Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science

Digestibility of fungi-based protein products fed to broiler chickens

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Abstract

Two fungal protein sources (X and Y) grown using wheat-DDGS were used in broiler chicken diet to evaluate the apparent ileal digestibility coefficient (AIDC) of crude (CP) and the amino acids (AA) cysteine, lysine, methionine, threonine as well as the apparent digestibility coefficient (ADC) of dry matter (DM), organic matter (OM) and gross energy (GE). Furthermore, the apparent metabolizable energy (AME), digestible CP and digestible AA in the two the fungal biomasses were evaluated.

A total of 280 one-day old Ross 308 broiler chicks were obtained from a local hatchery and randomly allotted to 35 pens in a closed house with controlled climate. Birds were fed a commercial crumbled organic starter diet from day 1 to 10, which was followed by a pelleted growing diet from day 11 to 28. Day 28-35 the chickens were fed experimental diets. A total of seven experimental diets were formulated by replacing 0 %, 10 %, 20 % and 30 % of the finisher diet with one of the two fungi-based potential feedstuffs (X or Y). Each experimental diet was randomly allotted to 5 pens. An indigestible marker, TiO_2 was supplemented to each diet at a level of 5g/kg to enable digestibility calculation. Body weight and feed intake were registered during the experiment. Faecal samples were collected on days 33 and 34 and ileal digesta samples were collected on day 35. Litter quality was assessed on day 33.

Results showed that AIDCs of CP, cystine, lysine, methionine and threonine in the fungal biomass X were 0.74, 0.68, 0.61, 0.70 and 0.67 respectively and in the fungal biomass Y they were 0.72, 0.60, 0.72, 0.75 and 0.70 respectively. The ADCs of DM, OM and GE in the fungal biomass X were 0.94, 0.94 and 0.78 respectively whereas these values were 0.89, 0.91 and 0.66 respectively in the fungal biomass Y. Digestible CP ($g\ kg^{-1}$ as is) in X and Y were 251 and 342 respectively. In the fungal biomass X, digestible cystine, lysine, methionine and threonine were 4.90, 5.01, 4.07 and 7.33 respectively and in Y corresponding values were 4.81, 11.79, 6.39 and 11.69 respectively. The calculated AME in the fungal biomasses X and Y was 14.7 and 16.1 MJ/kg DM respectively. Litter quality was good in all pens and no foot pad lesions were observed even for pens with high inclusion level of X and Y. Our findings suggest that both X and Y are potential sources of protein with good nutritional value and AME in broiler chicken diets.

Keywords: Broiler, alternative protein sources, ileal digestibility, fungi, wheat, DDGS.

Sammanfattning

I detta kycklingförsök utvärderades två svampbaserade proteinfodermedel (X och Y) som odlats på vetedrank med avseende skenbar ileal smältbarhetskoefficient (AIDC) av råprotein (RP), och aminosyrorna cystein, lysin, metionin och treonin samt skenbar smältbarhetskoefficient (ADC) av torrsubstans (TS), organisk substans (OS) och bruttoenergi (BE), dessutom beräknades den skenbart omsättbara energin (AME) samt smältbart RP och aminosyror.

I försöket ingick 280 kycklingar (Ross 308) som kom från ett lokalt kläckeri. Kycklingarna sattes in i ett klimatkontrollerat stall som daggamla och fördelades på 35 grupper med 8 djur per grupp. Kycklingarna utfodrades med ett kommersiellt ekologisk startfoder från dag 0-10, ett pelletrerat tillväxtfoder mellan dag 10-28. Dag 28-35 fick kycklingarna försöksfoder. Totalt 7 försöksfoder tillverkades genom att byta ut 0, 10, 20 och 30% av tillväxtfodret mot svampråvara X eller Y, varje foder gavs till 5 grupper. En markör (Titaniumdioxid) tillsattes (5 g/kg) till samtliga försöksfoder och användes för smältbarhetsberäkningar. Träckprov samlades under dag 33 och 34 och ileala digesta prover togs dag 35. Ströbäddsbedömningar utfördes dag 33.

Resultaten visade att AIDC av RP, cystein, lysin, metionin och treonin i svampprodukt X var 0.74, 0.68, 0.61, 0.70 och 0.67. Motsvarande värden för produkt Y var 0.72, 0.60, 0.72, 0.75 och 0.70. ADC av TS, OS, och BE i produkt X var 0.94, 0.94 och 0.78, och motsvarande värden för produkt Y var 0.89, 0.91 and 0.66. Mängden smältbart RP (g kg^{-1}) var 251 och 342 i X respektive Y. I produkt X var mängden (g kg^{-1}) smältbart cystein, lysin, metionin och treonin 4.90, 5.01, 4.07 och 7.33 och för produkt Y var motsvarande värden 4.81, 11.79, 6.39 och 11.69. AME i produkt X och Y beräknades till 14.7 respektive 16.1 MJ/kg TS. Ströbäddskvaliteten bedömdes god i samtliga grupper och inga fotskador observerades. Våra resultat indikerar att både produkt X och Y är proteinfodermedel med högt energiinnehåll och bra näringsvärden för slaktkycklingar, och har därför potential att användas som ingredienser i slaktkycklingfoder.

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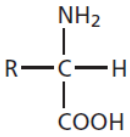
1 Introduction

Farm animals such as broiler chickens are in need for a daily amount of macro nutrients to provide fuel for metabolism. Carbohydrates, proteins and fats are the macro nutrients which are digested in the alimentary tract into smaller molecules before they are absorbed. However, micro nutrients such as vitamins and minerals are as important as macro nutrients and are essential for biological process in the body. Energy and protein in any feedstuff are given the most attention in feed evaluation systems. They are the key molecules for functions related to maintenance and production (Weurding, 2002)

The word "protein" is a derivative from the Greek word *proteios* which means of primary importance (McNab and Boorman, 2002). Proteins are composite organic compounds with high molecular weight containing carbon, hydrogen, nitrogen and generally sulfur, and are found in each living cell. Consisting of one or more long chains of polypeptides, proteins are polymers of amino acids. While over 200 amino acids have been recognized; only 20 amino acids are found as compounds of proteins (McDonald et al., 2011). Proteins differ from each other in amino acid sequence (the so called primary structure) and in how these amino acids are connected to each other (the so called secondary and tertiary structure). From a nutritional viewpoint, protein quality is distinguished as amino acids content (McNab and Boorman, 2002). Generally, amino acids have an amino group (–NH2), and an acidic carboxyl unit (–COOH) in their structure. In nature, most proteins consist of amino acids of the α-type and have the following general formula (McDonald et al., 2011):



Dietary protein has a basic function of providing sufficient amounts of required amino acids. Therefore, the quality of a feedstuff protein depends on both nitrogen content, the component amino acids and digestibility i.e. the use of specific amino acids after digestion and minimal unavoidable rates of oxidation (Ravindran and Bryden, 1999)

Using optimum levels of dietary protein or amino acids in poultry feed is vital for not only but most importantly the following reasons: (i) amino acids are critical nutrients for meat-type and layers, (ii) prices for protein concentrates are usually high and more expensive than energy feedstuff, (iii) the optimal use of amino acids will reduce the amount of nitrogen liberated into the environment by minimize the excretion of wastes containing nitrogen (McNab and Boorman, 2002).

At present, there is a greater than ever interest for introducing new ingredients in farm animals' nutrition that do not vie with human nutrition. The increasing human population and the changing in consumption patterns towards products of animal origin such as meat and milk result in increasing demand of animal feed. One of the potential new sources is microbial

biomass, which can be used as a substitute for traditional protein sources (van Kuijk et al., 2015).

Due to environmental concerns resulted from high level of pollution in the last decades, and the probable decline of global oil production in the near future, renewable sources for energy could be a potential alternative for fossil energy sources (Sarkar et al., 2012). by far, ethanol is the most common sustainable fuel that can substitute fuels derived from petroleum (Gray et al., 2006).

2 Literature review

2.1 Evaluation of potential feed sources

Energy and protein in any feedstuff are given the most attention in feed evaluation systems. They are the key molecules for functions related to maintenance and production (Weurding, 2002). The proximate analysis for any feedstuff gives a sequential knowledge of the potential nutrients but it does not measure the response of the animal. Therefore, most of the chemical analysis of the feedstuffs should be supported by biological tests in order to gain information of the bioavailability of the nutrients when fed to the animal (Leeson and Summers, 2001).

2.1.1 Measuring the digestibility

Digestibility of a feedstuff is defined as the amount that is not voided in the faeces and assumingly is absorbed by the animal. Digestibility is expressed as coefficient or percentage of dry matter (DM). In any digestibility trial, a known amount of the feedstuff under investigation is fed to the animal and faecal output is collected and measured (McDonald et al., 2011). The measuring of digestibility in poultry is somehow limited due to the fact that urine and faeces are excreted together from the cloaca and thus, the undigested nitrogen of the feedstuff is not separated from the metabolic nitrogen in the urine (Heuser, 1989).

2.1.2 Energy value of feedstuff

When describing energy, different measurements are used such as: gross energy (GE), digestible energy, apparent metabolizable energy and true metabolizable energy (Leeson and Summers, 2001). The correlation between these measurements is presented in figure 1. For poultry, apparent metabolizable energy (AME or ME) is mainly used to measure energy availability in feedstuffs and diets, and since feces and urine are excreted together via the cloaca, measuring the apparent digestible energy is not easily applicable without surgical intervention (NCR, 1994). If the retention of nitrogen is measured, the value of AME will be adjusted to a calculation of N equilibrium basis getting the so called nitrogen-corrected AME (AMEn or MEn) (Leeson and Summers, 2001; Sibbald, 1980). AMEn can be measured indirectly using equations that predict the AMEn from either physical or chemical parameters or both, including DM, GE, crude protein, crude fibre, ether extract, soluble sugars, starch, and tannin (for example in sorghum, the AMEn is calculated using the following equation: AMEn= 16.13g DM – 165.1g tannin) (Janssen, 1989; Sibbald, 1975). The three main methods to measure AMEn are: regression lines: when there are enough data allowing multiple regression (AMEn in barley= 9.258 DM – 9.258 ash + 6.810 starch), equation lines: when the data are not enough to carry out regression analysis or when the regression analysis is indecisive (AMEn in Cottonseed prods= 8.898 DM +19.72 crude fat -25.47 crude fibre), and digestibility coefficients: when data are not adequate for statistical analysis (Janssen, 1989). Although the latter assay is rapid, the predicted AMEn is usually overestimated due to inherent errors (Sibbald, 1980). One more indirect assay is feeding the chicken the tested ingredient and comparing their growth with a growth curve of chicks fed different levels of a compound of known AME content (Squibb, 1971). Growth assays, however, give variable data even if the

experimental conditions were stringent (Sibbald, 1980). Measuring AME can also be conducted using direct bioassays which involve feeding a diet for an acclimatization period followed an assay period (three or four days) where the excreta is collected. The difference in GE between the ingested feed and the excreta derived from this feed is equal to the AME (Sibbald, 1980).

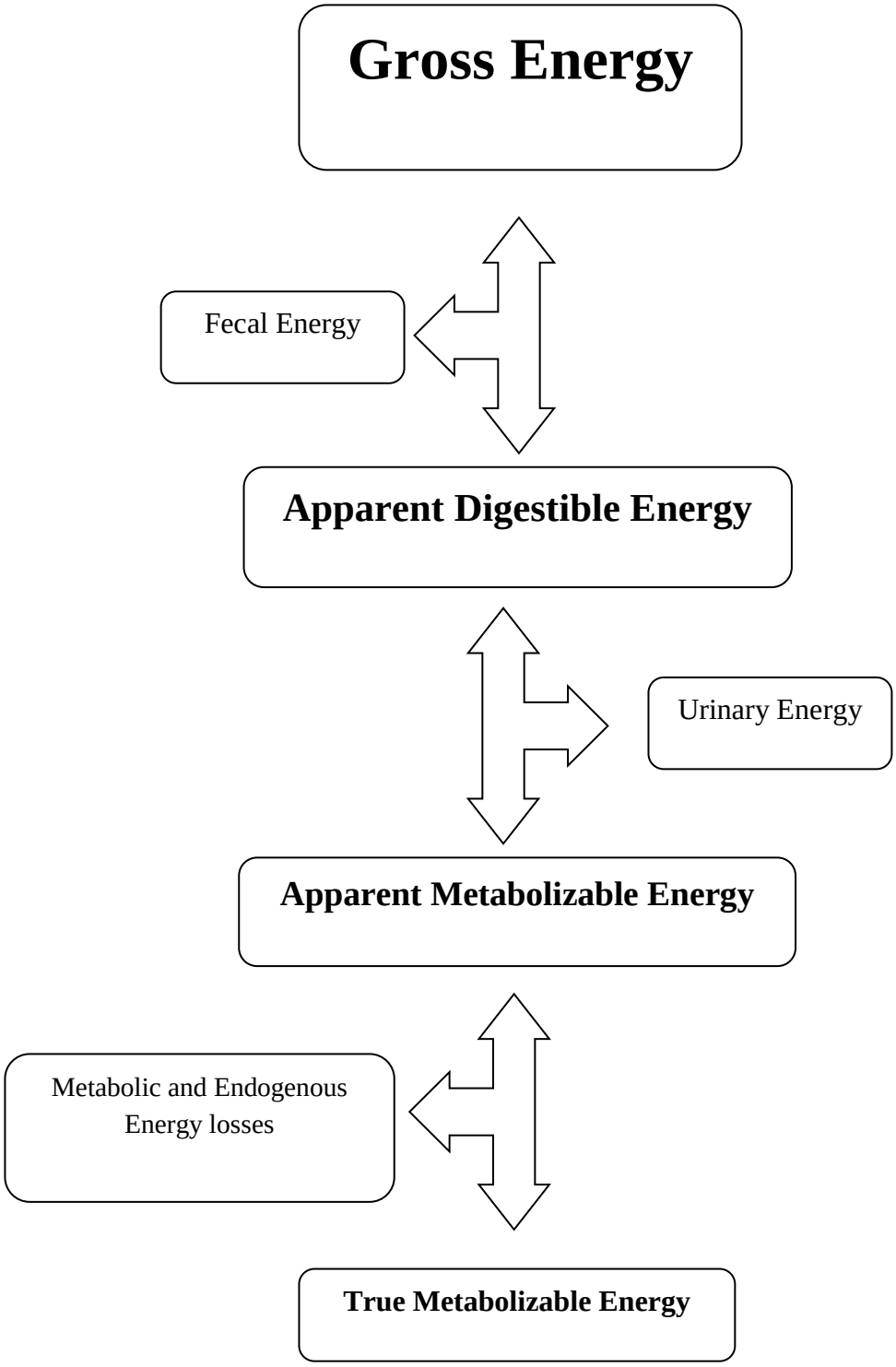


Figure 1.The partition of ingested energy in poultry

The most common technique used for measuring AME for new feedstuffs is a substitution by which a portion of basal diet is replaced by the new ingredient and then the AME is calculated by simultaneous linear regression (Larbier and Leclercq, 1994). Another method is to include the new feedstuff at different levels to extrapolate to an inclusion level of 100% (Potter et al., 1960). Despite the high reliability of these bioassays, a precise measuring for the feed consumption and total collection of the voided excreta can be difficult i.e. feed spillage and excreta contamination with feed (Sibbald, 1980). To preclude errors associated with imprecise measurements of ingested feed and excreta output, the use of inert markers such as insoluble ash, chromic oxide and titanium dioxide have been adapted (Scott and Boldaji, 1997; Short et al., 1996; Sibbald, 1980). The inert marker is incorporated in the feed and the AME or the digestibility is calculated by the ratio of the inert marker in both feed and excreta (Sales and Janssens, 2003).

2.1.3 Crude protein and amino acids

Amino acid digestibility assays have become the most preferential technique used by nutritionist to estimate amino acid availability. These assays are divided into two categories: excreta digestibility by which the collection of excreta from either intact or caecectomized birds is carried out, and ileal digestibility where the digesta from the ileum (the distal part of small intestine) is collected (Hoehler et al., 2005; Ravindran et al., 2005; Ravindran and Bryden, 1999). However, excreta digestibility assays are criticized for many reasons such as not separating amino acids in faeces from amino acids in urine, ignoring the contribution of hindgut microflora protein, using adult cockerels which are physiologically different from growing poultry and impairing animal welfare. Birds were force fed with a defined amount of test feedstuff by placing the it directly into the crop and then the birds were fasted to get sure that all the undigested feed are voided and the excreta were then collected on the assumption that all undigested components have been excreted (Hoehler et al., 2005; McNab and Boorman, 2002; Parsons, 1986). Using ileal digestibility assays could be the solution to avoid the drawbacks of excreta digestibility (Ravindran and Bryden, 1999). When using ileal digestibility assays an inert marker is required to calculate the ratio of amino acids between the diet and ileal digesta (Ravindran and Bryden, 1999).

2.2 The Apparent ileal digestibility of amino acids

The use of ileal digestibility of amino acids has been adapted to measure the nutritional value of protein in feedstuff (Ravindran and Bryden, 1999). Amino acids which can be utilized by the animal after digestion and absorption are named the bioavailable amino acids. In some instances, an amino acid (e.g. lysine in heat-treated feedstuff) could be absorbed in a form that is unavailable to the animal and it is lucid that undigested amino acids do not contribute to the animal but there is no method that can directly measure the bioavailability of amino acids (Moughan and Rutherfurd, 1996; Ravindran et al., 2005; Stein et al., 2007). The apparent ileal digestibility (AID) of amino acids can be defined as the net amount of fed amino acids that had disappeared from the alimentary tract at the proximal end of the distal ileum and ‘apparent’ as a word is used to emphasis on the amino acids from endogenous origin (Stein et al., 2007).

2.3 What is Distillers Dried grain with Solubles (DDGS)?

There is an increasing interest in ethanol production in Europe, and ethanol is now produced on an industrial scale by enzymatic breakdown of grain starch to glucose and a subsequent fermentation of the latter to ethanol. DDGS is the main by-product from this industry and in Europe mainly wheat-DDGS is produced (maize-DDGS in North America) (Cozannet et al., 2011). After grain fermentation and ethanol distillation, the whole stillage "which is in a form of slurry" will contain many nutritional compounds such as oil, protein, fibre, and the other unfermented compounds in addition to yeast cells (Kim et al., 2008). The amount of whole stillage produced per one liter of ethanol was estimated to be 20 liters resulting in more than a billion tons per year on global production basis (Davis et al., 2005). Centrifuging of the whole stillage results in a liquid fraction called thin stillage, and after a series of evaporations of the thin stillage, a syrup is produced which is dried to form the DDGS (Kim et al., 2008). A scheme for bio-ethanol production is shown in Figure 2. About 333 kg of DDGS are produced per 1000 kg grains used for bio-ethanol production (Bátori et al., 2015).

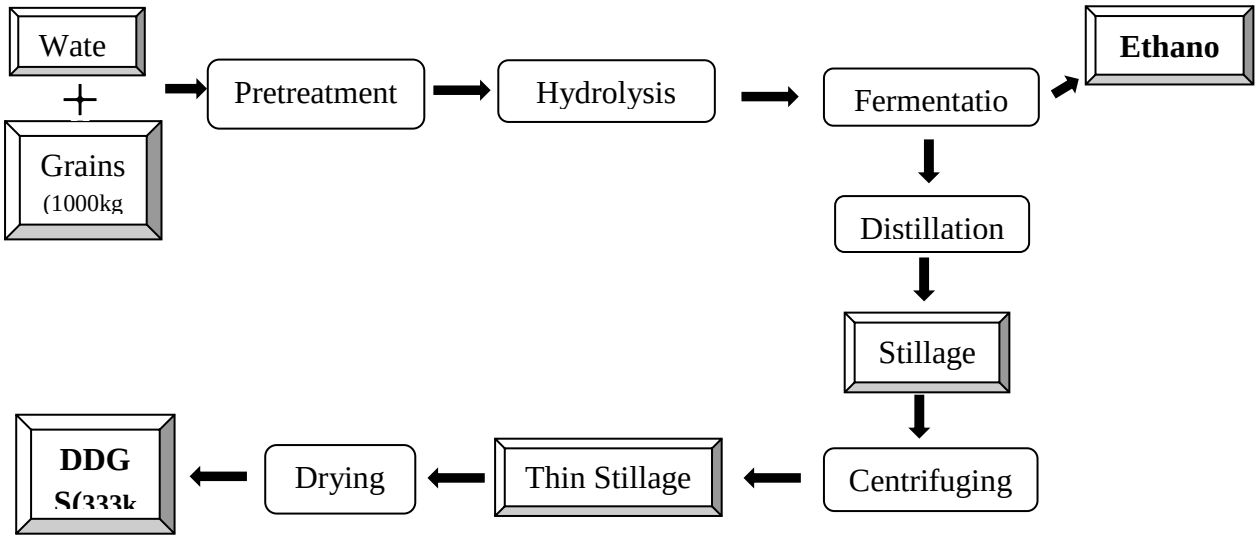


Figure 2*: A scheme for bio-ethanol production*

2.3.1 Composition and Chemical characteristics of wheat-DDGS

Since ethanol production depends mainly on the extracting, hydrolysing and fermenting of the grain's starch, it can be assumed that wheat-DDGS is in somehow equal to non-starch fractions of the grain. The wheat-DDGS composition depends principally on the composition of the grain. Thus, all the nutrients except for starch are expected to be around three fold more in wheat-DDGS than those in original wheat grains (Nyachoti et al., 2005). However, the chemical composition of wheat-DDGS is usually more variable than original grains (Noblet et

al., 2012) and differs among ethanol plants due to the grain processing methods i.e. previous dehulling, the process of fermentation, the quantity of solubles mixed with distillers grain, the drying temperature and duration and other possible separation (Belyea et al., 2004).

2.3.2 Energy value

As described before and due to the conversion of the starch fraction of the grain to ethanol, the concentrations of crude protein, amino acids, ether extract, crude fibre and minerals are tripled in the wheat-DDGS (Cozannet et al., 2010b).

Different values for GE of wheat-DDGS were reported. Nyachoti et al. (2005) reported that wheat-DDGS has a GE of 20.5 MJ/kg compared to wheat which has a GE equal to 16.9 MJ/kg. Comparably, Cozannet et al., (2010a) reported GE of 20.83 kg DM in wheat-DDGS. In a study by Thacker and Widyaratne, (2007), the GE of wheat-DDGS was 19.8 MJ/kg while it was 18.6 MJ/kg in wheat grain. The variability in calculated GE could be partially attributed to residual sugars in wheat-DDGS which differ among ethanol plants by the virtue of process used for grains (Noblet et al., 2012). Color of the wheat-DDGS could be an index for the digestible energy in monogastric animals and can be assessed by lighting score (L*) using a Minolta colorimeter that differentiates between dark (L* <50) and light products (L* >52). It was reported that AME of dark and light DDGS is equal to 9.45 and 10.55 respectively and the AMEn can be predicted by the lightening score (R^2 = 0.77). The lower AME of the dark samples was attributed to Maillard reactions due to overheating when drying the DDGS (Cozannet et al., 2010b).

2.3.3 Crude Protein and Amino Acids Composition

Cozannet et al. (2011) studied 19 samples of wheat-DDGS obtained from different ethanol plants in Europe. The results showed that crude protein (CP) content varies from 327 to 392 g/kg DM between the samples. More pronounced variation in the amino acid (AA) profile was found where levels of lysine and arginine in CP range from 0.83 to 3.01 % and 2.25 to 4.26% respectively, whereas the content of other AA was less variable. As a matter of fact, there are many factors which would lead to variation in CP and AA contents among sources. They include, but not limited to, adding of nitrogenous non-protein substances such as enzymes during ethanol process, temperature and duration of drying, yeast's AA contribution to the total AA together with the analytical assays utilized for estimating the chemical composition of DDGS (Kim et al., 2008). The apparent ileal digestibility of AA was negatively correlated to L* value, as the in case of AME, and these values were small and variable (Cozannet et al., 2010b).

2.3.4 Mineral Composition

As for other components, minerals in wheat-DDGS are generally three folds in comparison to wheat grain. However, this is specifically true in the case of potassium, calcium and phosphorus. For sodium and sulphur, the content in wheat-DDGS is more than expected (more than three folds) (Table 1). These higher contents could be explained by the use of sodium hydroxide (NaOH) and sulphuric acid (H_2SO_4) during the process of bio-ethanol (Noblet et al.,

2012). In wheat grain, phosphorus exists in the indigestible form of phytic phosphorus. However, wheat-DDGS has the possibility to be a source of phosphorus in poultry diets due to the fact that considerable amounts of phytic phosphorus is hydrolyzed by yeast phytase during the process of bio-ethanol production (Liu, 2011).

Table 1. *Mineral composition of wheat dried distiller's grain with solubles (Wheat-DDGS) and comparison with wheat (g kg -1 DM). Adapted from (Noblet et al., 2012)*

Mineral	Wheat	Wheat-DDGS
Sodium	0.1	3.6
Sulphur	1.7	6.5
Potassium	4.6	10.7
Calcium	0.8	2.2
Magnesium	1.2	2.9
Total Phosphorus	3.7	8.6

In pigs, wheat-DDGS phosphorus digestibility ranged between 50 to 62% (Nyachoti et al., 2005; Widyaratne and Zijlstra, 2007; Yanez et al., 2011). In a study by Adebiyi and Olukosi, (2015a) the phosphorus digestibility of wheat-DDGS in broilers was determined, it was found that about 95% of phosphorus in wheat-DDGS is available suggesting that wheat-DDGS is a conceivable source for phosphorous in poultry diets.

2.3.5 Non starch polysaccharides

Carbohydrates in plants can be divided into disaccharides, oligosaccharides and polysaccharides with the latter comprising starches and non-starch polysaccharides (NSP) (Bach Knudsen et al., 2012). According to their water solubility, NSP can be divided into soluble and insoluble (Bach Knudsen, 2001). The principle components of NSP are cellulose, (insoluble) arabinoxylans and β-glucans (soluble) (Cummings and Stephen, 2007).

Wheat grain contains arabinoxylans (5-8%), β-glucans (up to 1%) and cellulose (2-3%) (Choct et al., 2004). Although NSP decaying enzymes are used through ethanol process to enhance starch fermentation adequacy, NSP levels in DDGS increase threefold in comparison to the original grains (Widyaratne and Zijlstra, 2007).

It was noted that the main factor that limits DDGS inclusion at high levels in poultry diets is the adverse effect of NSP (Thacker and Widyaratne, 2007; Wang et al., 2007 a,b,c, 2008). Poultry fed diets containing high levels of NSP are more vulnerable to enteric disease such as necrotic enteritis (Kaldhusdal and Skjerve, 1996). In addition, NSP was reported to increase population of pathogenic bacteria in the gut at the expense of beneficial ones. Moreover, NSP was reported to increase moisture content if the faeces and cause wet litter (Bedford, 2006). Water-soluble NSP tend to form a gel-like medium that increases digesta viscosity and consequently slows down the digesta transition through the gastrointestinal tract and reduces nutrients absorption due to the physical separation between the nutrients and other enzymes within the gel-like medium (Adeola and Cowieson, 2011; Choct et al., 2004).

2.4 Improving the nutritional value of wheat-DDGS

2.4.1 The use of exogenous enzymes

Among enzymes used for monogastric animals, two categories of enzymes can be broadly recognized in the market: phytases and carbohydrases. In the latter category the two main enzymes are xylanases and glucanases. Phytase was found to improve phosphorus bioavailability in poultry diets containing wheat, corn and soybean (Martinez-Amezcua et al., 2006). Xylanases are used in wheat-based diet to reduce the disadvantageous effect of NSP (especially arabinoxylans) on nutrient utilization and production performance (Choct et al., 2004). In addition to phytases and carbohydrases, proteases are also available in the market (Adeola and Cowieson, 2011). Proteases were reported to enhance protein utilization by poultry (Adeola and Cowieson, 2011).

A series of experiments were done by Adebiyi and Olukosi, (2015a, b, c) to study the effect of adding different exogenous enzymes to diets containing wheat-DDGS fed to broiler chicken. It was found that adding protease to diets containing wheat-DDGS improved the apparent ileal digestibility of a wide range of amino acids (Table 2) (Adebiyi and Olukosi, 2015b). However, the AID of wheat-DDGS in the literature is rare and is not consistent and that could be attributed to different procedures between different plants producing ethanol that result in differences in chemical properties of DDGS (Adebiyi and Olukosi, 2015b; Cozannet et al., 2011; Matsuo, 2006). Results from two studies are shown in Table 2. On the other hand and irrespective of addition of phytase, the true ileal digestibility of phosphorus was about 0.95 (Adebiyi and Olukosi, 2015a). For both AME and AMEn, adding a mixture of enzymes (xylanase, amylase, and protease) tended to improve the diet AME and AMEn ($P < 0.10$) (Adebiyi and Olukosi, 2015b).

2.4.2 The use of microorganisms

Another way to improve the nutritional value of wheat-DDGS rather than adding exogenous enzymes is the use of microorganisms such as edible filamentous fungi to produce fungal biomass that is rich in protein with high nutritional value (Lennartsson et al., 2014). Filamentous fungi are famous for their ability to secret a wide range of enzymes empowering them to assimilate many complex substrates (Bátori et al., 2015). This is one reason why some of the filamentous fungi namely *Neurospora intermedia* and *Aspergillus oryzae* have been utilized to produce fermented food and enzymes used for the industries of feed, textile, beverage and paper and bulb (de Vries and Visser, 2001; Nout and Aidoo, 2002).

2.4.2.1 Producing protein-rich fungal biomass using ethanol by-products

A novel method to produce ethanol and protein-rich biomass from wheat-DDGS using *Neurospora intermedia* and *Aspergillus oryzae*, *respectively*, was investigated by Bátori et al. (2015). Their findings suggested that the use of this novel method in a typical ethanol plant that produces 200,000 m^3 ethanol/year can lead to a production of 12000 tons of fungal protein-rich biomass that can be used for animal nutrition and 44,000 m^3 of ethanol (22% improvement).

Table 2. *Apparent ileal digestibility coefficients of crude protein and amino acids in wheat-DDGS. Adapted from Adebiyi and Olukosi, (2015b) and Bandegan et al., (2009).*

	(Adebiyi and Olukosi, 2015b)		(Bandegan et al., 2009)
	Without protease	With protease	Without protease
Crude protein	0.49	0.60	0.67
Indispensable Amino acids			
Arginine	0.38	0.53	0.68
Histidine	0.52	0.56	0.64
Isoleucine	0.44	0.53	0.69
Leucine	0.5	0.59	0.74
Lysine	-0.28	0.02	0.36
Methionine	0.37	0.49	0.74
Phenylalanine	0.56	0.65	0.79
Threonine	0.37	0.42	0.55
Valine	0.44	0.54	0.65
Dispensable amino acids			
Alanine	0.35	0.45	0.61
Aspartate	0.34	0.31	0.43
Cystine	0.47	0.53	0.62
Glutamic acid	0.75	0.79	0.83
Glycine	0.49	0.48	0.57
Serine	0.54	0.65	0.66
Tyrosine	0.45	0.54	-

Lantmännen Agroetanol is the only large-scale producer of grain-based fuel ethanol in Sweden with a mission to produce ethanol and protein in a profitable and sustainable way. Based on 600 thousand tons of grain, 230 thousand m^3 of ethanol and 200 thousand tons of protein feed is produced every year (Lantmännen Agroethanol, 2016). Cultivation of *Neurospora intermedia* on industrial scale is now being tested at Lantmännen Agroethanol and two fungal biomasses have been produced: X and Y. The potential feedstuff Y has higher levels of protein lysine, methionine and threonine than X (Table 3). X is produced using two processed streams, a wet one containing *Neurospora intermedia* and a dry one consists of grain. On the other hand, Y is produced using only the wet stream. The expected production of the fungal biomass Y is 15 times less in comparison to the fungal biomass X using the same amount of the DDGS. The calculated approximate production capacity of X is 3000,000 tons/year while for Y is 200,000 tons/year. The nutritional composition of the two fungal biomasses used in this study is present in Table 3.

2.5 Fungi and their role in broiler nutrition

Fungi (fungus: singular) are one of the most abundant microorganisms in nature, but they are generally overlooked, underappreciated and sometimes misunderstood. The sudden appearance and disappearance, the connection with decomposing organic matter and the fantastic colors and shapes make the fungi to be considered a mystery. Four phylum have been

considered as true fungi in the kingdom of Fungi: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota. The phylum Ascomycota (or the ascomycetes) is the largest phylum and three subphyla are recognized for this phylum: Pezizomycotina, Saccharomycotina and Taphrinomycotina. Both *Aspergillus oryzae* and *Neurospora intermedia* fungi occur in the subphylum Pezizomycotina while *Saccharomyces cerevisiae* belongs to the subphylum Saccharomycotina (Stephenson, 2010).

"Single cell protein" (SCP) is a term that alludes to the dried cells of microorganism origin such as yeasts, bacteria, microalgae, and fungi which are grown in large-scale culture methods in order to be used as sources of protein in both human foods and animals feeds. Other nomenclatures such as "microbial biomass" and "microbial biomass protein," indicate the same meaning. Protein of these sources can be either directly consumed as a part of the cell itself, principally in animal feed, or processed into other products (fibers or meatlike products) for human consumption (Kuhad et al., 1997).

2.5.1 *Saccharomyces cerevisiae*

It was first reported by Eckles and Williams (1925) that *Saccharomyces cerevisiae* has a growth promoting effect in ruminants and nowadays commercial yeast products are used for animal feeding especially in ruminants diets (Gao et al., 2008). However, using *S.cerevisiae* at high inclusion levels in monogastric animals is limited due to the fact that *S.cerevisiae* has high levels of nucleic acids and low digestibility of cell wall (Alvarez and Enriquez, 1988). Consuming high levels of dietary nucleic acids in monogastics resulted in elevated plasma uric acid which consequently created toxicological effects and adversely influenced protein, fat, and carbohydrate metabolism (Rumsey et al., 1992). Results from studies using *S.cerevisiae* in broiler chicken nutrition are not consistent, and *S.cerevisiae* was used in these studies as an additive rather than a major ingredient of the diets (Gao et al., 2008; Madrigal et al., 1993; Onifade et al., 1998; Pizzolitto et al., 2013; Stanley et al., 2004; Yitbarek et al., 2013). Some studies reported that feeding yeast at levels of 1.5, 3.0 and 6.0 g/kg diet to broilers has positive effects on both body weight and feed conversation ratio (Onifade et al., 1998). However, Madrigal et al. (1993) did not find any positive effect of supplemented yeast at levels ranged from 5 to 20 g/kg diet on body weight of broilers. On the other hand, some studies reported that adding yeast to broiler chicken diets increases the body weight but does not affect feed conversation ratio (Kanat and Calialar 1996). Stanley et al. (2004) reported that yeast culture suppresses the pathogenic bacteria or increases the commensal microbes when adding 1g/kg diet. Gao et al. (2008) hypothesized that yeast culture in monogastrics has other effects rather than microbial ecology modulation and reported that it enhances the immune function and increases the digestibility of calcium and phosphorous when inclusion levels ranged from 0.2 to 7.5 g/kg diet. Furthermore, it has been reported that *S.cerevisiae* can be used as a mycotoxin adsorbent when added to feed (10^{10} cells/kg) or water (5×10^9 cells/L) or to both feed and water (Pizzolitto et al., 2013). Yitbarek et al. (2013) reported that diet supplemented with 2g/kg diet yeast products derived from *S.cerevisiae* (yeast derived macromolecules) positively affects the gut and reduces the mortality.

Table 3. *Proximate composition (g kg−1 as is), energy content (MJ kg−1 DM) and amino acid content (g kg−1 as is) of the fungal biomasses X and Y*

Chemical composion	Test ingredients	
	Fungal biomass X	Fungal biomass Y
Dry weight	941	939
Crude protein	340	474
Ash	23.3	32.2
Crude fat	73.5	146.3
Crude fibre	28	89
Total dietary fibre	131.2	179*
Water-soluble dietary fibre	20.0*	5.0*
Insoluble fibre	97*	174*
NDF	-	291
ADF	-	230
Calcium	0.91	0.5
Phosphorus	3.8	11
Gross energy	20.01	26.05
Indispensable Amino acids		
Arginine	13	20.9
Histidine	6.92	98.6
Isoleucine	12.9	18.4
Leucine	24.6	33.6
Lysine	8.17	16.6
Methionine	5.79	8.52
Phenylalanine	16.7	20.4
Threonine	10.9	16.8
Tryptophan	3.9	67.6
Valine	16	22.8
Dispensable amino acids		
Alanine	12.8	20.9
Aspartic acid	16.9	28.7
Cystine	7.18	7.99
Glutamic acid	95.8	92.2
Glycine	12.3	18.5
Proline	32.5	31.5
Serine	16.4	20.3
Tyrosine	10.3	17

**Values with asterisk were analysed from another batch of fungi.*
- not determined.

2.5.2 *Aspergillus* and *Neurospora*

Generally, filamentous fungi are characterized by their high ability to secret of proteins, enzymes and, high growth rates, easy to handle in large-scale production together with low-cost requirements for production in comparison to other microorganisms (Saleh et al., 2014).

To make traditional food derived from soybean, a fermentation process is used to produce fermented soy foods which are highly nutritious and digestible (Lee, 1998; Matsuo, 2006). *Aspergillus oryzae* is one of the fungi widely used for this fermentation process reducing trypsin inhibitor and decreasing the small-size peptide contents in soybean and soybean meal (Feng et al., 2007a; Hong et al., 2004).

Fermented soybean and soybean meal have been studies in poultry nutrition. The use of 10 cultures of *Aspergillus* to ferment full-fat soybean enhanced broiler growth and feed efficiency (Chah et al., 1975). Hirabayashi et al. (1998) reported that fermented soybean meal with *Aspergillus* improves weight gain and phosphorous retention in chicks. Likewise, it was reported that feeding fermented soybean meal with *Aspergillus* to broilers enhances the daily feed intake, daily body weight gain, the activity of the enzymes trypsin, lipase, and protease, and increases the villus height (Feng et al., 2007a, b). In addition, *Aspergillus oryzae* can be used to lessen the anti-nutritional effects of potential feedstuffs. Fermentation using *Aspergillus oryzae* and *Neurospora sitophila* of both Jatropha seed meal and jatropha seed cake could be one way to enhance their nutritional properties and to reduce the amount of toxins and anti-nutritive compounds (Kurniati, 2012; Wina et al., 2010).

2.5.3 Composition and Chemical Characteristics of Fungi

As previously explained, *S.cerevisiae* is not used as a source for protein in broilers diet and its inclusion is restricted as feed additives due to its high levels of nucleic acids and low digestibility of cell wall (Alvarez and Enriquez, 1988). Thus, information about the nutritional composition of the fungi is scarce. Yeasts such as *S.cerevisiae* contain 45 to 60% protein reliant on culture conditions with high levels of essential amino acids (their amino acid profile is similar to the one of fish meal) but their methionine content is lower than it is in bacteria (Langeland et al., 2016; Nasseri et al., 2011).

In general terms, fungi have a unique cell wall structure that differs from plant cell walls which are mainly comprised of cellulose. The main components of fungal cell walls are glycoproteins and polysaccharides (mainly glucan and chitin) that provide the fungal cell with mechanical resistance to endure the environmental changes in osmotic pressure (Bowman and Free, 2006).

The GE for intact and extracted *S.cerevisiae* was reported to be 19.9 and 18.1 MJ/kg DM with protein contents of 466 and 779 g/kg DM respectively (Table 4) (Vidakovic et al., 2015). The AMEn of *S.cerevisiae* in poultry is 8.3 MJ/kg DM (1990 kcal/kg) (NCR, 1994). Filamentous fungi like *Aspergillus oryzae* have lower protein content than yeasts and their amino acid profile is similar to fish meal (Langeland et al., 2016; Nasseri et al., 2011). Vidakovic et al. (2015) reported that the GE of *Aspergillus oryzae* is 21.6 MJ/kg DM and protein content of

497 g/kg DM (Table 4). Langeland et al. (2016) measured the ADC of GE and CP in *S.cerevisiae* (intact and extracted) and *Aspergillus oryzae* in Arctic charr and Eurasian perch. Results from this study are shown in Table 5.

Table 4. *Chemical composition (g kg^{-1} DM) and energy content (MJ kg^{-1} DM) of intact and extracted S. cerevisiae and Rhizopus oryzae. Adapted from Vidakovic et al. (2015).*

	Saccharomyces cerevisiae		*Rhizopus oryzae*	*Fish meal*
	intact	extracted		
Crude protein	466	779	479	745
Ether extract	10	2	94	127
Neutral detergent fibre	0	0	104	26
Gross energy	19.9	18.1	19.7	-
Indispensable amino acids				
Arginine	4.8	2.2	1.8	5.6
Histidine	2.2	1.3	1.5	2.4
Isoleucine	4.9	3.4	2.9	4.7
Leucine	6.9	4.7	3.8	7.6
Lysine	7.4	5	3.8	7.8
Methionine	2.1	1.7	1.7	3.0
Phenylalanine	4.1	2.7	2.1	4.1
Threonine	4.9	2.6	2.0	4.3
Valine	6.0	4.2	3.5	5.4
Sum	43.3	27.8	23.1	44.9

Table 5. *Apparent digestibility coefficient (ADC) of GE and CP of intact and extracted S. cerevisiae and Rhizopus oryzae. Adapted from Langeland et al. (2016)*

	Saccharomyces cerevisiae		*Rhizopus oryzae*
	intact	extracted	
ADC of GE in			
✓ Arctic charr	71	≥99	≥99
✓ Eurasian perch	80	96	88
ADC of CP in			
✓ Arctic charr	86	98	94
✓ Eurasian perch	90	98	89

3 Aim of the thesis

The aim of this thesis was to evaluate the two fungi-based protein ingredients X and Y in a digestibility trial in broiler chickens. As stated before, X and Y were derived the filamentous fungi *Neurospora intermedia*.

3.1 Hypothesis

The main hypotheses were:

i) The two fungi based protein products X and Y will have a higher digestibility than wheat-DDGS
ii) Differences in digestibility between these products will occur due to differences in protein, amino acids and fibre contents.
iii) Due to the fungal cell walls, high inclusion levels will increase excreta moisture and adversely affect litter quality.

4 Materials and methods

4.1 Ethical considerations

The experiment procedures were approved by the Ethical Committee of Uppsala region and in accordance with the regulations of Swedish animal welfare.

4.2 Birds and housing

The experiment was conducted between the 5th of April to 10th of May 2016, at the Livestock Research Centre of Swedish University of Agriculture Sciences (SLU) located in Funbo-Lövsta, Uppsala. A total of 280 one-day old Ross 308 broiler chicks were obtained from a local hatchery in Väderstad, Sweden. Birds were weighed in groups and allotted to 35 pens in a closed house with controlled climates. Each pen was equipped with three nipple drinkers and a feeder; wood shavings were used as bedding material. The house temperature was maintained at 33 ºC during the first three days and then gradually reduced until it reached 23 ºC on day 24. Then temperature was kept at 23 ºC until the end of the experiment. The birds received 24 hours of lighting on day one, which was then reduced by 1hour per day until total the illumination time was 19 hours.

4.3 Diets and experimental design

Feed and water were provided *ad libitum*. Birds were given a commercial crumbled organic starter diet from day 1 to 10 that did not include coccidiostats, which was followed by a pelleted growing diet from day 11 to 28. On day 28, seven experimental diets were formulated by replacing 0 %, 10 %, 20 % and 30 % of the growing diet with one of the two fungi-based feedstuffs (X or Y). The composition of the grower diet according to the manufacturer is presented in Table 6. As an indigestible marker, TiO_2 was supplemented to each diet at a level of 5g/kg. The proximate composition, gross energy and the content of some amino acids of the experimental diets are shown in Table 7. Before starting the experiment, the birds were individually weighed and the smallest bird per pen was removed so all groups consisted of 7 birds. The pens were then divided in 5 weight groups and the diets were distributed within each weight group with a total of 5 replicates (one in each weight group) per treatment. The average BW at day 28 was 1546.5 ± 23.31 and there were no significance differences ($P>0.05$) in weight between the different treatments at day 28.

4.4 Data collections

4.4.1 Live weights and feed consumption

The birds were weighed in groups at arrival and then once a week until day 21. In addition, they were individually weighed at day 28 and day 35. Feed consumption was calculated weekly by weighing the leftovers and substituted from the given feed.

Table 6. *The composition of the grower diet (%) as is fed*

Ingredient	Grower
Wheat	59.53
Soybean 46%	22.37
Wheat middlings	10.00
Soybean oil	4.00
Calcium	1.51
Monocalcium phosphate	0.69
Lysine	0.45
Potato protein	0.36
Premix	0.30
Salt	0.30
Methionine	0.25
Threonine	0.14
Sodium bicarbonate	0.10
Sum	100.00

Table 7. *Proximate composition (g kg^{-1} as is), energy content (MJ kg^{-1} DM) and amino acid content (g kg^{-1} as is) of the experimental diets*

	Experimental diet						
	Control	X10%	X20%	X30%	Y10%	Y20%	Y30%
Dry matter	897	907	906	918	905	902	905
Crude protein	203	219	231	255	229	262	287
Gross energy	18.9	19.2	19.3	19.6	19.5	20.0	20.7
Amino acids							
Cystine	3.2	3.44	4.14	4.51	3.73	4.31	4.73
Lysine	13.4	12.5	12.4	12.5	13.7	14.2	14.8
Methionine	5.38	5.02	5.45	5.55	5.46	5.96	6.39
Threonine	8.34	8.41	8.77	9.33	9.02	10.1	11.2

4.4.2 Total tract faecal samples

On day 33, the wood shavings and the solid floor in all pens were removed and the chickens were from that time housed on a wire mesh floor. Faeces were collected on days 33 and 34 of the experiment between 12:00 and 14:00 by placing plastic foil at the bottom of each cage under the wire mesh floor. During the collection period, an approximately 100 grams of faeces was collected from each pen and homogenized. Thereafter about 25 grams of the homogenized faeces was placed in a petri dish, frozen directly after collection at -20 ˚C, and subsequently removed to a -80 ˚C freezer to prepare it for freeze-drying.

4.4.3 Ileal digesta samples

On day 35, 5 to 6 birds from each pen were euthanized by injecting 2 ml of diluted sodium pentabarbitone intravenously. The birds were dissected and the content of ileum was collected

to a petri dish by gently pressing long through the ileum. The ileum was characterized by the part of the small intestine extending from Meckel's diverticulum to the ileocaecal junction. The Ileal digesta from birds in the same pen were pooled, frozen directly after collection at -20 ˚C and then moved to a -80 ˚C freezer to prepare it for freeze-drying.

4.4.4 Litter assessment

In order to check the litter moisture, a handful amount of the litter was taken and pressed between handgrip and fingers on day 33. A score scale from 1 (very good/dry) to 5 (very bad/very wet) and a scheme paper for the pen were used for the evaluation. In addition, DM in faeces was measured to relate to the litter moisture content

4.5 Sample preparation and analyses

The samples of experimental diets, ileal digesta and faeces were freeze dried (CoolSafe Superior 95/55-80 Superior, LaboGene's, Denmark) and then grounded using a coffee grinder (KG49, De'Longhi America Inc, USA) and finally stored in plastic containers for the following analysis.
All analyses were performed in the Animal nutrition laboratory at the Department of Animal Nutrition and Management, SLU, except for crude protein and amino acids which were sent to a commercial lab (Eurofins, Sweden).

4.5.1 Dry matter and ash determination

Dry matter (DM) content was determined by drying 250 mg of the samples at 103 °C in a laboratory oven (TS 9000, Termaks, Norway) overnight. Ash content was determined after incinerating the dried samples at 550 °C for 3 hours. All weights were scored using a scale with accuracy of 4 decimals.

4.5.2 Gross energy determination

The gross energy of experimental diets and faeces was determined with an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA) using 1g of samples that were manually pelleted and then placed in Cr-Ni-crucibles.

4.5.3 Crude protein and amino acids determination

Prepared samples of experimental diets and ileal digesta were sent to Eurofins laboratories (Eurofins, Sweden) where CP was analysed using the Dumas method and AA were analysed according to US ISO 13903:2005.

4.5.4 TiO2 determination

Titanium dioxide was analysed according to Short et al. (1996) with some minor modifications. Approximately 0.45 g of experimental diets samples, 0.150 g of both of ileal digesta and faeces samples were placed in glass tubes and ashed at 550 ˚C for 16 hours. After cooling, 10 ml of 7.4M H_2SO_4 was added to each tube and then boiled for 30 minutes on a digester with 20 heat

blocks (Tecator™ Digesters, Foss, Denmark) at 300 ˚C. Thereafter the temperature was set on 330 ˚C and left for another 60 minutes. After cooling for 15-20 minutes, about 15 ml Milli-Q water was added to each tube and the mix was then filtered into a 100 ml flask through filter paper. The tubes and the filter were then rinsed 4-5 times and 3-4 times respectively with Milli-Q water. As a final point, 20 ml of 30 % perhydrol was added to each flask and the volume diluted to 100 ml with Milli-Q water. The absorbance was read using a spectrophotometer (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher, Sweden) at wavelength 405 nm after pipetting 300 µl from the solution in each flask to a microtest plate with 96 wells. Each ileal digesta and faecal sample was run in duplicate and in triplicate for experimental diets samples and the absorbance was run in duplicate for each tube. Coefficients of variation (CV) were calculated for the pair wise determinations and a new determination was done when CV exceeded 6%. As references, a blank control and a sample of known TiO_2 concentration were used for each batch.

4.6 Calculations

4.6.1 The apparent ileal digestibility coefficient

The apparent ileal digestibility coefficient (AIDC) of CP and AA in the experimental diets was calculated using the following formula (Ravindran et al., 2005):

$$AIDC = \frac{\left(\frac{N}{TiO_2}\right)_D - \left(\frac{N}{TiO_2}\right)_I}{\left(\frac{N}{TiO_2}\right)_D}$$

Where, $\left(\frac{N}{TiO_2}\right)_D$ is the ratio of CP or AA to TiO_2 in diet; and $\left(\frac{N}{TiO_2}\right)_I$ is ratio of CP or AA to TiO_2 in ileal digesta.

The AIDC for CP and AA in the fungal biomass was calculated using the following equation (Bureau et al., 1999):

$$\mathrm{AIDC_{t.ingredient}} = \mathrm{AIDC_{t.diet}} + [(\mathrm{AIDC_{t.diet}} - \mathrm{AIDC_{c.diet}}) \times (0.7\mathrm{N_{c.diet}} \times 0.3\mathrm{N_{t.ingredient}})]$$

Where $AIDC_{t.ingredient}$= Apparent ileal digestibility coefficient of test ingredient; $AIDC_{t.diet}$: Apparent ileal digestibility coefficient for test diet; $AIDC_{c.diet}$: Apparent ileal digestibility coefficient of the control diet; $N_{c.diet}$: % nutrient in the control diet; $N_{t.ingredient}$: % nutrient in test ingredient (fungal biomass).

4.6.2 The apparent digestibility coefficient (ADC)

The apparent digestibility coefficient (ADC) of DM, organic matter (OM) and GE in control, X30 and Y30 diets was calculated using the following formula (Ravindran et al., 2005):

$$ADC = \frac{\left(\frac{N}{TiO_2}\right)_D - \left(\frac{N}{TiO_2}\right)_F}{\left(\frac{N}{TiO_2}\right)_D}$$

Where, $\left(\frac{N}{TiO_2}\right)_D$ is the ratio of DM, OM or GE to TiO_2 in diet; and $\left(\frac{N}{TiO_2}\right)_F$ is ratio of DM, OM or GE to TiO_2 in the faeces.

The ADC of DM, OM and GE in the fungal biomass was calculated using the following equation (Bureau et al., 1999):

$$\mathrm{ADC_{t.ingredient}} = \mathrm{ADC_{t.diet}} + [(\mathrm{ADC_{t.diet}} - \mathrm{ADC_{c.diet}}) \times (0.7\mathrm{N_{c.diet}} \times 0.3\mathrm{N_{t.ingredient}})]$$

Where $ADC_{t.ingredient}$: Apparent digestibility coefficient of test ingredient; $ADC_{t.diet}$: Apparent digestibility coefficient of test diet; $ADC_{c.diet}$: Apparent digestibility coefficient of control diet; $N_{c.diet}$: % DM, OM or (or kJ g^{-1} gross energy) in the control diet; $N_{t.ingredient}$: % DM, OM (or kJ g^{-1} DM gross energy) in test ingredient.

4.6.3 Digestible CP and AA and AME

Digestible CP and AA of the fungal biomasses were calculated the following equation:

$$Digestible_{t.ingredient} = AIDC_{t.ingredient} \times N_{t.ingredient}$$

Where $Digestibile_{t.ingredient}$: digestibility of test ingredient; $ADC_{t.ingredient}$: Apparent digestibility coefficient of test ingredient; $N_{t.ingredient}$: % nutrient in test ingredient.

AME was calculated using the following equation:

$$AME_{t.ingredient} = ADC_{t.ingredient} \times GE_{t.ingredient}$$

Where $AME_{t.ingredient}$: Apparent metabolizable energy of test ingredient; $ADC_{t.ingredient}$: Apparent digestibility coefficient of test ingredient; $GE_{t.ingredient}$: gross energy (KJ g^{-1} DM) in test ingredient.

5 Statistical analysis

SAS program V9.4 (SAS Institute Inc, Cary, NC, USA) was used to conduct the statistical analysis. All data were evaluated by analysis of variance using the GLM procedure with fixed effect of the experimental diet. AIDC and ADC in the test ingredients (X and Y) and in experimental diets, feed intake and FCR in the experimental diets were analysed. Litter condition results were not analysed because no differences between the pens were found. The effect of sex ratio was tested in the model but without significance and was therefore excluded. Values were considered significant different when *P*-value was < 0.05.

6 Results

6.1 Apparent Ileal digestibility coefficient

6.1.1 Apparent Ileal digestibility coefficient in the test fungi

AIDCs of CP, cystine, lysine, methionine and threonine in X and Y were calculated. Results showed that AIDCs of CP, methionine and threonine did not differ between the two fungal biomasses. However, significant differences between the test ingredients were found for AIDCs of cysteine and lysine. AIDC of cysteine was higher in the fungal biomass X than in the fungal biomass Y (*P*=0.0130, Table 8), whereas AIDC of lysine was lower in X than in Y (*P*=0.0238, Table 8).

Table 8. *Apparent Ileal digestibility coefficient of crude protein, cystine, lysine, methionine and threonine in the fungal biomasses X and Y*

	Test ingredients		SE	*P*-value
	Fungal biomass X	Fungal biomass Y		
Crude protein	0.74	0.72	0.0131	0.4002
Amino acids				
Cystine	0.68 [a]	0.60 [b]	0.0168	0.0094
Lysine	0.61 [b]	0.72 [a]	0.0274	0.0235
Methionine	0.70	0.75	0.0181	0.1045
Threonine	0.67	0.70	0.0203	0.4310

*Values within rows with different superscripts are significantly different (*P *< 0.05)*

When calculating the digestible CP and AA in both X and Y, we found that the fungal biomass Y has superiority on all the calculated values than X except for cystine which was nearly the same (Table 9).

Table 9. *Digestible crude protein and amino acids (g kg as is) in the fungal biomasses X and Y*

	Test ingredients	
	Fungal biomass X	Fungal biomass Y
Crude protein	251	342
Cystine	4.90	4.81
Lysine	5.01	11.97
Methionine	4.07	6.39
Threonine	7.33	11.69

6.1.2 Apparent Ileal digestibility coefficient in the diets

AIDCs of CP and AA were significantly higher in control diet than in the experimental diets (*P*<0.0001, Table 10). Diet X20 had significantly higher AIDC of CP than Y20 and Y30. Diet

Y10 had a significantly higher value than diets X10, Y20 and Y30. Diets X20, and Y10 had significantly higher AIDC of cystine than diets X10, X30, Y20 and Y30.

Table 10. *Apparent Ileal digestibility coefficient of crude protein, cysteine, lysine, methionine and threonine in the control and experimental diets*

	Experimental diet							SE	*P*-value
	Control	X10%	X20%	X30%	Y10%	Y20%	Y30%		
Crude protein	0.83 [a]	0.79 [cd]	0.81 [bc]	0.79 [bcd]	0.81 [b]	0.79 [d]	0.78 [de]	0.0064	<0.0001
Amino acids									
Cysteine	0.79 [a]	0.74 [b]	0.78 [a]	0.74 [b]	0.77 [a]	0.73 [b]	0.70 [c]	0.0064	<0.0001
Lysine	0.88 [a]	0.84 [c]	0.85 [bc]	0.83 [cd]	0.87 [b]	0.84 [c]	0.83 [c]	0.0057	<0.0001
Methionine	0.93 [a]	0.88 [ce]	0.88 [bc]	0.86 [d]	0.90 [b]	0.86 [de]	0.86 [d]	0.0050	<0.0001
Threonine	0.82 [a]	0.77 [c]	0.79 [b]	0.77 [c]	0.80 [b]	0.77 [c]	0.76 [c]	0.0065	<0.0001

**Values within rows with different superscripts are significantly different (P < 0.05*).

For lysine, the obtained AIDC in diets Y10 and X20 were higher than the corresponding values in diets X10, X30, Y20 and Y30.

AIDC of methionine was highest in the control diet (*P*<0.0001, Table 10). Diet Y10 had significantly higher value than these values of diets X10, X30, Y20 and Y30. The corresponding value of X10 was significantly higher than these of diets X30 and Y30 (Table 10).

The obtained AIDCs of threonine were highest in diets X20 and Y10 and were significantly higher than these values in other diets except for the control diet (Table 10).

6.2 Apparent digestibility coefficient

6.2.1 Apparent digestibility coefficient in the test fungi

For ADC of DM, OM and GE, the fungal biomass X had higher values than the fungal biomass Y (Table 11, *P* <0.0001).

AME in Y was significantly higher than X (*P* = 0.0005, Table 11) although ADC of GE in X was higher than in Y, but Y had higher GE than X (Table 3).

6.2.2 Apparent digestibility coefficient of DM, OM and GE in control, X30 and Y30 diets

The ADC of DM in control diet was significantly higher than both diets X30 and Y30 and diet X30 had a significantly higher value than Y30 (*P*<0.0001, Table 12). Diets control and X30 had higher ADCs of OM and GE than diet Y30 (*P*<0.0001, Table 12).

Table 11. *Apparent digestibility coefficient of dry matter, organic matter and gross energy and apparent metabolizable energy (MJ kg $^{-1}$ DM) in the fungal biomasses X and Y*

	Test ingredients		SE	*P*-value
	Fungal biomass X	Fungal biomass Y		
Dry matter	0.94 [a]	0.89 [b]	0.0040	<0.0001
Organic matter	0.94 [a]	0.91 [b]	0.0032	<0.0001
Gross energy	0.78 [a]	0.66 [b]	0.0095	<0.0001
AME	15.6 [b]	17.3 [a]	0.2175	0.0005

*Values within rows with different superscripts are significantly different (*P *< 0.05)*

Table 12. *Apparent digestibility coefficient of dry matter, organic matter and energy in the control, X30 and Y30 diets*

	Experimental diet			SE	*P*-value
	Control	X30%	Y30%		
Dry matter	0.94 [a]	0.93 [b]	0.92 [c]	0.0013	<0.0001
Organic matter	0.95 [a]	0.95 [a]	0.94 [b]	0.0009	<0.0001
Gross energy	0.76 [a]	0.76 [a]	0.70 [b]	0.0045	<0.0001

*Values within rows with different superscripts are significantly different (*P *< 0.05).*

6.3 Production parameters

6.3.1 Body weight, Feed intake, Feed conversion ratio (FCR) and growth rate

The average body weight for the birds and feed intake before giving the test diets are shown in Table 13. Body weight at the end of the experiment and growth rate during the experiment did not differ significantly between the different groups (Table 14). However, this is not the case for both FCR and feed intake. Birds fed diets Y30 had the lowest FCR and it was significantly lower compared to birds fed the control, X10 and X20 diets. Diet Y20 generated significantly lower FCR than diets X10 and X30. FCR in birds fed diet Y20 was lower than in birds given diet X10 (*P*=0.0212, Table 14). In regards of feed intake, all experimental diets were consumed normally except for the diet Y30 that resulted in lower feed intake (*P*=0.0002, Table 14).

6.3.2 Litter condition

Litter was on a good condition in all pens (the score mean was 1 for all diets). Faeces dry matter content in the different groups ranged between 19.8 % and 21.2 % for the diets Y10% and X30% respectively, but did not differ significantly (Table 15).

Table 13. *Body weight and feed intake before starting the experiment*

Parameter (g)	**Standard feed**	
	Mean value	SE
BW at day 7	147.8	7.14
BW at day 14	408.2	25.45
BW at day 21	867.8	42.72
BW at day 28	1546.5	66.07
Feed Intake day 1-10	238.2	12.34
Feed Intake day 10-28	1670.9	68.28

Table 14. *Some production parameters during the experiment period for the different diets*

Parameter	**Experimental diet**							SE	P-value
	Control	X10%	X20%	X30%	Y10%	Y20%	Y30%		
BW at Day 35 (g)	2410.48	2341.06	2321.46	2377.40	2346.09	2300.71	2242.89	83.92	0.86
FCR (28-35)	1.47 ^{bcd}	1.52 ^{d}	1.49 ^{cd}	1.36 ^{ab}	1.44 ^{abcd}	1.40 ^{ab}	1.33 ^{a}	0.0396	0.0212
Feed intake 28-35 (g)	1191.5 ^{a}	1165.6 ^{a}	1120.7 ^{a}	1127.1 ^{a}	1175.8 ^{a}	1098.5 ^{a}	986.9 ^{b}	26.96	0.0002
Growth rate 28-35 (g)	116.4	109.6	107.7	118.8	116.7	112.3	106.2	3.85	0.1828

**Values within rows with different superscripts are significantly different (*$P < 0.05$).

Table 15. *Dry matter content (%) in the faeces from different groups*

	Experimental diet							SE	*P*-value
	Control	X10%	X20%	X30%	Y10%	Y20%	Y30%		
DM %	20.1	20.5	20.6	21.2	19.8	20.5	20.8	0.402	0.3307

**Values within rows with different superscripts are significantly different (*$P < 0.05$).

7 Discussion

This study was done to evaluate the apparent digestibility of fungi based biomass as a source of protein in broiler chicken diets. This biomass was produced from wheat-DDGS using filamentous fungus, namely *Neurospora intermedia* which has the ability to secrete a wide range of enzymes that can degrade the complex structure of wheat-DDGS converting it to a protein-rich biomass with high nutritional value (Bátori et al., 2015; Lennartsson et al., 2014). The chemical composition of the two fungal biomasses X and Y shows a high content of CP (34% and 47.4% respectively) and good amino acid profile (Table 3). Furthermore, X and Y have relatively low levels of crude fibre (2.8% and 8.9% respectively). These chemical characteristics make these biomasses of interest as a protein source in chicken diets. Adebiyi and Olukosi, (2015c) reported the content of ADF and NDF in wheat DDGS to be 39.9% and 22.3% respectively. In our study, ADF and NDF content in Y was 29.1% and 23% respectively. This means that producing fungal biomass using wheat-DDGS reduced the level of ADF in the latter for more than 10%.

The findings of this study suggest that both X and Y can be a potential source of protein. The AIDCs of CP and most AA in wheat-DDGS is relatively low and differ between publications (Adebiyi and Olukosi, 2015b; Bandegan et al., 2009). Differences were found in CP and AA contents in wheat-DDGS among 19 samples of wheat-DDGS from 7 ethanol plants in Europe (Cozannet et al., 2011). These differences can be attributed to the different procedures between different plants such as adding of nitrogenous non-protein substances (enxymes), temperature and yeast (Kim et al., 2008). When comparing with the AIDCs in the test ingredients with results from a study on wheat-DDGS done by Adebiyi and Olukosi, (2015b), we find that these values are still not comparable even after adding proteases. The AIDC of CP and AA was higher especially for lysine which was in wheat-DDGS with enzymes equal to 0.02 while in X and Y this value was 0.61 and 0.72 respectively. On the other hand, when comparing AIDCs with results from Bandegan et al. (2009), we found that value of CP was equivalent to the values in X and Y (0.74, 0.74 and 0.72 respectively) and AIDC of cystine was comparable the value in Y (0.62 and 0.60 respectively). However, comparing with other studies would be bias since as mentioned there are differences in CP and AA between different ethanol plants. Thus, to conclude digestibility improvement, wheat-DDGS from the same plant should have been used in the same trial.

Results from this study suggest that inclusion of both X and Y had a negative effect on AIDC values of CP and AA compared to the control diet. However, this adverse effect did not increase with increasing inclusion level. Generally, the adverse effect was more pronounced in diet Y30 for all values in comparison to other diets. As the fungal biomass Y had a high content of CP (47.7 %), the diet Y30 had not only a very high content of CP (28.7%) but also a high AME in comparison to other experimental diets (Table 7). These high levels of energy and protein in Y30 could have affected feed intake and consequently FCR and resulted in this significant decrease (Cheng et al., 1997; Holsheimer and Veerkamp, 1992). In addition, the fungal biomass Y had a higher content of fibre (crude fiber, total dietary and insoluble fibres) than X (Table 3). The fibre content in Y is also higher than in soyabean meal the most common protein

feedstuff in poultry diets. In our study, NDF content in Y was equal to 29.1%, while in soyabean meal the corresponding value was reported to be highest 12.8% (Ravindran et al., 2014). This high fibre content could be attributed to the fungal cell walls that consist mainly of glucan and chitin (Bowman and Free, 2006). Chitinase (the enzyme which hydrolyse chitin) exists in the digestive tracts of adult chicken (*Gallus gallus*) but the prevailing amount is not enough to catalyze chitin hydrolysis (Jeuniaux and Cornelius, 1978). Though, the inclusion of chitin at level up to 2.8% in broiler diet has a positive effect on performance and chitin degradation may have some positive physiological effects like including antimicrobial activity, immune-enhancing activity, and the stimulation of proteoglycan biosynthesis (Chen et al., 2002; Khempaka et al., 2011, 2006). Even though the existence of these fungal cell walls would limit the inclusion of X and Y (especially Y) at high levels, the inclusion at low levels would possibly be beneficial (when chitin levels do not exceed 2.8%).

In general, digestible CP and AA in feedstuffs is used when formulating diets (*NRC*, 1994). Using that method is advantageous and may result in decreasing feed cost and reduce excretion of nitrogen to the environment (Kim, 2010). Therefore, when formulating broiler diets, ingredients are chosen depending on their availability (digestible CP and AA) rather than only meeting total levels of energy and amino acids (Beski et al., 2015).

When comparing digestible CP and AA (g/kg as fed basis) in X with other common protein-rich feedstuffs shown in Table 18, we can observe that digestible CP in X was 251 which is comparable to the corresponding values in sunflower meal (262). Digestible CP in Y was relatively high and higher than the corresponding values in many protein-rich feedstuffs in Table 18, but lower than this in soyabean meal (342 and 390, respectively). Digestible cystine in both X and Y was comparable to these values in soyabean meal, (4.90, 4.81 and 5.5, respectively). Digestible lysine in X was lower than most of the corresponding values in other protein-rich feedstuffs (Table 18). However, digestible lysine in Y was higher or comparable to most protein-rich feedstuffs but lower than this value in soyabean meal (11.97 and 25.5 respectively). Digestible methionine in X was 4.07 which is comparable to the corresponding value in soyabean (5.3), and in Y this value was 6.39 which is and higher than the corresponding value in soyabean meal, rapeseed meal, cottonseed meal (5.3, 5.8 and 4.7 respectively). The value of digestible threonine in the fungal biomass X was 7.33 which is lower than values in all protein-rich feedstuffs mentioned in Table 18 but chickpeas and faba beans (5.7 and 6.3 respectively). Furthermore, digestible threonine in the fungal biomass Y was 11.69 which is lower than the value in soyabean meal which is equal to15. In practice, methionine and cystine are the first limiting amino acids for growth and production in poultry and then comes lysine (Ravindran and Bryden, 1999). Based on this fact, Y would be a conceivable source of these limiting AA in addition to the higher digestible CP. However, since the calculated approximate production capacity of X is about 15 times more than Y, X is more likely to become a potential source of these AA than Y. Still, using Y as a protein source in the start feed for broilers where CP is included at high level (higher than growing and finisher feed) would perhaps be suitable.

When describing the available energy in a feedstuff to poultry, AME is mostly used (*NRC*, 1994). The calculated AME in the fungal biomasses X and Y was 15.6 and 17.3 MJ/kg DM respectively and this difference can be attributed to the crude fat content in X and Y (Table 3). Adebiyi and Olukosi, (2015c) reported that AME in wheat-DDGS with or without enzymes was 15.0 and 15.5 MJ/kg DM. Even though the relatively high AME, the inclusion of wheat-DDGS is still limited due to the SP high content (Thacker and Widyaratne, 2007; Wang et al., 2007 a,b,c, 2008) making X and Y superior sources of energy in broiler chicken diets.

The main hindering factor for inclusion DDGS at high levels in poultry diets are the high level of NSP and low digestibility of the CP and AA due to Maillard reaction in the drying process (especially lysine) (Adebiyi and Olukosi, 2015b; Cozannet et al., 2011; Thacker and Widyaratne, 2007; Wang et al., 2007a,b,c, 2008). Using wheat-DDGS as a substrate for producing a fungal based biomass will enhance their nutritional value and provide the market with new competitive protein-rich feedstuffs regardless of the relatively high fibre content in Y (Table 3). As stated before, the high proteins content allows lower inclusion levels of Y to meet the requirements especially in case of starter diets.

8 Conclusion

Both fungal biomasses X and Y can be potential sources of protein in broiler chicken diets with relatively high digestible and AA and high apparent metabolizable energy.

9 Future outlook

Additional analyses of these fungal biomasses, especially the NSP such as arabinoxylans, glucans, cellulose and chitin, are needed in order to optimize the use of these products in broiler nutrition and to include them at levels that do not have adverse effects on the animals and their productive parameters. Furthermore, studying methods for disrupting fungal cell walls or use exogenous enzymes to improve the digestibility and reduce the fibre content could be a way to allow higher inclusion levels.

Since biomass product X would be produced in a great deal higher amount than Y, finding a way to enhance the nutritional quality of X with maintained high production level is worth to be further studied.

The effects of these fungal biomasses on properties of the final product, i.e. meat quality are unknown. Thus, studying the sensory quality of the final product is of importance and this should be furtherly examined before using X or Y on large scale.

Correlation between intestinal microbiota and performance in broiler production has been studied. It was suggested that cecal microbiota profile reflects efficiency of feed digestion and nutrient utilization in the proximal intestine (duodenal loop to proximal ileum) (Dumonceaux et al., 2006; Rinttilä and Apajalahti, 2013). Therefore, studying the effect of fungal biomass on the intestinal microbiota would be beneficial toexplore.

Furthermore, investigating the possibility to include these fungal biomasses in laying hen and broiler breeder nutrition is of interest. Adult birds differ physiologically from growing poultry such as broiler chickens and can tolerate higher levels of fibre in their diets.

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11 Appendix

Table 16.*Crude protein and amino acids concentration (g kg^{-1} as is) in different feedstuffs. Adapted from Ravindran et al. (2005)*

	soyabean meal	sunflower meal	rapeseed meal	cottonseed meal	chickpeas	faba beans	blood meal	fish meal	meat meal	meat and bone meal
Crude protein	475	332	350	379	228	250	918	606	564	487
Indispensable amino acids										
Arginine	36.1	26.9	21.7	48.2	23.5	24.1	38.5	37.4	36.7	35.9
Histidine	13.8	8.2	11.2	12.9	6.4	6.6	59.6	14.9	13.4	10.3
Isoleucine	21.7	12.9	14.6	13.5	10.5	10.4	8.6	25.7	17.1	14.5
Leucine	37.2	21.2	25.2	24.2	16.8	18.6	118.9	43.9	38.2	30.7
Lysine	30.0	11.7	20.1	18.3	14	16.1	94.2	46.3	27.6	25.8
Methionine	6.4	7.0	6.8	6.6	2.8	1.6	-	15.3	9.4	6.7
Phenylalanine	24.8	14.9	14.4	22.6	12.7	10.6	67.1	24.5	33.3	16.4
Threonine	19.8	12.3	16.0	13.7	8.1	9.3	53.4	28.2	19.7	16.7
Valine	23.2	16.2	18.3	19.1	10.6	12.9	86.9	30.4	27.0	22.0
Dispensable amino acids										
Alanine	21.2	13.9	15.5	16.4	9.4	10.3	72.3	38.1	40.1	37.6
Aspartate	55.7	29.2	25.2	38.2	24.6	25.9	100.1	53.5	42.1	36.9
Cystine	7.3	5.6	8.0	6.4	3.2	2.9	-	-	8.3	3.4
Glutamic acid	87.3	62.4	62.7	80.6	35.7	38.7	83.2	75.6	69.2	61.2
Glycine	20.5	18.6	17.7	17.4	8.5	10.6	39.1	47.2	70.9	68.7
Serine	26.2	13.9	17.0	18.4	12.1	12.9	58.6	31.3	24.3	19.4
Tyrosine	18.9	9.0	11.0	12.9	6.1	8.2	29.6	19.8	13.7	12.0

Table 17. *Apparent ileal digestibility coefficients of crude protein and amino acids in different feedstuffs. Adapted from Ravindran et al. (2005)*

	soyabean meal	sunflower meal	rapeseed meal	cottonseed meal	chickpeas	faba beans	blood meal	fish meal	meat meal	meat and bone meal
Crude protein	0.82	0.79	0.79	0.74	0.73	0.7	0.87	0.79	0.64	0.61
Indispensable Amino acids										
Arginine	0.88	0.90	0.85	0.86	0.84	0.81	0.83	0.8	0.72	0.68
Histidine	0.82	0.77	0.81	0.73	0.77	0.72	0.88	0.76	0.65	0.62
Isoleucine	0.83	0.83	0.77	0.67	0.70	0.68	0.55	0.81	0.68	0.66
Leucine	0.83	0.83	0.78	0.69	0.70	0.7	0.87	0.83	0.67	0.66
Lysine	0.85	0.80	0.81	0.56	0.76	0.76	0.87	0.84	0.69	0.64
Methionine	0.83	0.96	0.86	0.71	0.72	0.63	-	0.83	0.75	0.72
Phenylalanine	0.84	0.85	0.80	0.78	0.78	0.72	0.88	0.81	0.71	0.70
Threonine	0.76	0.73	0.69	0.61	0.7	0.68	0.87	0.76	0.57	0.56
Valine	0.82	0.82	0.76	0.70	0.73	0.68	0.86	0.82	0.67	0.56
Dispensable amino acids										
Alanine	0.81	0.82	0.80	0.68	0.73	0.71	0.87	0.77	0.71	0.67
Aspartate	0.80	0.79	0.74	0.73	0.73	0.71	0.85	0.75	0.48	0.44
Cystine	0.75	0.92	0.77	0.72	0.58	0.58	-	0.57	0.38	0.36
Glutamic acid	0.86	0.88	0.85	0.83	0.78	0.75	0.82	0.8	0.67	0.63
Glycine	0.78	0.74	0.76	0.68	0.68	0.67	0.82	0.71	0.69	0.64
Serine	0.79	0.74	0.72	0.69	0.74	0.69	0.84	0.74	0.55	0.55
Tyrosine	0.84	0.84	0.77	0.75	0.72	0.70	0.83	0.77	0.67	0.62
Average	0.82	0.82	0.78	0.71	0.74	0.70	0.83	0.77	0.65	0.62

Table 18. *Digestible crude protein and amino acids (g kg−1 as is) in different feedstuffs. Adapted from Ravindran et al. (2005)*

	soyabean meal	sunflower meal	rapeseed meal	cottonseed meal	chickpeas	faba beans	blood meal	fish meal	meat meal	meat and bone meal
Crude protein	390	262	277	280	166	175	799	479	361	297
Indispensable Amino acids										
Arginine	31.8	24.2	18.4	41.5	19.7	19.5	32.0	29.9	26.4	24.4
Histidine	11.3	6.3	9.1	9.4	4.9	4.8	52.4	11.3	8.7	6.4
Isoleucine	18.0	10.7	11.2	9.0	7.4	7.1	4.7	20.8	11.6	9.6
Leucine	30.9	17.6	19.7	16.7	11.8	13.0	103.4	36.4	25.6	20.3
Lysine	25.5	9.4	16.3	10.2	10.6	12.2	82.0	38.9	19.0	16.5
Methionine	5.3	6.7	5.8	4.7	2.0	1.0	-	12.7	7.1	4.8
Phenylalanine	20.8	12.7	11.5	17.6	9.9	7.6	59.0	19.8	23.6	11.5
Threonine	15.0	9.0	11.0	8.4	5.7	6.3	46.5	21.4	11.2	9.4
Valine	19.0	13.3	13.9	13.4	7.7	8.8	74.7	24.9	18.1	12.3
Dispensable amino acids										
Alanine	17.2	11.4	12.4	11.2	6.9	7.3	62.9	29.3	28.5	25.2
Aspartate	44.6	23.1	18.6	27.9	18.0	18.4	85.1	40.1	20.2	16.2
Cystine	5.5	5.2	6.2	4.6	1.9	1.7	-	-	3.2	1.2
Glutamic acid	75.1	54.9	53.3	66.9	27.8	29.0	68.2	60.5	46.4	38.6
Glycine	16.0	13.8	13.5	11.8	5.8	7.1	32.1	33.5	48.9	44.0
Serine	20.7	10.3	12.2	12.7	9.0	8.9	49.2	23.2	13.4	10.7
Tyrosine	15.9	7.6	8.5	9.7	4.4	5.7	24.6	15.2	9.2	7.4

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