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Improving the Nutritive Value of Barley-Based Broiler Diets by Optimizing NSPase Efficacy through Grinding and Feeding Regimen

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ABSTRACT

It was assumed that the potential of exogenous non-starch polysaccharide degrading enzymes (NSPase: β-glucanase and xylanase) to degrade soluble non-starch polysaccharides (NSP: β-glucans and arabinoxylans) in barley-based broiler diets may be unlocked using fine feed grinding and intermittent feeding regimen due to easier, faster, and longer access of NSPase to the target molecules. The effects of NSPase supplementation together with feed grinding levels and feeding regimens on growth performance, nutrient digestibility and digestive tract characteristics of male broilers fed with formic acid acidified barley-based diets from 11 to 33 days of age were investigated in this study. Normal starch hulled barley was ground in a hammer mill to pass through the screen sizes of 2.0 mm (fine) and 6.0 mm (coarse). Finely and coarsely ground barley-based diets were manufactured either with or without NSPase supplementation. The four basal diets along with *ad libitum* (ADL) and intermittent feeding (INT) regimens were used to develop eight dietary treatments. A total of 1408 one-day-old male broilers (Ross 308) were allocated to 64 floor pens and divided into two halves to be fed either ADL or INT. ADL group of birds showed higher ($P < 0.001$) feed intake (FI) and body weight gain (BWG), without any improvement in feed conversion ratio (FCR). NSPase supplementation increased FI and BWG with coarse grinding ($P = 0.051$) and under INT feeding ($P = 0.063$), respectively. NSPase supplementation improved FCR with fine grinding ($P < 0.05$) and under INT feeding ($P < 0.05$). NSPase improved ($P < 0.05$) starch digestibility with coarse grinding. NSPase improved ($P = 0.012$) protein digestibility with fine grinding only under INT feeding regimen. In NSPase supplemented diets fed ADL, coarsely ground diet had higher ($P = 0.012$) protein digestibility as compared to finely ground diet. Coarsely ground diets increased gizzard dry matter contents ($P < 0.01$) and gizzard weight ($P < 0.05$) and reduced the gizzard pH ($P < 0.05$). INT feeding also increased gizzard weight ($P = 0.002$). Tenth percentile particle size ($P < 0.05$), median particle size ($P < 0.05$), ninetieth percentile particle size ($P < 0.001$), and volume weighted mean ($P < 0.01$) in jejunal digesta were smaller with fine grinding. Jejunal digesta viscosity was reduced by NSPase inclusion ($P < 0.001$) and with fine grinding ($P < 0.05$). Jejunal digesta viscosity and cecal prevalence of *C. perfringens* were numerically lower in broilers fed NSPase supplemented finely ground diet under INT feeding. These findings suggest that with fine grinding or/and INT feeding, the efficacy of NSPase in barley-based diets was improved. Future works aiming to study these interactions using barley varieties with low and high viscosity are encouraged.

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"He who does not thank the people is not thankful to Almighty God" (Muhammad PBUH)

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Abbreviations

ADL	*Ad libitum*
ADF	Acid detergent fiber
AME	Apparent metabolizable energy
AMEn	Nitrogen-corrected apparent metabolizable energy
ANF	Antinutritional factor
ANOVA	Analysis of variance
BWG	Body weight gain
CP	Crude protein
cP	Centipoise
d	Day
DM	Dry matter
DP	Degree of polymerization
EC	Enzyme commission number
EE	Ether extract
FCE	Feed conversion efficiency
FCR	Feed conversion ratio
FI	Feed intake
g	Gram
GIT	Gastrointestinal tract
GMD	Geometric mean diameter
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
H-COOH	Formic acid
HMSO	Hammer mill screen opening
HSD	Honestly significant difference
INT	Intermittent

kg	Kilogram
mg	Milligram
MJ	Mega Joule
mm	Millimeter
mmol	Millimole
NDF	Neutral detergent fiber
NE	Necrotic enteritis
NetB	Necrotic enteritis B-like toxin
NSP	Non-starch polysaccharide
NSPase	Non-starch polysaccharide-degrading enzyme
OH	Oat hulls
PDI	Pellet durability index
P-value	Probability
RO	Reversed osmosis
SCFA	Short chain fatty acid
SNE	Subclinical necrotic enteritis
T	Ton
TiO_2	Titanium dioxide
U	Unit
VFA	Volatile fatty acid
Vol. W.M.D	Volume weighted mean
WG	Weight gain
wk.	Week
α-toxin	Alpha toxin
β-glucan	Beta-glucan
β-glucanase	Beta-glucanase

1 Introduction

Modern broiler is an intensively raised meat-type chicken primarily for human consumption. It evolved from jungle fowl (*Gallus gallus*) less than a hundred years ago (Siegel, 2014). Broiler chickens were genetically selected for high growth performance (Everaert et al., 2022). Improved performance refers to increased feed intake (FI) and body weight gain (BWG), and reduced feed conversion ratio (FCR). FCR is an indicator of broiler efficiency that has been significantly improved over the years **Figure 1.1**. The efforts of researchers to achieve further improvements in broiler performance and reduction in the total production price are still ongoing.

Other than genetic selection, optimizations in nutrition and management are prerequisites to improve the performance of broiler chickens where feed covers around 70 % of the production cost of a finished (ready-to-sell) broiler (Ravindran, 2013b). Therefore, reducing the feed costs is one of the major areas of focus for poultry nutritionists and diet manufacturers. Of the total feed cost, purchase of the ingredients and feed processing carry the biggest share. Either increasing the efficiency of feed processing or the use of economically sustainable feed ingredients can cause a drop in the end-product cost. Even though diets for fast-growing-broilers are highly optimized worldwide, the need of the hour is to use sustainable feed ingredients. In modern broiler diet, cereal grains retain the biggest proportion (Ravindran, 2013a). Among cereals, barley is considered a sustainable cereal grain because of being comparatively low-priced (USDA, 2022), able to be cultivated in harsh climatic regions (Ullrich, 2010), and environmentally friendly (Sahabi et al., 2013). However, due to the presence of some antinutritional factors (ANF), its inclusion in broiler diets as a major cereal grain is usually discouraged. In barley grain, soluble non-starch polysaccharides (NSPs) especially β-glucans and arabinoxylans are viewed as the biggest ANF mainly because of their viscosity-producing properties in gastrointestinal tract (GIT) of broilers (McDonald et al., 1973). Increased digesta viscosity results in poor nutrient digestibility and therefore poor broiler performance (Leeson & Summers, 2009). Digesta viscosity can be reduced by supplementation of non-starch polysaccharide-degrading enzymes (NSPase) in broiler diet (Munyaka et al., 2016). β-glucanases and xylanases are NSPase that break down cereal cell walls by hydrolyzing β-glucans (Heng et al., 1997) and arabinoxylans (Berrin & Juge, 2008), respectively into smaller fragments. Particle size of cereal grains is another important parameter in broiler diets that can affect broiler performance (Nir et al., 1994).

Different grain particle sizes can be achieved by grinding through different hammer mill screen opening (HMSO) sizes (Siegert et al., 2018). Bigger HMSO size produces increased proportion of coarser particles and vice versa (Selle et al., 2019). Fine grinding increases the surface area of feed particles and thus theoretically increases the substrate availability to the endogenous enzymes. Coarse particles in broiler diets, on the other hand stimulate gizzard function and development (Svihus, 2011b). A well-developed gizzard further grinds the ingesta and improves nutrient bioavailability and digestibility (Hetland et al., 2002). It may be assumed that even finely-ground barley may contain enough hulls as structural components for contributing to gizzard development and fineness of the feed particles may allow easier and faster access to NSPase. Other than feed particle size, feed retention time in anterior GIT may also influence the function of NSPase. Svihus et al. (2010) postulated that the function of dietary enzymes may be improved by prolonging the retention time of feed in the anterior digestive tract by intermittent feeding rather than *ad libitum* feeding regimen. This is because broilers tend to transiently store feed in the crop in the former regimen (Sacranie et al., 2012), whereas tend to disuse crop as a feed storage organ in the latter one (Buyse et al., 1993; Klasing, 1999). Current literature is insufficient to study the effect of grinding level and feeding regimen on NSPase efficacy, therefore further research is needed to investigate these interactions.

The present study was an attempt to improve the use of barley as a major cereal grain in broiler diets using NSPase while investigating the effect of feed grinding level and feeding regimen on NSPase efficacy. Furthermore, the effect of dietary treatments on growth performance, nutrient digestibility, and prevalence of *Clostridium perfringens* in broilers was studied.

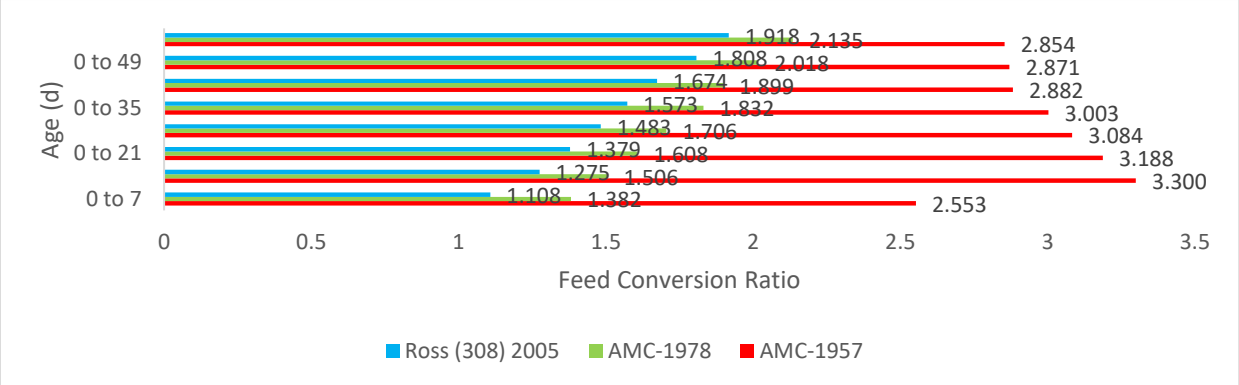

Figure 1.1 A comparison of cumulative FCR (feed intake/body weight gain) of University of Alberta Meat Control (AMC) strains unselected since 1957 and 1978, and Ross 308 broilers (2005). Modified from Zuidhof et al. (2014).

2 Literature Review

2.1 Barley

2.1.1 Background of Barley Crop

Barley (*Hordeum vulgare L.*) is a grass belonging to the family Poaceae and order Poales, and is the world's fourth most important cereal crop produced after maize, rice, and wheat; more than half (61.8% or 89.3 million tons) of world's barley is being produced by Europe per year (FAO, 2021). Barley crop can be grown on a vast range of climates, terrains and heights (Ullrich, 2010). Barley is typically classified into several types on the basis of harvesting season, morphology of spike or head, presence, or absence of an awn, or of a hull (Jacob & Pescatore, 2012; Verstegen et al., 2014). On the basis of harvesting season, barley can be divided into spring and winter barley. Generally, spring barley has more adaptability to climatic ranges and is known as the only suitable type for cultivation in the regions with long-lasting winters because of the absence of the requirement for vernalization (Verstegen et al., 2014). According to the arrangement of spikelet on a barley spike, barley types can be divided into six-rowed possessing three spikelets at each side of the node along the spike and two-rowed having one spikelet at each side of the node along the spike (Ullrich, 2010) (**Figure 2.1**). On the basis of awns, barley can be classified into long-awned, short-awned, awn-less, and hooded awn (Jacob & Pescatore, 2012). On the basis of having a tightly attached hull, barley varieties can be divided into hulled (normal) or hull-less (naked) barley (Bhatty, 1999). Structure of a barley kernel can be seen in **Figure 2.2**.

Figure 2.1 Two-rowed barley (a) and six-rowed barley (b). Source: Verstegen et al. (2014).

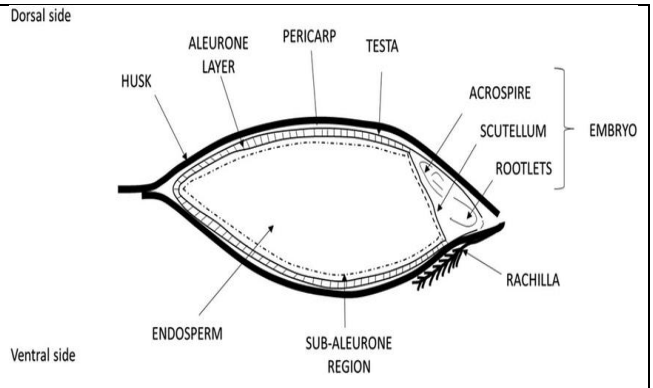

Figure 2.2 Structure of a barley kernel. Source: Filipowska et al. (2021).

2.1.2 Composition of Barley Kernel

Barley kernel is a rich source of macro- and micro- nutrients. Starch can be termed as the biggest energy source in poultry and may make up to half of proportion of dry matter (DM) in poultry diets (Svihus, 2014b). Around 58-77% of the DM of a normal barley grain consists of starch (Bhatty & Rossnagel, 1998; Svihus & Gullord, 2002). On DM basis, barley grain may contain 9.6 to 16% crude protein, 1.1 to 3.2% crude fat, and 1.7 to 4.2% ash (Guo et al., 2020; McDonald et al., 1973; Svihus & Gullord, 2002; Ullrich, 2010). Barley hull, which is tightly attached to the endosperm, constitutes around 10 to 14% of the grain weight (McDonald et al., 1973). The presence of hull dilutes the total digestible energy of normal barley (Yang et al., 1997).

2.1.3 Barley Starch – Properties and Digestion in Broilers

Tightly bound starch molecules make starch granules that are organelles in plant cells and act as sites for production and storage of starch; different plant cells contain different kinds of starch granules based on the shape, size, amylose-amylopectin ratio and crystalline to amorphous material ratio (Do & Singh, 2019; Laurentin & Edwards, 2013). Morphologically, starch granules are arranged in alternating semicrystalline layers with amorphous regions (Svihus et al., 2005), and because of the presence of tightly packed semicrystalline structures, starch granules are insoluble in water at room temperature (Ratnayake & Jackson, 2006). Starch can be classified into amylose and amylopectin. Amylose has long chains of glucose molecules linked with α- 1,4 glycosidic bonds and less branches with α- 1,6 glycosidic bonds, while amylopectin has short-chains of glucose molecules with lesser α- 1,4 bonds but highly branched with α- 1,6 glycosidic bonds (Bertoft, 2017). Plant enzymes can hydrolyze starch granules to generate glucose when the energy requirements are high (Jane, 2009). Starch in barley kernel is predominantly amylopectin 74 – 78%, whereas amylose constitutes 22 – 26% (Newman & Newman, 1992). Starch granules can be classified into A-type and B-type (Zhang et al., 2006). A-type starch granules are bigger with more amylose as compared to B-type granules that are smaller with less amylose (Liu et al., 2007). Champ (1985) reported in their review that the sources of A-type starch granules are cereals mainly and this type of starch is almost completely digestible in monogastric animals. In barley kernel, both biconvex large A-type starch granules, and spherical or polyhedral small B-type granules are present (Eskin & Shahidi, 2012). Another type of starch is resistant starch (RS) that evades enzymatic digestion, so is fermented by gut microbiota which produce short-chain

fatty acids (SCFA) as byproducts (Henningsson et al., 2003; van Munster et al., 1994). Usually, normal barley contains less than 5% resistant starch (Ahmed et al., 2016).

Starch digestion in broilers mainly depends upon various factors including starch source, starch type, feed processing, and presence of anti-nutrients (Carré, 2004; Gidley et al., 2011; Svihus et al., 2005). The broilers are capable of achieving relatively higher starch digestibility due to additional grinding in gizzard (Zaefarian et al., 2015). However, diets based on barley produce high digesta viscosity that result in relatively lower starch digestibility (Carré, 2004). Several mechanisms explaining lower starch digestibility of barley have been mentioned in section 2.1.4.4. Gelatinization is the heat treatment of starch in the presence of water which causes swelling of starch granules, which then break down into solubilized form (Morris, 1990; Ring et al., 1988). Increased starch gelatinization improves starch digestibility in broilers (Itani & Svihus, 2019). Even though pelleting of diets improves broiler performance it does not increase the extent of gelatinization to a very large extent (Moritz et al., 2005; Svihus et al., 2004). The extent of gelatinization can be increased by extrusion (Moritz et al., 2005). However, extrusion of broiler diets also increases the cost of feed production (Jones et al., 1995). Therefore, one efficient way of improving starch digestion of barley in broiler chickens could be to mitigate the causes of higher intestinal digesta viscosity in barley-based diets.

2.1.4 Limitations of Using Barley in Broiler Diets

Below are the major limitations of using barley in broiler diets and the proposed ways to overcome them.

2.1.4.1 Lysine Deficiency

Lysine is an essential and often first limiting amino acid in cereal grains. Like most cereal grains, barley is also deficient in lysine content (Tallberg, 1982). Efforts have been made by geneticists to develop high lysine-producing genetically modified varieties of barley, but those varieties are known to have an overall lower starch proportion and a total reduction in grain yield as compared to normal barley, which is unfavorable (Åman & Newman, 1986; Salomonsson et al., 1980). However, lysine is not a major concern in diets as it can be supplemented in crystalline form (McDonald et al., 1973).

2.1.4.2 Trypsin Inhibitors

The barley kernel also contains moderate quantity of heat-stable trypsin inhibitors (Mikola & Kirsi, 1972; Mikola & Suolinna, 1969; Sosulski et al., 1988). Generally, trypsin inhibitors are regarded as ANF due to their ability to inhibit the action of proteases. Barley trypsin inhibitors have been classified as weak inhibitors as compared to those found in legumes due to the lack of the significant inhibition activity of proteases (Mikola & Suolinna, 1969). Being weak inhibitors, these are not considered problematic for broilers.

2.1.4.3 Arabinoxylans

Arabinoxylans are soluble NSPs and are present in cell walls of barley kernel as hemicellulose (as well as in wheat, rye, and oats). Arabinoxylans are composed of a straight chain of β-1,4-glycosidic linked xylopyranosyl (xylan units) core with arabinofuranosyl substitutes (arabinose units) linked with α-1,2 or α-1,3 glycosidic bonds at O-3, O-4, and/or O-2,3 positions of xylan units (Chotinsky, 2015; Izydorczyk & Biliaderis, 1995; Zannini et al., 2022). Depending on the origin of the molecule, bonding position of arabinose substitutes with xylan core can make up to four kinds of structures (**Figure 2.3**). It has been stated that barley kernel contains all of the four structures (Izydorczyk & Dexter, 2008).

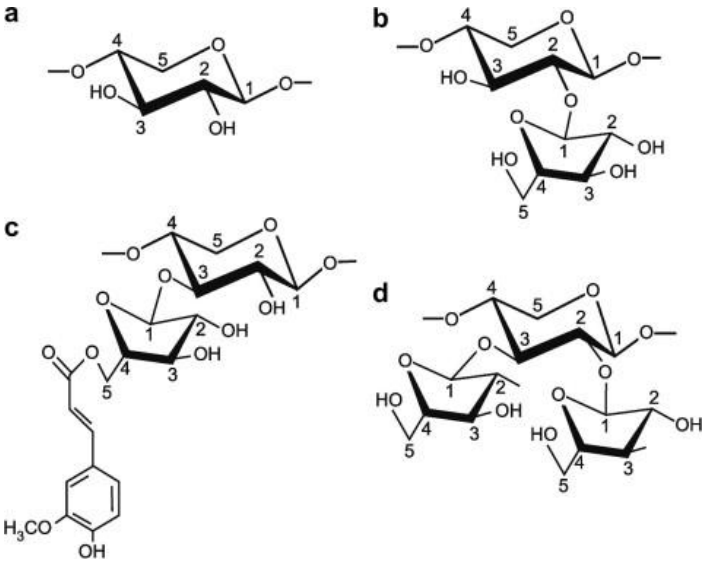

Figure 2.3 Structural constituents of arabinoxylans: (a) unsubstituted xylopyranosyl; (b) xylopyranosyl with single substitute at O-2; (c) xylopyranosyl with single substitute at O-3 with ferulic acid residue esterified to arabinofuranosyl, and (d) disubstituted xylopyranosyl at O-2,3. Source: Izydorczyk and Dexter (2008).

The arabinoxylan content of barley varies depending on the location within the grain. Barley grain (Henry, 1987), endosperm cell wall (Fincher, 1975), and aleurone cell wall (Bacic & Stone, 1981a, 1981b; Izydorczyk & Dexter, 2008; Jääskeläinen et al., 2013; Yadav & Hicks, 2015) on an average comprise of 6.6%, 25%, and 70% arabinoxylans, respectively. Barley arabinoxylans are soluble in aqueous solutions and increase their viscosity (Izydorczyk & Dexter, 2008). Increase in viscosity is probably due to their high molecular weight (Storsley et al., 2003) and the presence of stiff core with semi-flexible substitutes in their molecular structure (Fincher & Stone, 1986; Izydorczyk & Biliaderis, 1995). Arabinoxylans are identified as ANF in broiler diets because of their capability of developing viscous solutions in the GIT of birds that reduce the passage rate of digesta, rate of digestion and nutrient digestibility (Antoniou et al., 1981; Rodríguez et al., 2012). The relationship between high digesta viscosity and poor nutrient digestibility has been elucidated in section 2.1.4.4.

2.1.4.4 β-glucans

In the past, poultry nutritionists have been hesitant to include high proportions of barley in poultry diets not because of lysine deficiency, trypsin inhibitors or arabinoxylans but predominantly because of mixed linkage (1 → 3,1 → 4)-β-d-glucans, commonly known as β-glucans (Jacob & Pescatore, 2014; Johansson et al., 2008; Leeson & Summers, 2009; McDonald et al., 1973; McNab & Smithard, 1992). Like arabinoxylans, β-glucans are also soluble fibers or NSP. Barley grain (Leeson & Summers, 2009; Li et al., 2001), endosperm cell wall (Ballance & Manners, 1978; Fincher, 1975), and aleurone cell wall (Åman & Graham, 1987; Jääskeläinen et al., 2013) contain averagely 4-7%, 75% and 25% β-glucans, respectively. β-glucans in barley are structurally and functionally different than those present in bacterial and fungal cell walls. Barley β-glucans (**Figure 2.4**) are structurally unbranched, and are problematic when their high concentrations are present in poultry diets (Jacob & Pescatore, 2014; Schwartz & Vetvicka, 2021). In barley β-glucans, glucopyranosyl units are linked by two to three consecutive β-(1 → 4) bonds to form the spines that are separated by single β-(1 → 3) bond on terminals (Izydorczyk & Dexter, 2008). The presence of β-(1 → 3) glucopyranosyl (β-glucose) unit linkages induces flexibility and curves in the structure and affects the properties of the molecule (Buliga et al., 1986).

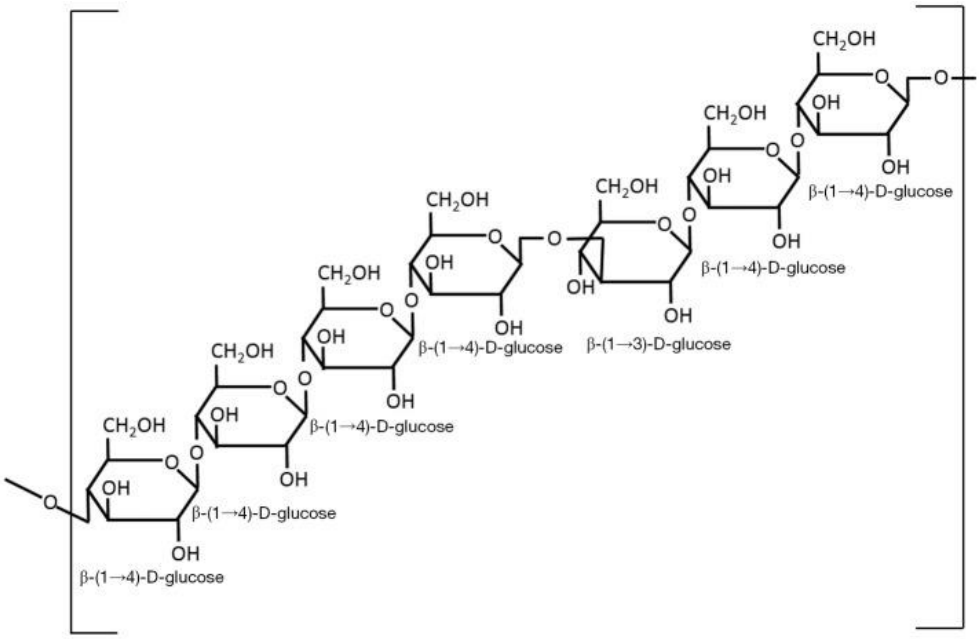

Figure 2.4 Cereal β-glucans. Source: Jacob and Pescatore (2014).

When treated with lichenase, β-glucans yield trisaccharide (DP3) and tetrasaccharide (DP4) (Johansson et al., 2000; Lazaridou et al., 2004; Wood, 2004). Structures of the substrate and the products can be seen in **Figure 2.5**.

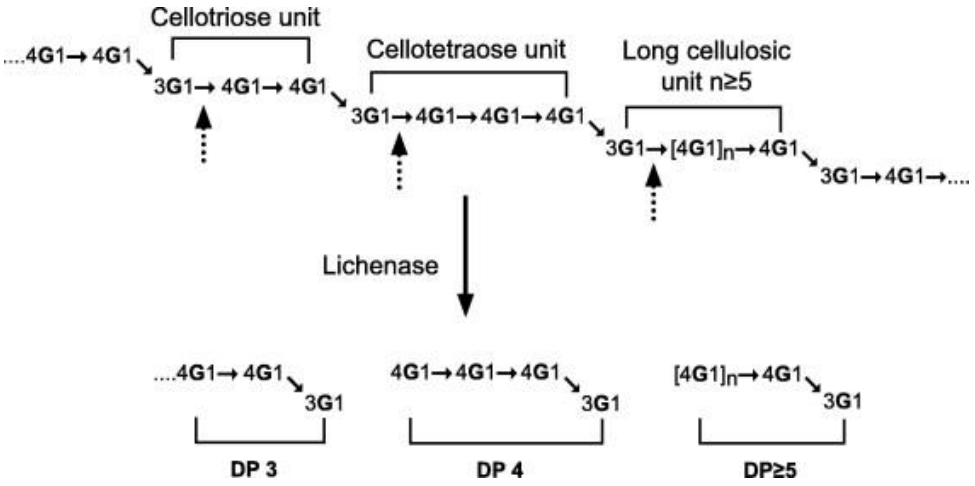

Figure 2.5 Trisaccharide (DP3) and tetrasaccharide (DP4) yielded from hydrolysis of beta-glucans with lichenase. Source: Izydorczyk and Dexter (2008).

On the basis of solubility in water at 40 - 65°C (Wettstein et al., 2000), cereal β-glucans can be sub-classified into water soluble and water insoluble fractions (Johansson et al., 2000). The

proportion of soluble and insoluble β-glucans in barley is 75% and 25%, respectively (Johansson et al., 2004). Insoluble β-glucans are also called matrix β-glucans as they provide structural integrity to endosperm cell walls along with other insoluble polysaccharides (Forrest & Wainwright, 1977; Miller & Fulcher, 1995). Whereas soluble β-glucans are located mainly in the inner layer of endosperm cell walls (Miller & Fulcher, 1995).

Insolubility of β-glucans is because of the presence of regularity in longer chains of trisaccharide units (Izawa et al., 1993), more β-(1 → 4) linkages (Izydorczyk et al., 1998), and lower molecular weight (Johansson et al., 2004). Whereas solubility of β-glucans is mainly due to the presence of irregularity in the consecutiveness of long chains of trisaccharide units in their structure, thus suggesting more β-(1 → 3) linkages between β-(1 → 4) linked chains (G. S. Buliga et al., 1986; Skendi et al., 2003). Moreover, soluble β-glucans possess higher molecular weight (Johansson et al., 2004). Since β-(1 → 3) linkages induce flexibility in the molecular structure of β-glucans, it can be suggested that increased molecular flexibility results in increased solubility.

Detailed description of soluble and insoluble β-glucans was important to differentiate between their viscosity and gelation producing properties. Viscosity and gelation are two distinct properties of barley β-glucans which are often wrongly interchangeably used to describe the formation of viscous digesta in the GIT of birds. Viscosity can be defined as the resistance to flow exhibited by a material, and increased digesta viscosity by β-glucans is usually troublesome. Gelation of β-glucans, on the other hand, is actually the tendency of consecutive β-(1 → 4) linkages to form interchain aggregations by hydrogen bonding, thus causing precipitation of β-glucans (Gaosong & Vasanthan, 2000; Izydorczyk et al., 1998). This precipitation occurs during malting process and causes filtration problems and is often termed as “gel-formation” (Jin et al., 2004). However, this precipitation or gel-formation requires special conditions such as increase in: β-glucans concentration (Tanaka & Sakuma, 1999), alcohol concentration (Gjertsen, 1966), and the number of freeze and thaw cycles (Vis & Lorenz, 1997). Moreover, it has been reported that the viscosity and gel producing properties of β-glucans are directly proportional to their water solubility and insolubility, respectively (Cui & Wood, 2000). These studies suggest that the major fraction in barley β-glucans is the soluble one and is responsible for increasing the viscosity of solutions while the minor insoluble fraction does not contribute to viscosity.

Barley β-glucans act as ANF for broiler chickens due to several underlying mechanisms and viscosity is one of them. Firstly, β-glucans in cell walls act as barrier for endogenous enzymes in

broiler GIT to reach the entrapped nutrients (Knudsen, 2014). Secondly, due to their high water-solubility, barley β-glucans are known to increase the intestinal digesta viscosity in broiler chickens (Gill et al., 2002; Henrion et al., 2019; Jacob & Pescatore, 2014; White et al., 1981). Highly viscous digesta forms the shape of a three dimensional net (Jacob & Pescatore, 2012). This net-like structure entraps starch, protein and fat in digesta and keeps enzymes inaccessible to nutrients (Bai et al., 2019). Moreover, it reduces the rate of mixing between digestive enzymes and digesta (Leeson & Summers, 2009). These conditions may hinder the transport of digested nutrients from the lumen of the intestine to the brush border of enterocytes (Leeson & Summers, 2009). Furthermore, β-glucans reduce the amylase and chymotrypsin activity *in vitro* (Dunaif & Schneeman, 1981), and amylase and lipase activity in broiler GIT (Almirall et al., 1995). Research has shown that elevated digesta viscosity has a detrimental impact on the digestibility of starch, protein, and particularly fat (Bedford & Apajalahti, 2022; Dänicke, 2001; Dänicke et al., 1997). Thirdly, β-glucans are known to bind with bile acids and form NSP-bile salt complexes, which may reduce the re-uptake of bile salts (Bielke et al., 2017; Gunness & Gidley, 2010). This action may result in increased excretion and hence lower concentrations of bile salts in the intestine (Salih et al., 1991), and may reduce fat digestibility of barley-based diets in broilers (Chotinsky, 2015). Fourthly, slower feed passage time due to increased viscosity results in the proliferation of pathogenic bacteria in the hindgut (Choct, 1997). The fourth mechanism has been discussed in detail in section 2.1.5. Lastly, the adverse effects of β-glucans are exerted on feces which are released as sticky droppings (Hofshagen & Kaldhusdal, 1992). Sticky droppings reduce the water holding capacity of litter material and wet litter significantly increases the chances of footpad dermatitis in the birds (Allain et al., 2009; Martland, 1985).

In summary, β-glucans in barley are the major contributors to its adverse effects like reduced nutrient digestibility, impaired energy utilization, compromised growth performance, and poor broiler health and welfare.

2.1.5 Relationship of Barley with *Clostridium perfringens* in Broiler Gut

Gut microbiota plays an important role in regulation of energy and may affect performance of the broilers (Apajalahti et al., 2012). However, the most important role of gut microbiota in broilers is to pre-dominate over pathogenic bacteria by occupying most of the epithelial surface area (Iqbal et al., 2020; Kamada et al., 2013; Zhu et al., 2002). Bacteria are the most numerous

microorganisms found in digestive system of a chicken (Gabriel et al., 2006). In anterior GIT of broiler chickens, *Lactobacilli* are the most common bacteria (Wang et al., 2017). Whereas the most abundant bacterial families in the ceca are Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, and Clostridiaceae (Oakley et al., 2014). Natural gut bacterial populations have developed a symbiotic relationship with host (Hooper, 2009). Therefore, having a dynamic balance between the beneficial and pathogenic bacteria is very important. This balance can be easily disrupted by abrupt changes in diet or environment (Pan & Yu, 2014; Yegani & Korver, 2008). In general, diet (Shang et al., 2018), and in specific, the type of principal cereal used in diets is one of the biggest factors that influence the gut microbiota diversity in chickens (Apajalahti, 2004; Hübener et al., 2002). Feed particles that are resistant to digestion by endogenous enzymes are fermented by bacteria in the hindgut. As a result of fermentation, SCFAs and volatile fatty acids (VFAs) are produced which can be absorbed directly into the epithelium and may be useful for the host (Apajalahti et al., 2004; Gabriel et al., 2006). Due to variations in nutrient compositions among diets, the nutrients available for fermentation may differ. As a result, the bacterial populations in the gut vary depending on the nutrient preferences of different bacterial strains (Hanning & Diaz-Sanchez, 2015).

Since the use of antimicrobial growth promotors has been banned in certain regions of the world, several broiler health issues have arisen in commercial poultry flocks with Necrotic Enteritis (NE) one of those issues. NE is a deadly disease in poultry that is caused by *Clostridium perfringens* (Fathima et al., 2022). The incidence of *C. perfringens* in poultry houses is well-documented (Craven et al., 2001). Even though the members of genus *Clostridium* are often present among intestinal microbiota of chickens (Barnes et al., 1972), not all of the species belonging to this genus are pathogenic (Apajalahti et al., 2012). *C. perfringens* itself may be present in the chicken gut but may not cause the disease under normal conditions (Gholamiandekhordi et al., 2006). Under unfavorable conditions, *C. perfringens* produce certain toxins that damage host cells and cause lesions (Petit et al., 1999). All *C. perfringens* strains possess the gene to produce alpha toxin (α-toxin) on the chromosome (Albini et al., 2008; Fathima et al., 2022). These reports imply that the detection of α-toxin producing gene in digesta may indicate the presence of *C. perfringens*. Moreover, the key virulent factor of pathogenic strains of *C. perfringens* is necrotic enteritis B-like toxin (NetB-toxin) (Keyburn et al., 2010; Keyburn et al., 2008). However, the prevalence of Net-B toxin-encoding gene has also been

reported in healthy chickens (Martin & Smyth, 2009; Yang et al., 2018). This suggests that the virulence of NetB-producing *C. perfringens* may depend upon certain predisposing factors. These predisposing factors are mucosal damage due to *Coccidia spp.* which is an intracellular parasite (Al-Sheikhly & Al-Saieg, 1980), and diets with high soluble NSP (Kaldhusdal & Hofshagen, 1992) and protein content (Drew et al., 2004). The correlation of barley with these predisposing factors is well-documented and can be noted in the following literature review. Firstly, as previously mentioned in section 2.1.4.4, soluble NSPs bind with bile salts in broiler intestine and form NSP-bile salt complexes (Chotinsky, 2015; Salih et al., 1991). According to Knarreborg et al. (2002), *C. perfringens* dominantly take part in the deconjugation of bile salts in broiler GIT. Secondly, low protein digestibility may be favorable for the growth of *C. perfringens* (Timbermont et al., 2011). Increased flow of undigested protein and amino acids leads to the production of alkaline metabolites that create a suitable environment for proliferation of pathogens like *C. perfringens* (Kiarie & Mills, 2019). Thirdly, cereal β-glucans have the capability to increase intestinal mucus secretion (Hu et al., 2015), and *C. perfringens* may use mucus as an energy source because of its mucolytic potential (Kiu & Hall, 2018; Timbermont et al., 2011). Fourthly, *C. perfringens* are obligatory anaerobes, so need the absence of oxygen to proliferate. It has been reported that increased digesta viscosity due to soluble NSPs decreases the oxygen tension in the intestines and makes conditions suitable for the growth of anaerobic pathogens (Choct, 1997). The pathogens from ileum may move to ceca during the inflow of digesta towards ceca. So, all the four factors i.e., formation of β-glucan-bile salt complexes, increased mucus secretion, reduced oxygen tension, and reduction in protein and amino acid digestibility could be the possible reasons behind higher cecal prevalence of *C. perfringens* in broilers fed barley-based diets. Incubation of *C. perfringens* with *in vitro* digested barley-based diet resulted in higher proliferation of *C. perfringens* as compared to corn-based diets in similar conditions (Annett et al., 2002). Cecal proliferation of *C. perfringens* is positively linked with the presence of lesions in the broiler GIT (Novoa-Garrido et al., 2006). A significant increase in the proportion of broilers with intestinal damage due to *C. perfringens* was seen when broilers were reared on barley-based diets as compared to corn-based diets (Kaldhusdal & Hofshagen, 1992). These studies suggest that broilers fed with barley-based diets may show a higher prevalence of *C. perfringens* as compared to those fed with diets having lower soluble NSP content.

2.1.6 Barley Inclusion in Broiler Diets

The application of barley grains can be found in different domains of utilization. However, most of the barley (60-70%) is used for animal feed (Newman & Newman, 2008). Conventionally, barley has been used in pig and beef cattle feeds, but rarely in poultry feeds. As barley-based diets impact broiler performance and nutritional parameters, the extent of adverse effects mainly depends upon the proportion of barley included in broiler diets. For example, it was shown that protein digestibility of broiler grower diets was significantly reduced with every 25% increase (from 0% to 75%) in barley inclusion (Rotter et al., 1990). Apparent metabolizable energy (AME) of barley in poultry is usually between 13.0 and 15.0 MJ/kg (Khalil et al., 2021; McDonald et al., 1973), and nitrogen-corrected AME (AMEn) is between 12.5 and 13.7 MJ/kg (Khalil et al., 2021). However, Fuente et al. (1995) has shown that AMEn of broiler diets significantly declined with every 10% increase (from 30% to 60%) in barley inclusion. The authors of the same study have reported that for every 1 cP increase in the intestinal digesta viscosity, 59 kJ AMEn was reduced. Another broiler study found significant reduction in FI and BWG for every 10% increase (from 0% to 30%) in barley inclusion in starters and growers (Sharifi et al., 2012). Among diets with zero, low, medium, and high barley inclusion, BWG and FCR were better with medium barley inclusion (starter: 7.5%, grower: 15%, finisher: 22.5% and withdrawal: 30%) (Toghyani et al., 2022). The reason for the improvement in growth performance with medium levels of barley inclusion could be that this level of inclusion did not significantly increase digesta viscosity, whereas the decline in performance by high inclusion of barley might be because of dilution of diets by barley hulls. In their study, Hetland et al. (2002) observed a reduction in the BWG when moderate (30%) or high (44%) levels of barley replaced wheat in the diet. The authors attributed this effect to the presence of hulls in barley that diluted the energy in diets. Perera et al. (2019) observed that the performance of broilers was greatest at 28% inclusion of barley. Even though the use of up to 28% barley in broiler diets does not significantly affect the broiler performance, it may still increase the prevalence of *C. perfringens*. This assumption can be supported by a study by Kaldhusdal and Hofshagen (1992) in which they found increased occurrence of sub-clinical necrotic enteritis (SNE) when barley-based (27% barley) diets were offered to broiler chickens and correlated SNE with poor performance.

These studies imply that high inclusion proportion of barley in broiler diets may be detrimental for growth performance mainly due to poor nutrient utilization because of the presence of

significant amounts of soluble NSPs, whereas it may be used in broiler diets at low and medium inclusion levels without affecting performance parameters yet increasing the risk of SNE by making the GIT conditions suitable for the growth of *C. perfringens* in broiler chickens.

2.2 Non-Starch Polysaccharide-Degrading Enzyme (NSPase)

2.2.1 NSPase in Broiler Nutrition - Background and Significance

With the inclining need of maximizing the nutrient bioavailability of barley-based diets in broilers mainly by eliminating the negative impacts of soluble NSP in barley, the need to use exogenous enzymes in the feed that can degrade these antinutrients has also arisen. Since broilers lack the typical enzymes required for the degradation of soluble NSPs, broiler diets need to be supplemented by exogenous enzymes (Paloheimo et al., 2010). Exogenous NSPase is an enzyme that can break down soluble NSP into smaller fragments thus eliminating their means of producing adverse effects in broiler GIT. NSPases can be derived from either certain bacteria or fungi and can be classified primarily into exoglucanases, endoglucanases, and xylanases (Beguin et al., 1992; Payne et al., 2015). *Trichoderma reesei* is a filamentous fungus well-known as a high-yielding producer of endoglucanases (Biely et al., 1991) and xylanases (Tenkanen et al., 1992). *T. reesei* can be genetically modified to achieve a higher production of NSPase as compared to its naturally occurring counterpart (Montenecourt & Eveleigh, 1977), and NSPase-producing genes from *T. reesei* can be cloned and expressed into *Escherichia coli* (bacteria) to get easy-to-purify endoglucanases with improved efficacy (Nakazawa et al., 2008).

The mechanism of action of NSPases is described as follows. Endoglucanases catalyze the hydrolysis of β-(1 → 4) glycosidic bonds of β-glucans randomly at multiple sites within the target molecules and make free chain ends available for the action of other cellulolytic enzymes (Annamalai et al., 2016). Exoglucanases, on the other hand, catalyze progressively from the non-reducing end of β-glucan chains (Beguin et al., 1992), and remove only one glucose molecule during each attack (Jin et al., 2004). This property makes them less potent as compared to endo β-glucanases. Endo 1,3(4) β-glucanases are known to hydrolyze both; β-(1 → 3) and β-(1 → 4) bonds simultaneously of barley β-glucans (Heng et al., 1997). Another type of NSPase are endo-1,4-beta-xylanases that attack arabinoxylan core glycosidic bonds between β-1,4 xylopyranosyl units at random sites and break them down into oligosaccharides (Berrin & Juge, 2008; Harris & Ramalingam, 2010).

It has been shown that the major mode of action of NSPases (β-glucanase and xylanase) from *T. reesei* is the hydrolysis of soluble NSP (Li et al., 2004). The capability of supplemented NSPases to break down β-glucans in broiler GIT and mitigation of their negative impacts on nutrient availability and broiler health is well-acknowledged (Chotinsky, 2015; Leeson & Summers, 2009; McDonald et al., 1973; Munyaka et al., 2016). NSPases may be beneficial for broiler nutrition in either of the three proposed modes of action: NSP hydrolysis, reduction in intestinal viscosity, and generation of prebiotics (Bedford, 2006; Bedford, 2018; O'Neill et al., 2014). These pathways can be elaborated in the following way: NSPase breaks down cereal cell walls and releases the trapped nutrients; reduction in the molecular size of NSPs reduces digesta viscosity, and this provides oligosaccharides as prebiotics that can be fermented by gut microbiota.

However, just the addition of NSPase in broiler diets may not be sufficient as certain intrinsic and extrinsic factors may affect the enzyme functionality. The efficiency and efficacy of enzymes whether exogenous or endogenous depends upon several factors such as pH, temperature, medium, time for action, and enzyme to substrate concentration. The optimum pH for endoglucanases and endo-1,4-β-xylanase from *T. reesei* was found to be between pH 4.0 and 5.0 (Macarrón et al., 1993; Payne et al., 2015; Rodionova et al., 2000; Törrönen et al., 1994). Taking their optimum pH into account, pH of the feed and the bird crop are important factors to consider that may affect NSPase efficacy. Usually, the pH of broiler diets is between 5.7 - 7.0 (Cox et al., 2013; Tabib et al., 1981). In an experiment conducted at our lab, the pH ranges of oat-based and barley-based broiler diets were found to be 5.70 - 6.00, and 6.40 - 6.75, respectively (unpublished data). Furthermore, the pH in the broiler crop ranges from 5.5 - 6.5 (Gao et al., 2008; Hinton et al., 2000; Kristoffersen et al., 2021; Mabelebele et al., 2014). Therefore, to achieve a lower pH in anterior GIT of broiler chickens, diets need to be acidified. Addition of weak acids such as organic acids should theoretically lower the feed pH (Abdollahi et al., 2020; Hajati, 2018). A study has shown that the addition of organic acids to broiler diet lowers the pH in crop, proventriculus and gizzard (Kim et al., 2015). Previously, broiler diets have been acidified with various organic acids to improve performance, nutrient utilization and for antimicrobial effects (Abdollahi et al., 2020; Adil et al., 2011; Kamal & Ragaa, 2014; Khooshechin et al., 2015; Van Immerseel et al., 2006). Among all organic acids, formic acid (H-COOH) is one of the most researched compound that is known for its antibacterial effect both in the feed and in the GIT due

to significant reduction in pH (Ricke et al., 2020). In a study by Ao et al. (2009), exogenous enzyme (α-galactosidase) improved AMEn of broiler diets only when the diets were acidified. In another study, the positive effect of diet acidification on the efficacy of exogenous phytase was demonstrated (Kristoffersen et al., 2021). These studies suggest a positive interaction between dietary organic acids and exogenous enzyme activity. However, the literature suggesting the effect of diet acidification on NSPase efficacy is scarce, thus necessitating further experimentation. Other than optimum pH, most NSPases need an optimum temperature (45 to 65°C) to function optimally (Gómez et al., 2016; Payne et al., 2015; Rodionova et al., 2000). The normal body temperature of a broiler chicken is between 41 and 42°C (Donkoh, 1989), which is quite close to the optimum temperature of NSPases. Svihus (2011a) has mentioned in their review that the efficacy of exogenous enzymes is not affected at body temperature in poultry. A possible reason for this could be the lack of significant difference between the optimum temperature for most exogenous enzymes and the body temperature of poultry. The naturally acquired exogenous enzymes are protein in nature and may be denatured by high-heat treatments like conditioning and pelleting in the feed manufacturing process. Therefore novel enzymes have been developed using genetic engineering to achieve stability at relatively higher temperatures (Amerah et al., 2011). A study showed that inclusion of heat-stable 1,3 - 1,4 β-glucanase in barley-based diet reduced intestinal viscosity as well as sticky droppings and improved BWG (Wettstein et al., 2000). Currently, most NSPase producers guarantee enzyme activity even after pelleting over 80°C. Moreover, enzymes require aqueous medium for function and considerable time for achieving optimal activity. Storage of feed in the crop as described later in section 2.4.2 can moisturize the feed and provide optimal time for the action of exogenous enzymes when it is used as a transient storage organ (Classen et al., 2016; Svihus, 2014a).

For poultry feed, commercial NSPases are available in liquid or powdered form. Actual dosage of NSPase in feed is still debatable as many studies use considerably lower dosages of NSPase to study their effect rather than using the optimum dose (Bedford, 2018). According to EFSA (Panel on Additives and Feed) (2022), the activity of xylanase is expressed in endo-pentosanase units (EPU), whereas that of β-glucanase is expressed in cellulase units (CU). The activity of various NSPases in different preparations vary and sufficient post-pelleting activity is ensured by the manufacturers. Dose of enzymes is usually expressed in units per kilogram diet (U/kg). It has been shown that the digesta viscosity was reduced and the broiler performance was improved

when a cocktail of xylanase (2500 U/kg) and β-glucanase (250 U/kg) was used in wheat-based diet (Munyaka et al., 2016). Since the β-glucan content in barley is higher than that in wheat, higher dose of β-glucanase may be required to cope with the substrate concentration. Generally, the inclusion percentage of NSPase in broiler diets varies from 0.01 to 0.5 % depending upon the type and proportion of cereals, and the type and activity of enzymes used (Coppedge et al., 2011; Coppedge et al., 2012; Derqaoui, 2022).

2.2.2 Effect of NSPase on Broiler Performance

The use of exogenous NSPase in barley-based broiler diets is very well-known due to its potential benefits (Chesson, 1993). There is a general accord on the positive effects of NSPase inclusion on broiler performance (Coppedge et al., 2012; Moftakharzadeh et al., 2017; Shirzadi et al., 2009; Toghyani et al., 2022; Williams et al., 2014). It has been shown that NSPase inclusion can improve the broiler performance in low-energy diets based on dried distiller's grains with solubles (DDGS) (Campasino et al., 2015). Mohajeri et al. (2022) and Woyengo et al. (2019) showed that NSPase supplementation improved AMEn of broiler diets. Poernama et al. (2021) found that NSPase addition in diets improved FI, BWG, FCR and performance index in broiler chickens. Another study showed improved BWG and FCR when barley-based diets were supplemented with NSPase in broiler growers (Gracia et al., 2003). In an experiment on broilers raised on wheat- and barley-based diets, NSPase addition to diets resulted in improved FI, BWG, FCR, and energy utilization, mainly by reducing the digesta viscosity and hindering the growth of facultative anaerobic bacteria and enteric *Escherichia coli* (Mathlouthi et al., 2002b). Choct et al. (1996) found high ileal fermentation activity with poor nutrient utilization due to the presence of NSP fractions in diet. Later on, it was confirmed that high ileal fermentation activity is unfavorable for nutrient digestibility in broilers as the ileal bacteria compete with the host for nutrient utilization (Choct et al., 1999). Moreover, it was found that the use of NSPase in the diet halted ileal fermentation and elevated cecal fermentation by positive alteration in the microbiota profile (Choct et al., 1996). Cecal fermentation activity may be better for the growth performance due to increased production of VFAs which may be utilized by the host (Apajalahti et al., 2012). As wet litter is detrimental for bird welfare and health (Ritz et al., 2009), NSPase can solve this problem if the causation behind this are soluble NSPs. It was shown that increased gut water content by barley-inclusion in diets increased the litter moisture, which was reduced by 30 to 40

% by NSPase supplementation (Toghyani et al., 2022). With reduced litter moisture improvement in broiler performance was seen (De-Jong et al., 2014).

Improvement in broiler performance by NSPase is seen mainly because of their capability of breaking down the soluble NSPs, thus improving the nutrient utilization principally by reduction in the digesta viscosity. Therefore, it may be hypothesized that NSPase supplementation in barley-based diets may improve growth performance of broilers.

2.2.3 Effect of NSPase on Jejunal Digesta Viscosity

Intestinal digesta viscosity is an important factor to consider when feeding barley-based diets that may affect nutrient digestion and availability in broiler chickens. Barley β-glucans are known to bind moisture in the intestines causing increased intestinal digesta viscosity (Henrion et al., 2019; Jacob & Pescatore, 2012; Jacob & Pescatore, 2014; Svihus et al., 2005; White et al., 1981). Increased intestinal digesta viscosity due to dietary β-glucans can reduce nutrient digestibility and nutrient absorption, while increasing the bird's susceptibility to the disease; stated Jacob and Pescatore (2014). It is evident from literature that supplementation of β-glucanase and xylanase in broiler diets reduces the intestinal digesta viscosity (Chiang et al., 2005; Choct et al., 1995; Dusel et al., 1998; García et al., 2008b; McDonald et al., 1973; Moftakharzadeh et al., 2017; Shirzadi et al., 2009; White et al., 1983). The ability of NSPases to reduce digesta viscosity has also been demonstrated with their addition to barley-based broiler diets (Almirall et al., 1995; Gracia et al., 2003; Józefiak et al., 2005; Józefiak et al., 2006). This can be explained by following reasoning. Soluble NSPs of large molecular size increase intestinal viscosity (Wettstein et al., 2000). Degradation of soluble NSPs by NSPases into smaller fragments reduces their molecule size and weight (Harris & Ramalingam, 2010; Heng et al., 1997). Reduction of molecular size reduces their solubility in aqueous solutions (Wood et al., 2000), which in turn reduces intestinal digesta viscosity. Given that high digesta viscosity is a primary factor contributing to poor nutrient digestibility in barley-based diets, it can be hypothesized that NSPase supplementation may potentially mitigate this issue, leading to improved nutrient digestibility and broiler performance.

However, contradictions are present in literature where the authors were unable to see the effect of NSPase supplementation on jejunal digesta viscosity. For example: it was found that xylanase supplementation in broiler diets did not appear to reduce intestinal digesta viscosity (Amerah et

al., 2008b), and a combination of β-glucanase and xylanase in barley-based diets showed no effect on intestinal digesta viscosity (Perera et al., 2020). The authors of the former study observed high variability in digesta viscosity among birds from same dietary treatments and reasoned the minimality of negative effects of digesta viscosity if it is below 10 centipoise (cP). The same could be a reason for nonsignificant effect of NSPase on digesta viscosity in the latter study; however, another possible explanation for this could be that the variety of barley used might have low concentrations of β-glucans which was evident from surprisingly low viscosity values even without NSPase supplementation.

These studies suggest that the lack of sufficient target molecules (β-glucans) in the cereal grains may be one major cause for the demonstration of minor or nonsignificant NSPase efficacy. On the other hand, from the general consensus of most of the studies, it can be concluded that exogenous NSPases are capable of reducing the intestinal digesta viscosity produced by barley-based diets, and this capability may depend on the variety of barley used.

2.2.4 Effect of NSPase on Nutrient Digestibility

Most nutrients in cereal grains are trapped in the cereal endosperm (Chesson, 2001), which is not fully prone to opening up by feed milling (Dhakal, 2022). Enzymatic hydrolysis of nutrients especially starch in barley endosperm is dependent on the cell wall degradation (Andriotis et al., 2016). NSPase addition can help in breaking the endosperm cell walls thus making the nutrients available for endogenous enzymes (Bedford, 2022). However, the biggest factor influencing nutrient digestibility is digesta viscosity which can also be controlled by NSPase. There are many studies that suggest the beneficial impacts of NSPase supplementation on nutrient digestibility against soluble NSP fractions in cereal grains especially barley. It has been shown that β-glucanase supplementation in barley-based diets increased amylase, lipase and trypsin activity in broiler chickens (Almirall et al., 1995). A number of studies have shown NSPase induced improvement in AMEn (Friesen et al., 1992; Mathlouthi et al., 2002b), and digestibilities of starch (Almirall et al., 1995; Hesselman & Åman, 1986), protein (Almirall et al., 1995; Friesen et al., 1992; Hesselman & Åman, 1986; Mathlouthi et al., 2002b), and fats (Almirall et al., 1995; Dänicke, 2001; Friesen et al., 1992; Mathlouthi et al., 2002b) of barley-based broiler diets.

The high activity of endogenous enzymes may be attributed to the reduction in digesta viscosity by NSPase, which in turn may result in improved nutrient digestibility. Starch and protein

digestibility may be improved due to cell-wall destruction and the release of nutrients by NSPase. Improved fat digestibility could be related to the destruction of β-glucans by β-glucanases, thereby preventing the conjugation of bile acid with β-glucans and facilitating their re-uptake into the circulation.

On the other hand, Perera et al. (2020) could not find any effect of NSPase (β-glucanase and xylanase) supplementation on nutrient digestibility in barley-based broiler diets. The same experiment showed that the jejunal viscosity remained unaffected with the use of NSPase which could be a possible reason for unimproved nutrient digestibility. This suggests inadequate activity of the NSPase enzymes, their inactivation during feed processing, or the use of barley variety with low amounts of soluble β-glucans.

It can therefore be concluded that NSPases generally possess the potential to improve the nutrient digestibility of barley-based broiler diets, however exceptions due to the type of the cultivar used and feed processing conditions need to be taken into consideration. Therefore, further investigations are vital to improve the efficacy of exogenous NSPase in barley-based broiler diets.

2.2.5 Effect of NSPase on *Clostridium perfringens*

Increased prevalence of *C. perfringens* in broiler chickens fed barley-based diets may be attributed to the presence of soluble NSPs in barley (Annett et al., 2002; Kaldhusdal & Hofshagen, 1992). The mechanism by which soluble NSPs can act as predisposing factors for NE has been elucidated in section 2.1.5. NSPs lose their antinutritive nature when hydrolyzed by NSPase. NSPases may reduce the prevalence of *C. perfringens* in broiler ceca mainly by reducing the extent of predisposing factors by which they support the proliferation of this bacterial species.

Coccidiosis is a disease that increases the prevalence of *C. perfringens* (Al-Sheikhly & Al-Saieg, 1980). Intestinal damage by coccidia spp. may allow *C. perfringens* to proliferate and exhibit pathogenic behavior (Al-Sheikhly & Truscott, 1977). Derqaoui (2022) observed a significant reduction in coccidia load and GIT lesions in broiler chickens when broiler diets were supplemented by a mixture of xylanase and β-glucanase. Mathlouthi et al. (2002b) reported a link between β-glucanase supplementation and reduced cecal pH, suggesting the degradation of β-glucans into fermentable sugars and the production of VFAs by cecal microbiota. They also suggested that the resulting lower cecal pH may inhibit the growth of pathogenic bacteria,

potentially leading to improved performance of the birds. Moreover, NSPases may reduce the prevalence of *C. perfringens* by reducing digesta viscosity thus increasing the oxygen tension (Sun et al., 2015). Increased oxygen tension may be detrimental for the growth of anaerobic bacteria such as *C. perfringens*. This may be a reason for reduced *C. perfringens* counts in broiler ceca with xylanase supplementation (Choct et al., 2006).

Since soluble NSPs increase digesta viscosity, mucus secretion, bile-salt conjugation, and coccidia load, the use of NSPase may limit these factors responsible for prevalence and proliferation of *C. perfringens* in broiler chickens. Therefore, it can be hypothesized that inclusion of NSPase in barley-based diets may reduce the prevalence of *C. perfringens* in broiler ceca.

2.3 Grinding of Feed Ingredients

2.3.1 Particle Size Reduction - Feed Processing and Nutrition

Reduction in particle size of ingredients is a mandatory step in feed production and is usually achieved by grinding main ingredients such as cereal grains using a hammer mill or a roller mill. Reduction in particle size ensures proper homogenization of feed ingredients during mixing. Hammer mills are the most common type of grinding mills used in feed processing. Particle size of ingredients can be controlled by the hammer mill screen opening (HMSO) size, which is directly proportional to geometric mean diameter (GMD) of particles in a pellet (Lott et al., 1992; Selle et al., 2019; Siegert et al., 2018). Ideally, optimum grinding level and mixing assure that each and every pellet produced is a representative of whole diet. Pelleted diets are appealing to feed manufacturers and broiler farmers due to their potential benefits over the mash diets like easier handling and storage, reduced dust production, and improved growth performance. (Abdollahi et al., 2013; Chewning et al., 2012; Lv et al., 2015; Reece et al., 1985; Zang et al., 2009).

The structure of a whole pellet is called its macrostructure (quality, shape, size, and hardness) and it affects feed intake of birds (Svihus, 2006). Therefore, the factors affecting pellet macrostructure are essential to consider during feed manufacturing. Though not to a great extent, HMSO size is one of them. According to Muramatsu et al. (2015), pellet quality is least affected by grinding level, while other factors such as diet composition and conditioning time and moisture affect pellet quality to a greater extent. Pellet quality is the ability of a pellet to remain

intact during handling and storage (Cramer et al., 2003), and is measured as pellet durability index (PDI) and pellet hardness (Amerah et al., 2007a). Therefore, the effect of ingredient particle size on the PDI varies greatly in literature. For example, many studies have shown improvement in PDI due to fine grinding (Chewning et al., 2012) (Angulo et al., 1996; Muramatsu et al., 2013; Svihus et al., 2004). One possible reason for improved PDI in these studies can be attributed to increased particle surface area and increased number of contact sites for bonding among particles (Muramatsu et al., 2015). On the contrary, some studies have shown increased PDI with coarser grinding (da-Silva et al., 2018; Reece et al., 1986), whereas Amerah et al. (2007b) could not find any significant effect grinding level on PDI of wheat-based pellets. Contradictions in literature suggest the possibility of other factors affecting pellet quality like grain type and fiber content. Moreover, it has been shown that increasing the inclusion of barley in broiler diets reduces the PDI (Toghyani et al., 2022). The authors attributed the reduction in PDI to the fibrous nature of barley and increasing oil supplementation in barley-based diets. Amerah et al. (2007a) suggested that the optimum particle size for corn-based broiler diets should be between 600 µm and 900 µm. However, the particle size is usually kept between 800 µm and 1000 µm to ensure high grinding rate and pellet quality (Rubio et al., 2020), and is usually achieved through HMSO sizes between 3.0-mm and 4.5-mm (Svihus, 2006). The GMD of barley ground through 2.0-mm and 8.0-mm screen sizes were 648 µm and 1,249 µm, respectively (Perera et al., 2020). Additionally, the GMD values of fine and coarse barley-based pelleted diets in the same study were 215 µm and 263 µm, respectively (Perera et al., 2020). However, in feed processing, there are more disadvantages of finer grinding over coarser grinding. It has been reported that fine grinding increases the cost of production significantly by increased use of energy (Al-Rabadi, 2013; Behnke, 1996; Svihus, 2006; Wondra et al., 1995) and reduced milling production rate (Al-Rabadi, 2013; Wondra et al., 1995). Moreover, fine grinding increases dust production during handling (Svihus, 2006). These reports suggest that pellet macrostructure is minimally affected by grinding level and its benefits may only be limited to feed handling and feed intake.

On the other hand, structure of the particles that make a pellet is called its microstructure and it is mainly responsible for the nutritional aspects of broilers including gizzard development and function (Svihus, 2006). Furthermore, particle size of ingredients is an important factor that may affect nutrient availability (Lyu et al., 2020). Usually finely ground pelleted diets are produced

for broilers. Theoretically, very fine and uniform grinding creates smaller microstructure and increases the surface area of the feed thus increasing the substrate concentration and consequently the rate of enzymatic digestion. Other than the feed production and quality problems, there are two possible nutritional problems that may arise with finely ground diets lacking in some coarse microstructure. First is the preference of broilers towards eating coarse feed particles (Portella et al., 1988). Second is the non-constructive role of fine feed particles towards gizzard development (Svihus, 2006). The first problem of selective pecking of feed particles by broilers can be controlled by pelleting. Either by coarse grinding of ingredients, or by the inclusion of particles like hulls, wood shavings or grit, the second problem can be resolved (Hetland et al., 2003). These studies indicate that the HMSO size should not be kept too small or too large in order to produce good quality pellets cost-effectively and to achieve nutritional benefits of the diet.

2.3.2 Effect of Grinding Level on Gizzard Function and Broiler Performance

The gizzard, also known as the ventriculus, is the posterior chamber of the gastric apparatus of the chicken, and the proventriculus is the anterior one. Both chambers are connected by an isthmus. Proventriculus is often termed as the glandular stomach because of active secretion of enzymes and hydrochloric acid (HCl), whereas gizzard is termed as the muscular stomach because of its vigorous grinding capacity (Duke, 1992; Farner, 2013). It has been reported that chickens are omnivorous but tend to be granivorous (Klasing, 1999), and that the birds prefer to eat coarser particles in all age stages (Portella et al., 1988). These findings suggest a unique function of the gizzard which is described as follows. Function of the gizzard is active grinding of ingesta to reduce its particle size and provide more surface area for endogenous enzymes to attack (Farner, 2013; Klasing, 1999). The gizzard is equipped with two pairs of smooth muscles and an inner surface containing rod-like projections that form a thick sand-like layer known as koilin (Klasing, 1999; Svihus, 2014a). The gizzard contracts four times per minute (Svihus, 2011b), and feed is ground by the friction of feed particles against each other and against the koilin layer (Klasing, 1999). The koilin layer also protects the mucosa from ulceration because of HCl and proteinases (Klasing, 1999).

Feed pellets quicky disintegrate in the anterior GIT of broiler chickens thus separating coarser and finer particles (Engberg et al., 2004; Svihus, 2006; Zaefarian et al., 2016). The gizzard tends to retain the coarse particles until they are finely ground (Hetland et al., 2002). This stimulates

the active use of muscles in the gizzard (Hetland et al., 2005). Stimulation of the gizzard muscles results in muscular hypertrophy, which is described by increased gizzard size and weight (Abdollahi et al., 2019). Increased gizzard size increases its capacity to hold the amount of ingesta and mainly coarse particles (Hetland et al., 2003; Nir et al., 1994). Increased amount of coarse particles in the gizzard increases the feed retention time in the gizzard (Nir et al., 1994). Such conditions in the gizzard stimulate the gastro-duodenal refluxes and may increase the contact of nutrients with gastric juices (Hetland et al., 2003). Therefore, a well-developed gizzard plays active role in regulating the digestive system function (Svihus, 2011b).

The effect of feed particle size on gizzard development has been extensively reviewed and there is a general agreement that inclusion of coarse particles in broiler diets increases the gizzard size. Studies have shown that coarser grinding of corn-based pelleted diets increases gizzard weight in broilers as compared to finer grinding of corn-based pelleted diets (Amerah et al., 2008a; Chewning et al., 2012; Kheravii et al., 2017; Nir et al., 1994). It has been shown that increasing the coarseness of diets increased gizzard size in 7-d old (Biggs & Parsons, 2009), 25-d old, (Svihus et al., 2010), 38-d old (Hetland et al., 2002). These findings indicate the presence of gizzard development capacity in broilers of all age groups. Following literature suggests the effect of increasing coarseness of various diets on gizzard development. Coarsely ground (HMSO: 6-mm) wheat-based diets increased gizzard size as compared to finely ground (HMSO: 2-mm) wheat-based diets (Péron et al., 2005). Whole wheat-based diet increased gizzard weight as compared to ground (HMSO: 3-mm) wheat-based diet (Hetland et al., 2002). Increased gizzard size was seen with feeding whole barley-based diets as compared to whole wheat-based diets in 38-d old broilers (Hetland et al., 2002). Inclusion of coarsely ground barley hulls in broiler diets increased gizzard size as compared to finely ground hulls (Sacranie et al., 2012). Coarsely ground (HMSO: 8-mm) barley-based diets increased gizzard size as compared to finely ground (HMSO: 2-mm) barley-based diets (Perera et al., 2020). These findings suggest that the major function of the gizzard is feed grinding, and the gizzard needs coarse particles for proper development as the grinding activity can be seen in birds of all age groups. Moreover, the extent of gizzard development by coarse microstructure highly varies and depends on the origin of the structural components other than the HMSO size (Hetland et al., 2004). Hulls, wood shavings and grit are some examples of structural components that can be added to broiler diets for stimulation of gizzard function. The extent of increase in gizzard size in broilers and layers by various structural

components has been shown in **Table 2.1**. According to these findings by (Hetland et al., 2003), inclusion of less than 4% wood shavings in diet caused a 50% increase in the gizzard weight whereas the inclusion of 40% whole wheat increased the gizzard weight only by 10% in layers. On the other hand, the same study showed that increase in gizzard weight by inclusion of oat hulls with grit in ground wheat- and whole wheat- based broiler diets was 59 and 33%, respectively. This finding suggests that the supplementation of structural components in a diet lacking adequate coarse microstructure stimulates gizzard to a greater extent as compared to a diet already containing sufficient coarse particles.

Table 2.1 Effect of structural component inclusion in wheat-based diets on relative weight of gizzard in 33-day old broilers and 29-week-old layers. Modified from Hetland et al. (2003).

Diet + Structural Components	**Experimental Birds**	**Relative Gizzard Weight[1]**	
		Ground wheat[2]	Whole wheat[3]
Basal diet	Broilers	2.06	2.79
Basal diet + OH[4]	Broilers	2.60	3.01
Basal diet + Grit[5]	Broilers	2.54	3.10
Basal diet + Grit + OH	Broilers	3.28	3.71
Basal diet	Layers	1.09	1.20
Basal diet + Wood shavings[6]	Layers	1.57	1.54

[1]Weight of gizzard relative to the body weight shown in percentage (%).
[2]Ground wheat (HMSO: 3-mm): 769 g/kg for broilers and 707.5 g/kg for layers.
[3]Whole wheat: 385 g/kg for broilers plus ground wheat and 400 g/kg for layers plus ground wheat.
[4]Oat hulls: 100 g/kg.
[5]Grit: 5 g (thrice a week).
[6]Wood Shavings: 10 g twice a week from 15 – 25 weeks of age; thrice a week from 26 – 29 weeks of age.

It is a general consensus that a coarse microstructure can influence broiler performance mainly by affecting the gizzard functionality. Amerah et al. (2008a) observed a significant improvement in broiler FCR when coarsely-ground (HMSO: 7-mm) diets were used as compared to finely-ground (HMSO: 1-mm) diets based on wheat and corn. Another broiler study found improvement in FCR with coarsely-ground (HMSO: 5-mm) corn-based diets than with finely-ground (HMSO: 3-mm) diets (Marx et al., 2021). Inclusion of 15% unground barley or oat hulls in wheat and corn based diets improved FI, BWG and FCE as compared to inclusion of same amount of finely-ground (HMSO: 1-mm) hulls (Sacranie et al., 2012). These studies attributed improved performance to prolonged feed retention time, increased gizzard activity and thereby improved nutrient digestion.

However, the effect of coarse particles on growth performance may depend on their origin and level of inclusion. It has been shown that increasing the inclusion of whole wheat from 35 to 50% in broiler diets reduced the BWG and FCE (Biggs & Parsons, 2009). In their study, Hetland et al. (2002) observed a reduction in FI and BWG in broilers when whole grains were offered instead of ground grains. The authors (Hetland et al., 2002) reasoned that the inclusion of whole grains might have induced satiety in birds, leading to reduced FI. They further suggested that active grinding of feed in the gizzard requires energy, which may explain why the final output was not significantly affected. Furthermore, many studies could not find any significance effect of different grinding levels on broiler performance (Lott et al., 1992; Lv et al., 2015; Reece et al., 1986; Rezaeipour & Gazani, 2014; Zang et al., 2009). This could be mainly because of lack of significant difference in HMSO size and/or further grinding during the pelleting process, thus resulting in an overall lack of significant coarseness in the coarsely ground diets. Another reason could be the difference in grain types. It has been stated that grinding of different grain types under same milling conditions may result in different particle size distributions (Amerah et al., 2007a; Healy et al., 1991). For example, wheat kernels with harder endosperm result in a more uneven particle size during grinding (Rose et al., 2001). Since a normal barley kernel contains a hard hull, it may produce even greater proportion of unevenly coarse particles. This claim can be supported by the study by Perera et al. (2019) in which increasing the proportion of barley in wheat-based broiler diets ground through HMSO size (3-mm) consistently increased the gizzard size. These findings suggest that grinding hulled barley grains through a smaller HMSO like 3-mm may produce hulls with sufficient coarseness for gizzard development. Moreover, it was shown that the extent of gizzard development by a diet containing 44% whole wheat and 44% ground oats was similar (Hetland et al., 2002). Therefore, it can be hypothesized that finely ground hulled barley-based diets may not require any additional coarseness due to the presence of hulls as structural components, which thereby may be better for growth performance.

2.3.3 Effect of Grinding Level on Jejunal Digesta Particle Size

The effect of feed grinding level on jejunal digesta particle size can be variable and mainly depends on the form of feeding. Generally, the effect of grinding on digesta particle size is more pronounced in mash diets whereas this effect seems to be lessened in pelleted diets. This is mainly because the friction inside the pellet die causes additional grinding of the mash (Abdollahi

et al., 2011). It was shown that the pelleting process tends to even out the particle size distribution of wheat-based diets ground using different types of mills and screen sizes (3-mm and 6.1-mm) (Svihus et al., 2004). Probably due to this reason, Amerah et al. (2007b) could not observe any significant effect of screen sizes (3-mm and 7-mm) on digesta particle size. Theoretically, this may not be the case with barley-based diets due to the presence of hulls. When ground through 3-mm screens, barley-based (hulled barley: 56%) diet significantly increased gizzard size as compared to wheat-based (wheat: 63%) diet (Perera et al., 2019). From this study, it appears that some of the barley hulls or kernels may retain their size even after pelleting. A broiler study showed that inclusion of whole barley grains in the wheat-based diet reduced the mean particle size in duodenum as compared to ground barley grains (Hetland et al., 2002). Another study showed similar results when birds were given access to wood-shavings in the litter (Hetland & Svihus, 2007). These findings suggest that the particles that retain their coarseness post-pelleting contribute to gizzard development and are retained in the gizzard until ground. However, the results of coarsely ground barley-based diets may be different than the results of diets containing whole barley. The presence of sufficient coarseness in finely ground barley-based diets may result in smaller particle size in the jejunum as compared to coarsely-ground barley-based diets. This could be due to the assumption that both, finely and coarsely ground barley-based diets can stimulate gizzard development due to the presence of hulls, but the gizzard might have to exert lesser effort in further grinding of finely-ground diet and may do it more efficiently thus producing a smaller jejunal digesta particle size. However, further investigations are necessitated in this regard.

2.3.4 Effect of Grinding Level on Jejunal Digesta Viscosity

It is generally considered that the pellet microstructure does not mainly contribute to the intestinal digesta viscosity in broilers. However, different concepts are pursued differently in this regard. The extent of viscosity production may depend upon the fineness of the particles post gizzard grinding and thus the ease in the release of soluble NSP fraction. Due to the unvaried proportion of fine particles in the duodenum produced by diets with finer or coarser microstructure, as demonstrated by Hetland et al. (2002), it may be difficult to propose their effect on digesta viscosity. For example, a study showed that inclusion of whole wheat (200g/kg) in wheat-based pelleted diet increased intestinal digesta viscosity (Taylor & Jones, 2004). This

might be due to increased proportion of fine particles in the duodenum because of the gizzard stimulatory effect of whole wheat. Another broiler study showed quite opposite results where finely ground (HMSO: 4-mm) wheat-based diet resulted in significantly higher (almost double) ileal digesta viscosity as compared to coarser (HMSO: 5-mm/6-mm/7 mm) wheat-based diets (Yasar, 2003). The author suggested that finely ground diet might have released higher amounts of soluble NSP that might have increased the viscosity. On the other hand, most of the broiler experiments were unable to produce any effect of particle size on digesta viscosity (Amerah et al., 2008b; Engberg et al., 2002; Perera et al., 2020; Tejeda & Kim, 2021; Wu et al., 2004). These studies suggest that the feed particle size might not be a major contributor to the jejunal digesta viscosity. However, further research is required to study this effect in barley-based diets.

2.3.5 Effect of Grinding Level on Nutrient Digestibility

The stimulation of gizzard by coarse particles and its relationship with digestion has been mentioned in section 2.3.2. Increased gizzard grinding efficiency by coarse microstructure in pellets is assumed to be positively linked with nutrient digestibility in birds. A well-developed gizzard may result in reduced digesta particle size in duodenum (Hetland et al., 2002). Reduction in particle size increases substrate availability for endogenous enzymes. Furthermore, the gizzard may retain coarse particles for an extended period, leading to increased secretion of pancreatic enzymes and bile, and stronger gastro-duodenal refluxes may result in increased contact of feed particles with digestive juices (Hetland et al., 2003). Many studies have shown that with increased coarseness of diets, the digestibilities of starch (Ruhnke et al., 2015; Selle et al., 2019) and protein (Kheravii et al., 2017; Perera et al., 2020; Selle et al., 2019) may be improved. Higher starch digestibility was achieved when whole diets based on whole barley were fed to broilers instead of ground barley-based diets (Hetland et al., 2002). Another study has shown improved starch digestibility by inclusion of oat hulls in broiler diets (Hetland et al., 2003). A study by Abdollahi et al. (2019) showed similar effects of oat hulls, wood shavings, and whole wheat inclusion on starch digestibility in broilers. However, the experiments by Hetland et al. (2003) and Svihus et al. (2017) failed to show the effect of grit inclusion on starch digestibility of broiler diets so further research to understand this mechanism is required. Even though some studies have found positive impact of coarser grinding on nutrient digestibility in broiler chickens, variations do exist in literature. For example, Péron et al. (2005) found a significant increase in

starch digestibility in broilers fed finely ground (HMSO: 2-mm) wheat-based diets as compared to coarsely ground (HMSO: 6-mm) wheat-based diets. The authors reasoned that due to the high hardness of wheat, the grinding efficiency of gizzard with coarsely ground diets could have been reduced, whereas easier access of endogenous enzymes to nutrients with finely ground diets could be reason of better starch digestibility. Pourazadi et al. (2020) found non-significant effects of fine (HMSO: 1-mm) and coarse (HMSO: 3-mm) grinding of each ingredient-based-diet on protein digestibility. A possible reason behind these results could be the lack of significant coarseness in the diets produced. Since the finely ground barley-based diets may provide adequate coarseness due to the presence of hulls, nutrient digestibility may be enhanced by increased gizzard function and easier availability of nutrients to endogenous enzymes. However, further investigations are required to reach a final conclusion.

2.3.6 Effect of Grinding Level on *Clostridium perfringens*

Diets with different coarseness may behave differently in the broiler GIT and may affect the prevalence of *C. perfringens* in the ceca mainly by affecting nutrient digestibility. A commonly accepted notion is that higher counts of *C. perfringens* in the ceca are associated with diets having poor nutrient digestibility. As the presence of coarse microstructure is positively linked with enhanced nutrient digestibility, fewer nutrients may be available for the pathogenic bacteria to feed on. This statement can be supported a study by Engberg et al. (2004) in which they observed the tendency of reduction in *Clostridium* spp. count in broiler ceca when whole wheat-based was fed as compared to wheat-based pelleted diets. Moreover, it was shown that wheat-based diet ground using a hammer mill (HMSO: 4.8-mm) showed much higher NE-associated mortality in broilers as compared to the diet ground with a roller mill (Branton et al., 1987). In the aforementioned study, the hammer mill produced lesser proportion of coarse particles as compared to the roller mill. This might have resulted in a non-stimulatory effect on gizzard and thereby poor nutrient utilization. According to Engberg et al. (2002), undigested nutrients may get utilized by *C. perfringens*, thus raising their counts. Thus, if finely ground barley-based diets succeed in generation of adequate gizzard stimulatory activity and enhance nutrient utilization, it may be assumed that the prevalence of *C. perfringens* in broiler ceca may get reduced. Since the effect of grinding level of barley-based diets on the prevalence of *C. perfringens* has not been previously studied, research is needed to reach a conclusion.

2.4 Feeding Regimens in Broiler Farming

2.4.1 Feeding Regimen - Background

Despite the reported advantages of NSPase supplementation in broiler diets, their efficacy in barley-based diets needs to be improved. The ability of NSPase to digest soluble NSP depends upon multiple factors such as their level of inclusion in feed, proportion of soluble NSP in feed, feed processing conditions, and the presence or absence of optimum conditions in the digestive tract of the animal (Svihus, 2011a). Feeding regimens influence the GIT functionality and may affect NSPase efficacy by affecting the conditions. Currently, most of the farms are using *ad libitum* feeding regimen in which the birds are free to feed most of the time of the day. Intermittent feeding, on the other hand, is another feeding regimen in which feeding is held most of the time and is released only during certain hours of the day. Intermittent feeding is usually controlled by regulating the photoperiod (lighting period) or scotoperiod (darkness period), and in ideal conditions it can be controlled by lifting the feeders or feed withdrawal. Intermittently-fed broilers develop a habit of eating much more at the start of dark period as compared to *Ad libitum* fed broilers who do not prefer to eat in the dark period (Rodrigues & Choct, 2019). It has been shown that broilers can quickly adapt to intermittent feeding (Svihus et al., 2013). Moreover, it is widely accepted that intermittent feeding may be beneficial for broiler birds, as it has been observed to positively affect the crop functionality (Buyse et al., 1993; Klasing, 1999; Svihus et al., 2013; Svihus et al., 2010).

2.4.2 Effect of Feeding Regimen on Crop Function and Broiler Performance

Bird crop, also known as ingulvies, is a diverticulated extension of esophagus. The inner lining (epithelium) of the crop is non-keratinized (Classen. et al., 2016), and possesses high degree of mucosal folds (Turk, 1982). These folds allow the crop to expand upon ingestion of large amounts of feed (Klasing, 1999). The contraction and relaxation of two muscular layers (inner circular, outer longitudinal) of crop wall help in peristalsis (Classen. et al., 2016; Turk, 1982). The crop can be used as a transient feed storage organ (Kierończyk et al., 2016; Klasing, 1999; Svihus, 2014a; Ziswiler & Farner, 1972). It the crop is empty, the feed material upon ingestion goes directly to the proventriculus (Klasing, 1999) or gizzard (Buyse et al., 1993). Once the proventriculus and the gizzard are filled, the feed starts to accumulate in the crop (Klasing, 1999).

However, the capacity of the crop to hold feed is much greater than that of the gizzard (Svihus, 2014a). That is why the crop is often termed as a transient feed storage organ.

Feed is thoroughly moistened in the crop by water, saliva and mucous (Klasing, 1999). Birds secrete minute amounts of salivary amylase (Rodeheaver & Wyatt, 1986). Lactobacilli strains isolated from crop also produce amylase (Champ et al., 1983). Amylases carry on starch hydrolysis in the crop to a smaller extent (Bolton, 1965). As a result of starch hydrolysis in the crop, glucose, fructose and maltose are produced (Pritchard, 1972). The glucose thus produced can be directly absorbed through crop epithelium (Kierończyk et al., 2016). Some sugars can undergo bacterial fermentation and result in the production of lactic acid and acetic acid as byproducts (Bayer et al., 1978; Bolton, 1965). The authors implied that other VFAs could also be produced in the crop. Sacranie et al. (2012) observed a strong effect of intermittent feeding on lowering the gizzard pH as compared to *ad libitum* feeding. They correlated it with stimulation of fermentation in the crop in intermittent feeding and production of VFAs causing a lower pH. The major fermenters in the broiler crop are *Lactobacilli* (Gong et al., 2007; Józefiak et al., 2006). It has been reported that some strains of *Lactobacilli* possess the capability to secrete β-glucanases that can hydrolyze β-glucans (Jonsson & Hemmingsson, 1991; Skrede et al., 2003; Skrede et al., 2002). Therefore, it can be assumed that the presence of such strains in the crop may trigger hydrolysis of β-glucans, which may be enhanced using intermittent feeding. Previously, extracellular 1,3-1,4-β endoglucanase-producing bacteria in the hindgut of broilers have been identified (Beckmann et al., 2006). However, due to the lack of solid evidence, the presence of β-glucanase-producing strains in the crop needs to be tested. Moreover, higher amounts of lactic acid and lower pH in the crop were observed when barley-based diets were supplemented with NSPase (Józefiak et al., 2006). This study indicates that exogenous NSPase may start working in the crop and that enzymes may work better in intermittent feeding regimen.

Buyse et al. (1993) has shown that intermittently fed broilers rapidly store large amount of feed in anterior portion of GIT during start of dark periods and then gradually release the digesta towards small intestine throughout the dark (fasting) period. Increasing the period of feed restriction increases the feed holding capacity of the crop by increasing its size (Fondevila et al., 2020). On the contrary, *ad libitum* fed broilers do not eat continuously and take frequent breaks between feed bouts (Svihus et al., 2013). This observation can be supported by studies in which *ad libitum* fed broilers did not fully use crop as a storage organ (Nielsen, 2004; Svihus et al., 2010). These

findings suggest differences in feeding habits and crop function of broilers in both feeding regimens.

Feeding regimens can affect the growth performance of the broiler chickens mainly by influencing the feeding behavior and functionality of the crop. The effect of feeding regimens on FI is variable. Some studies have shown higher FI in *ad libitum* fed broilers due to continuous availability of feed as compared to in intermittent feeding (Rahimi et al., 2005; Rodrigues et al., 2018; Rosebrough et al., 1989; Sacranie et al., 2017). Others have demonstrated the remarkable capacity of intermittently fed broilers to compensate for lesser feed access time by eating as much as *ad libitum* fed broilers (Sacranie et al., 2012; Svihus et al., 2013; Svihus et al., 2010). The BWG response in both feeding regimens may also be variable. For example, Sacranie et al. (2017) observed an increase in BWG of *ad libitum* fed broilers as compared to intermittently fed birds. In the previous study, improvement in BWG of broilers from the *ad libitum* fed group could be attributed to the increase in FI. On the other hand, many studies have shown improvement in BWG by intermittent feeding as compared to *ad libitum* feeding (Cave et al., 1985; Farghly et al., 2019; Ohtani & Leeson, 2000). Buyse et al. (1994) have shown that intermittently fed broilers show a slower initial growth with a higher compensatory growth later on. Higher compensatory growth improves FCR in broilers (Buyse et al., 1994). This is because the birds may find it challenging to adapt to intermittent feeding initially (Buyse et al., 1996), but later on get accustomed to making maximum use of the time in feed consumption (Sacranie et al., 2012). As the intermittently fed birds appear to store most of the feed in the crop in a short amount of time (Svihus et al., 2010), this moisturizes the feed well and increases its retention time in the anterior GIT (Svihus, 2014a). Softening of feed by moisturization and increased feed retention time may allow endogenous and bacterial enzymes to catalyze hydrolysis of target molecules to a greater extent. This, in turn may improve nutrient utilization, which is linked with improvement in FCR. This mechanism suggests improvement in FCR of the intermittently fed broilers despite their variable FI response, and several studies have affirmed this assumption (Deaton et al., 1978; Farghly et al., 2019; Rahimi et al., 2005; Svihus et al., 2013; Svihus et al., 2010; Yang et al., 2015). The authors attributed this improvement in broiler performance mainly to the enhanced crop functionality in the intermittently fed broiler chickens. Another study has shown the tendency of intermittent feeding to improve AME despite high FI and BWG by the *ad libitum* fed broilers (Sacranie et al., 2017). In the pervious study, this improvement in AME

nullified the difference in FCE between both regimens. A possible explanation for improvement in AME by intermittent feeding could be lower ME requirements of the intermittent group as demonstrated by (Ketelaars et al., 1986). Another broiler study could not find any significant effect of feeding regimens on broiler performance (Sacranie et al., 2012). These studies suggest the exceptional ability of the broiler chickens to adapt to the intermittent feeding regimen. Therefore, it can be postulated that intermittent feeding may improve broiler performance by positively influencing crop functionality.

2.4.3 Effect of Feeding Regimen on Jejunal Digesta Viscosity

Little progress has been made to study the effect of feeding regimens on jejunal digesta viscosity. It may be assumed that *Lactobacilli* working in the crop may break down β-glucans to some extent as mentioned in section 2.4.2, and thus may reduce the digesta viscosity. However, this proposal did not appear to work in a broiler study where increased jejunal digesta viscosity was seen in intermittently fed birds as compared to *Ad libitum* fed birds (Rodrigues et al., 2018). This finding may be explained by the fact that the viscosity values were recorded at the peak of NE infection in the birds which could have affected the results and the values may be different in healthy birds. In another study, feeding regimens did not appear to affect digesta viscosity (Chaudhary, 2013). Previous studies may be inadequate to prove the effect of feeding regimen on digesta viscosity and therefore, more research is needed in this regard.

2.4.4 Effect of Feeding Regimen on Nutrient Digestibility

The effect of feeding regimens on nutrient digestibility has not been extensively studied. Ingesta moisturization, digestion, and fermentation taking place in the crop may help in easier reduction of particle size in the gizzard, thus may be assumed to render increased proportion of fine particles in small intestine which may improve nutrient digestibility. But previous studies could not find any significant effect of intermittent feeding on starch digestibility of wheat-based diets (Sacranie et al., 2012; Svihus et al., 2010). The actual cause of lack of effect on starch digestibility is unknown. Buyse et al. (1996) suggested that improvement in growth performance in intermittently fed broilers may not be due to improvements in nutrient availability but due to reduction in maintenance energy requirements of broilers. However, these findings do not negate the chances of improved nutrient digestibility of barley-based diets under intermittent feeding regimen. Thus, more research is needed to reach a conclusion.

2.4.5 Effect of Feeding Regimen on *Clostridium perfringens*

Literature does not suggest any effect of feeding regimen on the prevalence of *C. perfringens* in broiler ceca. According to a study on sub-clinically NE-affected broilers, intermittently fed broilers showed increased endurance against the disease (Rodrigues et al., 2018). This may be because the FCE in intermittent fed broilers was not as much affected as in *ad libitum* fed broilers. This result can be supported by the fact that *ad libitum* feeding increases the maintenance of requirements of the birds (Ketelaars et al., 1986) and therefore, the birds usually get lower energy to endure against the disease. However, it may be assumed that the intermittent feeding program may lead to a decrease in digesta viscosity due to higher fermentation of β-glucans by *Lactobacilli*, resulting in improved nutrient digestibility and potentially reducing the prevalence of *C. perfringens*; nevertheless, additional research is vital to substantiate this hypothesis.

2.5 Interaction Between NSPase and Grinding Level

It is clear from the previous literature that increased nutrient digestibility by increased coarseness of the diets have been linked with a well-stimulated gizzard and increased endogenous enzyme activity post-gizzard grinding. However, changes in scenario with the inclusion of NSPase in finely ground barley-based diets may lead to a different outcome. For gizzard development and high grinding efficiency, appropriate proportion of structural components like hulls in finely-ground barley-based diets may be sufficient. Moreover, with fine grinding, the surface area of diets is increased. This may facilitate the activity of NSPase by providing easier and faster accessibility to the substrates. Increasing the substrate concentration has a positive effect on enzyme activity but is linked to the concentration of enzymes (Immanuel et al., 2006). However, literature offers contradictory views. In their studies, Wu et al. (2004) and Amerah et al. (2008b) observed better FCR with xylanase supplementation with coarse wheat-based diets as compared to diets with finely-ground wheat-based diets. This contradiction with the current assumption is justifiable as it could be due to the lack of adequate coarseness in the finely ground wheat-based diets and that xylanase might not have gotten enough time exhibit the activity pre-gizzard grinding due to *ad libitum* feeding. Moreover, a finely ground barley-based diet will have much more coarseness as compared to a finely ground wheat-based diet so opposite effects may be expected.

2.6 Interaction Between NSPase and Feeding Regimen

Hypothetically, intermittent feeding may improve NSPase functionality as literature in section 2.4.2 supports the onset of enzymatic (Pritchard, 1972) and microbial digestion (Józefiak et al., 2006) in the crop. Supplementation of NSPase in diet can enhance the fermentation activity by *Lactobacilli* (Józefiak et al., 2006). With increased retention time in the crop, the efficacy of exogenous NSPase and microbial enzymes to act on their respective substrates may increase. Literature on interaction of NSPase and feeding regimen is limited therefore it is difficult to conclude anything before the experiment. Moreover, some studies on the effect feeding regimen on phytase efficacy have been concluded but with contradictory results. For example, in a broiler study by Svihus et al. (2013), intermittent feeding did not improve exogenous enzyme (phytase) function. The authors reasoned that unimproved phytase efficacy in intermittent feeding might be due to reduction in gizzard pH, thus making conditions unsuitable for phytase action. On the other hand, Sacranie et al. (2017) observed improved nitrogen and mineral retention in broilers when phytase was used in intermittent feeding as compared to the use of phytase in *ad libitum* feeding regimen. Furthermore, Kristoffersen et al. (2021) showed improved phytase efficiency with increasing the feed retention time in crop using intermittent feeding and diet acidification suggesting the increased potential of intermittent feeding in improving the efficacy of exogenous enzymes. Moreover, Fondevila et al. (2020) observed that increasing the feed restriction time in intermittent feeding does not affect crop pH significantly. This suggests that the reduction in crop pH by diet acidification may not be affected by intermittent feeding. From the above-mentioned findings, it may be reasonable to hypothesize that intermittent feeding could potentially enhance NSPase activity through several mechanisms. These mechanisms may include increased feed moisturization, increased retention time of feed, earlier onset of NSPase action, and maintenance of stable pH levels. However, further research and experimentation would be required to validate this hypothesis and draw definitive conclusions.

2.7 Interaction Between Grinding Level and Feeding Regimen

It has been suggested that increased feed moisturization and prolonged retention time may onset the digestion in the crop (Classen. et al., 2016; Svihus, 2014a). Increasing feed retention time in the crop increases its moisture content reaching up to 60% within 90 minutes of feeding (Svihus et al., 2010). This may result in softening of ingesta (Classen et al., 2016). The presence of

structural components in the diet may increase the extent of grinding of the gizzard (Hetland et al., 2002). A well-developed gizzard may effectively grind softer feed particles and facilitate the reduction of particle size. This may increase their contact with digestive tract juices and enzymes, thus improving nutrient digestibility and thereby growth performance. These arguments may be supported by the studies reporting that softer wheat kernels with better hydration pattern may have higher nutrient digestibility (Wiseman, 2006), and that reduction in endosperm hardness and mean particle size of feed improves starch digestibility (Carré et al., 2005). Therefore, it may be suggested that finely ground barley-based diets fed under intermittent feeding regimen may show higher nutrient digestibility and growth performance as compared to coarsely ground barley-based diets fed under *ad libitum* feeding regimen. A study by Svihus et al. (2013) has shown that less initial adaptiveness of birds to the intermittent feeding resulted in lower BW, which by the addition of coarse oat hulls, was improved later on. However, some studies have shown that coarseness of the diets did not interact with the feeding regimen to improve broiler performance (Sacranie et al., 2012; Svihus et al., 2010). These studies were conducted using wheat-based diets, and results may be different with barley-based diets. Moreover, the absence of any negative impact due to the combined effects of increased diet coarseness and intermittent feeding on growth performance in these studies suggest that diets with coarse microstructure may be fed to broilers under intermittent feeding.

2.8 Interaction Between NSPase, Grinding Level and Feeding Regimen

The possibility of interactions of NSPase with grinding and feeding have been explained in sections 2.5 and 2.6, respectively. Svihus (2014a) stated that the function of crop and gizzard in broilers can be stimulated by intermittent feeding and structural components, respectively. The function of anterior GIT needs to be investigated by the use of different barley particle sizes and feeding regimens. It is also necessary to determine whether these factors work synergistically, antagonistically, or independently to influence the efficacy of exogenous NSPase for the improvement of nutrient digestibility and growth performance in broiler chickens. From the current literature, it is difficult to devise a conclusion, therefore, further investigations are required to study these interactions.

2.9 Research Question

Can barley-based diets be improved by NSPase supplementation using optimal feed particle size and feeding regimen?

2.10 Research Objective

The objective of the study was to optimize the use of barley (*Hordeum vulgare L.*) in broiler diets using NSPase, feed particle size and feeding regimen while mitigating its negative impacts on nutrient digestibility and growth performance.

2.11 Hypotheses

Following hypotheses were tested in the current study:

i. NSPase supplementation will interact in a two-way interaction with fine grinding or intermittent feeding regimen to improve broiler performance.
ii. NSPase supplementation will interact in a three-way interaction with fine grinding and intermittent feeding regimen to improve broiler performance.
iii. NSPase supplementation will interact in a two-way interaction with fine grinding or intermittent feeding regimen to improve nutrient digestibility.
iv. NSPase supplementation will interact in a three-way interaction with fine grinding and intermittent feeding regimen to improve nutrient digestibility.
v. Broilers fed with NSPase supplemented finely ground diet under intermittent feeding regimen will have lesser prevalence of *C. perfringens* in ceca as compared to those fed with coarsely ground diet without NSPase under *ad libitum* feeding regimen.

3 Research Methodology

3.1 Experimental Design

The experiment was designed as a three-factor full factorial with each factor consisting of two levels (2 x 2 x 2). Four diets having NSPase (supplemented, not supplemented) and hammer mill grinding screen opening size (2-mm, 6-mm) were divided into two halves between broilers reared under *ad libitum* and intermittent feeding regimens as shown in **Table 3.1**.

Table 3.1 Experimental design of the broiler experiment.

NSPase[1]	Grinding Level[2] (mm)	Feeding Regimen[3]
-	2	*Ad libitum*
+	2	*Ad libitum*
-	6	*Ad libitum*
+	6	*Ad libitum*
-	2	Intermittent
+	2	Intermittent
-	6	Intermittent
+	6	Intermittent

[1]NSPase: cocktail of beta-glucanase and xylanase supplementation; "**+**" supplemented, "-" not-supplemented).

[2]Hammer mill screen opening size/sieve size in millimeters.

[3]Half of the broilers were fed *ad libitum* and half intermittently.

3.2 Feed Manufacturing

Barley-based experimental diets were formulated (**Table 3.3**) with and without NSPase. To achieve optimum pH (4.0 – 5.0) for NSPase, optimal quantity of a commercial feed acidifier (formic acid) was required to be added in the diets. To find out the best inclusion percentage of formic acid, a controlled acidification trial was conducted at Chemical Analysis Lab for Livestock and Aquaculture (LabTek, NMBU). Samples (1 g) of the manufactured diets were ground up to 1-mm, dissolved into 5 ml Reversed Osmosis (RO) treated water and initial pH was noted. Later on, formic acid (conc. 85%) was added at various proportions to the samples, and the final pH was noted 45 minutes (**Table *3.2***). Concluding the acidification trial, 0.7% of formic acid was estimated to be suitable for experimental diets.

Table 3.2 pH of barley-based diets before and after addition of formic acid.

Volume (µl) of Formic Acid (conc. 85%)	Initial pH[1]	Final pH[2]
7	6.40	4.77
7	6.74	4.81
10	6.64	4.66
10	6.47	4.49
15	6.64	4.28
15	6.57	4.24

Samples (1 g) from manufactured barley-based pelleted diets were ground and dissolved in 5 ml RO treated water, and pH values before and after addition of formic acid (conc. 85%) were noted.
[1]pH before addition of formic acid; [2]pH after 45 minutes of formic acid addition.

Table 3.3 Calculated composition of experimental diets.

Ingredient	g/kg diet	Ingredient	g/kg diet
Wheat (2- or 6-mm)	163.8	L-threonine	0.86
Barley (2- or 6-mm)	541.2/541.1[1]	L-arginine	1.82
Ronozyme MultiGrain (GT) 100g/T[1]	0.0/0.1[1]	Limestone	8.70
Maize (2- or 6-mm)	31.8	Monocalcium phosphate	2.10
Soybean meal (2- or 6-mm)	110.9	Sodium chloride	4.40
Fish Meal	14.5	Mineral and vitamin premix[2]	5.34
Wheat gluten	24.1	Choline chloride ARGAVIS 70%	0.67
Potato protein concentrate	33.8	Phytase AXTR 5000 FTU/g 200g/T[3]	0.2
Soy oil	38.6	Titanium dioxide (TiO_2)	5.0
L-lysine HCl	3.35	Formic acid 85%	7.0
DL-methionine	1.83		

[1]Ronozyme MultiGrain (DSM Nutritional Products (Heerlen, Netherlands) was added at the expense of normal barley: a multi-component NSP-degrading enzyme derived from *T. reesei* (fungus) and contains heat-stable (up to 90°C pelleting temperature) endo-1.4-β-glucanase (EC 3.2.1.4; 800 U/g), endo-1.3(4)-β-glucanase (EC 3.2.1.6; 700 U/g), and endo-1.4-β-xylanase (EC 3.2.1.8; 2700 U/g).
[2]Mineral and vitamin premix provided the following components per kg diet: Iron (Fe) 53 mg; Manganese (Mn) 128 mg; Zinc (Zn) 82 mg; Copper (Cu) 15 mg; Iodine (I) 1 mg; Selenium (Se) 0.3 mg; retinyl acetate (vitamin A 2115) 4.1 mg; cholecalciferol (vitamin D 2116) 0.08 mg; dl-α-tocopheryl acetate (vitamin E 2221) 33 mg; menadione 4.9 mg; thiamine 3.2 mg; riboflavin 9.6 mg; niacin 48 mg; calcium pantothenate 24 mg; pyridoxine 9.6 mg; cobalamin 0.032 mg; biotin 0.3 mg; folic acid 2.6 mg.
[3]Quantum Blue (AB Vista, Marlborough, UK – IUB: 3.1.3.26) phytase was obtained from Escherichia coli and classified as 6-phytase.

Barley-based diets (**Table 3.1**) were manufactured at Centre for Feed Technology (FôrTek, NMBU). Barley, wheat, maize, and soybean meal were either finely or coarsely ground to pass through a 2-mm or 6-mm sieve, respectively, in a hammer mill (Münch-Edelstahl, Wuppertal, Germany licenced by Bliss, USA, 18.5 kW, 2870 RPM) before being mixed with other ingredients. The mash was steam-conditioned at 60°C (for fine diets) and 68°C (for coarse diets), in a double pass pellet-press conditioner (Münch-Edelstahl, Wuppertal, Germany) and then pelleted using a pellet-press (Münch- Edelstahl, Wuppertal, Germany, 1.2 t/h, 2 × 17 kW, RMP 350) equipped with a 3-mm-diameter die and 36-mm effective length. All diets contained 0.7% formic acid (85%) which was sprayed and mixed with the pellets in a twin-shaft paddle mixer (Dinnissen, Netherlands). Titanium dioxide (TiO_2) was used as an indigestible marker. Diet production parameters are presented in **Table 3.4**. The nutritional analysis of experimental diets was performed at LabTek (NMBU) and is given in **Table 3.5**.

Table 3.4 Production parameters of experimental diets.

Diet[1]	NSPase[2]	Grinding (mm)	Pellet Durability Index	pH[3]
1	-	2	92.15	4.86
2	+	2	92.05	4.79
3	-	6	89.05	4.87
4	+	6	88.10	4.89

[1]The capacity and post-pelleting temperature for all diets were 700 kg/h and 82°C, respectively.
[2]NSPase: Ronozyme MultiGrain (DSM Nutritional Products (Heerlen, Netherlands): a multi-component NSP-degrading enzyme derived from *T. reesei* (fungus) and contains heat-stable (up to 90°C pelleting temperature) endo-1.4-β-glucanase (EC 3.2.1.4; 800 U/g), endo-1.3(4)-β-glucanase (EC 3.2.1.6; 700 U/g), and endo-1.4-β-xylanase (EC 3.2.1.8; 2700 U/g), "-" not supplemented, "+" supplemented.
[3]Post-manufacturing pellet pH (pellets from each experimental diet were sprayed with 0.7% formic acid (conc. 85%) and ground up to 1-mm; a 1g homogenous sample from each diet was dissolved in 5 ml RO treated water and pH was noted after 45 minutes.

Table 3.5 Analyzed composition of experimental diets.

Component Analysis	g/kg feed	Component Analysis	g/kg feed
Dry Matter (DM)	894	**Essential amino acids**	
Crude Protein (CP)	201	Arginine	10.3
Apparent Metabolizable Energy (AME) MJ/kg	11.85	Histidine	4.4
Starch	373	Isoleucine	7.0
Soluble NSP	37.9	Leucine	12.7
Insoluble NSP	85.4	Lysine	11.3
Total NSP[1]	123.3	Methionine	4.2
Fat	53	Phenylalanine	7.3
Crude Fiber	49	Threonine	7.2
Neutral Detergent Fiber (NDF)	129	Valine	6.7
Acid Detergent Fiber (ADF)	66		
Ash	46		
Mineral Profile		**Non-essential amino acids**	
Calcium	6.3	Alanine	6.3
Phosphorus	4.5	Aspartic Acid	11.5
Sodium	2.3	Cysteine	2.7
Potassium	7.0	Glutamic Acid	38.6
Magnesium	1.4	Glycine	6.0
Iron (mg/kg)	211	Proline	13.7
Manganese (mg/kg)	167.5	Serine	7.0
Copper (mg/kg)	20.4	Tyrosine	4.2
Zinc (mg/kg)	134.6	Total Amino Acids	161.1

[1]NSP: non-starch polysaccharides

3.3 Broiler Rearing Conditions

A total of 1408 one-day-old male broilers (Ross 308) were allocated to 64 floor pens (2.4 x 0.95 m) bedded with wood shavings and kept on a commercial starter diet until d 10. The pens were present in a large room in the poultry house at the Centre for Livestock Production (SHF, NMBU). The room was environmentally controlled, and 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 had 23 h (hours) of light during the first three days, and 18 hours of light from 4:00 am to 10:00 pm until d (day) 10. From d 11, the birds were randomly distributed among eight dietary treatments with eight replicate pens each and 22 birds per pen. The birds had either *ad libitum* (ADL) or intermittent (INT) access to barley-based pelleted diets supplemented or not with NSPase, and with main ingredients either finely (2-mm) or coarsely (6-mm) ground. ADL and INT birds had 8 h darkness (10:00 pm to 2:00 am and 3:00 am to 7:00 am) interrupted with 1 h of light with access to feed. From 7:00 am to 10:00 pm, ADL birds had 15 h access to feed. INT birds were given four feeding bouts from 7:00 to 8:00 am, 11:00 to 12:00 pm, 3:30 to 4:30 pm and 8:00 to 10:00 pm. Between the feeding periods, feeders were raised high enough to ensure complete feed withdrawal. In total, ADL birds had 16 h and INT had 6 h of feed access. A photograph of broiler chickens in ADL and INT feeding regimens can be observed in **Figure 3.1**.

Figure 3.1 *Ad libitum* fed birds (left) showing non-continuous feeding behavior while intermittent fed birds (right) tending to consume feed as soon as the feeders are lifted down (PS: Birds in both the pens were offered same diet); Source: Author's own photograph (SHF, NMBU; June 15, 2022).

3.4 Dissection and Sampling

Body weight per cage was recorded on d 1, 11 and 33. Feed consumption was registered daily using automatic weighing scales mounted on each feeding bin. Mortality record was collected daily, and the weight of dead birds was recorded. FI was corrected for mortality to adjust FCR. Per treatment eight birds were killed for sample collection. INT-fed birds were killed on d 28 and on d 29. ADL-fed birds were also killed on d 28 but those killed on d 29 were (by mistake) taken from the same pens as those killed on d 28 due to an error, (i.e., not true replicate) so these were

excluded. The birds therefore were killed on d 32 instead. INT-fed birds had one hour access to feed then killing took place two hours after commencement of feeding, and every 15 minutes thereafter. ADL-fed birds were given five hours of access to feed then were killed continuously. The birds were killed by cervical dislocation and a plastic zip tie was placed on the birds' necks immediately to prevent loss of crop contents. The crop was then dissected out, and its contents were emptied in a 100 ml container and homogenized for pH measurement which was done by inserting the electrode of the pH meter directly into the sampling container. The gizzard was removed, freed from surrounding fat, and pH of contents was measured by inserting the electrode directly into the gizzard before recording the full and empty weight. Digesta from the jejunum and the ileum was placed in pre-weighed containers, and frozen at −20°C. Caecal contents were also collected and two samples of 1 ml each were transferred to two 2 ml cryotubes and frozen at -80℃ for microbiota analysis.

3.5 Sample Processing

3.5.1 Feed Samples

3.5.1.1 Sample Grinding

Feed samples were ground on a cutting mill (Pulverisette 19, Fritsch Industriestr. 8, 55743 Idar-Oberstein, Germany) through a 0.5 mm sieve.

3.5.1.2 Gross Energy

Gross energy was determined using an adiabatic bomb calorimeter (Parr 6400, Moline, USA) standardized with benzoic acid. The bomb calorimeter works under law of conservation of energy and measures the heat of combustion at a constant volume. Sample of known mass is placed in the chamber and completely burned in the presence of oxygen. The heat generated is transferred to water of known volume and the rise in the temperature of water along with its specific heat value is used to estimate the caloric count of the sample.

3.5.1.3 Dry Matter (DM)

Ground diet samples were weighed before and after drying in hot air oven at 105℃ over a period of 12 h. The loss in total weight was taken as moisture content and DM was calculated.

3.5.1.4 Ash Analysis

Ash content of the feed was determined by the mass remaining after six hours burning of pre-weighed feed sample in furnace at 550°C.

3.5.1.5 Nitrogen Analysis

Nitrogen content was determined by the Dumas method using Vario El Cube (Elementar Analysensysteme GmbH, Hanau, Germany). Dumas method involves total combustion of the sample in the presence of oxygen. The oxides of nitrogen produced resultantly are reduced using a copper wire and dried, whereas carbon dioxide gets trapped. Nitrogen is then measured by a thermal conductivity detector.

3.5.1.6 Amino Acid Profile

Amino acid concentration in the diets was determined using a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK) which works on the principle of continuous flow chromatography.

3.5.1.7 Mineral Profile Analysis

Phosphorus, Calcium, Potassium and Sodium were analysed spectrophotometrically after microwave digestion of sample (Start D Microwave digestion system (Milestone, Sorisole- Italy) and using MP-AES (Microwave Plasma Atomic Emission Spectrometer, Agilent Technologies, Santa Clara, United States), according to the method of Commission Regulation (EC) No 152/2009. 27 Jan 2009.

3.5.1.8 Ether Extract (EE) / Crude Fat Analysis

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

3.5.1.9 Starch Analysis

Starch analysis was performed using a modified (Mccleary et al., 1997) method of starch analysis by Megazyme (2019). The method includes hydrolysis of starch present in the sample into glucose using heat-stable α-amylase and amyloglucosidase at optimum temperatures. The absorbance value of glucose is measured using RX Daytona clinical chemistry analyzer (Randox Industries, Crumlin, UK). The value is then used to calculate starch percentage in the sample.

3.5.1.10 Crude Fiber Analysis / Detergent Analysis

Crude fiber, NDF and ADF content were determined using a fiber analyzer system (Ankom200; ANKOM Technologies, Fairport, NY, USA) with filter bags (Ankom F58; ANKOM Technologies). Sample is placed in the filter bag, which is then placed in the ANKOM instrument. The desired module is selected. The sample bag is treated with specific reagents as per the selected module and agitated. This solubilizes the unwanted components and filters them out. The resulting fiber is then rinsed and dried before automatic weighing.

3.5.1.11 Non-Starch Polysaccharide (NSP) Analysis

Total NSP (soluble and insoluble) were determined according to the method described by Englyst et al. (1994) in which starch from samples is enzymatically digested and removed while the NSP content is hydrolyzed into their constituent sugars which are then measured using a spectrophotometer.

3.5.1.12 Pellet Durability Index (PDI)

Pellet durability was measured using a Holmen pellet tester (Holmen Chemical Ltd., Borregaard Group, Norsolk, UK), as described by Zimonja and Svihus (2009).

3.5.1.13 Particle Size Analysis

Wet sieving of pelleted diets was performed to determine the particle size in pelleted diets as per the method described by Rodgers et al. (2012). Briefly, homogenous pellet sample is soaked in water for 2 h prior to sieving through a series of sieves arranged from top to bottom in descending sieve pore sizes. The sieves are continuously shaken under excess water. Sieving time is 3 x 3 min. After that the material from each sieve is dried in hot-air oven at 105℃ over a period of 12 h to get the actual mass of particles per sieve. The particle size distribution of pelleted diets can be seen in **Figure 3.2**.

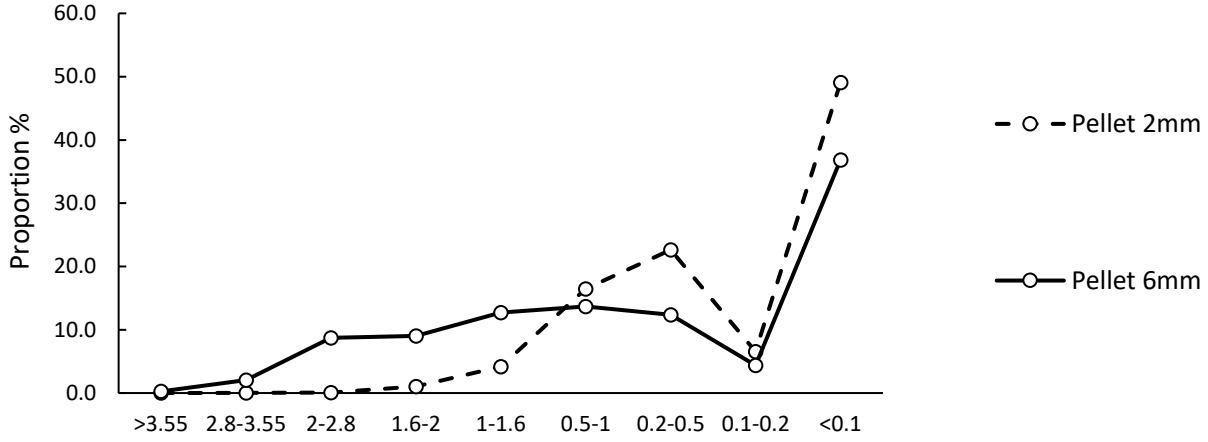

Figure 3.2 Particle size distribution of the barley-based pelleted diets measured by wet sieving (x-axis: size of sieve openings in mm, y-axis: proportion of particles retained).

3.5.2 Crop and Gizzard Samples

Crop and gizzard pH was recorded, and the contents were weighed on the sampling day. Contents from the crop were freeze dried whereas those from the gizzard were oven dried at 105℃ over a period of 12 hours to calculate DM.

3.5.3 Jejunal Samples

3.5.3.1 Jejunal Content Viscosity

Frozen jejunal samples were thawed by placing the containers in luke-warm water and around 5 ml per replicate was centrifuged (5810 R Eppendorf, Hamburg, Germany) at 10000 rpm for 10 minutes at 15℃. Approximately 1.0 - 1.5 ml supernatant per sample was collected in Eppendorf tubes using a micropipette. For viscosity analysis, 0.5 ml supernatant was analyzed on a Rheometer (Anton Paar MCR-301, Graz, Austria) with the temperature set to 40℃. The viscosity was obtained in centipoise (cP). Readings were recorded every 6 s and an average of 5 readings per sample was taken.

3.5.3.2 Jejunal Particle Size Distribution (Laser Diffraction Technique)

Frozen jejunal digesta samples were thawed at room temperature. Jejunal digesta (1 g) was dissolved in 150 ml RO-treated water using magnetic stirrer (C-Mag HS7, IKA-Werke®, Staufen, Germany) operating at 23℃ at 2.0 speed for 2 minutes. The solution was then poured into the

Hydro compartment of Mastersizer 2000 particle size analyzer (Malvern Panalytical, Malvern, UK) to measure particle size distribution. Mastersizer 2000 works on the principle of laser diffraction measurement. The particles pass through a focused beam of laser which is diffracted to an extent inverse to the particle size. The angular intensity of diffraction is then measured by photosensitive detectors. Particle size distribution along with volume weighted mean (Vol. W.M.D) were taken as results. Vol. W.M.D indicates the mean based on particle volume.

3.5.4 Ileal Samples

Ileal digesta samples were thawed at room temperature, weighed before and after overnight drying in hot air oven at 105℃ over a period of 12 hours. The loss in total weight was taken as moisture, and DM was calculated. Dried ileal digesta was ground up to 1-mm and homogenized samples were taken for starch, crude protein, and marker analysis.

3.5.4.1 Starch Analysis

For ileal starch digestibility, starch analysis was performed by method used for dietary starch analysis and is mentioned in section 3.5.1.9.

3.5.4.2 Crude Protein Analysis

Nitrogen analysis was performed using automated Kjeldahl's method. Samples were digested using concentrated sulfuric acid (H_2SO_4) and digestion was performed on Foss Automated Digestor (Foss Analytical AS, Hillerød, Denmark) whereas distillation and titration were carried on using Kjeltec^{TM} 8400 (Foss Analytical AS, Hillerød, Denmark). Results were acquired as nitrogen g/kg digesta sample and crude protein % was calculated.

3.5.4.3 Marker Analysis

Marker titanium oxide (TiO_2) in digesta samples was determined by method of marker analysis described by Short et al. (1996). Samples were burned in furnace at 550℃ overnight and ash was then dissolved and cooked in concentrated sulfuric acid (40% H_2SO_4) to remove any organic material present. The samples were then mixed with hydrogen peroxide (30% H_2O_2) and RO water, where titanium oxide reacted with sulfuric acid and hydrogen peroxide, and formed a yellow/orange colored titanium compound. The intensity of the yellow/orange color was then

analyzed at 410 nm on SpectraMax M2e (Molecular Devices, LLC.). The absorbance value was then used to calculate the concentration of TiO_2.

3.5.5 Cecal Samples

3.5.5.1 DNA Extraction and Quality Control

DNA extraction was performed according to an in-house protocol at the Norwegian Veterinary Institute (NVI) developed for the TEiCON project. Frozen cecal digesta samples were thawed at room temperature and 250 mg per sample was added to the dry bead tubes in PowerFecal Pro™ DNA Extraction Kit (Qiagen, Hilden, Germany). The lysis buffer was added to the tube containing samples and an empty tube as a negative control (empty dry bead tube). The samples were then homogenized using FastPrep-24™ Classic homogenizer (MP Biomedicals, CA, USA). DNA was extracted using PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) and the extraction was carried out on the automatic instrument QIAcube Connect (Qiagen, Hilden, Germany). The principle of lysis of cellular components was chemical (lysis buffer) and mechanical. The lysed and homogenized samples were then purified and extracted using a column-based system. The quantity and purity of DNA was tested using DeNovix QFX fluorometer (DeNovix Inc., DE, USA) and NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™, Massachusetts, USA), respectively.

3.5.5.2 Real-Time PCR

Real-Time PCR was used to detect the genes that encode for *C. perfringens* α-toxin and NetB-toxin in the DNA extracted from the cecal digesta samples. Primer sets and probes of α-toxin and NetB-toxin producing genes from NVI (**Table 3.6**) were added to nuclease free water along with fluorescent dye 2X Brilliant III QPCR Master Mix (Agilent Technologies, CA, USA) to make the master mix. The master mix and the extracted DNA were added to the desired wells of the PCR plate. Positive controls for α-toxin and NetB-toxin for generation of calibration curves were also added. The PCR plate was then sealed, centrifuged, and placed in the CFX 96™ Real-Time PCR machine (Bio-Rad Laboratories, CA, USA).

Table 3.6 Sequences of primer sets and probes of α-toxin and NetB-toxin producing genes.

Toxin	Gene	Primers and Probes	Nucleic acid sequences of primers and probes (5′-3′) with probe dyes
α-toxin	cpa		(Albini et al., 2008)
		Forward primer	AAGAACTAGTAGCTTACATATCAACTAGTGGTG
		Reverse primer	TTTCCTGGGTTGTCCATTTCC
		Probe	HEX-TTGGAATCA-ZEN-AAACAAAGGATGGAAAAACTCAAG-IBFQ
NetB-toxin	netB		(Schlegel et al., 2012)
		Forward primer	GGCGGTAATATATCTGTTGAAGG
		Reverse primer	ACCGTCCTTAGTCTCAAC
		Probe	FAM-ACTGCTGGT-ZEN-GCTGGAATAAATGCTTCA-IBFQ

The following CFX program was selected: first cycle was at 50℃ for 10 minutes, second cycle at 95℃ for 5 minutes, and then 48 cycles at 95℃ for 30 seconds, and 60℃ for 60 seconds. Real Time PCR works on the same principle as an ordinary thermal cycler except for an additional built-in spectrofluorometer. The machine measures the amount of genetic material to be detected by measuring the fluorescence of the dye bound to the genetic material, which is directly proportional to its quantity.

3.5.6 Calculations

3.5.6.1 Dry Matter

DM of crop, gizzard and ileal contents was calculated using the following formula:

DM% = Dry Sample Weight (g) / Wet Sample Weight (g) × 100

3.5.6.2 Starch

Starch % was calculated from absorbance value using the following formula:

% Starch = (glucose absorbance × 180 × 0.0073 × 162 × 100) / (mg sample × 180)

Note:

glucose absorbance = spectrophotometer reading (mmol/l)

180 = mol. mass of glucose (mg/mmol)

0.0073 = dilution factor (buffer + enzyme)

162/180 = glucose factor (conversion from glucose unit to starch)

mg sample = mass of sample used in milligram

100 = for calculation in %

3.5.6.3 Crude Protein

Crude protein was calculated from Kjeldahl's nitrogen using the following formula:

% Crude Protein = % Kjeldahl's Nitrogen × 6.25

3.5.6.4 Marker Calibration Curve

The calibration curve from LabTek, NMBU was used to determine the marker (TiO_2) concentration (**Figure 3.3**).

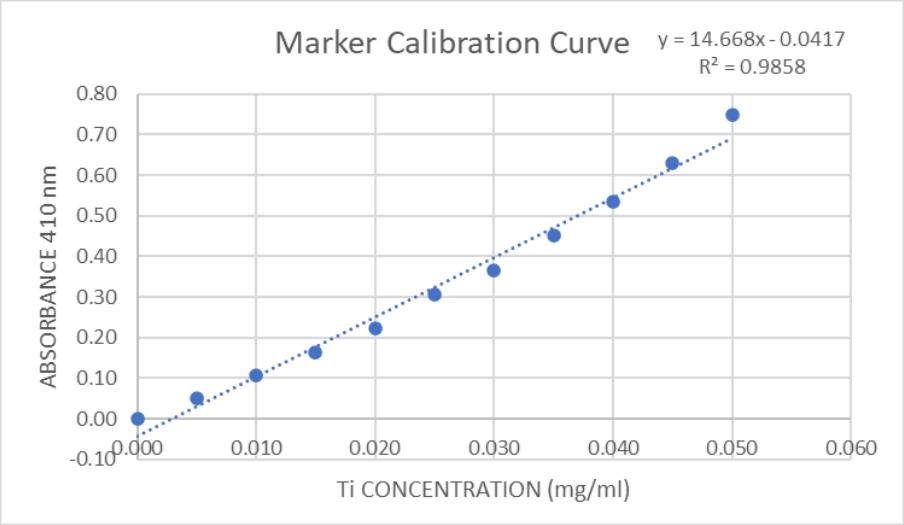

Figure 3.3 Titanium dioxide calibration curve for quantification of titanium dioxide from spectrophotometer absorbance reading (LabTek, NMBU).

3.5.6.5 Apparent Ileal Digestibility

Apparent ileal digestibility for both starch and protein were calculated using the following formula:

$$\textbf{Apparent Ileal Digestibility \%} = 100 - \frac{\textbf{(\% Marker in Feed} \times \textbf{\% Nutrient in Digesta)} \times 100}{\textbf{(\% Marker in Digesta} \times \textbf{\% Nutrient in Feed)}}$$

3.5.7 Statistical Analysis

The data were analyzed using three-way analysis of variance (ANOVA) to find out the main effects of grinding level, NSPase supplementation, and feeding regimen, and their possible interactions using General Linear Model in RStudio (2022.12.0 Build 353 © 2009-2022 Posit Software, PBC). Tukey's HSD (Honestly Significant Difference) test was used to determine significance of pair-wise means differences.

4 Results

4.1 Growth performance

Growth performance results have been shown in **Table 4.1**. No factor appeared to produce a significant main effect on livability of the birds. Broilers in ADL feeding regimen showed more ($P < 0.001$) FI than those in INT regimen. NSPase addition tended ($P = 0.051$) to increase FI with coarse grinding while the opposite effect was observed with fine grinding. Broilers in ADL feeding regimen showed improved ($P < 0.001$) live WG. NSPase increased ($P = 0.02$) live WG and tended ($P = 0.063$) to interact with feeding regimen because the effect of NSPase addition on BWG was larger under INT feeding. Although the ADL group consumed more feed and gained more weight as compared to INT group, no significant difference in FCR was seen between both groups. However, the two-way interaction ($P < 0.033$) between feeding regimen and NSPase showed that NSPase had a stronger beneficial effect on FCR with INT feeding than with ADL feeding regimen. Another two-way interaction ($P < 0.037$) was seen between NSPase and grinding indicating that NSPases were more effective with fine grinding than with coarse grinding.

4.2 Digestive Tract Characteristics

The results of digestive tract characteristics have been shown in **Table 4.2**. ADL-fed birds had more ($P = 0.007$) crop DM contents compared to INT-fed birds and there was a tendency ($P = 0.084$) for a higher crop DM contents with fine grinding. The pH of crop contents was not affected ($P > 0.05$) by any factor. Coarse grinding increased gizzard DM contents ($P < 0.009$) and weight of gizzard relative to the body weight ($P < 0.05$). Relative gizzard weight was also greater ($P = 0.002$) with INT feeding than with ADL feeding. The pH of gizzard contents was significantly lower ($P < 0.05$) with coarse grinding compared to fine grinding and was not affected by any other dietary treatments. NSPase addition ($P < 0.001$) and fine grinding ($P = 0.049$) reduced jejunal digesta viscosity. The main effects of NSPase supplementation and grinding on jejunal digesta viscosity were more obvious in INT group although no interactions ($P > 0.05$) were observed in this regard.

Table 4.1 Effect of NSPase supplementation, grinding level and feeding regimen on growth performance and nutrient digestibility of barley-based diets fed from day 11 in male broilers.

			Growth Performance (d 33)				Ileal Digestibility (d 28- 32)	
Feeding regimen	**Grinding**	**NSPase**	**Live Weight Gain**	**Feed Intake[1]**	**FCR[2]**	**Livability**	**Starch**	**Protein**
Ad libitum	2-mm	-	2333.0	3044.9	1.306 ab	97.2	99.5 a	82.4 ab
Ad libitum	2-mm	+	2356.3	3030.0	1.286 ab	100.0	99.3 a	81.0 b
Ad libitum	6-mm	-	2321.4	3047.4	1.314 ab	97.2	98.5 b	85.1 a
Ad libitum	6-mm	+	2319.0	3075.8	1.327 a	98.9	99.3 a	84.9 a
Intermittent	2-mm	-	2182.4	2886.9	1.324 a	98.7	99.5 a	81.2 b
Intermittent	2-mm	+	2255.1	2866.5	1.272 b	98.3	99.5 a	84.8 a
Intermittent	6-mm	-	2149.0	2824.6	1.314 ab	96.6	98.9 ab	83.2 ab
Intermittent	6-mm	+	2236.8	2892.6	1.293 ab	98.3	99.3 a	82.3 ab
√MSE[3]			73.28	65.09	0.03	3.55	0.69	1.86
Feeding regimen								
Ad libitum			2332.4 a	3049.5 a	1.308	98.3	99.1	83.4
Intermittent			2206.6 b	2867 b	1.3	98	99.3	82.9
Grinding								
2-mm			2284.9	2960.7	1.296 b	98.5	99.5 a	82.4 b
6-mm			2256.5	2959.3	1.312 a	97.7	99.0 b	83.9 a
NSPase								
-			2248.5 b	2953	1.314 a	97.4	99.1	83.0
+			2291.8 a	2966.2	1.295 b	98,9	99.3	83.4
P-value								
Feeding regimen			***	***	NS	NS	NS	NS
Grinding			NS	NS	*	NS	*	**
NSPase			*	NS	*	NS	NS	NS
Feeding regimen x Grinding			NS	NS	NS	NS	NS	**
Feeding regimen x NSPase			0.063	NS	*	NS	NS	0.069
Grinding x NSPase			NS	0.051	*	NS	*	NS
Feeding regimen x Grinding x NSPase			NS	NS	NS	NS	NS	*

Levels of significance: *P<0.05; **P<0.01; ***P<0.001; NS = Not Significant.

Means in a column not sharing a common letter (a, b, c, d) are significantly different (P < 0.05).

[1]Feed Intake: feed intake (FI) corrected for mortality; [2]FCR: feed conversion ratio = feed intake/live weight gain.

[3]√MSE: Square Root of Mean Square Error in Analysis of Variance.

Table 4.2 Effect of NSPase supplementation, grinding level and feeding regimen on digestive tract characteristics of 28 to 32 -day-old male broilers fed barley-based diets from day 11.

			Crop		Gizzard			Jejunum
Feeding regimen	**Grinding**	**NSPase**	**[1]Content DM (g)**	**pH**	**[2]Content DM (g)**	**pH**	**[3]Rel. wt.**	**Viscosity (cP)**
Ad libitum	2-mm	-	14.5	4.31	4.87	2.65	1.12	2.47
Ad libitum	2-mm	+	16.1	4.33	4.39	2.70	1.20	2.00
Ad libitum	6-mm	-	8.5	4.40	7.66	2.42	1.38	2.76
Ad libitum	6-mm	+	11.1	4.44	6.52	2.28	1.25	2.04
Intermittent	2-mm	-	7.9	4.39	4.21	2.81	1.33	2.27
Intermittent	2-mm	+	8.3	4.35	5.61	2.43	1.40	1.63
Intermittent	6-mm	-	6.6	4.41	5.90	2.46	1.37	2.64
Intermittent	6-mm	+	9.2	4.38	5.14	2.44	1.45	1.93
√MSE[4]			6.44	0.224	5.01	0.466	0.173	0.490
Feeding regimen								
Ad libitum			12.5 a	4.37	5.86	2.52	1.24 b	2.31
Intermittent			8.0 b	4.38	5.21	2.53	1.39 a	2.13
Grinding								
2-mm			11.7	4.34	4.77 b	2.64 a	1.25 b	2.08 b
6-mm			8.9	4.40	6.31 a	2.40 b	1.37 a	2.36 a
NSPase								
-			9.4	4.37	5.66	2.59	1.30	2.54 a
+			11.2	4.37	5.41	2.46	1.33	1.90 b
P-value								
Feeding regimen			**	NS	NS	NS	**	NS
Grinding			0.084	NS	**	*	*	*
NSPase			NS	NS	NS	NS	NS	***
Feeding regimen x Grinding			NS	NS	NS	NS	NS	NS
Feeding regimen x NSPase			NS	NS	NS	NS	NS	NS
Grinding x NSPase			NS	NS	NS	NS	NS	NS
Feeding regimen x Grinding x NSPase			NS	NS	NS	NS	NS	NS

Levels of significance: *P<0.05; **P<0.01; ***P<0.001; NS = Not Significant.

Means in a column not sharing a common letter (a, b, c, d) are significantly different (P < 0.05).

[1]Weight of crop contents on dry matter basis; [2]Weight of gizzard contents on dry matter basis; [3]Weight of gizzard relative to body weight.

[4]√MSE: Square Root of Mean Square Error in Analysis of Variance.

4.3 Nutrient Digestibility

Nutrient digestibility results have been shown in **Table 4.1**. NSPase supplementation with coarsely ground diet resulted in higher ($P < 0.05$) starch digestibility, resulting in a two-way interaction between NSPase and grinding level. In NSPase-supplemented diets fed ADL, coarse grinding resulted in higher ileal digestibility of protein than fine grinding, whereas under INT feeding, no difference between grinding levels was evident. This resulted in a three-way interaction ($P = 0.012$) between feeding regimen, grinding and NSPase. Moreover, this three-way interaction showed that NSPase supplementation in finely ground diet improved protein digestibility only in INT feeding, whereas under ADL feeding, no difference was seen in supplemented or non-supplemented diets. This suggests that the efficacy of NSPase to improve protein digestibility was dependent on both factors; fine grinding and INT feeding.

4.4 Jejunal Particle Size Distribution

Jejunal particle size distribution results can be seen in **Table 4.3**. The main effects of feeding regimen and NSPase were non-significant ($P > 0.05$) in all of the parameters of particle size distribution in jejunum. In coarsely ground diets, NSPase reduced ($P < 0.05$) the proportion of particles (<53 µm) under INT feeding while the opposite effect was seen under ADL feeding. As compared to coarse (6-mm) grinding, fine (2-mm) grinding tended to produce an increased ($P = 0.06$) percentage of fine particles (<53 µm). Coarse grinding produced bigger ($P < 0.05$) tenth percentile particle size as compared to fine grinding. With coarsely ground diets, NSPase inclusion resulted in a smaller tenth percentile particle size in the ADL group. This caused an interaction ($P < 0.05$) between NSPase and feeding regimen. Coarse grinding produced bigger ($P < 0.05$) median particle size as compared to fine grinding. NSPase tended ($P = 0.0702$) to reduce the median particle size only in ADL feeding. Coarse grinding resulted in bigger ($P < 0.001$) ninetieth percentile particle size as compared to fine grinding. Coarse grinding increased ($P = 0.0021$) the volume weighted mean as compared to fine grinding. NSPase tended ($P = 0.0659$) to reduce the volume weighted mean only in ADL feeding. Although the main effects of NSPase and feeding regimen were not significant, volume weighted mean appeared to be numerically lower in intermittent feeding.

Table 4.3 Effect of NSPase supplementation, grinding level and feeding regimen on jejunal particle size distribution measured by laser-diffraction in 28 to 32 -day-old male broilers fed barley-based diets from day 11.

Feeding Regimen	Grinding	NSPase	[1]particle <53µm %	[2]d(0.1) µm	[3]d(0.5) µm	[4]d(0.9) µm	[5]Vol.W.M.D. µm
Ad libitum	2-mm	-	16.17	29.24	236.60	881.0	360.96
Ad libitum	2-mm	+	17.28	28.60	216.7	749.1	314.11
Ad libitum	6-mm	-	14.47	34.36	263.3	1081	422.70
Ad libitum	6-mm	+	17.93	26.80	204.91	937.9	357.70
Intermittent	2-mm	-	18.62	26.05	191.6	723.8 a	304.14
Intermittent	2-mm	+	18.03	25.74	220.85	826.0	331.84
Intermittent	6-mm	-	17.18	28.09	263.35	990.9	382.43
Intermittent	6-mm	+	15.42	34.51	253.94	899.3	372.70
√MSE[6]			2.66	6.92	52.61	181.3	67.96
Feeding Regimen							
Ad libitum			16.46	29.75	230.4	912.8	363.9
Intermittent			17.31	28.60	232.5	860	347.8
Grinding							
2-mm			17.52	27.41 a	216.5 a	795.5 a	327.76 a
6-mm			16.25	30.94 b	246.4 b	977.4 b	383.88 b
NSPase							
-			16.61	29.43	238.72	919.78	367.56
+			17.16	28.91	224.12	853.05	344.09
P-value							
Feeding Regimen			NS	NS	NS	NS	NS
Grinding			0.0608	*	*	***	**
NSPase			NS	NS	NS	NS	NS
Feeding Regimen x Grinding			NS	NS	NS	NS	NS
Feeding Regimen x NSPase			*	*	0.0702	NS	0.0659
Grinding x NSPase			NS	NS	NS	NS	NS
Feeding Regimen x Grinding x NSPase			NS	0.0531	NS	NS	NS

Levels of significance: *P<0.05; **P<0.01; ***P<0.001; NS = Not Significant.

Means in a column not sharing a common letter (a, b, c, d) are significantly different (P < 0.05).

[1]Percentage of particles below 53µm; [2]tenth percentile particle size; [3]median particle size; [4]ninetieth percentile particle size; [5]Volume W.M.D (Volume Weighted Mean: mean based on particle volume); [6]√MSE: Square Root of Mean Square Error in Analysis of Variance.

4.5 *Clostridium perfringens* Toxin-Producing Genes

Results from *C. perfringens* toxin-producing gene analysis have been shown in **Table 4.4**. Since the detection of α-toxin producing gene can be ascribed as the detection of *C. perfringens*, the results show that diets supplemented with NSPase, and INT feeding resulted in lower prevalence of *C. perfringens* in ceca as compared to diets without NSPase and ADL feeding. On the other hand, broilers fed coarsely-ground diet without NSPase under ADL showed highest prevalence of *C. perfringens* and NetB-toxin. Overall, lower prevalence of NetB-toxin producing gene was seen as compared to that of α-toxin producing gene. NSPase supplementation in finely ground diets resulted in lowest (zero) prevalence of NetB-toxin.

Table 4.4 Effect of dietary treatments on the prevalence of *Clostridium perfringens* α-toxin and NetB-toxin producing genes in ceca of 28 to 32 -day-old male broilers fed barley-based diets from day 11.

Feeding Regimen	Grinding	NSPase	α-toxin[1]	NetB-toxin[2]
Ad libitum	2-mm	-	87.50	25.00
Ad libitum	2-mm	+	71.43	00.00
Ad libitum	6-mm	-	100.0	28.57
Ad libitum	6-mm	+	87.50	12.50
Intermittent	2-mm	-	75.00	25.00
Intermittent	2-mm	+	62.50	0.00
Intermittent	6-mm	-	66.67	16.67
Intermittent	6-mm	+	50.00	16.67
Feeding Regimen[3]				
Ad libitum			86.67	16.67
Intermittent			64.28	14.28
Grinding[4]				
2-mm			74.19	12.90
6-mm			77.77	18.50
NSPase[5]				
-			82.70	24.13
+			68.90	06.89

[1]Percentage of broiler chickens positive for α-toxin producing gene; [2]Percentage of broiler chickens positive for NetB-toxin producing gene; [3,4,5]Percentage of broiler chickens positive with toxin-producing genes in each level of feeding regimen, grinding and NSPase.

5 Discussion

The objective of this study was to enhance the potential of barley as a sustainable cereal grain in broiler diets through the application of NSPases along with different ingredient grinding levels and feeding regimens. The individual and interaction effects of NSPase, grinding level, and feeding regimen on growth performance, nutrient digestibility, digestive tract characteristics and the prevalence of *C. perfringens* in broilers were investigated.

5.1 Growth Performance

Higher FI in broilers under ADL feeding regimen than those in INT regimen is in accordance with the previous studies (Rodrigues et al., 2018; Rosebrough et al., 1989; Sacranie et al., 2017), and is attributed to the more uninterrupted feed access time. On the contrary, in many studies, feeding regimens did not appear to affect FI (Sacranie et al., 2012; Svihus et al., 2013; Svihus et al., 2010). Due to the implementation of adaptation period for shifting from ADL to INT feeding, the birds in these studies might have gotten accustomed to consuming as much feed as possible. Svihus et al. (2013) indicated towards the impressive capacity of birds to adapt to INT feeding by consuming and storing more feed. The lower FI of INT group from the present study was probably due to the lack of adaptation period. This has been shown previously when INT-fed broilers consumed lesser feed initially in the absence of an adaptation period (Buyse et al., 1996). Moreover, in the current study, addition of NSPase tended to increase FI with coarse grinding while the opposite effect was observed with fine grinding. This could be explained by lower efficacy of NSPase with coarse grinding due to difficult access to nutrients and higher efficacy of NSPase with fine grinding due to easier access to the nutrients. Low NSPase efficacy with coarsely ground diets can be seen from their higher digesta viscosity compared to finely ground diets as stated in the next section. This might have resulted in reduced energy utilization. When the energy requirements from diet are not met, the birds tend to eat more (Leeson et al., 1996). Increased FI as a compensation to low energy in diets may not necessarily affect BWG (Classen, 2017). This could be the possible reason why improvement in BWG was not seen despite improved FI when NSPase was supplemented in coarsely ground diets. Moreover, the assumption of improved energy efficiency by supplemented NSPase in the finely ground diet can be supported by the improvement in FCR.

Increased BWG of ADL-fed broilers could be expected as per FI results and this agrees with the previous studies (Buyse et al., 1996; Ghanima et al., 2021; Sacranie et al., 2017). The studies by Svihus et al. (2013) and Svihus et al. (2010) have shown that INT-fed birds have the capacity to gain as much weight as ADL-fed birds if the FI between both the groups is similar. In the present study, improvement of BWG with dietary supplementation of NSPase in INT-fed birds can be attributed to the possible enhancement of exogenous enzyme efficacy by increased feed retention in the crop as mentioned by Svihus (2011a). Previously, NSPase supplementation in barley-based diet increased BWG in broilers but their interaction with feeding regimens was not studied (Mathlouthi et al., 2002b). However, Sacranie et al. (2017) observed increased exogenous phytase function in INT-fed broilers which resulted in increased degradation of myo-inositol hexakisphosphate (IP6) in the anterior digestive tract. This suggests that regulation of crop function using INT feeding may enhance exogenous enzyme function. As the birds consume feed, the pellets disintegrate in the crop (Svihus, 2006), and thorough moisturization of feed takes place by water, saliva, and mucus (Klasing, 1999). NSPase may get sufficient time and ideal conditions to exhibit their activity in the aqueous medium. Improvement in energy utilization by increased NSPase efficacy due to enhanced crop use in INT feeding regimen might be a cause of improved BWG in the current experiment. This increase in BWG by dietary supplementation of NSPase in the INT group could have led to the improvement in FCR. Furthermore, increased efficacy of NSPase with finely ground diet was seen as an improvement in FCR. It may be because of increased surface area of the feed particles and thereby increased substrate concentration for enzymes in finely-ground diets (Amerah et al., 2007a). The activity of NSPase may get enhanced with easier access to the soluble NSPs. Active disintegration of cell wall components by NSPase releases the trapped nutrients (Meng et al., 2005) and reduces the digesta viscosity (García et al., 2008a). Reduction of digesta viscosity allows endogenous enzymes to attack the released nutrients, which results in improved energy utilization and thus FCR. Overall, the ADL group consumed more feed and gained more weight as compared to INT group. However, the feeding regimens discretely did not affect the FCR. This result is not in accordance with previous experiments in which the authors observed improved FCR or FCE in INT-fed broilers (Buyse et al., 1996; Deaton et al., 1978; Farghly et al., 2019; Svihus et al., 2013; Svihus et al., 2010; Yang et al., 2015). In these studies, INT feeding was controlled by photo- or scoto- period, while that in the current experiment was controlled by lifting the feeders. Lighting is the most critical

environmental factor for birds as it has direct effects on visual activity (feed selection, feed intake, exercise), circadian rhythms, body temperature and hormonal regulation of birds (Olanrewaju et al., 2006). There are two benefits linked with controlling INT feeding using scotoperiod. One is the reduced chances of leg abnormalities by preventing unnecessary exercise (Buckland et al., 1976; Buckland et al., 1973; Wilson et al., 1984). The other is the stimulation of melatonin (a hormone produced by pineal glands) secretion (Kliger et al., 2000; Pang et al., 1998; Zawilska et al., 2007). Melatonin increases growth hormone secretion (Zeman et al., 1999) and reduces the heat production in broilers (Zeman et al., 2001). This in turn may improve the growth performance of broilers. These postulations can be supported by a study in which melatonin supplementation improved growth performance in broilers (Osei et al., 1989). Therefore, it can be suggested that improved FCR by INT feeding in previously mentioned studies was possibly a synergistic effect of improved crop functionality and enhanced melatonin secretion. Since the objective of the current study was not to study the effect of lighting but to investigate the effect of INT feeding by prompting the crop function, photoperiod was kept constant between both feeding groups. However, implementation of one week adaptation period could have positively influenced the FI as seen in many of the aforementioned studies.

Overall, none of the factors appeared to affect the livability of broilers as expected. This was mainly due to the fact that the experiment was carried out in a highly controlled facility. In addition, none of the experimental treatments was expected to be associated with a risk of mortality. Higher FI resulted in increased BWG in ADL group as compared to INT group. The FCR results suggest improved NSPase efficacy with fine grinding, and with INT feeding.

5.2 Digestive Tract Characteristics

Many studies have shown that the birds under INT feeding consume high amounts of feed during the photoperiod, thus leading to increased DM in the crop as compared to ADL feeding (Sacranie et al., 2017; Svihus et al., 2010), and as the time passes, the crop releases its contents towards aborad direction (Buyse et al., 1993; Sacranie et al., 2017). However, in the current study, the lower DM contents in crop of INT-fed birds than in ADL-fed birds were mainly because the birds from INT group had access to feed for one hour and were killed after two hours of commencement of feeding and then after every 15 minutes, whereas those from ADL group were on continuous feed supply when killed. By the time of dissection, most of the crop contents in the

INT-fed birds might have left the crop. This could be supported by a study by Svihus et al. (2010), in which a three-fold decrease in the crop DM of INT-fed birds was observed within first two hours after a 15 minute access to feed.

In the current study, the results of diet acidification were evident from the low crop pH and were in accord to a previous study by Kristoffersen et al. (2022) in which formic acid was used to acidify the diets. However, the effect of dietary treatments on crop pH was not significant. It could be assumed that the addition of formic acid might have masked the effect of dietary treatments, or there was no further room for reduction in the crop pH. Reduction in gizzard pH by coarse grinding in the current experiment is in accordance with the previous studies (Nir et al., 1994; Sacranie et al., 2017; Svihus et al., 2013). As compared to finely-ground diets, coarsely ground diets had greater proportion of coarse particles (**Figure *3.2***). According to Zaefarian et al. (2016), longer retention of coarse particles in the gizzard may increase secretion of HCl. This might have led to a lower pH in the gizzard.

Increased quantity of DM in gizzard with coarsely ground diets than with finely ground diets is in accordance with several reports (Amerah et al., 2008a; Hetland et al., 2003; Sacranie et al., 2012; Svihus et al., 2010). According to Hetland et al. (2002), coarse particles are retained in gizzard until further grinding. A good proportion of particles in coarsely ground diets was supposed to be retained in the gizzard and the presence of hulls might have augmented this effect. Lesser quantity of DM in gizzard with finely-ground diets suggest easier grinding of finely ground diet as per the assumption and that fine particles do not stay in the gizzard for longer period of time. This assumption can be supported by following studies and arguments. Weight of the gizzard relative to the body weight, when increased by coarse grinding (HMSO: 7-mm) of wheat- and corn- based diets, was 1.0 and 1.26%, respectively (Amerah et al., 2008a). Another experiment showed that the relative gizzard weight was increased by inclusion of whole wheat (400 g/kg) in the pre-pelleted wheat-based layer diet and was 1.2% (Hetland et al., 2003). On the other hand, this parameter with finely-ground (HMSO: 2-mm) barley-based diets in the current experiment was 1.25%. This finding suggests that even the finely-ground barley-based diets may result in adequately developed gizzard due to the presence of hulls. Further increase in gizzard weight to 1.37% in broilers fed coarsely-ground (HMSO:6-mm) diets is in accord with previous studies, in which increasing the coarseness of diets increased gizzard weight by stimulation of the muscles during grinding action (Amerah et al., 2008a; Hetland et al., 2003; Perera et al., 2020; Sacranie et

al., 2012). Increased gizzard weight with INT feeding as compared to ADL feeding was peculiar and was not seen before (Fondevila et al., 2020; Sacranie et al., 2017; Sacranie et al., 2012; Svihus et al., 2013; Svihus et al., 2010). The presence of this effect may be explained by following line of reasoning. Firstly, fasting period of the INT group in the current experiment was not controlled by scotoperiod, but by complete feed withdrawal. Secondly, the pens of INT and ADL groups were partitioned with a perforated metal sheet as shown in the **Figure 3.1**. It is therefore possible that the INT group might have attempted to consume litter by imitating the ADL group's continuous feeding behavior. It has been shown that consumption of litter could be a reason for increased gizzard weight (Hetland et al., 2005). However, further investigations are necessary before making a conclusion.

Other than gizzard weight, better grinding efficiency with finely ground diets in the current experiment may also be supported by jejunal digesta particle size. The overall size of particles in jejunal digesta was smaller with fine grinding as compared with coarse grinding. This finding suggests that increase in the grinding capacity of gizzard beyond the limit by increased coarseness of the diets may not necessarily enhance its grinding efficiency. Other than that, it can be suggested that fine grinding of barley in a hammer mill may reduce the size of hulls to a greater extent as compared to particle size reduction of coarsely-ground diets in the gizzard. However, these results are in contrast with the studies of Svihus et al. (1997) and Hetland et al. (2002), in which inclusion of whole barley in diets resulted in reduced intestinal digesta particle size as compared to ground barley-based diets. The authors suggested that whole barley increases the gizzard grinding capacity to a greater extent and is more efficiently ground by the gizzard as compared to ground barley. One possible explanation for these contrasting results could be that the coarsely ground barley-based diets from the current study might have behaved differently as compared to the diets containing whole barley from the aforementioned studies, which might have stimulated gizzard to a higher extent. Moreover, active grinding in the gizzard requires energy (Hetland et al., 2002). Once the structural components develop the gizzard to the optimum size, further increase in the diet coarseness as in coarsely-ground barley-based diets may increase the maintenance energy requirements of the birds. An increase in the maintenance energy requirements by coarse grinding may mask its positive effects on the growth performance. This could be seen in a study by Hetland et al. (2003), in which addition of grit to a whole wheat-based diet containing oat hulls did not improve FCE. Furthermore, these arguments can be supported by

poor FCR despite high FI and high starch digestibility in coarsely ground diet with NSPase in the current study.

Even though pelleting of diets tends to even out the difference in particle size between diets produced through different grinding levels (Svihus et al., 2004), yet finely ground diets had 10% more fine particles (< 100 µm) than the coarsely ground diets (**Figure *3.2***). This could be due to the fact that Svihus et al. (2004) used wheat-based diets and that the barley hulls in the current study might have resisted further grinding during pelleting. However, this difference in fine particles almost diminished in the jejunum where the difference in the proportion of fine particles (< 53µm) between coarsely and finely ground diets was not so significant. This result is in accordance with the study by Hetland et al. (2002) in which the proportion of fine particles (< 40 µm) in duodenum was almost even between whole barley-based diets and ground barley-based diets. These findings suggest that during the grinding of very hard and coarse particles in the gizzard, their abrasion against the particles and the koilin layer results in the production of a great proportion of very fine particles. This argument could also be supported by the finding that the inclusion of grit in broiler diet increased the proportion of fine particles in duodenum (Hetland et al., 2003). In the current study, with NSPase supplementation, the proportion of fine particles (< 53µm) appeared to decrease in INT group and increase in ADL group. The exact cause of these results is not known. However, one possibility is the increased activity of NSPase in INT feeding might have disintegrated the cell walls in the anterior GIT to a greater extent. Disintegration of cell walls might have increased the extent of hydrolysis of nutrients by gastrointestinal juices and enzymes thus facilitating their faster absorption, thus leaving lesser proportion of finer particles in the jejunum. However, more research is needed to make a conclusion.

Jejunal digesta viscosity of broilers in the current study was lower than that reported by many of the broiler studies when barley-based diets were used in broilers without NSPase supplementation (Almirall et al., 1995; Cengiz et al., 2017; Gracia et al., 2003; Józefiak et al., 2006; Perera et al., 2020). This difference may be due to the variation in soluble NSP content of barley varieties, as reported by Svihus and Gullord (2002). Moreover, the notably high starch digestibility (discussed in section 5.3) in the diets even without NSPase supplementation indicate the possibility of the low soluble NSP content in the variety of barley used in the current experiment. Furthermore, reduced jejunal digesta viscosity with NSPase supplementation agrees with the results of many of the broiler studies (Almirall et al., 1995; Gracia et al., 2003; Józefiak et al., 2005; Józefiak et al.,

2006). Other than that, lower jejunal digesta viscosity due to fine grinding as compared to coarse grinding is in accordance with the studies by Engberg et al. (2004) and Wu et al. (2004), in which ground wheat-based diets resulted in lower intestinal digesta viscosity as compared to inclusion of whole wheat in broiler diets. However, this effect was not consistent with different wheat-types and at different intestinal sites, while in the current experiment, this effect was consistently seen in all of the finely ground diets. On the contrary, the study by Yasar (2003) showed higher ileal digesta viscosity with finely ground wheat-based diet as compared to coarsely ground wheat-based diet. High digesta viscosity in the previous study could be possibly due to ease in the release of arabinoxylans thus rapid formation of viscous solutions in the intestine. The possible causes of viscosity-reducing effect of fine grinding in the current study are described as follows. Major soluble NSP in wheat are arabinoxylans whereas in those barley are β-glucans and arabinoxylans (Bedford, 1995). Broiler crop is dominated by *Lactobacilli* as dominant fermenting bacteria (Józefiak et al., 2006), and some *Lactobacillus* strains are potent β-glucanase producers (Jonsson & Hemmingsson, 1991; Skrede et al., 2003). So, this peculiar effect in the current study could be explained as the facilitation in the release of cell wall components by fine grinding might have increased the degradation of β-glucans by microbial β-glucanases in the anterior GIT. This assumption of bacterial fermentation in the crop may be supported by the finding that the digesta viscosity in the birds from INT group was numerically lower than those from ADL group. On the other hand, the presence of DM in the crop of the ADL group birds indicates the possibility of minor fermentation activity in this group also. As a result of this, minor reduction in viscosity with finely ground diets fed ADL might have taken place. Another benefit of using NSPase with intermittent feeding may be explained as follows. Theoretically, it can be considered that soluble NSPs produce viscosity once they are released from the cell walls, and that happens during gizzard grinding. The supplemented NSPase in broilers fed ADL may reduce the viscosity in the small intestine once it is produced. On the other hand, upon pellet disintegration in the crop of INT-fed birds, the NSPase may start degrading the soluble NSPs even before the onset of grinding in gizzard and continue this process in the gizzard and small intestine (Józefiak et al., 2006). This suggests that NSPase in the INT group might have prevented the increase in viscosity whereas that in the ADL group might have reduced the viscosity after its production. The former may result in overall lower jejunal digesta viscosity.

The crop pH in this experiment was successfully optimized for NSPase function by the addition of formic acid. Lower gizzard pH with coarse grinding implies the increased secretion of HCl. Increased gizzard size and gizzard DM with coarse grinding were as per expectations. The argument of having well-developed gizzard with a high grinding efficiency due to presence of hulls in finely-ground diets could be supported by the comparison of gizzard size with previous studies and jejunal digesta particle size results in the present study. Increase in gizzard size by INT feeding might be caused by litter consumption during fasting periods thus stimulating the gizzard activity. Although there was no significant interaction effect, the function of NSPase to reduce jejunal digesta viscosity seemed to be highest with fine grinding possibly due to easier and faster access to soluble NSPs, and under INT feeding due to more time for enzyme action and possible bacterial fermentation in the crop. Lack of significant interaction effects in viscosity may be due to overall low soluble NSP content of the barley variety or small sample size; so further investigations are required in this regard.

5.3 Nutrient Digestibility

All dietary treatments in the current study resulted in remarkably high ileal starch digestibility with an average of 99.22%. Generally, the starch digestibility of normal barley-based diets in broilers is between 80 and 95% and is improved by NSPase addition (Almirall et al., 1995; Hesselman & Åman, 1986; Perera et al., 2020; Ravindran et al., 2007; Y. B. Wu et al., 2004). The difference in starch digestibility of barley-based diets may be again linked to the varieties of barley used. For example, a study reported that starch digestibility values of high and low viscosity barley in broiler diets were 85% and 88.5%, respectively (Hesselman & Åman, 1986). The same study showed that NSPase supplementation increased starch digestibility of high and low viscosity barley to 98.5% and 96.3%, respectively. To dig deep into interactions, the starch digestibility results were contrary to the expectations and showed improved starch digestibility by NSPase supplementation in coarsely-ground diet. Many studies suggest positive effects of NSPase addition on starch digestibility of broiler diets and attribute the results to reduction of viscosity and destruction of cell walls (Almirall et al., 1995; Bergh et al., 1999; Hesselman & Åman, 1986; Ravindran et al., 2007; Y. B. Wu et al., 2004). Moreover, some studies have linked increased gizzard size due to higher coarseness of diets with improved starch digestibility (Hetland et al., 2002; Mtei et al., 2019; Ruhnke et al., 2015; Selle et al., 2019). However, the

possible mechanism of improved starch digestibility with the interaction of NSPase with coarsely ground diets has not been thoroughly studied before and may be described as follows. The tenth percentile particle size in jejunal digesta with coarse and fine grinding was 30.94 and 27.41 µm, respectively. This is in accordance with the size of A-type starch granules in barley which is up to 30 µm (Andersson et al., 1999). However, particle size may not be the only factor that affects starch digestibility, but also lipid digestibility and pancreatic amylase activity (Hetland et al., 2003). This is because the dietary lipids may form amylose-lipid complexes and reduce starch digestibility (Bello-Perez et al., 2020). Increased concentration of bile acids is positively linked with lipid emulsification. Hetland et al. (2003) observed higher jejunal bile salt concentration and amylase activity by increased stimulation of gizzard by inclusion of oat hulls in the diets. This in turn, might have improved starch digestibility in the aforementioned study. Furthermore, according to Kim and Yoo (2020), xylanase can work synergistically with amylase. These arguments suggest the possible reasons for increased starch digestibility with NSPase supplementation in coarsely-ground diet. The finding implies that starch digestibility may be influenced by factors beyond particle size, such as the bile acid concentration and amylase activity that needs to be measured in future studies. Even though the starch digestibility was increased due to the interaction, the actual difference in digestibility results was relatively small (< 1%). Another possible reason for the absence of a positive effect on starch digestibility by NSPase supplementation in finely ground diet under INT feeding could be due to the lack of further room for increase in starch digestibility, and improvement to a higher extent may be expected with high viscosity barley. Moreover, the increase in total energy production by increased starch digestibility might be lesser than the energy consumption by gizzard during grinding of hard-to-grind coarse particles. This argument can be supported by the fact that the birds from this dietary treatment had the poorest FCR. Similar effects were seen by Hetland et al. (2002), when increasing the coarseness of barley-based diet increased starch digestibility but the FCE was surprisingly lower.

On the other hand, NSPase supplementation in finely ground diet improved protein digestibility only under INT feeding. Although these interactions have not been studied before, a number of broiler studies have shown improvement in protein digestibility of barley-based diets by NSPase supplementation (Almirall et al., 1995; Friesen et al., 1992; Hesselman & Åman, 1986; Mathlouthi et al., 2002a). Moreover, Hetland and Svihus (2001) have shown that NSPase

supplementation in wheat- or oat- based diets did not interact with varying coarseness of diets to affect protein digestibility. This finding suggests that the effect of NSPase to improve protein digestibility with fine grinding in the current study, was dependent on the INT feeding regimen. INT feeding might have played a key role in enhancement of NSPase efficacy by increased retention time with optimal conditions in the crop. Finely ground diet could have facilitated the accessibility of substrates to NSPase. As a result, this could have reduced the viscosity of jejunal digesta to a greater extent, thus creating space for endogenous proteases to digest proteins. These findings suggest better NSPase function together with fine grinding and INT feeding.

Higher protein digestibility with coarsely ground NSPase-supplemented diet as compared to finely ground NSPase-supplemented diet in the ADL-fed birds might be because of low gizzard pH in the former treatment. Diets with higher coarseness may result in increased HCl secretion and pepsin activity which may increase protein hydrolysis (Gabriel et al., 2003). Moreover, having no difference in protein digestibility between NSPase supplemented and non-supplemented coarsely ground diets under ADL or INT feeding regimen indicates that coarsely ground barley-based diets may not be suitable for NSPase efficacy.

These findings suggest that feeding finely ground barley-based diets under INT feeding regimen stimulate the anterior GIT to a desired extent and enhance NSPase efficacy, whereas coarsely ground barley-based diets under INT feeding regimen may be detrimental for effectiveness of NSPase probably due to ingesta overload on the anterior GIT.

5.4 *Clostridium perfringens*

C. perfringens may be normally present in the broiler gut (Gholamiandekhordi et al., 2006), therefore, taking the prevalence of NetB-toxin producing gene into account is of great importance to access their pathogenicity (Cheung et al., 2010). The prevalence of *C. perfringens* in the ceca was consistently lower in all diets supplemented with NSPase. This observation is in accordance with the results from Choct et al. (2006), in which xylanase supplementation in broiler diets reduced the *C. perfringens* counts in ceca. In another experiment, supplementation of carbohydrase (xylanase, glucanase and mannanase) in broiler diets reduced the *C. perfringens* counts in ileum (Sun et al., 2015). The authors attributed the reduction in *C. perfringens* counts to the reduction in digesta viscosity, faster digesta passage, increased nutrient digestibility and lower amounts of nutrients available for pathogenic bacteria. Moreover, xylanases break down

arabinoxylans into xylooligosaccharides. Xylooligosaccharides are known to reduce cecal pH by acting as prebiotics for cecal microbiota and enhance the production of SCFAs (Campbell et al., 1997). This drop in cecal pH by supplementation of NSPase impedes the growth of pathogenic bacteria in broilers fed with barley-based diets (Mathlouthi et al., 2002a). Moreover, broilers fed with finely ground diets with NSPase supplementation had zero prevalence of NetB-toxin producing gene. This finding suggests better NSPase functionality with fine grinding and thereby lowering digesta viscosity. Furthermore, lower prevalence of *C. perfringens* in the ceca of birds from the INT group might be due to numerically lower digesta viscosity in this group. The main mechanism of action of NSPase that reduces the soluble NSP-related increase in *C. perfringens* counts is the reduction in digesta viscosity (Bederska-Łojewska et al., 2017), thus resulting in reduced digesta transit time and increased oxygen tension (Sun et al., 2015). Moreover, the degradation of soluble NSPs may result in improved nutrient utilization by birds, lesser nutrients availability for pathogenic microbes, and lower secretion of mucus in the broiler GIT. Moreover, it has been shown the xylanases protect birds from *C. perfringens* infection by altering the mucin profile and reducing the permeability of digestive tract for *C. perfringens* (Liu et al., 2012). This may hinder these bacteria from getting nutrients from host cells and may lead to their lower counts in the intestine. Considering the prevalence results of *C. perfringens* and NetB-toxin producing gene together, it can be said that NSPase supplementation in finely ground barley-based diets under intermittent feeding resulted in lowest the prevalence of *C. perfringens*. On the other hand, despite high starch and protein digestibility in birds fed with coarsely ground diet without supplementation of NSPase under ADL feeding regimen, highest prevalence of *C. perfringens* was seen. Moreover, the highest prevalence of NetB-toxin producing gene in the birds from this dietary treatment indicates the presence of considerably higher counts of pathogenic *C. perfringens*. A possible reason behind this could be highest digesta viscosity with this treatment. This finding signifies the risk of practical utilization of this dietary treatment. However, just the prevalence data may not be sufficient to declare the preventive use of NSPase against *C. perfringens*, so further investigations are necessitated before reaching any conclusion. Moreover, the experiment was set up in a highly controlled environment. Therefore, higher prevalence of *C. perfringens* with same diets under field conditions may be expected.

To summarize this discussion, it can be said that INT feeding could have provided the exogenous NSPase with ideal conditions and longer time for action whereas fine grinding could have provided NSPase with easier soluble NSP targets. Hulls in the finely ground diets might have stimulated the gizzard activity to a desired extent while smaller feed particles might have prevented the overuse of gizzard thereby the loss of maintenance energy. Under INT feeding with finely ground diet, the destruction of cell walls structures by NSPase could have released the trapped nutrients and reduced the digesta viscosity while facilitating the access of endogenous proteases to the proteins. This in turn might have enhanced the protein digestibility. Increase in protein digestibility by NSPase along with reduction in maintenance energy requirement might have improved the BWG and thereby FCR in the INT group. Lesser availability of NSP-bile salt complexes, acidic cecal pH, increased oxygen tension, and less mucus secretion altogether due to efficient destruction of soluble NSPs might have limited the prevalence of *C. perfringens* in this dietary treatment. On the other hand, high protein, and starch digestibility of coarsely ground diets in ADL group did not lead to improvement in BWG or FCR, possibly due to increased maintenance energy requirements due to more difficulty in gizzard grinding. These results reject the hypothesis that NSPase will improve starch digestibility with fine grinding or INT feeding or both, and support following hypotheses: NSPase will improve broiler performance (FCR) with fine grinding or INT feeding, and NSPase will improve protein digestibility with fine grinding and INT feeding.

Overall, these findings suggest that offering finely ground barley-based diets in INT feeding may enhance NSPase efficacy as evident from improved protein digestibility and growth performance. However, in the absence of NSPases and INT feeding, increased stimulation of gizzard by coarsely ground barley-based diets may be sufficient for achieving higher starch and protein digestibility but may not necessarily improve broiler performance and may predispose the birds to the risk of *C. perfringens* infection.

6 Conclusion

In conclusion, the findings of the present study suggest that the increased NSPase efficacy either with fine grinding of barley-based diets or by intermittent feeding regimen can improve broiler performance. Moreover, finely ground hulled barley-based diets may stimulate gizzard function and may not require further coarseness. Furthermore, NSPase shows highest efficacy when supplemented with finely ground diet fed under intermittent feeding, thus improving protein digestibility, and reducing the prevalence of *C. perfringens* in broiler ceca. The interaction between NSPase, fine grinding and intermittent feeding on starch digestibility needs to be tested on barley varieties that differ on the basis of viscosity. These findings have important implications for the poultry industry, as they provide insights into strategies for optimizing barley-based broiler diets and improving bird health and welfare, while also promoting sustainable and cost-effective production practices.

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