EFFECT OF HEAT STRESS ON THE EFFICACY OF A CARBOHYDRASE ADMIXTURE IN GROWING PIGS FED WHEAT-BASED DIETS

by

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Dedicated to my family

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LIST OF APPREVIATIONS

Abbreviation	Description
AA	Amino acid
ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
AID	Apparent ileal digestibility
BUN	Blood urea nitrogen
CCK	Cholecystokinin
CF	Crude fiber
CP	Crude protein
CRP	C – reactive protein
DF	Dietary fiber
DM	Dry matter
DMI	Dry matter intake
G: F	Gain to feed ration
GE	Gross energy
GI	Gastrointestinal
GLP-1	Glucagon like peptide – 1
HS	Heat stress
HSP	Heat shock protein
LEfSe	Linear discriminant analysis effect size
ME	Metabolizable energy
NDF	Neutral detergent fiber
NE	Net energy
NEFA	Non esterified fatty acid
NSP	Non starch polysaccharide
OTU	Operational taxonomic unit
PYY	Peptide tyrosine tyrosine
RS	Resistant starch
SBP	Sugar beet pulp
SCFA	Short chain fatty acid
SGLT1	Sodium dependent glucose transporter 1
SID	Standardized ileal digestibility
TAG	Triacyl glyceride
TDF	Total dietary fiber
TER	Transepithelial resistance
TLR	Toll like receptor
TN	Thermoneutral
VFA	Volatile fatty acid
WB	Wheat bran
WM	Wheat middling

ABSTRACT

Carbohydrases have been used to improve fiber utilization in monogastric animals. However, their effects on animal performance and nutrient digestibility have been inconsistent in pigs. The efficacy of carbohydrases has been suggested to depend on enzyme activity and fiber composition, but recent evidence suggests other factors like environmental conditions may play significant role. The effect of heat stress (HS) on the efficacy of a multienzyme carbohydrase blend in growing pigs was investigated. It was hypothesized that HS alters the efficacy of carbohydrases. Ninety-six growing pigs (gilts:barrows; 1:1) (initial BW of 20.15±0.18 kg) were randomly assigned to 6 treatments, with 8 replicates of 2 pigs replicate pen in a 3×2 factorial arrangement: three levels of carbohydrase (0, 1X or 2X) and two room temperature conditions (20^{o}C constant or cyclical 28^{o}C night time and 35^{o}C daytime). The 1X is the recommended commercial dose of the enzyme (50g/tonne) and provides 1250 visco-units endo-β-1,4-xylanase, 4600 units α-L-arabinofuranosidase and 860 visco-units endo-1,3(4)-β-glucanase per kg of feed. The 2X dose was 100g/tonne. Pigs were fed ad libitum for 28 days and 1 pig per pen was sacrificed at d28. Heat stress increased the respiratory rate and skin temperature (P<0.001). There was no enzyme × temperature effect on response criteria. Enzyme treatment quadratically increased BW d28 (P=0.025), ADG (P=0.022) and average daily feed intake (ADFI) (P=0.032) with 1X being the highest. Heat stress reduced the BW at d14 (P=0.002) and d28 (P=0.006), average daily gain (ADG) (P=0.005) and ADFI (P<0.001). However, there was a trend of increased gain: feed ratio (G/F) (P=0.093) in the HS pigs compared to the thermoneutral (TN). Heat stress increased apparent jejunal digestibility (AJD) of energy (P=0.039) and apparent ileal digestibility (AID) of calcium (P=0.007). ADFI was positively correlated to ADG (r=0.57; P<0.001) but negatively correlated to G/F (r=-0.42; P=0.003). Enzyme supplementation increased in vitro viscosity at 3, 4 and 5 hours

(P value). Heat stress reduced serum glucose concentration on d1 (P=0.0002) but increased serum non esterified fatty acid (NEFA) concentration (P=0.002). A similar trend to NEFA was observed in triacylglycride (TAG). In the jejunum, carbohydrases had trend for increased villi height (P=0.07) while HS reduced villi height (P=0.02). Heat stress increased the jejunal mRNA abundance of IL1β in the jejunum (P<0.001). There was a trend for a reduction in ileal MUC2 (P=0.092), occludin (P=0.091) due to HS, with the trend increasing in PEPT1 (P=0.064). There was no effect of HS on alpha diversity of fecal microbiome, but sampling day affected beta diversity. There was an increase in the abundance of pathogenic bacteria (like Clostridium) in the HS group. Heat stress did not alter the efficacy of carbohydrase but both carbohydrase and HS modulate pig performance independently.

Key words: growing pig, carbohydrases, heat stress, gut microbiome

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The world population is increasing at an exponential rate with a concurrent increase in the need for food. Animal protein is one of the food needs. The problem, however, is that the land available for agriculture is decreasing in area due to increasing housing demand. This means food production system must be efficient in utilizing inputs to attain optimal productivity. Feed cost is the highest contributor to the cost of production in animal industry with carbohydrate being the energy source contributing the highest proportion to animal feed (Makkar, 2016). The increasing demand for cereal grains, the major energy source in animal feed, for human consumption and industrial uses, has made it difficult to reduce animal cost. The relative availability and low cost of cereal coproducts has led to an increase in their use in pig diets to reduce cost (Noblet and Goff, 2001). However, the fiber component of these coproducts is higher than in whole grains (Stein and Shurson, 2009).

Increased dietary fiber content in swine diet reduces growth rate of pigs due to their inability to enzymatically digest these fiber components. Instead, they are fermented, resulting in energy loss due to heat and gas production (Bach Knudsen, 2001). In addition, increasing dietary fiber content also cause a decrease in digestibility of other nutrients and result in poor performance (Noblet and Goff, 2001). Effect of dietary fiber on digestibility of nutrients vary according to the source and properties of the fiber (Wenk, 2001). Various methods have been used to improve the utilization of nutrients in diets high in dietary fiber. Strategies like the inclusion of exogenous fiber degrading carbohydrases (e.g. xylanase, β-glucanase and arabinofuranosidase) have been reported to improve digestibility of energy and protein in pig (Zijlstra et al., 2010). This may increase production in a

cost-effective way. Carbohydrases hydrolyze fiber in the small intestine, releasing energy and other nutrients within the matrix of the fiber. The released nutrients can then be absorbed in the foregut before they are fermented by the microbes in the hindgut (Bedford, 1995). Despite this potential of exogenous carbohydrases, their effect in pigs has not been consistent (Adeola and Cowieson, 2011).

Global warming has increased the burden of heat stress on animals. Heat stress causes important economic loss in pig production due to its adverse effect on animal performance. Pigs respond to heat stress through various physiological adaptations such as reduction in metabolic heat production and increase in body heat dissipation. However, the impact of these heat stress induced physiological changes on the efficacy of carbohydrases in fiber rich diets is still unclear. Therefore, the objective of the study conducted for this thesis was to determine the effect of a carbohydrase admixture on growth performance, digestibility of nutrient, intestinal histomorphology, gene expression, serum markers of heat stress and gut microbial community structure in growing pigs kept under thermoneutral and heat stress conditions.

1.2 The gastrointestinal tract

1.2.1 Anatomy and physiology of the gastrointestinal tract

The gastrointestinal (GI) tract is a continuous tube which contains organs necessary for digestion of food and processing of waste. It is the largest surface of the body interacting with the external environment. It serves as a barrier for the passage of potentially dangerous materials but allows the absorption of nutrients from the intestinal lumen. Anatomically, monogastric animal's GI tract consists of mouth, pharynx, esophagus, small intestine (duodenum, jejunum and ileum), large

intestine (cecum, colon and rectum), anus and the accessory glands like pancreas which secrete enzymes necessary for digestion. The GI tract has unique structural features that enables it to serve the body, with each part of the tract having distinct features that makes them able to perform their role. The wall of the GI tract has four concentric layers namely mucosa, submucosa, muscularis externa and serosa (Gartner and Hiatt, 2012) which may vary in their thickness depending on the part of the GI tract.

The GI tract contains enterocyte cells which absorbs nutrient and secretory cells like goblet cells and Paneth cells which secretes mucin and antimicrobial peptides which protect the GI tract (Falk et al., 1994; Bry et al., 1994). The GI tract also has the Peyer's patch which is considered as the immune sensor of the intestine. It extends from the mucosa to the submucosa (Jung et al., 2010) and is composed of lymphoid tissues that identify and destroy pathogens (Parkin and Cohen, 2001). They also possess enteroendocrine cells which secrete hormones and enzymes that help in digesting nutrients (Roth et al., 1990). The enterocytes are the dominant cells that make up the mucosa layer. The absorptive capacity of the gut is optimized by its several folding. First, are circular folds (kerckring valve), within which are smaller villi (with crypt as the depression between individual villi) which has tiny microvilli covered by glycocalyx which form the brush boarder through which nutrients are absorbed. These epithelial cells are joined by four different types of junctions namely gap junction, desmosomes, adherent junction and tight junction which selective allow passage of materials and maintain the integrity of the gut.

1.2.2 Nutrient digestion and absorption

The most important function of the GI tract is the digestion and absorption of nutrients from ingested feed. The various enzymes secreted in the GI tract hydrolyze complex molecules into

simpler forms which can perfuse the intestinal cell into the circulation. Digestion of feed starts in the mouth with salivary amylase which begins starch degradation and lipase which begins lipid hydrolysis. The digesta then moves to the stomach where pepsin and hydrochloric acid are secreted to start protein degradation. The small intestine (duodenum, jejunum and ileum) is where most digestion, absorption of nutrient and endocrine secretion take place. Bile is released from the gall bladder into the duodenum and aid the activity of lipase in digesting lipid, pancreatic amylase continues starch degradation while trypsin, chymotrypsin, elastase and carboxypeptidases degrade peptides and completes the luminal phase of digestion. In the jejunum and ileum, the mucosa cells secrete maltase, sucrase, lactase and trehalase which completes the digestion of starch while aminopeptidase completes peptide digestion. This is the mucosal phase which completes nutrient digestion. In the pig, digested nutrients absorbed are transported into circulation either through the portal system or lymphatic capillary surrounded by blood vessels called lacteal in case of the chylomicrons which are products of lipid digestion. Undigested and unabsorbed materials then move to the large intestine (colon and rectum) where microbial fermentation occur to degrade them into mostly volatile fatty acid (VFA) which are reabsorbed. The remaining undigested materials will then be excreted.

1.3 Dietary fiber

1.3.1 Definition of dietary fiber

The nutritional composition of dietary fiber is highly variable (Van Soest 1978), making it difficult for a single definition to capture the entire concept. Mccance and Lawrence (1929) first referred to fiber as unavailable carbohydrates in food which are mostly cellulose. This definition was great limiting as it did not account for a large amount of substances. In 1969, Southgate further classified

unavailable carbohydrates to include those not broken down by enzymes secreted in the gut but can be utilized by microbes. Trowell (1972) then defined these indigestible food components which are mostly in the plant cell wall as dietary fiber. These includes polysaccharides like cellulose, lignin and hemicellulose. This definition is based on the physiological function of the materials. However, storage polysaccharides like guar gum and mucilage with similar structure and physiological effect were excluded by this definition because they are not part of plant cell wall. Therefore, Trowell et al. (1976) broadened the definition to cover these materials. This definition is more encompassing as it covers undigestible materials in both grain and coproducts. DeVries (2003) reported that the American Association of Cereal Chemists (AACC) after its international meeting in Seattle in 1998 finally concluded that dietary fiber will be defined as the remains of plant components or related carbohydrates that are consumable which are not digested and absorbed in the small intestine but are fermented in the large intestine. This definition will be adopted for dietary fiber in this thesis.

1.3.2 Structure of dietary fiber

Non-starch polysaccharides (NSP) are the most dominant component of dietary fiber. The structural composition of NSP determines its functional properties which often influence its physiological effect and application in animal feed (Guo et al., 2017). The structure of NSP is influenced by the types of monosaccharides making up the chain, homogeneity or heterogeneity of the monosaccharides, degree of branching, length of the chain, configuration (α or β), patterns of glycosidic linkage and nature of charge (Choct, 1997).

There are varieties of monosaccharide that can form the chain of an NSP, they include pyranose (6 carbon ring sugar) and furanoses (5 carbon ring sugar) linked by either α or β glycosidic bond.

These monosaccharides can exist homogenously or heterogeneously with different degree of branching. An example are the β-glucans which have linear glucose polymers liked with β-(1,3), (1,4)-glycosidic bonds (Caprita et al., 2010). A more complex one is arabinoxylan (Figure 1.1.) which has a D-xylopyranose backbone linked by β-1,4-glucosidic bond. However, it has multiple L-arabinofuranose, ferulate and D-methyl glucuronic acid branching (Correia et al., 2011). There are several other NSP with other structural configurations, for example, cellulose has thousands of glucose units linked with β-1,4-glucosidic bond. The glucose units are arranged parallel to each and are liked by hydrogen bond forming a large dense polymer (Choct, 1997) which can trap several nutrients. Structural characteristics of dietary fiber that reduces the association of its molecules like branching, presence of charged groups like phosphate, sulphate and carboxylate increases its solubility.

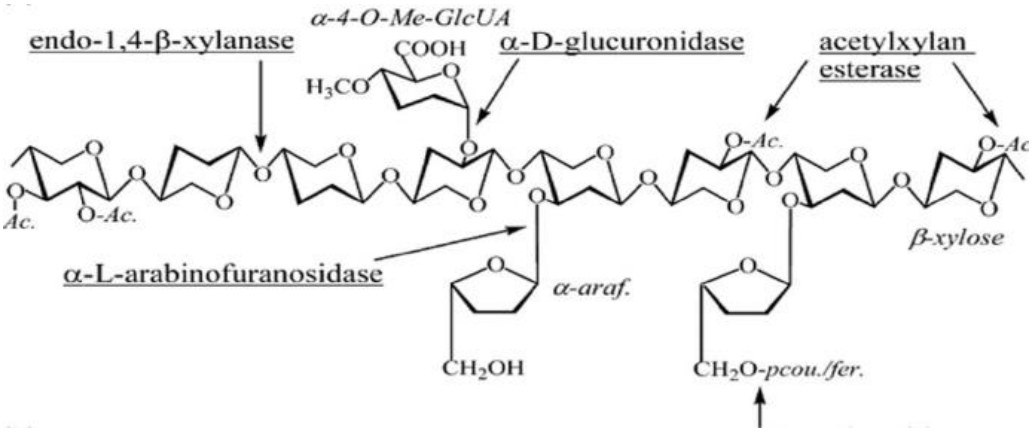

Figure 1.1. Structure of arabinoxylan (Collins et al 2005)

1.3.3 Classification of dietary fiber

Dietary fibers can be classified based of several features, including their solubility in water and chemical composition. However, due to the complexity of the structural components and physiological function of dietary fiber, it is difficult for any of the classifications to completely describe its wide range.

Based of solubility in water, dietary fiber is classified as:

i. Soluble fiber: they are readily dissolved in water. They include pectins, mucilages, gums and some hemicelluloses like β-glucans, mannans, glucomannans, arabinogalactan and oligofructans. Soluble fibers are highly fermentable and constitute most of the negative effect of impairment in performance and digestibility associated with dietary fiber in animal production.

ii. Insoluble fiber: they do not dissolve in water. They include cellulose, lignin and some hemicelluloses like xylan and arabinoxylan. Insoluble fibers are less fermentable and can induce the bulking effect associated with increasing digesta transit rate.

It is important to note that there is often an overlap between soluble and insoluble fiber in some of the NSP. For example, Marcotuli et al. (2016) in at a study of the structural variation in the content of arabinoxylan in wheat endosperm, reported that 37% of the arabinoxylan were water soluble with the remaining 63% insoluble. This suggests that both soluble and insoluble fraction can coexist in different NSP.

Based on chemical composition, the detergent fiber method can be used to classify NSP into neutral detergent fiber (NDF) which is obtained after digesting in neutral detergent and contains lignin, cellulose and hemicellulose and acid detergent fiber (ADF) which is obtained after

digestion in acid detergent and contains lignin and cellulose (Robertson and Horvath, 2001). The detergent fiber method is more repeatable and both NDF and ADF was used as a measure of fiber in this study (Figure 1.2.).

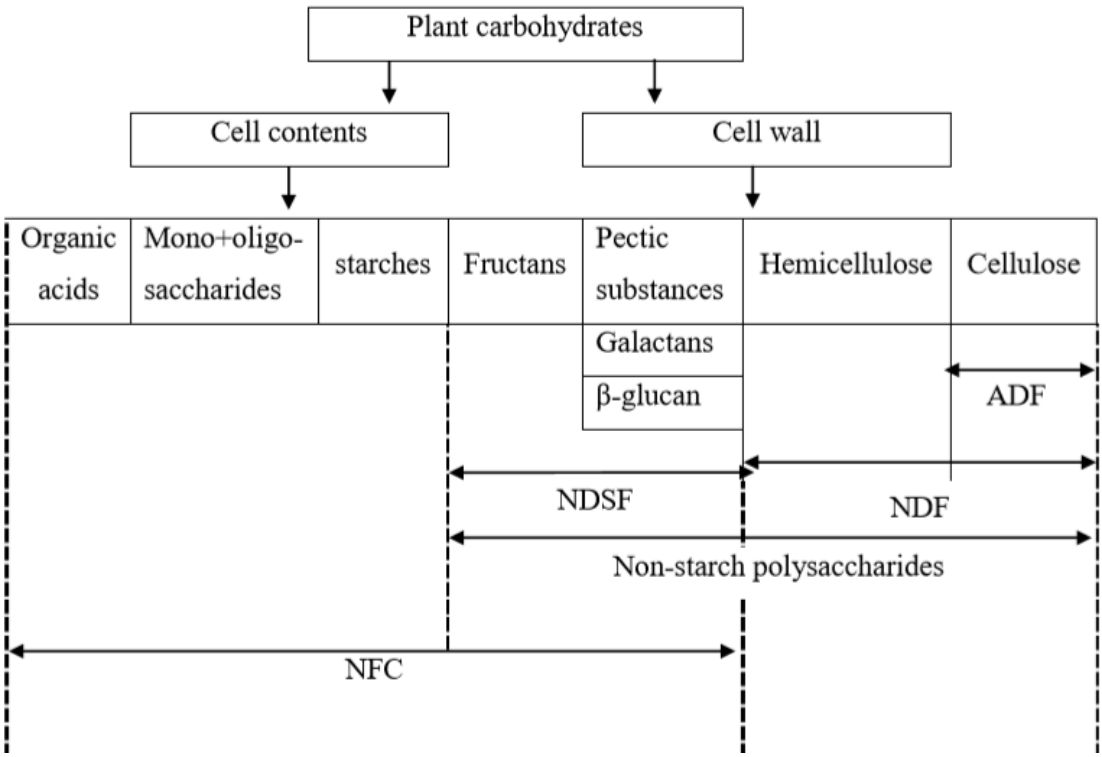

Figure 1.1. Classification of plant carbohydrates. ADF = acid detergent fiber, NDF = neutral detergent fiber, NDSF = neutral detergent-soluble fiber, NFC = non NDF carbohydrates. (NRC, 2012)

1.3.4 Dietary fiber in swine production

Cereal grain and legumes have been widely used to formulate diets that will meet the energy and protein need of pigs. The cell wall of these grains is high in NSP, serving as the first source of dietary fiber in pig diet. However, the increasing use of coproducts like rice bran, wheat bran, rice

husk, wheat middling and distiller dry grain with solubles (DDGS) to reduce cost has further increased the quantity of dietary fiber in pig diet. Arabinoxylans are the most predominant NSP in cereal grains and their coproducts (Jaworski et al., 2015) especially wheat (Figure 1.3.) with β-glucans also present in significant quantity. Wheat, wheat bran, wheat middling and rye (Table 1.1.) were the main source of dietary fiber in this study with arabinoxylan and β-glucan as focused NSP.

Table 1.1. Carbohydrate composition of the main fiber source used in growing pig diets in this thesis (DM basis, g/kg)

Item	Rye[2]	Wheat[2]	Wheat bran[2]	Wheat middlings[2]
Starch	613	618	169	168
Cellulose	15	13	64	67
NDF[1]	118	101	359	389
ADF[1]	40	32	122	67
NCP				
Soluble	42	19	38	12
Insoluble	94	62	243	227
Glucose	26	11	34	25
Arabinose	36	23	77	72
Mannose	5	2	5	3
Galactose	5	3	8	7
Xylose	61	36	144	116
Uronic acids	4	2	15	15
Total NSP	152	95	345	307
Lignin	21	18	69	73
Dietary fiber	174	112	414	381

ADF = acid detergent fiber; NDF = neutral detergent fiber; NCP = non-cellulosic polysaccharide; NSP = non-starch polysaccharides; total NSP = cellulose + NCP; dietary fiber = total NSP + lignin.
[1]According to NRC (2012)
[2]According to Jaworski et al. (2015)

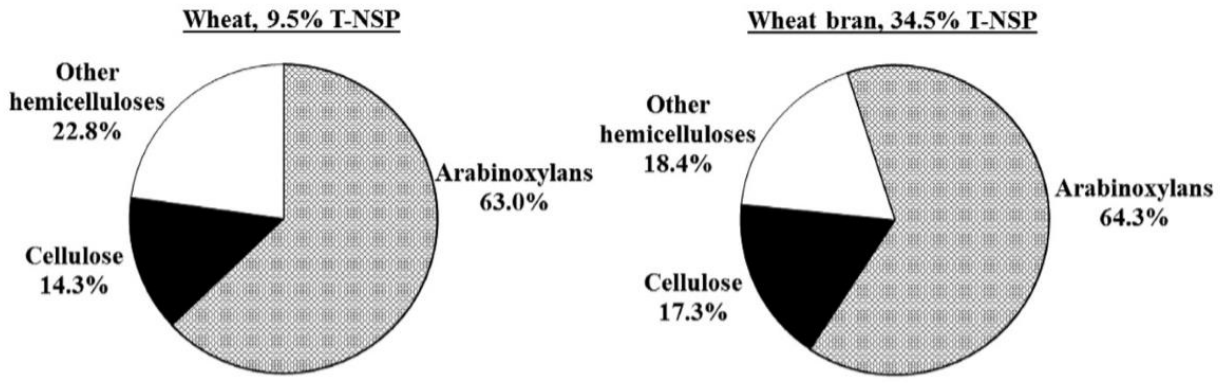

Figure 1.3. Composition of Non-starch polysaccharide in wheat and wheat bran as a proportion of total non-starch polysaccharides (T-NSP). (Jaworski et al. 2015)

1.3.5 Dietary fiber and growth performance in pigs

The general effect of dietary fiber in pig diet is decreased growth rate and body weight mostly because dietary fiber contains lower amount of energy compared to other carbohydrates, protein and lipid. Dietary fiber also increased endogenous loss and can also elicit a gut filling effect due to increased bulk which consequently lead to reduction in feed intake. Agyekum et al. (2014) reported a linear decrease in final body weight and average daily gain (ADG) in growing pigs fed graded level of wheat distiller dried grain with solubles (DDGS). Similarly, Avelar et al. (2010) showed a reduction in body weight gain, average daily feed intake (ADFI) and efficiency in weaned pigs fed wheat DDGS. However, Kerr et al. (2015) and Dunmire et al. (2018) reported that there was no effect of dietary fiber on ADG and ADFI in finishing pigs fed DDGS. The lack of negative effect of dietary fiber in finishing pigs could be due to increased gut and fermentation capacity.

These results suggest that the adverse effect of dietary fiber on body weight, ADF and ADFI may be limited to the growing phase, as the pigs develop enough gut capacity to cope during the

finishing phase. The use of different types of fiber may also be a cause of some variations in the response seen in different experiments. This is because different fiber types will induce different responses. Therefore, different methods of classifying dietary fiber should not be used exactly the same way when evaluating the effect of high dietary fiber (Agyekum and Nyachoti, 2017). Some of the contradictions between studies could also be explained by the difference in nutrient composition of the fiber used. Differences in amount of nutrients may result in different outcomes despite similar levels of fiber in the diets. Hence, it is important to use the same method to measure the amount of dietary fiber in diets when comparing the effect of different fiber type. Overall, the effect of dietary fiber on swine production appears to be reduction in body weight and ADG which appears to be associated with reduced caloric intake through reduced feed intake.

1.3.6 Dietary fiber and nutrient digestibility in pigs

Inclusion of dietary fiber in animal diet has been associated with reduction in the amount of energy in the diet. Classically, the presence of dietary fiber in animal feed is postulated to result in reduced nutrient uptake (Brownlee, 2011). There are several mechanisms proposed for this reduction in digestibility. However, like many other effects of fiber, its effect on digestibility will vary based on the physiochemical characteristics of the fiber. Diets high in NSP trap nutrients in their matrix hindering accessibility by digestive enzymes for their hydrolysis. Soluble fiber has been reported to increase the viscosity of digesta in the gut thereby reducing its transit time (Wenk, 2001). This increased viscosity is often associated with increase in the secretion of digestive enzyme which is metabolically demanding. This coupled with the potential limitation in the rate of mixing of digestive enzyme with substrate and the reduction in absorption of nutrient associated with increased viscosity often lead to a reduction in digestibility.

Owusu-Asiedu et al. (2006) reported that purified soluble and insoluble fiber from guar gum and cellulose increase the viscosity and retention time of digesta while they reduce passage rate and digestibility of nutrient. Wenk et al. (2001) stated that insoluble fiber reduces the transit time of digesta through the gut, which reduces the time available for digestive enzyme to hydrolyze feed component consequently reducing digestibility. Insoluble fiber from wheat bran was shown by Wilfart et al. (2007) to reduce the mean retention time in both the small and large intestine with a resulting reduction in nutrient digestibility. Result of reduced nutrient digestibility by fibrous diets in pigs have also been shown to be due to increase in endogenous loss of nutrient (Souffrant, 2001) through the sloughing of the epithelial layer and increased mucin production. Overall, the effect of dietary fiber (either soluble or insoluble) in animal diets is reduction in digestibility of nutrients through different mechanisms like directly trapping nutrients in its matrix or indirectly by interfering with accessibility of digestive enzymes to dietary components, reducing digesta transit time or impairing the absorptive layer of the intestinal wall.

1.3.7 Dietary fiber and physiology of the gastrointestinal tract

The physiology of the GI tract is affected by the nature and type of materials passing through it. The weight, volume and capacity of the GI tract has been shown to increase with increasing dietary fiber (Southgate, 1990). Fiber inclusion in diets has also been associated with a decrease in villi height, increase in crypt depth and thickness of the intestinal muscle layer often accompanied by impairment of microvilli of the brush border. The increase in muscle size was due to hypertrophy rather than hyperplasia. Several nerves innervate the cell of the GI tract and control intestinal motility in tandem with various hormones secreted by the gut. This underlines why the changes in gut morphology associated with dietary fiber are important for intestinal motility.

Parasympathetic vagal nerves innervate the whole GI tract except lower part of the colon which is controlled by sacral nerves (Edwards et al., 1997). The parasympathetic innervations are mostly excitatory while the sympathetic innervations tend to be inhibitory in the gut except in the sphincters (Camilleri and Ford, 1998). Motility in the GI tract is driven by luminal content sensed by the enteroendocrine cells and bulk by the mechanoreceptor cells along the GI tract (Cummings and Overduin, 2007). These factors drive motility to allow full digestion of dietary components. Dietary bulk is often sensed by the stretch receptors which drives the peristaltic contraction of the GI tract to break solid components into smaller particles for enzymatic digestion (Furness, 2000). The nutrients in the luminal content are also sensed by the intestinal cell which reduces motility and rate of gastric emptying to enable optimal nutrient absorption. This mechanism of negative feedback acts to reduce feed intake via the gut-brain axis (Konturek, 2004). Fiber increases dietary bulk and can have different effect on gut motility and subsequently appetite based on the characteristics of the fiber type. Both insoluble fiber which are poorly fermented and soluble fiber which increase viscosity and reduce digesta motility and gastric emptying have significant implication on appetite regulation due to their interaction with the GI tract. However, dietary fiber sources often exist in a mix of both soluble and insoluble fiber, and this may give more insight into the differences that exist in studies showing the effect of fiber on gut transit time and subsequently appetite regulation. Overall, dietary fiber may cause damage to the villi, increase the thickness of the muscles of the GI tract and increase the dietary bulk which often downregulate appetite through the stretching sensed by mechanoreceptors.

1.4 The gut microbiome

The GI tract is populated by a complex microbial community such as bacteria, protists and fungi, although bacteria are the most dominant. The population and complexity of microbial community in the GI tract increase towards the large intestine. The population of microbes in the gut of pig increase from $10^3 – 10^5$/g of stomach digesta to $10^9 – 10^{10}$/g digesta in distal part of the small intestine, increasing to $10^{10} – 10^{11}$/g of digesta in the large intestine (Jensen and Jorgensen, 1994). The bacteria community of the gut are mostly anaerobic which belong to about 50 generals with Firmicutes and Bacteroidetes being the two dominant phyla accounting for over 90% of the microbes, these phyla are subdivided into over 500 species (Sommer and Backhed, 2013). The different species occupy specific niche and relate with each other to maintain microbial homeostasis irrespective of their number. The acidic condition of the stomach supports the proliferation of few bacteria species while the small and large intestine with its nearly neutral pH support the proliferation of large number of microbes. The longer digesta resident time and slower transit rate in the large intestine also support the growth of large number of microbes (Sender et al., 2016) making the large intestine major site of microbial fermentation.

1.4.1 Dietary fiber and gut microbiome

Diet remains the major factor affecting activity of gut microbes (Bach Knudsen et al., 2012). Dietary fiber serves as substrate for microbial fermentation. It is a source of energy for gut microbes and it aids their proliferation (Gidenne, 2015). Dietary fiber can therefore cause a shift in gut microbiota, increasing the growth of specific bacteria species that utilize it (Williams et al., 2001). The major products of fiber fermentation by gut microbes are volatile fatty acid (VFA), ammonia, lactate and other gases like hydrogen, methane and carbon dioxide (Jensen and Jorgensen, 1994). VFA play important role in maintaining health environment in the gut, can serve

as energy source for intestinal cells and serve as signaling molecules (Frankel et al., 1994). Ziljstra et al. (2012) observed that VFA altered secretion of gastric enzymes and insulin release showing the potential important effect that they can have on overall digestive process. This potential of dietary fiber to modulate the gut microbiome has been applied in the use of probiotics to improve gut health in swine production. However, energy loss through the production of hydrogen, methane and carbon dioxide coupled with the heat produced during microbial fermentation and inefficient absorptive capacity of the large intestine for VFA makes hind gut fermentation less efficient compared to enzymatic digestion and absorption in the small intestine (Varel and Yen, 1997).

1.4.2 Impact gut microbiome on growth performance and nutrient digestibility in pigs

The VFA produced from microbial fermentation in the hindgut can contribute about 30% of growing pig's maintenance energy requirement (Yen et al., 1991) and even more energy for matured pigs (Varel, 1987). This is energy that would have been otherwise wasted in feces without microbial fermentation. However, the importance of gut microbes is not limited to energy provided from fermentation. As stated above, the VFA produced can serve as signaling molecule and also stimulate the secretion of digestive enzymes and improve gut health. Also, the stabilization of the gut microbial community can reduce the proliferation of pathogenic bacteria which helps to reduce activation of immune system, meaning more energy can be used for production. This cumulative benefit by the microbes in the gut can improve growth performance and digestibility. Wang et al. (2019), in a longitudinal study, showed distinct microbiome structure along different growth stage of pig. When feces from matured pigs were inoculated into piglets, the result showed improved growth rate in the piglet with fecal transplant. Tsai et al. (2019), in a study with weaned pigs transplanted with feces from healthy growing pig, reported a tendency for improved body weight gain in the fecal transplanted pigs. Although, more evidences are still needed to directly confirm

the potential benefit of microbiome on production, these results suggest a potential role of microbiome in improving animal performance.

1.5 Carbohydrases in swine diets

Dietary fiber in animal feed are poorly utilized by the animals because of the inability of endogenous enzymes to hydrolyze them. One of the methods used to improve the utilization of dietary fiber in animal diets is the inclusion of exogenous enzymes which can hydrolyze the bonds present in the complex carbohydrates of dietary fiber. The global exogenous enzyme market in the animal feed exceed $550 million saving the animal industry an estimated $3 to 5 billion yearly (Adeola and Cowieson, 2011). One of the major types of exogenous enzymes used in animal feed are carbohydrases.

1.5.1 What are exogenous carbohydrases?

Carbohydrases refer to all enzymes that catalyze the hydrolyses of carbohydrate polymers (Adeola and Cowieson, 2011). While some carbohydrases can be produced endogenously, e.g., amylase and maltase, there are a group of these enzymes that are not produced in the gut of the animal often referred to as exogenous carbohydrases. They have been supplemented in pig diets to improve the utilization of NSP. There are a variety of exogenous carbohydrases used in animal feed. Each carbohydrase is usually specific in the type of bond it hydrolyzes suggesting a specificity of substrate which depending on the abundance of such substrate in the diet can affect their effectiveness. This has increased the use of these exogenous enzymes in combination. Exogenous carbohydrases range from glucanases, which act on glucans, to cellulases which hydrolyze cellulose to glucose and xylanases which break the xylan backbone of NSP. There are also debranching enzymes like arabinofuranosidases which hydrolyze the arabinose branch in NSP.

Table 1.2 shows different exogenous carbohydrases and their substrate. Most of the enzymes listed in Table 1.2 hydrolyze carbohydrate polymers to smaller oligomers but rarely free sugars that can be directly metabolized by the animals (Adeola and Cowieson, 2011). The products of hydrolysis are either further broken down by endogenous gastric enzymes or fermented by gut microbial community.

Table 1.2. Commonly used exogenous dietary carbohydrase in pig diets

Enzyme	Enzyme Commission	Substrate	Targeted Ingredients
Xylanase	EC 3.2.1.8	Arabinoxylan	Corn, wheat, rye
β-glucanase	EC 3.2.1.6	β-glucan	Barley, oat
Arabinofuranosidase	EC 3.2.1.55	Arabinoxylan	Corn, wheat, rye
Pectinase	EC 3.2.1.15	Pectin	Beans, sugar beet pulp
Mannanase	EC 3.2.1.78	Mannan	Soybean, palm, copra
Cellulase	EC 3.2.1.4	Cellulose	Forages, brans
α-galactosidase	EC 3.2.1.22	Galactan	Beans

Combined from Adeola and Cowieson (2011) and Ohh (2011)

Most commercial carbohydrases are obtained from optimized fermentation system of genetically modified bacteria and fungi. In the pig, the activity of exogenous carbohydrases on their substrate starts once the diet becomes moist immediately after consumption. The objective is for the carbohydrase to act on the substrate before or in the small intestine to maximize nutrient absorption and utilization. The stomach is the first place where the feed ingested is temporarily resident. The stomach pH is acidic and the short resident time in this acidic condition may the efficacy of carbohydrases in pig. To achieve optimal efficiency, carbohydrases should be resistant to heat and

acidic pH. Examples of organisms used for exogenous carbohydrases include Aspergillus spp, Huminicola insolens, Bacillus licheniformis, Trichoderma spp, Escherichia coli, Pichia pastoris, etc.

1.5.2 Mode of action of exogenous carbohydrases

Three modes of action have been proposed for exogenous carbohydrases:

i. Most of the complex carbohydrates that pigs are unable to digest are present in the cell wall of grains. These complex carbohydrates like NSP traps other nutrients or interfere with their digestion by limiting the access to gastric enzymes. Carbohydrases have been proposed to break this NSP in the cell wall, releasing the nutrients entrapped there in and providing digestive enzymes access to these nutrients for digestion.

ii. Soluble fibers trap water in their matrix thereby increasing digesta viscosity. This limits the access of gastric enzymes to components of the diet for digestion and limit the rate of nutrient absorption by increasing the thickness of the glycocalyx of the brush boarder. Exogenous carbohydrases have been proposed to hydrolyze the bonds between soluble fiber reducing the viscosity in the digesta.

iii. Insoluble fiber is in the fibrous mesh which are poorly fermented by gut microbes. Exogenous carbohydrases have been proposed to hydrolyze these heavy polymers into smaller oligomers which are more effectively fermented in the gut. These smaller oligomers promote the proliferation of fiber fermenting bacteria which increase the efficiency of fiber utilization.

1.5.3 Effect of exogenous carbohydrases on growth performance in pigs

The effect of exogenous carbohydrase supplementation in diets on growth performance in pig has not been consistent (Adeola and Cowieson, 2011). Some studies have reported positive responses to exogenous carbohydrase supplementation mostly in NSP-rich diets (Li et al., 2018; Ndou et al., 2015; Kiarie et al., 2007). However, other studies did not record increase in body weight due to the enzymes (Moran et al., 2016; Agyekum et al., 2015; Woyengo et al., 2008). Adeola and

Cowieson (2011) suggested that the observed differences in results from these studies could be due to the differences in the quantity and type of dietary fiber source, animal age, level of limiting nutrients, and the level to which digestible nutrient content was increased by the enzyme. Young pigs possess limited ability to utilize fiber because their gut is still not fully developed and hind gut fermentation may not be optimal, making exogenous carbohydrase supplementation essential when they are fed diets high in dietary fiber. Zijlstra et al. (2004) and Ao et al. (2019) reported increased ADG and ADFI in young pigs (< 20kg) without increase in nutrient digestibility, suggesting the improvement in ADG may not be solely explained by increase in digestibility of nutrients but may also be influenced by increase in feed intake, improvement in gut health and microbial community structure.

1.5.4 Effect of exogenous carbohydrases on digestibility of nutrients in pigs

Similar to the effect of growth performance, the effect of exogenous carbohydrase supplementation on digestibility of nutrients has not been consistent. Several studies in pig have reported improvement in digestibility of DM (Olukosi et al., 2007), energy (Olukosi et al., 2007) and minerals (He et al., 2010), while others did not see the effect on DM (Woyengo et al., 2008), energy (Olukosi et al., 2007), CP (Nitrayova et al., 2009) and minerals (Nortey et al., 2008). The release of trapped nutrients and increase in volatile fatty acid (VFA) production is perhaps responsible for increased nutrient digestibility. However, this is sometimes masked by the increased endogenous losses from fiber consumption which may be responsible for the lack of effect of exogenous carbohydrases on nutrient digestibility observed in some studies. It has been suggested that exogenous carbohydrases shift VFA production and the absorption of monosaccharides that yield energy to the proximal intestine (Li et al., 1996). This will improve utilization of nutrients by ensuring availability of nutrients where it can be efficiently absorbed.

1.5.5 Effect of exogenous carbohydrases on gut microbiome

Exogenous carbohydrases can modulate the microbial population of the gut in two ways:

i. Release of oligosaccharides which are fermentable from the hydrolysis of NSP. These oligosaccharides can then be better fermented by specific bacteria, enhancing their proliferation thereby causing an alteration in the microbial community structure.

ii. Reduction in level of other fermentable substrates like protein and starch through the improved digestibility of nutrients in the small intestine (Bedford and Cowieson, 2012). Diet supplementation with exogenous carbohydrases can increase starch digestion in the small intestine of animals. This reduced level of undigested starch which is usually the easiest accessible substrate for microbes. The microbes instead ferment undigested NSP thereby increasing the population of NSP degrading bacteria and increase VFA production while reducing starch degrading bacteria.

These hypotheses were supported by results of experiments on cell wall degrading enzymes, where the population of Lactobacilli in the ileal digesta of pigs on a barley or oat basal diets and soyhull or wheat bran-based diets. Reduction in Lactobacillus was relative to the proportion of fiber degrading bacteria. Modulation of the microbial community was accompanied by an increase in the concentration of VFA in the colon (Li et al., 2020). Response to carbohydrase supplementation may be influenced by factors like the efficacy of the enzymes, the amount and type of substrates for the enzyme as well as factors intrinsic to the pig like age, health status and physiological status of the pig and the initial microbial population (Mendis et al., 2016).

1.6 Heat stress

Heat stress is a condition of increase ambient temperature load which is beyond the normal physiological range of an animal. Heat stress greatly affects the productivity of livestock industry (Baumgard and Rhoads, 2013), especially the swine industry because of the high sensitivity of pigs to increase temperature. It has been estimated that the US swine industry loss about 1 billion US dollars to heat stress and this figure is likely to rise with in increased incidence of global warming and adverse weather (US EPA, 2014).

1.6.1 Animal response to heat stress

When animals are exposed to temperature above their normal physiological range, they instigate measure to restore or maintain their core body temperature with its normal metabolic range. Some of these measures are energetically demanding and may divert energy mean for production purpose with resulting decline in animal productivity. Some of the first measures adopted by the animals include reduction in physical activity and reduction in feed intake which helps the animal to reduce metabolic heat production associated with digestion and absorption of nutrients (heat increment). Another important mechanism adopted by the animal is vasodilation of blood vessels and redistribution of blood to increase blood flow to the skin while blood flow to the GI tract is reduced. This aids in dissipation of excess body heat to the environment. While this is an important adaptive measure by the animal, it can lead to hypoxia and nutrient deprivation of intestinal cells since the blood carries oxygen and nutrient. This may cause impairment of the intestinal barrier which can cause increased permeability of endotoxin. Increased endotoxin in the circulation can elevated immune response and incidence of disease which are energetically demanding and costly for the animals.

1.6.2 Impact of heat stress on growth performance in pigs

Reduction in feed intake is one of the main strategies animals use to reduce heat production during heat stress (Baumgard and Rhoads, 2013). The reduction in feed intake is usually accompanied by a decrease in ADG since the amount of nutrient available to the animal is reduced (Renaudeau et al., 2001). The magnitude of the reduction in ADG depends on the weight of the animal with heavier animals having greater decline than lighter animals. The severity of the reduction in ADG also depends on the severity of the heat load. White et al. (2008) reported a reduction in final BW, ADG, ADFI and G: F in finishing pigs subjected to heat stress at 32.2^{o}C. Ma et al. (2019) reported similar decline in final BW, ADG and ADFI in finishing pigs at 35^{o}C, while G: F was not affected. In the two studies, ADG reduced by 40% and 62% respectively despite a lower reduction in ADFI (30% and 50%). This may suggest that despite feed intake been associated with the reduction in growth performance, the effect of heat stress on growth performance may be complicated. Results from Pearce et al. (2013) and Ma et al. (2019) show that under heat stress, growth rate and efficiency of pigs are higher compared to pair-fed thermoneutral pigs. This increase in efficiency is an energetically perplexing phenomenon but may be that heat stress greatly reduced maintenance cost (Johnson et al., 2015) as the animals reduce feed intake and physical activities. One other reason that can impact the severity of the decline in pig performance during heat stress is the type of diet. High dietary fiber for example will affect feed intake accompanied endogenous loss differently from diets not high in fiber. Result from Rauw et al. (2017) showed similar result on decline in growth performance in three genetic line of pig. This suggests that while the extent of the decline may vary by genetics, heat stress often causes significant reduction in animal performance.

1.6.3 Impact of heat stress on digestibility of nutrients in pigs

There is limited published literature on the effect of heat stress on nutrient digestibility in pigs. Heat stress has been associated with decrease in villi height and increased villi atrophy which may cause an impairment in the digestive and absorptive capacity of pigs. Morales et al. (2018) reported a reduction in apparent ileal digestibility (AID) of arginine and histidine in heat stressed pigs. Standardized ileal digestibility (SID) of arginine, histidine and leucine was also lower in heat stressed pig. Hao et al. (2014) also showed that heat stress reduced the apparent total tract digestibility of dry matter, gross energy, crude protein and ash. However, Habashy et al. (2017) and Koelkebeck et al. (1998) did not report any significant effect of heat stress on digestibility of nutrients in broilers and laying hens respectively. While these results may not be conclusive, they show that heat stress probably plays a significant role in nutrient digestibility.

1.6.4 Heat stress and gut health in pigs

The heat stress induced hypoxia in the intestinal cells leads to impairment in intestinal integrity. Pearce et al. (2014) reported that heat stress increased villi atrophy and villi autolysis suggesting severe damage to intestinal epithelium. Tight junction proteins are important barrier defenses and their redistribution during period of stress play a significant role in regulating intestinal barrier integrity (Zhang et al., 2012; Turner J. R., 2009).

Pearce et al. (2013) showed increased permeability of the intestine as measured by reduced transepithelial resistance (TER) in heat stressed pigs. This increased permeability corresponds with increase in glucose transporter 2 (GLUT2) and Na^+/K^+ ATPase activity. These results suggest that increased intestinal permeability associated with heat stress may not be only because of the redistribution of tight junction protein but also increase in activation of sodium dependent glucose

cotransporter 1 (SGLT1) and Na^+/K^+ ATPase which increase transcellular permeability. There was also an upregulation of membrane claudin 3 and cytosolic occluding, which agrees with previous 24 hours intestinal heat stress models where occludin was upregulated (Dokladny et al., 2008). The upregulation of, and redistribution of tight junction proteins, may show heat stress induced barrier enhancement to compensate for increased intestinal permeability. This notion is further supported by results showing heat stress induced expression of heat shock protein (HSP) is required for the upregulation of occludin. It is also possible that the detected occludin is not functionally attached to the zonula occludens-1 (ZO-1) and the tight junction complex which may indicate they are not able to significantly improve barrier integrity. While the tight junction complex is important for upregulating intestinal integrity, the mucus layer of the GI tract provides the first line of defense and it prevents the exposure of intestinal cells to toxins and mechanical injury.

1.6.5 Heat stress and the gut microbiome in pigs

Heat stress disrupts the physiology of the animal such as endocrine disorders, electrolyte imbalance and abnormalities in metabolism (He et al., 2018). Some of these physiological changes takes place within the GI tract which harbors a complex community of microbes. Crumeyrolle-Arias et al. (2014) reported that a lack of gut microbiota exacerbates acute stress-induced neuroendocrine and behavioral responses in rat suggesting the involvement of alteration in the microbiome in heat stress induced perturbation. Zhang et al. (2017) showed the decrease in the population of Bifidobacterium and Lactobacillus but enrichment of pathogenic E. coli, Salmonella and Clostridium in broilers under heat stress.

However, Wang et al. (2018) in another study in broiler chickens, reported heat stress enriched Clostridium, Faecalibacterium, Alistipes, Streptophyta, Rothia and Oscillibacter, while Streptococcus and Coprococcus were reduced. Zhu et al. (2019) reported that in laying hens, heat stress decreased the population of Firmicutes but increased Bacteroidetes, this change in microbiome composition correlated with alteration in metabolism pathways especially cysteine and methionine. However, there is paucity of information on the effect of heat stress on gut microbiome in pig. He at al. (2019), in a study in primiparous sows, reported that heat stress increased the abundance of the Clostridiales and Halomonas while Bacteroidales and Streptococcus were reduced. This suggests that heat stress may cause the proliferation of pathogenic microbes at the expense of beneficial ones.

1.6.6 Heat stress and activity of endogenous and exogenous digestive enzymes

There is paucity of literature on the effect heat stress on the activities of both endogenous and exogenous digestive enzymes in pig. Heat stress is associated with decreased feed intake, loss of appetite and change in intestinal morphology. The presence of feed bolus in the GI tract stimulates secretion of digestive enzymes either by the pancreas or the epithelial cells. It is therefore possible that a change in the environmental temperature may influence the secretion of digestive enzymes and their activity. This alteration may be involved in a compensatory mechanism to adjust to reduced nutrient availability when there is reduced feed intake or increased resident time when there is increased viscosity.

Routman et al. (2003), in a study in broiler chickens, reported that heat induced stress did not affect the level of alkaline phosphatase, pancreatic lipase and trypsin while the level of pancreatic amylase was significantly reduced by heat stress. The reduction in the level of amylase could be

due to increase in intestinal cell turnover. It should be noted that the activity of digestive enzymes could also be affected by the type of diet. This result suggests that the secretion of different digestive enzyme maybe differently regulated during period of heat stress. A modification of physiological condition of the gut has also been suggested to affect the activity of exogenous carbohydrases (Routman et al., 2003).

1.7 Summary of review and study objectives.

There is increase utilization of high-fiber ingredients in pig diets (Noblet and Goff, 2001). One of the factors significantly affecting gut physiology in livestock is dietary fiber (Lindberg, 2014). Fiber is not digested in the foregut of monogastric animals but can be fermented in the hindgut. However, it is associated with a relatively lower energy content and fiber consumption could lead to reduction the performance of animals (Noblet and Goff, 2001). One of the methods used to improve dietary fiber utilization is supplementation with exogenous carbohydrases which hydrolyzes the complex bonds in the fiber and make nutrients available for utilization in the small intestine (Bedford, 1995). In the swine industry, heat stress causes significant economic loss due to its adverse effect on performance of animals and its importance is becoming greater due to increasing occurrence of global warming. Pigs respond to heat stress through various physiological changes.

However, the impact of these physiological changes in modulating effect of carbohydrases on growth performance, digestibility of nutrient, intestinal integrity and gut microbiota is still unclear. Therefore, the objective of this study was to determine the effect of a carbohydrase admixture on growth performance, digestibility of nutrient, intestinal histomorphology, gene expression, serum markers of heat stress and gut microbial community structure in growing pigs kept under

thermoneutral and heat stress conditions. Results from this thesis increase our understanding of effect of heat stress on the efficacy of exogenous carbohydrases in pigs fed wheat-based high fiber diets.

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CHAPTER 2. EFFECT OF HEAT STRESS AND A MULTIENZYME CARBOHYDRASE ADMIXTURE ON THE GROWTH PERFOMANCE, NUTRIENT DIGESTIBILITY, BLOOD PARAMETERS, GENE EXPRESSION AND GUT MICROBIOME IN GROWING PIGS FED WHEAT-BASED DIETS

2.1 Abstract

Carbohydrases have been used to improve fiber utilization in monogastric animals with inconsistent results, especially in pigs. The efficacy of carbohydrases has been suggested to depend on enzyme activity and fiber composition, but recent evidence suggests other factors such as the environment might play significant role. The effect of heat stress (HS) on the efficacy of a multienzyme carbohydrase blend in growing pigs was investigated. It was hypothesized that HS alters the efficacy of carbohydrases. Ninety-six (barrows: gilts; 1:1) growing pigs (initial BW of 20.15±0.18 kg) were randomly assigned to 6 treatments, with 8 replicates of 2 pigs per pen in a 3×2 factorial arrangement: three levels of carbohydrase (0, 1X or 2X) at two environmental temperatures (20^{o}C or cyclical 28^{o}C nighttime and 35^{o}C daytime). The 1X dose is the recommended commercial dose (50g/tonne) and provides 1250 visco-units endo-β-1,4-xylanase, 4600 units α-L-arabinofuranosidase and 860 visco-units endo-1,3(4)-β-glucanase per kg of feed. The 2X was 100g/tonne. Pigs were fed ad libitum for 28 days and 1 pig per pen was sacrificed on d 28. Heat stress increased respiratory rate and skin temperature ($P < 0.001$). There was no enzyme × temperature effect on any response criteria. Enzyme treatment quadratically increased d 28 body weight (BW) ($P < 0.05$), average daily gain (ADG) ($P < 0.05$) and average daily feed intake (ADFI) ($P < 0.05$) with 1X being the highest. Heat stress reduced the BW at d 14 ($P < 0.01$) and d 28 ($P < 0.01$), ADG ($P < 0.01$) and ADFI (P<0.001). However, there was a trend of increased gain: feed (G/F) ($P < 0.1$) in the HS pigs. Heat stress increased apparent jejunal digestibility (AJD) of energy ($P < 0.05$) and apparent ileal digestibility (AID) of calcium ($P < 0.01$). The ADFI was positively

correlated to ADG (r=0.57; $P < 0.001$) but negatively correlated to G/F (r=-0.42; $P < 0.01$). Enzyme supplementation increased in vitro viscosity of diet at 3, 4 and 5 hours. Heat stress reduced serum glucose at d 1 ($P < 0.001$) but increased non esterified fatty acid (NEFA) at d 1 ($P < 0.01$) which became reduced at d 28. A trend similar to NEFA was observed for TAG. In the jejunum, there was a trend of increased villi height by carbohydrases ($P < 0.1$) while HS reduced villi height ($P < 0.05$). Heat stress increased the jejunal mRNA abundance of IL1β in the jejunum ($P < 0.001$). There was a trend for a reduction in ileal MUC2 ($P < 0.1$), occludin ($P < 0.1$) by HS, and a trend for increased PEPT1 ($P < 0.1$). There was no effect of HS on alpha diversity, but sampling day affected beta diversity with an increase in the abundance of pathogenic bacteria in the HS group. In conclusion, HS did not alter the efficacy of carbohydrases. Carbohydrases and HS modulate pig performance independently.

Key words: growing pig, carbohydrases, heat stress, gut microbiome

2.2 Introduction

There has been an increase in the use of use of plant coproducts or byproducts (Noblet and Goff, 2001) in animal feeds because they are cheaper than cereals and grains. However, these products typically have high dietary fiber component (Stein and Shurson, 2009), which is poorly digested in the small intestine where much of the nutrient absorption takes place but are fermented in the hind gut. One of the methods used to increase the foregut utilization of dietary fiber is the use of carbohydrases. Exogenous enzymes hydrolyze the β-1,4-glycosidic bond in fiber. However, results on the use of carbohydrases have been inconsistent in pigs in terms of effects on performance of pigs and nutrient digestibility. Several factors have been suggested for this inconsistency. These include the specificity of the enzyme activity and the composition of dietary non starch polysaccharide (NSP) which serve as substrate for the enzyme. However, recent evidence also

suggests that other factors like the environmental condition of the animals may alter metabolic and physiological condition of the animals and may influence the efficacy of the enzyme, but there is limited research on the effects of environmental conditions on the efficacy of carbohydrase in pigs.

This study was carried out to investigate the possible effect of heat stress on the efficacy of multienzyme carbohydrase blend on growth performance, nutrient digestibility, histomorphological characteristics of intestinal tissue, mRNA expression, serum metabolite and fecal microbial characteristics in growing pigs. It was hypothesized that heat stress will alter the efficacy of multienzyme carbohydrase in growing pig.

2.3 Materials and Methods

All animal procedures were approved by the Purdue University Animal Care and Use Committee. All pigs used in this study were obtained from the Purdue University Swine Research Unit.

2.3.1 Animals and Diets

A total of 96 growing pigs (Hampshire× Duroc× Yorkshire × Landrace, barrows: gilts = 1:1) with an average initial body weight of 20.15±0.18 kg were used in a randomized complete block design. There were eight replicates with two pigs as replicate. The study lasted for 28 days. Pigs were housed in floor pens and had ad libitum access to feed and water. Pigs were allocated to treatments based on their BW on d 0. Pigs were fed experimental diets in 2 phases of dietary treatments without titanium dioxide from day 0-day 14 and with titanium dioxide from day 14 to day 28. Titanium dioxide used in the diet at 0.5% for the determination of nutrient utilization (Wang and Adeola, 2018).

The experiment was arranged in 3×2 factorial arrangement with three levels of xylanase-arabinofuranosidase and β-glucanase admixture (Enzyme at 0, 1X or 2X). The 1X dose is the commercial dose inclusion (50g/tonne) and provides 1250 visco-units of endo-β-1,4-xylanase, 4600 units of α-L-arabinofuranosidase and 860 visco-units of endo-1,3(4)-β-glucanase per kg of feed (Table 2.1 - 2.2). The 2X was at an inclusion rate of 100g/tonne. The three dietary treatments were duplicated in two environmental temperature conditions; 2 rooms were maintained at 20ºC, while cyclical 28ºC from 2000 to 0600 hours and 35ºC from 0600 to 2000 hours (28/35ºC) was maintained in 2 other rooms. The 96 pigs were assigned to the 6 treatments stated above. There were 8 replicate pens per treatment at 2 pigs per pen (4 pens of gilts and 4 pens of barrows).

2.3.2 Sample collection

Body weight and feed intake were recorded on d 14 and d 28, and feed efficiency was calculated from body weight and feed intake and corrected for mortality on a pig-day basis. On d 14 and d 28, feces were collected through rectal palpation from one pig per pen to determine gut microbial diversity. On d 28, one pig per pen was euthanized by injecting 0.5 ml of Telazol ® [Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA, 100mg/ml in 5ml of xylazine (Veterinary Healthcare Solutions, Inc. Windsor, ON, Canada)] and asphyxiation with CO_2. Digesta from the mid jejunum and distal ileum were also collected from one pig from each pen to determine AID of nutrients. Mucosal samples were collected from the jejunum and distal ileum (from length of about 5 cm). The sections sampled were rinsed with distilled water before scrapping into 1.5µL micro centrifuge tubes containing Trizol (Invitrogen, Grand Island, NY, USA) with clean glass slides and stored at −80 °C until processed.

2.3.3 Analytical Methods

Digestibility Analysis

Diets, jejunal and ileal digesta were dried at 105°C in a drying oven (Precision Scientific Co., Chicago, IL) for 24h and ground to determine the DM content (AOAC, 2000). Gross energy was determined with a bomb calorimeter (Parr 1261 bomb calorimeter, Parr Instruments Co., Moline, IL, USA). Titanium concentration was determined after samples were ash and digested in sulfuric acid, hydrogen peroxide was then added to the mix. Titanium concentration was then determined by reading absorbance at 440 nm on a spectrophotometer (Spectronic 21D, Milton Roy Co., Rochester, NY) (Fenton and Fenton, 1979).

Similarly, for measurement Ca and P, samples were first digested in nitric and hydrochloric acid. P concentration was measured by reading absorbance in a spectrophotometer at 630 nm using the method described by Onyango et al. (2004). Concentration of Ca was determined using flame atomic absorption spectrometry (Varian FS240 AA Varian Inc., Palo Alto, CA, USA). Nitrogen content was determined with the combustion method on a TruMac nitrogen analyzer (Leco Corp., St. Joseph, MI). Apparent ileal digestibility (AID) or apparent jejunal digestibility (AJD) was calculated using the following equation:

AID (AJD), % = $[1-(Ti_i / Ti_o) \times (Y_o / Y_i)] \times 100$;

Where Ti_i and Ti_o are the titanium concentrations of the diet, jejunal and ileal output, respectively (mg/kg of DM); and Y_o and Y_i are the concentrations of nutrients in the jejunal or ileal output and diet, respectively (mg/kg of DM).

In vitro viscosity determination

The 3 dietary treatments were ground, 2 g of each dietary treatments were weighed into a 15 mL test tube. Distilled water (9 mL) was added to the feed samples and mixed. The mix was incubated in a water bath at a temperature of 38^{o}C. Viscosity measurements were conducted at 5 different time points (1, 2, 3, 4 and 5 hours) to mimic viscosity changes over time as a representation of the digesta passage in different segments of the pig digestive tract.

Sample mixtures were centrifuged at 3900 g for 5 minutes at 22^{o}C (Sigma 2-16pk, Sigma Osterode, Germany). Supernatants were recovered to new 15 mL tubes and placed in a water bath (Precision, GCA Corp., College Park, MD) that had been preheated to 40^{o}C until temperature of the sample equilibrated with that of the water bath. The viscosity, in centipoise of the supernatants, was then measured in triplicate with a viscometer (Vibro viscometer, model SV-1A, A&D Instrument Ltd, Oxfordshire, United Kingdom) according to manufacturer instruction.

RNA isolation and quantitative real-time PCR

Mucosa scrapings were homogenized in 1 mL of Trizol (Invitrogen, Grand Island, NY, USA) and homogenized using a VWR Power MAX homogenizer (Avantor, PA, USA). Total RNA was extracted from samples following the Trizol procedure. The concentration of RNA was quantified with a spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Rockland, DE). All samples had 260:280 nm ratios between 1.9 - 2.1. The quality of the RNA was also checked on 1% agarose gel. One μg of isolated RNA from each sample was reverse transcribed into cDNA using MMLV (Promega, Madison, WI, USA). All cDNA samples were diluted 10-fold with nuclease free water. RT-PCR was conducted with a Bio Rad CFX Connect machine (Bio Rad, California, USA). The PCR reaction mix consisted of 5 μL of cDNA, 0.5 μL of each of the gene specific forward and reverse primers (which had been diluted to 15μM working solution), and 10 μL of RT2 SYBR

Green Master Mix (Qiagen, Valencia, CA, USA) and nuclease-free water (Ambion, Austin, TX, USA) for a total reaction volume of 20 μL. Reactions were incubated at 95 °C for 3 min. Afterwards, samples were subjected to 40 cycles of an amplification protocol as follows: 10 s at 95 °C, 30 s at the variable annealing temperature for each gene (Table 2.3) and 95 °C for 10 s. Gene expression level of heat shock protein 70 and 90, interleukin (IL) 1 β, tumor necrosis factor (TNF) alpha, Mucin gene (MUC) 2, peptide transporter (PEPT) 1, sodium dependent glucose cotransporter (SGLT) 1, Galactoside 2-alpha-L-fucosyltransferase gene (FUT) 2, Claudin 4 and Occludin were analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous housekeeping control.

Analysis of serum

Serum glucose and non-esterified fatty acid (NEFA) concentrations were determined with Autokit kit Glucose and NEFA kit respectively (Wako Pure Chemical Industries Ltd., Chuo-Ku Osaka, Japan). Serum insulin concentration was determined using the porcine insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum triglyceride was determined with the triglyceride determination kit (Sigma–Aldrich, St Louis, MO) and blood urea nitrogen (BUN) concentration in the serum were determined by BUN colorimetric detection kit (Arbor Assays, Ann Arbor, Michigan) all according to manufacturer's instructions.

Histomorphology Analysis

The mid-jejunal and proximal ileal tissues were collected from a pig per pen. Excised intestinal segments stapled to a cardboard and fixed in 10% neutral buffered formalin (VWR International, Radnor, PA). Samples were then dehydrated with ethanol (VWR International, Radnor, PA), and then cleared wit Sub-X® (Polysciences, Inc., Warrington, PA) and fixed in paraffin (Polyfin

paraffin, Sigma Polysciences, St. Louis, MO). The segments (5μm) were then stained with hematoxylin and eosin at the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN, USA). Villus height and crypt depth were measured from 3 complete vertically oriented villi per slide. Villus height were taken from the apical portion of villus to the base, and crypt depth were taken from the base of the villus to the basolateral membrane. Subsequently, the villus height to crypt depth ratio was calculated. All measurements were performed under a binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

DNA extraction and 16s library preparation

Frozen fecal samples were thawed overnight at 4°C. Bacterial DNA was then extracted from the fecal samples with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA) according to manufacturer's instructions but using 200 mg of feces instead of 500 mg of soil originally described (Ariefdjohan et al., 2010). Samples were first homogenized before the extraction. The quality of the extracted DNA checked were verified on a 0.5 agarose gel after which it was quantified with by Hoechst 33258 dye (Thermo Fisher Scientific Inc., Waltham, MA) on a ND-3300 NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA). Calf thymus DNA was used as a standard. Two-step PCR was used to amplify DNA products for sequencing. The V3 and V4 region of 16S bacteria rRNA gene was amplified in the first step using primers V3_343F (5' TCT TTC CCT ACA CGA CGC TCT TCC GAT CTN NNN TAC GGR AGG CAG CAG 3') and V4_802R (5' GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCT ACC RGG GTA TCT AAT CC).

The PCR reaction mixture was made up of: 25 uL of 2x Q5 master mix (New England BioLabs, Inc., Ipswich, MA), 1.25 uL of 20 mg/mL bovine serum albumin (BSA), 1.25μL of 15 μM of each

primer and DNA template at 5μL of 1ng/μL. The reaction volume was made to a total of 50 uL with nuclease free water (Thermo Fisher Scientific Inc., Waltham, MA). The PCR reactions were carried out in a Eppendorf mastercycler (Eppendorf Hamburg, Germany) with the following program: an initial denaturation process at 95°C for 5 min, this was followed by 25 cycles of denaturation at 94°C for 30 s, then an annealing process at 58°C for 20 s, and extension process at 72°C for 20 s, and a final extension step at 72°C for 10 min. AMPure XP beads (Beckman Coulter Inc., Brea, CA) was used to purify the PCR products, 70% ethanol was then used to wash the purified PCR products before nuclease free water was used for final elution.

For the second step, the purified PCR products from the first step was further amplified through a second PCR reaction using a set of barcoded primers for library preparation according to suggested protocol by manufacturer (Illumina). The reaction mixture was made up of 24 uL PCR product from step 1, 26 uL of 2x Q5 master mix (New England BioLabs, Inc., Ipswich, MA) and 0.5 mM of each primer. The reaction program included an initial denaturation at 95°C for 5 min, which was followed by 5 cycles of denaturation at 94°C for 30 s, and then an annealing at 64°C for 20 s, and extension at 72°C for 20 s, and a final extension step at 72°C for 10 min. AMPure XP beads (Beckman Coulter Inc., Brea, CA) was used to purify the secondary PCR product was using the same protocol as in step 1. The second PCR amplicons were quantified as in step 1 but with a Quantiflour dsDNA dye (Promega, Madison, WI). The amplicons were pooled into a single 1.5 mL tube such that each library had equal final concentration.

Illumina Miseq sequencing

The V3 and V4 regions of the 16S bacteria rRNA of the pooled library was sequenced through the Illumina MiSeq sequencing platform. Paired end reads for V3 and V4 regions are about 300 bp

long and 500 bp long when reads are joined. Customized sequencing primers targeting the V3 and V4 regions of the 16S amplicon were used for the sequencing. The forward primer (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CC TAC GGG NGG CWG CAG-3') and reverse primer (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GG ACT ACH VGG GTA TCT AAT CC-3') were used during the procedure.

Quality filtering and sequence analysis

Results from the sequencing were analyzed based on the method described by Jones-Hall et al. (2015). Samples that contained very low numbers of sequencing reads, primer tags and low-quality reads were removed by the Illumina software. Paired-end sequences were merged, and high-quality reads were clustered into operational taxonomic units and analyzed based on the workflow of QIIME2 (version 2017.6.0). Amplicons were trimmed at the 12^{th} base of each sequence for both the forward and reverse sequence with Dada2. 5660 sequences were used for subsampling and 5 samples were lost due to low quality reads.

Bioinformatic analysis

The linear discriminant analysis effect size (LEfSe) method (Galaxy v1.0; Segata et al., 2011) was used to identify differentially abundant phyla, class, order, family and genera between groups under different conditions. The operational taxonomic units (OTU) were assigned using Silva data set (silva version 138) (Park et al., 2018). Subsequent comparisons were performed at a sampling depth of 5660 which was chosen by rarefaction as the lowest number of sequences from a single sample. The alpha diversity of richness was determined using Chao 1, Observed OTU, Evenness, Shannon and Faith phylogenetic diversity (PD), Simpson and Simpson evenness to compare diversity within a sample. Beta diversity was determined using phylogenetic distance (unweighted

and weighted Unifrac) (Hamady et al., 2010) and non-phylogenetic Bray Curtis distance and Jaccard. Principle coordinated analysis (PCoA) was also performed to assess beta-diversity.

2.3.4 Statistical analysis

Data were arranged in a 2×3 factorial arrangement, with 2 environmental temperatures and 3 levels of the enzyme blend. Data were arranged in randomized complete block design and were analyzed using the Proc GLM procedure of SAS (SAS Inst. Inc., Cary NC) with diet and temperature as the main effects and replicate as block. Results are reported as least square means and standard errors of the means. Means were considered significantly different at $P \leq 0.05$, with trend at $P \leq 0.1$ and means were separated using Tukey multiple comparison test. Significant mean differences were indicated with superscripts. For the relative abundance of microbes, parameters that meet the parametric test were analyzed by ANOVA while those that fail the parametric test assumptions were subjected to Krustral Wallis analysis (non-parametric equivalent to ANOVA). The Krustral Wallis analysis was also used for an overall comparison of the proportions of the treatments at different taxa level (phyla, class, order, family and genera) in fecal samples collected on d 14 and 28.

2.4 Results

2.4.1 Growth performance

There was no enzyme × temperature interactions effects on any performance parameters, therefore, all results are presented as main effects. Pigs in HS had lower BW on d 14 ($P < 0.01$) and d 28 ($P < 0.01$) compared to thermoneutral (TN) pigs (Table 2.4). Supplementation with 1X enzyme increased BW at day 28 by 7.7% whereas the improvement reduced in 2X diet fed pigs to 2.6% compared to the control. There were no effects of enzyme supplementation in pigs between d 0-

14, with increased feeding of the enzyme supplemented diet, 1X increased ADG and ADFI by 10.8% and 10.7% from d 14-28, and 10.4% and 7.0% from d 0-28. Enzyme treatment at 1X increased the BW of pigs on d 28 compared to control and 2X ($P < 0.05$). However, there was no effect of enzyme on d 14 BW. As illustrated in Table 2.4, there were no effects of enzyme on ADG, AFI and G/F but pigs in in the HS group had significantly lower ADG ($P < 0.01$) and ADFI ($P < 0.001$) in phase 1 (d 0-14). In phase 2 (d 14-28), ADFI was higher in the 1X carbohydrase treatment compared to the control and 2X ($P < 0.05$). There was a trend of increased ADG with 1X treatment ($P < 0.1$), but there was no effect of enzyme on G/F. There was a temperature effect on ADFI ($P < 0.001$), with the HS group having lower feed intake. Also, there was a trend for a higher G/F compared to the TN ($P < 0.1$).

For the entire duration of the study (d 0-28), the 1X enzyme treatment had higher ADFI compared to the control and 2X ($P < 0.05$) and had higher ADG ($P < 0.05$). The HS group had lower ADG ($P < 0.01$) and ADFI ($P < 0.001$) compared to the TN and there was also a trend of increased G/F in HS ($P < 0.1$). There was no carbohydrase effect on rectal and skin temperature and respiratory rate while pigs in HS had higher skin temperature ($P < 0.001$) and respiratory rate ($P < 0.001$). There was a quadratic effect of enzyme on ADFI for d 14 – 28 (period 2) ($P < 0.05$; Table 2.5) and ADG ($P < 0.05$) and ADFI ($P < 0.05$) in period 3 (d 0 – 28). The control group had a trend for a difference from the enzyme groups (both 1X and 2X) for period 1 (ADG; $P < 0.1$) and period 3 (ADG; $P < 0.1$).

2.4.2 Nutrient digestibility

There was no enzyme × temperature interaction effects on jejunal and ileal digestibility of nutrients. Therefore, all results are presented as main effects. There were also no effects of enzyme on both

jejunal and ileal digestibility. Heat stress led to significantly higher ileal calcium digestibility ($P < 0.01$; Table 2.5) and jejunal energy digestibility ($P < 0.05$) compared to the TN. There were no effects of temperature on digestibility of other nutrients.

2.4.3 Correlation between performance and nutrient digestibility

There was a trend for a significant correlation between AID of energy and ADG ($P < 0.1$; Table 2.6) and G/F ($P < 0.1$). There was no correlation between AID of nitrogen, calcium and phosphorus digestibility and ADF and G/F. There was also no correlation between AJD of nutrients and ADF and G/F (Table 2.7). However, there was a strong positive correlation between ADFI and ADG ($P < 0.001$, Table 2.8) and a strong negative correlation between ADFI and G/F ($P < 0.01$).

2.4.4 In vitro viscosity

Carbohydrase did not affect the *in vitro* viscosity of feed samples after 1 hour of incubation. However, there was a trend of increasing viscosity in both the 1X ($P < 0.1$; Table 2.9) and 2X group after 2 hours of incubation compared to the control. After 3 ($P < 0.01$) and 4 hours ($P < 0.01$), the 2X group had higher viscosity compared to the control. After 5 hours of incubation, there was significant difference in the viscosity of the three enzyme groups with the 2X having the highest viscosity and the control group having the lowest viscosity ($P < 0.01$). There was no effect of enzyme on NDF composition across the incubation period (Table 2.10).

2.4.5 mRNA expression of cytokine, tight junction proteins and nutrient transporters

There was no enzyme × temperature interactions and treatment effects on ileal gene expression (Table 2.11). There were trends of significant decrease in the ileal gene expression of MUC 2 ($P < 0.1$; Table 2.12) and occludin ($P < 0.1$) in the HS compared to the TN, while PEPT1 had a trend

for increase in HS ($P < 0.1$). There was a trend for an enzyme × temperature interaction effect on jejunal SGLT1 gene expression ($P < 0.1$; Table 2.13). There were no effects of enzyme on the jejunal gene expression, but HS has a significant increase in the expression of IL1β ($P < 0.001$; Table 2.14).

2.4.6 Serum metabolic and stress parameters

Serum parameters were measured in blood collected on d 1, 14 and 28. The d 1 serum represents acute heat stress while d14 and d28 represent chronic heat stress. There was no enzyme × Temperature interactions effect on all serum parameters. Both enzyme supplemented groups (1X and 2X) showed a trend for reduced TAG at d 14 ($P < 0.1$; Table 2.15). Carbohydrase treatment did not have significant effect on other serum parameters. Heat stress caused elevated glucose concentration at d 1 ($P < 0.001$). Insulin level, however, was not affected. Heat stress caused elevated NEFA at d 1 ($P < 0.01$; Table 2.15), this was reversed on d 28 with the serum NEFA significantly lower compared to the TN ($P < 0.05$). There was a trend for an increase in the serum concentration of TAG in HS at d 1 ($P < 0.01$), with further significant increase at d 14 ($P < 0.05$). Heat stress resulted in a trend for increased serum BUN at d 1 ($P < 0.1$).

2.4.7 Histomorphology

There was a trend for enzyme × temperature interaction effect in the jejunal villus height ($P < 0.1$; Table 2.16). This trend was not observed in the jejunal crypt, villi/crypt ratio and all ileal histological parameter. Heat stress reduced jejunal and ileal villi height compared to the TN ($P < 0.05$; Table 2.17), while there was a trend of increased villi height in the 1X group compared to the control and the 2X ($P <= 0.1$; Table 2.17).

2.4.8 Gut microbiome

There was no significant enzyme effect on Chao1, richness, Shannon diversity indices, Evenness diversity indices, Faith diversity indices and Observed OTUs, Simpson and Simpson evenness. (Figure 2.1) There were also no significant temperature effect on alpha diversity indices (Figure 2.2). Although, there was no significant day effect on Observed OTUs, Chao 1 and Faith diversity indices, there was a significantly higher evenness diversity index on d 28 ($P < 0.05$; Figure 2.3D). The assessment of beta diversity differences is illustrated in Tables 2.18 - 2.20. PERMANOVA analysis of Bray Curtis, Jaccard, Unweighted and weighted UniFrac revealed there were no differences among treatment groups but there was differential clustering among temperature groups according to Jaccard ($P < 0.05$). There was also differential clustering by day according to Bray Curtis ($P < 0.001$), Jaccard ($P < 0.001$) and Unweighted UniFrac ($P < 0.01$). PCoA plot of unweighted UniFrac distance further revealed a difference in clustering (Fig 2.4). As illustrated in Figure 2.5, the abundance of the two dominant phyla were reduced in d28. LEfSe analysis showed that 21 bacterial taxa were differentially enriched due to temperature treatment (Fig 2.6), 81 bacteria taxa by experiment day (Fig 2.7) and 7 bacteria taxa by experiment day and then treatment groups (Fig 2.8).

2.5 Discussion

The interplay between the diet and host's GI tract and its functionality is complex and it is affected by the type of diet. With the increasing rate of inclusion of dietary fiber in animal diet, this is even more important. The composition and type of dietary fiber influences the process of digestion in the gut, the morphology of the gut, functions of the intestinal epithelium and the gut microbiota. Dietary fiber includes all polysaccharides resistant to digestion and absorption in the foregut but are either completely or partial fermentation in the hindgut. Non-starch polysaccharides are

structural components of the cell wall of cereals and can also be found in cereal by-products. They interfere with digestion by reducing the access of digestive enzymes to nutrients in the endosperm of cereals. Exogenous enzymes that can hydrolyze the cell wall matrix of plant may improve the access of the digestive enzymes to nutrients in the endosperm and facilitate the release of nutrients trapped or incorporated into the cell wall.

2.5.1 Growth performance

The increase in the performance with feeding of carbohydrase supplemented diets is consistent with result from Lu (2018) in weanling pigs fed corn-soy diet supplemented with xylanase. Growing pigs, unlike weanling pigs, have a more developed GIT and can better utilize high fiber diet, this may explain the non-significant improvement in performance from d 0-14 in the present study since all diets were similar and the only difference is the inclusion of multienzyme carbohydrases which may require prolonged feeding for its effectiveness. Similar observations were reported by Zijlstra et al. (2004) in weanling pigs fed wheat-based diet supplemented with graded level of xylanase and β-glucanase enzyme blend with no increase in BW at d 7 and ADG from d 0-7. However, there was increase in BW d 14, d 21 and d 28 while increased ADG was recorded from d 8-14 and d 1-28. Interestingly, the result from this study shows that the effect of carbohydrases on BW, ADG and ADFI is quadratic rather than linear in a dose dependent manner suggesting that at higher dose of carbohydrases, there can be increased hydrolysis of arabinoxylan in the GIT which may negatively impact voluntary feed intake by either releasing entrapped nutrient which can cause nutrient imbalance (Zijlstra et al., 2004) or breaking of the insoluble fiber to the soluble fiber which will increase viscosity.

Result from the *in vitro* incubation of experimental diets further showed that 2X carbohydrases supplementation increased viscosity over time, with 3-5 hours post incubation which mimics the digesta transit time in the small intestine most significant. The increased viscosity may explain the reduction in feed intake with the higher dosage of the enzyme. Lu (2018) reported similar anorexic effect of xylanase in weaning pigs while Zijlstra et al. (2004) also reported a positive quadratic response in ADFI and ADG to carbohydrase in growing pigs. Increased viscosity may reduce digesta transit time and reduce the access of digestive enzyme to digesta which may impair digestion and growth. The bulking effect may also trigger the release of PYY, a gut satiety peptide which has been reported to be induced by xylanase supplementation in broilers (Singh et al., 2012), which may reduce voluntary feed intake. It is however important to note that the effect of carbohydrase on digesta viscosity, its impact on voluntary feed intake and the optimal level required in pig diet needs further investigation.

As expected, HS significantly reduced the BW at d 14 and d 28 with corresponding decrease in ADG and ADFI. Under high thermal load, animals reduce metabolic heat production, a high proportion of which comes from digestion. Hence, it is understandable that pigs reduce ADFI under heat stress. This is important because excessive temperature has greater impact on pigs because of their poor ability to dissipate heat due to a lack of functional sweat glands. However, pigs in the HS group had a trend for increased feed efficiency in phases 2 and 3 suggesting that they utilized the feed consumed for weight gain more efficiently. Although it may appear that heat stress decreases efficiency of protein deposition in pigs, this depends on the severity of the heat stress and size of the pigs. Mild heat stress leads to increased efficiency compared to severe heat stress which reduces efficiency with large finishing pigs having reduced efficiency due to reduced

energy intake in relation to their requirement (Baumgard and Rhoad, 2013). Recent evidence suggests that heat stress reduces basal metabolic rate in pigs which subsequently increases ADG and G/F (Pearce et al., 2013). This may explain the increased gain to feed in the present study. The reduction in feed intake in the HS group might have also led to reduced mucin secretion, hence reduction in endogenous losses which may increase efficiency.

2.5.2 Nutrient digestibility

Carbohydrase supplementation in poultry and swine diet has been used to reduce the antinutritive effects of fiber, improve performance and increase nutrient digestibility (Kiarie at al., 2016). Unlike poultry where consistent improvement has been observed with carbohydrases, the efficacy of carbohydrases in improving digestibility in pigs has been inconsistent. Results of this study showed that carbohydrase supplementation did not affect nutrient digestibility both in the jejunum and in the ileum. Carbohydrases are expected to hydrolyze dietary arabinoxylans and β-glucans in the small intestine to improve absorption of monosaccharides. However, the primary products of hydrolysis by carbohydrases are oligosaccharides and pentose sugars which are not well absorbed in the small intestine but can serve as substrates for better microbial fermentation. This may explain the lack of significant difference in both jejunal and ileal digestibility even though there was improvement in performance. This idea is consistent will result reported by Petry et al. (2019) who showed that xylanase increased the ATTD of energy but not the AID of nutrients in barrows.

Changes in voluntary feed intake often influences nutrient digestibility, with nutrient digestibility studies in pigs commonly carried out with equalized feed intake. Studies in pigs with equalized feed intake showed improvement in AID and ATTD of nutrients (Baidoo et al., 1998) whereas studies in which pigs were allowed voluntary feed intake did not show any effect of carbohydrases

on nutrient digestibility (Baas and Thacker 1996). Similarly, in this study there was no effect of carbohydrases on both AJD and AID. This may be because the animals were fed ad libitum with enzyme supplementation increasing voluntary feed intake. Therefore, it is possible that the improvement in nutrient digestibility expected from carbohydrases supplementation may have been negated by the reduction in nutrient digestibility with a higher level of feed intake.

Heat stress increases the body temperature of pigs and as a mechanism for dissipating heat, animals' direct blood from the GI tract to the skin. Reduced blood flow to the GI tract may cause oxidative stress in the intestinal epithelium. The combination of hypoxia, nutrient deficiency and oxidative stress leads to cell death of epithelial cells on the villi (Liu et al., 2009). Increased cell death increases endogenous loss of nutrients and can cause a decrease in the length of the villi due to heat stress. This can impair digestion and absorption of nutrients. However, recent studies have suggested that under mild condition of heat stress, the reduction in the feed intake can cause an increase in nutrient digestibility. Kellner at al. (2016) also reported an increase in ATTD of energy in pigs under heat stress. This is similar to the result of increased AJD and AID of energy and calcium. Increased energy digestibility may be a mechanism by which the pigs compensate for reduced energy intake owning to reduction in the voluntary feed intake. Result from this study may also be influenced by the reduction in the amount of endogenous loses in pigs under heat stress due to reduction in the feed intake of the highly fibrous diet which is associated with sloughing intestinal lining. Interestingly, there was a strong positive correlation between ADG and ADFI while there was a strong negative correlation between G/F and ADFI. However, there was weak correlation between either ADG or G/F and both apparent jejunal and ileal digestibility

coefficients. This suggests that feed intake may be the main driver of the improvement in performance due to both carbohydrases and temperature.

2.5.3 mRNA expression of cytokine, tight junction proteins and nutrient transporters

Nutrient transporters located at the surface of the enterocytes in the small intestine are important for absorption of nutrient. SGLT1 facilitates the Na^+-dependent uptake of glucose and galactose across the brush border membrane (Garriga et al., 2006) and it is the main mediator of glucose absorption in the small intestine (Hediger and Rhoads, 1994). PEPT1 mediates the transport of di and tripeptides into the epithelial cells (Leibach and Ganapathy, 1996). Intestinal enterocytes respond to fluctuations in luminal nutrients by modifying the gene expression of intestinal nutrient transporters (Dyer et al., 2005). In the present study, there was no interaction effect of temperature and enzyme treatment in the ileum, enzyme treatment in both ileum and jejunum, and temperature in jejunal gene expression of both SGLT1 and PEPT1. While carbohydrases have been hypothesized to increase the release of nutrients in the small intestine by liberating nutrients trapped in the cell wall and releasing nutrients from their hydrolysis, the primary product of carbohydrase hydrolysis are oligosaccharides which cannot be digested or absorbed in the small intestine but are better fermented in the hindgut and the amount of nutrient trapped in the cell wall may also not be enough to alter expression of nutrient transporters. This may explain the lack of differenced observed in the expression of primary glucose and peptide transporters SGLT1 and PEPT1 by the enzyme.

However, there was a trend of increased ileal expression of PEPT1 in the heat stressed pigs. This may be a compensation mechanism to reduced nutrient intake caused by reduced feed intake in the heat stressed pigs. There was a trend of interaction in the SGLT1 expression in the jejunum with

a reduction in SGLT1 expression of the 1X group in the thermoneutral pigs while it increased in the heat stressed pigs. This may be associated with differential feed consumption in both temperature groups. Gal-Garber et al. (2003) reported that a reduced affinity and activity of SGLT1 accompanied an increase in the SGLT1 gene in starved chicken which suggests that increased nutrient absorption may not necessarily accompany the increase in the SGLT1 expression in the heat stressed pigs.

Despite the effect of both enzyme and temperature treatment on the BW, ADG, ADFI and G/F in the study, there were no effects of the treatments on most nutrient digestibility responses suggesting the possible involvement of other mechanism in the improvement observed in the performance parameters. One of such mechanism is the improvement of the intestinal barrier function, the intestinal barrier is made of epithelial cells bound together by different proteins overlaid by mucus secretion (France and Turner, 2017). The paracellular space between neighboring epithelial cell is sealed by tight junction proteins making them an important regulator of paracellular permeability (Gunzel and Yu, 2013). Reduction in the protein abundance or mRNA expression of these tight junction proteins could cause an impairment in paracellular permeability which in turn can result in increased permeability of luminal antigens and pathogens which induces immune activation (Hu et al., 2013).

The activation of immune system diverts energy and other nutrients from growth and production to defense system leading to reduced growth performance (Huntley et al., 2018). There were no effect of the interaction of enzyme and temperature or enzyme alone on the gene expression of both ileal and jejunal tight junction protein. Although an increase in the ileal expression of claudin

3 was observed by Li et al. (2018), the study was in weanling pigs as opposed to growing pigs in the present study. This reason for this is not clear but it may be due to increased capacity of growing pigs to handle fiber more than weaning pigs irrespective fiber supplementation. Heat stress reduced the expression of both ileal occludin and MUC2. This is contrary to result obtained by Peace et al. (2013) that heat stress upregulated the abundance of both claudin 3 and occludin. Increased intestinal permeability was also observed in the same study suggesting that the increase in the protein abundance tight junction proteins may be a mechanism to compensate for increased permeability. Although, the increased expression of the occludin was in the cytosol and not on the membrane which may be why there was no significant improvement in intestinal integrity. However, MUC2 which indicate the mucin content of the gut was not measured in the study by Pearce et al. (2013), mucins are the first line of defense again exposure of intestinal cells to toxins. The trend for reduced gene expression of MUC2 in heat stressed pig may indicate severe exposure of intestinal cells which may have caused the damage to tight junction which was shown in the trend for reduced expression of occludin in the heat stress group in the ileum. Increased gene expression of ileal MUC2 may also be due to increased amount of fibrous feed interacting with the lining of the gut indicating increased endogenous loss rather than improvement in intestinal integrity.

Evidence from Wenk, (2001) suggested that a minimal amount of fiber is required for optimal gut function. However, effects of different types of fiber is unclear. Activation of immune system is energy and nutrient costly and takes precedence over production functions (Huntley et al., 2018). Although dietary fiber has been shown to modulate immune response in pig through alteration of the intestinal microbiome and their metabolites (Chen et al., 2013), the effect and mechanisms are

not fully understood and whether carbohydrases which alter the structure of dietary fiber indirectly modulate immune response is even less known. Understanding the modulatory effect of carbohydrases under both thermo-neutral and heat stress condition on the immune system may explain some of the effect of carbohydases on growth performance. The lack of effect of temperature and enzyme on mRNA expression of cytokines in both ileum and jejunum may be because pigs were not clinically infected with pathogens and the immune system were not activated (Li et al., 2018), suggesting that carbohydrases themselves may not regulate inflammatory response. However, a different result might be obtained under inflammatory challenge. In the jejunum, heat stress increased the expression of IL1β. This may be due to increased intestinal inflammation owing to increased permeability and cell turn over during heat stress. This is consistent with reduction in the ileal expression of occludin observed. Heat stress has been shown to cause local and systemic inflammation in the intestine of both human and animal models (Leon, 2007) which is linked to increased permeability (Pearce et al., 2013).

2.5.4 Serum metabolic and stress parameters

Glucose is the primary product carbohydrate digestion absorbed into blood stream from the intestinal lumen by SGLT1. The concentration of glucose in the blood is influenced by the amount of luminal glucose and metabolism of glucose which is regulated by insulin. While postprandial concentration of glucose in the blood can be influenced by feed intake, the post absorptive concentration of blood glucose and insulin are under tight homeostatic regulation. In the present study, neither enzyme nor temperature nor their interaction affected both serum glucose and insulin concentration. Effect of carbohydrases on the regulation of serum glucose and insulin concentration has been inconsistent. Gao (2001) reported that xylanase supplementation did not affect plasma glucose of broilers despite significant increase in the concentration of glucose in the

digesta. Lee et al. (2017) however reported increased serum glucose concentration in broilers fed xylanase after 3 weeks, suggesting a possible improvement in glucose absorption. However, the result from Lee et al. (2017) should be viewed with caution due to its small sample size. Luo at al. (2009) also reported no effect of xylanase on plasma concentration of glucose and insulin in broilers. Similar lack of effect on blood glucose and insulin has been reported for other exogenous enzymes. Lu et al. (2019) also reported no effect of phytase on serum concentration of glucose and insulin in pigs. The reduction in glucose concentration after acute heat stress (d 1) observed in this study may be due to utilization of blood glucose for energy after a steep decline in feed intake within 24 hours of HS. Although, it appears that pigs maintain tight homeostatic regulation of serum concentrations of glucose and insulin when fed carbohydrases, additional investigations are still needed in this area.

There was no enzyme × temperature and enzyme effect for serum NEFA, TAG and BUN. Interestingly, serum NEFA concentration increased after acute stress but reduced at d 28. This pattern is similar to result obtain in pigs after chronic and systemic exposure to heat stress by Pearce et al. (2013) and Qu and Ajuwon, (2018). During period of acute heat stress, sudden adaptation measure resorted to by pigs often leads to negative energy balance one of which is reflected in the reduced serum glucose concentration. Therefore, pigs mobilize free fatty acid from the adipose tissue to serve as energy source through beta oxidation (Vitoria Sanz Fernandez et al., 2015). With prolonged HS, increased heat stress adaptation leads to increased lipogenesis in adipose tissue, causing a decline in blood concentration of NEFA. Similar to what was observed in the NEFA concentration, the concentration of TAG increased during acute heat stress but reduced gradually as stress with chronic HS. However, unlike NEFA, the concentration of TAG

at d 28 was not significantly different between HS and TN. This may indicate that concentration of TAG is not a sensitive enough indicator of the transition from lipolytic to lipogenic state by the animal since phosphoenolpyruvate carboxykinase 1 (PCK1) and glycerol kinase (GK) increase glyceroneogenesis by sequestering free fatty acid in TAG in adipose tissue (Qu and Ajuwon, 2018).

Urea excreted in urine is the primary nitrogenous end product produced by amino acid catabolism in pigs and there was a strong linear relationship between concentration of BUN and rate of N excretion (Kohn et al., 2005). Therefore, BUN is an important indicator of protein catabolism. It is known that heat stress increases muscle catabolism (Pearce at al., 2013; Cruzen et al., 2017; Ganesan et al., 2017). There was a trend of elevation in the concentration of serum BUN after acute heat stress but not chronic heat stress. Qu and Ajuwon (2018) reported increase in BUN after both acute and chronic heat stress while Pearce et al. (2013) reported increased BUN after heat stress showing the possibility of increased protein catabolism during heat stress. Increased trend for BUN in HS pig may suggest increased protein catabolism as a response to the sudden increase in the heat load and steep reduction in feed intake. Increased protein catabolism may be used be the pigs to meet their maintenance requirement suggesting that the increased catabolism may be a survival response.

2.5.5 Histomorphology

The small intestine is major site of digestion and absorption of feed materials. The physical properties of the intestinal surface are impacted by the nature and type of materials passing through the gut as well as the physiological condition of the animals. Dietary level of NSP can affect intestinal morphology (Montagne et al., 2003). Diet high in NSP content from wheat and barley impaired villus height and villus height to crypt depth ratio in the ileum of pigs (Willamil et al.,

2012). The use of exogenous enzymes can mitigate the negative effect of NSP in wheat and barley on intestinal morphology (Willamil et al., 2012). Also during heat stress, there is intestinal cellular hypoxia (Hall et al., 2001), ATP depletion, acidosis and cellular dysfunction caused by the shunting of blood flow from the GIT to the periphery to increase heat dissipation which often lead to necrosis and shedding of intestinal epithelial cells (Gisolfi, 2000). The increase trend of jejunal villi height of the 1X supplemented pigs suggest reduced impairments of the villi by the NSP due to its hydrolysis by the carbohydrase admixture. The decrease in the villi height of the HS pigs in both the ileum and jejunum suggest increase in damage to the villi due to blood flow reducing the availability of oxygen to the enterocytes. This is a typical deleterious effect of HS on epithelial cells.

A trend of enzyme x temperature interaction was observed for jejunal villi height. Although there is no information on carbohydrase effect on jejunal villi height under heat stress, there appears to be an enzyme dose effects on jejunal villi height because the 2X dose of the enzyme resulted in lower villi height than the control and 1X. The lack of the same response in the heat stress group might indicate that higher temperature might have blunted this does specific enzyme effect. Although the reason for the apparent dose-specific effect of enzyme in the thermoneutral temperature is not clear, a similar result of increased intestinal villi height in broilers fed wheat-based diet supplemented with xylanase to 1000U/kg was observed before the villi height reduced at 5000U/kg xylanase under thermoneutral temperature (Luo et al., 2009). The apparent reduction in jejunal villi height at higher dose of the enzyme in the thermoneutral temperature mirrors the effect of the enzyme on animal performance suggesting that that carbohydrase supplementation may be more deleterious at high dosage. Passos et al. (2015) however did not record any effect of

xylanase supplementation on intestinal morphology of pigs fed corn-soybean meal-based diet, suggesting the effect of carbohydrase on intestinal morphology is substrate specific and may vary among different dietary ingredients. However, as expected, heat stress decreased villi height in both the jejunum and ileum of pigs. Similar result was reported of reduced intestinal villi height was reported by Yu et al. (2010) in pigs under heat stress. A further analysis in Yu et al. (2010) showed that microvilli height was shorter, mitochondrial were swollen, number of lysosomes was increased, and enterocyte tight junction was altered suggesting that heat stress caused marked damage to intestinal cells. Although these additional analyses were not done in this experiment, heat stress may have caused similar damage to enterocytes in this study, leading to reduced jejunal and ileal villi height.

2.5.6 Gut microbiome

Dietary components such as carbohydrates play a critical role in shaping the composition and functionality of the gut microbiota (Conlon and Bird, 2014). Carbohydrases hydrolyze β-1,3-glycosidic bong in the NSP components of cereal-based diets to improve their utilization and subsequently improve digestibility and performance in the pig industry (Munyaka et al., 2016). The pentose sugars released from this NSP hydrolysis are not well absorbed but can be fermented by microbes in the hind gut of pigs, often causing changes in the fiber structure and properties. These changes may result in alteration of nutrient availability and the gut environment that may modulate the gut microbiota, which in turn can improve NSP fermentation, animal performance and health. A previous study in our group (Lu, 2018) showed that xylanase did not alter bacterial community structure 6 weeks after weaning. Pigs are subjected to multitude of stressors post weaning which could affect the action of carbohydrase, with environmental temperature been one of such stressors.

The lack of effect of carbohydrases, heat stress or their interaction on the alpha diversity and beta diversity suggested limited effects of treatment on overall microbiome composition. However, the significant increase in evenness on d 28 (alpha diversity) at d28 (Figure 2.5) indicated that the time on treatment might play a significant role. Similarly, although sampling date significantly affected beta diversity with significant p-value for PERMANOVA (Table 2.26), some of the differences may be due to the dispersion in the sample due to the significant p-value of the PERMDISP (Table 2.26). A minimal difference in clustering between the two sampling days by PCoA plot of unweighted UniFrac (Figure 2.7) may further indicate that some minor clustering might be occurring, albeit this appears inconsequential. The lack of significant effect of carbohydrase effect on microbiome composition agrees with the results of Zhang et al. (2018) and Hang et al. (2018) who also reported lack of effect of different types of xylanase in growing and weanling pigs respectively on alpha and beta diversity. Pigs generally develop a stable bacteria community structure 3 weeks after weaning (Frese et al., 20015), and it was possible that a stable community was already established at the time treatments were introduced since the pigs were already about 10 weeks old. In addition, effects of the carbohydrases may not have been strong because the animals were fed diets with similar base ingredients.

Although there was no difference in the overall community structure, results indicate that abundance of specific bacterial groups might have been differentially regulated. For example, the abundance of phylum Bacteroidetes and Firmicutes were significantly higher in d 14 compared to d 28 (Figure 2.8). Bacteroidetes have been reported to have a higher gene encoding for glycoside hydrolases like β-xylosidases, endo-1,4-β-xylanases and α-N-arabinofuranosidases and

polysaccharide lyases per genome compared to other bacteria phyla in the gut making it them the primary degraders of complex polysaccharides in the pig gut (El Kaoutari et al., 2013; Frese et al., 2015). Contrary to expectation that with prolonged inclusion fiber in pig ration, the abundances of Bacteroidetes would increase (Chen et al., 2019b), the abundance of Bacteroidetes decreased from d14 to d28 in this study. The exact reason for this reduction is not clear but age of the animals probably played a major role.

There was increased abundance of 5 genera in the TN pigs compared to HS pigs (Figure 2.9). Some of the upregulated general were responsible for fiber breakdown and increased efficiency of nutrient utilization. These include Alloprevotella which has been shown to be associated with increased fat accumulation in mice (Zhou et al., 2019). Eubacterium is also responsible for converting unsaturated fatty acid to saturated fatty acid in the gut, a process which has been shown to be important for fiber breakdown (Walker, 2018); and Ruminococcus which associated with fermentation of resistant starch (Scott et al., 2013). The abundance of 16 genera were increased in the HS pig (Figure 2.9). A few of these could be pathogenic Clostridiaceae_1 (Zhang et al., 2019; Grzeskowiak et al., 2019) which is associated with diarrhea and other intestinal inflammation related diseases. Others include symbiotic bacteria taxa like Oxalobacter that are responsible to degrading oxalate which then contributes to maintaining healthy gut environment. Additional groups include fiber degrading Ruminococcaceae and Lachnospiraceae (Zhang et al., 2018).

At the genus level, the abundance 48 bacteria taxa were increased at d14 while 33 were increased at d28 (Figure 2.10). Most of the bacterial taxa on both days were mostly commensal bacterial like Ruminococcaceae, Lachnospiraceae, Stretococcaceae, Coriobactaria and Oscillibacter (De et al.,

2020) which suggest a normal gut microbiome community development. A closer look on the differentially abundant bacteria taxa by sampling day showed the varying abundance by enzyme×temperature (Figure 2.11). At d 28, Streptococcus (Figure 2.12), Lachnospiraceae (2.15) and Eubacterium xyanophilum (Figure 2.15) a highly abundant in both CON and 2X of the HS stress group. These two bacteria taxa are commensal bacteria with Eubacterium xyanophilum also possessing fiber degrading characteristics. While the exact implication of the increased abundance of these taxa is not clear, it may hold some potential in unlocking some of the mechanism of carbohydrases activity in pigs.

The abundance of Erysipelotrichaceae (Figure 2.14) was significantly reduced in the enzyme group under HS at d28. This is particularly important because this bacteria taxon is associated with intestinal inflammation and related gastrointestinal disease (Kaakoush, 2015). This is not consistent with previous result of increased inflammatory cytokine mRNA expression. The reason for this is unclear and it requires further investigation.

2.6 Conclusion

In conclusion, results from this study suggest that heat stress does not affect the efficacy multienzyme carbohydrases. Both heat stress and carbohydrases impact animal performance independently. Effects of heat stress and carbohydrases on animal performance could be through their independent impact on digestibility, gut barrier integrity, immune activation and gut microbiome.

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Table 2.1. Ingredient composition of experimental diets.

	Diet		
	CON	1X	2X
Ingredients			
Wheat	198	188	178
Rapeseed Meal	56	56	56
Soybean Meal	150	150	150
Rye	260	260	260
Wheat Bran	100	100	100
Wheat Middlings	100	100	100
Soy Oil	80	80	80
L-Lysine	4	4	4
L-Threonine	1	1	1
DL-Methionine	1	1	1
L-Tryptophan	0	0	0
Limestone	11	11	11
MCP	6	6	6
Salt	4	4	4
Vitamin Premix	2.5	2.5	2.5
Mineral Premix	1.5	1.5	1.5
Xylanase Enzyme	0	10	20
Titanium dioxide premix	25	25	25
TOTAL	1000	1000	1000

CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture

1. Premix composition per kg DIET: Vit. A, 7500 IU; Vit. D3, 1500 IU; Vit E,20 IU; Vit B1,0.5 mg; Riboflavin, 3.0 mg; d-pantothenic acid, 10.0 mg; Vit B6, 1.0 mg; Vit B12, 15ug; Vit PP, 15.0 mg; Vit K3 1.0mg; Fe, 80mg; I, 0.50mg; Cu, 15mg; Mn, 40mg; Zn, 80mg; Se, 0.25mg.
2. Carbohydrase premix made to 0.05g carbohydrase/g premix to be added at 10, 20 g/kg to provide 0.5 and 1.0 g/kg
3. Titanium dioxide premix made at 5g titanium dioxide/g premix to be added at 25g/kg for each treatment to provide 75g/kg

Table 2.2. Calculated and analyzed composition of experimental diets.

	Diet		
	CON	1X	2X
Chemical composition (%)			
DE (kcal/kg)	3575	3575	3575
ME (kcal/kg)	3433	3433	3433
NE (kcal/kg)	2597	2597	2596
Ca	0.67	0.67	0.67
Total P	0.66	0.65	0.65
CP	19.04	19.03	19.03
Starch	33.46	33.46	33.46
Fat	0.99	0.99	0.99
Cellulose			
NDF	14.72	14.72	14.72
ADF	5.32	5.32	5.32
Amino acids composition			
Arg	1.15	1.15	1.15
His	0.47	0.47	0.47
Ile	0.69	0.69	0.69
Leu	1.24	1.24	1.24
Lys	1.09	1.09	1.09
Met	0.37	0.37	0.37
Cyst	0.76	0.76	0.76
Met+Cyst	1.13	1.13	1.13
Phe	0.83	0.83	0.83
Thr	0.76	0.76	0.76
Trp	0.23	0.23	0.23
Valine	0.83	0.83	0.83
Analyzed			
GE	4734	4763	4757
CP	21.25	21.5	21.19
Ca	0.7	0.74	0.68
P	0.72	0.73	0.71

CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. NDF: Neutral detergent fiber, ADF: Acid detergent fiber

Table 2.3. Primer sequences used for quantitative PCR.

Gene	Primer Sequence (5' - 3')	Annealing Temperature
HSP 70	F – TTCGTGGACAGAAGCCACAG	55
	R – TTGCTAGGATCTCCACCCGA	
HSP 90	F – GTCGAAAAGGTGGTTGTGTCG	55
	R – TTTGCTGTCCAGCCGTATGT	
IL 1 β	F – CCAAAGAGGGACATGGAGAA	55.7
	R – GGGCTTTTGTTCTGCTTGAG	
TNF α	F – CGTCGCCCACGTTGTAGCCAAT	55.7
	R – GCCCATCTGTCGGCACCACC	
MUC 2	F – AACCAGAAGCTGGTCCTGAA	55
	R – TGTCAGCCATCGTAGGAAAT	
PEPT 1	F – CAGACTTCGACCACAACGGA	55
	R – TTATCCCGCCAGTACCCAGA	
SGLT 1	F – GTGCAGTCAGCACAAAGTGG	55
	R – CCCGGTTCCATAGGCAAACT	
FUT 2	F – CGAGTGGATTGGGATCGAGG	55
	R – AAGAAGAATGGGGAGCCGAG	
Claudin 4	F – CTCTCGGACACCTTCCCAAG	55
	R – GCAGTGGGGAAGGTCAAAGG	
Occludin	F – CTACTCGTCCAACGGGAAAG	61
	R – ACGCCTCCAAGTTACCACTG	
GAPDH	F – GTTTGTGATGGGCGTGAAC	55
	R – ATGGACCGTGGTCATGAGT	

Table 2.4. Effect of carbohydrase supplementation and environmental temperature on performance of pigs fed wheat-based diets.

	Carbohydrase Level				Temperature			P-value			
	CON	1X	2X	SEM	TN	HS	SEM	Tem	Enz	Enz Linear	Enz Qudratic
BW d14, Kg	28.5	29	29	0.30	30	28	0.25	0.002	0.162	0.505	0.486
BW d28, Kg	39.5[a]	41[b]	40[a]	0.48	41	39	0.39	0.006	0.025	0.450	0.152
Period 1, 0-14 days											
ADG, g	589	649	632	21.27	664	582	17.37	0.002	0.141	0.165	0.152
ADFI, g	1177	1212	1185	22.97	1265	1117	18.76	0.0001	0.542	0.806	0.323
G/F	505	539	532	15.90	527	524	12.98	0.712	0.381	0.214	0.452
Period 2, 15-28 days											
ADG, g	760	842	784	26.57	813	777	21.70	0.261	0.097	0.646	0.120
ADFI, g	1294[a]	1433[b]	1290[a]	42.27	1424	1253	34.51	0.001	0.033	0.956	0.025
G/F	588	610	622	30.57	572	641	24.96	0.058	0.719	0.489	0.899
Period total, 0-28 days											
ADG, g	675	745	708	17.03	738	679	13.90	0.005	0.022	0.287	0.049
ADFI, g	1235[a]	1322[b]	1238[a]	25.55	1344	1185	20.68	0.0001	0.032	0.958	0.032
G/F	548	570	575	14.75	549	579	12.05	0.093	0.395	0.292	0.690
Rectal Temp	40	40	40	0.05	40	40	0.04	0.286	0.582	0.749	0.310
Skin Temp	37.5	37	37	0.16	36.3	38.0	0.13	0.0001	0.261	0.110	0.681
Resp Rate	34.5	35	35.5	0.52	31.3	38.7	0.43	0.0001	0.448	0.192	0.879

Data are means of 16 replicates per treatment in carbohydrase level and 24 replicates per treatment among temperature. Means with different superscript are different ($P<0.05$). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture, TN= Thermoneutral, HS= Heat stress, Tem= Temperature, Enz= Carbohydrase treatment.
Linear contrast between enzyme groups, Enz Quadratic= Quadratic contrast between enzyme groups

Table 2.5. Effect of carbohydrase supplementation and environmental temperature on ileal and jejunal digestibility coefficient of pigs fed wheat-based diets.

	Carbohydrase Level				Temperature				
	CON	1X	2X	SEM	TN	HS	SEM	Tem	Enz
Ileum									
Energy	0.689	0.714	0.712	0.04	0.665	0.745	0.03	0.333	0.583
Nitrogen	0.715	0.762	0.747	0.04	0.692	0.790	0.03	0.288	0.693
Phosphorus	0.510	0.514	0.482	0.03	0.464	0.540	0.03	0.329	0.605
Calcium	0.604	0.629	0.591	0.04	0.538	0.678	0.03	0.007	0.892
Jejunum									
Energy	0.680	0.716	0.676	0.02	0.665	0.716	0.02	0.039	0.551
Nitrogen	0.693	0.736	0.710	0.03	0.688	0.738	0.03	0.264	0.684
Phosphorus	0.554	0.497	0.530	0.02	0.523	0.531	0.02	0.656	0.426
Calcium	0.645	0.678	0.665	0.02	0.659	0.666	0.02	0.618	0.289

Data are means of 16 replicates per treatment in carbohydrase level and 24 replicates per treatment among temperature. Means with different superscript are different ($P<0.05$). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture, TN= Thermoneutral, HS= Heat stress, Tem= Temperature, Enz= Carbohydrase treatment.

Table 2.6. Correlation between performance and ileal digestibility coefficients.

	Energy Cor Coeff	P-value	N Cor Coeff	P-value	P Cor Coeff	P-value	Ca Cor Coeff	P-value
ADG	0.27	0.097	0.24	0.141	0.25	0.138	0.09	0.585
G: F	0.27	0.097	0.16	0.331	0.07	0.696	0.16	0.337

Statistical significance at P>0.05. Cor Coeff= Correlation coefficient, N= Nitrogen, P= Phosphorus, Ca= Calcium, ADG= Average daily gain, G: F= Gain to feed ratio.

Table 2.7. Correlation between digestibility and jejunal digestibility coefficients.

	Energy Cor Coeff	P-value	N Cor Coeff	P-value	P Cor Coeff	P-value	Ca Cor Coeff	P-value
ADG	0.26	0.112	0.18	0.282	0.10	0.539	0.03	0.869
G: F	0.17	0.294	0.08	0.629	0.08	0.635	0.16	0.335

Statistical significance at P>0.05. Cor Coeff= Correlation coefficient, N= Nitrogen, P= Phosphorus, Ca= Calcium, ADG= Average daily gain, G: F= Gain to feed ratio.

Table 2.8. Correlation with performance data.

	ADFI Cor Coeff	P-value
ADG	0.5719	0.0001
GF	-0.423	0.0027

Statistical significance at P>0.05. Cor Coeff= Correlation coefficient, ADFI= Average daily feed intake, ADG= Average daily gain, G: F= Gain to feed ratio.

Table 2.9. Effect of carbohydrase level on in vitro feed viscosity (mPa-s) over a time.

Treatment	Control	1X	2X	SEM	P-value
1 hour	3.46	3.39	3.41	0.037	0.514
2 hours	3.50[b]	3.66[a]	3.69[a]	0.041	0.087
3 hours	3.56[b]	3.60[b]	3.74[a]	0.013	0.004
4 hours	3.71[b]	3.85[b]	4.00[a]	0.023	0.007
5 hours	3.82[c]	3.93[b]	4.08[a]	0.014	0.002

Data are means of 3 replicates per treatment. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture

Table 2.10. Effect of carbohydrase level on in vitro ADF (%) over time.

Treatment	Control	1X	2X	SEM	P-value
1 hour	18.44	17.68	19.54	0.515	0.176
2 hours	19.21	18.11	19.89	0.5	0.178
3 hours	19.43	19.84	19.43	0.165	0.275
4 hours	20.78	20.42	21.49	0.88	0.713
5 hours	21.18	21.92	21.88	0.391	0.430

Data are means of 3 replicates per treatment. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture

Table 2.11. Interaction effect of carbohydrase level and environmental temperature on ileal gene expression of pigs fed wheat-based diets.

	Thermoneutral			Heat stress			
Trt	CON	1X	2X	CON	1X	2X	P-value
HSP70	1.077±0.42	0.823±0.46	0.786±0.42	1.534±0.37	1.511±0.39	1.30±0.46	0.960
HSP90	0.862±0.15	0.738±0.15	0.659±0.16	0.841±0.16	0.916±0.14	0.667±0.14	0.771
MUC2	1.197±0.24	1.192±0.23	1.212±0.24	0.712±0.24	1.112±0.23	0.786±0.23	0.643
IL1β	1.416±0.36	1.400±0.29	1.367±0.29	1.463±0.31	1.390±0.29	1.361±0.29	0.995
TNFα	1.010±0.21	1.192±0.21	1.149±0.21	1.362±0.19	1.208±0.18	1.041±0.18	0.488
PEPT1	0.689±0.26	0.910±0.26	0.812±0.24	0.916±0.24	1.637±0.24	1.033±0.24	0.521
SGLT1	0.912±0.26	1.030±0.26	0.926±0.22	1.022±0.24	1.195±0.24	1.152±0.24	0.971
FUT2	2.258±1.35	2.513±1.48	3.610±1.48	1.616±1.65	4.796±1.48	2.718±1.17	0.486
Claudin 4	2.493±0.60	2.686±0.65	2.684±0.65	2.414±0.60	2.649±0.65	2.121±0.55	0.893
Occludin	0.801±0.15	0.889±0.17	0.844±0.14	0.558±0.13	0.734±0.13	0.627±0.14	0.954

Data are means of 8 replicates per treatment ± Standard error. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. HSP 70= Heat shock protein 70, HSP 90= Heat shock protein 90, MUC2= Mucin 2, IL1 β = Interleukin 1β, TNFα = Tumor necrosis factor α, PEPT1= Peptide transporter type 1, SGLT1= Sodium dependent glucose cotransporter type 1, FUT2= Fucosyltransferase type 2.

Table 2.12. Effect of carbohydrase supplementation and environmental temperature on ileal gene expression of pigs fed wheat-based diets.

	Carbohydrase Level			Temperature		P-value	
	CON	1X	2X	TN	HS	Enz	Tem
HSP70	1.305±0.28	1.167±0.3	1.043±0.31	0.895±0.25	1.448±0.24	0.823	0.120
HSP90	0.852±0.11	0.827±0.1	0.663±0.11	0.753±0.09	0.808±0.09	0.413	0.658
MUC2	0.955±0.17	1.152±0.16	0.999±0.17	1.200±0.14	0.870±0.13	0.673	0.092
IL1β	1.439±0.24	1.395±0.2	1.364±0.2	1.394±0.18	1.405±0.17	0.972	0.967
TNFα	1.186±0.14	1.200±0.14	1.095±0.14	1.117±0.12	1.204±0.11	0.841	0.589
PEPT1	0.803±0.18	1.273±0.18	0.922±0.17	0.804±0.15	1.195±0.14	0.171	0.064
SGLT1	0.967±0.17	1.112±0.17	1.039±0.16	0.956±0.14	1.123±0.14	0.842	0.402
FUT2	1.937±1.07	3.654±1.05	3.164±0.94	2.793±0.83	3.043±0.84	0.504	0.834
Claudin 4	2.454±0.42	2.667±0.46	2.403±0.43	2.621±0.37	2.395±0.35	0.907	0.657
Occludin	0.679±0.10	0.812±0.11	0.736±0.10	0.845±0.09	0.640±0.08	0.668	0.091

Data are means of 16 replicates per treatment ± Standard error in carbohydrase level and 24 replicates per treatment± Standard error among temperature. Means with different superscript are different ($P<0.05$). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. HSP70= Heat shock protein 70, HSP90= Heat shock protein 90, MUC2= Mucin 2, IL1β = Interleukin 1β, TNFα = Tumor necrosis factor α, PEPT1= Peptide transporter type 1, SGLT1= Sodium dependent glucose cotransporter type 1, FUT2= Fucosyltransferase type 2.

Table 2.13. Interaction effect of temperature and carbohydrase level on jejunal gene expression of pigs fed wheat-based diets.

	Thermoneutral			Heat stress			
Trt	CON	1X	2X	CON	1X	2X	P-value
HSP70	1.614±0.35	0.789±0.41	1.376±0.44	1.726±0.41	1.148±0.41	1.456±0.38	0.931
HSP90	1.251±0.21	0.754±0.22	1.315±0.21	1.451±0.24	1.310±0.21	1.144±0.22	0.258
MUC2	1.233±0.2	1.075±0.21	1.350±0.23	1.187±0.21	0.772±0.25	1.092±0.23	0.814
IL1β	1.024±0.2	0.909±0.2	0.956±0.22	1.575±0.19	1.596±0.24	1.736±0.24	0.862
TNFα	1.284±0.3	1.239±0.3	1.202±0.27	1.556±0.24	1.493±0.27	1.576±0.3	0.975
PEPT1	0.663±0.19	0.506±0.17	0.702±0.16	0.639±0.	1.170±0.17	0.715±0.19	0.112
SGLT1	0.755±0.12	0.491±0.11	0.645±0.11	0.564±0.1	0.793±0.1	0.543±0.11	0.058
FUT2	1.552±0.76	1.524±0.69	1.934±0.76	1.921±0.64	2.318±0.76	1.418±0.64	0.649
Claudin 4	1.438±0.34	1.567±0.31	1.758±0.37	1.629±0.34	1.660±0.31	1.037±0.37	0.385
Occludin	1.541±0.26	1.259±0.26	1.921±0.26	1.557±0.25	1.578±0.25	1.608±0.25	0.47219

Data are means of 8 replicates per treatment ± Standard error. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. HSP 70= Heat shock protein 70, HSP 90= Heat shock protein 90, MUC2= Mucin 2, IL1 β = Interleukin 1β, TNFα = Tumor necrosis factor α, PEPT1= Peptide transporter type 1, SGLT1= Sodium dependent glucose cotransporter type 1, FUT2= Fucosyltransferase type 2.

Table 2.14. Effect of carbohydrase supplementation and environmental temperature on jejunal gene expression of pigs fed wheat-based diets.

	Carbohydrase Level			Temperature		P-value	
	CON	1X	2X	TN	HS	Enz	Tem
HSP70	1.670±0.27	0.969±0.29	1.416±0.29	1.260±0.23	1.443±0.23	0.215	0.578
HSP90	1.351±0.16	1.032±0.15	1.230±0.15	1.107±0.12	1.302±0.13	0.354	0.286
MUC2	1.210±0.14	0.924±0.16	1.221±0.16	1.220±0.12	1.017±0.13	0.341	0.271
IL1β	1.299±0.14	1.253±0.16	1.346±0.16	0.963±0.12	1.636±0.13	0.918	0.0006
TNFα	1.420±0.19	1.366±0.2	1.389±0.2	1.241±0.17	1.542±0.16	0.982	0.203
PEPT1	0.651±0.14	0.838±0.12	0.708±0.13	0.624±0.1	0.841±0.11	0.576	0.152
SGLT1	0.659±0.08	0.642±0.07	0.594±0.08	0.630±0.06	0.633±0.06	0.820	0.974
FUT2	1.736±0.5	1.921±0.51	1.676±0.5	1.670±0.43	1.885±0.39	0.939	0.714
Claudin 4	1.533±0.24	1.614±0.22	1.397±0.26	1.588±0.2	1.442±0.2	0.820	0.604
Occludin	1.549±0.18	1.419±0.18	1.764±0.18	1.574±0.15	1.581±0.14	0.403	0.972

Data are means of 16 replicates per treatment ± Standard error in carbohydrase level and 24 replicates per treatment ± Standard error among temperature. Means with different superscript are different ($P<0.05$). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. HSP70= Heat shock protein 70, HSP90= Heat shock protein 90, MUC2= Mucin 2, IL1 β = Interleukin 1β, TNFα = Tumor necrosis factor α, PEPT1= Peptide transporter type 1, SGLT1= Sodium dependent glucose cotransporter type 1, FUT2= Fucosyltransferase type 2.

Table 2.15. Effect of carbohydrase supplementation and environmental temperature on serum parameters of pigs fed wheat-based diets

	Carbohydrase Level			Temperature		P-value	
	CON	1X	2X	TN	HS	Enz	Tem
Glucose (mg/dL)							
d 1	81.75±4.02	85.40±4.15	85.58±4.02	93.92±3.39	74.57±3.25	0.754	0.0002
d 14	66.90±5.33	67.61±5.33	65.36±5.51	68.78±4.35	64.47±4.45	0.957	0.492
d 28	71.69±9.49	82.75±9.21	79.71±9.12	76.80±7.65	79.30±7.50	0.693	0.816
Insulin (μg/L)							
d 1	0.23±0.05	0.20±0.05	0.20±0.05	0.21±0.04	0.21±0.04	0.876	0.941
d 14	0.23±0.04	0.24±0.04	0.25±0.04	0.26±0.03	0.22±0.03	0.915	0.364
d 28	0.21±0.03	0.21±0.03	0.21±0.03	0.19±0.02	0.23±0.02	0.999	0.231
NEFA (mmol/L)							
d 1	0.22±0.05	0.22±0.05	0.24±0.5	0.13±0.02	0.32±0.04	0.895	0.002
d 14	0.11±0.01	0.1±0.01	0.1±0.01	0.1±0.005	0.1±0.005	0.584	0.961
d 28	0.14±0.03	0.16±0.03	0.17±0.03	0.21±0.03	0.11±0.03	0.801	0.015
TAG (mmol/L)							
d 1	0.48±0.11	0.59±0.11	0.78±0.11	0.51±0.08	0.72±0.09	0.163	0.010
d 14	0.54±0.03	0.44±0.03	0.44±0.03	0.42±0.02	0.53±0.03	0.058	0.012
d 28	0.39±0.04	0.29±0.04	0.34±0.04	0.38±0.03	0.31±0.04	0.212	0.132
BUN (mg/dL)							
d 1	12.43±2.29	14.48±2.02	16.07±2.02	12.25±1.76	16.40±1.70	0.498	0.099
d 14	8.03±0.74	7.19±0.74	7.21±0.74	7.02±0.61	7.94±0.61	0.661	0.625
d 28	10.38±1.76	10.93±1.63	12.47±1.63	11.68±1.36	10.83±1.36	0.659	0.663

Data are means of 16 replicates per treatment ± Standard error and 24 replicates per treatment ± Standard error among temperature. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture. NEFA= Non esterified fatty acid, TAG= triacyl-glycerol, BUN= Blood urea nitrogen.

Table 2.16. Interaction effect of temperature and carbohydrase supplementation on intestinal histomorphometric characteristics in pigs fed wheat-based diets.

	Thermoneutral			Heat stress				P-value
Trt	CON	1X	2X	CON	1X	2X	SEM	
Jejunum								
Villus Height (μm)	584.96^{ab}	668.97^{a}	564.19^{b}	551.85^{b}	560.85^{b}	564.21^{b}	23.90	0.080
Crypt Depth (μm)	234.73	258.46	232.71	216.15	224.80	246.98	16.45	0.340
V/C Ratio	2.56	2.67	2.50	2.66	2.54	2.38	0.15	0.670
Ileum								
Villus Height (μm)	584.29	602.98	585.56	527.56	571.11	535.09	21.29	0.830
Crypt Depth (μm)	216.61	224.81	241.99	202.06	236.48	215.55	14.91	0.430
V/C Ratio	2.77	2.77	2.49	2.66	2.46	2.50	0.11	0.350

Data are means of 8 replicates per treatment. Means with different superscript are different (P<0.05). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture

Table 2.17. Effect of carbohydrase supplementation and environmental temperature on intestinal histomorphometric characteristics in pigs fed wheat-based diets.

	Carbohydrase level				Temperature			P-value	
	CON	1X	2X	SEM	TN	HS	SEM	Enz	Tem
Jejunum									
Villus Height (μm)	568.40	614.91	564.20	16.90	606.04	558.97	13.80	0.070	0.020
Crypt Depth (μm)	225.44	241.63	239.85	11.63	241.96	229.31	9.50	0.560	0.350
V/C Ratio	2.61	2.60	2.44	0.11	2.58	2.53	0.09	0.440	0.680
Ileum									
Villus Height (μm)	555.92	587.04	560.32	15.05	590.94	544.58	12.29	0.300	0.010
Crypt Depth (μm)	209.33	230.64	228.77	10.54	227.80	218.03	8.61	0.300	0.430
V/C Ratio	2.71	2.62	2.49	0.08	2.68	2.54	0.06	0.150	0.140

Data are means of 16 replicates per treatment and 24 replicates per treatment ± Standard error among temperature. Means with different superscript are different ($P<0.05$). CON= Control, 1X= 50g/tonne carbohydrase admixture, 2X= 100g/tonne of carbohydrase admixture.

Table 2.18. Effect of carbohydrases on beta diversity

	PERMANOVA	PERMDISP
Bray Curtis	0.848	0.187
Jaccard	0.610	0.184
Unweighted Unifrac	0.713	0.460
Weighted Unifrac	0.962	0.327

Table 2.19. Effect of heat stress beta diversity

	PERMANOVA	PERMDISP
Bray Curtis	0.075	0.550
Jaccard	0.030	0.303
Unweighted Unifrac	0.128	0.369
Weighted Unifrac	0.066	0.660

Table 2.20. Effect of sampling day on beta diversity

	PERMANOVA	PERMDISP
Bray Curtis	0.001	0.017
Jaccard	0.001	0.009
Unweighted Unifrac	0.002	0.001
Weighted Unifrac	0.054	0.028

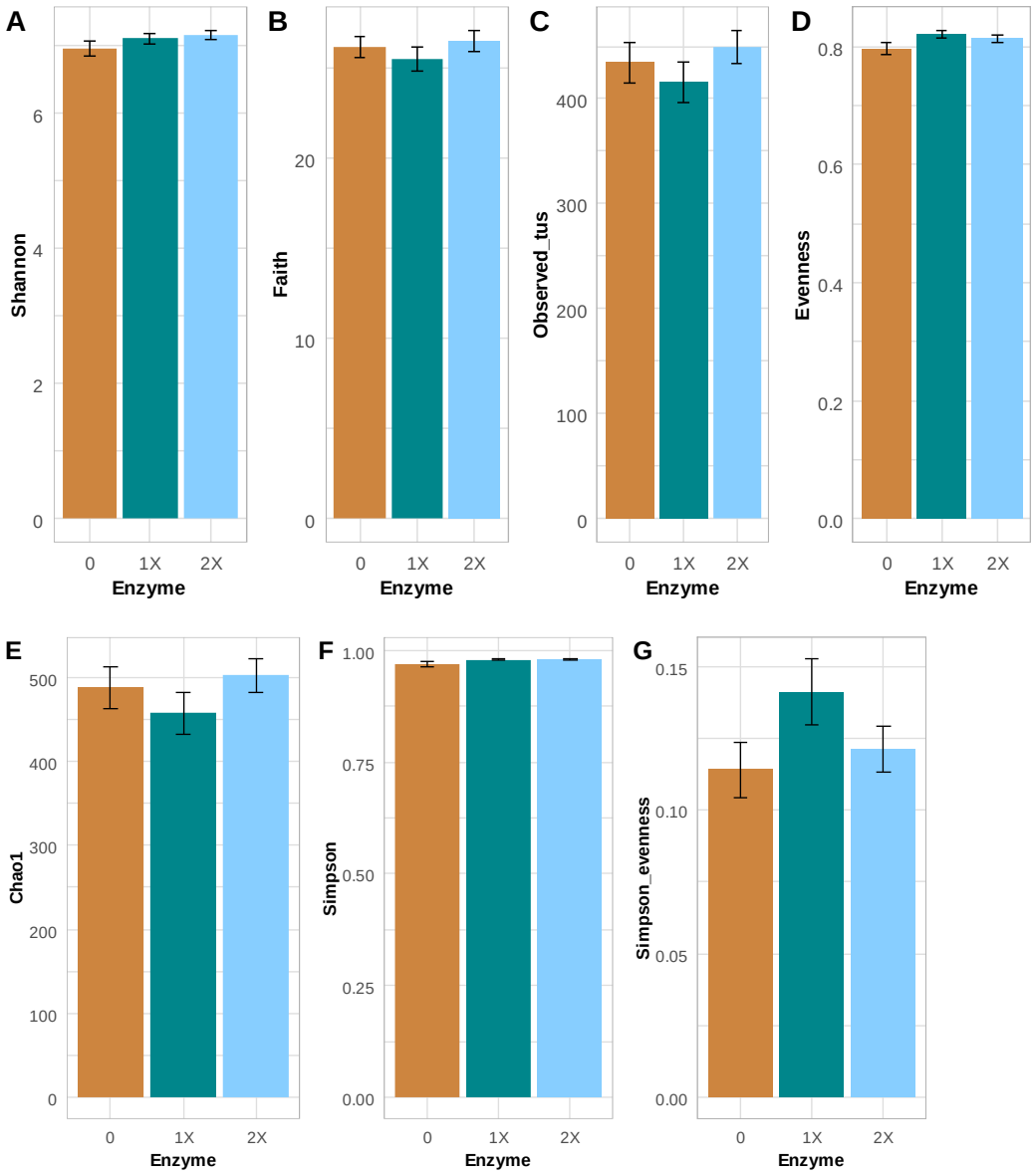

Figure 2.1. Effect of carbohydrase treatment on alpha diversity. (A) Shanon, (B) Faith, (C) Observed OUT, (D) Evenness, (E) Chao1, (F) Simpson and (G) Simpson evenness.

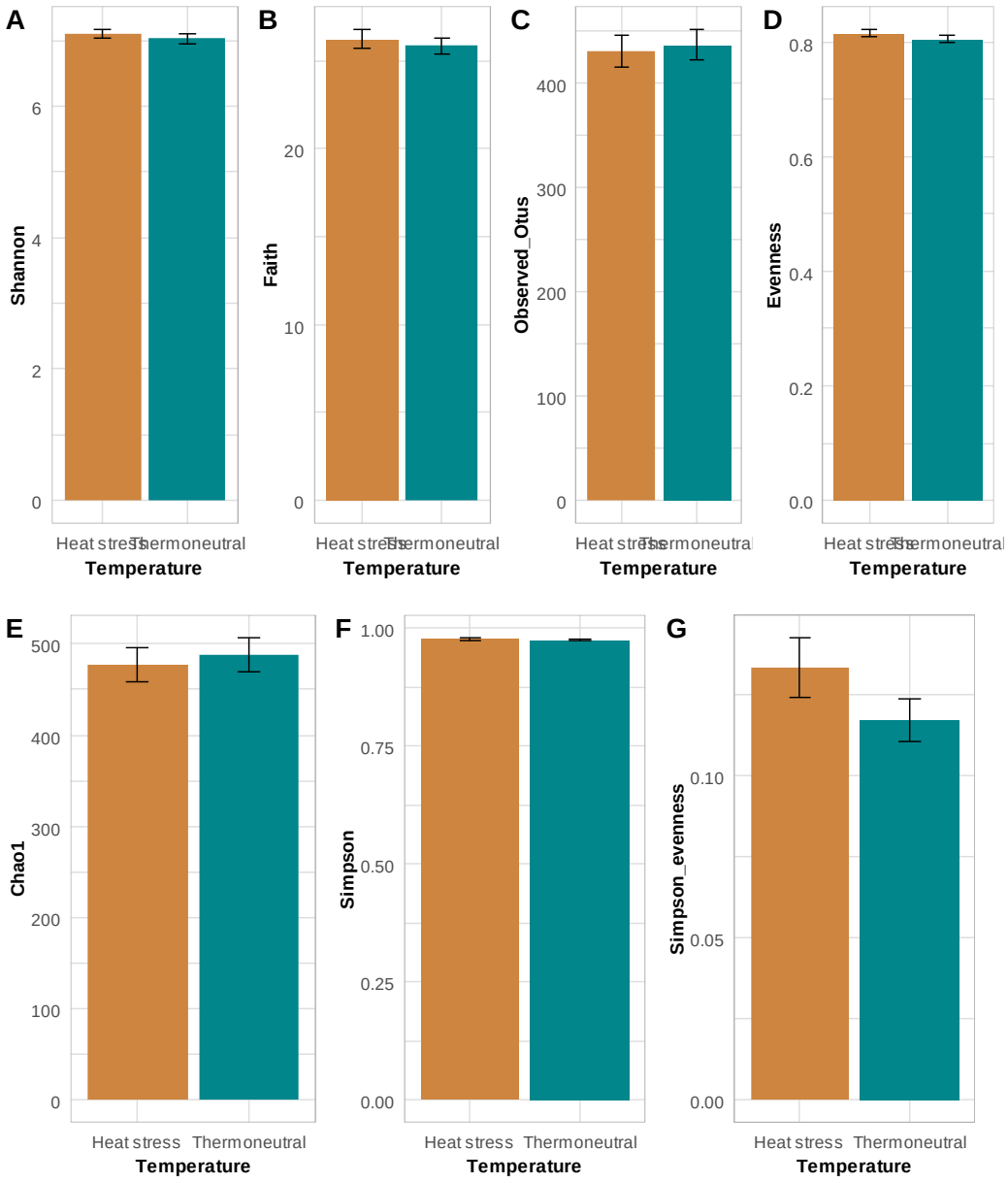

Figure 2.2. Effect of heat stress on alpha diversity. (A) Shanon, (B) Faith, (C) Observed OUT, (D) Evenness, (E) Chao1, (F) Simpson and (G) Simpson evenness.

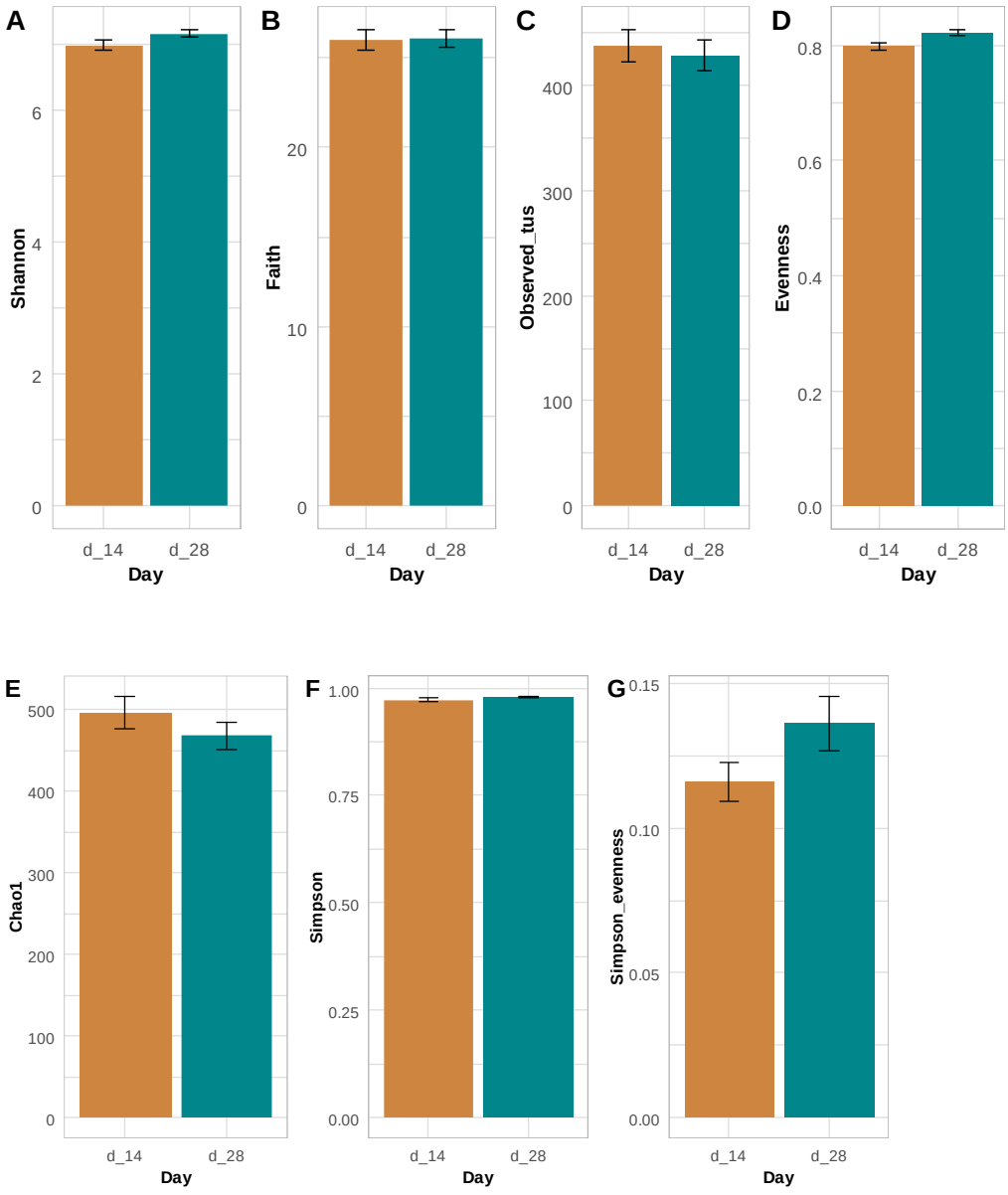

Figure 2.3. Effect of sampling day on alpha diversity. (A) Shanon, (B) Faith, (C) Observed OUT, (D) Evenness, (E) Chao1, (F) Simpson and (G) Simpson evenness.

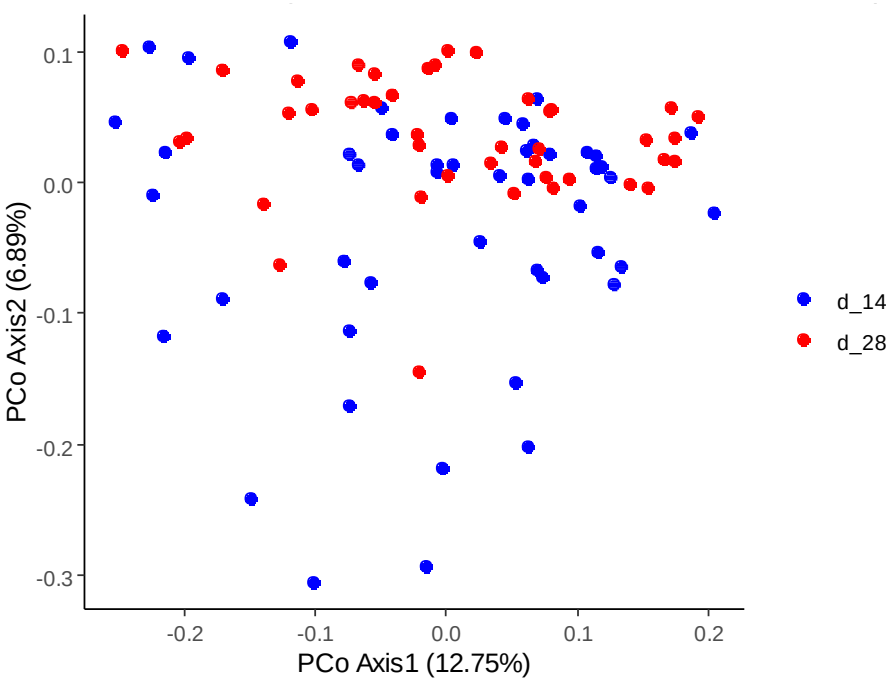

Figure 2.4. PCoA of unweighted UniFrac distance between fecal samples by sampling day.

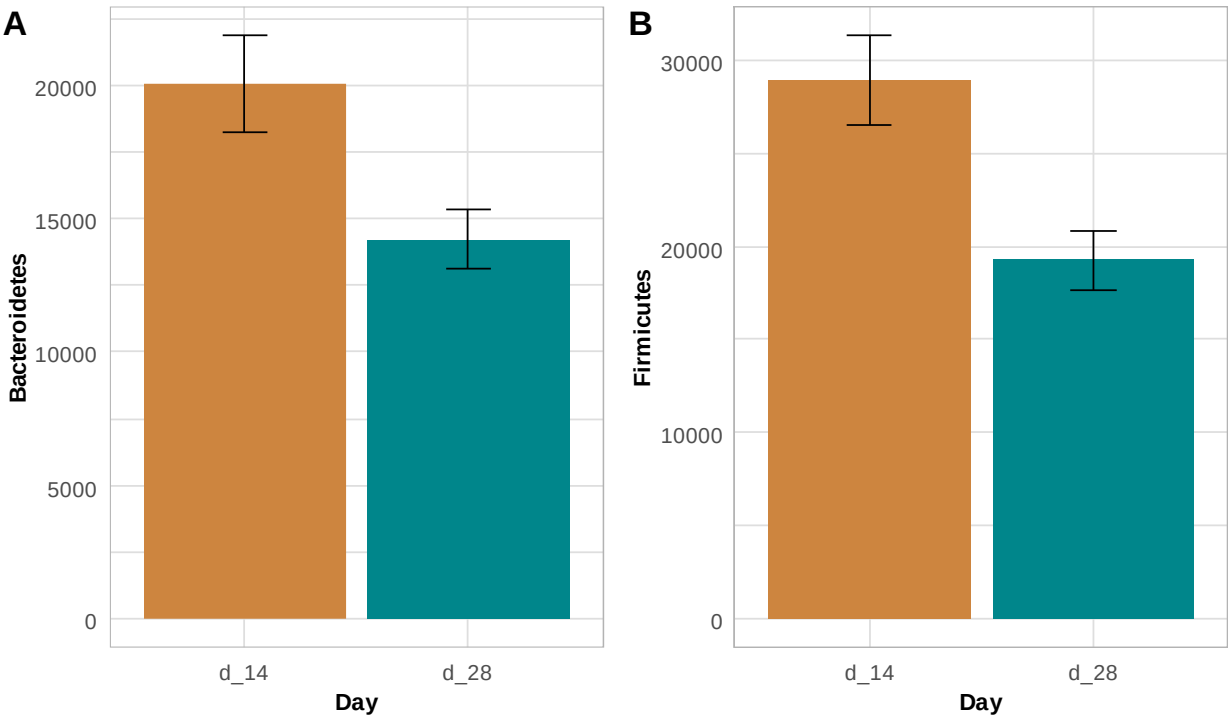

Figure 2.5. Relative abundance of the two dominant bacteria phyla. (A) Bacteroidetes, (B) Firmicutes.

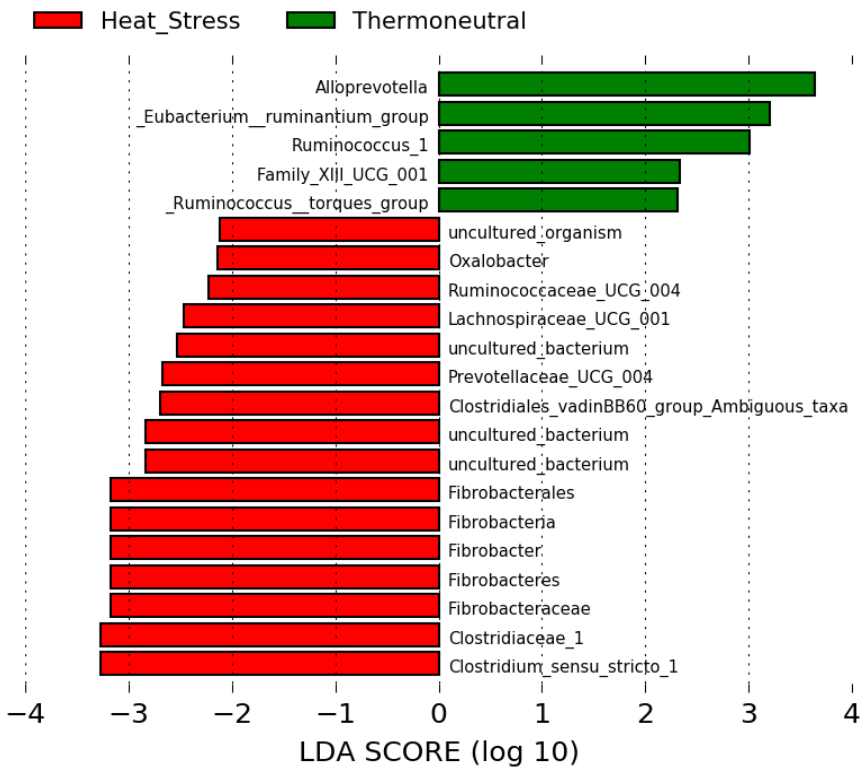

Figure 2.6. Differentially abundant bacteria taxa between temperature groups.

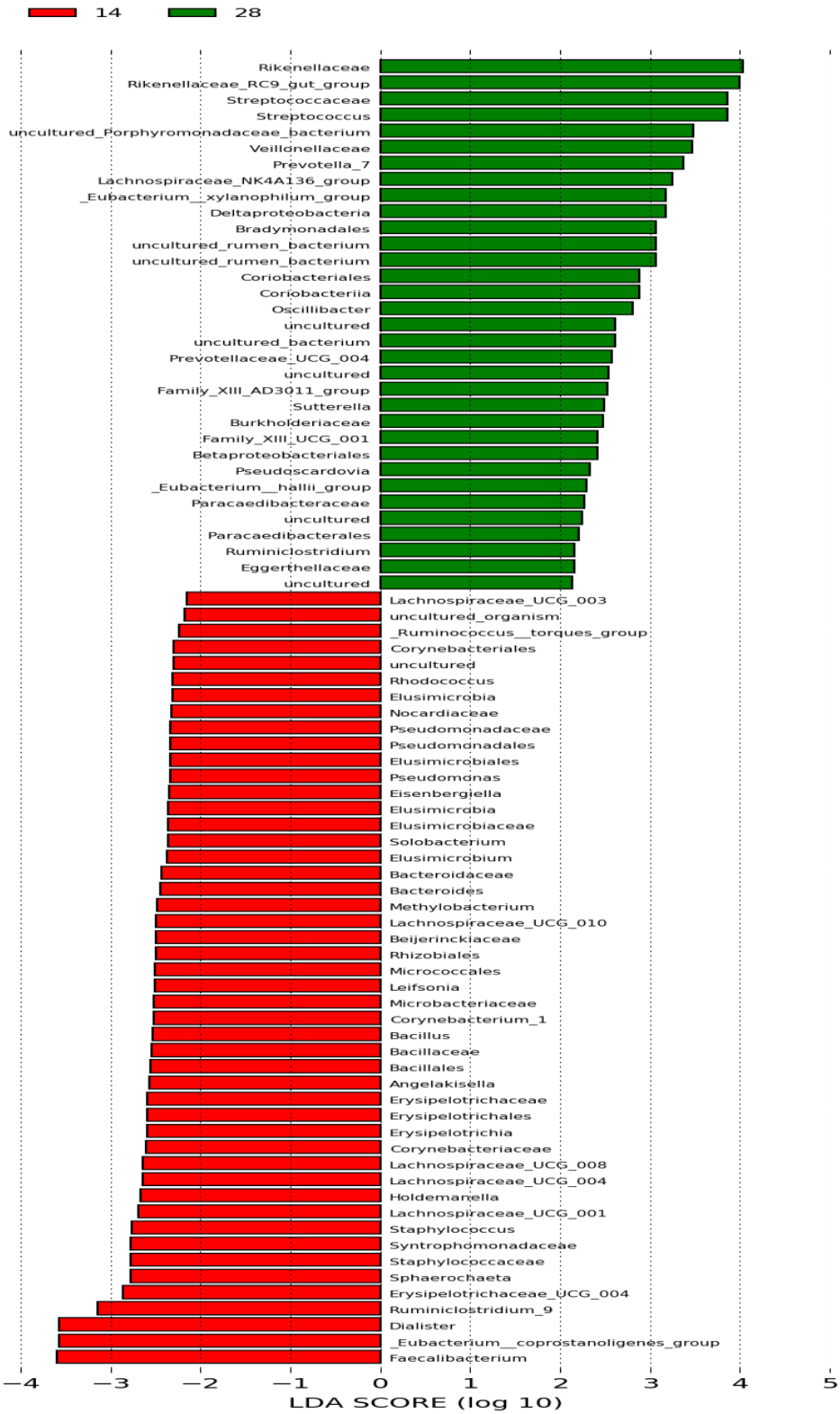

Figure 2.7. Differentially abundant bacteria taxa among groups by experimental day.

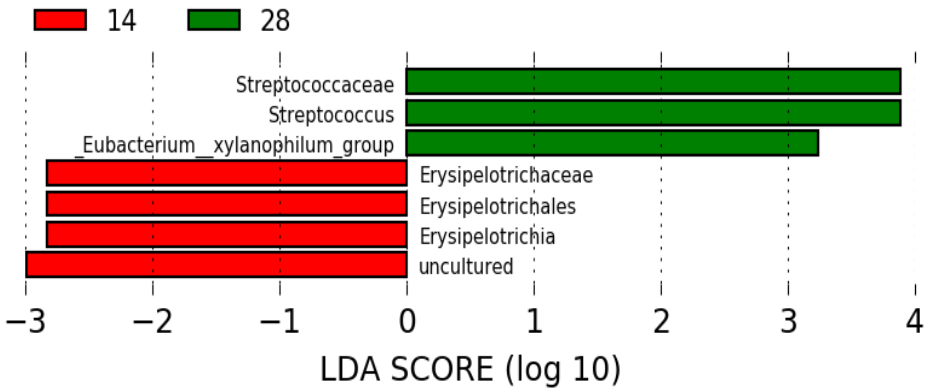

Figure 2.8. Differentially abundant bacteria taxa by sampling days and then treatment groups

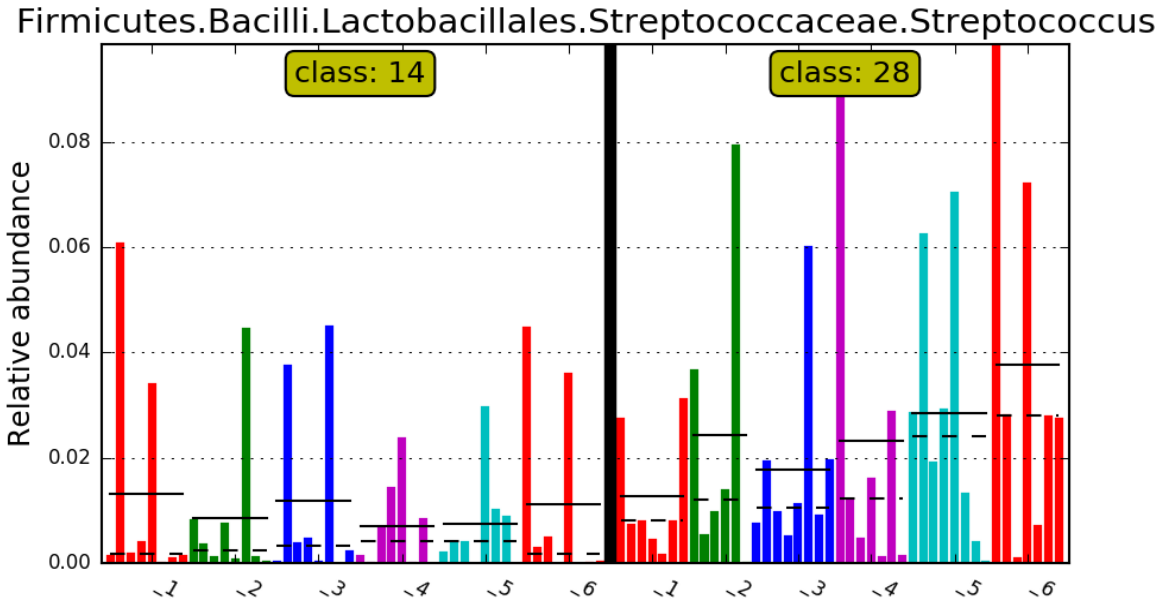

Figure 2.9. Differentially abundant bacteria taxa by enzyme×temperature at the two sampling days.

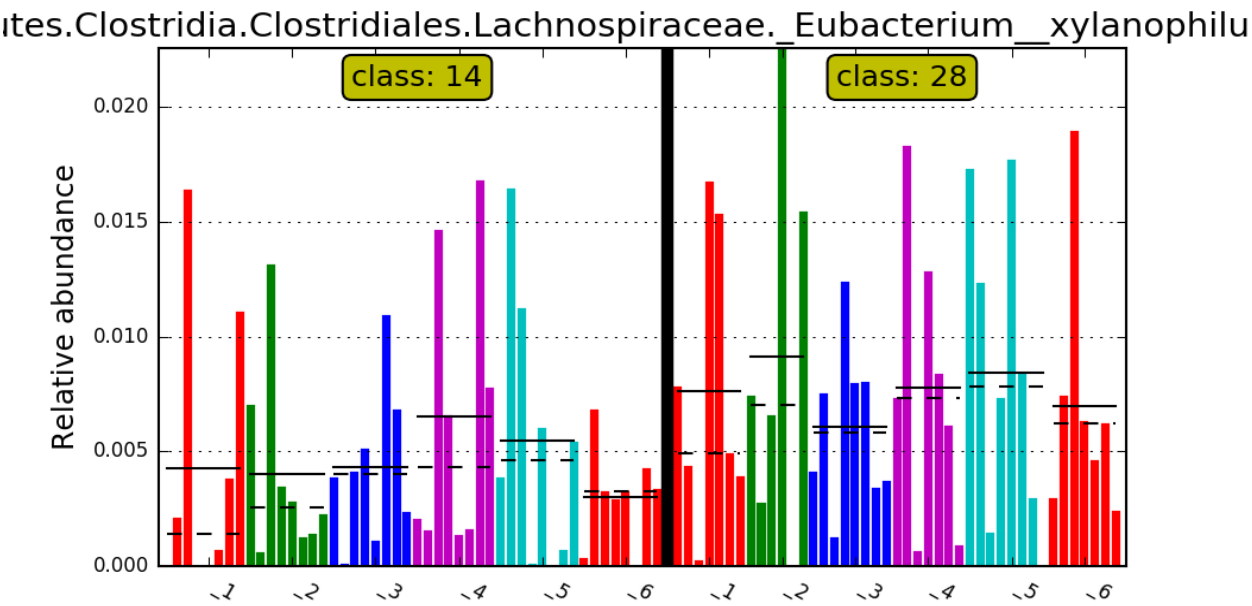

Figure 2.10. Abundance of Eubacterium xylanophilum by enzyme×temperature at the two sampling days.

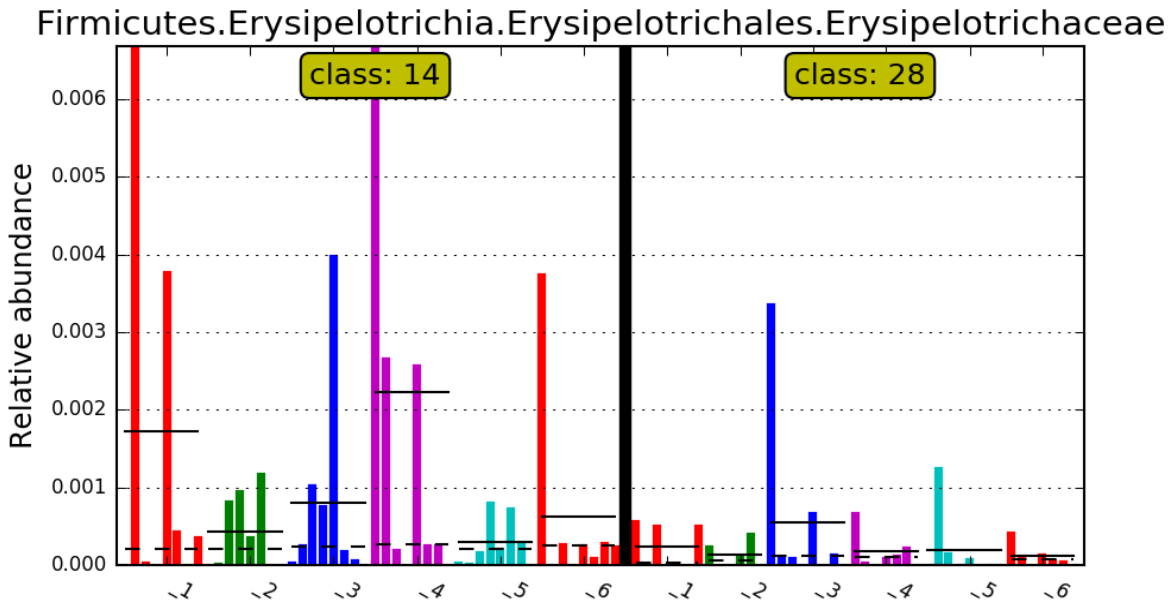

Figure 2.11. Abundance of Erysipelotrichaceae by enzyme×temperature at the two sampling days.

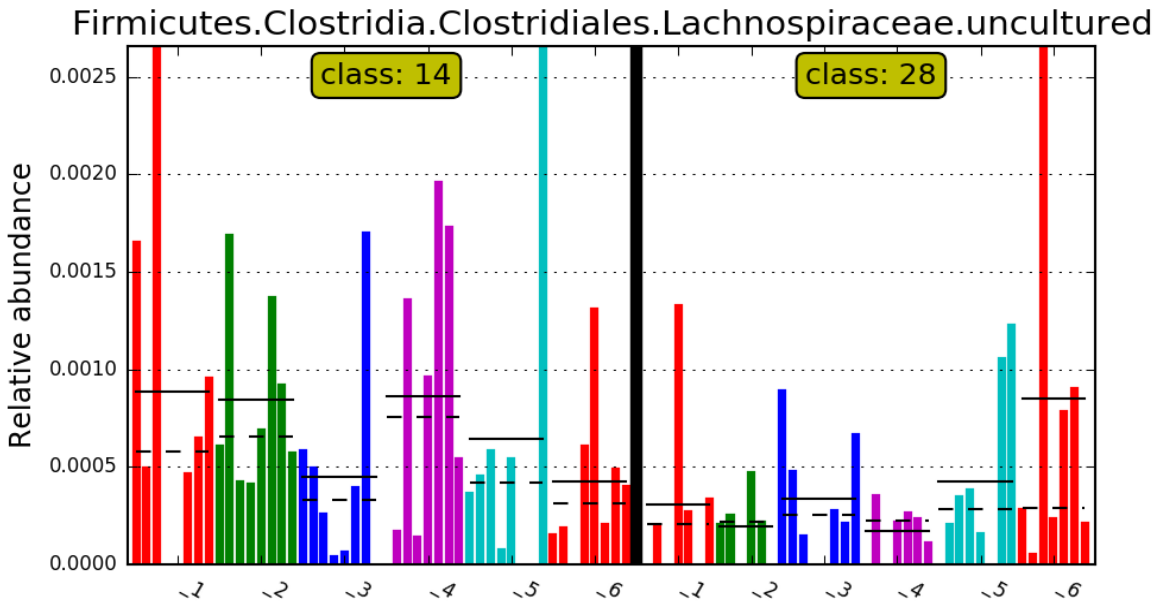

Figure 2.12. Abundance of Lachnospiraceae by enzyme×temperature at the two sampling days.

CHAPTER 3. SUMMARY

There is an increase in the quantity of dietary fiber used in swine diets due to increase in the use of cereal coproducts. The dietary fiber component of feed cannot be digested efficiently in the foregut but are fermented to VFA in the hindgut by microbes, a process that is less efficient due to energy loss as heat and gases like methane, hydrogen and carbon dioxide. There has been effort to increase foregut utilization of dietary fiber, with the use of exogenous carbohydrases in pig production being one. Despite the potential of exogenous carbohydrases, their effects on animal performance have not been consistent. In the swine industry, heat stress causes significant economic loss due to its adverse effect on performance of animals. With higher global warming, heat stress is bound to affect animals more adversely. Given the increasing use of exogenous carbohydrases in swine production, this study was conducted to investigate the impact of heat stress on the efficacy of the carbohydrases with a view to understanding how ambient temperature may alter the effect of these enzymes on animal performance. Specific responses considered were impact on growth performance, nutrient digestibility, serum metabolites, intestinal integrity and gut microbial community structure.

Results presented in chapter 2 showed that carbohydrase admixture had a quadratic effect on growth performance, in which performance improved at 1X but reduced at 2X. This effect was possibly driven by differences in feed intake between the enzyme levels. However, nutrient digestibility was not affected. Carbohydrase supplementation increased *in vitro* viscosity of feed in a dose dependent manner, suggesting that at high dosage carbohydrase may increase viscosity which could have negative effect of feed intake and consequently growth performance. Although carbohydrase supplementation did not affect overall microbial community structure, there was an

enrichment of specific fiber degrading microbes like Streptococcus and Eubacterium xylanophilu. Heat stress, on the other hand, reduced growth performance and might also have impaired gut health.

A major limitation of the study was that enzyme activity in the feed was not confirmed. Therefore, it was difficult to ascertain that projected enzyme activity levels were actually in the feed. Another limitation was that enzyme formulation was not well matched to substrate levels in the feed. A better substrate-enzyme matching could have resulted in better optimization of response to the enzyme and animal performance.

Overall, results from this study indicate that both heat stress and enzyme affected feed intake. Therefore, it was difficult to separate the extent to which differences in feed intake played a major role on the observed effects of carbohydrases and heat stress on growth performance. In the future a pair-feeding experiment should be done in which the feed intake of animals under heat stress and thermoneutral and those with/without enzyme supplementation are matched. Additionally, the *in vitro* experiment indicated that viscosity of the diet with 2X enzyme level was higher than 1X and control. In future experiments, it will be critical to determine the viscosity of digesta in animals as this might be useful for optimizing the level of enzyme supplementation.

In conclusion, results from this study indicate that heat stress does not affect the efficacy multienzyme carbohydrases, and it provides key insights into the effect of carbohydrases and heat stress on performance and physiology of growing pigs.

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