DIETARY ANALYSIS OF BARLEY CROP FOR ANIMAL FEED SUPPLEMENTATION IN POULTRY FARMING

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Abstract

Barley contains dietary fibres such as beta-glucan, which are not easily hydrolysable by the avian gastrointestinal enzymes but are linked to possible prebiotic properties. Prebiotics are non-digestible food ingredients that selectively encourage the growth of beneficial (probiotic) bacteria in the animal gut. A major advantage of prebiotics is their potential to compensate for the reduction in prophylactic antibiotic use. It is suggested that incorporating prebiotics into animal feed as supplements can modulate animal guts towards ensuring greater immunity against pathogens.

The source and physicochemical condition of a prebiotic is key to its functionality. In the case of cereals, location on the grain and extraction method of prebiotics plays a vital role on its viability. Eight varieties of barley were investigated in this study, with grains separated into nine fractions, FR1 – FR9, by pearling, and each fraction analysed for its biochemistry and how it affects the growth of a probiotic bacterium - *Lactobacillus acidophilus* in a simulated poultry gut. Results showed an increase of beta-glucan from FR1 to FR9. The reverse was the case for protein. FR6 – FR8, supported the highest growth of *L. acidophilus,* with high amounts of beta-glucan. Multiple regression analysis, showed a strong correlation between bacterial growth patterns observed and beta-glucan in FR1 – FR8. However, FR9 with a high beta-glucan content, supported a relatively low amount of bacterial growth, which was attributed to the presence of unavailable nutrients in this fraction.

This research contributes information on the precise distribution of potentially prebiotic substances in eight barley varieties, with FR6 – FR9 standing out. This could form the basis for further research on the prebiotic property of barley in terms of prebiotic structure and mode of action, for use in poultry feed supplementation.

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Glossary

AACC	American Association of Cereal Chemists
AGPS	Antibiotic Growth Promoters
AME	Apparent Metabolisable Energy
DDGS	Distillers Dry Grains with Solubles
DF	Dietary Fibre
DP	Degree of Polymirization
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
FAO	Food and Agricultural Organisation of the United Nations
FOS	Fructo-oligosaccharide
GIT	Gastrointestinal Tract
GOS	Galacto-oligosaccharide
NARS	National Animal Research Support
NAS	National Academy of Sciences
NDO	Non-digestible Oligosaccharide
NRC	National Research Council
PPM	Parts Per Million
PUFA	Polyunsaturated Fatty Acids
RDI	Recommended Daily Intake

SCFA	Short Chain Fatty Acids
TOS	Transgalacto-oligosaccharide
USDA	United States Department of Agriculture
VRE	Vancomycin-resistant *Enterococci*

1.0. Chapter One: General Introduction

1.1. Overview

The poultry industry is an important supplier of nutrients, especially protein, to human diet. However, it is plagued with zoonotic microorganisms such as *Campylobacter* and *Salmonella* which cause food poisoning in humans. These microorganisms are naturally occurring and controlling their invasion has been very challenging even with the use of prophylactic antibiotics (Choct, 2001). Zoonotic microorganisms, aside from the fact that they can cause morbidity and mortality in animals, also cause diseases to humans who consume contaminated animal products, which could lead to hospitalisation and even death. Food poisoning by contaminated chicken products is a very common occurrence. In 2009, 17,000 people were admitted for food poisoning in England and Wales, 88 of these died. In 2011, 72,000 laboratory-confirmed cases of *Campylobacter* poisoning were also reported in England, and this number increased to 500,000 cases in 2013, with 60% to 80% attributed to chicken sources (Takatsuki, 2013). Farmers have experienced huge economic losses due to food poisoning relating to zoonotic microorganisms such as *Salmonella*, *Campylobacter* and *Escherichia coli* as well as a results of other poultry diseases like bird flu (FAO, 2015). Poultry disease is responsible for about 20 percent loss of the gross value of poultry production in developed industries. This figure is a lot higher in developing countries (FAO, 2015).

For decades, antibiotics have been used at sub-therapeutic levels (incorporated in animal feed as additives) as a means of preventing pathogenic colonisation of farm animals. These antibiotics have improved the health status and growth rates of farm animals and have been termed antibiotic growth promoters (AGPs) (Gaggìa et al., 2010). This prophylactic antibiotic use has been combined with very strict hygiene

control at all levels of animal production, to ensure safe animal production, and has been considerably effective for the past fifty years (Castanon, 2007). However, concerns about the development of antibiotic resistant microorganisms brought about a ban on their use in animal breeding in Europe (Choct, 2001). The withdrawal of sub-therapeutic antibiotics, however, increases the risk of farm animals being colonised by pathogens (Edens, 2003). The need for a continuous production of farm animals safe for consumption has put the animal industry under immense pressure to search for alternatives. One area of current research in this regard is the provision of more natural alternatives that can be incorporated in animal feed as supplements, which can modulate animal guts towards ensuring greater immunity against harmful pathogens. The nature of an alternative to antibiotics in animal feed is however, very important because it should have a significant and sustainable beneficial impact on animal production, be safe for animal and human consumption, easy to apply and store, and provide a substantial return on investment (Cheng, 2014). Examples of these alternatives include prebiotics, probiotics, organic acids and enzymes (Choct, 2001). Prebiotics and probiotics are derived from naturally-occurring sources and their use does not generally lead to any serious repercussions. For instance, prebiotics are mostly carbohydrates derived from plants such as cereals, onions, Jerusalem artichokes and chicory roots (Hajati and Rezaei, 2010).

This research work is centred on assessing one of these alternatives – prebiotics, with barley serving as the possible source, focusing on its effect in poultry feed, in terms of microbial proliferation relative to positive poultry health. Prebiotics are 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and or activity of one or a limited number of bacteria in the colon' (Gibson

and Roberfroid, 1995). In other words, the presence of prebiotics in animal feed, impacts positively on animal health by encouraging the growth of 'probiotics'.

Probiotics are microorganisms that include the group of lactic acid producing bacteria, which are normal constituents of the animal gut flora. They can also be intentionally introduced into the gut, as they are helpful in maintaining an appropriate balance of the intestinal microbiota. They ferment a range of feedstuff to produce short chain fatty acids, which in turn prevent the proliferation of harmful bacteria in the gut by lowering the gut pH (Choct, 2001). They are officially defined as 'live microorganisms which when administered in adequate amounts, confer a health benefit on the host' (Gaggìa et al., 2010) Examples of the most common probiotics include *Lactobacillus* and *Bifidobacterium*; others include organisms of the genus *Enterococcus*, *Bacillus and Saccharomyces.* Their presence in poultry gut has been linked with several health benefits such as inhibiting the growth of pathogens (Abudabos et al., 2015). These organisms have a long history of safe use and some of such organisms have been officially approved by the European food standard agency as probiotics (EFSA-ECDC, 2009). This research will focus on how prebiotics from barley can positively affect the growth of a probiotic species of *Lactobacillus* - *L*. *acidophilus*.

As mentioned earlier, prebiotics are mostly carbohydrates and some proven examples include fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), transgalacto-oligosaccharides (TOS) and lactulose (Gaggìa et al., 2010), most of which are available commercially (Fahey et al., 2011). In poultry birds, diets containing FOS has shown positive effects in body weight gain (Yang et al., 2008). The list of potential prebiotics is, however, very large and current research seeks to prove their value. Potential prebiotics of interest in this research, include beta-glucan

and resistant starch obtained from barley. Beta-glucan and resistant starch belong to a class of food substances known as dietary fibre (DF). In nutritional terms, DF has been defined as any polysaccharide that reaches the hindgut undigested, which is a primary property of prebiotic substances. Dietary fibres have also been defined as the edible part of plants or analogous carbohydrates, which resist hydrolysis by alimentary tract enzymes (Charalampopoulos et al., 2002). DF are fermented by probiotic gut microbes to produce short chain fatty acids (SCFA), such as butyrate, acetate, lactate, propionate, valerate and isovalerate. A study by Tsukahara and Ushida (2000) reported a higher SCFA production in birds fed plant based diets, compared to those fed animal based diets due to the fact that the cecal microbiota of birds are not adapted to animal base diets. DF have been linked to various health benefits in poultry and have also been shown to confer some anti-nutritive effects when included in poultry diets. Both effects are dependant on dosage and administration. Dietary fibre in barley is classified either as water soluble, examples of which are beta-glucan and arabinoxylan; or as water insoluble such as lignin, cellulose and hemicellulose (Charalampopoulos et al., 2002). The inclusion of dietary fibre in poultry diets from various sources have been reported to modify the composition and quality of gut microflora population, both *in-vitro* and *in-vivo* (Dunkley et al., 2007; Jiménez-Moreno et al., 2011).

In general, the consumption of barley has been described as beneficial to animal health. Feeding whole grain barley to poultry is a common management practice in Europe, Canada and Australia (Biggs et al., 2007), and it has been noted that feeding whole barley to male chicks improved feed digestibility. Taylor and Jones (2004) reported that the inclusion of 20% whole barley in feed mix before pelleting led to an overall better performance and alteration in gut structure, showing a

smaller proventriculus and a larger gizzard. The gizzard governs many physiological aspects of the gastrointestinal tract (GIT) in poultry, including synchronization of digestion and absorption processes (Svihus, 2011). A healthy gizzard therefore ensures feed particle size reduction, excellent feed flow, gastroduodenal refluxes and enhanced digestive secretions. More importantly, however, this research is concerned with the prebiotic property barley can confer on poultry feed in terms of proliferation of poultry gut microbes. Barley grains and germinated barley feed stuff have been cited as a possible sources of prebiotics (Patterson and Burkholder, 2003).

The health of poultry is most commonly directly proportional to the state of its gastrointestinal tract (GIT). The GIT is the organ with the largest surface area and metabolic capacity in poultry, just like other animals (Brouns et al., 2002). The gut serves as a reservoir of various microorganisms which impacts on poultry health and can pose significant health risks to humans who consume poultry birds. A healthy gut depends on a delicate balance between diet, mucosa (digestive epithelium and overlying mucus layer), and the microbiota. These three interact in a dynamic equilibrium that ensures an efficient functioning of the digestive system (Yegani and Korver, 2008). Poultry feed serves as a source of nutrient to the bird, as well as to the gut microorganisms. Provision of balanced feed formulations is therefore key to the control of microorganisms in poultry, as well as in the development of the gastrointestinal tract. Poultry chicks show slower intestinal development with delayed access to feed compared to those fed immediately after hatching (Potturi *et al*., 2005). In the post hatch period, intake of feed triggers rapid development of the GIT, with the small intestine developing more rapidly than the

rest of the body mass. During this period, the chick transits from utilizing energy from the yolk to the usage of exogenous carbohydrate feed (Noy and Sklan, 1999). The digestive tract of poultry birds is short, compared to other domestic animals. It therefore takes a relatively short time for the feed to pass through the digestive tract, hence, a short time is available for nutrients to be absorbed. To compensate for this, birds require easily digestible nutrients in their diets. Poultry farmers sometimes administer exogenous nutrients such as arginine and β-hydroxy-β-methyl-butyrate into the amniotic fluid at days 17 and 18 of incubation to enhance the development of intestinal villi and the chick's ability and capacity to digest disaccharides (Yegani and Korver, 2008).The frequency and type of feed is therefore critical for the morphological, biochemical and molecular changes of the intestine (Geyra et al., 2001).

Poultry feed these days are no longer just intended to satisfy hunger but to provide required nutrients in the right amount, to improve the physical of birds, and also prevent nutrition related diseases (Roberfroid, 2000). In the past, there was the tendency for farmers to overformulate diets due to insufficient information on nutrient requirement .This practise is however no longer acceptable because it is wasteful and can cause pollution.

Poultry feed, like most animal feed, is tending towards functional food these days. Mark-Herbert (2004) defined functional foods as 'food products fortified with special constituents that possess advantageous physiological effects that improve general body condition and decrease the risk of some diseases'. There are however conflicting arguments for what qualifies as a functional food. A study by Spence (2006), said that probiotics and prebiotics in poultry feed can be classified or labelled as 'enriched products'. Other studies have classified cereals as functional foods with

prebiotic properties (Vitaglione et al., 2008). This has been attributed to the relationship between the consumption of cereal whole grain and the reduced risk of many diseases. ‘Functional cereals’ have also been defined as cereals containing high amounts of fibres such as beta-glucan, arabinoxylan, oligosaccharides and resistant starch (Bigliardi and Galati, 2013).

In Europe, the main cereals used in poultry diet are wheat and barley. Wheat and barley are both lower in feeding value (about 70-80%) than maize which is typically used in the United states (Jacob and Pescatore, 2012). The research reported in this project, however, focuses on the use of barley in poultry feed as a possible source of prebiotics, not as a direct feed. On a general note, the inclusion of barley in poultry diet has been reported to be both beneficial and antinutritive, due to the presence of dietary fibres which are not hydrolyzable by the avian gastrointestinal enzymes. An important member of the dietary fibre family is beta-glucan. Among all cereal grains, barley and oat contain the highest levels of beta glucan. It is usually concentrated in the inner aleurone and subaleurone cell wall layer of barley (Arzu Baman, 1999). The antinutritive property of barley does not make it an excellent choice for poultry feed, however, its use as a feed supplement can confer some health benefits. This is because poultry have a certain need for dietary fibre and when the diet does not provide minimal amount of this nutrient, birds can show abnormal behaviour, including litter consumption and feather pecking (Hetland et al., 2005). The level of fibre required for optimal performance depends on the source of fibre, age of bird and the trait being studied (Mateos et al., 2012). Broilers particularly require a minimal amount of fibre in their diet to optimize litter quality, feed intake, nutrient digestibility and production performance (Hetland et al., 2003). Inclusion of barley in chicken feed has been studied extensively with results

showing various effects based on levels of inclusion, method of inclusion and the barley cultivar used (Jacob and Pescatore, 2014).

1.2. Barley

Barley is a cereal that has been grown mainly for feed and malting purposes for decades and varieties that have not met the malting standards are also listed as feed grade. Currently, barley is also grown for animal feed and other uses such as ethanol production, due to the fact that it can be grown cheaply and easily. Compared to maize and wheat, barley has a higher photosynthetic activity, thus reducing the level of nitrogen fertlizer required for its production (Karley et al., 2011). It is a short season and early maturing crop adapted to a wide variety of climates. The deep root system of barley ensures nutrients and water uptake even in very dry climates (Jacob and Pescatore, 2014). Its has also been reported that barley distillers dried grain with solubles (DDGS) contain higher amounts of proteins and lysine, making it a better source of DDGS than maize (Moreau et al., 2011).

Varieties of barley are classified in several ways, based on seasons, as winter or spring barley, based on number of seeds on stalk, as two-rowed or six-rowed barley; and based on presence of hull, as hull or hull-less varieties (Jadhav et al., 1998). The nutritional composition of barley is, usually, dependent on the geographical location, growing conditions, cultivars, condition of harvest and storage. These factors have lead to varied reports on the nutritional compostion of barley. However, on a general note, barley grain has been reported to be made up of about 60 % starch and 22 % fibre (Knudsen, 1997). Other components include non starch polysaccharides, proteins and lipids. The lipid content of barley is about 2 to 3 %,

mostly located in the germ (Åman et al., 1985). Protein amounts depend largely on the cultivar. Six-row cultivars have higher amounts of about 13.5%, compared to about 11% of two row cultivars (Jacob and Pescatore, 2012). Other substances of importances in barley as mentioned earlier, are the non starch polysaccharides also known as DF.

Figure 1.1 below shows the different layers of the barley kernel. The barley kernel is divided into the husk, pericarp, testa and aleurone layers, which make up about 14% of the kernel; and the germ and endosperm regions, which make up the remaining 86%, with the endosperm comprising about 80-85% of the entire kernel. As shown in the figure below, the chemical components of barley are heterogeneously distributed across the grain. Currently the different layers of a barley grain can be fractionated by processing technologies such as milling, sieving and debranning or pearling (Charalampopoulos et al., 2002) and each fraction studied independently.

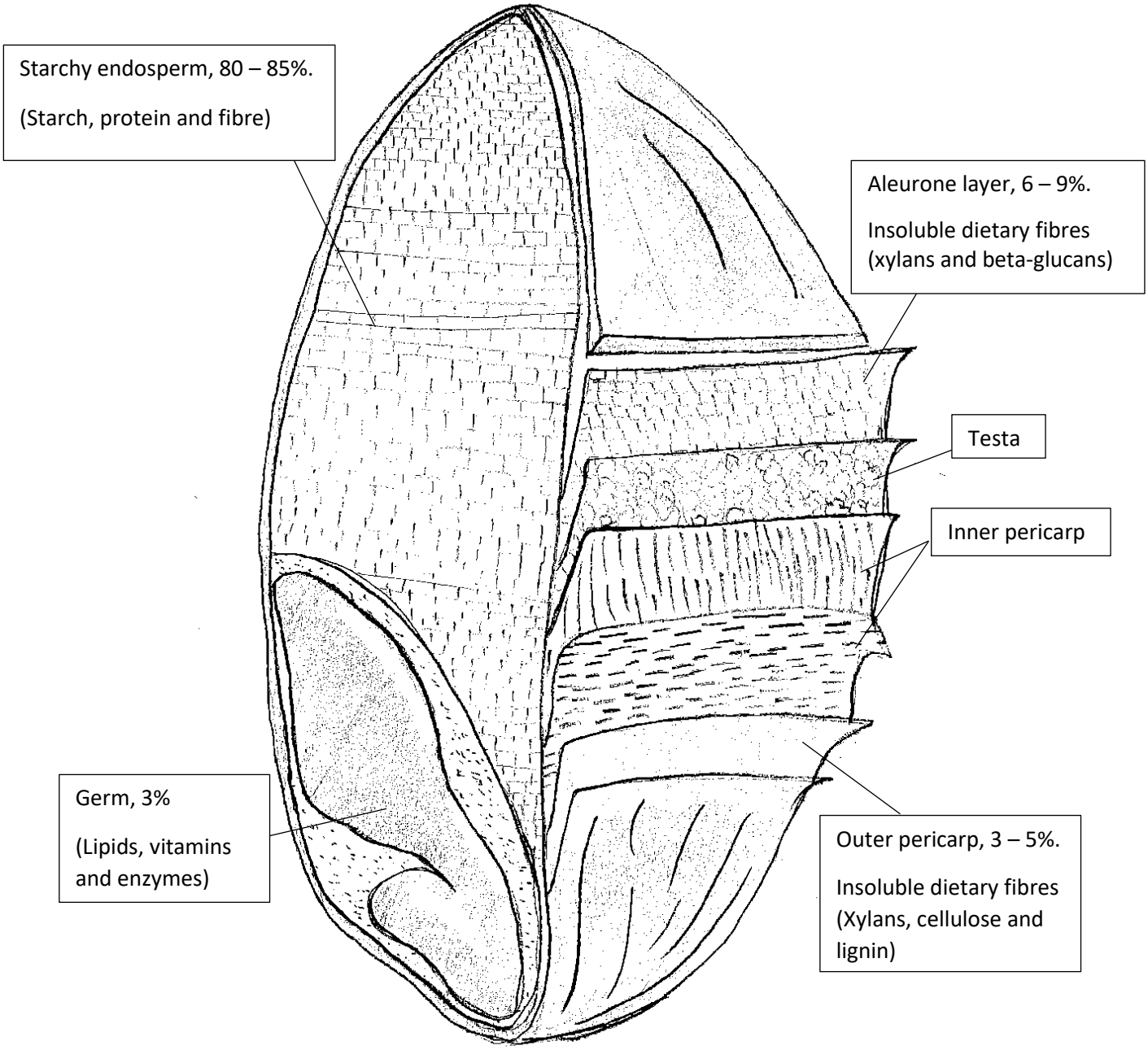


Figure 1.1: Layers of a barley grain

In this research, eight varieties of two-rowed barley (Pearl, Propino, Concerto, Cassata, Maris otter, Munton, Chevalier and Tipple), were analysed for their chemical content, and how three out of eight varieties affect the growth of *Lactobacillus acidophilus* in a simulated poultry gut *in-vitro* in relation to prebiotic properties. Whole grains of these varieties were systematically fractionated by pearling into nine fractions (FR1 - FR9) and each fraction analysed biochemically for the presence of possible prebiotic substances. This method of fractionation was employed because the chemical components in barley grains, as mentioned ealier, are heterogeneously distributed, hence the need of a proper mapping techinique to pin point fractions of the grain containing prebiotic properties of interest. The effect of fractions from three varieties obtained on the growth of *Lactobacillus acidophilus* (a probiotic microorganism) was monitored thereafter to determine which fractions of the grain encouraged the growth of *Lactobacillus acidophilus*.

Lactobacillus acidophilus is an internationally certified probiotic (Hill et al., 2014) commonly found in poultry birds. Studies have shown that its growth is supported by prebiotics and dietary fibres (Charalampopoulos et al., 2002). However, it shows the lowest growth rates compared with other *Lactobacillus,* as a result, it is not commonly used in growth studies (Charalampopoulos et al., 2002; Lee and Salminen, 1995). The growth of *Lactobacillus acidophilus* in this study will therefore be of interest due to its difficult growth requirements, as fractions that support its growth will possess the essential nutrients and excellent growth conditions required by *Lactobacillus acidophilus*, which could then be related to other species of *Lactobacillus*.

1.3. Research Questions:

1. How relevant are the dietary components of barley grain to its ability to affect poultry health positively by encouraging the growth of probiotics when used as a feed supplement?

2. What is the prebiotic potential of barley varieties (with specific emphasis on grain fractions) and how do they affect the growth of *Lactobacillus acidophilus* (a probiotic gut microbe) in a simulated poultry gut?

1.4. Aims

1. Determination of beta-glucan, resistant starch, sugar, protein and FAN content in nine fractions of eight barley varieties. This was done to determine if different fractions vary in their concentration of these components across the barley grain.
2. Determination of the effect of barley fraction on the growth of *Lactobacillus acidophilus* in a simulated poultry gut. This was done with the aim of determining which fraction (FR1 – FR9) encourages the best bacterial growth and how this relates to its chemical composition.

2.1. The method used in point '2' above will separate chemical components (nutrients) in barley fraction extracts that are easily digested from those that are not, using a model poultry digestive system. This is because undigested chemical compounds in feed items could be possibly prebiotic, hence the aim to determine how these undigested compounds affect the growth of the test probiotic bacteria.

3. Determination of growth pattern of the test bacteria in extracts containing both digested and undigested nutrients for comparison with growth patterns obtained from barley fractions containing only undigested nutrients, with the aim of determining prebiotic effects.
4. Correlation of bacterial growth patterns of interest with the chemical compositions of growth media (extracts) with the aim of determining possible causes of growth patterns.

2.0. Chapter Two: Literature Review

2.1. The Poultry Gut

The poultry gut is made of five distinct regions – the crop, proventriculus, gizzard, small intestine (duodenum, jejunum and ileum) and the large intestine (caecum colon and rectum) Figure 2.1. These regions contain a diverse community of bacteria, fungi, protozoa and viruses. Among all the regions of the gastrointestinal tract, the duodenum has the lowest population of bacteria and the caecum the highest. The caecum is an organ of interest in this research. This specialized organ is located at a blind end between the small intestine and the large intestine. It provides a nutrient rich habitat for millions of bacteria, thus providing a site for bacterial fermentation of undigested food particles, which may be prebiotic in nature. Most researchers suggest that the caeca is the primary site of fermentation in poultry GIT (Jamroz et al., 2002; Marounek et al., 2005). The shape, size and capacity of this organ varies amongst poultry birds. For instance, grouse species have a caecum that amounts to about 24% of the body weight, while chicken caeca makes only 1% of the body weight (Redig, 1989).

Over 200 different bacteria species have been isolated from the poultry gut, most of which are strict anaerobes (Józefiak et al., 2004). Microbes of poultry GIT can be generally divided into potentially pathogenic or beneficial. Pathogenic bacteria are usually involved in intestinal infection, putrefaction and toxin formation, while beneficial organisms are involved in enzyme production, stimulation of the immune system and inhibition of the growth of harmful microbes (Jeurissen et al., 2002). The poultry microbiota, is therefore presumed to play a multidimensional role including digestion, metabolism, pathogen exclusion, immune stimulation and vitamin

synthesis (Mulder et al., 1997; Salminen et al., 1998), such that a disruption of the system may affect the health of the bird negatively.

Digestion in chickens starts in the mouth and oesophagus (figure 2.1), with the secretion of amylase by the salivary gland, which breaks down complex carbohydrates (Jacob and Pescator, 2013). However, very little digestion takes place here, due to the minute amount of enzyme secreted and the low retention time. The digesta is then moved to the crop, where it is stored for a while and softened by microbial enzymes. The crop is dominated by *Lactobacilli* and serves as the first major defence against pathogens. *Lactobacillus* in the crop reduces the passage of pathogens further down the digestive tract (Classen et al., 2016). Digestion therefore begins primarily in the proventriculus, which is also known as the true stomach. Hydrochloric acid and pepsin from the intestinal wall are added at this point to the digesta to lower the pH and initiate protein digestion. The digesta moves on to the gizzard for proper grinding and mixing, and then to the small intestine where the final enzyme digestion takes place with the addition of pancreatic juice, bile and bicarbonate. Pancreatic juice containing pepsin further breaks down proteins, while bicarbonate counters the effect of the hydrochloric acid from earlier stages of digestion thus increasing the digesta pH to a strong alkaline. Lipase from the pancreas, is responsible for lipid digestion. The digestion process to this point takes about two hours and is almost complete. Nutrients are ready to be absorbed, while undigested food passes on to the caeca where they are digested by resident bacteria, which could be probiotic or pathogenic. Prebiotics, will be selectively digested by probiotics while non prebiotic undigested nutrients will be digested by either probiotic or pathogenic microbes. The entire digestion process takes about six to eight hours (Jacob and Pescator, 2013).

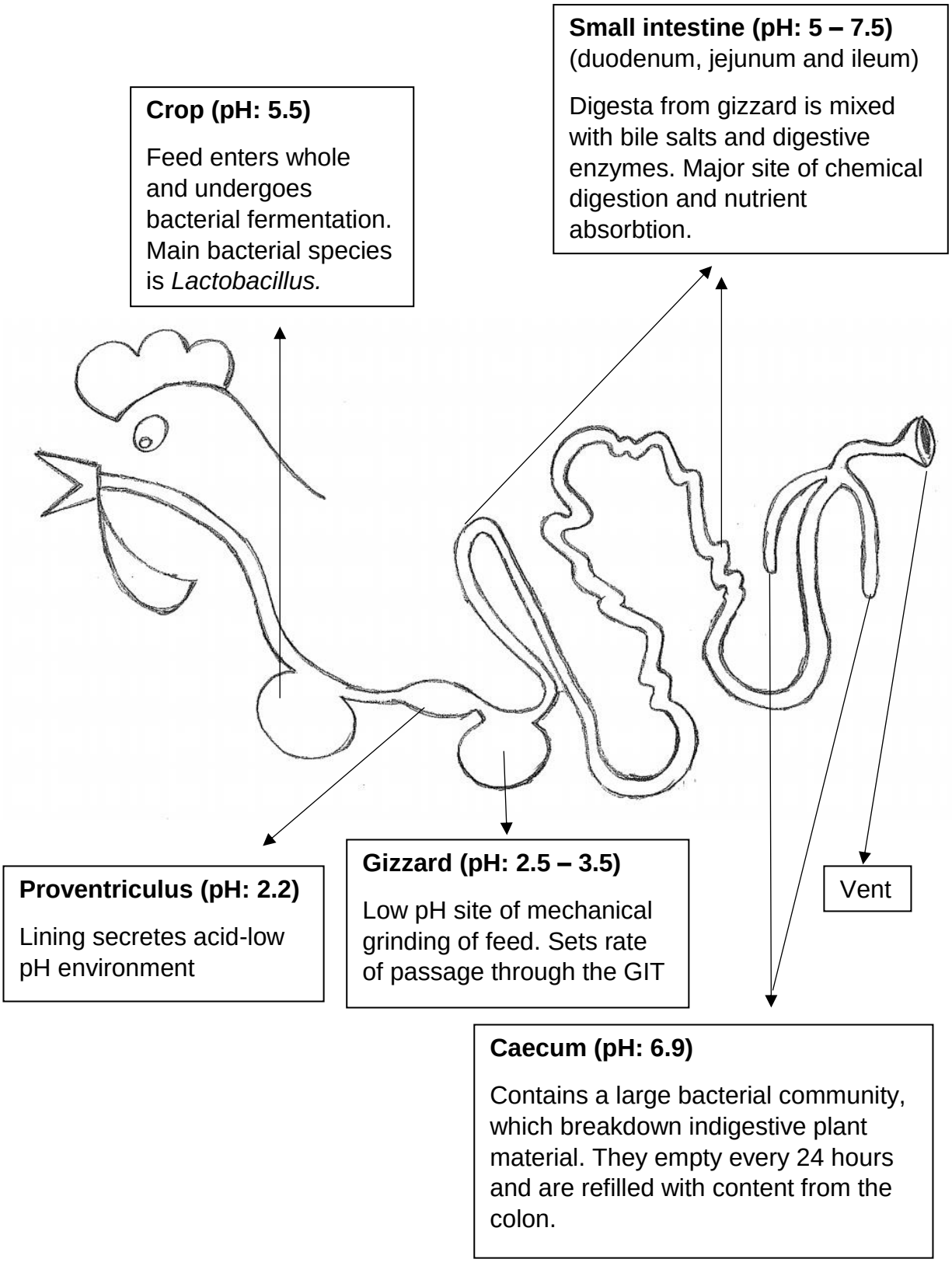


Figure 2.1: The poultry gastrointestinal tract.

The alimentary tract of a newly hatched chick is usually sterile. In poultry, the absence of a normal flora in the intestine is considered a major factor in the susceptibility of chicks to bacterial infections. Newly hatched chicks rapidly gain microbes from their parents and the surrounding environment. The health of the parent bird and type of organisms present in the environment is very vital to the health and survival of the chick. If chicks gain beneficial bacteria, they form a protective barrier, which lines the gut, thus preventing the growth of other harmful bacteria. This principle is commonly known as competitive exclusion. The theory suggests that the commensal microbes will dominate attachment sites on the gut cells, as a result, reducing the opportunity for attachment and colonisation by pathogens (Edens, 2003). Another proposed mechanism is that the intestinal microbiota is able to secrete compounds, such as volatile fatty acids, organic acids and antimicrobial compounds known as bacteriocins that either inhibit the growth of pathogens or make the environment unsuitable for them (Edens, 2003). Some of the microorganisms gained by chicks in the early days could, however be potentially pathogenic to poultry. For example, *Clostridium*, which causes necrotic enteritis, are usually isolated from young birds. Necrotic enteritis, is quite common in poultry production regions of the world (McDevitt *et al*., 2006). Toxins produced by the bacterium are responsible for intestinal necrotic enteritis. *Clostridium perfrigens* is widespread and can contaminate the hatchery, growing and breeder houses as well as processing plants (Craven et al., 2003). The lesions of necrotic enteritis are usually very serious and destructive to the poultry gut (Long et al., 1974). *Clostridium perfrigens* as mentioned earlier, is considered as part of the normal poultry gut microbiota. Other predisposing factors must therefore be present to produce clinical necrotic enteritis. Damage to the Intestinal mucosal caused by the

parasitic coccidiosis in poultry is usually considered as one of the most important predisposing factors (McDevitt et al., 2006). *Salmonella*, *Campylobacter* and *Escherichia coli* are also regularly isolated from healthy older birds. These bacteria, however, do not always produce intestinal disturbances due to a healthy balance of the intestinal microbiota. In fact, intestinal disorders in poultry are usually caused by disturbances resulting from antibiotic treatment, hence, the need for more natural disease prevention feed supplements. Apajalahti, (2004), have shown that one day after hatching, bacterial densities in the ileum and caecum of broiler chicks reach 10^8 and 10^9 cells per gram of digesta respectively. The number of microbes reach 10^9 per gram of ileal digesta and 10^{11} per gram of caeca digesta during the first three days post hatch and remain relatively stable for the following 30 days.

A healthy gut consists predominantly of *Enterobacteriaceae*, *Enterococcus* and *Lactobacillus* in the first few days of life (Van der Wielen *et al*., 2001). *Lactobacillus* species appear in significant amounts at four days of life, up to 10^8 – 10^{10}/ g (Józefiak *et al*., 2004). *Bacteriodes* and *Eubacterium* are established after two weeks (Józefiak *et al*., 2004). *Lactobacillus* species dominant in poultry birds are *Lactobacillus acidophilus*, *Lactobacillus salivarium* and *Lactobacillus fermentum* which are all certified probiotic organisms (Józefiak *et al*., 2004). During the first 2 to 4 days post hatch, *Streptococcus* and *Enterobacteriaceae* colonise the small intestine and caecum. In the caeca of juvenile birds, the bacteria population is different from that found in the small intestine. Actually, as early as three days of age the number of *Enterobacteriaceae* and *Enterococci* in the caeca start to decline probably due to the increase in volatile fatty acids (acetate, butyrate and propionate), produced by *Lactobacillus* and other probiotics. A study by Amit-romach *et al*, (2004) showed that analysis of the microbial luminal contents of

different small intestine sites examined indicated that among six bacteria species examined, only *Lactobacillus* was consistently detected in all intestinal regions. Poultry diet plays a key role in the proliferation of beneficial and pathogenic microorganisms. Substances in poultry diet that serve as energy sources for beneficial probiotic bacteria, are dietary fibres from plant sources. Examples as mentioned earlier include resistant starch and beta-glucan.

Traditionally, in the UK, the definition of dietary fibre includes only non-starch polysaccharides like beta-glucan and lignin, and did not include resistant starch (Sharma *et al*., 2008). However, currently, naturally occurring resistant starch, such as found in whole grains, and other plants and food items, are considered dietary fibres in the UK. Also resistant starches added to foods for health benefits are classified as functional fibre under the American Association of Cereal Chemists, (AACC) and the National Academy of sciences (NAS) definitions, 2002 (Sajilata *et al., 2006)*. In the early stages of prebiotic research, resistant starch, identified as retrograded starch was described as the fraction of starch that could not be hydrolysed without prior chemical dispersion (Englyst and Englyst, 2005). It is often regarded as colonic food, instead of a prebiotic due to the fact that it is not fermented solely by probiotic bacteria (Ogueke *et al*., 2010). Resistant starch positively influences the functioning of the digestive tract and microbiota, just like other prebiotics, though its fermentation rate is slower than other prebiotics. Overall, it was argued that since resistant starch behaves physiologically as a fibre, it should be retained in the total dietary fibre assay (Haralampu, 2000). Resistant starch has also been described as starches which are not broken down by digestive enzymes for a variety of reasons. Depending on the reason, they are classified as RS1, RS2, RS3 and RS4. RS1 results due to physical inaccessiblity of starch granules to

digestive enzymes while RS2 are resistant starches which results due to insufficient preprocessing that produces ungelatinized resistant granules. RS3 are obtained when starchy foods are cooked and allowed to cool while RS4 result due to chemical modification of starchy substances in food items. The type of resistant starch obtained therefore determines its fermentative property by gut microbes.

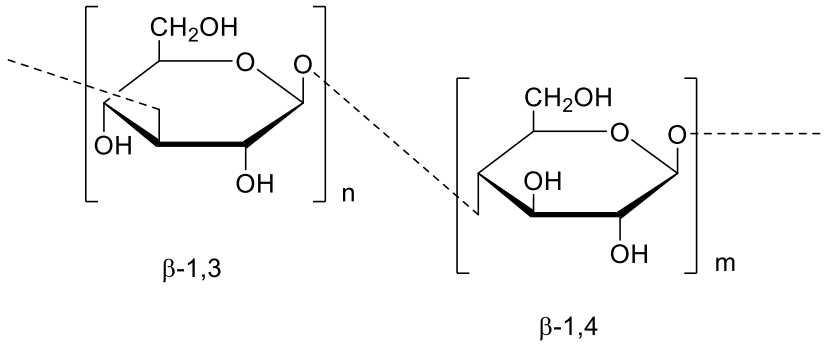


Figure 2.2: Barley beta-glucan.

Beta-glucans are linear or branched polysaccharides of D-glucose monomers linked together by beta-glycosidic bonds and has been termed as possible prebiotic substances. Cereal beta-glucans are usually linear (figure 2.2), while those from bacterial origins are usually highly branched (Vannucci *et al*, 2013). Generally, beta-glucans have immuno modulating effects, however, its source, determines the degree of its effect (Bohn and BeMiller, 1995; Eccles, 2005). It has been reported that beta-glucan having branched chains are more effective than those with a linear structure (Bohn and BeMiller, 1995). Both beta-glucan and resistant starch are fermented by probiotics to produce SCFA. However, the profiles of SCFA produced are slightly different. Resistant starch is known to produce large amounts of butyrates, which is the prime energy substrate of the colonocyte, more than any

other prebiotic (Brouns *et al*., 2002). Van der Wielan *et al* (2001) demonstrated the production of SCFA by the fermentation activities of poultry cecal probiotics.

SCFA serve as additional souces of energy to poultry and are linked to low gut pH which inhibits the growth of pathogenic micro organisms. Insoluble dietary fibre also has an abrasive action as it passes along the GIT, causing endogenous losses of mucin and thus modifying the composition of commensal and pathogenic bacteria capable of adhering to the mucus layer (Montagne et al., 2003a), (Apajalahti, 2004). SCFA production in poultry gut have also been reported to have a toxic effect on *Enterobacteriaceae* and to cause a 50-80% reduction in *Salmonella* (McHan and Shotts, 1993). Apajalahti (2004) reported that barley based diets increased the number of probiotics such as *Lactobacillus* while corn and sorghum based diets increased *Enterococcus* in broiler chicken. Oat based diets were reported to encourage the growth of *Escherichia coli* and *Lactococcus*, while rye based diets increased the number of *Streptococcus* in poultry gut.

Several studies have reported that dietary fibres have a significant effect on poultry gut anatomy, development and function. For instance, it has been widely reported that the ingestion of dietary fiber has lead to an increase in size and length of the digestive organ of poultry birds (Iji *et al*., 2001). A report by Steven and Hume (1998) showed that high amounts of plant fibre and chitin in poultry diet resulted in birds with relatively larger digestive tracts. In his study, he measured the relative length of intestinal segments in 644 specimens, representing 24 orders, 51 families, 124 genera and 166 species of birds and concluded that the most developed caeca were found in granivores and species whose diet contained high levels of plant fibre or chitin. Duke (1984) also reported from a study that high-fibre fed turkey had a 25 % increase in cecal size. The increase of the digestive organ often occurs as a result

of the viscocity created by dietary fibre in the poultry gut. Increased digesta viscosity in the small intestine, leads to high rates of villus cell losses (atrophy) which results in increased crypt-cell production. Increased crypt depth further leads to increase in size of the digestive organ. Mixed linked beta-glucan, obtained from barley and oat, have shown this effect largely in poultry (Bedford and Classen, 1993; Montagne et al., 2003a). The effect of the importance of the viscous property of prebiotics have been proven in several studied where changes in the morphology of the small intestine in poultry have been observed, when poultry birds are feed with diets supplemented with water soluble synthetic viscous polysaccharides (Klis and Voorst, 1993; Smits et al., 1998). Increase in size of the digestive organ, has beneficial effects on poultry health in that it provides a larger surface area, thus ensuring enhanced nutrient absorption. Villus height and crypt depth correlates positively with empty body-weight gain and dry matter intake (Montagne et al., 2003a). Another advantage of dietary fiber in poultry diet, is the positive effect it has on gizzard development and functionality including enhancement of digestive secretions, such as hydrochrolic acid, bile acids and other endogenous enzymes. The synchronization of the entire digestive and absorption process is controlled by the gizzard (Hetland and Svihus, 2001; Svihus, 2011).

2.2. The Poultry Industry

Poultry products contribute greatly to human nutrition while providing employment and income for commercial and backyard farmers. Traditionally, poultry birds were raised in small-scaled backyard farms with household waste, materials from the environment and crop residues serving as feed. This method developed into semi commercial production systems, housing about 50 - 500 birds. Birds in this system

were also fed farm mixings of locally available items (Ravindran, 2013). In both methods, there are usually cases of disease outbreak due to poor hygiene and lack of information on good cultural practices. The poultry industry has however grown to be a multifaceted production system, with an expanded scope and size of farming practises. Currently, there exist large commercial farms that operate highly mechanised integrated production units, which cover breeding flock, hatcheries, feed mills, disease controlled production units, slaughtering and packaging plants. Also included in some production lines, are distribution centres. A vast knowledge on the interdependence of these units has made poultry farming very successful in the world today (Ravindran, 2013). Also available are constant updates on modern methods of hygienic cultural practises, feed requirements as well as disease control measures.

The poultry industry is one of the largest in livestock production, producing meat and eggs for millions of people both in developed and developing countries. About 58 billion chickens are slaughtered for meat annually in the world (FAO, 2013). Poultry products are a rich source of amino acids like lysine, threonine, methionine and cysteine. They also serve as a source of lutein which functions in the prevention of cataract which is a major cause of blindness in developing countries (Farrell, 2013). Poultry meat has also been reported to be the cheapest of all livestock and is seen as a healthier option compared to red meat (Farrell, 2013) because of the following reasons. Fat from chicken is made of desirable mono-saturated fat and is a good source of essential polyunsaturated fatty acids (PUFAs) like omega (n) – 3 fatty acids, with just about one third of its entire meat, made of less healthy saturated fat. It also does not contain trans-fat which contributes largely to coronary heart disease. Poultry meat also supplies vitamins and minerals. 100 g of chicken meat

can supply the recommended dietary intake (RDIs) of niacin for an adult, and also, a hen's egg contains about 53µg iodine/100g edible portion, which is approximately 33 percent of the RDI for an adult (Farrell, 2013).

Poultry products are a good source of high quality protein for low-income earners whose diets are typically high in carbohydrates but low in protein. In 2012, poultry meat production was recorded as 103.5 million tonnes globally (FAO, 2013). This figure was higher than bovine meat (beef and veal) which recorded 67.5 million tonnes. The industry saw a steady growth with an annual average of 4 percent globally between 2005 and 2012 (FAO, 2013), contributing its fair share to global meat production. The poultry industry is contributing millions of dollars to various countries economy, suppling considerably healthy products. It is expected that global meat consumption per person will reach 36.3kg by 2023, with poultry meat contributing 72 percent which is 2.4kg more than the average in 2012 (FAO, 2013). Also, the average age for meat chicken to reach the market weight of 2 kg has steadily decreased from 63 days in 1976 to 35 days in 2009 (Ravindran *et al*., 2009). The steady growth of the poultry industry can be attributed to demand for cheaper and healthier meat, advances in breeding techniques and genetic progress in poultry strains for meat and egg production. Also in recent times, the introduction of mechanized poultry production, integrated poultry production and a better knowledge on feed requirement in the line of functional feed has added more value to the poultry industry.

2.3. Poultry Feed

Poultry feed represents the most important and largest expenditure in poultry production, covering about 70 % of total production cost (Ravindran, 2013). Getting

feed formulation right to a large extent determines profit margins in poultry production. The principal role of feed is to provide nutrients that birds will digest and utilize for growth and development. However, with increased demand for healthier birds and consumer satisfaction, poultry feed is no longer just intended to satisfy hunger and growth requirements but also to improve the health status of birds while preventing nutrition related diseases (Roberfroid, 2000). Without the right feed formulation therefore, poultry will underperform, causing problems for farmers and consumers alike. Figure 2.2 below describes the essential components required for the efficient functioning of the poultry gut.

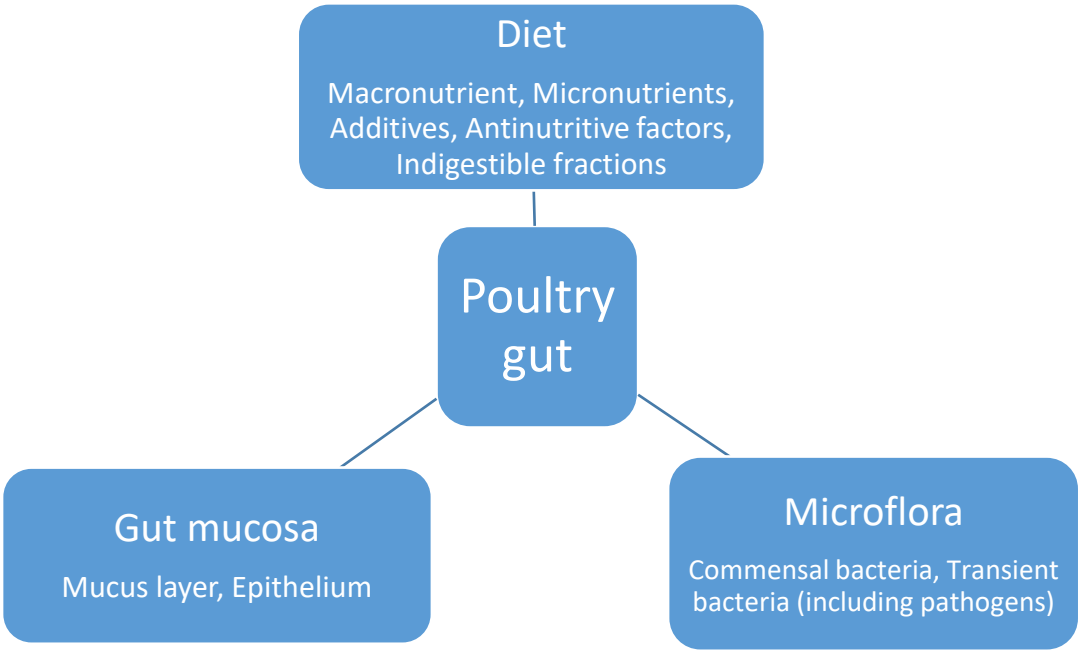


Figure 2.3: Schematic representation of the gut ecosystem (Montagne et al., 2003b). Each element interacts with the other in order to maintain a dynamic equilibrium, ensuring functioning of the digestive system and lack of pathology, a state defined as gut health.

Poultry farmers therefore always seek the best balanced diet formulation at the least cost while maintaining the standards set by National Research Council (NRC). The NRC is the operational arm of the National Academy of Sciences (NAS), established in 1863. The NRC is the benchmark for research, judicial and regulatory information concerning poultry feed requirements. The basic feed requirements for poultry were first published by the NRC in 1944 and have been reviewed frequently since then (NRC, 1994). Information from the NRC on animal feed is used globally as gold standard for regulatory purposes. Figures published were derived from a compilation of an extensive literature review on experimentally determined levels of nutrients requirements for poultry feed by various researches and studies published over the years prior to its first publication. The poultry nutrient requirements published by NRC in 1994 is currently viewed as the minimal nutrient requirement for poultry and they include carbohydrates, proteins/amino acids, fats, vitamins, minerals, water and non-nutritive feed additives (NRC, 1994). All of which will supply energy and building blocks for growth and egg production, as well as prevent deficiency symptoms (Applegate and Angel, 2014). Figures published by NRC have also evolved further over the years and now cover a wider spectrum including nutrient requirement per unit of diet, return on investment on nutrient used, disease prevention and nutrient responses (Applegate and Angel, 2014).

Lessons and Summers, (2001) defined poultry nutrient requirement as 'the minimum amount of nutrient required to produce the best weight gain, feed efficiency and the lack of any signs of nutritional deficiency'. With a wide range of ingredients available, extensive mathematical calculations are now required to ensure poultry feed meets all health and nutrient requirement standards (see chapter 6). In 2010, the USDA in a bid to build a systematic integrated approach to better animal research and

development launched the National Animal Research Support (NARS) project. One of its key objectives was to focus on developing and improving technologies which will provide software platforms with feed ingredients data bases and modelling information for feed compositions which will enable farmers calculate feed requirements mathematically (Applegate and Angel, 2014) for the best production results. The scope of poultry nutrition has increased to include ideas from biology, immunology, microbiology, molecular biology and biotechnology. The focus is also now on ensuring production of healthy birds with highly efficient feed conversion rates. It has been observed that the presence of indigestible nutrients are the major cause of inefficient feed conversions rates (Ravindran and Son, 2011). These indigestible nutrients have however, been found to possess some health benefits (Gaggìa et al., 2010). Poultry have high metabolic rates, hence, have high nutritional needs. It is worthy to note that several factors influence minimum nutrient needs of poultry birds. These include genetics, sex, variety and stage of production as well as environmental factors. This has led to the current concept of 'nutrient responses' in addition to nutrient requirements (Applegate and Angel, 2014). Poultry feed is therefore currently modelled on responses based on desired end products. This approach is gradually replacing static requirements presented in tabular forms. For instance, research has shown that chicken meat and eggs can be enriched further with essential nutrients unlike any other type of meat. A principle where very small changes in poultry diet constituents, in terms of additives, could show huge positive differences in poultry health, meat and egg qualities. Farmers have keyed into this principle and are producing poultry feed tailored to specific needs. A study by Yu *et al*, (2008) showed that the selenium content of chicken breast meat increased from 8.6 µg to 41 µg/100 g by the addition of 0.24 mg organic selenium per kilogram of

feed (Yu *et al* 2008). Selenium is a powerful anti-oxidant that plays a major role in the prevention of cancer. Another study by Surai, (2000), showed that supplementing the diet of laying birds with 0.4 mg organic selenium per kilogram of feed increased selenium content of 100 g edible egg from 20 µg to about 60 µg. The RDI of selenium is 55 µg for an adult (Farrell, 2013). Also, inexpensive supplementation of poultry diet with 5 mg potassium iodide increased iodine content of a 60 g egg from 26 µg to 88 µg, which is more than 50% of an adults RDI (Röttger *et al*., 2011). About one billion people suffer from iodine deficiency – a major cause of goitre and mental retardation (Farrell, 2013). In the light of the above, poultry feed are being developed to test the effect of prebiotics and probiotics substances with desirable qualities on poultry health and products, despite the problem of variation of nutrient composition and formulation versus nutrient delivered to birds. These new feed models are designed from already existing basic feed requirements, which are then developed and analysed as alternative feeding regimes under existing husbandry condition and are used as a means of comparing potential versus actual performance (Applegate and Angel, 2014). Current feed models also have various challenges because of differences in individual responses with a particular environment and feed management system.

2.3.1. Carbohydrates

The major source of energy for poultry is dietary carbohydrates mostly in the form of starch, which is derived mostly from maize in the United States. Farmers in the United States are the world's largest producers of poultry. Others sources of energy include wheat, sorghum and barley, which are common in other parts of the world (Moran, 1985a). Maize is used commonly as an energy source because of its high

digestibility and palatability, with the added advantage of being free of anti-nutritive factors. In recent times, a lot of maize has been diverted for use in the biofuel industry thus cutting the supply of maize to the poultry industry. However, by products from the biofuel industries are recycled back to the poultry industry as feed. For example, distillers dried grains with soluble, DDGS, are recycled back as a good source of amino acids (Ravindran, 2013). Energy requirement is a very important factor to consider in poultry feed formulation. This is because it determines largely the feed cost. The appropriate amount of energy ensures the lowest feed cost per unit of poultry product (NRC, 1994). Most poultry birds eat just enough to meet their energy needs hence energy concentration serves as a basis for setting other nutrient requirements (Leeson and Summers, 2001). Some studies have shown that feed intake may not necessarily be proportional to changes in energy concentration (Leeson and Summers, 2001; Thomas et al., 1961). Energy in poultry feed is classified as metabolizable energy (AME) which is the amount of energy available in the feed for use by birds to meet their daily metabolic needs. Energy needs, unlike most other nutrient requirements, vary with amount of physical activity and environmental temperature. The amount of energy in poultry feed might also affects the intake of other nutrients because high-energy content usually decreases feed intake (NRC, 1994).

Other sources of energy such as barley contain high amounts of polysaccharides and oligosaccharides, most of which are not easily digested by poultry hence, are not classified as good energy sources and therefore used commonly with enzymes by farmers. Some of these polysaccharides have been linked to some negative effects in the digestive process. For instance, beta-glucan, a non-starch polysaccharide in barley increases the viscosity of the digesta, which further

interferes with nutrient utilization (Bedford *et al*., 1991). However these polysaccharides have been termed possible prebiotics, and have been linked to encouraging the growth of probiotic bacteria in poultry gut (Bigliardi and Galati, 2013). The poor performance of growing birds fed barley based diets has been known for decades and was initially associated with the high fibre content of the hull of the grain, however, hull-less varieties have shown similar effects (Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). It has been shown that beta-glucan was responsible for this effect (White *et al*., 1981). Beta-glucan has a high water holding capacity, which results in gel formation, thus increases the intestinal viscosity of the digesta. The level of viscosity in a barley feed is indicative of the amount of beta-glucan present. White *et al* (1981) isolated beta-glucan from barley, added it to a maize based diet, and reported a reduction in growth performance as well as an increase in the viscosity of the digesta samples collected. Although various reports have linked increased viscosity with barley-based diets with water-soluble beta-glucan, Gohl *et al* (1978) reported that soluble arabinoxylan might also be an associated factor. Fuente *et al* (1995) indicated that poultry digesta viscosity accounts for 97% of the variations in the AME in barley-based diets. The viscous nature of the digesta could also cause an increase moisture content of poultry litter as more time is required for the complete mixing of a viscous intestinal content. This is usually not possible due to the relatively short nature of the poultry digestive tract and the flow rate of the digesta, which results in sticky droppings and a reduction of the air quality of the poultry house.

High intestinal viscosity is often linked with digestive health problems in poultry. A decrease in digesta passage rates makes colonisation with potentially pathogenic bacteria easier (Yegani and Korver, 2008). Chicken fed barley-based diets have

shown increased incidences of necrotic enteritis associated with increased levels of *Clostridium perfrigens* in the gut (Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). Necrotic enteritis is a breakdown of the intestinal wall commonly caused by *Clostridium perfrigens*, an anaerobic bacterium often found in the small intestinal tract of poultry. At low levels, *Clostridium perfrigens* does not pose any health risk to poultry, however, at higher levels necrotic enteritis can result in serious poultry disease. Antibiotics have been used prophylactically and therapeutically to check the problem in the past (Immerseel *et al*., 2004), however, with the ban on prophylactic antibiotics in poultry feed and increased cost of biosecurity, poultry farmers are turning to functional food as alternatives to control poultry diseases. Other studies, conversely have reported various advantages of including barley in poultry diet. These advantages are however dependent on the amount and level of inclusion. It is commonly accepted that an increase in dietary fibre reduces feed intake in poultry. However, different authors have demonstrated that the inclusion of moderate amounts of insoluble dietary fibre does not affect voluntary feed intake in broilers (González-Alvarado *et al*., 2007; Jiménez-Moreno *et al*., 2011, 2009), in turkey (Sklan *et al*., 2003) and laying hens (Almirall *et al*., 1995). The inclusion of fibre in poultry diets has shown enhanced intestinal function and modification of the composition and quality of the gut microbiota population both *in vitro* and *in vivo* (Dunkley *et al*., 2007; Jiménez-Moreno *et al*., 2011).

Current research on the topic of functional feed has reported conflicting arguments on what qualifies as a functional feed, its usage and labelling. A recent review of functional foods revealed that research in this area is driven by already existing nutritional information and directives on food additives regulation (Bigliardi and Galati, 2013). Of all functional food researched, prebiotics and probiotics have taken

centre stage. Cereals contains high amounts of indigestible substances and have been studied widely for their potential to act as functional foods (Vitaglione *et al*., 2008). These cereals are known as 'functional cereals' and they have been defined as cereals containing high amounts of dietary fibres such as beta-glucan and arabinoxylan; oligosacharrides such as galacto- and fructo- oligosacharrides; and resistant starch (Bigliardi and Galati, 2013). Cereals studied include oat and barley, which contain about 11.5 % - 27.1 % dietary fibre, with barley usually containing the highest amounts. The research is driven by the relationship between the consumption of cereal whole grain and the reduced risk of many diseases (Vitaglione *et al*., 2008). The beneficial effect of cereals is also attributed to the presence of associated phenolic compounds such as hydroxycinnamic acids which are released by the degradation of cereals by intestinal microbes. These phenolic compounds are absorbed through the intestine into the blood stream where they then exerts health benefits (Vitaglione *et al*., 2008).

Barley can be included in poultry feed as an energy and fibre source. However, the amount of fibre and non-starch polysaccharides needs to be controlled to obtain beneficial effects. For instance, it has been shown that soaking barley grains in water before inclusion in poultry diets, causes positive effects on bird performance. The positive effect was linked to the reduction of water-soluble beta-glucan and the activation of endogenous enzymes in cereal grains capable of degrading them (Annison and Choct, 1991). Water soaking can reduce beta-glucan content as this effect was also noticed with high moisture storage (Svihus *et al*., 1997). High moisture storage has however been linked to fungal contamination (Jacob and Pescatore, 2012). Barley based diets can also be pelleted to enhance its quality, as pelleting was reported to reduce digesta viscosity, caused by the presence of beta-

glucan, by 45% (Pettersson *et al*., 1991). This research also recommends that using fractionation techniques, fractions of barley grains containing high amounts of beta-glucan can be removed and later reintroduced as supplements, at the right amounts in poultry feed to obtain the best results. Fractionation of barley grains can be easily achieved by modern techniques of pearling. In barley botanical components, the majority of dietary fibres generally occur in decreasing amount from the outer pericarp to the endosperm (except arabinoxylan, which is also a major component of endosperm cell wall material) (Izydorczyk and Dexter, 2008).

2.3.2. Fats

Fats in poultry diets are sources of energy. Oxidation of fat yields large amounts of energy in animal cells. Their presence therefore ensures a healthy supply of energy. Other advantages of fats are that they improve the palatability of the diet and increase intestinal retention time, allowing for complete digestion and absorption of nutrients (Sell *et al*., 1987). There are no specific requirements for fat in poultry diet however requirements for linoleic acid, an essential fatty acid have been demonstrated (Ensminger *et al*., 1990). Sources of fats include vegetable oils such as soy and corn oil. Some feeding trails indicated that addition of fat improved the performance of chicks on barley-based diets (Edney *et al*., 1989). In these studies, barley was used to replace maize on a weight for weight basis. The improved performance seen was reported to be most likely due to increased energy content in the diet with the addition of fat, rather than a reduction on the effects of beta-glucan present in barley.

2.3.3. Proteins and Amino Acids

Protein and amino acids are required by poultry for development of bone, muscles, organs and other body parts. They supply building blocks for structural tissues. Nutritionally, amino acids are classified as essential or non-essential. Essential amino acids need to be provided in poultry feed, while non-essential amino acids can be synthesized by birds. Essential amino acids include lysine, methionine, threonine, tryptophan, isoleucine, histidine, valine, phenylalanine and arginine with particular requirement depending on the bird type. For instances, broiler chickens have very high demands to meet growth needs, since they are reared for meat (NRC, 1975). Sources of protein could either be plants or animals. An excellent source of plant protein for poultry is soybean, which contains about 40 to 48 percent crude protein with a right balance of essential amino acids. Fish meal and bone meal are sources of animal proteins (Ravindran *et al*., 2009). There are also commercially available amino acids such as methionine, lysine, threonine and tryptophan that can be purchased and included in poultry diets (Ravindran, 2013).

The digestion and almost complete absorption of protein takes place primarily in the small intestine, however, amino acid residues from undigested feed also pass to the caeca, where they are fermented by resident bacteria, which replace the protein residues with bacterial proteins with different amino acid profiles, thus aiding amino acid synthesis. The origin of protein residues that reach the caeca depends on a number of factors including the presence of anti-nutritive substances in the poultry feed, which could inhibit protein digestion. Protein sources in poultry diets are a heterogeneous mixtures of various proteins, which are digested at different rates in the small intestine. They also have various linkages with other carbohydrates and lipids in the diet, such that the composition and interactions of these substances

determine the entire digestive process, which in turn determines feed conversions rates (Hughes and Choct, 1999). Non-starch polysaccharides and phytate are examples of anti-nutritive factors that depress protein digestion and utilisation due to the high viscosity the cause in the poultry gut (Bryden and Li, 2010; Hughes and Choct, 1999). It has been reported also that not all protein that reach the caeca are from feed sources but rather of endogenous origin such as remains of digestive enzymes or shed mucosal cells caused movement of fibrous feed along the digestive tract (Bryden and Li, 2010). Table 2.1 below summarises nutritional components of cereals used in poultry feed.

Table 2.1. Nutritional components of cereal grains (per 100g)

Cereal grain	Energy (Kcal)	Water (g)	Carbohydrate (g)	Protein (g)	Fat (g)	Minerals (g)
Barley (pearled)	352	10.09	77.72	9.91	1.16	1.11
Corn (field)	365	10.37	74.26	9.42	4.47	1.20
Millet	378	8.67	72.85	11.02	4.22	3.25
Oats (Oatmeal)	384	8.80	67.00	16.00	6.30	1.90
Rye	335	10.95	69.76	14.76	2.50	2.02
Sorghum	339	9.20	74.63	11.30	3.30	1.57
Wheat (hard red winter)	327	13.10	71.18	12.61	1.54	1.57

Source: Gebhardt et al, 1978. Composition of foods. Agricultural hand book no.8-20, US department of agriculture.

2.3.4. Non-Nutritive Feed Additives

Also included in poultry diets, are a range of non-nutritive additives, which function in ensuring improved health and performance of poultry birds. Examples include enzymes, antibiotics, probiotics, prebiotics, etc. Examples of non-nutritive feed additives are summarized in table 2.1 and 2.2.

2.3.4.1. Enzymes

Poultry birds do not produce non-starch polysaccharide degrading enzymes and are therefore unable to hydrolyse non-starch polysaccharides present in the grains they consume. Non-starch polysaccharides dissolve in water to produce sticky substances, producing a gel-like mixture in poultry gut thus, leading to a reduction in gut performance (Campbell *et al*., 1989; Ward, 1995). Enzymes are used as feed additives to overcome the anti-nutritive effects of arabinoxylans (in wheat and triticale), beta-glucans (in barley) and phytate (in all plant feedstuffs) thus, reducing digesta viscosity, improving the overall nutrient availability and feed value which encourages a balanced growth of microorganisms in the gut (Khattak *et al*., 2006). Enzyme supplementation improved broiler performance with both high and low-viscosity barley cultivars with a more positive response recorded in high viscosity extract cultivars (Campbell *et al*., 1989). The enzymes are typically of fungal or bacterial origin (Bedford and Apajalahti, 2000).

Poultry feed enzymes increase the feeding value of barley, reduced the variations in available nutrients and the incidence of wet litter common in birds fed barley-based diets. The beneficial effects of enzyme supplementation include non-starch polysaccharide hydrolysis of grains, eliminating the nutrient-encapsulating effect of the cell wall, which then results in the release of some available sugars (Guenter,

1993). The aleurone layer of barley is multicellular, unlike other cereals with single cell layers (Jacob and Pescatore, 2012). Supplementing barley-based diets with enzymes can also alter the microbiota in the digestive tract. Jozefiak *et al* (2010) reported lower numbers of the potentially pathogenic *Enterobacteriaceae* and increased number of *Bifidobacteria* with the addition of beta-glucanase to barley based poultry diet. Mathelouthi *et al*, (2002), reported that the addition of beta-glucanase in barley-based broiler diets reduces the viscosity of the intestinal contents and slowed the growth of *Escherichia coli*. Rodriguez *et al*, (2012) compared supplementing barley-based broiler diets with a feed enzyme cocktail (xylanase and beta-glucanase), a prebiotic (inulin), a probiotic (*Enterococcufaecium*) and a probiotic and prebiotic combination, and concluded that they all had beneficial effects on the intestinal microflora of the broilers.

Table 2.2: Non-Nutritive Feed Additives Commonly used in Poultry Feed Formulations

Additive	Example	Reasons for use
Enzymes	Xylanases, beta glucanases, phytase proteases	To overcome the anti-nutritive effects of arabinoxylans (in wheat and triticale), β glucans (in barley) or phytate and proteases (in all plant feedstuffs); to improve the overall nutrient availability and feed value
Antibiotics[1]	Avilamycin, viginiamycin, zinc bacitracin, avoparcin, tylosin, spiramycin	To control harmful bacterial species in the gut; to improve production efficiency; as a prophylactic measure against necrotic enteritis
Coccidiostats	Moneensin, salinomycin, narasin	To prevent and control the clinical symptoms of coccidiosis
Pigments	Xanthophyll (natural and synthetic)	To increase yolk colour in eggs and to improve the skin colour and appearance of carcasses
Antioxidants	Butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), ethoxyquin	To prevent auto-oxidation of fats and oils in the diet
Antifungals		To control mould growth in feed; to bind and mitigate the negative effects of mycotoxins

[1] The use of avoparcin, zinc bacitracin, spiramycin, virginiamycin and tylosin phosphate as animal feed additives was banned in the European Union in 1998. Envisaging a total ban on in-feed antibiotic use, a multitude of feed additives (individually and in combination) are currently being tested.

Table 2.3: Non-Nutritive Feed Additives Commonly Used in Poultry Feed Formulations (cont.)

Probiotics	*Lactobacillus*, *Bifidobacteria*	To provide beneficial lactic acid species
Prebiotics	FOS, GOS, inulin	To bind harmful bacterial
Organic acids	Propionic acid, diformate	To lower gut pH and prevent the growth of harmful bacterial
Botanicals	Herbs, spices, plant extracts, essential oils	To prevent the growth of harmful bacteria
Antimicrobial proteins/peptides	Lysozyme, lactacin F, lactoferrin, α-lactalbumin	To prevent the growth of harmful bacteria

[1] The use of avoparcin, zinc bacitracin, spiramycin, virginiamycin and tylosin phosphate as animal feed additives was banned in the European Union in 1998. Envisaging a total ban on in-feed antibiotic use, a multitude of feed additives (individually and in combination) are currently being tested.

2.3.4.2. Antibiotics (Sub-Therapeutic)

Control of zoonotic microorganisms has been a major challenge for animal breeders. For decades, antibiotics have been used at sub-therapeutic levels as a means of preventing pathogenic colonisation. Poultry feed is usually not sterile, containing a lot of foreign bodies such as bacteria and antigens. The gut lining serves as the only interface between the bird and these foreign materials, which like all foreign materials, whether pathogenic or not, are able to stimulate immune responses (Huang *et al*., 2005). Early passage of feed through the gastrointestinal tract therefore stimulates the proliferation of stem cells, to withstand environmental

antigens, thus creating a wide repertoire of antibodies (Uni, 1998). Different bacteria species have different nutrient and growth requirements. The nutrient and chemical composition of poultry digesta determines to a large extent, the microbial composition (Apajalahti, 2004). In the 1940s, it was discovered that animals that were fed dried mycelia of *Streptomyces* containing chlortetracycline residues, showed better growth rates. This improved growth performance was attributed to observed better animal health status because of the prevention of pathogenic invasion and improvement in feed conversion efficiency. This led to further research and the conclusion that antibiotics ensured efficient nutrient availability and absorption by causing the host intestinal epithelium to be thinner thus reducing intestinal microbial load (Ohimain and Ofongo, 2012), which implies a reduction of harmful bacteria, thus promoting animal health. Antibiotics have therefore been included as an additive in animal feed for use as growth promoters (AGPs) since the 1940s until 2006, when a ban was placed on their use (Richards *et al*., 2005).

The use of antibiotics at sub-therapeutic levels came with little or no initial concern because of an argument which stated that the residue of antibiotics in animal produce was not enough to cause allergic or toxic reactions in humans and was therefore negligible (Edens, 2003). As a result, their use was approved by governments all over the world for the past 50 years without any reservations, up until 2006. For example, bacitracin and lincomycin were classified by the World Health Organisation as neither critical nor highly important antimicrobials (FAO/WHO, 2006). These antibiotics therefore, according to a report are of limited or no importance to human medicine; hence their use will not pose any negative effect on humans (Smith, 2011). The use of antibiotics as AGPs has recorded tremendous success in the poultry industry. Choct (2001) reported 3-5% increase

in growth and feed conversion efficiency with the application of AGPs. It was also reported that the use of AGPs in poultry has been effective in the prevention of necrotic enteric (Immerseel *et al*., 2004). In 1951, the Food and Drug Administration agency of the United States approved the use of antibiotics without prescription as a feed additive for farm animals (Edens, 2003). States in the European Union also adopted similar regulations that approved the use of antibiotics as an animal feed additive between 1950 and 1960 (Castanon, 2007). Below is a table showing antibiotics that were approved nationally for inclusion in animal feed without veterinary approval in the European Union until the imposition of the ban.

Table 2.4: Antibiotics, which were permitted as, feed additives only in national poultry feeds in Europe

Antibiotic	Species of Animal	Maximum Content and Period of Authorisation	Legislative Reference
Bacitracin manganese, neomycin, tylosin, sotramycin	Only national restriction	November 25, 1970 – June 30, 1976	Directives 70/524, 75/296
Hygromycin B	Only national restriction	November 25, 1970 – December 31, 1976	Directives 70/524, 75/296, 76/603
Erythromycin	Only national restriction	November 25, 1970 – June 23, 1976	Directive 70/524, 75/296, 76/603
Erythromycin	Chicken for fattening	20ppm, June 24, 1976 – December 31, 1978	Directives 76/603, 78/58

Source: (Castanon, 2007)

Table 2.5: Antibiotics, which were permitted as, feed additives only in national poultry feeds in Europe (cont.)

Bacitracin-methylene-disalicylate	Poultry (excluding ducks, geese and laying hens) up to 10wks old	20ppm, April 29, 1975 – December 31, 1977	Directives 75/267, 75/296, 76/603
Lincomycine	Poultry (excluding ducks, geese and laying hens) up to 10wks old	10ppm, March 5, 1974 – June 30, 1981	Directives 74/180, 75/296, 76/603, 78/58, 79/139, 79/553, 80/618, 80/1139
Mocimycin	Chickens for fattening	5ppm, August 1, 1978 – November 30, 1983	Directives 78/743, 80/1156, 82/91, 82/822
Nosiheptide	Chickens for fattening	10ppm, November 16, 1977 – December 3, 1986	Directives 79/1011, 80/1156, 82/91, 82/822, 83/466, 84/349, 85/342, 85/520
Ardacin	Chickens for fattening	7ppm, January 20, 1995 – November 30, 1997	Directives 94/77, 95/55, 96/66, 97/72

Source: (Castanon, 2007)

2.3.4.2.1. The Ban on Sub-Therapeutic Antibiotics in Animal Breeding

Antibiotics approved for use as AGPs were those that can be absorbed by the digestive tract and were administered in minute and manageable amounts. Their continuous use however created some concerns in some quarters. There was fear over the very possible risk of development of antibiotic resistant microorganisms in farm animals, with possible transfer of these resistant microbes along the food chain through meat and milk (Mathur and Singh, 2005; Salyers *et al*., 2004). As a result, the World Health Organisation (1997) and the Economic and Social Committee of the European Union (1998) concluded that the continuous use of antibiotics at sub-therapeutic levels in farm animals reared for food, was a public health issue (Castanon, 2007). In 1999, about 30 percent of all antibiotics used in Europe went into animal feeds amounting to an estimated 3.52 million kilograms of antibiotics entering into the human system through chicken and pork alone (Edens, 2003). Several other reports emerged that emphasized the risk. For example the occurrence of vancomycin-resistant *Enterococci* (VRE) in Australia was linked to the use of glycopeptide avoparcin as a growth promoter (Collignon, 1999). Other examples include reports of antibiotic resistant microorganisms found in Czech Republic poultry industry, as summarised in table 2.6.

Table 2.6: Antibiotic resistance of *E. coli* (128 strains), *Staphylococcus* (88 strains), and *Enterococcus* (223 strains) found in Czech Republic poultry industry

Antibiotic	***E.coli***	***Staphylococcus***	***Enterococcus***
Amikacin	8/128	-	-
Ampicillin	65/128	-	3/228
Ampicillin/Sulbactam	0/128	4/88	3/228
Aztreonam	8/128	-	-
Cefazolin	8/128	-	-
Cefpirome	8/128	-	-
Cefoperazone	8/128	-	-
Cefoperazone/sulbactam	8/128	-	-
Cefotaxime	8/128	-	-
Ceftazidime	8/128	-	-
Cefuroxime	8/128	-	-
Cefoxitin	8/128	-	-
Ciprofloxacin	13/128	-	-
Chloramphenicol	11/128	3/88	16/228
Clindamycin	-	17/88	-

Table 2.7: Antibiotic resistance of *E. coli* (128 strains), *Staphylococcus* (88 strains), and *Enterococcus* (223 strains) found in Czech Republic poultry industry

Antibiotic	***E.coli***	***Staphylococcus***	***Enterococcus***
Erythromycin	-	35/88	135/228
Gentamycin	8/128	-	16/228
Merpenem	8/128	-	-
Netilmicin	8/128	-	-
Nitrofurantoin	-	-	78/228
Ofloxacin	13/128	12/88	117/228
Oxacilin	-	4/88	-
Piperacillin	48/128	-	-
Piperacillin/tazobactam	0/128	-	-
Streptomycin	-	-	51/228
Telcoplanin	-	0/88	12/228
Tetracycline	125/128	13/88	183/228
Trimethoprim/sulfamethoxazole	18/128	-	-
Vancomycin	-	0/88	12/228

Source: Kolar et al., 2002.

In January 2006, a ban was placed on the use of antibiotics as growth promoters due to the associated risk of selecting for antibiotic resistance in microbial flora (European Union (a), 2003; European Union (b), 2003). Some negative side effects were expected from the removal of AGPs since there were no ready alternatives towards prevention of pathogenic colonisation of healthy farm animals. These negative effects included a massive drop in animal productivity due to increased animal mortality from diseases, reduction of feed conversion efficiency, poor growth as well as increase in the use of therapeutic antibiotics on diseased animals (Casewell *et al*., 2003). It was also estimated, that US consumers would pay $1.2-2.5 billion more due to expected increased cost of production (Gill and Best, 1998). A report by Danish integrated Antimicrobial Resistance Monitoring and Research Program in 2008, revealed that the use of therapeutic antibiotics in all food animals increased by 110% between 1998 and 2008 due to a gradual phasing out of AGPS (Smith, 2011). The blanket ban on all AGPs has since been described as unwise, unscientific, unwarranted and counterproductive (Smith, 2011). The removal of AGPs has put the animal breeding industry under immense pressure to provide alternative feed additives toward ensuring animal health. Currently, research in this regard is centred on more natural alternative that could be implemented at primary levels of animal production. Research on the modulation of animal gut microbiota using alternative feed additives like probiotic, prebiotics, organic acids, and enzymes towards prevention of invasion by pathogens is currently creating room for fascinating possibilities like the total replacement of in-feed antibiotics with equally competent natural alternatives (Gaggìa *et al*., 2010).

2.3.4.2.2. Alternative to AGPs

The poultry industry like every other food processing industry is expected to deliver healthy products that are safe and free from contamination to consumers irrespective of the ban on prophylactic antibiotics. Despite efforts to achieve safe poultry products delivery, there have been many outbreaks of food poisoning and cases of food borne diseases resulting from consumption of contaminated poultry products over the years (Takatsuki, 2013). It is common knowledge that *Campylobacter* and *Salmonella* are the major causes of contamination of poultry products. Contaminated products when consumed by humans, cause illnesses and infections known as zoonosis. The spread of *Campylobacter is* very rapid in chicken; therefore, reduction in the number of infected birds helps reduce associated risk to consumers. Campylobacteriosis has been reported to be the most frequently reported zoonotic disease in humans in the European Union (Hugas *et al*., 2009; Westrell *et al*., 2009). The European food safety authority (EFSA) also reported that poultry is the major source of sporadic campylobacteriosis in humans (EFSA-ECDC, 2009). In 2007, one year after the ban of AGPS, 151,995 people were affected by *Salmonella* in Europe (EFSA-ECDC, 2009). In Europe, 26 % of fresh broiler meat tested positive to *Campylobacter* in 2007 (Westrell *et al*., 2009). A recent report by the Food Standards Agency, United Kingdom stated that about 73% of fresh shop-bought chicken tested positive for the food poisoning bug *Campylobacter* in a year-long study by the Agency (Food Standards Agency, 2015). As a result, a lot of effort is being put into the search for alternative solutions to the problem contamination of poultry products by pathogens, outside the use of prophylactic antibiotics. It has been noted however, that advances in studies of these alternatives requires a multi-disciplinary approach of dieticians, nutritionist, microbiologists, clinicians and

immunologists to produce relevant and applicable results. Prebiotics as an alternative, has however been reported to have many positive effects on poultry intestinal health, mucosa integrity, feed intake and microbial growth rates (Mateos et al., 2012).

2.3.4.3. Prebiotics

Prebiotics, as defined earlier, are 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and or activity of one or a limited number of bacteria in the colon' (Gibson and Roberfroid, 1995). Prebiotics have also been defined as 'carbohydrates that selectively stimulate some or all of the beneficial organisms in the microbial balance which in turn affects the host in a beneficial way' (Kolida and Gibson, 2011). In simple terms, prebiotics can be said to be dietary substances, usually carbohydrates that encourage the growth of beneficial lactic acid bacteria in the animal gut by serving as an energy source. There are however other ideas that suggest a modification of the definition of prebiotics. It has been suggested that prebiotics should be named and defined according to their specific function in the animal gut by the addition of specific qualifiers. For example, bifidogenic prebiotic, immune-modulating prebiotic, anticancer prebiotic, anti-adhesive prebiotic, etc. (Fahey *et al*., 2011). Certain companies define prebiotics as 'oligosaccharides that are fermented in the colon'. Known sources of prebiotics include plants like chicory, bananas, cereals, onions, leek, garlic, asparagus and Jerusalem artichoke. Large amounts of prebiotics extracted from chicory roots are available commercially. Plant cellulose and chitin are the most abundant polysaccharide in nature and oligosaccharides derived from these are common in most food ingredients (Fahey *et al*., 2011). Information on other plant sources of prebiotics are currently gathering momentum.

For a substrate to qualify as a prebiotic, it must pass unhydrolysed and unabsorbed in the upper part of the gastrointestinal tract to the caecum, colon or large intestine where it can serve as an energy source for microbes. In addition, it must be able to alter the microbial flora to favour a healthier composition and must be a selective substrate for a limited number of bacteria commensal to the caecum/colon. The ability to induce systemic effects that are beneficial to the host's health is a must for prebiotic substances, which must also have a chemical structure that can be documented (Hajati and Rezaei, 2010). Table 2.8 below, summarises the properties of a prebiotic substance.

Table 2.8: Prebiotic and Gastrointestinal Function

Region of GI tract	Prebiotic character
Upper GI tract	Resistant to digestion Retarded gastric emptying Increased oro-caecal transit time Reduced glucose adsorption and low glycaemic index Hyperplasia of small intestine epithelium Stimulation of secretion of intestinal hormonal peptides
Lower GI tract	Acting as food for colonic microbiota Acting as substrate for colonic fermentation Production of fermentation end products (mainly SCFAs) Stimulation of saccharolytic fermentation Acidification of colonic epithelium Stimulation of secretion of colonic hormonal peptides Bulking effect on stool production Regularization of stool production (frequency and consistency) Acceleration of caeco-anal transit.

Source: Gaggia et al., 2010.

Carbohydrates that have qualified as prebiotics are mainly non-digestible oligosaccharides (NDOs) and fibres. Table 2.9 shows a list of some commercially available prebiotics. Several substances possessing the criteria mentioned above are qualified to be called prebiotics, while others are still undergoing further testing. Table 2.10 shows a list of proven prebiotics and potential prebiotic substrates.

Table 2.9: List of Some Commercially Available Prebiotic.

Commercial Prebiotic Carbohydrates			
Prebiotics	Chemical Structure	Degree polymerization (DP)	Purity (%)
NutraFlora P-95	Gluα1-2-[βFru-1-2]n	2–4	97% FOS[b]
Raftilose P95	Gluα1-2-[βFru-1-2]n	2–7	95% FOS[b]
Raftilose P95	Gluα1-2-[βFru1-2]n	2–7	95% FOS[b]
Inulin-S	Gluα1-2-[βFru-1-2]n	2–60	>99% Inulin[b]
Raftiline HP	Gluα1-2-[βFru-1-2]n	>23 (average)	>99% Inulin[b]
Purified GOS	Gluα1-4-[βGal-1-4]n	2–4	>99% GOS[c]

Where Glu = Glucose; Fru = Fructose; Gal = Galactose; b = based on manufacturer's analysis and c = approximate composition after purification.

Table 2.10: Proven Prebiotics and Potential Prebiotics

Proven Prebiotics	Fructooligosaccharides(FOS, oligofructose and inuline) Galactooligosaccharides (GOS) Transgalcto-oligosaccharides (TOS) Lactulose (Gaggia et al., 2010).
Potential Candidate Prebiotic Substrates	Germinated barley feed stuff Gentio-oligosaccharides Gluco-oligosaccharides Gluconi acid Hemicellulose rich substrate Isomalto-oligosaccharides Lactoferrin derived peptide Lactosucrose Mannan oligosaccharide Melibiose oligosaccharides N-acetylchito-oligosaccharide Polydextrose Resistant starch Beta-glucan Soybean oligosaccharides Sugar alcohols Xylo-oligosaccharides Glycooligosaccharide Lactitol Multooligosaccharide Staclyose Raffinose (Patterson & Burkholder, 2003).

2.3.4.3.1. Mode of Action of Prebiotics

Prebiotics function by lowering the gut pH through lactic acid production as a result of the fermentation of probiotics in the gut. Low gut pH discourages the growth of most pathogenic microorganisms (Gibson and Roberfroid, 1995).The terminal sugars of prebiotics can interfere with receptors on pathogens thus preventing them from being attached to the intestinal wall (Morgan *et al*., 1992; Bengmark, 2001). Prebiotics also stimulate the immune system by increasing anti-inflammatory cytokines and decrease pro-inflammatory cytokines (Monsan and Paul, 1995).

2.3.4.3.2. Prebiotics in poultry

Prebiotic supplementation of poultry diets modifies fermentation profiles of birds by increasing the total SCFA which in turn decreases the intestinal pH (Zduńczyk et al., 2006). Prebiotic research on poultry has been performed since 1990, with a large database of research accessible in the area. Prebiotics in broiler diets have been shown to increase intestinal *Lactobacilli* counts in poultry (Baurhoo et al., 2007; Xu et al., 2003). Other researchers have reported increased *Bifidobacteria* and decreased *Clostridia*, *Salmonella* and *Streptococci* in poultry as a result of prebiotic supplementation (Cao et al., 2005; Spring et al., 2000).

A literature review on the inclusion of prebiotics in poultry diets revealed a lot of data on the inclusion of proven prebiotics in poultry diets but data on inclusion of possible prebiotics were limited. Proven prebiotics have been reported to have some positive effect on body weight gain and feed conversion ratio in poultry birds. Some examples are summarised in the table 2.11 below.

Table 2.11: Effect of Prebiotics on Poultry Diet

Parameter	Prebiotics	Response (%)	Reference
Weight gain (g/bird)	FOS	+5	Li *et al*., 2008
		+2	(Biggs *et al*., 2007)
		+6	(Zhang *et al*., 2003)
		+8	(Xu *et al*., 2003)
	Transgalactooligosaccharide	0	(Biggs *et al*., 2007)
	Stachoyse	-3	(Jiang *et al*., 2006)
	Chitosan	+2	(Huang *et al*., 2005)
	Isomaltooligosaccharide	+5	(Zhang *et al*., 2003)
Body weight (g/bird)	FOS	+3	(Yusrizal and Chen, 2003)
		+1	(Waldroup *et al*., 1993)
Feed conversion ratio	FOS	+2	Li *et al*., 2008
		+3	(Biggs et al., 2007)
		0	(Yusrizal and Chen, 2003)
		+6	(Zhang *et al*., 2003)
		+6	(Xu *et al*., 2003)
		-1	(Waldroup *et al*., 1993)
		-1	(Biggs *et al*., 2007)
	Transgalactooligosaccharide	+3	(Jiang *et al*., 2006)
	Stachoyse	+4	(Huang *et al*., 2005)

Source: (Yang et al., 2009)

On a general note, barley consumption has been linked with various health benefits due to the presence of beta-glucan, thus the need for proper investigation into its mode of action (Jacob and Pescatore, 2014). One mode of action of beta-glucan at exerting health benefits is its ability to increase the viscosity of poultry intestinal digesta. Varying reports on the effectiveness of beta-glucan could be attributed to difference in terms of source or changes in its structure during processing. The modification or alteration of this character during extraction or food preparation, could therefore affect its effectiveness. As a result, commercial producers of beta-glucan concentrates always seek the best extraction methods to ensure preservation of the bioactive component of cereal grains. A study by Vasanthan and Temelli (2008) analysed fractionation and extraction technologies for beta-glucan in cereals and reported that fractions or particles obtained during dry fractionation, depending on the extent of grain size reduction, vary in beta-glucan content. Dry fractionation and wet separation technologies were evaluated in the study, which stated that dry fractionation processing involve grain particle size reduction to produce meal and flour, which could then be further used in wet processing technologies for separation and concentration of desired chemical component. According to the same report, the choice of separation methods are dependent on the type of cereal used and the aim of the processing. Pearled grains are commonly used as ingredients in soups while fractions obtained from pearling are used as raw materials for animal feed formulations. Pearling as a dry fractionation method was reported to be more suitable for barley grain fractionation compared to oat grains. Other researchers have also reported on the suitability of this method (Sumner *et al*., 1985; Zheng *et al*., 2000). Oat grains have been reported to exhibit extensive grain breakages during pearling. Also, challenges on separation

of pearled grains from pearled fractions have been reported in oat, due to its relatively high lipid content. This fact makes extraction of beta-glucan from oat a bit difficult, as dry milling technologies like pearling are usually the starting procedure for other separation processes like wet processing, targeted at beta-glucan extraction especially for experiments involving grain fraction effects.

Various studies have provided data on the inclusion and effect of wheat, oat and barley fractions in poultry diets. However, comparing all three cereals mentioned, studies on the inclusion of barley fractions are limited compared to studies on wheat and oat. A study by Svihus and Gullord (2002), on the effect of chemical content and nutritional value of wheat, barley and oat in poultry, showed similarities in the AME values of the three cereals. However, a modification of their chemical composition, with the addition of enzymes produced varying beneficial effects on poultry health. It was concluded in the study that AME values might have a limiting effect on predicting the nutritional value of cereals in broiler chickens. AME values have limited the use of cereals like barley in poultry feed. Several studies have shown varying AME values with the addition of barley to poultry feed, which has led to the underutilisation of barley in poultry feed. As mentioned earlier, this effect is due to the viscous nature of barley.

In a related study, Amerah (2009), analysed the effect of diluting a wheat-based poultry diet with insoluble fibre sources on bird performance, nutrient utilisation, digestive tract development and ileal microbiota profile of broiler chicken and concluded that AME was unaffected by the dietary treatment. The results also showed that microbial compositions were however, affected by the treatment with magnitude of the effect dependant on the particle size of the fibre used. In order words, the inclusion of fibre with prebiotic effect is beneficial to poultry. However,

proper knowledge on source, type and level of inclusion is critical to achieving the best results. Mateos (2012), reported that fibre is essential in poultry diets and recommended the inclusion of moderate amounts of 2 – 3% fibre in poultry diet, which could be in the form of coarse, insoluble fibre such as oat hull. The study analysed responses of high levels of dietary fibre sources varying in physical and chemical characteristics. Jørgensen *et al* (1996) also studied the effect of how the source and level of inclusion of a dietary fibre affects development of the gastrointestinal tract and energy metabolism in broiler chicken. Results from the study revealed that the length of the GIT increased with the level of fibre inclusion. The effect was particularly high for the weight and length of the caecum. In relation to the fibre sources, oat bran showed the best results, compared to wheat bran and pea fibre. The levels of digestibility of all nutrients however decreased with increasing fibre levels.

The positive effect of prebiotics have also been demonstrated in their ability to replace in feed antimicrobials. Baurhoo *et al* (2007) evaluated the effect of lignin and MOS as a potential alternative to antibiotic growth promoters in broiler chickens. Results from the study revealed that broiler performance was similar in birds fed sub therapeutic antibiotics compared to those fed antibiotic free feeds containing either MOS or lignin. Positive effects were also recorded in gut structure and microbiota for birds fed MOS and lignin. There was increased jejunum villi height and a higher number of goblet cells per villus as well as a higher *Lactobacillus* count in the caeca of the treatment groups.

Despite the positive results obtained by the inclusion of dietary fibres or prebiotics in poultry feed, repeat experiments have been shown to be inconsistent. Some researchers purchase commercially available prebiotics without a clear attention to

the source and specific structure of the prebiotics purchased. Such that commercial prebiotic from different supplies might yield different results. In the case of cereal fractions or cereal obtained dietary fibres, the variety of the cereal as well as the location on the cereal of the dietary fibre may also affects its functionality as a prebiotic substance. Also dietary fibre extraction methods might also affect their effectiveness.

Some reviews on the effect of most proven prebiotics on the performance of poultry as mentioned earlier has shown inconsistent results. In a recent study by Murshed *et al* (2015), commercially purchased prebiotic and probiotics were used individually to replace an AGP (neoxyval) in broiler diet. The results showed a similar performance among all groups. However, a combination of the probiotic and prebiotics showed an impaired broiler performance compared to feeding the additives individually. Conversely, other studies have shown that, the combination of probiotics and prebiotics are more effective when compared with individual additives (Awad *et al*., 2009; Dizaji *et al*., 2012; Li *et al*., 2008; Saiyed *et al*., 2015). Awad *et al* (2009) also reported the beneficial effects of a synbiotic application of prebiotics and probiotics in a study on the effects of dietary inclusion of probiotics and symbiotic on growth performance, organ weight and intestinal histomorphology of broiler chickens. Sarangi *et al*, (2016), obtained a different result in a study aimed at investigating the effects of dietary supplementation of prebiotics, probiotics and synbiotics on growth performance and carcass characteristics on broiler chicken, where dietary inclusion of prebiotics, probiotics and a synbiotic combination has no significant effect on growth parameters compared to the control group. The prebiotic used was yeast cell derived.

These inconsistencies have made difficult the prediction of standard recommendations for designing *in vivo* trails (Hajati and Rezaei, 2010; Jacob and Pescatore, 2012; Ricke, 2015). Various reasons have been cited for these inconsistencies. They include level of inclusion of prebiotic, purity of prebiotics, source of prebiotic and length of adaptation time and exposure of the poultry microbes to prebiotics (Bailey *et al*., 1991; Catald-Gregori *et al*., 2008; Choi *et al*., 1994). Other factors mentioned include prebiotic-feed interactions, type of microbes present prior to feeding prebiotic and type of responses being considered in terms of growth versus disease prevention (Ricke, 2015). Another major factor being considered is the prebiotic-probiotic combination. All of these have brought about a call for researchers in prebiotic studies to go back to the basics, in order to create a streamlined detailed database of information. Attention is being called to the sources and specific structure of prebiotics in order to understand precisely, their functionality.

2.4. Current situation

This research focuses on the prebiotic market. Of all the functional food researched, probiotics and prebiotics have taken centre stage, dominating the functional food market in Europe, Japan and USA (Kumar *et al*., 2015).

The prebiotic market has been dominated by research for human applications. However, due to the current demand for production of healthier animals with less drug use, more research has gone into the feeding of prebiotic substances to poultry (Hajati and Rezaei, 2010; Hume, 2011; Józefiak *et al*., 2004; Patterson and Burkholder, 2003; Ricke *et al*., 2013). Most of this research has been focused on

proven prebiotics. Inulin and FOS are classic examples of such prebiotics and have been administered in a variety of experimental studies (Mussatto and Mancilha, 2007; Patterson and Burkholder, 2003; Roberfroid, 2007; Rossi *et al*., 2005) with varying results. There is still a considerable gap between the use of in-feed antimicrobials and their possible replacement with prebiotics in the near future (Cheng *et al*., 2014).

Despite these uncertainties, prebiotic additives do hold promise and have advantages for use in alternative and non-conventional poultry operations. Several substances have been and are currently being assessed. Shakouri (2006), analysed the effect of different non-starch polysaccharides on the performance and intestinal microflora of young broiler chicken and concluded that addition of non-starch polysaccharides increased feed intake. The study specifically analysed the effect of cellulose and pectin, with results stating that pectin significantly increased the number of anaerobes in the poultry duodenum. Also reported was that cellulose influences positively feed conversion ratio and final body weight gain. Another study by Charalampopoulous *et al* (2003) examined the effect of cereal extracts on the viability of lactic acid bacteria under conditions simulating the gastrointestinal tract and concluded that the absence of cereal extracts showed a significant decrease in probiotic bacteria, especially *Lactobacillus plantarium*. Lactic acid bacteria used in the study include *Lactobacillus plantarium*, *Lactobacillus acidophilus* and *Lactobacillus reuteri*. Also stated was that the presence of malt, wheat and barley extracts exhibited a protective effect on the viability of the lactic acid bacteria studied.

The addition of prebiotics in poultry diets is also currently been applied in combination with other feed additives like enzymes, to achieve improved results. A

study by Jozefiak *et al* (2010) to determine the effect of beta-glucanase and xylanase supplementation on barley and rye-based diets on cecal microbiota of broiler chickens showed that, irrespective of the method applied; cereal type and enzyme supplementation influenced the microbiota in broiler chicken caeca in beneficial ways such as reducing potentially pathogenic *Enterobacteriaceae* populations. Jimenez-Moreno *et al* (2009), studied the effect of a combination of dietary fibre and fat on the performance and digestive properties of broilers from day old to twenty one days and reported that the inclusion of moderate amounts of fibre in diets of young chicks, improved performance and nutrient digestibility, especially when used in combination with saturated fats.

Several factors are, however, inhibiting the progress of research in prebiotic studies, the first being the ability to identify and establish a hierarchy for which possible factors should be considered as of primary importance versus the others, in order to establish a cause and effect model. However, some basic issues have been identified and need to be addressed to develop a strategy for effectively deploying prebiotics in animal production. These include detailed knowledge on the source of the prebiotic, extraction/production process, as well as level of inclusion. All of which lack standard recommendations. The research into prebiotics is still at its infancy, with much research yet to be streamlined such that there is no proper regulations on production and extraction of prebiotics from natural or organic sources (Ricke, 2015) nor are there regulation on further modifications of candidate prebiotic compounds using standard processing techniques. For example, the application of thermal processing to create more specific prebiotic structures with specific antimicrobial effects have been recommended and is yet to be tested and approved (Hernandez-Hernandez *et al*., 2012; Ricke, 2015).

The source and physical form of cereal component in a feed item is critical to its effectiveness as a prebiotic in terms of its effect on the morphological and physiological characteristics of the intestinal tract as well as on its effect on the gut microbiota (Brunsgaard, 1998; Engberg *et al*., 2004). For instance, high molecular weight and high solubility are physicochemical characteristics critical to the functionality of beta-glucan as a viscous dietary fibre. Depending on the source, there are clear differences between beta-glucans in their solubility, molecular mass, tertiary structure, degree of branching, polymer charge and solution conformation, all of which in turn alter their immune modulating effect (Bohn and BeMiller, 1995). Beta-glucans having a beta - (1, 3) chain with beta – (1, 6) branching are more effective than beta - (1, 3) linear chain alone (Bohn and BeMiller, 1995). It has also been reported that cereal beta-glucans from barley are more beneficial than oat beta-glucan in inhibiting the early stages of atherosclerosis, with the barley polysaccharides showing greater physiological potency than the respective oat beta-glucans of similar molecular weight (Lazaridou *et al*., 2011). The mode of action of this disease preventing property of barley beta-glucan is still under investigation. A report by Jacob and Pescator (2014) stated that barley beta-glucan had a negative effect on poultry immunological performance, compared to Yeast beta-glucan, which showed enhanced immune function in many animals, including poultry. The adverse effects of barley beta-glucan was linked to its propensity to increase intestinal viscosity. Which has led to the suggestion that barley beta-glucan could have other effect on the immune system, if the digestive tract is by-passed, by its possible use as an adjuvant for vaccines (Jacob and Pescatore, 2014).

Since the launch of the prebiotic concept about 20 years ago, prebiotic producers have published various health claims on their beneficial effects on gut microbiota,

gut development, etc. The GALT, commensal bacteria, mucus and host epithelial cells interact with each other, forming a delicate and dynamic equilibrium within the alimentary tract that ensures efficient functioning of the digestive system. The value of a prebiotic is said to lie in its capacity either to stabilise or to perturb this equilibrium. However, given the set criteria for these claims, the EFSA has concluded that evidence presented thus far does not satisfy the stated claims, based on the fact that an increase in gut microbes as a beneficial physiological effect is not clear cut. This according to EFSA is due to the fact that there is no clear evidence of cause and effect relationship between prebiotics and the health claims. As a result, under current legislation prebiotics cannot be categorically linked to the said health claims in both humans and animals (Delcour *et al*., 2016; EFSA Panel on Dietetic Products, 2010). One prominent reported effect of prebiotics is its ability to increase production of SCFA in poultry gut. SCFA as mentioned earlier has been linked to several health benefits. However, it has been argued that the mere demonstration of functional changes in the gut, such as fibre induced SCFA production, is not enough for regulatory bodies to grant health claims. A gap has thus been identified in the application of prebiotics, in that; similar effects are not obtained with the administration of prebiotics compared to the equivalent amounts of SCFA produced. Hence, the lack of a direct link between fermentable fibres and colonic SCFA production. There exist only a level of association.

To fill this gap, researchers are calling for technologies such as isotope monitoring that will allow for proper SCFA tracking, where SCFA produced, can be linked with their fibre sources. However, such studies need to be preceded by characterization of fibre sources in terms of monosaccharide composition, structure and physicochemical properties (Delcour *et al*., 2016). Research in prebiotic study is

therefore gearing towards studies in proper distinction between different prebiotic fibres, such that researchers will no longer just focus on increased number of probiotics as a marker for beneficial effects. There is therefore a need to develop the database of prebiotic substances in terms source, structure and functionality, which can be achieved by detailed grain fractionation and biochemical analysis, as, is the case with cereals.Cereal hulls have been used with various degrees of pearling in prebiotic studies, with the lack of a proper standardization. Research in this area needs to refocus on attention to details in terms of pearling and cereal fraction effects, hence this study. Proper information on this can then be transferred into already existing models for predicting feed requirements.

Researchers as mentioned earlier recommend the inclusion of moderate amounts of dietary fibre in poultry diet. However, proper information on established dosage of inclusion is limited. Also limited is information on the combination of fraction or dietary fibres from barley and other cereals to obtain outstanding health benefits. Currently, invention of novel prebiotics have been linked to synergistic mixtures of prebiotics because as established earlier, small changes in poultry diet often produces large effect in poultry products obtained. An example is the synergism of GOS and polyfructose or nutritional compositions containing them. This was reported in an invention which found that when GOS and inulin, a polyfructose when administered together, they provide a number of beneficial effects including an optimal fermentation pattern, with increase in desirable total SCFA production and a decreased gas formation (Speelmans *et al*., 2013). Previously, it has been reported widely that intake of prebiotics like GOS and inulin individually, increases *Bifidobacterial* counts (Boehm *et al*., 2002) and produces other side effects like formation of gases which results in fluctuation and abdominal pains. The synergy of

GOS and inulin was novel in that they do not significantly increase the number of *Bifidobacteria,* while increasing the amount of desirable SCFA. This benefit was achieved when the prebiotics were administered together, indicating that they acted synergistically. This invention with Patent number 20130210763 is an example where minute changes in the combination of prebiotics led to large differences in produced results. This discovery has made possible the prescription of prebiotic application with no side effects of abdominal pain, flatulence or constipation (Speelmans *et al*., 2013). More of these types of results can only be achieved by detailed studies of prebiotic sources in terms of constituents and possible combinations. Hence, this study, which seeks minute fraction effects across a barley grain on the growth of the probiotic *Lactobacillus acidophilus* and fractions of interest recommended for further study.

Also worthy to note is that research in the inclusion of prebiotics and probiotics in masking or replacing animal medication is growing in the animal production industry. In 2013, seven years after the ban of sub-therapeutic antibiotic in animal production, a U.S patent number 20130171204 by DuBourdieu (2013) describes a soft food composition for masking solid medication in animal feed with probiotics and prebiotics. The invention relates to a food product and feed supplements for animal that improve animal health by providing one, all or various combinations or sub combinations of a drug agent plus probiotic, prebiotics, vitamins, minerals and enzymes. The prebiotic could selected from the group consisting of inulin, lactulose, lactitol, a FOS, GOS, etc., included in an amount of about 0.5% to about 6% w/w. The patent application emphasized the fact that similar preparations from earlier patents lacked the inclusion of prebiotic and probiotics, (Farber and Farber, 2009; Huber *et al*., 2005; Kalbe *et al*., 2011). In the same year, another invention by Culver

et al (2013) was registered, which relates to dietary supplements for enhancing animal health. The invented supplement was made up of three components: fatty acid component supplied by sunflower, the GI component supplied by prebiotics and probiotic and the breed specific component made of immunoglobulin with a Patent number 20130216521.

Despite the 'fibre gaps' in prebiotic study, a lot of research has been invested in determining the effectiveness of prebiotic substances. Roberfroid (2007), stated that the effectiveness of a prebiotic substance can be measured by its prebiotic index (PI), which is defined as the increase in probiotics expressed as the absolute number of 'new' CFU/g of faeces (E) divided by the dose (g) of prebiotic ingested (A). PI is commonly used as a standard for comparison of different prebiotic effects among prebiotic carbohydrates. This can be used in the selection of prebiotic substances, prior to further testing.

Another equation was also use to describe PI by Roberfroid (2007) as

PI = (Bif/total) – Bac/total) + (Lact/total) – (Clos/total)

The bacterial groups incorporated into the PI equation include *Bifidobacteria*, *Lactobacilli*, *Clostridia* and *Bacteriodes*.

Where,

Bif = *Bifidobacterial* numbers at sample time/number at inoculation

Bac = *Bacteroides* numbers at sample time/number at inoculation

Lact = *Lactobacilli* numbers at sample time/number at inoculation

Clos = Clostridia numbers at sample time/number at inoculation

The equation assumes increase of probiotics like *Bifidobacteria* and *Lactobacillus* to be a positive while increases of *Clostridia* and *Bacteriodes* to be negative. This is done to ensure that the increase of probiotics is expressed relative to total bacterial growth. Rycroft *et al* (2001) compared the effectiveness of commercially available prebiotics using the PI method, and concluded that galactose containing oligosaccharides were more effective than the fructose containing oligosaccharides and inulin on *Bifidobacteria* and *Lactobacilli*. He concluded that GOS and lactulose demonstrated the greatest prebiotic effect. Similar results were obtained by Palframan *et al* (2003).

Currently, however, the measurement of PI like most other prebiotic studies, is still subject to errors due to variations in microbial counts and inter-individual variations. There is hope that more research will bring about accurate quantitative methods of determining prebiotic index.

In this research, eight varieties of barley were analysed, with grain fractionation technique to obtain precise grain mapping. Each fraction was analysed separately with careful attention to extraction techniques to obtain chemical components with high physicochemical properties in order to fulfil the specific aims of this research.

3.0. Chapter Three: Materials and Methods

3.1. Methods Summaried

Experiments were conducted to determine the chemical composition of eight two-rowed, hulled varieties of barley – Pearl, Propino, Cassata, Concerto, Maris Otter, Munton, Chevalier and Tipple, and how fractions of these varieties affect the growth of *Lactobacillus acidophilus* in a simulated poultry gut in relation to prebiotic properties.

Chemical components of interest of this study include beta-glucan, resistant starch, protein, free amino nitrogen (FAN) and sugars, while the microorganism of interest is *Lactobacillus acidophilus*. *Salmonella enteritidis* was also used in this research in a mixed culture experiment to determine growth patterns of probiotic and pathogenic microorganisms in a simulated poultry gut.

100 g of each barley variety was subjected to an abrasive scouring process (pearling) that sequentially removes the outer layer of the barley kernel producing pearling fractions every 10 seconds, using a Sakate TM05 pearling machine (Screen slot: 1mm; gap between the roll and screen: 16 mm; baffle thickness: 3 mm) Sakate International (Australia). A total of nine fractions were obtained and labelled FR1 to FR9. The first eight fractions were separated 10 seconds apart while the ninth fraction was the left over kernel after pearling for 80 seconds. The fractions were further milled separately using a coffee blender and the powder sieved through a 0.05 µm aperture sieve to obtain very fine powder. 1000 grains of Propino, Concerto and Maris Otter were also weighed in triplicate.

Microscopic images of pearled grains were obtained using a Hitachi model 7021 S – 3000 N scanning electron microscope, Oxford analytical instruments (England).

Beta-glucan content (w/w) was measured using K-BGLU 07/11 assay kits, (a modification of AOAC method 995.16, AACC method 32-23.01 and ICC standard method No. 166), Megazyme International (Ireland). Resistant starch content (w/w) was determined using K-RSTAR assay kit (AOAC method 2002.02, AACC method 32-40.01) Megazyme International (Ireland). Free amino nitrogen (FAN) was determined using micro titre well ninhydrin assay method (Abernathy & Starcher, 2009). The amount of simple sugars present in each fraction was measured using high performance liquid chromatography (HPLC). Protein was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, England). A microbial culture method, under variable biochemical conditions simulating the gastrointestinal tract of a poultry bird, adopted from John Kirkpatrick Skeeles poultry health laboratory, University of Arkansas (Latorre et al, 2015; Bedford and Classen, 1993) was used to determine intestinal growth of *Lactobacillus acidophilus* and *Salmonella* Enteritidis in relation to prebiotic properties on fractions of barley. *Salmonella* Enteritidis was obtained from Public health England culture collection in flame sealed glass ampoule containing 0.15 g freeze-dried bacteria culture. *Lactobacillus acidophilus* was obtained from National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK).

Data obtained were subjected to analysis of variance (ANOVA) and correlation analysis using IBM SPSS statistics 21 software.

3.1.1. Materials

3.1.1.1. Barley

Propino, Pearl, Concerto, Cassata, Munton, Maris Otter, Chevalier and Tipple are all two-row barley varieties supplied by the Brewlab, Sunderland.

3.1.1.2. Microorganism

Lactobacillus acidophilus was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK). They were isolated from a location along the human alimentary canal. *Salmonella* Enteritidis was obtained from Public health England culture collection in flame sealed glass ampoule containing 0.15 g freeze-dried bacteria culture.

3.1.1.3. Chemicals

Beta-glucan K-BGLU 07/11 assay kits and resistant starch K-RSTAR assay kit were purchased from Megazyme International (Ireland). Protein BCA assay kit was purchased from Thermo Fisher Scientific, United Kingdom. All other reagents were purchased from Sigma-Aldrich, United Kigndom.

3.2. Beta-glucan: McCleary Method.

Mixed-linkage beta-glucan in barley samples were determined using a streamlined method, a modified version of AOAC method 995.16, AACC method 32-23.01 and ICC method No.166 recommended/standard methods. The experiment involved two major steps, an extraction step followed by a measurement step.

3.2.1. General Principle

Samples were suspended and hydrated in a buffer solution of pH 6.5 and then incubated with purified lichenase enzyme and filtered. An aliquot of the filtrate was then hydrolysed to completion with purified beta-glucosidase. The D-glucose produced is assayed using a glucose oxidase reagent.

3.2.2. Procedure

100 g of each sample was weighed into a 15 mL centrifuge tube and moistened with 0.2 mL aqueous ethanol (50% v/v) to aid dispersion. 4.0 mL sodium phosphate buffer (20 mM, pH 6.5) was added and contents stirred on a vortex mixer. The tubes were immediately incubated for 3 minutes in a boiling water bath with intermittent vigorous stirring on a vortex mixer. The contents of the tube were then allowed to equilibrate for 5 minutes at 50°C. After which 0.2 mL lichenase was added to each tube and vortexed. The tubes were then incubated for a further 1 hour with intermittent vigorous stirring (3-4 times). 5.0 mL sodium acetate buffer (200 mM, pH 4.0) was then added, and the contents stirred again, followed by a 5 minutes equilibration step. The tubes were then centrifuged at 1000 g for 10 minutes. 0.1 mL of the supernatant from each tube was then transferred carefully to the bottom of glass test tubes in triplicates. 0.1 mL of beta-glucosidase was added to two of the tubes (the reaction) and to the third (the blank), 0.1 mL of sodium acetate buffer (50 mM, pH 4.0) was added. All tubes were incubated at 50°C for 10 minutes. After which 3.0 mL GOPOD reagent (glucose determining reagent) was added to all the tubes and incubated for a further 20 minutes. 100 µL of the resultant solution was measured at 510 nm in a photo spectrometer alongside 100 µL of the blank reagent and glucose standard in triplicate within 1 hour (Glucose standard was supplied by the kit). ODs were converted to beta-glucan amounts using Mega-Calc™, supplied by Megazyme.

3.3. Resistant Starch: AOAC method 2002.02, AACC Method 32-40.01

3.3.1. Procedure

100 g each of FR4 to FR9 were weighed into a 15 mL centrifuge tube. (These fractions were chosen based on results from SEM images as fractions that may contain starch. To each tube was added 4.0 mL solution 2 [1 gram pancreatic α-amylase in 100 mL sodium maleate buffer plus 1 mL amyloglucosidase (AMG) solution] and mixed well on a vortex mixer. The tubes were then tightly capped and incubated in a continuous motion water bath (horizontal motion, 100 forward and 100 reverse stroke/minutes) at 37°C for exactly 16 hours to solubilise and hydrolyse D-glucose. The tubes were removed from the water bath and excess water on the surface wiped with paper towel. The solution was then treated with 4.0 mL ethanol (99% v/v), to terminate the above reaction, stirred vigorously on a vortex mixer and then centrifuged at 1500 g for 10 minutes, with tubes uncapped. The supernatant was decanted into a volumetric flask and pellet washed with 2 mL ethanol (50% v/v) and mixed well. The re-suspended pellets were further treated with 6 mL ethanol (50% v/v), mixed again and centrifuged at 1500 g for 10 minutes. The supernatant produced was added again to the relevant volumetric flask. The ethanol wash (50% v/v) was repeated a second time and the supernatant collected each time. The pellet contains resistant starch while the supernatant contains the soluble starch.

Pellets from the above were re-suspended in 2 mL 2M KOH solution using a magnetic stirrer for 20 minutes in an ice/water bath (vortex mixing is not advised as it may cause the starch to emulsify). 8 mL sodium acetate buffer (1.2 M, pH 3.8) was added to the mixture on the magnetic stirrer, after which 0.1 mL AMG solution was immediately added to each tube and mixed well to quantitatively hydrolysed

glucose. The tubes were then incubated in a water bath at 50°C for 30 minutes with intermittent stirring on a vortex mixer. The tubes containing FR4-FR7 were then centrifuged at 1500 g for 10 minute (SEM images suggest samples may contain less than 10% starch). For FR8 and FR9, the resulting solutions after incubation, were transferred into a clean volumetric flasks, the volumes adjusted to 100 mL (as these fractions may contain more than 10% starch according to SEM images) using distilled water and mixed well (As advised by the standard method). Aliquots of all samples were then centrifuged at 1500 g for 10 minutes. 0.1 mL of supernatants from all tubes were then transferred into glass test tubes and 3.0 mL GOPOD reagent (glucose determining reagent) added and incubated for a further 20 minutes at 50°C. Absorbance was then measured at 510 nm. And the starch concentrations calculated using Mega Calc™.

3.4. Free Amino Nitrogen (FAN): Micro Titre Well Ninhydrin Assay Outlined by the 1977 European Brewery Convention (EBC).

3.4.1. Procedure

100 g sample was weighed into a 15 mL centrifuge tube and moistened with 0.2 mL aqueous ethanol (50%v/v). 4.0 mL sodium phosphate buffer (20 mM, pH 6.5) was then added and the contents stirred on vortex mixer. The tubes were immediately incubated for 3 minutes in a boiling water bath with intermittent vigorous stirring on vortex. The tubes were allowed to cool and then centrifuged at 1500 g for 10 minutes, and the supernatant collected for measurements. 2 µL supernatant was

then transferred into a 96 well micro titre plate to which was added 2 µL glycine nitrogen standard solution (107.2 mg of glycine dissolved in 80 mL distilled water and the final volume adjusted to 100 mL).100 µL working reagent (25 µL $SnCl_2$ + 1 mL of ninhydrin stock solution) was added and the plate heated for 10 minutes at 104^oC in an oven. The absorbance was then measured at 575nm (Abernathy et al., 2009). FAN was measured using the following formula.

FAN = (Sample OD – Distilled water OD) / (FAN standard OD – Distilled water OD)

3.5. Protein: Thermo Scientific™ Pierce™ BCA Protein Assay: A Detergent-Compatible Formulation Based on Bicinchoninic acid (BCA) for the Colorimetric Detection and Quantitation of Total Protein.

3.5.1. Principle

This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.

3.5.2. Procedure

Standard curve was prepared for a protein range of 25 to 2000 µg/mL, using bovine serum albumin (BSA) as stock solution.100 g sample was weighed into a 15 mL centrifuge tube and 0.2 mL aqueous ethanol (50%v/v) added to aid dispersion. 4.0 mL sodium phosphate buffer (20 mM pH 6.5) was added and contents stirred on vortex mixer. The tubes were immediately incubated for 60 seconds in a boiling water bath. The contents were the stirred vigorously on a vortex and further incubated for 2 minutes at 100^oC and stirred again, allowed to cool and then

centrifuged at 1500 g for 10 minutes and the supernatant used for protein measurements.

25 µL each of standard and sample solutions were transferred into a 96 well micro titre plate, and then 200 µl of the working reagent added and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37^{o}C for 30 minutes and then cooled to room temperature and absorbance measured at 562nm.

The measurement of each standard and sample were subtracted from the blank measurements. A standard curve was plotted for each BSA standard versus its concentration in µg/mL and protein concentrations of samples determined from the standard curve equation.

3.6. Sugars: Analysis using Hitachi Lachrom Elite® Liquid chromatography system.

3.6.1. Experimental conditions

Hitachi LaChrom NH2, 5 µm, 4.6 x 250 mm HPLC column was used in this experiment. The pump (L-2130) has a mobile phase made up of 75 % acetone nitride and a flow rate of 1 mL/minute and an auto sampler (L-2200) with an injection volume of 20 µL. The oven was set at 40^{o}C. Sugar concentrations ware measured using a refractive index detector (L-2490). A calibration curve was created using a 5 sugar mixture at 5 %, 2 %, 1 % and 0.5 % of glucose, fructose, sucrose, maltose and maltotriose.

3.7. Simulated Poultry Gut Method

Two methods were used in this experiment as described below. The first method produced inconsistent results, so a second method, which was a modification and optimization of the first method, was adopted. Three varieties of barley Propino, Concerto and Maris Otter, were selected randomly for this experiment to reduce the sample size, increase manageability and accuracy.

3.7.1. Principle

Samples were mixed with 0.03M hydrochloric acid and incubated at 40^{o}C for 30 minutes. After which pepsin in 1.5M hydrochloric acid was added, the sample was mixed again and incubated for a further 45 minutes to simulate the proventriculus. After which, pancreatin and sodium bicarbonate were added to the same sample tube, mixed and allowed to incubate for a further one hour and the final product centrifuged to obtain dissolved nutrients to simulate the small intestine. The supernatant from the small intestine was dialysed to separate digested nutrients from undigested ones. Undigested nutrients were then used in bacterial growth studies to simulate what occurs in the caeca. The undigested nutrient are hypothesized to be prebiotic in nature.

3.7.2. Bacteria Preparation

Lactobacillus acidophilus and *Salmonella enteritidis* were cultured at 37^{o}C overnight in 10 mL MRS and nutrient broth respectively. These overnight cultures were then centrifuged at 4000 rpm for 15 minutes, the supernatant discarded and the pellet re-suspended in 10 mL saline and this washing step repeated a second time. The final concentration was adjusted 4 X 10^{5} cells/mL, to make a start culture.

3.8. Method One - Mixed Culture Drop Plate Count Method

A mixed culture method, to determine the effect, as a prebiotic, of extracts from nine barley fractions, FR1 to FR9, on microbial populations under variable biochemical conditions simulating the gastrointestinal tract of a poultry bird, using *Lactobacillus acidophilus* and *Salmonella enteritidis*. The method was adapted from John Kirkpatrick Skeeles poultry health laboratory, University of Arkansas, United States that was a modification of in vitro digestion by Bedford & Classen, (1993).

3.8.1. Procedure

1 gram barley fraction was measured into a 15mL centrifuge tube. 4 mL 0.03M HCL was then added to each tube and mixed thoroughly and incubated at 40^{o}C for 30 minutes. To the same tube was added 1 mL pepsin (6U/µL in 1.5M HCL) and the contents mixed thoroughly. The tubes were incubated at 40^{o}C for 45 minutes. 1.3 mL 8 X pancreatin (0.06mg/µL in 1.0M $NaHCO_3$) was then added to the same tube and allowed to settle.

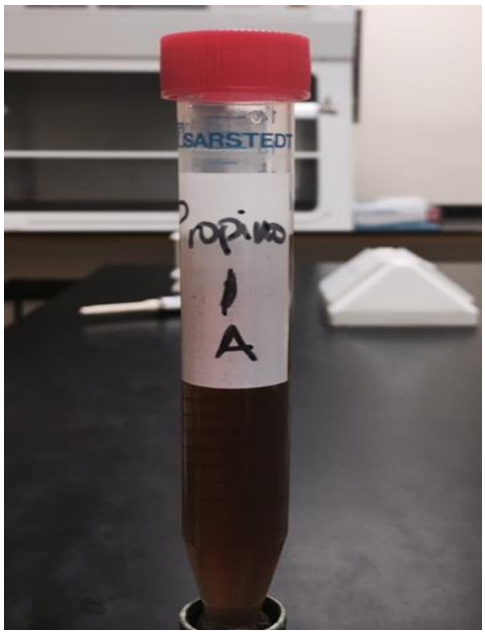


Figure 3.1. Simulated poultry gut

The tubes from above were then centrifuged at 4000 rpm for 15 minutes, and the supernatant dialysed to separate micro-molecules from macromolecules to simulate nutrient absorption using a Pierce™ 96-well micro-dialysis plate for four hours. 100 µL of supernatant was transferred into the micro dialysis tube and the tube placed in 2 mL water for separation process. Macromolecules are hypothesized to be prebiotic in nature. At the end of the separation, 90 µL of dialysed solution containing micro molecules and macro molecules where transferred into a micro-titre plate. 5 µL *Lactobacillus acidophilus* and 5 µL *Salmonella enteritidis* of the start culture was added to each well and incubated at 40^{o}C for 2 hours. 10µL sample was taken from each well, serial diluted to 10^{-4} and inoculated on XLT-4 and MRS agar plates selective for *Salmonella and Lactobacillus* respectively, using a drop plate method and incubated at 37^{o}C for 24 hours.

The micro-titre plate from above was further incubated anaerobically at 40^{o}C for 4 hours to simulate the caeca, after which 10µL sample was taken from each tube, serial diluted to 10^{-4}, plated onto XLT- 4 and MRS agar at 37^{o}C and colonies counted after 24 hours.

3.9. Method 2 - Micro-Titre Plate Method

A single culture method was used to determine the effect, as a prebiotic, of extracts from nine barley fractions, FR1 to FR9, on microbial populations under variable biochemical conditions simulating the gastrointestinal tract of a poultry bird, using *Lactobacillus acidophilus.* A modification of the simulated poultry gut method was adapted from John Kirkpatrick Skeeles Poultry Health Laboratory, University of

Arkansas, United States which was a modification of the in-vitro digestion model of Bedford & Classen, (1993).

3.9.1. Procedure

1 gram barley fraction was measured into a 15mL centrifuge tube. 4 mL 0.03M HCL was then added to each tube and mixed thoroughly and incubated at 40^{o}C for 30 minutes. To the same tube was added 1 mL pepsin (6U/µL in 1.5M HCL) and the contents mixed thoroughly. The tubes were incubated at 40^{o}C for 45 minutes. 1.3 mL 8 X pancreatin (0.06mg/µL in 1.0M $NaHCO_3$) was then added to the same tube and allowed to settle. The tubes were then centrifuged at 6000rmp for 15 minutes, and the supernatant dialysed to separate micro-molecules from macromolecules to simulate nutrient absorption using a Pierce™ 96-well micro-dialysis plate for four hours. 100 µL of supernatant was transfer into the micro dialysis tube and the tubes placed in 2 mL water for separation of digested molecules (micro molecule) from undigested molecule (macro molecule, which could possibly be prebiotic).

At the end of the separation, 90 µL of dialysed solution containing macro molecules where transferred into a 96 well micro-titre plate. 10 µL *Lactobacillus acidophilus* start culture was added to each well and the growth monitored in a spectrophotometer at 600nm for 10 hours. 90 µL undialyzed samples were also transferred to a micro titre plate and growth monitored for 10 hours and used to compare growth patterns of *Lactobacillus acidophilus* in the dialysed sample.

4.0. Chapter Four: Chemical Analysis

Details of all statistical analysis available in appendix, including F-ratios and degree of freedom for p-values, as well as standard deviations and R-squared values.

4.1. Pearling Results

Table 4.1 below, shows the cumulative weights of fractions obtained from the pearling experiment. At the end of the pearling cycle (i.e. after 80 seconds), approximately, 15% of the surface layers of most grains have been abraded. After 80 seconds of pearling, varieties showed differences in the amounts abraded, as well as for fractions obtained per step of the pearling cycle. These differences could possibly be attributed to degree of toughness of the grain per variety. For instance, Concerto produced a relatively high pearled fraction of 17.36%, while Maris Otter, produced a lower amount of 11.40% after 80 seconds of pearling.

Table 4.1: Cumulative pearling fraction mean weights for eight barley varieties from 80 seconds pearling cycle. Fractions were obtained 10 seconds apart for FR1 to FR8. FR9 is the leftover pearled grain after pearling for 80 seconds. 100 g samples were used for each cycle in triplicate. (See appendix 1 for standard deviations)

	Cumulative fraction weights (%)							
	Pearl	Propino	Cassata	Concerto	Maris Otter	Munton	Chevalier	Tipple
FR1	5.27	5.71	5.24	6.29	4.19	3.53	2.08	5.94
FR2	8.00	7.16	7.89	8.59	7.07	7.00	5.01	7.98
FR3	9.25	8.30	9.15	9.90	8.20	8.90	8.42	9.10
FR4	10.42	9.46	10.17	11.20	8.97	10.07	10.30	10.12
FR5	11.58	10.73	11.25	12.65	9.67	11.01	11.27	11.16
FR6	12.57	11.79	12.15	14.04	10.25	11.89	11.95	12.07
FR7	13.60	13.01	13.15	15.68	10.77	12.75	12.57	13.04
FR8	14.79	14.26	14.18	17.36	11.40	13.59	13.25	14.00
FR9	85.20	85.75	85.64	82.83	88.61	86.75	86.75	86.00

Table 4.1 shows that after 10 seconds of pearling, pearling fractions obtained from five out of eight varieties was approximately 5 – 6%, except for Chevalier, Maris Otter and Munton which showed lower amounts of 2.08%, 4.19% and 3.53% respectively. Fractions obtained from this step were the highest in the entire pearling cycle. This fraction, labelled FR1 is the outermost layer of the grain, known as the husk. This region is made up of mainly fibrous substances (figure 4.1), that are not tightly attached to the rest of the grain. In fact, for hulless barley varieties, this portion of the grain falls off freely during harvesting and threshing. The husk is made of two distinct overlapping layers called lemma and palea. FR2, which is the next layer to FR1, is also part of the husk, containing similar chemical components. However, pearling fractions obtained from this layer, were smaller in amount compared to FR1 except for Chevalier, where the reverse was the case. Pearling fractions from FR2 on average were 2.55 g compared to 4.78 g obtained from FR1. The amount of pearling fraction obtained after FR2 reduced greatly (table 4.1), indicating the complete removal of the highly fibrous hull, not tightly attached to the rest of the grain. After 30 seconds of pearling, the amount of pearling fraction obtained was approximately 1.0 g per pearling step compared to approximately 5.0g and 3.0g for FR1 and FR2 respectively. According to literature, FR3 corresponds with the pericarp region of the grain, which is cemented to the rest of the grain by a thick three-layered cutin. Cutin is the polymer that separates the different layers of the grain. After 30 and 50 seconds, the cumulative pearling fractions obtained were approximately 8-9% and 10-12% respectively for all eight varieties. The slight variations observed can be attributed to the grain sizes. 1000 grain weight measurements show 46.56kg, 51.36kg and 40.63kg for Concerto, Propino and Maris Otter respectively

SEM images (Figure 4.1 and Figure 4.2) were obtained for whole grain, pearled grains and pearling fraction after each pearling step. The images were labelled whole grain, FR1 after 10 seconds of pearling, upto FR8 after 80 seconds of pearling. A summary of all the images suggest that the bran layer of the grain (hull, pericarp, testa and aleurone) was removed after 80 seconds of pearling, corresponding to approximately 15% grain weight. Starch granules were clearly exposed after 80 seconds of pearling (figure 4.2). There were however, some breakages during the pearling process, however, this did not affect the consistency of fractions obtained in replicates.

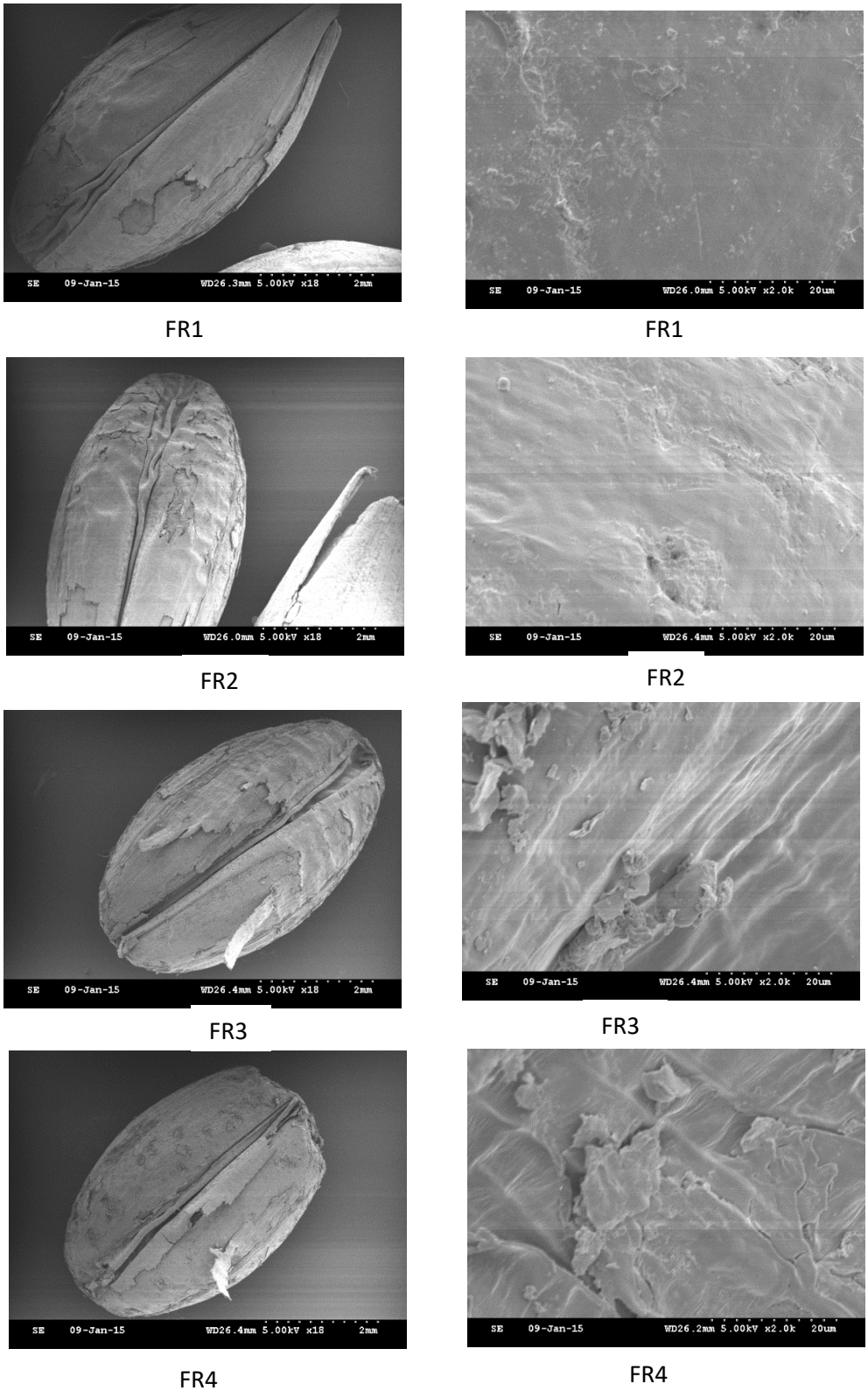
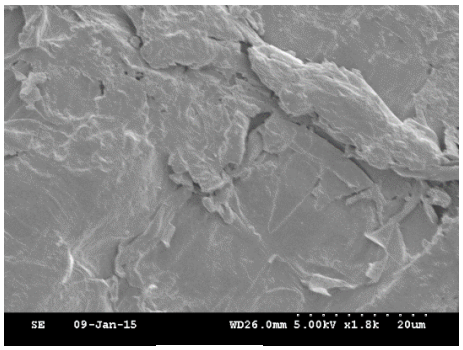


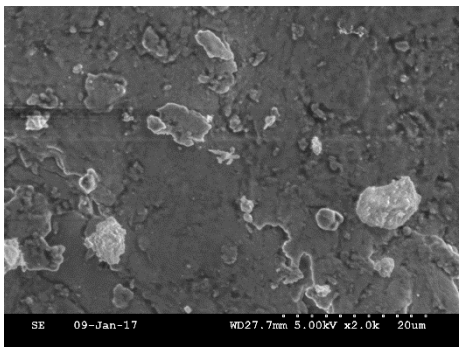
Figure 4.1. SEM images of pearled Chevalier after 10,20,30 and 40 seconds of pearling. Magnification: 18X, bar 2mm (left); 2000X, bar 20µm (right).

FR5

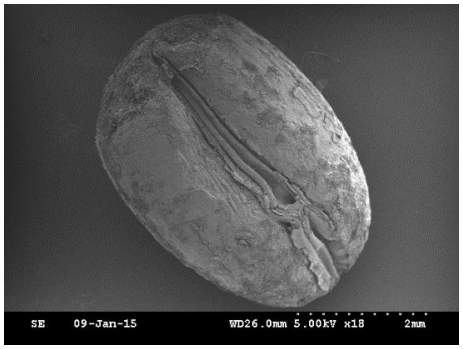


FR5

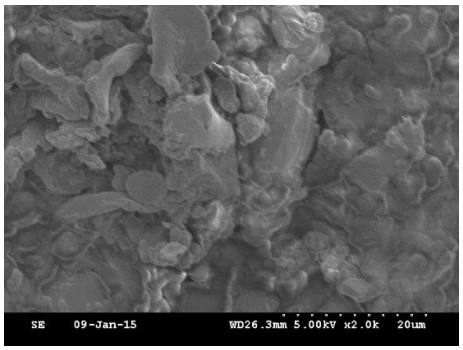
FR6



FR6

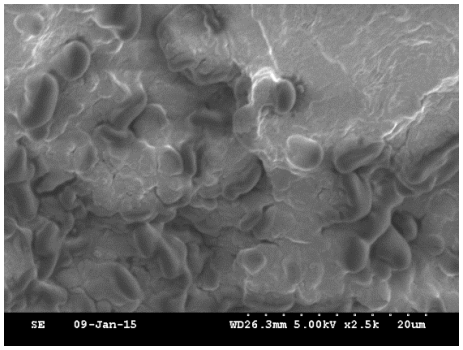


FR7



FR7

FR8



FR8

Figure 4.2. SEM images of pearled Chevalier after 50, 60, 70 and 80 seconds of pearling. Magnification: 18X, bar 2mm (left); 2000X, bar 20µm (right).

The images show the gradual removal of the outer layer of the grain with time. After 10 seconds the grain appeared huskless (Figure 4.1) which corresponds with about 5% grain weight loss. During the pearling process, some grain surfaces did not appear smooth, as some breakages were observed which might have been due to the abrasive scouring process or as a result of lack of moisture. The grains were not moisture treated or tempered before pearling. Some studies recommend 12-15% moisture treatment before pearling (Wang et al., 1997). However, a repeat of pearling moisture treated grains yielded similar images. The tempering step for barley consists of adjusting the moisture content of the kernel by soaking grains in water in percentages relative to the grain weight, followed by a rest period of 24 hours prior to pearling (Kent and Evers, 1994). A report by Wang *et al* (1997) stated that tempering cereals to 12.5% and 15% moisture had no consistent effect on rates of grain mass removal by abrasion.

4.2. Beta-glucan

Fractions obtained from pearling eight barley varieties were tested for beta-glucan content. The results showed that beta-glucan was present in all fractions of all eight varieties. The amounts ranged between 2 and 25 % (w /w) per fraction are presented in below in table 4.2. Total beta-glucan per variety ranged between 11 and 20 mg (w/w) per variety. Tipple and Propino had the highest total beta-glucan content of 19.58 mg (w/w) and 18.77 mg (w/w) respectively. Next were Concerto, Pearl and Cassata with 17.32 mg (w/w), 16.06 mg (w/w), and 16.93 mg (w/w) respectively, while Munton, Chevalier and Maris Otter had low amounts of 14.27 mg (w/w), 11.42 mg (w/w) and 11.68 mg (w/w) respectively.

Table 4.2: Total beta-glucan content (percentage) per pearling fractions of eight barley varieties in triplicate. (See appendix 2.0 for standard deviations in mg/100mg)

	Pearl	Propino	Cassata	Concerto	Maris Otter	Munton	Chevalier	Tipple
FR1	3.28	2.62	3.43	3.38	3.55	6.41	2.95	2.73
FR2	4.78	5.46	5.33	6.06	3.76	4.05	3.47	5.16
FR3	8.54	9.12	8.69	9.61	7.57	7.00	4.95	9.06
FR4	10.37	10.46	10.74	10.73	9.52	9.14	6.42	10.41
FR5	10.80	12.27	11.88	11.54	10.24	11.55	9.05	12.37
FR6	13.10	14.30	14.44	13.06	15.49	14.07	15.00	13.94
FR7	14.74	15.36	15.10	15.19	15.49	15.21	15.74	15.60
FR8	16.53	15.52	15.45	14.62	15.13	16.43	17.37	16.24
FR9	17.85	14.89	14.93	15.80	19.25	16.14	25.05	14.49

Beta-glucan in barley is usually unevenly distributed (Yeung and Vasanthan, 2001). A summary of the results, showed a gradual increase of beta-glucan from FR1 to FR9 across all varieties (figure 4.3), with the highest amounts of 14 – 25% in FR7-FR9. Variety differences were however observed in the distribution of beta-glucan across the grain. FR1 in all varieties recorded the lowest beta-glucan content ranging between 2 – 3% except for Munton which had a slightly high beta-glucan content of 6.41% in FR1 (table 4.2). On average, beta-glucan content rose from 2% in FR1 to a range of 7-12% in FR3-FR5 across all varieties, with the exception of Chevalier, which showed exceptionally low amounts of beta-glucan in FR1-FR4, ranging between 4 and 9%. In most varieties, the amounts of beta-glucan present in FR1 to FR6 were significantly different one from the other at $P \leq 0.05$, with a few exceptions. In three out of eight varieties, beta-glucan content in FR7 was not significantly different from FR8 as was the case with Cassata, Maris Otter and Propino. In addition, amounts present in FR8 were not significantly different from FR9 at $P \leq 0.05$ in four out of eight varieties (see appendix 2.0).

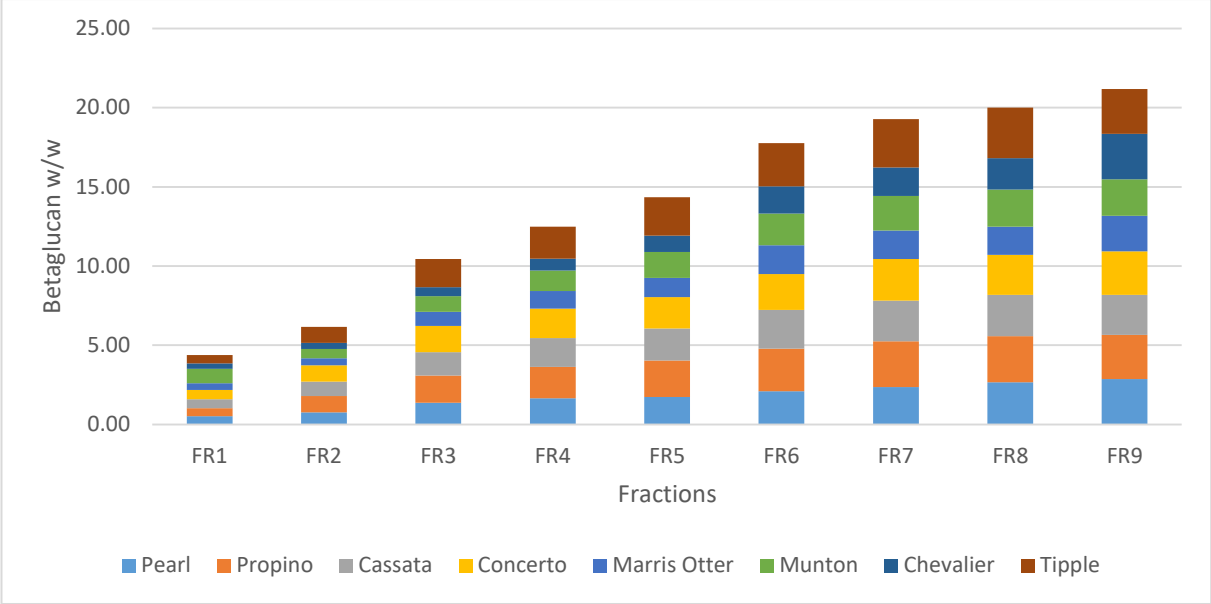


Figure 4.3: Beta-glucan content (w/w) in FR1 – FR9 of eight barley varieties.

Varieties showed differences and similarities in terms of beta-glucan content. Tipple and Propino were very similar in terms of total beta-glucan content as well as in amounts present per fraction of the grain. Both varieties had very high total beta-glucan content with the highest amount present in FR8, which was significantly higher than FR9 at $P \leq 0.05$. However, amounts present in FR8 was not significantly higher than FR7 at $P \leq 0.05$ in Propino. FR1 in Tipple and Propino, were the lowest of all varieties with 2.62% and 2.73% respectively, while FR2-FR6 contained the fraction average similar to other varieties ranging between 5 and 15%. There was however, no significant difference in the amounts present in FR3 and FR4 in Tipple at $P \leq 0.05$.

Maris Otter and Chevalier, on the other hand, showed very low total beta-glucan content of 11.68 mg (w/w) and 11.42 mg (w/w). For both varieties, beta-glucan content in FR1-FR5 was relatively lower than the fraction average of other varieties. Their beta-glucan content however rose considerably after FR5, with amounts in

FR6-FR9 relatively higher than other varieties. In fact, Maris otter and Chevalier recorded the highest beta-glucan content in FR8 and FR9 compared to all other varieties, with chevalier recording the highest amounts of 17% and 25% in FR8 and FR9 respectively.

Concerto was a variety that stood out from the other varieties, in that; it recorded the lowest beta-glucan content in FR8. An amount that was lower than amounts obtained in FR7 and FR9 of all varieties tested. Beta-glucan content of other fractions of Concerto, were however, similar to the fraction averages of other varieties. It had a total beta-glucan content of 17.32 mg (w/w), which was lower than Tipple and Propino but higher than the other varieties.

Pearl, Cassata and Munton were moderately high in beta-glucan content compared to other varieties. Pearl and Cassata were quite similar in total beta-glucan content, with both varieties, having approximately 16.00 mg (w/w) beta-glucan content, while Munton had a total beta-glucan content of 14.27 mg (w/w). However, there were variations in the distribution of beta-glucan across these three varieties. Amounts in FR9 was significantly higher than all other fractions in Pearl at $P \leq 0.05$, while FR8 was the highest in Cassata and Munton. FR8 was however not significantly higher than FR6, FR7 and FR9 in Cassata at $P \leq 0.05$. Munton at 6.4%, recorded the highest beta-glucan content in FR1 amongst all varieties tested, while Pearl and Cassata recorded about 3%, similar to other varieties. FR2 in Munton at 4.5% was however slightly lower than Pearl and Cassata which were 4.78% and 5.33% respectively. Despite the differences mentioned above, all varieties still followed the general pattern of a gradual increase in beta-glucan content from FR1 to FR9. Differences in amount of beta-glucan are discussed in relation to pearling fraction size and location on grain, are discussed below.

4.3. Resistant Starch

The table 4.3 below shows the total resistant starch present in FR4 – FR9 of all varieties. SEM images of FR1-FR6 clearly did not show any starch granules, therefore, the test for resistant starch was performed for FR4 – FR9.

Table 4.3: Total resistant starch content (percentage) in six pearling fractions (FR4 – FR9) of eight barley varieties. (See appendix 3.1 for standard deviations).

	Pearl	Propino	Cassata	Concerto	Maris Otter	Munton	Chevalier	Tipple
FR4	4.47	4.19	4.24	4.76	3.34	4.49	4.38	4.59
FR5	4.37	4.40	4.24	4.59	3.42	4.41	4.56	4.69
FR6	4.47	4.28	4.43	4.59	4.12	4.53	4.49	4.59
FR7	4.35	4.40	4.55	4.81	3.92	4.34	4.40	4.54
FR8	39.86	41.36	40.21	39.81	32.08	39.38	38.07	40.80
FR9	42.46	41.37	42.31	41.46	53.12	42.85	44.08	40.80

The table 4.3 above show the total amount of resistant starch present in all eight varieties with the largest amount present in the endosperm region of the grain - FR8 and FR9. Of all varieties tested, the total amount of resistant starch present was approximately 1.1 mg (w/w) per fraction in FR4 to FR7 which is about 4 %. SEM images of these regions, showed no starch granules (figure 4.1 and 4.2). It has been widely reported that the amount of starch increases with degree of pearling, suggesting that the outer tissue layers of barley grains are made of non-starch material (Yeung and Vasanthan, 2001). Results from the table above show a similar pattern. The resistant starch content in FR8 and FR9 ranged between 9 and 11 mg (w/w), which was approximately 30 – 45 % with no major variety differences. The amount measured in FR9 of Marris Otter was however 17.1 mg (w/w) i.e. 53.1%. Barley used in the experiment was milled, and subjected to several processing steps

of hot water extraction, cooling and enzyme treatment to obtain total resistant starch content. Results for FR4 – FR7 clearly showed that very little or possibly no resistant starch was present in these fractions, while FR8 and FR9 contained large amounts of resistant starches. The total amount of resistant starch was approximately 25.00 mg (w/w) in all varieties tested.

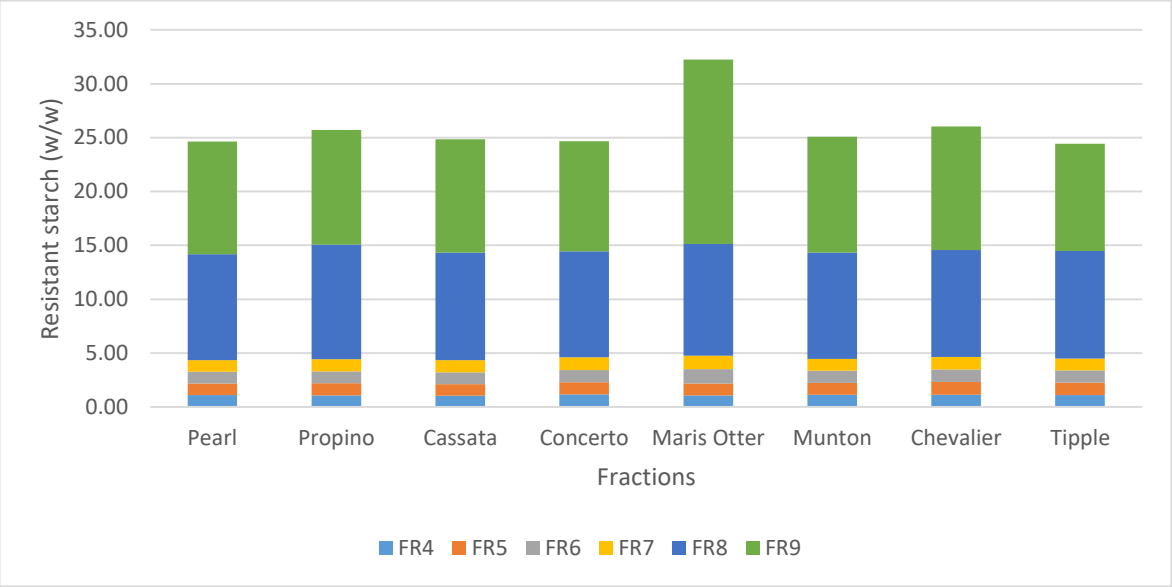


Figure 4.4: Mean percentage resistant starch content (w/w) in FR4 – FR9 of eight barley varieties in triplicate. FR4-FR7 contained < 10% resistant starch while FR8 and FR9 contained >10% resistant starch.

4.4. Protein

The total protein per variety ranged between 8.2 - 10.2 mg/mL. Cassata had the highest total protein content of 10.3 mg/mL and the lowest were Pearl and Propino, both with approximately 8.6 mg/mL. Percentage protein per fraction is presented in table (4.4) below. FR1 had a low protein content of approximately 10 -11%. This amount was however higher than amounts present in FR9 with a range of 2 and 4%. There were however, some variety difference around these ranges. For instance, Maris Otter and Chevalier have protein content slightly lower than 10% in FR1.

Table 4.4: Percentage protein per pearling fraction of eight barley varieties.

	Pearl	Propino	Cassasta	Concerto	Maris otter	Munton	Chevalier	Tipple
FR1	10.83	10.17	10.90	11.64	9.26	10.23	8.26	10.93
FR2	13.65	15.02	13.94	15.74	10.29	13.05	10.23	14.62
FR3	15.69	15.51	16.27	16.30	13.63	14.44	12.61	14.52
FR4	14.36	14.96	13.56	12.51	15.65	13.82	14.96	14.78
FR5	12.64	12.17	12.61	12.21	14.89	14.42	13.65	13.40
FR6	9.86	10.09	9.85	10.87	11.65	10.41	12.16	10.23
FR7	10.41	9.57	10.35	8.97	10.43	9.67	11.73	9.78
FR8	9.31	9.40	8.90	8.39	10.39	9.54	12.18	9.12
FR9	3.26	3.11	3.63	3.37	3.81	4.41	4.22	2.63

A summary of the result in figure (4.5) below shows a gradual rise in protein content from FR1 to FR3, where it peaks and then begins to decrease to its lowest amounts in FR9. There was a sharp decrease in protein content after FR5 and another sharp decrease after FR8. The figure below also shows that the highest amounts of protein were concentrated between FR2 and FR5, with FR3 standing out as having the highest protein content.

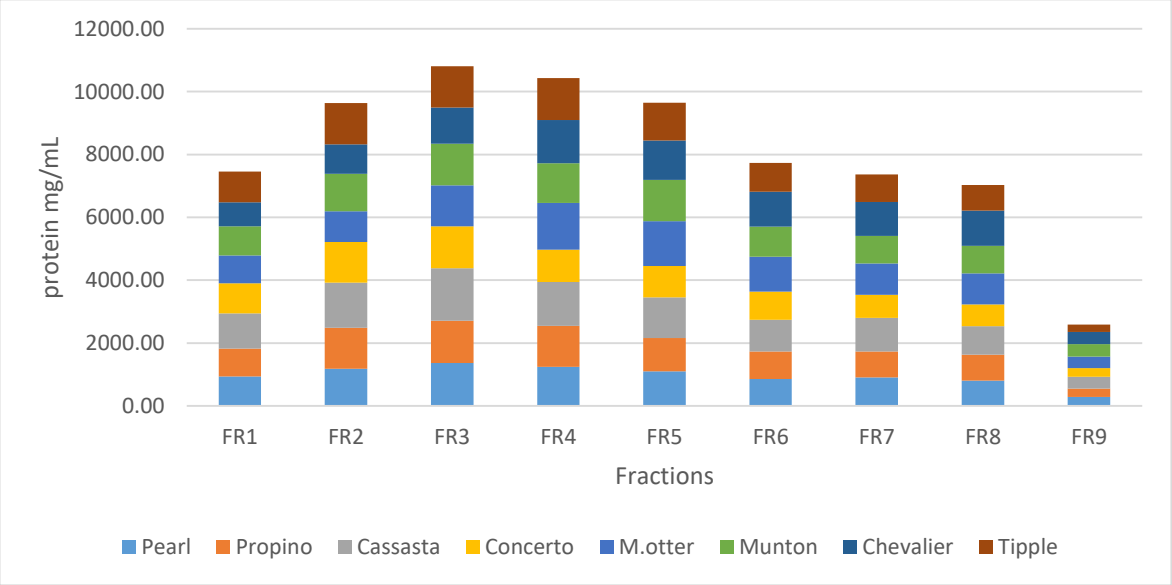


Figure 4.5: Protein content (µg/mL) in nine fractions (FR1-FR9) of eight barley varieties

In terms of the highest amount of protein per fraction, there were slight variety differences. FR3 had the highest protein content in Propino, Concerto, Pearl, Cassata and Munton, i.e. five out of eight varieties, while, FR4 was highest in Maris Otter, Tipple and Chevalier. For all varieties, protein content in FR6, FR7 and FR8 were quite similar, i.e., smaller than FR2 - FR5 but greater than FR9. The protein content in FR9, as expected was high because it is the endosperm region of the grain, made up of mainly starch granules and proteins. However, they are termed storage starches and proteins and are usually unavailable. Statistical analysis of results obtained showed that protein amount was highest in FR3 and FR4, amounts that were not significantly different one from the other at $P \leq 0.05$. Next were FR2 and FR5. FR9 was significantly lower than all other fractions at $P \leq 0.05$, followed by FR1, FR6, FR7 and FR8 were all not significantly different at $P \leq 0.05$ one from the other.

4.5. Free Amino Nitrogen (FAN)

A summary of FAN results showed that Pearl and Concerto recorded the lowest amounts at 0.015 mg/mL and 0.017 mg/mL. The highest varieties were Propino, Cassata, Tipple and Maris Otter with 0.023 mg/mL, 0.022 mg/mL, 0.022 mg/mL and 0.021 mg/mL FAN content respectively. Chevalier and Munton had similar amounts of 0.020 mg/mL and 0.020 mg/mL. Amounts per fraction ranged between 0.001 mg/mL and 0.003 mg/mL.

Figure (4.6) below showed a slightly higher FAN content in FR1-FR5 compared to FR6-FR9. FR9 contained the lowest amount in all varieties tested. Significant differences at $P \leq 0.05$ across fractions were observed in Propino, Concerto and Maris Otter, see appendix 3.0.

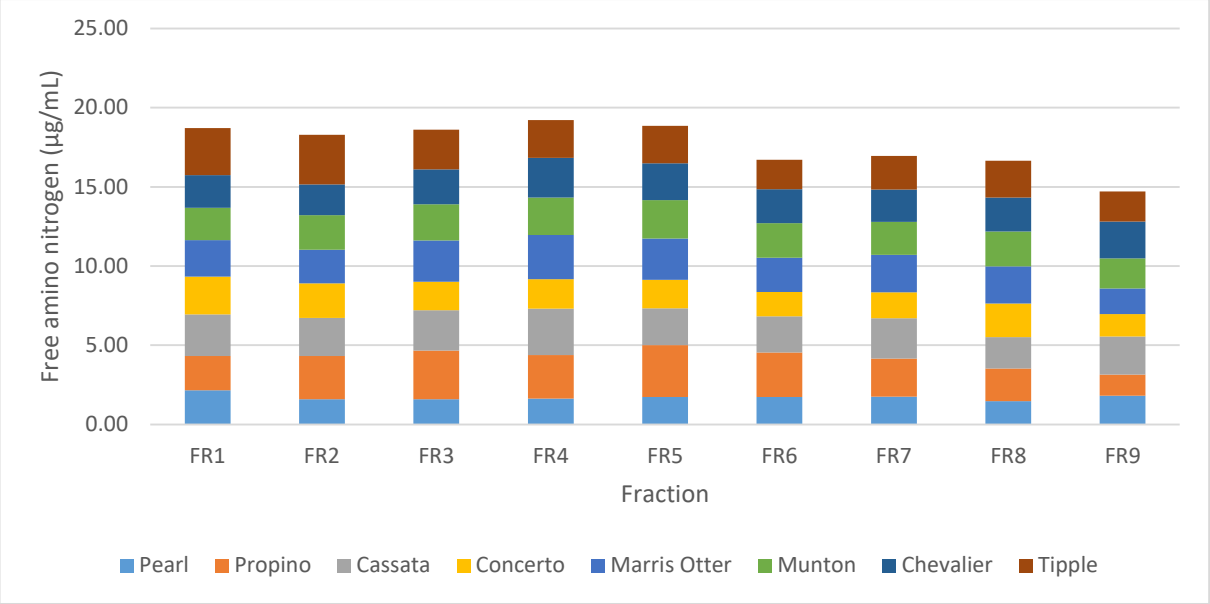


Figure 4.6: Free amino nitrogen content (µg/mL) in FR1 – FR9 of eight barley varieties.

4.6. Sugars

Table 4.5 shows total amount of fructose, glucose, sucrose, maltose and maltotriose, in all eight varieties of barley tested. Values showed differences in amount of sugars present in all varieties.

Table 4.5: Total sugar (mg/mL) content in eight barley varieties.

	Simple sugars (mg/mL)				
	Fructose	Glucose	Sucrose	Maltose	Maltotriose
Pearl	1.2196	3.4332	11.6235	6.0339	8.6391
Propino	1.0810	2.2495	10.3445	3.6453	9.0753
Cassata	1.4450	3.1082	12.6594	4.8463	7.3368
Concerto	1.4156	2.0585	10.4178	4.9509	13.3632
Maris Otter	1.5449	3.7540	5.6384	6.1140	15.8171
Munton	1.4352	2.7552	10.1420	3.7553	7.3995
Chevalier	0.3791	2.7367	12.2632	5.1173	9.3940
Tipple	2.8246	2.2296	10.3181	4.0361	17.6841

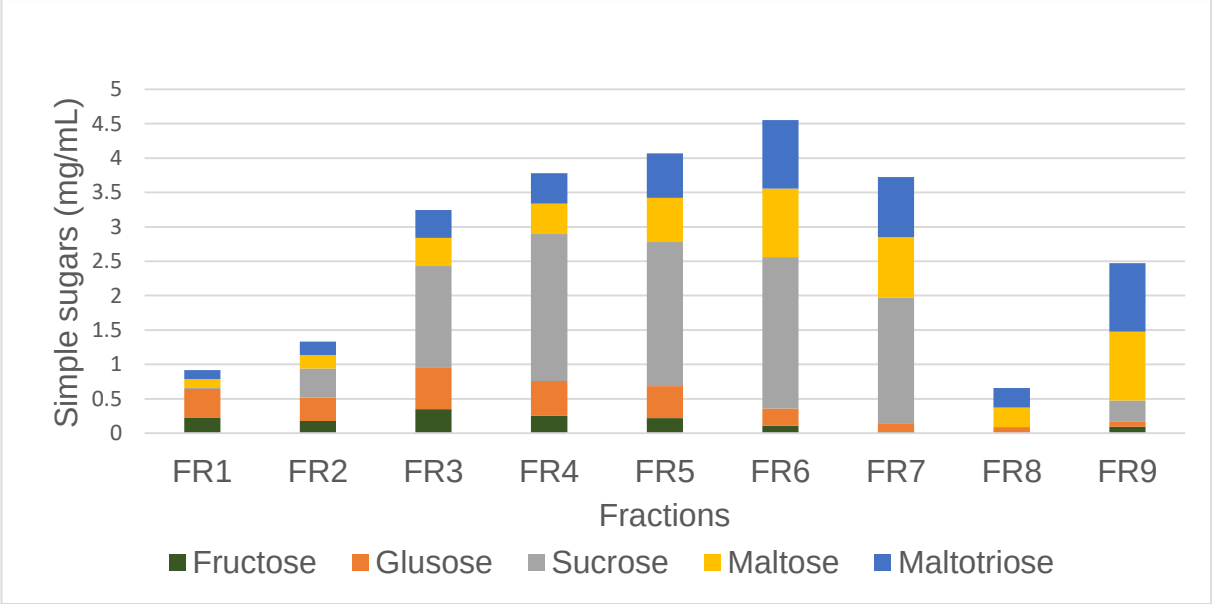


Figure 4.7: Sugars content (mg/mL) of FR1 – FR9 of eight barley varieties.

The above graph shows the distribution of sugars in FR1-FR9. Glucose and maltose were found in every fraction with glucose amounts decreasing from FR1 to FR9 while maltose increased. Similar results were obtained by Gohl *et al* (1978). Sucrose was found in high amount in FR3-FR7, but unexpected absence in FR8 of all eight varieties tested. Minute amounts were however detected in FR1 and FR9. These sugars were however removed by dialysis before extracts were used in experiments aimed at determining prebiotic properties as only complex sugars possess prebiotic properties. The sugars were however relevant to growth patterns observed when un-dialyzed extracts were used.

4.7. Discussion

In this study, barley grains were fractionated and mapped for the presence of beta-glucan, resistant starch, protein, free amino nitrogen and sugars. Fractionation was achieved by pearling. Pearling is the gradual and sequential removal of grain tissue

starting at the husk, followed by the pericarp, testa, aleurone layer, and finally terminating in the endosperm (Marconi *et al*., 2000; Wang *et al*., 1997). A study by Wang (1997) showed that the first 30% pearling fraction of a barley grain is made of the hull, pericarp, testa, aleurone and sub-aleurone layers, all of which vary in chemical composition. Fractions or pearling fines are collected between time lapses, weighed and represented as a percentage of the original grain weight. In this study, fractions of barley grain were fractionated 10 seconds apart in order to obtain a clear picture of the different layers of the grain, which would provide a relatively accurate account of chemical components distribution across the grain. A review of reports of pearling barley have shown similar results to those obtained in this study. Lampi *et al* (2004) reported in a pearling experiment that after 30 and 90 seconds of pearling, fractions obtained from a barley were 7% and 14.6% respectively. In this current research, after 80 seconds of pearling, approximately 15% fractions were obtained from hulled varieties. In another experiment by Wang *et al* (1997), using a Satake abrasive mill equipped with a medium abrasive roller stone, produced 2.7 - 4.9% fractions after 10 seconds of pearling, similar to amounts obtained in this research where approximately 5% fractions were produced after 10 seconds of pearling in eight barley varieties.

The amount of pearling fraction obtained per pearling step, across the kernel, determines the amount of protein, beta-glucan and resistant starch contained per fraction, hence, the variety differences observed, which has been linked to grain size in relation to hull/endosperm ratio per variety. Pre-pearling steps such as moisture treatment or tempering are commonly used to prepare grains prior to pearling to treat hardness of hull and prevent breakages. However, concerns about the effect pre pearling steps could have on the chemical composition of fractions

prevented grain tempering in this research. A report by Wang *et al* (1997) showed that tempering grain with moisture had an effect on protein and starch removal from the grain. He stated that increased tempering from 12.5 to 15.0% grain moisture reduced starch content in the bran during abrasion. The same report also stated that protein content of the outer hull fractions removed in the first abrasion steps were low (6.7 – 12%) due to grain tempering. Grain tempering also leads to a loss of water soluble beta-glucan. The effectiveness of grain tempering have also been questioned in some quarters. Using 3D magnetic resonance imaging, Song *et al* (1998) showed water penetration during grain tempering. His results showed an uneven water distribution during tempering especially around the germ and crease regions. He concluded that tempering increased the plasticity of the outer layers of the grain thus making them more difficult to separate. Results from images obtained from his study showed high water concentration in the germ and bran layer immediately after moisture treatment and an uneven moisture distribution in the endosperm, at the end of the treatment. In another report Andersson (2008) recommended a 20 minutes grain tempering, which allows water to penetrate only the outer layers of the grain, allowing for easy separation, as opposed to conventional tempering times of 12 – 36 hours, which causes the fusion of the seed coat and the aleurone layer, thus making separation difficult.

The ability of researchers to fractionate barley grains has been employed in the production of functional foods with desired chemical component. Liu *et al*, (2009), employed a dry fractionation (pearling and milling) method to produce barley meals varying in protein, beta-glucan and starch content. The degree of pearling over time determines the chemical composition of fractions obtained. Van Donkelaar *et al* (2015) in his work titled ‘pearling barley to alter the composition of the raw material

before brewing', reported that the 5% outer layer of barley grain contains 15% insoluble arabinoxylans, 23% insoluble fibre, 19% ash, 11% polyphenol and about 0.20% starch. The knowledge of the chemical composition of the different layers of cereal grains, especially barley, is creating more nutritional applications of the crop, which was considered relatively under-utilised with regards to its potential as an ingredient in both human and animal feeds. Another report by Zheng *et al* (2002) stated that beta-glucan content increased with degree of pearling to about 25% of the grain weight, where it peaks. High amounts of beta-glucan have also been reported in the aleurone layer of barley grain (Summers *et al*, 1985, Marconi *et al*, 2000). This corresponds with results obtained in this study where beta-glucan was highest in FR7 and FR8 which are the aleurone layer of the grain, as suggested by SEM images.

Beta-glucan and resistant starch are health-promoting compounds. Their presence in any food substance is therefore very important. Barley grains contain high amounts of beta-glucan, higher than most other cereals, hence the interest in barley for possible prebiotic property. Results from this current study have shown the presence of beta-glucan, resistant starch, protein and free amino nitrogen in all barley varieties tested, all, existing in varying amounts across varieties and fractions. There was, however, a consistent pattern in the distribution of these chemical components across all grains of all varieties. For instance, there was a consistent increase in beta-glucan and resistant starch, from the husk to the endosperm region (FR1-FR9) in all varieties. The reverse was the case for protein, which showed a consistent gradual decrease from FR5 to FR9, after an initial increase from FR1, which peaked at FR3 and FR4. Free amino nitrogen which is a

measure of the concentration of small peptides and amino acids were almost evenly distributed across all fractions.

SEM images have shown that FR1-FR8 are the bran layer of the grain while FR9 is the starchy endosperm. The bran is divided into the outer bran (FR1-FR5) and the inner bran (FR6-FR8). A breakdown of results obtained from this study, revealed low beta-glucan, low resistant starch and high protein content in the outer bran, while the inner bran had a high beta-glucan, high resistant starch and low protein content. FR9, which is the starchy endosperm, was high in beta-glucan and resistant starch but very low in protein. These results correspond with the literature review on chemical components of barley grains. Similar results were obtained in a recent study, where six varieties of barley were tested for beta-glucan content and extractability in kernel, inner/outer bran and sifted flour (Djurle et al., 2016). The study showed that SLU 7 (a variety with a shrunken endosperm) had the highest beta-glucan content of 9.2%, while other varieties ranged between 3.3 and 6.6%. Varying amounts of beta-glucan were equally obtained across different varieties in the current study, ranging between 11- 20 %. These differences amongst varieties according to Djurle (2016) could be attributed to the ratio of the hull to the endosperm portion. A variety with a shrunken endosperm, obviously has a larger hull portion which usually relates to high dietary fibre content, hence a higher beta-glucan content. It was also stated in the same report that producing sifted flour for all varieties, reduced their dietary fibre content which further supports the fact that beta-glucan is higher in the fibrous portion of the grain. In the current research, the barley varieties used also showed difference in a thousand grain weight, with 46.56kg, 51.36kg and 40.63kg for Concerto, Propino and Maris Otter respectively,

suggesting possible difference in bran versus endosperm ratios, hence differences in chemical compositions.

Djurle (2016) carried out further analysis on the bran fractions of these six varieties to determine beta-glucan distribution in the outer and inner bran. Analysis of Gustav (a hulled variety) showed a higher beta-glucan content (5.6%) in the inner bran, compared to 2.1% in the outer bran and 3.6% in sifted flour. These results are similar to results from this study (table 4.2) which shows low beta-glucan content in FR1 – FR5 (outer bran) and high beta-glucan content in FR6 - FR8 and FR9, which corresponds with inner bran and flour portions of the grain. However, the flour portion (FR9) in this current study was not sieved and hence, might still contain fractions of the inner bran. Table 4.2 showed high amount of beta-glucan in FR9, which was slightly higher than FR8 in some varieties and slightly lower in others.

5.0. Chapter Five: Microbial Analysis Results

5.1. Extracts Containing Undigested Nutrients (Method One)

Fractions obtained from pearling were subjected to a digestion process in a simulated poultry gut, with digested nutrients serving as energy source for *Lactobacillus acidophilus* and *Salmonella enteritidis* over a 6-hour growth period (see methods). Samples were collected from the simulated poultry gut at two hours and six hours after bacterial inoculation and plated on growth agar and colonies counted 24 hours later. Amount of growth was analysed by calculating the difference in growth between the two hours and six hours after bacterial inoculation. Result for all three varieties showing growth pattern of fractions is summarised in figure 5.1 for *Lactobacillus acidophilus* and figure 5.2 for *Salmonella enteritidis*, for possible prebiotic effect (extracts containing undigested nutrients) and figure 5.3 and figure 5.4 for *Lactobacillus acidophilus* and *Salmonella enteritidis* growth in for solution containing digested nutrients only respectively.

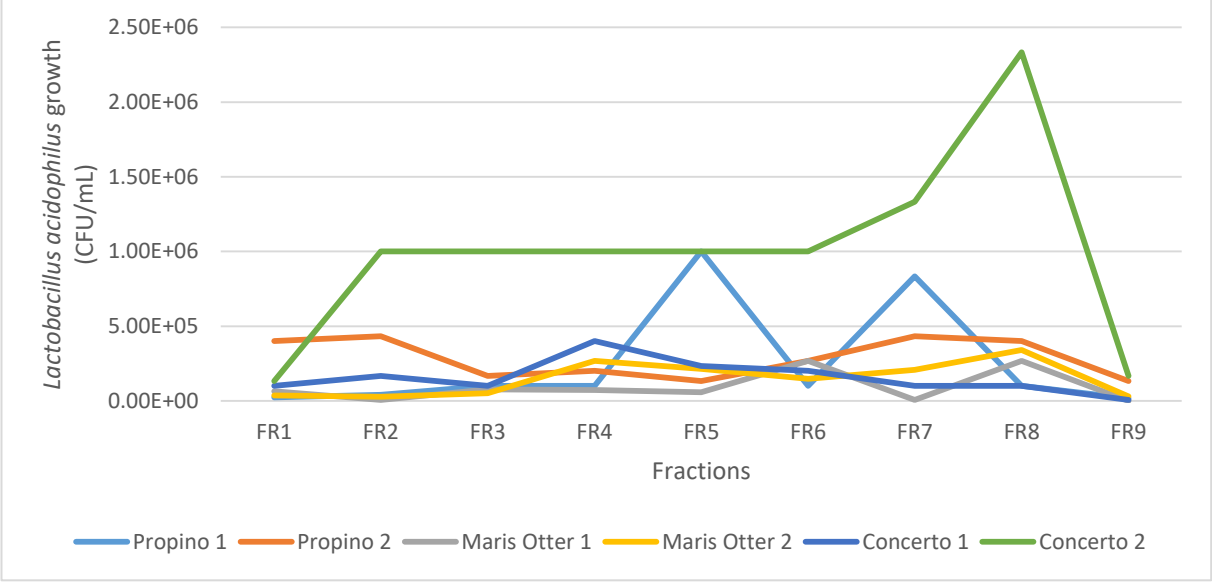


Figure 5.1: Growth patterns of *Lactobacillus acidophilus* on Propino, Marris Otter and Concerto extracts containing undigested nutrient. (n = 6)

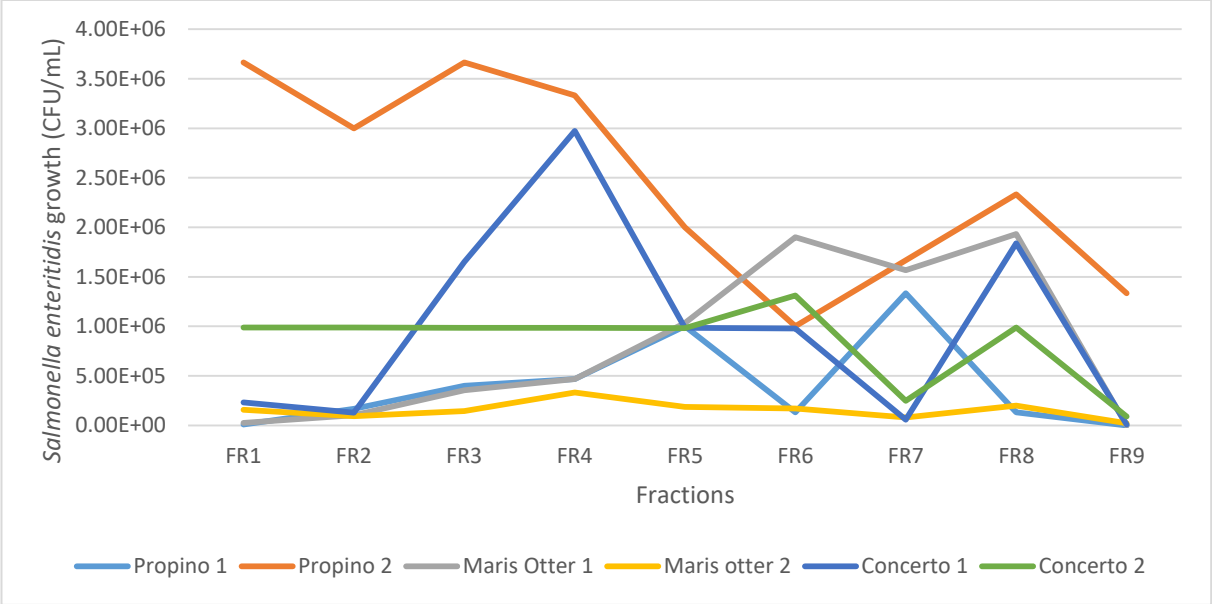


Figure 5.2: Growth patterns of *Salmonella enteritidis* on Propino, Maris Otter and Concerto extracts containing undigested nutrients. (n = 6).

The test bacteria grew on each fraction extract, with FR9 showing the lowest amount of growth for both *L. acidophilus* and *Salmonella* Enteritidis across all varieties. FR1, FR2 and FR3 also showed relatively low growth except for the second replicate of Propino, which might be an outlier. Growth rates of other fractions (FR4, FR5, FR6, FR7 and FR8) varied greatly, with no particular fraction or variety showing any exceptionally consistent growth patterns between replicates and across varieties. Growth for these fractions were, however, observably higher than FR1, FR2, FR3 and FR9 across replicates and varieties. Results from the statistical analysis was consistent with the observed results. There were inconsistencies between replicates and across varieties at $P \leq 0.05$. In Propino for instance, there was no significant difference across FR1-FR9 in one replicate while the second replicate of the same experiment showed significant differences across FR1-FR9 at $P \leq 0.05$. However, bacterial growth in FR9 was low across all experiments with the amount significantly

different from other fractions in some varieties and replicates but not significantly different in others at P ≤ 0.05, with no consistent pattern observed.

Figure 5.3 and 5.4 below, show the growth patterns of *Lactobacillus acidophilus* and *Salmonella enteritidis* on digested nutrients obtained from dialysed extracts of FR1-FR9 in a simulated poultry gut. These nutrient extract supported growth of the test bacteria but there was no consistent growth pattern, except for FR1 and FR9, which recorded very low bacterial growth in both experiments. No other consistent fraction effect was observed, similar to results obtained in Figure 5.1 and figure 5.2. Results from Propino was discussed as an example in 5.1.1 below.

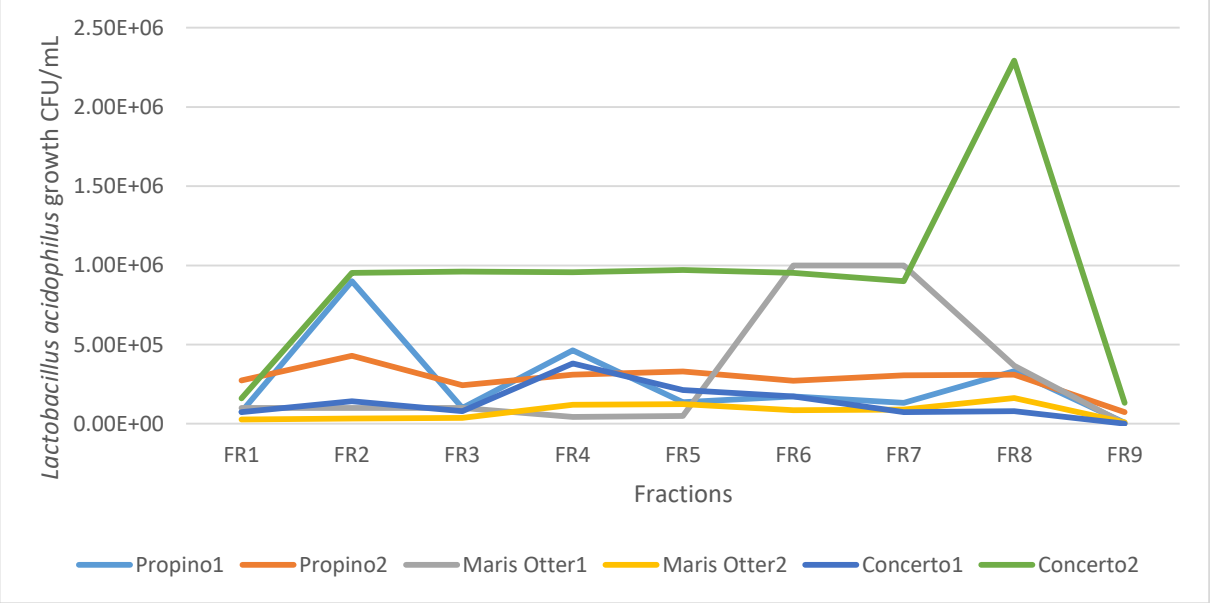


Figure 5.3: Growth pattern of *Lactobacillus acidophilus* on Propino, Marris Otter and Concerto extracts containing digested nutrients. No consistent growth patterns across fraction replicates was observed (n=6)

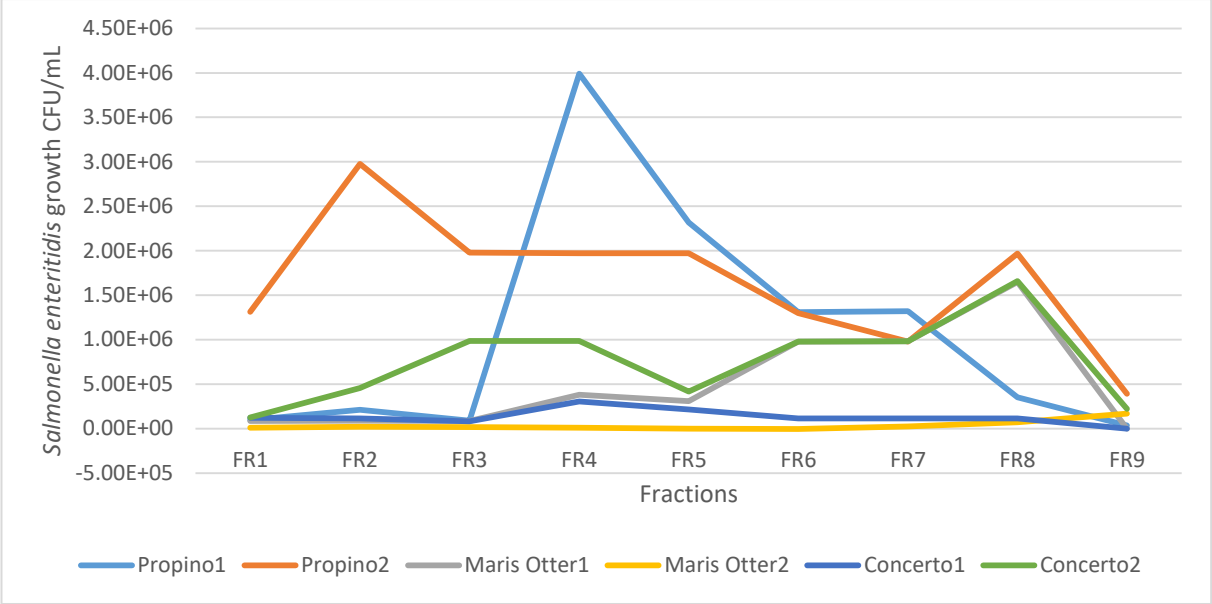


Figure 5.4: Growth pattern of *Lactobacillus acidophilus* on Propino, Marris Otter and Concerto extracts containing digested nutrient. No similar consistent growth patterns across fraction replicates was observed (n=6).

The results just like those obtained with extract containing undigested nutrients (figure 5.1 and 5.2) were inconsistent, thus making inferences on fractions or variety effects impossible.

5.1.1. Propino

Growth results using Propino fractions showed FR5 and FR7 recording a significantly high bacterial growth, approximately 1.0 x 10^6 CFU/mL higher than all other fractions, which were mostly below 2.0 x 10^5 CFU/mL for *Lactobacillus acidophilus.* FR5 and FR7 also supported high growth (about 1.0 x 10^6) of *Salmonella enteritidis* while other fractions recorded less than 5.0 x 10^5 CFU/mL (figure 5.6). A repeat of the experiment however revealed a different result, with FR1, FR2, FR7 and FR8, showing the highest amount of growth, with no significant differences between them for *Lactobacillus acidophilus* at about 4.0 x10^5 CFU/mL.

While other fractions were below 2.0 x 10^5 CFU/mL. FR1, FR2, FR3 and FR4 supported the highest growth for *Salmonella enteritidis* in the same experiment, recording growth values of approximately 3.0 x 10^6 CFU/mL. Growth for FR7 and FR8 were also moderately high, recording values of about 2.0 x 10^6 in the repeat experiment (Figure 5.5). The growth rate of *Salmonella enteritidis* was generally higher than *Lactobacillus acidophilus*. This inconsistent trend was also the case for Maris Otter and Concerto – see appendix 1 and 2. These varying results make it difficult to draw inferences as to which fraction encouraged the best bacterial growth. However, results for FR9 were consistently low, recording growth amounts below 5.0 x 10^3 CFU/mL in most experiments. This portion of barley grain is the endosperm portion of the grain, left over after pearling for 80 seconds, and consist mainly of starch granules. The digestion process used in this experiment does not provide enzymes needed to break down storage starch, thus, possibly, making less nutrient available from FR9. Poultry birds, as mentioned earlier, also lack NSP degrading enzymes, so farmers add enzymes to poultry feed mix to aid break down of starch, into forms available for absorption. Due to the inconsistencies observed in the above results, experimental design was modified and adopted to check the variations (see materials and methods) and the new results presented in ‘5.2’ below.

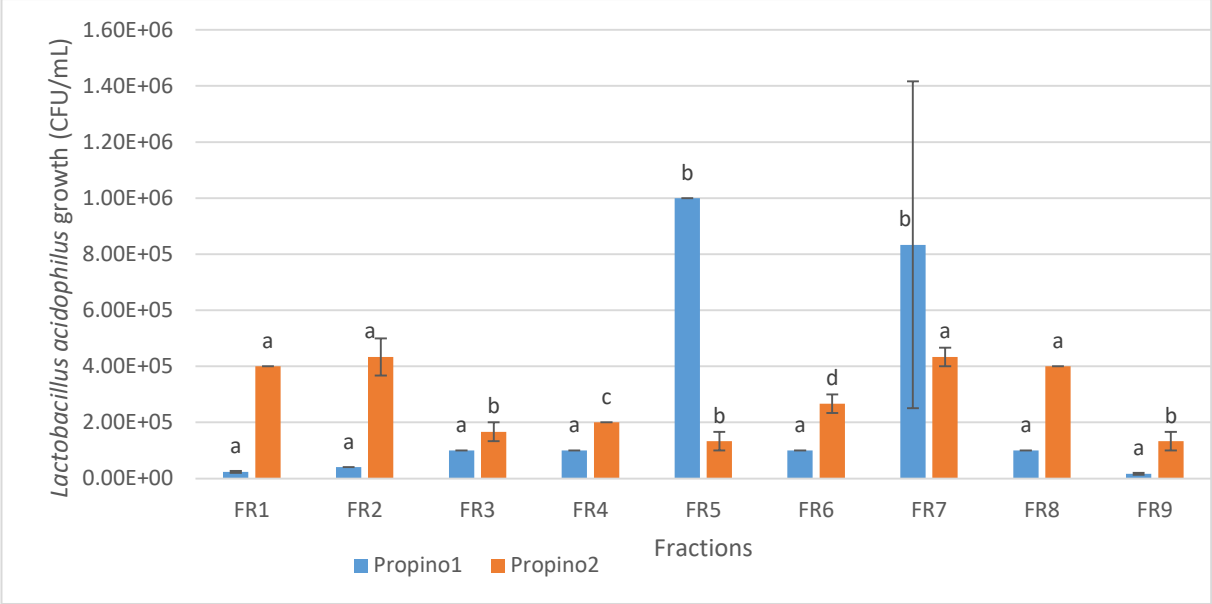


Figure 5.5: Growth (CFU/mL) of *Lactobacillus acidophilus* in Propino extract containing undigested nutrient over 8 hours. Same colour bars with similar letters are not significantly different at P ≤ 0.05.

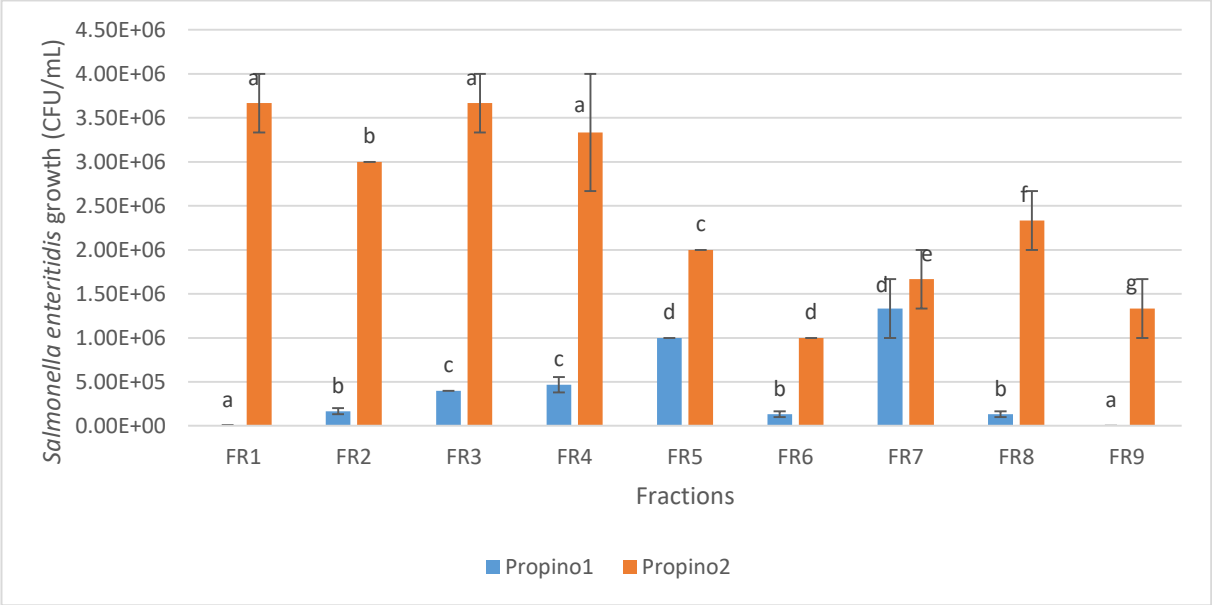


Figure 5.6: Growth (CFU/mL) of *Salmonella enteritidis* in Propino extract containing undigested nutrients over 8 hours. Same colour bars with similar letters are not significantly different at P ≤ 0.05.

Maris Otter and Concerto extracts also showed inconsistent results. See appendix 5.0 for Maris Otter and Concerto growth results.

5.2. Simulated Poultry Gut (Procedure 2).

5.2.1. Extracts Containing Undigested Nutrients (Possible Prebiotics)

On a general note, from all results obtained, bacterial growth rate followed a similar pattern in all three replicates (figure 5.7). FR1, FR2 and FR9 recorded the lowest growth rates, with FR9 showing the significantly lowest growth rate at $P \leq 0.05$. The highest growth was recorded by FR6, FR7 and FR8 in most replicates. Growth from these fractions were also significantly higher that all other fractions at $P \leq 0.05$. FR3, FR4 and FR5 generally showed growth higher than FR1, FR2 and FR9 but lower than FR6, FR7 and FR8. Amount of Growth in FR3 and FR4 were not significantly different one from another in three out of three replicates at $P \leq 0.05$. Amount of growth in FR5 was also not significantly different from FR3 and FR4 in one out of three replicates but significantly different in two out of three replicates at $P \leq 0.05$, being higher in one replicate and lower in the second.

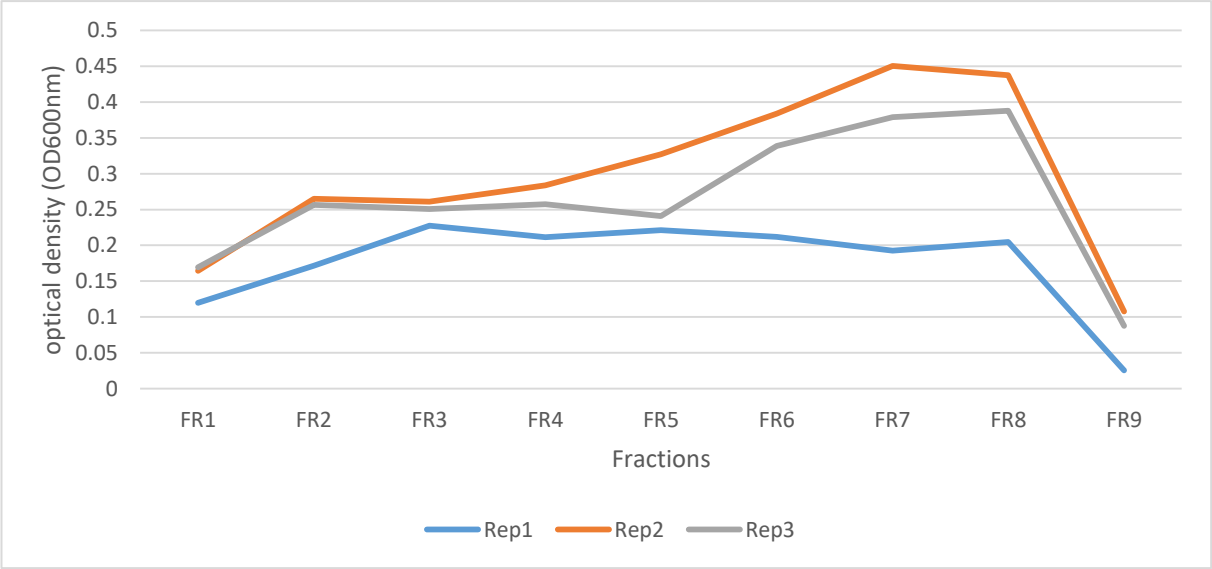


Figure 5.7: Growth pattern of *Lactobacillus acidophilus* on Propino, Maris Otter and Concerto dialysed extracts. (I.e. extracts containing undigested nutrients) over 10 hours in triplicates measured in optical density (OD_{600nm}), showing a consistent growth pattern across replicates. Each line represents all three varieties in triplicate (n=9).

Based on the results from the second experimental design, barley fractions can be divided into three groups based on how they supported the growth of *Lactobacillus acidophilus*. Group 1 consist of FR1 and FR9, which showed low growth; group 2, which are FR2, FR3, FR4 and FR5, showed moderate growth while group 3 made up of FR6, FR7 and FR8 showed high growth. Each group showed Slight variations across varieties, however, FR5 also showed consistently high amount of growth in the moderate group. FR1 corresponds with the outer husk of the barley grain, FR2 – FR6, the pericarp and testa region, FR7 – FR8, the aleurone layer while FR9 corresponds with the starchy endosperm. Varieties are discussed below, based on similarities of growth patterns.

5.2.1.1. Concerto and Propino

Results from Concerto showed that bacterial growth was significantly higher in FR6, FR7 and FR8 in two out of three replicates (figure 5.8). FR9 showed the lowest growth rate in all replicates at $P \leq 0.05$, followed by FR1. All other fractions (FR2, FR3, FR4 and FR5) showed relatively moderate growth with FR5 recording the highest amount of growth rate in this group except for the first replicate where FR3 was highest. Propino recorded a similar result with FR8 and FR9 recording the highest growth in two out of three replicates (figure 5.9). For Propino also, FR9 and FR1 significantly recorded the lowest amount of growth, while FR3 instead of FR5 showed a high growth rate in the moderate growth rate group.

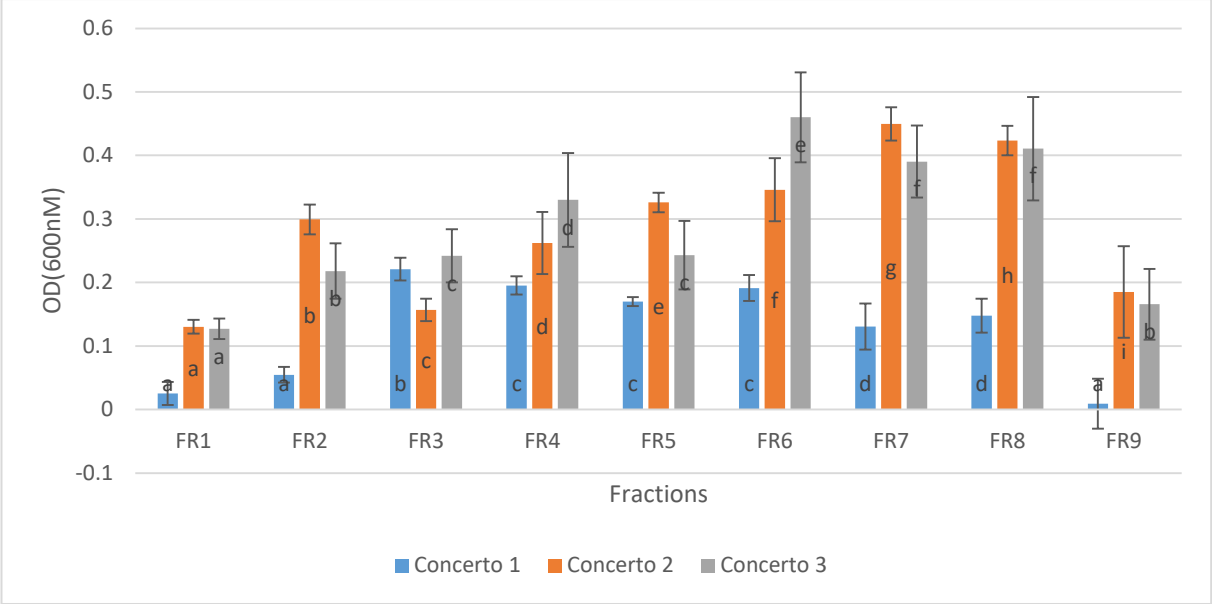


Figure 5.8: *Lactobacillus acidophilus* growth in Concerto dialysed extracts. (I.e. extracts containing undigested nutrients) over 10 hours measured in optical density (OD_{600nm}) in triplicate. Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

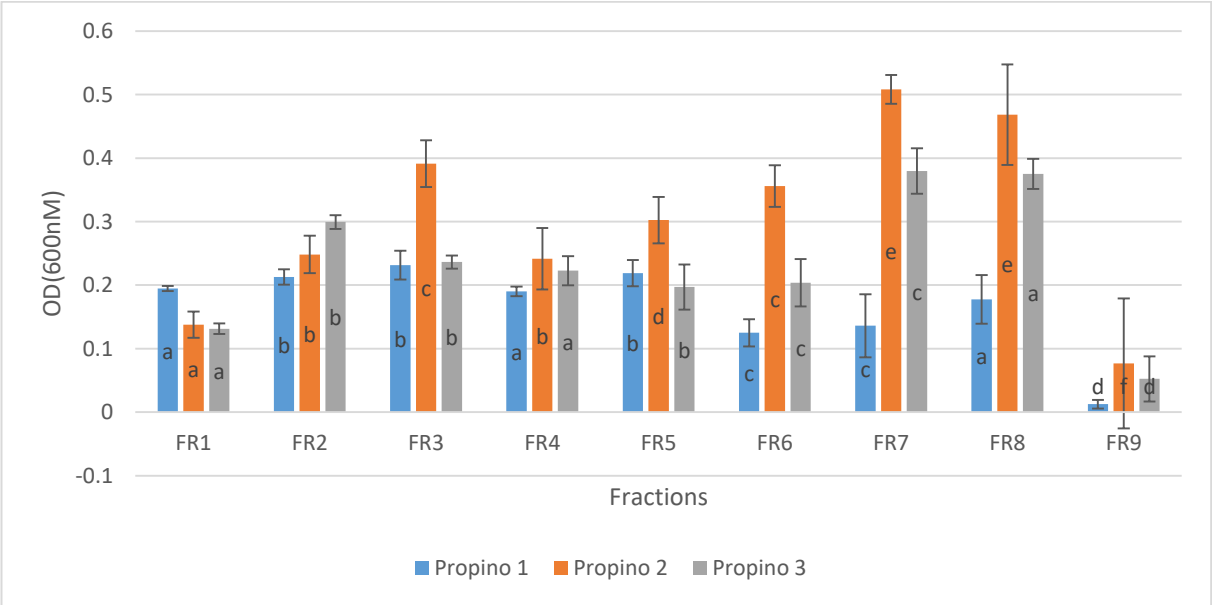


Figure 5.9: *Lactobacillus acidophilus* growth in Propino dialysed extracts. (I.e. extracts containing undigested nutrients) over 10 hours measured in optical density (OD_{600nm}) in triplicate. Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

5.2.1.2. Maris Otter

Results from Maris otter showed bacterial growth to be highest in FR6, FR7 and FR8 in all three replicates (figure 5.10) which was about four times higher than the growth of FR9, which recorded the lowest growth at $P \leq 0.05$. FR1 also supported low bacterial growth, recording about half as much growth as FR6, FR7 and FR8. FR2, FR3, FR4 and FR5 recorded relatively moderate growth rate across all three replicates, however FR5 also showed the highest growth rate in this group as was also the case with Propino and Concerto.

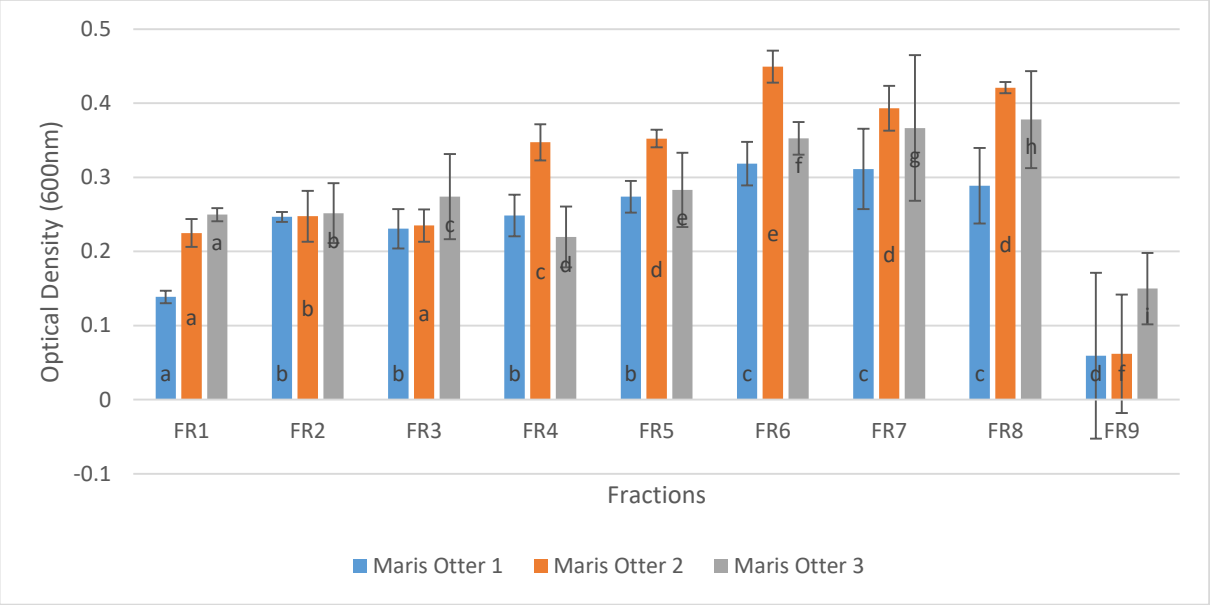


Figure 5.10: *Lactobacillus acidophilus* growth in Maris Otter dialysed extracts. (I.e. extracts containing undigested nutrients) over 10 hours measured in optical density (OD_{600nm}) in triplicate. Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

5.2.2. Extracts Containing Digested and Undigested Nutrients

Figure 5.11 below, shows a summary of the growth pattern of Lactobacillus acidophilus on undialyzed extracts from Propino, Concerto and Maris otter. These extracts contain both digested and undigested nutrients. The growth pattern on these extracts followed a similar pattern across all varieties tested. FR1 and FR9 supported low bacterial growth. FR1 and FR9 have consistently showed low bacterial growth, as was observed also with dialysed extracts containing undigested nutrients (figure 5.7), suggesting a low nutrient availability in these fractions. FR6 also showed low bacterial growth across all varieties with undialyzed extracts but this fraction supported moderate bacterial growth in dialysed extracts containing undigested nutrients. The amount of growth in FR6 was however significantly lower than FR2, FR3, FR4, FR5, FR7 and FR8 in one out of three varieties at $P \leq 0.05$. A summary of results obtained from all other fractions, showed a similar growth pattern across all fractions, with slight variations across varieties. FR2 – FR7 all contained relatively high amounts of sucrose and growth in these fractions, except FR6, were higher than FR1 and FR9, with FR2 showing the highest growth in two out of three varieties. However, on a general note, there were no outstanding fraction effects between FR2, FR3, FR4, FR5, FR7 and FR8. Further statistical analysis of Propino, Concerto and Maris Otter revealed that FAN, protein, glucose and fructose were responsible for the growth pattern observed in Propino and Concerto, while FAN, protein and sucrose were strongly correlated with the growth pattern observed in Maris otter as seen in table 5.1 below.

Table 5.1: Pearson correlation of bacterial growth pattern (OD600) versus chemical composition in Propino, Concerto and Maris Otter.

	Propino OD	Concerto OD	Maris Otter OD
OD	1.000	1.000	1.000
FAN	0.769	0.362	0.508
Protein	0.859	0.841	0.569
Resistant Starch	-0.702	-0.624	-0.444
Beta-glucan	-0.532	-0.351	0.112
Glucose	-0.459	0.281	-0.339
Fructose	0.652	0.474	-0.071
Sucrose	0.179	0.354	0.473
Maltose	-0.675	-0.431	0.043
Maltotriose	-0.675	-0.431	0.043

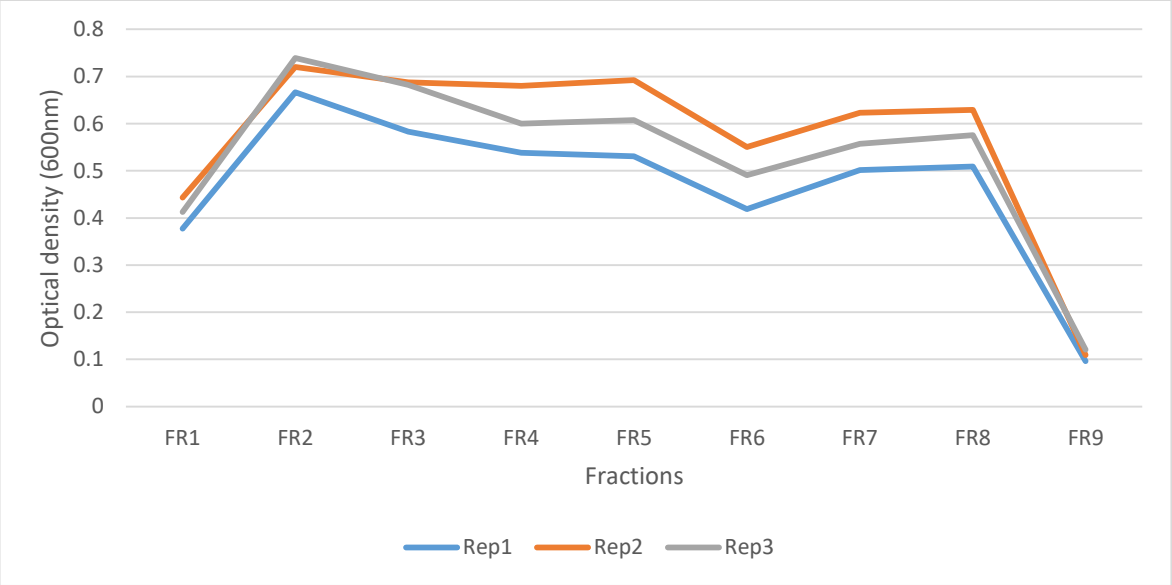


Figure 5.11: Growth pattern of *Lactobacillus acidophilus* on Propino, Maris Otter and Concerto undialyzed extracts. (I.e. extracts containing digested and undigested nutrients) over 10 hours in triplicates measured in optical density (OD_{600nm}), showing a consistent growth pattern across replicates (n=9).

5.2.2.1. Propino

The growth of *Lactobacillus acidophilus* on undialyzed Propino extracts, did not show any outstanding fraction effects, except for FR9 which showed very low bacterial growth (figure 5.12). FR1, which usually records low growth, however

supported bacterial growth in amounts similar to FR2 to FR8. Fraction effects where however noticed on Propino extracts containing dialysed samples containing undigested nutrients (figure 5.9).

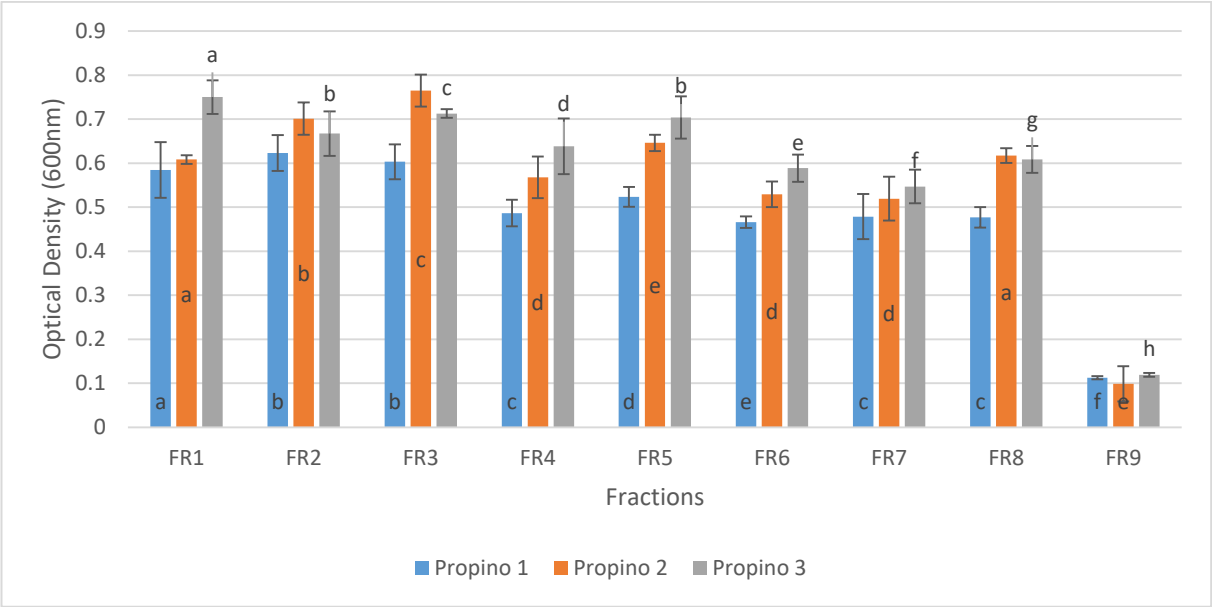


Figure 5.12: *Lactobacillus acidophilus* growth in Propino undialyzed extracts. (I.e. extracts containing digested and undigested nutrients) over 10 hours measured in optical density (OD_{600nm}) in triplicate. Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

5.2.2.2. Concerto

Figure 5.13 below showed low growth of *Lactobacillus acidophilus* on FR1 and FR9, both of which were significantly lower than other fractions at $P \leq 0.05$. FR2 recorded the highest amount of growth in replicates, an amount significantly higher than all other fractions. FR3 to FR8 showed similar growth. Except for FR2, there were also no major fraction effects on the growth of *Lactobacillus acidophilus* on concerto undialyzed extracts. Fraction effects were however noticed in dialysed extracts containing undigested nutrients (figure 5.8).

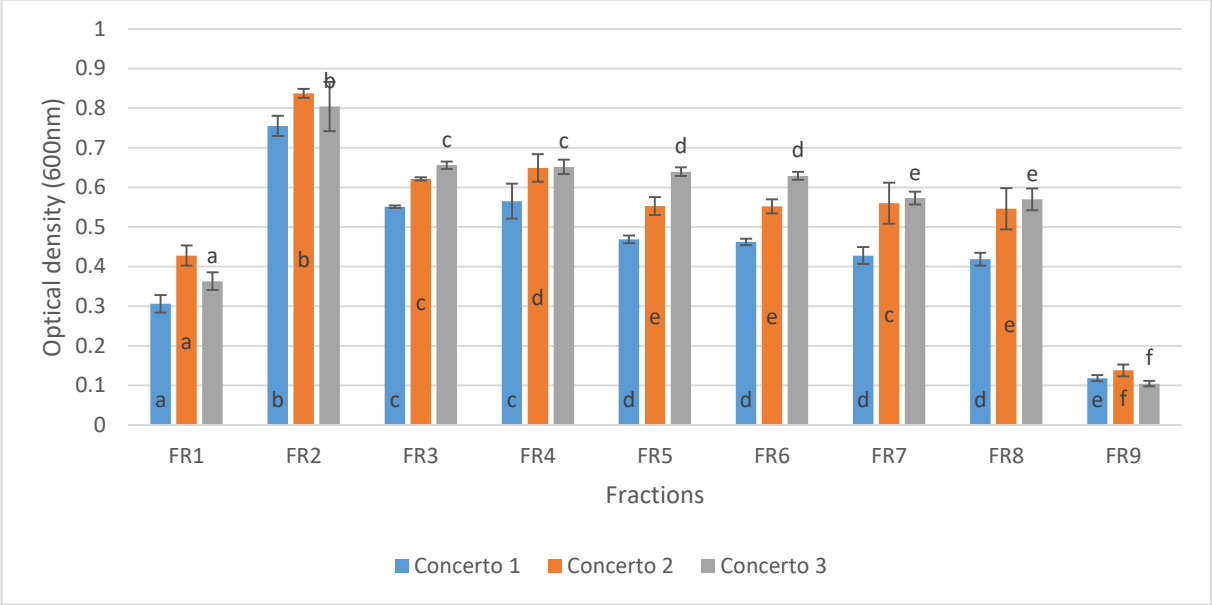


Figure 5.13: *Lactobacillus acidophilus* growth in Concerto undialyzed extracts. (I.e. extracts containing digested and undigested nutrients) over 10 hours measured in optical density (OD_{600nm}). Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

5.2.2.3. Maris Otter

The growth of *Lactobacillus acidophilus* on Maris Otter undialysed extracts showed similar results as Propino and Concerto. FR1 and FR9 showed the significantly lowest growth at $P \leq 0.05$. The amount of growth in FR6 was higher than FR1 and FR9, but significantly lower that amounts in other fractions at $P \leq 0.05$. FR2, FR3, FR4, FR5, FR7 and FR8 all supported moderate to high bacterial growth, with no outstanding differences between them.

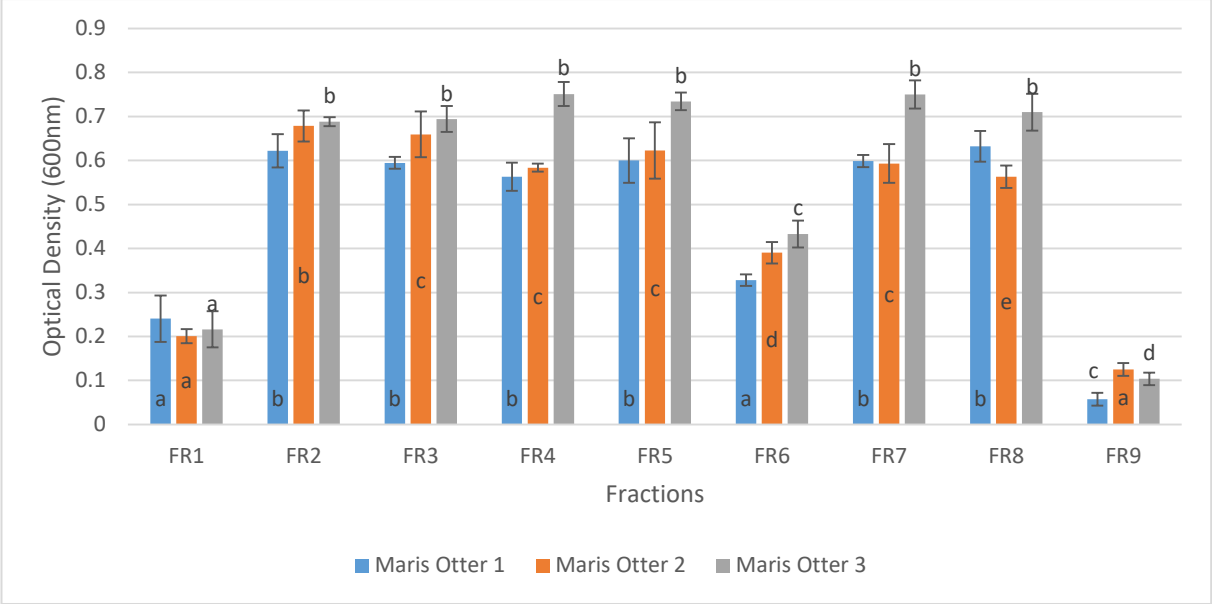


Figure 5.14: *Lactobacillus acidophilus* growth in Maris Otter undialyzed extracts (i.e. extracts containing digested and undigested nutrients) over 10 hours measured in optical density (OD_{600nm}). Same colour bars with similar letters are not significantly different at $P \leq 0.05$.

5.3. Discussion

Pearling barley produces fractions with varying biochemical properties that had varying effects on bacterial growth in the simulated poultry gut model used in this research. FR1 – FR9 showed different bacterial growth patterns for *Lactobacillus acidophilus* and *Salmonella enteritidis*, though results were inconsistent in the mixed culture plate-count method, FR9 consistently supported very low bacterial growth in all experiments.

The mixed culture plate-count method was first chosen as the experimental design, due to the large sample size. All eight varieties of barley were used in this experiment, making a total 864 sample (i.e. 8 varieties * 9 fractions * 3 replicate * 2 agar plates (XLT – 4 agar and MRS agar, selective for *Salmonella enteritidis* and *Lactobacillus acidophilus* respectively)* 2 (undigested nutrients and digested

nutrients). Also considered, was the ability to differentiate *Lactobacillus* from *Salmonella*, which could be easily achieved using selective growth agar, compared to broth cultures, where mixed cultures cannot be easily separated and enumerated. A drop plate method was also adapted, as it required less time and effort to dispense drops compared to a spread plate method. Unlike spread plates however, growth might have been limited by the free space available around each cell. Colony counting was achieved easily and faster, however the results obtained were inconsistent. Figure 5.15 below shows the drop plate-count method used in this research. The growth of *Salmonella enteritidis* appeared as black colonies, which were easily enumerated at 10^{-4} dilution. In most of the experiment conducted, technical replicates gave similar results, while biological replicates were very different.

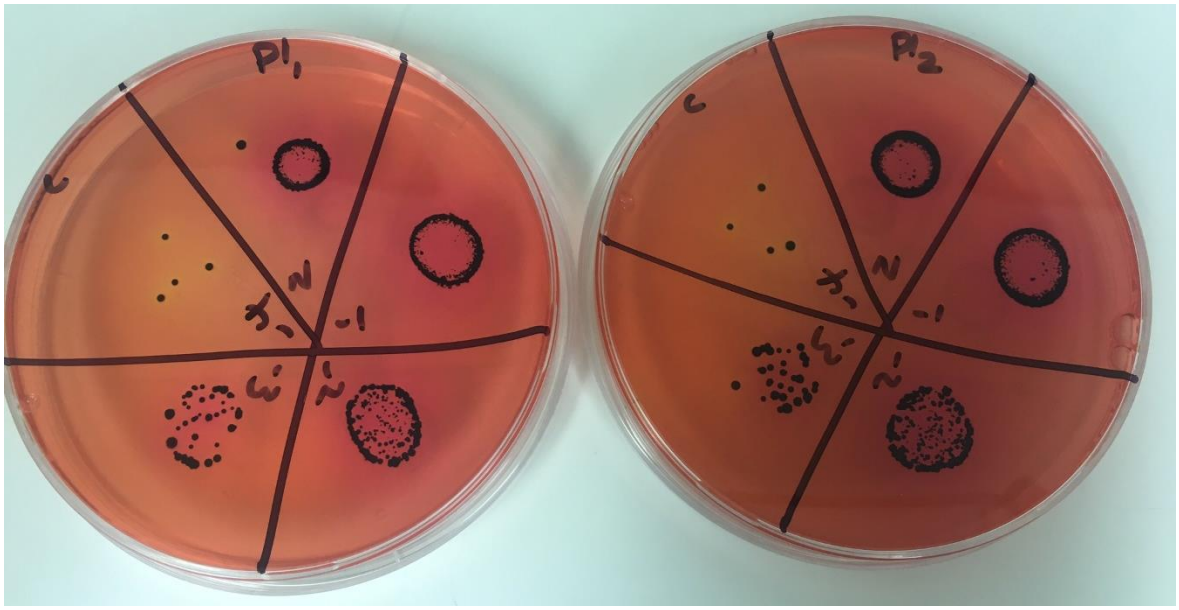


Figure 5.15: Drop plate method used showing growth of *Salmonella enteritidis* on selective XLT- 4 agar.

Bacterial growth on agar plates depends on a number of factors. Growth may be guaranteed, but the consistency might not be. There is a constant interaction between cells and existing environmental conditions in an agar plate such as space, temperature, nutrient composition and agar depth, all of which are essential at determining the growth rates of bacterial cells. The inconsistency noted in the plate-count method used might have been as a result of one or a combination of the following factors. XLT- 4 agar used in this research contained a supplement, which could not be autoclaved. As a result, the agar was produced manually by hand pouring; a technique, which could affects agar depth and consistency. The components of the growth media used, might have also differed slightly in composition due production/preparation techniques and storage conditions. Agar used in this experiment were from different production dates and batches as supplied by the University of Sunderland Microbiology Laboratory. Another factor considered was the location of agar plates in the incubator, which might have affected prevailing temperature. Agar plates were piled upon each other in different arrangements at different times, which led to condensations on some plates. The presence of growth inhibitors might have affected the result also. The XLT- 4 agar contained substances that inhibited the growth of *Lactobacillus.* These substances might have affected the pH of the agar at varying degrees, thus leading to inconsistent results. In addition, the accumulated dead *Lactobacillus* cells could also have had a negative effect the growth patterns at *Salmonella*, acting like growth inhibitors. These factors might have affected growth patterns in this study, especially, where expected growth differences were marginal. However, no conclusions were made, as the above mentioned factors were no analysed experimentally.

In the light of the above a single culture method using a 96 well micro titre plate method was adopted. The mixed culture method was discarded because attempts to modify it involved extensive testing, modification and optimization, which was beyond the scope of the current research. However, the method is recommended for further research as microorganism do not grow in isolation. A modification and optimization of the mix culture method will also provide more insights to microbial interaction *in vitro*. The new method adopted involved using barley extract as bacterial growth medium broth. Broth cultures have less predisposing factor to bacterial growth irregularities compared to agar plates. As long as nutrients are available in a broth media that does not contain any growth inhibitors, bacteria will grow exponentially. The method, involving the use of a spectrophotometer which constantly monitored bacteria growth, taking reading in optical density every 30 minutes. As expected, results obtained were consistent across biological and technical replicates.

Results from the plate count method, though inconsistent, showed a better growth of *Salmonella enteritidis* than *Lactobacillus acidophilus* in all replicates. This was expected, as *Lactobacillus acidophilus* has been reported to have high nutritional needs (Morishita et al., 1981). In a recent study by Charalampopoulos *et al* (2002), *Lactobacillus acidophilus* exhibited the poorest growth amongst a collection of *Lactobacillus* species. In addition, culture preparation of *Lactobacillus acidophilus* in this study was more difficult to maintain than *Salmonella enteritidis*. 48-hour culture of *Salmonella enteritidis* survive better than *Lactobacillus acidophilus*. Despite these inconsistencies, FR9 constantly supported low bacterial growth for both *Lactobacillus acidophilus* and *Salmonella enteritidis.*

The new method, compared growth of *Lactobacillus acidophilus* on FR1 to FR9 dialysed extracts containing undigested nutrient with FR1 to FR9 undialysed extracts that contained a combination on digested and undigested nutrients. The results showed fraction effect in extracts containing undigested nutrient while no outstanding fraction effects was notice in extracts containing both digested and undigested nutrients. FR6, FR7 and FR8 showed high bacterial growth in extracts containing undigested nutrients, a result that is of great interest in this research. These fractions were shown to contain high amounts of beta-glucan and low amount of protein compared to all other fractions. FR9 however, also contained high amounts of beta-glucan and low protein but the growth of *Lactobacillus acidophilus* in this fraction was consistently low. FR9 is the starchy endosperm region of the grain, which is made up of mainly carbohydrates. These carbohydrates are reserve carbohydrates known as storage starches. They are critical for providing energy for the embryo during grain biosynthesis. These starches are composed of two α-glucan polymers, amylose and amylopectin, packed in as crystalline granules. Also present in the endosperm are a variety of storage proteins which are contained in endoplasmic membranes as insoluble accretions. Other types of proteins in the endosperm are protease inhibitors, α-amylase inhibitors, ribosome inactivating proteins with the primary function of protecting the grain from pathogens and predators. These all combine to make digestion of the endosperm difficult before germination is activated (Lopes and Larkins, 1993). This could be the possible reason why FR9 does not contain available digested nutrients, thus supporting very low bacterial growth. As a recommendation for further research, enzyme treatment of this fraction could be a prior step to microbial growth analysis in order to yield better bacterial growth. Results from extracts containing a mixture of digested and

undigested nutrients, did not show outstanding growth in FR6 – FR8. A summary of the results from this experiment showed that fractions which exhibited similar bacterial growth patterns contained similar chemical components, with a few exceptions. For instance FR2, FR3, FR4 and FR5 which supported moderate bacterial growth, all contained relatively moderate amount of beta-glucan and a high amount of protein. Also, FR1 which contained relatively low amount of beta-glucan, protein and resistant starch, supported very low bacterial growth across all varieties. The exception however was FR9 which had high amounts of beta-glucan and resistant starch, yet, supported very low bacterial growth, for the possible reasons mentioned above. Also, as mentioned earlier, FR6, FR7 and FR8, which supported high bacterial growth, all contained high amount of beta-glucan and relatively low amount of protein. A mere observation of the results suggested that beta-glucan was responsible for the growth patterns observed, as fractions that contained high amounts of beta-glucan, encouraged high bacterial growth. However, a further analysis of the results using multiple regression proved otherwise. Results from Maris otter and Propino, showed that proteins and FAN were correlated with the observed growth patterns, while beta-glucan was the factor responsible for the growth pattern observed in Concerto (table 5.2). Maris Otter and Propino, however do not contain similar amount of total beta-glucan or protein. Maris otter contained 11.68mg (w/w) beta-glucan while Propino contained 18.77 mg (w/w). Also, the amount of protein present in Maris Otter was 9.5 mg/mL while Propino contained 8.6 mg/mL. Also worthy to note, is the fact that the distribution of these components differed slightly across the varieties, thus, a clear conclusion cannot be derived from the results.

Table 5.2: Pearson correlation of bacterial growth pattern (OD600) versus chemical composition in Propino, Concerto and Maris Otter.

	Propino OD	Concerto OD	Maris Otter OD
OD	1.000	1.000	1.000
FAN	0.506	-0.281	0.508
Protein	0.516	0.018	0.569
Resistant Starch	-0.234	0.006	-0.444
Beta-glucan	0.199	0.564	0.112

Based on the results obtained however, beta-glucan, protein and FAN, can be concluded to be chemical components of interest that could positively affect the growth of *Lactobacillus* in this research. However, a clear conclusion cannot be drawn on what combinations of these chemical components were responsible for the growth patterns observed. As a result, the type, amount and combination of beta-glucan, FAN and protein present in FR6, FR7 and FR8, are worthy of further investigation as these fractions encouraged the highest growth of *Lactobacillus acidophilus*. This is in accordance with concerns that has been raised by various researchers as to how differences in the types, amounts and combinations of chemical components yield inconsistent results in the test for prebiotic properties in various substances. Issues have been raised on extraction methods used, location of chemical component on the grain as well as combinations of prevailing chemical components of extract used in a given experiment. The research in the development of prebiotics is therefore driving towards standardization of possible prebiotic substances, in terms of the source, extraction methods, precise chemical structure and best application methods. All of which are explored in discussions below.

6.0. Chapter Six: Discussion

The source and physicochemical condition of a prebiotic substance is key to its functionality. As discussed in the introduction of this thesis, cereal crops are currently providing healthy substances that could be incorporated into functional feeds in poultry as prebiotics. In this research, grain fractions from eight varieties of barley were investigated, with positive results obtained on their effect on the growth of a probiotic bacteria. Fractions FR6 – FR8 supported high growth of *Lactobacillus acidophilus* compared to all other fractions. These fractions, FR6 – FR8 alongside FR1 – FR5 are usually removed and discarded during processing of barley grains for human consumption to produce easy to cook pearled barley known as pot barley, as well as in the production of fine barley flour. These fractions could therefore be easily channelled to functional feed production. Grain fractionation techniques targeted at specific fractions of the grain can therefore be incorporated into barley food production processes. This idea however can only be recommended to industries after further testing and analysis of fractions of interest and clear cut benefits established.

Barley grain extracts contain potentially prebiotic compounds with functionality that can be explored positively in terms of their ability to grow and deliver probiotic bacteria to the poultry gut. The dietary manipulation of fermentation in the hindgut by the use of these compounds, commonly known as dietary fibres, to improve the ability of commensal microbes to exclude enteric pathogens and thereby improve animal gut health is generally gaining momentum, with various *in-vitro* and *in-vivo* studies. Results from FR6 – FR8 emphasizes the positive effect of potentially prebiotic substances on the growth of the probiotic bacteria in this study. Grain fraction effects were observed on the growth of *L.acidophilus* when a dialysis step

was used to select only potentially prebiotic substances, for used as growth media in this study. Fractions containing potential prebiotics encouraged high *L.acidophilus* growth, as was the case for FR6 – FR8. No fraction effect was observed when un-dialysed grain extract was used as the growth media. Un-dialysed grain extracts usually contains a mixture of simple sugars and potential prebiotic substances. As a result, the test bacteria would readily use up available simple sugars before attempting to digest more complex substances like prebiotics, thus masking the presence, if any, of potentially prebiotic substances.

For most prebiotic activity from plant sources, the active component is believed to be long chain beta-glucan (Lam and Chi-Keung Cheung, 2013). In this research, barley fractions containing high amounts of beta-glucan, FR6 – FR8 encouraged the highest growth of the test probiotic bacteria. These fractions also contained low amounts of protein, suggesting beta-glucan to be responsible for the observed growth pattern. Multiple regression analysis of all nine fractions however, showed a strong correlation between protein, FAN and the growth pattern observed. This was an unexpected result which prompted the need for further analysis. A close observation of the results made obvious the fact although FR9 contained high amounts of beta-glucan and resistant starch, it still supported low microbial growth, due to presence of unavailable nutrients (see 5.3). A further multiple regression analysis of FR1 – FR8, without FR9, showed a strong correlation between growth patterns observed and beta-glucan across all varieties (see appendix 7.3). These results implies that the presence of unavailable nutrients in FR9 might have affected the initial multiple regression analysis. Also worthy to note is the fact that further processing of FR9 might be necessary, in order to obtain the full benefits of its nutrient composition. A lot of research is currently available on enzyme treatments

in this regard for better nutrient availability. Enzyme treatment can therefore be incorporated into treatments as a follow up to this study. Most poultry farmers add various enzymes such as betaglucanase, protease, phytase, etc to poultry feed, to ensure nutrient availability.

In this study, pearling as a dry fractionation method was used for grain separation. Fractions were separated 10 seconds apart to ensure proper mapping of the different layers of the grain, in terms of chemical component determination. Results from this study showed that fractions varied in weight and chemical compositions. Also noted were differences in total chemical content per variety but there was a consistent pattern in the distribution of chemical substances across the grain. There was a clear fraction effect in terms of chemical composition, without any major overlaps, which was also clearly reflected in the microbial analysis. Similar fractions produced consistent fraction effects on the test probiotic, across varieties, with FR6 – FR8 producing the best fraction effects. In a similar study, Kedia et al (2008) separated layers of oat grains 5, 20 and 35 seconds apart using the same pearling machine as the current study, in order to obtain 1%, 1-3% and 3-4.5% fractions of the grain. Results from the study recommended 1-3% grain fraction as best for the growth of the test *Lactobacillus* spp. Results from the study showed a range effect which is less precise compared to the 10 seconds separation technique used in the current study. A review on dry fractionation of barley for biochemical analysis revealed various degrees of fractionations, depending on the method used and the aim of the analyses. A study by Zheng *et al* (2011) reported the pearling of nine hull-less barley varieties for analysis of beta-glucan distribution across the grains. The grains were pearled to remove 70% of the entire weight at 10% intervals. Results showed that 80% of the grain beta-glucan was distributed evenly throughout the

endosperm with the outer 20% of the grain showing low beta-glucan content. Another study by Zheng *et al* (2011) on the distribution and molecular characterization of beta-glucan from hull-less barley, stated that grains from six hull-less barley varieties were roller milled to produce bran, shorts and flour fractions to determine distribution of beta-glucan. Results obtained like most other studies showed variety differences with beta-glucan ranging from 4.96% to 7.62% for all six varieties. Highest beta-glucan content was recorded in the short fraction, followed by the bran and the least amount recorded in the flour. The results also revealed differences in the molecular weight of beta-glucan from the different fractions, which is a vital information relevant to the functionality of the fraction as a prebiotic. Beta-glucan from the flour fraction had the highest molecular weight, followed by the shorts and bran fractions respectively. A further analysis of beta-glucan obtained, showed that there was no significant differences in intrinsic viscosities of purified beta-glucans obtained from the short, bran or flour fractions. The grains used in the study were moisture treated and the bran fraction made of the first 20% of the grain, the short was made of the next 10% while the flour was the remaining 70%. The study concluded by recommending the roller mill method for obtaining barley fractions for use in the food, feed and commercial industries. These results go to prove that similar biochemical substances from the same grain might differ in size and structure and as a result, might differ in their functionality. This brings to bear, a need for detailed study on prebiotic sources with the aim of standardizing information on source specifics, for instance how beta-glucan from the aleurone layer of barley differs from beta-glucan from the endosperm region of the grain. Results from the current study also showed differences in the total beta-glucan content per variety, while the relative fraction amounts were similar in all varieties,

which as mentioned above, were also reflected in the microbial analysis. Determination molecular weight of beta-glucan was however not part of the current study but is however recommended for further studies to determine if growth patterns observed were due to the concentration of beta-glucan and/or differences in molecular weight.

Another study on the source of beta-glucan by Zhao and Cheung (2011), showed that beta-glucan obtained from barley, sea weed, bacteria, and mushroom sclerotia, all showed similar effect, irrespective of differences in glycosidic linkages and molecular weights. These beta-glucans were incubated with pure cultures of *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium adolescentis* for a 24 hour batch fermentation in order to evaluate bifinogenic effect with inulin as the positive control (Zhao and Cheung, 2011). The results showed that the utilization of all beta-glucan isolated from different sources was comparable to that of inulin (Zhao and Cheung, 2011). However, further *in-vitro* and *in-vivo* trials are require to prove these facts, as other conflicting results have also reported, as mentioned earlier in this report. Concerns has also been raised on methods of beta-glucan extraction, as a possible cause of inconsistent results.

Extraction of beta-glucan has been reported to be a very difficult task that requires special attention to obtain consistent extracts (Ahmad *et al*., 2012). Also of importance might be how extraction from different regions of the grain affect their final structures. Factors like the presence indigenous enzymes and other polysaccharides like arabinoxylans in the beta-glucan source could also influence the extraction and purification of beta-glucan (Jadhav *et al*., 1998). Various extraction processes are available for the extraction of beta-glucan , however, hot water and acid/enzymatic processing has been reported to be the best extraction

method to produce beta-glucan with high purity and better physicochemical property (Lazaridou *et al*., 2003), which was the extraction method used in this research. A consistent pattern in terms of concentration was observed across FR1 – FR9 for eight out of eight varieties used in this study. This above mention extraction method was used in the biochemical analysis, while the microbial analysis, involved a digestion process similar to what is obtained in a poultry gut, which lacks prebiotic hydrolysing enzymes. Potentially prebiotic substances in FR1- FR9, will therefore most likely exist in undigested forms which will provide relevant information on the mode of action of probiotics on prebiotic in terms of fermentation processes. An information that can be compared on further analysis with digestion processes involving enzyme use, where there is a possible initial digestion of prebiotic substances. This will expose further the mode of action of probiotics on prebiotics in terms of the effect of prebiotic structure on fermentation process.

Most prebiotics studies are conducted with commercially available prebiotics, with limited information of cereal fraction effects. This research therefore provides basic and fundamental information on the location of potentially prebiotic substances on the barley varieties used in this study, and how they affect the growth of *Lactobacillus acidophilus.* Proper information on positive properties of barley fractions can lead to recommendation of these fractions for used in animal feed, without any need for further industrial processing, such as extraction of active ingredient, which could negatively influence the viability of the active prebiotic component.

Information from this study, can be used for further analysis to determine specific factors and conditions responsible for the observed effect. For instance, beta-glucan showed a strong correlation with the observed growth pattern. However, as

mentioned earlier, other factors might also have influenced the results. Hence the need for a detailed analysis of the fractions of interest which are FR6 – FR9. FR2 – FR5, also supported moderate probiotic growth and can be analysed alongside FR6 – FR9. Factors such as concentration, molecular weight, nutrient availability and combinations can be considered.

Over the years, research on prebiotic has led to various inventions and development of commercial growth models for predicting feed requirements and supplements for the best results in poultry with these models tailored and fine-tuned to fit the age, size, type and medical conditions of animals. Mathematical equations are used and have been used for the past 100 years to describe animal systems and feed requirements (Black, 2014). The first equations used, were termed static and performed very basic predictions of animal feed requirements, based on recommended nutrient requirements by the NRC. However, with increased computerization, the number of equations used in animal production have increased tremendously with equations now described as dynamic such that they allow for the inclusion of robust scientific theories and biological discoveries, which allow for flexibility and more effective applications in animal production (Ferguson, 2015). In addition, computer models simulating animal systems are used currently to test feed equations (Dumas *et al*., 2008) after which the technology are transfer to *in-vitro* systems. Research in animal feed and animal feed supplements has led to the development of several hypotheses. Most of which can now be readily evaluated using mathematical models rather than traditional subjective approach and results readily obtained (Emmans, 1989, 1981; Gous *et al*., 2006). These models have evolved over the years, such that results obtained are used to refine hypothesis

which are then further tested before expensive experiments are conducted, based on the hypothesis (Black, 2014).

These equations have been used to demonstrate and quantify how well animal systems are understood and are available as a tools of integrating current knowledge to assist decision making in terms of prioritising research activities and direct applications. Most models are able to predict animal performance under a range of circumstances with reasonable accuracy. However, most poultry farmers have been reluctant to use these models for the day to day running of their farms such that limited success has been recorded in the use of simulation models in animal production (Ferguson, 2015; Newman *et al*., 2000; Rivera-Torres, 2015). Research in the field is still ongoing, with components of several models being upgraded and improved to include current challenges and trends such as disease representation (Eugeni Roura and David Torrallardona, 2009; Sandberg et al., 2006) and prebiotic and probiotics inclusion. However, the level of standard applications are still very low.

The poultry industry will benefit from the inclusion of prebiotic substances in poultry feed, which are natural remedy, compared to in feed antibiotics. Since the ban on prophylactic antibiotics in poultry farming, more natural means of preventing disease has been recorded. The British Poultry Council, (2016) reported an overall 44% reduction in industrial antibiotic use between 2012 and 2015. Also, Finland has recorded 0 % prophylactic antibiotic use on all broiler farms since 2010 (British Poultry Council, 2016). The above successes were achieved mainly by very strict biosecurity systems. The addition of prebiotic substances to poultry feed toward positive poultry gut health, together with strict biosecurity will therefore be of great value to the poultry industry.

7.0. Conclusion and Recommendations

7.1. Conclusion

This research sought to determine how relevant the dietary components of barley grains are to its ability to affect poultry health positively as a prebiotic, with possible application as a feed supplement in poultry feed by the following steps

Originality

- Eight barley varieties were selected at random and used in this study. Thus, covering a wide range of possible variety differences as opposed to using a single variety or a mere representation of barley without taking variety differences into account.
- The grains were fractionated using a pearling technique which separated fractions 10 seconds apart, providing a detailed grain mapping as opposed to less precise grain fractionation or poorly defined grain fractions.
- Fractions were analyzed in a simulated poultry gut containing a unique dialysis step that selected only potentially prebiotic substances, which was very relevant to the aim of the study.
- Results from dialyzed samples were also compared to un-dialyzed samples, for effective determination of possible prebiotic effect.

Analysis of barley varieties used in this research confirmed the following points:

- The eight barley varieties analyzed contained potentially prebiotic substance such beta-glucan in all fractions of the grain in varying amounts.

- Fraction effects were observed when potentially prebiotic extracts were used exclusively as growth media for the test probiotic, compared to undialysed barley extracts, were no fraction effects were observed.
- A combination of high beta-glucan, low protein and FAN encouraged the highest growth of *Lactobacillus acidophilus* in FR6, FR7 and FR8 containing potentially prebiotic extracts only.

Differences in chemical composition of barley varieties has been said to be an advantage because varieties can be chosen for various processes based on their chemical compositions (Yu *et al*., 2012). These differences relate to the amount and distribution of beta-glucan, protein, resistant starch, free amino nitrogen and other chemical component across the kernel. Their location on the grain determines their extractability and hence, usage (Djurle *et al*., 2016). In this research, barley grains were successfully fractionated, producing consistent fractions, with interesting distribution of protein, beta-glucan, FAN and resistant starch, which all affected the growth of *Lactobacillus acidophilus* in different ways.

Extraction methods used in this research proved effective and relatively accurate, as biological replicates produced consistent results. Extracts from FR6, FR7 and FR8, were of particular interest as they supported the highest growth of the test probiotic, and may therefore contain potentially prebiotic substances in the right combinations. Results from all fractions analysed in this research are equally important, as they will contribute specific information on the distribution of the mentioned chemical components in the eight barley varieties analysed, which will form a basis for further investigation into the specific prebiotic property of barley crops in poultry feed supplementation. The following points are therefore recommended.

7.2. Recommendations

- Further chemical analysis of FR6, FR7, FR8 and FR9 dialyzed extracts for structural specifics on beta-glucan, resistant starch FAN and protein, as well as the presence of other polysaccharides like FOS and MOS, arabinoxylan and other sugars, to determine chemical component responsible for the outstanding growth patterns.
- Further enzymatic pretreatment of FR9, prior to chemical and microbial analysis to ensure nutrient availability.
- Inclusion of FR6 – FR8 in poultry feed for *in-vivo* studies
- Further analysis of FR1 – FR9 to determine factors responsible growth patterns observed
- Determination of prebiotic index of FR1 – FR9 using a mixed culture method in a simulated poultry gut.

Reference

Abernathy, D.G., Spedding, G., Starcher, B., 2009. Analysis of Protein and Total Usable Nitrogen in Beer and Wine Using a Microwell Ninhydrin Assay. J. Inst. Brew. 115, 122–127.

Abudabos, A.M., Al-Batshan, H.A., Murshed, M.A., 2015. Effects of prebiotics and probiotics on the performance and bacterial colonization of broiler chickens. South Afr. J. Anim. Sci. 45, 419–428.

Ahmad, A., Anjum, F.M., Zahoor, T., Nawaz, H., Dilshad, S.M.R., 2012. Beta glucan: a valuable functional ingredient in foods. Crit. Rev. Food Sci. Nutr. 52, 201–212.

Almirall, M., Francesch, M., Perez-Vendrell, A.M., Brufau, J., Esteve-Garcia, E., 1995. The differences in intestinal viscosity produced by barley and beta-glucanase alter digesta enzyme activities and ileal nutrient digestibilities more in broiler chicks than in cocks. J. Nutr. 125, 947–955.

Åman, P., Hesselman, K., Tilly, A.-C., 1985. The variation in chemical composition of Swedish barleys. J. Cereal Sci. 3, 73–77.

Amerah, A.M., Ravindran, V., Lentle, R.G., 2009. Influence of insoluble fibre and whole wheat inclusion on the performance, digestive tract development and ileal microbiota profile of broiler chickens. Br. Poult. Sci. 50, 366–375.

Amit-Romach, E., Sklan, D., Uni, Z., 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poult. Sci. 83, 1093–1098.

Andersson, A.A.M., Lampi, A.-M., Nyström, L., Piironen, V., Li, L., Ward, J.L., Gebruers, K., Courtin, C.M., Delcour, J.A., Boros, D., Fraś, A., Dynkowska, W., Rakszegi, M., Bedo, Z., Shewry, P.R., Aman, P., 2008. Phytochemical and dietary fiber components in barley varieties in the Health Grain Diversity Screen. J. Agric. Food Chem. 56, 9767–9776.

Annison, G., Choct, M., 1991. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. Worlds Poult. Sci. J. 47, 232–242.

Apajalahti, J.H., 2004. Structure and dietary modulation of intestinal microbial communities, in: Proceedings of the Second Mid-Atlantic Nutrition Conference. University of Maryland, College Park, MD. pp. 69–76.

Applegate, T.J., Angel, R., 2014. Nutrient requirements of poultry publication: History and need for an update. J. Appl. Poult. Res. 23, 567–575.

Arzu Baman, H.K., 1999. Properties and Composition of Turkish Flat Bread (Bazlama) Supplemented with Barley Flour and Wheat Bran. Cereal Chem. 76(4), 506-511

Awad, W.A., Ghareeb, K., Abdel-Raheem, S., Böhm, J., 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88, 49–56.

Bailey, J.S., Blankenship, L.C., Cox, N.A., 1991. Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. Poult. Sci. 70, 2433–2438.

Baurhoo, B., Letellier, A., Zhao, X., Ruiz-Feria, C.A., 2007. Cecal Populations of Lactobacilli and Bifidobacteria and Escherichia coli Populations After In Vivo Escherichia coli Challenge in Birds Fed Diets with Purified Lignin or Mannanoligosaccharides. Poult. Sci. 86, 2509–2516.

Bedford, M.R., Apajalahti, J., 2000. Microbial interactions in the response to exogenous enzyme utilization. 299–314.

Bedford, M.R., Classen, H.L., 1993. An in vitro assay for prediction of broiler intestinal viscosity and growth when fed rye-based diets in the presence of exogenous enzymes. Poult. Sci. 72, 137–143.

Bedford, M.R., Classen, H.L., Campbell, G.L., 1991. The Effect of Pelleting, Salt, and Pentosanase on the Viscosity of Intestinal Contents and the Performance of Broilers Fed Rye. Poult. Sci. 70, 1571–1577.

Biggs, P., Parsons, C.M., Fahey, G.C., 2007. The Effects of Several Oligosaccharides on Growth Performance, Nutrient Digestibilities, and Cecal Microbial Populations in Young Chicks. Poult. Sci. 86, 2327–2336.

Bigliardi, B., Galati, F., 2013. Innovation trends in the food industry: The case of functional foods. Trends Food Sci. Technol. 31, 118–129.

Black, J.L., 2014. Brief history and future of animal simulation models for science and application. Anim. Prod. Sci. 54, 1883–1895.

Boehm, G., Lidestri, M., Casetta, P., Jelinek, J., Negretti, F., Stahl, B., Marini, A., 2002. Supplementation of a bovine milk formula with an oligosaccharide

mixture increases counts of faecal bifidobacteria in preterm infants. ResearchGate 86, 178–181.

Bohn, J.A., BeMiller, J.N., 1995. (1→3)-β-d-Glucans as biological response modifiers: a review of structure-functional activity relationships. Carbohydr. Polym., Frontiers in Carbohydrate Research 28, 3–14.

British Poultry Council, 2016. Leading the way in the responsible use of antibiotics. [WWW Document]. URL http://www.britishpoultry.org.uk/fight-against-amr-global-commitment-on-the-responsible-use-of-antimicrobials-in-poultry/ (accessed 01.05.17).

Brouns, F., Kettlitz, B., Arrigoni, E., 2002. Resistant starch and "the butyrate revolution." Trends Food Sci. Technol. 13, 251–261.

Brunsgaard, G., 1998. Effects of Cereal Type and Feed Particle Size on Morphological Characteristics, Epithelial Cell Proliferation, and Lectin Binding Patterns in the Large Intestine of Pigs. ResearchGate 76, 2787–98.

Bryden, W.L., Li, X., 2010. Amino acid digestibility and poultry feed formulation: expression, limitations and application. Rev. Bras. Zootec. 39, 279–287.

Campbell, G.L., Rossnagel, B.G., Classen, H.L., Thacker, P.A., 1989. Genotypic and environmental differences in extract viscosity of barley and their relationship to its nutritive value for broiler chickens. Anim. Feed Sci. Technol. 26, 221–230

Cao, B.H., Karasawa, Y., Guo, Y.M., 2005. Effects of Green Tea Polyphenols and Fructo-oligosaccharides in Semi-purified Diets on Broilers' Performance and Caecal Microflora and Their Metabolites. Asian-Australas. J. Anim. Sci. 18, 85–89.

Castanon, J.I.R., 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86, 2466–2471.

Catald-Gregori, P., Mallet, S., Travel, A., Orengo, J., Lessire, M., 2008. Efficiency of a prebiotic and a plant extract alone or in combination on broiler performance and intestinal physiology. Can. J. Anim. Sci. 88, 623–629.

Charalampopoulos, D., Pandiella, S.S., Webb, C., 2003. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int. J. Food Microbiol. 82, 133–141.

Charalampopoulos, D., Wang, R., Pandiella, S.S., Webb, C., 2002. Application of cereals and cereal components in functional foods: a review. Int. J. Food Microbiol., Notermans Special Issue 79, 131–141.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., Yuan, Z., 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front. Microbiol. 5, 217.

Choct, M., 2001. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Tech. Bull. AN30 1–6.

Choi, K.H., Namkung, H., Paik, I.K. (Chungang U., 1994. Effects of dietary fructooligosaccharides on the suppression of intestinal colonization of Salmonella typhimurium in broiler chickens. Korean J. Anim. Sci. 36, 271-284.

Classen, H.L., Apajalahti, J., Svihus, B., Choct, M., 2016. The role of the crop in poultry production. Poult. Sci. J. 72, 459–472.

Collignon, P.J., 1999. Vancomycin-resistant enterococci and use of avoparcin in animal feed: is there a link? Med. J. Aust. 171, 144–146.

Craven, S.E., Cox, N.A., Bailey, J.S., Cosby, D.E., 2003. Incidence and tracking of Clostridium perfringens through an integrated broiler chicken operation. Avian Dis. 47, 707–711.

Culver, M.F., Weber, A., Draper, M., Myers, T., 2013. Dietary supplement for enhancing animal health. US20130216521 A1.

Delcour, J.A., Aman, P., Courtin, C.M., Hamaker, B.R., Verbeke, K., 2016. Prebiotics, Fermentable Dietary Fiber, and Health Claims. J. Adv. Nutr. Int. Rev. 7, 1–4.

Dizaji, B.R., Hejazi, S., Zakeri, A., 2012. Effects of dietary supplementations of prebiotics, probiotics, synbiotics and acidifiers on growth performance and organs weights of broiler chicken. Eur. J. Exp. Biol. 2(6), 2125-2129.

Djurle, S., Andersson, A.A.M., Andersson, R., 2016. Milling and extrusion of six barley varieties, effects on dietary fibre and starch content and composition. J. Cereal Sci. 72, 146-152.

DuBourdieu, D.J., Sinha, A.K., Lall, R., Gor, T.A., Raval, D.C., 2013. Soft food composition with probiotics and prebiotics for masking medications. US20130171204 A1.

Duke, G.E., Eccleston, E., Kirkwood, S., Louis, C.F., Bedbury, H.P., 1984. Cellulose digestion by domestic turkeys fed low or high fiber diets. J. Nutr. 114, 95–102.

Dumas, A., Dijkstra, J., France, J., 2008. Mathematical modelling in animal nutrition: a centenary review. J. Agric. Sci. 146, 123–142.

Dunkley, K.D., Dunkley, C.S., Njongmeta, N.L., Callaway, T.R., Hume, M.E., Kubena, L.F., Nisbet, D.J., Ricke, S.C., 2007. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. Poult. Sci. 86, 801–810.

Edens, F.W., 2003. An alternative for antibiotic se in poultry: probiotics. Rev. Bras. Ciênc. Avícola 5, 75–97.

Edney, M.J., Campbell, G.L., Classen, H.L., 1989. The effect of β-glucanase supplementation on nutrient digestibility and growth in broilers given diets containing barley, oat groats or wheat. Anim. Feed Sci. Technol. 25, 193–200.

EFSA-ECDC, 2009. Zoonotic infections in Europe in 2007: a summary of the EFSA-ECDC annual report [WWW Document]. URL http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19100 (accessed 22.08.15).

EFSA Panel on Dietetic Products, N. and A. (NDA), 2010. Scientific Opinion on the substantiation of health claims related to various food(s)/food constituents(s) and increasing numbers of gastro-intestinal microorganisms (ID 760, 761, 779, 780, 779, 1905), and decreasing potentially pathogenic gastro-intestinal microorganisms (ID 760, 761, 779, 780, 779, 1905) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA J. 8, n/a–n/a.

Emmans, G.C., 1989. The growth of turkeys. Recent Adv. Turk. Sci. 21, 135-166.

Emmans, G.C., 1981. A model of the growth and feed intake of ad libitum fed animals, particularly poultry. Comput. Anim. Prod. 103–110.

Engberg, R.M., Hedemann, M.S., Steenfeldt, S., Jensen, B.B., 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 83, 925–938.

Englyst, K.N., Englyst, H.N., 2005. Carbohydrate bioavailability. Br. J. Nutr. 94, 1–11.

Ensminger, M.., Oldfield, J.., W.w, H., Ensminger, M.., 1990. Feeds and nutrition digest: formerly, Feeds and nutrition-abridged. Clovis, Calif., pp 794.

Eugeni Roura, David Torrallardona (Eds.), 2009. Voluntary feed intake in pigs. Wageningen Academic Publishers, pp. 368.

European Union (a), 2003. Commission Regulation (EC) No 1334/2003 of 25 July 2003 amending the conditions for authorisation of a number of additives in feedingstuffs belonging to the group of trace elements. Off. J. Eur. Union 46, 11–13.

European Union (b), 2003. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (1). Off. J. Eur. Union 46, 29–43.

Fahey, G., Heimbach, J., Scott, K., Reid, G., Guarner, F., 2011. Probiotic and prebiotic claims in Europe: seeking a clear roadmap. Br J Nutr. 106, 1765-1767.

FAO, 2015. Poultry and Animal Health : FAO's Animal Production and Health Division [WWW Document]. URL http://www.fao.org/ag/againfo/themes/en/poultry/animal_health.html (accessed 9.22.15).

FAO, 2013. FAOSTAT -World +total chicken production [WWW Document]. URL http://faostat3.fao.org/download/Q/QA/E (accessed 22.09.15).

FAO/WHO, 2006. Report of a joint FAO/WHO working group on drafting guidelined for the evaluation of probiotics in food. FAO Food Nutr. Pap. 85.

Farber, M., Farber, J., 2009. Delivery systems for functional ingredients. US7531192 B2.

Farrell, D., 2013. Poultry development review. The role of poultry in human nutrition. FAO 1–2.

Ferguson, N.S., 2015. Commercial application of integrated models to improve performance and profitability in finishing pigs, in: Sakomura, N.K., Gous, R., Kyriazakis, I., Hauschild, L. (Eds.), Nutritional Modelling for Pigs and Poultry. CABI, Wallingford, pp. 141–156.

Food Standards Agency, 2015. Campylobacter survey: cumulative results from the full 12 months (Q1 - Q4) | Food Standards Agency [WWW Document]. URL http://www.food.gov.uk/news-updates/news/2015/14003/campylobacter-survey-results-12months (accessed 21.09.15).

Fuente, J.M., Pérez^de Ayala, P., Villamide, M.J., 1995. Effect of dietary enzyme on the metabolizable energy of diets with increasing levels of barley fed to broilers at different ages. Anim. Feed Sci. Technol. 56, 45–53.

Gaggìa, F., Mattarelli, P., Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol., 141, Supplement, S15–S28.

Gebhardt, S.E., Cutrufelli, R., Matthew, R.H., 1978. Composition of foods. Agricultural hand book no. 8-20, 32-55.

Geyra, A., Uni, Z., Sklan, D., 2001. Enterocyte dynamics and mucosal development in the posthatch chick. Poult. Sci. 80, 776–782.

Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125, 1401–1412.

Gohl, B., Nilsson, M., Thomke, S., 1978. Distribution of soluble carbohydrates in barley grain at late stage of maturity and relation to viscosity. Cereal chem. 55(3), 341-347.

González-Alvarado, J.M., Jiménez-Moreno, E., Lázaro, R., Mateos, G.G., 2007. Effect of Type of Cereal, Heat Processing of the Cereal, and Inclusion of Fiber in the Diet on Productive Performance and Digestive Traits of Broilers. Poult. Sci. 86, 1705–1715.

Gous, R., Fisher, C., Morris, T.R., 2006. Mechanistic Modelling in Pig and Poultry Production. CABI. pp. 321

Guenter, W., 1993. Impact of Feed Enzymes on Nutrient Utilization of Ingredients in Growing Poultry. J. Appl. Poult. Res. 2, 82–84.

Hajati, H., Rezaei, M., 2010. The application of prebiotics in poultry production. Int. J. Poult. Sci. 9, 298-304.

Hetland, H., Svihus, B., 2001. Effect of oat hulls on performance, gut capacity and feed passage time in broiler chickens. Br. Poult. Sci. 42, 354–361.

Hetland, H., Svihus, B., Choct, M., 2005. Role of Insoluble Fiber on Gizzard Activity in Layers. J. Appl. Poult. Res. 14, 38–46.

Hetland, H., Svihus, B., Krogdahl, A., 2003. Effects of oat hulls and wood shavings on digestion in broilers and layers fed diets based on whole or ground wheat. Br. Poult. Sci. 44, 275–282.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514.

Huang, R.L., Yin, Y.L., Wu, G.Y., Zhang, Y.G., Li, T.J., Li, L.L., Li, M.X., Tang, Z.R., Zhang, J., Wang, B., He, J.H., Nie, X.Z., 2005. Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers. Poult. Sci. 84, 1383–1388.

Huber, G.R., Jones, D.R., Kuenzi, J.C., Kuenzi, K.D., Cabrera, F.A., 2005. Animal feeds including heartworm-prevention drugs. US6866862 B2.

Hugas, M., Tsigarida, E., Robinson, T., Calistri, P., 2009. The EFSA Scientific Panel on Biological Hazards first mandate: May 2003–May 2006. Insight into foodborne zoonoses. Trends Food Sci. Technol. 20, 188–193.

Hughes, R.J., Choct, M., 1999. Chemical and physical characteristics of grains related to variability in energy and amino acid availability in poultry. Aust. J. Agric. Res. 50(5), 689-702.

Hume, M.E., 2011. Historic perspective: prebiotics, probiotics, and other alternatives to antibiotics. Poult. Sci. 90, 2663–2669.

Iji, P.A., Saki, A.A., Tivey, D.R., 2001. Intestinal development and body growth of broiler chicks on diets supplemented with non-starch polysaccharides. Anim. Feed Sci. Technol. 89, 175–188.

Immerseel, F.V., Buck, J.D., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R., 2004. Clostridium perfringens in poultry: an emerging threat for animal and public health. Avian Pathol. 33, 537–549.

Izydorczyk, M.S., Dexter, J.E., 2008. Barley β-glucans and arabinoxylans: molecular structure, physicochemical properties, and uses in food products. Food Res Int. Food Res. Int. - FOOD RES INT 41, 850–868.

Jacob, J.P., Pescatore, A.J., 2014. Barley β-glucan in poultry diets. Ann. Transl. Med. 2, 20.

Jacob, J.P., Pescatore, A.J., 2012. Using barley in poultry diets—A review. J. Appl. Poult. Res. 21, 915–940.

Jadhav, S.J., Lutz, S.E., Ghorpade, V.M., Salunkhe, D.K., 1998. Barley: Chemistry and Value-Added Processing. Crit. Rev. Food Sci. Nutr. 38, 123–171.

Jamroz, D., Wiliczkiewicz, A., Orda, J., Wertelecki, T., Skorupińska, J., 2002. Aspects of development of digestive activity of intestine in young chickens, ducks and geese. J. Anim. Physiol. Anim. Nutr. 86, 353–366.

Jeurissen, S.H.M., Lewis, F., van der Klis, J.D., Mroz, Z., Rebel, J.M.J., Huurne, A.A.H.M. ter, 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. Curr. Issues Intest. Microbiol. 3, 1–14.

Jiang, H.Q., Gong, L.M., Ma, Y.X., He, Y.H., Li, D.F., Zhai, H.X., 2006. Effect of stachyose supplementation on growth performance, nutrient digestibility and caecal fermentation characteristics in broilers. Br. Poult. Sci. 47, 516–522.

Jiménez-Moreno, E., Chamorro, S., Frikha, M., Safaa, H.M., Lázaro, R., Mateos, G.G., 2011. Effects of increasing levels of pea hulls in the diet on productive performance, development of the gastrointestinal tract, and nutrient retention of broilers from one to eighteen days of age. Anim. Feed Sci. Technol. 168, 100–112.

Jiménez-Moreno, E., González-Alvarado, J.M., González-Serrano, A., Lázaro, R., Mateos, G.G., 2009. Effect of dietary fiber and fat on performance and digestive traits of broilers from one to twenty-one days of age. Poult. Sci. 88, 2562–2574.

Jørgensen, H., Zhao, X.Q., Knudsen, K.E., Eggum, B.O., 1996. The influence of dietary fibre source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. Br. J. Nutr. 75, 379–395.

Józefiak, D., Rutkowski, A., Frątczak, M., Boros, D., 2004. The effect of dietary fibre fractions from different cereals and microbial enzymes supplementation on

performance ileal viscosity and short-chain fatty acids concentration in caeca of broiler chickens. ResearchGate 13, 487–496.

Jozefiak, D., Rutkowski, A., Kaczmarek, S., Jensen, B.B., Engberg, R.M., Højberg, O., 2010. Effect of β -glucanase and xylanase supplementation of barley- and rye-based diets on caecal microbiota of broiler chickens. Br. Poult. Sci. 51, 546–557.

Józefiak, D., Rutkowski, A., Martin, S.A., 2004. Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Technol. 113, 1–15.

Kalbe, J., Geißler, K., Träubel, M., Harder, A., Samson-Himmelstjerna, G. von, 2011. Endoparasiticidal agents for voluntary oral ingestion by animals. US7914816 B2.

Kaldhusdal, M., Hofshagen, M., 1992. Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. Poult. Sci. 71, 1145–1153.

Karley, A.J., Valentine, T.A., Squire, G.R., 2011. Dwarf alleles differentially affect barley root traits influencing nitrogen acquisition under low nutrient supply. J. Exp. Bot. 62, 3917–3927.

Kent, N.L., Evers, A.D., 1994. Technology of Cereals: An Introduction for Students of Food Science and Agriculture. Woodhead Publishing. pp. 352.

Klis, J.D.V.D., Voorst, A.V., 1993. The Effect of Carboxy Methyl Cellulose (A Soluble Polysaccharide) on the Rate of Marker Excretion from the Gastrointestinal Tract of Broilers. Poult. Sci. 72, 503–512.

Knudsen, K.E.B., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67, 319–338.

Kolida, S., Gibson, G.R., 2011. Synbiotics in health and disease. Annu. Rev. Food Sci. Technol. 2, 373–393.

Kumar, H., Salminen, S., Verhagen, H., Rowland, I., Heimbach, J., Bañares, S., Young, T., Nomoto, K., Lalonde, M., 2015. Novel probiotics and prebiotics: road to the market. Curr. Opin. Biotechnol., 32, 99–103.

Lam, K.L., Chi-Keung Cheung, P., 2013. Non-digestible long chain beta-glucans as novel prebiotics. Bioact. Carbohydr. Diet. Fibre 2, 45–64.

Lampi, A.M., Moreau, R.A., Piironen, V., Hicks, K.B., 2004. Pearling barley and rye to produce phytosterol-rich fractions. Lipids 39, 783–787.

Latorre, J.D., Hernandez-Velasco, X., Kuttappan, V.A., 2015. Selection of *Bacillus* spp. For cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and Clostridium perfrigens proliferation using an *in vitro* digestive model in different poultry diets. Front. In Vet Science 2, 25.

Lazaridou, A., Biliaderis, C.G., Izydorczyk, M.S., 2003. Molecular size effects on rheological properties of oat β-glucans in solution and gels. Food Hydrocoll. 17, 693–712.

Lazaridou, A., Papoutsi, Z., Biliaderis, C.G., Moutsatsou, P., 2011. Effect of oat and barley β-glucans on inhibition of cytokine-induced adhesion molecule expression in human aortic endothelial cells: Molecular structure–function relations. Carbohydr. Polym. 84(1), 153-161.

Leeson, S., Summers, J.D., 2001. Scott's Nutrition of the Chicken: v. 4, 4th Revised edition edition. ed. University Books, Canada, Guelph, Ont. pp. 608.

Lee, Y.-K., Salminen, S., 1995. The coming of age of probiotics. Trends Food Sci. Technol. 6, 241–245.

Liu, K., Barrows, F.T., Obert, D., 2009. Dry fractionation methods to produce barley meals varying in protein, beta-glucan, and starch contents. J. Food Sci. 74, C487–499.

Li, X., Li, Q., Xu, C., 2008. Effects of Supplementation of Fructooligosaccharide and/or Bacillus Subtilis to Diets on Performance and on Intestinal Microflora in Broilers. Arch. Tierz. 51(1), 64-70.

Long, J.R., Pettit, J.R., Barnum, D.A., 1974. Necrotic enteritis in broiler chickens. II. Pathology and proposed pathogenesis. Can. J. Comp. Med. Rev. Can. Médecine Comparée 38, 467–474.

Lopes, M.A., Larkins, B.A., 1993. Endosperm origin, development, and function. Plant Cell 5, 1383–1399.

Marconi, E., Graziano, M., Cubadda, R., 2000. Composition and Utilization of Barley Pearling By-Products for Making Functional Pastas Rich in Dietary Fiber and β-Glucans. Cereal Chem. 77, 133–139.

Mark-Herbert, C., 2004. Innovation of a new product category — functional foods. Technovation 24, 713–719.

Marounek, M., Skivan, M., Bezina, P., Hoza, I., 2005. Digestive Organs, Caecal Metabolites and Fermentation Pattern in Coypus (Myocastor coypus) and Rabbits (Oryctolagus cuniculus). Acta Vet. Brno. 74, 3-7.

Mateos, G.G., Jiménez-Moreno, E., Serrano, M.P., Lázaro, R.P., 2012. Poultry response to high levels of dietary fiber sources varying in physical and chemical characteristics. J. Appl. Poult. Res. 21, 156–174.

Mathlouthi, N., Mallet, S., Saulnier, L., Quemener, B., Larbier, M., 2002. Effects of xylanase and β-glucanase addition on performance, nutrient digestibility, and physico-chemical conditions in the small intestine contents and caecal microflora of broiler chickens fed a wheat and barley-based diet. Anim. Res. 51, 12.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria--a review. Int. J. Food Microbiol. 105, 281–295.

McDevitt, R. m., Brooker, J. d., Acamovic, T., Sparks, N. h. c., 2006. Necrotic enteritis; a continuing challenge for the poultry industry. Worlds Poult. Sci. J. 62, 221–247.

McHan, F., Shotts, E.B., 1993. Effect of short-chain fatty acids on the growth of Salmonella typhimurium in an in vitro system. Avian Dis. 37, 396–398.

Montagne, L., Pluske, J.R., Hampson, D.J., 2003a. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol. 108, 95–117.

Montagne, L., Pluske, J.R., Hampson, D.J., 2003b. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol. 108, 95–117.

Moreau, R., Nghiem, N.P., Johnston, D., Hicks, K., 2011. Ethanol Production from Starch-Rich Crops Other than Corn and the Composition and Value of the Resulting DDGS, in: Distillers Grains. AOCS Publishing, pp. 103–117.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., Yura, T., 1981. Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. J. Bacteriol. 148, 64–71.

Mulder, R.W., Havenaar, R., Veld, J.H.J.H. in't, 1997. Intervention Strategies: The use of Probiotics and Competitive Exclusion Microfloras against Contamination with Pathogens in Pigs and Poultry, in: Fuller, R. (Ed.), Probiotics 2. Springer Netherlands, pp. 187–207.

Murshed, M.A., Abudabos, A.M., Murshed, M.A., Abudabos, A.M., 2015. Effects of the Dietary Inclusion of a Probiotic, a Prebiotic or their Combinations on the Growth Performance of Broiler Chickens. Rev. Bras. Ciênc. Avícola 17, 99–103.

Mussatto, S.I., Mancilha, I.M., 2007. Non-digestible oligosaccharides: A review. Carbohydr. Polym. 68, 587–597.

Newman, S., Lynch, T., Plummer, A.A., 2000. Success and Failure of Decision Support Systems: Learning as We Go. J Anim. Sci. 77, 1-12.

Noy, Y., Sklan, D., 1999. Energy utilization in newly hatched chicks. Poult. Sci. 78, 1750–1756.

NRC, 1975. The 1975 US National Academy of Sciences / National Research Council Report [WWW Document]. Stoat. URL http://scienceblogs.com/stoat/2011/07/11/the-1975-us-national-academy-o/ (accessed 08.01.17).

NRC, C., 1994. Nutrient Requirements of Poultry: Ninth Revised Edition. National Academies press. pp, 176.

Ogueke, C.C., Nwosu, J.N., Owuamanam, C.I., Iwouno, J.N., 2010. Ugba, the Fermented African Oilbean Seeds; its Production, Chemical Composition, Preservation, Safety and Health Benefits. ResearchGate 13, 489–96.

Ohimain, E.I., Ofongo, R.T., 2012. The effect of probiotic and prebiotic feed supplementation on chicken health and gut microflora: a review. Int J Anim Vet Adv 4, 135–143.

Palframan, R., Gibson, G. r., Rastall, R. a., 2003. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. Lett. Appl. Microbiol. 37, 281–284.

Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82, 627–631.

Pettersson, D., Graham, H., Åman, P., 1991. The nutritive value for broiler chickens of pelleting and enzyme supplementation of a diet containing barley, wheat and rye. Anim. Feed Sci. Technol. 33, 1–14.

Potturi, P.V.L., Patterson, J.A., Applegate, T.J., 2005. Effects of delayed placement on intestinal characteristics in turkey poults. Poult. Sci. 84, 816–824.

Ravindran, V., 2013. “Main ingredients used in poultry feed formulations.” Poultry development review [WWW Document]. URL http://www.fao.org/3/contents/86dd3cfa-0096-52d3-a9c0-5762921dc2a9/i3531e.pdf#page=73 (accessed 08.01.17).

Ravindran, V., Morel, P.C.H., Rutherfurd, S.M., Thomas, D.V., 2009. Endogenous flow of amino acids in the avian ileum as influenced by increasing dietary peptide concentrations. Br. J. Nutr. 101, 822–828.

Ravindran, V., Son, J.-H., 2011. Feed enzyme technology: present status and future developments. Recent Pat. Food Nutr. Agric. 3, 102–109.

Redig, P.T., 1989. The avian ceca: obligate combustion chambers or facultative afterburners? The conditioning influence of diet. J. Exp. Zool. Suppl. Publ. Auspices Am. Soc. Zool. Div. Comp. Physiol. Biochem. Wistar Inst. Anat. Biol. 3, 66–69.

Richards, J.D., Gong, J., de Lange, C.F.M., 2005. The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: Current understanding, possible modulations, and new technologies for ecological studies. Can. J. Anim. Sci. 85, 421–435.

Ricke, S.C., 2015. Potential of fructooligosaccharide prebiotics in alternative and nonconventional poultry production systems. Poult. Sci. 94, 1411–1418.

Ricke, S.C., Dunkley, C.S., Durant, J.A., 2013. A review on development of novel strategies for controlling Salmonella Enteritidis colonization in laying hens: fiber-based molt diets. Poult. Sci. 92, 502–525.

Riddell, C., Kong, X.M., 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis. 36, 499–503.

Rivera-Torres, V., 2015. Challenges associated with the application of poultry models: the case of turkeys, in: Sakomura, N.K., Gous, R., Kyriazakis, I., Hauschild, L. (Eds.), Nutritional Modelling for Pigs and Poultry. CABI, Wallingford, pp. 96–105.

Roberfroid, M., 2007. Prebiotics: the concept revisited. J. Nutr. 137, 830S–7S.

Roberfroid, M.B., 2000. A European consensus of scientific concepts of functional foods. Nutr. Burbank Los Angel. Cty. Calif 16, 689–691.

Rodríguez, M.L., Rebolé, A., Velasco, S., Ortiz, L.T., Treviño, J., Alzueta, C., 2012. Wheat- and barley-based diets with or without additives influence broiler chicken performance, nutrient digestibility and intestinal microflora. J. Sci. Food Agric. 92, 184–190.

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., Matteuzzi, D., 2005. Fermentation of Fructooligosaccharides and Inulin by Bifidobacteria: a Comparative Study of Pure and Fecal Cultures. Appl. Environ. Microbiol. 71, 6150–6158.

Röttger, A.S., Halle, I., Wagner, H., Breves, G., Flachowsky, G., 2011. The effect of various iodine supplementations and two different iodine sources on performance and iodine concentrations in different tissues of broilers. Br. Poult. Sci. 52, 115–123.

Rycroft, C.E., Jones, M.R., Gibson, G.R., Rastall, R.A., 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol. 91, 878–887.

Saiyed, M.A., Joshi, R.S., Savaliya, F.P., Patel, A.B., Mishra, R.K., Bhagora, N.J., 2015. Study on inclusion of probiotic, prebiotic and its combination in broiler diet and their effect on carcass characteristics and economics of commercial broilers. Vet. World 8, 225–231.

Sajilata, M. g., Singhal, R.S., Kulkarni, P.R., 2006. Resistant Starch–A Review. Compr. Rev. Food Sci. 5, 1–17.

Salminen, S., Bouley, C., Boutron-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M., Rowland, I., 1998. Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80 Suppl 1, S147–171.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 12, 412–416.

Sandberg, F.B., Emmans, G.C., Kyriazakis, I., 2006. A model for predicting feed intake of growing animals during exposure to pathogens. J. Anim. Sci. 84, 1552–1566.

Sarangi, N.R., Babu, L.K., Kumar, A., Pradhan, C.R., Pati, P.K., Mishra, J.P., 2016. Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on

growth performance and carcass characteristics of broiler chickens. Vet. World 9, 313–319.

Sell, J.L., Angel, C.R., Escribano, F., 1987. Influence of Supplemental Fat on Weights of Eggs and Yolks During Early Egg Production. Poult. Sci. 66, 1807–1812.

Shakouri, M.D., H. Kermanshahi, M. Mohsenzadeh., 2006. Effect of Different non Starch Polysaccharides in Semi Purified Diets on Performance and Intestinal Microflora of Young Broiler Chickens. Int. J. Poult. Sci. 5, 557–561.

Sharma, A., Yadav, B.S., Ritika, 2008. Resistant Starch: Physiological Roles and Food Applications. Food Rev. Int. 24, 193–234.

Sklan, D., Smirnov, A., Plavnik, I., 2003. The effect of dietary fibre on the small intestines and apparent digestion in the turkey. Br. Poult. Sci. 44, 735–740.

Smith, J.A., 2011. Experiences with drug-free broiler production. Poult. Sci. 90, 2670–2678.

Smits, C.H., Veldman, A., Verkade, H.J., Beynen, A.C., 1998. The inhibitory effect of carboxymethyl cellulose with high viscosity on lipid absorption in broiler chickens coincides with reduced bile salt concentration and raised microbial numbers in the small intestine. Poult. Sci. 77, 1534–1539.

Song, H.P., Delwiche, S.R., Line, M.J., 1998. Moisture Distribution in a Mature Soft Wheat Grain by Three-Dimensional Magnetic Resonance Imaging. J. Cereal Sci. 2, 191–197.

Speelmans, G., Govers, M.J.A.P., Knol, J., Tol, E.A.F.V., 2013. Synergism of gos and polyfructose. US20130210763 A1.

Spence, J.T., 2006. Challenges related to the composition of functional foods. J. Food Compos. Anal., 28th US National Nutrient Databank Conference28th US National Nutrient Databank Conference 19, Supplement, S4–S6.

Spring, P., Wenk, C., Dawson, K.A., Newman, K.E., 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poult. Sci. 79, 205–211.

Stevens, C.E., Hume, I.D., 1998. Contributions of Microbes in Vertebrate Gastrointestinal Tract to Production and Conservation of Nutrients. Physiol. Rev. 78, 393–427.

Sumner, A.K., Gebre-Egziabher, A., Tyler, R.T., Rossnagel, B.G., 1985. Composition and Properties of Pearled and Fines Fractions from Hulled and Hull-less Barley. Cereal Chem 62, 112–116.

Surai, P.F., 2000. Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. Br. Poult. Sci. 41, 235–243.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. Worlds Poult. Sci. J. 67, 207–224.

Svihus, B., Gullord, M., 2002. Effect of chemical content and physical characteristics on nutritional value of wheat, barley and oats for poultry. Anim. Feed Sci. Technol. 102, 71–92.

Svihus, B., Herstad, O., Newman, C.W., 1997. Effect of high-moisture storage of barley, oats, and wheat on chemical content and nutritional value for broiler chickens. Acta Agric. Scand. Sect. — Anim. Sci. 47, 39–47.

Takatsuki, K., 2013. Poultry industry “must act” over food poisoning bug [WWW Document]. BBC News. URL http://www.bbc.co.uk/news/uk-21029389 (accessed 24.08.15).

Taylor, R.D., Jones, G.P.D., 2004. The incorporation of whole grain into pelleted broiler chicken diets. II. Gastrointestinal and digesta characteristics. Br. Poult. Sci. 45, 237–246.

Thomas, J.M., Jensen, L.S., McGinnis, J., 1961. Interference with the Nutritional Improvement of Water-Treated Barley by Antibiotics. Poult. Sci. 40, 1204–1208.

Tsukahara, T., Ushida, K., 2000. Effects of animal or plant protein diets on cecal fermentation in guinea pigs (Cavia porcellus), rats (Rattus norvegicus) and chicks (Gallus gallus domesticus). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 127, 139–146.

Uni, Z., 1998. IMPACT OF EARLY NUTRITION ON POULTRY: REYIEW OF PRESENTATIONS - 452.full.pdf [WWW Document]. URL http://japr.oxfordjournals.org/content/7/4/452.full.pdf (accessed 16.09.15).

Van der Wielen, P.W.J.J., Biesterveld, S., Lipman, L.J.A., van Knapen, F., 2001. Inhibition of a Glucose-Limited Sequencing Fed-Batch Culture of Salmonella enterica Serovar Enteritidis by Volatile Fatty Acids Representative of the Ceca of Broiler Chickens. Appl. Environ. Microbiol. 67, 1979–1982.

Van Donkelaar, L.H.G., Noordman, T.R., Boom, R.M., van der Goot, A.-J., 2015. Pearling barley to alter the composition of the raw material before brewing. J. Food Eng. 150, 44–49.

Vasanthan, T., Temelli, F., 2008. Grain fractionation technologies for cereal beta-glucan concentration. Food Res. Int., Cereal Foods 41, 876–881.

Vitaglione, P., Napolitano, A., Fogliano, V., 2008. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. Trends Food Sci. Technol. 19, 451–463.

Waldroup, A.L., Skinner, J.T., Hierholzer, R.E., Waldroup, P.W., 1993. An evaluation of fructooligosaccharide in diets for broiler chickens and effects on *Salmonella* contamination of carcasses. Poult. Sci. 72, 643–650.

Wang, S., Sosulski, K., Sosulski, F., Ingledew, M., 1997. Effect of sequential abrasion on starch composition of five cereals for ethanol fermentation. Food Res. Int. 30, 603–609.

Ward, N., 1995. With dietary modifications, wheat can be used for poultry. Feedstuffs 7, 14-16.

Westrell, T., Ciampa, N., Boelaert, F., Helwigh, B., Korsgaard, H., Chríel, M., Ammon, A., Mäkelä, P., 2009. Zoonotic infections in Europe in 2007: a summary of the EFSA-ECDC annual report. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 14, 785–794.

White, W.B., Bird, H.R., Sunde, M.L., Prentice, N., Burger, W.C., Marlett, J.A., 1981. The viscosity interaction of barley beta-glucan with Trichoderma viride cellulase in the chick intestine. Poult. Sci. 60, 1043–1048.

Xu, Z.R., Hu, C.H., Xia, M.S., Zhan, X.A., Wang, M.Q., 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82, 1030–1036.

Yang, Y., Iji, P. a., Choct, M., 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds Poult. Sci. J. 65, 97–114.

Yang, Y., Iji, P.A., Kocher, A., Thomson, E., Mikkelsen, L.L., Choct, M., 2008. Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilisation, nutrient digestibility and intestinal microflora. Br. Poult. Sci. 49, 186–194.

Yegani, M., Korver, D.R., 2008. Factors affecting intestinal health in poultry. Poult. Sci. 87, 2052–2063.

Yeung, J., Vasanthan, T., 2001. Pearling of hull-less barley: product composition and gel color of pearled barley flours as affected by the degree of pearling. J. Agric. Food Chem. 49, 331–335.

Yu, L.L., Tsao, R., Shahidi, F., 2012. Cereals and Pulses: Nutraceutical Properties and Health Benefits. John Wiley & Sons. pp 328

Yusrizal, Chen, T.., 2003. Effect of Adding Chicory Fructans in Feed on Broiler Growth Performance, Serum Cholesterol and Intestinal Length. Int. J. Poult. Sci. 2, 214–219.

Zduńczyk, Z., Juśkiewicz, J., Jankowski, J., Mikulski, D., 2006. Performance and Gastrointestinal Tract Metabolism of Turkeys Fed Diets with Different Contents of Fructooligosaccharides. Poult. Sci. 85, 886–891.

Zhang, W.F., Li, D.F., Lu, W.Q., Yi, G.F., 2003. Effects of isomalto-oligosaccharides on broiler performance and intestinal microflora. Poult. Sci. 82, 657–663.

Zhao, J., Cheung, P.C.K., 2011. Fermentation of β-glucans derived from different sources by bifidobacteria: evaluation of their bifidogenic effect. J. Agric. Food Chem. 59, 5986–5992.

Zheng, G.H., Rossnagel, B.G., Tyler, R.T., Bhatty, R.S., 2000. Distribution of β-Glucan in the Grain of Hull-less Barley. Cereal Chem. 77, 140–144.

Zheng, X., Li, L., Wang, Q., 2011. Distribution and Molecular Characterization of β-Glucans from Hull-Less Barley Bran, Shorts and Flour. Int. J. Mol. Sci. 12, 1563–1574.

Appendix

1.0. Pearling

Appendix 1.1: Cumulative pearling fraction mean weights for eight barley varieties from 80 seconds pearling cycle. Fractions were obtained 10 seconds apart for FR1 to FR8. FR9 is the leftover pearled grain after pearling for 80 seconds. 100 g samples were used for each cycle in triplicate.

	Cumulative fraction weights (%)							
	Pearl	**Propino**	**Cassasta**	**Concerto**	**Maris otter**	**Munton**	**Chevalier**	**Tipple**
FR1	5.27±0.02	5.71±0.03	5.24±0.09	6.29±0.20	4.19±0.04	3.53±0.08	2.08±0.18	5.94±0.21
FR2	2.73±0.04	1.45±0.04	2.65±0.06	2.30±0.15	2.88±0.03	3.47±0.07	2.93±0.23	2.04±0.27
FR3	1.25±0.04	1.14±0.05	1.26±0.06	1.31±0.01	1.13±0.03	1.90±0.02	3.41±0.05	1.12±0.04
FR4	1.17±0.05	1.16±0.05	1.02±0.07	1.30±0.04	0.77±0.05	1.17±0.10	1.88±0.06	1.02±0.04
FR5	1.16±0.03	1.27±0.03	1.08±0.09	1.45±0.04	0.70±0.06	0.94±0.06	0.97±0.02	1.04±0.04
FR6	0.99±0.02	1.06±0.02	0.90±0.04	1.39±0.02	0.58±0.03	0.88±0.12	0.68±0.01	0.91±0.05
FR7	1.03±0.05	1.22±0.03	1.00±0.04	1.64±0.05	0.52±0.01	0.86±0.02	0.62±0.01	0.97±0.02
FR8	1.19±0.06	1.25±0.06	1.03±0.02	1.68±0.04	0.63±0.01	0.84±0.04	0.68±0.02	0.96±0.03
FR9	85.20±0.12	85.75±0.05	85.64±0.04	82.83±0.11	88.61±0.04	86.75±0.17	86.75±0.05	86.00±0.08

2.0. Betaglucan

Appendix 2.1: Total beta-glucan content (mg/100mg) per pearling fractions of eight barley varieties in triplicate.

	Betaglucan mg/100mg							
	Pearl	**Propino**	**Cassasta**	**Concerto**	**M.otter**	**Munton**	**Chevalier**	**Tipple**
FR1	0.57±0.05	0.49±0.01	0.58±0.04	0.59±0.04	0.41±0.04	0.91±0.04	0.34±0.00	0.54±0.09
FR2	0.77±0.08	1.03±0.11	0.90±0.01	1.05±0.01	0.44±0.02	0.58±0.07	0.40±0.00	1.01±0.05
FR3	1.37±0.02	1.71±0.05	1.47±0.05	1.67±0.05	0.88±0.07	1.00±0.10	0.57±0.06	1.77±0.02
FR4	1.67±0.06	1.96±0.06	1.82±0.06	1.86±0.02	1.11±0.10	1.3±0.05	0.73±0.01	2.04±0.01
FR5	1.74±0.01	2.30±0.03	2.01±0.04	2.00±0.04	1.20±0.05	1.65±0.10	1.03±0.00	2.42±0.02
FR6	2.10±0.06	2.69±0.01	2.44±0.17	2.26±0.06	1.81±0.03	2.01±0.06	1.71±0.07	2.73±0.08
FR7	2.37±0.01	2.88±0.04	2.56±0.09	2.63±0.04	1.81±0.07	2.17±0.15	1.80±0.08	3.05±0.16
FR8	2.66±0.05	2.91±0.08	2.61±0.06	2.53±0.12	1.77±0.11	2.35±0.11	1.98±0.11	3.18±0.09
FR9	2.87±0.08	2.78±0.05	2.53±0.01	2.74±0.04	2.25±0.08	2.30±0.01	2.86±0.14	2.84±0.17
TOTAL	16.12	18.75	16.92	17.33	11.68	14.27	11.42	19.58

Appendix 2.2. One way anova of beta-glucan analysis.

Appendix 2.2.1. Descriptive (beta-glucan)

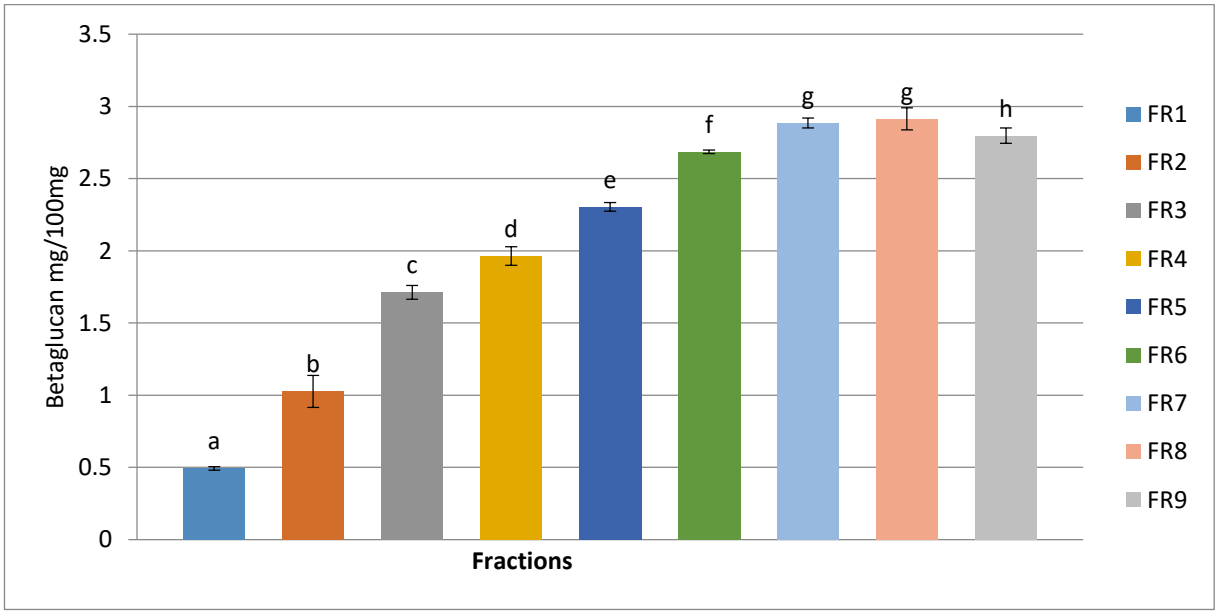
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
FR1	16	.5484	.17086	.04271	.4574	.6395	.34	.95
FR2	16	.7712	.26613	.06653	.6294	.9130	.40	1.14
FR3	16	1.3052	.43056	.10764	1.0757	1.5346	.51	1.79
FR4	16	1.5617	.45193	.11298	1.3209	1.8026	.72	2.04
FR5	16	1.7939	.47914	.11978	1.5386	2.0492	1.01	2.44
FR6	16	2.2199	.37666	.09416	2.0192	2.4206	1.65	2.81
FR7	16	2.4093	.45821	.11455	2.1651	2.6535	1.72	3.21
FR8	16	2.4994	.45730	.11433	2.2557	2.7430	1.66	3.27
FR9	16	2.6475	.26325	.06581	2.5072	2.7878	2.16	3.01
Total	144	1.7507	.81419	.06785	1.6166	1.8848	.34	3.27

Appendix 2.2.2. Test of Homogeneity of Variances (beta-glucan)

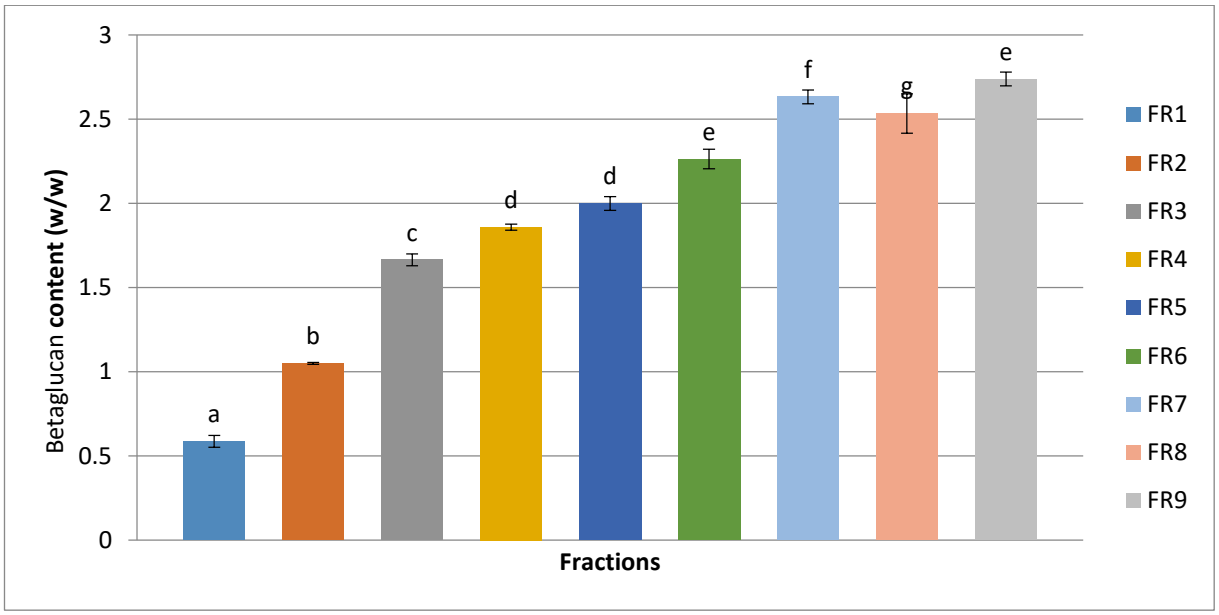
Levene Statistic	df1	df2	Sig.
3.455	8	135	.001

Appendix 2.2.3. ANOVA (betaglucan)

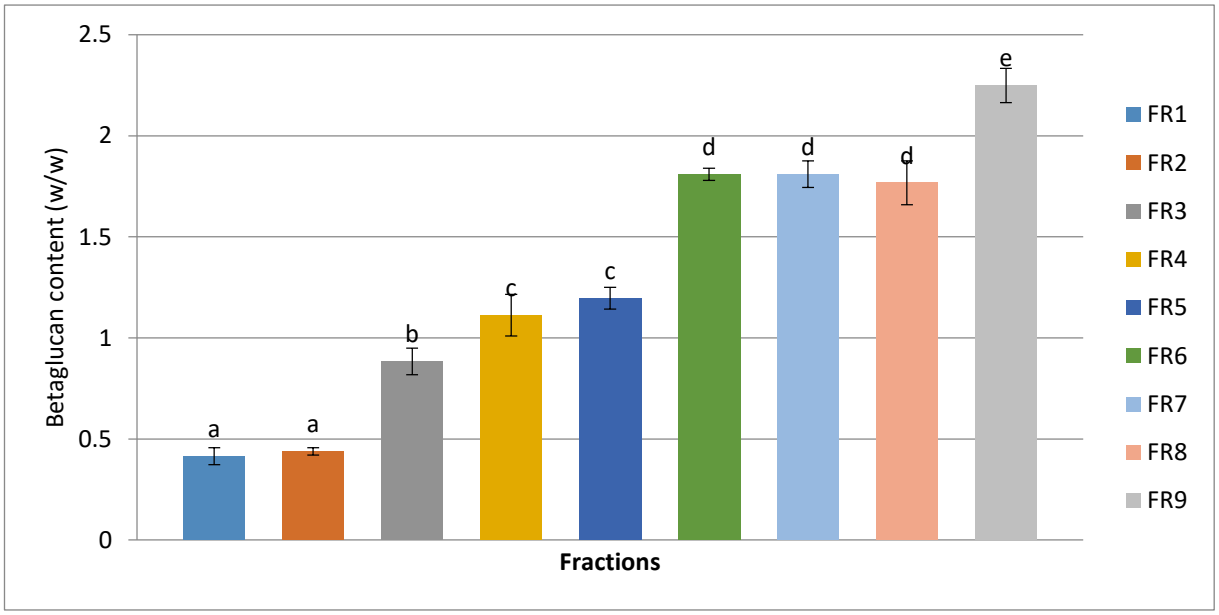
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	74.554	8	9.319	62.153	.000
Within Groups	20.242	135	.150		
Total	94.796	143			



Appendix 2.3. Beta-glucan content in Propino extracts. Bar with similar letters are not significantly different at $p \leq 0.05$.



Appendix 2.4. Beta-glucan content in Concerto extracts. Bar with similar letters are not significantly different at $p \leq 0.05$.



Appendix 2.5. Beta-glucan content in Maris Otter extracts. Bar with similar letters are not significantly different at $p \leq 0.05$.

3.0. Resistant starch

Appendix 3.1.Total resistant starch content (mg/100mg) in six pearling fractions (FR4 – FR9) of eight barley varieties.

Resistant Starch mg/100mg								
	Pearl	Propino	Cassasta	Concerto	M.otter	Munton	Chevalier	Tipple
FR4[d]	1.10±0.05[defg]	1.08±0.06[defg]	1.05±0.05[defg]	1.17[defg]	1.08[defg]	1.13±0.02[defg]	1.14±0.05[defg]	1.12±0.02[defg]
FR5[e]	1.08±0.07[defg]	1.13[defg]	1.05±0.01[defg]	1.13±0.02[defg]	1.10[defg]	1.11±0.01[defg]	1.19±0.08[defg]	1.15±0.02[defg]
FR6[f]	1.10±0.05[defg]	1.10[defg]	1.10±0.02[defg]	1.13[defg]	1.33±0.24[defg]	1.14±0.03[defg]	1.17±0.04[defg]	1.12[defg]
FR7[g]	1.07±0.02[defg]	1.13±0.01[defg]	1.13±0.02[defg]	1.19±0.04[defg]	1.26±0.03[defg]	1.09±0.03[defg]	1.15[defg]	1.11±0.02[defg]
FR8[h]	9.82±0.04[h]	10.63±0.62[h]	10.00±0.21[hi]	9.82±[h]	10.35±0.09[hi]	9.88±0.09[h]	9.91±0.06[h]	9.97±0.11[hi]
FR9[i]	10.46±0.09[i]	10.64±0.03[i]	10.52±0.38[hi]	10.23±0.21[i]	17.13±6.30[hi]	10.75±0.03[i]	11.48±0.58[i]	9.97±0.23[hi]

Appendix 3.2. One way anova of resistant starch analysis.

Appendix 3.2.1. Descriptives (resistant starch)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
FR4	8	1.1014	.06651	.02352	1.0458	1.1570	1.01	1.18
FR5	8	1.0985	.05339	.01888	1.0539	1.1431	1.01	1.15
FR6	8	1.1090	.03031	.01071	1.0836	1.1343	1.06	1.15
FR7	8	1.1299	.04937	.01746	1.0887	1.1712	1.06	1.22
FR8	8	10.0688	.50546	.17871	9.6463	10.4914	9.67	11.25
FR9	8	10.4613	.28698	.10146	10.2214	10.7012	10.02	10.90
Total	48	4.1615	4.36902	.63061	2.8929	5.4301	1.01	11.25

Appendix 3.2.2. Test of Homogeneity of Variances (resistant starch)

Levene Statistic	df1	df2	Sig.
4.797	5	42	.001

Appendix 3.2.3. ANOVA (resistant starch)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	894.710	5	178.942	3080.927	.000
Within Groups	2.439	42	.058		
Total	897.150	47			

4.0. Proteins

Appendix 4.1. One way anova of protein analysis.

Appendix 4.1.1. Descriptives (protein)

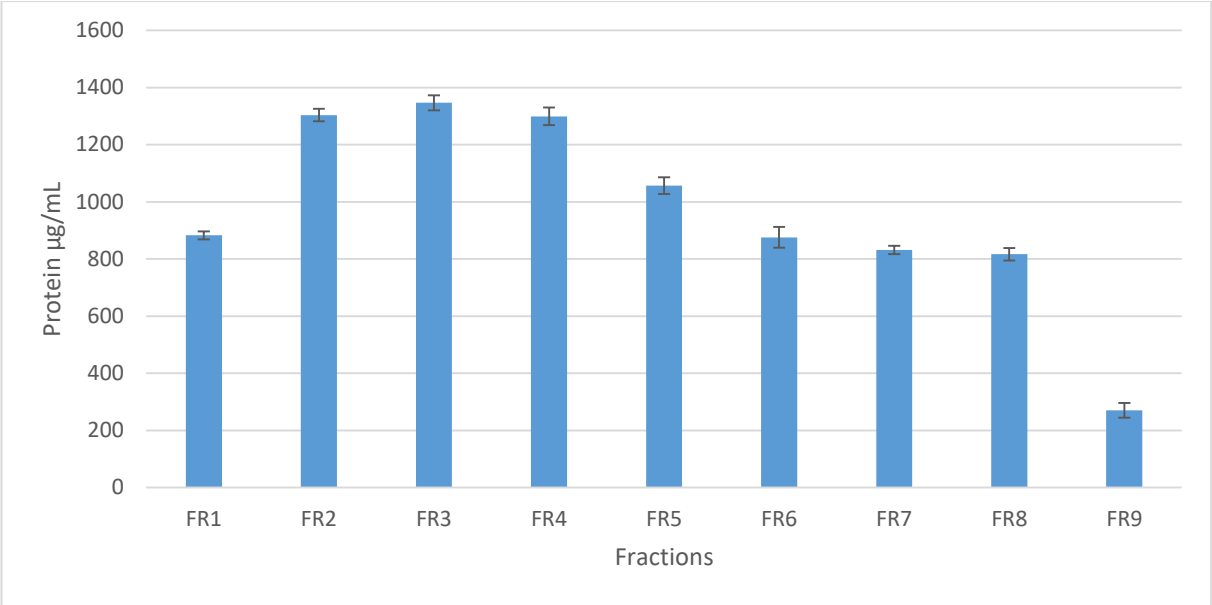
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
FR1	8	932.2813	102.59298	36.27210	846.5114	1018.0511	756.92	1119.00
FR2	8	1205.0417	170.02655	60.11346	1062.8959	1347.1874	937.75	1431.08
FR3	8	1350.3542	144.48728	51.08397	1229.5598	1471.1486	1156.08	1671.08
FR4	8	1303.2188	136.77269	48.35645	1188.8739	1417.5636	1027.75	1494.83
FR5	8	1206.0833	143.50523	50.73676	1086.1100	1326.0567	1002.75	1421.92
FR6	8	966.8646	102.51341	36.24396	881.1612	1052.5679	856.08	1114.42
FR7	8	921.0833	116.40212	41.15437	823.7687	1018.3979	736.50	1074.83
FR8	8	878.6354	130.36435	46.09076	769.6481	987.6227	689.00	1116.50
FR9	8	323.9479	63.90890	22.59521	270.5187	377.3771	236.50	403.17
Total	72	1009.7234	319.98135	37.71016	934.5314	1084.9153	236.50	1671.08

Appendix 4.1.2. Test of Homogeneity of Variances (protein)

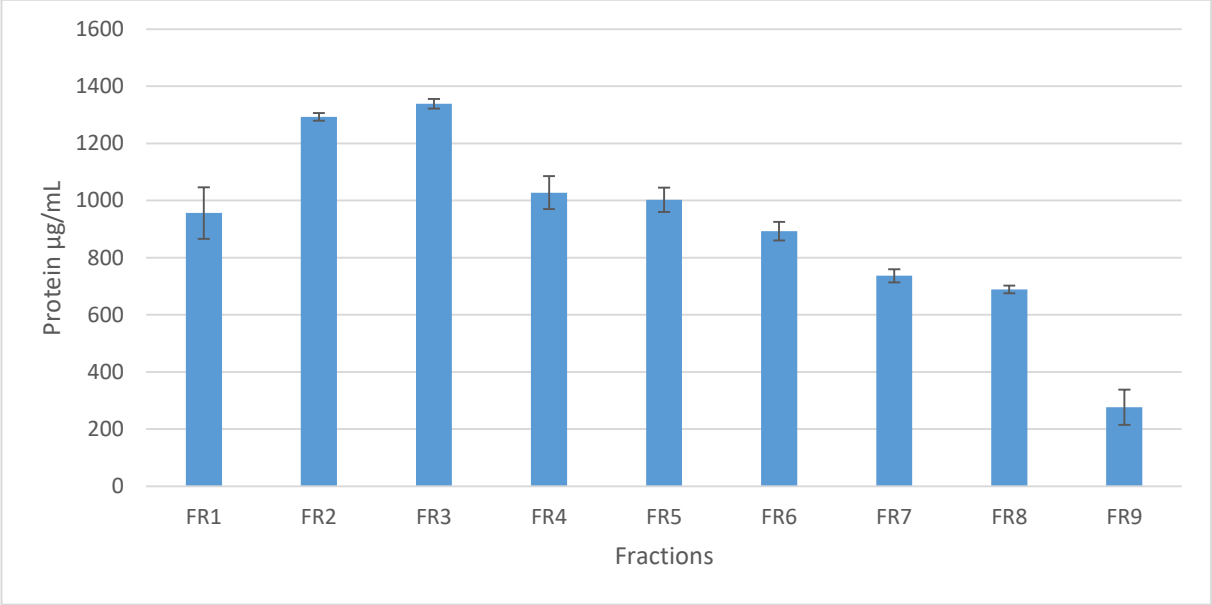
Levene Statistic	df1	df2	Sig.
.657	8	63	.727

Appendix 4.1.3. ANOVA (protein)

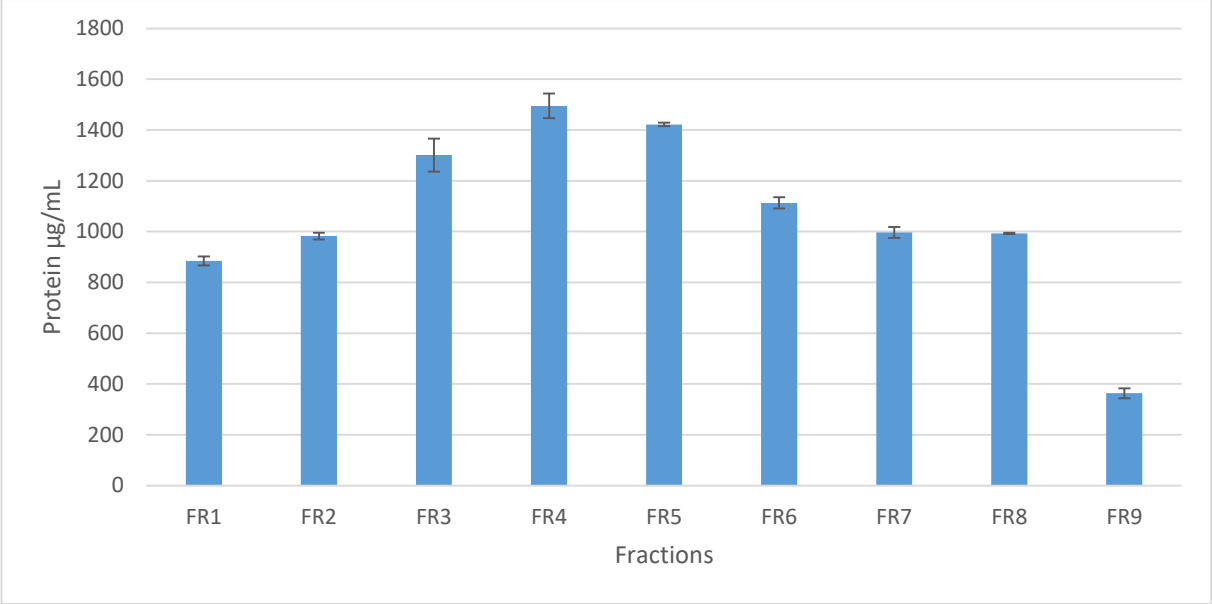
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6256308.699	8	782038.587	48.624	.000
Within Groups	1013243.728	63	16083.234		
Total	7269552.426	71			



Appendix 4.2. Protein content in Propino extracts



Appendix 4.3. Protein content in Concerto extracts



Appendix 4.4. Protein content in Maris Otter extracts

5.0. Free amino nitrogen (FAN)

Appendix 5.1. One way anova of FAN analysis.

Appendix 5.1.1. Descriptives (FAN)

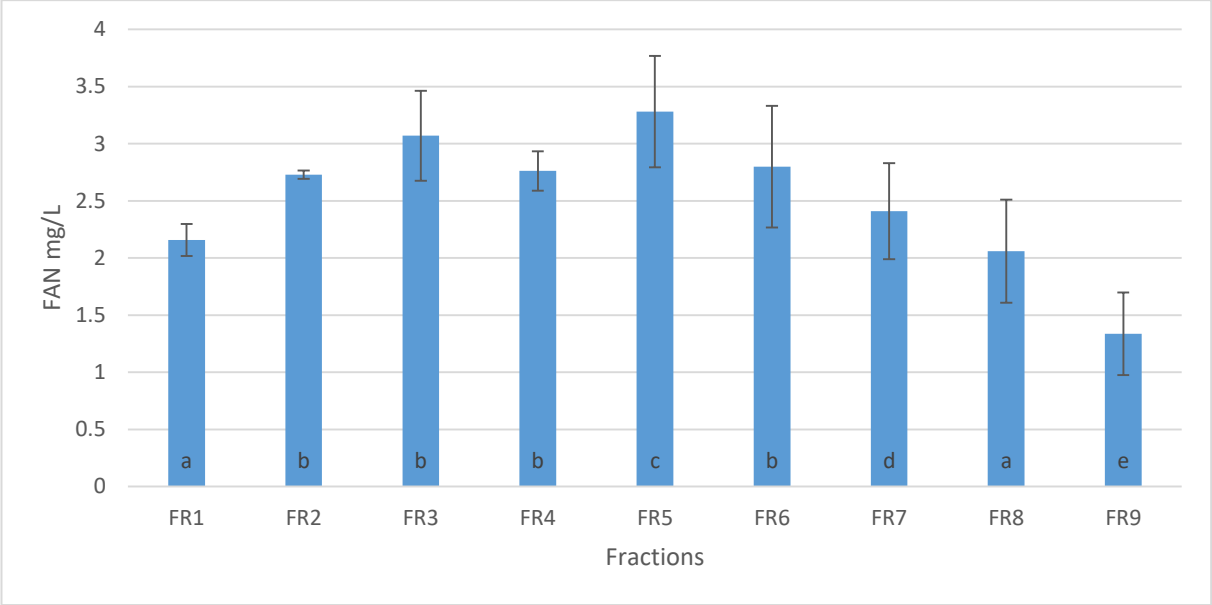
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
FR1	8	2.3398	.31889	.11274	2.0732	2.6064	2.04	2.98
FR2	8	2.2861	.47515	.16799	1.8888	2.6833	1.60	3.14
FR3	8	2.3268	.46836	.16559	1.9353	2.7184	1.59	3.07
FR4	8	2.4030	.45574	.16113	2.0219	2.7840	1.63	2.93
FR5	8	2.3564	.48314	.17082	1.9525	2.7603	1.73	3.28
FR6	8	2.0895	.38407	.13579	1.7684	2.4106	1.54	2.80
FR7	8	2.1187	.31637	.11185	1.8542	2.3832	1.63	2.54
FR8	8	2.0823	.27741	.09808	1.8503	2.3142	1.47	2.34
FR9	8	1.8380	.38530	.13623	1.5159	2.1601	1.34	2.41
Total	72	2.2045	.41799	.04926	2.1063	2.3027	1.34	3.28

Appendix 5.1.2. Test of Homogeneity of Variances (FAN)

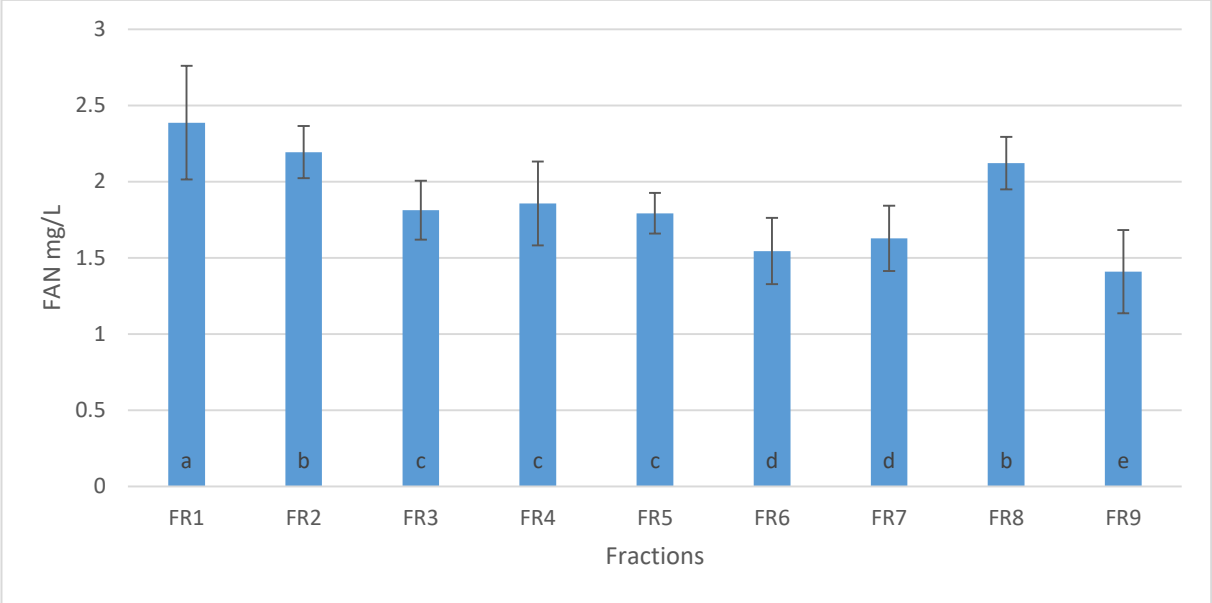
Levene Statistic	df1	df2	Sig.
.430	8	63	.899

Appendix 5.1.3. ANOVA (FAN)

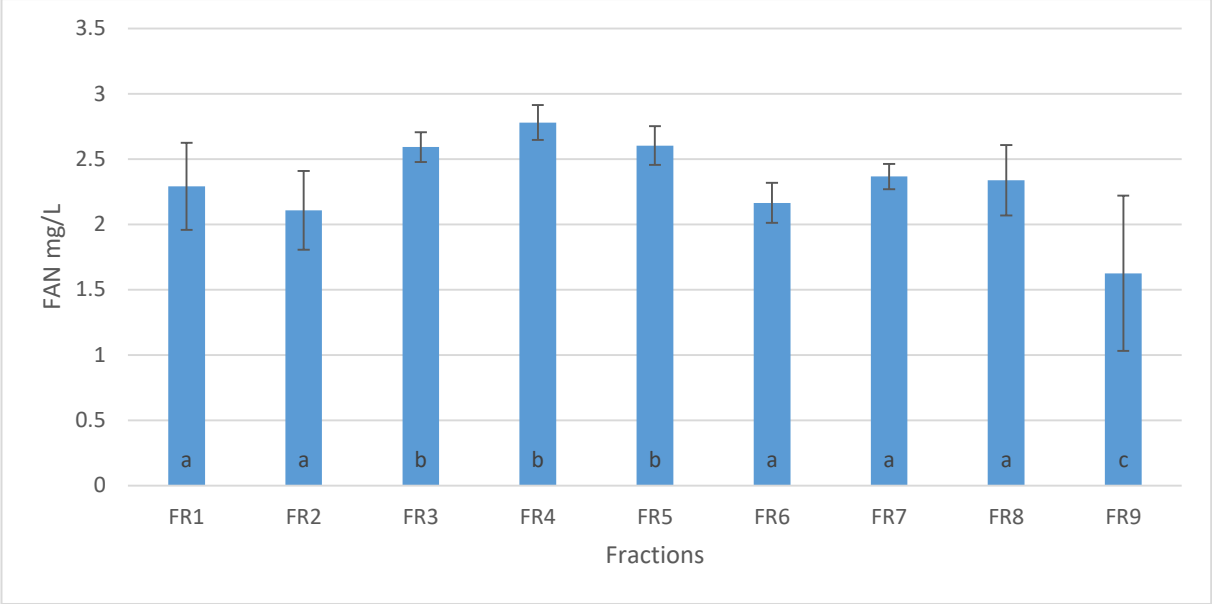
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.178	8	.272	1.677	.122
Within Groups	10.227	63	.162		
Total	12.405	71			



Appendix 5.2. FAN content in Propino extracts. Fractions with similar alphabets are not significantly different at p ≤ 0.05.



Appendix 5.3. FAN content in Concerto extracts. Fractions with similar alphabets are not significantly different at p ≤ 0.05.

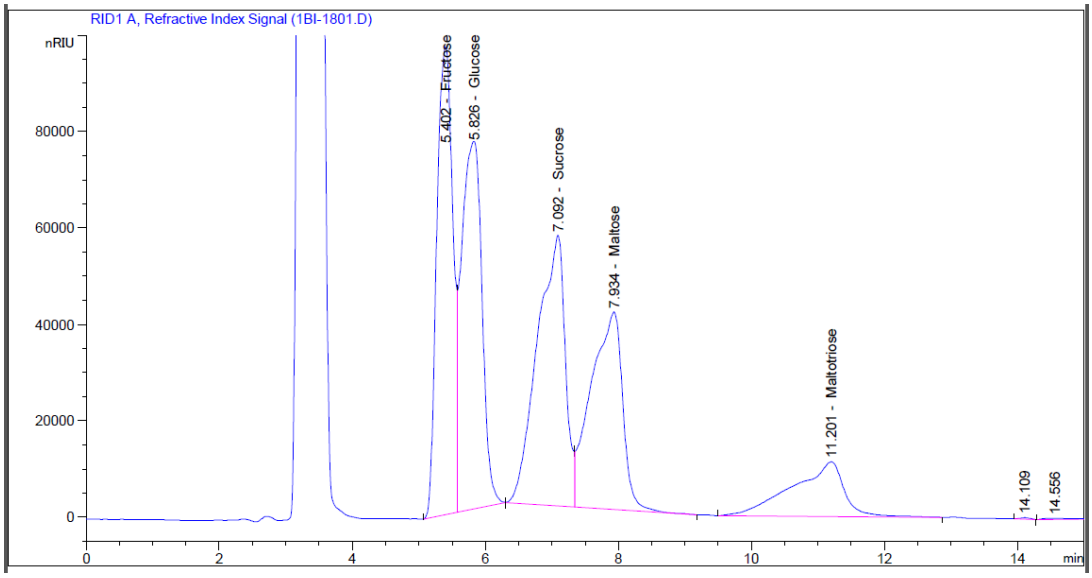


Appendix 5.4. FAN content in Maris Otter extracts. Fractions with similar alphabets are not significantly different at $p \leq 0.05$

6.0. Sugars

Appendix 6.1: Retention time of fructose, glucose, sucrose, maltose and maltotriose

Sugar	R^2 Value	Retention Time (minutes)
Fructose	0.99240	5.503
Glucose	0.99320	5.991
Sucrose	0.99188	7.337
Maltose	0.99453	8.246
Maltotriose	0.99394	11.686



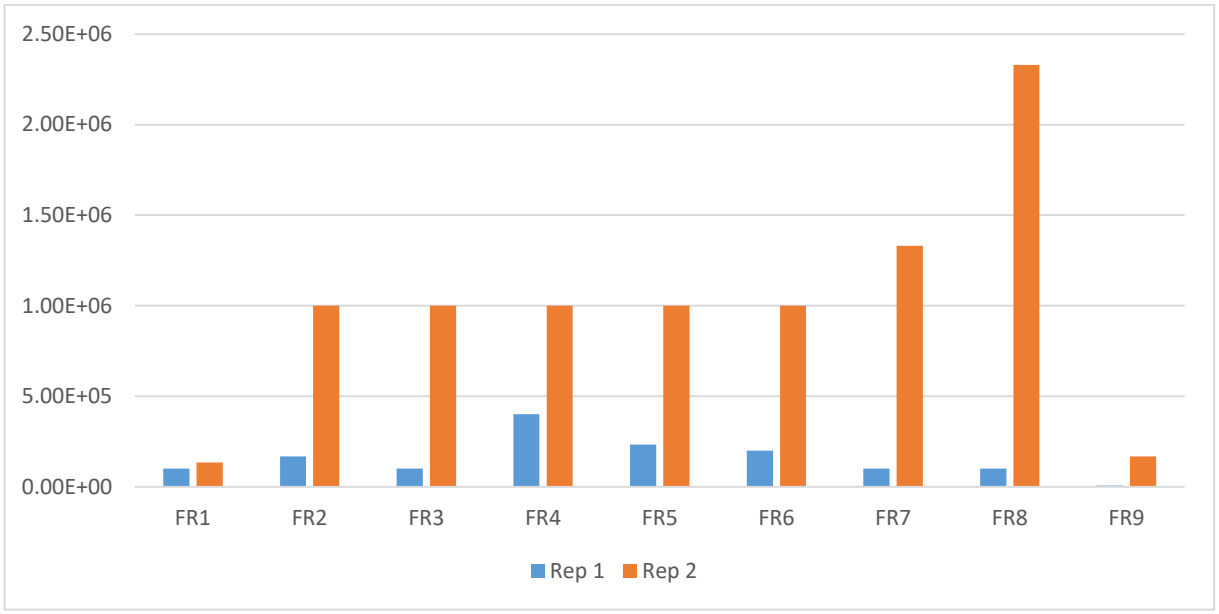
Appendix 6.2. 1% standard solution of fructose, glucose, sucrose, maltose and maltotriose.

Appendix 6.3: Simple sugar in FR1 – FR9 of eight barley varieties.

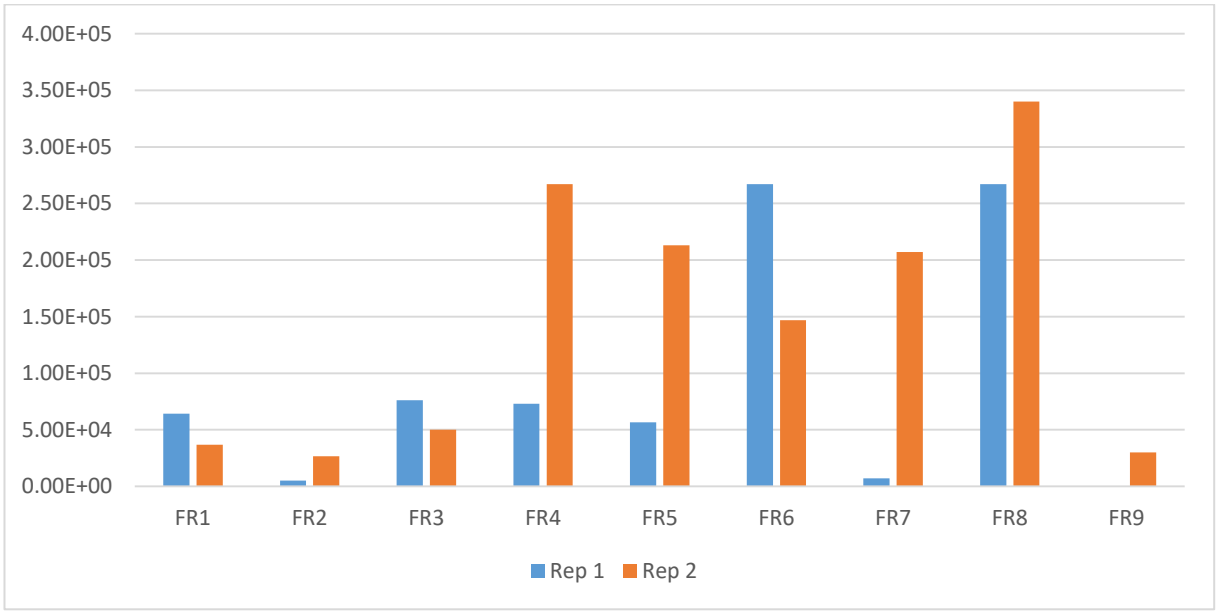
	Fructose	Glucose	Sucrose	Maltose	Maltotriose
FR1	0.2287	0.4118	0.0144	0.1313	0.1313
FR2	0.1772	0.3415	0.4211	0.1970	0.1970
FR3	0.3482	0.6115	1.4717	0.4072	0.4072
FR4	0.2531	0.5075	2.1354	0.4427	0.4427
FR5	0.2199	0.4663	2.0921	0.6450	0.6450
FR6	0.1062	0.2500	2.2054	0.9947	0.9947
FR7	0.0134	0.1240	1.8329	0.8774	0.8774
FR8	0.0000	0.0887	0.0000	0.2848	0.2848
FR9	0.0937	0.0764	0.3055	0.9994	0.9994

7.0. Bacterial growth

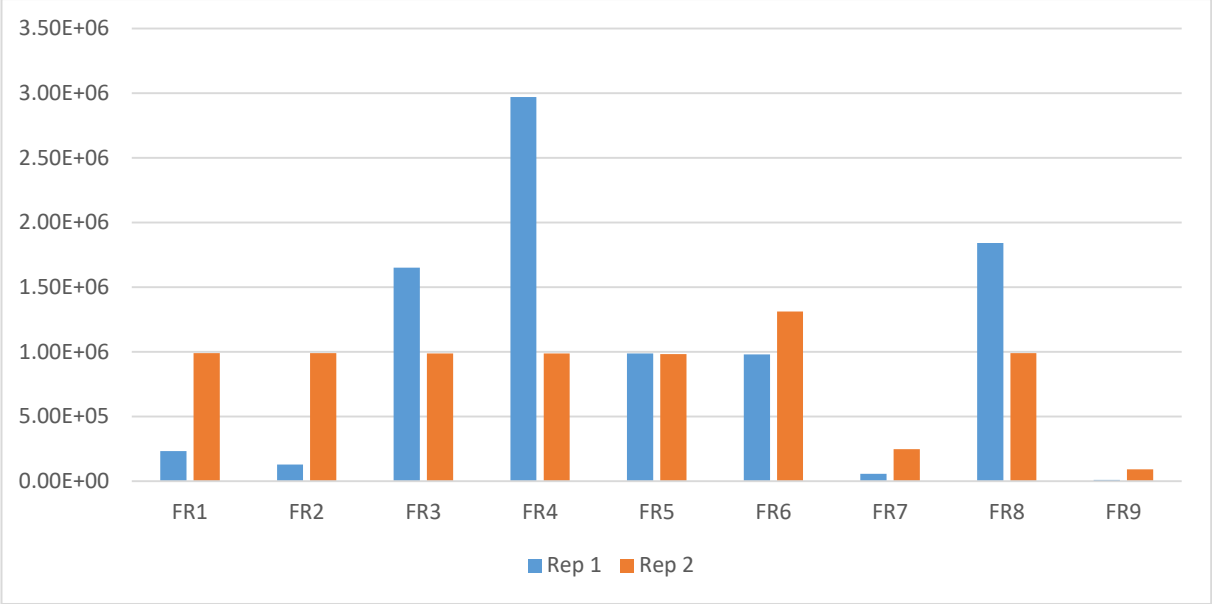
7.1. Method 1



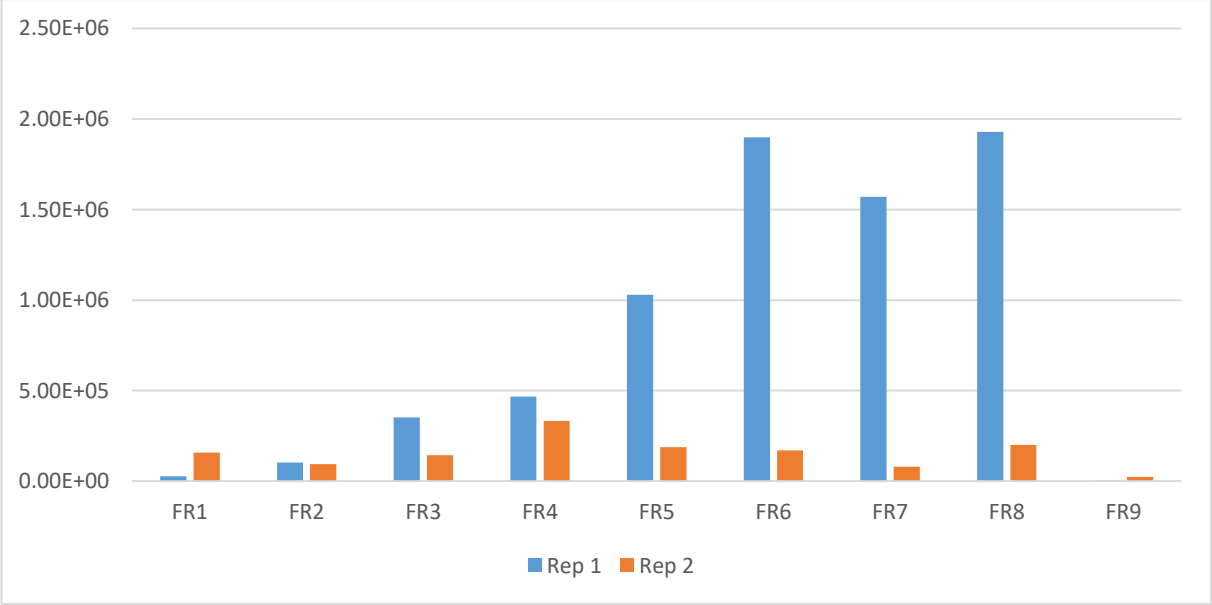
Appendix 7.1.1. Growth (CFU/mL) of *Lactobacillus acidophilus* in Concerto extract containing undigested nutrient over 8 hours. (Method 1)



Appendix 7.1.2. Growth (CFU/mL) of *Lactobacillus acidophilus* in Maris Otter extract containing undigested nutrient over 8 hours. (Method 1).



Appendix 7.1.3. Growth (CFU/mL) of *Salmonella enteritidis* in Concerto extract containing undigested nutrient over 8 hours. (Method 1).



Appendix 7.1.4. Growth (CFU/mL) of *Salmonella enteritidis* in Maris Otter extract containing undigested nutrient over 8 hours. (Method 1)

7.2. Method two

Appendix 7.2.1. Test of Homogeneity of Variances (Propino OD)

Levene Statistic	df1	df2	Sig.
2.795	8	18	.033

Appendix 7.2.2. ANOVA (Propino)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.202	8	.025	2.595	.044
Within Groups	.176	18	.010		
Total	.378	26			

Appendix 7.2.3. Test of Homogeneity of Variances (Concerto)

Levene Statistic	df1	df2	Sig.
1.595	8	18	.195

Appendix 7.2.4. ANOVA (Concerto)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.188	8	.023	1.882	.126
Within Groups	.224	18	.012		
Total	.412	26			

Appendix 7.2.4. Test of Homogeneity of Variances (Marisotter)

Levene Statistic	df1	df2	Sig.
1.917	8	18	.120

Appendix 7.2.5. ANOVA (Maris Otter)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.196	8	.025	9.258	.000
Within Groups	.048	18	.003		
Total	.244	26			

Appendix 7.3. MULTIPLE REGRESSION

Appendix 7.3.1: Pearson correlation of bacterial growth pattern (OD600) versus chemical composition in Propino, Concerto and Maris Otter (FR1 – FR8)

	Propino OD	Concerto OD	Maris Otter OD
OD	1.000	1.000	1.000
FAN	-.142	-.722	-.173
Protein	-.174	-.572	-.044
Resistant Starch	.553	.513	.554
Beta-glucan	.704	.961	.966

Appendix 7.3.2. Propino

Appendix 7.3.2.1. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.745[a]	.555	.110	.08664	1.767

a. Predictors: (Constant), BETAGLUCAN, FAN, STARCH, PROTEIN

b. Dependent Variable: PROPINOOD (FR1 – FR9)

Appendix 7.3.2.2. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.807[a]	.651	.187	.05711	2.690

a. Predictors: (Constant), BETAGLUCAN, FAN, PROTEIN, STARCH

b. Dependent Variable: PROPINOOD (FR1 FR8)

Appendix 7.3.3. Concerto

Appendix 7.3.3.1. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.966[a]	.933	.844	.03269	3.470

a. Predictors: (Constant), BETAGLUCAN, STARCH, PROTEIN, FAN

b. Dependent Variable: CONCERTOOD (FR1 – FR8)

Appendix 7.3.3.2. ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.045	4	.011	10.472	.041[b]
	Residual	.003	3	.001		
	Total	.048	7			

a. Dependent Variable: CONCERTOOD (FR1 – FR8)

b. Predictors: (Constant), BETAGLUCAN, STARCH, PROTEIN, FAN

Appendix 7.3.3.3. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.928[a]	.862	.724	.04646	3.238

a. Predictors: (Constant), BETAGLUCAN, STARCH, PROTEIN, FAN

b. Dependent Variable: CONCERTOOD (FR1-FR9)

Appendix 7.3.3.4. ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.054	4	.013	6.244	.052[b]
	Residual	.009	4	.002		
	Total	.063	8			

a. Dependent Variable: CONCERTOOD (FR1 – FR9)

b. Predictors: (Constant), BETAGLUCAN, STARCH, PROTEIN, FAN

Appendix 7.3.4. Maris Otter

Appendix 7.3.4.1. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.994[a]	.988	.972	.01065	3.308

a. Predictors: (Constant), BETAGLUCAN, FAN, STARCH, PROTEIN

b. Dependent Variable: MARISOTTEROD (FR1 – FR8)

Appendix 7.3.4.1. ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.028	4	.007	60.685	.003[b]
	Residual	.000	3	.000		
	Total	.028	7			

a. Dependent Variable: MARISOTTEROD (FR1 – FR8)

b. Predictors: (Constant), BETAGLUCAN, FAN, STARCH, PROTEIN

Appendix 7.3.4.2. Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.779[a]	.607	.215	.08014	2.331

a. Predictors: (Constant), BETAGLUCAN, PROTEIN, STARCH, FAN

b. Dependent Variable: MARISOTTEROD (FR1 – FR9)

Appendix 7.3.4.3. ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.040	4	.010	1.547	.341[b]
	Residual	.026	4	.006		
	Total	.065	8			

a. Dependent Variable: MARISOTTEROD (FR1 –FR9)

b. Predictors: (Constant), BETAGLUCAN, PROTEIN, STARCH, FAN