
Batch 2 Starter B Trt 2	17856	4221	
Batch 2 Starter B Trt 3	26020	6225	
Batch 2 Starter B Trt 4	43363	4503	
Batch 2 Starter B Trt 5	31606	4028	
Batch 2 Starter B Trt 6	51039	1802	25%
Batch 2 Starter B Trt 8	41303	6960	
Batch 2 Starter B Trt 9	33755	5342	
Batch 2 Starter B Trt 10	38811	8459	
Batch 2 Starter B Trt 11	42273	4995	
Batch 2 Starter B Trt 12	37857	3025	
Batch 2 Starter B Trt 13	31473	5387	
Batch 2 Starter A Phytase Test	243	N/A	
Batch 2 Starter A Retention	1114	N/A	

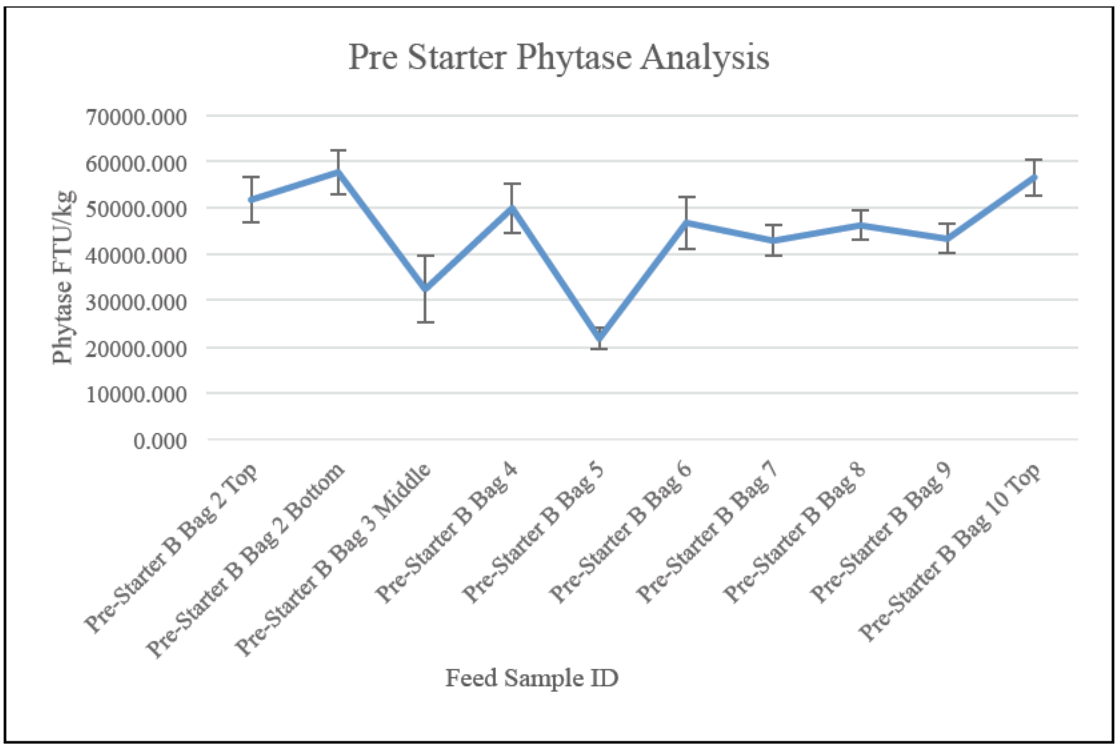


Figure 1. Graph showing the Phytase content in samples taken throughout the bagging of the treated Pre-Starter Ration (Group B).

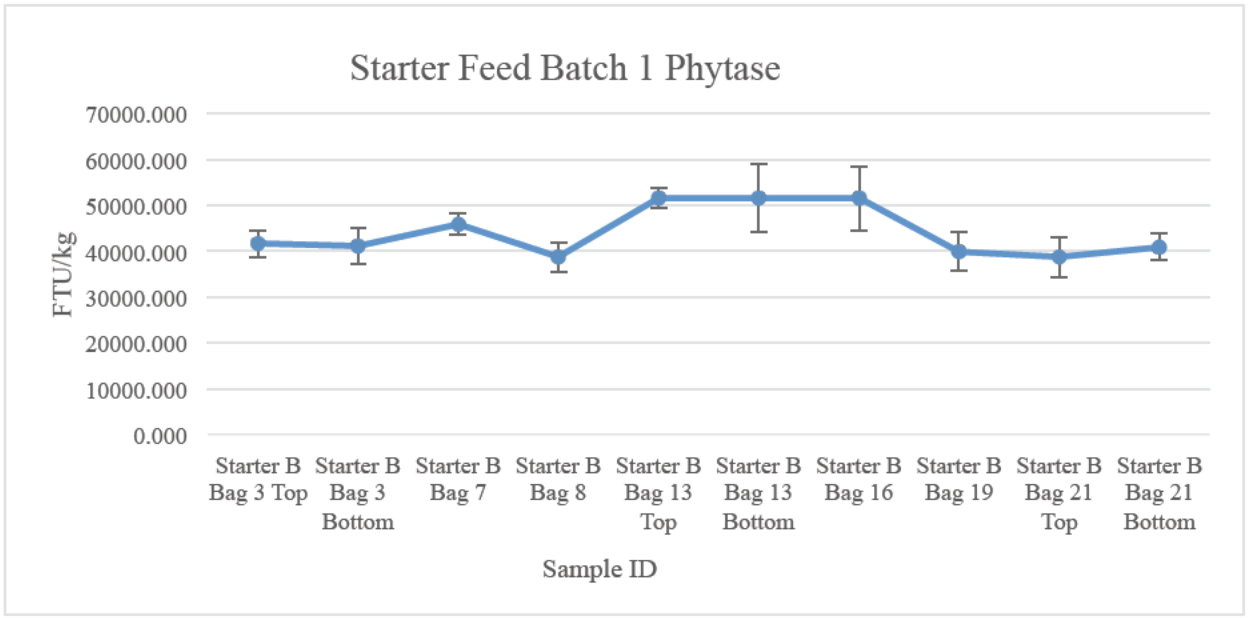


Figure 2. Graph showing the Phytase content in samples taken throughout the bagging of the first mixing (August 04, 2016) of treated Starter Ration (Group B).

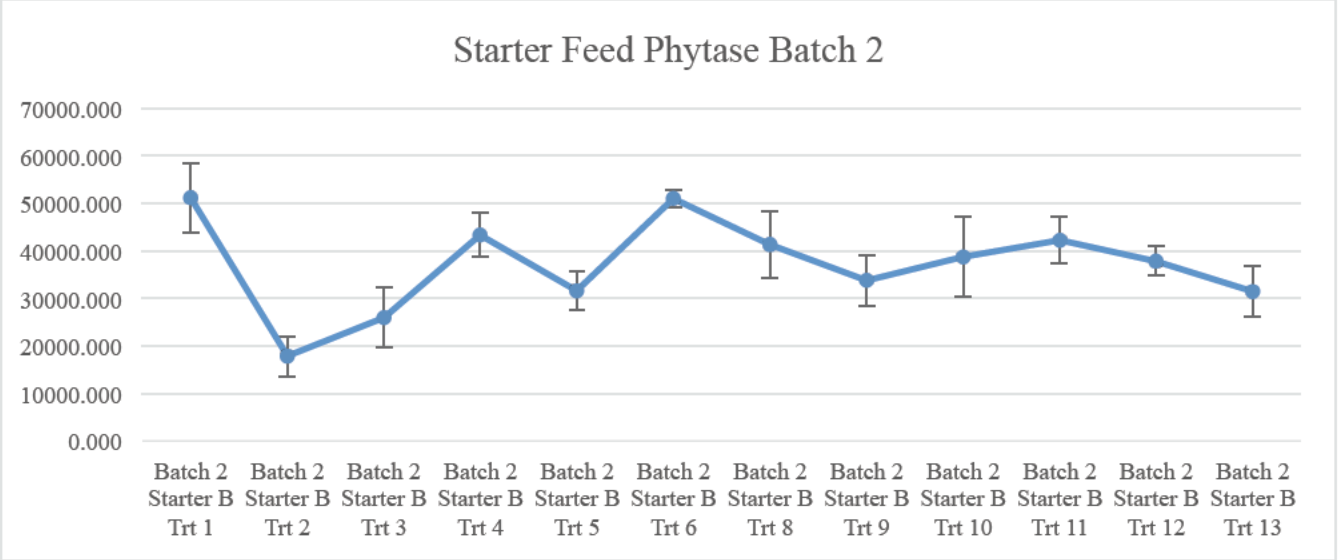


Figure 3. Graph showing the Phytase content in samples taken throughout the bagging of the second mixing (September 19, 2016) of treated Starter Ration (Group B).

Table 6.

Proximate Analysis Results in %(mass/mass)										
	Crude Ash		Moisture	Dry-matter	Crude Fat		Crude Fiber		Crude Protein	
Sample ID	AR*	DM*	AR*	AR*	AR*	DM*	AR*	DM*	AR*	DM*
Pre-Starter A	5.810	6.367	8.74	91.26	5.17	5.67	2.18	2.39	21.33	21.33
Pre-Starter B	5.814	6.371	8.74	91.26	4.85	5.31	2.22	2.43	21.39	21.39
Starter A (31Aug16 mixing)	5.743	6.378	9.95	90.05	4.58	5.09	2.34	2.60	20.75	20.75
Starter B (31Aug16 mixing)	5.433	6.020	9.74	90.26	4.72	5.23	2.54	2.81	19.73	19.73
Starter A (19Sep16 mixing)	5.967	6.643	10.18	89.82	4.29	4.78	2.27	2.53	20.01	22.28
Starter B (19Sep16 mixing)	5.829	6.471	9.93	90.07	4.35	4.83	2.61	2.89	22.32	24.78
Average	5.77	6.38	9.55	90.45	4.66	5.15	2.36	2.61	20.92	21.71
Std Dev	0.18	0.20	0.64	0.64	0.33	0.33	0.18	0.20	0.96	1.72
%CV	3.10	3.19	6.71	0.71	7.05	6.41	7.47	7.78	4.60	7.94

*AR = As Received; DM = Dry Matter basis

Feed Consumption Results

Feed was weighed prior to adding feed to a feeder to allow for calculation of feed intake. The average daily intake of feed was calculated for the period of time that the pre-starter ration was fed and for the period of time that the starter ration was fed. Animal weights along with the feed weight information were utilized to calculated average daily gain.

Average Daily Intake and Average Daily Gain are in **Table 7**. Full results of feed totals, daily intake, daily gain, and weight to feed ratios are in **Tables 8-11**.

Table 7. Average Daily Intake and Average Daily Gain by feed ration and group.

Feed Ration	Animal Group	Average Daily Intake (lbs/pig/day)	Average daily gain (lbs/pig/day)	Weight to Feed ratio
Pre-starter	A	0.75	0.54	0.74
Pre-starter	B	0.72	0.54	0.75
Starter	A	2.32	1.46	0.63
Starter	B	2.35	1.46	0.62

Table 8. Feed totals and average daily intake on pre-starter ration.

Pre - Starter Feed Conversions					
Pen	**Group**	**Feed Total (lbs)**	**Feed Remaining (lbs)**	**Feed/Day**	**Average Daily Intake (lbs/pig/day)**
4	A	24	6.0	1.38	0.69
5	A	24	1.8	1.71	0.85
8	A	24	7.6	1.26	0.63
10	A	24	3.6	1.57	0.78
11	A	24	4.8	1.48	0.74
14	A	24	0.6	1.80	0.90
16	A	24	6.8	1.32	0.66
17	A	24	5.2	1.45	0.72
18	A	24	9.6	1.11	0.55
20	A	24	2.6	1.65	0.82
1	B	24	6.2	1.37	0.68
2	B	24	4.2	1.52	0.76
3	B	24	5.2	1.45	0.72
6	B	24	4.2	1.52	0.76
7	B	24	3.0	1.62	0.81
9	B	24	6.2	1.37	0.68
12	B	24	11.0	1.00	0.50
13	B	24	1.8	1.71	0.85
15	B	24	2.4	1.66	0.83
19	B	24	8.0	1.23	0.62

Table 9. Animal Weights, Average Daily Gain, and Gain to Feed ratio on Pre-starter Ration.

Animal Weights on Pre – Starter Feed						
Pen	**Group**	**Animal ID**	**Day 0 (lbs)**	**Day 13 (lbs)**	**Average Daily Gain (lbs)**	**Gain:Feed**
4	A	274	9.4	16.2	0.52	0.76
4	A	301	9.4	15.8	0.49	0.71
5	A	269	10.4	18.0	0.58	0.69
5	A	291	10.4	18.8	0.65	0.76

8	A	292	10.4	14.4	0.31	0.49
8	A	297	10.6	17.8	0.55	0.88
10	A	279	11.2	17.6	0.49	0.63
10	A	280	10.8	18.4	0.58	0.75
11	A	283	11.6	18.0	0.49	0.67
11	A	285	13.0	19.4	0.49	0.67
14	A	271	9.4	19.8	0.80	0.89
14	A	272	9.4	19.2	0.75	0.84
16	A	265	10.0	14.2	0.32	0.49
16	A	278	10.2	17.4	0.55	0.84
17	A	295	11.2	17.8	0.51	0.71
17	A	300	11.4	20.6	0.71	0.98
18	A	267	8.6	14.4	0.45	0.81
18	A	276	9.0	14.0	0.38	0.70
20	A	288	9.8	17.0	0.55	0.68
20	A	299	9.6	19.0	0.72	0.88
1	B	270	9.2	16.0	0.52	0.77
1	B	294	9.2	16.0	0.52	0.77
2	B	284	10.4	19.2	0.68	0.89
2	B	296	10.4	20.4	0.77	1.01
3	B	264	10.2	15.8	0.43	0.60
3	B	293	9.8	17.8	0.62	0.85
6	B	262	10.4	15.8	0.42	0.55
6	B	298	10.2	18.0	0.60	0.79
7	B	263	9.0	16.4	0.57	0.70
7	B	281	9.0	16.2	0.55	0.68
9	B	261	10.6	15.6	0.38	0.57
9	B	282	11.0	15.6	0.35	0.52
12	B	275	9.8	15.2	0.42	0.83
12	B	277	9.6	15.0	0.42	0.83
13	B	287	11.0	19.0	0.62	0.72
13	B	290	11.2	20.4	0.71	0.83
15	B	268	11.4	21.8	0.80	0.96
15	B	289	11.8	19.6	0.60	0.72
19	B	273	9.6	16.0	0.49	0.79
19	B	286	9.4	14.6	0.40	0.65

Table 10. Feed totals and average daily intake on starter ration.

Starter Feed Conversions					
Pen	**Group**	**Feed Total (lbs)**	**Feed Remaining (lbs)**	**Feed/Day**	**Average Daily Intake (lbs/pig/day)**
4	A	144	9.2	4.49	2.25
5	A	144	8.4	4.52	2.26
8	A	144	3.4	4.69	2.34
10	A	144	4.0	4.67	2.33
11	A	144	6.8	4.57	2.29
14	A	160	1.0	5.30	2.65

16	A	136	6.8	4.31	2.15
17	A	152	7.0	4.83	2.42
18	A	136	7.2	4.29	2.15
20	A	144	2.2	4.73	2.36
1	B	152	9.4	4.75	2.38
2	B	168	2.8	5.51	2.75
3	B	144	9.4	4.49	2.24
6	B	160	9.0	5.03	2.52
7	B	152	8.0	4.80	2.40
9	B	128	6.2	4.06	2.03
12	B	128	7.6	4.01	2.01
13	B	152	2.0	5.00	2.50
15	B	152	6.8	4.84	2.42
19	B	144	9.2	4.49	2.25

Table 11. Animal Weights, Average Daily Gain, and Gain to Feed ratio on starter ration.

Animal Weights on Starter Feed						
Pen	**Group**	**Animal ID**	**Day 13 (lbs)**	**Day 43 (lbs)**	**Average Daily Gain (lbs)**	**Gain:Feed**
4	A	274	16.2	58.8	1.42	0.63
4	A	301	15.8	55.0	1.31	0.58
5	A	269	18.0	59.0	1.37	0.60
5	A	291	18.8	56.4	1.25	0.55
8	A	292	14.4	59.0	1.49	0.64
8	A	297	17.8	64.4	1.55	0.66
10	A	279	17.6	58.6	1.37	0.59
10	A	280	18.4	61.6	1.44	0.62
11	A	283	18.0	66.4	1.61	0.70
11	A	285	19.4	61.4	1.40	0.61
14	A	271	19.8	66.8	1.57	0.59

14	A	272	19.2	67.2	1.60	0.60
16	A	265	14.2	57.6	1.45	0.67
16	A	278	17.4	60.4	1.43	0.67
17	A	295	17.8	67.0	1.64	0.68
17	A	300	20.6	68.0	1.58	0.65
18	A	267	14.4	53.6	1.31	0.61
18	A	276	14.0	55.4	1.38	0.64
20	A	288	17.0	53.2	1.21	0.51
20	A	299	19.0	71.2	1.74	0.74
1	B	270	16.0	68.6	1.75	0.74
1	B	294	16.0	52.8	1.23	0.52
2	B	284	19.2	70.6	1.71	0.62
2	B	296	20.4	72.0	1.72	0.63
3	B	264	15.8	56.2	1.35	0.60
3	B	293	17.8	58.2	1.35	0.60
6	B	262	15.8	61.2	1.51	0.60
6	B	298	18.0	62.6	1.49	0.59
7	B	263	16.4	60.4	1.47	0.61
7	B	281	16.2	56.6	1.35	0.56
9	B	261	15.6	49.2	1.12	0.55
9	B	282	15.6	57.6	1.40	0.69
12	B	275	15.2	58.2	1.43	0.71
12	B	277	15.0	53.2	1.27	0.63
13	B	287	19.0	64.0	1.50	0.60
13	B	290	20.4	71.2	1.69	0.68
15	B	268	21.8	72.2	1.68	0.69
15	B	289	19.6	66.6	1.57	0.65
19	B	273	16.0	53.2	1.24	0.55
19	B	286	14.6	56.8	1.41	0.63

Hematology and Blood Chemistry Analysis. Hematology testing was performed on blood samples at two blood collections, August 17, 2016 and September 29, 2016. Hematology testing included White Blood Cells counts (WBC), Red Blood Cell counts (RBC), Hematocrit (HCT) and Platelets. The results of hematology testing by animal and group are in **Tables 12 – 15**. The reference ranges for blood cell analysis can be found in **Table 13**. Blood chemistry testing included glucose, ALT, Albumin, Phosphorus. The results of blood chemistry testing are in **Tables 16-18**.

Table 12. Blood cell analysis results including white blood cell count, red blood cell count, hematocrit, and platelets. Red cells in the table indicate higher values than the reference range and blue cells in the table indicate lower values than the reference range. The reference ranges can be found in Table 13.

Blood cell analysis			=low value		= high value	
Animal ID	**Group**	**Collection Date**	**WBC**	**RBC**	**HCT**	**Platelets**
265	A	17-Aug-16	10.71	5.55	43.7	256
265	A	29-Sep-16	13.53	7.61	53.0	181
267	A	17-Aug-16	6.22	5.87	47.3	332
267	A	29-Sep-16	15.04	7.49	47.6	202
269	A	17-Aug-16	9.24	4.56	37.5	656
269	A	29-Sep-16	15.41	7.87	51.6	205
271	A	17-Aug-16	9.18	5.90	46.3	595
271	A	29-Sep-16	13.99	6.76	45.1	193
272	A	17-Aug-16	9.41	5.97	39.6	435
272	A	29-Sep-16	15.48	6.97	43.8	363
274	A	17-Aug-16	11.16	6.48	46.6	232
274	A	29-Sep-16	19.35	6.57	43.6	139
276	A	17-Aug-16	7.33	5.10	43.2	413
276	A	29-Sep-16	14.54	7.45	49.5	242
278	A	17-Aug-16	11.15	5.86	44.3	583
278	A	29-Sep-16	12.10	7.10	45.5	230
279	A	17-Aug-16	9.01	5.98	43.1	493
279	A	29-Sep-16	13.88	7.41	45.3	233
280	A	17-Aug-16	8.92	6.63	44.7	616
280	A	29-Sep-16	12.27	6.67	44.1	250
283	A	17-Aug-16	7.79	5.93	42.4	358
283	A	29-Sep-16	17.36	7.39	47.0	112
285	A	17-Aug-16	7.99	6.00	45.2	425
285	A	29-Sep-16	20.40	7.30	47.7	264
288	A	17-Aug-16	6.84	5.91	46.0	480
288	A	29-Sep-16	13.10	7.31	47.2	235
291	A	17-Aug-16	10.60	5.50	38.3	585
291	A	29-Sep-16	16.62	6.92	43.2	210
292	A	17-Aug-16	7.29	6.26	48.4	337
292	A	29-Sep-16	14.27	7.02	44.2	118
295	A	17-Aug-16	7.17	5.59	38.9	399
295	A	29-Sep-16	16.21	6.52	43.5	196
297	A	17-Aug-16	14.24	6.21	42.8	575
297	A	29-Sep-16	16.04	7.32	45.4	357
299	A	17-Aug-16	10.28	6.33	44.1	423
299	A	29-Sep-16	19.16	6.94	47.5	177
300	A	17-Aug-16	11.95	5.66	44.8	454

300	A	29-Sep-16	17.96	6.47	45.6	168
301	A	17-Aug-16	7.64	4.56	37.1	619
301	A	29-Sep-16	14.38	7.23	49.8	360
261	B	17-Aug-16	5.75	5.49	39.3	588
261	B	29-Sep-16	21.49	7.55	48.0	553
262	B	17-Aug-16	7.53	5.96	46.0	298
262	B	29-Sep-16	11.83	7.10	47.0	203
263	B	17-Aug-16	6.52	6.10	43.5	393
263	B	29-Sep-16	16.41	6.81	42.4	237
264	B	17-Aug-16	4.88	5.58	42.2	272
264	B	29-Sep-16	17.76	7.42	46.0	217
268	B	17-Aug-16	12.40	6.04	45.8	422
268	B	29-Sep-16	13.45	7.18	46.0	224
270	B	17-Aug-16	24.71	5.80	41.1	295
270	B	29-Sep-16	18.69	6.82	41.8	175
273	B	17-Aug-16	8.01	5.20	44.8	326
273	B	29-Sep-16	13.63	7.89	50.4	205
275	B	17-Aug-16	clotted	clotted	clotted	clotted
275	B	29-Sep-16	clotted	clotted	clotted	clotted
277	B	17-Aug-16	7.50	5.55	46.6	347
277	B	29-Sep-16	12.51	7.31	48.9	143
281	B	17-Aug-16	9.64	6.13	46.7	347
281	B	29-Sep-16	17.48	7.24	45.4	185
282	B	17-Aug-16	8.57	5.61	41.5	707
282	B	29-Sep-16	11.75	6.92	45.1	352
284	B	17-Aug-16	6.87	6.49	47.8	293
284	B	29-Sep-16	16.55	7.22	47.8	156
286	B	17-Aug-16	10.47	6.13	45.2	458
286	B	29-Sep-16	17.38	6.74	44.1	190
287	B	17-Aug-16	9.69	6.20	45.9	474
287	B	29-Sep-16	13.53	7.19	45.9	152
289	B	17-Aug-16	7.68	5.84	43.6	608
289	B	29-Sep-16	14.31	6.59	44.5	217
290	B	17-Aug-16	6.31	5.31	39.4	647
290	B	29-Sep-16	15.70	7.04	46.5	201
293	B	17-Aug-16	7.47	5.00	39.5	517
293	B	29-Sep-16	15.14	6.48	41.5	211
294	B	17-Aug-16	8.78	6.03	43.1	420
294	B	29-Sep-16	18.56	7.21	45.9	306
296	B	17-Aug-16	17.15	5.97	41.0	450
296	B	29-Sep-16	19.23	6.89	43.8	239
298	B	17-Aug-16	clotted	clotted	clotted	clotted
298	B	29-Sep-16	10.44	7.52	48.0	116

Table 13. White blood cell count, red blood cell count, hematocrit, and platelet reference ranges.

Blood Cell Reference Ranges				
Date	**WBC** $x10^3/ul$	**RBC** $x10^6/ul$	**HCT** %	**Platelets** $x10^3/ul$
17-Aug-16	9.6 - 25.2	4.87 - 7.88	28.2 - 39.8	374 - 1081
29-Sep-16	11.4 - 28.9	5.88 - 8.19	32.3 - 42.6	119 - 523

Table 14. Average blood cell counts by group on August 17, 2016.

Group	WBC	RBC	HCT	Platelets
A	9.21	5.79	43.2	463
B	9.44	5.80	46.5	437

Table 15. Average blood cell counts by group on September 29, 2016

Group	WBC	RBC	HCT	Platelets
A	15.55	7.12	46.5	222
B	15.57	7.11	45.7	225

Table 16. Blood Chemistry Results.

Blood Chemistry Results			= low value		= high value		
Animal ID	**Group**	**Collection Date**	**Glucose** 65.0 - 150.0 mg/dl	**ALT** 25.0 - 90.0 IU/L	**Albumin** 3.0 - 4.5 gm/dl	**Phos** 4.5 - 9.0 mg/dl	**CK** 100.0 - 2500.0 IU/L
265	A	17-Aug-16	123	44	2.9	10.2	376
265	A	29-Sep-16	134	51	4.2	11.4	4412
267	A	17-Aug-16	136	75	4.2	13.1	10908
267	A	29-Sep-16	115	61	3.7	12.2	6753
269	A	17-Aug-16	145	45	3.4	9.8	760
269	A	29-Sep-16	119	45	4.1	10.4	4165
271	A	17-Aug-16	147	60	3.2	9.7	633
271	A	29-Sep-16	113	55	4.0	10.8	866
272	A	17-Aug-16	114	56	2.9	11.0	377
272	A	29-Sep-16	112	47	3.7	11.0	2811
274	A	17-Aug-16	125	51	3.2	10.2	661
274	A	29-Sep-16	114	61	3.4	9.3	2933
276	A	17-Aug-16	136	46	3.4	10.7	723
276	A	29-Sep-16	131	54	3.7	11.5	3429
278	A	17-Aug-16	125	54	3.3	9.8	461
278	A	29-Sep-16	106	51	3.8	10.9	1237
279	A	17-Aug-16	119	49	3.6	10.1	407

279	A	29-Sep-16	118	47	3.7	10.6	2559
280	A	17-Aug-16	96	58	2.9	10.3	229
280	A	29-Sep-16	111	48	3.6	10.2	1336
283	A	17-Aug-16	108	54	3.3	11.4	680
283	A	29-Sep-16	106	52	3.8	10.0	902
285	A	17-Aug-16	114	44	3.6	9.8	495
285	A	29-Sep-16	118	60	4.1	10.8	8262
288	A	17-Aug-16	124	39	3.8	10.7	601
288	A	29-Sep-16	127	41	3.7	10.2	474
291	A	17-Aug-16	95	47	3.4	11.9	2853
291	A	29-Sep-16	108	36	3.4	9.7	2976
292	A	17-Aug-16	143	46	3.9	11.2	649
292	A	29-Sep-16	125	46	3.5	10.7	643
295	A	17-Aug-16	128	40	3.6	10.3	776
295	A	29-Sep-16	112	43	3.4	10.1	691
297	A	17-Aug-16	116	58	3.7	11.7	3284
297	A	29-Sep-16	110	58	4.1	10.7	5625
299	A	17-Aug-16	125	60	2.6	9.6	378
299	A	29-Sep-16	122	50	3.9	11.2	841
300	A	17-Aug-16	123	55	3.4	10.8	812
300	A	29-Sep-16	117	50	3.7	11.3	4725
301	A	17-Aug-16	132	42	3.8	11.3	848
301	A	29-Sep-16	112	42	4.3	10.8	1533
261	B	17-Aug-16	153	34	3.7	11.5	2404
261	B	29-Sep-16	103	54	3.9	10.6	555
262	B	17-Aug-16	120	46	3.1	10.2	334
262	B	29-Sep-16	122	67	3.9	10.4	4482
263	B	17-Aug-16	115	58	3.1	10.5	719
263	B	29-Sep-16	104	48	3.5	10.4	2603
264	B	17-Aug-16	129	51	3.3	10.2	823
264	B	29-Sep-16	115	53	3.4	9.3	1154
268	B	17-Aug-16	131	59	3.5	9.6	1519
268	B	29-Sep-16	108	71	3.9	10.2	3021
270	B	17-Aug-16	89	54	3.2	10.0	358
270	B	29-Sep-16	103	66	3.6	8.2	1301
273	B	17-Aug-16	139	49	4.0	10.1	910
273	B	29-Sep-16	102	49	3.9	11.1	2868
275	B	17-Aug-16	140	49	3.4	10.6	611
275	B	29-Sep-16	120	65	3.9	10.5	750
277	B	17-Aug-16	118	45	3.8	10.5	907
277	B	29-Sep-16	117	65	4.0	10.6	13704
281	B	17-Aug-16	112	53	3.5	10.1	823
281	B	29-Sep-16	96	55	3.8	9.7	554

282	B	17-Aug-16	111	35	2.9	10.3	364
282	B	29-Sep-16	119	54	3.7	9.9	1249
284	B	17-Aug-16	121	43	3.1	11.1	375
284	B	29-Sep-16	117	50	3.7	9.4	1348
286	B	17-Aug-16	119	51	2.5	10.2	325
286	B	29-Sep-16	128	49	3.5	11.0	536
287	B	17-Aug-16	103	50	3.1	10.9	357
287	B	29-Sep-16	127	50	3.9	11.2	569
289	B	17-Aug-16	122	56	3.4	10.2	599
289	B	29-Sep-16	123	59	3.6	10.9	1206
290	B	17-Aug-16	123	37	4.0	11.0	608
290	B	29-Sep-16	118	60	4.3	11.1	1019
293	B	17-Aug-16	122	50	3.4	9.9	777
293	B	29-Sep-16	112	55	3.3	9.1	1200
294	B	17-Aug-16	99	40	3.3	10.6	545
294	B	29-Sep-16	109	50	3.7	9.6	953
296	B	17-Aug-16	111	55	2.4	10.7	219
296	B	29-Sep-16	113	69	3.5	9.5	2569
298	B	17-Aug-16	126	50	3.5	10.3	560
298	B	29-Sep-16	119	58	4.2	9.5	2392

Table 17. Blood Chemistry average by group on August 17, 2016.

Group	Glu	ALT	ALB	Phos	CK
A	123.7	51.2	3.4	10.7	1345.6
B	120.2	48.3	3.3	10.4	706.9

Table 18. Blood Chemistry average by group on September 29, 2016.

Group	Glu	ALT	ALB	Phos	CK
A	116.5	49.9	3.8	10.7	2858.7
B	113.8	57.4	3.8	10.1	2201.7

Necropsy Findings. At necropsy, the kidney, bladder, and joints were observed for abnormalities and the kidney was collected where an abnormality in the kidney was observed. Bladder and joint fluid was collected where the veterinarian determined it was needed. Testing of tissue was not performed. Kidney was collected from animals 263, 264, 268, 270, 271, 272, 278, 279, 280, 284, 287, 289, 290, 292, 297, 299. Bladder tissue was collected from animal 267 and 277. Joint fluid was collected from animal 263 and 279. Necropsy samples that were collected for each animal are shown in **Table 19**.

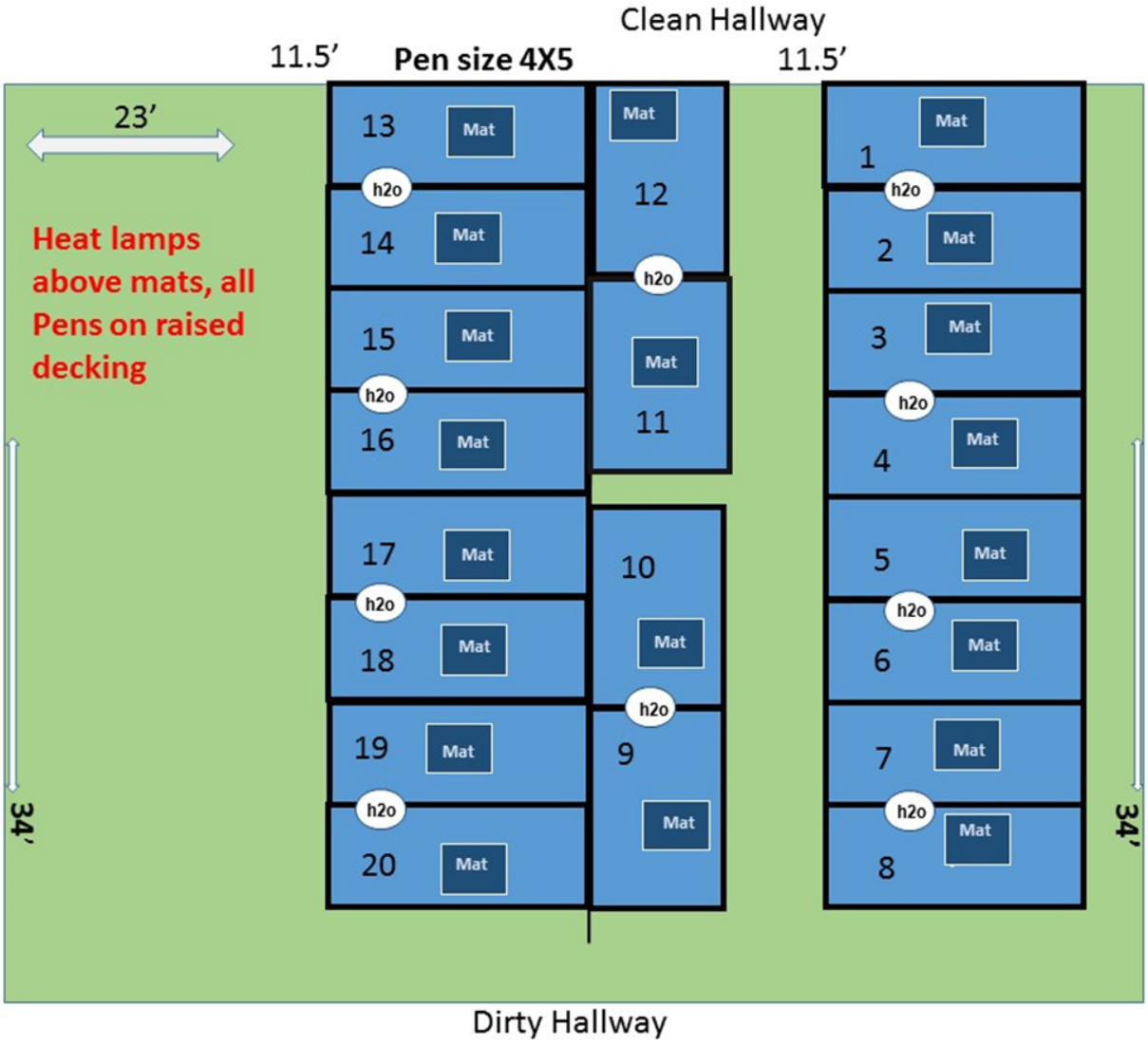
Table 19. Necropsy samples that were collected for each animal.

Necropsy Findings (N=normal)						
Animal ID	**Group**	**Kidney**	**Bladder**	**Joints**	**Other**	**Samples Collected**
265	**A**	N	N	N	N	
267	**A**	N	Abnormal	N	N	Bladder
269	**A**	N	N	N	N	
271	**A**	Abnormal	N	N	N	Left Kidney
272	**A**	*	N	Abnormal	N	Left Kidney
274	**A**	N	N	Abnormal	N	
276	**A**	N	N	N	N	
278	**A**	Abnormal	N	N	N	Right Kidney
279	**A**	Abnormal	N	Abnormal	N	Joint Fluid*, Right Kidney
280	**A**	Abnormal	N	Abnormal	N	Right Kidney
283	**A**	N	N	Abnormal	N	
285	**A**	N	N	Abnormal	N	
288	**A**	N	N	N	N	
291	**A**	N	N	N	N	
292	**A**	Abnormal	Abnormal	N	N	Right Kidney
295	**A**	N	N	N	N	
297	**A**	Abnormal	N	Abnormal	N	Right Kidney
299	**A**	Abnormal	N	Abnormal	N	Left Kidney
300	**A**	N	N	N	N	
301	**A**	N	Abnormal	N	N	
261	**B**	N	Abnormal	N	N	
262	**B**	N	N	N	N	
263	**B**	Abnormal	N	Abnormal	N	Joint Fluid**, Right Kidney
264	**B**	Abnormal	N	Abnormal	N	Left Kidney
268	**B**	Abnormal	N	N	N	Right Kidney
270	**B**	Abnormal	N	Abnormal	N	Right Kidney
273	**B**	N	N	N	N	
275	**B**	N	N	Abnormal	N	
277	**B**	N	Abnormal	Abnormal	N	Bladder
281	**B**	N	N	Abnormal	N	
282	**B**	N	N	N	N	
284	**B**	Abnormal	N	Abnormal	N	Right Kidney
286	**B**	N	N	N	Umbilical abscess	
287	**B**	Abnormal	N	N	N	Right Kidney
289	**B**	Abnormal	N	Abnormal	N	Left Kidney
290	**B**	Abnormal	N	N	N	Right Kidney
293	**B**	N	N	Abnormal	N	

294	**B**	N	N	N	N	
296	**B**	N	N	N	N	
298	**B**	N	N	N	N	

*See Note-to-File #9, dated 07Nov16

Appendix 1. (b) (4) Diagram.



Appendix 1: Study report for a 10X tolerance dose swine feeding trial

Appendix 2. The email communication containing the tracking number for shipment inadvertently shipped without a Chain of Custody is below. The samples were received at Agrivida on September 01, 2016 at 10:14 am by (b) (4) .

Dear Jon,

My records indicate that I owed you a retention sample of all of the feeds used in the study, and they are being shipped this afternoon.

I've listed the tracking number below, please confirm you've received all of the samples required for the protocol.

Piglets and feeders were weighed yesterday and switched over to the starter feed mix.

Tracking number: (b) (4)

(b) (4)

Appendix 1: Study report for a 10X tolerance dose swine feeding trial

Appendix 3. Quality Assurance Statement

Study Title

GraINzyme phytase Phy02 10x Tolerance Swine STudy

Study Number

16-17

This study was inspected and audited and the results reported to the Investigator and Management of (b) (4) on the following dates:

Date of Inspection/Audit	Subject of Inspection/Audit	Date Reported to Investigator	Date Reported to Management
August 30, 2016	Phase Inspection	02Sep16	02Sep16
September 1-2, 2016	Data review	02Sep16	02Sep16
October 07, 2016	Data review	11Oct16	11Oct16
October 10, 2016	Data review	11Oct16	11Oct16
October 21, 2016	Data review	21Oct16	21Oct16
November 04, 2016	Data review	04Nov16	04Nov16
November 22, 2016	Data review	02Dec16	02Dec16
December 02, 2016	Data review	02Dec16	02Dec16
September 26-27, 2017	Report review	29Sep17	29Sep17

The raw data, records, and report were audited and the report was found to accurately reflect the raw data. The raw data was forwarded to Agrivida.

Prepared By:

(b) (4)

29 Sep 17
Date

☐ **AMENDMENT** ☒ **DEVIATION - 7**

Study Investigator: (b) (4)

Study Number: 16-17

Study Location: (b) (4)

Monitor or Study Director: (b) (4)

Date: 26Sep17

Re: Stability Testing

Section: 6.0 states that "Stability testing has been performed by Agrivida. A summary of stability of test article will be provided in the final report." Section 18.2 states that "final study report will include test article stability results."

DEVIATION:

The final report does not include test article stability results.

REASON:

The sponsor indicated that stability results are not a concern for this study as feed was not pelleted.

EFFECT: (for deviation only)

This change is reflected on the GLP compliance statement.

(b) (4)

27 Sep 17
Date

26 Sep 17
Date

(b) (4)

8.2 Appendix 2

Phytase activities measured in the feeds used in the GraINzyme® Phytase tolerance study and in swine performance trials.

The protocol used to determine the phytase activity in Phy02 phytase product material for all results presented in this document is a modification of the standard method for the determination of phytase activity in feed (AOAC 2000.12). The standard protocol for the determination of phytase activity is appropriate for feed materials containing 200 – 400 FTU/kg feed and since the Phy02 product material has over 10 times more phytase activity than this range, the assay was modified to account for this difference. Prior to analysis, the product material is milled so that the particle sizes are less than or equal to 0.5 mm. 20 g of milled material is shaken for 1 hour at room temperature in 200 mL of 25 mM sodium borate, pH 10 buffer, 0.01% Tween 20. A 2 mL sample is taken and centrifuged at 12,000×g for 10 min. The product supernatants are diluted in phytase assay buffer (250 mM sodium acetate, pH 5.5, 1 mM calcium chloride, 0.01% Tween 20) so that the target absorbance at 415nm is between 0.3 and 1.1. To test protein extract activity, 75 µL of the diluted mixtures is dispensed into individual wells of a 2 mL 96-deep-well block. One hundred and fifty µL of freshly prepared phytic acid (9.1 mM dodecasodium salt from (b) (4) , prepared in assay buffer) is added to each well. Negative controls, which serve to correct sample background absorbance, have no protein extract in the wells before addition of the stop solution. Plates are sealed and incubated for 60 min at 37°C. One hundred and fifty µL of stop solution (20 mM ammonium molybdate, 5 mM ammonium vanadate, 4% nitric acid) is added to each well, mixed thoroughly via pipetting, and allowed to incubate at room temperature for 10 minutes. Seventy-five µL of the diluted protein extract is dispensed into negative control wells and mixed. Plates are centrifuged at 3000×g for 10 minutes, and 100 µL of the clarified supernatants are transferred to the wells of a flat-bottom 96-well plate. Absorbance at 415 nm from each sample is compared to that of negative controls and potassium phosphate standards. A standard curve is prepared by mixing 50 µL of potassium phosphate standards (0-1.44 mM, prepared in assay buffer) with 100 µL of freshly prepared phytic acid, followed by 100 µL of stop solution.

The phytase activity in feed samples was measured using a modified version of the standard phytase protocol (AOAC 2000.12). After mixing of the diets, a 500g sample of each of the diets in the mash form was collected. Subsequently, the mash diets were pelleted in a California Pellet Mill at 65°C and a 500g sample of each of the diets after pelleting was collected. All feed samples were shipped to the Agrivida, Inc. laboratory in Medford, MA where the phytase activity of each sample was determined. The feed samples were milled in a knife mill and sieved with a 1mm screen. Two 20 g samples of each milled feed sample were extracted at room temperature with 100ml of prewarmed (65°C) extraction buffer (30 mM Sodium Carbonate/Bicarbonate pH 10.8). Each extract diluted 25- to 100-fold in assay buffer (250 mM sodium acetate, pH5.5, 1mM calcium chloride, 0.01% Tween 20)

and 75 mL of the diluted extracts or 75ml of buffer-only controls were dispensed into individual wells of a round-bottom 96-well plate. 150 mL of freshly prepared, prewarmed (65°C), phytic acid (9.1 mM dodecasodium salt from (b) (4) prepared in assay buffer) was added to each well. Plates were sealed and incubated for 60 min at 65°C. 150 mL of stop solution (20 mM ammonium molybdate, 5 mM ammonium vanadate, 4% nitric acid) was added to each well, mixed thoroughly via pipetting, and allowed to incubate at room temperature for 10 min. Plates were centrifuged at 3000×G for 10 minutes, and 100 mL of the clarified supernatants were transferred to the wells of a flat-bottom 96-well plate. Absorbance at 415 nm from each sample was compared to that of negative controls (buffer-only, no enzyme) and potassium phosphate standards. The standard curve is prepared by mixing 50 ml of potassium phosphate standards (0-1.44 mM, prepared in assay buffer) with 100 mL of freshly prepared phytic acid, followed by 100 mL of stop solution.

8.2.1 Tolerance of weaned piglets to GraINzyme® Phytase

Weaned piglets were fed a high dose of GraINzyme® Phytase (target of 60,000 FTU/kg feed) for 43 days. Ten samples of GraINzyme® Phytase treated feed from the pre-starter and starter diets were collected and the phytase activity determined. The average phytase activity in 10 samples of these feeds is reported in the table below.

Feed Type	Average (FTU/kg)	St Dev
Pre-starter feed	44,926	10,929
Starter feed	44,134	5,500

8.2.2 Study 1. Swine trial conducted at the (b) (4)

Feed samples (20g each) were collected in duplicate and extracted with 100 ml of buffer at room temperature. The phytase assays were conducted at 37 °C. The results from the three different feeds for each of the phases of the trial are presented below.

Treatment Group	Target Dose FTU/kg	Phytase Activity After Diet Preparation			
		FTU/kg	stdev	% Target Dose	CV
Pos. Control	0	ND	-	-	-
Neg. Control	0	ND	-	-	-
NC+ 500Phy02	500	405	119	81	0.29
NC+1000Phy02	1000	884	223	88	0.25
NC+2000Phy02	2000	1603	186	80	0.12
NC+4000Phy02	4000	3938	900	98	0.23

8.2.3 Study 2. Swine trial conducted at the (b) (4)

A sample of each feed was assayed in triplicate and the averages for phytase activity (FTU/kg) are presented below.

	Target	FTU/kg	STDev
PC	0	ND*	ND
NC	0	ND	ND
AxtraPhy	500	464	114
AxtraPhy	1000	1086	348
GZ 0-1mm	500	143	139
GZ 0-1mm	1000	495	128
GZ 1-2.3mm	500	141	103
GZ 1-2.3mm	1000	584	145

*Not Detected

8.2.4 Study 3. Swine trial conducted at (b) (4)

Feed samples were collected in duplicate and a 20g aliquot was extracted with 100 ml of buffer at room temperature. The phytase assays were conducted at 37 °C. The results from the three different feeds for each of the phases of the trial are presented below.

Phase 2 Feed		**Phytase Activity After Diet Preparation**			
Treatment Group	**Target Dose FTU/kg**	**FTU/kg**	**stdev**	**% Target Dose**	**CV**
Pos. Control	0	43	9		
Neg. Control	0	4	28		
NC+ 500Phy02	500	212	40	42	0.19
NC+1000Phy02	1000	390	63	39	0.16
NC+2000Phy02	2000	1636	44	82	0.03
NC+4000Phy02	4000	2792	355	70	0.13

Appendix 2: Phytase activities measured in feeds

Phase 3 Feed		Phytase Activity After Diet Preparation			
Treatment Group	Target Dose FTU/kg	FTU/kg	stdev	% Target Dose	CV
Pos. Control	0	ND*	-	-	
Neg. Control	0	ND	-	-	
NC+ 500Phy02	500	376	52	75	0.14
NC+1000Phy02	1000	989	45	99	0.05
NC+2000Phy02	2000	2222	91	111	0.04
NC+4000Phy02	4000	3529	360	88	0.10

*Not Determined

Phase 4 Feed		Phytase Activity After Diet Preparation			
Treatment Group	Target Dose FTU/kg	FTU/kg	stdev	% Target Dose	CV
Pos. Control	0	69	67	-	-
Neg. Control	0	26	16	-	-
NC+ 500Phy02	500	658	163	132	0.25
NC+1000Phy02	1000	814	50	81	0.06
NC+2000Phy02	2000	1712	44	86	0.03
NC+4000Phy02	4000	3006	283	75	0.09

8.2.4 Reference

AOAC 2000.12 (2001). Phytase activity in feed. J. AOAC Internatl. **84**:629.

8.3 Appendix 3

Study report for a swine feeding trial conducted at the (b) (4) (Swine Trial 1)

Research Report

Effect of GraINzyme phytase in diets fed to growing pigs

(b) (4)

July 29, 2016

ABSTRACT: The objective of this experiment was to determine the effects of graded inclusion levels of GraINzyme phytase in diets fed to growing pigs. A total of 60 growing pigs (30 barrows and 30 gilts) with an initial BW of 10.78 ± 0.67 kg were randomly allotted to 6 dietary treatments in 2 phases with 10 replicate pens per treatment. There was 1 pig per pen. The experiment was conducted for 28 d. Six diets were formulated for each phase: Positive control, negative control, and negative control plus 500, 1,000, 2,000, or 4,000 phytase units (FTU) of GraINzyme phytase (Agrivida, Boston, MA). Concentrations of Ca and P were reduced by 0.20 and 0.18% respectively in the negative control diet compared with the positive control diet. Pigs were offered their respective diets on an ad libitum basis. Daily feed allotments were recorded. Pig weights were recorded at the beginning of the experiment and on the last d of the experiment. During the last 3 d of the experiment, a fecal sample was collected from all pigs by anal stimulation. On the last d of the experiment, all pigs were euthanized via captive bolt penetration and the right femur was removed. Results indicate there was a reduction in final BW, ADG, ADFI, G:F, bone ash (% and g), and bone Ca (g) and bone P in pigs fed the negative control diet compared with pigs fed the positive control ($P < 0.05$). Linear and quadratic increases ($P < 0.05$) in BW, ADG, ADFI, G:F, ATTD of Ca, ATTD of P, bone ash (%), bone ash (g), bone Ca, and bone P were observed due to phytase supplementation indicating that it is possible that additional benefits may be observed by inclusion of a greater concentration of phytase than the maximum concentration used in this experiment. However, adding 4,000 FTU of phytase to the negative control diets resulted in growth performance, bone ash (% and g) and bone Ca and P (g) that were not different from that of pigs fed the control diet. In conclusion, the novel phytase GraINzyme is effective in improving growth performance, Ca and P digestibility, and concentrations of bone ash, bone Ca, and bone P in pigs fed diets that are deficient in Ca and P. Results also indicated that 0.18% P and 0.20% Ca may be fully replaced by 4,000 FTU of GraINzyme in diets fed to growing pigs.

INTRODUCTION

Corn is one of the most important feed ingredients in swine diets and has 60 to 70% of the total phosphorous bound to phytate (Humer et al., 2013). However, P in this form is not available for pigs and poultry, because they do not have enough endogenous phytase to digest dietary phytate (Nelson, 1967). Therefore, swine and poultry diets are supplemented with inorganic phosphorous. But high phytate P excretion in manure is a potential source of environmental pollution. In addition, phytate in diets may form insoluble salts with several divalent cations such as Ca, Zn, Mg, and Cu, and therefore, inhibit the absorption of these minerals (Adeola, 1995; Kornegay, 2001).

Phytase catalyzes the hydrolysis of inorganic phosphate from phytate, which usually increases the digestibility of Ca and P (Brady et al., 2002) and reduces the antinutritional effects of phytate (Lim et al., 2000). Phytases can be obtained from plants,

animals, or microorganisms (bacteria, yeasts, and fungi). The effects of microbial phytase can vary due to the level of phytase supplementation (Carlson and Poulsen, 2003), the physiological status of the animal, and the origin of the phytase (Paditz et al., 2004). In addition, there are some advantages of using a genetically modified phytase compared with microbial phytase: 1) foreign genes can be easily transferred and expressed in plants; 2) plants have large biomass accumulation and use solar energy; and 3) phytases in the plants are not contaminated with animal pathogens (Zhang et al., 2000).

In this experiment, a novel corn-expressed E.coli phytase, GraINzyme phytase, will be evaluated for generating data on growth performance (ADG, ADFI, and G:F), digestibility of Ca and P, and bone ash in weaned piglets. The objective is, therefore, to determine the effect of graded inclusion levels of GraINzyme phytase in diets fed to pigs.

MATERIALS AND METHODS

Six dietary treatments were used during the 28 d experiment (Tables 1 and 2). Diet 1 was a positive control diet in which all nutrients were supplied according to current recommendations (NRC, 2012). Diet 2 was the negative control diet and this diet was similar to the positive control diet with the exception that inclusion of Ca was reduced by 0.20 percentage units, and inclusion of digestible P was reduced by 0.18 percentage units. Diets 3 to 6 are fed diets that were similar to the negative control diet, but 500, 1,000, 2,000, or 4,000 units of phytase (FTU) were included in these diets. All diets contained an indigestible marker (titanium dioxide).

A total of 60 weanling pigs (30 barrows and 30 gilts) that were the offspring of L 359 males and C-46 females (b) (4)) were used. Pigs were 5 weeks old and had a BW of 10.78 ± 0.67 kg at the start of the experiment. There was 1 pig per pen and 10 replicate pigs (5 barrows and 5 gilts) per treatment. Pigs were offered their respective diets on an ad libitum basis, and water was freely available as well throughout the experiment. Daily feed allotments were recorded. Pig weights were recorded at the beginning of the experiment and on the last d of the experiment. During the last 3 d of the experiment, a fecal sample was collected from all pigs by anal stimulation. Samples from the 3 d were pooled for each pig.

On the last d of the experiment, feeders were emptied and the amount of feed left in each feeder was recorded and subtracted from total feed allotments to calculate feed disappearance in each pen. On the last d of the experiment, all pigs were euthanized via captive bolt penetration and the right femur was removed. This bone was broken and soaked in ether for three d to remove the bone marrow. Bone weights were recorded, and bones were analyzed for dry matter (2h at 135˚C) and total bone ash (24h at 600˚C).

All diets were analyzed for dry matter, ash, gross energy, crude protein, NDF and ADF, Ca, P, and titanium dioxide, and phytase were also analyzed in all diets. All fecal samples were dried in a forced air oven and ground through a 1 mm screen. A subsample

was then analyzed for titanium dioxide, DM, ash, Ca, and P. All analyses were conducted according to AOAC procedures.

Statistical Analyses

Data were analyzed using the Proc Mixed procedure of SAS® (version 9.3, SAS Institute; Cary, USA). An ANOVA was conducted with diet, sex, and the interaction between sex and diet as main effects and replicate as random effect. However, there were no interactions between treatment and sex and there were no main effects of sex on any variables. The final model, therefore, only analyzed effects of treatment. Linear and quadratic contrasts were also used to determine the responses to inclusion of graded levels of phytase to the negative control diet. Means were calculated using the LS Means statement in SAS. The pig was the experimental unit and an alpha level of 0.05 was used for the determination significance among means.

RESULTS AND DISCUSSION

Growth Performance

There was a reduction ($P < 0.01$) in final BW and ADG of pigs fed the negative control diet compared with pigs fed the positive control diet (21.54 vs. 28.40 kg), but pigs fed the negative control diet with 1,000, 2,000 or 4,000 GraINzyme had a final BW and ADG that were not different from pigs fed the positive control diet, and linear and quadratic increases ($P < 0.05$) in final BW and ADG were observed as GraINzyme was added to the diets. Pigs fed the negative control diet also had reduced ($P < 0.05$) ADFI, G:F, and ATTD of Ca compared with pigs fed the positive control diet, but addition of any level of GraINzyme resulted in ADFI, G:F and ATTD of Ca that were not different from values observed for pigs fed the positive control diet. Average daily feed intake increased (linear, $P < 0.001$) and G:F and ATTD of Ca also increased (linear and quadratic, $P < 0.05$) as GraINzyme was added to the diet. The ATTD of P was also reduced ($P < 0.05$) for pigs fed the negative control diet compared with pigs fed the positive control diet, but pigs fed the diets with 500 or 1,000 FTU of GraINzyme had ATTD of P that was not different from that of pigs fed the positive control diet. In contrast, pigs fed diets with 2,000 or 4,000 FTU of GraINzyme had ATTD of P that was greater ($P < 0.05$) than that of pigs fed the positive control diet and the response in ATTD of P for addition of GraINzyme to the diets was both linear and quadratic ($P < 0.001$).

Bone Ash, Bone Calcium, and Bone Phosphorus

Pigs fed the negative control diet had less bone ash (% and g) compared with pigs fed the positive control diet (33.48 % and 7.49 g vs. 42.7 % and 14.80 g; $P < 0.01$). However, pigs fed the diet with 1,000 FTU GraINzyme had bone ash that was greater ($P < 0.05$) than pigs fed the negative control diet, but less ($P < 0.05$) than pigs fed the positive control diet, but pigs fed the diets with 2,000 or 4,000 FTU of GraINzyme had bone ash (%) that was not different from that of pigs fed the positive control diet. Likewise, pigs fed the diet with 4,000 FTU of GraINzyme had bone ash (g) that was not

different from that of pigs fed the positive control diet. Bone ash (% and g) in femurs increased linearly ($P < 0.01$) and quadratically ($P < 0.05$) as the concentration of GraINzyme phytase in the diets increased.

The percentages of Ca and P in bone ash were not affected by dietary treatments. However, the total amount (g) of Ca and P was less ($P < 0.001$) in bone ash from pigs fed the negative control diet compared with pigs fed the positive control diet, but addition of GraINzyme phytase to the negative control diet increased ($P < 0.05$) the amount of Ca and P in bone ash and pigs fed the diet with 1,000 FTU of GraINzyme had bone ash Ca and P (g) that were greater ($P < 0.0$) than for pigs fed the negative control diet, but less ($P < 0.05$) than for pigs fed the positive control diet. However, pigs fed the diets with 2,000 or 4,000 FTU of GraINzyme had bone Ca and P that were not different from the positive control diet and the response in bone ash concentrations of Ca and P to addition of GraINzyme was both linear ($P < 0.01$) and quadratic ($P < 0.05$).

These observations indicate that dietary changes in digestible Ca and P primarily will result in changes in the size of the bones and therefore also in the quantities of Ca and P that are stored in the bones, whereas the composition of the bone ash in terms of percent of Ca and P in bone ash is less affected by the availability of absorbed Ca and P. This indicates that the composition of bone ash is relatively stable regardless of the dietary provision of Ca and P, whereas the size of the bones is directly affected by dietary Ca and P.

There were no differences between pigs fed 4,000 FTU and pigs fed the positive control diet for bone ash, bone Ca, and bone P (% and g), which indicates that 4,000 FTU of GraINzyme phytase can effectively replace 0.18% P and 0.20% Ca. This is a result of the increased ATTD of Ca and P that was observed as GraINzyme was added to the diets and inclusion of GraINzyme is therefore expected to reduce the excretion of P from the pigs.

Conclusions

Addition of the novel phytase GraINzyme to a negative control diet to pigs linearly increased growth performance of pigs, ATTD of Ca and P, and bone ash, bone Ca and bone P. Because the responses were linear, it is possible that additional responses can be obtained if more GraINzyme is included in the diets and that more dietary Ca and P may be replaced by the enzyme. Results also demonstrated that 4,000 FTU of GraINzyme may completely replace 0.18% P and 0.20% Ca in the diets.

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Table 1. Composition of experimental diets as-fed basis[1]

Ingredient, %	Positive control	Negative control
Ground corn	64.35	66.05
Soybean meal	29.75	29.75
Soybean oil	2.00	1.35
Limestone	1.16	1.01
Monocalcium phosphate	1.00	0.10
L-lysine HCL	0.42	0.42
DL-methionine	0.10	0.10
L-threonine	0.12	0.12
Titanium dioxide	0.40	0.40
Sodium chloride	0.40	0.40
Vitamin-mineral premix[2]	0.30	0.30

[1]Four additional diets that were similar to the negative control diet with the exception that 500, 1,000, 2,000, or 4,000 units of GraINzyme phytase (Agrivida, Boston, MA) were added to these diets.

[2]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

Table 2. Analyzed composition of experimental diets, as-fed basis[1]

Item	Positive	Negative	500 FTU[2]	1,000 FTU	2,000 FTU	4,000 FTU
DM, %	86.54	86.61	86.55	86.49	86.56	86.41
Ash, %	5.60	5.18	4.65	4.62	4.64	4.63
GE, kcal/kg	3,817	4,570	3,923	3,886	3,874	3,851
CP, %	19.68	20.10	16.67	17.44	18.53	19.56
NDF, %	7.22	8.36	7.20	7.96	7.33	7.67
ADF, %	1.53	1.65	1.54	1.43	1.58	1.45
Ca, %	0.84	0.52	0.58	0.62	0.58	0.57
P, %	0.58	0.44	0.41	0.38	0.41	0.39
Phytase, FTU	0	0	170	440	1,200	1,500

[1]All diets were formulated to contain 2,494 kcal NE per kg and the following quantities of standardized ileal digestible AA: Arg, 1.17%; His, 0.47%; Ile, 0.72%; Leu, 1.49%; Lys, 1.23%; Met, 0.37%; Phe, 0.84%; Thr, 0.73%; Trp, 0.21%; and Val, 0.78%.
[2] FTU = phytase units.

Table 3. Growth performance, apparent total tract digestibility (ATTD) of Ca and P, and bone mineralization of pigs fed diets containing 0 to 4000 phytase units (FTU) of GraINzyme phytase for 28 days[1,2,3]

	Treatments						Pooled SEM	*P*-Value		
	Control +	Control -	500 FTU	1000 FTU	2000 FTU	4000 FTU		Treatment	Linear	Quadratic
Initial BW, kg	10.77	10.81	10.82	10.81	10.74	10.74	0.22	1.000	0.766	0.955
Final BW, kg	28.40^{a}	21.54^{c}	24.41^{bc}	26.55^{ab}	26.87^{ab}	29.40^{a}	0.84	<.0001	<.0001	0.027
ADG, g/d	600.14^{ab}	382.54^{c}	480.47^{bc}	562.00^{ab}	575.89^{ab}	637.22^{a}	30.40	<.0001	<.0001	0.009
ADFI, g/d	1,028.61^{ab}	847.74^{b}	955.71^{ab}	1,025.39^{ab}	1,070.18^{a}	1,118.46^{a}	47.73	0.003	0.0002	0.054
G:F	0.584^{a}	0.482^{b}	0.529^{ab}	0.549^{a}	0.553^{a}	0.568^{a}	0.015	0.0001	0.0002	0.020
ATTD Ca, %	65.96^{a}	51.61^{b}	65.49^{a}	72.36^{a}	74.96^{a}	73.53^{a}	2.88	<.0001	<.0001	<.0001
ATTD P, %	53.64^{b}	40.82^{c}	52.53^{b}	50.29^{b}	63.82^{a}	63.05^{a}	1.64	<.0001	<.0001	<.0001
Bone ash[4], %	42.71^{a}	33.48^{d}	35.79^{cd}	37.73^{bc}	40.30^{ab}	42.58^{a}	0.79	<.0001	<.0001	0.008
Bone ash, g	14.80^{a}	7.49^{e}	8.89^{de}	10.58^{cd}	12.26^{bc}	14.31^{ab}	0.57	<.0001	<.0001	0.018
Bone Ca, %	34.50	34.13	34.15	34.31	34.80	34.16	0.51	0.9131	0.852	0.327
Bone Ca, g	5.10^{a}	2.57^{d}	3.05^{cd}	3.64^{bc}	4.27^{ab}	4.91^{a}	0.22	<.0001	<.0001	0.025
Bone P, %	16.96	16.48	16.76	16.64	16.94	16.81	0.25	0.7425	0.370	0.411
Bone P, g	2.51^{a}	1.24^{d}	1.50^{cd}	1.76^{bc}	2.08^{ab}	2.42^{a}	0.11	<.0001	<.0001	0.029

$^{a-e}$ Means within a row lacking a common superscript letter are different ($P < 0.05$).
[1]Data are means of 10 observations per treatment, except for the negative control, which had only 9 observations.
[2]There was no interaction between treatment and sex, so the interaction was removed from the model.
[3]The effect of sex was not significant so sex was removed from the final model and only the main effects of GraINzyme is presented.
[4]Bone ash as percent of the weight of dried defatted bone.

8.4 Appendix 4

Study report for a swine feeding trial conducted at the (b) (4) (Swine Trial 2)

Research report

Effects of particle size of GraINzyme and of Axtra phytase in restoring P digestibility and bone ash in pigs fed low-P diets

(b) (4)

March 28, 2017

INTRODUCTION

Phytase is a commonly used enzyme that reduces the anti-nutritional effects of phytate and increases the release of P in diets fed to pigs (Olukosi and Adeola, 2013; She et al., 2015). Phytase also increases the digestibility of Ca because phytate may bind dietary Ca, making it inaccessible to the pig (González-Vega et al., 2015). However, inclusion of microbial phytase will partly ameliorate this problem and phytase is, therefore, usually included in diets for pigs (Esmaeilipour et al., 2012; Olukosi and Adeola, 2013).

A novel corn-expressed E. coli phytase, GraINzyme phytase, has been developed by the company Agrivida. Recent research has documented the effectiveness of this phytase, but there are no data that demonstrate the comparative effects of GraINzyme and other commercial phytases. It is also not clear what the optimum particle size of GraINzyme is. Therefore, the objective of this research was to test the hypothesis that GraINzyme phytase is equally efficient regardless of the particle size and that results that are comparable to that of the commercial phytase AxtraPhy can be achieved with GraINzyme when fed to growing pigs.

MATERIALS AND METHODS

Eight dietary treatments were used and pigs were fed diets during a 28 d experiment starting 2 week post weaning (Tables 1). Treatment 1 is a positive control treatment in which all nutrients were supplied according to current recommendations (NRC, 2012). Treatment 2 was the negative control treatment and this diet was similar to the treatment 1 diet with the exception that inclusion of Ca was reduced by 0.20 percentage units, and inclusion of digestible P was reduced by 0.15 percentage units. Diets for treatments 3 and 4 were similar to the negative control diet with the exception that 500 or 1,000 phytase units (FTU) per kg from AxtraPhy (Danisco Animal Nutrition, Marlborough, UK) were added to these diets. Likewise, diets for treatments 5 and 6 were similar to the negative control diet with the exception that 500 or 1,000 FTU of GraINzyme ground to a particle size of < 1mm were used, whereas 500 or 1,000 FTU of GraINzyme ground to a particle size between 1 mm and 2.3 mm were used in diets 7 and 8. All diets contained vitamins and minerals except Ca and P according to requirements and all diets also contained an indigestible marker (titanium dioxide).

A total of 64 weanling pigs (32 barrows and 32 gilts) that were the offspring of L 359 males and C-46 females (b) (4) were used. Pigs were 5 weeks old and had a body weight of 11.15 ± 0.85 kg at the start of the experiment. There was 1 pig per pen and 8 replicate pens per treatment for a total of 64 pens. Pigs were offered their respective diets on an ad libitum basis, and water was freely available throughout the experiment. Daily feed allotments

were recorded. Pig weights were recorded at the beginning of the experiment and on the last day of the experiment.

On the last day of the experiment, feeders were emptied and the amount of feed left in each feeder was recorded and subtracted from total feed allotments to calculate feed disappearance in each pen. All pigs were euthanized via captive bolt penetration and the right femur was removed. This bone was autoclaved, soft tissue was removed, and the bone was broken and soaked in ether to remove the bone marrow. Bone weights were recorded, and bones were analyzed for dry matter (2h at 135˚C) and total bone ash (16h at 600˚C). Bone ash was analyzed for Ca and P.

All diets were analyzed for dry matter, ash, GE, CP, Ca, P, and phytase was also analyzed in all diets (Table 2). All analyses were conducted according to AOAC procedures.

At the conclusion of the experiment, data for pig weights and feed disappearances were summarized and ADG, ADFI, and G:F were calculated. Data for bone ash were summarized as well within each treatment group. Likewise, data for concentrations of Ca and P in bones were summarized.

Data were analyzed by ANOVA using the PROC MIXED of SAS in a complete randomized design with the pen as the experimental unit. The statistical model included the fixed effect of dietary treatment and the random effect of replicate. Least square means were calculated for each independent variable. When diet was significant ($P < 0.05$) or tended to be a significant ($P < 0.10$) source of variation, means were separated using the PDIFF with the Tukey adjustment option of SAS. A contrast analysis was used to determine if there were differences between the positive control and the negative control. Additional contrast analyses were used to determine if there were differences between the 2 particle sizes of GraINzyme, between GraINzyme and AxtraPhy, and between the controls and the 2 phytases.

RESULTS AND DISCUSSION

Growth Performance

There were differences in the ADG, G:F, and final BW among all treatments ($P < 0.05$), however, for ADFI no differences among diets were observed (Table 3). The ADG, G:F, and final BW were greater ($P < 0.05$) for pigs fed the positive control than for pigs fed the negative control. This observation is in agreement with data indicating that reduced Ca and P in diets affects ADG and final BW of growing-finishing pigs (Shelton et al., 2004). Differences between the 2 particle sizes of GraINzyme were not observed. The ADG and final BW for pigs fed the 2 diets with AxtraPhy were greater ($P < 0.05$) than in pigs fed the 4 diets with GraINzyme. However, for G:F, no differences between the 2 enzymes were observed. Pigs fed the positive control diet also had greater ($P < 0.05$) ADG and final BW than pigs fed the phytase supplemented diets indicating that the reduction in dietary P and Ca in the

negative control diet was greater than the release of P and Ca by the phytase enzymes. Likewise, pigs fed the positive control had greater G:F than pigs fed the GraINzyme diets, but no differences between the positive control and AxtraPhy in G:F were observed. For ADG, G:F, and final BW, the negative control was less (P < 0.05) than the AxtraPhy diets, and for G:F, the negative control was less (P < 0.05) than the GraINzyme diets.

Bone Ash, Bone Calcium, and Bone Phosphorus

The concentration of bone ash (%) in pigs fed the negative control diets was less (P < 0.05) than in pig fed the positive control diet (Table 4). Addition of 1,000 FTU of AxtraPhy or 1,000 FTU of GraINzyme ground to less than 1 mm to the negative control diet increased (P < 0.05) the percentage of bone ash. However, the percentage of Ca and P in the bone ash was not different among treatments indicating that a deficiency of Ca or P does not change the composition of the bones.

Bone ash, bone Ca, and bone P measured in g were greater (P < 0.05) in the positive control than in the negative control diet and confirms that the negative control diet was formulated to not support maximum bone tissue synthesis. Bone ash and bone P measured in g were greater (P < 0.05) in pigs fed diets with 1,000FTU AxtraPhy than in pigs fed diets with GraINzyme. However, bone ash and bone P in pigs fed diets with 500FTU of AxtraPhy were no different from values in pigs fed diets with GraINzyme. No differences between the 2 particle sizes of GraINzyme were observed for bone ash, bone Ca, and bone P. However, pigs fed the positive control diet had greater (P < 0.05) bone ash, bone P, and bone Ca measured in g than pigs fed any of the phytase diets, which confirms results from the growth performance part of the experiment indicating that neither of the phytase enzymes were able to release enough P and Ca to compensate for the reduction in the negative control diet compared with the positive control diet. However, both AstraPhy and the 2 sources of GraINzyme increased (P < 0.05) bone ash, bone Ca, and bone P measured in g compared with the negative control diet. These observations indicate that a reduction in Ca and P levels in diets affects the size of the bones and as a consequence the amount of Ca and P that is stored in the bones. Likewise, results confirmed that phytase enzymes increases the availability of dietary Ca and P.

Conclusions

Inclusion of GraINzyme to a negative control increases the G:F, bone ash, bone Ca, and bone P compared with pigs fed a similar diet without GraINzyme. However, GraINzyme seems to be less efficient in releasing P and Ca than AxtraPhy. The particle size of GraINzyme did not affect any variable evaluated.

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Table 1. Composition of experimental diets as-fed basis[1]

Ingredient, %	Positive control	Negative control
Ground corn	64.35	66.05
Soybean meal	29.75	29.75
Choice white grease	2.00	1.35
Limestone	1.16	1.01
Monocalcium phosphate	1.00	0.10
L-lysine HCL	0.42	0.42
DL-methionine	0.10	0.10
L-threonine	0.12	0.12
Sodium chloride	0.40	0.40
Vitamin-mineral premix[2]	0.30	0.30

[1]Six additional diets that are similar to the negative control diet with the exception that 500, or 1,000 FTU of Axtra Phytase, or 500 or 1,000 FTU of GraINzyme ground to a particle size of < 1mm,or 500 or 1,000 FTU of GraINzyme growing to a particle size between 1 mm and 2.3 mm were used in diets.

[2]Provide the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D_3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

Appendix 4: Final Report Swine Trial 2

Table 2. Analysed nutrient composition of experimental diets, as-fed basis

Item	Positive control	Negative control	AxtraPhy, 500 FTU	AxtraPhy, 1,000 FTU	GraINzyme, 500 FTU <1mm	GraINzyme, 1,000 FTU <1mm	GraINzyme, 500 FTU 1-2.3mm	GraINzyme, 1,000 FTU 1-2.3mm
DM, %	88.35	87.97	87.79	87.81	87.76	87.77	87.86	87.76
Ash, %	2.53	2.50	2.65	2.48	2.77	2.46	2.42	4.38
GE, kcal/kg	3,942	3,933	3,955	3,968	4,012	4,092	3,976	3,987
CP, %	18.55	17.45	19.28	18.71	16.23	19.11	19.08	18.23
Ca, %	0.761	0.561	0.601	0.577	0.478	0.552	0.550	0.549
P, %	0.594	0.403	0.399	0.405	0.389	0.406	0.394	0.412
Phytase FTU[2]	82	<70	390	810	350	700	210	950

[1]All diets were formulated to contain 2,494 kcal NE per kg and the following quantities of standardized ileal digestible AA: Arg, 1.17%; His, 0.47%; Ile, 0.72%; Leu, 1.49%; Lys, 1.23%; Met, 0.37%; Phe, 0.84%; Thr, 0.73%; Trp, 0.21%; and Val, 0.78%.
[2] FTU = phytase units.

Table 3. Growth performance of pigs fed experimental diets[1]

	Treatment									
Item	Positive control	Negative control	AxtraPhy, 500 FTU	AxtraPhy, 1,000 FTU	GraINzyme, 500 FTU <1mm	GraINzyme, 1,000 FTU <1mm	GraINzyme, 500 FTU 1-2.3mm	GraINzyme, 1,000 FTU 1-2.3mm	SEM	*P*-value
day 0-28, kg										
Initial BW[2]	11.26	11.31	11.04	11.23	11.21	11.18	11.03	10.98	0.32	0.780
ADG[3,4,6,7,9]	0.75[a]	0.56[c]	0.70[ab]	0.65[abc]	0.61[bc]	0.67[abc]	0.58[bc]	0.62[bc]	0.04	<0.001
ADFI[2]	1.25	1.20	1.33	1.19	1.13	1.32	1.15	1.21	0.06	0.107
G:F[3,6,8,9]	0.60[a]	0.47[c]	0.53[abc]	0.55[ab]	0.55[ab]	0.52[bc]	0.50[bc]	0.51[bc]	0.02	<0.001
Final BW[3,4,6,7,9]	32.26[a]	26.99[c]	30.67[ab]	29.53[abc]	28.41[bc]	29.81[abc]	27.25[bc]	28.39[bc]	1.16	<0.001

[a-b] Means within a row lacking a common superscript letter are different ($P < 0.05$).
[1]Each least squares mean represents 9 or 10 observations.
[2]No differences in any contrast were observed.
[3]Contrast between negative control and positive control different ($P < 0.05$).
[4]Contrast between AxtraPhy and GraINzyme different ($P < 0.05$).
[5]Contrast between <1mm and 1-2.3mm particle size different ($P < 0.05$).
[6]Contrast between AxtraPhy and negative control different ($P < 0.05$).
[7]Contrast between AxtraPhy and positive control different ($P < 0.05$).
[8]Contrast between GraINzyme and negative control different ($P < 0.05$).
[9]Contrast between GraINzyme and positive control different ($P < 0.05$).

Table 4. Bone ash, Ca, and P in pigs fed experimental diets

	Treatment									
Item	Positive control	Negative control	AxtraPhy, 500 FTU	AxtraPhy, 1,000 FTU	GraINzyme, 500 FTU <1mm	GraINzyme, 1,000 FTU <1mm	GraINzyme, 500 FTU 1-2.3mm	GraINzyme, 1,000 FTU 1-2.3mm	SEM	*P*-value
Ash, g[1,2,4,5,6,7]	16.71[a]	7.35[d]	11.15[c]	13.63[b]	9.56[c]	11.16[c]	9.35[c]	10.48[c]	0.52	0.001
Ash, %[1,2,4,5,6,7]	49.98[a]	41.64[d]	44.91[bcd]	48.52[ab]	42.80[cd]	46.11[bc]	43.62[cd]	44.93[bcd]	0.88	0.001
Ca, g[1,2,4,5,6,7]	6.10[a]	2.67[d]	4.03[c]	4.82[b]	3.47[c]	4.05[bc]	3.44[cd]	3.78[c]	0.21	0.001
Ca, %	36.53	36.39	36.08	36.75	36.28	36.28	36.68	36.12	0.36	0.863
P, g[1,2,4,5,6,7]	2.83[a]	1.23[d]	1.88[c]	2.25[b]	1.60[c]	1.87[c]	1.59[c]	1.75[c]	0.09	0.001
P, %	16.96	16.67	16.85	17.11	16.72	16.74	16.92	16.74	0.18	0.710

[a-d] Means within a row lacking a common superscript letter are different ($P < 0.05$).
[1]Contrast between negative control and positive control different ($P < 0.05$).
[2]Contrast between AxtraPhy and GraINzyme different ($P < 0.05$).
[3]Contrast between <1mm and 1-2.3mm particle size different ($P > 0.05$).
[4]Contrast between AxtraPhy and negative control different ($P < 0.05$).
[5]Contrast between AxtraPhy and positive control different ($P < 0.05$).
[6]Contrast between GraINzyme and negative control different ($P < 0.05$).
[7]Contrast between GraINzyme and positive control different ($P < 0.05$).

8.5 Appendix 5

Study report for a swine feeding trial conducted by (b) (4) (Swine Trial 3)

Project Title

Effects of Adding a Novel Corn-Expressed Phytase (GraINzyme) on the Bone Characteristics, Apparent Phosphorous Digestibility, and Growth Performance of Nursery Pigs Fed Low-Phosphorous Diets.

Sponsor

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(b) (4)

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Introduction

A portion of the phosphorous (P) contained in the corn-soybean meal diets fed to pigs is un-available for absorption and use because it is bound with Phytate. Pigs produce low levels of Phytase during digestion (releasing a portion of this P); however, not at high enough levels to fully utilize it. To overcome this, and meet the P requirement of the pig, supplemental P is added (increasing diet costs), and the

excess P secreted in the feces. Because P level is of concern environmentally in swine waste, reduction in P excretion is of benefit. Having adequate available P in the diet is essential for the growth and well-being of growing pigs, and diets inadequate have a negative effect on the pigs.

Supplementing pig diets with additional Phytase has been shown to make the Phytate-bound P available for absorption and use by pigs. Supplementing pig diets with Phytase has gained wide acceptance in pig production worldwide. Therefore, the objective of this study was to test the hypothesis that supplementing corn-soybean meal based nursery pig diets with low levels of a novel corn-expressed phytase (GraINzyme), containing high levels of phytase, results in increased phytate bound P release, making it available for utilization by the pig. The proposed response parameters were: 1) Bone Mineralization, 2) Apparent-P Digestibility, and 3) Growth Performance (Average Daily Gain [ADG], Average Daily Feed Intake [ADFI], and Feed:Gain Ratio [FCONV]).

Materials and Methods

Abstract: At weaning, a group of 382 pigs (½ barrows and ½ gilts) were purchased from a commercial swine operation ((b) (4)). Pigs were received and placed on a common medicated commercial Phase II diet (Lean Start 2 Complete, Hubbard Feeds, Mankato, MN) for 7 days (Phase I diets are for early weaned pigs [<15 days of age]). At the end of the transition (acclimation) phase, pigs were weighed, and randomized into sixty single sex pens (see Deviation #1), with each pen containing six pigs. The non-allocates (twenty-two) were removed to other non-study pens within the nursery. The pens were fed one of six treatment diets: Positive Control, Negative Control, or one of four treatments containing GraINzyme formulated to levels of 500, 1000, 2000, or 4000 FTUs of Phytase/kg. The pigs were fed for two 14-day phases (P2, P3) and one 13-day phase (P4) [See amendment #1], with feed disappearance determined for each phase. Pig body weights (individual) were collected at the beginning and end of the study, and at each phase change. These data were used to calculate ADG, ADFI, and FCONV for each phase and the entire experimental feeding period. Diets fed during the third phase contained Chromic-oxide (Cr_2O_3) and fecal samples were collected 7-days after the initiation of P4 feeding to determine apparent-P digestibility. At the end of the live phase, four pigs per pen were humanely euthanized (captive-bolt gun) and the 4th metacarpal of the right foot collected and assayed for bone-breaking strength, de-fated/dried weight, and bone ash weight. These data were used to calculate percentage bone ash. Data were analyzed as a RCBD using the GLM Procedure of SAS (Ver 9.2, Cary, NC). LS Means were separated using the PDIFF option, and were considered significant if both the P Value of the effect in the model and the LS Means differences were $P < .05$. In addition, orthogonal contrasts for FTU inclusion rate (linear, quadratic, cubic and quartic) were evaluated. None of the pigs fed the test article entered the human food chain.

Pig Source – At weaning (21±3 days), 382 commercial pigs (See Deviation #1 - approximately ½ barrows and ½ gilts) were purchased by (b) (4) from (b) (4) The pigs were composed of (b) (4) genetics. The farm was PRRS, APP, and PEVD negative, and Myco positive. In addition to traditional pre-weaning activities and treatments (iron injection, tail docking, castration, etc.), the pigs were administered a 1 cc dose of Fostera ™ PCV MH (Zoetis) while on the sow as part of a two shot regimen to control Myco and PRCV. The second shot was administered at the end of the P1 phase (three weeks after arrival). Pigs were transported to the research site in a bedded-commercial truck (approximately 200 miles).

Research Facility Description – The (b) (4) was a 30' by 100' tunnel ventilated commercial research nursery containing 64-pen, and a plastic floor. Each pen was 5'5" x 5'5", and contained, one adjustable-height spiglett water lixit, one 15" Smidley stainless steel feeder, and contained one 8" gruel pan for three days after arrival. The building was ventilated using a multi-stage ventilation controller, and was managed in accordance with (b) (4) internal SOP's. The building contained radiant tube heaters, exhaust fans, vent boards, misters, and a curtain controlled air inlet. All scales used in the study were certified to be accurate by a licensed scale within 90 days of the initiation of the study. Check weights were used to verify each scales accuracy before and after is use (on an activity basis).

Daily building observations included minimum and maximum temperature and humidity on a daily basis (approximately every 24-hour period). A facility diagram is included in the final study records.

Arrival and 1st 7 Days Activities – Upon arrival, the pigs were unloaded and received into pens (n = 39) and each pen contained either 9 or 10 pigs after receipt and sorting. Pigs were visually sorted by weight to enhance acclimation. The pigs were placed on a commercially available common acclimation diet (Lean Start II®, Hubbard Feeds, Mankato, MN). This diet was a medicated diet (CTC and Tiamulin) formulated to meet the nutrient requirements of weaned pigs. For the first three days after arrival, a gruel pan (an 8" pan containing a mixture of water and feed) was made available within each pen to help the pigs transition to solid feed from their dam's milk. Pans were monitored twice per day to assure gruel availability. Pigs were tagged with bi-lateral ear tags (one in each ear) as a means for individual identification. Any missing or lost tags were replaced during the experimental period. At the end of the 7-day acclimation period, all pigs were visually inspected and weighed. Any pig found unsuitable for inclusion (rough haired, injured, non-castrated, un-thrifty) was excluded from the pool of potential study candidates.

Treatments – Six dietary treatments were created from three basal diets (one per phase) and the six treatments evaluated over three experimental feeding phases.

Tmt 1 – Positive Control – Adequate available P as defined by the NRC (available P = .40, .32 and .32 for Phases 2, 3 and 4, respectively) were created by adding 14.5,

14.0 and 14.0 lbs/ton of Mono-Calcium Phosphate to the basal diet for Phases 2, 3, and 4, respectively
Tmt 2 – Negative Control – Inadequate available P as defined by the NRC. (available P = .250, .174, and .174 for Phases 2, 3 and 4, respectively) were created by adding 8.0, 7.8, and 7.8 lbs of Corn/ton of the basal, and 6.5, 6.2 and 6.2 lb of Limestone/ton of the basal for Phases 2, 3, and 4, respectively.
Tmt 3 – Same as Tmt 2 + 500 FTU's of Phytase/kg created by adding 98.6 grams of the GraINzyme corn/ton of the basal for Phases 2, 3, and 4, respectively (reducing regular corn by the same amount).
Tmt 4 – Same as Tmt 2 + 1000 FTU's of Phytase/kg created by adding 197.2 grams of the GraINzyme corn/ton of the basal diet for Phases 2, 3, and 4, respectively (reducing regular corn by the same amount).

Tmt 5 – Same as Tmt 2 + 2000 FTU's of Phytase/kg created by adding 394.4 grams of the GraINzyme corn/ton of the basal diet for Phases 2, 3, and 4, respectively (reducing regular corn by the same amount).

Tmt 6 – Same as Tmt 2 + 4000 FTU's of Phytase/kg created by adding 788.9 grams of the GraINzyme corn/ton of the basal diet for Phases 2, 3, and 4, respectively (reducing regular corn by the same amount).

The GraINzyme contained 4,600 FTUs/gr phytase.

Treatment diets were manufactured using a "basal aliquot" approach A basal diet was created for each phase. An aliquot of the basal had additional P added (using mono-calcium phosphate) to create Tmt 1, no additional available P added to create Tmt 2, but limestone added at equal Ca concentration from the added calcium phosphate in Tmt 1, and Tmts 3 through 6 with the appropriate amount(s) of the GraINzyme corn added to Tmt 2. Any replacements necessary to equalize the dilution of added materials was done using an equal weight of corn (replaced GraINzyme) and Tmts 2 through 6 (difference between phosphate and limestone addition). During the creation of the Phase four basal, Cr_2O_3 was added to the diet at a rate of 0.4% to serve as an indigestible marker for determining apparent P digestibility. Diets were formulated by the designee of (b) (4) .

The formulations used to create the basal diets, and their formulated nutrient values are presented in Appendix 1. Appendix 2 shows the final batch size and additions fused to create each treatment.

Feed Form and Manufacture– Basal diets were created and sent to a single bin from which aliquots were brought to the mixer for the appropriate additions to create the treatments. After the additions were made and mixed, the feed was pelleted (approximately 165° F) and bagged. Feed was transported to the research facility and stored in a 53' dry van trailer.

Feed was manufactured at the (b) (4) The mill contained a one-ton Weigh-Tronix vertical mixer, and a 3-ton per hour Sprout-Waldron pellet mill. The flow rate and steam pressure of the pelleting process were adjusted on an as-needed basis during the manufacture process, but was kept below 175 ºF. The investigator provided the mill with the pre-weighed additions for making each treatment.

Feed Sampling –

Basal Diets - A grab sample (approximately 2 lb) was collected during the early, middle and late potions of the mixing of each basal diet. These three samples were mixed to create one 6 lb grab sample representing the basal diet.
Treatment Diets – A grab sample (approximately 2 lb) was collected during the early, middle and late portions of the bagging process for each treatment. These three samples were mixed to create a 6 lb grab sample representing each treatment diet.

Feed Testing and Approval – All diets were approved by the sponsor prior to feeding. All nutrient testing of the feed (not associated with the apparent digestibility portion of the study) was done at (b) (4) using the following procedures

Basal Diets	Method
Moisture	AOAC 925.10
Protein	AOAC 990.03
Crude Fat	AOAC 920.39
Total Lysine	AOAC 975.44 modified

Treatments	Method
Calcium	AOAC 965.17/985.01 modified
Phosphorus	AOAC 965.17/985.01 modified

Treatment samples were sent to (b) (4) for evaluation of the FTUs level in the treatment feeds. The investigator supplied the sponsor with the feed assay values. The sponsor was final authority as to the rejection or acceptance of the feed prepared. No treated feed was fed to the pigs prior to permission being granted by the sponsor.

Apparent Digestibility Assay – The feed samples from each Phase 4 treatment were additionally assayed for P and Cr at the (b) (4) using AOAC 965/985.01 modified. The fecal samples were similarly tested to determine apparent digestibility using the following equation:

$$\text{Digestibility \%} = 100 - [(Cr_{diet} * Nut_{feces})/(Cr_{feces} * Nut_{diet})] * 100:$$

Where:

Cr_{diet} = Chromium Concentration in the diet (mg/kg),
Nut_{feces} = Nutrient concentration in the feces (g.kg),
Cr_{feces} = Chromium concentration in the feces (mg/kg), and
Nut_{diet} = Nutrient concentration in the diet (g/kg).

Experimental Design – The study followed a Randomized Complete Block Design. Blocks were formed using initial pig weight (Study Day 0) and pen location within the building. Each block contained 12 pens, and six pens contained barrows and six pens contained gilts (See Deviation #1). Within each sex and each block, one pen was assigned to each treatment diet.

Study Activity Schedule –

Study Day	Event
-7	Pig Arrival, Visual Sorting
-5	Tagging
-4	Gruel Pans Removal
0	Weigh, Allocate, Feed P2 Diets
14	Weigh, Vaccinate, P2 Feed Weigh back, Feed P3 Diets
28	Weigh, P3 Feed Weigh back, Feed P4 Diets
35	Collect Fecal Samples
41	Weigh, P4 Feed Weigh back, Euthanize, Collect Feet

Observations were collected daily for minimum and maximum temperature and humidity. Daily animal health observations were made and recorded by exception. The pigs were managed in accordance with site SOP's, excluding the use of therapeutic interventions. No health related matters arose that warranted (b) (4) (site veterinarian) involvement. Individual animals were not removed from the study due to poor performance, and removals were only done to protect animal welfare and to prevent suffering. No therapeutic interventions were administered. When animal removal was required, the removed pig was weighed at the time of removal. All aspects of the study were conducted in accordance with the Guide for Care and Use of Agricultural Animals in Research and Teaching (FASS, 3rd Edition, January 2010).

Feed placements to pens was done on an "as needed" basis and the pigs were provided *ad libitum* access to feed and water.

Fecal Collections - On study day 35, four pigs per pen had fecal samples collected. Samples were collected by stimulating the anus to the pig until it defecated. The four samples were placed into a plastic bag and mixed. The corner of the bag was cut with scissors, and two representative samples "ejected" onto waxed paper. The wax paper was rolled and the sample placed into a 50 ml Falcon tube and frozen at -25 C

(approximately). The samples were freeze dried at the (b) (4) and delivered in their dried state to (b) (4).

Metacarpal Collections –At the end of the live phase (Study day 41) all pigs fed diets containing the GraINzyme corn were humanely euthanized using a captive bolt gun. Four pigs from each pen (including PC and NC pens) were randomly selected for the collection of the 3rd and 4th metacarpals from their right front foot. At the time of euthanasia, the right foot was collected and placed into a pre-labeled zip-lock bag, and placed on ice. The feet were frozen at -25 C until dissection. Upon dissection, the metacarpals were placed into two separate pre-labeled 50 ml falcon tubes (one for the 3rd and one for the 4th) and returned to the freezer. After all the bones were dissected and refrozen, the 4th metacarpal was shipped on dry-ice to (b) (4) for testing and the 3rd metacarpal retained until permission for disposal is granted by the sponsor.

Bone Assays – The 4th metacarpal bone from each foot was evaluated for bone breaking strength (BBS) and percent ash. BBS was determined (HD 250 Texture Machine, Texture Technologies Corporation, Scarsdale, NY) using a 3-point bend rig with a load cell capacity of 250 kg and cross-head speed of 100 mm/min. After determining BBS, fat was extracted by a 48-h Soxhlet extraction in ethyl alcohol followed by a 48-h extraction with diethyl ether. The bones were dried at 110°C for 24 h and weighed. The dry, defatted bones were dry-ashed in a muffle furnace at 560°C for 48 h and ash weight determined. The percent ash weight was determined as:

[((Ash Weight + Crucible Weight)-(crucible weight))/((De-fatted, Dried Weight + Crucible Weight)-(crucible weight))]*100.

Adverse Events – The Investigator did detect any Adverse Events during the study. An Adverse Event was defined as an unintended and unexpected severe event that would impact the health and welfare of the pigs after feeding of the test article. The definition of "severe" was be defined by the Investigator based upon his pig production experience, and excluded normal "production" losses.

Statistical Analysis – Pen will serve as the experimental unit and data were analyzed as a General Linear Model using SAS 9.2 (Cary, NC) with the model:

$$Y_{ijk} = \mu + B_i + S_j + T_k + ST_{jk} + E_{ijkl},$$

Where:

μ = the mean,
B_i = the effect of the i^{th} block,
S_j = the effect of the j^{th} sex,
T_k = the effect of the k^{th} dietary treatment,
ST_{jk} = the effect of the j^{th} sex and k^{th} dietary treatment interaction, and
E_{ijkl} = residual error.

The sex*treatment interaction was found to be significant (P<.05) for a few dependent variables and it was left in the model. Least squares means were separated (where appropriate) using the PDIFF option. Additionally orthogonal linear, quadratic, cubic and quartic contrasts for FTU inclusion rate were evaluated.

Randomization/Allocation - All randomization was done using the =rand() function of Excel, and then sorting in ascending random number, and assigning parameters in a sequential manner. The randomization process is included in the study records. Pens were assigned to blocks based on location within the building, and the blocks randomized and assigned a sequential designated number. The lowest numbered block received the lightest weight pigs, and highest number the heaviest pigs. Within each block, pens were assigned a random number and sorted, and pens with lowest six random numbers were assigned as barrow pens, and the highest six assigned as gilts pens. Within block and sex, pen was assigned a random number and sorted, and treatment assigned in sequential order. Once each pen was randomly assigned a block, sex and treatment designation, each line was copied six times to represent the entire population allocated to the study. Next, the data were sorted by sex, block and treatment, and group designations assigned. Group 1 was assigned to the first "space" in Tmt 1, 2, 3, 4, 5, and 6 within block 1, and group 2, the second pig within each treatment, etc, until all "spaces" were given a group designation (the first space of block 2 will be assigned group 7). All lines were assigned anther random number, and the data sorted by random number within group.

The pigs were weighed on study day 0, and sorted by sex and weight. Within sex, the potential allocates were "centered" (removing the heaviest and lightest pigs) from the list, and the individual pigs "merged" into their corresponding pen.

Blinding – All personnel collecting data at the research site were blinded, but the investigator was not. The investigator only collected data when it was essential and could not be collected in a timely manner by those who were blinded.

Training – Training occurred for all employees collecting data on the study, and is documented in the study records.

Data Collection – Data was collected in a timely manner on Investigator approved forms and kept safe from damage and loss. All data collected was recorded in ink, dated and attributable, and corrected using corrections codes. A list of correction codes is included in the Final Report.

Copies of Records – A certified copy of all data collected as part of the study has retained by the research site, and will be maintained for a period of not less than five years.

Animal Accountability – The outcome for all tagged pigs was documented.

Results and Discussion

Feed Assays -

As shown in Table 1, the nutrient profiles of the basal diets were close to the formulated target levels.

Table 1 – AG1601 Basal Diet Feed Nutrient Assays

	Phase 2	Phase 3	Phase 4
Moitsure (%)	12.00	12.66	12.61
Protein (%)	24.00	22.92	22.43
Crude Fat (%)	5.65	6.20	6.76
Total Lysine (%)	1.64	1.57	1.55
Crude Fiber (%)	2.60	3.00	2.70

Table 2 contains the assayed levels of Ca, P and FTU for each treatment feed for all three phases. The sponsor approved the use of these feeds after determining they were close enough to targeted levels to be suitable for testing the hypothesis.

Table 2 – AG1601 Ca, P and FTU Assay Values

	P2			P3			P4		
Tmt	Ca %	P %	FTU	Ca %	P %	FTU	Ca %	P %	FTU
1	0.779	0.70	<60	0.626	0.64	63	0.656	0.61	<60
2	0.703	0.55	<60	0.566	0.50	<60	0.604	0.48	<60
3	0.702	0.54	304	0.557	0.49	705	0.566	0.49	235
4	0.736	0.54	902	0.634	0.48	961	0.530	0.47	354
5	0.629	0.55	1850	0.571	0.47	1240	0.603	0.46	2170
6	0.679	0.55	4710	0.560	0.49	2590	0.594	0.48	3330

Statistical Analysis

As a consequence of not receiving enough barrows for all pens to be singled sexed (See Deviation #1), six barrow pens in one block were filled with 3 barrows and 3 gilts. It was expected that no significant sex*treatment interactions would be detected, and that this effect could be removed from the model; however, three independent variables (ADFI0t14, ADFI0t41, and Bone De-Fatted Weight) showed significant ($P < .05$) sex*treatment interactions. To investigate the affect of these six "mixed-sexed" pens had on the results, they were deleted from the data set, and the

analysis re-run. Their removal had no "substantial" impact on this interaction, and the data from these pens were included in the analysis and designated as borrow pens. For the ADFI data, because pen intake was collected, it was not possible to create an experimental unit of sex within pen as a means to overcome this. As will be discussed further in the text, the Investigator does not believe that including these pens in the analysis affected the ability to reach valid conclusions regarding the hypothesis being tested.

The P Values (Protected F Test) and the R-Squares for the model used to evaluate the independent variables are presented in Table 3. Treatment effects were significant (P < .05) for all variables except initial weight (Wt0), Feed Conversion days 14 to 28 (FCONV14t28), and days 0 to 41 (FCONV0t41), and Bone Dry Matter.

Table 3. AG1601 P Values for Statistical Analysis

Source	Df	Wt0 (lb)	Wt14 (lb)	WT28 (lb)	WT41 (lb)	ADG (lb/day) 0t14	ADG (lb/day) 14t28	ADG (lb/day) 28t41	ADG (lb/day) 0t41
block	4	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
sex	1	<.0001	0.0006	0.0455	0.0008	0.2087	0.8575	0.0008	0.0184
trt	5	0.9996	0.0026	<.0001	<.0001	0.0002	0.0021	<.0001	<.0001
sex*trt	5	0.9994	0.4941	0.4387	0.1633	0.3153	0.7503	0.4653	0.1658
R-Square		0.9784	0.9262	0.8950	0.9307	0.6809	0.6385	0.8794	0.8786

Source	Df	ADFI (lb/day) 0t14	ADFI (lb/day) 14t28	ADFI (lb/day) 28t41	ADFI (lb/day) 0t41	FCONV (fd/gain) 0t14	FCONV (fd/gain) 14t28	FCONV (fd/gain) 28t41	FCONV (fd/gain) 0t41
block	4	<.0001	<.0001	<.0001	<.0001	0.2323	0.4973	0.6477	0.6523
sex	1	0.2352	0.8414	0.0060	0.0122	0.4296	0.5434	0.9257	0.9529
trt	5	0.0007	<.0001	<.0001	<.0001	0.0364	0.6765	0.0248	0.6335
sex*trt	5	0.0233	0.1607	0.2578	0.0337	0.7660	0.5483	0.4529	0.6388
R-Square		0.8089	0.8583	0.8351	0.9050	0.3350	0.2002	0.3301	0.1751

Source	Df	aDig P (%)	Bone Brk Str gr	Bone Brk Str kg	Bone De-Fat Wt gr	Bone De-Fat Dry Wt gr	Bone Ash Wt gr	Bone DM %	Bone De-Ash %
block	4	0.5147	<.0001	<.0001	<.0001	<.0001	<.0001	0.1841	0.0011
sex	1	0.2403	0.2438	0.2438	<.0001	<.0001	0.0002	0.5742	0.1546
trt	5	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.5426	<.0001
sex*trt	5	0.7566	0.4216	0.4216	0.0069	0.0559	0.1659	0.1180	0.7543
R-Square		0.8351	0.6944	0.6944	0.9014	0.9036	0.9292	0.3158	0.8145

As previously noted, there were three significant sex*treatment interactions. Sex affects accounted for a significant amount of variation (P < .05) for a number the independent variables. Sex Least Squares Means are presented in Table 4.

Table 4. AG1601 Sex Least Squares Means

Sex	Wt0 (lb)		Wt14 (lb)		WT28 (lb)		WT41 (lb)		ADG (lb/day) 0t14	ADG (lb/day) 14t28	ADG (lb/day) 28t41		ADG (lb/day) 0t41	
Barrow	14.96	a	23.11	a	35.29	a	57.52	a	0.58	0.86	1.70	a	1.04	a
Gilt	14.19	b	22.01	b	34.22	b	55.17	b	0.56	0.86	1.61	b	1.00	b
Std Err	0.080		0.208		0.367		0.458		0.013	0.021	0.018		0.011	

Sex	ADFI (lb/day) 0t14		ADFI (lb/day) 14t28		ADFI (lb/day) 28t41		ADFI (lb/day) 0t41		FCONV (fd/gain) 0t14	FCONV (fd/gain) 14t28	FCONV (fd/gain) 28t41	FCONV (fd/gain) 0t41
Barrow	0.71	b	1.35		2.63	b	1.54	b	1.23	1.61	1.66	1.56
Gilt	0.69	a	1.35		2.48	a	1.48	a	1.26	1.58	1.67	1.56
Std Err	0.010		0.016		0.036		0.015		0.020	0.037	0.025	0.021

Sex	aDig P %	Bone Brk Str gr	Bone Brk Str kg	Bone De-Fat Wt gr		Bone De-Fat Wt gr		Bone Ash Wt gr		Bone DM %	Bone Ash %
Barrow	55.08	42551.62	42.55	3.96	a	3.38	a	1.52	a	85.58	44.78
Gilt	52.68	40785.14	40.79	3.65	b	3.13	b	1.42	b	85.79	45.25
Std Err	1.410	1057.394	1.057	0.044		0.037		0.017		0.258	0.232

[a,b] LS Means with different superscripts are different (P < .05)

The Least Squares means for the Treatment Effects and the orthogonal contrasts are presented in Table 5. At the initiation of the experimental feeding period, the pigs had similar (P > .05) weights. After 14 days (Wt14), the NC pigs were lighter than the other pigs (P < .05), and they remained lighter than the other pigs through the end of the experimental feeding period (P < .05). At the end the fourth week of feeding (Phase 3 – Wt28), the linear response to increasing FTU's by adding the GraINzyme was significant (P < .0001) and quadratic and cubic responses approached significance (P = .06). By the end of the study (WT41) the quadratic and cubic responses were significant (P <.003). Because the pigs began the study having similar weights (P > .05), the differences in live weight are attributable to differences (P < .05) in growth rate, and these differences generally followed the same pattern of that of Body Weight across the three experimental feeding periods Over the entire experimental feeding period (ADG0t41), pigs fed the NC diets had the lowest (P < .05) ADG , and pigs fed the NC+4000 FTU's of Phytase supplied by GraINsyme had the highest (P < .05) ADG, resulting in a cubic response (P<.003) for increasing FTU's. Even though there is no statistical difference (P > .05) between the PC and the NC+500 FTU (Treatment 3) the data suggest that in order for the NC to become "fully" equal with the PC, that somewhere between 500 and 1000 FTUs would have been needed to have been added to the NC diet.

Because ADG is largely driven by ADFI, it is not surprising that ADFI followed a similar pattern to ADG; however, the significant sex*treatment interactions (P < .05) were not detected in either WT or ADG. Figures 1 and 2 reveal that the barrows fed treatment 3 feed (NC+500 FTU's Phytase) consumed more feed both during the first

two weeks and thus over the entire experimental feeding period that would have been anticipated. No biological reason (hypothesis) can be set forth to explain the reason for this.

Table 5. Least Squares Treatment Means and P Values for Orthogonal Contrasts.

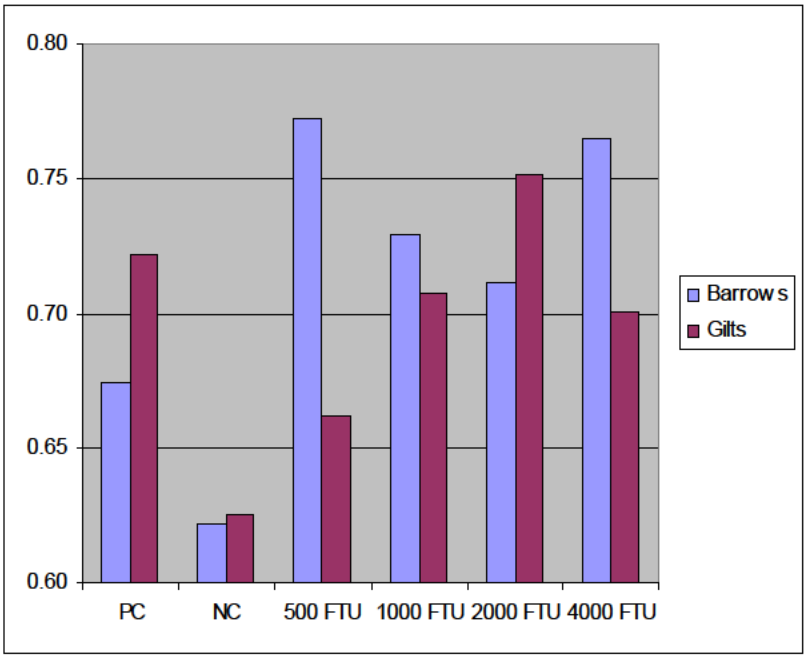
Trt	Wt0 (lb)	Wt14 (lb)	WT28 (lb)	WT41 (lb)	ADG (lb/day) 0t14	ADG (lb/day) 14t28	ADG (lb/day) 28t41	ADG (lb/day) 0t41
PC	14.59	22.48 [a]	35.00 [b]	57.13 [bc]	0.56 [b]	0.88 [ab]	1.70 [ab]	1.04
NC	14.58	21.23 [b]	31.76 [c]	50.35 [d]	0.48 [c]	0.74 [c]	1.43 [c]	0.87
500 FTU	14.57	22.43 [a]	34.03 [b]	55.30 [c]	0.56 [b]	0.82 [bc]	1.61 [b]	0.99
1000 FTU	14.60	22.70 [a]	35.15 [b]	57.35 [bc]	0.58 [ab]	0.88 [ab]	1.71 [a]	1.04
2000 FTU	14.53	23.12 [a]	35.40 [ab]	57.57 [b]	0.61 [ab]	0.88 [ab]	1.71 [a]	1.05
4000 FTU	14.57	23.40 [a]	37.19 [a]	60.36 [a]	0.63 [a]	0.97 [a]	1.78 [a]	1.12
Std Err	0.138	0.359	0.636	0.793	0.022	0.036	0.031	0.019
Lin FTU	0.9276	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Quad FTU	0.9006	0.0313	0.0609	0.0009	0.0109	0.2867	0.0002	0.0008
Cubic FTU	0.8192	0.2579	0.0697	0.0020	0.2224	0.1981	0.0022	0.0022
Quart FTU	0.8366	0.5655	0.9852	0.8262	0.4448	0.7155	0.9202	0.8232

Trt	ADFI (lb/day) 0t14	ADFI (lb/day) 14t28	ADFI (lb/day) 28t41	ADFI (lb/day) 0t41	FCONV (fd/gain) 0t14	FCONV (fd/gain) 14t28	FCONV (fd/gain) 28t41	FCONV (fd/gain) 0t41
PC	0.70 [a]	1.34 [bc]	2.55 [bc]	1.51 [bc]	1.26 [abc]	1.53	1.62 [a]	1.52
NC	0.62 [b]	1.18 [d]	2.08 [d]	1.28 [d]	1.32 [c]	1.60	1.57 [a]	1.52
500 FTU	0.72 [a]	1.29 [c]	2.46 [c]	1.47 [c]	1.28 [bc]	1.69	1.66 [a]	1.58
1000 FTU	0.72 [a]	1.39 [b]	2.62 [bc]	1.55 [b]	1.24 [abc]	1.60	1.66 [a]	1.56
2000 FTU	0.73 [a]	1.38 [b]	2.66 [b]	1.56 [b]	1.20 [ab]	1.58	1.69 [ab]	1.56
4000 FTU	0.73 [a]	1.52 [a]	2.94 [a]	1.70 [a]	1.16 [a]	1.58	1.79 [b]	1.60
Std Err	0.018	0.027	0.062	0.025	0.034	0.064	0.043	0.036
Lin FTU	0.0021	<.0001	<.0001	<.0001	0.0014	0.4682	0.0014	0.2598
Quad FTU	0.0039	0.0314	0.0028	0.0002	0.2724	0.9233	0.7136	0.9838
Cubic FTU	0.0388	0.0063	0.0022	0.0002	0.9786	0.503	0.3199	0.4075
Quart FTU	0.1965	0.3295	0.8156	0.8909	0.8523	0.3204	0.5356	0.5313

Trt	aDig P %	Bone Brk Str gr	Bone Brk Str kg	Bone De-Fat Wt gr	Bone De-Fat Dry Wt gr	Bone Ash Wt gr	Bone DM %	Bone Ash %
PC	38.33 [d]	44845 [a]	44.84 [a]	3.92 [b]	3.37 [b]	1.56 [b]	85.82	46.46
NC	31.53 [d]	31038 [c]	31.04 [c]	3.16 [c]	2.71 [d]	1.11 [d]	85.68	40.68
500 FTU	56.95 [c]	39617 [b]	39.62 [b]	3.74 [b]	3.15 [c]	1.41 [c]	84.88	44.87
1000 FTU	57.99 [c]	44934 [a]	44.93 [a]	3.91 [b]	3.36 [b]	1.51 [b]	85.92	44.90
2000 FTU	65.32 [b]	44587 [ab]	44.59 [ab]	3.86 [b]	3.32 [bc]	1.52 [b]	85.97	45.84
4000 FTU	73.17 [a]	44989 [a]	44.99 [a]	4.24 [a]	3.64 [a]	1.72 [a]	85.82	47.33
Std Err	2.442	1831	1.831	0.076	0.065	0.030	0.446	0.402
Lin FTU	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.4083	<.0001
Quad FTU	<.0001	0.0002	0.0002	0.0018	0.0012	<.0001	0.5929	<.0001
Cubic FTU	0.0004	0.0183	0.0183	<.0001	0.0001	<.0001	0.4271	0.0001
Quart FTU	0.0110	0.7439	0.7439	0.5280	0.9294	0.3797	0.1328	0.0090

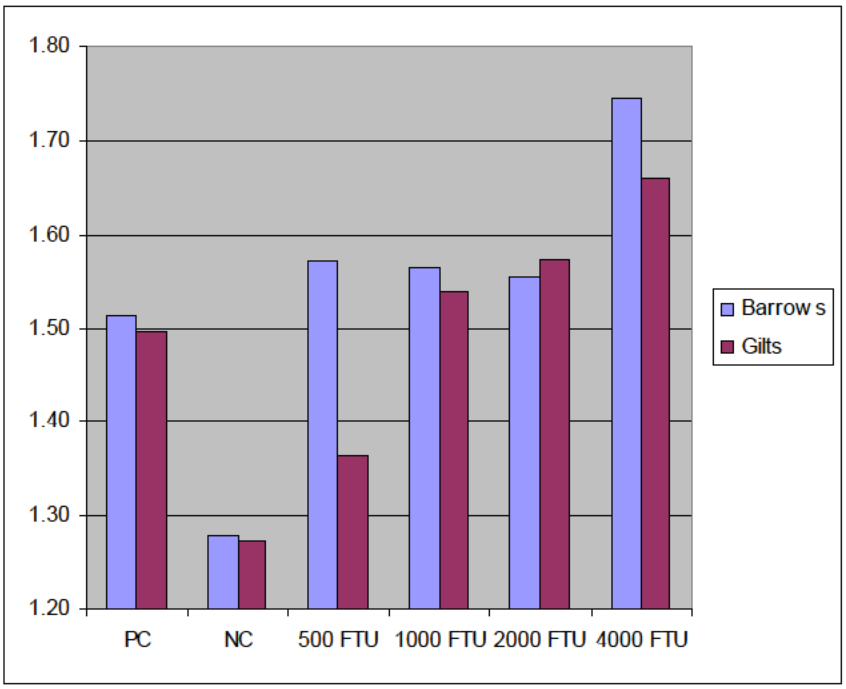
[a,b,c,d] LS Means with different superscripts are different (P < >05).

Figure 1. AG1601 ADFI0t14 Sex*Treatment LS Means



If one excludes the barrows receiving the Treatment 3 feed, the pattern of both sexes exhibit the significant ($P < .05$) linear, quadratic, and cubic effects of increasing FTU's of Phytase by increasing GraINzyme inclusions.

Figure 2. AG1601 ADFI0t41 Sex*Treatment LS Means



FCONV is more complex to explain as being directly affected by the GraINzyme inclusion. The reason for this is that as pigs become heavier, they become less efficient in converting feed to live gain, due to an increasing proportion of nutrients being shifted toward maintenance. This creates additional variation in the data, making detecting differences and drawing conclusion regarding the impact of increasing FTU's on FCONV more difficult.

However, the Apparent Digestibility results suggest that the responses seen in the live-animal performance resulted from the pigs' increasing P absorption ($P < .001$) as FTU level in the diet was increase through the GraINzyme. The effect that this increased absorption of P by the pigs is supported by the Bone Parameter results. Improvements were detected in Bone Breaking Strength ($P < .001$), De-fatted Bone Green Weight ($P < .001$), De-fatted Bone Dry Weight ($P < .001$), Bone Ash Weight ($P < .001$) and Bone Percent Ash ($P < .001$) as FTU level increase.

Conclusions -

The data suggested that the FTU's in GraINzyme increase the availability of P when added to nursery pig diets containing low aP levels.. These conclusions are supported by all the response parameters evaluated (Live Performance, P aDigestibility, and Bone Characteristics).

Appendix 1 – Basal Diet Formulations and Formulated Nutrient Values

Phase 2 Diet & Treatment Formulations

							Treatment Creations (lb addtions/ton to basal)					
Ingredients	PC lbs	PC %	NC lbs	NC %	Basal lb	Basal (%)	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	715.35	35.77	723.35	36.17	715.35	36.0287	0.000	8.000	7.783	7.566	7.131	6.262
Soybean meal, 48%	600.000	30.000	600.000	30.000	600.00	30.2191	0.000	0.000	0.000	0.000	0.000	0.000
Corn DDGS, >6 and <9% Oil	300.000	15.000	300.000	15.000	300.00	15.1095	0.000	0.000	0.000	0.000	0.000	0.000
Poultry Fat	50.000	2.500	50.000	2.500	50.00	2.5183	0.000	0.000	0.000	0.000	0.000	0.000
Monocalcium P	14.500	0.725		0.000	0.00	0.0000	14.500	0.000	0.000	0.000	0.000	0.000
Limestone	20.500	1.025	27.000	1.350	20.50	1.0325	0.000	6.500	6.500	6.500	6.500	6.500
Salt	7.000	0.350	7.000	0.350	7.00	0.3526	0.000	0.000	0.000	0.000	0.000	0.000
L-Lysine	7.300	0.365	7.300	0.365	7.30	0.3677	0.000	0.000	0.000	0.000	0.000	0.000
DL-Methionine	3.050	0.153	3.050	0.153	3.05	0.1536	0.000	0.000	0.000	0.000	0.000	0.000
L-Threonine	1.200	0.060	1.200	0.060	1.20	0.0604	0.000	0.000	0.000	0.000	0.000	0.000
L-Tryptophan		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
ZnO		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Copper Sulfate		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Trace Mineral Premix (NB-8534)	3.000	0.150	3.000	0.150	3.00	0.1511	0.000	0.000	0.000	0.000	0.000	0.000
Vitamin Premix (NB-6508)	5.000	0.250	5.000	0.250	5.00	0.2518	0.000	0.000	0.000	0.000	0.000	0.000
Plasma (AP-920)	30.000	1.500	30.000	1.500	30.00	1.5110	0.000	0.000	0.000	0.000	0.000	0.000
Fish Meal, Menhaden		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Whey Powder	240.000	12.000	240.000	12.000	240.00	12.0876	0.000	0.000	0.000	0.000	0.000	0.000
Corn Phytase (GralNzyme™)		0.000		0.000	0.00	0.0000	0.000	0.000	0.217	0.434	0.869	1.738
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.60	0.0302	0.000	0.000	0.000	0.000	0.000	0.000
Denaguard 10		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Chlortetracycline 100 (ADM)		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Tylan 40 Sulfa-G	2.500	0.125	2.500	0.125	2.50	0.1259	0.000	0.000	0.000	0.000	0.000	0.000
Tylan 40 Elanco		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Lactose		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Cr2O3		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Total	2000.0	100.0	2000.0	100.0	1985.50	100.00	14.500	14.500	14.500	14.500	14.500	14.500
Calculated Nutrient Values												
NSNG ME (kcal/kg)	3413		3426									

Total Lysine (%)	1.608	1.609
SID Lysine (%)	1.422	1.422
Total P (%)	0.654	0.502
Available P (%)	0.403	0.250
Ca (%)	0.756	0.756
Ca/P	1.16	1.51

Phase 3 Diet & Treatment Formulations

							Treatment Creations (lb addtions/ton to basal)					
Ingredients	PC lbs	PC %	NC lbs	NC %	Basal lb	Basal (%)	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	955.70	47.79	963.50	48.18	955.70	48.1219	0.000	7.800	7.583	7.366	6.931	6.062
Soybean meal, 48%	632.000	31.600	632.000	31.600	632.00	31.8228	0.000	0.000	0.000	0.000	0.000	0.000
Corn DDGS, >6 and <9% Oil	300.000	15.000	300.000	15.000	300.00	15.1057	0.000	0.000	0.000	0.000	0.000	0.000
Poultry Fat	50.000	2.500	50.000	2.500	50.00	2.5176	0.000	0.000	0.000	0.000	0.000	0.000
Monocalcium P	14.000	0.700		0.000	0.00	0.0000	14.000	0.000	0.000	0.000	0.000	0.000
Limestone	19.000	0.950	25.200	1.260	19.00	0.9567	0.000	6.200	6.200	6.200	6.200	6.200
Salt	10.000	0.500	10.000	0.500	10.00	0.5035	0.000	0.000	0.000	0.000	0.000	0.000
L-Lysine	6.900	0.345	6.900	0.345	6.90	0.3474	0.000	0.000	0.000	0.000	0.000	0.000
DL-Methionine	2.120	0.106	2.120	0.106	2.12	0.1067	0.000	0.000	0.000	0.000	0.000	0.000
L-Threonine	1.180	0.059	1.180	0.059	1.18	0.0594	0.000	0.000	0.000	0.000	0.000	0.000
L-Tryptophan		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
ZnO		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Copper Sulfate		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Trace Mineral Premix (NB-8534)	3.000	0.150	3.000	0.150	3.00	0.1511	0.000	0.000	0.000	0.000	0.000	0.000
Vitamin Premix (NB-6508)	5.000	0.250	5.000	0.250	5.00	0.2518	0.000	0.000	0.000	0.000	0.000	0.000
Plasma (AP-920)		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Fish Meal, Menhaden		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Whey Powder		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Corn Phytase (GralNzyme™)		0.000		0.000	0.00	0.0000	0.000	0.000	0.217	0.434	0.869	1.738
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.60	0.0302	0.000	0.000	0.000	0.000	0.000	0.000
Denaguard 10		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Chlortetracycline 100 (ADM)		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Tylan 40 Sulfa-G		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000

Tylan 40 Elanco	0.500	0.025	0.500	0.025	0.50	0.0252	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Lactose		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Cr2O3		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Total	2000.0	100.0	2000.0	100.0	1986.00	100.00	14.000	14.000	14.000	14.000	14.000	14.000
Calculated Nutrient Values												
NSNG ME (kcal/kg)	3400		52178									
CP (%)	23.636		23.668									
Total Lysine (%)	1.462		1.463									
SID Lysine (%)	1.275		1.276									
Total P (%)	0.586		0.440									
Available P (%)	0.322		0.174									
Ca (%)	0.654		0.653									
Ca/P	1.12		1.49									

Phase 4 Diet & Treatment Formulations

							Treatment Creations (lb addtions/ton to basal)					
Ingredients	PC lbs	PC %	NC lbs	NC %	Basal lb	Basal (%)	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	947.86	47.39	955.66	47.78	947.86	47.7271	0.000	7.800	7.587	7.375	6.950	6.099
Soybean meal, 48%	632.000	31.600	632.000	31.600	632.00	31.8228	0.000	0.000	0.000	0.000	0.000	0.000
Corn DDGS, >6 and <9% Oil	300.000	15.000	300.000	15.000	300.00	15.1057	0.000	0.000	0.000	0.000	0.000	0.000
Poultry Fat	50.000	2.500	50.000	2.500	50.00	2.5176	0.000	0.000	0.000	0.000	0.000	0.000
Monocalcium P	14.000	0.700		0.000	0.00	0.0000	14.000	0.000	0.000	0.000	0.000	0.000
Limestone	19.000	0.950	25.200	1.260	19.00	0.9567	0.000	6.200	6.200	6.200	6.200	6.200
Salt	10.000	0.500	10.000	0.500	10.00	0.5035	0.000	0.000	0.000	0.000	0.000	0.000
L-Lysine	6.800	0.340	6.800	0.340	6.80	0.3424	0.000	0.000	0.000	0.000	0.000	0.000
DL-Methionine	2.100	0.105	2.100	0.105	2.10	0.1057	0.000	0.000	0.000	0.000	0.000	0.000
L-Threonine	1.140	0.057	1.140	0.057	1.14	0.0574	0.000	0.000	0.000	0.000	0.000	0.000
L-Tryptophan		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
ZnO		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Copper Sulfate		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Trace Mineral Premix (NB-8534)	3.000	0.150	3.000	0.150	3.00	0.1511	0.000	0.000	0.000	0.000	0.000	0.000
Vitamin Premix (NB-6508)	5.000	0.250	5.000	0.250	5.00	0.2518	0.000	0.000	0.000	0.000	0.000	0.000
Plasma (AP-920)		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000

Fish Meal, Menhaden		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Whey Powder		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Corn Phytase (GralNzyme™)		0.000		0.000	0.00	0.0000	0.000	0.000	0.213	0.425	0.850	1.701
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.60	0.0302	0.000	0.000	0.000	0.000	0.000	0.000
Denaguard 10		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Chlortetracycline 100 (ADM)		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Tylan 40 Sulfa-G		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Tylan 40 Elanco	0.500	0.025	0.500	0.025	0.50	0.0252	0.000	0.000	0.000	0.000	0.000	0.000
Milk, Lactose		0.000		0.000	0.00	0.0000	0.000	0.000	0.000	0.000	0.000	0.000
Cr2O3	8.000	0.400	8.000	0.400	8.00	0.4028	0.000	0.000	0.000	0.000	0.000	0.000
Total	2000.0	100.0	2000.0	100.0	1986.00	100.00	14.000	14.000	14.000	14.000	14.000	14.000
Calculated Nutrient Values												
NSNG ME (kcal/kg)	3387		52090									
CP (%)	23.596		23.628									
Total Lysine (%)	1.457		1.458									
SID Lysine (%)	1.270		1.271									
Total P (%)	0.585		0.439									
Available P (%)	0.321		0.174									
Ca (%)	0.654		0.653									
Ca/P	1.12		1.49									

Appendix 2 – Summary of Actual Additions for Creating Tmts

Phase 2 Basal Amount 1290.575

Tmt Batch Size = 1300 # Batches = 1

Treatment Creations (lb addtions/Final Amount to basal)

Ingredients	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	0.000	5.200	5.059	4.918	4.635	4.071
Monocalcium P	9.425	0.000	0.000	0.000	0.000	0.000
Limestone	0.000	4.225	4.225	4.225	4.225	4.225
Corn Phytase (GralNzyme™)	0.000	0.000	0.141	0.282	0.565	1.129
Corn Phytase gr (GralNzyme™)	0.000	0.000	64.094	128.189	256.377	512.755

Phase 3 Basal Amount 1787.400

Tmt Batch Size = 1800 # Batches = 1

Treatment Creations (lb addtions/Final Amount to basal)

Ingredients	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	0.000	7.020	6.825	6.629	6.238	5.456
Monocalcium P	12.600	0.000	0.000	0.000	0.000	0.000
Limestone	0.000	5.580	5.580	5.580	5.580	5.580
Corn Phytase (GralNzyme™)	0.000	0.000	0.195	0.391	0.782	1.564
Corn Phytase gr (GralNzyme™)	0.000	0.000	88.746	177.492	354.984	709.968

Phase 4 Basal Amount 1191.600

Tmt Batch Size = 1200 # Batches = 2

Treatment Creations (lb addtions/Final Amount to basal)

Ingredients	PC Tmt1	NC Tmt 2	Tmt 3	Tmt 4	Tmt 5	Tmt 6
Corn, Yellow Dent	0.000	4.680	4.550	4.419	4.159	3.637
Monocalcium P	8.400	0.000	0.000	0.000	0.000	0.000
Limestone	0.000	3.720	3.720	3.720	3.720	3.720
Corn Phytase (GralNzyme™)	0.000	0.000	0.130	0.261	0.521	1.043
Corn Phytase gr (GralNzyme™)	0.000	0.000	59.164	118.328	236.656	473.312

8.6 Appendix 6

Study report for a swine feeding trial conducted by the (b) (4) (Swine Trial 4)

Corn-expressed phytase nursery pig study

Starting Date: 04/13/2017 (~6 to 7 kg, 21 d of age)
Ending Date: 05/25/2017 (~22.7 kg, 42 days on test)

Title: Effects of a corn-expressed phytase on growth performance and bone ash of nursery pigs.

Objective:

1) To determine the effect of increasing dietary (from 500 to 1,500 FTU/kg) corn-expressed phytase on growth performance and bone characteristics in nursery pigs fed phosphorus and Ca deficient diets.
2) To compare the effect of using corn-expressed phytase and commercial phytase (HiPhos) on growth performance and bone characteristics in nursery pigs fed phosphorus and Ca deficient diets.

Primary Investigators: (b) (4)
(b) (4)

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Mailing Address: (b) (4)

INTRODUCTION

Phytate is the main storage form of phosphorus in cereal grains and plant based protein supplements. However, pigs lack the endogenous enzyme to digest phytate which leads to poor availability of dietary phytate phosphorus as well as higher excretion levels of phosphorus. This is an environmental concern since increased buildup of soil phosphorus as a result of manure land application can lead to increased phosphorus runoff and negatively impact water quality. In addition, phytate has been shown to reduce availability of other nutrients. Superdosing of phytase at very high dietary levels has been shown to enhance growth performance beyond the expected improvement in performance as a result of release of phytate phosphorus, but inclusion of higher dietary phytase is somewhat expensive. Utilization of corn-expressed phytase has potential for reducing the cost of superdosing.

In a previous trial done by our lab (unpublished), pigs fed 1,000 FTU/kg Corn-expressed phytase restored weight gain, final BW, and bone characteristics when compared to positive control without adding Corn-expressed phytase.

Therefore, the objectives of this study are:

1. To determine the effect of increasing dietary (from 500 to 1,500 FTU/kg) corn-expressed phytase on growth performance and bone characteristics in nursery pigs fed phosphorus and Ca deficient diets.
2. To compare the effect of using corn-expressed phytase and HiPhos (10,000GT) on growth performance and bone characteristics in nursery pigs fed phosphorus and Ca deficient diets.

MATERIALS AND METHODS

Nursery phase:

Weanling pigs (n=288, 21 d) at the (b) (4) were selected and transferred to the (b) (4) .

Allotment to Treatments:

The pigs were individually weighed and sorted at weaning and allowed a 7-day adaption on a phase 1 diet. To avoid the confounding effect of initial weight, pigs were assigned to 8 blocks of 36 pigs. There was a total of 8 replicates per treatment with pigs housed 6 pigs/pen. Sex within block was balanced such that each treatment was represented by equal number of each sex within block. Pigs remained in the same pens throughout the experiment.

Dietary Phases:

The study consisted of four dietary phases:

- Phase 1: 6-6.7 kg (7 d)
- Phase 2: 6.7-7.9 kg (7 d)
- Phase 3: 7.9-13.9 kg (14 d)
- Phase 4: 13.9-23.0 kg (14 d)

Treatment regime during phase 1:

A common phase 1 diet (Table 1) with 5% Spray dried porcine plasma (SDPP) was provided for phase 1.

Treatment Regimes during phase 2, 3 & 4:

Treatment 1: (PC): Moderately complex industry diet devoid of corn-expressed phytase
Treatment 2: (NC) Negative control diet identical to the PC diet but with 0.15% and 0.10% reduction on aP and Ca
Treatment 3: Negative control diet with 500 FTU corn-expressed phytase/kg of diet
Treatment 4: Negative control diet with 1,000 FTU corn-expressed phytase/kg of diet
Treatment 5: Negative control diet with 1,500 FTU corn-expressed phytase/kg of diet
Treatment 6: Negative control diet with 500 FTU/kg of HiPhos (10,000 FTU/g)

Diets Formulation, Requirements, Mixing and Sampling:

Dietary formulation were provided by (b) (4) . Diets for the study are presented in Tables 1, 2, 3 and 4. Throughout the study, diets were formulated to represent moderately complex diets similar to those used in industry. Feed was provided in mash form.

Housing and Environment:

Pigs were housed in the conventional nursery facility, with wire floored pens and equipped with propane-fueled heaters, a two-hole nursery feeder and a cup waterer designed to minimize waste in each pen. Ambient minimum room temperature was initially set at 84°F and dropped 2°F weekly until temperature reached 78°F. Pigs had *ad libitum* access to water and feed during all phases.

Standard Measurements:

At the start of the study and at the end of each phase throughout the study, individual pig weights and pen feed intake was measured in order to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) by phase. At study termination the front left feet were removed for subsequent isolation of the metacarpal and bone ash was determined.

Feed samples

Feed samples were obtained for each batch of mixed feed. These were accumulated for each phase, subsampled to one composite sample/treatment/phase, and shipped with

proper identification of the ration number to research sponsor for subsequent nutrient analysis.

Animal Care:

The pigs in this study were cared for according to typical commercial management procedures. This experiment was carried out in accordance with the Animal Care Protocol #17027 for this swine experiment issued by the (b) (4) Any animal suffering from minor illness was reported to the Study Director and treated. All medical treatments were recorded. Any animal that died or became ill was weighed and removed from the study. An animal removal form was completed detailing the reason for removal, date, time and animal disposition.

Data Analysis

The data were analysed using the MIXED procedures of SAS. Treatment is the fixed effect. An orthogonal contrast was used to determine the effects of increasing corn-expressed phytase on performance and bone characteristics. Probability values of $P \leq 0.05$ were considered as a statistically significant difference, with $0.05 < P \leq 0.10$ considered a statistical trend.

Results:

Overall results in this study were very good and only 4 pigs were removed as a result of poor performance during the study. Note that the pigs were fed a common diet during phase 1 (Table 1) and the study with dietary treatments was initiated at the beginning of phase 2.

In all phases, pigs fed the positive control diet had numerically improved ADG when compared to those fed the negative control diet although differences were not significant ($P > 0.10$, Table 5). However, ADG increased linearly during phase 2 ($P < 0.05$), phase 4 ($P < 0.01$) and for the overall study ($P < 0.01$) with increasing levels of GraINzyme from 0 to 1,500 FTU/kg of diet (Figure 1). The linear improvement in ADG with increasing GraINzyme during the combined phase 2 and phase 3 periods approached significance ($P = 0.10$). Average daily gain in pigs fed the highest dose of GraINzyme (1,500 FTU/kg of diet) was higher in phase 4 ($P < 0.08$) when compared to those fed the negative control diet. Overall ADG was higher ($P = 0.10$) in pigs fed all GraINzyme levels compared to those fed the negative control diet. Similarly, ADG in pigs fed the highest level of GraINzyme was numerically higher than that observed in pigs fed the positive control diet in all phases and overall, although differences were not significant ($P > 0.10$). ADG in pigs fed HiPhos at 500 FTU/kg was similar in all phases to daily gain observed in pigs fed the positive control and GralNzyme at 500 FTU/kg diet ($P > 0.10$) and ADG was not significantly improved over that observed in pigs fed the negative control diet ($P > 0.10$). As might be expected based on ADG, BW at study completion increased with increasing dietary level of GraINzyme from 0 to 1,500 FTU ($P < 0.05$; Figure 2). BW was also improved in pigs fed GraINzyme at 1,500 FTU compared to pigs fed the negative control diet although differences were not significant ($P > 0.10$).

Average daily feed intake was similar among all treatments in all phases ($P > 0.35$; Figure 3) with the exception of phase 4 where ADFI increased linearly ($P < 0.01$) with increasing level of GraINzyme from 0 to 1,500 FTU/kg of feed. ADFI in phase 4 also tended

to be higher in pigs fed 1000 or 1500 FTU/kg GralNzyme when compared to those fed the negative control diet ($P < 0.10$).

Feed efficiency was similar among pigs fed the negative control and positive control diets. However, G:F increased linearly with increasing GraINzyme from 0 to 1,500 FTU/kg of diet in phase 3, the combined phase 2 and 3 periods and for the overall study ($P < 0.05$; Figure 4). Efficiency was also numerically higher in pigs fed the highest level of GraINzyme in all phases when compared to pigs fed the negative control diet although differences were not significant ($P > 0.14$). Efficiency was also numerically higher in pigs fed the highest level of GraINzyme in all phases with the exception of phase 4 when compared to those fed the positive control diet.

Effect of GraINzyme on metacarpal bone characteristics (Table 6) indicates that bone length and bone ash weight (Figure 5) tended to increase linearly with increasing level of GraINzyme from 0 to 1,500 FTU/kg of feed ($P < 0.10$). Percent ash increased both linearly and quadratically (Figure 5; $P < 0.001$) with increasing level of GraINzyme from 0 to 1,500 FTU/kg of feed. In addition, fresh bone weight, weight of ash, and % ash in pigs fed the highest dose of GraINzyme (1,500 FTU/kg of diet) was higher than observed in pigs fed the negative control diet ($P < 0.01$). Pigs fed the highest dose of GraINzyme (1,500 FTU/kg of diet) had similar bone ash weight ($P > 0.05$) but lower percent ash ($P < 0.05$) when compared to those fed the positive control diet. However, the opposite was observed with pigs fed 1000 FTU/kg GraINzyme, in which percent bone ash was similar ($P > 0.05$) to positive control pigs. Weight and percent of bone ash were higher in pigs fed HiPhos at 500 FTU/kg of diet when compared to those fed the negative diet but lower when compared to pigs fed the positive control diet ($P < 0.05$). Pigs fed 500 FTU/kg GraINzyme had higher percent bone ash ($P < 0.05$) than the equivalent dose of HiPhos.

This study demonstrates that increasing inclusion of GraINzyme from no inclusion to 1,500 FTU/kg of diet substantially improves ADG and G:F. With the exception of phase 3 where feed intake increased with increasing GraINzyme, it appears that the differences in gain were due to an improved efficiency and not increased intake. The improved efficiency and intake in phase 3 resulted in numerically superior ADG and BW. GralNzyme is as effective as HiPhos on growth phenotypes when supplement at the same level.

Table 1. Diet composition of Phase 1

	PC + 0 FTU/kg	
PIC C29 x PIC380		
Trial:	BW(lb)	8-12
Delivery Date:	10	lb Ave.
Ingredients	lbs	%
Corn, Yellow Dent	841.99	42.10
Soybean meal, 48%, high	380.000	19.000
Corn DDGS, >6 and <9% Oil		0.000
Poultry Fat	60.000	3.000
Monocalcium P	13.800	0.690
Limestone	11.700	0.585
Salt	5.000	0.250
L-Lysine	4.400	0.220
DL-Methionine	3.200	0.160
L-Threonine	0.760	0.038
L-Tryptophan	0.550	0.028
ZnO		0.000
Copper Sulfate		0.000
Trace Mineral Premix (NB	3.000	0.150
Vitamin Premix (NB-6508	5.000	0.250
Plasma (AP-920)	100.000	5.000
Fish Meal, Menhaden	100.000	5.000
Milk, Whey Powder	400.000	20.000
Corn Phytase (GralNzyme™)		0.000
Ethoxiquin (Quinguard)	0.600	0.030
Milk, Lactose	70.000	3.500
TiO2	0.000	0.000
Commercial Phytase) (	0.000	0.000
Total	2000.0	100.0

Table 2. Diet composition of Phase 2

(b) (4)	PC + 0 FTU/kg		NC		NC+500 FTU/kg		NC+ 1000 GralNyme		NC + 1500 FTU/kg		C + 500 FTU/kg HIPh	
PIC C29 x PIC380												
Trial:	BW(lb)	12-16	BW(lb)	12-16	BW(lb)	12-16	BW(lb)	12-16	BW(lb)	12-16	BW(lb)	12-16
Delivery Date:	14	lb Ave.	14	lb Ave.	14	lb Ave.	14	lb Ave.	14	lb Ave.	14	lb Ave.
Ingredients	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%
Corn, Yellow Dent	818.10	40.91	840.41	42.02	839.93	42.00	839.46	41.97	838.98	41.95	840.35	42.02
Soybean meal, 48%, high	553.000	27.650	553.000	27.650	553.000	27.650	553.000	27.650	553.000	27.650	553.000	27.650
Corn DDGS, >6 and <9%	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000
Poultry Fat	60.000	3.000	51.000	2.550	51.000	2.550	51.000	2.550	51.000	2.550	51.000	2.550
Monocalcium P	23.500	1.175	9.200	0.460	9.200	0.460	9.200	0.460	9.200	0.460	9.200	0.460
Limestone	15.750	0.788	16.900	0.845	16.900	0.845	16.900	0.845	16.900	0.845	16.900	0.845
Salt	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500
L-Lysine	6.290	0.315	6.240	0.312	6.240	0.312	6.240	0.312	6.240	0.312	6.240	0.312
DL-Methionine	3.040	0.152	2.980	0.149	2.980	0.149	2.980	0.149	2.980	0.149	2.980	0.149
L-Threonine	1.170	0.059	1.130	0.057	1.130	0.057	1.130	0.057	1.130	0.057	1.130	0.057
L-Tryptophan	0.550	0.028	0.540	0.027	0.540	0.027	0.540	0.027	0.540	0.027	0.540	0.027
Trace Mineral Premix (NB	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150
Vitamin Premix (NB-6508	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250
Plasma (AP-920)	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500
Fish Meal, Menhaden	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500
Milk, Whey Powder	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000	200.000	10.000
Corn Phytase (GralNzyme™)		0.000		0.000	0.476	0.0238	0.952	0.0476	1.428	0.0714		0.000
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030
TiO2		0.000		0.000		0.000		0.000		0.000		0.000
Commercial Phytase)		0.000		0.000		0.000		0.000		0.000	0.060	0.0030
Total	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0

Table 3. Diet composition of Phase 3

(b) (4)	PC + 0 FTU/kg		NC		NC+500 FTU/kg		NC+ 1000 GralNyme		NC + 1500 FTU/kg		C + 500 FTU/kg HlPh	
PIC C29 x PIC380												
Trial:	BW(lb)	16-25	BW(lb)	16-25	BW(lb)	16-25	BW(lb)	16-25	BW(lb)	16-25	BW(lb)	16-25
Delivery Date:	21	lb Ave.	21	lb Ave.	21	lb Ave.	21	lb Ave.	21	lb Ave.	21	lb Ave.
Ingredients	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%
Corn, Yellow Dent	884.08	44.20	907.45	45.37	906.97	45.35	906.50	45.32	906.02	45.30	907.39	45.37
Soybean meal, 48%, high	628.000	31.400	628.000	31.400	628.000	31.400	628.000	31.400	628.000	31.400	628.000	31.400
Corn DDGS, >6 and <9%	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000
Poultry Fat	60.000	3.000	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500
Monocalcium P	14.300	0.715		0.000		0.000		0.000		0.000		0.000
Limestone	17.200	0.860	18.200	0.910	18.200	0.910	18.200	0.910	18.200	0.910	18.200	0.910
Salt	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500
L-Lysine	7.200	0.360	7.200	0.360	7.200	0.360	7.200	0.360	7.200	0.360	7.200	0.360
DL-Methionine	2.700	0.135	2.650	0.133	2.650	0.133	2.650	0.133	2.650	0.133	2.650	0.133
L-Threonine	1.550	0.078	1.530	0.077	1.530	0.077	1.530	0.077	1.530	0.077	1.530	0.077
L-Tryptophan	0.370	0.019	0.370	0.019	0.370	0.019	0.370	0.019	0.370	0.019	0.370	0.019
Trace Mineral Premix (NB	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150
Vitamin Premix (NB-6508	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250
Plasma (AP-920)	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500
Fish Meal, Menhaden	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500	50.000	2.500
Corn Phytase (GralNzyme™)		0.000		0.000	0.476	0.0238	0.952	0.0476	1.428	0.0714		0.000
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030
TiO2	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300
Commercial Phytase)		0.000		0.000		0.000		0.000		0.000	0.060	0.0030
Total	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0

Table 4. Diet composition of Phase 4

(b) (4)	PC + 0 FTU/kg		NC		NC+500 FTU/kg		NC+ 1000 GralNyme		NC + 1500 FTU/kg		C + 500 FTU/kg HIPh	
PIC C29 x PIC380												
Trial:	BW(lb)	25-50	BW(lb)	25-50	BW(lb)	25-50	BW(lb)	25-50	BW(lb)	25-50	BW(lb)	25-50
Delivery Date:	38	lb Ave.	38	lb Ave.	38	lb Ave.	38	lb Ave.	38	lb Ave.	38	lb Ave.
Ingredients	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%	lbs	%
Corn, Yellow Dent	959.12	47.96	981.38	49.07	980.90	49.05	980.43	49.02	979.95	49.00	981.32	49.07
Soybean meal, 48%, high	611.000	30.550	611.000	30.550	611.000	30.550	611.000	30.550	611.000	30.550	611.000	30.550
Corn DDGS, >6 and <9%	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000	300.000	15.000
Poultry Fat	60.000	3.000	51.000	2.550	51.000	2.550	51.000	2.550	51.000	2.550	51.000	2.550
Monocalcium P	14.200	0.710		0.000		0.000		0.000		0.000		0.000
Limestone	18.900	0.945	20.000	1.000	20.000	1.000	20.000	1.000	20.000	1.000	20.000	1.000
Salt	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500	10.000	0.500
L-Lysine	7.800	0.390	7.750	0.388	7.750	0.388	7.750	0.388	7.750	0.388	7.750	0.388
DL-Methionine	2.450	0.123	2.400	0.120	2.400	0.120	2.400	0.120	2.400	0.120	2.400	0.120
L-Threonine	1.600	0.080	1.550	0.078	1.550	0.078	1.550	0.078	1.550	0.078	1.550	0.078
L-Tryptophan	0.330	0.017	0.320	0.016	0.320	0.016	0.320	0.016	0.320	0.016	0.320	0.016
Trace Mineral Premix (NB	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150	3.000	0.150
Vitamin Premix (NB-6508	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250	5.000	0.250
Corn Phytase (GralNzyme™)		0.000		0.000	0.476	0.0238	0.952	0.0476	1.428	0.0714		0.000
Ethoxiquin (Quinguard)	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030	0.600	0.030
TiO2	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300	6.000	0.300
Commercial Phytase		0.000		0.000		0.000		0.000		0.000	0.060	0.0030
Total	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0	2000.0	100.0

Table 5. Effect of GralNzyme on growth performance in nursery pigs (LS means)

	PC	NC	NC + GralNzyme			NC + HiPhos		*P* - Value		
FTU/kg			500	1,000	1,500	500	SEM	Treatment	Linear[1]	Quadratic[1]
ADG, kg/d										
Phase 2	0.172	0.143	0.176	0.180	0.187	0.170	0.014	0.3694	0.0412	0.3682
Phase 3	0.419	0.401	0.430	0.438	0.434	0.412	0.019	0.6901	0.2013	0.3596
Phase 4	0.633^{xy}	0.579^{x}	0.640^{xy}	0.634^{xy}	0.682^{y}	0.635^{xy}	0.023	0.0788	0.0044	0.7594
Phase 2&3	0.337	0.315	0.345	0.352	0.351	0.331	0.015	0.5256	0.1016	0.3182
Overall	0.455^{xy}	0.421^{x}	0.463^{y}	0.465^{y}	0.483^{y}	0.453^{xy}	0.015	0.1016	0.0063	0.4157
BW, kg										
Initial	6.67	6.70	6.66	6.73	6.64	6.64	0.28	0.9846	0.8205	0.8112
End of phase 2	7.88	7.70	7.89	7.99	7.95	7.83	0.28	0.8578	0.2530	0.4900
End of phase 3	13.75	13.32	13.91	14.12	14.02	13.60	0.47	0.6930	0.1799	0.3643
End of phase 4	22.61	21.43	22.87	23.00	23.56	22.49	0.67	0.1846	0.0135	0.4355
ADFI, kg/d										
Phase 2	0.273	0.209	0.256	0.243	0.246	0.240	0.020	0.3520	0.2792	0.2747
Phase 3	0.707	0.661	0.697	0.629	0.631	0.646	0.045	0.6582	0.3956	0.6755
Phase 4	0.992^{xyz}	0.913^{x}	0.999^{xyz}	1.018^{yz}	1.056^{z}	0.952^{xy}	0.033	0.0603	0.0041	0.4675
Phase 2&3	0.563	0.511	0.550	0.501	0.503	0.511	0.033	0.6041	0.5999	0.5479
Overall	0.734	0.671	0.730	0.707	0.724	0.687	0.028	0.5198	0.2752	0.4477
G:F										
Phase 2	0.672	0.674	0.685	0.750	0.750	0.722	0.046	0.5870	0.1282	0.9011
Phase 3	0.623	0.621	0.631	0.705	0.712	0.653	0.041	0.2419	0.0312	0.9675
Phase 4	0.665	0.638	0.641	0.637	0.654	0.667	0.017	0.6936	0.5962	0.6807
Phase 2&3	0.624	0.627	0.639	0.709	0.717	0.662	0.035	0.1382	0.0172	0.9517
Overall	0.644	0.630	0.638	0.666	0.679	0.663	0.019	0.4122	0.0416	0.9043

[x,y,z]LS means with different superscripts tend to be different (P ≤ 0.10).

[1.] IML procedure of SAS was used to estimate coefficient which then being used in orthogonal contrast analysis for NC, NC+GralNzyme 500 FTU/kg, NC+GralNzyme 1,000 FTU/kg, and NC+GralNzyme 1,500 FTU/kg.

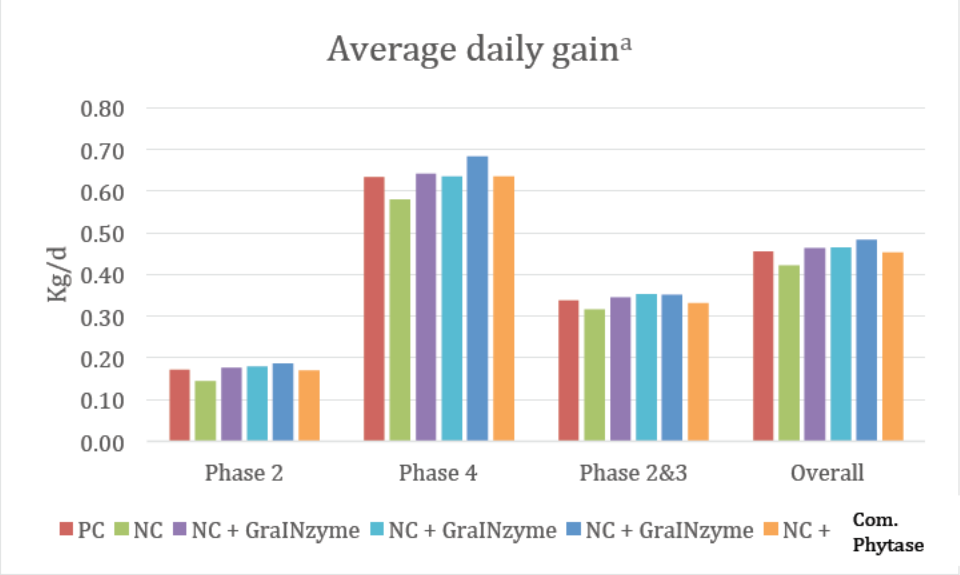
Table 6. Effect of GralNzyme on metacarpal bone characteristics in nursery pigs (LS means)

	PC	NC	NC + GraINzyme			NC + HiPhos		*P* - Value		
FTU/kg			500	1,000	1,500	500	SEM	Treatment	Linear[1]	Quadratic[1]
Length, mm	36.87	36.31	36.57	36.75	37.00	36.39	0.31	0.3862	0.0627	0.9796
Width, mm	9.248	9.113	9.148	9.048	9.365	9.300	4.149	0.4510	0.2403	0.2592
Fresh bone, g	4.314^{bc}	3.872^{a}	4.091^{ab}	4.090^{ab}	4.393^{c}	4.134^{b}	0.110	0.0030	0.6360	0.1876
Ash, g	1.122^{d}	0.840^{a}	0.984^{bc}	1.037^{c}	1.098^{cd}	0.951^{b}	0.029	<0.0001	0.0878	0.3522
Ash, %	26.010^{e}	21.610^{a}	24.070^{c}	25.450^{de}	25.090^{d}	23.080^{b}	0.284	<0.0001	<0.0001	<0.0001

[a.b.c.d.e.] LS means with different superscripts are significantly different (P < 0.05).

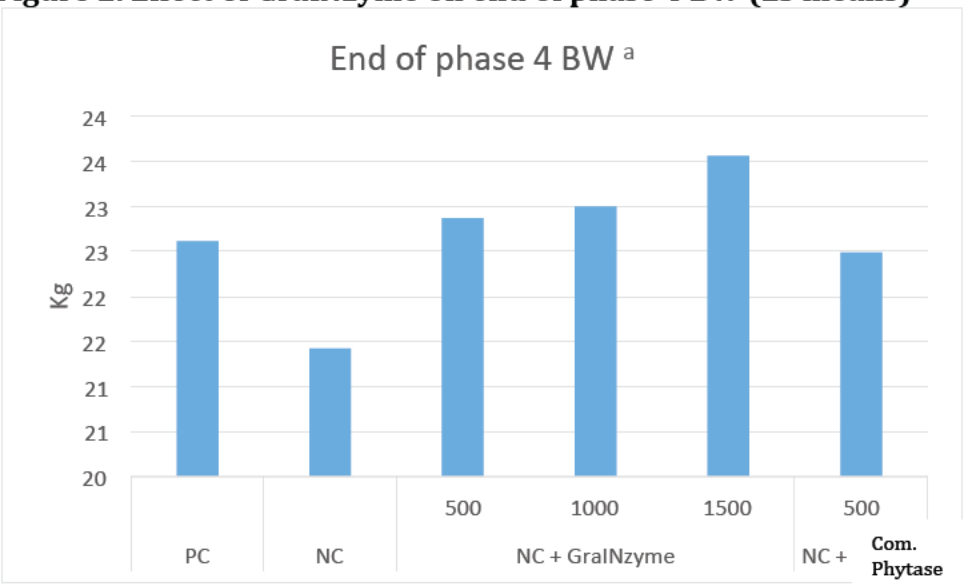
[1.] IML procedure of SAS was used to estimate coefficient which then being used in orthogonal contrast analysis for NC, NC+GralNzyme 500 FTU/kg, NC+GralNzyme 1,000 FTU/kg, and NC+GralNzyme 1,500 FTU

Figure 1. Effect of GraINzyme on average daily gain (LS means)



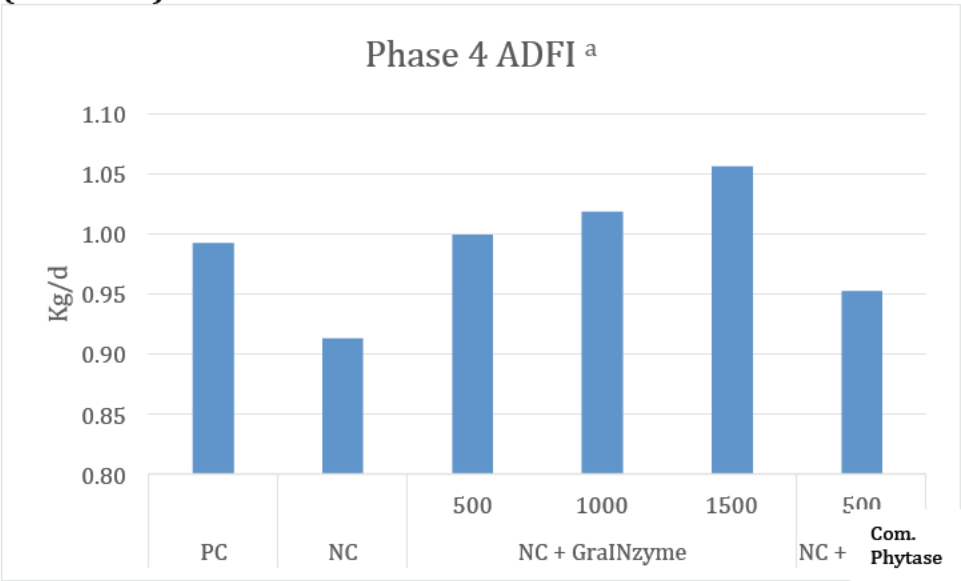
[a] Increasing Grainzyme from 0 in the NC to 1,500 FTU/kg increased ADG linearly in phase 2 ($P < 0.05$), phase 4 ($P < 0.01$) and for the overall study ($P < 0.01$).

Figure 2. Effect of GraINzyme on end of phase 4 BW (LS means)



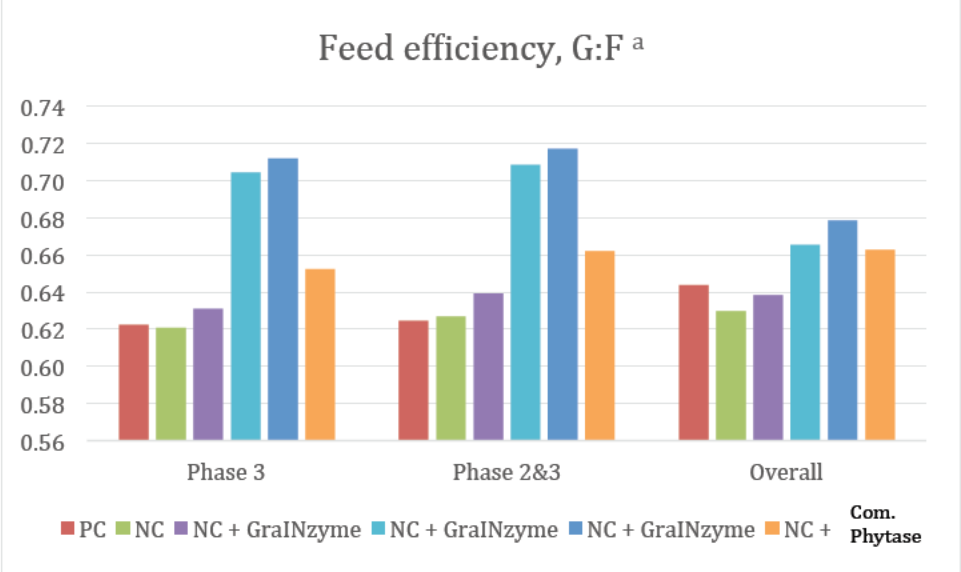
[a a] Increasing Grainzyme from 0 in the NC to 1,500 FTU/kg increased BW linearly at study completion ($P < 0.05$).

Figure 3. Effect of GraINzyme on phase 4 average daily feed intake (LS means)



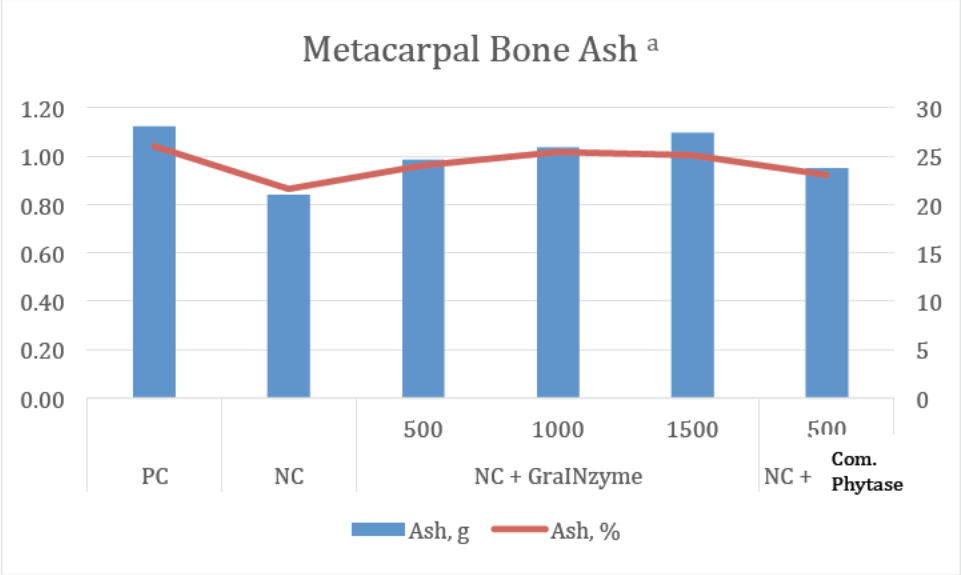
[a] Increasing Grainzyme from 0 in the NC to 1,500 FTU/kg linearly increased ADFI in phase 4 ($P < 0.01$).

Figure 4. Effect of GraINzyme Feed efficiency (LS means)



[a] Increasing GraINzyme from 0 in the NC to 1,500 FTU/kg increased G:F linearly in phase 3, the combined phase 2 and 3 phases and for the overall study ($P < 0.05$).

Figure 5. Effect of GraINzyme on metacarpal bone ash (LS means)



[a] Increasing Grainzyme from 0 in the NC to 1,500 FTU/kg tended to linearly increase g of bone ash ($P < 0.10$) and linearly and quadratically increased % ash ($P < 0.001$).

8.7 Appendix 7

Characterization of the Phy02 enzyme in three typical product batches

8.7.1 Production of three typical product batches

Three separate representative product batches of the Phy02 phytase were produced from grain of the maize event PY203. The product batch numbers, location of planting and dates of planting and harvest are shown in Table 1. Planting the seed and harvest of the grain were performed using commonly used agronomic practices for maize. Cultivation of the Phy02 producing maize also utilized common agronomic practices for maize including the use of fertilizers, herbicides and pesticides approved for use on maize. (b) (4)
(b) (4)

Table 1. Planting locations and dates for the production of three representative Phy02 phytase product batches.

	Phy02 Product Batches		
Product Batch No.	AV_Phy02_0043	AV_Phy02_0049	AV_Phy02_0050
Planting Location	Field; (b) (4)	Field; (b) (4)	Greenhouse, (b) (4)
Planting Date	12 June 2015	12 June 2015	25 May 2015
Harvest Date	1 October 2015	14 October 2015	21 September 2015

Each of the three representative Phy02 phytase product batches were analyzed to demonstrate that they meet the purity, chemical and microbial specifications established for enzyme preparations, as outlined in the Food Chemical Codex (FCC 2001), and the specifications established for enzymes used in food processing, as proposed by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO 2001). Physical, chemical, and microbial characteristics were determined for each of the Phy02 phytase product batches by (b) (4) The results of these analyses are presented in Table 2.

Examination of the results of the analysis of key product characteristics as presented in Table 2 demonstrate that all three Phy02 phytase product batches meet or exceed all JECFA specifications established for enzyme preparations that are used in food and/or feed with the exception of total bacterial count and the number of coliform colony forming units (cfu). All three product batches had no detectible presence of either *Salmonella* or *E. coli* bacteria. Coliform bacteria are defined as rod-shaped Gram

negative, non-spore forming and motile or non-motile bacteria that can ferment lactose with the production of acid and gas when incubated at 35–37°C (Brenner, 1992; Bettelheim, 1992). While coliforms themselves are not normally causes of serious illness, their presence has been used to indicate that other pathogenic organisms of fecal origin may be present (Krentz et al., 2013). Typical genera in the coliform group include: *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Escherichia* (Brenner, 1992; Bettelheim, 1992).

The JECFA specifications for food enzyme preparations have been traditionally applied to enzyme products that are produced by sterile fermentation followed by purification of the enzyme in a sanitary laboratory environment. Under these conditions it is feasible to produce a purified enzyme product that meets the JECFA specifications for the presence of microbes in the product. However, the Phy02 phytase product is produced in the same manner as the production of maize grain that is widely used as a major component of human food and animal feed. It is produced in agricultural fields in the environment where bacteria are present in the soil, air and water and on the surfaces of plants, including the maize that produces the Phy02 phytase containing grain. Therefore it is reasonable to expect that the Phy02 phytase product would contain levels of bacterial presence that is typical for maize grain produced by typical agricultural practices. Two of the three Phy02 phytase product batches exceeded the JECFA specification of 30 cfu/g product for coliform bacteria with coliform numbers of 300 and 6,700/g (Table 2). However, these numbers are consistent with studies of microbial presence in maize grain and in animal feed. Tabib et al. (1981) surveyed feeds and feed ingredients, including maize, in the feed of broilers, layers and turkeys and found that the numbers of coliform bacteria ranged from 450 – 910,000 cfu/g. Similar studies have also reported equivalent levels of coliform bacterial in cattle feed (Sanderson *et al*., 2005) and tortillas made from corn meal (Gomez-Aldapa *et al*., 2013). From these reports it is evident that the level of coliform bacteria in two of the three Phy02 product batches is similar to those reported as normal for maize grain and other commonly used feed ingredients. Since the numbers of coliform bacteria found to be present in two of the three Phy02 phytase product batches are typical for those found in maize grain and other animal feed ingredients and since known pathogenic bacteria such as *Salmonella* and *E. coli* were absent from the product batches, the higher level of coliforms in the Phy02 product compared to the JECFA specifications for food enzyme products is considered to be safe.

Table 2. Physical, chemical, and microbial characteristics of three independent Phy02 phytase product batches compared to JECFA specifications for enzyme preparations used in food and feed.

			Phytase Phy02 Product Batch			
	Method	**Unit**	**AV_Phy02_0043**	**AV_Phy02_0049**	**AV_Phy02_0050**	**JECFA Specification Limit**
Physical Characteristics						
Phytase Activity	Agrivida, Inc. SOP	FTU/g	(b) (4)	(b) (4)	(b) (4)	NA
	Agrivida, Inc. SOP	FTU/mg protein				NA
Density	USP 616	g/ml	0.6	0.6	0.6	NA
Micron particle size	MF-2051 Evaluating Particle Size, KSU 2002	micron	2,704.00	2,705.00	2,690.00	NA
Chemical Characteristics						
Cadmium	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	30 max
Mercury	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	30 max
Lead	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	5 max
Arsenic	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	<0.010	<0.010	<0.010	3 max
Microbial Characteristics						
Coliforms	AOAC 991.14	cfu/g	6,700	300	10	30 max
Salmonella	AOAC 2003.09	#/25g	negative	negative	negative	Absent
Aerobic Plate Count	BAM Chapter 3	cfu/g	97,000	6,300	86,000	50,000 max
E. coli	U.S. Pharmacopeia Chapter 62	#/10g	negative	negative	negative	Absent
Aflatoxin	Commercial Test Kit (ELISA)	ppb	<5	<5	<5	Nondetectible
T-2 Toxin	Commercial Test Kit (ELISA)	ppb	<25	<25	<25	Nondetectible
Ochratoxin	Commercial Test Kit (ELISA)	ppb	<2	<2	<2	Nondetectible
Sterigmatocystin	Eurofins Internal method	ug/kg	<10	<10	<10	Nondetectible

8.7.2 Characterization of the Phy02 phytase in three typical product batches

For the purpose of characterizing the Phy02 phytase product, characteristics of the Phy02 phytase in protein extracts prepared from grain derived from three representative Phy02 phytase product batches (Lot numbers AV_Phy02_0043, AV_Phy02_0049, and AV_Phy02_0050) were assessed. The molecular weight, immunoreactivity, intactness and phytase activity of the Phy02 phytase protein in the three product batches were evaluated and the results are contained in a report presented in Appendix 5. In all three product batches the Phy02 protein was shown to have an approximate molecular weight of 46,000 kDa which is very close to the expected size of 45,684 kDa for the mature Phy02 phytase protein including the endoplasmic retention signal from maize. In addition, the Phy02 protein from each production batch reacted with a phytase specific rabbit polyclonal antibody to demonstrate the expected immunoreactivity of the Phy02 phytase protein. The phytase activities of the three product batches were measured using protocols described in Appendix 2 and were determined to be:

AV_PHY02_0043	6,454 FTU/g
AV_PHY02_0049	4,049 FTU/g
AV_PHY02_0050	4,890 FTU/g

8.7.2.1 Determination of specific activity of Phy02

The phytase activity and specific activity of the phytase relative to total soluble protein was determined in grain from three separate product batches of Phy02 phytase. The amount of total soluble protein in the aqueous protein extracts of flour produced from the grain was determined by two different methods, the Bradford method (Kruger, 1996) and the BCA method (Walker, 1996). Three grams of milled flour from each product batch was placed in 35 mL of 25 mM sodium borate, pH 10 buffer, 0.01% Tween 20 for 1 hr at room temperature. The samples were shaken on a tabletop shaker at maximum speed and 2 mL was centrifuged at 12,000×g for 10 min. Supernatants were transferred to phytase assay buffer (250 mM sodium acetate, pH 5.5, 1 mM calcium chloride, 0.01% Tween 20) prior to analysis for proteins by either method. Three separate determinations were performed for each extract using each of the two methods and all results for each extract were averaged. The specific activity for each test substance was calculated from the phytase activity determined for each batch (FTU/g) divided by the average amount of protein/g determined for each sample by the two protein quantitation methods. The specific phytase activities of the test substances from the three product batches analyzed expressed in FTU phytase activity/mg protein are:

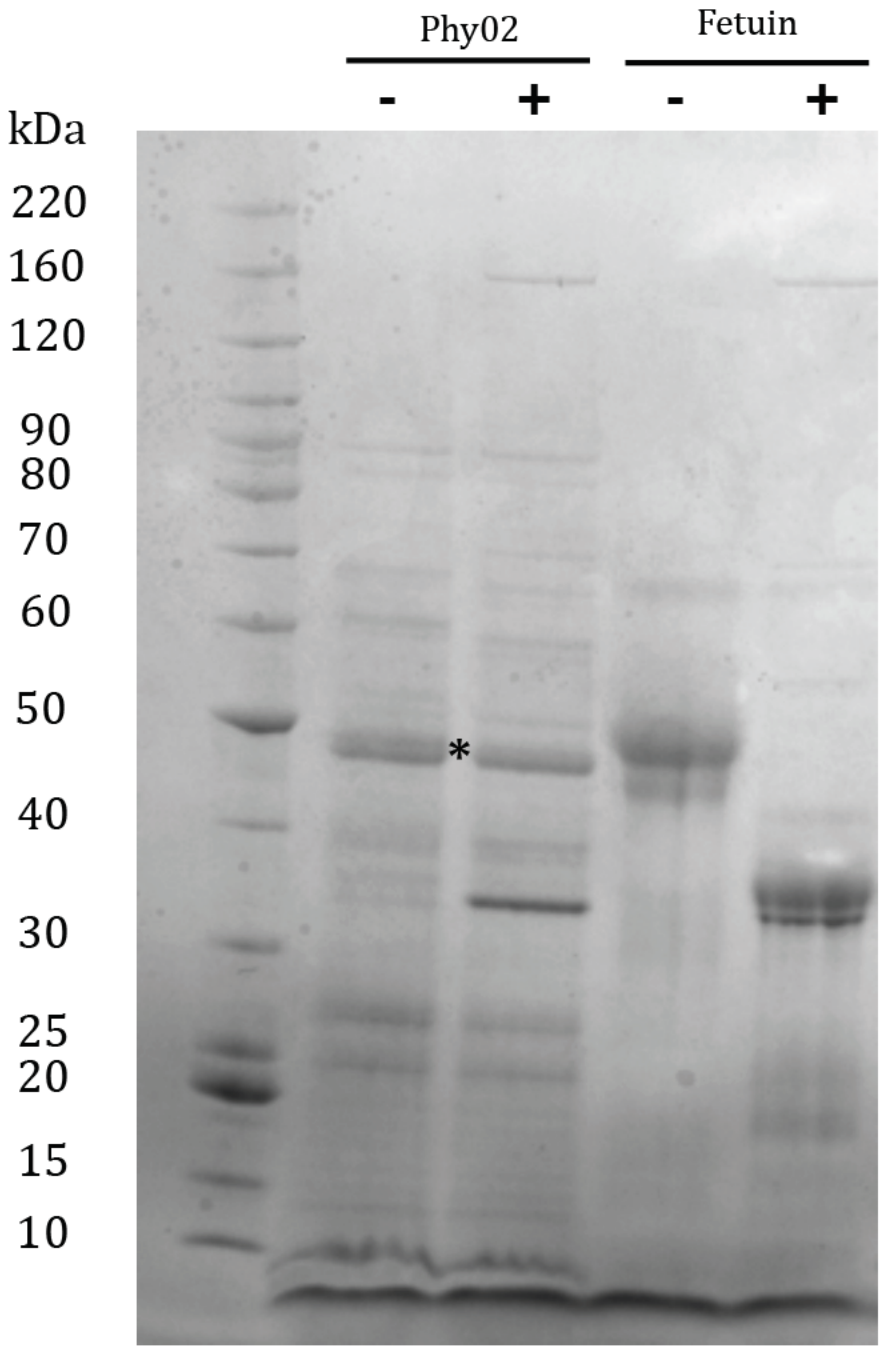
AV_PHY02_0043	(b) (4)
AV_PHY02_0049	

AV_PHY02_0050 (b) (4)

8.7.2.2 Glycosylation of maize-produced Phy02 phytase

The glycosylation status of the Phy02 phytase protein produced by maize was examined using a Protein Deglycosylation Kit obtained from (b) (4) and the protocol supplied with the kit. Briefly, the Phy02 phytase protein in an extract produced from Phy02 product batch AV_Phy02_0049 (§4.4) was treated with the enzymes PNGase F and O-Glycosidase that remove N-linked and O-linked glycosyl groups, respectively. After treatment with these deglycosylating enzymes, treated and untreated protein extracts were examined by SDS-PAGE and the apparent size of the Phy02 protein in each was compared. In the case of glycosylated proteins, removal of the glycosyl moieties results in an apparent reduction in the size of the protein on SDS-PAGE gels. SDS-PAGE gels containing total protein from enzyme treated and untreated extracts from Phy02 containing maize grain are shown in Figure 1 and show that there is no change in the apparent size of the Phy02 protein with and without enzyme treatment. This result demonstrates that the Phy02 phytase protein produced in the grain of maize is not glycosylated.

Figure 1. Comparison by SDS-PAGE of the apparent size of the Phy02 phytase protein (indicated by an asterisk) from grain extracts with (+) and without (-) treatment with deglycosylation enzymes. The control protein, fetuin, that contains sialylated N-linked and O-linked glycans, is shown before (+) and after (-) treatment in the right two lanes. The reduction in the apparent size of the fetuin protein after treatment with deglycosylating enzymes demonstrates that the deglycosylation reaction was functional. Protein molecular weight standards are included in the left lane and their sizes in kDa are indicated on the left of the gel.



8.7.2.3 Confirmation of the amino acid sequence of Phy02 phytase

The Phy02 gene coding sequence includes at the N-terminus the coding sequence for the γ-zein seed storage protein signal sequence of *Zea mays* that directs proteins to the endoplasmic reticulum (Geli *et al*., 1994). The 19 amino acid signal sequence of the γ-zein protein is typically cleaved from peptides during transport into the endoplasmic reticulum to generate the mature protein (Esen *et al*., 1982). Prat et al. (1985) noted that the 19 amino acid signal sequence of γ-zein has structural features commonly found among eukaryotic signal peptides (Walter *et al*., 1984). By comparing the N-terminal amino acid sequence of γ-zein with the coding sequence that includes the signal peptide Esen *et al*. (1982) determined that the signal peptide is cleaved upon transport into the endoplasmic reticulum immediately following the sequence Ser-Ala-Thr-Ser. The γ-zein signal peptide has been successfully used to target numerous heterologous proteins to the endoplasmic reticulum (de Virgilio *et al*., 2008; Harrison *et al*., 2011; Torrent *et al*., 2009).

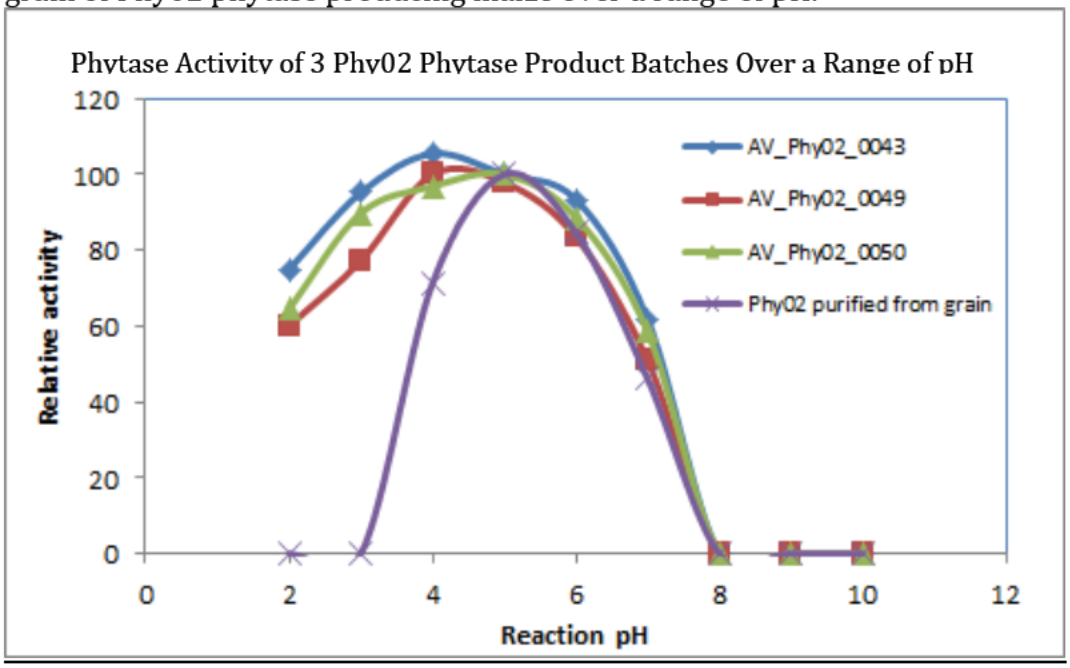
Protein from extracts of the representative Phy02 phytase product batch AV_PHY02_0049 (§3.0) were run on an SDS-PAGE gel and transferred to a PVDF membrane that was stained with Coomassie Blue without heating to visualize the protein bands. The band corresponding to the correct molecular weight of the Phy02 phytase was excised and the N-terminal amino acid sequence of the protein was determined by Edman degradation by (b) (4)). The predicted cleave site of the γ-zein signal peptide is immediately after the serine residue at position 19 of the Phy02 phytase protein (Esen *et al*., 1982). The results demonstrated that the γ-zein signal peptide is cleaved at two different locations within the Phy02 preprotein since there were two different amino acid residues at the N-terminus of the mature Phy02 phytase protein. The N-terminal amino acid sequence of the mature Phy02 phytase protein was shown to be either AQSEP or SEPEL. In the case of the Phy02 phytase, it appears that the site of cleavage of the γ-zein signal peptide is not precise and cleavage may also occur between residues 21 (Q, glutamine) and 22 (S, serine) to produce a mature protein that begins with the sequence SEPEL. These results confirm that the mature Phy02 phytase protein that is produced in the grain of maize has the N-terminal amino acid sequence that is expected from the coding sequence of the phy02 gene with the exception of the slight variability due to variable cleavage of the γ-zein signal peptide at the C-terminus of either residue 19 or 21 of the Phy02 phytase preprotein.

8.7.2.4 Optimal reaction pH for Phy02 phytase

The phytase activity in protein extracts from three independent Phy02 phytase product batches (Lot numbers AV_PHY02_0043, AV_PHY02_0049, and AV_PHY02_0050) and of maize purified Phy02 phytase was determined over a range of pH to determine the pH optimum for phytase activity. The phytase enzymatic reactions were performed in 10x CCH (42.8 g/L citric acid, 92.1 g/L CHES, 79.4 g/L HEPES, pH 3) buffer that was diluted to 1x CCH buffer using either 1N HCl or 1N NaOH to adjust the pH from 2 to 10. Extracts of flour from Phy02 producing maize

grain were diluted 500-fold in each 1x CCH buffer. Phytic acid substrate was prepared at a concentration of 9.1 mM and was dissolved in each of the 1x CCH buffers with different pH to ensure that upon mixing enzyme solution with the substrate the reaction pH did not change. Prior to analyses the pH of the phytic acid substrate solution and each reaction buffer was verified with a standardized pH meter. Phytase reactions were initiated by adding diluted protein extract to the corresponding pH-adjusted substrate followed by incubation of the reaction mixtures for 60 minutes at 37°C. Reaction pH was monitored with colorpHast pH indicator strips (EM Science) following addition of enzyme**.** The results of the analyses of phytase activity are shown in Figure 13. The activities of the Phy02 phytase in the protein extracts from three Phy02 product batches and that of purified Phy02 phytase protein as a percent of activity of the Phy02 phytase at its pH optimum of pH 4.0 – 5.0 are presented. The results demonstrate that the phytase activity in the extracts from the three different product batches have nearly identical activity profiles over the range of pH tested with highest activity at pH 4.0 - 5.0. Above pH 6 the activity of the Phy02 phytase from the different test materials is lost rapidly and is absent at pH 8 (Figure 2). The activity of the purified Phy02 phytase is similar to that of the Phy02 phytases from the product extracts except that its activity is more sensitive to pH lower than pH 4. The phytase activity in the product extracts demonstrated 60 – 80% relative activity at pH 2 whereas the purified Phy02 phytase had no activity at pH 4. A comparison of the pH profile of maize produced Phy02 phytase from this study with that reported for the *E. coli* AppA phytase reveals many similarities between these related phytases (Lim *et al*., 2000). Both proteins exhibit a broad pH profile with maximum activity occurring at pH 4.5, and both retain significant activity in the acidic pH range. At pH above neutral, AppA and Phy02 phytases lose their enzymatic activity.

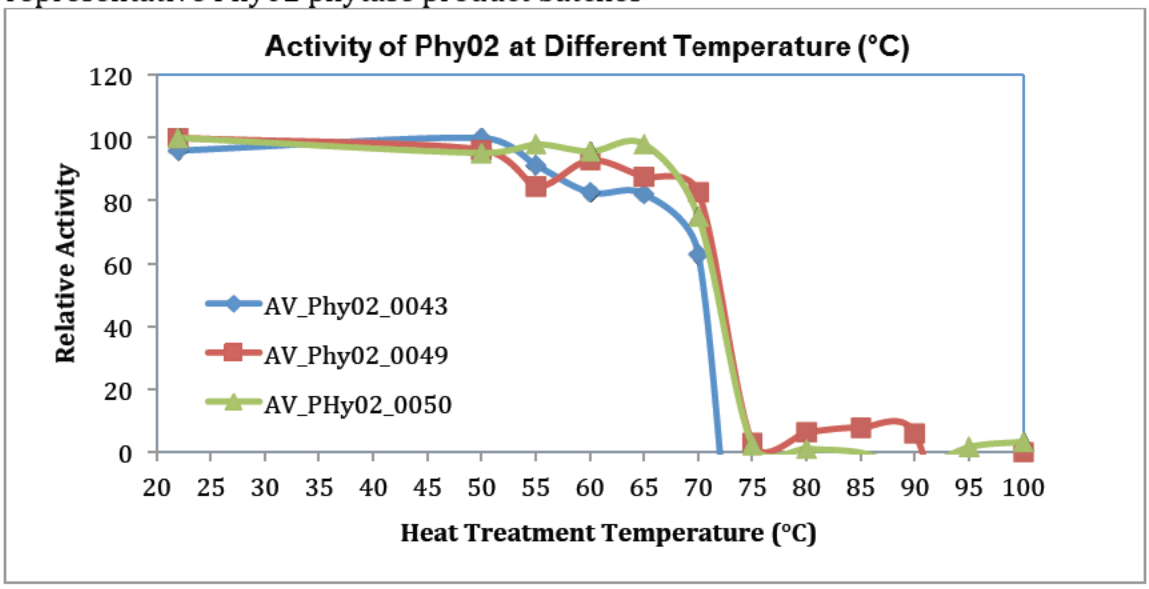
Figure 2. Relative phytase activity of protein extracts from three independent Phy02 phytase product batches and of a Phy02 phytase protein purified from the grain of Phy02 phytase producing maize over a range of pH.



8.7.2.5 Thermal optimum of Phy02 phytase

The phytase activity in protein extracts from three independent Phy02 phytase product batches (Lot numbers AV_PHY02_0043, AV_PHY02_0049, and AV_PHY02_0050) was determined over a range of temperatures to determine the temperature optimum for phytase activity. Protein extracts prepared from flour from each of the Phy02 phytase products were diluted 10-fold using phytase assay buffer. 400 µl of diluted protein was placed in a Thermo-Shaker MSC-100 at temperatures of 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100°C. Heat treatment at each temperature was carried out for 5 min with shaking at 1000 rpm. The temperature of sample wells was checked using a Dual Channel Digital Thermometer (Fisher Scientific). After heat treatment, the protein was further diluted in phytase assay buffer prior to analysis for phytase activity. The relative phytase activity of the Phy02 phytase in each of the Phy02 product batch extracts at the different temperatures is presented in Figure 3. Phy02 demonstrated 100% activity at temperatures from 50 to 55°C relative to its optimal temperature for activity of 22°C. Activity decreased only slightly at 60°C and 65°C and at 70°C the activity in the 3 samples tested ranged from 63 to 85%. At temperatures above 70°C the phytase activity of all samples was reduced drastically and at 75°C none retained significant phytase activity.

Figure 3. Relative phytase activities at different temperatures of three representative Phy02 phytase product batches



8.7.2.6 Enzymatic side activities of Phy02 phytase

Protein extracts from grain derived from Phy02 phytase product batch AV_Phy02_0049 and from conventional maize grain not engineered to produce the Phy02 phytase were tested for the presence of other significant enzymatic activities. The enzymatic activities that were tested included protease, α-amylase, xylanase, cellulase, and glucanase. The detectible enzymatic activities of the Phy02 and non-Phy02 producing grain were compared for each enzyme tested. The results presented in Table 3 show that in general there were low levels of activity for each of the enzymes tested but there were no differences between the activities present in the Phy02 and non-Phy02 phytase producing grains. The presence of low levels of endogenous enzymatic activity for these enzymes in normal maize grain is expected and therefore, the fact that there was not a significant difference in the activities of these enzymes in Phy02 producing and nonproducing grain indicates that the Phy02 phytase does not demonstrate significant levels of activity for the enzyme activities tested.

Table 3. Enzymatic side activities in protein extracts of Phy02 producing (Phy02) and Phy02 nonproducing grain (Control). In each case the activity values shown are standard activity units of the enzyme and are the average of three determinations. Control reactions with each enzyme that included its typical substrate were run to ensure that the enzyme and the reaction were functioning.

Enzyme	Phy02		Control	
	Activity	Std Dev	Activity	Std Dev
Amylase	0.014	0.007	0.026	0.010
Xylanase	0.025	0.037	0.120	0.002
Cellulase	0.041	0.037	0.015	0.011
Glucanase	0.052	0.003	0.017	0.000
Protease	0.025	0.023	0.039	0.008

8.7.2.7 References

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14 August 2018

RE: Supplemental information to Agrivida Inc.'s GRAS notice for the use of GraINzyme® Phytase in swine feed

Dear Dr. Tang,

This letter contains supplementary information to Agrivida's recent GRAS notice for use of GraINzyme® phytase in swine feed that was submitted to CVM and dated 14 June 2018. In a teleconference with CVM and Agrivida personnel on 24 July 2018, CVM suggested additional information that would support Agrivida's conclusion that its GraINzyme® phytase product is GRAS for use in swine feed. Based on the recommendations of CVM during this teleconference, we are submitting this letter to provide the supplemental information that further supports Agrivida's conclusion of the GRAS status of its GraINzyme® phytase product for swine feed. It is Agrivida's understanding that with the addition of this supplemental information the GRAS notice for swine is complete, includes all required sections, has been properly formatted, and the information enclosed supports our determination that GraINzyme® phytase satisfies the criteria necessary to be Generally Regarded as Safe.

The following information is provided to address the suggestions of CVM for additional supplementary information to support Agrivida's conclusion of the GRAS status of GraINzyme® phytase for use in swine feed. Information within this document that Agrivida Inc. considers to be Confidential Business Information is shaded in grey (eg., CBI) If you have any questions related to this information, please contact me.

Jim Ligon
VP, Regulatory Affairs
Agrivida, Inc.

Supplementary Information Supporting Agrivida Inc.'s GRAS Notice for the Use of GraINzyme® Phytase Phy02 in Swine Feed

1. Verification that Quantum Phytase is also known as Nov9X, as described in Agrivida's GRAS notification

Several employees at Agrivida previously worked at Syngenta on the Quantum/ Nov9X program and know that Nov9X was the original name given to the mutant *Escherichia coli*-derived phytase enzyme, which later was given the tradename Quantum. Current Agrivida employees, who formerly worked at Syngenta and know this include (b) (6), and (b) (6). Personal communications between Agrivida and Syngenta have also verified that the Nov9X phytase is also called Quantum and the two names refer to the same enzyme. These personal communications were between (b) (6) (Syngenta Biotechnology), (b) (6) (formerly employed at Syngenta), and (b) (6) (formerly employed at Syngenta) and members of the Agrivida team.

In Agrivida's GRAS notification it is stated that Nov9X and Quantum refer to the same phytase enzyme. In addition, the fact that Nov9X is Quantum is stated in several publicly available technical and literature resources including:

- "GraINzyme® Phytase. A phytase feed enzyme produced by *Zea mays* expressing a phytase gene derived from *Escherichia coli* K12." James M. Ligon. Summary of Data Supporting a Notification of GRAS Status, filed with FDA CVM, May 6, 2016.
    - This reference is publically available at: https://www.fda.gov/downloads/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/UCM581398.pdf
    - Page 8 states:

      "The NOV9X phytase is the active phytase in the commercial phytase product named Quantum that is produced by the yeast *Pichia pastoris* and that has been approved by FDA Center for Veterinary Medicine (CVM) for inclusion in animal diets since 2008."
- "Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from the European Commission on the safety and efficacy of the product Quantum Phytase 5000 L and Quantum Phytase 2500 D (6-phytase) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening and piglets (weaned)." *The EFSA Journal* (2008) 627:1-27.
    - This reference is publically available at: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2008.627
    - Page 2 states:

      "This additive is produced by fermentation of yeast *Pichia pastoris* sNOV9Xpp27, which is genetically modified to contain a synthetic gene encoding a thermotolerant 6-phytase."

      In the above, note the inclusion of "NOV9X" in the *P. pastoris* strain name.
    - Page 7 states:

"A synthetic gene encoding 6-phytase (NOV9X) was introduced into the recipient organism." and,
"A synthetic gene encoding the NOV9X phytase was cloned into *P. pastoris* expression vector pPIC9 to produce a fusion protein in which the *Saccharomyces cerevisiae* a-mating factor pre-pro-peptide secretion signal is in frame with the N-terminus of the 6-phytase."

These passages describe the gene and associated protein that was used to make the Quantum product and thereby disclose that indeed the commercial product, Quantum, contains the Nov9X phytase.

- "Biotechnology in the development of improved phytases." Robert Speight. 27th Annual Australian Poultry Science Symposium, 14 – 17, February 2016.
  - This reference is publically available at: http://eprints.qut.edu.au/94164/1/Speight%20APSS%20Final.pdf
  - Page 7 states:
    "These were then combined to produce a protein termed Phy9X that is also referred to as NOV9X in the patent literature (Lanahan et al. 2006) and Quantum phytase registration documents (EFSA, 2008)."
  - This reference was also published as:
    - Zootecnica International, October, 2017. "Biotechnology in the development of improved phytases." R. E. Speight, QUT. Page 43.
    - This second publication of this reference is publically available at: https://zootecnicainternational.com/wp-content/uploads/2017/11/Zootecnica-International-English-10-October.pdf

- "Recombinant Thermotolerant Phytase Produced in E. coli." A.S. Axambayeva, A.V. Shustov. CBU International Conference on Innovation, Technology Transfer and Education, 25 – 27 March, September 2015.
  - This reference is publically available at: https://www.researchgate.net/publication/283851690_RECOMBINANT_THERMOTOLERANT_PHYTASE_PRODUCED_IN_ECOLI (DOI: http://dx.doi.org/10.12955/cbup.v3.631)
  - Page 417 states:
    "The Nov9X is a generic name of the enzyme available on the market under brands Quantum (Syngenta Animal Nutrition, USA) and Quantum Blue Phytase (AB Vista, Germany)."

The above mentioned personal communications and publications confirm that the Nov9X and Quantum phytases are identical to one another.

2. Variability of the enzyme kinetic parameters among phytase enzymes

Phytases comprise a class of enzymes produced by many divergent species, whose K_m values span at least four orders of magnitude, including from 7.2 μM (*Lilium longiflorum*) to 2400 μM (soybean) (Rao, *et al.*, 2009). K_{cat} values are similarly as variable, also spanning four orders of magnitude, including from 2.6 s^{-1} (*A. niger* APase) to 1274 s^{-1} (*E. coli*) (Lei, *et al.*, 2013; Menezes-Blackburn, *et*

al., 2015). Most commercial phytases are histidine acid phosphatases, and all share a common active site sequence motif (Arg-His-Gly-X-Arg-X-Pro) (Oh, *et al.*, 2004). Despite their divergent origins, commercially available phytases have a narrower range of kinetic parameters as described in Table 1 (Menezes-Blackburn, *et al.*, 2015; Lei *et al.*, 2013).

As shown in Table 1, all commercial phytase enzymes have K_m and K_{cat} values that span two orders of magnitude. The reported K_m values span a 12.5X range between Natuphos and Ronozyme HiPhos. Even within *E. coli* phytases, the variability in K_m is 4X between Optiphos and Phyzyme. The variability in K_{cat} values is similar, ranging 7.5X between Natuphos and Quantum Blue. Within *E. coli* phytases the variability is smaller, spanning 1.5X between OptiPhos and Quantum Blue. Despite these differences in kinetic parameters, as further discussed below, many of these enzymes perform very similarly in swine, as evaluated by bone ash and growth performance.

Table 1. Summary of Commercial Phytase Kinetic Parameters

Enzyme	Organism Source	K_m (μM)	k_{cat} (s^{-1})	Reference
Quantum	*E. coli*	257	1012	Menezes-Blackburn, et. al., 2015
Quantum Blue	*E. coli*	178	1274	Menezes-Blackburn, et. al., 2015
Phyzyme	*E. coli*	302	984	Menezes-Blackburn, et. al., 2015
OptiPhos	*E. coli*	74	840	Lei et. al., 2013
AxtraPHY	*Buttiauxella* sp.	311	768	Menezes-Blackburn, et. al., 2015
Ronozyme HiPhos	*Citrobacter braakii*	427	1061	Menezes-Blackburn, et. al., 2015
Ronozyme NP	*P. lycii*	98	824	Menezes-Blackburn, et. al., 2015
Natuphos	*A. niger*	142	170	Menezes-Blackburn, et. al., 2015
		34	170	Lei et. al., 2013
Finase P/L	*A. niger*	103	628	Lei et. al., 2013

The GraINzyme® Phy02, Quantum and Quantum Blue phytases were all derived from the native AppA phytase of *E.coli* by Gene Site Saturation mutagenesis (Garrett et al., 2004). The number of amino acid substitutions relative to the original AppA phytase in these phytases is 8 (Quantum), 16 (Phy02), and 17 (Quantum Blue). Therefore, the Phy02 phytase is intermediate in terms of amino acid substitutions relative to the Quantum and Quantum Blue phytases. Because the amino acid sequences of Phy02, Quantum, and Quantum Blue phytases are nearly identical, it would be expected that the enzymes would perform very similarly. Table 2 presents the measured K_m and K_{cat} values for Quantum, Quantum Blue, and Phy02 that were determined by Agrivida.

Table 2. Summary of Measured Phytase Kinetic Parameters

Enzyme	Organism Source	K_m (μM)	k_{cat} (s^{-1})
Quantum	*E. coli*	789	767
Phy02	*E. coli*	515	529
Quantum Blue	*E. coli*	494	261

In Table 1 and Table 2, the ratio of K_m values between Quantum and Quantum Blue is 1.4X and 1.6X, respectively, demonstrating relative agreement for the K_m values reported in Table 1 and measured in Table 2. Conversely, the K_{cat} values are less similar. While the values measured by Agrivida, Inc. that are presented in Table 2 are the same order of magnitude as the values reported by others in Table 1, the absolute value of these measurements are different. These differences are likely due to the fact that the kinetic values reported in the literature that are presented in Table 1 were mostly determined with impure enzyme preparations whereas those determined by Agrivida, Inc. and presented in Table 2 were with enzyme preparations that were more highly purified. In addition, these differences are the result of different experimental conditions used by the different laboratories involved. However, because the measured Phy02 kinetic parameters were between those measured for Quantum and Quantum Blue (Table 2), and given the high level of variability across commercial phytases, it is reasonable to expect that Phy02 would perform within the variability of Quantum and Quantum Blue and well within the performance of all commercial phytases. Given the measured similarity in kinetic constants, and the near identity of amino acid sequences, it is reasonable to expect Phy02 to perform in a manner that is substantially equivalent to Quantum or Quantum Blue. This conclusion is further substantiated in the performance similarity we've observed in our studies and studies conducted in the literature, as described below.

Quantum, Quantum Blue, and Phy02 phytases all share the identical, intact, active site in the enzyme and based on our *in vitro* and *in vivo* experiments, all have similar levels of activity. Given that the active site is maintained, this data strongly suggests that amino acid changes outside of the active site are well tolerated by the enzyme and do not lead to inactivation or other unanticipated effects. Indeed, Natuphos (*A. niger* phytase), also has the identically maintained active site and possesses an even lower level of sequence identity (<25% amino acid identity relative to the AppA phytase from *E. coli*), with many changes throughout the enzyme, and still functions similarly in *in vitro* studies and performs very similarly in feeding studies, further suggesting that mutations outside the active site do not interfere with enzyme function or *in vivo* performance.

In summary, the variation in enzyme kinetic parameters measured for Quantum, Quantum Blue and Phy02 phytases is well within the kinetic variation that has been measured for other commercially used phytases (Table 1). Indeed, the variation among these *E. coli* AppA derived phytases is much lower than for the larger group of phytases listed in Table 1 (Table 2). The similarity in the kinetic characteristics among Quantum, Quantum Blue, and Phy02 and the fact that the K_m and K_{cat} values

for Phy02 are between those of Quantum and Quantum Blue provide further support that these three enzymes are substantially equivalent.

3. Published literature demonstrates the functionality of the commercial phytases Quantum and Quantum Blue

Phytase has been used in monogastric diets since 1991, and its efficacy and safe use in swine are well established (Selle and Ravindran, 2008). With regard to the bioefficacy of phytase in swine feed, phytases are in general supplemented according to their activity determined at standard conditions (pH 5.5, 37°C, 5 mmol/L sodium phytate) (Menezes-Blackburn, *et al.*, 2015), and many commercial phytases perform similarly when dosed at the same level in swine feed. Given the high similarity of the amino acid sequences, measured kinetic parameters, pH optima, and thermal tolerance between the Phy02 phytase and other commercial phytases and publicly studied phytases, the results from swine trials that are presented in Agrivida's GRAS notice for use in swine support a conclusion that Phy02 is substantially equivalent to other known phytases and satisfies the criteria to be Generally Recognized as Safe.

For example, Venum *et. al.* (2006) concluded that Quantum and Ronozyme were equivalent at 500 FTU/kg in a low-P diet based on growth performance, bone strength and ash weight, and the apparent absorption of P, Ca, Mg, N, GE, and DM. They stated "Pigs fed our low-P diets containing 500 U of *E. coli* (Quantum) or *P. lycii* (Ronozyme) phytase/kg had growth performance and bone strength values similar to those reported in other experiments for weanling pigs fed low-P diets containing 500 U of either *P. lycii* or an *E. coli*-derived phytase". They also referenced results using other similar phytases, stating "Different *E. coli*-derived phytase products were also efficacious at concentrations from 250 to 1,200 U/kg of low-P diet fed to weanling swine, with increased growth performance, plasma inorganic P concentrations, and bone strength, and reduced plasma alkaline phosphatase activities". Finally, similar to our results, Venum *et. al.* (2006) observed dose-dependent linear improvements in bone characteristics when using Quantum: "There were linear and quadratic increases ($P < 0.001$) in metacarpal breaking strength, fresh and fat-free dry bone weight, bone ash weight, and most bone length and width measurements with increasing dietary concentration of *E. coli* phytase."

These results were further substantiated in other publications, including Guggenbuhl *et. al.* (2007), that compared Quantum, Ronozyme, and Natuphos. They concluded that "The effect of including 500 u/kg of *E. coli* (Quantum) phytase on P digestibility was similar to those induced by the *A. niger* (Natuphos) and *P. lycii* (Ronozyme) phytases at their recommended levels of 500 and 750 U/kg, respectively." Consistent with Venum *et. al.* (2006) and the results developed by Agrivida, Inc., they further stated, "The effect of the *E. coli* (Quantum) phytase at 500 U/kg appeared to be very similar to 500 u/kg of *A. niger* (Natuphos) and 750 U/kg of *P. lycii* (Ronozyme)." In another report on the functionality of Quantum phytase, Beaulieu *et al.* (2007) carried out a study involving "four levels (250, 500, 1000 and 2000 FTU kg–1) of an experimental *E.coli*-derived phytase (2900 FTU g–1,

Quantum)" with weanling and growing pigs, and found that "the percent bone ash was similar between genders and tended to increase ($P < 0.10$) in response to phytase (linearly)."

The functionality of the commercial phytase Quantum was also reported in other publications, including the following:

- "Comparative Effects of Three Phytases on Phosphorus and Calcium Digestibility in the Growing Pig," P. Guggenbuhl, A. Pinon Quintana, C.S. Nunes. Livestock Science, 2007, 109:258-260. https://doi.org/10.1016/j.livsci.2007.01.109.

    - Page 258 states:
      "The inclusion levels were 250 (Q1) and 500 U/kg (Q2) for an *E. coli* phytase (Quantum), 500 U/kg (Nat) for *A. niger* (Natuphos) and 750 U/kg (Ron) for *P. lycii* (Ronozyme P)."
    - Page 258 states:
      "P digestibility was improved by 13.8, 18.6, 18.3, and 17.9 percentage units by Q1 (Quantum 250 U/kg), Q2 (Quantum 500 U/kg), Nat (Natuphos 500 U/kg), and Ron (Ronozyme 750 U/kg), respectively."
    - Page 259 states:
      "In all phytase supplemented diets the faecal P was significantly reduced by 19% with Q1, by 21% with Ron, by 23% with Nat and by 24% with Q2 (Table 2)."

In addition to the above publications that support the functionality of Quantum phytase in swine, the following publications provide support for the functionality of Quantum Blue in swine:

- "Performance and bone characteristics of growing pigs fed diets marginally deficient in available phosphorus and a novel microbial phytase." T. T. Santos , C. L. Walk , P. Wilcock , G. Cordero , and J. Chewning. 2014. Can. J. Animal Sci. 94: 493497. http://www.nrcresearchpress.com/doi/full/10.4141/cjas2013-190 - .W3GGjthKit8h

    - Page 494 states:
      "The phytase used was a novel, intrinsically thermostable, Escherichia coli 6-phytase expressed in Trichoderma reesei and contained a declared activity of 5000 FTU g1 (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK)."
    - Page 496 states:
      "Phytase supplementation at 500 FTU kg1 improved bone ash comparable to the PC indicating this novel phytase was efficacious at hydrolysing phytate and providing a source of avP and Ca."

- "Effect of dietary phytase level on intestinal phytate degradation and bone mineralization in growing pigs." I. Kühn, M. Schollenberger, and K. Männer. J. Anim. Sci. 2016.94:264–267. https://academic.oup.com/jas/article-abstract/94/suppl_3/264/4731418.

    - Page 265 states:

"Positive control (PC) diets met current nutrient requirements for fattening pigs whereas negative control (NC) diets were reduced in minerals (Ca, P, and Na) and fed without or with phytase at 500 (NC500) and 2,000 (NC2000) phytase units (FTU)/kg feed (modified, Escherichia coli–derived 6-phytase; Quantum Blue; AB Vista, Marlborough, UK)."

- Page 266 states:
  "Phytase application improved all bone minerals except Se to the level of those analyzed in PC pigs or above (Fe and Zn; $P < 0.05$)."
- Page 266 states:
  "The improved bone mineralization demonstrates that phytase application is a suitable tool to support bone mineralization in pigs, especially when added at higher levels."

- "Improved mineral utilisation in grower-finisher pigs fed a diet supplemented with graded amounts of two phytases." P. Guggenbuhl, E. Perez Calvo and F. Fru. (2015) *Animal Production Science* 55(12): 1560-1560. https://doi.org/10.1071/ANv55n12Ab069

  - Page 1560 states:
    "The aim of this study was to evaluate the effects on P and calcium (Ca) utilisation, plasma indices and bone strength of a *C. braakii*-(Ronozyme HiPhos) and an *E. coli*-(Quantum Blue) derived 6-phytase at high dosages in grower-finisher pigs."

  - Page 1560 states:
    "Compared to the MC treatment group (neg. control), bone ash and breaking force were improved ($P < 0.05$) in all phytase groups."

- "Scientific opinion on the efficacy and safety of Quantum® Blue (6-phytase) as a feed additive for poultry (except laying hens) and pigs." EFSA Journal 2013, 11(10):3364. https://www.efsa.europa.eu/en/efsajournal/pub/3364.

  - Page 22 states:
    "At the end of the experiment, 12 piglets from each of the nc0, nc500, nc1000 [nc = neg. control diet + units of Quantum Blue] and pc0 treatment groups were killed. Digesta samples from the ileum were collected and metacarpal bones III and IV from the left front foot and metatarsal bones III and IV from left hind foot were collected. Digesta and faecal samples were analysed for dry matter, organic matter, ash, phosphorus and calcium. In the ileal samples, phytate digestibility was also measured. Bones were subject to strength determinations and ash, phosphorus and calcium analysis."
  - Page 23 states:
    "Ileal digestibility of phosphorus (45, 60, 60 and 52 % in the nc0, nc500, nc1000 and pc0 group, respectively), bone ash (33, 35, 37, 36 % in the nc0, nc500, nc1000 and pc0 group, respectively) and phosphorus in bones (5.6, 6.0, 6.3 and 6.1 % in the nc0, nc500, nc1000 and pc0 group, respectively) were significantly higher in the nc500 and nc1000 groups than in the nc0 group."
  - Page 23 states:

> "The results of the three trials showed that the supplementation of the feed with Quantum® Blue phytase at the minimum recommended dose of 250 FTU/kg resulted in a significant increase in phosphorus digestibility in two trials (trial 1 and 2). A dose of 500 FTU/kg diet resulted in a significant increase in phosphorus digestibility and bone mineralisation in another trial."

The above described publications describe studies that clearly demonstrate the functionality of both the Quantum and Quantum Blue phytases in terms of increasing bone strength and/or bone ash and minerals. The functionality of these phytases as reported is consistent with the fact that these phytases have been used effectively in the diets of poultry and swine for a cumulative period of over 10 years. Taken together, these points demonstrate that the Quantum and Quantum Blue phytases are generally recognized to be functional and effective in the diets of poultry and swine.

4. Statement concerning information considered by Agrivida, Inc. to be Confidential Business Information

In an email dated 26 July 2018, Dr. Lei Tang from CVM noted that Part 6 of the swine GRAS notice submitted by Agrivida that identified information considered by Agrivida to be Confidential Business Information (CBI) there was no explanation of how qualified experts could reach a conclusion of the GRAS status without having access to the information that is identified as CBI. Primarily, the information in the current swine GRAS notice that Agrivida, Inc. considers to be confidential consists of the identity of the two commercial phytase enzymes to which Agrivida, Inc. has compared the GralNzyme® Phy02 phytase for the purposes of establishing substantial equivalence with these phytase enzymes. Qualified experts that examine Agrivida's GRAS notice without the knowledge of the identities of the two commercial phytases would be able to determine that based on the information presented the GralNzyme® Phy02 phytase is substantially equivalent to two currently used commercial phytases that are well known to be functional and safe when used in swine feed. This information is sufficient for experts to conclude that if the GralNzyme® Phy02 phytase is substantially equivalent to the two safe and functional commercial phytases, then it is equally as safe and functional as these commercial enzymes. The actual identities of the two commercial enzymes, e.g. trade names, are not required for qualified experts to reach this conclusion. Based on this, Agrivida, Inc. believes that the information related to the identities of the two commercial enzymes that is considered by Agrivida, Inc. to be CBI is not necessary for qualified experts to conclude that the GralNzyme® Phy02 phytase is substantially equivalent to two commercial phytases that are safe and functional in swine and therefore, the GralNzyme® Phy02 phytase is equally as safe and functional when included in swine feed.

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T-5

From: jim.ligon@agrivida.com
To: Tang, Lei
Cc: Michael Raab R. Ph.D.; Phil Lessard
Subject: Re: GRAS notice AGRN #27
Date: Friday, March 01, 2019 10:00:42 AM
Attachments: GRASN Swine_Phy02_Amendment_28Feb19.pdf
Broomhead_JAS 2018_GZ Phy functionality swine.pdf

Dear Dr. Tang,

As discussed in our meeting with you earlier this week, Agrivida, Inc. is submitting an amendment to the GRAS Notification (AGRN #27) submitted to CVM for the use of GraINzyme® Phytase in swine feed. Agrivida inadvertently included the incorrect experimental incubation temperature for determining Phy02 phytase activity in feed mixtures that is described in Appendix 2 (p. 94 and 95) of the original notice. The correct temperature of the assay is 37°C, not 65°C as stated in the original Notification. In addition, while reviewing the information presented in the final paragraph on page 94 that continues to page 95 of the original Notification, Dr. Lessard noticed that some of the volumes of reagents, etc. are listed in mL (milliliter) instead of µL (microliter). We believe that in converting the text to a standard font that the µL cited in the document were converted to mL. In order to correct these errors, I am submitting to you as an amendment to the notification a corrected version of pages 94 and 95 in the attached file. In this version of pages 94 and 95 the mL have been changed to µL where appropriate and the assay temperature is corrected to 37°C.

In addition, Agrivida has recently published a study that demonstrates the functionality of the GraINzyme® Phytase in swine. It has been published in the Journal of Animal Science and I have attached a copy of it for your information (Broomhead *et al.*, 2018). This paper reports that the inclusion of the GraINzyme® Phytase in swine feed at a range of doses results in increased bone ash and bone breaking strength compared to negative controls, as well as similar improvement in animal performance characteristics such as body weight gain and feed conversion ratios. The results reported are consistent with, and support, the claims of functionality of GraINzyme® Phytase in the GRAS Notification in swine feed.

If you have further questions related to Agrivida's GRAS Notification for the use of GraINzyme® Phytase in swine feed, please feel free to contact me

Sincerely,

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VP, Regulatory Affairs and Stewardship
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A phytase feed enzyme produced by *Zea mays* expressing a phytase gene derived from *Escherichia coli* K12

AMENDMENT to a NOTIFICATION of GRAS STATUS for USE in SWINE FEED

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March 1, 2019

This document contains corrections of the GRAS Notification that Agrivida, Inc. has submitted for the use of GraINzyme® Phytase in the feed of swine. It contains corrected versions of pages 94 and 95 of the original notification.

8.2 Appendix 2

Phytase activities measured in the feeds used in the GraINzyme® Phytase tolerance study and in swine performance trials.

The protocol used to determine the phytase activity in Phy02 phytase product material for all results presented in this document is a modification of the standard method for the determination of phytase activity in feed (AOAC 2000.12). The standard protocol for the determination of phytase activity is appropriate for feed materials containing 200 – 400 FTU/kg feed and since the Phy02 product material has over 10 times more phytase activity than this range, the assay was modified to account for this difference. Prior to analysis, the product material is milled so that the particle sizes are less than or equal to 0.5 mm. 20 g of milled material is shaken for 1 hour at room temperature in 200 mL of 25 mM sodium borate, pH 10 buffer, 0.01% Tween 20. A 2 mL sample is taken and centrifuged at 12,000×g for 10 min. The product supernatants are diluted in phytase assay buffer (250 mM sodium acetate, pH 5.5, 1 mM calcium chloride, 0.01% Tween 20) so that the target absorbance at 415nm is between 0.3 and 1.1. To test protein extract activity, 75 µL of the diluted mixtures is dispensed into individual wells of a 2 mL 96-deep-well block. One hundred and fifty µL of freshly prepared phytic acid (9.1 mM dodecasodium salt from (b) (4) prepared in assay buffer) is added to each well. Negative controls, which serve to correct sample background absorbance, have no protein extract in the wells before addition of the stop solution. Plates are sealed and incubated for 60 min at 37°C. One hundred and fifty µL of stop solution (20 mM ammonium molybdate, 5 mM ammonium vanadate, 4% nitric acid) is added to each well, mixed thoroughly via pipetting, and allowed to incubate at room temperature for 10 minutes. Seventy-five µL of the diluted protein extract is dispensed into negative control wells and mixed. Plates are centrifuged at 3000×g for 10 minutes, and 100 µL of the clarified supernatants are transferred to the wells of a flat-bottom 96-well plate. Absorbance at 415 nm from each sample is compared to that of negative controls and potassium phosphate standards. A standard curve is prepared by mixing 50 µL of potassium phosphate standards (0-1.44 mM, prepared in assay buffer) with 100 µL of freshly prepared phytic acid, followed by 100 µL of stop solution.

The phytase activity in feed samples was measured using a modified version of the standard phytase protocol (AOAC 2000.12). After mixing of the diets, a 500g sample of each of the diets in the mash form was collected. Subsequently, the mash diets were pelleted in a California Pellet Mill at 37°C and a 500g sample of each of the diets after pelleting was collected. All feed samples were shipped to the Agrivida, Inc. laboratory in Medford, MA where the phytase activity of each sample was determined. The feed samples were milled in a knife mill and sieved with a 1mm screen. Two 20 g samples of each milled feed sample were extracted at room temperature with 100ml of prewarmed (37°C) extraction buffer (30 mM Sodium Carbonate/Bicarbonate pH 10.8). Each extract diluted 25- to 100-fold in assay buffer (250 mM sodium acetate, pH5.5, 1mM calcium chloride, 0.01% Tween 20)

and 75 µL of the diluted extracts or 75µl of buffer-only controls were dispensed into individual wells of a round-bottom 96-well plate. 150 µL of freshly prepared, prewarmed (37°C), phytic acid (9.1 mM dodecasodium salt from (b) (4) prepared in assay buffer) was added to each well. Plates were sealed and incubated for 60 min at 37°C. 150 µL of stop solution (20 mM ammonium molybdate, 5 mM ammonium vanadate, 4% nitric acid) was added to each well, mixed thoroughly via pipetting, and allowed to incubate at room temperature for 10 min. Plates were centrifuged at 3000×G for 10 minutes, and 100 µL of the clarified supernatants were transferred to the wells of a flat-bottom 96-well plate. Absorbance at 415 nm from each sample was compared to that of negative controls (buffer-only, no enzyme) and potassium phosphate standards. The standard curve is prepared by mixing 50 µl of potassium phosphate standards (0-1.44 mM, prepared in assay buffer) with 100 µL of freshly prepared phytic acid, followed by 100 µL of stop solution.

8.2.1 Tolerance of weaned piglets to GraINzyme® Phytase

Weaned piglets were fed a high dose of GraINzyme® Phytase (target of 60,000 FTU/kg feed) for 43 days. Ten samples of GraINzyme® Phytase treated feed from the pre-starter and starter diets were collected and the phytase activity determined. The average phytase activity in 10 samples of these feeds is reported in the table below.

Feed Type	Average (FTU/kg)	St Dev
Pre-starter feed	44,926	10,929
Starter feed	44,134	5,500

8.2.2 Study 1. Swine trial conducted at the (b) (4)

Feed samples (20g each) were collected in duplicate and extracted with 100 ml of buffer at room temperature. The phytase assays were conducted at 37 °C. The results from the three different feeds for each of the phases of the trial are presented below.

Treatment Group	Target Dose FTU/kg	Phytase Activity After Diet Preparation			
		FTU/kg	stdev	% Target Dose	CV
Pos. Control	0	ND	-	-	-
Neg. Control	0	ND	-	-	-
NC+ 500Phy02	500	405	119	81	0.29
NC+1000Phy02	1000	884	223	88	0.25
NC+2000Phy02	2000	1603	186	80	0.12
NC+4000Phy02	4000	3938	900	98	0.23