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Starch Digestion in Broiler Chickens: A Literature Study and an *In vitro* Comparison with Pigs

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ABSTRACT

Starch is a quantitatively important source of energy in poultry diets. Despite high loads of starch in the diet, poultry species can utilize a variety of starch sources very efficiently. Pancreatic α-amylase is the major responsible enzyme for starch digestion in birds and mammals. Considering high starch digestion capacity of broiler chickens, a hypothesis was made that a fixed certain amount of chicken pancreas is more effective at degrading starch than the same amount of pancreas from pigs. To test this hypothesis, amylase activity of the pancreatic tissues from 9 broiler chickens and 9 pigs was determined by *in vitro* starch digestibility analysis. The method was adapted from the total starch determination analysis with some modifications to the procedure. Homogenates of all pancreas tissues were prepared to be used as a source of α-amylase during starch digestibility analysis. Sieved wheat flour was used as a starch substrate. Results of the study showed that there is no systematic difference between the same certain amounts of pig and poultry pancreases on their ability to digest starch. However, correction of the pancreas weights for the body weights of the species revealed that on average chickens have twice larger (P<.01) pancreases than pigs relative to their body weights. Specific amylase activities (mg starch digested/min/mg protein) were also not significantly different between species. Large individual variation was observed in enzyme activities within both species.

Furthermore, enzyme kinetics of the starch digestion was also analyzed and discussed. The literature on enzyme related factors of starch digestion was extensively reviewed and presented in this thesis.

Keywords: starch digestion, pancreas, amylase, broiler chicken, pig, enzyme activity

CONTENTS

1. INTRODUCTION

Today's modern poultry breeds exhibit extreme production performance compared to the ones in 1950s. For instance, in 1956, a broiler breed needed 84 days to reach 1,82 kg, 10 years later this period was shortened to 60 days, and as a result of intensive selection, in 2000 it took a broiler 34 days to reach the same weight (Hafez and Hauck, 2005 as cited in (Buzała et al., 2015)).

Rapid growth of broiler chickens requires adequate amounts of energy to be supplied through the diet alongside with protein, particularly essential amino acids. Most of the energy needed for fast growth of broilers comes from starch which is quantitatively the major nutrient in broiler diets. It provides around 60% of apparent metabolizable energy (AME) content of poultry feed (Cowieson, 2005) and broiler diets may comprise up to 50% of starch on dry matter (DM) basis (Svihus, 2014b). Several studies (Krogdahl, 1985, Krogdahl and Sell, 1989, Noy and Sklan, 1995, Wiseman et al., 1998, Tancharoenrat et al., 2013) reported physiological limitations for lipid digestion in young birds. Impeded fat digestion was related to either low pancreatic lipase availability or limited bile secretion by previously mentioned authors. Although fats and oils possess about twice higher energy density (37 kJ/g) for the same mass of most carbohydrates (17 kJ/g), yet, starch remains the primary source of energy for poultry.

Starch is a reserve carbohydrate that is present in many plants. The main sources of starch in commercial broilers are cereal grains such as wheat (starch content[1] ~70% DM), maize (~65% DM) and barley (~60% DM). In cereal grains, starch is stored inside the granules in the endosperm. The granules consist of semi-crystalline and amorphous layers and exist in various sizes and shapes. Amylose and amylopectin are the main building components of layers and both are polymers of glucose units (Svihus et al., 2005).

α-amylase is the major responsible enzyme for starch digestion in birds and mammals. Although, there is some α-amylase activity present in the saliva of humans and pigs (McDonald, 2002), in poultry, saliva does not contain any α-amylase and pancreatic α-amylase is the only starch degrading enzyme (Moran, 1982, Wiseman, 2006).

There are several factors affecting starch digestion in poultry. Those factors can be grouped in two categories: feed related factors: such as feed processing, particle size, starch/granule characteristics in the feed, presence of fibers, antinutritional factors (ANFs), etc. and non-feed related factors: bird's genetics, age. Recent review by Zaefarian et al. (2015) explicitly discusses those factors causing variation in starch

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[1] http://www.feedipedia.org

digestibility in broiler chickens fed cereal diets. In fact, pancreatic α-amylase activity does not take place among factors affecting starch digestibility. Moran (1982) postulated that pancreatic α-amylase secretion is never insufficient to support starch digestion in fowl and fowl is capable to increase the amount of pancreatic amylase secretion to keep up with high levels of starch intake (Moran (1985) as cited in (Zaefarian et al., 2015)). This statement was later supported by Noy and Sklan (1995) where they quantified the amount of pancreatic enzymes secreted into the duodenum from 4 days to 21 day post-hatch and revealed that amylase secretion increased more compared to the other enzymes. However, according to the study amylase and protease synthesis during the first days of post-hatch can be limited. Gracia et al. (2003) observed significant increase in starch digestibility when α-amylase was supplemented to corn-soybean meal diets of broilers, hinting that endogenous α-amylase can be limiting for optimal starch digestion. Kaczmarek et al. (2014) investigated the effect of amylase supplementation of starch digestibility and growth performance of broiler chicks fed corn-soybean diet. Contrary to the earlier studies no effect of amylase supplementation on starch digestibility and growth performance was observed compared to the control (no enzyme) group. Authors recurrently concluded that digestive enzyme deficiency in young chickens may not be as evident as thought. Generally, poor starch digestibility in broilers is not attributed to inadequate α-amylase concentration in the small intestine (Wiseman, 2006).

Looking at starch digestion as a simple hydrolysis reaction, then another key factor would be time required for complete hydrolysis. Too short exposure time to α-amylase may be one of the reasons for suboptimal starch digestion (Svihus, 2011b). Feed intake level and particle size of the diet have shown considerable impact on feed retention time in the digestive tract and hence on the starch digestibility. Svihus (2011b) clearly illustrated declining total tract starch digestibility for increasing feed consumption of broiler chickens receiving wheat based diet. This result was previously supported by Svihus (2006) where he observed decreased AME values (due to lower starch digestibility) for broilers overconsuming pelleted wheat-based diet. High levels of undigested starch in the excreta was detected by Péron et al. (2005) where they provided finely ground wheat diet to broiler chickens immediately after food-deprivation, again relating reduced starch digestibility to feed overload and consequently fast transit of feed through the digestive tract. Feeding with whole cereals was also believed to decrease passage rate of feed through upper digestive tract, however this hypothesis was not proved by Svihus et al. (2002), although Svihus and Hetland (2001), Hetland et al. (2002), Svihus et al. (2004) observed improved starch digestibility when broilers were fed unground cereals. Some of those aforementioned aspects of starch digestion in chickens will be extensively discussed later with more emphasis on enzyme related factors.

Pancreas is known to contain several enzymes as well as amylase (Berdutina et al., 2000). Comparative study of pancreatic amylase activities between red jungle fowl and a broiler breed has been done by Kadhim et al. (2011). Although significant differences were found in the enzyme activities between two breeds, these differences were generally associated with differences in body weight. However, no literature was found comparing pancreatic amylase activity of broilers with the activity of amylase from pig pancreas. The aim of this study, was to compare the efficiency of chicken and pig pancreatic amylases at degrading starch. Considering, very high starch digestion capacity of broilers a hypothesis was made that a fixed certain amount of chicken pancreas is more effective at degrading starch than the same amount of pancreas from pigs. To test this hypothesis, amylase activity of the collected chicken and pig pancreatic tissues were determined by *in vitro* starch digestibility analysis in our lab. The literature on enzyme activity, and other related factors of starch digestion were also reviewed and presented herein.

2. LITERATURE REVIEW

2.1. Distinctiveness of the digestive system of poultry *(chicken example)*

Chickens are commonly known as granivorous poultry species. Although, Klasing classifies chickens as omnivorous (Klasing, 1999, Klasing, 2005), it is a fact that cereal grains make up most of the poultry diets and poultry possesses distinctive digestive anatomy to efficiently utilize even whole seed grains.

Unlike pigs and some other monogastrics, chickens cannot reduce feed size in the mouth. Ingested feed moves down to crop which serves mainly as a storage organ in birds. Considerable moisturization takes place in the crop, which may aid grinding and enzymatic digestion further down the digestive tract (Svihus, 2014a). Crop is not thought to have a direct significant contribution to enzymic digestion, since no enzyme secreted by the crop wall (Svihus, 2014a). However, Bolton (1965) investigated the digestion process in the crop and reported extensive hydrolysis of starch (more than 20 %) into sugars in the crop. Thereafter, Champ et al. (1983) identified three *Lactobacillus* strains from isolated chicken crop that produce amylase, hinting to the fact that some microbial pre-digestion occurs in the crop. According to Bolton (1965) some absorption of sugars and microbial fermentation of sugars to lactic and other acids also takes place in the crop. Fermentation of simple sugars into acids by crop *Lactobacillus* species have been well established (Sarra et al., 1992, Hilmi et al., 2007, Classen et al., 2016). In addition, it must be noted that crop has a noticeable storage function under the situations of intermittent feeding, hence transient store of the feed in the crop is not always the case for poultry which has ample access to feed throughout a day (Svihus, 2014a).

Distinct to the pigs, chickens' gastric region consists of two compartments called proventriculus (glandular stomach) and ventriculus or gizzard (muscular stomach) (Klasing, 1999). Enzymic digestion initiates when the feed meets proventriculus. The walls of the proventriculus secrete gastric juices: Hydrochloric acid (HCl), pepsinogen (precursor of pepsin) and mucus. HCl and pepsinogen secreted by the gastric glands initiates digestion of proteins by the action of later formed pepsin, and mucus secreted by the tubular glands (Klasing, 1999) forms a protective coat to prevent gastric wall from the damage of HCl and pepsin (Duke, 1994). However, due to the small volume of the proventriculus, retention time of the feed is very short here and thus, gizzard is the main site where initial digestion takes place by the action of gastric juices (Svihus, 2011a).

Gizzard has strongly myolinated muscles and a koilin layer, which aids in the grinding process due to its sand-paper-like surface (Svihus, 2014a). Particle size reduction occurs when the feed leaves

proventriculus and enters gizzard. Thus, the main function of the gizzard is grinding and mixing of the feed which first action increases the surface area of the feed and the latter facilitates the action of digestive fluids on feed particles. Refluxes from gizzard to proventriculus allows additional secretions to be added to the feed (Duke, 1992 as cited in (Svihus, 2011a)).

Total tract retention time is lower in chickens compared with pigs, nevertheless, nutrient digestibility is quite comparable with that of pigs (Moran and Practice, 1982). Average retention time in the stomach region of chickens varies between half an hour and hour. Svihus et al. (2002) observed that significant amounts of feed left gizzard in 30 min of feeding. Moreover, selective retention in the gizzard results very short retention time of fine particles compared to coarse ones (Svihus, 2011a). When expressed as a percentage of total tract retention time, the gastric retention time is quite similar between pigs and poultry (Moran and Practice, 1982).

The gastric juice secreted by the proventriculus has been stated to have pH value around 2. However, depending on the chemical characteristics of the feed, the pH in the proventriculus and gizzard of broiler chickens has been reported to have an average value of 3-4 for normal pelleted diets (Svihus, 2011a). This is consistent with the results observed by Jiménez-Moreno et al. (2009) where broilers were fed raw or heat treated corn or rice with inclusion of some fibers. As feed passes to the duodenum pH rises to higher levels above 6 (Svihus, 2014a) due to bicarbonate secretion from the pancreas (Fuller, 1991).

Small intestine is the site where enzymatic starch digestion initiates in the chicken. Total length of the small intestine of the bird (1-2 m) is quite a bit shorter than in pigs (15-20 m). Also, addition of moisture to intestinal chyme is less in chickens compared with pigs, resulting in a higher dry matter content of the intestinal chyme (Moran and Practice, 1982).

Luminal cavity of the jejunum is the major site for starch digestion, since pancreatic duct opens up in the beginning of the jejunum (Osman, 1982). Pancreatic α-amylase is the only enzyme responsible for starch digestion in the fowl (Moran, 1982) since, chicken saliva does not contain α-amylase activity (Jerrett and Goodge, 1973, Benkel et al., 1997). Although saliva of the pigs contains α-amylase, its activity is negligible (McDonald, 2002). Hence, in monogastric animals, the major digestion of starch initiates in the small intestine by the action of pancreatic α-amylase, and then followed by brush border enzymes dextrinase and glucoamylase (Svihus et al., 2005).

2.2. Starch structure, characteristics, and digestibility

Starch is the most abundant component in the diet of the poultry and other domestic animals. Apart from animal related factors of starch digestion, digestibility of different native starches is closely related to their structural and chemical characteristics. Therefore, it is crucial to have a basic knowledge about their structure and composition.

Naturally starch is stored inside the granules which size and shape differ among plants. In cereals, starch granules are accumulated in the endosperm. Granules consist of several layers which are composed of two types of α-glucan: amylose and amylopectin (Buléon et al., 1998). They together represent approximately 98–99% of the dry weight (Tester et al., 2004). Both are the polymers of glucose units bound together with glycosidic bonds. Amylose is made of α-D-glucose units bound to each other with α $(1{\rightarrow}4)$ glycosidic bonds (99%). Therefore, amylose has a linear structure compared to amylopectin which is highly branched. Besides α $(1{\rightarrow}4)$ linkages (95%) of glucose units in amylopectin, α $(1{\rightarrow}6)$ glycosidic bonds (5%) (Tester et al., 2004) occur every 20-25 glucose units (Sajilata et al., 2006). This causes amylopectin to get highly branched structure.

The proportion of amylose and amylopectin fraction depends on the source of the starch. Normally amylopectin makes up to 70% and amylose 20-30% of starch (Svihus et al., 2005). Tightly packed structure of amylose limits the accessibility of the digestive enzymes in ungelatinized starches with high amylose content (e.g raw potato, banana) (Sajilata et al., 2006). High degree of crystallinity (high amylose content) is associated with lower catalytic efficiencies and hydrolysis rate (Tahir et al., 2010). Moreover, starches with high amylose content are also associated with high amounts of lipid formation (such as fatty acids) on the surface of granules which can reduce the rate of enzymatic digestion (Svihus et al., 2005). This will be discussed below in more detail. Amylose content may considerably differ depending on the type and variety of cereals. In a wheat, amylose has been reported to vary within a range of 30-310 g/kg which is smaller than barley (30-460 g/kg) and maize (0-700 g/kg). This smaller range for wheat is due to the polyploid genomes which buffer variation in amylose content (as stated in (Svihus et al., 2005)). Amylopectin ratio in wheat was reported to be 36 % (Morsi and Sterling 1966, Morrison et.al 1994) and 39 % (Yusuph et.al 2003) as stated in (Tester et al., 2004)).

Starch granule size can be another factor affecting digestibility of native starches. This is due to the relationship between surface area and starch volume, which affects contact between starch substrate and enzyme, that decreases as the granule size increases (Svihus et al., 2005, Dona et al., 2010). The granules may vary in size from 1 to 50 µm. Cereals with smaller granules e.g rice 8 µm, have higher starch

digestibility than the cereals with larger granules e.g wheat (22 µm) and potato (38 µm) (as stated in (Svihus et al., 2005)).

There are some other non-starch components such as lipids and proteins in the starch granules that have potential to interfere with starch digestion. For instance, lipid-starch complexes can reduce the access of enzymes to starch. Lipids may further hamper digestibility due to their hydrophobic nature, thus reducing the water access to granules and impeding the extent of gelatinization (Svihus et al., 2005). Other than lipids, proteins found on the surface of granules (friabilin) may impair starch digestibility through interactions during milling (Svihus et al., 2005). Matrix protein of the endosperm (gluten) may also contribute to low digestibility of wheat starch (Wiseman, 2006, Svihus, 2011b, Svihus, 2014b).

The granular architecture and, more specifically, the surface organization of starch granules provide barriers to the diffusion and adsorption of the enzymes, which is proposed to be one of the main actors determining the kinetics and degree of hydrolysis (Slaughter et al., 2001, Tahir et al., 2010, Zhang et al., 2013, Zhang et al., 2015, Dhital et al., 2017).

2.3. Action of α-amylase and amyloglucosidase on starch

The term "*amylase*" is generally defined as the enzyme which hydrolyzes the *O*-glycosyl linkage of starch (Kuriki and Imanaka, 1999). α-amylase belongs to the endoamylase group of starch converting enzymes. Endoamylases cleave α, 1-4 glycosidic bonds in the inner part of the amylose and amylopectin chains. The breakdown products of α-amylase are oligosaccharides (e.g. maltose and maltotriose) and limit dextrins with varying length (Van Der Maarel et al., 2002). However, there is also a possibility of glucose appearence in the digesta due to action of pancreatic α-amylase. In an extensive study of the metabolism of short-chain oligosaccharides by use of porcine pancreatic α-amylase, (Robyt and French, 1970) observed that 70% of maltotetraose was cleaved to two molecules of maltose and 30% was cleaved to glucose and maltotriose (as cited in (Kaufman and Tietz, 1980).

Using scanning electron microscopy technique Lynn and Cochrane (1997) observed wheat starch digestion by the action of pancreatic α-amylase. According to their observations digestion initiated through the channels on the surface of the central disc of the lenticular wheat starch granule and from those channels digestion extended towards the interior of the granule before breaking apart the surface of the granule (as stated in (Svihus et al., 2005)). Hydrolysis proceeds very rapidly in a radial direction with the formation of new channels. Granular starch digestion occurs by a 'side-by-side' mechanism involving the simultaneous digestion of crystalline and amorphous regions (Zhang et al., 2006), although the

nonordered structure has been generally thought to be more easily digested (Gallant et al., 1992) as stated in (Zhang et al., 2013)).

Amyloglucosidase was thought to act primarily on α-amylase breakdown products (Kaufman and Tietz, 1980), rapidly converting them to glucose. However, lately several authors (Miao et al., 2011, Brewer et al., 2012, Zhang et al., 2013, Warren et al., 2015) have indicated synergistic action of α-amylase and amyloglucosidase on attacking starch granules. As amyloglucosidase is also capable of hydrolyzing α-(1→6) linkages, which α-amylase cannot. As in *in-vivo* digestion brush border enzymes maltase-glucoamylase and sucrase-isomaltase can commit the same function (Nichols et al., 2003, Diaz-Sotomayor et al., 2013) as cited in (Warren et al., 2015). This direct attack mechanism of amyloglucosidase on starch granules was more evident in *in-vitro* digestibility trials of starchy foods with high amylopectin content. Presumably, this was due to more branched structure of amylopectin containing many α-(1→6) linkages.

This has important impact on interpretation of the results of *in-vitro* digestibility studies, as different concentrations of enzymes may lead to doubtful estimations of the rate and extent of starch digestion (Warren et al., 2015). Considering this fact, starch digestibility assay in our experiment was divided into two successive steps as, after digestion of the substrate with pancreatic homogenate unreacted starch was removed from the digesta, so that amyloglucosidase could only act on amylase products.

2.4. Kinetics of enzymic starch digestion

Kinetics of starch digestion in enzymic systems principally depends on two main factors: the physicochemical architecture and molecular characteristics of the starch granule (amylose to amylopectin ratio, starch granule size, etc.) and hydration (gelatinization) level of starch which increases the availability of starch chains to digestive enzymes, thus affecting rate of hydrolysis (Dona et al., 2010). Enzymic digestion of native starch is a pseudo-first order kinetic process, which the initial digestion curve is linear with a constant rate at earlier time points until the substrate is not significantly depleted (Warren et al., 2015). As the reaction continues the rate shows decay curve (Warren et al., 2015, Dhital et al., 2017) and the kinetics of the hydrolysis can be studied by fitting first-order reaction equation:

$$C_t = C_\infty (1 - e^{-kt}) \tag{1}$$

where C_t is the concentration of product at a given time (t), C_∞ is the concentration of product at the end of the reaction, and k is the digestibility rate constant. For ease of interpretation, C_t may be expressed as

the amount of starch digested as a percentage of the total starch content of sample, calculated assuming that all polysaccharide is converted to maltose (Edwards et al., 2014).

2.5. Enzyme related factors of starch digestion in chickens

α-amylase isozymes. α-amylase is single chained enzyme synthesized in the pancreas and salivary glands and it exist at least in two isoamylase forms (Rodeheaver and Wyatt, 1984b). Lehrner and Malacinski (1975) discovered three electrophoretically distinct pancreatic α-amylase isozymes in chicken pancreatic tissues. In their study, they showed amylase to exist in the highest concentration compared to other proteins. The results of the purification analysis of amylase isozymes showed that amylase proteins accounted for 20-30% of the extractable proteins, which is higher than the rabbit (5%) and the rat (10.9%). During the structural analysis of amylase isozymes Lehrner and Malacinski (1975) observed post-translational modifications of amylase isozymes in crude pancreatic homogenates which was suggested to be temperature, buffer, and pH dependent. However, authors stated that amylolytic activity of those modified amylases (nongenetic isozymes) remained unchanged.

Effect of genotype. Several authors concluded that the levels of digestive enzymes are mainly determined by the genetic stock (Nitsan, 1989, Nitsan et al., 1991b, O'Sullivan et al., 1992). Gapusan et al. (1990) investigated the functional properties of two chicken allozymes (designated as Amy^F and Amy^S) *in-vitro*. Statistical analysis of the study indicates significant differences between two of them in terms of specific activities, substrate specificities and inhibitor sensitivities. Amy^S showed significant specific activity (maltose units per microgram of amylase) than Amy^F. Activity of both amylases were negatively affected by the α-amylase inhibitors from wheat, however, Amy^S was less sensitive to protein inhibitors from wheat than Amy^F. This observation suggests that wheat utilization efficiency may differ between amylase isozymes, depending on their ratio in the medium. Morever, in the study both amylases utilized different substrates in the following order: amylopectin > potato starch > dextrin > glycogen > amylose. Since amylose has the tendency to form helical coils of glucose when suspended in water (Conn et. Al 1987 as stated in (Gapusan et al., 1990)) hence becomes more resistant to action of amylase. However, Amy^F was better able to attack α, 1-4 linkages of amylose than Amy^S.

Yardley et al. (1988) examined amylase activity variation in three chicken flocks by sex, genotype (Amy^S, Amy^F) and tissues. Significant differences in mean pancreatic amylase activities (maltose units per μg protein) were found among three flocks. Variation among individuals within a flock is also reported in the paper. However, there were no significant effects of sex and genotype on amylase activity. Highest

amylase activity was observed in the pancreatic tissue, the lower activity in the intestine and the lowest activity was observed in the serum.

Effect of feed intake. Rodeheaver and Wyatt (1984a) studied the effect of feed intake on serum and pancreatic α-amylase activities (units $\times 10^2$ per g wet weight) in broiler chickens. Pancreatic α-amylase levels elevated almost twofold in 20-day old male broiler chicks that were deprived from feed for 24 hours. The results of the study showes that pancreatic enzyme activity (concentration) was not reduced by long-term feed restriction but it increased instead. Feeding for 24 hours following feed restriction caused a sixfold reduction (from 440 to 77) in pancreatic α-amylase activity which was less than one-half of the control value. This is explained as the compensatory effect to starvation and high need for glucose, thus feed intake causes excessive release of α-amylase from the pancreas compared to the normal conditions, which results in low levels of α-amylase in the pancreas itself. It is proposed that there is a certain basal secretion of α-amylase from the chicken pancreas independent on feed intake. During feeding periods, α-amylase secretion increases from the pancreas, however due to the release of the enzymes to the intestine the level of α-amylase in the pancreas decreases. Without feed stimulation of the pancreas activity, secreted α-amylase is accumulated in the pancreas, which results in increased levels of α-amylase in the pancreas. This type of reverse relationship between amylase levels in the pancreas and intestines of chickens was also noticed by Osman and Tanios (1983). Contrary to these findings, (Kadhim et al. (2011), Kadhim et al. (2014)) did not find any correlation between the reduction of enzyme activities in the intestinal content and any accumulation of these enzymes in the pancreatic tissue of the red jungle fowl, Malaysian village chicken and the commercial broiler breed.

Nitsan et al. (1974) investigated enzymatic adaptability of young chicks in digesting and metabolizing excessive amounts of nutrients under force-feeding and subsequent fasting conditions. Pancreatic lipase, together with lipase and chymotrypsin significantly increased in the force-fed groups compared to the *ad-lib*-fed group. Amylase activity (expressed as units/g) also increased in the small intestine contents of the force-fed group, whilst other enzyme activities remained the same when expressed as units/g. However, the specific activities of enzymes per g of tissue or chyme were the same in both groups despite the bigger pancreas and high amount of feed in the intestines of the force-fed group. Similar results were observed by O'Sullivan et al. (1992) where chicks from high-weight lines had elevated overall enzyme levels after a mild feed restriction compared with those provided *ad libitum* access to feed.

Kokue and Hayama (1972) studied exocrine pancreatic secretion in starved chickens and observed that the chicken is able to secrete the same volume of pancreatic juice after 72 hr starvation period as that of

the chicken starved for 24 hours. Feeding and sham feeding resulted in rapid increase in the volume of secretions of the starved chicken. However, immediate increase was not observed in the vagotomized chicken, although small delayed increase was noted. It is concluded that there is continuous synthesis (not release) of pancreatic juice regardless of extraneous control mechanisms. Control by the parasympathetic nervous system is suggested to be the reason for continuous pancreatic secretions.

Regulation. Murai et al. (2000) investigated the effects of different neurotransmitters and gut hormones on pancreatic amylase secretion response in pancreatic acini *in vitro*. The researchers postulated that, neural regulation of pancreatic enzyme secretion is more important than the humoral regulation. Based on their findings cholecystokinin (CCK) at physiological concentrations did not influence amylase release from isolated pancreatic acini of the chicken. This is in accordance with the findings of Niess et al. (1972) where they did not observe significant effect CCK regulation on the pancreatic amylase secretion when chickens were fed heated and unheated soybean proteins.

Effect of age. The level of α-amylase activity in the pancreas increases with the age of the bird. Rodeheaver and Wyatt (1986) arrived at this conclusion where they examined pancreatic α-amylase activity by the amylochrome method in different broilers at different ages. According to their observations, male broilers at 7-weeks of age exhibited nearly 5 times increase in pancreatic α-amylase activity compared to the activity of 11 days of age young chicks. At 20 days of age a moderate activity level of pancreatic α-amylase was noticed. Progressive increase in the levels of activity is speculated to be the normal response to increased metabolic needs of birds.

Similar tendency was monitored by Krogdahl and Sell (1989) where they measured different enzyme activities in the pancreatic tissue in young turkeys and revealed that activity of amylase increased rapidly during the first 14 days after hatch, whereas protease and lipase activities increased after 14 days. Opposite to that Nitsan et al. (1991b) observed declining pancreatic amylase activity with increasing age in the three different chicken populations. In the previous study by Nitsan et al. (1991a) the specific activity of amylase did not change during the first 2 days but later increased continuously up to 17 days of age and was 5-fold that at hatching.

Effect of diet composition. Several studies have shown that amylase activity of the birds can change with diet as well as age. Hulan and Bird (1972) observed significant increase (P<0.001) in the amylase content of the pancreatic juice per mg of protein when chickens received high amounts of carbohydrates. Krogdahl and Sell (1989) monitored similar type of influence of feed composition to the levels of enzyme activity,

thus high carbohydrate and protein intake significantly increased amylase and protease activities of pancreatic juice of young turkeys. Pubols (1991) reported the amylase to be the most abundant enzyme in the chicken pancreas. The results of the ion-exchange chromatography showed that amylase was about 28.9%, chymotrypsinogens about 20% and trypsinogen was about 10% of total protein from the chicken pancreata.

2.6. Evaluation of analytical parameters for measuring α-amylase activity

Such as other enzyme proteins, functional properties of α-amylase enzymes are also affected by extrinsic factors such as pH, reaction temperature, storage temperature and duration prior to analysis, and presence of certain ions for enzyme functioning. All these factors have significant contribution to the results of enzyme assays thus, standardization of those parameters for assayed samples is necessary to keep comparability of experimental results (Rodeheaver and Wyatt, 1984b).

pH and temperature. In general, α-amylases have relatively alkaline pH optima. Porcine pancreatic α-amylase has the pH optima 6.9 for the majority of substrates (Ishikawa et al., 1991). Buonocore et al. (1977) investigated the effect of pH on the activity of the chicken pancreatic amylase and reported that enzyme activity stayed unaffected at alkaline pH values even after 24 h at 4 ^{0}C. But at pH values lower than 7 activity noticeably decreased after 30 min. The effect of temperature on enzyme activity was tested at the temperature range 20-60 ^{0}C. Maximum enzyme activity was observed at 43 ^{0}C at pH 7.5.

Storage temperature and duration. Rodeheaver and Wyatt (1984b) evaluated different analytical techniques for quantification of α-amylase activity in serum samples. In the study poultry serum samples were kept at different temperatures and durations and later samples were assayed for serum α-amylase activity by three different (amylotube, amylochrome and Bernfeld 1955) techniques. Serum samples kept at 4^{0}C showed large variation in α-amylase activity depending on the assay method used. The technique of Bernfeld (1955) demonstrated significant decrease in activity after 24 hr and the lowest activity was observed on the 4th day of storage. In contrary to that amylotube method showed consistent increase in α-amylase activity through 8 day of storage at 4^{0}C. Serum α-amylase activities as measured by amylochrome method did not significantly change during 8 day of storage at 4^{0}C. Freezing serum samples at -80^{0}C significantly increased (by 32%) α-amylase activity compared to the fresh samples. It must be noted that serum α-amylase is of pancreatic origin (Rodeheaver and Wyatt, 1986).

Rodeheaver and Wyatt (1986) observed more than 35-fold increase in the α-amylase activity (measured by amylochrome method) when pancreas samples were sonicated after homogenization compared to the samples that only followed homogenization. It confirms the intracellular nature of the pancreatic α-amylase, indicating that the enzyme is secreted and stored in the zymogen granules of the pancreas and subsequently released into the duodenum.

Solovyev and Gisbert (2016) tested the stability of digestive enzyme activities of gilthead sea bream stored at -20^0C from 1 day up to 720 days. Up until 270 days, the change in α-amylase activity was still not significant when samples were stored at -20^0C and authors recommended not to preserve samples at -20^0C for digestive analysis more than 270 days.

Effect of ions. All amylases are believed to contain inorganic ions. Calcium has been found associated with all amylases. Removal of this ion results in decreased enzyme activity which can be restored upon re-addition of calcium ions (Vallee et al., 1959). In contrary, Buonocore et al. (1977) monitored irreversible nature of the enzyme inactivation by the removal of calcium ions. It is generally believed that calcium does not directly participate in th+e catalytic activity but it is involved in stabilizing the tertiary structure (Buisson et al., 1987). Other than calcium, chloride ions also required for maximum enzyme activity (Buonocore et al., 1977, Karn and Malacinski, 1978, Osman, 1982).

In-vitro analyses provide great advantages for speculation of *in-vivo* processes in animals. However, all those aforementioned factors must be carefully considered prior to simulation of *in-vivo* starch digestion to ensure the reliability of the results.

Summary

The pancreas has quantitively important role in starch digestion in pigs and poultry. Literature shows that amylase in many species is continuously produced and stored in the pancreas until it is released. Several factors such as age, diet, genotype, feed intake, etc. have shown to influence the level of α-amylase activity in the pancreas. Moreover, synthesis and secretion of α-amylase appears to be differently regulated between pigs and poultry. These aspects are important to take into consideration in the studies of pancreas functionality.

3. MATERIALS AND METHODS

All laboratory work was conducted at three different labs at the Department of Animal and Aquaculture Sciences of the Norwegian University of Life Sciences (NMBU) during the months of February and March of 2017.

3.1. Background information on dissected animals

Chicken pancreases were obtained from an experimental trial that was conducted at the Animal Production Experimental Centre, at NMBU, Ås, Norway, between the November 12th and December 4th 2015. The experiment was a part of a comprehensive study, where the effect of different types of grit on broiler chickens' (Ross 308) performance was investigated. All birds had *ad-libitum* access to commercial pelleted broiler diets from the Norwegian feed company Norgesfôr. The birds were fed starter diet from day 0-11, grower diet from day 11-18. From 18-21 days of age birds got access to a mixed diet consisting of 15% whole wheat and 85% protein-rich starter diet. 12 dissected chickens were selected from the birds which 4 of them got zeolite, 4 marble and the rest for 4 got granite treatment. All birds were dissected at 21 day of age.

Pig pancreases were obtained from another experiment and belonged to the animals of the control group. Pigs were fed with finisher diet and the main ingredient composition is given in table 1. According to the information was obtained, animals were full-stomach before slaughtering. All pigs were dissected at 17 weeks of age.

Table 1. Main ingredient composition of broiler starter diet and pig finisher diet (%).

	Broiler starter diet	Pig finisher diet
Wheat	35.4	23
Maize	25.0	-
Barley	-	40.9
Oat	-	13.9
Soybean meal	23.3	13
Full-fat extruded soya	6.3	-
Rapeseeds	2	-
Rapeseed expeller	1.5	-
Lard	-	4.8
Lysine	13.3	0.3
Methionine	6.3	0.6
Metabolizable energy (MJ/kg)	12.3	-
Net energy (MJ/kg)	-	9.4
Crude protein	23.5	14

3.2. Collection and storage of pancreases

Dissection and collection of all pancreases were conducted by a PhD student Khaled Itani. All pancreases were freed from surrounding fat tissue after dissection. After dissection pig pancreases were stored in a freezer at -80^0 C for 12 months and chicken pancreases were stored in a freezer room at -20^0 C for 14-month period until enzyme activity analysis. Only 9 pancreases from each species (18 in total) were analyzed for α-amylase activity.

3.3. Materials

50 mM Tris-HCl buffer pH 8 and 0.2 M acetate buffer pH 6.1, (containing 200 mM $CaCl_2$ and 0.5 mM $MgCl_2$) were prepared and kept in the fridge to be used during pancreatic homogenate preparation and starch digestibility analysis. Fungal amyloglucosidase (*Aspergillus niger*) was obtained from Megazyme® and had an activity of 3260 U/mL as defined by the manufacturer. One unit was defined by the manufacturer as the amount of enzyme required to release one μmole of D-glucose reducing-sugar equivalents per minute from soluble starch at pH 4.5 and 40^oC.[2] Ordinary sieved wheat flour was obtained from the shop and used as a starch source in *in-vitro* digestibility analysis.

3.4. Pancreatic homogenate preparation

3.4.1 Sampling

The simplified sketch of pancreatic homogenate procedure is illustrated in figure 2. Each pancreas was taken out from the freezer and weighed before preparation of a pancreatic homogenate. Immediately after weighing, entire chicken pancreas was chopped into small fragments with a scalpel while pancreas was still frozen. Pig pancreases possessed extremely huge mass and volume in contrast with chicken pancreases. To avoid any possible methodical errors due to the great difference in the size of pancreases, only representative subsamples were taken from pig pancreases. For this, each pig pancreas was divided into half by a longitudinal cut (Figure 1a). Thin fragments were taken throughout the longitudinal cross-section of a pancreas with the scalpel (Figure 1b). Fragments were then collected and weighed. Weight of the subsamples were kept in the weight range of chicken pancreases.
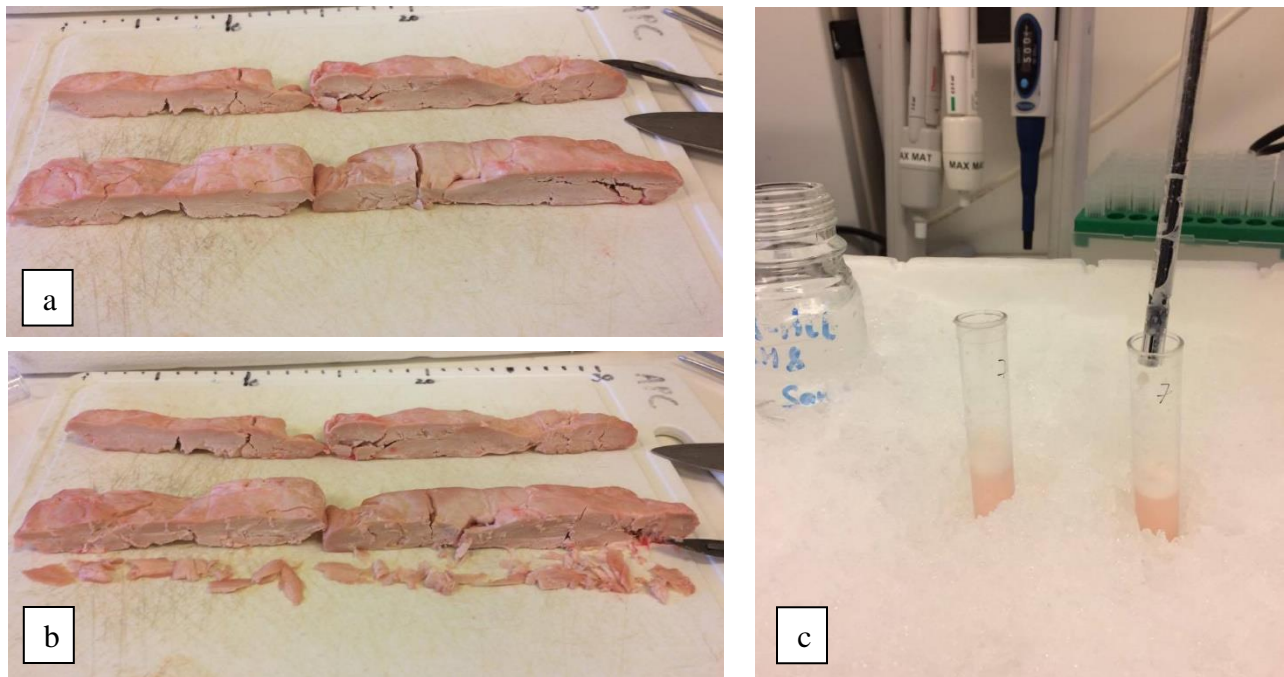

Figure 1. Sampling and preparation of pancreatic homogenates. a, b – sampling, c- homogenization.

3.4.2. Homogenization (enzyme extraction)

All pancreas samples followed the same subsequent steps during homogenate preparation. Chopped fragments were placed into 15 ml propylene tubes and to this was added ice-cold (1:5 wt/vol) 50 mM Tris-HCl buffer pH 8 (Pinheiro et al., 2004). The volume (ml) of the buffer was adjusted with the pipette according to the weight (g) of a pancreas in order to keep the weight/volume ratio same for all pancreases. See the example in Table 2.

Table 2. Buffer volumes corresponding to different pancreatic tissue weights. 1:5 wt/vol ratio.

Pancreas weight (g)	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0
Buffer volume (ml)	7.5	8	8.5	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	14	14.5	15

Propylene tubes were placed on ice and tissues were homogenized with "*QIAGEN" TissueRuptor* handheld homogenizer for about 50 seconds until all tissue fragments disappeared (Figure 1c). Homogenates were centrifuged at 4000 RPM 3^0 C for 12 minutes. Aliquots of supernatant were divided into several small (1.5 ml) portions, (so that no repeated freezing and thawing of the same sample took place throughout analysis) snap frozen in liquid nitrogen and stored at -80^0 C for later α-amylase activity measurements and for total protein analysis.

3.5. Determination of α-amylase activity

3.5.1 *Principle*

Determination of pancreatic α-amylase activity was based on *in-vitro* starch digestibility analysis with pancreatic homogenates. Overall each digestibility assay can be divided into two main succesive steps. The first step was starch digestion with pancreatic α-amylase. This step involves *in-vitro* starch digestibility trials with pancreatic tissue homogenates. The procedure was adapted from the total starch determination analysis by McCleary et al. (1997). However, as a major modification to the procedure, pancreatic tissue homogenates were used (instead of thermostable α-amylase) to hydrolyze starch into shorter oligomers. In the second step, those oligomers were further hydrolyzed into glucose monomers by fungal amyloglucosidase. Glucose concentration of the final solution was measured in the spectrophotometer. Glucose values were translated into starch and thus the percentage of digested starch was calculated at different durations of starch hydrolysis reaction. Amylolytic activity of pancreases were expressed as the amount (mg) of starch hydrolyzed per min per mg of protein.

3.5.2 Procedure

Each pancreas was tested for the enzyme activity in two parallels. For the sake of simplicity, the assay procedure is described below as a single setup (see also figure 2).

Blanks. Parallel to the enzyme activity analysis "blank sample" analysis was run on every assay round. All assay parameters were kept identical for blank samples, but no pancreatic tissue homogenate was used. Instead of pancreatic tissue homogenate only homogenate buffer (Tris-HCl buffer pH 8) was added in 0.7 ml volume.

Starch digestibility analysis with a single pancreatic homogenate. Approximately 100 mg of wheat flour was accurately weighed into 15 ml glass tube with a screw cap. 9 ml 0.2 M acetate buffer pH 6.1 containing 200 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Warren et al., 2015) was added into the tubes. A pancreatic tissue homogenate was taken out from the freezer, thawed and was added into the tube in 0.7 ml accurate volume. The mixture was incubated in water bath at 40^0 C for 150 minutes. Tube contents were mixed on a vortex mixer every 10 min. Aliquots (1.2 ml) were taken at time intervals between 0 (before incubation) and 150 min (0, 15, 30, 60, 90, 120, 150) and centrifuged (3000 RPM for 5 min) to remove any unreacted starch residue. Removal of unreacted starch at this step was important to avoid any possible amylolytic action of amyloglucosidase (Warren et al., 2015) in the further step. Supernatant (1 ml) were then carefully

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transferred to a new tube and immediately placed into boiling water to inactivate the action of pancreatic α-amylase (Slaughter et al., 2001). From that 0.925 ml was added into 5 ml propylene tubes containing 3 ml 200 mM acetate buffer pH 4.5 (McCleary et al., 1997) and 0.075 ml fungal amyloglucosidase (Megazyme®) mixture to degrade all amylase breakdown products to glucose. This was then incubated in water bath at 50^0 C for 30 min. Tube contents were mixed on a vortex mixer every 10 min. Then tube was placed into boiling water for 5 min to deactivate amyloglucosidase. After boiling, the tube was centrifuged at 5300 RPM for 10 min to remove coagulated enzyme proteins from the solution. 300 μl sample was taken from the supernatant and analyzed for glucose concentration in MaxMat spectrophotometer.

Measurement principle of the spectrophotometer is based on colorimetric glucose-hexokinase method. (Richterich and Dauwalder, 1971). Required reagents for glucose measurements were obtained from DIALAB®. Test principle of the method is given below as described by the manufacturer:

$$ATP + Glucose \xrightarrow{hexokinase} Glucose\text{-}6\text{-} phosphate + ADP$$

$$Glucose\text{-}6\text{-} phosphate + NAD^+ \xrightarrow{G6PDH} 6\text{-Glucose phosphate ester} + NADH + H^+$$
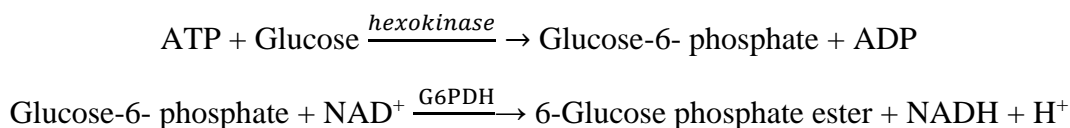
Glucose in the sample, under the catalysis of hexokinase and glucose-6 - phosphate dehydrogenase (G6PDH), react with ATP to produce glucose - 6 - phosphate and adenosine diphosphate. Glucose - 6 – phosphoric acid is oxidized to 6 - phosphate glucose in ester, meanwhile, NAD+ in the reagent is reduced to NADH, cause the increase of light absorbance value at 340 nm. NADH volume is proportional to the amount of glucose. By measuring the change of absorbance value at 340 nm, the concentration of glucose can be calculated:

$$Glucose\ concentration\ (mmol/L) = \frac{Absorbence\ value\ of\ sample\ tube}{Absorbence\ value\ of\ calibration\ tube} \times Concentration\ of\ Calibrator\ (mmol/L)$$

Starch digestibility. Average of all blank values at each duration were subtracted from the glucose values of starch digestibility analysis to obtain glucose concentration reflecting only starch hydrolysis by the action of pancreatic α-amylase. In other words, since the assay procedure did not include removal of free-sugars from the substrate, it was believed that pre-existing free-glucose in wheat flour would overestimate starch digestibility.

Digestibility of starch was calculated according to the following formula:

$$\text{Starch digestibility (\%)} = \frac{G \times 180 \times 0.9 \times 0.001 \times df \times V}{S} \times 100\ \%$$

Where: G = glucometer reading (mmol/L), 180 = molecular weight of glucose, 0.9 – factor to convert glucose concentration into starch, 0.001 = conversion factor from L to ml, df = dilution factor in the digestion step with amyloglucosidase, V = volume (ml) of the digesta with pancreatic α-amylase, S = the amount of starch added (mg) into the digesta.

Wheat flour from the shop was analyzed for the starch content in our lab and it was determined to contain 74.7 % starch on as is basis.

Relative weight of pancreases. Body weights of the animals before dissection were obtained from previous experiments. Relative pancreas weights were estimated by expressing pancreas weights as a percentage of animals' body weights.
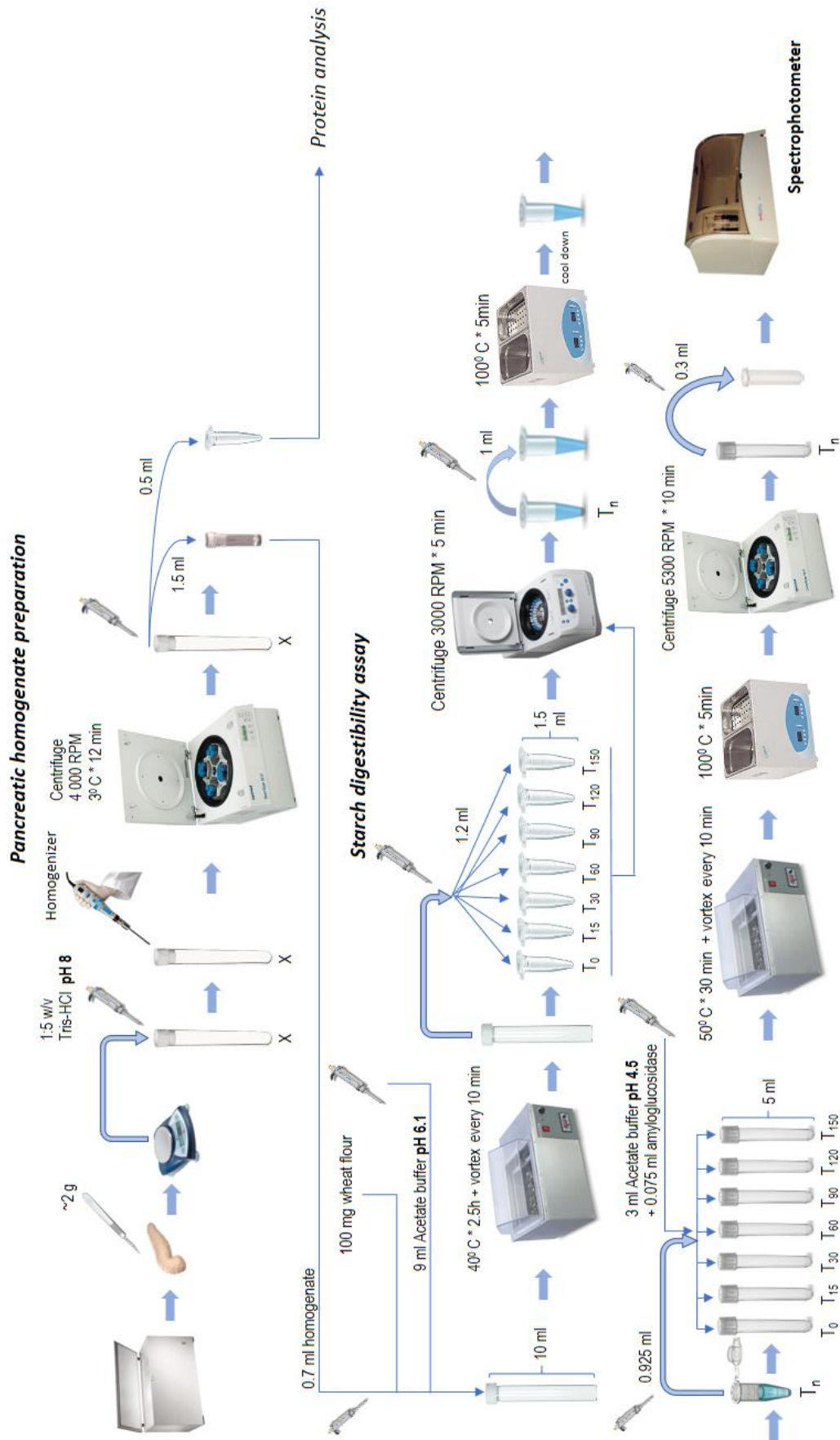
Figure 2. Sketch of the pancreatic homogenate preparation procedure and starch digestibility assay.

3.6. Protein analysis

To determine the protein concentration of the pancreatic homogenate solutions protein analyses were conducted by me at the *Cigene* lab of the university under the supervision of PhD student Ragnhild Ånestad. Analyses were run according to the Quick Start™ Bradford protein assay (Bradford, 1976). After the analysis known concentration of homogenate solutions were used to calculate the soluble protein content of the pancreases.

3.6.1. Principle

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored (Bradford, 1976).

3.6.2. Selecting a protein standard

Bovine serum albumin (BSA) protein with a known protein concentration (2 mg/ml) were used to develop a standard curve for the assay. Since pancreatic homogenate samples were made in Tris-HCl buffer pH 8, the same buffer was used as a diluent (instead of water) throughout the assay. The compatibility of the buffer with the assay was tested against double-distilled water. Despite the slight dissimilarity in the curves it was believed that using Tris-HCl buffer as a diluent will not affect the comparability of the samples (Appendix B fig. 1.)

3.6.3. Assay protocol

Analysis were conducted according to the microassay protocol[3] in 300 μl microplates. The linear range of this assay for BSA is 1.25-10 μg/ml, therefore, samples were diluted several times to find appropriate protein concentration fitting to the linear range of a standard curve (Appendix B fig. 2). Final dilution factor was determined at 1:3000 homogenate to buffer ratio. Standard and sample protein solution were pipetted into the microplate wells in 150 μl volumes and to this was added 150 μl dye reagent (Coomassie Brilliant Blue G-250) to get the color response. Microplate was then placed into spectrophotometer and statistical means were obtained from a software (SoftMax). All protein solutions were assayed in

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[3] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf

triplicates. All results (raw data) are presented in the Appendix B table 1. The mean values were multiplied by the dilution factor to obtain the protein concentration of the pancreatic homogenates.

Average amylolytic activity. Average digestion rate (%/min) for each pancreas was first determined by dividing the final digestibility values by the total reaction time (150 min). Percentages were converted into mg of starch and values were corrected for the protein content of the pancreases. Average amylolytic activity (specific activity) of the pancreases were expressed as mg of starch hydrolyzed per minute per mg of protein (León et al., 2014).

3.6. Enzyme kinetics

The rate of starch digestion is very important for determining the glycemic responses of starchy foods for humans. Based on this phenomenon Englyst et al. (1992) classified starch sources in three categories as: rapidly digestible, slowly digestible and resistant starch. Thus, it is well established that in starch containing foods digestion of the different starch fractions occurs at different rates (k). Poulsen et al. (2003) developed a method called "Logarithm of Slope" (LOS) analysis which was later used by many to examine those changes in reaction rates. The slope is sensitive to changes in k occurring during a reaction and these changes are revealed by discontinuities in the linear plot (Butterworth et al., 2012). LOS analysis enable scientists to determine the reaction rate constants (k) at divergent phases of reactions and true reaction end points (C_∞). Since a thorough understanding of starch digestion kinetics was not the focus of this thesis, LOS plots were only obtained to reveal occurrence of distinct phases during starch digestion in our experiment. A LOS plot was obtained by expressing the first derivative of the first-order equation (see section 2.6 eq. 1) in logarithmic form:

$$ln\left(\frac{\Delta C}{\Delta t}\right) = -kt + \ln(C_\infty\, k) \qquad (2)$$

where $\ln(\Delta C/\Delta t)$ represents the logarithm of the slope, and the equation describes a linear relationship between LOS and time of amylolysis, t (as described by Edwards et al. (2014). Thus, a plot of natural logarithms of $(C_2 - C_1)/(t_2 - t_1)$, $(C_3 - C_2)/(t_3 - t_2)$, etc. against t is linear with a slope of -k (Butterworth et al., 2012). Because logarithms are plotted, C values do not have to be concentrations. They can be any measured parameter that is directly related to concentration (Butterworth et al., 2012). Hence, starch digestibility values (%) were employed instead of concentrations.

3.7. Data analysis

Statistical analysis of the data was performed in IBM SPSS Statistics 23 software. Starch digestibility values were subjected to one-way ANOVA using General Linear Model (GLM) procedure. The variance in relative pancreas weights of the species was analyzed by independent sample t tests. Simple linear regression analysis was performed to examine the relationship between enzyme activity and protein content of the pancreases. Mean values and standard deviations were calculated and illustrated by using math and graphical functions of Microsoft Office Excel 365. Plots of the LOS (Butterworth et al., 2012, Edwards et al., 2014) of the experimentally obtained digestibility curves were generated in Microsoft Office Excel 365 to establish whether amylolysis followed a single-phase or two-phase hydrolysis reaction.

4. RESULTS

The main objective of this thesis work was to test the hypothesis whether chicken α-amylase was more effective at degrading starch than pig α-amylase for the same amount of pancreatic tissue. For this, we conducted *in-vitro* wheat starch digestibility analysis with 9 pig and 9 chicken pancreatic homogenates and the results of the analysis are presented below.
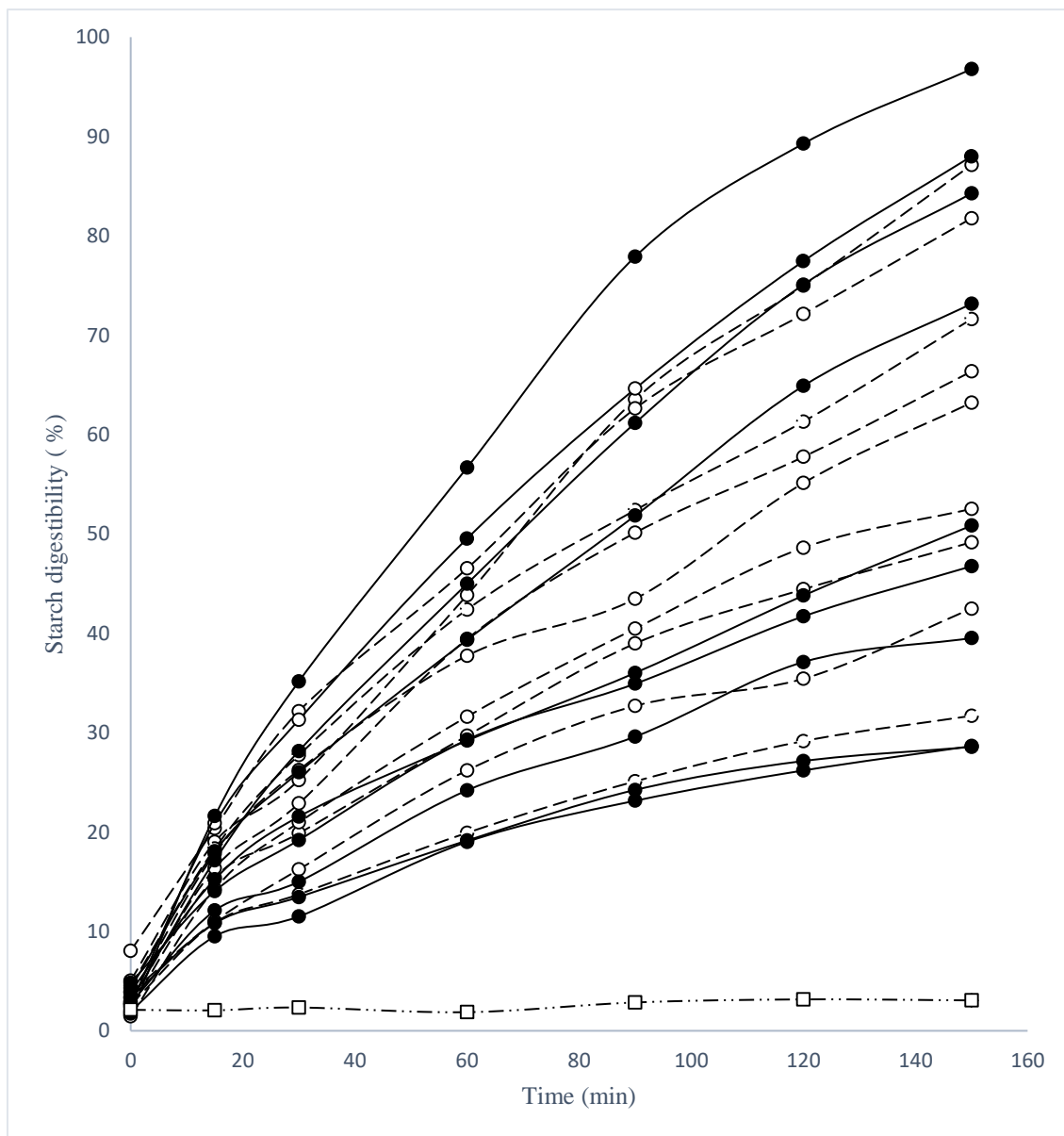

Figure 3. Starch digestibility curves at different times (○ pig, ● chicken, □ blank)

4.1 Kinetics of starch digestion

Blanks. Results of the blank values at all digestibility trials are given in Appendix A fig. 1. As would be expected, in the absence of α-amylase, the release of glucose from the wheat flour was nearly zero. However, very low glucose (1-2 %) was detected in the blank samples where no pancreatic solution was added. There was no significant (P>0.05) increase in glucose at increasing reaction times in the blank samples. High variability in glucose values was noticed between measurements at all durations (CV % ranging from 33 % to 95%, Appendix A fig.1)

Reaction kinetics at low and high enzyme concentrations. Digestibility analysis revealed large variability in starch digestibility among individuals from both species. However, for both species during the first 30 minutes on average ~22 % of starch was digested. From 30 to 60 min ~12 %, 60-90 min ~10 %, 90-120 min~8 %, and during the last half an hour ~7 % of starch was hydrolyzed.

LOS of the highest (96 %) and the lowest (28 %) digestibility curves were plotted to observe enzyme kinetics at two different enzyme concentrations. LOS plots revealed two distinct linear ($R^2 > 0.9$) phases for both reactions which can be characterized by two different rate constants (figure 4).
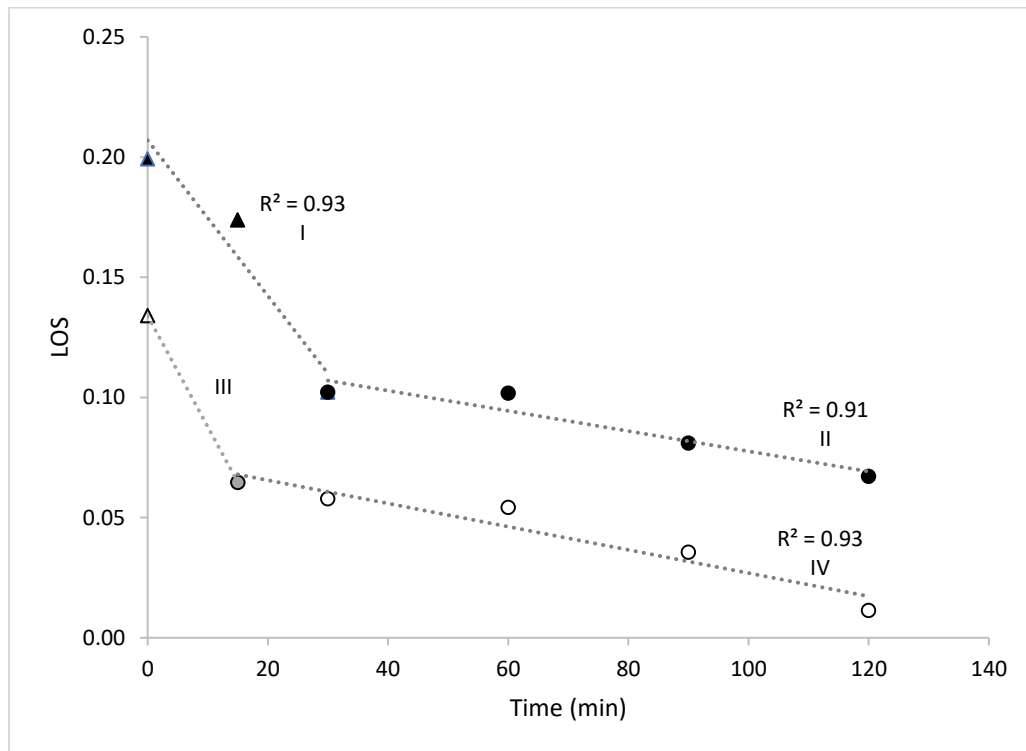

Figure 4. Logarithm of slope (LOS) plots of the highest (upper line) and the lowest (lower line) starch digestibility curves. (I, III – rapid phases, II, IV – slow phases)

The point at which the slower phase becomes predominant is represented by the intersection between the two linear phases. At high enzyme concentration, the slower phase initiates 30 minutes after the commencement of incubation, whilst at low enzyme concentration this occurs earlier, as 10-15 min after the onset of incubation. These results revealed that digestion of the wheat flour in our experiment followed two: rapid and slow phases, independent of the enzyme concentration.

4.2 Amylase activity of the pancreases

As can be seen from the figure 3 both species showed large variation in the rate and extent of wheat starch digestion. Besides large individual variation within the species remarkable variation was present between the species. Variation among chickens was even greater than pigs (figure 5). The maximum and minimum digestibility values were obtained from the digestion with chicken pancreases which were 96.8 % and 28.6 % respectively. The values for pig pancreases varied within the range from 31.7 % to 87.1 % of starch digestibility. Average starch digestibility at the end of the incubation was 61 % for pigs and 60 % for chickens (insignificant difference at $\alpha >0.05$).
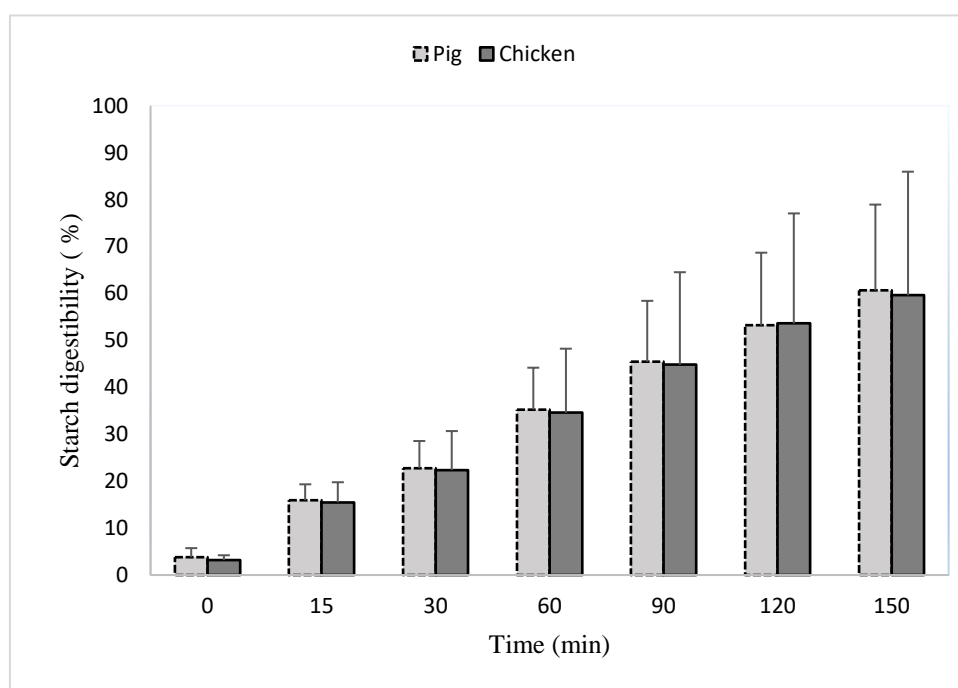
Figure 5. Average starch digestibility. Values are shown as means + S.D.

As expected, both species showed systematic increase (P<0.05) in starch digestion over time. However, average starch digestibility at each time duration was not significantly different (P>0.05) between two species (Figure 5). The results of the GLM procedure also did not show any significance (P>0.05) for the effect of species on starch digestibility (see Appendix C, table 2) These results indicated that there is no

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systematic difference between same certain amounts of pig and poultry pancreases on their ability to digest starch.
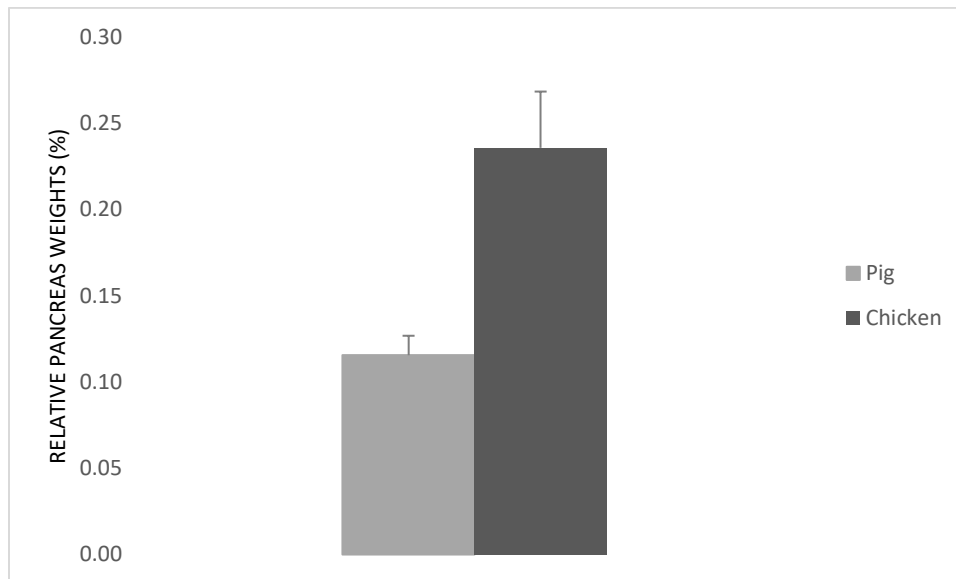
Figure 6. Averages of the relative pancreas weights for both species. Values are shown as means + SD, P<0.01.

Correction of the pancreas weights for the body weights of the animals showed that average of the relative pancreas weights for the pigs was 0.12 %, whilst for chicken this value was 0.24 % with relatively large standard deviation (figure 6). Independent sample t test showed that the difference in the mean values was significant at 0.01 significance level (Appendix C table 3).

4.3 Protein analysis

The results of the protein analysis are presented in the figure 7. From the plot, it can be seen that soluble protein content of the pancreases showed considerable variation among individuals within the species. This variation was even larger among chickens with values ranging from 6 % to 16%. For pig pancreases values lied in between 11-14%. Simple linear regression analysis (Appendix C table 1) showed weak correlation (r=0.4 for pig, r=0.1 for chicken) between soluble protein content of the pancreases and the total starch digestibility. However, correlations were not statistically significant for both species. Interestingly, the pancreas with the lowest protein content (6 %) exhibited the highest starch digestibility (96 %).
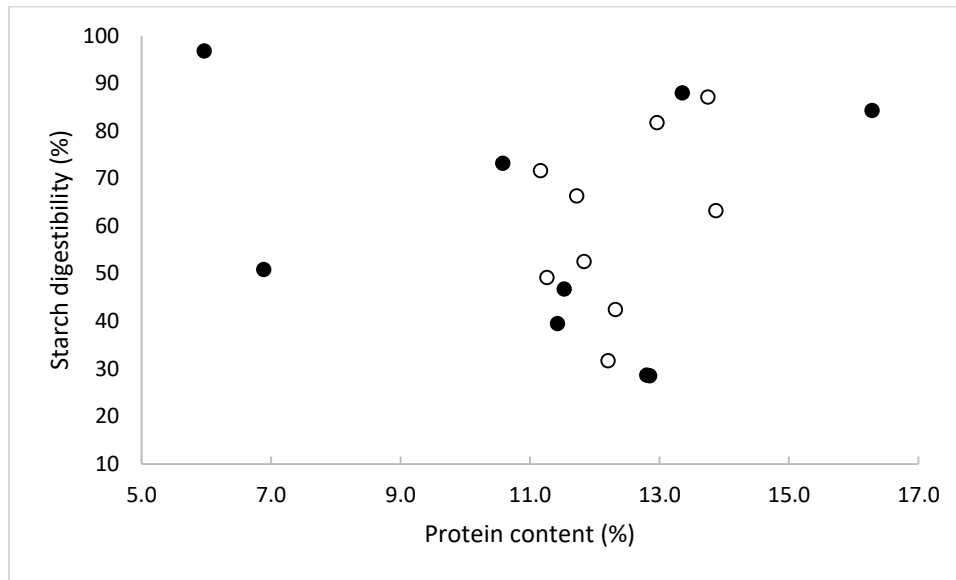
Figure 7. Relationship between starch digestibility and soluble protein content of the pancreases (○ pig, ● chicken) R_{pig}=0.4 for pig, $R_{chicken}$=0.1. Correlations are not statistically significant (P>0.05)

Overall protein data showed that soluble protein content of the pancreases was not a potent predictor of α-amylase activity in our experiment.

It must be mentioned that extraordinarily low protein value of the pancreas of the chicken individual #8 is not thought to be due to any practical or methodical errors during analysis. Results displayed consistent low values for that pancreas after several attempts in protein quantification analysis.

4.4 Amylolytic activity *(Specific activity)*

Once amylase activities were corrected for the protein content of the pancreases very diverse results were obtained (figure 8). The chicken pancreas with the highest α-amylase activity (individual #8) had the lowest protein content, hence correction for the protein content showed extremely high amylolytic activity for that pancreas.

Average amylolytic activities (mg starch/min/ mg of protein) of the pancreases showed higher value (0.021) for chickens compared to pigs (0.017). Although this difference was insignificant at α =0.05. Obviously, higher average amylolytic activity value of the chickens was highly influenced by the extremely high value of the chicken #8 (figure 9).

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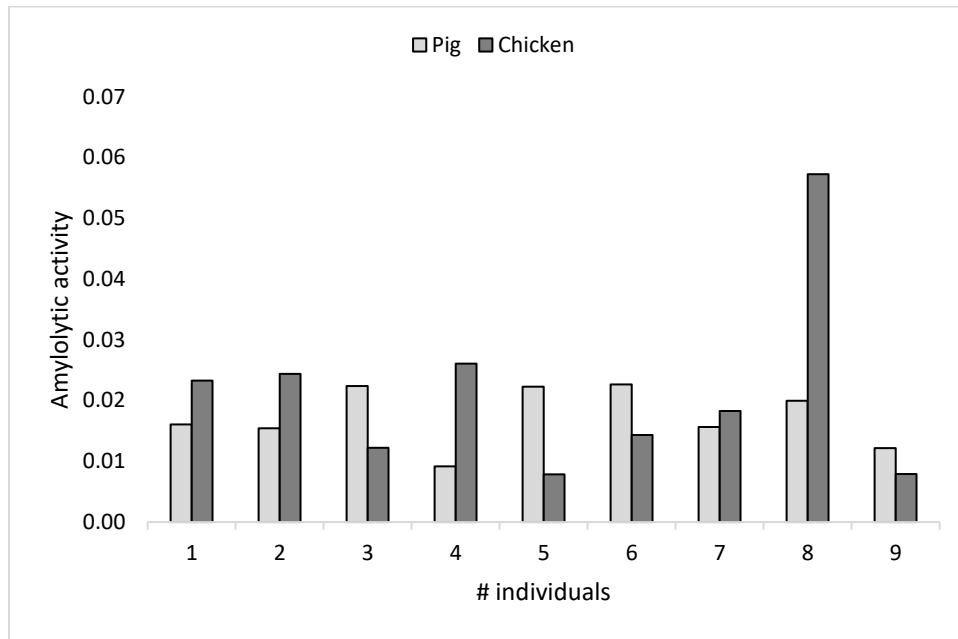
Figure 8. Amylolytic activity as expressed mg starch digested/min/mg protein
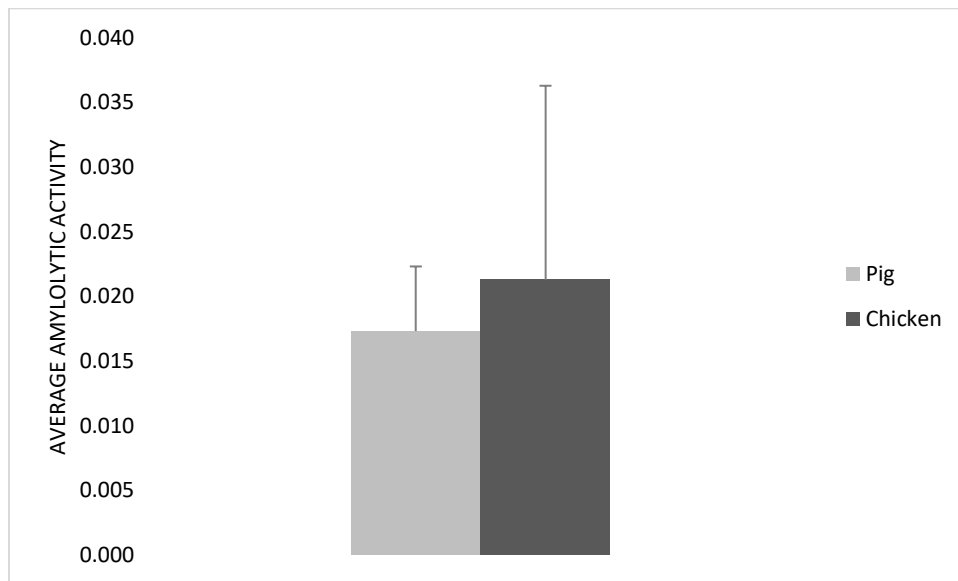
Figure 9. Average amylolytic activity (mg starch digested/min/mg protein). Values are shown as means + S.D. P>0.05

5. DISCUSSION

5.1 Kinetics of starch digestion

Although, the digestibility assay in this current study was not designed in a way to study the kinetics of starch digestion, yet, the digestibility curves allowed to observe some patterns of enzymic starch hydrolysis.

***Blanks.*** Looking at the blank data, it would be hard to comprehend any possible action of intrinsic α-amylase (Major et al., 2001) in the wheat flour. Since, there was no significant increase in glucose at increasing reaction times in the blank samples. Moreover, large variation between replicates may also indicate that measurements were highly sensitive to practical errors. However, compared to the samples with pancreatic tissues, blank samples showed fairly steady low levels of glucose throughout the reaction which is more attributable to the level of pre-existing free glucose in the wheat flour.

Reaction kinetics at low and high enzyme concentrations

First of all, it is more logical to think that varying digestibility values in our experiment is more likely to be due to varying α-amylase concentration in the pancreatic tissues. Since the same starch source was used and the assay parameters were kept identical throughout the experiment.

The reaction of α-amylase on soluble (gelatinized) starches is a single-phase first-order reaction (Butterworth et al., 2012, Edwards et al., 2014) and can be described by traditional Michaelis-Menten kinetics where, the rate of the reaction is directly proportional to the enzyme concentration. Thus, as the reaction proceeds and the substrate is depleted the reaction rate shows an exponential decay curve (Slaughter et al., 2001, Dhital et al., 2017). However, hydrolysis reaction of insoluble native (ungelatinized) starches is rather complex due to their granular architecture. The shape of the curve (logarithmic or linear) is affected by the level of enzyme activities. Butterworth et al. (2012) stated that when starch or starch-containing foods are digested *in-vitro* with relatively high concentrations of pancreatic amylase and for long time periods, i.e., extending over several hours, the rate of the reaction decreases as the time is extended and plots of the concentration of product formed (or quantity of starch digested) against time are logarithmic. Slaughter et al. (2001) investigated the action of porcine pancreatic amylase on native starches and observed that the rate of amylolysis of native starch suspensions was essentially linear for up to 2 h but the rate over the first 60 s or so was often higher than over the remaining time course when digestion products were measured by reducing sugar assay. Independent of the enzyme concentration, the similar pattern was also observed for the digestibility curves in our experiment. Thus,

computed linear lines to digestibility curves up to 2 hours provided a very good fit with $R^2 > 0.9$ (the graph is not shown in this thesis). However, declining digestibility values throughout sampling times makes it clear that lines are not of true linear form.

The binding step of α-amylase to starch granules is of kinetic significance when the enzyme is acting on particulate starch, for instance, α-amylase of *Bacillus subtilis* has been shown to adsorb to crystalline starchy materials and the binding is a prerequisite for catalysis (Leloup et al. (1991) as cited in (Slaughter et al., 2001)). Thus, for the enzymes acting on insoluble substrates the rate is not directly proportional to the enzyme concentration (Slaughter et al., 2001) and usually reaction occurs in two phases: rapid and slow phases.

Edwards et al. (2014) monitored a single-phase hydrolysis reaction for wheat flour. In contrary, the results of the LOS analysis of the lowest and the highest digestibility curves revealed two distinct phases of wheat starch digestion in our experiment. At high enzyme concentration, rapid hydrolysis phase lasted up to ~30 minutes and followed by a slow phase for the rest of the reaction time. This was in accordance with the findings of Butterworth et al. (2012) where they observed the rapid phase lasting 20-30 minutes during *in-vitro* digestion of native wheat and pea starches. At low enzyme concentration, relatively short (10-15 min) rapid phase was observed and the onset of the slow phase shifted to earlier time points. Although discontinuity occurred in both LOS plots, it was more evident for higher starch digestibility curve. Overall, LOS plots showed that digestibility curves in our experiment were of discontinuous semi-logarithmic form.

Despite the fact, that digestibility curves at relatively low enzyme concentrations seems to level out close to the end of the reaction time (2.5 h), obviously, that decay in the digestion rate cannot be linked to substrate depletion, since for most of the trials considerable amounts of starch remained undigested after the end of the reaction. Surprisingly, even at high enzyme concentrations where almost more than 90% of starch was digested, curves did not appear to plateau after 2.5 hours. Explanation for this remains a bit unclear, since the reaction time was restricted to 150 minutes which considerably limits the amount of information that may be obtained about the progress of the reaction.

Warren et al. (2015) postulated that at low enzyme activities the observed digestion curves are essentially linear form, where insufficient substrate is converted to the product during the time course of the reaction that results in significant decay in overall rate of reaction. According to the definition of a first-order reaction, it is a reaction that proceeds at a rate that depends linearly on only one reactant concentration.

Hence, fitting starch hydrolysis to a first-order reaction would only be plausible when the amount of enzyme in the system relative to the substrate is not a limiting factor for hydrolysis. Slaughter et al. (2001) postulated that fraction of enzyme molecules bound productively to the starch granules is minor compared with the total amount of enzyme in the system. Interpreting this in a different way, it could also be said that at relatively high concentrations of α-amylase in the system, the rate of the reaction is primarily dependent on starch concentration. The more the starch, the more productive binding and hydrolysis. This has an important implication to the assay method that was used in our experiment. Thus, the removal of considerable amount of digesta (>12 % of the total) at every sampling time (0, 15, …, 150 min) would have also resulted in removal of substantial amount of starch from the system. As a result of this, it is reasonable to speculate impairment in the hydrolysis rate throughout sampling procedure and in overall rate of the digestion.

High-substrate inhibition. Inhibition of enzymic processes is normally either competitive, where the inhibitor (in this case maltose) binds to the enzyme competitively with the substrate, or non-competitive, in which case the inhibitor has identical affinities for the enzyme and the enzyme–substrate complex, decreasing the maximum velocity of the reaction (Dona et al., 2010). Both types of inhibition of starch hydrolysis can occur during *in vitro* enzymic digestion of starch. One of the special forms of uncompetitive inhibition is where the inhibitor binds with enzyme-substrate complex and decreases the reaction rate at high substrate concentrations (Dona et al., 2010). This type of inhibition mechanism will not be further discussed here, since product inhibition of starch digestion appears to be more probable for the digestion kinetics than substrate inhibition in our experiment.

Product inhibition. Oligosaccharides (e.g., maltose and maltotriose) are known to be competitive inhibitors of a-amylase (Leloup et al., 1991, Elödi et al., 1972, Butterworth et al., 2012). During prolonged periods of starch digestion maltose which is the main product of amylase digestion may accumulate and inhibition of starch hydrolysis can become significant (Elödi et al., 1972). However, during the initial stages of starch digestion, maltose levels in the digesta is not sufficient to have a significant inhibitory effect on α-amylase action (Warren et al., 2015). Morever, it was hypothesized that under certain conditions, especially at high concentrations of maltose, two maltose molecules can bind to the active site of the porcine pancreatic α-amylase (Warren et al., 2012). In the study by Warren et al. (2012) where they investigated maltose inhibition during the hydrolysis of maize starch by porcine pancreatic amylase and concluded that, even when maltose concentration is sufficiently high, the affinity of the binding of the second molecule to the active site is approximately 6.5-fold weaker than the first binding. Because of the

relatively low affinity for maltose, authors postulated that inhibition by maltose of the initial stage of starch-amylase interaction normally does not play an important role in regulating intestinal digestion of starch (Warren et al., 2012). Considering these findings, its logical to think of any potent inhibitory effect of maltose on starch digestion during our experiment. Commonly, in *in-vitro* digestibility assays, combination of α-amylase (or pancreatin, containing α-amylase) and fungal amyloglucosidase are used. In such cases, amyloglucosidase rapidly converts all α-amylase products into glucose which has an advantage of eliminating inhibitory effect of maltose accumulation during prolonged digestibility trials (Warren et al., 2015). Digestion steps with α-amylase and amyloglucosidase were isolated in our experiment. Since no combination of these enzymes was used, declining rate of digestion over prolonged durations can partly be due to accumulation of α-amylase breakdown products and particularly maltose.

Enzyme activity analysis of pancreatic tissues by means of *in-vitro* digestibility assays offer great advantages for the study of enzyme kinetics. However, comparability of the obtained data with literature data is often challenged because of the differences in assay systems. Several aspects must be carefully considered while establishing methods to achieve results that are broadly open for discussion.

5.2 Amylase activity of the pancreases

Generally, amylase activity of the pancreas refers to the level of α-amylase (concentration of α-amylase) in the pancreatic tissue and should not be confused with the specific activity of the α-amylase where the activity of the enzyme is corrected for the protein content of the enzyme containing solution.

Ultimate purpose of the current study was to test the starch digestion capacity of the same amount of pancreatic tissue from chickens and pigs and the results of the starch digestibility analysis showed that there is no significant difference (P>0.05) in starch digestion capacity between the two species. Because of the exclusive design of the study it was challenging to find comparable data in the literature to explain the reasons for large variation in amylase activity among individuals from both species. Extensive literature review indicated that amylase activity is usually studied under specific conditions (e.g. exposure to heat) or treatments (diet, age, genotype etc.). It must be mentioned that experiments which pancreases were obtained, were not designed for pancreatic enzyme activity studies. Nevertheless, results can be discussed assuming that the selected pigs and chickens for the present study were quite representative for the population of pigs and poults in general.

Somewhat similar to our study Kadhim et al. (2011) investigated enzyme activity of the pancreas in two breeds of chicken that differ in growth rate. Comparative data on enzyme activities (units/gram of pancreas) showed that level of amylase in the pancreas of commercial broiler chicken (CBC- characterized by high-growth rate) was significantly ($P<0.05$) higher than the red jungle fowl (RJF - characterized by low-growth rate) at different ages from hatching to 120 days. However, when results were expressed in relative basis as units/100 g of body weight no significant difference was detected after 20 days between the two breeds. Similarly, absolute activities (U/g) of duodenal and jejunal amylase of CBC were significantly higher than RJF at all ages, but when the results were expressed as U/100 g BW this relationship was reversed, except for 1 day after hatching. In other words, data failed to follow the rapid gain for body weight of CBC when amylase activity was corrected for body weight, except for the 1 day post-hatching. Moreover, although absolute amylase activities (U/g pancreas) increased for increasing age, however, relative amylase activities (U/100 g BW) showed increase up to 10 days post-hatch and reached to the peak level and then declined with age for both strains. At 10 days post-hatch amylase activity of CBC was approximately 3 times higher ($p<0.05$) than RJF. Authors, postulated that the body requirements had important effect on the synthesis of enzymes, however major source of differences in enzyme activities was due to the genetic stock of two chickens. Identical pattern of differences in absolute and relative amylase activities was observed when Kadhim et al. (2014) studied differences in enzyme activities in pancreas and intestinal contents of Malaysian village chicken (MVC - characterized by low-growth rate) and CBC (high-growth rate). As in the previous study, CBC showed significantly higher amylase activities per g of pancreatic tissue or intestinal content compared to MVC, but once the results were corrected for body weight no significant difference in amylase activities was present between the two breeds. These findings elucidate that high levels of amylase activity in the pancreas of broiler chickens is paralleled by high growth rate which shows that synthesis of the pancreatic enzymes is adjusted according to bird's body requirements.

In order to reveal whether the similar relationship existed among birds and pigs in our study, the pancreas weights were corrected for the body weight of the animals and thus relative pancreas weights were obtained. Weights of the animals (appendix A table 2) did not vary to a large extent within both species (since individuals from both species were killed at the same age) thus, it was not possible to observe changes in the relative pancreas weights in intra-species level. However, results of the relative pancreas weights between two species revealed that on average chickens had twice larger ($P<.01$) pancreases than pigs relative to their body weights. This seems to be logical considering high growth rate of the broiler chickens and thus relatively high body requirements for the enzymes. Moreover, maintenance energy

requirements for the same body weight of birds can be presumed to be relatively higher than pigs depending on their body composition (ratio of fat to lean body tissue) and furthermore, considering the fact that body temperature of the birds is relatively higher than pigs. These are just hypothesized subjective opinions suggested to explain significant differences in relative pancreas weights of pigs and chickens.

Feed restriction. The reverse relationship between amylase levels in the pancreas and in the intestine, was briefly discussed in the literature review part (see section 2.5). According to the information received, dissected broiler chickens in the present study were deprived from feed at day 20 from 9 pm until 7 am next day. On that day birds were given *ad-libitum* access to feed again until they were dissected at about 12 am. It can be speculated that during ~4 hours birds would have normalized the secretion of pancreatic enzymes. However, there are uncertainties regarding the rapidity in the response of pancreas secretions to the feed intake in chickens. For instance, Pinheiro et al. (2004) studied the effect of 30% feed restriction in broiler chickens from 7 to 14 days old chickens and revealed adaptation of digestive enzymes during and after feed restriction. Thus, birds lowered the secretion of enzymes during the restriction period. However, amylase, trypsin and lipase levels increased in feed restricted birds immediately after feed restriction compared to non-feed restricted group. The increase is considered to be one of the factors contributing to compensation in delayed growth due to energy restriction (Pinheiro et al., 2004). Rodeheaver and Wyatt (1984a) on the other hand, observed a six-fold reduction in pancreatic amylase activity after feeding for 24 hours following feed restriction (starvation) which was less than one-half of the control value. Based on this record, it can be stated that even 24 hours of feeding subsequent to starvation is not enough to return the pancreas back to its regular physiological condition. Moreover, there is a likelihood that, providing *ad-libitum* access to feed after starvation led to excessive feed intake which in turn stimulated unusually high secretion of pancreatin resulting in lower amylase activity in the pancreas itself.

Effect of age. The effect of age on pancreatic amylase activities in birds (see section 2.5) and pigs was studied by many. Laws and Moore (1963) observed interesting phenomenon in chicks, thus, high levels of pancreatic amylase activity were found in the newly-hatched chick but these levels decreased during the first 20 days after hatching and then remained constant. This finding with the chick is in contrast to those reported for pigs. Pig amylase activity is low at birth but increases remarkably during the following 35 days (Laws and Moore, 1963). However, some studies (Lindemann et al., 1986, Owsley et al., 1986) reported transient decrease in the activity of enzymes immediately after weaning and the total activity of all enzymes increased with time post-weaning. Not much literature is known on the pancreatic enzyme

activity of older pigs. Kvanitski (1951) assessed enzyme activity of the pancreatic juice from 20 days to 200 days old pigs and found a decrease in proteolytic and lipolytic activity of the pancreatic juice throughout increasing age, however amylase levels stayed relatively constant. Weström et.al (1987) studied the development of pancreatic enzymes in pigs from 10 days up to 6 month of age. According to their observations all enzyme activities increased over increasing age (as stated in (Makkink and Verstegen, 1990). From these literature, it can be summarized that, enzyme activities for pigs and poultry develops with increasing age of the animals with some fluctuations at certain stages of their lifespan. Moreover, reports also show that feeding regimen, diet composition interacts with age dependency (Makkink and Verstegen, 1990).

The pigs and the birds selected for our experiment were dissected at the same age (all pigs at 17 weeks of age and all chicks at 21 days). Thus, individual variation in pancreatic amylase activity within species cannot be explained by the effect of age. However, difference in the ages of the dissected pigs and chickens emerges a question whether the chosen animals were suitable for comparative study or not. Chickens in our experiment were killed at 3 weeks of age well before they reached sexual maturation at 5-6 month of age. However, pigs were slaughtered at about 4 month of age which was fairly closer to maturity age (5-6 month (Reiland, 1977)) compared to birds. Since, none of the animals have reached to maturity age we cannot not expect dramatic shifts in the physiological status of the individuals. However, literature shows that pancreatic amylase activity of the animals until sexual maturity can be irregular with age. Hence, it is arguable whether pancreatic amylase activities of chickens and pigs in our experiment are representative for the entire population of pigs and chickens.

Environmental conditions. The conditions which animals are kept can also change their pancreatic enzyme output (Longland, 1991). Osman and Tanios (1983) postulated that pancreas plays an important role during the adaptation of chickens to heat through the regulation of intestinal level of amylase. In the study, broilers exposed to heat for 15 days increased biosynthesis of amylase in the pancreas, but not its secretion to the intestine thus, levels of amylase in the intestine declined. Reverse relationship of amylase levels in the pancreas and small intestine was monitored by the authors. However, in the same study heat acclimatized laying hens lowered the amylase activity both in the intestine and pancreas. Previous observation in broilers agree with the report by Routman et al. (2003) where they evaluated the effect of temperature exposure (26 and 35ºC) and different diet energy levels (2,900 and 3,200 kcal ME/kg) on the activity of digestive enzymes in broiler chickens. Based on their findings, pancreas amylase activity was affected by energy level of the diet and by thermal stress. Heat stressed birds had a higher enzymatic

activity (p<0.05) than control birds. Furthermore, energy restricted birds also increased (p<0.01) their pancreatic amylase compared to non-feed restricted group. Authors however, did not monitor the intestinal enzyme activity.

In contrary, Szabo *et.al* (1976) found that piglets kept at high (38-40 °C) temperature had lower lipase and α-amylase levels in pancreatic homogenates than piglets kept at lower (0-2 °C) temperature (Makkink and Verstegen, 1990, Longland, 1991). Low enzyme activities in general can be explained as a physiological adjustment to reduce body's heat production by reducing the feed intake and consequently the synthesis of the enzymes that are needed for digesting main energy sources such as lipids and carbohydrates. However, Makkink and Verstegen (1990) claims that, in previously mentioned study feeding was *ad-lib*, so that feed intake could have affected the results. Heat acclimatization does not seem very appropriate to explain individual variation in pancreatic amylase activity within species in our experiment. Presumably all animals from both species were kept under same normal ambient temperatures that would not disturb the synthesis of pancreatic amylase and other enzymes.

5.3 Protein analysis

The Bradford protein assay is widely used assay to determine the concentration of protein in solution. The assay is based on binding of Coomassie Brilliant Blue (CBB) G-250 dye to proteins which causes a change in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored (Bradford, 1976). Binding probably involves hydrophobic interactions between the anionic form of the dye and basic (Arg, His, Lys)/aromatic (Phe, Tyr, Trp) amino acid residues on the protein (Compton and Jones, 1985).

Nevertheless, the assay is not free of disadvantages. The high dependence of the assay on protein composition presents a major problem to the broad use of CBB binding as a quantitative protein assay. It is, however, quite useful as a general, sensitive, semi-quantitative assay for proteins (Sapan et al., 1999).

It is generally believed that the assay monitors formation of a soluble blue complex (Marshall and Williams, 1993). However, Marshall and Williams (1992) observed that insoluble proteins could also contribute to the color formation during analysis. They demonstrated loss in color yield after centrifugation and filtration of the initial assay mixture. This indicates that centrifugation speed and duration has an important impact on removal of insoluble proteins and thus on accuracy of determination of soluble proteins. Centrifugation speed and duration varies in the published literature. Several authors

used rather high centrifugation speed of the pancreatic homogenates prior to enzyme analysis (30 000×g for 1 h (Jensen et al., 1998), 27,000 ×g for 10 min (Owsley et al., 1986), 14,000 x g for 30 min (Routman et al., 2003)) and obtained sufficient results in enzyme activities. The speed was restricted to 4000 RPM (~2700 ×g for 12 min) in our experiment which was the maximum speed of the centrifuge. Thus, there is a possibility that analyzed samples might have contained insoluble proteins to a little extent which would have interacted with color formation. This might have some consequences on accuracy of protein quantification in general, however, it should not affect the comparability of the data.

α-amylase and other enzymes of pancreatic origin are considered as water-soluble enzymes (Schmid, 1979), except some derivatives of α-amylase enzymes (Ledingham and Hornby, 1969). Since pancreas contains several enzymes and non-enzyme proteins, variation in the protein content can be due to varying amounts of other enzymes (lipase, colipase, nuclease, proteases, etc.) assuming that concentration of non-enzyme proteins in the pancreatic tissue is relatively constant.

5.4 Specific activity of α-amylase

Diet composition. Pancreatic amylase is very sensitive to any changes in the amount of starch intake (Corring, 1980). Numerous studies have shown diet induced changes in the activity of several enzymes of the pancreas in rats, pigs and birds. Desnuelle et al. (1962) and Abdeljlil et al. (1963) observed some adaptations to a new diet in rats and stated that adaptation of the pancreas to dietary changes starts immediately or almost immediately. Desnuelle et al. (1962) showed that specific amylase activity in the rat pancreatic tissue increased six-fold when animals, adapted to a 20% starch diet, received food containing 56 % of starch. Increasing the daily starch intake from 160 g to 600 g, caused 30 % average increase of the specific amylase activity in pigs (Corring 1975 as cited in (Corring, 1980). Such responses in the pigs can occur very rapidly with a change in the diet composition. Nunes and Corring (1979) found that, when a high carbohydrate diet was fed instead of a high lipid diet pancreatic amylase increased significantly within two hours of the diet change (Longland, 1991).

In their study, Abdeljlil et al. (1963) found statistically significant variations in the specific activities of several enzymes of rat pancreas after one day in the change of the nutrient composition of the diet. As a matter of fact, specific activities of the fresh pancreas homogenates were high for amylase when animals received high-starch diet and similarly, chymotrypsinogen was higher when animals were given casein-rich diet. Lipase behaved differently. It was low in the casein-rich diet and high on the others. The specific lipase activity in pig pancreatic juice was 7 times higher when the daily amount of triglyceride intake

increased from 30 to 220 g, however adaptation of the lipase to the fat content of diet is much slower compared to other pancreatic enzymes (Corring, 1980).

The effect of diet can be quite marked on both levels of digestive enzyme activities and on the amount of digestive tract secretions (Longland, 1991). Level of trypsin appear to stay relatively irresponsive to diet induced changes, except the presence of some anti-nutritional factors (trypsin inhibitors) in the diet. Corring (1980) and Schumann et al. (1983) showed that the overall pancreatic secretion increased when the diet contained untoasted soybean meal compared to toasted soybean meal. Zebrowska et al. (1983) performed experiments with growing pigs (ca 35 kg) on two diets: diet A (purified): casein, wheat starch, sucrose, soya oil and diet B (cereals): barley meal, soybean meal. With diet B they found higher pancreatic juice secretion (2182 versus 1204 g per 24 hours), but no difference in enzyme activities (Makkink and Verstegen, 1990). Similarly, Nitsan and Gertler (1972) reported that amylase was affected more than the proteolytic enzymes by the quality of the diet, and responded more of the methionine supplementation of the heated and raw soybean diet. The diet of both species contained soybean meal in our experiment. The inclusion level of soybean meal in the diet of chickens was reasonable higher (23.3%) compared to pigs (13%). Considering previously mentioned findings, it is also possible to imagine stimulation of amylase or overall pacreatic secretions due to soybean meal in the diet of both species. However, it would be difficult to speculate the magnitude of the influence for both type of animals.

Any alteration in the type or quantity of dietary proteins leads to an adjustment of specific and total enzyme activities in the pancreatic tissue and pancreatic juice (Corring, 1980). This statement also indicates that amylase activity in the pancreas is not dictated by the amount of starch substrate per se. Presence of other protein polypeptides may also affect synthesis and secretion of amylase from the pancreatic tissue. Corring et al. (1972) demonstrated that relative amounts of dietary protein and starch may affect the degree of variation in the specific and total activities of amylase in the pig pancreatic tissue. At very high levels of only starch intake (81 % starch, 0 % protein) specific amylase activity was not as high as in the feeding regimen where diet contained considerable amount of protein (30% protein, 41 % starch), although starch content was reduced by half. Similarly, the ratio of carbohydrate, protein and fat in the diet could also influence lipase activity in pigs (Corring, 1980). Pekas et al. (1966) showed that secretion of amylase, protease and lipase was about 5 times higher when heat treated soya-protein were fed to 7 weeks old piglets compared to milk protein. This finding indicates again that not only the quantity but also the quality of proteins has tremendous effect on enzyme activities of the pancreas.

Feed intake. Effect of feed intake on pancreatic enzymes activities was studied by León et al. (2014). In the study, effect of starvation on pancreatic amylase activity was also monitored. Newly hatched broiler chickens were assigned to three different dietary treatments from hatching to 48 hours: fasting, hydrated balanced feed (HBF: 54% cornmeal, 35% soybean meal, 6% soybean oil) and commercial hydrating supplement (CHS: soy flour 40%, corn flour 15%, corn syrup 18%, water 25%). After 48 hours, birds were fed with commercial feed and specific enzyme activities (mg starch degraded/min/mg protein) were measured immediately, 48 and 72 hours after hatching. The specific activity of pancreatic amylase peaked to the highest level at 48 hours in the fasting birds which was about three times higher than the birds receiving feed. However, a reduction in the amylase activity was detected 72 hours after hatching. On the other hand, specific activity of lipase and trypsin followed the opposite pattern, remained relatively stable up to 48 h and increased at 72 h in the fasting birds. In birds receiving HBF and CHS pancreatic enzyme activity was reduced in the first 3 days of life of broiler chickens.

Palo et al. (1995) reported decrease in relative and specific activities of pancreatic amylase and protease in the partially feed restricted birds compared to *ad-libitum* fed birds. Authors explained it as an adaptation to the reduced feed intake and thus reduced substrate levels in the small intestine. Their observations were in agreement with the report by Corring (1980) indicating that pancreatic amylase and protease secretions, as well as their concentrations in pancreatic tissue were proportional to the amount of the substrate. These responses also depend on the duration of the restriction period. Corring (1980) reviewed the literature on the adaptation of digestive enzymes to the diet in pigs and indicated that when dietary restriction is not too severe, the biosynthesis of all digestive enzymes markedly decreases (Palo et al., 1995).

Surprisingly, in spite of the fact that the diet, feeding, age, genetic stock, environmental conditions were pretty standardized for birds and pigs, large variation was present in pancreatic amylase activities among animals from both species. Although there is inconclusiveness in the literature in regard to the effect of the feed intake and diet composition on pancreatic enzyme activities, it can be assumed that the large variation in the pancreatic amylase activity is not very abnormal situation for the birds. Varying concentration of amylase in chickens can primarily be the reason of varying extent of pancreatic emptying caused by the effect of the feed restriction. Furthermore, *ad-libitum* feeding after feed restriction may have resulted in varying amount of feed being consumed by the birds that would have consequently caused dissimilar stimulation of synthesis and secretion of pancreatic enzymes.

Storage conditions. The measurement of amylase activity in the pancreas encounters several problems, e.g., standardization of methods of analysis, enzyme activity changes in stored pancreatic juice and

interference by microbial contamination of the homogenates (Makkink and Verstegen, 1990). Some of the challenges mentioned by Makkink and Verstegen (1990) were also experienced in the present study. Although, enzyme assays were quite standardized for all pancreases throughout the experiment, differences in storage conditions of the pancreases prior to enzyme activity analysis may as well impeach the reliability of the comparative data. Effect of storage conditions on amylase activity was explicitly discussed in the literature review (see section 2.6) and will be briefly reconsidered here.

Low (1982) analyzed digesta samples for trypsin, chymotrypsin and peptidases within a month of collection and found that storage at $-20^0\,C$ for less than 3 months did not influence enzyme activities in the samples (Makkink and Verstegen, 1990). Legg and Spencer (1975) studied the stability of pancreatic enzymes in human duodenal fluid to storage temperature and pH. They found that activity of amylase remained relatively constant when samples were kept at $-20^0\,C$ over a month period. At pH < 5 enzyme activities substantially decreased within 4 weeks of storage at -20^0C. Corring (personal communication, 1988) recommended storage of pancreatic juice at -80 °C to prevent loss of enzyme activities (Makkink and Verstegen, 1990).

It is difficult to conclude any significant decrease in the amylase activity of the chicken pancreases based on the reviewed literature. Since most of the studies evaluating enzyme activities of the samples stored at $-20^0\,C$ restricted the duration of storage to less than 3 months.

6. CONCLUSION

The hypothesis that, pancreatic tissue of broiler chickens resulting in more efficient digestion of starch was falsified in our experiment.

The *in-vitro* digestibility assay used in this experiment can be further improved in future studies. Thus, amyloglucosidase and amylase digestion steps can be combined, digesta sample volumes can be reduced and total reaction time can be extended.

More carefully controlled studies are needed in the future to study pancreatic amylase activity and pancreases that are used must be standardized more on feeding and diet composition between species.

Storage conditions as well as assay methods should be standardized to facilitate comparison of the research data.

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APPENDIX A

Table 1. Results of the starch digestibility analysis. Raw data for each assay round. Values represent glucose concentration (mmol/L) of the digesta at different reaction times (min).

Sample #	Chicken 1			Pig 1	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.54	0.51	0.18	0.64	0.55
15	2.51	2.54	0.22	2.12	2.26
30	3.76	3.64	0.34	3.14	3.15
60	5.72	5.59	0.19	4.31	4.41
90	7.42	7.41	0.44	4.55	5.63
120	8.76	8.97	0.46	6.24	6.58
150	10.05	9.98	0.41	6.95	7.63

Sample #	Chicken 2			Pig 2	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.61	0.8	0.15	0.42	0.58
15	2.15	2.28	0.14	1.87	1.98
30	3.16	3.08	0.24	2.47	2.42
60	4.61	4.46	0.24	3.47	3.48
90	6.05	5.97	0.17	4.42	4.77
120	7.59	7.38	0.36	5.26	5.21
150	8.48	8.29	0.22	5.71	5.78

Sample #	Chicken 3			Pig 3	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.52	0.55	0.11	0.77	0.8
15	1.5	1.62	0.26	2.26	2.38
30	1.92	1.9	0.27	3.09	2.98
60	2.84	2.9	0.36	4.64	5.42
90	3.53	3.6	0.41	7.21	7.38
120	4.49	4.37	0.45	8.39	8.8
150	4.85	4.52	0.39	9.81	10.03

Sample #	Chicken 4			Pig 4	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.49	0.84	0.34	0.51	0.53
15	1.81	2	0.16	1.42	1.43
30	2.63	-------	0.31	1.75	1.79
60	3.59	3.25	0.26	2.4	-------
90	4.27	-------	-------	3.17	2.97
120	5.27	5.06	0.56	3.6	3.51
150	6.04	5.82	0.74	3.99	3.66

Sample #	Chicken 5			Pig 5	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.54	0.66	0.59	1.09	1.14
15	1.37	1.47	0.45	2.46	2.48
30	1.78	1.7	0.24	3.79	3.8
60	2.29	2.34	0.22	5.2	5.45
90	3.1	2.85	0.42	7.27	7.12
120	3.33	3.34	0.27	8.35	8.21
150	3.62	3.34	0.32	9.5	9.16

Sample #	Chicken 6			Pig 7	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.71	0.81	0.57	0.77	0.68
15	1.74	1.81	0.22	2.22	2.27
30	2.35	2.39	0.38	3.25	3.37
60	3.41	3.45	-------	4.85	4.89
90	4.07	4.23	0.23	-------	6.07
120	4.84	5.03	0.19	7.08	7.1
150	5.48	-------	0.31	8.18	8.25

Sample #	Chicken 7			Pig 7		Sample #	Chicken 8			Pig 8	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2	Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.52	0.58	0	0.34	0.44	**0**	0.38	0.46	0	0.59	0.52
15	2.11	2.12	0.19	1.9	1.7	**15**	2.61	2.6	0.17	1.99	2.07
30	3.37	3.34	0.32	2.47	2.66	**30**	4.1	4.15	0.12	2.7	2.85
60	5.29	5.02	0.13	3.66	3.71	**60**	6.14	6.74	0.05	4.6	4.49
90	7	7.07	0.23	4.79	4.73	**90**	8.86	8.89	0.34	5.62	6.02
120	8.62	8.59	0.35	5.73	5.66	**120**	9.67	10.66	0.37	6.67	6.73
150	9.77	9.44	0.19	6.01	6.22	**150**	10.53	11.43	0.22	7.61	7.66

Sample #	Chicken 9			Pig 9	
Time	Parallel 1	Parallel 2	Blank	Parallel 1	Parallel 2
0	0.51	0.38	0.15	0.61	0.69
15	1.21	1.34	0.23	1.51	1.37
30	1.54	1.51	0.1	2.02	2.07
60	2.23	2.37	0.2	3.02	3.16
90	2.95	2.76	0.27	3.94	3.87
120	3.22	3.24	0.12	4.29	4.2
150	3.49	3.49	0.23	5.01	5.01

------- *data was lost*
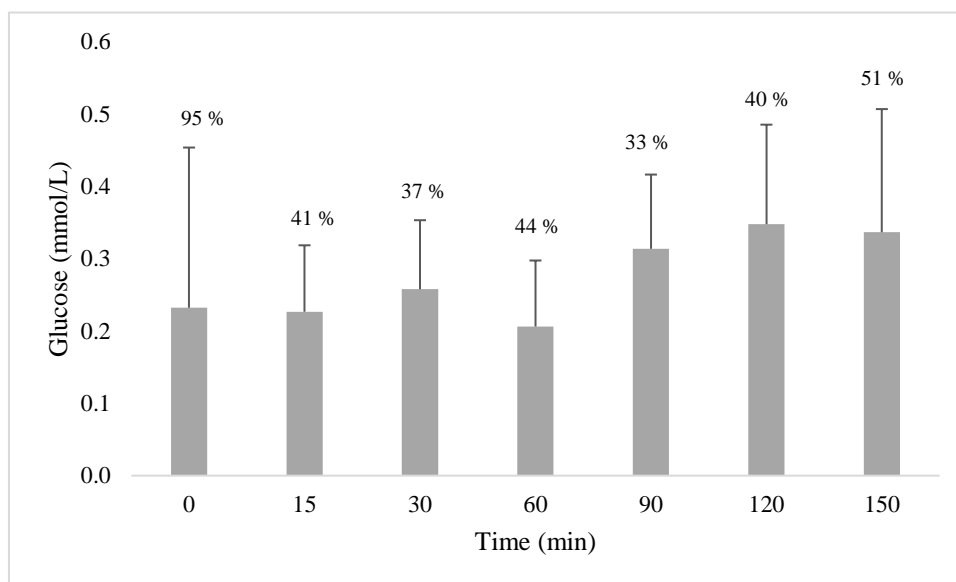
Figure 1. Average blank values at different times. Values are shown as means + SD + CV%

Table 2. Body weights (BW) and pancreas weights (PW) of the two species.

#	Pig		Chicken	
	BW $\times 10^3$ (g)	PW (g)	BW* (g)	PW (g)
1	108.5	128	Zeolite 1107	2.1
2	108.9	139		2.8
3	109.4	125		2.7
4	111.1	137	Marble 996	2.7
5	114.5	141		2.3
6	115.5	126		2.5
7	107.3	129		2.8
8	115.9	104	Granite 1070	2.2
9	112.1	137		2.1
Average	**111.5**	**130**	**1050**	**2.4**
SD	**3.2**	**11**	**53**	**0**

* Average body weights of the chickens for different treatment groups

APPENDIX B
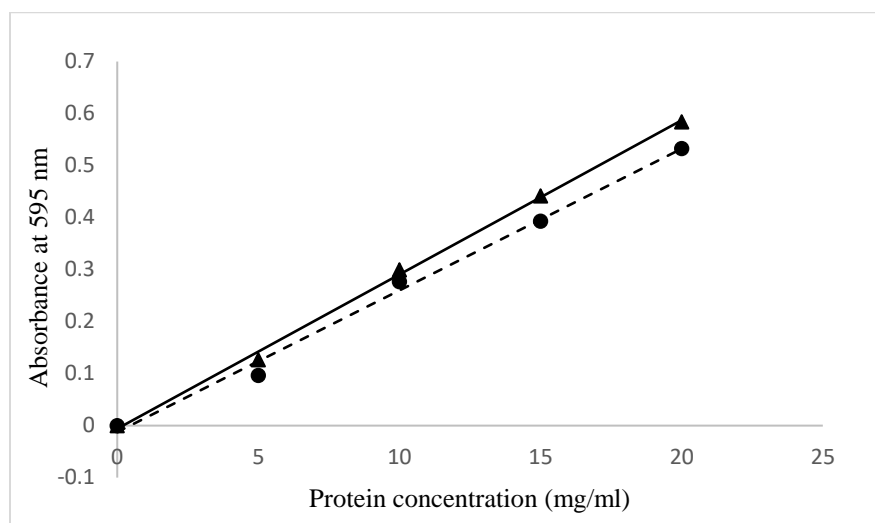
Figure 1. Standard curves with H_2O (--●--) and Tris-HCl buffer (—▲—)
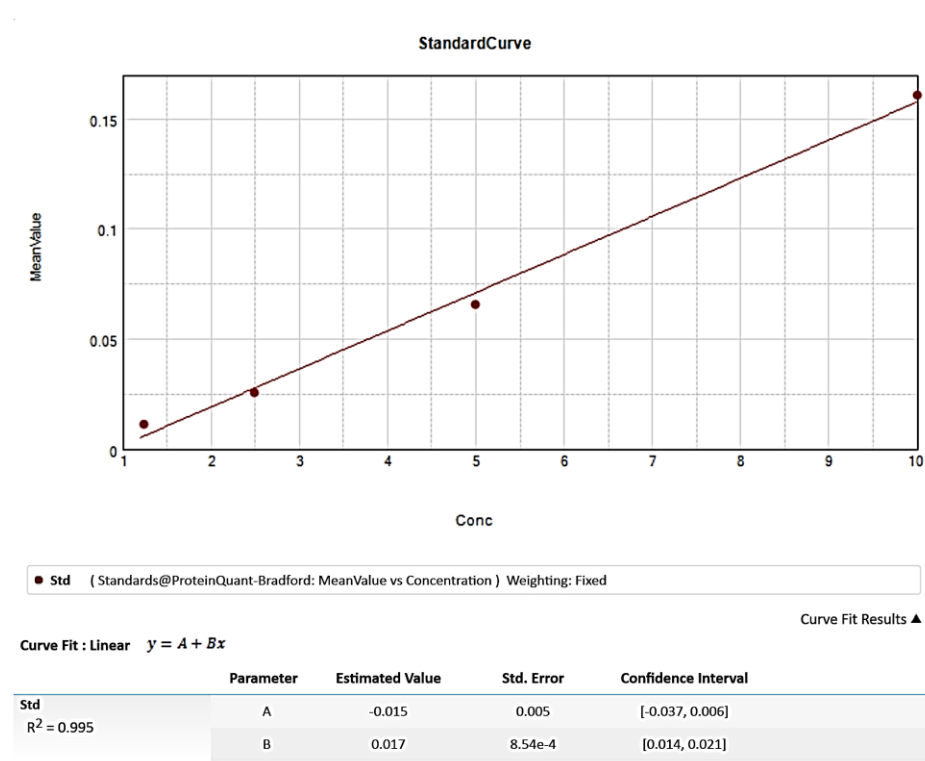

Figure 2. BSA standard curve. Output from the software (SoftMax).

Table 1. Results of the protein analysis. Mean results are given as μg/ml unit of protein.

Sample	Well	Values (absorbance at 595nm)	Result	MeanResult	Std.Dev.	CV%
1	A3	0.145	9.277	9.248	0.258	2.789
	A4	0.14	8.977			
	A5	0.149	9.491			
2	B3	0.119	7.771	7.506	0.255	3.393
	B4	0.114	7.483			
	B5	0.11	7.264			
3	C3	0.145	9.266	9.164	0.182	1.981
	C4	0.145	9.271			
	C5	0.14	8.954			
4	D3	0.127	8.21	8.137	0.262	3.215
	D4	0.129	8.354			
	D5	0.12	7.846			
5	E3	0.136	8.752	8.64	0.099	1.149
	E4	0.134	8.608			
	E5	0.133	8.562			
6	F3	0.114	7.471	7.44	0.107	1.441
	F4	0.111	7.321			
	F5	0.115	7.529			
7	G3	0.123	7.979	7.89	0.195	2.466
	G4	0.124	8.025			
	G5	0.117	7.667			
8	H3	0.114	7.5	7.815	0.665	8.503
	H4	0.112	7.367			
	H5	0.133	8.579			
9	A6	0.127	8.198	8.212	0.084	1.029
	A7	0.128	8.302			
	A8	0.126	8.135			
10	B6	0.143	9.139	8.9	0.229	2.569
	B7	0.138	8.879			
	B8	0.135	8.683			
11	C6	0.111	7.298	7.052	0.286	4.053
	C7	0.101	6.739			
	C8	0.108	7.119			
12	D6	0.118	7.731	7.617	0.497	6.529
	D7	0.124	8.048			
	D8	0.107	7.073			
13	E6	0.067	4.742	4.592	0.145	3.148
	E7	0.064	4.581			
	E8	0.062	4.454			

	F6	0.132	8.498			
14	F7	0.134	8.631	8.564	0.066	0.775
	F8	0.133	8.562			
	G6	0.121	7.875			
15	G7	0.116	7.569	7.687	0.165	2.145
	G8	0.116	7.615			
	H6	0.168	10.592			
16	H7	0.16	10.125	10.302	0.253	2.46
	H8	0.161	10.189			
	C9	0.13	8.412			
16a[4]	C10	0.136	8.723	8.685	0.256	2.948
	C11	0.139	8.919			
	A9	0.057	4.194			
17	A10	0.053	3.963	3.977	0.211	5.303
	A11	0.05	3.773			
	B9	0.133	8.55			
18	B10	0.133	8.585	8.535	0.059	0.694
	B11	0.131	8.469			

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[4] Sample was diluted more (1:3750) to fit to the standard curve

Table 1. Results of the simple linear regression analyses (output from SPSS). Relationship between starch digestibility and protein content of the pancreas $P > 0.05$. (Species 1= pig, Species 2=chicken)

Model Summary[b,c]

Model	R		R Square	Adjusted R Square	Std. Error of the Estimate
	Species =1.00 (Selected)	Species ~= 1.00 (Unselected)			
1	.402[a]	.	.161	.042	17.89733

a. Predictors: (Constant), Protein pancreas
b. Unless noted otherwise, statistics are based only on cases for which Species =1.00.
c. Dependent Variable: Starch digestibility

Model Summary[b,c]

Model	R		R Square	Adjusted R Square	Std. Error of the Estimate
	Species =2.00 (Selected)	Species ~= 2.00 (Unselected)			
1	.131[a]	.	.017	-.123	27.91072

a. Predictors: (Constant), Protein pancreas
b. Unless noted otherwise, statistics are based only on cases for which Species =2.00.
c. Dependent Variable: Starch digestibility

Table 2. General linear model (GLM) (output from SPSS). Starch digestibility as a dependent variable, time and species as fixed factors.

Dependent Variable: Starch digestibility

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	46464.930[a]	7	6637.847	35.287	.000
Intercept	142542.776	1	142542.776	757.755	.000
Time	46457.219	6	7742.870	41.161	.000
Species	7.711	1	7.711	.041	.840
Error	22197.218	118	188.112		
Total	211204.924	126			
Corrected Total	68662.149	125			

a. R Squared = .677 (Adjusted R Squared = .658)

Table 3. Independent sample t test (output from SPSS) to compare the means of relative pancreas weights of pigs and broiler chickens. (Species 1= pig, Species 2=chicken)

Group Statistics

	Species	N	Mean	Std. Deviation	Std. Error Mean
Relative pancreas weights	1.00	9	.1156	.01130	.00377
	2.00	9	.2356	.03087	.01029

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means							
										99% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Relative pancreas weights	Equal variances assumed	8.286	.011	-10.952	16	.000	-.12000	.01096	-.15200	-.08800
	Equal variances not assumed			-10.952	10.108	.000	-.12000	.01096	-.15464	-.08536

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