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# Degradation and digestion of wheat and legume starch in broiler chickens as affected by pelleting and extrusion

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Feed Manufacturing Technology

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**ABSTRACT**

Starch is a quantitatively most important source of energy in broiler chicken diets. Diets may contain up to 50% starch on a dry matter basis. Despite high starch contents in the diet, broiler chickens can utilize a variety of starch sources very efficiently. Dietary starch is the major energy source for broiler chickens, so any knowledge about its digestive behavior can be important. Pancreatic α-amylase is the major responsible enzyme for starch digestion in birds. Susceptibility of starch to α-amylase may differ from source to source.

According to previous studies, the rate and extend of starch digestion in legumes is lower than cereals. To test this hypothesis, digestibility trial with 200 broiler chickens was studied. Starch digestion was determined using the slaughter technique, which involves the removal of the small intestine from the recently killed chicken, with manual collection of the contents. Site and extent of starch digestion of pelleted and extruded wheat (WS) and air classified faba bean starch (FBS) fraction based diet examined in 30 day-broiler chickens. The digesta samples were collected from the upper and lower parts of both jejunum and ileum. In addition to starch digestibility analysis, light microscopy imaging was performed to evaluate the rate of starch digestion of the broiler chickens fed with pelleted and extruded diets. Scanning electron microscopy (SEM) imaging was also performed to compare starch morphology in pelleted diets and digesta samples taken from upper jejunum and ileum. The effect of starch digestion rate on the nitrogen digestion was also evaluated.

Results of the light microscopy imaging study showed that starch from the pelleted faba bean starch fraction (FBS-P) based-diet was digested more slowly, whereas starch from pelleted wheat (WS) based-diet was digested most rapidly. On the other hand, starch from the extruded diets was digested and absorb mainly in the upper part of the jejunum. SEM images also showed that starch in digesta samples taken from the upper jejunum and ileum of the broiler chickens fed with faba bean starch fraction (FBS) based-diet remained undigested compared to wheat based-diet. Furthermore, extend and rate of the starch digestion were discussed for both cereal and legume based diets. The literature study on different starch sources and factors affecting starch digestion were extensively reviewed and presented in this thesis.

***Keywords:*** Starch digestion, broiler chickens, amylase, wheat, faba bean starch fraction

**CONTENTS**

## 1. INTRODUCTION

Broiler chickens have been submitted to an intensive genetic selection that has been increasing the growing rate and made them the fastest growing farmed species. Recent improvements in growth rate and feed conversion efficiency of broiler chickens are remarkable. Growth and feed conversion ratio (FCR) of genetically selected broiler were studied lines with lines that had not been subject to selection since 1957 and used diets similar to those fed in 2003 and in 1957 (Havenstein et al., 2003). They reported that a broiler breeder needed 84 d to reach 1.82 kg in 1956, and it took a broiler 34d to reach the same weight in 2003. Due to the production demands of broiler chickens, high-energy is an essential requirement in their diets. Starch is a key ingredient and principal energy source in broiler chicken diets.

The main sources of starch for broiler chickens are predominantly maize, wheat, and other cereals, starch from tubers (e.g., cassava) and legumes (e.g., beans, peas) may be used in poultry diets. Wheat is a major ingredient in broiler diets due to the high protein and starch content, and its abundance. Faba bean (*Vicia faba L*.) is an emerging, rotational pulse crop cultivated worldwide to provide dietary energy in livestock feeds. Raw dehulled faba bean was studied as a protein source in broiler chickens (Gous, 2011) and in laying hens (Magoda and Gous, 2011). The effect of the extrusion of faba bean (dehulled) on broiler performance was evaluated (Diaz et al., 2006). However, the utilization of faba bean as sources of protein for the poultry was limited by the presence of antinutritional factors (ANFs). Therefore, faba bean can be fractionated using air classification method, and resulted fractions that may have higher value for animals with high nutritional demands such as weaned pigs, and broiler chickens. For swine, even though high starch content constitutes in feed, relatively little is known about the kinetics of starch digestion in weaned pigs (Van Kempen et al., 2007 as cited in (Wierenga et al., 2008)), especially for extruded, fractionated faba bean starch. In grower pigs, ileal starch digestion appears lower for faba bean than wheat as cited in (Wierenga et al., 2008). Similar results can be expected for broiler chickens. In an earlier study, the effect of extrusion of whole faba bean on broiler performance were evaluated (Diaz et al., 2006). Accordingly, extrusion tended to reduce ANFs present in faba bean, and faba bean *in vitro* α- amylase digestibility increased after extrusion. Extrusion lead starch gelatinization and enhance starch digestion, therefore differences in the kinetics of starch digestion might be small among extruded feedstuffs as stated in (Sun et al., 2006).

In monogastric animals like broiler chickens, feed evaluation rests on digested nutrients. Digestion coefficients of nutrients at the lower ileum give information about the amount of nutrients available to the animal. However, it does not give information about the site or the synchronization of availability of different nutrients. Most starch is digested in the upper part of the small intestine. Diets with rapidly digestible starch may result in elevated plasma glucose levels when other nutrients are not yet absorbed, which may have consequences for protein utilization. Diets with similar amounts of digestible nutrients, but differences in digestion kinetics, may result in different performances.

The objective of this research was to investigate starch digestion extend and kinetics of wheat (WS) and faba bean starch fraction (FBS) based diet produced by both pellet press and extrusion for broiler chickens. To test this hypothesis, imagining of both pelleted diets and digesta samples were studied to compare the digestion of wheat and faba bean starch sources for broiler chickens. Digesta samples of broiler chickens were collected from the different section of small intestine, and extent of the starch digestion was studied. Beside starch digestion, nitrogen digestion was also studied to test if there is any effect of starch digestion kinetics on protein digestion. Diets produced by different processes were also analyzed to measure the extent of starch gelatinization. α-amylase and trypsin enzyme activities were also evaluated. The literature on different starch sources, and other related factors of starch digestion were also reviewed and presented herein.

## 2. LITERATURE REVIEW

### 2.1. Digestive system of broiler chicken

The general principle of the digestion in poultry is similar to other animal species. Overall, feed is ingested, moisturized, and ground into small particles, acidified, and hydrolyzed by endogenous enzymes. The macronutrients are broken down into monosaccharides, dipeptides and amino acids, free fatty acids, and monoglycerides, which will be absorbed in the small intestine (McDonald, 2002). The digestive system of the chicken, which is a typical avian digestive system, has some unique features (Figure 1). Therefore, bird specific features are in existence, and the function of each features will be described in the following sections.

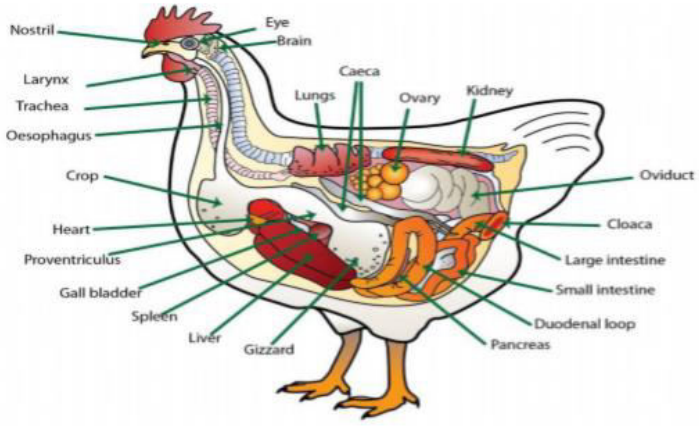



**Figure 1**. The digestive system of chicken (Jacob et al., 2011)

In opposition to pigs, chickens cannot chew their feed in their mouth; therefore, the feed material is swallowed without any remarkable grinding. Their mouth contains glands that secrete saliva consisting of electrolytes, enzymes and antibacterial substances, and the function of saliva is to moisten the feed for easier swallowing. Pig saliva contains α-amylase, however its activity is low (McDonald, 2002). The pH of pig's saliva (~7.3) is slightly above the optimal pH value for the activity of α-amylase (McDonald, 2002).

Although the secretion of saliva does occur in the chicken, saliva does not contain amylase and time exposed to saliva in the mouth is short (Duke, 1986 as cited in (Svihus, 2006)). Quantitatively, salivary α-amylase make contributions insignificantly to the starch degradation, since α-amylase is an acid labile and rapidly degraded in the stomach, gizzard of pigs and chickens where the pH typically will be in the range of 2-4 (Knudsen et al., 2006).

The esophagus connects the mouth with the rest of the digestive tract. It carries feed material from mouth to crop and from crop to proventriculus. After feed is swallowed, it can either enter the crop or pass directly into the proventriculus or gizzard when this section of the digestive tract is empty (Chaplin et al., 1992). The crop located just outside the body cavity in the neck region, is an out pocketing of the esophagus. Swallowed feed material with water are stored in the crop until they are transferred to the rest of the digestive tract (Jacob et al., 2011). The capacity of the gizzard is generally limited to a maximum of 5 to 10 g of feed, and if more feed is consumed, storage is required in the crop (Svihus, 2014). Therefore, the main role of the crop is to provide storage for ingested feed. According to Nielsen et al. (2004), commercial broilers that were fed *ad libitum* have shown that they eat in a semi continuous pattern. Under *ad libitum* feeding, the

crop is not used with its maximum capacity. Consequently, *ad libitum* feeding will conceivable oppose the usage of the crop.

Since the enzyme secretion and nutrient absorption has not been reported, it can be stated that the crop do not have any direct nutritional roles in terms of digestion as mentioned by (Svihus, 2014). Still, significant moisturizing takes place revealing better grinding and therefore affecting enzymatic digestion for the digestive tract (Svihus, 2014). Svihus et al. (2010) reported that the contents in the crop are moistened gradually reaching up to 50% moisture within approximately 60 min. Moistened feed in the crop may be softened, exogenous and microbial enzymes may be activated. The storage time in the crop may have an important role to determine the efficacy of enzyme activity (Svihus, 2014).

Previous research done by Champ et al. (1983) showed that some primary microbial digestion occurs in the crop. Champ et al. (1983) isolated three different *Lactobacillus* strains from the chicken crop, which could produce amylase. The crop is stated as the first major defense against pathogens, and a *Lactobacilli* dominated microbiota may be able to reduce the passage of pathogenic microorganisms through the digestive tract (Classen et al., 2016). This defense is achieved by promoting *Lactobacilli* fermentation resulting the production of lactic acid and other volatile fatty acids and decreasing pH in the crop. In several experiments, pH had been found to be above 6 (Ptak et al., 2015), whereas a pH between 4.5 and 5.8 had been observed in other experiments (Svihus et al., 2013, Józefiak et al., 2012, Amerah et al., 2014). So overall, some variations in pH had been observed in the crop. Feed for non-ruminants usually have pH values around 6 (Onyango et al., 2005 as cited in (Kierończyk et al., 2016)). Therefore, it may be assumed that when the content begins to be stored, the pH will be at a similar level (Ao et al., 2008). However, longer retention time in the crop is associated with a higher fermentation. Higher fermentation will result in the production of organic acids by microorganisms and pH in the crop will be reduced. Thereby, different retention times result different levels of fermentation in the crop, and it may explain these pH variations among the experiments. Therefore, crop does have some nutritional roles, and its functionality depends on feeding systems, which subsequently will influence dietary effects.

Unlike pigs, chickens have two compartments called proventriculus and ventriculus or gizzard. The proventriculus and gizzard are the true stomach compartments where nutrient digestion initially begins. Enzymatic digestion begins when the feed reaches to proventriculus. The walls

of the proventriculus secrete gastric juices which consist of a viscous fluid composed of hydrochloric acid, pepsinogen, and mucus. The hydrochloric acid and pepsinogen secretion will start the digestion of proteins by the gastric glands, which will be converted later into its active form called pepsin. Mucus secreted by the tubular glands protects the gastric wall from the hydrochloric acid (Svihus, 2011b). Gastric juices and ingested material are mixed via muscular movements in the gizzard. Consumed feed and the digestive juices from the salivary glands and proventriculus pass into the gizzard for grinding, mixing, and mashing. Main grinding process of feed material takes place in the gizzard rather than mouth in chickens. Grinding is achieved by strongly myolinated muscles and sandpaper like gizzard surface (Svihus, 2014). Hence, the function of the gizzard is highly critical for the digestion. Average retention time in the stomach region of chickens varies between half an hour and an hour (Svihus, 2014). The gastric juice secreted by the proventriculus has been stated to have a pH value around 2 (Duke, 1986). Depending on the characteristics of the feed, the pH in the proventriculus and gizzard of broiler chickens have been reported to have an average value of 3-4 for normal pelleted diets (Svihus, 2011b as cited in (Kierończyk et al., 2016)). Therefore, physical properties of feed material have a great role for the hydrochloric acid secretion, which eventually affects the nutrient digestion in the gizzard.

The small intestine is the main site for most of the nutrient digestion and absorption. The small intestine consists of the duodenum, and the lower small intestine. The lower small intestine is composed of two parts, namely jejunum and ileum. The first part of this segment, namely duodenum receives digestive enzymes and bicarbonate from the pancreas and bile from the liver via the gallbladder. Despite ending at the outlet of the pancreatic and bile ducts, the acidic contents from the gizzard mix with bile and pancreatic juices in this section through gastro-duodenal refluxes. The high endogenous secretion also assures the dilution of the feed residue to a level of ~0.10 in pigs and 0.15–0.20 in poultry, which helps the polar solution penetrate the feed particles thus it ensures an efficient cleavage of starch (Knudsen et al., 2006). As feed passes to the duodenum, pH rises due to bicarbonate secretion from the pancreas, and digestion starts. Secretion of bicarbonate acts as a lubricant, and it protects the duodenal wall from the hydrochloric acid entering from the stomach. Bile is secreted by the liver and passes to the duodenum through the bile duct. Bile is stored in the gallbladder until it is required. The bile salts play an important role in digestion by activating pancreatic lipase and emulsifying the fats. The pancreas is a gland has secretory functions such as insulin production and digestive

enzyme production. Pancreatic juice is secreted into the duodenum through the pancreatic duct. Luminal cavity of the jejunum is the major site for starch digestion (Osman, 1982).

The ileum is the last segment of the small intestine. Although some nutrient digestion and absorption may result in, this segment is mainly thought to have a role in absorption. For instance, Zimonja and Svihus (2009) found that the ileum might have a significant role for digestion and absorption of starch in especially fast growing animals namely broiler chickens. According to their study, starch digestibility of pelleted wheat diet increased from 81 to 98% from ileum to excreta, and Svihus et al. (2004) observed starch digestibility increased from 91 to 99% from the anterior third to the posterior third of the ileum.

Some physiological differences between pigs and poultry exist. For example, the length of the small intestine of the bird (around 2 m) is quite a bit shorter than in pigs (De Verdal et al., 2011). For pigs at birth, the small intestine is about 2 m long. At weaning, it has more than 6.5 m long. The small intestine of fully grown pigs is 16-21 m (Lindberg and Ogle, 2001). Furthermore, the addition of moisture into intestinal chyme is less in chickens compared with pigs (Moran Jr, 1982).

For chickens, pancreatic α-amylase is the main enzyme for starch digestion. No enzymatic hydrolysis of starch occurs prior to the stomach. After the ingested feed has passed the crop and proventriculus, it passes into the gizzard. In the gizzard, the feed is ground before it passes to the small intestine. Pancreatic α- amylase is secreted in the lumen of the small intestine. The optimal pH for α-amylase is 6.9 (Weurding, 2002). The major digestion of starch initiates in the small intestine via pancreatic α- amylase, and then followed by brush border enzymes. Despite the relatively small digestive tract (~2m) compared to pigs, the starch digestion capacity of chickens is very high, attaining values close to 100%, and being above that of pigs for instance, even at young ages (Zelenka and Ceresnakova, 2005) in association with a high secretion of amylolytic enzymes in the duodenum.

Except pigeons, a pair of ceca is found in domesticated poultry species. The pair of ceca is located at the juncture of the ileum and colon. Pigs have a shorter caecum and longer colon compared to poultry. Some of the remaining water in the digested material is absorbed in ceca. Another important function of the ceca is the fermentation. There is a complex population of aerobic and obligate anaerobic bacteria, including *Lactobacilli*, *Streptococci, Coliforms*,

*Bacteroides*, *Clostridia* and yeasts (McDonald, 2002). The review by Józefiak et al. (2004) states the overview of the fermentation in the avian cecum and discuss in detail about the benefits of this fermentation. The role of ceca will be given in a short summary here. During this fermentation, the ceca produce several volatile fatty acids as well as vitamins (Coates et al., 1968). The volatile fatty acids are absorbed and contribute to the energy supply. Because the ceca are located so close to the end of the digestive tract, few of the produced nutrients are absorbed (Jacob et al., 2011). Therefore, the one of the main functions of the ceca is electrolyte and water absorption.

As discussed previously, the main site of absorption of digested nutrients is the small intestine. The remaining feed material, which is resistant to the action of enzymes, will move further to large intestine. The digestive enzymes will not digest cellulose, hemicellulose, and lignin. In addition, untreated starch or certain starches are resistant to the hydrolysis by amylase. The glands of large intestine are mainly mucous glands, and no enzyme secretion has been observed here (Jacob et al., 2011). However, the hydrolysis of remaining materials occurs due to microbial activity. Therefore, the large intestine plays an important role for capturing remaining nutrients, and water in the digesta.

In the cloaca, the digestive wastes mix with wastes from the urinary system. Chickens usually void fecal material as digestive waste with uric acid crystals on the outer surface (Jacob et al., 2011). Chickens do not urinate. The waste materials, or feces, voided from the large intestine via the anus consists of water, undigested feed materials, digestive secretions, epithelial cells from the tract, inorganic salts, bacterial and products of microbial disintegration.

### 2.2. Action of α-amylase

α-amylase belongs to the endoamylases, which cleave α-(1→4) glycosidic bonds in the inner part of the amylose and amylopectin chains (Weurding, 2002). During starch digestion by α-amylase, amylose is broken down to maltose and maltotriose. Whereas amylopectin is degraded to maltose, maltotriose and α-dextrins. α-amylase attaches to the substrate at a random position along α-(1→4) chain (Figure 2).

Enzymatic hydrolysis requires binding of amylolytic enzymes to starch molecules. Porcine pancreatic α-amylase has five subsites to bind substrates and from its hydrolysis product profile (maltose, maltotriose, and other dextrins with DP 2-7), it requires binding to at least three

glucose units before cleaving an α-(1→4) glycosidic linkage (Zhang et al., 2006a). Based on this study, α-amylases possess less specificity for smaller glycosidic oligosaccharides. In the case of these small molecules, only two or three catalytic sites are occupied and therefore, it is impossible to span a cleaving site α-amylase does not possess the specificity for α-(1→6) bonds at the branching points in amylopectin and the ability to break α-(1→4) linkages adjacent to the branching point is prevented by its spherical structure (Gray, 1992 as cited in (Weurding, 2002)). The end products of amylopectin are α-dextrins that contain α-(1→6) linkages. These end products cannot pass the intestinal wall, therefore these molecules must be degraded further to glucose molecules. α-amylase is the only carbohydrase, which dissolves in the fluid in the lumen of the small intestine. Moreover, 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) hydrolyzed by oligosaccharidases, which are located in the intestinal surface brush border membrane.

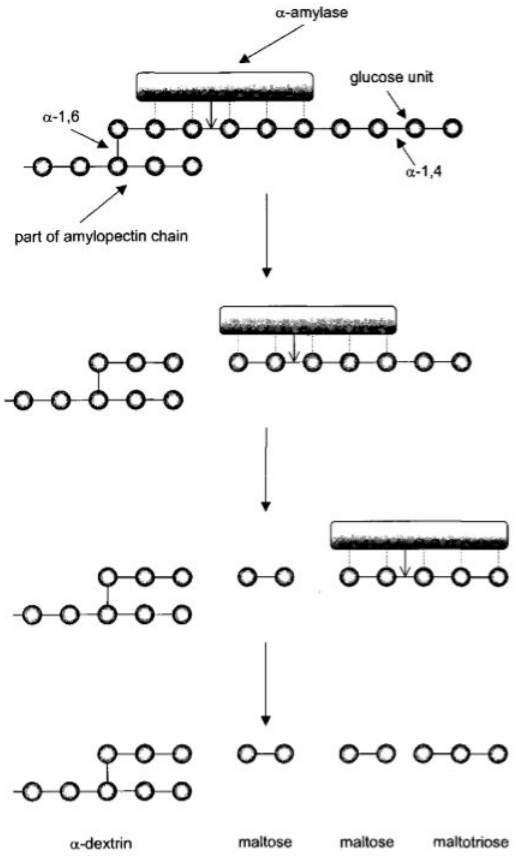



Figure 2. Schematic presentation of the initial enzymatic hydrolysis of amylopectin by α-amylase (Weurding, 2002)

Amyloglucosidase was thought to act primarily on α- amylase breakdown products by converting them to glucose (Kaufman and Tietz, 1980). However, recent study indicated synergistic action of α-amylase and amyloglucosidase on attacking starch granules (Warren et al., 2015). Amyloglucosidase is also capable of hydrolyzing α-(1→6) linkages, however α-amylase cannot. Microbial amyloglucosidase are commonly used *in vitro* trials as a final step to convert α-limit dextrins and maltose to glucose (Dhital et al., 2017). Dhital et al. (2017) emphasized that excess amount of amyloglucosidase was used *in vitro* to ensure complete conversion of α-amylase reaction products to glucose immediately, for ease of measurement as glucose. Despite the multiple stages involved in reaction pathways, the kinetics of starch digestion, either with amylase or in combination with amyloglucosidase, often show simple decay curves with apparent first-order behavior (Zhang et al., 2013). In contrast to *in vitro* trials, mucosal enzymes hydrolyze the products of amylase action to glucose *in vivo* as described below. To conclude, the direct attack mechanism of these two enzymes on starch granules was more evident in *in vitro* digestibility trials. Therefore, it is a fact that the main enzyme for starch digestion is α-amylase *in vivo*.

Lynn and Cochrane (1997) observed wheat starch digestion by the action of pancreatic α-amylase via scanning electron microscopy technique. According to their results, digestion initiated through the channels on the surface of the starch granule and from those channels digestion extended towards the interior of the granule. 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., 2006a). Therefore, understanding of starch structure is very important to understand starch digestion in broiler chickens.

### 2.3. Cereal and Legume Starch Characteristics and their effects on digestion

Starch is a complex carbohydrate produced by plants to store energy generated through photosynthesis. Starch is especially abundant in legumes, tubers, and cereals; all these are highly consumed by feed industry worldwide. Due to the high availability, it is considered as the main energy source for broiler chickens. The main sources of starch in commercial broilers are cereal grains such as wheat (starch content ~60% DM), maize (~65% DM) and barley (~60% DM). Among cereal starch sources, wheat is one of the most important cereals used for animal

feeds. The wheat grain (Figure 3) contains 2-3% germ, 13-17% bran and 80-85% mealy endosperm (DM basis) (Belderok et al., 2000).

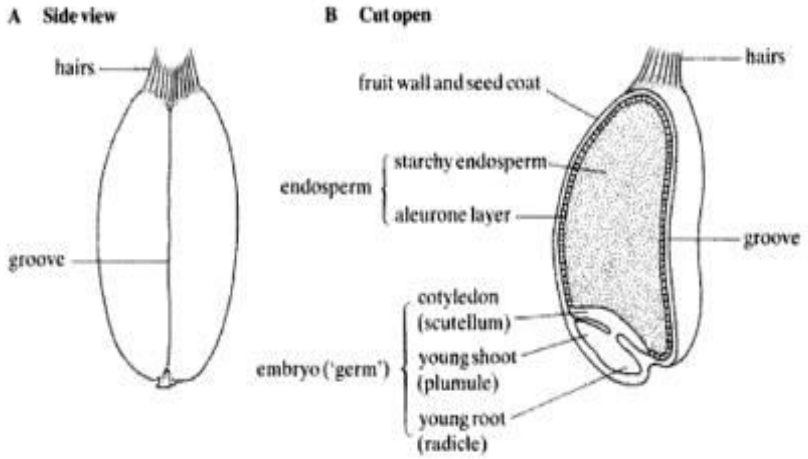



**Figure 3**. Structure of a wheat grain. The seed is endospermic (Roberts, 1986)

Bran is the outer layers of wheat grain which is made up of different layers to protect the main part of the grain. The bran comprises water insoluble fiber essentially cellulose and pentosans, polymers based on xylose and arabinose, which are tightly bound to proteins (Belderok et al., 2000). Therefore, milling is applied to separated bran from the starchy endosperm. As shown in figure 3, the endosperm is surrounded by the pericarp and seed coat. The outer endosperm, the aleurone layer is rich in proteins and enzymes, which play a vital role in the germination process (Belderok et al., 2000). The inner endosperm without the aleurone layer referred to as starchy endosperm (Belderok et al., 2000). The endosperm mainly contains reserves needed for the growth of the seedling, and therefore it is rich in starch to yield energy.

On the other hand, legumes are valuable source of nutrients. Starch is the most abundant carbohydrate in the seed (22–45%) (Hoover and Sosulski, 1997). Faba bean (Vicia faba *L*.) is a major feed legume, because of the high nutritional value of its seeds, which are rich in protein and starch. It is a protein rich legume seed well adapted to most climatic areas of Europe and widely used for feed, and due to high adaptation in different climates, faba bean is grown worldwide (Crépon et al., 2010). It contributes to the sustainability of cropping systems through its ability by biologically fixing nitrogen; its capacity to reduce fossil energy consumption; and providing protein rich food and feed (Jensen et al., 2010).

In cereals, endosperm acts as a food store for the developing seed, however in non-endospermic seeds, the endosperm is used up in the early stages of seed development so the food is stored in the cotyledons (Bewley and Black, 2014). Therefore, the organization of cereal grains and legume seeds is quite different (Figure 3 and 4). The starch granules in beans are present in the cotyledon cells and are embedded in the protein matrix of the cellular contents (Berg et al., 2010).

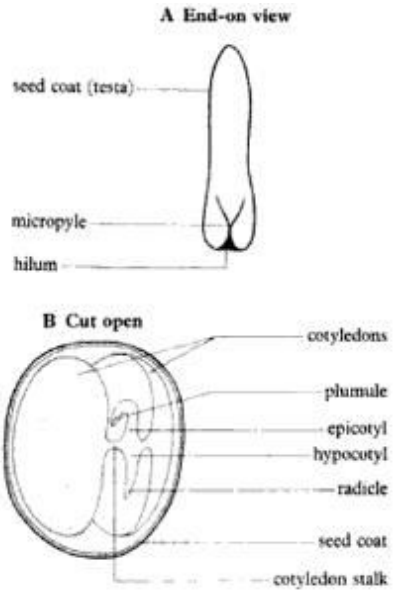


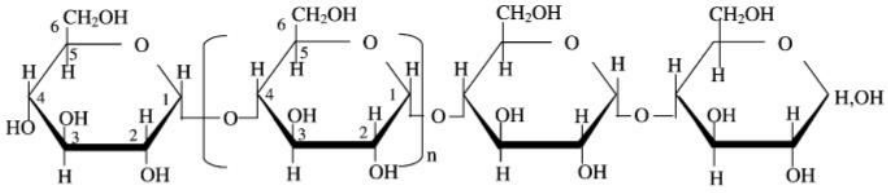

**Figure 4.** Structure of bean seed, a non-endospermic seed (Roberts, 1986)

Starch is the major faba bean seed component, reported as mean content 423 g/kg DM basis (Créponet al., 2010). The classic faba bean cultivars contain tannin; an anti-nutritional factor that primarily reduce nutrient digestibility and compromise growth performance (Wierenga et al., 2008). Fortunately, the fractionation of faba bean components has been explored. New varieties have been bred to reduce levels of anti-nutritional qualities. Demand for high protein or high starch fractions in animal nutrition has made faba bean valuable in the animal nutrition. Air classification not only separates pulse flour into protein and starch, but also enriches the fractions. In this study, starch content of the fraction of air classified faba bean was enriched from 376 g/kg DM to 744 g/kg DM after air classification.

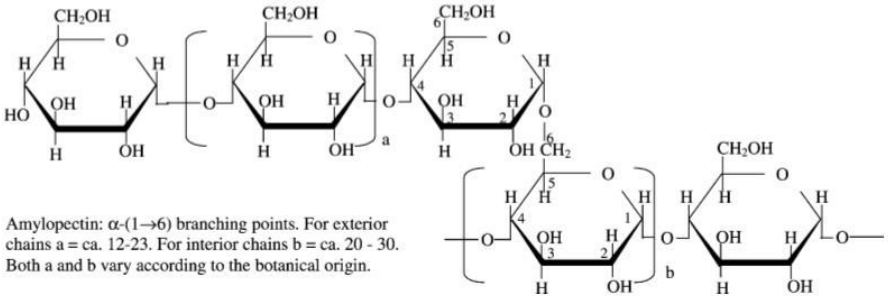
The rate of starch digestion in legumes is lower both *in vitro* and *in vivo*, than that of cereals (Weurding et al., 2001a, Chung et al., 2009). Overall mechanisms involved in limiting amylase digestion rates of starches, can be classified into two groups such as barriers that slow down or prevent access of enzyme to starch; and starch structural features that slow down or prevent

enzymatic action. Differences in the *in vivo* digestibility of starches can be affected by many structural factors depending on the starch source such as size/distribution of the granules, extent of molecular association between starch components, amylose/amylopectin ratio, degree of crystallinity, types of crystalline polymorphic form, amylose–lipid complexes, dietary fiber components, and anti-nutrient factors in the feed. In this section, structural composition of starch will be explained and differences in both cereal and legume starch sources, which affect the digestion rate, will be discussed.

Native starch is stored in granules, which are variable in size and shape. Starch granules consist of several layers, which are composed of two types of α-glucan: namely, amylose and amylopectin (Tester et al., 2004b) (Figure 5). Both are the polymers of glucose units bound together with glycosidic bonds. Amylose consists of long linear chains of α-(1→4) linked glucose residues with relatively few α-(1→6) linked branches whereas amylopectin is a highly branched molecule of shorter α-(1→4) linked glucose molecules and more α-(1→6) branches (Banks and Muir, 1980). Molecular weight of amylose is around 100 kDa, whereas amylopectin is a much larger molecule than amylose with a molecular weight in between $10^4$ –$10^6$ kDa (Buléon et al., 1998).



Amylose: α-(1→4)-glucan; average n = ca. 1000. The linear molecule may carry a few occasional moderately long chains linked α-(1→6).



Amylopectin: α-(1→6) branching points. For exterior chains a = ca. 12-23. For interior chains b = ca. 20 - 30. Both a and b vary according to the botanical origin.

**Figure 5.** Structure of amylose and amylopectin (Tester et al., 2004b).

Amylose has a linear structure compared to amylopectin, which is highly branched. The proportion of amylose and amylopectin fraction depends on the source of the starch. Most starches consist of about 75 % semi-crystalline amylopectin and about 25% amorphous amylose (Svihus et al., 2005). For cereal sources, the proportion of amylose in barley starch varies from 30 to 460 g/kg (Vasanthan and Bhatty, 1996, Åkerberg et al., 1998, Andersson et al., 1999) and in maize from 0 to 700 g/kg (Jenkins and Donald, 1995, Kishida et al., 2001). In wheat, a range from 30 to 310 g/kg has been reported (Mohammadkhani et al., 1998, Peng et al., 1999, Abdel-Aal et al., 2002). The amylose content of legume starches varies from normal to high in the range of 170-510 g/kg (Wani et al., 2016). In addition, the proportion of amylose in faba bean ranges from 170-420 g/kg (Gunasekera et al., 1999, Haase and Shi, 1991, Morrison and Laignelet, 1983). High degree of crystallinity (high amylose content) is associated with a lower hydrolysis rate (Tahir et al., 2010). It is believed that amylase attacks the regions of starch polysaccharide that are non- crystalline and thus the degree of crystallinity will have an influence on the rate of digestion of the starch granules (Tahir et al., 2010). Opposite to Tahir et al. (2010), Zhang et al. (2006a) reported that both crystalline and amorphous regions of maize starch were digested with ease by α-amylase, thus leaving the question open of the susceptibility of crystalline regions to digestion.

The amylopectin molecule forms regions with low and high levels of branches. In highly branched regions, side-chains of amylopectin are grouped by forming crystalline clusters. Side chains of the amylopectin molecule can be divided in A, B, and C chains. C-chains constitute the backbones of the amylopectin molecules, to which B-chains are linked that carry one or more branches. B-chains are entitled with an additional number based on their participation in side chain clusters. B1-chains participate in one cluster, B2- and B3- chains participate in two or three clusters. A-chains are present at the outside of the branched molecule and have only one α-(1→6) linkage to B1-chains (Figure 6). A-chains are believed to correspond with side chains with a degree of polymerization of DP (6–12), B1 chains with DP (13–24), B3 chains with DP (25–36), and C-chains with DP> (36) (Pérez and Bertoft, 2010). Differences with the side chain distribution among the starch sources are given in Table 1.

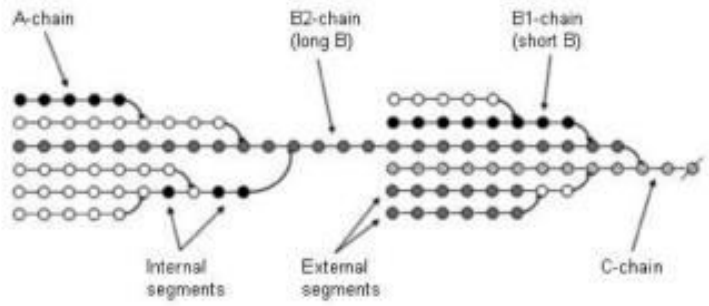



**Figure 6.** Basic labelling of chains in amylopectin. Circles denotes glucosyl residues, horizontal lines (1→4) and bent arrows (1→6) linkages. The reducing- end residue is to the right (Pérez and Bertoft, 2010).

**Table.1.** Characteristics of amylopectin of different starch sources

| Starch source | Amylopectin chain length distribution (%) | | | | References |
|---|---|---|---|---|---|
| | DP (6–12) | DP (13–24) | DP (25–36) | DP (37–50) | |
| Pea (smooth) | 16.2–25.4 | 48.5–59.9 | 13.9–16.0 | 16.4–19.4 | (Ambigaipalan et al., 2011) |
| Lentil | 26.0–26.9 | 57.8–58.4 | 15.6 | - | (Ambigaipalan et al., 2011) |
| Faba bean | 20.38 | 53.39 | 14.83 | 11.40 | (Ambigaipalan et al., 2011) |
| Black bean mean | 19.62 | 53.81 | 16.01 | 10.56 | (Ambigaipalan et al., 2011) |
| Wheat-Regular | 9.9 | 31.2 | 18.8 | 40.2 | (Srichuwong et al., 2005) |
| Corn | 5.1 | 31.4 | 56.7 | 6.8 | (Srichuwong et al., 2005) |

Short chains DP (6–12) of amylopectin present in the amorphous region might be easily attacked by enzymes, since they are unable to form stable double helices (Chung et al., 2008b, Zhang et al., 2006b). Slowly digestible starch consists of imperfect crystallites and amorphous components and positively correlated with intermediate amylopectin chains DP (13–36) (Shin et al., 2004, Zhang et al., 2006b, Benmoussa et al., 2007). Long amylopectin branch chains (DP > 37) used to connect adjacent clusters can also lead to high slowly digestible starch content (Benmoussa et al., 2007, Chung et al., 2010). Resistant starch mainly consists of a crystalline structure tightly packed in a radial pattern. The longer amylopectin chains and amylose chains of legume starches can form more stable helices that can be further stabilized by hydrogen bonds and distributed over the entire crystalline regions (Singh et al., 2010). Thus this structural feature of legume starch may limit its accessibility of digestive enzymes (Singh et al., 2010).

Clustered amylopectin side chains and amylose chains are organized in the helix conformation that later forms crystalline structures divided into three types: A, B and C. In A-type crystalline starch, glucose helices are packed densely, whereas B-type crystalline starch is packed less dense, by leaving space for water molecules in between the branches (Tang et al., 2000). A-

type and B-type differ in their packing of double helices and water content as shown in Figure 8. C-type crystalline starch consists of a combination of A- and B-type crystallites. Most cereal starches give the A-type, some tubers (such as potato and lesser yam), and cereal starches rich in amylose yield the B-type; legume starches generally have a C-type pattern (Pérez and Bertoft, 2010).The arrangement in A-type or B-type crystallites markedly influence digestibility. Generally, a higher susceptibility of A-type crystallites to hydrolysis compared to B-type crystallites has been reported (Srichuwong et al., 2005, Zhang et al., 2006b).

Jane et al. (1997) explained the differences between A- and B-type starches susceptibility towards α- amylase. In A-type starches, there were many short A- chains of amylopectin derived from branch linkages located inside the crystalline regions containing α-(1→6) linked branched points and the short double helices were more susceptible to α- amylase hydrolysis. In B-type starches, more branch points were clustered in the amorphous region, and there were fewer short branch chains. Overall, the crystalline structure of B-type starches are predominant. Hence, B-type starches are more resistant to α-amylolysis. In addition, shorter double helices and interior crystallites in A-type starches are more readily digestible and exhibit a high amount of readily digestible starch and slowly digestible starch compared to B-type starches (Lehmann et al., 2007).

Bogracheva et al. (1998) described the chemical structure of different crystalline polymorphs. Regarding to C-type starches, they had shown that the B- polymorph was situated in the center of all granules and was surrounded by the A- polymorph in pea starch. More recent research done by Wang et al. (2009) also showed that B-type polymorph existed at the center part of the granules, which was surrounded by the A-type polymorph in the peripheral part of granules (Figure 7).

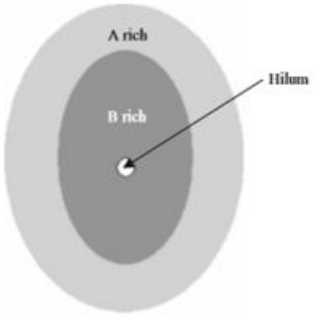



**Figure 7.** Drawing of C-type starch (e.g, Chinese yam starch) (Wang et al., 2009)

Ratnayake et al. (2001) had shown by studies on starches from different cultivars of field peas that resistance to α-amylase increases with increase in B- polymorph content. Afterwards, Gerard et al. (2001) had also agreed that orientational distribution and packing of B-type crystallites within the granule could be a factor influencing resistance towards α-amylase. Overall, it can be stated that legume starches (C-type) are more resistant against enzymatic hydrolysis than cereal starches (A-type).

Starch is deposited in alternating amorphous and crystalline layers by forming growth rings during starch biosynthesis. The semi-crystalline layer is believed to consist of alternating crystalline layers of double helical α-glucans extending from branches of amylopectin, and the amorphous layers of amylopectin branch points, and starch granules are made up of alternating amorphous and crystalline shells, which are between 100 and 400 nm thick (Gallant et al., 1997).

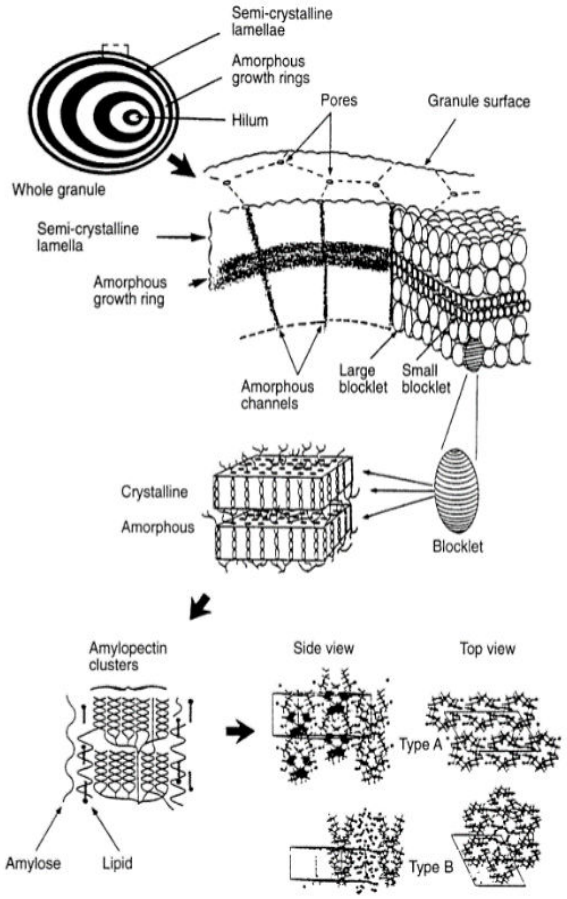



**Figure 8.** The idealized structure of a starch granule redrawn from (Gallant et al., 1997).The dots in the type A and B amylopectin lattices correspond to positions of water molecules deduced from X-ray scattering data (Tang et al., 2000)

Starch granules in different plants show distinctive morphology, ranging from round, oval, lenticular or polyhedral, and sizes from 1 μm to more than 100 μm in diameter. Granule size distributions are usually classified as unimodal or bimodal. According to previous investigations, a bimodal size distribution of large and small granules is characteristic of wheat starches as well as those from rye and barley (Eliasson and Larsson, 1993, Buléon et al., 1998). The proportion of small and large granules differs among raw material and its genotypes. On the other hand, oat, rice and maize starch have unimodal granule-size distribution.

In the previous study (Raeker et al., 1998), it had been reported that wheat starch actually showed a trimodal granule distribution rather than a bimodal. An intermediate granule (underdeveloped A-type) was mentioned as constituting the third group. However, wheat endosperm is mostly reported to contain just two types of starch granules. A-type granules are disc-like or lenticular in shape with a diameter of >10 μm, while the B-type starch granules are less than 10 μm in diameter and spherical or polygonal in shape (Vermeylen et al., 2005, Ao and Jane, 2007, Kim and Huber, 2008, Wang et al., 2014). In wheat, A-type granules contribute to more than 70% total weight of the starch (Peng et al., 1999, Shinde et al., 2003) whereas B-type granules comprise up to 90% of granules in number (Raeker et al., 1998).

The granule shape and size of cereal and legume starches are listed in Table 2 and 3. In particular, faba bean starch granules were oval, and spherical shaped, and its granule size varies between 9-24 μm in width and 11-48 μm in length (Wani et al., 2016). Faba bean starch showed unimodal size distribution (Cai et al., 2014). In general, larger sized granules exist in legumes compared to cereal grains.

**Table 2**. Shape, size and distribution of starch granules of cereal sources (Tester et al., 2004a)

| Starch source | Size (μm) | Shape | Distribution |
|---|---|---|---|
| Wheat | 15–35, 2–10 | Lenticular (A-type), spherical (B-type) | Bimodal |
| Barley | 15–25, 2–5 | Lenticular (A-type), spherical (B-type) | Bimodal |
| Maize (waxy and normal) | 2–30 | Spherical/ Polyhedral | Unimodal |
| Rice | 3–8 | Polyhedral | Unimodal |
| Oat | 3-10 | Polyhedral | Unimodal |

**Table 3.** Shape, size and distribution of legume starches (Wani et al., 2016)

| Starch source | Size (μm) | Shape | Distribution |
|---|---|---|---|
| Kidney bean | 9-60 | Oval, round, elliptical, irregular | Unimodal |
| Chick pea | 9-30 | Oval, spherical | Unimodal |
| Green gram | 7-49 | Oval, round elliptical, irregular | Unimodal |
| Faba bean | 9-48 | Oval, round, irregular | Unimodal |
| Lentil | 6-37 | Oval, spherical, elliptical | Unimodal |
| Pinto bean | 6-42 | Oval, round, irregular | Unimodal |

The granule size, distribution and shape are considered important for the functional properties of the starch. The effect of starch granule size on the digestibility stated in (Svihus et al., 2005), showed cereals with smaller granule size (e.g, rice, average 8 μm) have higher starch digestibility than the cereals with larger granules (e.g, wheat, average 22 μm). The distribution of different size granule fraction of different faba bean cultivars was examined and according to results, 70 % of the faba bean granules were in the range of 17.3-30.19 μm, 20 % of the granules in the range of 34.67-60.2 μm, and less than 10 % were in the range of 8.7-15 um (Ambigaipalan et al., 2011).

Since, starch hydrolysis involves an enzyme in solution by acting on starch granules; the surface area for the enzyme is a critical kinetic parameter. As in the case of granule size, the starch size distribution may affect important physicochemical properties, thus may affect the digestion of starch. According to recent research done by (Chiotelli and Le Meste, 2002), the thermomechanical behavior of separated large (A-type) and small (B-type) starch granules from wheat was examined. Accordingly, A- and B-type starch granules have significantly different gelatinization and rheological properties. The B-type granules have higher gelatinization temperature and lower gelatinization enthalpy than A-type granules. This lower enthalpy value for the gelatinization of B-type granules suggests a lower proportion of organized structures in small granules than large granules; another speculation can be a lower stability of the crystalline regions. Therefore, starch granule size distribution may affect its thermomechanical properties, such as gelatinization and enzyme susceptibility.

Internal structure, morphology, and the surface characteristics of starch granules had been extensively examined by many researchers (Fannon et al., 1992, Fannon et al., 1993, Kaur et al., 2002). Pores at the surfaces of starch granules were observed for some maize, sorghum

and millet starch granules, and along the equatorial groove of large wheat, barley and rye starch granules (Huber and BeMiller, 2000). In the study done by Fannon et al. (1993), it had been proposed that granule pores might be more than surface features such as pores that might be openings to interior channels.

Hilum is defined as the core of the starch and the central area of the granule around the hilum is believed to be the least organized region of the starch granule, since gelatinization, enzymatic attack (maize), acid-catalyzed hydrolysis (maize) and cavitation all originate there (Huber and BeMiller, 2000). Hence, as microstructural features of granules such as pores, channels and cavities have the potential to influence reactions by connecting the cavity at the hilum to the granule exterior and increasing the surface area available for enzymes into the granule matrix, especially into the less organized region surrounding the hilum. Hereby, a greater understanding of these microstructural features is required.

In A-polymorphic starches, interior channels were reported to be lined with proteins and lipids and had apparent diameters ranging from 0.007 to 0.1 mm, whereas pores, the opening of channels, were larger in diameters varying from 0.1 to 0.3 mm (Han et al., 2005, Benmoussa et al., 2010, Naguleswaran et al., 2011). These surface pores and interior channels of starch granules provide easy access for α-amylase with a radius of approximately 3–4 nm to diffuse inside the granules (Dhital et al., 2017). Dhital et al. (2010) showed the specific digestion pattern for the easy access of enzymes to the less organized hilum area inside the maize granules (Figure 9a, b). However, for granules lacking pores and channels such as potato starch, the enzymes initiate the digestion from the surface towards the granule interior with a different pattern shown as (Figure 9c, d). Cereal starches contain peripheral pores and channels, which allows the penetration of enzymes into the interior of granules. In conclusion, these surface pores of cereal starches may be main site for initial enzyme attack by allowing enzyme molecules directly into the interior of granules.

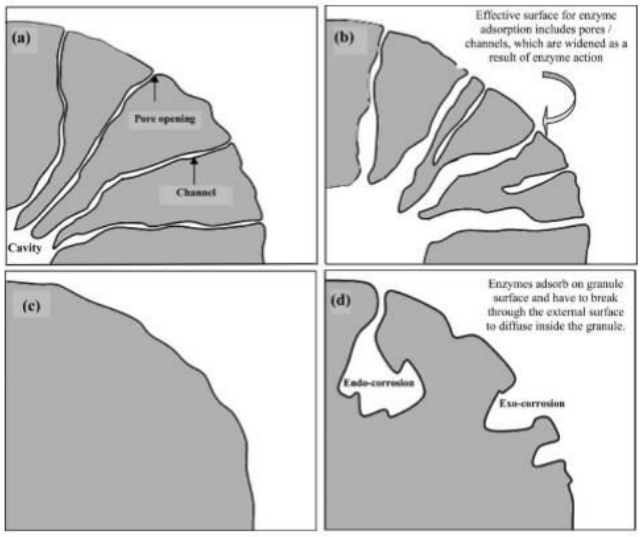



**Figure 9.** Model illustrating diffusion of amylase and its catalytic patterns in maize and potato starches (Dhital et al., 2010): (a) Maize starch showing pores, channels and cavity, (b) maize starch hydrolyzed by amylase with enlarged pores, channels and cavity, (c) potato starch lacking pores, channels and cavity, (d) and potato starch exo and endo-corroded by amylase

In addition to amylose and amylopectin, starch granules contain non-starch components, which are associated with the starch granule. Among these, proteins and lipids are by far the most abundant and important for the properties of starches, and the quantities of protein and lipid associated with the starch depends on the botanical origin of the starch (Svihus et al., 2005). Non-starch components such as lipids and proteins in the starch granules have potential to interfere with starch digestion (Svihus et al., 2005).

Starch granules usually contain 3 g or less protein/kg (Cornell et al., 1994, Vasanthan and Bhatty, 1996, Abdel-Aal et al., 2002 as cited in (Svihus et al., 2005)). Proteins, which are associated with starch granules from different sources, generally exist in two forms, as storage proteins (e.g., gluten and gliadin) or starch granule associated proteins. The storage proteins, which remain on the surface of starch granules, have a molecular weight in the range of 5-60 kDa, while internal granule-associated proteins have a molecular weight in the range of 60-150 kDa (Baldwin, 2001). In wheat, the 15 kDa protein called friabilin found on the surface of starch granules has been associated with an important quality characteristic such as endosperm hardness, which affects milling quality (Baldwin, 2001). Storage protein of the endosperm may also contribute to low digestibility of wheat starch (Al-Marzooqi et al., 2009). Proteins found on the surface of granules may impair starch digestibility, because surface proteins associated with starch granules provide barriers to the diffusion and absorption of the enzymes, which are

proposed to be one of the main actors determining the kinetics and degree of hydrolysis (Dhital et al., 2017).

Lipids are rare in many root and tuber, and pulse starches, but more abundant in cereals ranging from 5 to 10g/kg (Hoover and Vasanthan, 1994, Vasanthan and Bhatty, 1996, Buléon et al., 1998, Sahlström et al., 1998, Andersson et al., 1999 as cited in (Svihus et al., 2005)). Thus, important components in cereal starches may be lipids. Lipids are present in the form of free fatty acids (mostly palmitic and linoleic acids) or lysophospholipids (mainly in cereals), which are associated with amylose (Baldwin et al., 1997). The amount of lipid-complexed amylose ranges from 15 to 55% of the amylose fraction in cereal starches as cited in Tester and Qi (2004). A significant portion of these lipids is found on the surface of the starch granule (Baldwin et al., 1997). These components may also represent a challenge during digestion. Lipid and starch complexes may influence digestion by reducing contact between enzyme and substrate. Starches with high amylose content are also associated with high amounts of lipid formation on the surface of granules (Svihus et al., 2005). Therefore, lipids may impair digestibility due to their hydrophobic properties by reducing the water access on the granule surfaces (Svihus et al., 2005).

Mineral fractions are also important non-starch components. Most cereal starches contain 0.01-0.07 % phosphorus that is mainly in the form of phospholipids (Dhital et al., 2011), whereas tuber starches such as potato contain 0.09% phosphorus in the form of starch phosphate monoesters (Singh et al., 2003). Mineral fractions are negligible in starch from cereals. On the other hand, legume starches contain varying amount of phosphate monoester groups (Jane et al., 1996 as cited in (Singh et al., 2008)). Starch phosphate is enclosed to the amylopectin fraction, and mainly associated in the amorphous region of the native starch granules (Blennow et al., 2000). Thus, it may be a major obstacle to swelling and subsequent digestion (Blennow et al., 2000). Starches from legumes showed better shear stability than wheat starches due to higher peak viscosity and lower breakdown in legumes (Singh et al., 2008).

Lastly, dietary fibers are important fractions. Dietary fibers are divided into two groups, namely soluble and insoluble dietary fiber. Beta-glucans and arabinoxylans are soluble dietary fibers. Cellulose, hemicellulose and lignin are insoluble dietary fiber, which are insoluble in water. Svihus and Gullord (2002) compared 16 samples of Norwegian wheats, and reported variation in dietary fiber content between 20-26 g/kg. In most grain legumes, the content ranges of

dietary fiber from 80 to 270 g/kg, with soluble fiber in the range 33–138 g/kg. The non-starch polysaccharide content of faba beans consists mainly of cellulose (89–115 g/kg DM), with lower levels of hemicellulose (21–57g/kg DM) (Salgado et al., 2002a, Salgado et al., 2002b). Soluble dietary fiber can increase the viscosity of intestinal contents, and consequently they affect the digesta passage rate and efficacy of digestion (Svihus and Gullord, 2002). Soluble dietary fraction such as arabinoxylans (mainly in wheat) can lower the digestibility of starch (Maisonnier et al., 2001). Supplementation of exogenous xylanase to wheat-based diet is known to increase starch digestibility (Svihus and Gullord, 2002). Legumes such as faba beans have been used in the poultry and swine diets (Jezierny et al., 2010). The use of grain legumes in animal diets could be impaired by the presence of anti-nutritional factors, and non-starch polysaccharide content (Adamidou et al., 2011). By using air-classification, the negative effect of non-starch polysaccharides may be eliminated for faba bean starch (FBS) fraction based diet.

In conclusion, the reduced bioavailability of legume starches has been attributed to the presence of intact non-starch compounds enclosing starch granules, absence of pores on the starch granules, high content of viscous soluble dietary fiber components, presence of a large number of anti-nutrients, larger granule size, high levels of amylose, and C-type crystallites.

### 2.4. Effect of feed processes

Dry fractionation (Figure 10) is a process, which employs both milling and air classification. Air classification is not a new method; air classification of legumes has been investigated since 1970s. In starch-rich legumes, the cotyledon cells consist of starch granules (±20μm) enclosed with protein matrix (1-3 μm) that are surrounded by a fiber cell wall (Pelgrom et al., 2013). The starch granules are released via milling and the protein matrix is fragmented in smaller particles than the starch granules (Figure 11). Subsequently, the particles and fragments are separated based upon either size, density or both by air classification. In substance, air classification separates the smaller protein rich fragments from the larger starch granules and fiber rich fragments. In this study, air classification is used to obtained faba bean starch fraction.

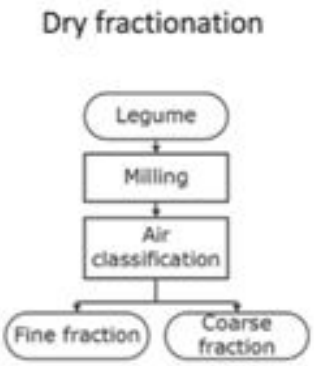



**Figure 10.** Schematic illustration of dry fractionation process (Schutyser et al., 2015).

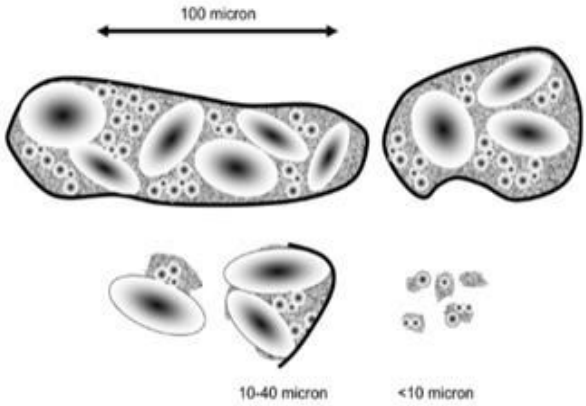



**Figure 11.** Schematic drawing of typical cells present in the endosperm of wheat related fragments containing high starch (10-40 μm) and high protein concentration (<10μm) (Schutyser and Van der Goot, 2011).

Almost all feeds used in commercial poultry production are subjected to some of feed processing. The widely used processing methods in feed manufacturing are grinding, batching, mixing, thermal treatment (e.g., pellet press, expander, and extruder), and cooling, drying. Hammer and roller mills are the most common equipment, which are used to reduce the particle size of feed ingredients. Via hammer mills, a set of hammers are rotated at high speed and particle size reduction is performed by impacting force. Size distribution of particles produced in a hammer mill varies widely around the geometric mean, with some large and many small sized particles (Svihus et al., 2004). Via roller mills, size reduction achieved by compression force between rotation roll pairs. Generally, roller mill gives more uniform particle size distribution. Feed for layers is commonly fed as a mash feed, while other poultry feed are passed through a conditioner followed by pelleting. Pelleting is used because not only it is cost effective, but also pelleting process improves hygienic quality of feed (Abdollahi et al., 2013). In addition, pelleting reduces feed wastages (Jensen, 2000). For broiler feed, pelleting is also widely used due to

higher feed intake and weight gain observed by Engberg et al. (2002) and Svihus et al. (2004). The major effects of processing are the structural changes of the feed. Thermomechanical processes such as pelleting and extrusion will cause heat induced chemical changes of some components in the feed (Svihus et al., 2006). Both the physical and chemical changes take place during processing may have a large impact on the performance of birds, both directly through the effect on feed digestibility and indirectly through effects on feed intake pattern and gut function (Svihus et al., 2006). In this section, the comparison of pelleting and extrusion process will be covered, and their effect on the starch digestibility will be discussed.

In the typical pelleting process, the process consists of batching, grinding and mixing, conditioning, pelleting and cooling. The dry feed ingredients are conditioned with saturated steam. Via saturated steam, the temperature of mash feed will rise up to 80 °C. Utilizing steam provides different benefits such as lubrication of feed through die, heating the mash and most importantly inducing chemical changes. Right after conditioning process, the feed enters the pellet press. Pellet pressing is a thermomechanical process, which the mash feed material is forces through cylindrical holes in die and the mash feed material is shaped into pellets. While the rolls are turned during the feed between rolls, die creates friction. Because of this friction, pellets leave the pellet mil at temperatures varying from 80 to 90 °C and containing as much as 150-170 g/kg moisture (Zimonja et al., 2007). Therefore, cooling process is needed to reduce the moisture to 100-120 g/kg (Zimonja et al., 2007). A stream of ambient air is usually used to remove heat and moisture from the hot pellets by cooling process.

Extrusion process is generally used for pet food and fish feed production. The typical extrusion process consist is batching, grinding, mixing, conveying, extrusion cooking, drying, pumping and coating. Extrusion process may be defined as a high temperature short time process (HTST) in which moistened; feed materials are plasticized and cooked in a tube by the help of moisture, temperature, pressure and mechanical shear. The mechanical shear force applied through the extruder can disrupt complex structure of the feed material, has also positive impact in terms of denaturing harmful enzymes, inactivation of anti-nutritional factors and sterilize the final product. The moisture addition and a properly configured extruder barrel will result in a final pressure prior to the extruder die up to 40 atmospheres, a temperature of 100 to 140 °C and a moisture content of 20 to 30 % (Zimonja, 2015).Therefore, dryer process must be used to decrease the moisture content of the final product. It has been reported by different studies that the extrusion process has effects on feed nutritional quality under different extruder condition (temperature,

feed moisture, screw speed, screw configuration and pressure) and raw material characteristics (Al-Marzooqi and Wiseman, 2009, Van den Einde et al., 2004). Therefore, any variation of the process parameters may influent the nutritional quality and nutrient digestibility of the final feed.

Gelatinization is a term used to describe the molecular events associated with heating starch in excess amount of water. In detail, when starch is exposed to high temperatures and high water addition (<30%), the granular structure starts to disintegrate. Heating aqueous suspensions of starch causes hydrogen bond to weaken to a point where water can be absorbed by the starch granule. The granule will swell, lose birefringence, and lose its crystallinity. Briefly, starch structure is converted from a semi-crystalline to an amorphous form. As the gelatinized starch molecule begins to cool, a gel is formed, which can increase the pellet quality (Zimonja and Svihus, 2009). Overall, starch gelatinization takes place at the different range of temperatures dependent on several parameters. This starch gelatinization may be stated as a complicated process. The degree of gelatinization is based on many properties of starch, such as the amylose/ amylopectin ratio, the water/starch ratio, and the starch/protein ratio (Singh et al., 2003). In this study, two different starch sources were used to compare starch digestion in broiler chickens, therefore starch features and water /starch ratio will be discussed further.

Structural features of starch with different processing conditions may affect starch gelatinization. For instance, starch gelatinization temperature may vary depending on starch sources. Gelatinization temperature can be determined by differential scanning calorimetry (DSC). Stevens and Elton (1971) firstly used DSC for measuring gelatinization and retrogradation of starch. In principle, DSC is a thermal analysis technique for measuring the temperature and heat flows associated with phase transitions, as a function of time and temperature. During starch gelatinization, the starch granules absorbs energy, and the extent of gelatinization varies from source to source. DSC measures the temperature at which irreversible changes occur inside the granule. DSC has become a common method to measure the extent of gelatinization. Beside the extent of gelatinization, DSC method will also determine the thermal transition parameters such as onset (To), peak (Tp), conclusion (Tc) temperatures and enthalpy change (ΔH). DSC measures the gelatinization temperatures and the heat energy required for gelatinization is thought to mainly related to characteristics of the starch granule features (e.g., degree of crystallinity) as cited in (Singh et al., 2003).

At excess water, gelatinization temperature for most cereal starches ranges between 50 °C and 70 °C and most starch sources will gelatinize upon heating to above 80 °C (Svihus et al., 2005). Depending on the starch sources, gelatinization temperature range may vary (Table 4 for cereal sources, Table 5 for legume sources). The onset, peak and conclusion temperatures of gelatinization of wheat were depending on the different cultivars, To, Tp and Tc of faba bean are 55.4 °C, 60.8 °C and 67.6°C, respectively. However, enthalpy change (ΔH) values for cereal and legumes sources are very different. In general, cereal starch sources have higher enthalpy change compared to legume starch sources.

**Table 4:** DSC thermal properties of starches separated from different cereals (Singh et al., 2003).

| Cereal | To [°C] | Tp [°C] | Tc [°C] | ΔH gel [J/g] |
|---|---|---|---|---|
| Normal corn | 62.3 | 67.7 | 84.3 | 14 |
| Normal corn | 64.1 | 69.4 | 74.9 | 12.3 |
| Normal corn | 65.7 | 71 | - | 12 |
| Waxy corn | 66.6 | 73.6 | - | 14.2 |
| Waxy corn | 64.2 | 69.2 | 74.6 | 15.4 |
| High amylose corn | 66.8 | 73.7 | - | 13.7 |
| Rice | 62 | 67.4 | 97.5 | 11 |
| Rice | 57.7 | 65.1 | - | 11.5 |
| Rice | 66-67.26 | 69.74-71.94 | 74.8-78.04 | 8.16 |
| Rice | 70.3 | 76.2 | 80.2 | 13.2 |
| Waxy rice | 66.1-74.9 | 70.4-78.8 | - | 7.7-12.1 |
| Wheat | 51.2 | 56 | 76 | 9 |
| Wheat | 46-52.4 | 52.2-57.6 | 57.8-66.1 | 14.8-17.9 |
| Wheat | 57.1 | 61.6 | 66.2 | 10.7 |

**Table 5.** DSC thermal properties of starches separated from different legumes (Singh et al., 2008).

| Legume starch | Cultivar | To [°C] | Tp [°C] | Tc [°C] | ΔH gel [J/g] |
|---|---|---|---|---|---|
| Faba bean | PG-03 | 55.4 | 60.8 | 67.6 | 3.6 |
| Kidney bean | Chakrata | 68.3 | 73.4 | 79.1 | 3.0 |
| Chick pea | GL-769 | 60.2 | 68.6 | 77.3 | 2.6 |
| Chick pea | GPF-2 | 59.3 | 66.6 | 76.5 | 4.2 |
| Chick pea | PBG-1 | 60.2 | 67.9 | 76.1 | 4.1 |
| Blackgram | UG-916 | 70.6 | 74.6 | 79.6 | 1.6 |
| Blackgram | UG-562 | 66.8 | 71.4 | 77 | 1.7 |
| Blackgram | UG-1008 | 68.7 | 72.8 | 77.3 | 1.7 |
| Pigeon pea | AL-201 | 72.5 | 77.7 | 83.4 | 2.6 |

Gelatinization starts at the hilum of the granule and swells rapidly to the periphery (Chen et al., 2015). Gelatinization occurs initially in the amorphous regions, because hydrogen bonding is weakened in these areas as opposed to the crystalline regions of the starch granule (Singh et

al., 2003). Gelatinization temperatures and enthalpies associated with gelatinization endotherms vary between the starches from different sources (Table 4& 5). High degree of crystallinity provides structural stability and makes the granule more resistant towards gelatinization, and the differences in transition temperatures between the different starches may be caused by the differences in the degree of crystallinity (Singh et al., 2003). The gelatinization and swelling properties are affected by the molecular structure of amylopectin (unit chain length, extent of branching, molecular weight), starch composition (amylose/ amylopectin ratio), and granule architecture (crystalline/amorphous ratio) (Miao et al., 2009). Tp gives a measure of crystallite quality and enthalpy of gelatinization (ΔH) gives an overall measure of crystallinity indicates the loss of molecular order within the granule (Cooke and Gidley, 1992, Hoover and Vasanthan, 1994, Tester and Morrison, 1990 as cited in (Singh et al., 2006)). Moreover, enthalpy of gelatinization (ΔH) is related to the characteristics of starch granules, such as crystallinity degree and granule size (Bogracheva et al., 2006).

The structure of different starch sources was discussed earlier in this thesis. In more details, the effect of these features on the gelatinization process will be discussed further. As mentioned earlier, the side chains of the amylopectin molecule in the starch granule are packed into two polymorphous forms of crystallites as A-type (e.g., cereal starches) and B-type (mostly root and tuber starches). A mixed type of packing, which includes both A- and B-types, is called C-type crystallites (e.g., legume starches). In this respect, wheat starch is termed A-type, potato starch B-type, and faba bean starch C-type. The differences in crystallite structures are thought to be the main reason for the different physicochemical properties such as gelatinization temperature (Genkina et al., 2007). Since A- and B-type crystallite structures show some differences with significant variations of bound water attached to the double helices within the crystallites, Genkina et al. (2007) reviewed that this structural specificity gave different crystallite densities for A-type being denser than B-type crystallites. This difference in crystal density was showed as the main reason for the different gelatinization temperatures. They also stated that a higher gelatinization temperature characterized starch with A-type crystallite due to higher density. If it can be generally accepted, it can be suggested that higher gelatinization temperature is required for A-type due to higher density. This suggestion is in line with the conclusions states from a comparison of the gelatinization temperature of some A-type (maize) and B-type starches (potato) but, it was not supported by the comparison of B-type (potato) starch and other A-type starches (barley) (Genkina et al., 2007). Therefore, it remains unclear that the difference in crystalline density may not be the only factor to speculate the effect of the starch

feature on the physicochemical property. Therefore, differences between gelatinization temperatures may depend on other starch structure features.

Amylose and amylopectin content within the various starch sources affect the gelatinization process as cited by Lehmann et al. (2007). Starches high in amylopectin are easy to swell as compare to starch rich in amylose. For maize and pea starches, sources with high amylose content are more resistant to gelatinization compared to the ones having the moderate to high content of amylopectin during the processing (Themeier et al., 2005). For barley, swelling process occurs at a lower rate for high-amylose content and at a higher rate for high-amylopectin content than for normal source (Vasanthan and Bhatty, 1996, Chiotelli and LeMeste, 2002 as cited in (Svihus et al., 2005)). In contrast to these findings, Swanston et al. (2001) found that gelatinization temperature was higher both for high-amylopectin and high-amylose barleys compared to normal barley. These findings shows that there are other factors may influence gelatinization process for different starch sources.

Granule shape, percentage of large and small granules and presence of phosphate esters have been reported to affect the gelatinization enthalpy values of starches (Singh et al., 2003). Yamin (1999) reported that a starch with low To and broad gelatinization range might have irregularly shaped granules. The variation in To, ΔH and gelatinization temperature range in starches from different cultivars may be due to different amounts of longer chains in amylopectin chains (Table 6) (Yoo and Jane, 2002, Yamin et al., 1999). Longer chains in amylopectin structure require a higher temperature to dissociate completely than that required for shorter chains (Yamin et al., 1999).

For wheat, Chiotelli and Le Meste (2002) investigated the thermomechanical behavior of A-type (large) and B-type (small) starch granules separated from wheat. Accordingly, B-type (small) granules showed slightly higher gelatinization temperature and lower gelatinization enthalpy than that of the A-type (large) granules. Therefore, A- and B-type starch granules in mature wheat endosperm have different gelatinization characteristics such as temperature orders (Table 7).

**Table 6.** Thermal properties and amylopectin chain length distribution of native wheat starches (Yoo and Jane, 2002).

| Sample | To [°C] | Tp [°C] | Tc [°C] | ΔH gel [J/g] | Amylose content (%) | DP6–9 | DP6–12 | DP13–24 | DP25–36 | DP≥37 |
|---|---|---|---|---|---|---|---|---|---|---|
| Waxy | | | | | <0.2 | 4.6 | 21.5 | 45 | 14.8 | 18.7 |
| Wheat Kanto 107 | 55.7 | 61.4 | 67.6 | 13.6 | 21.5 | 4.6 | 21.3 | 45.2 | 15.2 | 18.3 |
| | 57.5 | 62.1 | 67 | 11.8 | | | | | | |
| Centura | 55.6 | 59.1 | 63.1 | 10.7 | 26.2 | 4.2 | 20.2 | 44.2 | 15.6 | 20.2 |
| Wheat[A] | 54.9 | 58.9 | 63.8 | 10.6 | 26.6 | 4.8 | 22.4 | 46.1 | 14.4 | 17.1 |

A: Commercial wheat cultivar

**Table 7.** Gelatinization properties of wheat (Ao and Jane, 2007).

| Cereal | To [°C] | Tp [°C] | Tc [°C] | ΔH gel [J/g] |
|---|---|---|---|---|
| Wheat | 61.7 | 65.3 | 69.3 | 12.4 |
| Wheat A-granule | 61.2 | 64.3 | 68.1 | 11.7 |
| Wheat B-granule | 57.9 | 64.7 | 67.5 | 12.1 |

Under processing conditions where water is present in limited amount, the differences in gelatinization behavior can be expected. Therefore, the water/ starch ratio may be the limiting factor influencing starch gelatinization (Svihus et al., 2005). In earlier studies done by Lund (1984) and Jacobs and Delcour (1998), it was reported a minimum ratio of 0.3:1 (water/starch ratio) as a precondition to initiate starch gelatinization during heat treatment (Svihus and Zimonja, 2011). During steam conditioning and pelleting, the extent of starch gelatinization is usually found to vary between 50 and 300 g starch/kg (Skoch et al., 1983, Goelema et al., 1999, Svihus et al., 2004, Moritz et al., 2005, Zimonja et al., 2007, Zimonja et al., 2008, Zimonja et al., 2009 as cited in (Svihus et al., 2011)). Therefore, starch is gelatinized to some extent during the pellet process. This low extent of gelatinization remarks that steam conditioning and pelleting will not have an apparent effect on starch digestibility. During extrusion processing, up to 180 g water/kg is added and the diet is subjected to temperatures higher than 110 °C under high pressure compared to pelleting (Svihus et al., 2005). Consequently, extrusion process usually results in a more complete gelatinization and disintegration of starch granules.

### 2.5. Starch digestion rate

Among the cereals, the extent of wheat starch digestion is relatively well studied in broiler chicken nutrition (Choct et al., 1999, Steenfeldt et al., 1998). Far less information is available on

the site and rate of wheat starch digestion; however wheat is generally proposed to be rapidly digested in the proximal sections of the small intestine (Gutierrez del Alamo et al., 2009, Weurding et al., 2001a). Even fecal starch digestibility of wheat grain is nearly complete; the differences in starch digestion rate among wheat cultivars have been found (Gutierrez del Alamo et al., 2009). Del Alamo et al. (2009) studied rate of starch digestion in three wheat cultivars, each from two origins. In their study, starch digestibility ranged from 41.9 to 56.1% in the proximal jejunum (PJ), 77.4 to 80.0% in the distal jejunum (DJ), 92.9 to 95.2% in the proximal ileum (PI), and 95.2 to 96.1% in the distal ileum (DI). Weurding et al. (2001a) determined starch digestibility in one wheat sample and reported values of 88.2, 92.9, and 94.4% for the DJ, PI, and DI, respectively. Differences in values could be possibly due to differences in wheat cultivars affected by growing environment and genotype by affecting the rate of starch digestion and subsequent broiler performance (Gutierrez del Alamo et al., 2009). Yet, it is still not clear if different growing conditions of wheat cultivars have an effect on starch digestion kinetics similarly as their physicochemical properties (McCracken et al., 2002, Pirgozliev et al., 2003). However, the study done by Del Alamo et al. (2009) found that different wheat cultivars had different starch digestion kinetics, which affected the broiler chicken performance. Therefore, the rate of starch digestion could be a critical consideration for better animal performance.

In the study done by Knudsen et al. (2006), the digestibility of the different starch sources such as A-type (wheat and barley), B-type (potato), and C-type (peas and faba beans) was studied in growing pigs. Legume starches (C-type) were less digestible than cereal starches (Knudsen et al., 2006). Starch digestibility of raw cereal mix was 0.96, whereas the starch digestibility of faba bean starch was around 0.84. The crystalline nature of potato starch (B-type) makes it the least digestible starch for growing pigs with values in the range of 0.39 for raw and 0.983 for gelatinized form. As expected, starch digestibility in the raw form of starches was lower compared to their gelatinized form.

Weurding et al. (2001a) studied the starch digestion rate of different feedstuffs, which covered a wide range in starch characteristics (e.g., starch structure, amylose content and granule size) in broiler chickens. According to the results, 90% of digested starch in cereal grains was digested before the ileum and 98% before the posterior ileum. These values were lower for common beans (50 and 87%, respectively), peas (71 and 91%, respectively), horse beans (70 and 92%, respectively) and potato starch (60 and 77%, respectively). A large difference showed a gradual

starch digestion along the small intestine of the broiler chickens. Tapioca starch digestion was almost complete in the upper small intestine. A substantial proportion of ingested starch from peas and beans was digested in the ileum (23-36%). A smaller proportion of potato starch (13%) and cereal starch (6-13%) was digested in this part of the small intestine. Depending on the starch source and possibly starch structural features, the rate of the starch digestion differs as shown in this study.

Factors not directly related to starch features may also affect the starch digestibility (Weurding et al., 2001a). Starch granules can be encapsulated by a rigid protein matrix or by cell walls reducing the accessibility of starch granules to enzymatic attack (Classen, 1996). Furthermore, other ingredients of the diet may also affect starch digestion. Soluble non starch polysaccharide in the diet increase digesta viscosity, possibly impairing starch digestion (Annison et al., 1991). Starch digestion is also affected by animal related factors such as age, feed intake, the passage rate and absorption capacity (Weurding et al., 2001a). Therefore, these factors must be also take into account.

## 2.6. Nitrogen digestion along the intestinal tract of broiler chickens

The gastrointestinal tract of pigs and poultry consumes approximately 20% of dietary energy density for the digestion and absorption of nutrients (Cant et al. 1996 as cited in (Liu et al., 2013)). As explained in McDonald (2002), glucose is produced as a result of starch hydrolysis, and absorbed from the small intestine. Afterwards, specific glucose carrier, which depends on the presence of sodium in the lumen, transports it across the intestinal wall. Absorbed glucose is oxidized and it serves as an energy source for the gut wall. The remainder glucose is transported by the portal vein and supply energy for other tissues. Changes in blood glucose concentrations trigger insulin secretion, which influences both amino acid catabolism and protein synthesis (Grizard et al. 1999 as cited in (Liu et al., 2013)). Therefore, the balance of starch and protein digestion dynamics is emphasized to be critical for depositing net protein efficiently.

Rate and site of starch digestion are considered important in animal nutrition because of their effects on other nutrients (Seal et al., 2003), enterocyte nutrition and function (Regmi et al., 2011), microbial fermentation in the distal intestine and digestive tract (Regmi et al., 2011), and

bird performance (Weurding et al., 2003). Slowly digested and undigested starch may also influence microbial fermentation in the distal intestine, and mainly in the ceca (Weurding et al., 2001b). Regardless of digestive tract location, short chain fatty acids may affect lumen pH, the distribution of microbial community, and minimize the occurrence of pathogenic microorganism (van der Wielen et al., 2000).

Weurding (2002) showed that broilers grew more efficiently with slowly digested starch. Weurding (2002) stated that a better synchronization of energy with protein availability and more continuous supply of glucose to the chicken intestinal lumen was achieved by slowly digestible starch. Wheat starch has been considered to be rapidly digested (Weurding et al., 2001b) and the research from the current study is in general agreement.

Studies suggest that slowly digestible starch in broiler diets is considered to be beneficial in terms of animal performance such as better feed conversion ratio (Weurding, 2002, Weurding et al., 2003, Del Alamo et al., 2009). Weurding (2002) concluded that slowly digestible starch digested in the lower small intestine improved feed conversion ratio. Broiler chickens grew faster and more efficiently with diet containing slowly digestible starch (Weurding et al., 2003). More recently, Liu et al. (2013) stated that feed conversion efficiency and weight gain were correlated with both starch and nitrogen digestion kinetics but not with their ileal digestibility coefficients. In conclusion, starch digestion rate is thought to affect bird metabolism and the efficiency of energy utilization, and thereby animal performance (Weurding et al., 2003).

## 3. MATERIALS AND METHODS

In this study, diets were produced in the Centre of Feed technology (FôrTek), at the University of Life science (NMBU) in Ås, Norway. Animal experiment and laboratory work were conducted at the Poznań University of Life Sciences (Poznań, Poland). Formulation of diets, animal experiment, sampling and statistical analysis of the results were conducted by a PhD student Khaled Itani.

### 3.1. Formulation of Experimental Diets

Four soybean meal-based diets were processed via two different thermochemical processes. These processes were pelleting and extrusion. Cereal based diet had wheat, and legume based diet had faba bean starch fraction as a starch source. The experimental diets composition and chemical compositions were shown in Table 8.

**Table 8**. Experimental diets composition, analysed and calculated nutrient content

| Ingredients, g/kg (as fed) | Wheat | Faba bean starch |
|---|---|---|
| Wheat (WS) | 582 | -- |
| Faba bean starch fraction (FBS) | -- | 512 |
| Soybean meal [1] | 274 | 275.6 |
| Cellulose powder [2] | -- | 70 |
| Rapeseed oil | 75 | 76 |
| Limestone | 14.77 | 15.04 |
| Monocalcium phosphate | 16.79 | 22.28 |
| L-Lysine | 8 | 1 |
| DL-Methionine | 6.09 | 5.61 |
| L-Threonine | 4 | 3.6 |
| Sodium chloride | 4.76 | 4.29 |
| Titanium dioxide | 5 | 5 |
| Choline chloride | 1.96 | 1.95 |
| Mineral & Vitamin premix [3] | 6.13 | 6.13 |
| Enzyme (Rovabio) [4] | 1.5 | 1.5 |
| *Analysis* | Pelleted - Extruded | Pelleted - Extruded |
| Dry matter | 904 - 934 | 906 - 923 |
| Starch gelatinisation [5] | 209 - 715 | 207 - 943 |
| Gross energy (MJ/kg DM) | 19.7 | 19.6 |
| Starch (g/kg DM) | 370 | 374 |
| Crude Protein (g/kg DM) | 239 | 237 |
| Fat (g/kg DM) | 90 | 90 |
| NDF (g/kg DM) | 110 | 118 |
| Lysine (g/kg DM) | 16 | 15 |
| Methionine (g/kg DM) | 7.8 | 7.8 |
| Threonine (g/kg DM) | 9.6 | 10.3 |
| *Calculated nutrient content* | | |
| Metabolisable energy (MJ/kg) | 12.6 | 12.7 |
| Calcium (g/kg) | 9.7 | 10.5 |
| Available Phosphorous (g/kg) | 5.0 | 5.4 |

[1] Ground to pass a 1-mm screen

[2] SANACEL® 150, CFF GmbH & Co. KG, Gehren. Germany.

[3] Mineral and vitamin premix provided the following per kg diet: Fe, 50 mg; Mn, 122 mg; Zn, 80 mg; Cu, 14 mg; I, 0·72 mg; Se, 0·28 mg, retinyl acetate, 5.72 mg; cholecalciferol, 0.15 mg; dl-α-tocopheryl acetate, 78 mg; menadione, 8 mg; thiamine, 5 mg; riboflavin, 24 mg; niacin, 32 mg; calcium pantothenate, 24 mg; pyridoxine, 13 mg; cobalamin, 0.03 mg; biotin, 0.5 mg; folic acid, 4 mg.

[4] Enzyme Rovabio Excel Ap T-Flex, Adisseo, France provided the following per kg diet: Endo-1,4-β-xylanase: 33 000 visco units; Endo-1,3(4)-β-glucanase: 45 000 visco units; Endo-1,4-β-glucanase (cellulase) >9600 DNS units + 16 other enzyme activities obtained from a fermentation broth of Penicillium funiculosum.

[5] Starch gelatinisation: g/kg of total starch

### 3.2. Production of main ingredients for trial

Production of faba bean starch fraction and grinding of wheat were conducted by FeedMileage, Norway. The faba beans were dehulled by a multistep process starting with grinding whole beans trough a roller mill (DT900-12; CPM-Roskamp, Waterloo, IA, United States) with 8 mm gap between rolls. The cracked beans were then passed through pre-clenar a Type Vibam 1013 (Damas A/S, Faaborg, Denmark) to remove dust, then smaller particles were removed by a Triør Type Hotyp 520 (Damas A/S, Faaborg, Denmark), and finally uncracked beans and splitted beans with hulls were removed by a vibration table (Sorla SB, Damas A/S, Faaborg, Denmark). The dehulled beans were milled with a Contraplex 630 C pin mill (Hosokawa Alpine, Augsburg, Germany) and air classified into a light (protein) fraction and a heavy (starch) fraction by using an Air Classifier 500 ATP (Hosokawa Alpine, Augsburg, Germany) (Table 9).

**Table 9.** Chemical composition of whole faba bean, faba bean protein (FBP) fraction, and faba bean starch (FBS) fraction

| Sample Name | Dry matter g/kg DM | Crude protein g/kg DM | Starch g/kg DM | NDF g/kg DM | Crude fat g/kg DM | Ash g/kg DM |
|---|---|---|---|---|---|---|
| Whole faba bean | 856,47 | 293,06 | 376,74 | 176,20 | 19,98 | 44,43 |
| FBP | 924,53 | 632,41 | 87,88 | 98,13 | 32,99 | 65,78 |
| FBS | 902,15 | 176,66 | 744,42 | 21,69 | 7,94 | 21,35 |

After air classification, starch content of the faba bean starch (FBS) fraction increased from 376, 74 g/kg to 744, 42 g/kg (DM). On the other hand, protein content decreased from 293, 06 g/kg to 176, 66 g/kg (DM). One of the advantages of air classification of whole faba bean was to decrease the NDF content significantly.

The wheat was also pin-milled by the same procedure described above. Particle size distribution of wheat (WS) and faba bean starch (FBS) fraction was shown in Figure 12. Particle size determination showed differences in particle size distribution between wheat (WS) and faba bean starch (FBS) fraction passed through the same screen size in the same pin mill. Grinding wheat resulted in slightly coarser particles compared with faba bean starch (FBS) fraction.

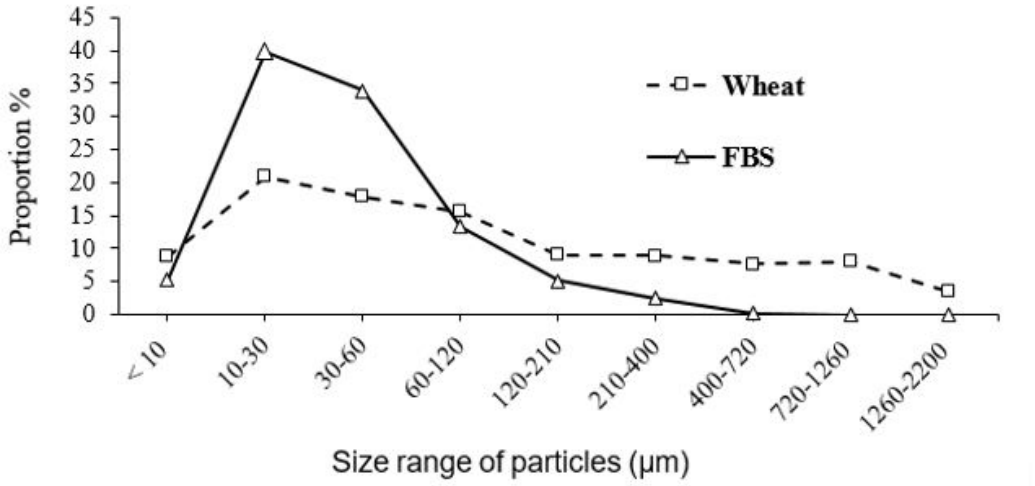



**Figure 12.** Particle size distribution of the main starch sources. Wheat and air classified faba bean starch (FBS) fraction

### 3.3. Feed production process

Experimental diets were processed at the Centre for Feed Technology (Fôrtek), Norwegian University of Life Sciences, Ås, Norway. Pelleted diets were produced continuously on 22th of January 2018. Extruded diets were produced consecutively on 23th of January 2018. Before milling, macro ingredients of the formulated diet were weighted using a large weighing scale. For micro ingredients, the premixes were prepared manually by mixing vitamin, amino acids and micro, mineral, premix, enzyme and marker. The soybean meal was ground to pass through a 1-mm sieve in a hammer mill (Münch-Edelstahl, Wuppertal, Germany licensed by Bliss, USA, 18.5kW, 3000 RPM) before being mixed with other ingredients. The premix mixtures were added manually during a second mixing cycle into the mixer. Each weighing 250 kg mixed in a 400-liter mixer Model 1992 OB-1078) (Twin shaft paddle, Tatham of England, Norway, 7.5 kW). After dry mixing, rapeseed oil was sprayed via nozzle (angle 65, size 05, Unijet, spraying systems Co, Wheaton, Illinois, USA) on the mash with a pressure of 4 bars for 7.6 min. The mixing time after oil addition was 2 minutes. The diets contained titanium dioxide ($TiO_2$) as a digestibility marker and cellulose powder was used to balance the diets for fibre content.

For pelleted diets, the mash feed was sent through the twin pass/double conditioner (Twin Pass, Muench, Germany, 1.2 t/h, 2 x 1.8m x 30cm). There was 4% steam added at 81℃ in 36

seconds (retention time) before it was processed in a pellet press (Muench, Germany, 1.2t/h max. capacity, 2 x 18.5 kW) equipped with 3 mm die (42 mmm thickness). The boiler steam pressure was set at 8 bar and pressure at pellet press was set at 2.3 bar. The roller and die distance in the pellet press were 0.5 mm. During the pelleting progress, processing parameters were recorded shown in Table 10. The production capacity of the wheat (WS) and faba bean starch (FBS) fraction based diet were 400 and 200 kg/h respectively. Immediately after the pelleting, the temperature of feed was measured manually with a thermometer in an insulating box. Post-pelleting temperatures were 89 and 94°C whereas specific energy consumption values (kWh/t) were 38 and 77 for the wheat (WS) and faba bean starch (FBS) fraction based diet, respectively. The pelleted feed was sent to the cooler directly. A counter-flow cooling system was used for 45 minutes, which used ambient air to reduce temperature of the products (Miltenz, New Zealand, capacity 1.2t/h). Pelleted product after cooling was packed as 25 kg in each bag.

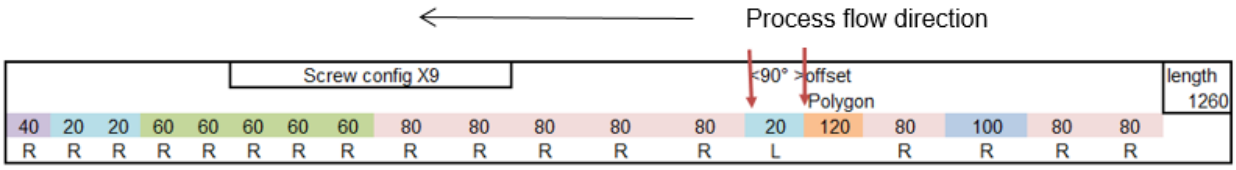
**Table 10.** Pelleting process parameters for wheat (WS) and faba bean starch (FBS) fraction based diets

| Diet | | WS | FBS |
|---|---|---|---|
| Grinding | | | |
| Screen size | mm | 1 | 1 |
| Bulk density(mixed mash) | g/L | 683.5 | 607.5 |
| Pelleting | | | |
| Die specification | mm | 3 | 3 |
| Feeder speed | % | 18 | 7 |
| Capacity | kg/h | 400 | 200 |
| Conditioner temperature | °C | 81 | 81 |
| Motor load | % | 16 | 20 |
| Amperes motor 1 | amp | 13 | 13 |
| Amperes motor 2 | amp | 12 | 12 |
| Steam pressure | bar | 2.3 | 2.3 |
| Steam consumption | kg/h | 29.5 | 18.5 |
| Post Pelleting temperature | °C | 89 | 94 |
| Specific energy consumption | kWh/t | 38 | 77 |

For extruded diets, the mixed mash feeds were passed through a Bühler (BTCT) (Bühler, Uzwil, Switzerland) pre-conditioner where water and steam are added in diets. Conditioner temperature of of the wheat (WS) and the faba bean starch fraction (FBS) based diet were 89 and 92 °C, respectively. All diets were extruded in a twin-screw co-rotating extruder (Bühler BCTG 62/20 D,5-barrel sections, driven by a 72kW electrical motor) fitted with 12 dies x 3 mm

and with a feeder rate of 145 kg/h for the wheat- and FBS-based diet, respectively. Screw configuration of extruder was shown as Figure 13, and process parameters were shown in Table 11.

The temperatures in the five sections of the extruder were 92, 112, 95, 90, and 64°C for wheat (WS) based diet and 95, 110, 100, 96, and 64°C for faba bean starch fraction (FBS) based diet. Specific mechanical energy values (KWh/t) were 65 and 62, and die temperatures were 91 and 95 °C for wheat (WS) based diet and faba bean starch fraction (FBS) based diet, respectively. Extruded feed subsequently dried in a batch dryer for 40 min. The obtained extruded pellets were air-dried in a bed dryer fixed with electrical fans to achieve final dry matter (DM). Additional drying for extruded pellets were performed manually, approximately 40 kg, batch dryers/coolers made by “FôrTek”. The fan was a W2E 300-DA 01 W-160(EBM-Papst, Mulfingen, Germany) with capacity 2550 m³/h. The heater was “Viking” (Viking, Denmark), power 10 kW with max air temperature 60°C. Drying was performed at temperature 60°C, 45 minutes for diets. Cooling was performed in the same dryers/coolers by switching off heater, at room temperature 20°C, 5 minutes for each diet. Moisture content during extrusion was kept at around 290 g/kg by addition of steam and water (ambient temperature) in amounts of 60 g/kg and 100 g/kg in the conditioner. During pelleting, around 43 g/kg of steam were added in the conditioner to achieve average total moisture of 150 g/kg.




R - Right turn of the element, L - Left (backward) turn of the element, Numbers are representing the length of the Archimedean Spiral (screw alike) for each screw element

**Figure 13.** Screw configuration of the extruder

**Table 11.** Extrusion parameters for wheat (WS) based and faba bean starch (FBS) fraction based diet

| Diet | | WS | FBS |
|---|---|---|---|
| Die size | | 3 | 3 |
| Number of sizes | | 12 | 12 |
| Calibration | kg/h | 287 | 211 |
| Feeder | kg/h | 145 | 145 |
| Conditioner temperature | °C | 89 | 92 |
| Conditioner steam | kg/h | 8.5 | 8.4 |
| Conditioner water | kg/h | 13 | 13 |
| Barrel 1 | °C | 94.7 | 97.6 |
| Barrel 2 | °C | 113.3 | 109.8 |
| Barrel 3 | °C | 93 | 102.1 |
| Barrel 4 | °C | 89.2 | 96.9 |
| Barrel 5 | °C | 65 | 63,6 |
| Die temperature | °C | 91 | 95 |
| Die pressure | bar | 30 | 30.1 |
| Screw speed | rpm | 475 | 475 |
| Torque | Nm | 192 | 206 |
| Drive power | kW | 9.4 | 10.4 |
| SME | Wh/kg | 59.7 | 62 |
| Knife speed | rpm | 650 | 550 |
| Number of knives | | 6 | 6 |

Representative samples were taken from batches of feed by sampling from different but equally spaced locations. Three samples from each diet were taken after mixing process from the waiting hopper (before conditioning, after mixing). The mixed samples were taken directly from different spots and mixed together in a bucket to achieve representative samples, finally transferred into plastic bags for further investigations. Three representative pellet samples from each diet were taken directly from the filled bags with grain sampler (A/S rationel cornsieve, Esbjerg/Denmark). Before each diet was processed, the system was flushed by 20 kg of the new batch to avoid contamination. Via this flushing, next diet sample was taken without any contamination. The extruded feed samples for each diet were taken after drying process. About 1 kg of each diet was collected for chemical and physical analysis. The samples were kept in 4°C for further assessments.

### 3.4. Birds, housing and management

A total of 400 one-day-old male broilers (Ross 308) were randomly allocated to 40 floor pens (1 x 1 m) that were bedded with chopped wheat straw (7-15 cm length) and contained 10 birds each. The pens were arranged in the centre of an environmentally controlled broiler house (PIAST PASZE Sp. z o.o., Experimental Unit Olszowa, Poland) that contained 9000 birds of the same age as those in the experiment. A temperature of 33°C was maintained during the first week, then reduced by 3-4°C weekly to a minimum temperature of 21°C. The birds were maintained on a commercial pelleted diet produced by Piast Pasze factory (Lewkowiec, Poland) until 16 d, and fresh water was provided *ad libitum* throughout the experimental period. At 17 d, the 400 birds were randomly distributed among 4 dietary treatments using 10 replicate pens per treatment and 5 birds per pen. Treatments consisted of a control diet with wheat as the main starch source, and a diet in which the wheat was replaced by faba bean starch fraction. These diets were either steam-pelleted or extruded, thus constituting a 2 x 2 factorial experiment.

### 3.5. Sample collection

At 30 d, 20 birds (2 birds/replicate pen) per treatment were weighed, killed by cervical dislocation. Next, using clamping forceps, the jejunum and ileum were clamped at three points (start, middle, end) to prevent the passage of contents along the intestine, then weighed. The jejunum was defined as the segment from the end of the duodenal loop to Meckel's diverticulum, and the ileum as the section from Meckel's diverticulum to the ileocecal junction. Each of the two segments were then divided into two parts of equal length: upper and lower jejunum (UJ and LJ), upper and lower ileum (UI and LI) and the contents of each part were expressed by gentle manipulation into a pre-weighed plastic container and stored at −20°C until chemical analysis and SEM imaging.

About 500 mg of fresh digesta samples from the UJ, LJ, UJ and LI were transferred to a 2 mL Sarstedt tube containing 1.6 ml fixation solution (1.25% glutaraldehyde, 2% paraformaldehyde in 0.1 M PIPES-buffer at pH 7.4) and kept at 4°C for 48 h. After centrifugation at 3600 rpm for four minutes, the fixation solution was carefully removed using disposable pipette and then a 1.5 ml of buffer solution (0.4 M PIPES-buffer at pH 7.4) was added to each tube, vortexed. Samples stored at 4°C until then used for light microscopy analysis.

To measure enzyme activity, around 200 mg of fresh digesta from the LJ were transferred to a 2 mL Sarstedt tube, frozen on dry ice then stored at − 80 °C until analysis.

### 3.6. Physical Analysis of diets

Physical characteristics of broiler feed were measured in terms of durability, pellet dimension, particle size distribution, durability and hardness tests at Feed lab, NMBU. Durability was measured using Holmen pellet tester (NHP200). Surface attrition was measured as the pellets were conveyed at high air velocity with reference to time. Attrition of surface occurs when pellets hit pipe walls, bends, and other pellets. 100g pellets were taken and the Holmen was run with a die size setting as 3 mm for pelleted diets, and 4 mm for extruded diets. The dust was collected automatically by the machine via sieving. After test, the final weight of the diets was recorded. Pellet durability index (percentage) was calculated using the following formula. Two replications were taken for measurement.

PDI (%) = final weight of pellets after Holmen (g)/ weight of pellets before Holmen (g)*(100)

Hardness was measured on a Lloyd texture analyzer (Model 1000R, Hampshire, UK) equipped with a 50N load cell using a compression speed. The hardness value was given in force (N) at breakage point. Diameter and length of 30 pellets were recorded to take the average for each treatment, and then 15 pellets from each diet were chosen to test hardness according to these averages. The analyzed physical pellet quality results such as hardness, durability, pellet length, and diameter were shown in Table 12.

**Table 12.** Physical quality of pelleted and extruded wheat and faba bean starch based diets

| Treatment | Process | Hardness(N) | PDI(%) | Length(mm) | Diameter(mm) |
|---|---|---|---|---|---|
| WS | Pelleting | 63.92 ± 2.45 | 94.3 | 7.14 ± 0.139 | 2.91 ± 0.003 |
| FBS | Pelleting | 98.95 ± 6.27 | 91.6 ± 0.2 | 6.65 ± 0.138 | 2.92 ± 0.004 |
| WS | Extrusion | 39.45 ± 0.97 | 92.7 ± 0.1 | 5.45 ± 0.081 | 4.08 ± 0.021 |
| FBS | Extrusion | 59.19 ± 1.72 | 99.1 | 5.23 ± 0.121 | 3.95 ± 0.023 |

The greatest load and variation among the diets were observed (Table 12). Pelleted faba bean starch (FBS) fraction based diet had the highest hardness of 98.95 N and extruded wheat (WS) based diet had the lowest hardness of 39.45N. The highest pellet durability (PDI) was measured (99.1 %) for extruded faba bean starch (FBS) fraction based diet. Among the pelleted diets, durability of faba bean starch (FBS) fraction based diet was lower than wheat (WS) based diet (91.6 and 94.3 % respectively). Although, expansion was avoided to obtain equal pellet size dimensions, slight expansion was observed for extruded diets. Extruded wheat (WS) based diet had 36 % of expansion and faba bean starch (FBS) fraction based diet had 31.7 % of expansion. Length of pelleted diets (WS and FBS) were 7.14 mm and 6.65 mm respectively, whereas length of extruded diets were shorter, 5.45 mm and 5.23 mm respectively.

To measure the particle size distribution, wet laser diffraction method was used at Feedlab, NMBU. Particle size analysis of wheat, faba bean starch fraction and feed samples was carried out using a Malvern Mastersizer Hydro 2000 SM (Malvern Instruments Ltd., Malvern WR14 1XZ, Worcestershire, UK) as described by Hetland et al. (2002). System adjustments and corrections were conducted before each measurement. Water was used as the dispersant.

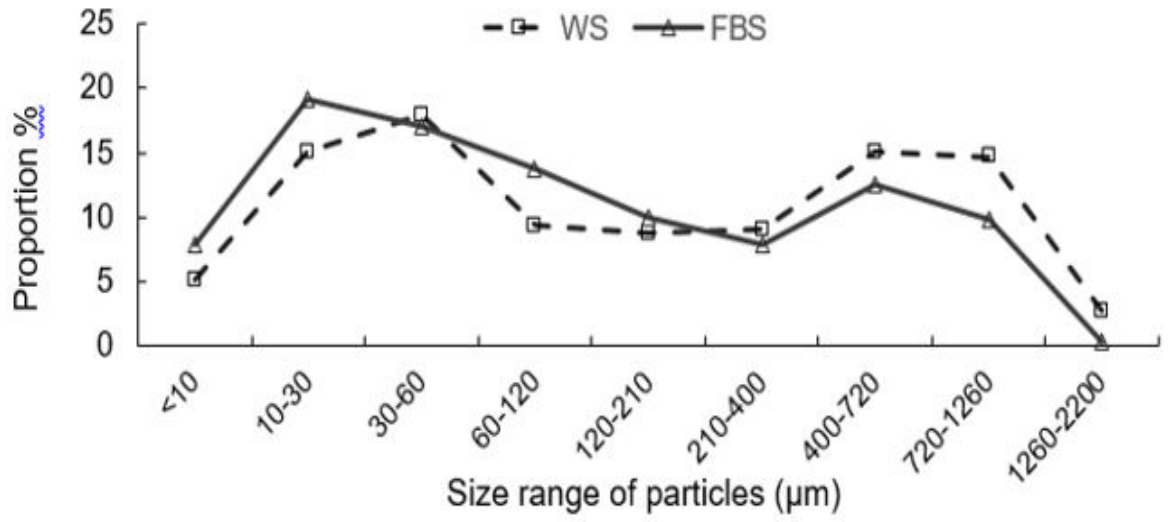



**Figure 14.** Particle-size distribution of the wheat (WS) and faba bean starch (FBS) based diets

In addition, experimental diets were grinded via hammer mill by using 1 mm sieve. Generally, cereals used for poultry are usually finely ground in a hammer mill fitted with a screen between 3 and 4.5 mm in size. Therefore, the particle size distribution of both starch sources and

experimental diets were finely grinded. Particle size distribution of the FBS and WS diets indicated differences in particle size distribution (Figure14). FBS-diets were finely grinded compared to WS-diets.

### 3.7. Chemical analyses

Feed and digesta samples were analyzed by the LabTek group for dry matter, ash, crude protein (Kjeldahl-N×6.25), starch, NDF, crude fat, starch, gross energy, titan, amino acids, protein digestibility, and enzyme analysis at the Department of Animal an Aquaculture Science (IHA), NMBU, Ås, Norway. Representative feed samples (n=3) were ground on a cutting mill (Pulverisette 19, Fritsch Industriestr. 8, 55743 Idar-Oberstein, Germany) through a 0.5 mm sieve.

Gross energy (GE) was determined using an adiabatic bomb calorimeter (Parr 6400, Moline, USA) standardized with benzoic acid.

Dry matter and ash content of the feed were determined after drying overnight at 105°C and after 6 h ashing at 550°C, respectively.

Nitrogen content was determined by the Dumas method using a Vario El Cube (Elementar Analysensysteme GmbH, Hanau, Germany 2016). Amino acids except tryptophan in the diets were analysed on a Biochrom 30 Amino Acid Analyzer178 (Biochrom Ltd., Cambridge, UK).

Ether extract was determined after extraction with 80% petroleum ether and 20% acetone in an Accelerated Solvent Extractor from Dionex (ASE200; Sunnyvale, CA, USA).

NDF content was determined using a fibre analyser system (Ankom200; ANKOM Technologies, Fairport, NY, United States) with filter bags (Ankom F58; ANKOM Technologies).

Starch content was analysed enzymatically based on the use of thermostable α-amylase and amyloglucosidase (McCleary et al., 1994).

$TiO_2$ content was determined as described by Short et al. (1996). Freeze-dried jejunal and ileal contents were pulverized using a mortar and pestle, and the contents from two birds per replicate-pen were pooled and analysed in duplicates for starch (without 80 % ethanol washing), nitrogen and $TiO_2$ as described above.

Intestinal samples from the lower jejunum were taken from one bird per replicate-pen and were prepared as described by Pérez de Nanclares et al. (2017) for enzyme activities analysis. Amylase and trypsin activities were assayed colorimetrically using amylase and trypsin commercial assay kits (Abcam, Cambridge, UK) according to manufacturer's instructions. Activities of amylase and trypsin were expressed as unit/g jejunal chyme.

The degree of starch gelatinisation (DG; as a proportion of total starch) was measured by differential scanning calorimetry (DSC 823e Module, Mettler-Toledo, Switzerland) as described by Kraugerud and Svihus (2011).

### 3.8. Imaging analysis

#### 3.8.1. Light microscopy

Digesta samples were taken for 4 dietary treatments with 10 replicate pens per treatment. In total, 40 samples were taken. Digesta samples taken from upper jejunum, lower jejunum, upper ileum and lower ileum of the broiler chickens fed with pellet and extruded diets were fixed and suspended in buffer solution (0.4 M PIPES). Starch granules in the digesta samples were observed under a light microscope (magnification ×10) using Leica DM6B Light microscope equipped with LAS X analysis program, which was used for image capture and counting.

100 µl of digesta sample from a defined intestinal segment (UJ, LJ, UI, LI) was placed on a glass slide and a 100 µl of iodine solution was added. The sample was then covered with a cover slip. The slide area was divided into 10 areas and images were taken from the five sequential areas in order to obtain a representative number of starch granules for 100 µl digesta samples. Per sample, five images were taken and, overall the starch granule results were given as an average number of these five individual images. All the samples were observed at room temperature. After images were taken, starch granules were counted. Based on the images, and literature, the starch granules higher than 15 µm in length and diameter were counted. The

scale bar was added on the right corner of the images to be able to count and evaluated the granules morphology. In figure 15, the principle of counting for this study was shown. The counting principle was conducted for each five image, and the average starch granule was calculated.

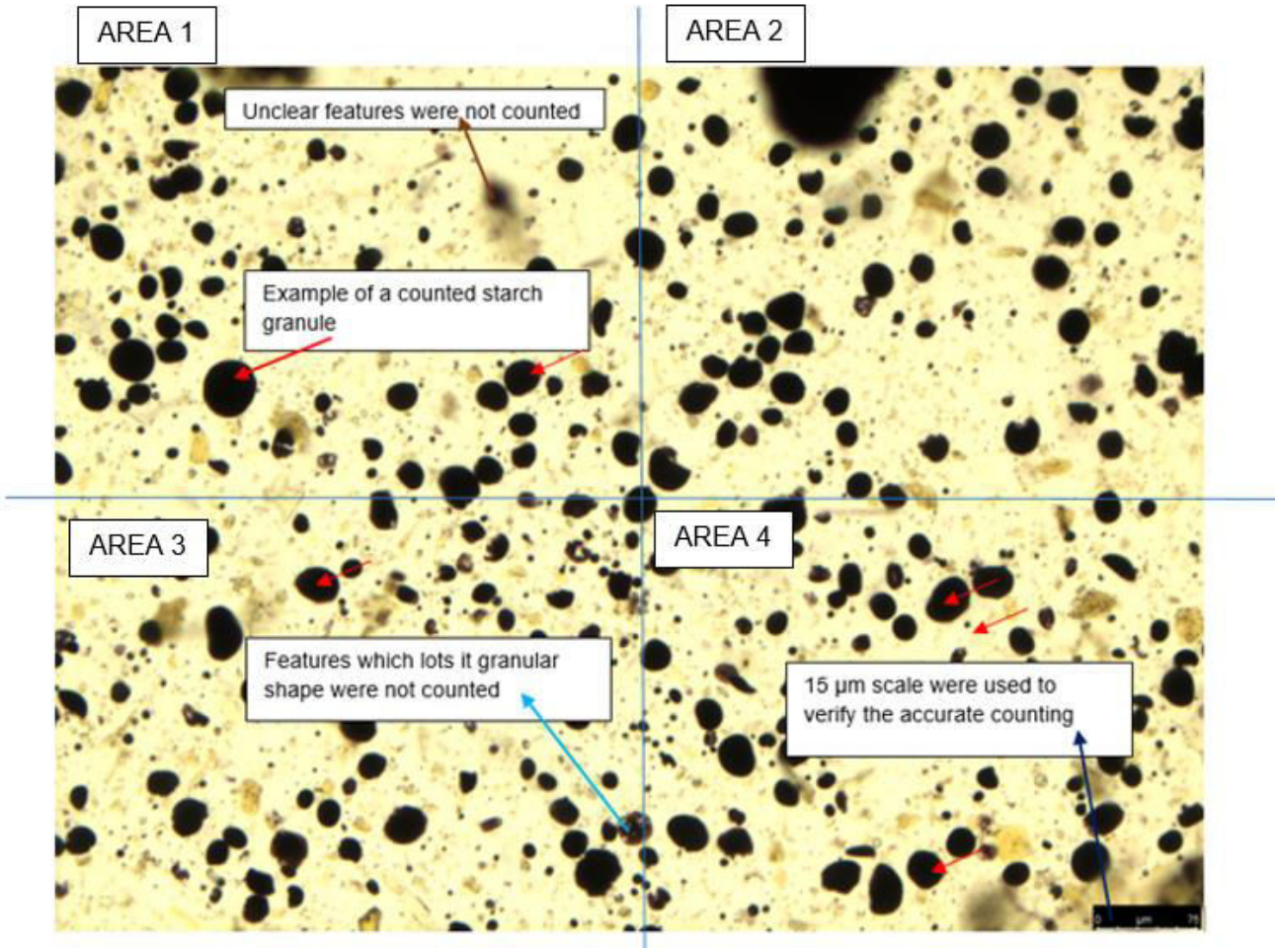



**Figure 15.** The light microscopy image of the digesta sample from the upper jejunum of the broiler chicken fed with pelleted wheat based diet. The image were divided into 4 different areas to make counting visually easier. The example of starch granules were counted and neglected were shown with the arrows and the explanations.

### 3.8.2. Scanning Electron Microscopy (SEM)

For SEM images, pelleted wheat (WS) and faba bean starch (FBS) fraction based diet were used and also freeze-dried digesta samples taken from upper jejunum and upper ileum were used. Freeze-dried digesta samples were mounted onto aluminum stubs (Agar Scientific, Essex, UK) and coated with gold palladium mixture using the sputter coater (Quorum

Technologies Ltd, Newhaven, U.K.), before being viewed and photographed in the SEM unit. The samples were examined using a Zeiss EVO 50 scanning electron microscope, operated at 20-25 kV (Zeiss, Jena, Germany).

### 3.9. Statistical analysis

Statistical analyses were carried out by Ph D Khaled Itani using the statistical software R version 2.3.2. A two-way analysis of variance (ANOVA) was performed to determine the main effects and interactions of starch sources and processing methods on growth parameters, nutrient digestibility and enzyme activities. Means were separated by Tukey post-hoc test and differences were considered significant at $P < 0.05$.

## 4. RESULTS

### 4.1. Birds Performance

**Table 13.** Body weight gain, feed intake, and feed efficiency of broiler chickens fed experimental diets for 30-d-old male broilers[1]

| Diet | Process | Body weight (g) (1-29days) | Feed intake (g) (1-29 days) | Feed conversion ratio (1-29 days) |
|---|---|---|---|---|
| WS | Pelleting | 2,201$^{b}$ | 2,715$^{c}$ | 1.24$^{a}$ |
| FBS | Pelleting | 2,209$^{ab}$ | 2,820$^{b}$ | 1.28$^{a}$ |
| WS | Extrusion | 2,270$^{ab}$ | 2,795$^{bc}$ | 1.23$^{a}$ |
| FBP | Extrusion | 2,294$^{a}$ | 2,924$^{a}$ | 1.27$^{a}$ |
| SEM | | 16.7 | 21.1 | 0.009 |
| P-value | | 0.124 | <0.05 | 0.21 |

Values are means of 10 replicate cages of 2 birds each.
* √MSE: square root of means square error in the analysis of variance.
$^{a}$ $^{b}$ =Means within column followed by different letters are significantly different (P 0.05).

The chickens stayed healthy and mortality was not observed during the 30 days experiment. The effect of diets (pelleted and extruded WS, FBS) on broiler chickens as body weight (BW), feed intake (FI), and feed conversion ratio (FCR) is shown in table 13.

## 4.2. Starch digestibility

**Table 14.** The effect of starch source and processing method on starch digestion along the intestinal tract of 30-d-old male broilers[1]

| | | Jejunum | | Ileum | |
|---|---|---|---|---|---|
| **Starch source** | **Processing** | Upper | Lower | Upper | Lower |
| WS | Pelleting | 0.921 **a** | 0.947 **a** | 0.981 **a** | 0.998 **a** |
| FBS | Pelleting | 0.826 **b** | 0.912 **b** | 0.940 **b** | 0.972 **c** |
| WS | Extrusion | 0.879 **ab** | 0.973 **a** | 0.994 **a** | 0.994 **ab** |
| FBS | Extrusion | 0.902 **a** | 0.971 **a** | 0.985 **a** | 0.987 **b** |
| | √**MSE*** | 0.051 | 0.027 | 0.020 | 0.006 |
| **Diet** | | | | | |
| WS | | 0.900 | 0.960 | 0.988 | 0.996 |
| FBS | | 0.864 | 0.942 | 0.962 | 0.980 |
| **Processing** | | | | | |
| Pelleting | | 0.873 | 0.930 | 0.959 | 0.985 |
| Extrusion | | 0.891 | 0.972 | 0.989 | 0.991 |
| *P-value* | | | | | |
| **Diet** | | 0.0315 | 0.0377 | 0.0006 | <.0001 |
| **Processing** | | 0.2994 | <.0001 | <.0001 | 0.0092 |
| **Diet x Processing** | | **0.0008** | **0.0569** | **0.0177** | **<.0001** |

[1] Values are means of 10 replicate cages of 2 birds each.
*√MSE: square root of means square error in the analysis of variance.
a,b,c Means within column followed by different letters are significantly different ($P < 0.05$).

Significant differences were found in AID of starch for both different diets and processes (Table 14). Numerical values are showing lower AID for starch in broiler chickens fed with the FBS source (Table 14). AID of starch was higher (P<0.05) in broiler chickens receiving the pelleted wheat based diet. Both diet and processing had an effect on starch digestion along the intestinal tract of broiler chickens, and interaction between diet and processing was also found (P<0.05).

## 4.3. Enzyme activity

**Table 15.** The effect of starch source (wheat 'WS' and faba bean starch 'FBS' fraction) and processing methods (pelleting and extrusion) on the activities of digestive enzymes in the digesta collected from the lower jejunum

| Starch source | Processing | Amylase (U/g chyme) | Trypsin (U/g chyme) |
|---|---|---|---|
| WS | Pelleting | 64.7 | 4.1 |
| FBS | Pelleting | 82.9 | 3.1 |
| WS | Extrusion | 54.8 | 4.8 |
| FBS | Extrusion | 77.1 | 3.9 |
| | √MSE[2] | 32.2 | 0.9 |
| **Starch source** | | | |
| WS | | 59.7 | 4.4 |
| FBS | | 80.0 | 3.5 |
| **Processing** | | | |
| Pelleting | | 73.8 | 3.6 |
| Extrusion | | 66.0 | 4.3 |
| *P-value* | | | |
| **Starch source** | | **0.0545** | **0.0030** |
| **Processing** | | 0.4442 | **0.0188** |
| **Starch source x Processing** | | 0.8404 | 0.9591 |

[1] Values are means of 10 replicate cages of 1 bird each
[2] √MSE: square root of means square error in the analysis of variance.

Amylase and trypsin enzyme activity for both pelleted and extruded diets was shown in table 15. Pelleted FBS had the highest amylase activity (82.9 U/g chyme) followed by extruded FBS (77.1 U/g chime), pelleted WS (64.7 U/g chime), and extruded WS (54.8 U/g chyme). Starch source had significant effect on amylase activity ($P<0.05$), however processing resulted lower amylase activity for both starch sources. Extruded diets contained lower amylase enzyme activity in chyme compared with pelleted diets.

Extruded WS had the highest trypsin activity (4.8 U/g chyme) followed by pelleted WS (4.1 U/g chime), extruded FBS (3.9 U/g chyme), and pelleted FBS (43.1 U/g chyme). Starch source and process had significant effect on trypsin activity ($P<0.05$). Extruded diets contained higher trypsin enzyme activity in chyme compared with pelleted diets

### 4.4. Imaging analysis

#### 4.4.1. Light microscopy

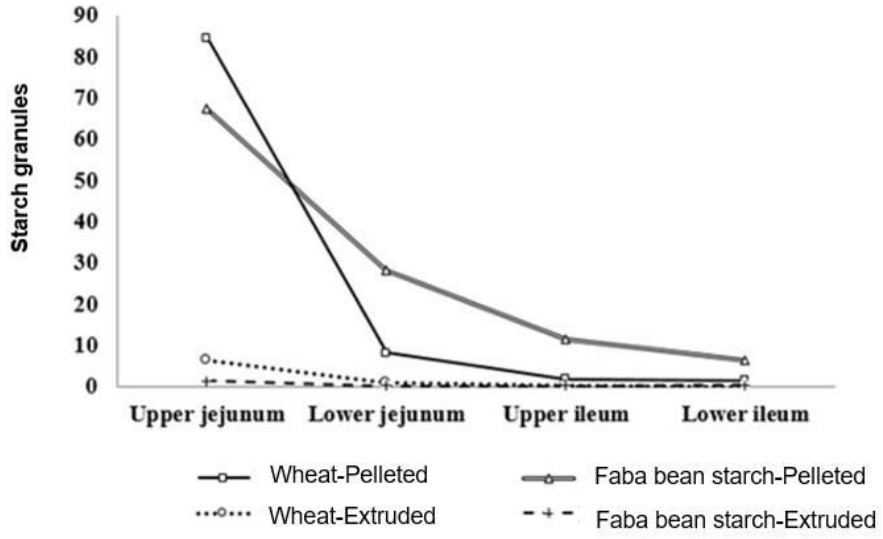



**Figure 16.** Expression of number of starch granules/mm² along the small intestine of the broiler chickens fed with pelleted and extruded wheat (WS) and faba bean starch (FBS) based diets

According to light microscopy images, pelleted diet with faba bean starch was more gradually digested than pelleted wheat-based diet along the small intestine. In addition, almost no signification amount of starch granules were observed for extruded diets in the lower section of small intestine, which showed that the absorption occurred in the upper jejunum. The light microscopy images for digesta samples taken from the small intestine of broiler chickens fed with pelleted and extruded diets was shown in Appendix. For simplification, one representative image was selected and shown among five images for each sample image.

#### 4.4.2. Scanning Electron Microscopy (SEM)

For pelleted diets, physical entrapment of starches and proteins in faba bean diets was observed by SEM images (Figure.17). Some of the starch granules were pointed out with red arrows. SEM images for wheat based diet (Figure 17, A) and faba bean based diet (Figure 17, B) showed clearly the oval-shaped and circular starch granules and non-starch compounds which could be protein matrix (pointed with blue arrows) that was attached to starch granules. These non-starch compounds encapsulates starch granules especially pelleted faba bean starch fraction based diet (FBS-P), thereby preventing their access to amylase (as shown with

yellow circle) in Figure17, b. In case of pelleting process, appearance of starch granules remained intact due to low starch gelatinization.

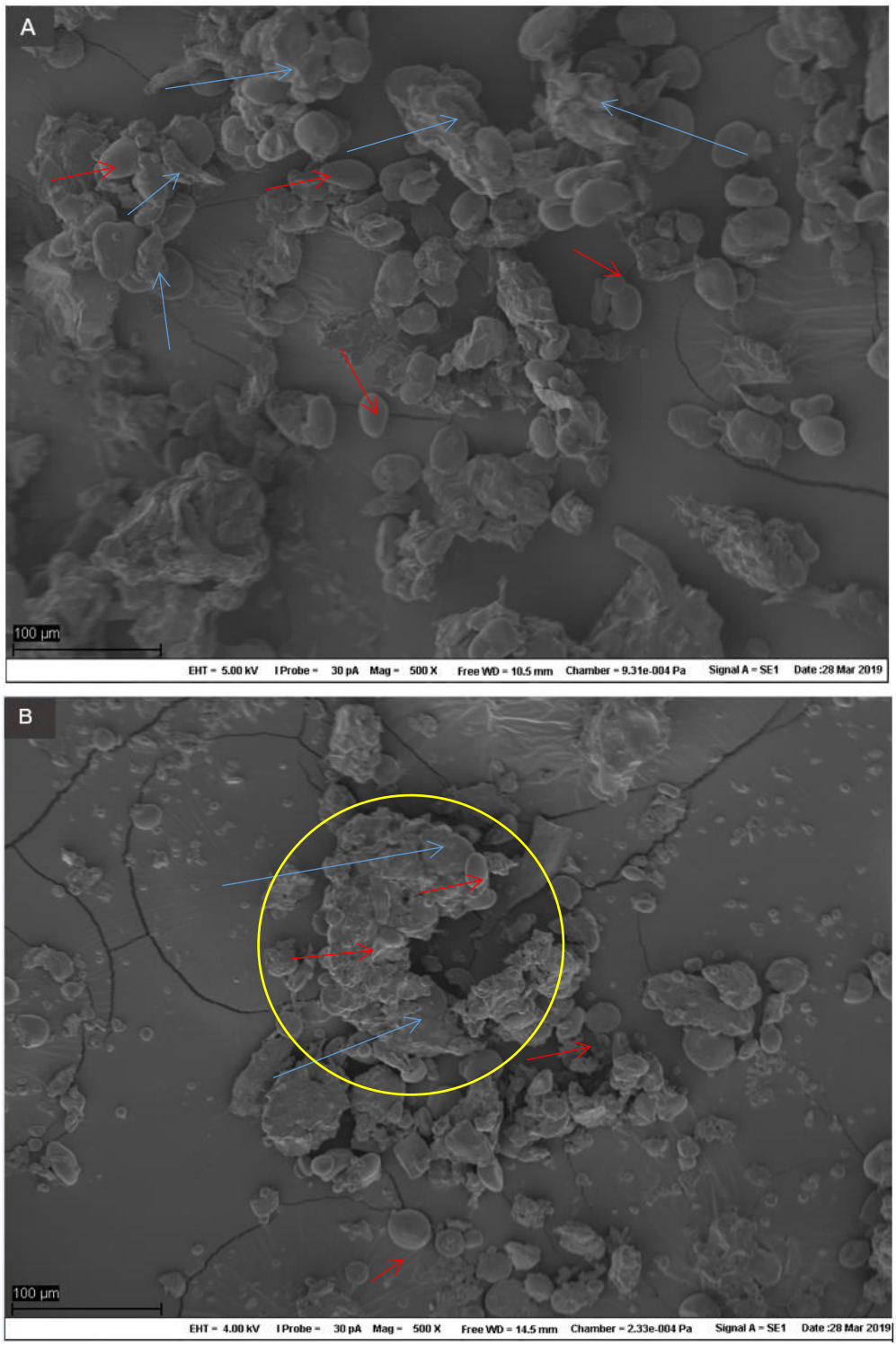



**Figure 17**. Scanning electron microscopy (SEM) images of starch granules from pelleted diets
(A) Wheat based diet; (B) Faba bean starch fraction based diet
Scale bar in images: 100 µm

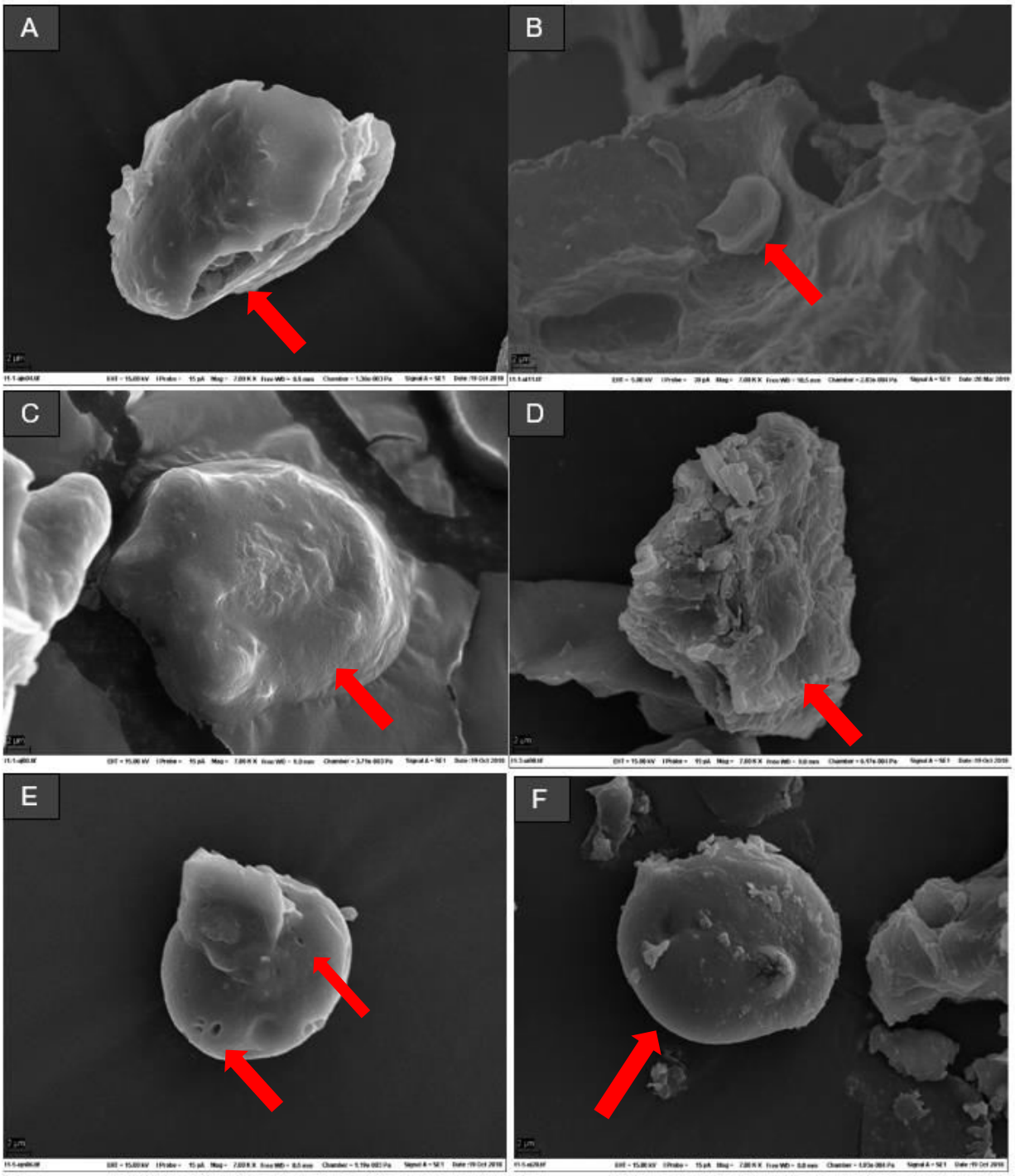



**Figure 18**. Scanning electron microscopy (SEM) images of starch granules in freeze- dried digesta samples from upper jejunum and upper ileum of the broiler chickens fed with pelleted wheat-based diets (Scale bar in images: 2 µm)
(A) Wheat starch from upper jejunum of 1. Pen/bird (B) Wheat starch from upper ileum of the 1. Pen/bird
(C) Wheat starch from upper jejunum of 3. Pen/bird (D) Wheat starch from upper ileum of 3.Pen/bird
(E) Wheat starch from upper jejunum of 5. Pen/bird (F) Wheat starch from Upper ileum of the 5. Pen/bird

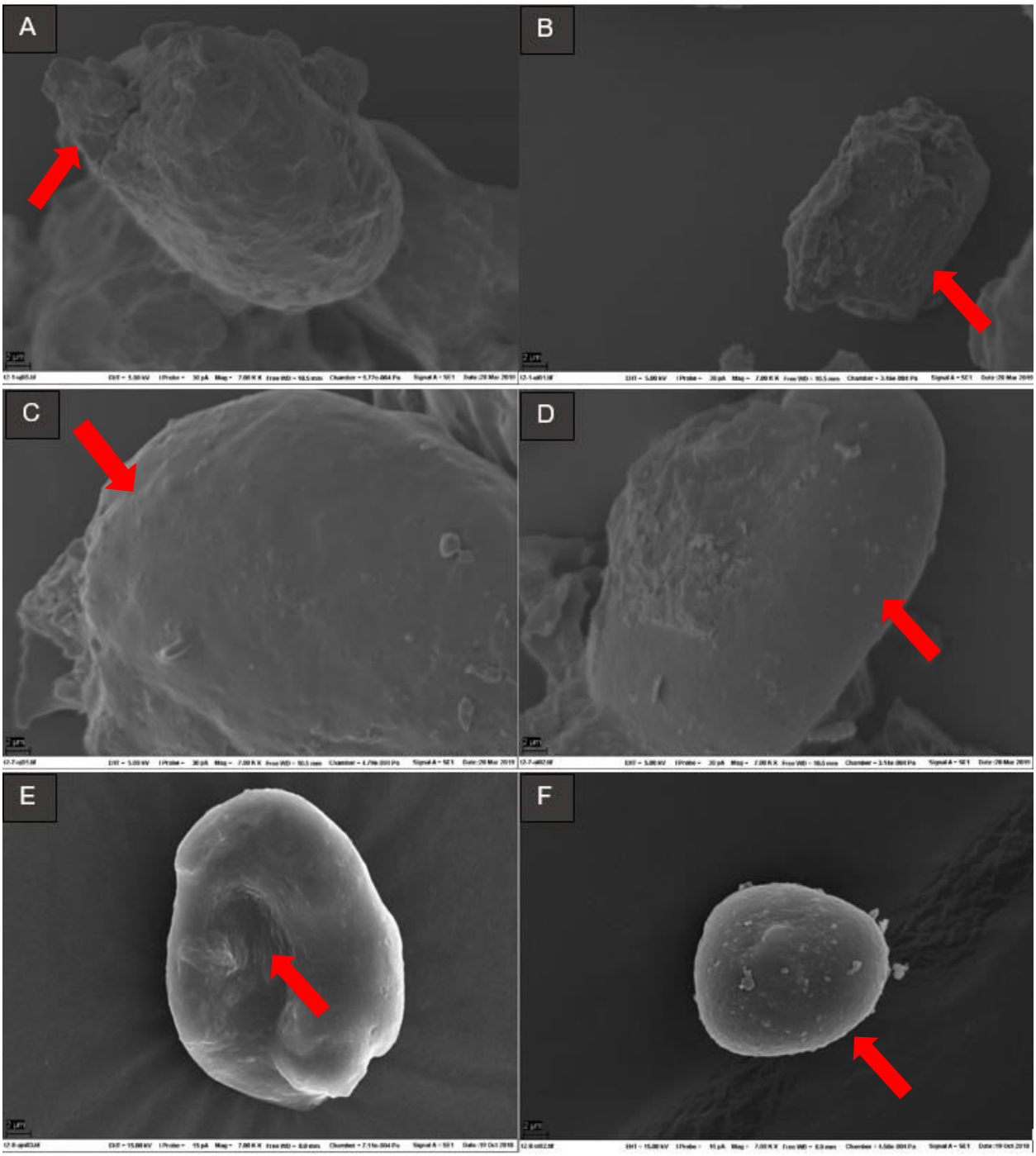



**Figure 19.** Scanning electron microscopy (SEM) images of starch granules in freeze- dried digesta samples from upper jejunum and upper ileum of the broiler chickens fed with pelleted faba bean starch based diets (Scale bar in images: 2 µm)
(A) Faba bean starch from upper jejunum of 1. Pen/bird (B) Faba bean starch from upper ileum of the 1. Pen/Bird (C) Faba bean starch from upper jejunum of 7. Pen/ bird (D) Faba bean starch from upper ileum of 7. Pen/bird (E) Faba bean starch from upper jejunum of 8. Pen/bird (F) Faba bean starch from Upper ileum of the 8. Pen/ bird

SEM images for freeze-dried digesta samples taken from the broiler chickens fed with wheat-based diets revealed that starch granules in the upper jejunum preserved their granular shape, whereas starch granules in the upper ileum lost their granular structure. Freeze-dried digesta samples taken from upper ileum of the broiler chicken were shown in Figure 18, B and D. Starch granules were not easy to find due to the low starch content. In image B shown in Figure 18 could be a small wheat starch granule (B-type) remained inside the endosperm of the wheat. Image in Figure 18, D showed a non-starch compound, which could be a part of protein structure. On the other hand, Image Figure 18, F showed that even in upper ileum intact wheat starch granules were remained. The holes on the surface of the wheat starch were also observed (image Figure 18, E) which were not observed for faba bean starch.

SEM images for freeze-dried digesta samples taken from the broiler chickens fed with faba bean starch based diets revealed that starch granules in the upper jejunum preserved their granular shape, but also starch granules in the upper ileum kept their granular structure (image in Figure 19, D and F). The structure shown in Figure 19, B image could be a starch granule surrounded by some protein matrix or a protein structure. It is not evident and obvious as the starch granule shown in images Figure 19, D and F. In addition, the starch granule sizes were larger than wheat starch granules, which were also supported by light microscopy. Starch granule shown in image Figure 19, C had a 40 µm diameter.

### 4.5. Nitrogen digestion along the intestinal tract of broiler chickens

**Table 16.** The effect of starch source and processing method on nitrogen digestion along the intestinal tract of 30-d-old male broilers[1]

| | | Jejunum | | Ileum | |
|---|---|---|---|---|---|
| **Starch source** | **Processing** | Upper | Lower | Upper | Lower |
| WS | Pelleting | 0.370 | 0.582 | 0.711 | 0.813 |
| FBS | Pelleting | 0.305 | 0.552 | 0.735 | 0.832 |
| WS | Extrusion | 0.255 | 0.538 | 0.737 | 0.823 |
| FBS | Extrusion | 0.254 | 0.588 | 0.733 | 0.848 |
| | **√MSE*** | 0.096 | 0.068 | 0.046 | 0.030 |
| **Diet** | | | | | |
| WS | | 0.309 | 0.560 | 0.725 | 0.818 |
| FBS | | 0.279 | 0.570 | 0.734 | 0.840 |
| **Processing** | | | | | |
| Pelleting | | 0.338 | 0.567 | 0.724 | 0.822 |
| Extrusion | | 0.255 | 0.563 | 0.735 | 0.836 |
| *P-value* | | | | | |
| **Diet** | | 0.3406 | 0.6505 | 0.5374 | **0.0266** |
| **Processing** | | **0.0119** | 0.8531 | 0.4370 | 0.1695 |
| **Diet x Processing** | | 0.2984 | **0.0738** | 0.3463 | 0.7743 |

[1] Values are means of 10 replicate cages of 2 birds each.
*√MSE: square root of means square error in the analysis of variance.

The effect of starch source and processing method on nitrogen digestion along the small intestine for broiler chicken were shown in Table 16. Processing had no effect on nitrogen digestion except in the upper jejunum ($P<0.05$). In addition, starch source had no effect on nitrogen digestion in all section of small intestine, except in the lower part of ileum. No interaction between diet and processing was observed to affect the nitrogen digestion along the small intestine. Extruded FBS had highest nitrogen digestion following pelleted FBS (0.832), extruded WS (0.823), and pelleted WS (0.813).

## 5. DISCUSSION

### 5.1. Birds Performance

There were no significant differences of body weight of the broiler chickens fed with different diets ($P= 0.124$) (Table 13). Body weight of the broiler chickens fed with pelleted wheat and faba bean starch based diet was not significantly different. Same results were also observed with the extruded diets. Starch source had no significant effect on body weight. Extruded FBS had highest body weight (2,294 g) following extruded WS (2,270 g), pelleted FBS (2,209 g), and pelleted WS (2,201 g) during 1-29 days. Extrusion process did not improve the body weight of the broiler chickens fed with wheat starch sources.

Feed intake values for broiler chickens fed with pelleted wheat and faba bean based diets were significantly different ($P<0.05$). Therefore, starch source had an effect on feed intake. Feed intake in broiler chickens fed with pelleted wheat-based diet (2,715 g) was lower than pelleted faba bean starch fraction based diet (2,820 g). Feed intake in broiler chickens fed with extruded faba bean starch fraction based diet was (2,924 g), which was the highest. This can be attributed to chemical changes during extrusion, increased nutrient intake by changing in the physical form of the feed. The pellet quality namely pellet durability index was higher in extruded faba bean starch fraction based diet (99.1%). However, it is not evident to state that feed intake is affected by only physical quality of the feed.

Broiler chickens fed with four different diets had similar FCR values at the end of the experimental period (Table 13). Numerically, pelleted FBS had highest FCR (1.28) following extruded FBS (1.27), pelleted WS (1.24), and extruded FBS (1.23) during 1 – 29 days. Feed conversion ratio (FCR) for broiler chickens was similar between all complete diets. According to

Gous (2011), broiler chickens fed with pelleted faba bean (250 g/kg inclusion level) had FCR value of 1.57. This study showed that air classification of faba bean made its inclusion more convenient and it could improve the feed conversion ratio in broiler chickens. The results did not show an increased performance when diets are extruded in terms of feed conversion ratio. This was mainly related to a similar feed intake among the diets, which in turn did not increase the body weight and therefore reduced the proportion of energy used for gain. Pelleting is widely used for broiler chicken feed production, and pelleting process is known to improve weight gain, feed intake and feed efficiency in broilers (Abdollahi et al., 2013).

In conclusion, these data indicate that diets based on faba bean starch (FBS) fraction have no negative effect on broiler chickens performance. Chickens fed with pelleted diets based on wheat were to have observed similar performance in chickens fed with pelleted faba bean starch diet in terms of weight gain and feed efficiency. The particle size of the faba bean starch fraction based diets was finer compared to wheat-based diets. It could be stated that pelleting tended to even out differences in particle distribution and extend of gelatinization levels between diets and improves nutrient utilization (Svihus et al., 2004).

### 5.2. Starch digestibility along the intestinal tract of broiler chickens

Starch digestibility is often considered as complete; however, several authors have reported incomplete starch digestion in broiler chickens (Mollah et al., 1983, Rogel et al., 1987, Choct et al., 1995, Yutste et a1., 1991). The rate and extend of starch digestion have been reported to be affected by both physicochemical properties of starch (starch structure, granule shape, size and dimension, surface area, degree of crystalline, non-starch components and antinutritional factors, and also other animal related factors (Weurding, 2002).

Wheat, considered as a good source of nutrient for broiler chickens, is a major ingredient in broiler diets in many countries due to its high starch content. Digestibility studies with wheat usually reveals a starch digestibility between 0.93 and 0.98 (Choct et al., 1999, Steenfeldt et al., 1998, Edney et al., 1989). In some studies, lower starch digestibility (0.82) had been reported (Mollah et al., 1983, Rogel et al., 1987, Choct et al., 1995 as cited in (Svihus, 2011a)), the lower values were related to antinutritive effect of soluble fibre. Low starch digestibility values of wheat diets had been confirmed in different experiments (Maisonnier et al., 2001, Marron et al., 2001,

Svihus, 2011, Svihus and Hetland, 2001, Weurding et al., 2001b, Carré et al., 2002, Hetland et al., 2002, Amerah et al., 2009, Murphy et al., 2009, Zimonja et al., 2009, Svihus et al., 2010). When fibre degrading enzymes were added to the wheat diets, starch digestibility increased to an average value of 0.93 (Svihus, 2001). This indicates that fibre interferes with starch digestibility. In this study, starch digestibility of pelleted wheat based diet was almost complete (0.998) at the lower ileum. Therefore, antinutritive effect of soluble fiber did not seem to be a problem in terms of total starch digestibility for this experiment.

The study done by Péron et al. (2005) examined the effect of particle size of coarse wheat pellet diet and fine wheat pelleted diet to evaluate animal performance and starch digestion. In general, feed ingredients such as cereal grains and grain legumes are subjected to some type of particle size reduction prior to thermomechanical process. It is generally thought that smaller particles with an increased surface area will allow increased access to digestive enzymes and therefore enhance nutrient digestion (Amerah et al., 2007). In this study done by Péron et al. (2005), fine wheat pelleted diet were ground by hammer mill with 1 mm sieve. The positive effect of fine grinding on wheat starch digestibility were observed, however fine grinding did not result in starch digestibility values close to 100%. Based on this study, the starch digestibility was (0.925) for pelleted wheat-based diet. Thus, it remains to be unknown if more intense grinding would be necessary to achieve complete starch digestion or other factors are involved in incomplete starch digestion. In this study, whole wheat was reground by pin mill, this could improve the destruction of endosperm resulting higher nutrient access resulting higher starch digestibility.

For pelleted diets, the starch digestibility in broiler chickens was affected by starch sources. ($P < 0.05$) (Table 14). In agreement with previous researches, starch digestibility of legume source was lower compared to cereal source (Weurding et al., 2001b, Sandhu and Lim, 2008, Chung et al., 2009). Several factors such as structural properties of the starch and feed processing may influence the rate and extent of starch digestion in the small intestine as stated by Zimonja et al., 2007. Although complete gelatinization via extrusion process improved starch digestibility of wheat based diets exhibiting low digestibility, Zimonja and Svihus (2009) did not detect any significant improvements in starch digestibility via pelleting for broiler chickens. In this study, low starch gelatinization levels were observed for pelleted diets compared to extruded diets. Via pellet press, starch gelatinization extend was limited (209 g/kg and 207 g/kg for WS-P and FBS-P respectively). At the lower ileum, starch digestibility of wheat-based diet was almost complete

(0.998), however starch digestibility of faba bean starch fraction based diet was lower (0.972). Therefore, the explanation for this could be the differences in type of polymorphic forms, granule size, surface features, amylose/amylopectin ratio, and degree of crystallinity, non-starch compounds and anti-nutritional factors in starch sources used in this study. Based on the literature review, legume starch is often considered to be less digestible, and digested by pancreatic α-amylase more slowly than cereal starch.

Different structural features of starches also affect their susceptibility for amylase (Tester and Qi, 2004). Therefore, enzyme activity is also very important. Amylase enzyme activity were inflected by starch source for pelleted diets ($P<0.05$). Amylase activity was 64.6 (U/g chyme) for WS, and 82.9 (U/g chyme) for FBS pelleted diets. Broiler chickens fed with pelleted faba bean starch based diet had the highest amylase enzyme activity. Thus, the results showed that the susceptibility of the starch sources were significantly different for broiler chickens. The susceptibility of legume starches known to contain both A- and B-type crystalline polymorphs in varying proportion towards α -amylase have not understood well as describe in the literature review section (Zhou et al., 2004). However, it is generally accepted that legume starches are more resistant to amylase. Therefore higher amylase activity for faba bean starch fraction based diets is an agreement with this statement.

It is widely known that the shape and size of starch granules varies (Tester and Karkalas, 2002, Tester et al., 2004b, Wani et al., 2016) with botanical source as given in Table 2 and 3. Many authors have indicated how important size and hence the surface area to volume ratio is of different starch sources with respect to evaluate the rate and extent of granular starch digestion (Slaughter et al., 2001, Kong et al., 2003, Tester et al., 2004a, Tester and Qi, 2004). Kong et al. (2003) also stated that porcine pancreatic α-amylase activity on native starch granules was more accurately described as a function of surface area rather than that of substrate concentration. The rate and extent of α-amylase hydrolysis of starches from different botanical sources had been quantified by Ring et al (1988) where they showed that wheat>maize>pea>potato, which reflected by increasing granule size as cited in Tester et al. (2006). Tester and Qi (2004) had shown that the diameter of waxy barley starch granules was negatively correlated with the amount of α-amylase hydrolysis. Based on light microscopy investigations, wheat starch granules ranged from 15 to 40 μm and faba bean starch granules had larger size interval ranged from 15 up to 65 μm. Elementally, the average starch granule of 25 μm (WS), 40 μm (FBS) size is almost 3000 times larger than the size of amylase, which has

a hydrodynamic radius of 3-4 nm (Payan et al., 1980). In the recent review (Qi and Tester, 2016), surface area , volume and surface area/ volume ratio for rice (3-8 μm) , maize (5-25 μm) and potato starch (15-100 μm) were calculated. Based on the calculation, large surface area to volume ratio of the small granules provided very different substrate accessibility compared to large granules. Thus, the granule is likely to provide many potential sites for adsorption of enzymes.

Many different authors have discussed surface features of starch granules such as pores, channels, smooth surface, and it is recognized that surface features of native granules do impact in the way amylase enzymes attach and progress hydrolysis of granules. Granules lacking pores and channels in legume starches, the enzymes initiate the digestion with slower rate from the surface towards the granule interior. Surface of granules is impermeable to amylase owing to higher local concentration (Jane, 2007). Based on SEM investigations, presence of surface holes (Figure 18, E) had been observed for wheat starch granule, which was taken from the digesta of broiler chicken fed with pellet wheat based diet. This could be one of the reason resulted with lower amylase activity and relatively higher starch digestibility for wheat- based diet.

Factors not directly related to starch itself may also affect its digestibility. Starch granules can be encapsulated by a rigid protein matrix or by cell walls from the same feedstuff. This may reduce the accessibility of starch granules to enzymatic attack. Via microscopy analysis of ileal contents examined by (Pérron et al., 2007), it was found that a large part of the undigested starch was entrapped in cell wall material, particularly from areas of the endosperm close to the aleurone layer. Milling is highly crucial for the destruction of cell walls. In Figure 18, B, a part of the endosperm were detected and the composition (which pointed out) could be B-type (<10) small wheat granule remained inside the cell wall.

In conclusion, the reason for the higher starch digestibility in pelleted wheat based diet could be explain that faba bean starch was C-type starch granules, which were more complex structure and less susceptible for amylase. The granule size in faba bean starch were higher in general than wheat starch, which could be resulted in slower enzymatic reaction. The fine grinding gave high starch digestibility in wheat-based diet by destruction the endosperm resulting a better access to amylase. Therefore, pelleted wheat based diet were more easily digested.

For extruded diets, the starch digestibility was not significantly different for wheat-based diet than FBS-fraction based diet (Table 14). As stated by (Hejdysz et al., 2016), extrusion process improved nutrient digestibility of faba bean cultivars. Not much is known about the effect of extrusion process on faba bean cultivars in broiler chicken diets. However (Alonso et al., 2000) and Diaz et al. (2006) reported positive effects of extrusion process on starch digestibility of grain legumes. Accordingly, starch crystallinity after extrusion was lost which improved the accessibility of starch for α-amylase also cited by Chauhan et al. (2003). The high starch digestibility values of extruded faba bean could be caused by decreasing level of resistant starch though heat induced changes in starch structure (Pérez- Navarrete et al., 2006). Legume starches are known to be more resistance to digestive enzymes for their C-type diffraction pattern, but these patterns are lost during the extrusion process since the starch is gelatinized and probably degraded with screw speed, shear stress forces exerted by the screw of the extruder and residence time destroying the granules (Pérez- Navarrete et al., 2006).

Extent of starch gelatinization is dependent on the processing method (pelleting, steam flaking, expanding, extrusion) and operating variables within the processing method (level of water addition, temperature and retention time) (Gilpin et al., 2002). Therefore, gelatinization results of extruded diets were higher than pelleted diets as expected. Although gelatinization results of extruded diets were higher than pelleted diets, starch source influenced gelatinization level of extruded diets. Gelatinization of WS-E diet were 715 g/kg, on the other hand gelatinization of FBS-E diet were more complete 943 g/kg. During extrusion, steam addition, screw speed and die pressure were equal for the diets, however among the non-adjustable variables such as barrel temperatures in extruder, torque, SME values were different for two sources. Possible explanation for this difference could be the finer particle size distribution of faba bean starch fraction based diet absorbed more steam readily and gave different rheological properties during extrusion.

Dry fractionation is applied to legumes successfully as described earlier. Uniform granules of the starch granules in legumes, it can be expected that milling can lead to a better separation compared to cereals, which contains a bimodal starch granule size distribution (Schutyser and Van der Goot, 2011). Therefore, starch granule size largely determines the potential for dry separation. Based on light microscopy images, uniform starch granule size distribution was supported this information for faba bean starch.

Particle size distribution of faba bean starch fraction was shown in Figure 12, and it has particle size not larger than 720 µm. Whole faba bean contained 376, 7 g/kg DM starch, which was lower compared to these cultivars (438 g/kg DM in Merlin cultivar., and 394 g/kg DM in Olga cultivar) (Hejdysz et al., 2016). After fractionation, starch content of FBS fraction was increased to 744, 42 g/kg DM. In this study done by Bergthaller et al. (2001), the efficiency of air classification was evaluated by calculation of a recovery factor defined as the protein content of the fine fraction divided by the protein content of the feed. Based on this, starch recovery factor of air classification of faba bean was 1.98. According to study done by Coda et al. (2015), starch recovery factor for air classified faba bean were 1.56.

Dijkink et al. (2007) examined the adhesion of small particles (protein bodies) to the larger starch granules via SEM image. The adhesion of protein particles to the larger starch granules resulted in a lower yield of protein fraction and lower purity of the pea starch fraction. Protein recovery factor of air classification of Whole faba bean for this study was 2.16. Starch content in the protein fraction was 87.88 g/kg DM which was lower than (Coda et al., 2015) (233.8 g/kg DM), but higher than (Gunewardena et al., 2010) (1.39 g/kg DM). Therefore, separation process is important step to obtain efficient fractions. Fat may have a negative effect on the separation of the constituents of the legumes as it impairs powder dispersibility which explains why soy has been reported to be unsuitable for dry fractionation (Elkowicz and Sosulski, 1982, Sosulski and Youngs, 1979 as cited in (Schutyser et al., 2015)). Crude fat content of whole faba bean was 20 g/kg DM. In conclusion, air classified faba bean used in this study was achieved efficiently. However, smaller protein bodies could be expected in the starch fraction due to the highly organized cotyledon tissue structure, which consists of proteins, and starches are neatly packed. In images (Figure 17, A and B) taken by SEM, some of the starch granules were labeled, and non-starch components could be entrapped the starch granule surfaces which could have an effect on the starch digestibility in broiler chickens. SEM images showed that non-starch components were surrounding the starch granules especially for FBS-P diet. Moreover, the reasoning may not be solely dependent on plant cell wall material, but may also contain protein matrices that encapsulates FBS-P starch granules, thereby preventing their susceptibility to the amylase.

Amylase enzyme activity were not affected by processing for extruded diets (P= 0.4442). Greater extend of gelatinization were observed for extruded diets compared to pelleted diets. Therefore, lower amylase activity could be explained by the greater starch gelatinization.

Amylase activity for extruded WS diet was 54.8 (U/g chyme), contrarily amylase activity for extruded FBS diet was 77.1 (U/g chyme). For that matter, greater level of starch gelatinization of may enhance the surface of contact between the substrate and amylase enzyme.

For all diets, starch was gradually digested along the small intestine with less than 4% undigested starch entering the large intestine. For pelleted diets, starch source had an effect of the rate of the starch digestion ($P<0.05$). Therefore, it can be stated that starch digestion rate was different for wheat and faba bean starch in broiler chickens. Most of the differences for the pelleted wheat-based diet occurred in the lower jejunum and upper ileum. On the other hand, most of the differences for the pelleted faba bean starch based diet occurred in the upper jejunum and lower ileum. In opposite to pelleted diets, starch digestion rate did not influenced by extrusion processing ($P=0.2994$) among the extruded diets. The interaction between starch source and processing was observed ($P<0.05$). Although, the starch digestion rate was significantly different for pelleted diets, faba bean starch based diet produced via extrusion process resulted the similar starch digestion rate as the wheat based diet.

The aim of the light microscopy imaging was to observe morphology of the starch granules, compare both starch sources in terms of their kinetics by quantifying them, and evaluate if the thermomechanical processes had an effect on the starch digestion kinetics in the small intestine of broiler chickens. For pelleted diets, FBS- fraction based diet was more gradually digested than wheat-based diet along the small intestine. Most of the differences among the pelleted diets occurred in the jejunum and were responsible for the different kinetics of starch digestion observed. However, the rate of the starch digestion for extruded diets was similar for both starch sources. The main advantage of extrusion was to obtain a high level of starch gelatinization and disruption of crystalline structure. Probably the crystalline structure of starch granules after extrusion was lost with a great extend for both starch sources which improved then the accessibility of starch for amylase enzyme, as also stated in (Tester et al., 2004a)

According to results of starch digestibility, the amount of starch digested at specific sites of the small intestine differed for wheat and faba bean based pelleted diets. Differences in site of starch digestion may have metabolic consequences that affect feed utilization (Liu et al., 2013). The digestion pattern of dietary protein was not affected by starch source except in the lower ileum, and by processing except in the upper jejunum. The results were not significant to

interpret the effect of starch digestion rate on the nitrogen digestion along the small intestine of broiler chickens.

## 6. CONCLUSION

In conclusion, the results of the present study showed that air classified faba bean starch fraction based diet produced by pelleting resulted with lower starch digestibility for the broiler chicken compared to pelleted wheat based diet. Overall, broilers chickens fed with pelleted wheat and faba bean starch fraction based diet had similar feed conversion ratio during 1-29 days. Based on light microscopy images, pelleted faba bean starch fraction based diet had more gradually digested compared to wheat based diet. Enzyme activities were also influenced by starch source. On the other hand, extrusion processing had a major impact on the starch digestion in the small intestine of broiler chickens. The great level of star gelatinization was observed for extruded diets compared to pelleted diets. Based on light microscopy images, nearly all starch granules gelatinized and dissolved so all of the starch granules were able to detectable in the upper jejunum of the small intestine.

Nitrogen digestibility was not significantly related to starch source or processing along the small intestine. In the lower ileum, nitrogen digestibility was related to diet, whereas it was related to processing in the upper jejunum. Therefore, the data was not easy to interpret or speculate if the starch digestion along the small intestine had an effect on nitrogen digestion.

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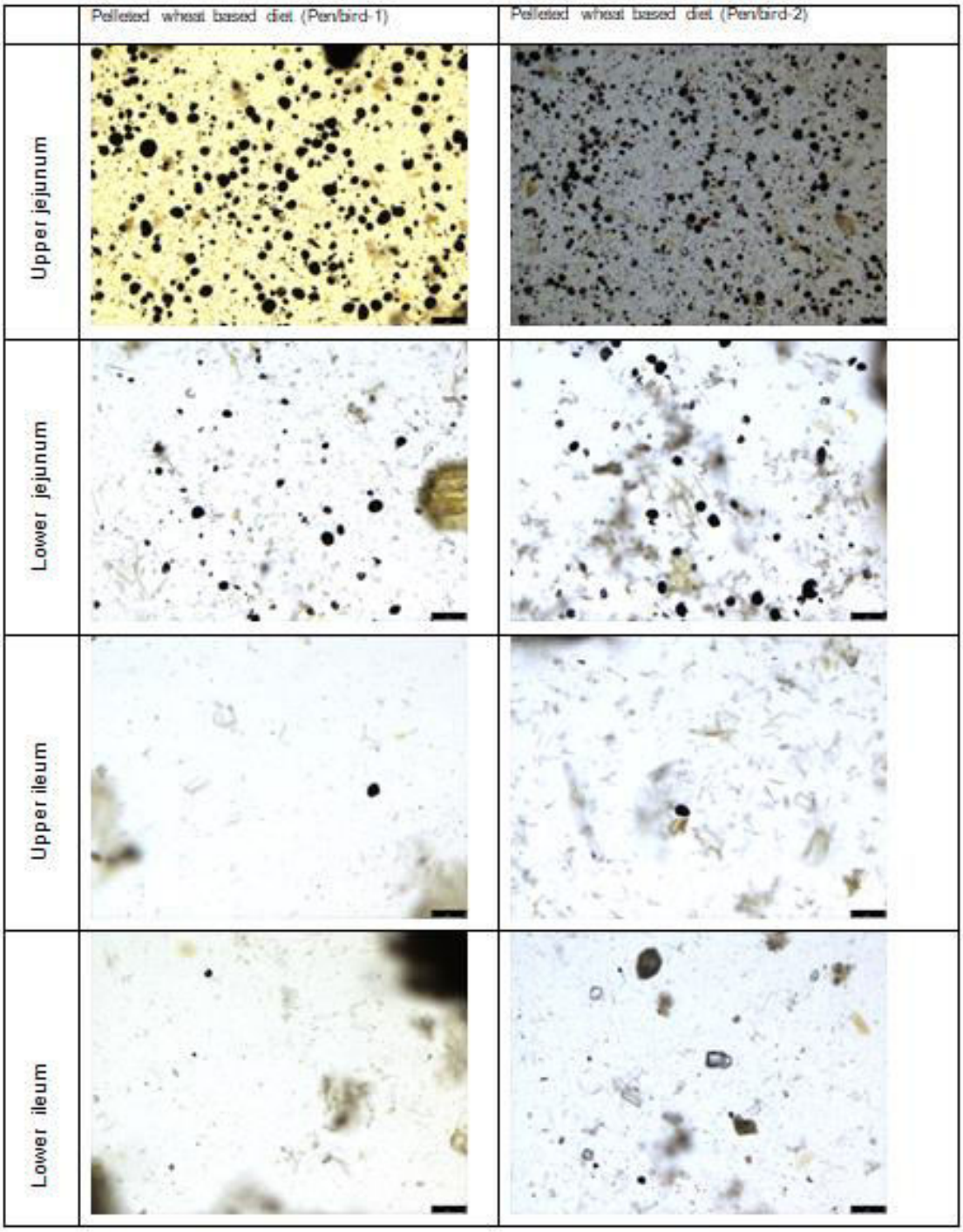
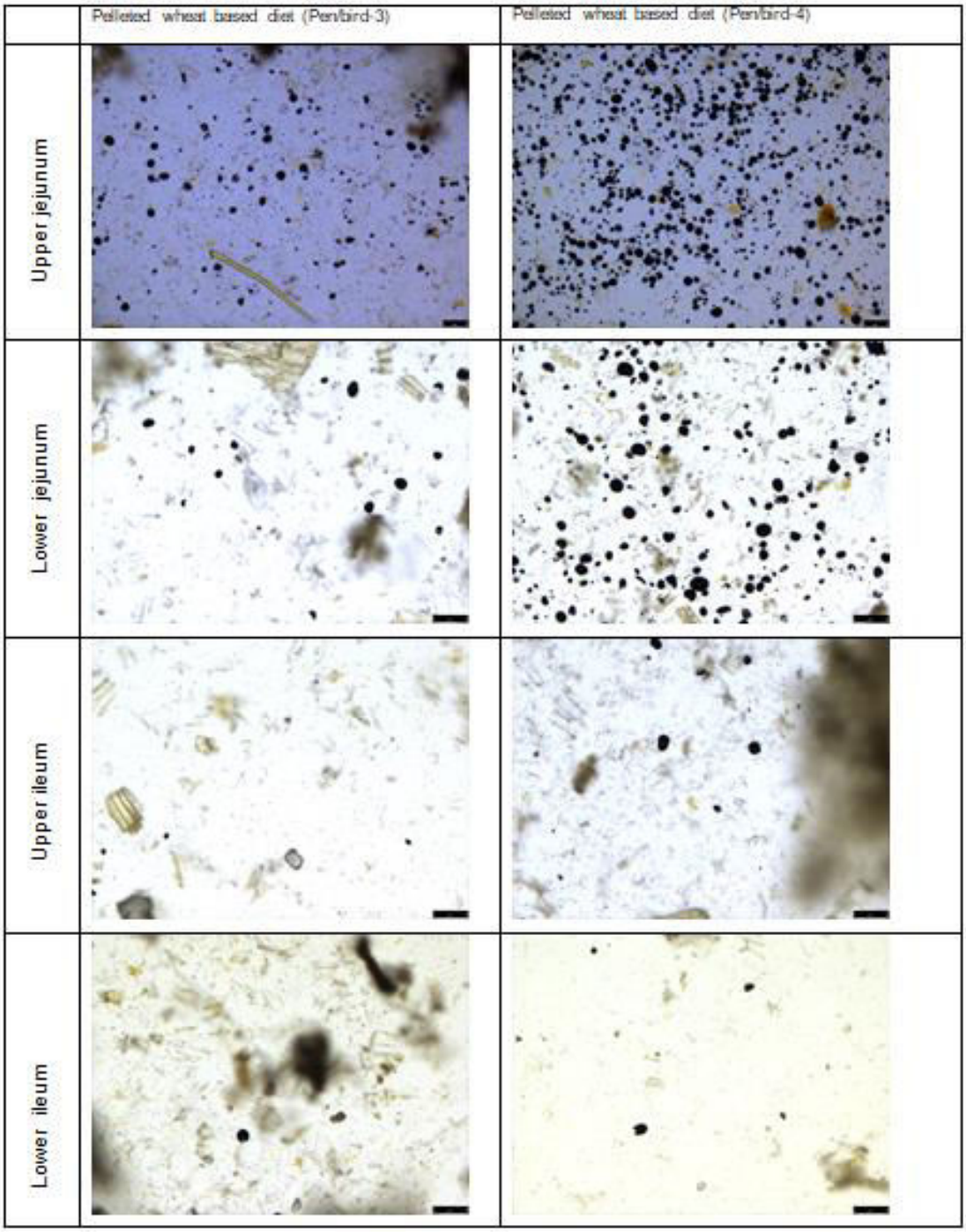
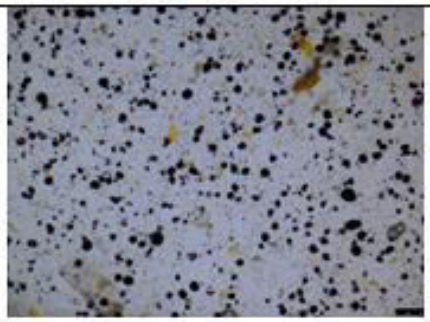
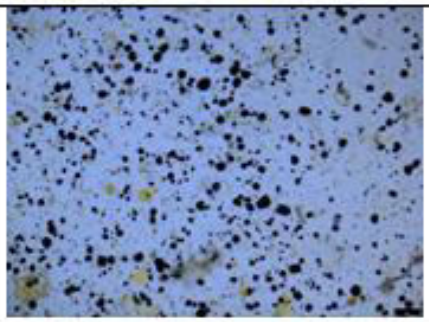
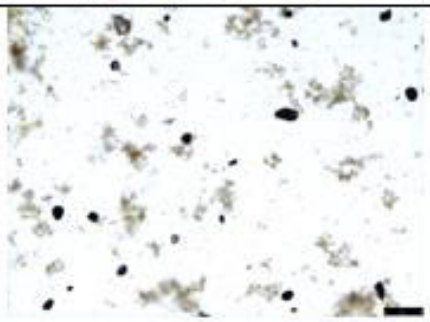
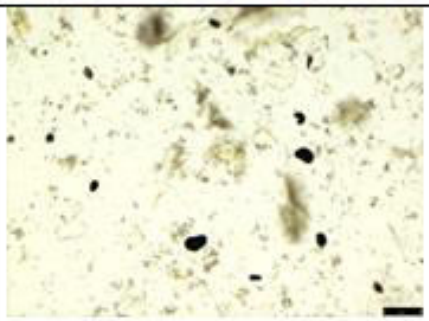
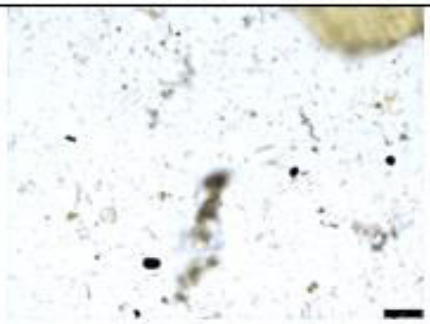
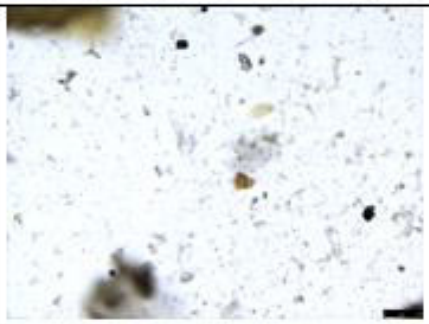
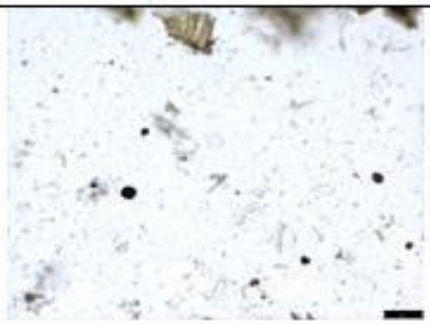
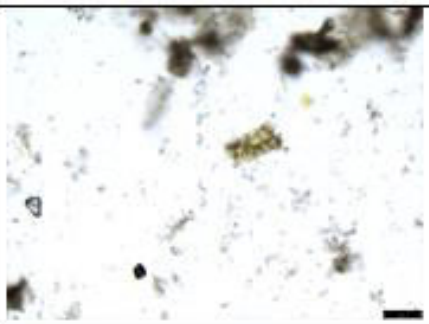
## 8. APPENDIX


Pelleted wheat based diet (Penfbird-1)
Pelleted wheat based diet (Penfbird-2)
Upper jejunum
Lower jejunum
Upper ileum
Lower ileum


Pelleted wheat based diet (Pen/bird-3)
Pelleted wheat based diet (Pen/bird-4)
Upper jejunum
Lower jejunum
Upper ileum
Lower ileum

| | Pelleted wheat based diet (Pen/bird-5) | Pelleted wheat based diet (Pen/bird-6) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

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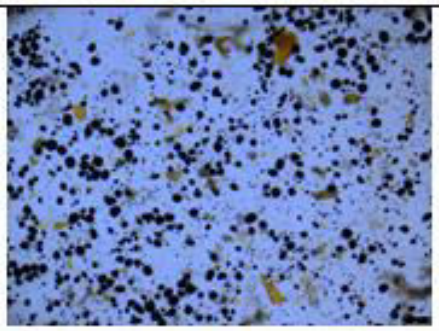
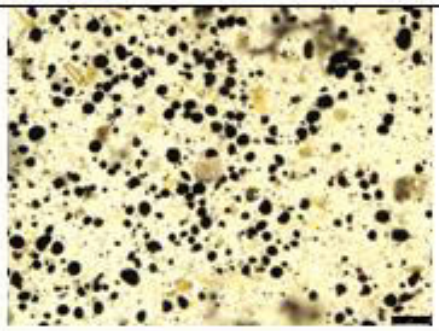
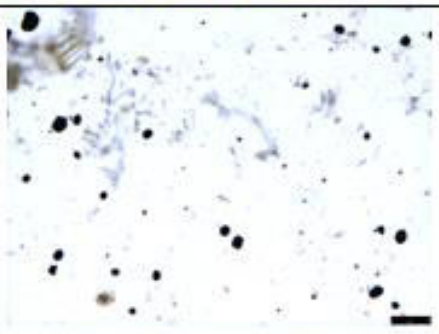
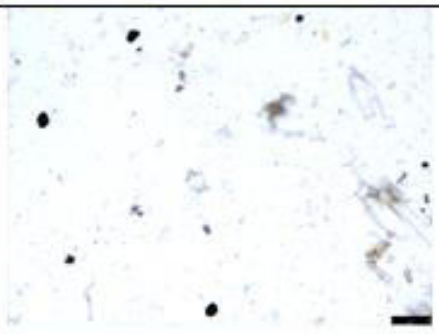
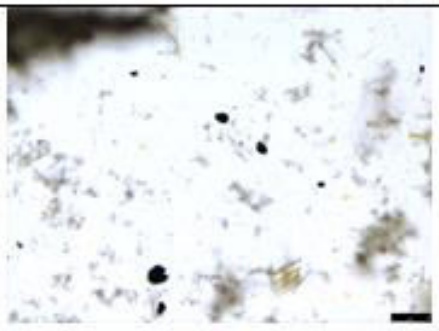
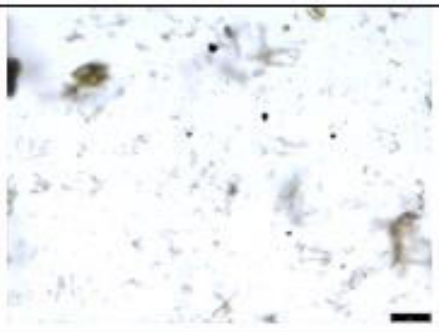
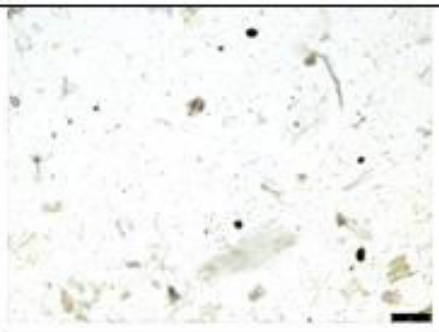
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

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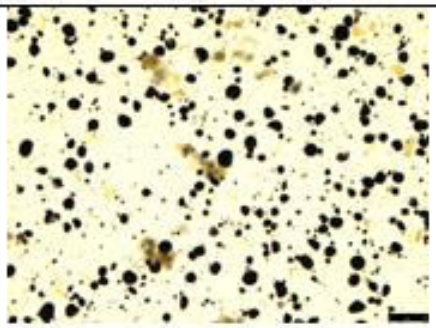
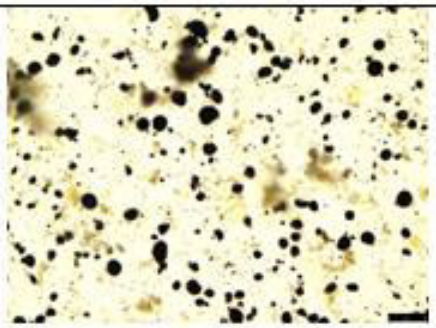
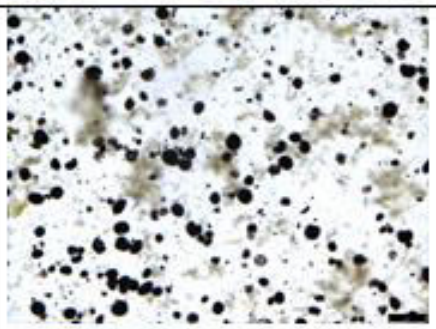
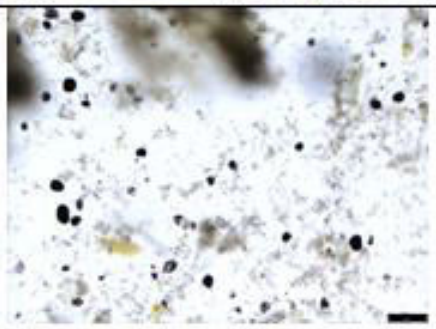
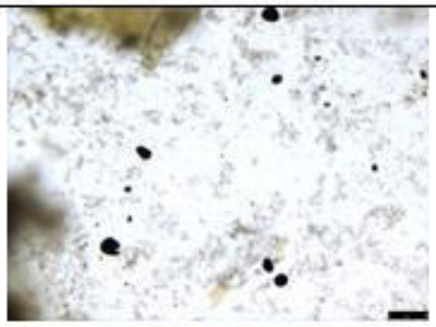
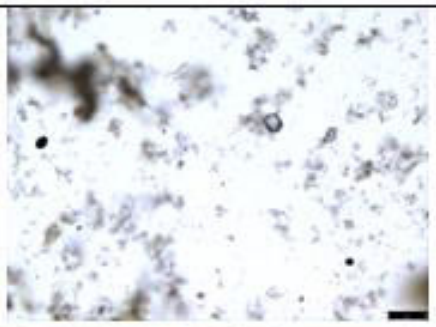
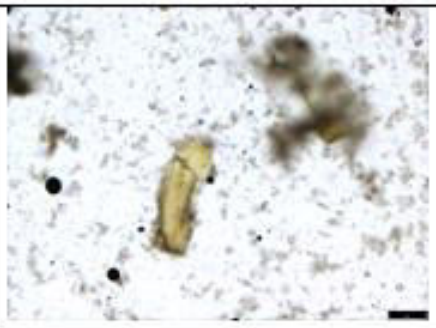
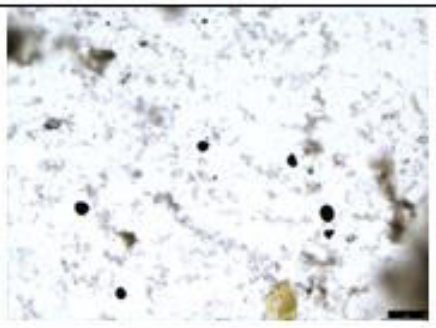
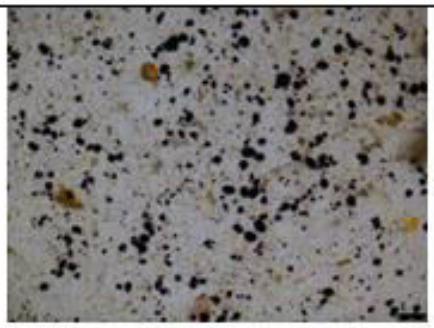
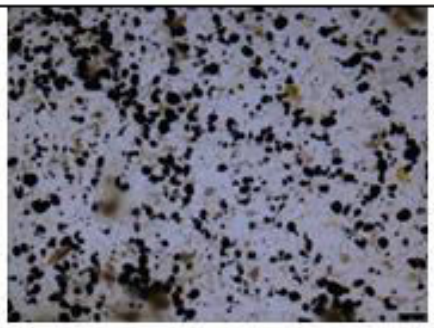
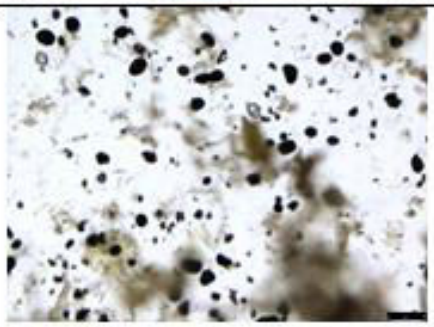
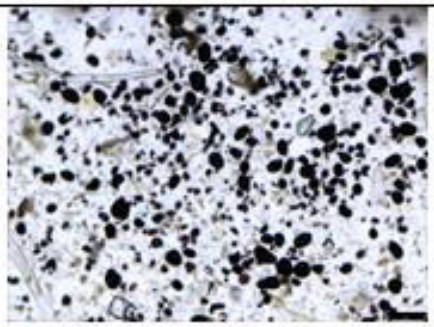
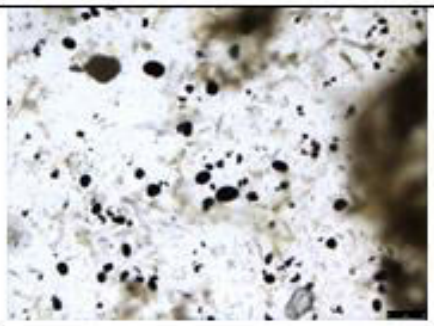
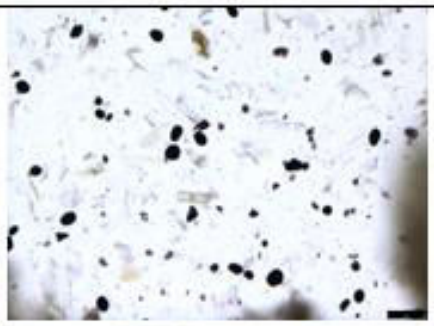
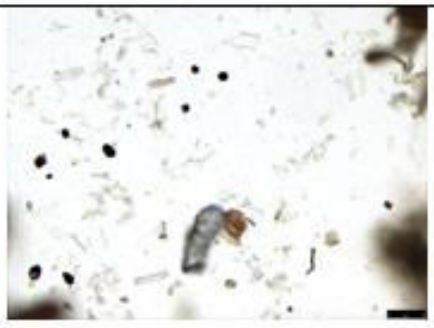
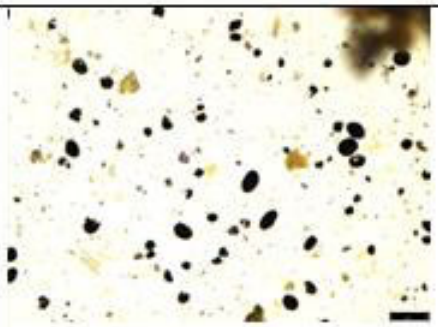
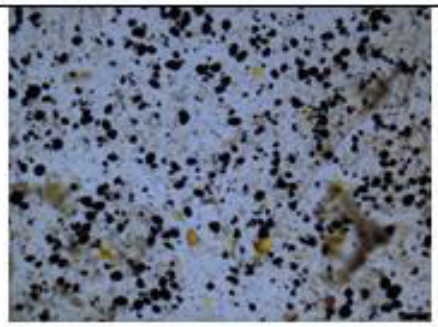
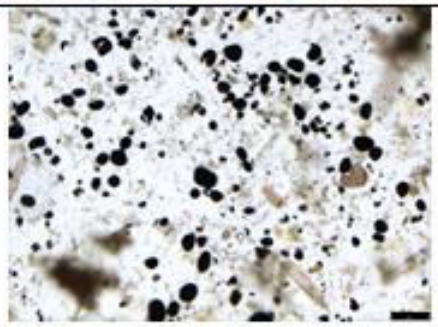
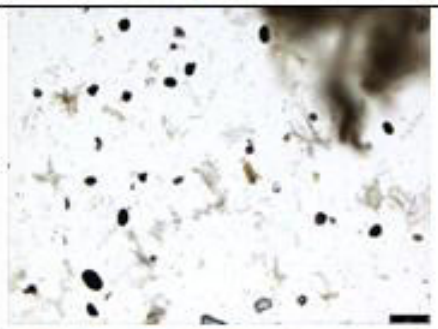
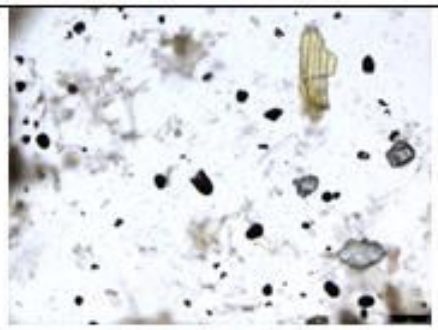
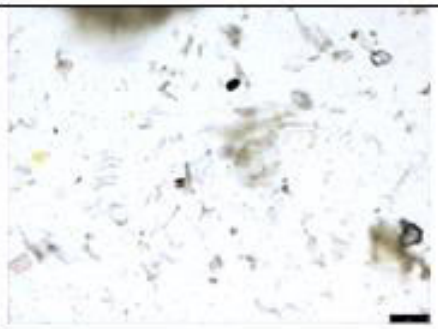
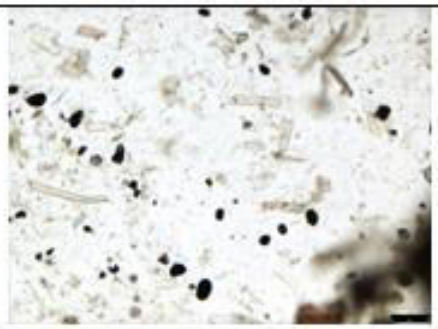
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Pelleted Faba bean starch based diet (Pen/bird-1) | Pelleted Faba bean starch based diet (Pen/bird-2) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Pelleted Faba bean starch based diet (Pen/bird-3) | Pelleted Faba bean starch based diet (Pen/bird-4) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

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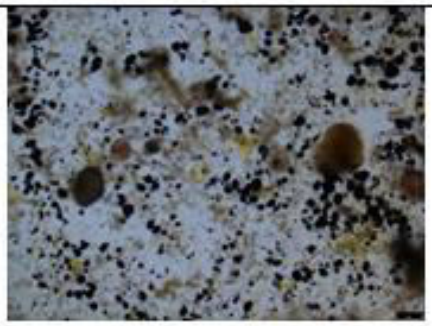
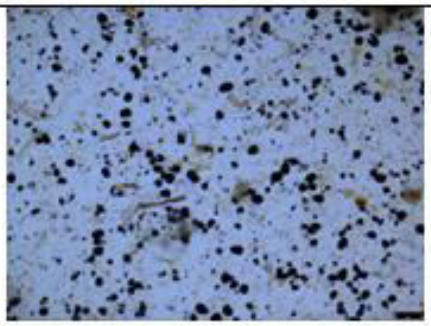
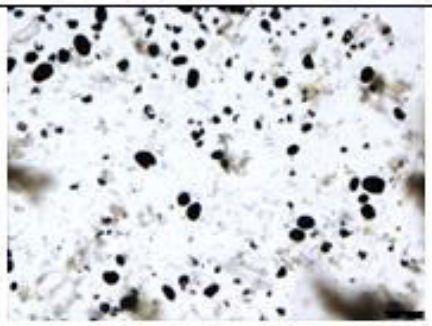
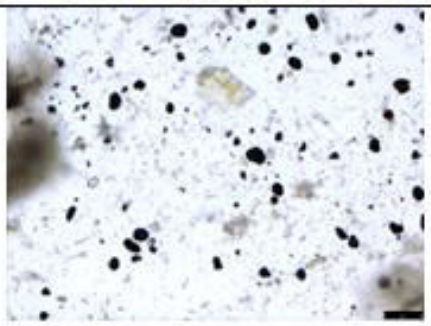
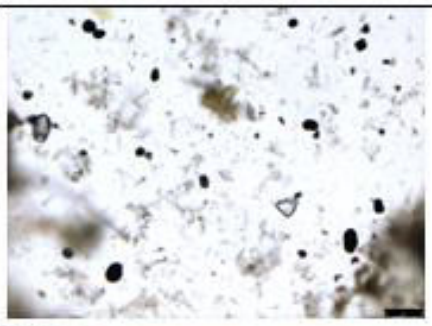
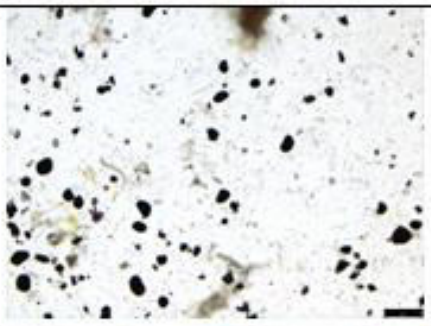
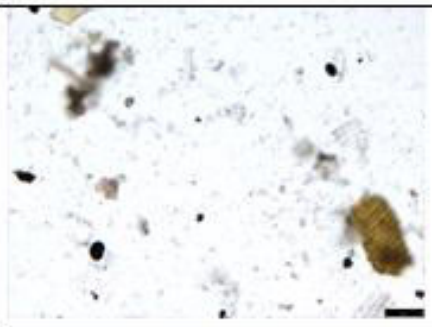
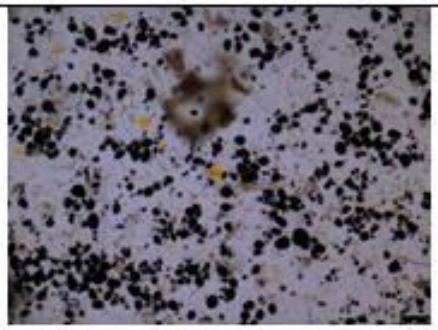
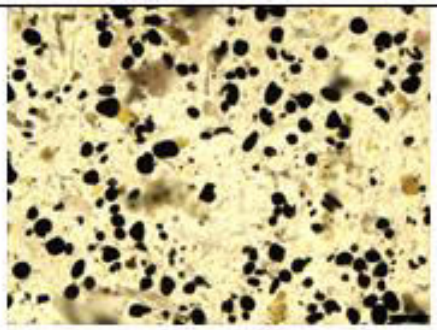
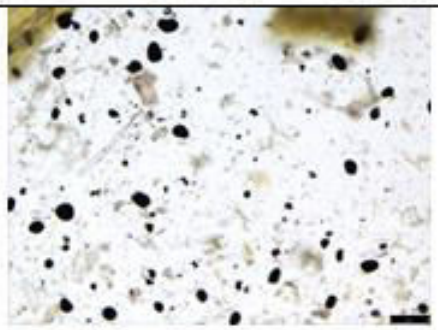
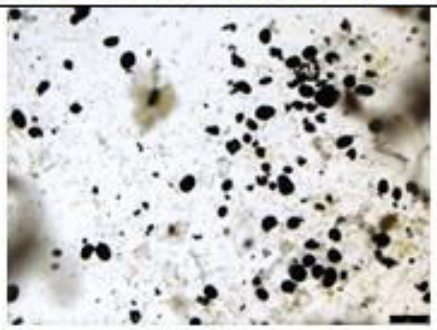
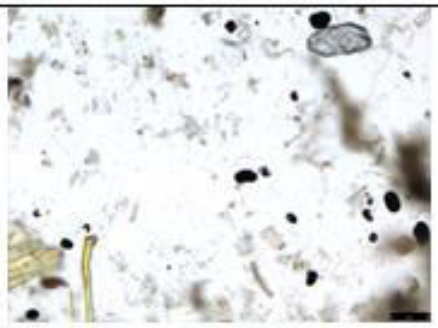
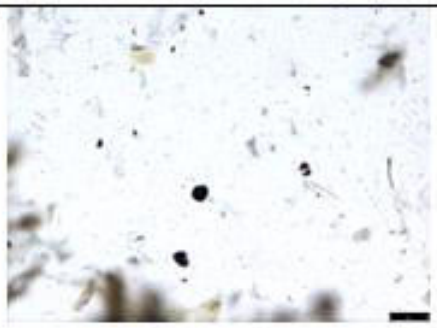
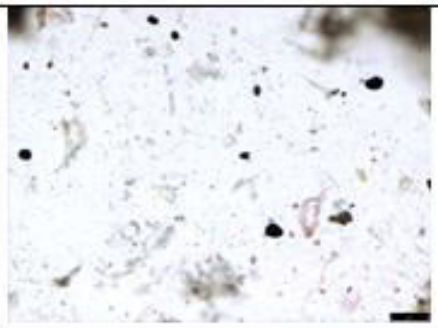
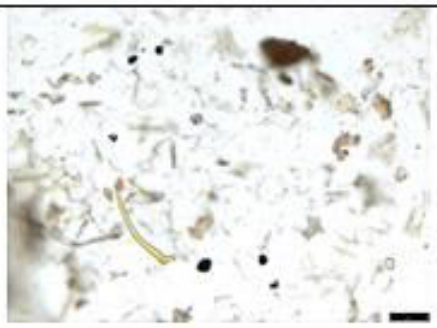
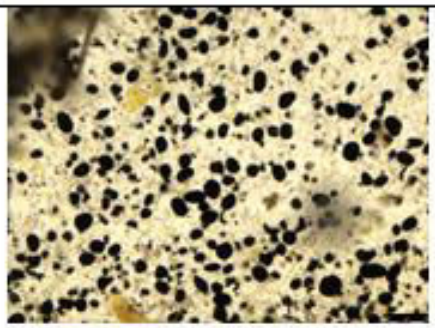
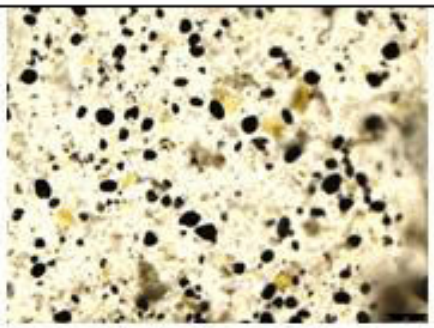
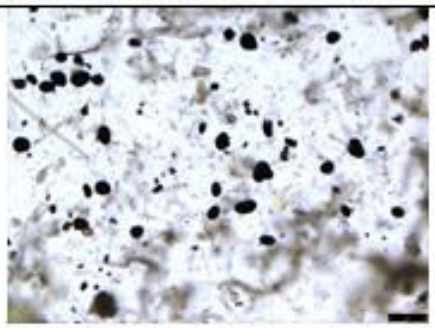
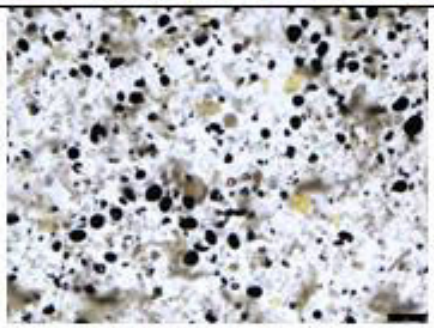
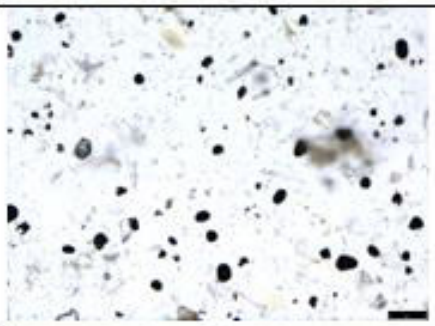
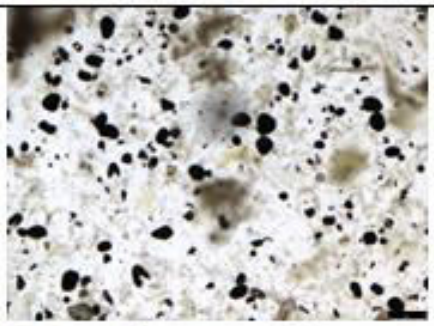
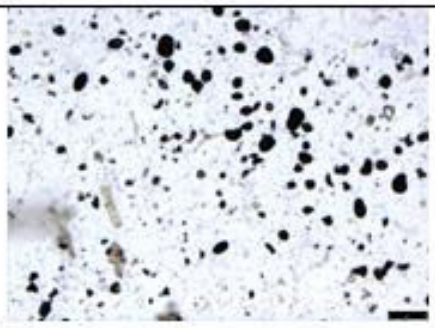
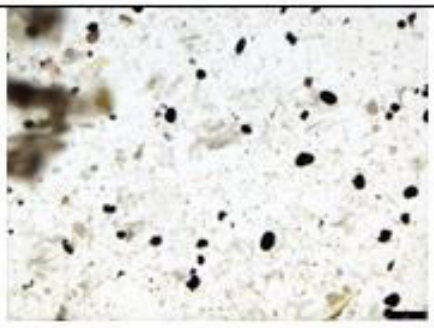
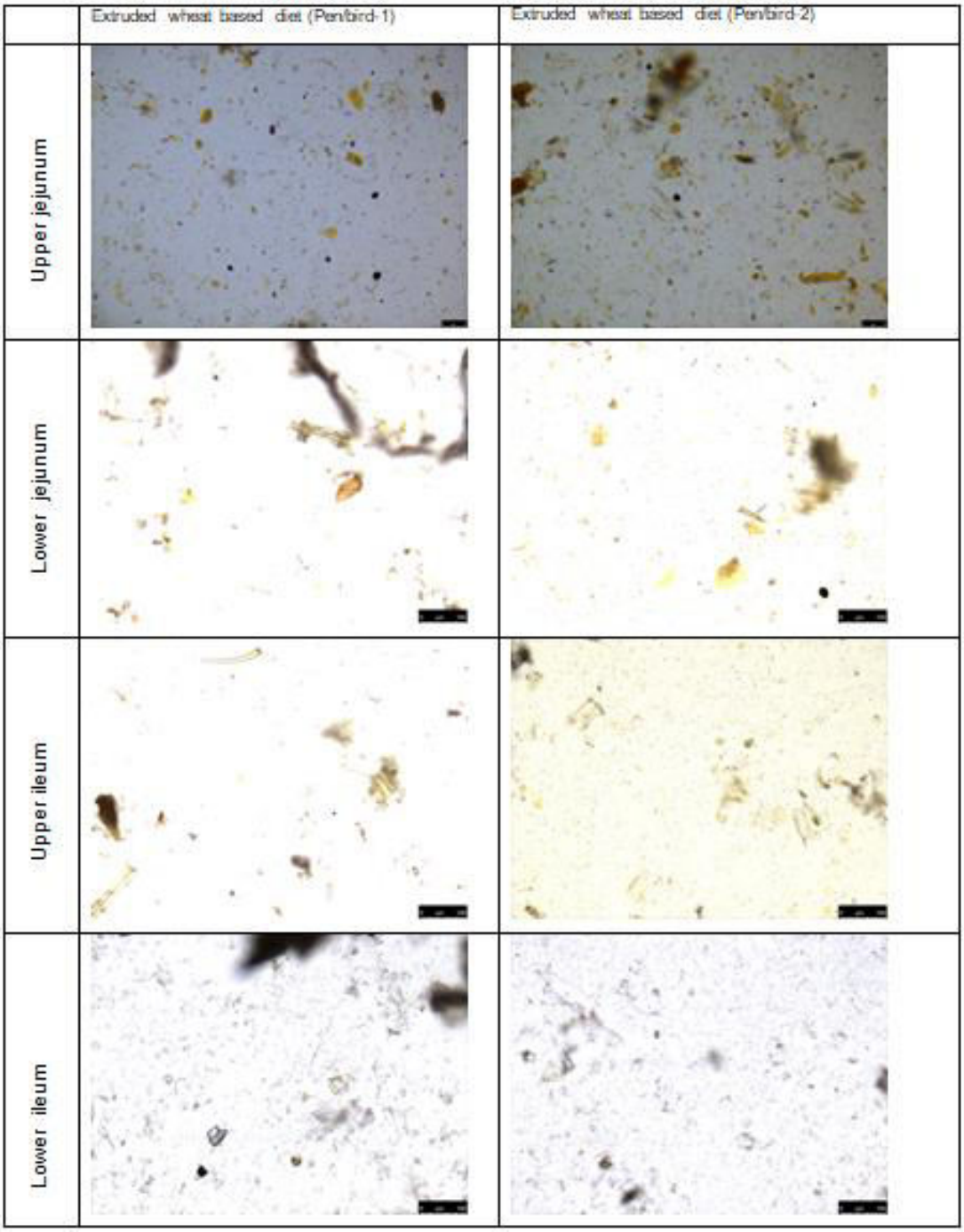
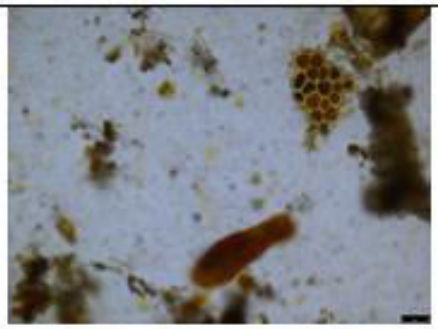
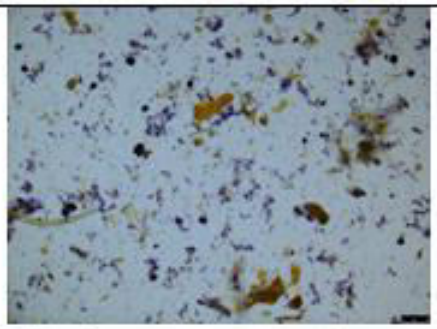
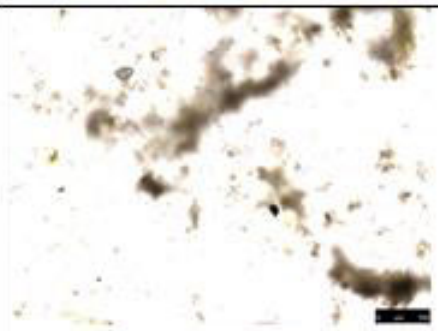
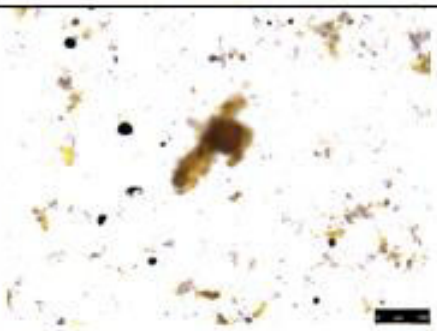
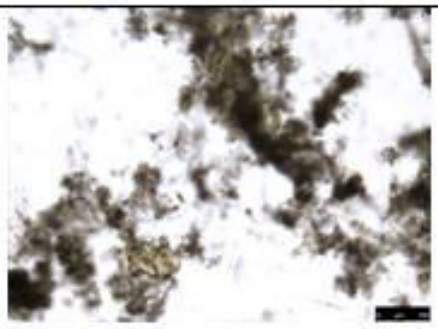
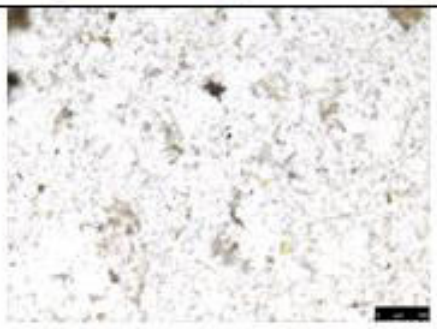
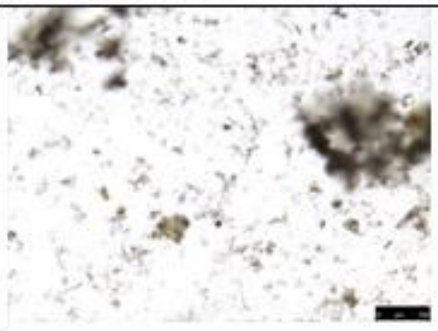
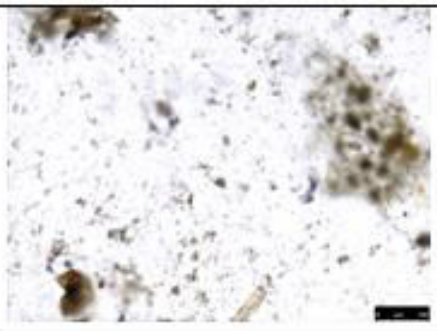
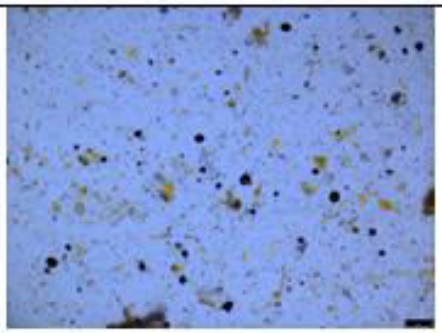
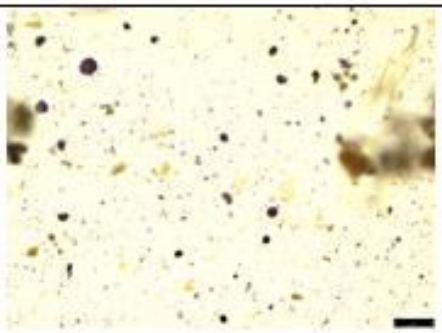
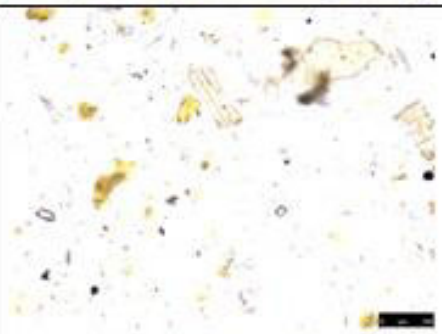
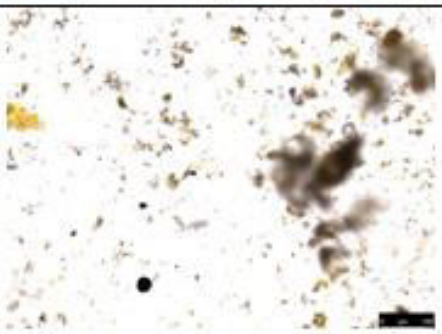
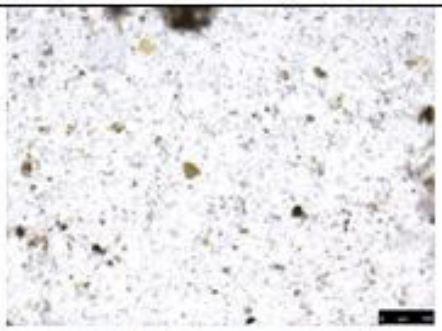
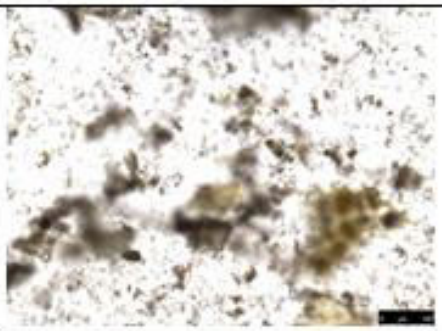
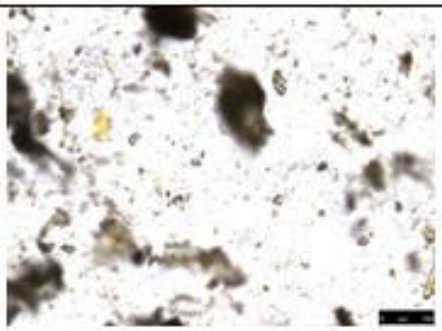
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

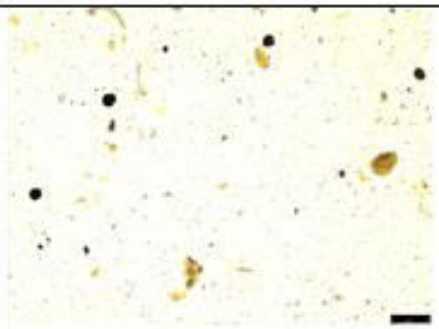
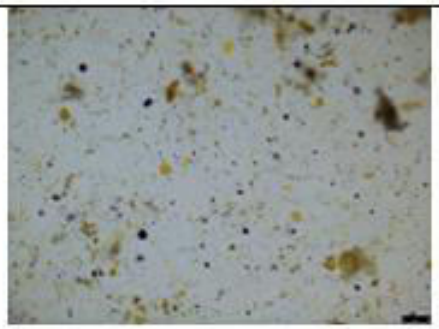
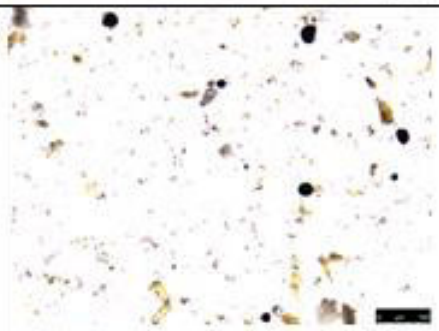
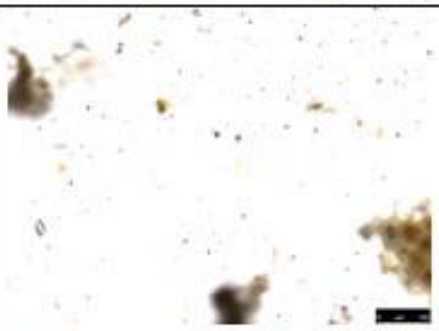
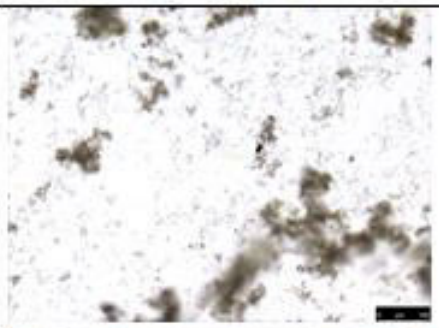
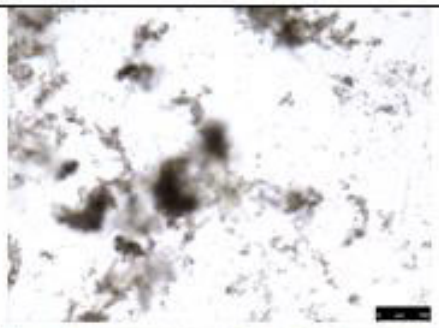
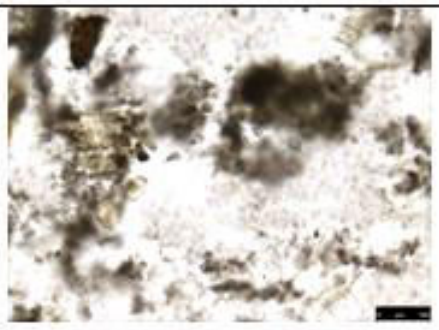
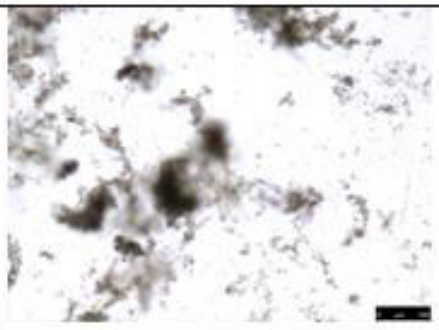
| | Pelleted Faba bean starch based diet (Peribird-7) | Pelleted Faba bean starch based diet (Peribird-8) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

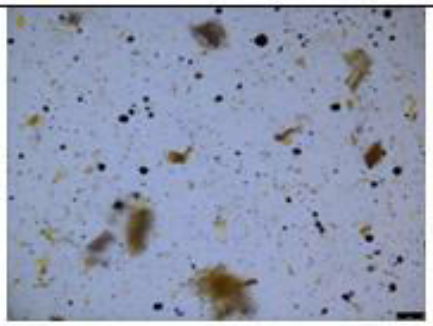
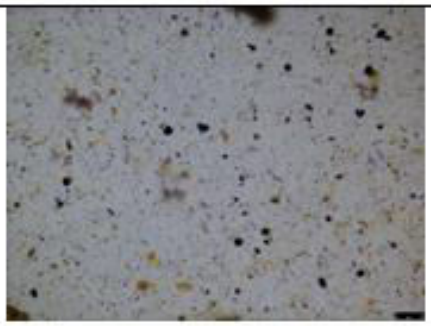
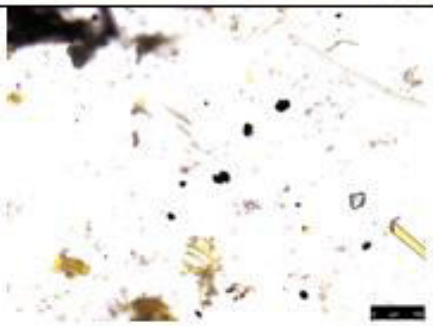
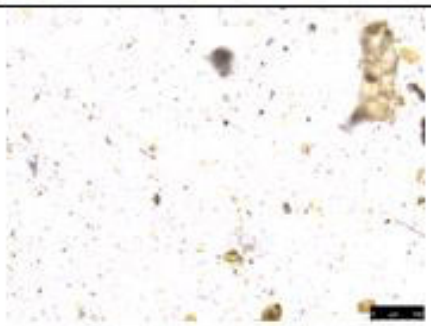
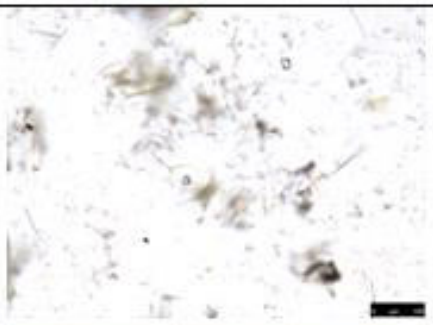
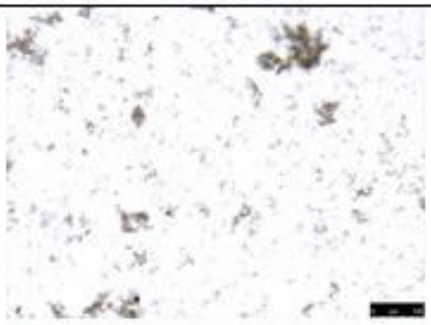
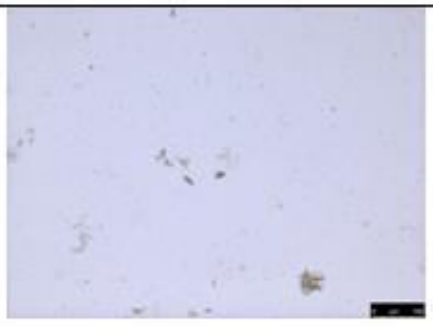
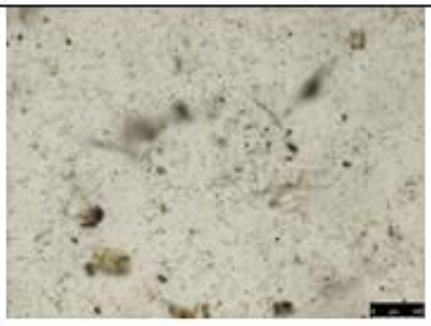
| | Pelleted Faba bean starch based diet (Pen/bird-9) | Pelleted Faba bean starch based diet (Pen/bird-10) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

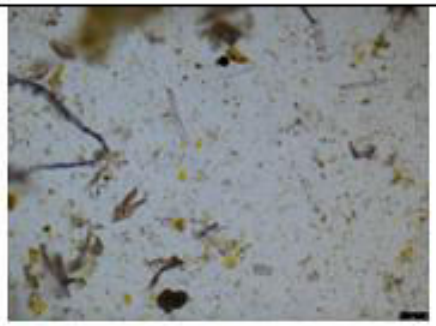
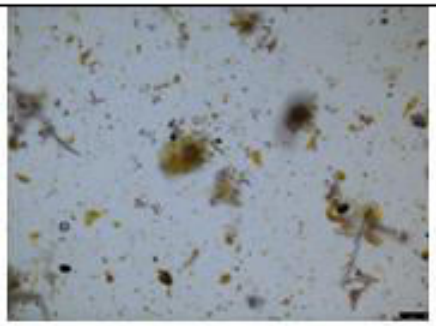
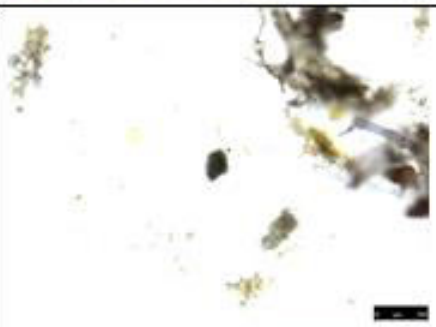
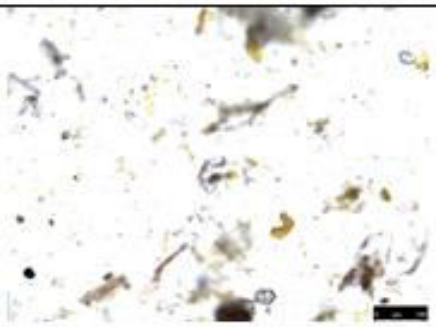
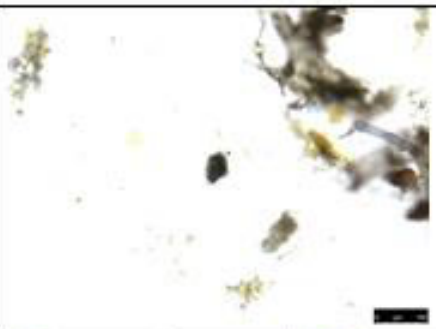
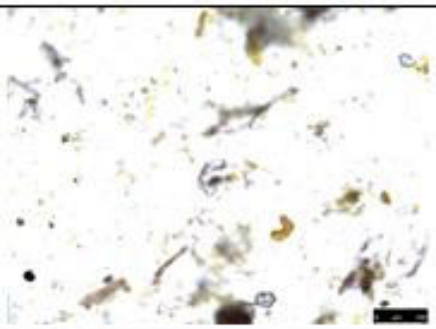
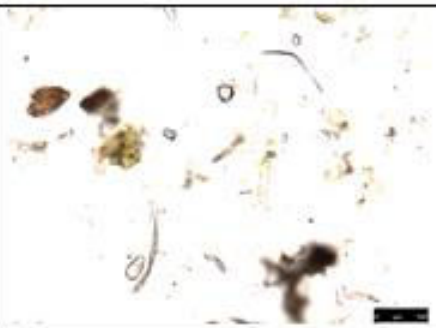
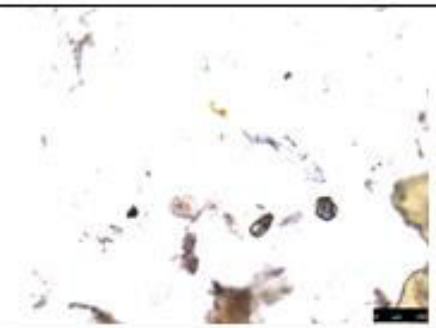

Extruded wheat based diet (Penibird-1)
Extruded wheat based diet (Penibird-2)
Upper jejunum
Lower jejunum
Upper ileum
Lower ileum

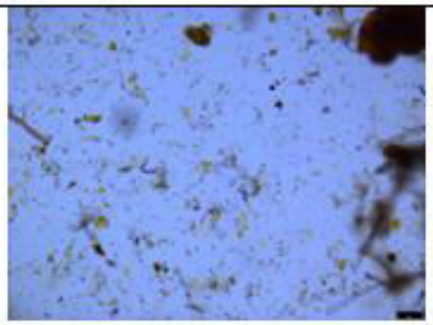
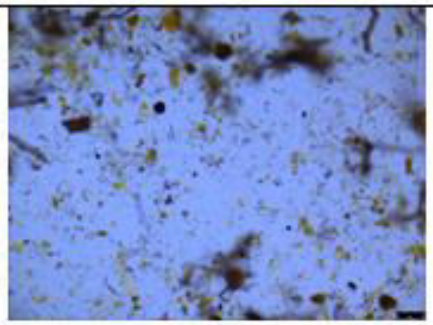
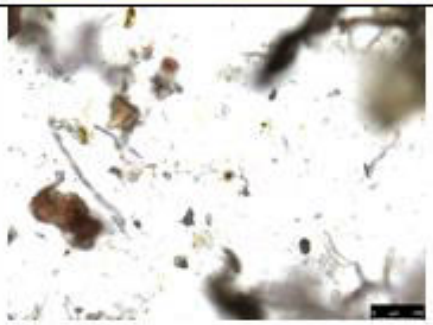
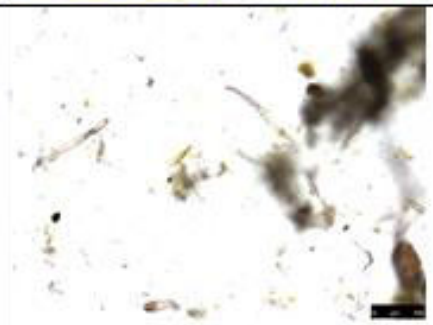
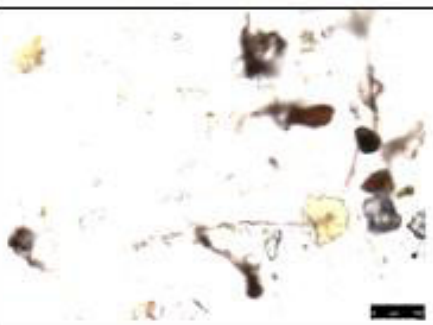
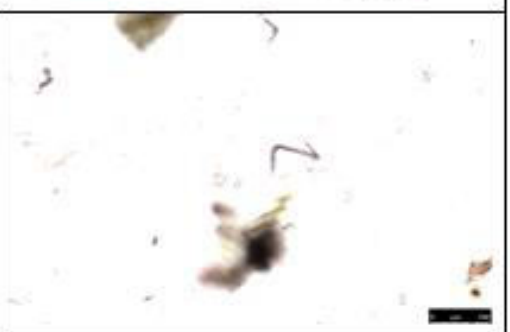
| | Extruded wheat based diet (Pen/bird-3) | Extruded wheat based diet (Pen/bird-4) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

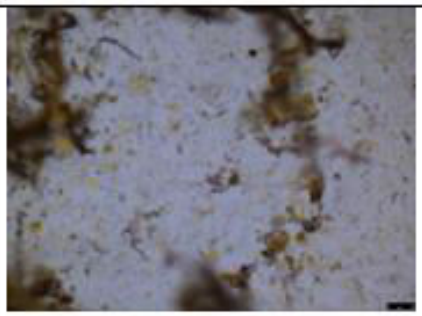
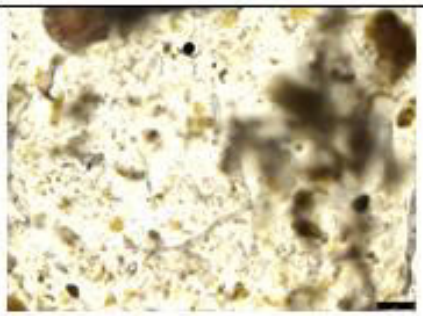
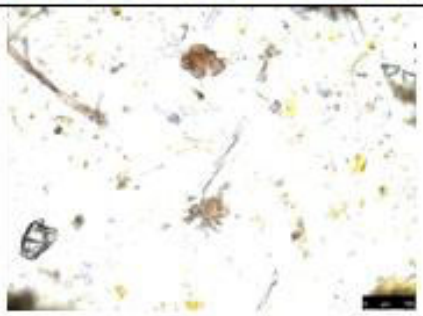
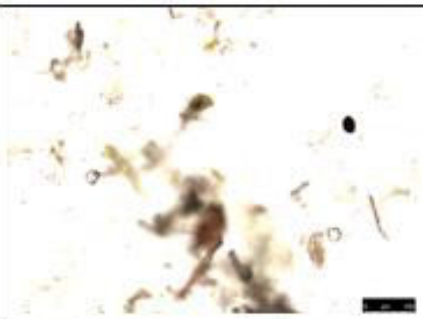
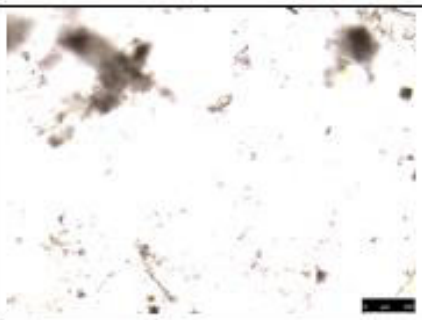
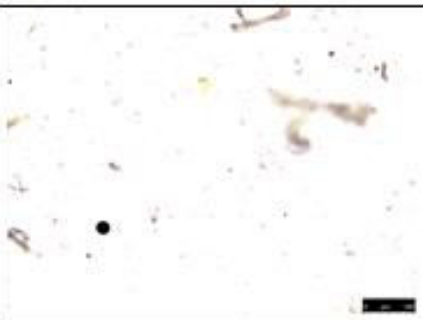
| | Extruded wheat based diet (Peri/bird-5) | Extruded wheat based diet (Peri/bird-6) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

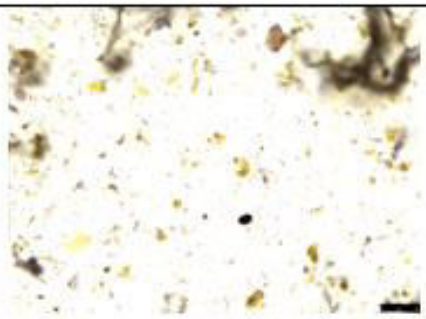
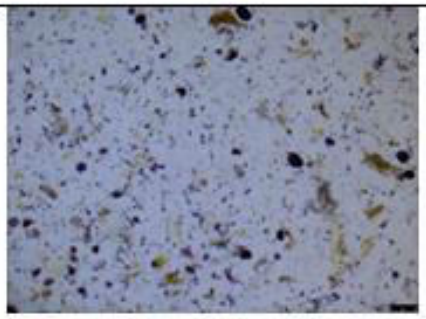
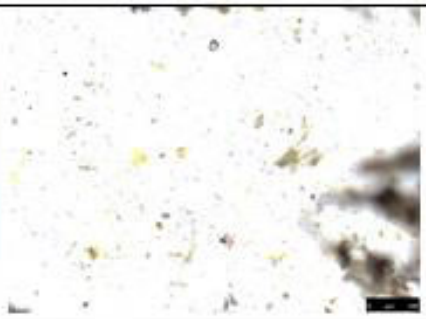
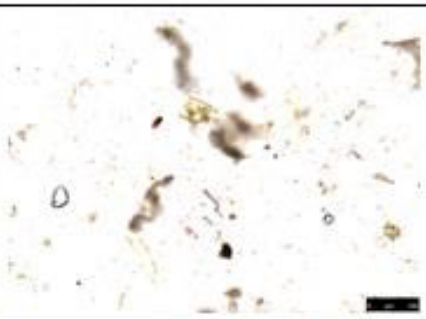
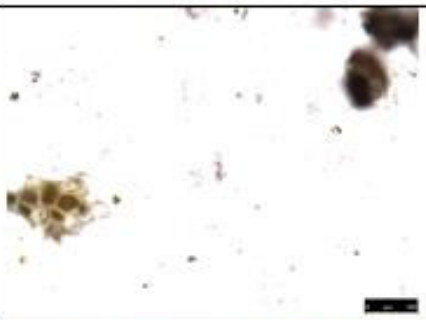
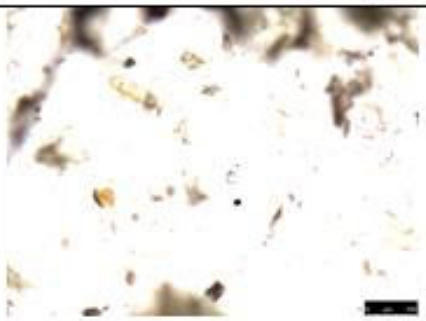
| | Extruded wheat based diet (Pen/bird-7) | Extruded wheat based diet (Pen/bird-8) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

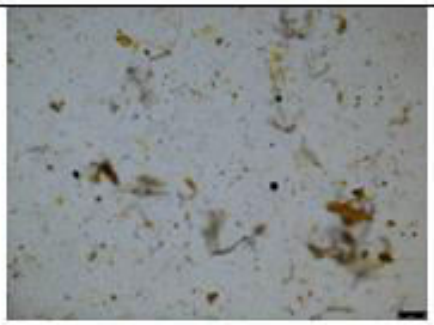
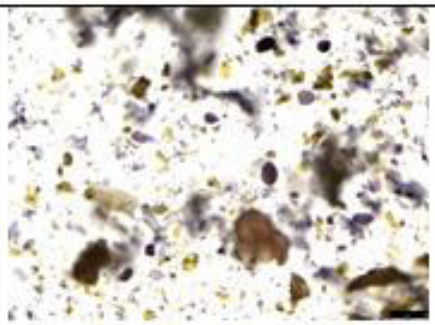
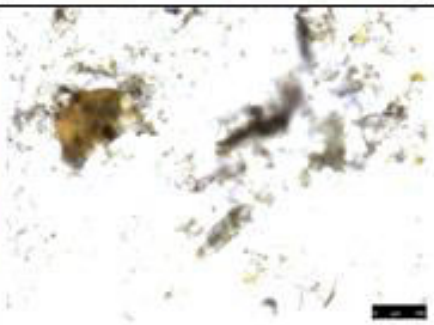
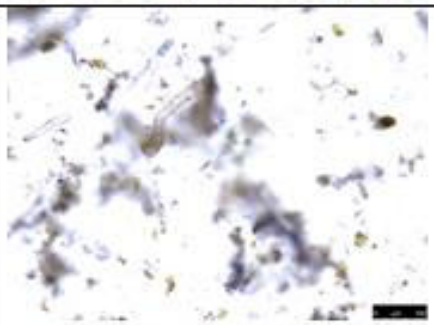
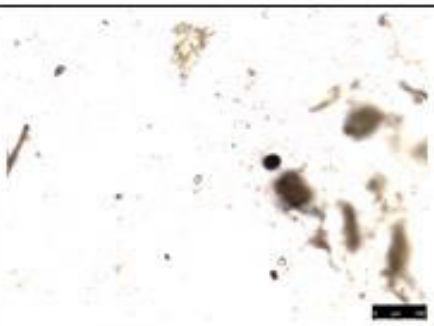
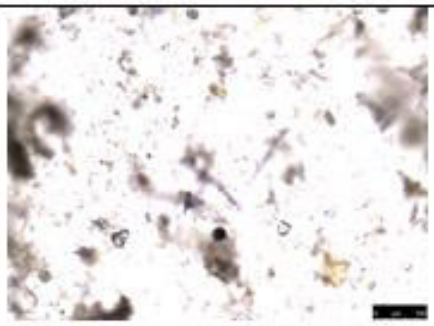
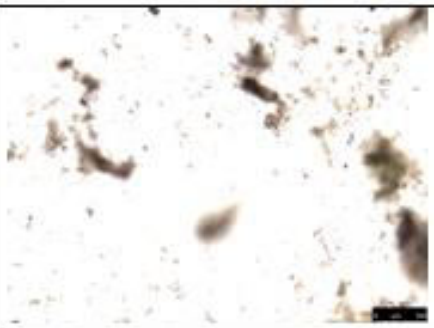
| | Extruded wheat based diet (Penfbird-9) | Extruded wheat based diet (Penfbird-10) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

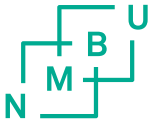
| | Extruded faba bean starch based diet (Pen/bird-1) | Extruded faba based diet (Pen/bird-2) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Extruded faba bean starch based diet (Penfbird-3) | Extruded faba based diet (Penfbird-4) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Extruded faba bean starch based diet (Peniaird-5) | Extruded faba based diet (Peniaird-6) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Extruded faba bean starch based diet (Penibird-7) | Extruded faba based diet (Penibird-8) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

| | Extruded faba bean starch based diet (Penbird-9) | Extruded faba based diet (Penbird-10) |
|---|---|---|
| Upper jejunum |  |  |
| Lower jejunum |  |  |
| Upper ileum |  |  |
| Lower ileum |  |  |

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